AN EXPERIMENTAL MODEL OF PORPHYRIA

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by

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Several aspects of the work described in this thesis have been published.

ABSTRACTS:

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Odber, J., Gillespie, J.S., Moore, M.R. and Goldberg, A (1991) The effects of haem synthesis blocking agents on the responses of some rat isolated nerve/muscle preparations. Br. J. Pharmacol. (in press)

1. The porphyrias are a group of disorders of haem metabolism, due to an enzymatic defect in the haem biosynthetic pathway. Two current hypotheses regarding the underlying causes of the neuropathy of the acute type of porphyria were examined. Firstly, are the haem precursors δ-aminolaevulinic acid (ALA) and porphobilinogen (PBG) neurotoxic? Secondly, is acute porphyric neuropathy a consequence of a reduction in essential haemoproteins?

2. The responses of a variety of *in vitro* rabbit nerve/muscle preparations, whose responses are mediated by different neurotransmitters, were unaffected by ALA in concentrations ranging from 1μM. to 10mM. In isolated nerve/muscle preparations taken from rats that had received porphyrinogenic drug treatment, which disrupted haem biosynthesis, 10nM. to 300μM. ALA did not alter the responses of the muscles to electrical field stimulation of their intrinsic nerves.

3. 30μM. to 1mM. PBG did not significantly alter the response of the rat anococcygeus muscle to electrical field stimulation of the intrinsic inhibitory nerves.

4. The results of these experiments provide no evidence that ALA or PBG are neurotoxic.

5. In the second group of experiments the porphyrinogenic drugs (succinylacetone; allylisopropylacetamide; DDC and its 4-ethyl analogue 4-ethyl DDC; phenobarbitone; lead) were employed, in various combinations, for periods ranging from 3 to 44 days, in an attempt to produce a haem deficiency neuropathy, in rats, by inhibiting haem synthesis.

6. A range of rat *in vitro* nerve/muscle preparations were examined, the responses of which are mediated by different neurotransmitters, one of which, the nitrergically-mediated inhibitory response of the anococcygeus muscle results from activation of a cytosolic haemoprotein, guanylate cyclase.

7. The results of this group of experiments provide no evidence that the porphyrinogenic compounds employed, in this study, reduce essential haemoproteins to levels where a neuropathy ensues.

8. The third group of experiments examined liver, blood and brain haemoproteins, following porphyrinogenic drug treatment for periods ranging between 14 and 44 days. Hepatic respiratory cytochrome levels and catalase activity, blood haemoglobin content and catalase activity and brain respiratory cytochromes were measured. These tissues give a measure of haemoproteins in the two major haem containing organs, the liver and blood and a measure of haemoproteins in neural tissue where a deficit, in these cytochromes, could lead to neuropathy.

9. Treatments which included the use of either 4-ethyl DDC or N-methyl protoporphyrin, both of which inhibit hepatic ferrochelatase, caused a significant reduction in hepatic haemoproteins, but were ineffective in reducing blood or brain levels. Lead treatment did cause a reduction in whole blood haemoglobin content and a rise in catalase activity, but was also incapable of reducing brain respiratory cytochrome levels.

10. The failure of these porphyrinogenic compounds to alter brain haemoproteins may be due to their inability to cross the blood brain barrier.

11. The last group of experiments examined both hepatic and brain mitochondrial function following porphyrinogenic treatment, which was known, from the previous group of experiments, to reduce hepatic respiratory cytochromes. Additionlly, to circumvent the blood brain barrier, the ferrochelatase inhibitor, N-methyl protoporphyrin was administered directly into the ventricular system. Treatments which significantly reduced hepatic respiratory cytochromes also caused a reduction in the Respiratory Control Ratio (RCR) in liver mitochondria, while all other respiratory parameters were unaltered.

All brain mitochondrial function parameters were unaltered by these systemic treatments. Central administration of N-methyl protoporphyrin, caused a reduction in brain mitochondrial RCRs, while all other respiratory parameters in this tissue remained unaltered. Hepatic mitochondrial function was unaffected by centrally-administered N-methyl protoporphyrin.

12. The results of these experiments show that some porphyrinogenic drugs are capable of altering some aspects of mitochondrial function, in this case the Respiratory Control Ratio (RCR). Although systemically-administered compounds were unable to alter brain mitochondrial function, while doing so in liver, N-methyl protoporphyin did reduce RCR's in brain mitochondria when administered centrally. This latter observation suggests that when porphyrinogenic drugs gain access to neural tissue they can exert similar effects.

13. The failure, in this study, to produce a neuropharmacological model of acute porphyria is most probably due to the inability of the porphyrinogenic compounds employed to reduce neural respiratory cytochromes to levels where a functional deficit occurs. This problem may be overcome by a longer period of treatment. The results of the present set of experiments indicate that succinylacetone is not a suitable compound for use in *in vivo* haemoprotein depletion. N-methyl protoporphyrin at larger concentrations than used in this study may be more effective in producing a model of a haem deficiency neuropathy.

CHAPTER 1

GENERAL INTRODUCTION

The porphyrias are a group of heterogeneous disorders in which there is a defect in the metabolic pathway leading to the synthesis of haem. They are members of a group of diseases which occur due to an inborn error of metabolism (Garrod 1923). This error results in either the imperfect synthesis of enzymes, producing an aberrant structure, or in the decreased synthesis of a perfect compound. There is a type of porpyria associated with an enzymatic deficit at every level in the haem pathway, except the first step. The biochemical correlate of this group of diseases is an accumulation of the haem precursors prior to the site of defect and the clinical symptoms are indicative of an underlying central and peripheral neuropathy. The link between the biochemical defect and the clinical symptoms, however, remains obscure.

1.1 HAEM BIOSYNTHESIS.

The principle sites of haem synthesis are the haemopoietic and hepatic tissue (Berk et.al. 1974) but haem synthesis is also maintained in other tissues including rat brain, heart, adrenal gland and testes and the mouse harderian gland (De Matteis et. al. 1981a; De Matteis and Ray 1982; Percy and Shanley 1979; Briggs et.al. 1976; Condie et.al. 1976; Tofilon and Piper 1980; Margolis 1971).

The formation of the haem molecule requires eight molecules of glycine and eight molecules of succinic acid and is synthesized in a sequence of eight enzymatically catalysed steps either in the cytosol or the mitochondria (figure 1). The first evidence that protoporphyrin, the immediate precursor of haem, was synthesized from glycine and succinate came from the 1946 experiments of Shemin and Rittenberg. These experiments demonstrated that glycine provides all four nitrogen atoms, the methene bridge carbon atoms and the α-carbon in each pyrrole ring. The activated form of succinate, coenzyme A, takes part in this reaction and is the donor of 26 of the 34 carbon atoms of the protoporphyrin molecule. Glycine and succinate condense to form the five carbon amino ketone, δ-aminolaevulinic acid (ALA) (Shemin and Russel 1953). ALA is the precursor of the tetrapyrrole nucleus of the porphyrins (the iron chelate), the corrin ring of vitamin B_{12} (the cobalt chelate) and plant and bacterial chlorophyl (the magnesium

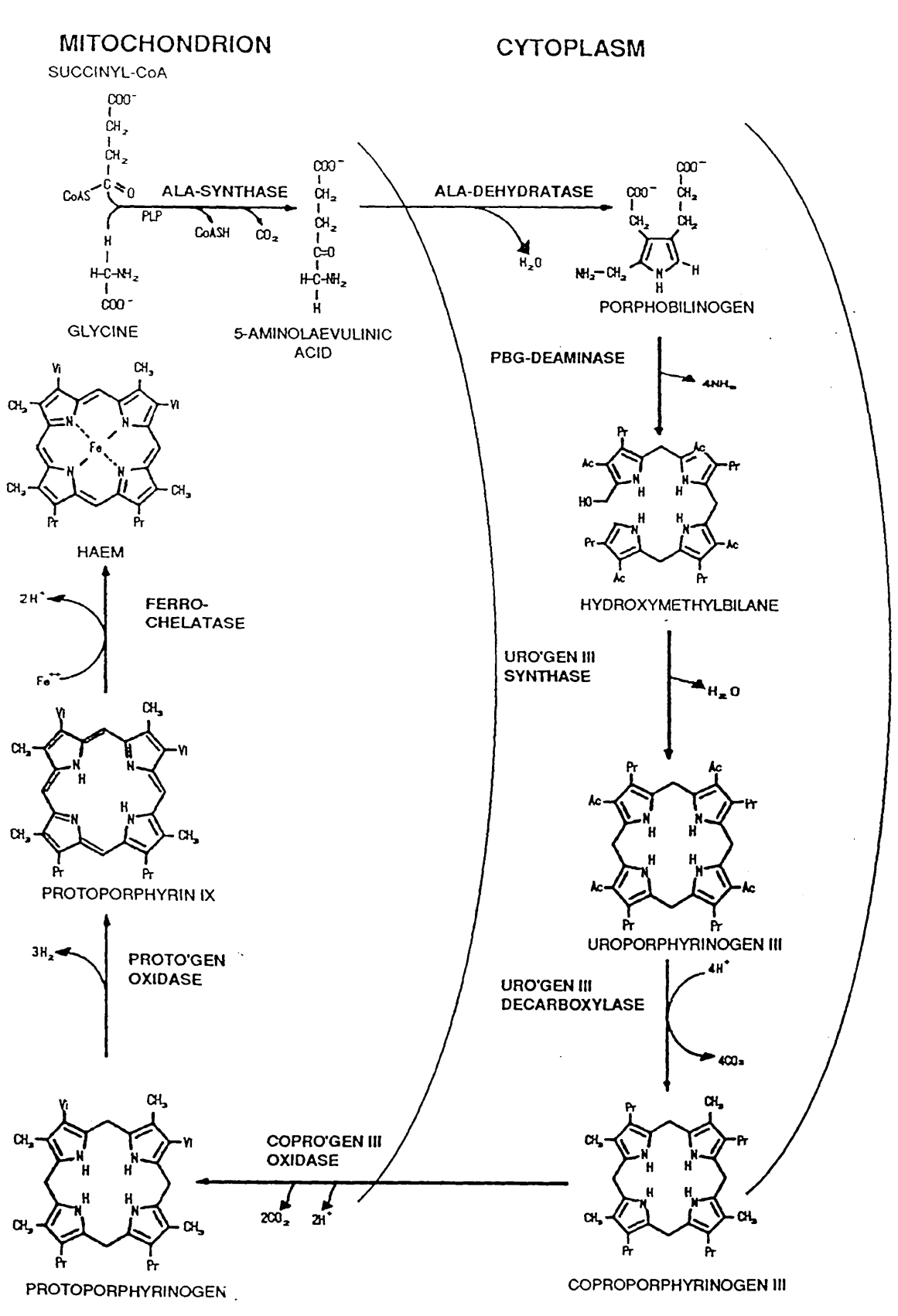

Figure 1: The haem biosynthetic pathway.

chelate). The condensation of glycine and succinyl CoA is catalysed by the pyridoxal containing enzyme ALA synthase (ALAS) (Gibson et.al. 1958). This enzyme is synthesized in the cytoplasm and transported into the mitochondria where it is free or bound loosely to the inner mitochondrial membrane (Patten and Beattie 1973). The biological half-life of ALAS is short. Tschudy et.al. 1965 reporting a half-life of 1hr. in rat liver while shorter half-lives for both mitochondrial and cytoplasmic enzymes have been observed e.g. 35min. for the mitochondrial and 20min. for the cytoplasmic enzyme (Beale and Granick 1978; Kikuchi and Hyashi 1981). This difference probably reflects both cytosolic degradation of the enzyme and transport of the cytosolic enzyme into the mitochondria. In both mouse and chick embro liver the half-lives of both cytoplasmic and mitochondrial synthase is approximately 3hrs. (Gayathri et.al. 1973; Sassa and Granick 1970). Glycine forms a Schiff base with a pyridoxal-enzyme complex. A proton is then removed from the methylene carbon atom of the glycine and the succinyl group from the succinyl CoA is transferred to form alpha-amino-β-ketoadipic acid. This intermediate is decarboxylated, a proton inserted and ALA is released (Zaman et.al. 1973; Akhtar et.al. 1976; Abboud et.al. 1974). The findings that the activity of the enzyme is normally very low, that increased ALAS activity is concomitant with increased porphyrin synthesis and that the activity of subsequent enzymes in the pathway is increased following increased ALAS activity implicate the enzyme as the rate limiting catalyst in the synthesis of haem (Gibson et.al. 1958; Granick 1966; Granick and Sassa 1971).

ALA passes into the cytoplasm where two molecules condense to form the monopyrrole porphobilinogen (PBG) with the removal of two water molecules. This reaction is catalysed by the enzyme ALA dehydratase. A covalent bond is formed between the ε-amino group of a lysine amino acid of the enzyme and the keto group of the ALA molecule. Condensation takes place with a second ALA molecule and following molecular rearrangement PBG is formed. This enzyme requires sulphydryl groups for its activity and its inhibition by chelators such as EDTA suggests that it is a metalloenzyme (Wilson et.al. 1972). The metal co-factor was identified as zinc and the

enzyme requires this element in the proportion of 1 atom of zinc per active site. (Cheh and Neilands 1973; Tsukamoto et.al. 1979).

Under the concerted action of the next three enzymes in the pathway, PBG deaminase uroporphyrinogen 1 synthase and uroporphyrinogen III cosynthase, four molecules of PBG condense to form uroporphyrinogen III, the first tetrapyrrole nucleus of the haem biosynthetic pathway. The complex of the two enzymes is known as porphobilinogenase and in the absence of uroporphyrinogen III cosynthase PBG is converted to the physiologically inactive isomer uroporphyrinogen I. PBG deaminase catalyses the assembly of the four PBG molecules into a linear tetrapyrrole, hydroxymethylbilane, and the cosynthase facilitates the ring closure (Battersby et.al. 1979). In normal cells the activity of the cosynthase exceeds that of the uroporphyrinogen I synthase. Therefore, under normal conditions, only the type III isomer is formed.

Uroporphyrinogen III is converted to coproporphyrin III by the catalytic action of the cytoplasmic enzyme uroporphyrinogen decarboxylase. Four carboxyl carbons, one from each of the four pyrrole rings, are removed and the side chains converted into methyl groups. These decarboxylations occur in a sequential manner with uroporphyrinogen III, starting with the D ring followed by the A, B and finally the C ring (Jackson et.al.1977) and although the type III isomer is the preferred substrate, all four isomers of uroporphyrinogen can be decarboxylated (Kawanishi et.al. 1983; Smith and Francis 1979; 1981).

The conversion of coproporphyrinogen III to protoporphyrinogen is catalysed by a mitochondrial enzyme situated in the intermembrane space, coproporphyrinogen oxidase (Elder and Evans 1978; Yoshinaga and Sano 1980a; 1980b). During this reaction propionic acid side chains on rings A and B are oxidatively decarboxylated to vinyl groups.

The penultimate step in the modification of the tetrapyrrole nucleus is the oxidation of protoporphyrinogen to protoporphyrin, a reaction catalysed by the mitochondrial enzyme protoporphyrinogen oxidase, although this reaction can proceed uncatalysed. This reaction

involves the removal of six hydrogen atoms from the porphyrinogen nucleus (Maines 1984).

Ferrochelatase, the last enzyme in the haem biosynthetic pathway is an inner mitochondrial membrane protein which catalyses the insertion of a reduced Fe^{2+} ion into the centre of the tetrapyrrole ring. The enzyme is bound to the inner mitochondrial membrane and the conversion of Fe^{3+} to Fe^{2+} takes place in the inner membrane in the proximity of ferrochelatase (Barns et.al. 1972). The source of this iron is proposed to be from an innermitochondrial pool which is not associated with cytochromes or iron-sulphur proteins (Tangeras 1980). The activity of the enzyme is inhibited by hemin and is sulphydryl group dependent since the presence of glutathione or dithiothreitol activates the purified enzyme. The action of these SH-compounds on ferrochelatase is not only to protect the SH groups of the enzyme but also to maintain the substrate, iron, in the reduced form (Porra and Jones 1963a; 1963b) and to protect against phospholipid peroxidation of the enzyme and the substrate (Peterson et.al. 1980; Dailey and Fleming 1986).

1.2 NOMENCLATURE OF THE PORPHYRINS.

The porphyrins are tetrapyrroles in which the four rings A,B,C and D are attached through four methene bridges. Uroporphyrin, the first cyclic tetrapyrrole formed during the process of haem biosynthesis, has four acetic acid and four propionate side chains, therefore, four isomers of this compound are possible depending on the arrangement of the chains around the tetrapyrrole nucleus. These are designated types I, II, III and IV. Similarly, coproporphyrin, the second tetrapyrrole to be formed, has four methyl and four propionate side chains, producing four possible isomeric forms. Protoporphyrin has four methyl, two vinyl and two propionate side chains and subsequently has fifteen possible isomeric forms. However, only types I and III of the uroporphyrin and coproporphyrin and type IX isomer of protoporphyrin occur in nature (Maines 1984). Haem is the neutral divalent ferro-protoporphyrin 1X whereas haemin is the positively charged ferri-protoporphyrin 1X. Haemin exists usually as the chloride salt. Both haem and haemin are poorly soluble at

physiological pH. When the alkaline product of haemin, haematin, is titrated with acid it gives rise to the neutral precipitate.

1.3 REGULATION OF HAEM SYNTHESIS.

Evidence indicates that ALA synthase is the rate limiting enzyme of the haem biosynthetic pathway in liver (Granick and Urata 1963); the activity of this enzyme is very low compared to other enzymes in the pathway (Hutton and Gross 1970); the turn over rate is very rapid (Tschudy et.al. 1965; Sassa and Granick 1970; Gayathri et.al. 1973); the administration of ALA results in the induction of the haem degradative enzyme, haem oxygenase (Bissel and Hammaker 1976a); haem, the end-product of the pathway, represses ALAS (Bissel and Hammaker 1976b). PBG deaminase and ferrochelatase, two enzymes with low catalytic activity (Hutton and Gross 1970; Jones and Jones 1969) , may also have minor roles to play in the regulation of the pathway.

Haem synthesis is controlled by the production and rate of activity of ALAS . The production and activity of this enzyme is in turn regulated by the end product of the pathway, haem. Haem exerts a negative-feedback inhibitory action on the enzyme at four levels, activity, transcription, translation and transfer from the cytosol into the mitochondria. The role of haem in the direct inhibition of ALAS activity is thought to be unphysiological. The concentration of haem required to inhibit enzyme activity was above 10^{-5}M (Aoki et.al. 1971; Granick and Kappas 1971) and Scholnick et.al. 1972 showed that the K_i of exogenous haemin was $2x10^{-5}$M. These values are much greater than the concentration required to inhibit the synthesis of the enzyme, $2x10^{-8}$M (Srivastava et.al. 1980). However, Whiting and Elliot in 1972 suggested that due to the close proximity, in the mitochondrial inner membrane, of ferrochelatase and ALAS the concentration of haem may rise sufficiently for it to exert a repressive action on ALAS activity *in vivo.*

Granick in 1966 was the first to suggest that haem inhibits ALAS at the transcriptional level. The differential effects of haem administered *in vivo,* where ALAS synthesis is inhibited and when added *in vitro* to liver homogenate supernatants, where synthesis is

unaffected indicates that this substance inhibits ALAS RNA synthesis (Whiting 1976). The reduction of ALAS by haem follows the same kinetics as that of the mRNA synthesis inhibitor actinomycin D (Srivastava 1980; Yamamoto et.al 1982) and the ability of haemin-treated rat liver to direct ALAS synthesis in a cultured polysome system was significantly reduced when compared to that of control livers (Yamamoto 1982). The half-life of ALAS mRNA is also decreased by haem (Hamilton et.al. 1991).

Haem inhibits ALAS production at the post-transcriptional level. Radioactive exogenous haem is detected on ribosomes but not on nuclear membranes contiguous with a reduction in ALAS activity which Padmanaban et.al. 1973 suggests indicates that haem inhibits the production of the enzyme at the protein synthesis level. Haem reduces the synthesis of ALAS in actinomycin-treated chick hepatocytes in a similar manner to that of the protein synthesis inhibitor cyclohexamide (Sassa and Granick 1970) and haem has a role to play in the inhibition of ALAS peptide chain synthesis (Yamomoto et.al. 1983).

ALAS, like many other mitochondrial proteins, is synthesized on cytosolic ribosomes and translocated into the mitochondrion (Ohashi and Sinohara 1978; Hayashi et.al. 1983). Haemin inhibition of translocation of the cytosolic enzyme into the mitochondria has been reported in rat (Hayashi et.al. 1980) and in chick embryo livers (Hayashi et.al. 1983; Srivastava et.al. 1983). Andrew et.al. (1990) suggest that haem inhibits translocation by binding to the cytosolic ALAS physically inhibiting translocation.

The idea of a regulatory "free" haem pool has been postulated by a number of researchers (Granick et.al 1975; Israels et.al.1975; Muller-Eberhard and Vincent 1985). It is suggested that this free haem pool consists of either recently synthesized haem which has not yet been bound to proteins or haem that has just been released from its binding protein or apoprotein. Utilising the ability of the rate limiting enzyme of the tyrosine degradative pathway, tryptophan pyrrolase, to maintain a dynamic equilibrium with free haem Badaway in 1978 estimated that the free haem pool concentration was in the region of 10^{-7}M, very close to the haem concentration which inhibits

ALAS synthesis (Granick et.al.1975). Kappas et.al. (1989) suggest the possible existence of three different regulatory free haem pools in the mitochondria, the cytosol and the endoplasmic reticulum regulating the production of mitochondrially synthesized cytochromes, ALAS and haem oxygenase synthesis and haem oxygenase activity, respectively.

The inhibitory effects of haem on the overall activity of ALAS have been well documented in hepatic tissue and a similar inhibitory action occurs in the brain (De Matteis and Ray 1982) The regulatory action of this compound in other tissues is not so clear. In contrast with liver, haem increases ALAS activity in leukaemia cells (Hoffman et.al. 1980), stimulates haemoglobin formation in mouse bone marrow cultures (Porter et.al. 1979) and in 19 day old fetal rat liver, which is mostly erythroid tissue at this stage, haem has no effect on ALAS activity (Woods and Murthy 1975). This differential tissue effect of haem on its own synthesis may be indicative of the existence of more than one form of the ALAS enzyme (Bishop 1990).

Most of the haem synthesized is transported out of the mitochondria to the sites of haemoprotein synthesis. The bulk of the haem migrates to the microsomes (Israels et.al.1975). The majority of apoproteins, including mitochondrial apocytochrome c and a proportion of the cytochrome oxidase apoprotein are synthesized on cytoplasmic ribosomes and complex with their haem moiety there or in the cytoplasm. Catalase apoprotein complexes with haem mainly in the peroxisomes (Lazarow and DeDuve 1973a; 1973b).

Granick and Gilder in 1947 classified the haemoproteins into five groups: those which 1. transport oxygen - haemoglobin and myoglobin; 2. transport electrons - the mitochondrial cytochromes; 3. activate oxygen - cytochrome oxidase, tryptophan pyrrolase, cytochrome P-450; 4. activate hydrogen peroxide- peroxidases; 5. decompose hydrogen peroxide - catalase.

Except for haemoglobin, which is present only in erythrocytes and their precursors and myoglobin which is present only in muscle, the other haemoproteins exist in most mammalian cells.

When the biological life of the haem has reached its end it is broken down in a series of enzymatic steps to bilirubin and excreted or enters the enterohepatic system. Haem oxygenase, a microsomal enzyme, catalyses the conversion of haem to biliverdin 1X and CO. Haem oxygenase is the rate limiting enzyme of haem degradation (Tenhunen 1972). Biliverdin is then converted, in mammals, to bilirubin by the enzyme biliverdin reductase.

Haem refers to the complex of a ferrous Fe atom linked to the four nitrogen atoms of the tetrapyrrole nucleus. However, there are several types of haem. The most common are the b type haems which constitute the prosthetic moiety of haemoproteins such as haemoglobin, myoglobin, catalase, peroxidase, the mitochondrial b cytochromes and the microsomal cytochromes, P-450 and b_5. Haem types c and a form the prosthetic moieties of the other mitochondrial cytochromes, c, c_1, a and a_3. The three types of haem differ in the nature of the substituent side chains of the metalloporphyrin. (Lemberg and Barret 1973).

1.4 HAEMOPROTEINS.

1.4.1. Haemoglobin

Haemoglobin is the most abundant haemoprotein in the mammalian body. Berk et.al. (1974) quote figures of around 500-700g of haemoglobin in the circulation of the 70kg. male. In mammals haemoglobin is contained within the erythrocytes. It is the oxygen carrier of blood and has an important role to play in the transport of CO_2 and in the regulation of blood pH. Oxygen molecules bind cooperatively to the haem moieties in the haemoglobin molecule, a process which is also pH and 2,3-bisphosphoglycerate-dependent (Stryer 1988). Oxygen, which is an essential component in the body's energy production by the process of oxidative phosphorylation, is transported to every cell in the body. Once synthesized, haemoglobin remains stable for the life of the erythrocyte, 120 days in man and 60 days in the rat (Tait 1978). Haemoglobin is a haemoprotein made up of four globin chains each attached to a haem prosthetic group. This haemoprotein is produced primarily in the erythroblasts, in the bone marrow, and in circulating reticulocytes, the immediate

precursors of erythrocytes. By the time the reticulocytes have developed into mature erythrocytes they possess their quota of haemoglobin and have lost all of their mitochondria (Keele et.al. 1984). In adults the major form of haemoglobin consists of 2α and 2β globin chains although in 2% of adult haemoglobin δ chains replace the β chains . Fetal haemoglobin, which has a higher affinity for oxygen than the mother's, has ζ chains which are similar to α chains and ε/γ chains which are like β chains. These fetal globin chains are replaced during maturation with the appropriate adult forms. The haem prosthetic groups in these different forms of haemoglobin are, however, always the same (type b) (Maines 1984). Globin production is regulated by haem availability as haem depletion activates an inhibitor of globin polypeptide chain initiation, the hemin-controlled repressor (HCR) (Bruns and London 1965; Freedman et.al. 1974). The iron free precursor Protoporphyrin 1X is incapable of maintaining globin synthesis and therefore haemoglobin production (Zucker and Schulman 1968) and, in turn, haem synthesis is reduced when globin synthesis is inhibited (Grayzel et.al. 1967). Haem deficiency states may be due to iron deficiency (Douglas and Adamson 1975), decreased haem synthesizing enzymes (e.g. Sideroblastic anaemia, Vogler and Mingioli 1968; Bottomley 1990) or chemically-induced with substances like benzene (Forte et.al. 1976). The symptoms of long lasting anaemias, such as pernicious anaemia, include weakness, lassitude, shortness of breath, tingling in the hands and feet and sometimes diarrhoea. In severe cases peripheral neuropathy and demyelination of the nerve tracts in the spinal cord have led to degeneration affecting both afferent and efferent pathways. Severe mental disturbances may also occur (Keele et.al.1984).

1.4.2. Catalase.

Catalase is a haemoprotein found in all mammalian and non-mammalian cells containing a respiratory cytochrome system (Lemberg 1949). The enzyme is a protein which contains four haem groups (DeDuve and Baudhuin 1966). Catalase content is highest in liver and kidney and lowest in connective tissue. In the liver cell it is mainly localised in the peroxisomes (DeDuve and Baudhuin 1966) and mitochondria (Neubert et.al. 1962). The peroxisomes were first observed by Rouiller and Benhard in 1956. These intracellular

organelles contain a variety of enzymes which produce hydrogen peroxide as a product of their function. The peroxisomes contain uricase, D-amino acid oxidase and L-α-OH-acid oxidase in addition to high concentrations of catalase (De Duve and Baudhuin 1966). The mitochondrial respiratory chain also produces hydrogen peroxide during the process of respiration and this is in turn decomposed by catalase (Boveris and Chance 1973). Catalase represents approximately 11-16% of the total peroxisomal protein (Leighton et.al. 1969) and the biological half-life of rat liver catalase is approximately two days (Poole et.al. 1969). Employing radioactive labelling techniques Lazarow and DeDuve (1973a;1973b) studied the synthetic process of rat hepatic catalase. The catalase apoprotein is synthesized outside of the peroxisomes and is translocated into an extraperoxisomal pool which is rapidly taken up into the peroxisomes. The combination with haem takes place inside the peroxisome. Haem is transported from the mitochondria and into the peroxisome where it is attached to a monomeric intermediate which then aggregate to form the tetrameric active form of catalase. The catalytic breakdown of hydrogen peroxide by catalase is initiated by the combination of one molecule of catalase with one molecule of hydrogen peroxide to form COMPLEX I. This complex is formed by the reaction of the H_2O_2 with the iron atom of the haem moiety (Chance 1949). The primary complex then reacts with a second molecule of H_2O_2, a reaction which achieves the catalytic destruction of the H_2O_2.

Reaction 1: $P\text{-}Fe^{3+}\text{-}OH + H_2O_2 = P\text{-}Fe^{3+}\text{-}OOH + H_2O$

Reaction 2: $P\text{-}Fe^{3+}\text{-}OOH + H_2O_2 = P\text{-}Fe^{3+}\text{-}OH + H_2O + O_2$

This second reaction is the catalytic reaction unique to catalase. There are, however, two additional routes available for decomposition of the primary complex: Its spontaneous decomposition and the peroxidative action of hydrogen donors such as alkyl peroxides.

Mature erythrocytes are also rich in catalase which is present in a free form within the blood cell (Aebi et.al. 1968). In the erythrocyte, catalase is only one of the enzyme systems capable of protecting haemoglobin against oxidation to methaemoglobin. In addition to

catalase, glutathione peroxidase and methaemoglobin reductase prevent the accumulation of methaemoglobin either by preventing the oxidation of haemoglobin by peroxide or by reducing the methaemoglobin to haemoglobin as fast as it is formed. Since the mature erythrocyte has no cytochrome system and is metabolically not very active, Nichols (1965) suggests that H_2O_2 within the erythrocyte is low and erythrocyte catalase is there merely to provide intermittent protection in situations where the red blood cell may be exposed to relatively high levels of external tissue peroxides. The contribution of catalase towards the protection of the erythrocyte is therefore thought to be minimal, the main protection being provided by glutathione peroxidase. In support of a minor role for erythrocyte catalase is the observation that people who are acatalaemic, mostly Japanese, pursue a normal life with only occasional appearance of oral gangrene probably due to their inability to beak down bacterially generated hydrogen peroxide. This hydrogen peroxide may in turn oxidise the haemoglobin reaching the lesion causing necrosis in the infected area (Takahara 1968). On the other hand, Boveris and Chance in 1973 suggested that erythrocyte catalase may have a role to play in the decomposition of mitochondrially-produced hydrogen peroxide in tissues that do not contain peroxisomes, such as brain, lung and heart.

1.4.3. Mitochondrial cytochromes.

When the electrons of the energy rich molecules nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$), formed during glycolysis, fatty acid oxidation and the citric acid cycle are donated to molecular oxygen the free energy released is used to generate adenosine-5'-triphosphate (ATP). Oxidative phosphorylation is the process by which ATP is formed as electrons are transfered from these energy rich sources to oxygen in a sequence of redox steps. The electrons are carried by a series of carriers known as the electron transport chain which is driven by the difference in electrode potential between the NADH or $FADH_2$ relative to that of oxygen. As the electrons move down the chain protons are pumped into the intermitochondrial space producing a proton motive force across the inner membrane. When these protons flow back through a protein complex ATP synthase, they drive the synthesis of ATP (Mitchell

1961;1979). The same complex can in fact pump protons using the energy from ATP hydrolysis. The respiratory cytochromes are haemoproteins that undergo oxidation-reduction changes in their haem prosthetic group. Three different types of haem are found in the respiratory chain, cytochromes a, b and c. These cytochromes along with the other components of the respiratory chain (electron transfer chain) are situated on the inner mitochondrial membrane. The main function of this inner membrane is energy transduction. This electron transfer chain has been broken down into four multicomponent complexes designated Complex I (NADH-ubiquinone reductase), Complex II (succinate-ubiquinone reductase), Complex III (ubiquinone cytochrome c oxidoreductase or bc_1 complex) and Complex IV (cytochrome c oxidase or merely cytochrome oxidase) (Van Gelder 1966). The other components of the electron transfer chain are ubiquinone and the soluble cytochrome c. Complex III contains 2 b haems (which do not have identical properties) and 1 c_1 haem and Complex IV contains 2 a haems (a,a_3). The spatial relationship of the electron transfer chain components allows the electrons to pass along the chain down their electrode potential gradient.

The electrons are first passed to a hydrophobic quinone, ubiquinone. The bc1 complex catalyses the transfer of electrons from a reduced ubiquinone to cytochrome c. The c cytochromes, unlike the a and b-types are covalently linked to the protein by thioether bridges. Cytochrome oxidase catalyses the transfer of electrons from reduced cytochrome c to molecular oxygen.

Cytochrome c oxidase (cytochrome oxidase) is regarded as one of the most important enzymes in nature as it participates in the terminal oxidative step in energy metabolism (Capaldi et.al. 1983). Cytochrome oxidase is present in all aerobic organisms. It contains four prosthetic groups two a-type haems (a and a_3) and two copper atoms (Cu_a and Cu_{a3}) (Malmstrom 1979). Van Gelder and Muijsers (1966) estimated that the ratio of cytochrome $a:a_3$ is 1:1. Cytochrome oxidase catalyses a net addition of four electrons to molecular oxygen. Chance et. al. (1975) suggest that the overall equation for this reaction is:

$$a_3^{2+}Cu^{+} + O_2 + 4H^{+} \longrightarrow a_3^{2+}Cu^{+} + 2H_2O$$

the reaction proceeding from a $\longrightarrow$ a_3 $\longrightarrow$ O_2. Although this sequence of events has been disputed over the years, especially in favour of a concerted reaction rather than a sequential reaction (Okunuki 1966), an unequivocal mechanism has not, as yet, emerged.

An interesting feature of the mitochondria is that they possess bacterial-like ribosomes which are capable of maintaining limited protein synthesis (Attardi and Ojala 1971). Utilising chloramphenicol, the bacterial protein synthesis inhibitor, and the cytosolic protein synthesis inhibitor cyclohexamide to differentiate between the site of protein synthesis, it was discovered that cytochrome b and part of the cytochrome oxidase molecule are synthesized on the mitochondrial ribosomes whereas the c cytochromes are transported into the mitochondria from cytoplasmic ribosomes (Schatz and Mason 1974; Tzagoloff et.al. 1979). Apocytochrome c is synthesised on the cytosolic ribosomes and transported to the intermembrane space, via specific outer membrane receptors, where it combines with haem to form functional cytochrome c. Three subunits of cytochrome oxidase are synthesised in the mitochondria and inserted into the membrane while the other components are translocated into the mitochondria from the cytosol. A deficiency in mitochondrial cytochromes is one of the factors contributing to a group of disorders, the encephalomyopathies, where patients manifest neuropathic symptoms.

1.4.4. CYTOCHROME P-450

The microsomal metabolising enzymes cytochrome P-450 catalyse the oxidation of a large number of compounds including endogenous steroids, cholesterol and fatty acids in addition to a wide range of xenobiotics such as morphine, polycyclic hydrocarbons, insecticides and barbiturates. 32% of rat hepatic haem is in the form of cytochrome P-450 (Meyer and Marver 1971).

1.4.5. OTHER HAEMOPROTEINS

Haem is also an essential component in tryptophan pyrrolase the degradative enzyme of tryptophan, and a necessary cofactor in prostaglandin oxidation by endoperoxide synthase, the oxidation of

indoleamine as indoleamine 2,3-dioxygenase and in the production of cyclic GMP as guanylate cyclase.

1.5. THE PORPHYRIAS.

The porphyrias are a group of inherited and acquired disorders of haem biosynthesis due to defects in the synthetic enzymes (figure 2). Porphyrins and their precursors are overproduced in all forms of the disease although each type manifests it own characteristic pattern of precursor accumulation. The first reported case of what is now thought to have been porphyria was that recorded by Stockvis just over a century ago (1889) who examined an elderly lady who passed a dark red urine which was found to contain a chemical which Stockvis named haematoporphyrin.

1.6 CLASSIFICATION OF THE PORPHYRIAS.

The diseases of porphyrin metabolism were first classified by Hans Gunther in 1911 and 1922. He observed 14 cases where acute symptoms arose spontaneously, which he termed "haematoporphyria acuta" . 56 cases he noted were associated with the ingestion of the sedative Sulphonal, Trional or Veronal and these he called " haematoporphyria acuta toxica". In addition, Gunther defined and named a condition where the predominating symtoms were due to skin photosensitivity and this he called "haematoporphyria congenita". Gunther's classification included a group "haematoporphyria chronica", which although showing a similarity to "haematoporphria congenita" the skin photosensitivity symptom did not occur until later in life. Gunther observed that the symptoms of what he called congenital porphyria persisted throughout the life of the patient and Garrod in 1923 credits Gunther with the first recognition that the disease was due to an inborn error of metabolism. Gunther noted that acute haematoporphyria may also be inherited and observed that patients liable to develop either acute or congenital haematoporphyria posses certain physical and mental characteristics, such as dark hair, pigmented skin, insomnia and neurosis. In the study of the clinical features of acute haematoporphyria Gunther observed that a cluster of symptoms were

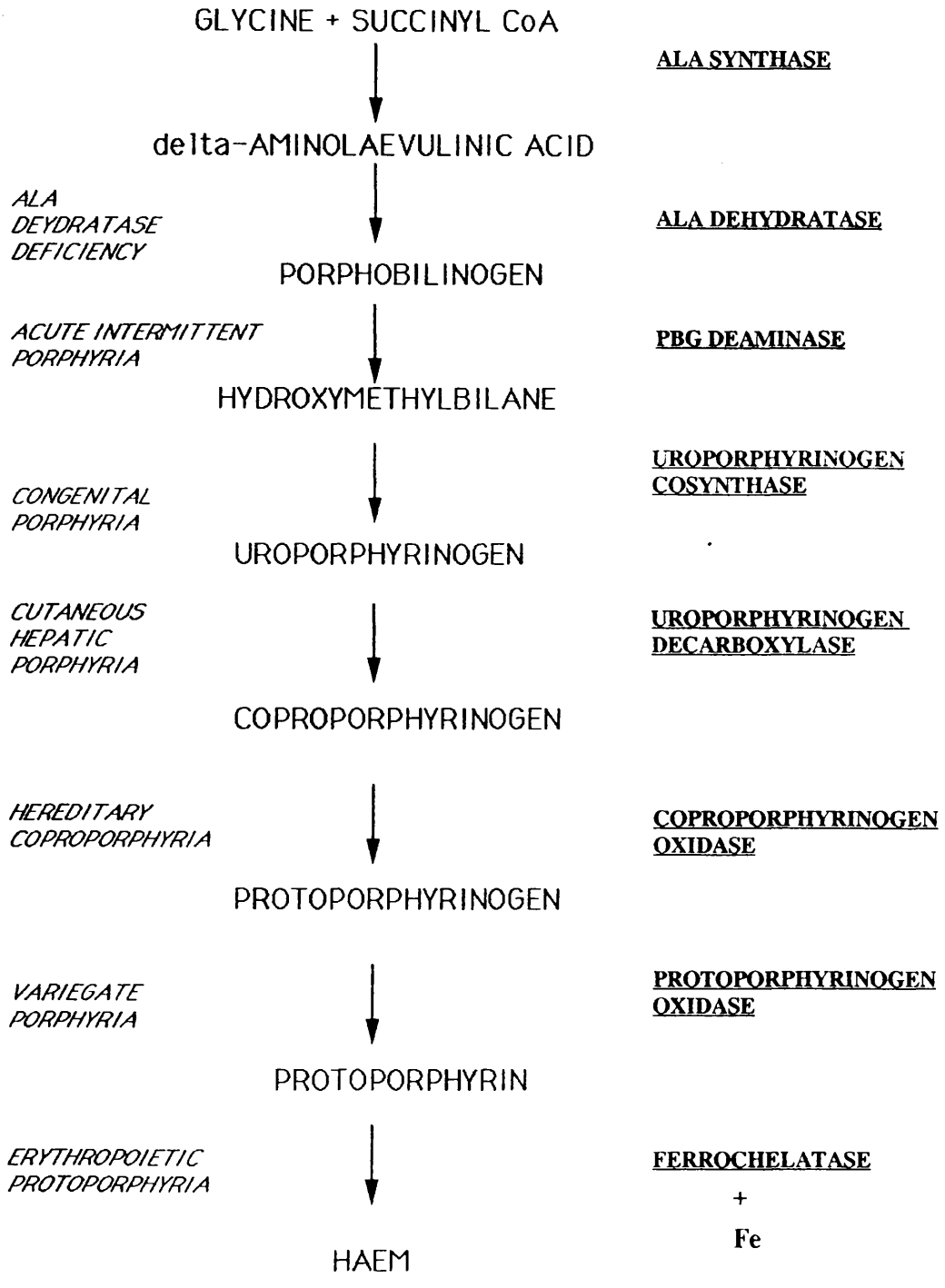

Figure 2: The haem biosynthetic pathway and the level of enzymatic defect of the porphyrias.

commonly exhibited, namely, abdominal pain, constipation and vomiting.

Following this initial classification of the porphyrias several groups have redefined the various types of the disease as knowledge regarding them accrued. Waldenstrom (1937) classified the porphyrias into three main groups; "porphyria congenita" which was equivalent to Gunther's 1911 "haematoporphyria congenita"; "porphyria cutanea tarda" which encompassed Gunther's chronic haematoporphyria patients and who manifest acute attacks of abdominal pain. This group he later revised to include both symptomatic ("porphyria cutanea tarda symptomatica"), environmentally-induced by circumstances such as alcoholic cirrosis and hepatoma, and hereditary ("porphyria cutanea tarda hereditaria") forms of the disease (Waldensrom 1957). Waldenstrom's third group "porphyria acuta" he subdivided into abdominal, nervous, latent and classical forms.

Further classifications includes Schmid et.al.'s 1954 classification based on liver and bone marrow porphyrin content. These he named "porphyria hepatica" and "porphyria erythropoietica". These two major groups were further subdivided. The hepatic form into an intermittent acute, exhibiting abdominal and or nervous symptoms, a cutanea tarda type where skin photosensitisation occurred later in life and a mixed type which included patients who manifest symptoms which fell into both of the previous catagories.

Goldberg and Rimington (1962) extended Schmid et.al.'s 1954 classification to include drug induced porphyrias in addition to congenital erythropoeitic porphyria, showing skin photosensitisation, acute intermittent porphyria, with neurovisceral symptoms but no skin photosensitisation, and finally a cutaneous hepatic type which they subdivided into hereditary and acquired forms.

The gradual elucidation of the haem biosynthetic pathway and a more comprehensive examination of the widely variable symptoms of the porphyrias has led to a re-evaluation of the disorders and in addition to the tissue type classification (hepatic and erythropoetic) the porphyrias may be broadly divided into the non-acute and the acute

porphyrias. The non-acute porphyrias are characterised by skin photosensitisation brought about by the action of light on the overproduced porphyrins which accumulate in the skin, while the acute porphyrias possess symptoms indicative of an underlying central, autonomic and somatic neuropathy.

There is a type of porphyria associated with each enzyme in the haem biosynthetic pathway except at the level of the rate limiting enzyme, ALAS (figure 2). The nonacute porphyrias are due to enzymatic defects at the levels of uroporphyrinogen decarboxylase, uroporphyrinogen cosynthase and ferrochelatase. The acute porphyrias are acute intermittent porphyria (AIP), variegate porphyria, hereditary ALA dehydratase deficiency and hereditary coproporphyria.

The most common form of acute porphyria is the acute intermittent type (Goldberg et.al. 1987) and is due to a deficiency in the enzyme PBG deaminase of about 50% (Strand et.al. 1970; Meyer et.al. 1972).

1.7 NEUROPATHY OF ACUTE INTERMITTENT PORPHYRIA.

The existance of a central neuropathy in AIP is indicated by both behavioural and histological evidence. Behavioural changes have been recorded in the majority of AIP cases. These include a study of 25 patients by Ridley (1969) where 22 manifest psychiatric symptoms which included insomnia, confusion, hallucinations, delusions, depression and emotional disturbances. Baker and Watson (1945) described a patient as irritable and listless. Waldenstrom (1957) and Goldberg (1959) in studies of 321 and 50 cases of AIP respectively reported psychiatric symptoms in 55% and 58% of the patients studied and more recently Gorchein and Webber (1987) observed neuropsychiatric disturbances in an AIP patient. Post mortem histological examination of fatal AIP cases provides supportive structural evidence for the existence of a central neuropathy. Baker and Watson (1945) found evidence of cerebral lesions, especially in the cranial nerves, the facial, hypoglossal and the dorsal nucleus of the vagus being most affected. The involvement of the latter is most likely the underlying cause of death in AIP due to respiratory insufficiency. Although there was no observed change in the cerebral grey matter Baker and Watson noted perivascular demyelination of

the cerebral white matter. On a neuropathological study of five fatal cases of AIP Gibson and Goldberg in 1956 similarly found small foci of demyelination in the cerebral white matter in addition to chromatolysis in cortical and basal nuclei cells. Histological evidence of foci of perivascular demyelination was also found on autopsy of an AIP case by Stozel et.al. in 1987. Neuronal loss, gliosis and vacuolisation have also been reported in the supraoptic and paraventricular nuclei of the hypothalamus (Stein et.al. 1972; Perlroth et.al. 1966; Tschudy et.al. 1975). EEG abnormalities have been observed in AIP patients (Ridley 1969). Central neuropathy is therefore a hallmark of AIP. By the same token, the incidence of AIP is significantly higher in the psychiatric population than in the general population (Kaebling et.al. 1961; Tishler et.al. 1985; Goldberg et.al. 1987).

The symptoms of AIP also suggest the existence of an underlying autonomic neuropathy. Abdominal pain, either local or general, is the most common complaint of AIP sufferers (Berlin and Cotton 1950; Waldenstrom 1957; Goldberg 1959; Baker and Watson 1945; Goldberg and Rimington 1962; Stein and Tschudy 1970; Stolzel et.al. 1987). Other gastrointestinal symptoms include nausea, vomiting, constipation, diarrhoea and abdominal distension. The existence of an autonomic cardioneuropathy is manifest by the frequency of symptoms such as tachycardia and hypertension (Ridley et.al. 1968; Baker and Watson 1945; Stein and Tschudy 1970; Allen and Rees 1980; Yeung Laiwah et.al.1985). In a series of objective autonomic cardiovascular function tests on AIP patients both in attack and in remission parasympathetic cardiovascular reflexes (valsalva manoeuvre, heart rate response on standing and heart rate variation during deep breathing) were all abnormal as was the sympathetically-mediated blood pressure response to sustained hand grip (Yeung Laiwah et. al. 1985). Although in several cases some of the cardiovascular abnormalities persisted in remission most were reversible analagous with that of the subjective abdominal and muscular pain. The universality of the autononic neuropathy is implied by other accompanying features of AIP attacks such as excessive sweating and urinary retention problems (Goldberg and Rimington 1962; Stein and Tschudy 1970).

Motor neuropathy is extremely common in AIP and both functional and histopathological evidence exists. Muscle weakness and cramp-like pains have been reported in the majority of AIP cases (Goldberg 1959; Ridley 1969; Stein and Tschudy 1970; Anzil and Dozic 1978; Gorchein and Webber 1987). This weakness although starting in the legs frequently involves all four limbs and may occur symmetrically, asymmetrically or focally (Baker and Watson 1945; Ridley 1969; Anzil and Dozic 1978; Poser and Edwards 1978). Histological examination of peripheral nerves from AIP patients have confirmed the existence of pathological conditions including oedema, demyelination, thinned and irregular axons, axonal vacuolisation and chromatolysis in the anterior horn cells (Denny-Brown and Sciarra 1945; Gibson and Goldberg 1956; Baker and Watson 1945; Ridley 1969). Cranial nerves V11, X11 and X show signs of degeneration (Baker and Watson 1945). Defective vision observed in some patients may be the result of optic nerve neuropathy (Ridley 1969).

Sensory neuropathy is frequently found in AIP patients (Goldberg 1959; Baker and Watson 1945; Sorensen and With 1971) and may be exhibited as sensory loss, paresthesia and numbness.

The cause of the porphyrias are known. They are due to an enzymatic defect in the biosynthesis of haem. However, the underlying causes of the neurological dysfunction so widely evident in the acute porphyrias remains obscure. The link between the known defect in haem synthesis and the observed neurogenic disorder continues to be a subject of controversy. A number of hypotheses have been suggested as the correlate between the biochemical disorder and the clinical symptoms of the acute porphyrias. These hypotheses include a depletion of pyridoxal phosphate (Cavanagh 1967; Cavanagh and Ridley 1967); depletion of glycine (Piper et al. 1973), depletion of zinc (Peters et.al. 1974) or the accumulation of abnormal porphyrin products such as porphobilin (Feldman et.al. 1971). The two most compelling theories regarding the aetiology of acute porphyric neuropathy is that either the haem precursors ALA and /or PBG, which accumulate during acute porphyric attacks are neurotoxic or that a deficiency in the end product of the pathway, haem, in neural tissue

results in a reduction of essential haemoproteins. The latter two hypotheses will be dealt with in more detail.

1.8 PHARMACOLOGY OF HAEM PRECURSORS.

ALA and PBG are over produced in all types of acute porphyria except hereditary ALA dehydratase deficiency and these precursors can be detected in the blood, urine and CSF (Moore et. al. 1979; Gorchein and Webber 1987). In hereditary ALA dehydratase deficiency and hereditary tyrosinaemia, where the enzymatic inhibition by the abnormal metabolite succinylacetone occurs at the level of ALAD, only ALA is over produced (Lindblad et.al 1977; Bird et.al. 1979; Doss et. al 1979). Yeung Laiwah and his colleagues in 1987 in a critical overview of the pathogenesis of acute porphyria suggest that acute porphyric neuropathy may be the result of neurotoxicity due to an accumulation of the porphyrin precursors ALA and or PBG. The cornerstone of this hypothesis is the observation that the onset of the symptoms of acute porphyria is nearly always accompanied by an increased excretion of ALA and PBG either together or alone (Becker and Kramer 1977). However, some patients have elevated ALA and PBG during remission and the excretion of these precursors do not correlate well with the clinical severity of the disease (Ackner et.al. 1961; Gorchein and Webber 1987).

These haem intermediates may accumulate as a result of disordered haem biosynthesis either within the neural tissue itself or by the nervous systems uptake of extraneuronally produced precursors. Brain uptake of ALA occurrs in rodents (Becker et.al. 1974; McGillion et.al.1974) and in chick cultured cerebral hemisphere and glial cells (Percy et.al. 1981). Both ALA and PBG have been found in the cerebrospinal fluid of AIP patients during periods of crises (Sweeney et.al. 1970; Percy and Shanley 1977; Gorchein and Webber 1987). The concept of a pharmacological role for the porphyrin precursors ALA and PBG has received much attention over the years. PBG possesses pharmacological effects *in vitro* , inhibiting the K^+- stimulated release of Ach from the rat phrenic nerve diaphragm preparation

(Feldman et.al. 1971). A neurotoxic role for PBG does not seem likely, however, on the basis that hereditary ALAD deficiency and hereditary tyrosinaemia patients manifest a syndrome congruent with the acute porphyrias yet PBG is not an overproduced precursor in these conditions. Shanley et.al.(1975) reported only transient excitatory behaviour following central administration of PBG and Goldberg et.al. (1954) found no evidence of pharmacological activity from PBG when applied *in vitro* to isolated tissues or following intravenous administration to rabbits and cats. Controversy still exists, however, over the putative role of ALA in the development of porphyric neuropathy. There exists a considerable body of evidence indicating that ALA may be neurotoxic. Changes in the activity of mice occurred following administration of ALA systemically (McGillion et.al.1973; Cutler et.al. 1979) while central administration produced transient activity alterations (Shanley et.al. 1975; Pierach and Edwards 1978). However, ALA failed to produce any porphyric-like symptoms when administered orally to humans (Berlin et.al. 1956; Meyer et.al. 1972) or intraperitoneally to rats and cockerels (Berlin et.al. 1956) *In vitro* studies on the effects of ALA are numerous including the inhibitory effect on cray fish stretch receptor neuones, (Ditcher et.al. 1977); decreased motor nerve conduction velocity (Sima et.al.1981); reduced muscle resting membrane potential (Becker et.al. 1975); postsynaptic inhibition of frog gastrocnemius muscle (Cutler et.al. 1978); a decrease in Na^+/K^+ and Mg^{2+} ATPase activity in cultured chick and rat brain neuronal membranes (Russel et.al.1983); a reduction in erythrocyte and brain ATPase activity (Becker et.al. 1971); stabilisation of nerve and muscle excitable membranes (Feldman 1968); a decrease in ventral root potentials in the hamster evoked by dorsal root stimulation (Jordan et.al. 1990). However, in a large proportion of these studies large concentrations of ALA in the mMolar range were required to elicit effects. Cutler and colleagues in a series of experiments on rat and rabbit intestinal preparations have implicated ALA as the possible mediator of acute porphyric neuropathy. The *in vitro* effects of ALA appear to be species specific. In rabbit gut preparations, ALA at high doses of 0.2mM (Cutler et.al.1982), 3-4.5mM (Cutler et.al.1990) and 3-6mM (Cutler and Arrol 1987) exert an inhibitory action on the tone and the contraction amplitude of the inherent rythmic activity, with a subsequent

prostaglandin-mediated rebound contracture. These inhibitory responses are attenuated by prazosin while unaffected by yohimbine and propranolol suggesting that ALA effects are mediated via α-receptors (Cutler et.al. 1985). The concentrations of ALA required, however, to elicit pharmacological events in rabbit intestine are much greater than those which would exist during acute porphyric attacks (9-12μM Gorchein and Webber 1987). Similar large concentrations of ALA (3-6mM, Cutler and Arrol 1987) were required to initiate an inhibition of contraction amplitude in human taeni coli. Rat small intestine appears to be more sensitive to the effects of ALA. At low doses (10nM-50nM Cutler et.al.1991) ALA augments the contractile responses of the gut, an augmentation which is enhanced by the $GABA_A$ receptor antagonist, bicuculine, while the action of 0.3x10μM ALA and above was reduced by bicuculine. The authors suggest that the effects of ALA at concentrations which could exist during acute porphyric attacks may be due to their action on GABA autoreceptors, a suggestion also proposed by Brennan and Cantrill (1979) to account for ALA's inhibition of GABA release from rat synaptosomal preparations. Therefore, disparity in the literature concerning a pharmacological role for ALA in the aetiology of acute porphyric neuropathy may be due to ALA's widely variable differential species and tissue action.

1.9. HAEM DEFICIENCY IN ACUTE PORPHYRIA.

The concept of a haem deficient state underlying the neuropathy of acute porphyria has been proposed by Shanley et.al. in 1977 and Yeung Laiwah et.al. in 1987.

The liver and erythropoietic tissues are the major sites of haem synthesis (Berk et.al..1974). The total liver content of haem in rats has been estimated at 70nmoles/g wet weight of which 43% is present in the mitochondrial cytochromes, 32% in cytochrome P-450, 17% in cytochrome b5 and 7% in hepatic catalase (Meyer and Marver 1971; Nichols and Elliot 1974). However, haem synthesizing enzymes are present in other tissues including rat brain, heart, adrenal gland and testes and the mouse harderian gland (Percy and Shanley 1979;

De Matteis et.al. 1981a; Briggs et.al. 1976; Condie et.al. 1976; Tofilon and Piper 1980; Margolis 1971). A state of haem deficiency in neural tissue could lead to the development of a neuropathy. De Matteis and his colleagues in 1981a and 1982 demonstrated that rat brain synthesizes its own haem and the pathway is regulated by end-product inhibition by haem on the regulatory enzyme of the pathway ALAS, although the activity of this enzyme was much lower in the brain than liver (Maines 1980; De Matteis et.al. 1981a) The brain possesses mitochondrial cytochromes (Chepelinsky and Arnaiz 1970; Bull et.al. 1979) and cytochrome P-450 mediated function (Cohn et.al. 1977; Nabeshima et.al. 1981). The enzymatic defect in PBG deaminase of AIP patients has been identified in the liver (Miyagi et.al.1971), erythrocytes (Strand et.al. 1972), lymphocytes (Sassa et.al.1978), fibroblasts and amniotic cells (Sassa et.al.1975). There is no evidence to date, however, that the same enzymatic defect is present in neural tissue of such patients although it would not be unreasonable to assume that such a defect does exist. Assuming that PBG deaminase activity is depressed in the neurones of AIP sufferers, an additional precipitating factor could compromise the synthesis of haem to the extent that a reduction in neuronal haemoproteins is consequential to a reduction in the free haem pool. The existence of a regulatory free haem pool in rat brain has been supported by evidence from DeMatteis and Ray (1982) who demonstrated that intraventricullary-administered hematin inhibited the rise in ALAS activity caused by succinylacetone. Impaired hepatic haemoproteins have been observed in patients with AIP, particularly in the functioning of the haem containing metabolising enzymes cytochrome P-450. Salicylamide and antipyrine metabolism is reduced in some AIP patients (Song et.al.1974; Anderson et.al.1976). Evidence of depression in the activity of the mitochondrial haemoprotein cytochrome oxidase has also been observed (Goldberg et.al.1985). A deficiency in neural mitochondrial cytochromes could well lead to the development of a clinical neuropathy (Labbe 1967).

The neurone is dependent on mitochondrially produced energy for functions such as ion pumping, repair, transmitter synthesis and axonal transport. The latter is dependent on adequate oxidative phosphorylation which in turn is reliant upon normally functioning

mitochondria (Ochs and Hollingsworth 1971). Jakobsen et.al. 1986 suggest that porphyric neuropathy may be classified as one of a group of diseases caused by a deficiency in axonal transport due to an insufficiency in the respiratory cytochromes. A deficiency of mitochondrial cytochromes has a role to play in the clinical symptoms of the mitochondrial encephalomyopathies. The term "encephalomyopathy" was used to describe a multisystem disease associated with "structurally and/or functionally abnormal mitochondria in the brain and/or muscle" the clinical symptoms of which include muscle weekness, dementia, ataxia, seizures and a sensory neuropathy (Shapira et.al. 1977; Petty et.al. 1986). Deficiencies in mitochondrial cytochrome b (Spiro et.al. 1970; Morgan-Hughes et.al. 1982) and cytochrome oxidase (Willems et.al. 1977; Petty et.al. 1986) were seen in patients diagnosed as possessing this group of diseases. The clinical manifestations of AIP bear a close similarity to some of the symptoms of the diseases which are classified under the rubric of mitochondrial encephalomyopathies and a deficit in mitochondrial cytochrome oxidase has been identified in muscle tissue of patients with confirmed AIP even during remission (Goldberg et.al. 1985). A malfunction in energy metabolism in the neurones would account for both the acute symptoms of the attacks and of the longer lasting structural changes.

The successful therapeutic use of exogenous haem in the form of haematin provides support for the thesis that porphyric neuropathy is a consequence of a haem deficient environment. Haemin can functionally reconstitute apocytochrome P-450 from rat liver homogenates and hepatic tryptophan pyrrolase (Farrell and Correia 1980; Bornheim et.al. 1985; Badaway and Evans 1975). Haematin has been employed therapeutically by several groups and although the treatment outcome is variable biochemical improvement is seen in most cases and clinical improvement results in almost 50% of cases (McColl et al. 1981). Haematin treatment, however, is not without adverse side effects, particularly phlebitis, (McColl et. al. 1981; Pierach 1982; Tokola 1988) prolonged blood clotting times and platelet aggregation (Glueck et.al. 1983). Goetsch and Bissel in 1986 observed that haematin is unstable over a 24hr. period, this

breakdown showing marked temperature dependency. Additionally, the metabolites of the haemin have no therapeutic value and it is these metabolites and not the haematin itself which are causing the coagulation effects. These problems were alleviated by the development of a stable form of haematin, haem arginate (Tenhunen et.al. 1987). This compound is the reaction product of haemin and L-arginine in a mixture of propylene glycol, ethanol and water. Haem arginate proves just as therapeutically successful as freshly prepared haematin in reducing both haem precursor levels and clinical symptoms but is without the side effects of haematin (Tokola 1988; Tokola et. al. 1986; Fontaine et.al. 1987; Mustajoki et.al. 1986;1989). Haem arginate improves the abnormal antipyrine clearance in porphyric patients indicating that exogenous haem can functionally restore cytochrome P-450 metabolising capacity (Tokola et. al 1988). The therapeutic value of haem arginate may, therefore, be due to its ability to reconstitue haem deficient haemoproteins. However, this compound could be exerting its effect by virtue of its ability to reduce porphyrin precursor levels which may be neurotoxic. Nevertheless, these two hypotheses concerning the aetiology of acute porphyric neuropathy may not be mutually exclusive and both precursor toxicity and haem deficiency together may constitute the link between the biochemical, clinical and neurological phenomena associated with acute porphyria.

1.10. HAEM SYNTHESIS BLOCKING AGENTS.

It has been recognised for over 100 years that some chemical agents have the ability to disrupt haem metabolism. Figure 3 shows the haem biosynthetic pathway and the level at which some chemicals can interfere with haem synthesis.

Stockvis (1889) recorded the first case of acute porphyria in an elderly lady who passed dark red urine, containing hematoporphyrin, following Sulphonal treatment. Since this report there has been a plethora of compounds which have been attributed with porphyrinogenic properties. Sedormid (allylisopropylacetyl urea) produced a condition in both rabbits and rats which in many ways

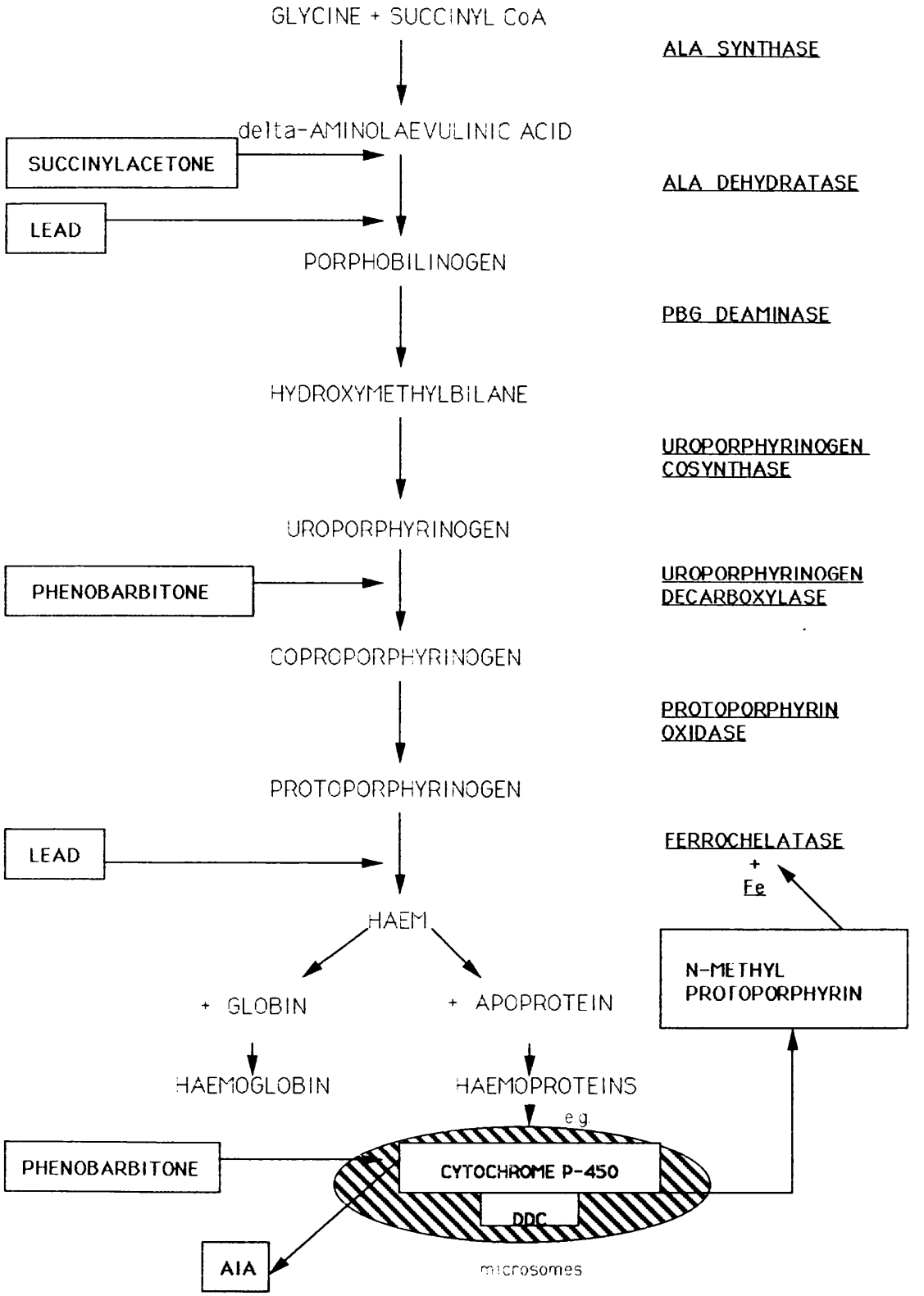

Figure 3: The haem biosynthetic pathway and the level at which porphyrinogenic agents disturb the pathway.

resembled the biochemical profile of acute intermittent porphyria (Schmid and Schwartz 1952). An analogue of sedormid, allylisopropylacetamide (AIA) induced a similar biochemical porphyrinogenesis in rabbits (Goldberg 1954a). An experimental porphyria was observed in rabbits on administration of the substituted dihydropyridine, 3,5-diethoxycarbonyl 1,4-dihydrocollidine (DDC) (Soloman and Figge 1959). The cause of an outbreak of cutaneous porphyria in Turkey between 1955 and 1959 was identified as being due to the contamination of seed grain with the fungicide hexochlorobenzene, an epidemic that affected over 3,000 people with a mortality rate of 10% (Cam 1963). The importance of barbiturates in relation to human acute porphyria was suspected fairly soon after the introduction of these drugs into clinical therapy. Dobrschansky in 1906 described a typical case of acute porphyria in a patient receiving treatment with 5,-5-diethyl barbituric acid. The toxic effects of lead were first recorded in the 4th. century B.C. by Hippocrates who described an abnormal colic in a lead worker, and the Roman's recognition of lead toxicity prompted the use of primitive protective measures (see Hunter 1962). Succinylacetone (4-6 dioxoheptanoic acid) an abnormal metabolite produced in patients with hereditary tyrosinaemia exerts an enzymatic block on the haem pathway at the level of ALAD.

1.10.1. 3,5-DIETHOXYCARBONYL 1-4, DIHYDRO 2,4,6-TRIMETHYL COLLIDINE (DDC)

The substituted dihydrocollidine, 3,5-diethoxycarbonyl 1-4, dihydro 2,4,6 trimethyl collidine (DDC) lowers ferrochelatase activity in rodents (Onisawa and Labbe 1963; Tephly et. al. 1979; De Matteis et.al. 1973), in 17 day old chick liver cells (Rifkind 1979) and in chick embryo hepatocytes (Cole et.al. 1981). Analogues of DDC, differing in their 4-alkyl substituents, possess differential inhibitory activity. Both the 4-methyl and its 4-ethyl analogue produce a marked inhibitory action on the hepatic chelatase enzyme whereas the 4-desmethyl analogue is completely inactive (Cole et.al.

1980). In a more comprehensive examination of the porphyrinogenic action of DDC, and its analogues, in their ability to block ferrochelatase activity in cultured chick embryo hepatic cells Marks et. al. (1987) demonstrated that the 4-methyl, 4-ethyl and 4-propyl analogues were the most potent inhibitors, an action which decreased as the length of the 4-alkyl chain increased reaching total inactivity with the isobutyl analogue.

DDC does not directly inhibit ferrochelatase. It has a profound inhibitory action on the enzyme when administered *in vivo* to rats and mice but lacks this action when added to a homogenate or to an enzyme preparation (Onisawa and Labbe 1963) . The authors suggested that *in vivo* the DDC may be converted to a totally different compound, which in turn is inhibitory for ferrochelatase. In support of this suggestion is the observation that 2-diethylaminoethyl 3,3-diphenylpropylacetate (SKF 525-A), an inhibitor of the drug metabolising enzymes, cytochrome P-450 (Tephly et.al. 1980) prevents the decrease in ferrochelatase activity (De Matteis et. al. 1973). Further evidence implicating cytochrome P-450 in the activation of DDC into a ferrochelatase inhibitor comes from the observation that DDC was not very effective at inhibiting the enzyme in newborn animals where hepatic cytochrome P-450 is low (see De Matteis et.al.1987) Additionally, DDC causes a rapid loss of hepatic cytochrome P-450 (Wada et. al.1968).

Evidence, therefore, indicates that DDC decreases ferrochelatase activity *in vivo* after being metabolised to an active compound with inhibitory capabilities. Cytochrome P-450 is a mediator in this blocking of ferrochelatase as the quantity and activity of this enzyme declines at the same time as the occurrence of ferrochelatase inactivity and inhibitors of cytochrome P-450 protect the ferrochelatase from inhibition.

A porphyrin was extracted from the livers of mice, treated with DDC, which could inhibit ferrochelatase activity (Tephly et. al.1979). De Matteis et. al. (1980a; 1980b) demonstrated that this porphyrin, a green pigment, extracted from murine livers produced a ferrochelatase enzyme block which once initiated could not be reversed by the addition of substrate and concluded that the

inhibition was irreversible. Following DDC administration, a green pigment was extracted from rat liver by Ortiz de Montellano et. al. (1981a) and was fractionated into four components by high pressure liquid chromatography. Ortiz de Montellano et. al. (1981a; 1981b) identified these fractions as the four regioisomers of N-methyl protoporphyrin 1X all of which possessed hepatic ferrochelatase inhibitory activity in the rat. Utilising the discovery by Loev and Snader (1965) that during the oxidative process leading to aromatisation, a number of dihydropyridines could loose their 4-alkyl group in a reactive form and transfer this to a suitable nucleophile, De Matteis et.al. (1987) suggested that this could explain the inability of DDC's oxidised analogue 3,5 diethoxycarbonyl collidine to promote the formation of N-methyl protoporphyrin *in vivo*, explaining the drugs failure to block ferrochelatase. Further studies on N-alkylated protoporphyrin, either isolated from livers from animals treated with DDC (McClusky et.al. 1986) or with chemically synthesised porphyrins (De Matteis et.al. 1980b) confirmed these compounds to be potent inhibitors of ferrochelatase.

The 4-ethyl DDC compound has received considerable attention regarding its ability to inhibit the ferrochelatase enzyme. As with DDC this analogue has been shown to cause the formation of a green pigment in the livers of mice and the chemical isolated from this tissue was found to be 4-ethyl protoporphyrin 1X (De Matteis et. al. 1981b). Augusto (1982) established that the 4-ethyl group is lost as a radical as a result of the compound's oxidation by cytochrome P-450. The oxidation of the nitrogen in 4-ethyl DDC by cytochrome P-450 proceeds in a one-electron step releasing the ethyl radical into the cytosol where it can react with any of the nitrogen atoms on the tetrapyrrole moiety of the cytochrome P-450. This reaction causes the ring on which the alkyl group attaches itself to be pulled out of the plane of symmetry of the haem moiety (De Matteis et. al. 1982) resulting in the release of the alkylated protoporphyrin from the cytochrome P-450 apoprotein. In contrast to the situation with the products of DDC metabolism, where all the regioisomers have a similar degree of inhibitory action, the four regioisomers of 4-ethyl protoporphyrin 1X products are not equally potent in their ability to block ferrochelatase, the A and B regioisomers possessing a 300

times greater inhibitory action than the C and D isomer. (Ortiz de Montellano et.al. 1980a; Ortiz de Montellano et. al. 1981c; De Matteis et. al. 1983). The various cytochrome P-450 isoenzymes can alter the proportion of regioisomers formed from 4-ethyl DDC, Phenobarbital-inducable isoenzymes increasing the proportion of the A ring isomer at the expense of the C and D ring isomers (De Matteis et. al. 1983).

Once these alkylated porphyrins are formed it is assumed that the porphyrin is taken up into the mitochondria where it binds to the ferrochelatase enzyme. N-methyl protoporphyrin is a high affinity, tight-binding inhibitor of the enzyme (Dailey and Fleming 1983).

DDC and its analogues, therefore, exert a dual assault on the haem biosynthetic pathway. Firstly, they destroy cytochrome P-450, a haem containing protein, creating a demand for increased haem production to replace this enzymatic moiety and secondly the pathway is compromised by the N-alkyl protoporphyrin metabolite's ability to inhibit the activity of the last enzyme in the haem biosynthetic pathway. Mackie and Marks (1989) argue that both cytochrome P-450 destruction and ferrochelatase inactivation have a role to play in the disruption of the haem pathway, demonstrating that N-ethyl DDC and 4-isobutyl DDC, an analogue lacking ferrochelatase inhibitory effects, cause a synergistic induction of ALAS.

1.10.2. ALLYLISOPROPYLACETAMIDE.

Following the first observation by Goldberg (1954b) that the barbiturates containing allyl groups were the most potent in producing a rise in rabbit urinary porphyrins, it was found that the allyl containing, non-hypnotic analogue of sedormid, allylisopropylacetamide also produced a biochemical porphyrinuria (Goldberg 1954a). An abnormal green pigment accumulated in rabbit livers following treatment with AIA (Schwartz and Ikeda 1955) and it was subsequently determined that the AIA molecule contributed in some way to the composition of this green pigment as the pigment

acquires the ^{14}C labelling in animals treated with ^{14}C-labelled AIA (Ortiz de Montellano et. al. 1978). As is the case with DDC cytochrome P-450 is a vital component in the formation of the pigment in response to AIA administration. An increase in hepatic ALA production was found in AIA treated mice concommitant with a reduction in hepatic haem, particularly in the microsomal fraction (Wada et.al.1968), a finding confirmed by De Matteis (1971). The destruction of cytochrome P-450 by AIA requires oxygen and is dependent on the presence of NADPH, the cofactor essential for the metabolism of drugs by the liver microsomal fraction, a process which is inhibited *in vivo* by the cytochrome P-450 inhibitor SKF-525A (Ortiz de Montallano et. al., 1979; De Matteis, 1971; Ortiz de Montellano and Mico, 1981). The latter authors suggest that the inactivation of cytochrome P-450 by AIA is a "suicidal" or "mechanism-based" process.

Phenobarbitone-inducible isoenzymes appear to be the main target for AIA destruction (Waxman and Walsh 1982). In contrast to the phenobarbital-inducible cytochrome P-450 subspecies attacked by AIA the 3-methyl cholanthrene-inducible subspecies is relatively resistant to destruction by AIA. This inactivation of cytochrome P-450 by AIA involves prosthetic haem alkylation with the production of an N-alkylated protoporphyrin 1X, the green pigment observed in treated liver tissue and identified by NMR spectroscopy (Ortiz de Montellano et. al. 1978;1979). The formation of a drug-porphyrin adduct, as a result of cytochrome P-450 metabolism, strips the haemoprotein of its prosthetic haem moeity , in a similar manner to that of DDC metabolism, leaving the apoprotein intact (Ortiz de Montellano and Mico 1981; Ortiz de Montellano et. al. 1983) The cytochrome P-450 apoprotein left after the isoenzymes destruction by AIA remains intact as Farrell and Correia (1980) and Bornheim et. al. (1985) demonstrated that the cytochrome P-450 apoprotein can be functionally reconstituted by exogenous haem.

This production of a green pigment in the livers of animals treated with AIA is, therefore, very similar to the mechanism of pigment production resulting from the administration of DDC and its analogues. Both groups of compounds are acted upon by cytochrome P-450 and in the process the haem moeity of the isoenzyme is alkylated

and stripped away from its apoprotein leaving an alkylated protoporphyrin 1X, the intact apoprotein and the residual part of the drug. However, in contrast to the alkylated protoporphyrins formed by DDC and some of its analogues, the porphyrin formed by AIA's interaction with cytochrome P-450 has no inhibitory action on ferrochelatase (De Matteis and Gibbs 1980). The porphyrinogenic action of AIA occurs only as the result of this compounds ability to destroy the haemoprotein cytochrome P-450. It has been proposed that this drug mediated alkylation and loss of cytochrome P-450 prosthetic haem permits the apocytochrome to utilise haem from the "free" or uncommitted regulatory haem pool with which the apoprotein is in intimate contact thereby creating a demand for increased haem production (Correia et.al.1979; De Matteis 1978).

1.10.3. SUCCINYLACETONE.

4,6-Dioxoheptanoic acid (succinylacetone) is a seven carbon keto acid (figure 4) discovered in the urine of patients suffering from hereditary tyrosinaemia as a result of an enzymetic deficiency in the tyrosine degradative pathway at the level of the enzyme fumarylacetoacetase (Lindblad et. al. 1977). This enzyme normally has a multiple catalytic action in the conversion of fumaryl acetoacetic acid to fumaric acid and acetoacetic acid to succinic acid and acetoacetic acid A deficiency in this enzyme, however, diverts the conversion of fumaryl acetoacetic acid to succinyl acetoacetic acid which in turn is metabolised via a different pathway to succinylacetone (Sassa and Kappas 1983). Patients with this disorder have low ALAD activity (Lindblad et. al. 1977) and Sassa and Kappas (1983) demonstrated that both urine from patients with tyrosinaemia and succinylacetone itself profoundly inhibits ALAD isolated from human erythrocytes, mouse and bovine liver. Subsequent to the inhibition of ALAD, succinylacetone decreases total cellular haem and the haemoprotein cytochrome P-450 in cultured chick hepatocytes (Sassa and Kappas 1983) and inhibits ^{14}C-labelled glycine incorporation into haem by over 90% in cultured rabbit reticulocytes. This block is overcome by the addition of porphobilinogen but not delta-aminolaevulinic acid, indicating that the site of inhibition was ALAD. ^{59}Fe incorporation into haem is also profoundly inhibited although ^{59}Fe uptake into the reticulocytes is increased (Ponka et.al.

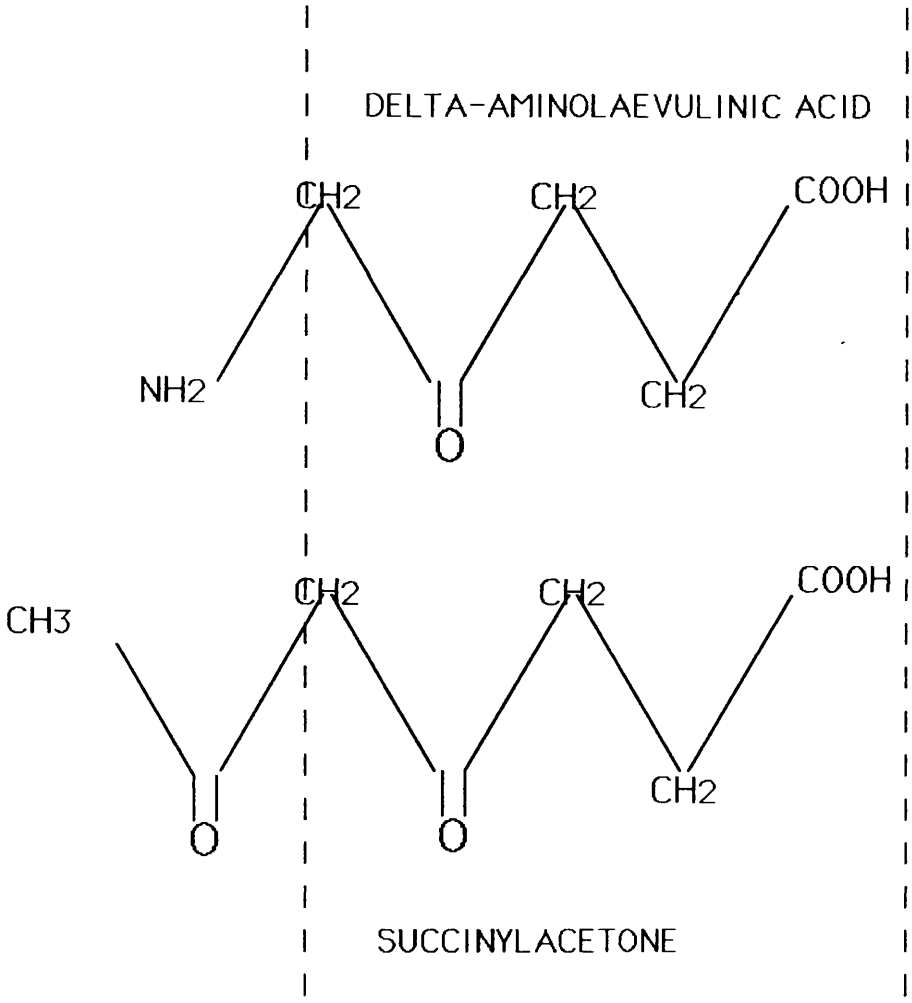

Figure 4: A comparison of the structures of ALA and the inhibitor of ALA dehydratase, succinylacetone.

1982), this latter observation being consistant with other work which indicates that iron uptake by reticulocytes is feedback-inhibited by intracellular haem (Ponka and Neuwirt 1969; Ponka et.al. 1974; Schulman et.al. 1974).

Succinylacetone irreversibly inhibits hepatic ALAD and evidence indicates that the inhibitor reacts with the catalytic site of the enzyme. Succinylacetone is a structural analogue of ALA in which the amino group of the latter is replaced by an acetyl group (figure 4) Increasing concentrations of ALA at levels which do not increase PBG formation in the uninhibited enzyme do increase PBG formation in the presence of a fixed concentration of inhibitor, indicating that the substrate is competing with the inhibitor for reaction with the enzymatically active site (Tschudy et.al.1981). Nandi and Shemin (1968) showed that labelled ALA reacts with ALAD to form a Schiff base and that the ALA could be irreversibly bound to the enzyme by sodium borohydride reduction of the Schiff base to a secondary amine. Utilising this method as an investigative tool Tschudy et.al. (1981) demonstrated that succinylacetone prevented the formation of the Schiff base between ALA and the active site of the enzyme and suggested that the succinylacetone itself combines with the enzymatic site. The kinetics of inhibition of ALAD by succinylacetone is characteristic of an irreversible inhibitor in that inhibition increases progressively with time, either when succinylacetone and the ALA substrate are added simultaneously or when succinylacetone is added to the enzymatic medium prior to the substrate. Further supportive evidence for succinylacetone's irreversible inhibition of ALAD comes from the fact that the activity of the inhibited enzyme is not restored after dialysis against water or buffer containing the sulphydryl protecting agent dithiothreitol, which protects the SH groups necessary for enzymatic activity, a situation which was the same in both the presence or absence of Zinc (a necessary cofactor in the enzymatic activity of the enzyme) (Tschudy et.al.1981).

Barnard et.al. (1977) and Battle et.al. (1978) suggest that under normal circumstances two ALA molecules align themselves at two adjacent sites on the enzyme and when alignment is complete, aromatisation to PBG proceeds. Labelling experiments carried out by Tschudy et.al. (1981) indicate that only one site need combine with

succinylacetone to inhibit formation of PBG. The ALAS induction observed following ALAD inhibition by succinylacetone is thought to occur when inhibition is sufficient to diminish haem synthesis to the extent that the negative-feedback repression of haem on ALAS is alleviated.

Succinylacetone has been identified as an inhibitor of ALAD in a variety of tissues. Ebert et.al. (1985) and Weinbach and Ebert (1985) demonstrated an inhibition of the growth of L1210 leukemia cells by succinylacetone which they proposed was due to a decrease in haem production. In these studies haematoporphyrin uptake into the cells was also increased indicating that a decrease in intracellular haem results in a lifting of the repressive action of haem in porphyrin uptake. The erythropoietic system of rabbits is similarly subject to inhibition by this compound at the level of ALAD. Haem synthesis is inhibited in both control and erythropoietin-stimulated bone marrow cells in a dose dependent fashion, a blockade which is partially overcome by porphobilinogen or protoporphyrin 1X (Beru et.al. 1983).

Patients with this disorder of the tyrosine degradative pathway excrete excessive amounts of delta-aminolaevulinic acid (ALA) in their urine (Gentz et.al. 1969; Kang 1970) and a clinical syndrome resembling that of acute intermittent porphyria is evident (Strife et. al. 1977).

1.10.4. LEAD.

In 1895, Stockvis found an increase in urinary porphyrins in lead poisoned rabbits and since then a wealth of studies have also shown that lead poisoning results in the increased excretion of porphyrins and their precursors (Goldberg 1968;1972). Lead causes a marked disruption in haem biosynthesis by inhibiting at least three enzymes in the pathway (Campbell et.al. 1977). Porphyrin precursors which have been shown to increase following exposure to lead include ALA (Haeger 1957; Haeger-Aronsen 1960); coproporphyrin (Duesberg 1931; Grotepass 1932); PBG (Gibson et. al. 1968); protoporphyrin 1X (Moore and Goldberg 1974; Lamola and Yamane 1974)

ALAD and ferrochelatase are enzymes which are adversely affected by lead (Goldberg1968). Campbell et.al. in 1977 also found a marked inhibition of ALAD, ferrochelatase and coproporphyrinogen oxidase activity in patients suffering from lead poisoning, an inhibition which occurred concomitantly with an increased ALAS activity. Further evidence of lead's ALAD inhibitory action comes from Millar et.al. (1972) and Beattie et.al. (1972).

Although the precise mechanism by which lead inhibits ALAD is not as yet known, Moore et.al. (1987) suggest that lead binds to the sulphydryl groups, necessary for enzyme function (Wilson et.al. 1972) or that lead may replace the Zinc co-factor, the other prerequisite for ALAD activity (Cheh and Neilands 1973). In support of this latter hypothesis is the observation by Finelli et.al. in 1975 that ALAD inactivation in erythrocytes exposed to lead can be reversed by the addition of Zinc. Sulphydryl groups are also necessary for normal ferrochelatase activity and Porra and Jones (1963a;1963b) suggest that lead may interfere with these essential groups in a similar manner to that suggested for ALAD. The mechanism by which lead interferes with coproporphyrinogen oxidase still remains obscure.

In addition to the accumulation of haem precursors in the blood and urine upon lead poisoning there is also evidence of a resulting haem deficit. ALAS is increased, a phenomenon which is regarded as an indication of a reduction in the uncommitted haem pool and a subsequent release of ALAS from its repressive negative-feedback control by haem. The activity of the haemoprotein, cytochrome P-450 is compromised in some patients as a consequence of lead exposure Meredith et.al. (1977) observing a decrease in cytochrome P-450 activity, as measured by the isoenzyme's ability to metabolise the xenobiotic phenazone (antipyrine) while in lead-exposed rats an analagous impaired drug metabolism and depressed hepatic cytochrome P-450 level was evident (Alvares et.al. 1972; Scoppa et.al. 1973 and Goldberg et.al. 1978).

There is ample evidence, therefore, that lead exposure has detrimental effects on the haem biosynthetic pathway and many of the clinical features of lead poisoning, such as neuropathy, abdominal

pain and constipation are similar to those of the acute porphyrias, which are also caused by defects in the enzymes of the haem biosynthetic pathway.

1.10.5. PHENOBARBITONE

In 1954(b) Goldberg observed that a variety of barbiturate analogues were capable of raising porphyrin excretion in rabbits and DeVerneuil et. al. in (1983) showed that phenobarbital significantly reduces uroporphyrinogen decarboxylase activity in chick hepatic cells. It has been known for many years that phenobarbitone is a potent inducer of the microsomal metabolising enzymes, cytochrome P-450 (Remmer 1959; Waxman and Walsh 1982). It is this last action of phenobarbitone which exerts the greatest stress on the haem biosynthetic pathway and the barbiturates are a major class of drug reported to be unsafe for use in patients diagnosed as porphyric (Moore et.al. 1987). Additionally, phenobarbitone increases the amount of alkylated protopophyrins formed, in the liver, from DDC and AIA (De Matteis et.al. 1982b).

The normal biosynthesis of haem can, therefore, be disturbed either genetically, as in porphyria, by an enzymatic defect or by chemical intervention at the synthetic enzyme level or by disturbance of the pathway's equilibrium.

1.11. Main aims of the thesis.

The biochemical profile of the porphyrias are explained by a genetic defect in haem synthetic enzymes. However, the aetiology of the symptoms of acute porphyria still defies elucidation. This study examines two hypotheses regarding the underlying causes of the clinical symptoms of the acute porphyrias. Firstly, the accumulating precursors may be neurotoxic. The effects of the haem precursors

will be examined for evidence of toxicity. Secondly, porphyric neuropathy may be due to a state of neural haem deficiency. This second hypothesis will be examined by the use of an animal model of porphyria employing porphyrinogenic chemicals to inhibit haem synthesis. Evidence of a developing neuropathy will be sought following treatment with these agents which are known to reduce haem availability. As the control of haem may not be the same in different tissues a range of haemoproteins will be measured in the liver, blood and the brain. The liver and blood are the two main haemoprotein containing tissues and the brain is important as it is the manifestation of defects in neural tissue which causes acute porphyric neuropathy.

CHAPTER 2

THE PHARMACOLOGY OF HAEM PRECURSORS.

INTRODUCTION.

The evidence already cited in the general introduction demonstrates the controversy that exists over the putative neurotoxic role of the haem precursors ALA and PBG. Only sparse evidence exists implicating PBG as a neurotoxic agent. On the other hand ALA does elicit pharmacological effects both at levels which could exist during acute porphyric attacks, (Russel et.al 1983 (10μM); Cutler et.al.1978 (3-38μM); Cutler et.al. 1991 (10-50nM); Jordan et.al.1990 (50μM)) and at large unphysiological concentrations, (Becker et. al. 1971 (2mM); Ditcher et.al. 1977 (0.5-5mM); Cutler et.al. 1985 (0.23-7.6mM); Cutler and Arrol 1987 (3-6mM); Cutler et.al. 1990 (3-4.5mM); Russel et. al. 1983 (1mM); Feldman et. al. 1968 (6mM)). There are also species and tissue differential effects of this compound. In rabbits and human intestinal preparations ALA induces a reduction in tone and contracture amplitude whereas in the rat the same agent causes an increase in intestinal tone, an effect also elicited in rabbit gastric fundus in large concentrations (0.1-3mM) (Cutler et. al. 1990). In the Crayfish stretch receptor neurones 1-2mM of ALA are required before effects are seen (Ditcher et. al. 1977) whereas in hamster spinal cord neurones 50μM of ALA inhibits transmission (Jordan et.al. 1990). ALA at 10μM reduces Na^+/K^+ ATPase activity in cultured neurones whereas a larger concentration of 1mM is required to inhibit Mg^{2+} ATPase activity in the same tissue (Russel et.al. 1983).

Different types of nerves may, therefore, possess a differential sensitivity to the neurotoxic action of ALA. The present study examines the effects of ALA and to a lesser extent, PBG on a variety of *in vitro* nerve/ muscle preparations where not only are the nerves anatomically distinct but the muscle response is mediated by different neurotransmitters. The contraction of mammalian intestinal smooth muscle is mediated by cholinergic muscarinic neurotransmission while inhibition of this tissue is sympathetically controlled by the transmitter noradrenaline. A range of smooth muscles elicit an inhibitory response which is mediated by an as yet unknown transmitter which is neither adrenergic nor cholinergic (NANC). In the anococcygeus, this NANC inhibitory response is mediated by nitric oxide acting post-synaptically on the haemoprotein, guanylate cyclase. In the vas deferens, however, and possibly the taenia coli, the NANC motor and inhibitory responses

appear to be mediated by the purine, adenosine triphosphate (ATP), acting, in the vas deferens and some vascular tissue, in concert with noradrenaline.

The effects of ALA were examined on six different rabbit tissues, the distal colon preparation, perfused ear artery, anococcygeus, taenia coli, jejunum and vas deferens. The haem precursor PBG was examined for pharmacological action on the NANC-mediated inhibitory response of the rat anococcygeus muscle. The possibility that ALA would show toxicity only in tissue already depleted of haem was examined in the anococcygeus and vas deferens from rats pretreated with porphyrinogenic agents. These treatments are dealt with in more detail in chapter 3 of this thesis.

CHAPTER 2

THE PHARMACOLOGY OF HAEM PRECURSORS.

METHODS.

2.1.1. Physiological salt solution:

Krebs' solution with the following composition (mM) was used throughout the investigation:

NaCl, 118.4; $NaHCO_3$, 25.0; NaH_2PO_4, 1.13; KCl, 4.7; $CaCl_2$, 2.7; $MgCl_2$, 1.3; glucose 11.0; pH 7.4. The solution was bubbled with a mixture of 95% O_2: 5% CO_2 obtained from Air Products, Scotland.

2.1.2. Chemicals:

δ-Aminolaevulinic acid HCl (ALA); Porphobilinogen (PBG); Histamine acid phosphate; Carbamylcholine chloride; were obtained from Sigma Co.Ltd. and guanethidine monophosphate from Ciba Laboratories, England.

ALA was prepared daily as a stock solution dissolved in water and brought to a pH of 7.0 with 0.5M NaOH. Porphobilinogen was prepared as a stock solution and frozen in aliquots.

2.1.3. TISSUE PREPARATION.

In the first part of the present study the effect of haem precursors were examined on 8 different isolated innervated muscle preparations .

All tissues were set up in isolated organ baths containing Kreb's solution at 37^0C and gassed with 95% O_2/5% CO_2.

2.1.3.1 RABBIT TISSUE

Rabbits of either sex were killed by $C0_2$ asphyxiation and exanguinated.

a) Distal colon:

This tissue was set up as reported by Garry and Gillespie (1954). The abdominal skin was incised by a midline cut. The muscle wall was opened, the symphysis pubis split with a sharp scalpal blade and the pelvic ring forcibly opened by dislocation of the sacro-ileac joints. The filaments of origin of the pelvic nerves, which convey the pre-

synaptic parasympathetic outflow from the 2nd., 3rd. and 4th. sacral nerve rootes were identified on either side of the colon. The two pelvic nerves were ligatured separately as close to their origin from the sacral roots as possible using fine thread. The lumbar colonic nerves, which convey the post-ganglionic sympathetic outflow from the inferior mesenteric ganglion to the colon, were ligatured at their origin from the ganglion, the ligature including the lumbar colonic vein. The colon itself was then cut through, the lower section about 1cm. caudal to the pelvic nerves, the upper section 4-5cm. rostral to this. The upper cut was continued through the mesocolon above and parallel to the inferior mesenteric artery following it to its origin from the aorta. The inferior mesenteric artery was cut through and the preparation removed to a Petri dish containing oxygenated Kreb's solution. Faecal pellets were removed gently from the anal end. The preparation was suspended as a Magnus preparation in a 50ml. isolated organ bath. The caudal end of the colon was fixed by a loop of thread to a hook and the cranial end attached by a thread to an isotonic strain gauge. With the preparation suspended above the organ bath the ligatures attached to the nerves were threaded by means of a fine needle through the condom rubber diaphragm of the two fluid filled electrodes. These electrodes consisted of two Ag/AgCl ring electrodes (15mm. diameter) recessed in a Perspex shell. The two halves of the electrode fitted tightly into each other separated by a rubber diaphragm. The Kreb's solution of the bath had free access to the first chamber whereas the second was filled with Kreb's prior to sealing with a Perspex disc. Each set of electrodes was cleaned and chlorided in 0.1M HCl each morning prior to the start of the experiment. Both pelvic nerves were placed in the lower electrode and the lumbar colonic nerves in the upper electrodes. The nerves were pulled gently into place, the closed half cell of each electrode filled with Kreb's and sealed with the Perspex disc. The ligature attached to the nerves was caught between the Perspex shell of the electrode and this disc, thereby fixing the nerve in position across the electrodes. The whole assembly was racked down into the warmed Kreb's solution and 2gm. of initial tension placed on the tissue. The preparation was left for at least 30 min. to equilibrate before the start of the experiment.

b) Anococcygeus muscle:

The rabbit anococcygei are a pair of well developed smooth muscles related to the terminal colon. (Langley and Anderson 1896). This muscle possesses a rather sparse motor adrenergic innervation and an NANC inhibitory innervation (Creed et. al. 1977). The muscles originate from the upper coccygeal vertebrae. They lie behind the terminal colon about 1cm. from the anal margin pass on either side of the colon with some fibres ending within the longitudinal external muscle of the colon. The muscles were identified lying on the lateral aspect of the distal colon, ligatured and dissected away from the distal colon for a few mm. The colon was cut through at a point approximately 6cm cranial to this point and the colon dissected away from the underlying mesocolon until the point of origin of the two anococcygei muscles was identified at the coccygeal vertebrae. The muscles were ligatured at their point of origin and dissected out of the animal. Each muscle was passed through a Ag/AgCl ring electrode and its lower end tied to a similar hook electrode which acted as the lower fixed attachment. A thread from the other end of the tissue was connected to an isometric strain gauge, the muscle placed under an initial tension of 1g. and left to equilibrate for 30 min. prior to the start of the experiment.

c) Ear artery:

The central ear artery of the rabbit receives a noradrenergic innervation with ATP as a candidate for a cotransmitter in the pressor response of this tissue. (Kennedy et. al. 1986)

The ears were removed from the rabbit and shaved. The central ear artery was identified and a score made laterally on either side of the vessel with a scalpel blade. The skin above the artery was peeled away exposing the underlying vascular bed. The artery was dissected out of the ear and placed in a Petri dish containing Kreb's solution. The vessel was cannulated with a plastic cannula whose diameter was sufficient not to contribute significantly to the total resistance of the system. 2-3cm. lengths of artery were threaded through a pair of Ag/AgCl ring electrodes, placed in a 25ml. organ bath containing

Kreb's and perfused with warm Kreb's solution at a rate of 3ml./min. The perfusion pressure was measured by a Statham pressure transducer on a side arm.

d) Taenia coli:

The Taenia coli is a condensation of the longitudinal muscle of the caeum. The taenia caeci receives both exitatory parasympathetic nerves and a sparse inhibitory sympathetic innervation. In addition, like many other parts of the gastrointestinal tract, the taenia receives an inhibitory NANC innervation whose transmitter is as yet unknown.

Strips of this longitudinal muscle of the rabbit caecum were cleaned, the mucosa removed and the lateral portions trimmed away to leave a 3mm. broad medial strip of muscle. 2cm. portions were drawn through a single Ag/AgCl ring electrode and attached to a lower hook electrode. A thread from the cranial end of the tissue was attached to an isometric strain gauge, the tissue placed under an initial 1g. of tension in a Kreb's filled 10ml. organ bath and left to equilibrate for 30min. before the start of the experiment.

e) Vas deferens.

The rabbit vas deferens receives a dense noradrenergic innervation and responds to field stimulation like the rat vas deferens. Each vas deferens was ligatured close to the epidydimus and again near the junction with the prostate, cut and removed to a Petri dish containing Kreb's solution. The tissue was cleared of any residual connective tissue and blood vessels. A 2.5cm. portion of the epidydimal end of the tissue was attached to a hook electrode similar to that used for stimulation of the anococcygeus and taenia coli muscle and a thread connected the cranial end of the tissue to an isometric strain gauge. The tissue was placed under an initial tension of 1gm and left to equilibrate for 30min. before the start of the experiment.

e) Jejunum.

The abdomen was opened and the jejunum identified. The intestine was cut at a point 5-10cm. below the stomach and a length of

intestine taken from here caudally towards the caecum. 2-3cm. lengths were cut, freed from mesenteric attachments and cleaned by flushing the lumen with Kreb's solution. A thread was tied at each end, the lower one forming a loop which was secured to a hook at the bottom of a 25ml. organ bath. The upper thread was attached to an isometric strain gauge.

f) Rat anococcygeus and vas deferens.

These tissues were prepared as described in chapter 3 methods section.

2.1.4. Experimental procedure.

In the presence of $3x10^{-5}M$ guanethidine, the tone of the rabbit anococcygeus and the taenia coli muscles were raised, with $3x10^{-6}M$ histamine and $3x10^{-6}M$ carbachol respectively.

In all tissues field stimulation was by 20-200 pulses at supramaximal voltage at frequencies between 0.1 and 64 HZ depending on the frequency sensitivity of the tissue.

CHAPTER 2

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RESULTS.

2.2.1. The effects of ALA on the contractile responses of the rabbit distal colon to pelvic nerve stimulation:

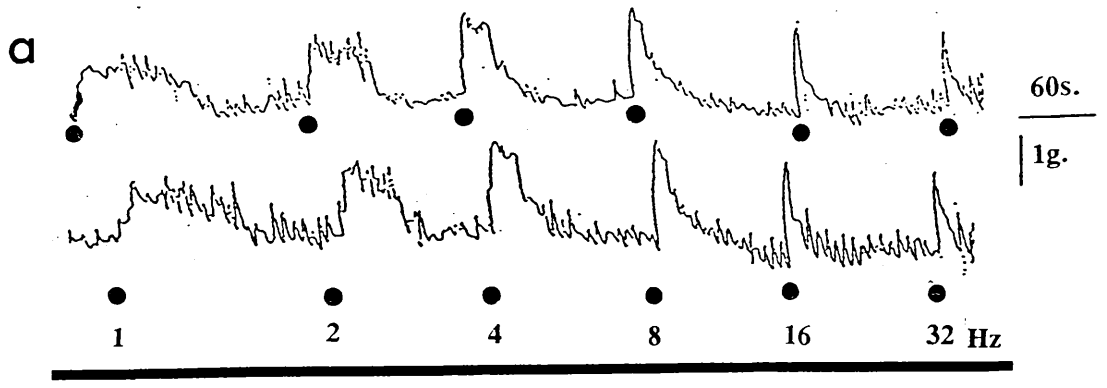
During the equilibration period the initial 2g. of resting tension decayed to approximately 1-1.5g. The tissue exhibits an inherent rhythmic activity. Field stimulation of the pelvic nerves, which innervate the distal colon, with 100 pulses (0.5ms. duration, supramaximal voltage) at 1-32 Hz produced a frequency-dependent, cholinergically-mediated contraction of the rabbit distal colon. The effects of the haem precursor δ-aminolaevulinic acid (ALA) on the responses of this tissue were examined at concentrations of 1, 10, 100μM and 1mM. As the effects of increasing concentrations of ALA were examined on the same tissues, following a control period for each concentration, a two way analysis of variance on the control responses alone was carried out (repeated trials as the variable). This statistical analysis revealed that there were no significant differences among control responses over the course of the experiment. ALA, at all concentrations examined, did not significantly alter the responses of the distal colon to pelvic nerve stimulation (figures 5a, 6a). Each drug-exposed response group was statistically compared to its own control period (paired t-tests).

2.2.2. The effects of ALA on the inhibitory response of the rabbit distal colon to lumbar colonic nerve stimulation:

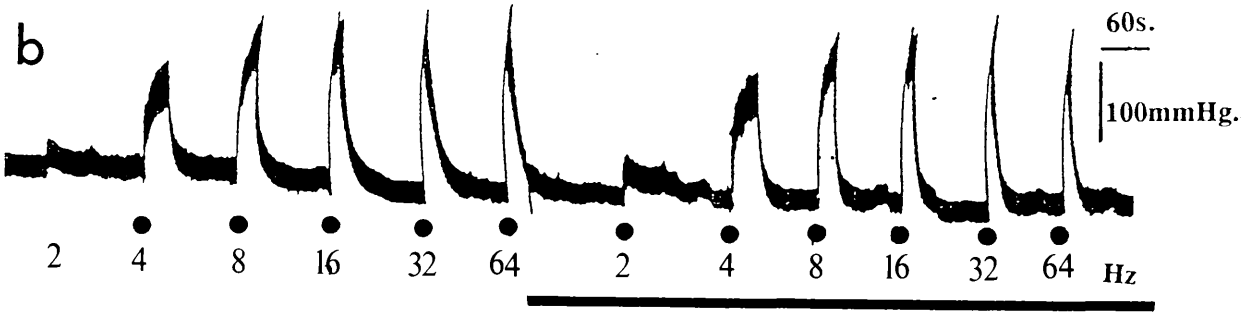
Stimulation of the lumbar colonic nerves innervating the rabbit distal colon (20s. stimulation period of 4, 8 and 16 Hz) produces an inhibitory effect on the rhythmic contractile activity of this tissue. ALA at concentrations ranging from 1μM-1mM had no effect on the inhibition produced by this sympathetically-mediated response on a limited number of preparations (figure 8b).

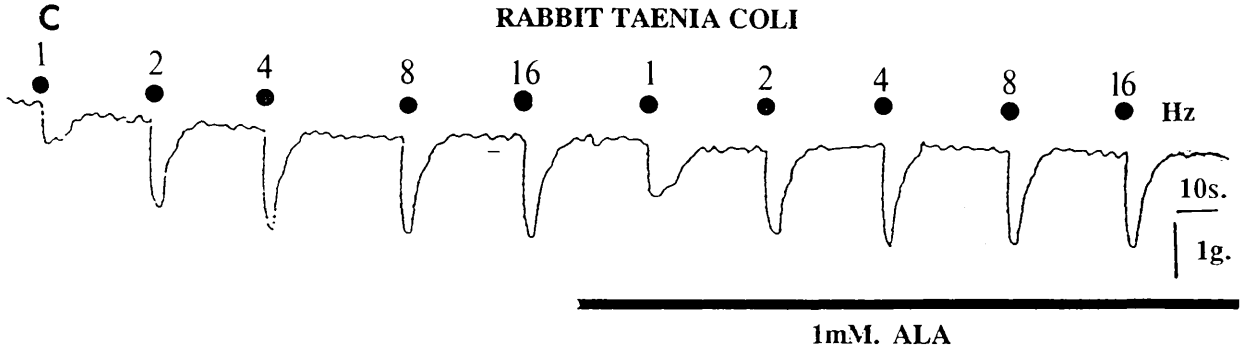
Figure 5: Shows the lack of effect of 1mM. ALA on the responses of four tissues to nerve stimulation, at the frequencies shown. a) the response of the distal colon to pelvic nerve stimulation; b) the pressor response of the perfused rabbit central ear artery to intramural nerve stimulation; c) the response of the taenia coli to intrinsic inhibitory nerve stimulation; d) the response of the anococcygeus muscle to intrinsic inhibitory nerve stimulation. The time bars apply during stimulation periods only, the interstimulus interval is 3 miutes for each tissue. The black bar below each trace indicates the presence of 1mM. ALA.

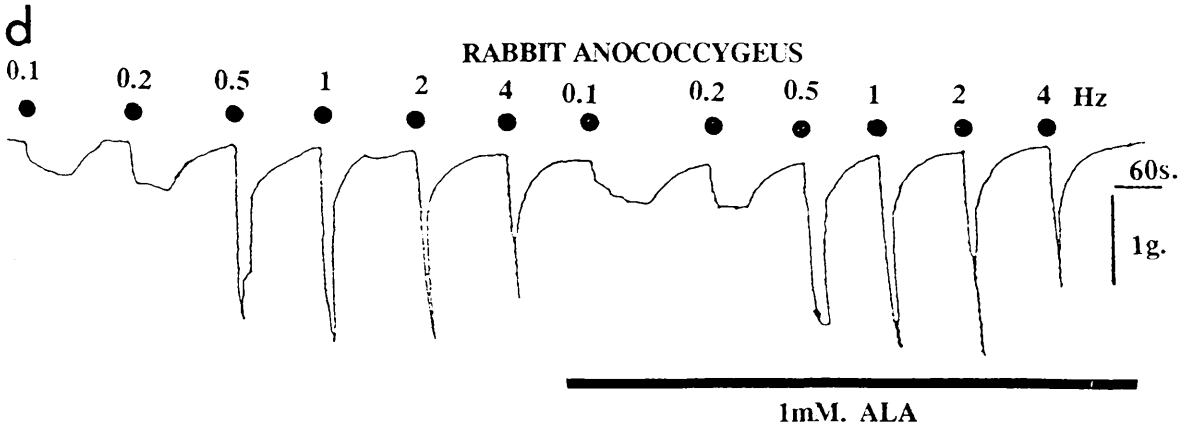
RABBIT DISTAL COLON/ PELVIC NERVE STIMULATION



PERFUSED RABBIT CENTRAL EAR ARTERY







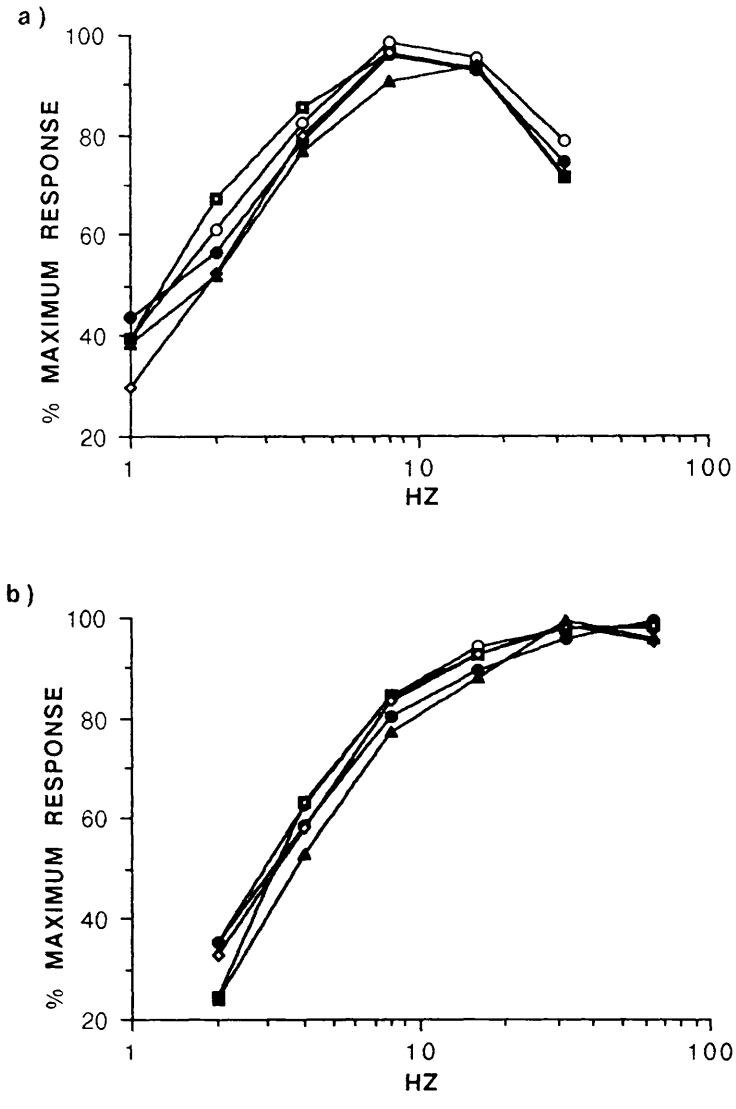


Figure 6: Shows the lack of effect of ALA on the responses of a) the rabbit distal colon to pelvic nerve stimulation and b) the perfused rabbit ear artery to field stimulation of the periarterial nerve. For clarity, the graphs show the mean values only and standard deviations for all points are tabulated below. There are no statistically significant differences.

Distal colon:

- CONTROL, 2.6, 3.14, 1.9, 0.6, 1.78, 2.46
- 10-6M ALA, 6.5, 4.4, 5.4, 2.8, 4.8, 7.0
- 10-5M ALA, 8.5, 8.5, 4.9, 1.6, 4.6, 1.8
- 10-4M ALA, 5.2, 6.6, 4.5, 2.6, 5.7, 8.7
- 10-3M ALA, 5.7, 2.7, 10, 3.6, 6.4, 7.1

Ear artery:

- CONTROL, 5.7, 3.2, 2.1, 1.3, 0.53, 0.61
- 10-6M ALA, 7.5, 2.6, 2.9, 2.6, 3.4, 0.57
- 10-5M ALA, 6.7, 5.4, 4.6, 1.4, 1.8, 0.9
- 10-4M ALA, 5.8, 7.4, 8.2, 3.8, 1.3, 2.8
- 10-3M ALA, 8.0, 10, 10, 6.9, 0.63, 2.4

2.2.3. The effect of ALA on the pressor responses of the rabbit ear artery to intramural nerve stimulation:

Upon equilibration the perfusion pressure of this tissue settled down to a resting pressure of 20 +/- 4mm.Hg. Electrical field stimulation of the periarterial nerves (0.5ms duration, supramaximal voltage, 200 pulses at 2-64Hz) elicitated a frequency-dependent vasoconstriction (figure 5b, 6b). The effects of increasing concentrations of ALA were examined on these responses following a control frequency response period. A two-way analysis of variance on the repeated control response periods determined that there were no sgnificant differences in these responses with time. ALA at all concentrations examined did not significantly alter pressor responses of this tissue to electrical field stimulation of the intramural nerves.

2.2.4. The effects of ALA on the inhibitory response of the rabbit taenia coli to intramural nerve stimulation:

Carbachol ($3x10^{-5}M$) produces a contraction (approximately 70% of maximum) of the isolated longitudinal muscle of the rabbit caecum. In the presence of $3x10^{-5}M$ guanethidine, which acts as a sympathetic neurone blocker, electrical field stimulation of the intrinsic nerves supplying this tissue (0.5ms. duration, supramaximal voltage, 20 pulses at 1-16Hz) produces a frequency-dependent non-adrenergic non-cholinergically-mediated inhibition of the induced tone. The effects of ALA in increasing concentrations (1μM-1mM) were examined on these inhibitory responses. A two-way analysis of variance on repeated control periods determined that there were no significant difference in the control responses with time. ALA at all concentrations examined produced no significant alteration in the responses of this tissue to NANC-mediated inhibition of induced tone (Figure 5c,7a).

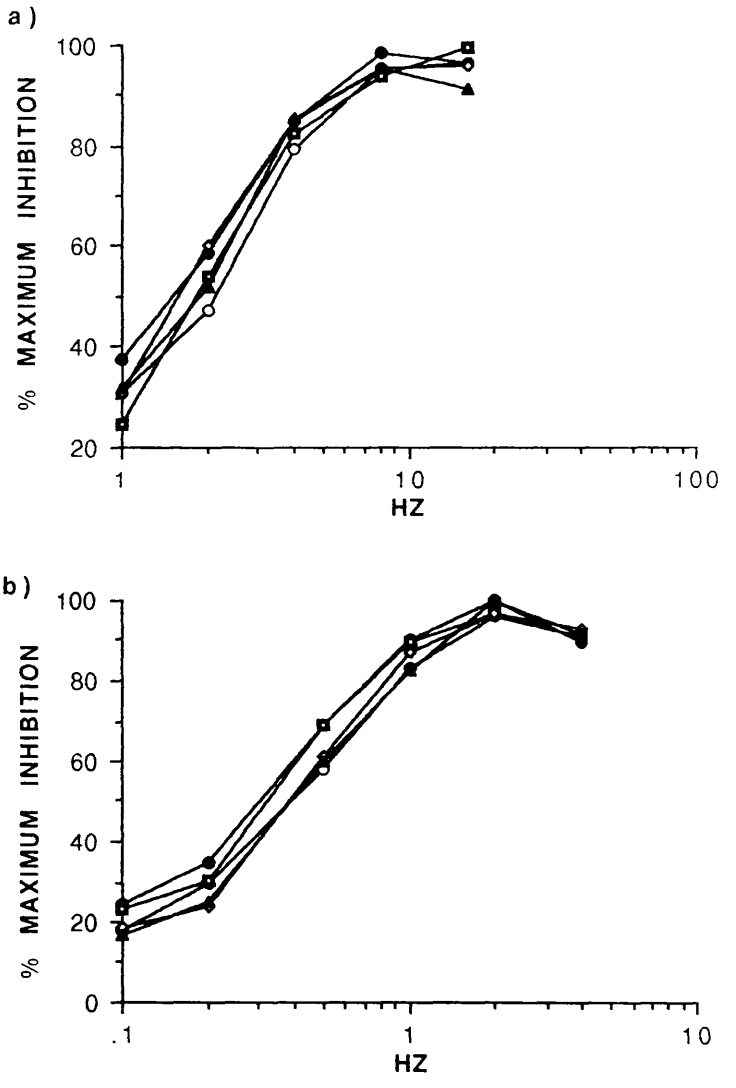


Figure 7: Shows the lack of effect of ALA on the responses of a) the rabbit taenia coli and b) the anococcygeus to intrinsic nerve stimulation. For clarity, the graphs show the mean values only and standard deviations for all points are tabulated below. There are no statistically significant differences.

Taenia coli:
- CONTROL, 3.6, 4, 3.4, 1.1, 1
- 10-6M ALA, 6.4, 5.9, 6.6, 0.7, 3.5
- 10-5M ALA, 8, 9.5, 8.3, 3.3, 0.62
- 10-4M ALA, 8, 8.2, 8, 2.2, 1.6
- 10-3M ALA, 5.8, 8.7, 7.1, 2.9, 5.7

Anococcygeus:
- CONTROL, 1.6, 2.5, 3.9, 3.4, 1.6,1.7
- 10-6M ALA, 3.7, 4.7, 5.5, 2.7, 4.4,4.8
- 10-5M ALA, 2.2, 5.4, 7.2, 4.8, 3.6,2.4
- 10-4M ALA, 3.5, 4.4, 5.6, 3.6, 2.6,3.7
- 10-3M ALA, 5.8, 4.3, 6.5, 6, 1.1,1.7

2.2.5. The effects of ALA on the inhibitory response of the rabbit anococcygeus muscle:

Histamine ($3x10^{-5}M$) induces tone in the isolated rabbit anococcygeus muscle (approximately 70% of maximum). In the presence of guanethidine ($3x10^{-5}M$) field stimulation of the intrinsic nerves innervating this tissue (0.5ms. duration, supramaximal voltage, 20 pulses at 0.1-4Hz) produces an NANC-mediated frequency-dependent inhibition of the induced tone. ALA in concentrations ranging between 1μM-1mM had no adverse effect on the responses of this muscle to NANC nerve stimulation when drug-exposed frequency responses were compared to drug-free control responses (Figure 5d, 7b). A two-way analysis of variance on repeated control periods determined that there were no significant difference in the control responses with time

2.2.6. The effects of ALA on the contractile response of the rabbit isolated vas deferens to field stimulation:

Electrical field stimulation of the rabbit vas deferens (0.5ms. duration ,100 pulses, 16Hz) produces a biphasic response (figure 8a). The initial "fast" component is purinergically-mediated and the "sustained" secondary component is noradrenergically-mediated. The effects of ALA at concentrations ranging from 1μM-1mM were examined on a limited number of preparations. ALA produced no difference in either component of this response.

2.2.7. The effects of ALA on the intrinsic rhythmic activity of the rabbit jejunum:

When 2g. of tension was placed on lengths of rabbit jejunum the tissue exhibited rhythmic activity. ALA at concentrations up to 1mM had no adverse effect on this rhythmic activity. 10mM of ALA in 9 out of 18 cases caused a small transitory reduction in the amplitude of contraction while the remaining 7 preparations showed a small increase and 2 tissues were unaffected by this large concentration

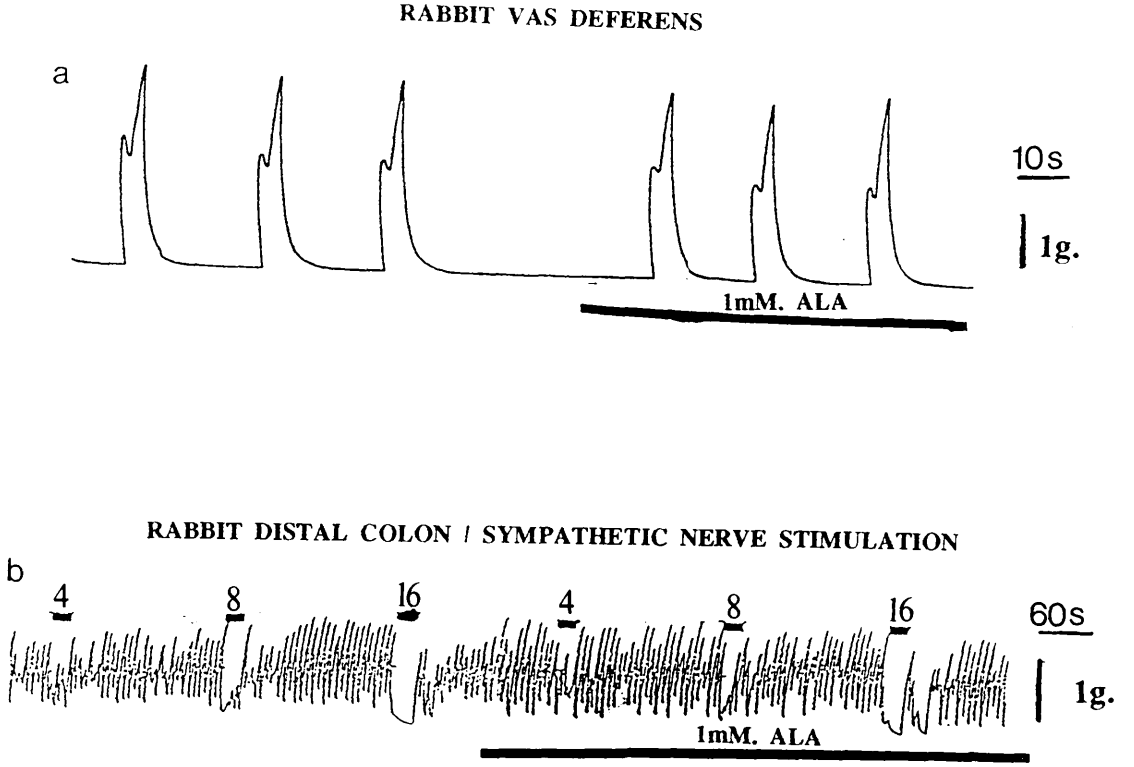


Figure 8: The effects of 1mM. ALA, in a single experiment, on the responses of a) the rabbit vas deferens to field stimulation (100 pulses at 16Hz) and b) the distal colon to extrinsic sympathetic nerve stimulation (20s. stimulation at 4-16Hz). The dark line below each trace indicates the presence of 1mM. ALA.

of ALA (figure 9). The mean change in contraction amplitude of the tissue was -1.5%. Overall, ALA exerted no major effects on the inherent activity of this tissue.

2.2.8. The effects of ALA on tissues from rats treated with porphyrinogenic drugs:

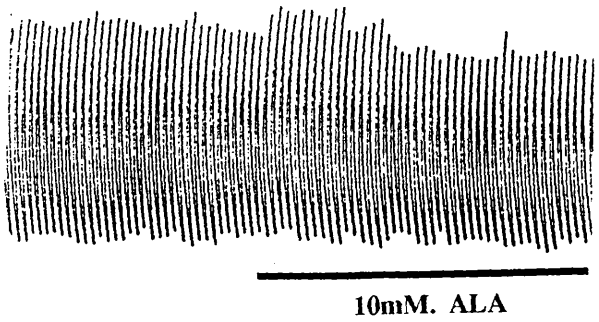
a. Anococcygeus:

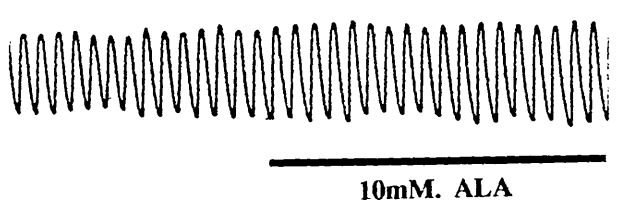
Treatment 8 , described in the methods section of chapter 3 consisting of 3 alternate days intraperitoneal administration of the porphyrinogenic drugs 4-ethyl DDC and succinylacetone, caused a significant increase, above control levels, in the urinary excretion of the haem precursor, δ-aminolaevulinic acid (figure 16). Electrical field stimulation of the intrinsic nerves of the isolated anococcygeus muscles from these rats (0.5ms. duration, supramaximal voltage, 50 pulses at 0.5-32Hz) (described in chapter 3) induces a noradrenergically-mediated frequency dependent contraction of the muscle (figure 10a,12). These contractions in control tissues were indistinguishable from contractions in treated animals and ALA in concentrations of 10nM, 100nM, 1μM, 10μM and 300μM had no significant effect on the responses of the tissue at stimulation frequencies of 1 and 8Hz (figure 11a, 12).

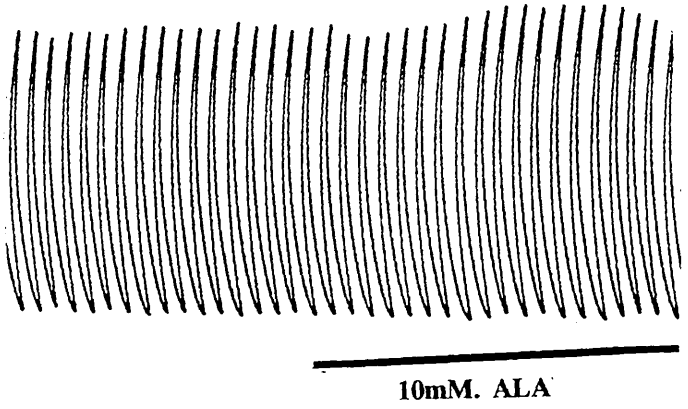
b. Vas Deferens:

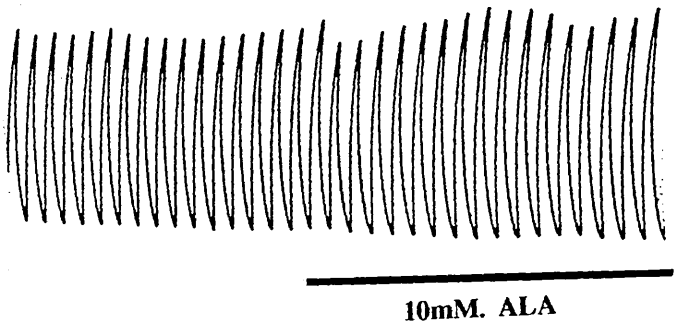
Field stimulation of the intrinsic nerves of the rat vas deferens (0.5ms. duration, supramaximal voltage, 20s. train at frequencies of 1-16Hz) (described in chapter 3) induces a frequency-dependent biphasic motor response (figures 10b, 10c, 12). The initial "fast" component is purinergically-mediated and the secondary "sustained" component is noradrenergically mediated (Swedin 1971; Sneddon and Westfall 1984). The haem precursor ALA at concentrations ranging from 10nM to 300μM produced no significant effect on the responses of either component of these contractile responses at either 1 or 8 Hz (figures 11b, 11c,12).

Figure 9: Shows the effects of 10mM. ALA, in a selection of experiments, on the rabbit jejunal preparations. The black bar below each trace indicates the presence of 10mM. ALA. The response varies from a slight reduction, through no effect to a slight stimulation of the spontaneous rhythmic activity of the intestinal preparations.


10S
1g.
10mM. ALA


10S
1g.
10mM. ALA


10S
1g.
10mM. ALA


10S
1g.
10mM. ALA

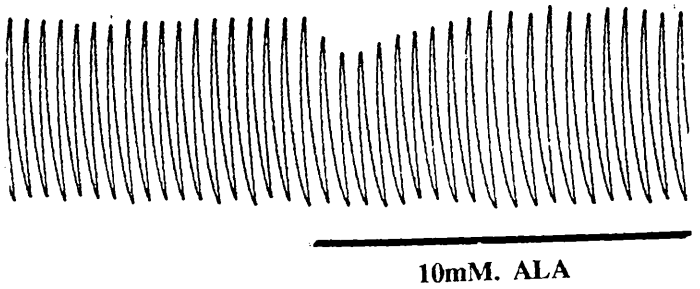
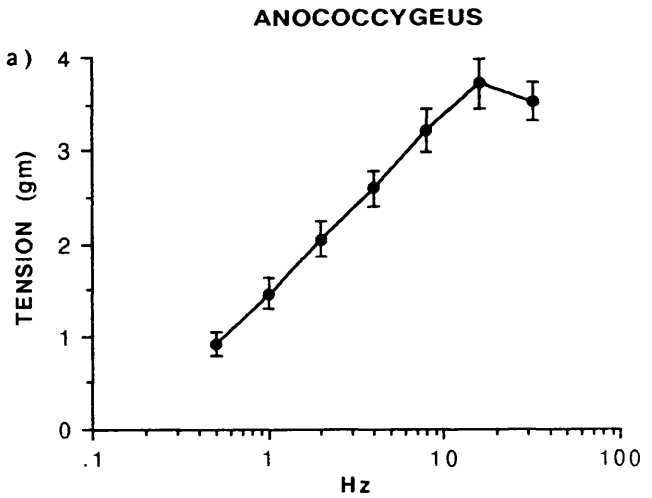
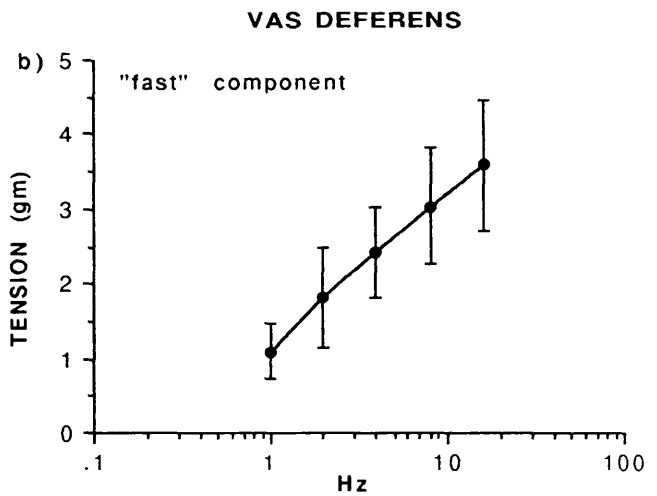

10S
1g.
10mM. ALA

Figure 10: Shows the mean +/- S.E.M. responses of the rat a) anococcygeus motor response, b) the vas deferens "fast" component and c) the vas deferens "sustained" component (n=6, all cases), in tissues from animals that had received 3 alternate days treatment with 300mg./kg. 4-ethyl DDC and two doses of 40mg./kg. succinylacetone.


ANOCOCCYGEUS
a)
TENSION (gm)
0
1
2
3
4
.1
1
10
100
Hz


VAS DEFERENS
b)
"fast" component
TENSION (gm)
0
1
2
3
4
5
.1
1
10
100
Hz

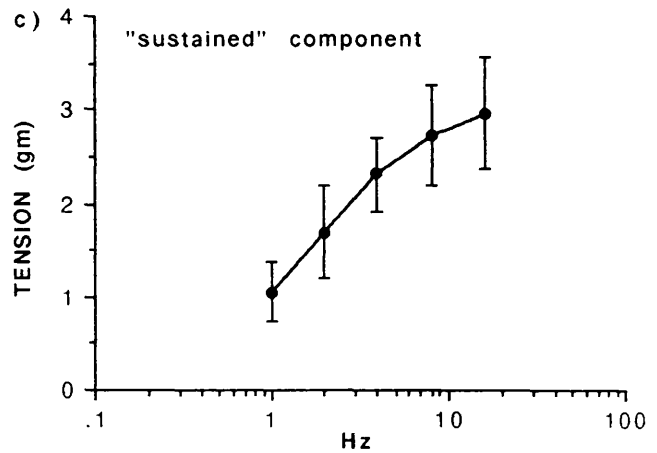
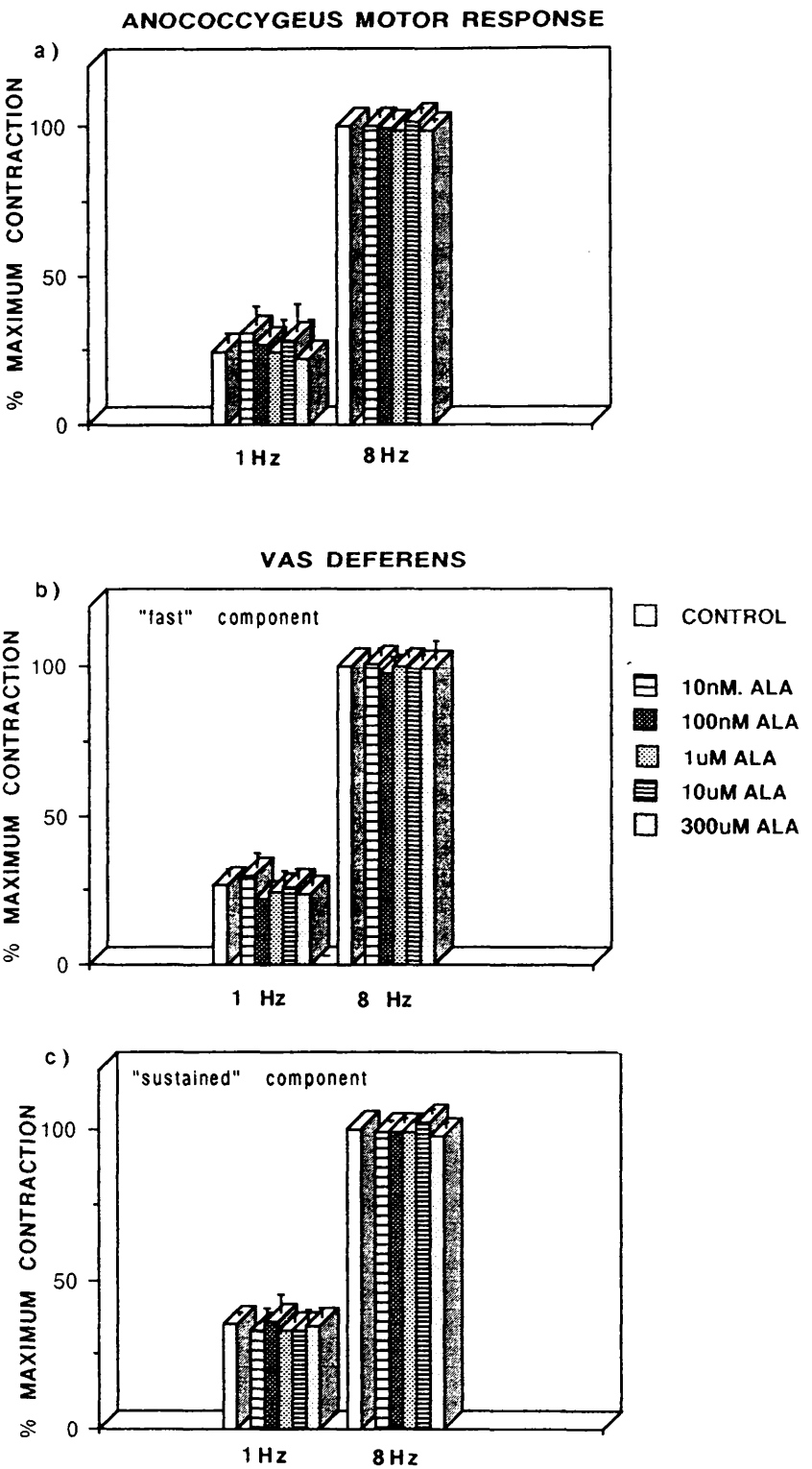

c)
"sustained" component
TENSION (gm)
0
1
2
3
4
.1
1
10
100
Hz

Figure 11: Shows a summary diagram of the effects of ALA at concentrations of 10nM, 100nM, 1μM, 10μM and 300μM (n=6, all concentrations) on the % of maximum response of a) the anococygeus motor response at 1 and 8Hz, b) the vas deferens "fast" component response at 1 and 8Hz and c) the vas deferens " sustained" component response at 1 and 8Hz. The response of control tissues at 8Hz were taken as 100% maximum response. ALA has no effect on any of the responses.


ANOCOCCYGEUS MOTOR RESPONSE
a)
% MAXIMUM CONTRACTION
100
50
0
1 Hz
8 Hz
VAS DEFERENS
b)
"fast" component
% MAXIMUM CONTRACTION
100
50
0
1 Hz
8 Hz
CONTROL
10nM. ALA
100nM ALA
1uM ALA
10uM ALA
300uM ALA
c)
"sustained" component
% MAXIMUM CONTRACTION
100
50
0
1 Hz
8 Hz

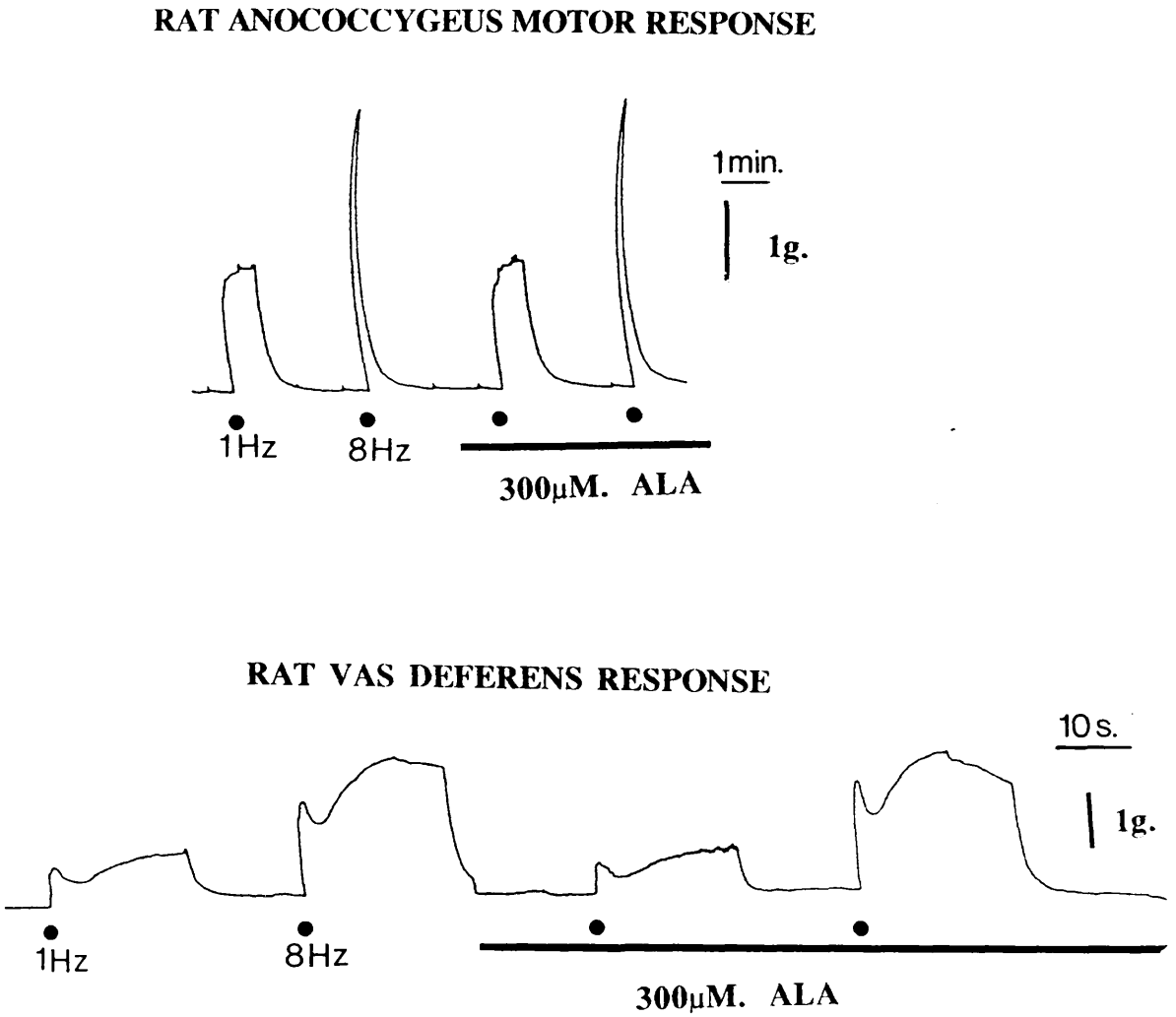


Figure 12: The responses of a) a single anococcygeus muscle to stimulation with 50 pulses at 1 and 8Hz and b) of a single vas deferens to a 20s. train of pulses at 1 and 8Hz before and in the presence of 300μM. of ALA. The black bar indicates the presence of ALA. ALA had no effect on the responses of these two preparations.

2.2.9. The effects of porphobilinogen on rat isolated tissues:

Since the literature contains little evidence for toxicity by porphobilinogen, the experiments were confined to a single tissue, the anococcygus of the rat.

a) Anococcygeus:

As well as blocking adrenergic neurones, guanethidine ($3x10^{-5}M$) induces tone in the rat anococcygeus muscle by releasing the preformed store of noradrenaline. Field stimulation of the intrinsic nerves of this tissue (0.5ms. duration, supramaximal voltage, 20 pulses 0.5-8Hz), when the tone is raised, produces a NANC-mediated frequency-dependent relaxation of the induced tone (Gillespie 1972). Porphobilinogen in concentrations of 30 and 100μM had no significant effect on the nerve-induced inhibition of this tissue (figure 13). PBG, at the highest concentration used in this study (1mM) caused a small transient drop in induced tone (9% ± 3.3%) which returned to its original level within the interstimulus interval period of 4min. This phenomenon ocurred in 2/3rds. of the preparations (figure 14).

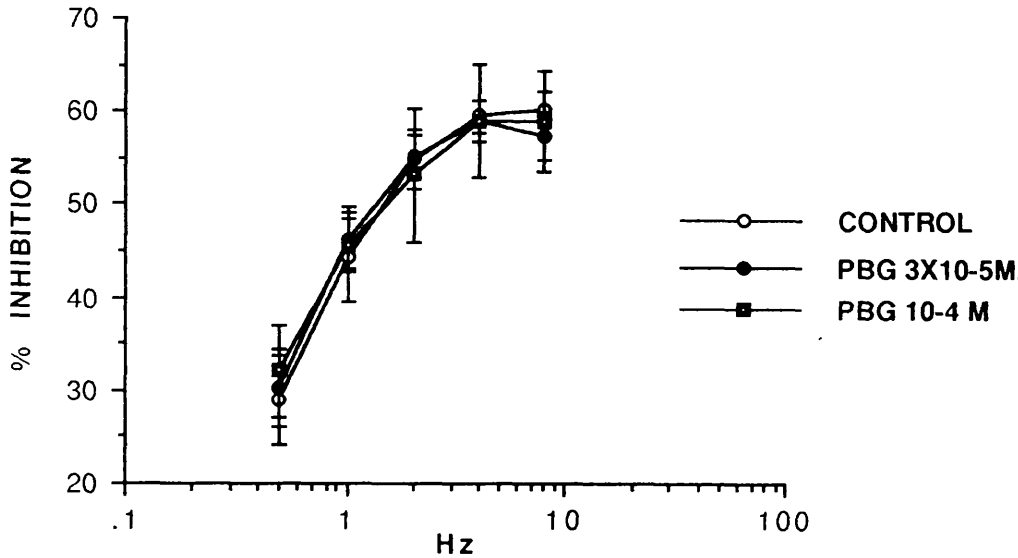


Figure 13: Shows a graph of the mean inhibitory response of the rat anococcygeus muscle preparation. Porphobilinogen (PBG) at concentrations of 10-4M. (n=6) and 3x10-5M (n=6) had no significant effect on the response of this muscle to field stimulation with 20 pulses at 0.5-8Hz.

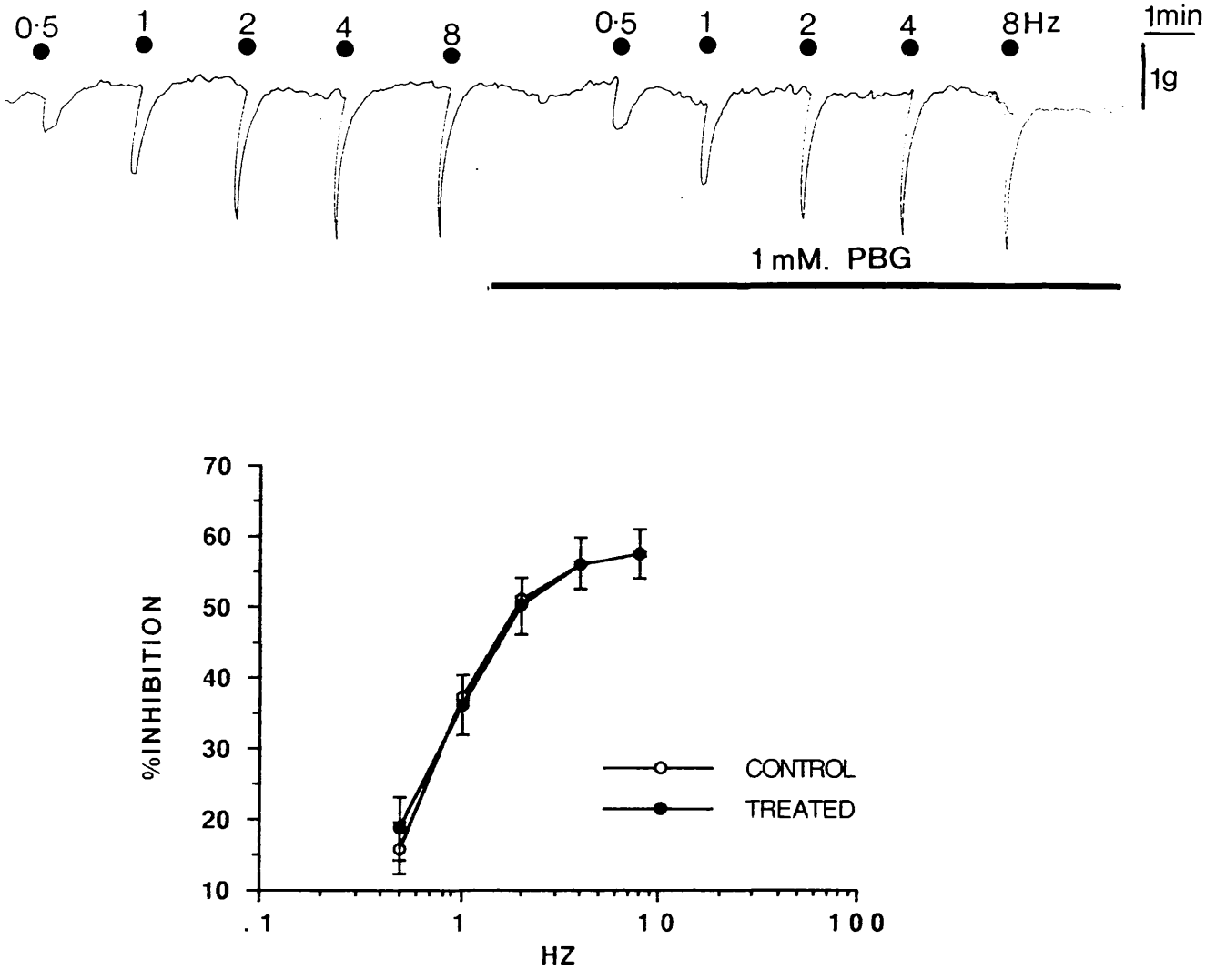


Figure 14: The upper trace shows a single experiment on the effect of 10^{-3}M porphobilinogen on the inhibitory responses of the rat anococcygeus muscle to stimulation with 20 pulses at 0.5-8 Hz. The presence of PBG is indicated by the black bar. The lower graph is a summary diagram, mean (+/- S.E.M.), of the nerve-stimulated inhibitory responses in the presence (treated) and absence (control) of 10^{-3}M. PBG (n=6).

CHAPTER 2

THE PHARMACOLOGY OF HAEM PRECURSORS.

DISCUSSION.

The hypothesis that acute porphyric neuropathy is the result of the neurotoxic actions of the haem precursors which accumulate during acute attacks has received much consideration over the last few decades. There is, however, no unequivocal outcome regarding the role of these precursors in the aetiology of the symptoms of this disease. Although Sweeney et.al. (1970) have reported measuring plasma ALA levels in the region of0·1mM. in porphyric patients, other researchers have recorded much lower levels during acute porphyric attacks. 2μM-5μM. ALA were measured in the plasma of porphyrics by Bonkowsky et.al. (1971) and levels of 120nM.-7μM. by Percy and Shanley (1977). The highest recorded plasma level measured by Gorchein and Webber (1987) in porphyric patients was 12μM. whereas the levels during a severe crisis were between 3.6μM. and 4.2μM. Cerebrospinal fluid (CSF) levels of this precursor are much lower. Again, Sweeney et.al. (1970) have recorded the highest CSF ALA concentrations (2.1μM.) whereas lower levels have been reported by Bonkowsky (1971) of 0.29μM and 0.2μM. by Gorchein and Webber (1987). The last group of authors also reported that during the severest attack, in which the patient was comatose, the highest CSF ALA concentration measured was 192nM.

Some in vitro tissue preparations are adversely affected by relatively low concentrations of the haem precursor ALA, (Russel 1983 et. al. (10μM.); Cutler et.al. 1978 (3-38μM.); Cutler et.al. 1991(10-50nM.); Jordan et.al. 1990 (50μM.)) whereas in other preparations ALA has either no effect or is only effective at very high concentrations, which probably do not occurr even during the severest acute porphyric attack.

The aim of this first part of the study was to examine the effects of a range of concentrations of ALA on the responses of a variety of innervated muscle. These preparations included noradrenergically-mediated responses (distal colon inhibitory response, vas differens secondary component), cholinergically-mediated responses (distal colon motor response), purinergically-mediated responses (vas deferens initial response and part of the ear artery pressor response) and NANC-mediated responses (inhibitory response of the rabbit anococcygeus and taenia coli muscles). This range of innervated

preparations whose responses were mediated by different neurotransmitter systems was chosen to examine the possibility of a differential tissue sensitivity to the effects of ALA.

In this study, none of the ALA concentrations produced a significant effect on any of the preparations. Although McGillion (1974) found a slight hypotensive effect on intravenous administration of 500-1000mg of ALA to anaesthetised rats, he also observed that ALA had no significant effects on the blood pressure of pithed rats nor on the noradrenergically and neuronally stimulated responses of the isolated perfused rabbit ear artery. The findings reported in the present study and those of Edwards et.al. in 1984 who failed to find cardiovascular effects of ALA *in vivo* in the rat, measuring the blood pressure and heart rate response to noradrenaline, acetylcholine and isoprenaline, argue against a cardiovascular role for ALA in the aetiology of acute porphyric neuropathy.

In those reports in the literature where low ALA concentrations, which could occur during porphyric attacks, did exert effects on *in vitro* preparations, the effects may be mediated by an interaction between ALA and the inhibitory transmitter, γ-aminobutyric acid (GABA). Hyperpolarisation of frog motoneurones by 50-400μM. of ALA was blocked by the GABA antagonist, picrotoxin (Nicoll 1976), 100μM. ALA inhibited GABA uptake into synaptosomes while at much lower levels of 1μM. it inhibited K^+ stimulated release of GABA from synaptosomes (Brennan and Cantrill 1979). The latter authors suggest that the inhibition of GABA release is mediated via ALA's interaction with GABA autoreceptors. 50μM. of ALA inhibited polysynaptic transmission in the hamster spinal cord and Jordan et. al. (1990) suggested that GABA had a role to play in these effects. However, Becker et.al. (1980) reported only small inhibitory effects of ALA on GABA metabolism at concentrations ranging from 100μM. to 1mM. and suggest that the role of ALA in acute porphyric attacks is not mediated via an effect on GABA synthesis and metabolism while Russel et.al. (1983) ruled out a possible role for GABA-mediated inhibitory effects of ALA on neuronal Na^+ K^+ ATPase activity. GABA was also implicated as a possible mediator of the effects of ALA in rat jejunual preparations reported by Cutler and her colleagues in 1991. Although 1-3mM. of ALA was required to increase the tone and

contracture amplitude of this tissue, the $GABA_A$ antagonist, bicuculline enhanced the responsiveness of the preparation to 10-50nM. of ALA. This effect of ALA is, therefore, proposed to be mediated via these postsynaptic GABA sites. Although ALA was reported to enhance the amplitude and degree of contraction of the rat jejunum, it decreases these parameters in rabbit jejunal preparations (Cutler et.al. 1990). In the studies reported in the present thesis, the role of ALA at GABA sites was not examined but if GABA receptors are ubiquitous in the mammalian myenteric plexus, as Saffrey et. al. (1983) suggests ALA would be expected to exert qualitative if not quantitatively similar effects in these two tissues. However, possible species differences in receptor type and population make this finding not totally surprising. In the rabbit jejunal preparations studied in the present series of experiments, ALA at a concentration of 10mM. produced only minor and variable effects on the inherent rythmic activity of the muscle. Although inhibition was seen in rabbit jejunal preparations by Cutler et.al. (1990) at lower concentrations than reported here (1.5-6mM.) the levels of ALA required to elicit effects in this tissue were still in the mMolar range, concentrations which have never been recorded during even the severest porphyric attack. The observation by Cutler and Arrol (1987) that the effects of ALA (1.5-6mM.) on human taenia coli are similar to that of the haem precursor in rabbit tissue suggest that the responses of rabbit tissue to ALA may give a more valuble index of the role of ALA in human acute porphyric attacks. Although the results reported here provide no evidence for the thesis that ALA is neurotoxic they do not rule out the possibility that in some tissue ALA may alter function via interaction with GABA receptors. However, a definitive role for ALA in the GABAergic system has not, as yet, been elucidated.

In order to investigate the possibility that ALA exerts neurotoxic action in a haem deficient environment, the effects of the precursor were examined following treatment which is known to decrease haemoprotein content and which causes a rise in excreted ALA (figure 16). This increased ALA excretion is thought to be evidence of depletion of the free haem pool. In this group of animals urinary excretion was increased by a factor of approximately 40 over

excretion in control animals. Urinary excretion has been reported to be between 10 and 100 times greater than serum concentrations (Percy and Shanley 1977) therefore, the serum levels of ALA in these animals would be roughly in the region of 5-50μM, comparable with the highest concentrations evidenced in human patients during an acute attack. In vitro examination of ALA on the motor responses of the anococcygeus and the vas deferens from these animals provided further evidence for a lack of ALA toxicity even in circumstances where haem synthesis has been disrupted.

The lack of effect of the haem precursor ALA reported in this study and the findings that ALA elicits little or no effect in vivo (Berlin et.al. 1956; Meyer et.al. 1972; Shanley et.al. 1975; Edwards et.al. 1984) argue against a neurotoxic role for the haem precursor ALA in the aetiology of the neurological symptoms of acute porphyria.

The case for a neurotoxic role for the haem precursor porphobilinogen is not strong. Although PBG does elicit an effect on the K^+-stimulated release of acetylcholine from the rat phrenic nerve-diaphragm preparation a neurotoxic role for PBG seems unlikely. In hereditary ALAD deficiency and hereditary tyrosinaemia, diseases which both elicit symptoms commensurate with the symptoms of acute porphyria, PBG is not an overproduced precursor. Serum PBG of porphyric patients is generally higher than that of ALA (Percy and Shanley (1977), 0.2μM.-20μM.; Bonkowsky et.al. (1971), 15μM.) In vitro and in vivo studies by Goldberg et. al. in 1954 showed that PBG had no effect on a variety of physiological systems. The blood pressure, respiratory and cardiovascular responses to acetylcholine, nicotine, adrenaline, noradrenaline and vagal stimulation were unaltered by intravenously administered PBG. Infusion of PBG-containing sterilised urine from patients in attack into anaesthetised cats and rabbits caused no changes in the same physiological parameters. PBG was similarly ineffective in altering the responses of a variety of isolated muscle preparations from rabbit or guinea-pig. In isolated rabbit jejunal preparations, 1.1mM. PBG was ineffective in producing any alteration in the responses of this preparation (Arrol 1986). In the experiments reported here, 30μM. and 100μM. PBG did not alter the responses of the rat anococcygeus muscle to inhibitory nerve stimulation. At a concentrations of 1mM., although PBG caused a

small and transient drop in the induced tone of the muscle, the responses to intrinsic nerve stimulation were unaltered. The transient drop in tone caused by this high dose of PBG would probably not be a contributory factor in the neuropathy of AIP as this was an acute effect of drug addition and not a sustained phenomenon.

The results of this part of the study provide no evidence for a neurotoxic role for the haem precursors ALA or PBG in the symptoms of acute porphyric neuropathy.

CHAPTER 3

THE EFFECTS OF PORPHYRINOGENIC DRUGS ON NERVE AND MUSCLE.

INTRODUCTION.

The use of chemical agents as experimental tools to study the aetiology and treatment of disease states is a common practice in developing models of human disorders. Over a century ago Claude Bernard pointed out that "foreign chemicals can be employed as instruments to analyse the most delicate vital processes; much can be learned about the physiological processes themselves by the careful study of the mechanisms by which these are altered by chemicals." (cited by De Matteis and Aldridge 1978). A variety of chemicals may be used as experimental tools with which to study haem biosynthesis and the human disease states of porphyria (figure 3). Some of these compounds (lead, succinylacetone, N-methyl protoporphyrin 1X and phenobarbitone) directly inhibit haem biosynthetic enzymes at the same level as the genetic defects which are the basis of the disorders in humans (ALAD, ferrochelatase and uroporphyrinogen decarboxylase). Other chemicals such as DDC and its analogues, AIA and phenobarbitone are used, not to mimic a genetic defect, but to create an environment similar to that which may exist as a result of the genetic block in the pathway. The existence of a haem deficient state has been suggested as the underlying cause of acute porphyric neuropathy. All the agents mentioned above in some way contribute to the development of such an environment by partially blocking the synthesis of haem (succinylacetone, lead, N-alkylated protoporphyrin 1X), by destruction of haem already synthesized (AIA, DDC and 4-ethyl DDC) or by diverting newly synthesized haem into the increased production of one particular haemoprotein (phenobarbitone). The last two manipulations disrupt the equilibrium of synthesis and when combined with a chemical block in the pathway provide a means of producing a model of a haem deficient state.

The aim of this part of the study was to produce an animal model of the proposed haem deficiency of porphyria. Following treatment with porphyrinogenic agents, either alone or in combinations, the functional capacity of tissues can be compared with biochemical correlates. Urinary ALA excretion provides an indication of a change in haem availability whereas the responses of nerve and muscle tissue gives a measure of functional capacity. In this part of the study, following short, medium or long term treatment with these

porphyrinogenic drugs, a range of nerve/muscle preparations were examined for evidence of a developing neuropthy. The tissues examined consisted of cholinergically-mediated skeletal muscle contraction, noradrenergically-mediated motor responses, NANC-mediated inhibitory responses and purinergically-mediated responses. The examination of a wide range of tissues covers the possibility of differential sensitivity of these systems to a state of haem deficiency. Since the intention was to deplete haem proteins in nerve and muscle, the duration of drug administration was linked to to the turnover time of haem proteins which varies from a few hours, in the case of tryptophan pyrrolase and cytochrome P-450 to 60 days for rat haemoglobin. A range of treatment durations from 3 days to 44 days was, therefore, included.

In this section the hypothesis that porphyric neuropathy is due to a deficiency in haemoproteins essential for normal nerve function was, therefore, assessed using a chemically produced animal model.

CHAPTER 3

THE EFFECTS OF PORPHYRINOGENIC DRUGS ON NERVE AND MUSCLE.

METHODS.

3.2.1. DRUGS:

4,6-Dioxoheptanoic acid (succinylacetone); phenobarbitone (the powder was dissolved in 0.1 M NaOH and brought to pH 9. Dropping below this pH brought the compound out of solution); Ammonium hydroxide (BDH, Scotland); ethanol (BDH, Scotland); lead tetraacetate; Ethylacetoacetate; Prop ionaldehyde; Dimethyl sulfoxide (DMSO); Glycerine trinitrate; sodium hydroxide. Unless stated otherwise all the above chemicals were obtained from Sigma Co. Ltd. England. Diethoxycarbonyl 2,4,6-trimethyldihydropyridine (DDC) and Allylisopropylacetamide were gifts from Anthony Gibbs, MRC Toxicology Unit, Carsholton. 4-ethyl DDC was synthesized as described below. A source of 4-ethyl DDC was also obtained from Professor G.S. Marks, University of Alberta, Canada.

3.2.2. SYNTHESIS OF 4-ETHYL DIETHOXYCARBONYL 2,6-DIMETHYL 4-ETHYL DIHYDROPYRIDINE (4-ETHYL DDC).

3.2.2.1. Reagents required to make 1 mole of 4-ethyl DDC:

1. Absolute ethanol		60ml.
2. Ethylacetoacetate (M.W. 130.14)	(0.2 mole)	25.4ml.
3. Pro p ionaldehyde (M.W. 58.08)	(0.1 mole)	7.2ml.
4. Ammonium hydroxide (M.W. 35.05) (28-30% NH_3 by weight)	(0.1 mole)	6.74ml.

3.2.2.2. Method:

The reagents were added in the order listed above to a round bottom flask in a fume cupboard and refluxed at a gentle boil for 3-4hrs. The

resultant solution was poured into a 500ml. Erlenmeyer flask and 250ml. of cold distilled water added. The reaction flask was rinsed with cold 95% ethanol and added to the reaction mixture which was allowed to stand at 4^0C for 3 days after scratching the bottom of the flask and seeding with a few crystals of 4-ethyl DDC. The crystals were harvested by filtration and washed with cold 40% ethanol. They were allowed to air dry and then placed in an open Petri dish in a vacuum desiccator for a further 4 days. The 4-ethyl DDC crystals were then recrystalised from water and ethanol. 50ml. of 95% ethanol was added to the compound and heated to a gentle boil. Boiling distilled water was added dropwise. After each addition of water the cystals come out of solution temporarily and the water was added until the point of saturation is reached. A few drops of 95% ethanol was then added to clear the solution. The liquid was cooled on ice, the bottom of the vessel scratched and left to stand overnight at 4^0C. The crystals formed were again harvested by filtration, washed with cold 40% ethanol and air dried. Vacuum desiccating then took place in an open Petri dish overnight. This recrystalisation procedure was carried out once more before final harvesting of the 4-ethyl DDC crystals. The melting point of the crystals was determined (110^0C). Gas chromatographic analysis of the synthesized product was compared with that of a sample of 4-ethyl DDC from the external source.

3.2.3. Treatments:

Male wistar rats (bred in the Pharmacology Animal Unit of Glasgow University) ranging from 200-300gm. at the start of the experiments served as subjects in these experiments. All injections in this group of experiments were administered intraperitoneally in a volume of 1ml/kg. except for the DDC, 4-ethyl DDC and the AIA which were administered in DMSO vehicle in a volume of 0.5ml/kg. Nine combinations of drug and treatment duration were tested.

1) Animals were administered succinylacetone at a dose of 40mg./kg. twice daily for 3 days.

2) A combination of succinylacetone (40mg./kg), allylisopropylacetamide (200mg./kg) and phenobarbitone (80mg./kg.) were administered daily over a period of 9 days.

3) Animals received 30 days of succinylacetone (20mg./kg.) followed by 6 days of phenobarbitone (80mg./kg.) and a final dose of 300mg./kg. 4-ethyl DDC. Four days elapsed after the 4-ethyl DDC before the animals were sacrificed. Succinylacetone administration was continued throughout the treatment period .

4) This treatment was the same as 3) above except that DDC replaced 4-ethyl DDC.

5) Animals in this group received 30 days of daily succinylacetone (20mg./kg.) followed by 10 days of 4-ethyl DDC at 25mg./kg., phenobarbitone (80mg./kg.) for 4 days with a final dose of 300mg./kg. of 4-ethyl DDC. Succinylacetone administration was continued throughout the treatment period.

6) This treatment regime was similar to that of 5) above except DDC replaced 4 ethyl-DDC.

7) Lead tetraacetate was administered at a dose of 50μMoles per day for 14 days.

8) Animals were injected with DDC (200mg/kg) on three alternate days. On these days succinylacetone was also administered twice daily at a concentration of 40 mg/kg.

Following the last injection in all the above groups the animals were placed in metabolic cages and their urine collected over the 24 hrs immediately prior to sacrifice. Urinary ALA levels were determined.

9) Three groups of mice weighing between 30-50gm. were treated for 6 days with succinylacetone at a concentration of 40mg/kg. In addition, the animals received AIA (200mg/kg), DDC (25mg/kg) or 4-ethyl DDC (25mg/kg).

3.2.4. RAT TISSUE:

Animal were stunned, bled and innervated muscle preparations removed in the following order.

a) Phrenic nerve/diaphragm:

The rat diaphragm receives a motor cholinergic innervation via the phrenic nerve and ATP is a putative co-transmitter in producing the contractile response of this preparation.

A midline incission was made over the sternum and the skin and pectoral muscles reflected to expose the ribs. The upper abdomen was opened and a pair of strong scissors inserted from there severing the ribs on either side of the sternum. The sternum was removed. The rib cage on either side was removed by cutting just above and parallel to the first rib and then through the ribs cranially near the vetebrae. The two lungs were removed from the thoracic cavity and the right and left phrenic nerves identified as they passed behind the heart. Each nerve was ligatured close to the heart and carefully freed down to their entry into the diaphragm. The whole diaphragm plus the phrenic nerves were removed to a Petri dish containing Kreb's and a mideline incission divided the diaphragm into two innervated preparations. The phrenic nerve was placed over a pair of Ag/AgCl ring electrodes recessed in a Perspex electrode assembly. The diaphragm was laid on top of the electrode assembly and the lower ribs, to which the diaphragm was connected, pressed onto securing pins. A ligature connected the apex of the hemidiaphragm to an isometric strain gauge. The whole preparation was transfered to a 50ml. isolated organ bath containing warm Kreb's solution and the muscle placed under an initial 4gm. of tension. The preparation was left to equilibrate for 30min. before the start of the experiment.

b) Vas deferens:

The rat vas diferens receives a dense adrenergic innervation from sympathetic nerves originating in the lumbar vertebrae L1-L4. (McGrath 1978). Two transmitters are released from the nerves

innervating this tissue and the response is mediated by the combined actions of noradrenaline (Swedin 1971; McGrath 1978; Sneddon and Westfall 1984) and ATP (Sneddon and Westfall 1984; Meldrum and Burnstock 1983).

The abdomen was opened by a mid-line incision, the testicles pushed into the abdominal cavity and the epididymi identified. The vas deferens were tied and cut at their junction with the epidydimus. The tissue was freed of connective tissue along its length to its junction with the prostate. This prostatic end was tied, cut and the two vas deferens removed to a Petri dish containing Kreb's solution. The epidydimal half of the vas deferens was used. The epididymal end of the tissue was secured to a hook electrode and the tissue passed through a single Ag/AgCl ring electrode in a 10ml. organ bath containing Kreb's solution at 37^{o}C. A thread connected the other end of the tissue to an isometric strain gauge. The tissue was placed under an initial 1g. of tension and left to equilibrate for 30 min.

c) Anococcygeus:

The rat anococcygeus muscle possesses a dense noradrenergic motor innervation and an NANC inhibitory innervation (Gillespie 1972).

The paired rat anococcygeus muscles originate from the first two coccygeal vertebrae in the mid-line of the pelvic cavity. They pass on either side of the colon, merge onto the ventral surface of the colon and join to form a longitudinal bar which passes into the skin of the peritoneum. The muscles form a short transverse bar merging on the ventral surface of the colon. The extrinsic nerves pass in a branch of the perineal nerve on either side to enter each muscle just short of the formation of the ventral bar.

The abdomen was opened, the symphysis pubis split and the pelvic arch forced apart. The anococcygeus muscles were identified behind the colon and the right and left muscles ligatured at the ventral bar. The two muscles were separated at this point, freed from the colon and cleaned up to their origin from the coccygeal vertebrae. This end was tied, cut and the muscles transfered to a Petri dish containing Kreb's solution. Two preparations approximately 2cm. long and 2-3mm. wide resulted. Each muscle was drawn through a Ag/AgCl ring

electrode, one end of the muscle secured to a similar hook electrode and a thread attached the other end to an isometric strain gauge. The whole assembly was mounted in a 10ml. organ bath and 1gm. of initial tension placed on the tissue. The preparation was left to equilibrate for 30 min.

d) Tail artery:

The pressor response of the rat tail artery, like that of the rabbit central ear artery, is mediated by the co-transmitters noradrenaline and ATP (Sneddon and Burnstock 1984).

The tail was severed from the body and the skin peeled from the tail. The channel in which the tail artery lies was identified on the ventral surface of the tail. The artery was ligatured at the base of the tail, cleaned and removed from the channel in which it lies. The tissue was transfered to a Petri dish and the artery cannulated with a plastic cannulae whose diameter was large enough not to contribute significantly to the total peripheral resistance of the system. 2cm. portions of the artery were drawn through a pair of Ag/AgCl ring electrodes. The cannula was attached to a Watson and Marlow Peristaltic Pump and the tissue perfused at 3ml./min. with warmed Kreb's soltution. The whole assembly was placed in a 25ml. organ bath containing Kreb's and the perfusion pressure measured by a Statham pressure transducer attached to a side arm of the assembly.

3.2.5. Mouse tissue.

Mice were killed by a blow to the head and exanginated. The vas deferens were dissected out of the animal and prepared in a similar manner to that described for the rat vas deferens.

In all tissues in this section field stimulation was by 20-100 pulses at supramaximal voltage between 0.5 and 32 HZ depending on the frequency sensitivity of the tissue.

3.2.6. Urinary ALA was determined by the method of Mauzerall and Granick (1956).

CHAPTER 3

THE EFFECTS OF PORPHYRINOGENIC DRUGS ON NERVE AND MUSCLE.

RESULTS.

Chapter 3 PORPHYRINOGENIC TREATMENTS.

All injections were made intraperitoneally. succinylacetone and lead tetra acetate injections were administered in a volume of 1ml/kg. in distilled water vehicle. Phenobarbitone was dissolved in 0.1M NaOH and brought to pH 9 with 0.1MHCl and was also administered in a volume of 1ml/kg. AIA, DDC and 4-ethyl DDC were administered in a volume of 0.5ml/kg. in dimethyl sulfoxide (DMSO).

TREATMENT 1.1: Rats received succinylacetone at a dose of 40mg/kg. twice daily for 3 days.

TREATMENT 1.2: 9 days administration of a combination of AIA (200mg/kg.), phenobarbitone (80mg/kg.) and succinylacetone (40mg/kg).

TREATMENT 1.3: 30 days of succinylacetone (20mg/kg.) followed by 6 days of phenobarbitone (80mg/kg.) then 1 injection of 300mg/kg. of 4-ethyl DDC. Succinylacetone (20mg/kg.) was administered throughout the treatment and the animals were sacrificed 4 days after 4-ethyl DDC administration.

TREATMENT 1.4: 30 days of succinylacetone (20mg/kg.) followed by 6 days of phenobarbitone (80mg/kg.) then 1 injection of 300mg/kg. of DDC. Succinylacetone (20mg/kg.) was administered throughout the treatment and the animals were sacrificed 4 days after DDC administration.

TREATMENT 1.5: 30 days of succinylacetone (20mg/kg.) followed by 10 days administration of 4-ethyl DDC (25mg/kg.) then 4 days of phenobarbitone (80mg/kg.) with a further 300mg/kg of 4-ethyl DDC 24 hours prior to killing.

TREATMENT 1.6: 30 days of succinylacetone (20mg/kg.) followed by 10 days administration of DDC (25mg/kg.) then 4 days of phenobarbitone (80mg/kg.) with a further 300mg/kg of DDC 24 hours prior to killing.

TREATMENT 1.7: for 14 days rats received 50μmoles of lead tetra acetate.

TREATMENT 1.8: rats received 200mg/kg. 4-ethyl DDC on 3 alternative days in addition to two injections of succinylacetone (40mg/kg.).

TREATMENT 1.9: Three groups of mice received 6 days of intraperitoneally-administered succinylacetone (40mg./kg.) in addition to either, AIA (200mg./kg.), DDC (25mg./kg.) or 4-ethyl DDC (25mg./kg.)

3.3.1. Synthesis of 4-ethyl DDC:

Gas chromatography and mass spectrography of the synthesized 4-ethyl DDC was identical to that of 4-ethyl DDC synthesized in the laboratory of Professor G. Marks, University of Alberta, Canada where this compound is produced on a regular basis (figure 15). The compound synthesized was, therefore, 4-ethyl DDC.

3.3.2. Urinary ALA levels:

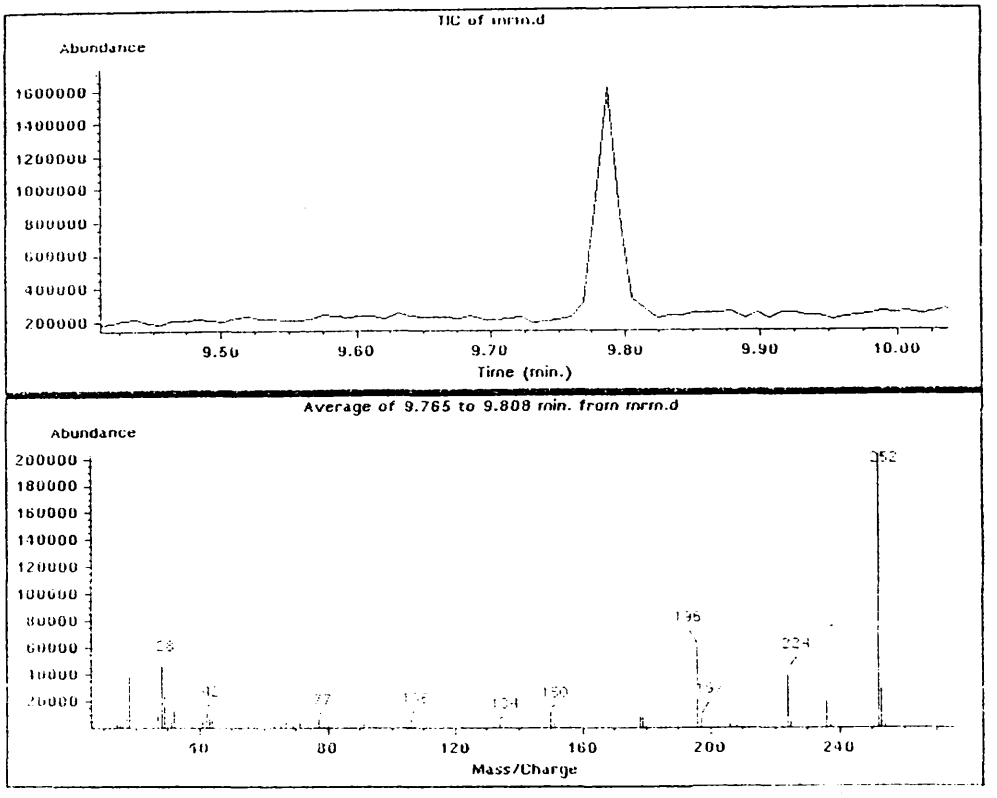
Rats that received porphyrinogenic treatments 1-8 excreted significantly increased concentrations of the urinary haem precursor ALA (figure 16). 3 Days treatment with succinylacetone was the most potent inducer of ALA excretion (1000μM. ALA/24hr.) with treatment 1.2, 9 days of succinylacetone, AIA and phenobarbitone the next most potent in inducing increased ALA excretion (572μM. ALA/24hr.). The long term combinations of porphyrinogenic drugs, although to a smaller extent, also caused a significant increase in ALA excretion. Although significantly increasing urinary ALA (62μM./24hrs compared to normal rat values of 12μM./24hrs), lead tetraacetate was least effective in its ability to increase urinary ALA excretion. These increases in urinary excretion of ALA were taken as an indication of the effectiveness of the drugs in reducing free haem pool which normally exerts a negative feedback inhibition on ALA synthase.

3.3.3. Neurogenic mediation of the responses of the isolated innervated muscle preparations:

Electrical field stimulation of the intrinsic nerves of the anococcygeus muscle, the vas deferens and the tail artery induced a motor response. This contraction was inhibited by the neuronal blocking agent, tetrodotoxin ($3x10^{-7}$M), confirming that the responses were neurogenically-mediated (figure 17).

Figure 15: Figure a) shows a gas chromatograph (top trace) and a mass spectrogaph (bottom trace) of a sample of 4-ethyl DDC from an external source of regularly synthesized compound. Figure b) shows a gas chromatograph (top trace) and a mass spectrograph (bottom trace) of the 4-ethyl DDC synthesized in this laboratory by the method specified. Both samples are identical.

a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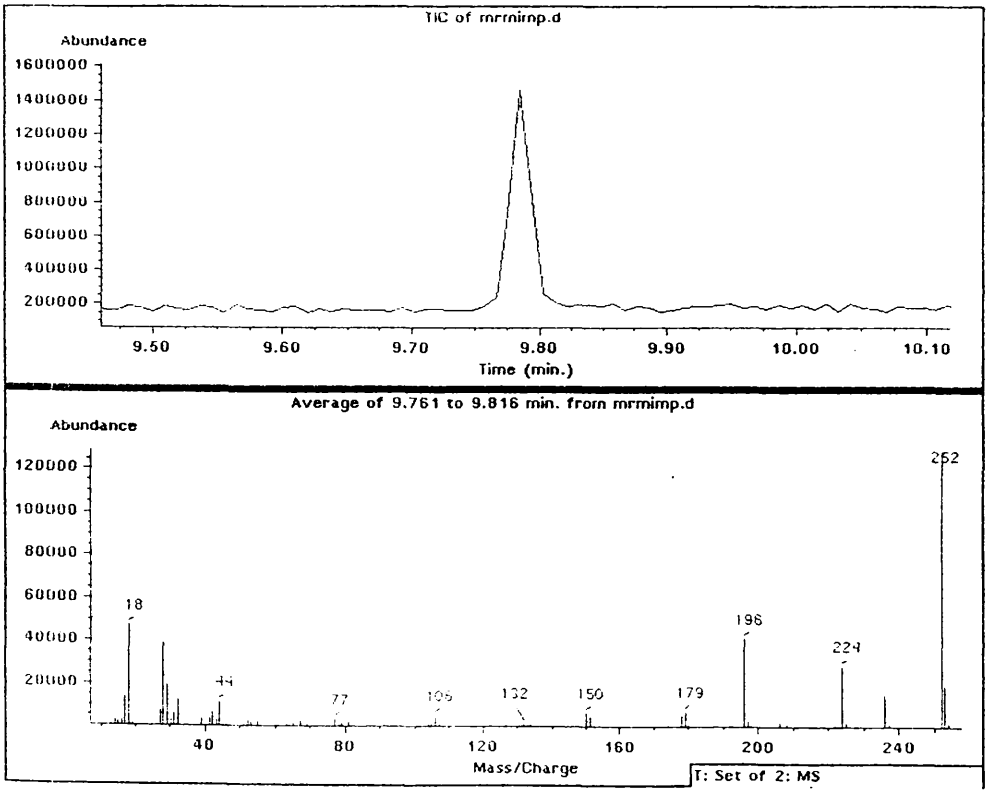
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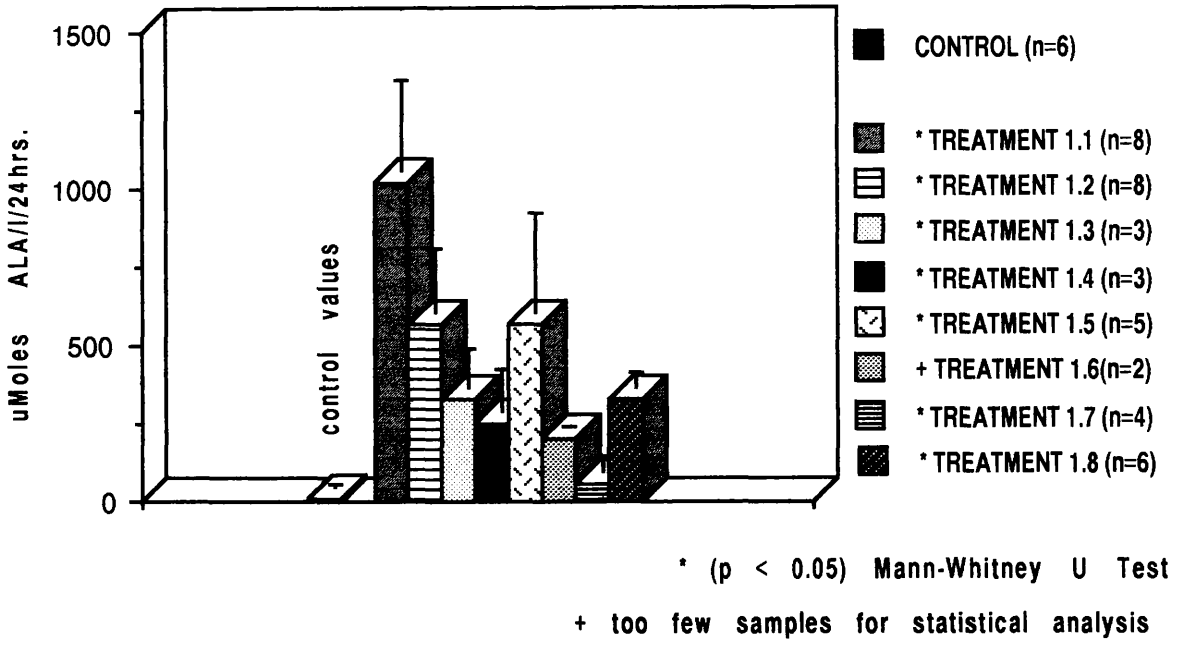


Figure 16: The mean urinary excretion of ALA (μMoles/l/day) of animals that had received 8 different porphyrinogenic treatments.

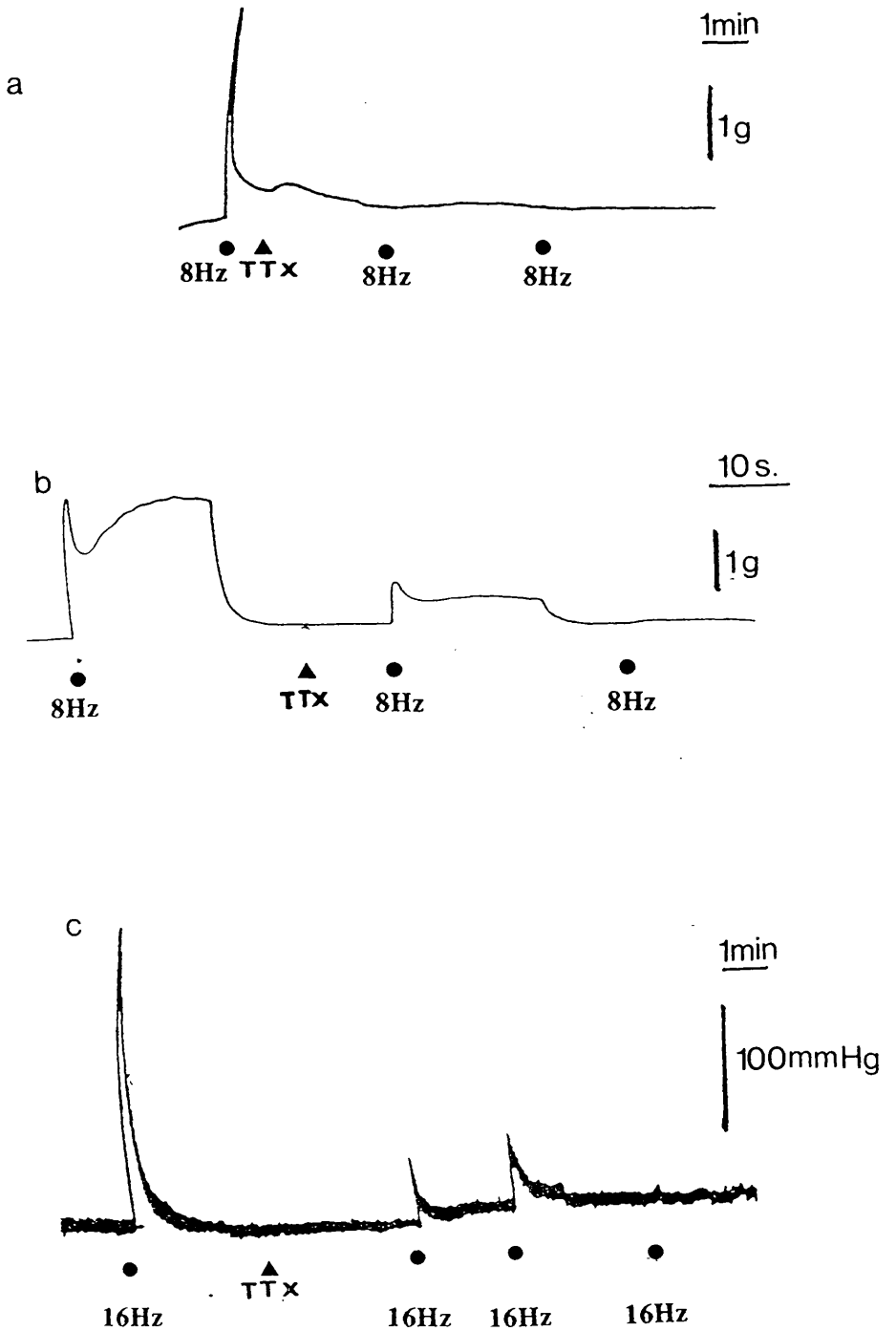


Figure 17 :The effects of $3x10^{-7}$M tetrodotoxin (TTX), in single experiments on the responses of a) the rat anococcygeus muscle to stimulation with 50 pulses at 8Hz, b) the rat vas deferens to a 20s. train of pulses at 8Hz and c) the perfused rat tail artery to stimulation with 100 pulses at 16Hz.

3.3.4. The effects 3 days succinylacetone treatment (1.1) on the responses of a range of innervated muscle preparations:

3.3.4.1 The effects of agonists:

a) The effects of phenylephrine on the responses of the rat annococygeus muscle:

During the equilibration period, following the set up of the anococcygeus muscle, the initial 1g. tension placed on the muscle decayed to approximately 0.5-0.75g. Phenylephrine in concentrations ranging from $3x10^{-7}M$ to $3x10^{-5}M$ caused a dose-dependent contraction of the muscle which reached a maximum at $10^{-5}M$ (figure18). The responses of tissues from animals treated for 3 days with succinylacetone (treatment 1.1) did not differ significantly from the responses of tissues from control animals (figure 18).

b. The effect of phenylephrine on the rat vas deferens muscle:

$3x10^{-5}M$ phenylephrine induced a mean contraction of 1.09+/ 0.1g. in tissues from treated animals which does not differ significanlty from the responses of tissues from control animals 1.30 +/- 0.1g. (figure 19b).

c. The effects of sodium nitroprusside on the responses of the anococcygeus muscle:

When the tone of the anococcygeus muscle is raised with $3x10^{-5}M$ guanethidine, $10^{-7}M$ sodium nitroprusside causes a reduction in the induced tone of approximately 50%. This reduction in muscle tone is similarly propossed to be mediated via activation of guanylate cyclase (Waldman and Murad 1987). This compound caused a reduction in the guanethidine-induced tone in tissues from treated animals which did not differ significantly from the effect produced in tissues from control animals (figure 20).

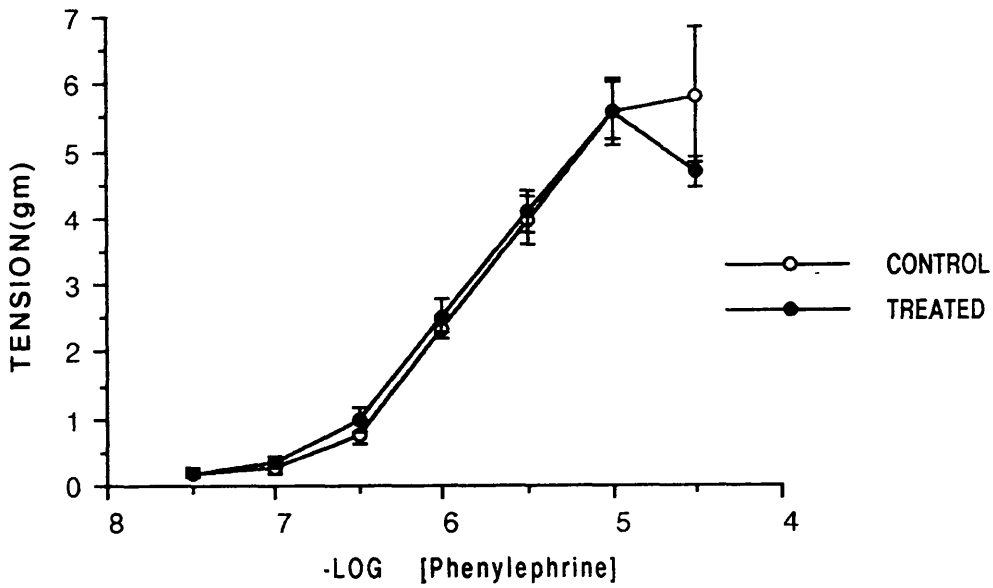


Figure 18: Shows the mean +/- S.E.M. (gm. of tension) response of the rat anococcygeus muscle to concentrations of phenylephrine ranging from 3x10-8M. to 3x10-5M., in tissues from control animals (n=6) and in tissues from animals that had received twice daily succinylacetone administration (40mg/kg) for 3 days (n=6). This treatment had no effect on the responses of the anococcygeus muscle to the agonist phenylephrine.

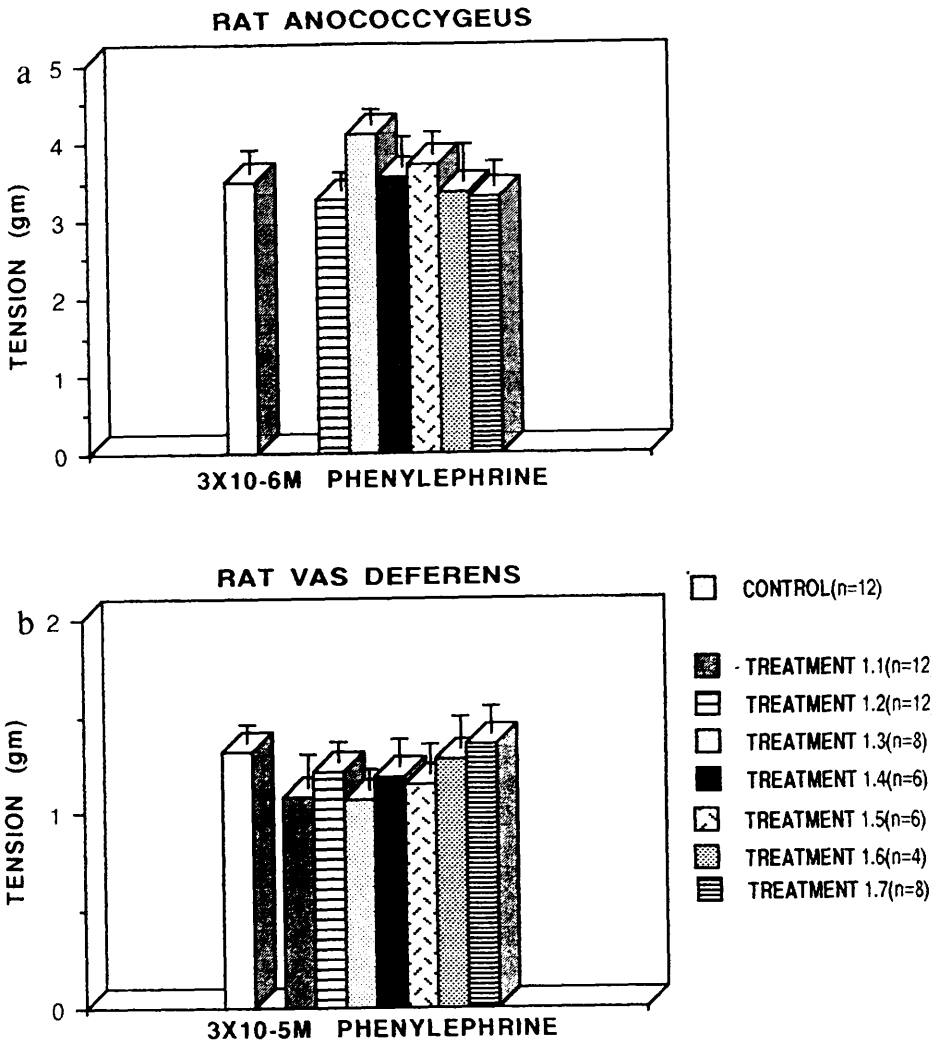


Figure 19 : The top graph shows the mean +/- S.E.M. contraction, in gm., of the rat anococcygeus muscle to $3x10^{-6}M$ phenylephrine in tissues from animals in 6 treatment groups. The lower graph shows the mean +/- S.E.M. of the contraction elicited in the vas deferens by $3x10^{-5}M$ phenylephrine in 7 treatment groups. None of the porphyrinogenic drug treatments altered the responses of either tissue to the noradrenergic agonist phenylephrine.

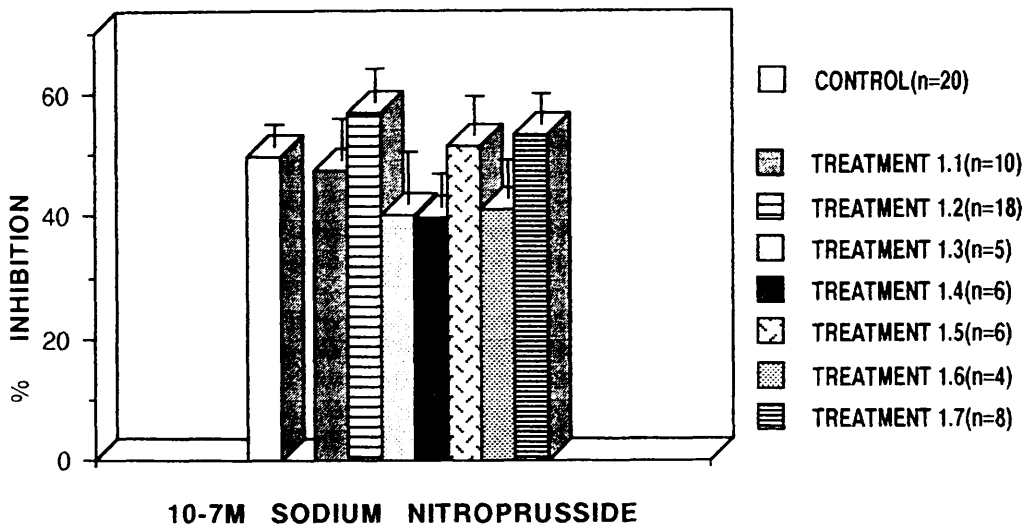


Figure 20: The mean (+/- S.E.M.) % inhibition of guanethidine-induced tone of the rat anococcygeus muscle caused by 10^{-7}M. sodium nitroprusside in tissues from animals of the 7 treatment groups. None of the porphyrinogenic drug treatments significantly altered the response of the anococcygeus to this activator of soluble guanylate cyclase.

3.3.4.2. Electrical stimulation of the nerve/muscle preparations:

d. Anococcygeus Motor Response:

During the equilibration period, following the set up of the anococcygeus muscle, the initial tension of 1g. placed on the tissue decayed to approximately 0.5 to 0.75g. Electrical field stimulation of the intrinsic nerves of the anococcygeus muscle (0.5ms. duration, supramaximal voltage, 50 pulses at 0.5-32Hz) induced a noradrenergically-mediated frequency-dependent contraction of the muscle (Gillespie 1972). Porphyrinogenic treatment 1.1 (3 days of twice daily succinylacetone at 40mg/kg.) had no significant effect on the responses of this tissue when compared to tissues from control animals (figures 21, 22a).

e. Anococcygeus Inhibitory Response:

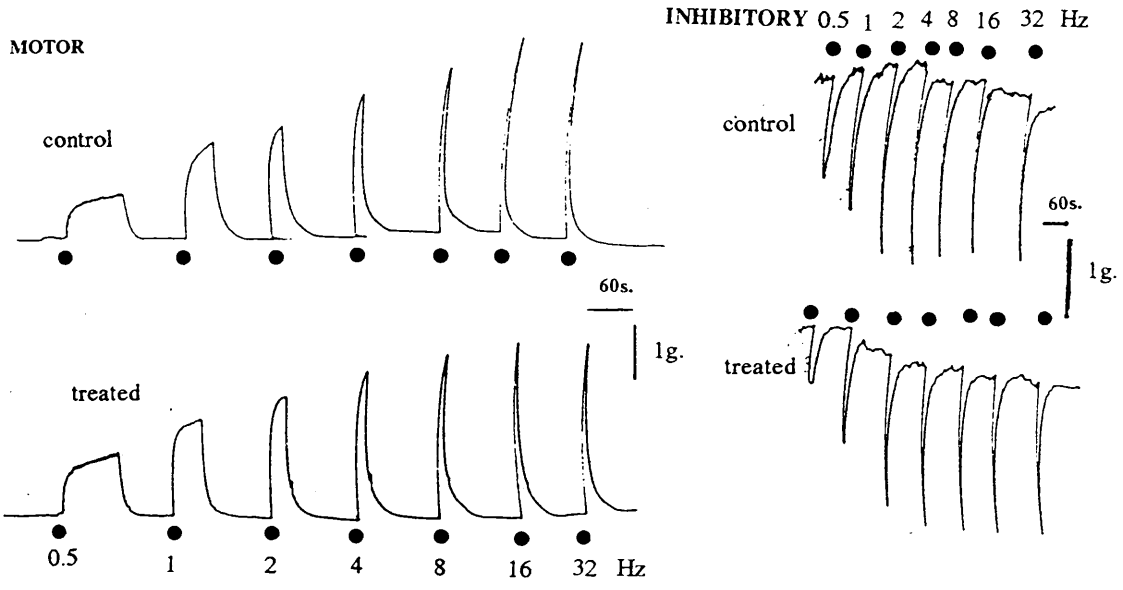
When the tone of this tissue is raised by $3x10^{-5}M$ guanethidine an NANC-mediated inhibitory response is revieled (Gillespie 1972). Upon field stimulation of the intrinsic nerves of the tissue (0.5ms. duration, supramaximal voltage, 20 pulses 0.5-32Hz) a frequency-dependent inhibition of the induced tone results. The porphyrinogenic treatment in this group did not significantly alter the inhibitory response of this tissue when compared to control tissues (figures 21, 22b).

f. Vas Deferens motor response:

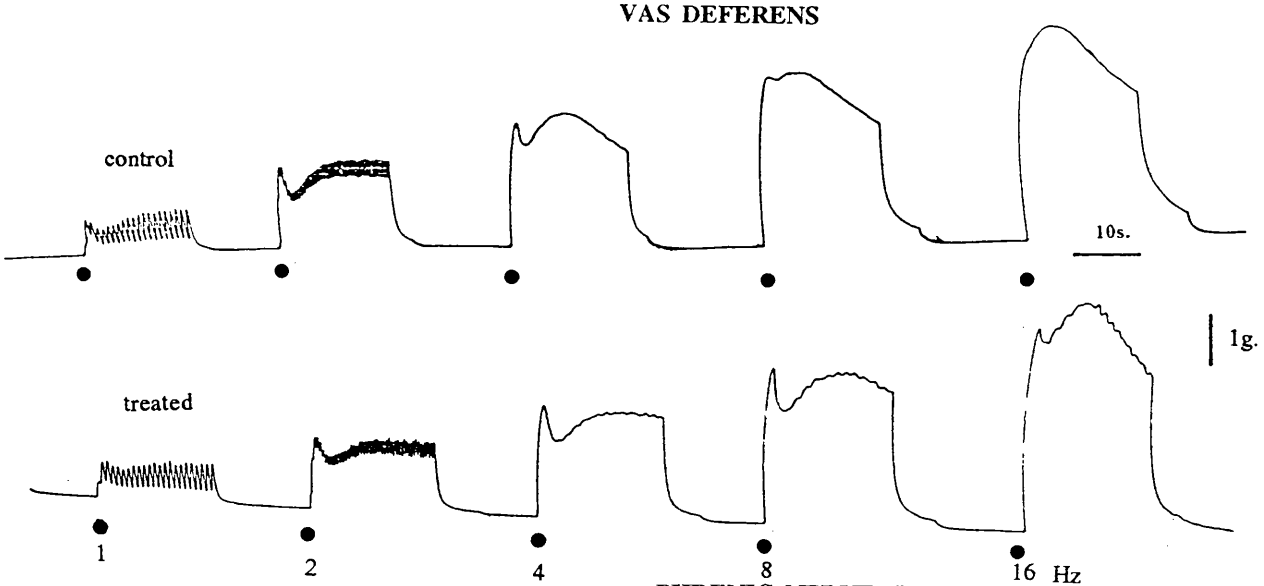
During the equilibration period the initial 1g. of induced tension decayed to 0.5-0.75g. Field stimulation of the intrinsic nerves (0.5ms. duration, supramaximal voltage, 20s. train of 1-16Hz) produced a frequency-dependent byphasic response. This response consisted of an initial purinergically-mediated "fast" component and a secondary noradrenergically-mediated "sustained" component (Swedin 1971; Sneddon and Westfall 1984). Neither of these components were adverseley affected by the porphyrinogenic treatment of this group when compared to control tissue responses (figures 21, 23a, 23b).

Figure 21: The effects of porphyrinogenic treatment 1.1 (3 days of twice daily succinylacetone 40mg/kg) on the five tissue responses, in individual experiments (bottom traces), compared to the responses from individual control tissue responses (top traces). The time bars apply during the stimulation period only and the interstimulus interval in all cases was 3 min. Stimulation frequencies are shown above or below each response. For clarity only the frequencies for one tissue are shown, but both control and treated tissues received the same stimulation parameters. The top left hand traces show the motor response of the rat anococcygeus muscle to stimulation with 50 pulses and the traces on the top right are the inhibitory response of the anococcygeus muscle to stimulation with 20 pulses. The middle traces show the response of the rat vas deferens to field stimulation with a 20s. train of pulses. The bottom left hand traces show the response of the perfused rat tail artery to field stimulation with 100 pulses. The bottom right hand traces shows the response of the rat hemidiaphragm to phrenic nerve stimulation with 50 pulses. This treatment did not significantly alter the responses of any of these tissues to nerve stimulation.

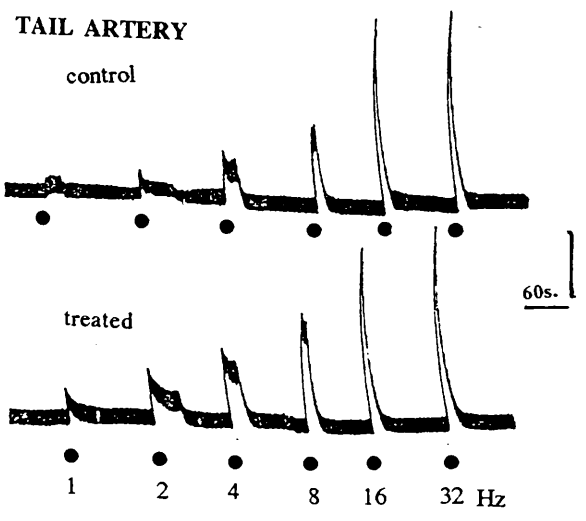
ANOCOCCYGEUS



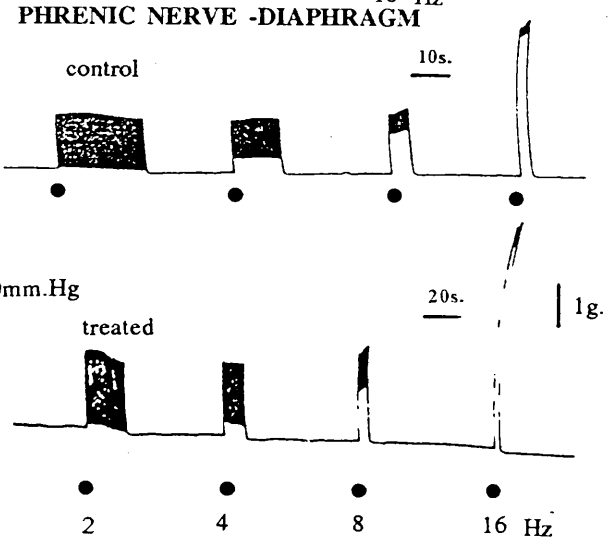
VAS DEFERENS



TAIL ARTERY



PHRENIC NERVE -DIAPHRAGM



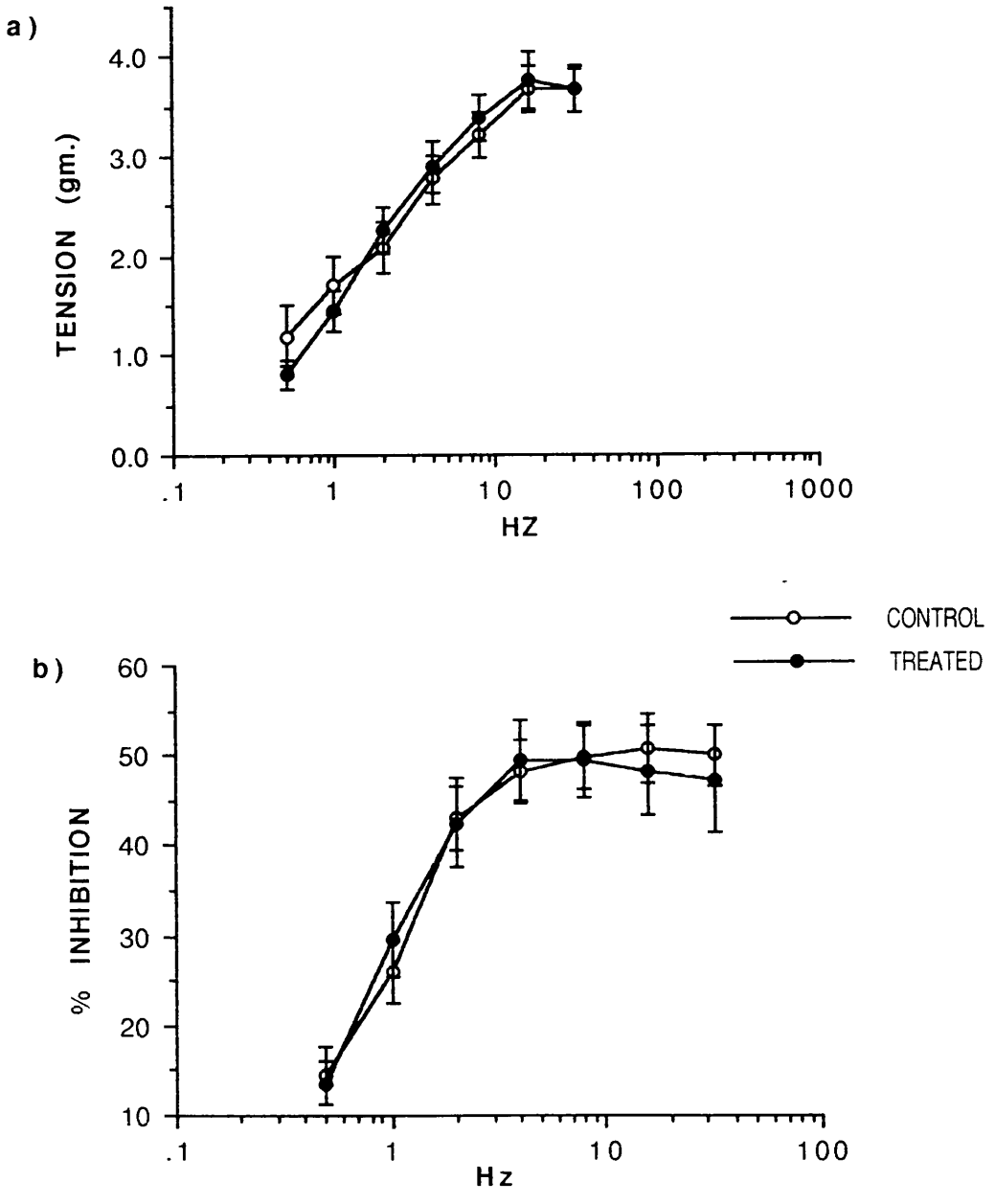


Figure 22: Graph a) shows the mean +/- S.E.M. of the motor response the rat anococoocygeus muscle, in gm. tension, to stimulation with 50 pulses at 0.5-32 Hz in tissues from control animals (n=14) and in tissues from animals that had received 3 days twice daily succinylacetone (40mg/kg.) treatment (n=14). Graph b) shows the inhibitory response of the anococcygeus muscle to intrinsic nerve stimulation with 20 pulses at 0.5-32 Hz in tissues from control animals (n= 14) and treated animals (n=10)

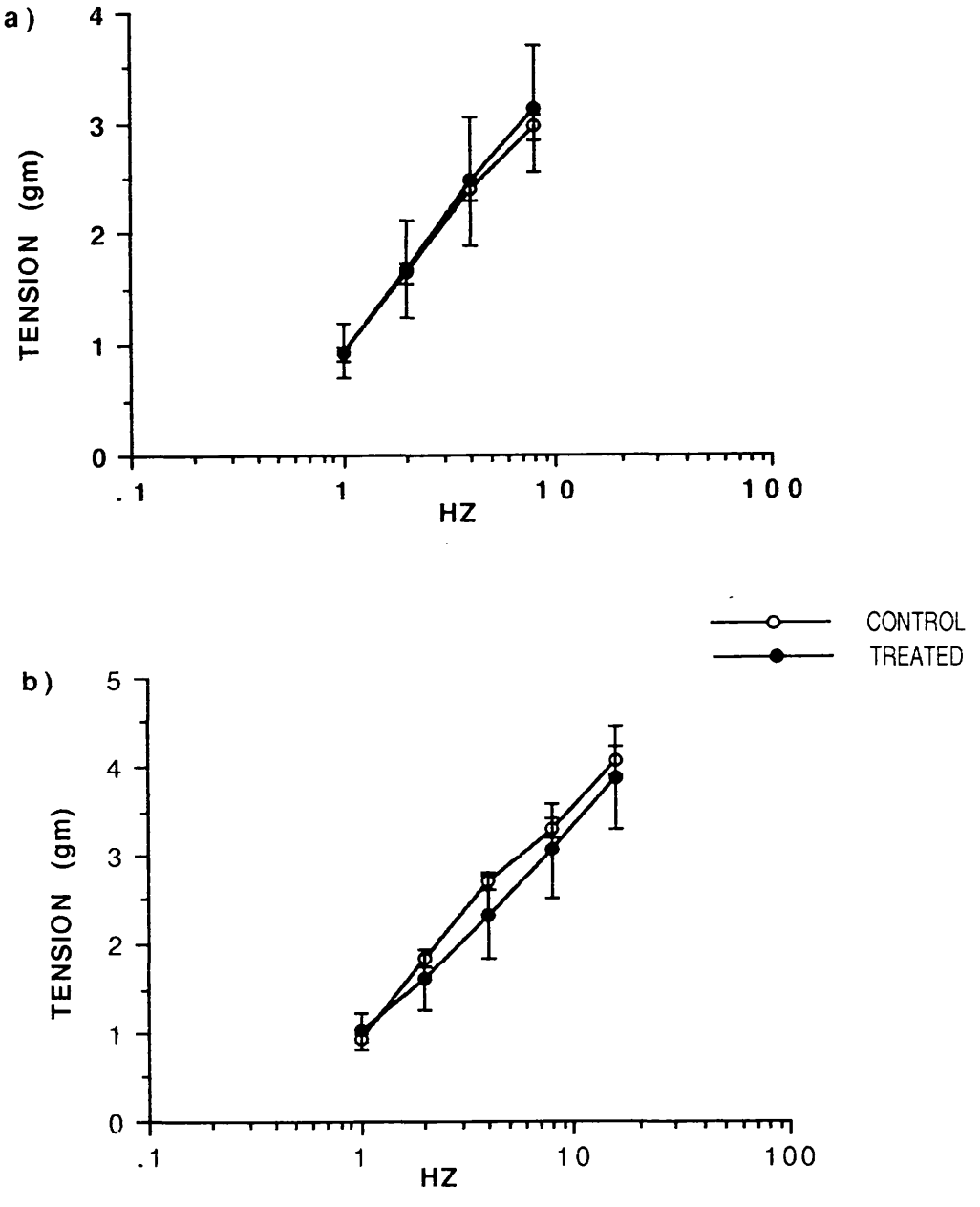


Figure 23: Graph a) shows the mean +/- S.E.M. of the "fast" component of the rat vas deferens motor response, in gm. tension, to stimulation for 20s. at 1-8 Hz in tissues from control animals (n=20) and in tissues from animals that had received 3 days of succinylacetone (40mg/kg.) twice daily (n=6). Graph b) shows the mean "sustained" component of the rat vas deferens response to intrinsic nerve stimulation with a 20s. train of pulses at 1-16 Hz in tissues from control animals (n=20) and treated animals (n=6).

g. Tail artery pressor response:

During the equilibration period the rat tail artery settled down with a perfusion pressure of approximately 25mm. Hg. Electrical field stimulation of the intrinsic nerves of this tissue (0.5ms. duration, supramaximal voltage, 100 pulses at 1-64Hz) produced a frequency-dependent motor response, mediated by the co-transmitters Adenosine triphosphate (ATP) and noradrenaline (NA) (Sneddon and Burnstock 1984). The porphyrinogenic treatment of this group of animals did not significantly alter the response of the isolated rat tail artery to field stimulation when compared to the responses from tissues from control animals (figures 21, 24a).

h. Phrenic nerve diaphragm:

Upon equilibration, the initial tension of 4g. placed on the hemidiaphragm decayed to approximately 2g. Electrical stimulation of the phrenic nerve (0.5ms. duration, supramaximal voltage, 50 pulses at 2-16Hz) produced a frequency-dependent rapid contraction of the diaphragm. In addition to the cholinergically-mediated contraction of the rat diaphragm to phrenic nerve stimulation, ATP has been proposed as a putative co-transmitter at this neuromuscular junction (Silinsky and Hubbard 1973). The responses of the hemidiaphragm preparations from animals that had received porphyrinogenic treatment did not differ significantly from the responses of the tissue from control animals (figures 21, 24b).

3.3.5. The effects of drug treatment 1.2 (9 days succinylacetone, AIA and Phenobarbitone) on the responses of a range of innervated muscle preparations:

3.3.5.1. The effects of agonists:

a. The effect of phenylephrine on the rat anococcygeus muscle:

At a concentration of $3x10^{-6}M$, phenylephrine induces a contraction of the anococcygeus muscles, from treated animals, of 3.27+/- 0.3g.(about 70% of the maximum possible response (figure 18) which does

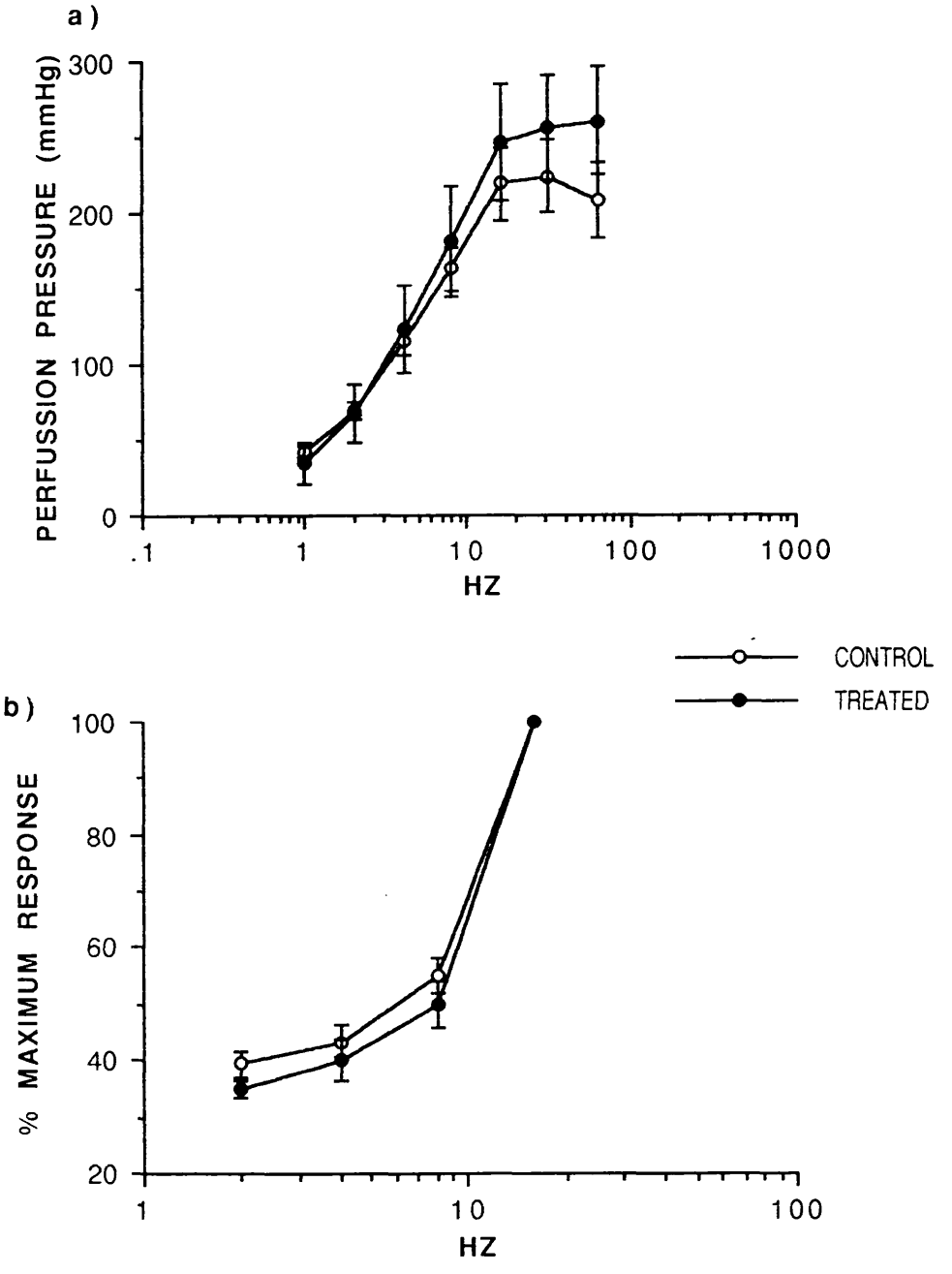


Figure 24: Graph a) shows the mean (+/- S.E.M.) pressor response of the perfused tail artery to intramural nerve stimulation with 100 pulses at 1-64 Hz in tissues from control animals (n=6) and from animals that had been treated for 3 days with twice daily succinylacetone (40mg/kg.). Graph b) shows the mean (+/- S.E.M.) of the response of the rat hemidiaphragm to phrenic nerve stimulation as the % of the maximum response achieved at 16 Hz in control animal tissue (n=8) and in tissues from treated animals (n=6). The responses of these two tissue were not affected by this porphyrinogenic drug treatment.

not differ significantly from the contraction of 3.48 +/- 0.3g. that this compound elicits in tissues from control animals (figure 19).

b. The effect of glycerine trinitrate on the response of the anococcygeus muscle:

When the tone of the anococcygeus muscle is raised by $3x10^{-5}M$ guanethidine, glycerine trinitrate (GTN) in concentrations ranging from $10^{-9}M$ to $10^{-6}M$ produces a dose-dependent reduction in the induced tone. The action of this nitrovasodilator is mediated via the formation of nitric oxide from the glycerine trinitrate which in turn activates the haemoprotein, guanylate cyclase causing a relaxation of the muscle (Marks 1987). The response of tissues from treated animals, to GTN, did not differ significantly from the responses in control animals (figure 25).

c. The effects of sodium nitroprusside on the responses of the anococcygeus muscle:

When the tone of the anococcygeus muscle is raised with $3x10^{-5}M$ guanethidine, $10^{-7}M$ sodium nitroprusside causes a reduction in the induced tone of approximately 50%. This reduction in muscle tone is similarly proposed to be due to activation of guanylate cyclase (Waldman and Murad 1987). This compound caused a reduction in the guanethidine-induced tone in tissues from treated animals which did not differ significantly from the effect produced in tissues from control animals (figure 20).

d. The effect of phenylephrine on the rat vas deferens muscle:

3x10-5M phenylephrine induced a mean contraction of 1.21+/ 0.08g. in tissues from treated animals which does not differ significanlty from the responses of tissues from control animals 1.30 +/- 0.1g. (figure 19).

3.3.5.2. Electrical stimulation of the nerve/muscle preparations:

The stimulation parameters were the same as those specified for Treatment Group 1.1, 3 days of succinylacetone.

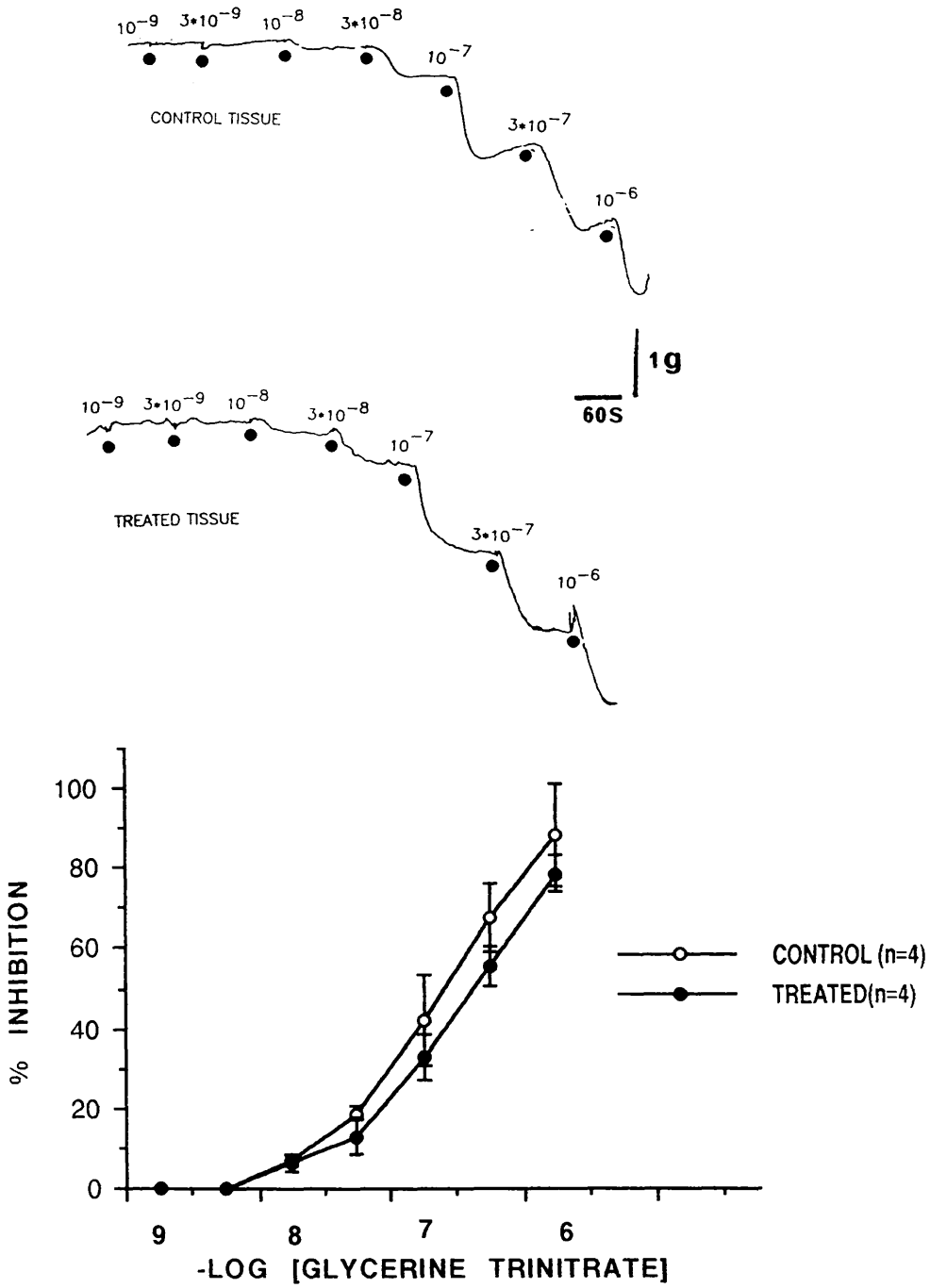


Figure 25: The effect of increasing concentrations of glycerine trinitrate (10^{-9}M to 10^{-6}M) on the guanethidine-induced tone (3x 10^{-5}M) in the rat anococcygeus muscle in a single experiment on a tissue from a control animal and a treated animal tissue. Treatment lasted 9 days (treatment 1.2, succinylacetone, phenobarbitone, AIA). The bottom graph shows the mean (+/- S.E.M.) % inhibition of induced tone in both control tissues and in treated animal tissues (n=4, in both cases) caused by glycerine trinitrate. This porphyrinogenic drug treatment did not significantly alter the responses of tissues to glycerine trinitrate.

e. Anococcygeus motor response:

Field stimulation of the intrinsic nerves of the anococcygeus muscle from treated animals produced a frequency-dependent contraction which did not differ significantly from the responses of control tissues (figures 26, 27a).

f. Anococcygeus inhibitory response:

Following contraction with $3x10^{-5}M$ guanethidine, field stimulation of the intrinsic nerves of the anococcygeus muscle from treated animals produced a frequency-dependent inhibitory response which did not significantly differ from the responses elicited in control tissues (figures 26, 27b).

g. Vas deferens motor response:

Electrical stimulation of the intrinsic nerves of the rat vas deferens, from animals treated with this porphyrinogenic drug combination, produced a frequency-dependent contraction of the tissue. Neither component of the biphasic response differed significantly from the responses elicited from control tissues (figures 26, 28a, 28b).

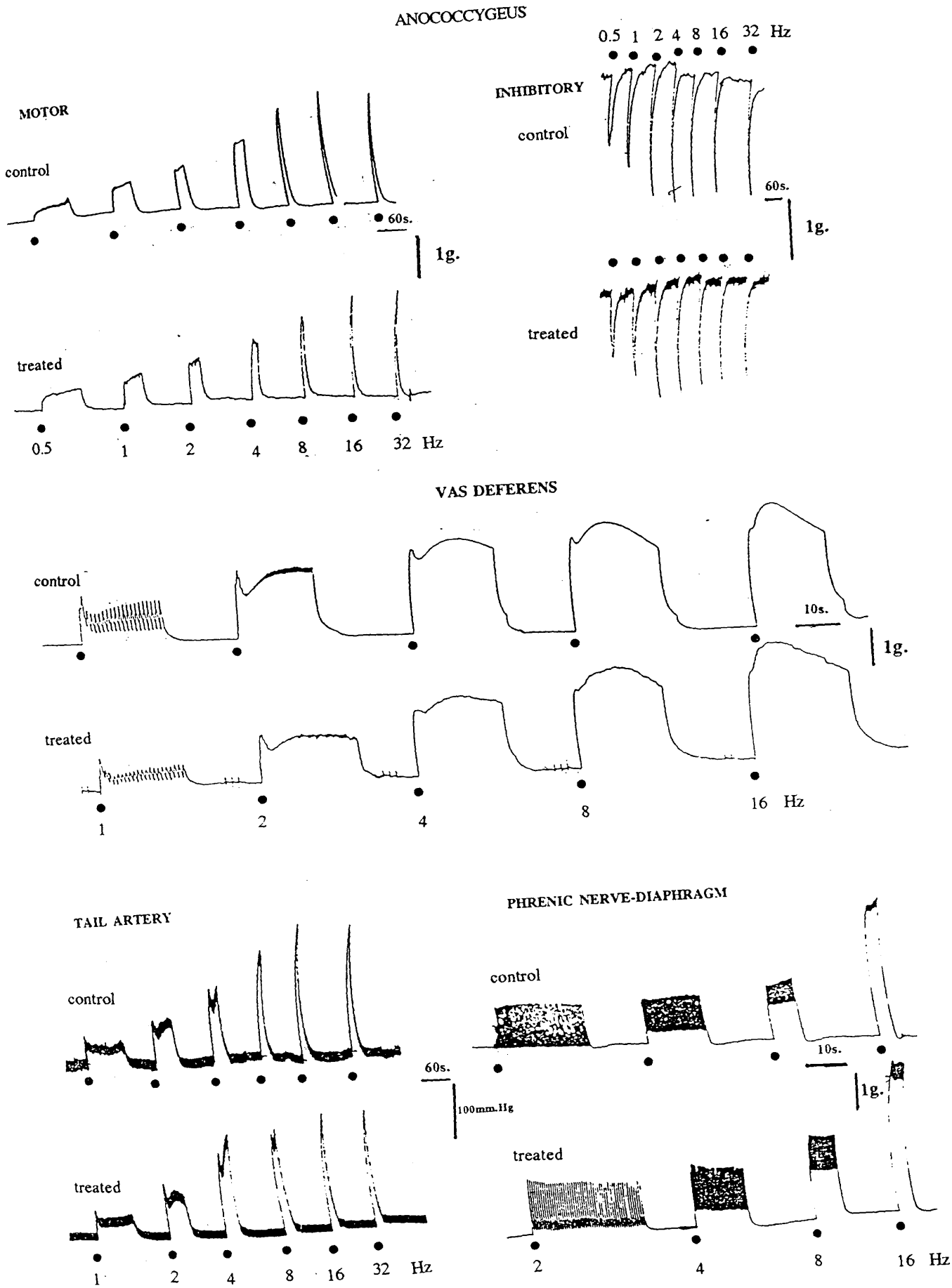
h. Tail artery pressor response:

Electrical field stimulation of the perfused rat tail artery isolated from treated animals produced a frequency-dependent pressor response which did not significantly differ from the responses of tissues from control animals (figures 26, 29a).

i. Phrenic nerve diaphragm:

On stimulation of the phrenic nerve the diaphragm contracts in a ferquency-dependent manner. The response of tissues isolated from treated animals did not differ significantly from the responses of tissues isolated from control animals (figures 26, 29b).

Figure 26: The effects of prophyrinogenic treatment 1.2 (9 days of succinylacetone, phenobarbitone and AIA) on the five tissue responses, in individual experiments (bottom traces), compared to the responses from individual control tissue responses (top traces). The time bars apply during stimulation periods only and the interstimulus interval in all cases was 3 min. Stimulation frequencies are shown above or below each response. For clarity only the frequencies for one tissue are shown but both control and treated tissues received the same stimulation parameters. The top right hand traces show the motor response of the rat anococcygeus muscle to stimulation with 50 pulses and the traces on the top right are the inhibitory response of the anococcygeus muscle to stimulation with 20 pulses. The middle traces show the response of the rat vas deferens to field stimulation with a 20s. train of pulses. The bottom left hand traces show the response of the perfused rat tail artery to field stimulation with 100 pulses. The bottom right hand graph shows the response of the rat hemidiaphragm to phrenic nerve stimulation with 50 pulses. This treatment did not significantly alter the responses of these tissues to nerve stimulation.


ANOCOCCYGEUS
MOTOR
control
60s.
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0.5 1 2 4 8 16 32 Hz
INHIBITORY
control
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VAS DEFERENS
control
10s.
1g.
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1 2 4 8 16 Hz
TAIL ARTERY
control
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PHRENIC NERVE-DIAPHRAGM
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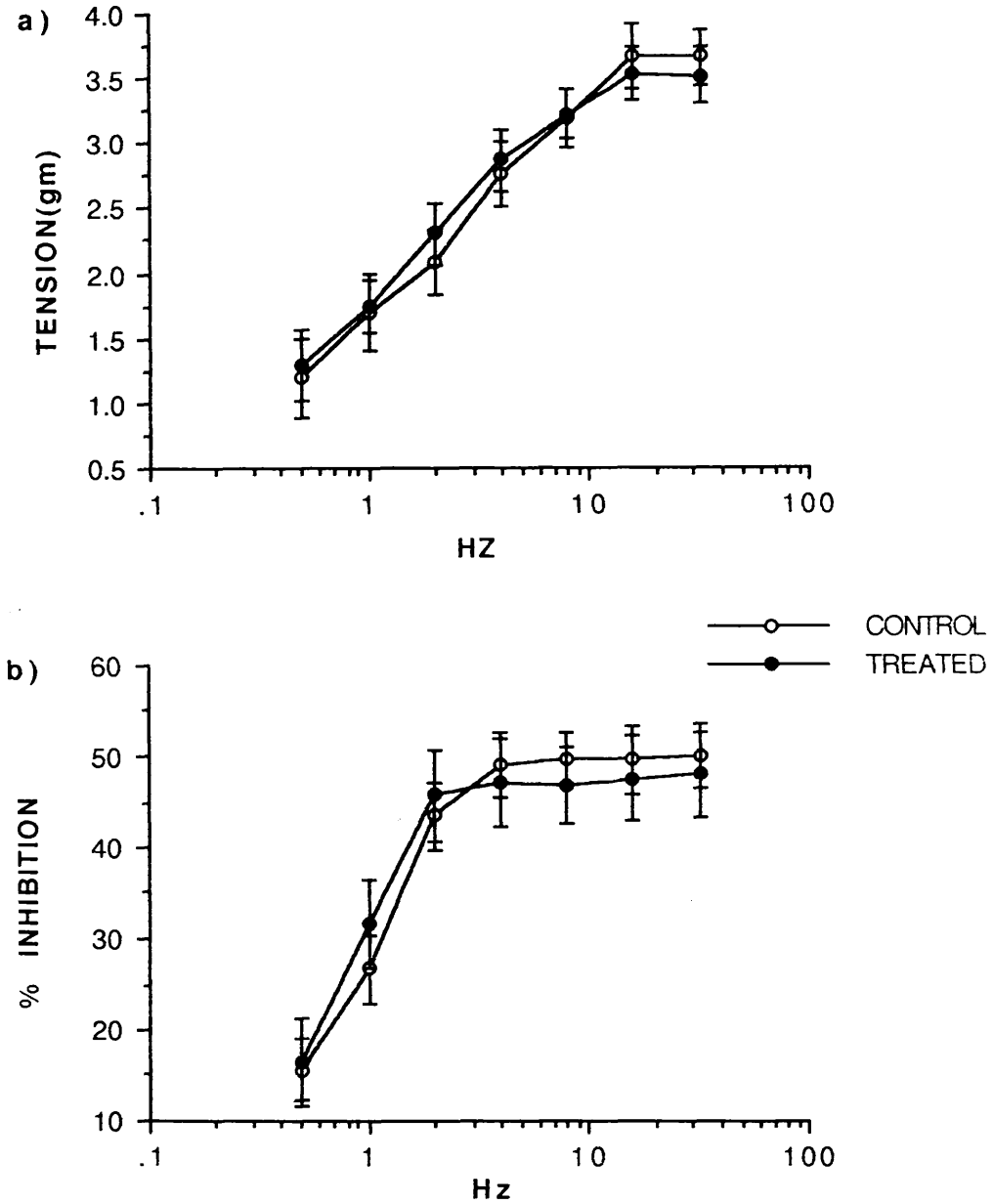


Figure 27: Graph a) shows the mean +/- S.E.M. of the motor response of the rat anococoocygeus muscle, in gm. tension, to stimulation with 50 pulses at 0.5-32 Hz in tissues from control animals (n=14) and in tissues from animals that had received 9 days of treatment 1.2 (succinylacetone, phenobarbitone, AIA) (n=10). Graph b) shows the inhibitory response of the anococcygeus muscle to intrinsic nerve stimulation with 20 pulses at 0.5-32 Hz in tissues from control animals (n=14) and treated animals (n=10). There were no significant differences.

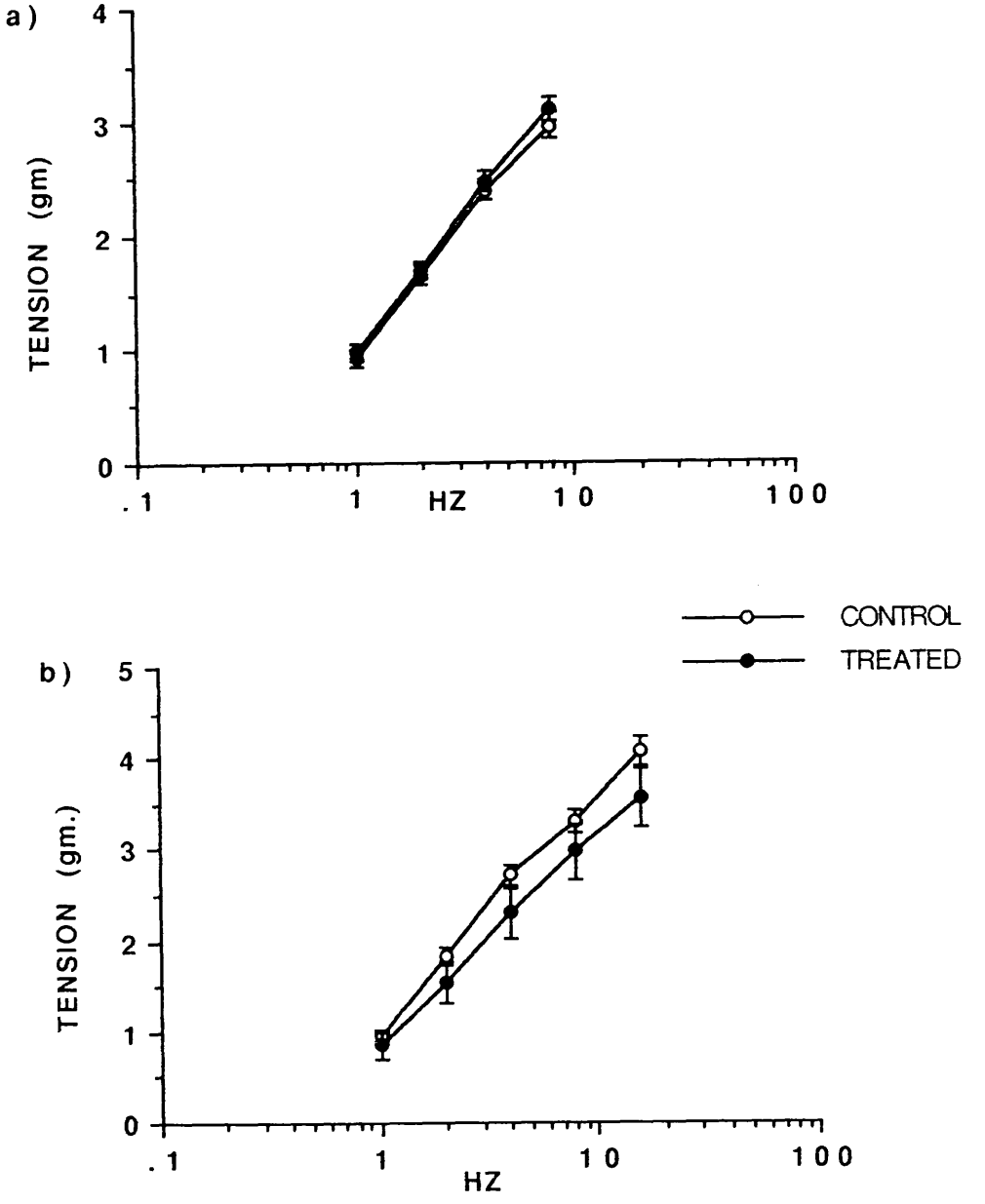


Figure 28: The top graph a) shows the mean +/- S.E.M. of the "fast" component of the rat vas deferens motor response, in gm. tension, to stimulation for 20s. at 1-8 Hz in tissues from control animals (n=20) and in tissues from animals that had received treatment 1.2 for 9 days (succinylacetone, phenobarbitone, AIA) (n=10). Graph b) shows the "sustained" component of the rat vas deferens response to intrinsic nerve stimulation with a 20s. train of pulses at 1-16 Hz in tissues from control animals (n=20) and treated animals (n=10). There were no significant differences.

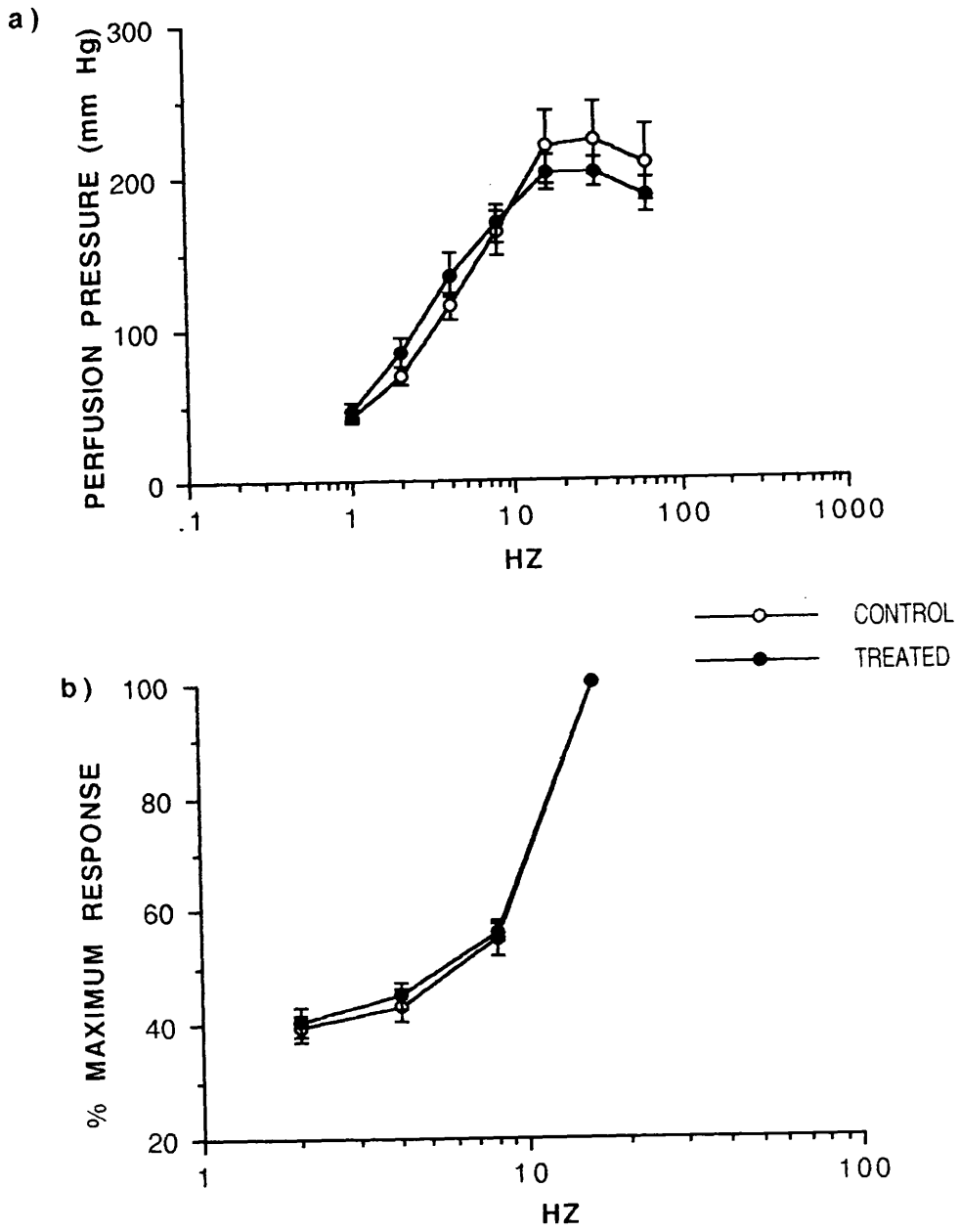


Figure 29: The top graph shows the mean +/- S.E.M. pressor response of the perfused rat tail artery to intramural nerve stimulation with 100 pulses at 1-64 Hz in tissues from control animals (n=8) and from animals that had been treated for 9 days with treatment combination 1.2 (succinylacetone, phenobarbitone, AIA) (n=6). The bottom trace shows the mean +/- S.E.M. of the response of the rat hemidiaphragm to phrenic nerve stimulation as the % of the maximum response achieved at 16 Hz in control animal tissue (n=8) and in tissues from treated animals (n=8). There were no significant differences.

3.3.6. The effects of porphyrinogenic treatment 1.3 (30 days succinylacetone; 6 days phenobarbitone; 1 injection of 4-ethyl DDC) on the responses of a range of innervated muscle preparations:

3.3.6.1. The effects of agonists:

a. The effect of phenylephrine on the rat anococcygeus muscle:

At a concentration of $3x10^{-6}M$, phenylephrine induced a contraction of the anococcygeus, from treated animals, of approximately 3.77 +/- 0.58g., which did not differ significantly from the contraction of 3.48 +/- 0.3g. that this compound elicited in tissues from control animals (figure 19).

b. The effects of sodium nitroprusside on the responses of the anococcygeus muscle:

When the tone of the anococcygeus muscle was raised with $3x10^{-5}M$ guanethidine $10^{-7}M$ sodium nitroprusside caused a reduction in the induced tone of approximately 50%. The effects of this compound in tissues from treated animals did not differ significantly from the effect produced in tissues from control animals (figure 20).

c. The effect of phenylephrine on the rat vas deferens muscle:

$3x10^{-5}M$ phenylephrine induced a mean contraction of 1.19+/- 0.14g. in tissues from treated animals which did not differ significantly from the responses of tissues from control animals to this concentration of phenylephrine (1.30 +/- 0.1g) (figure 19).

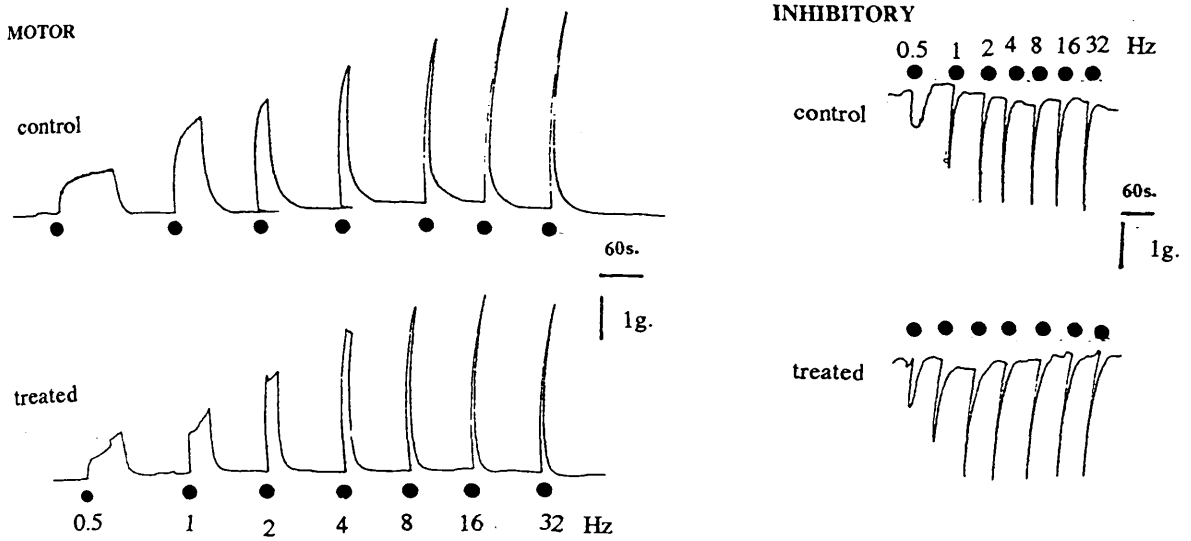
3.3.6.2. Electrical stimulation of nerve/muscle preparations:

d. Anococcygeus motor response:

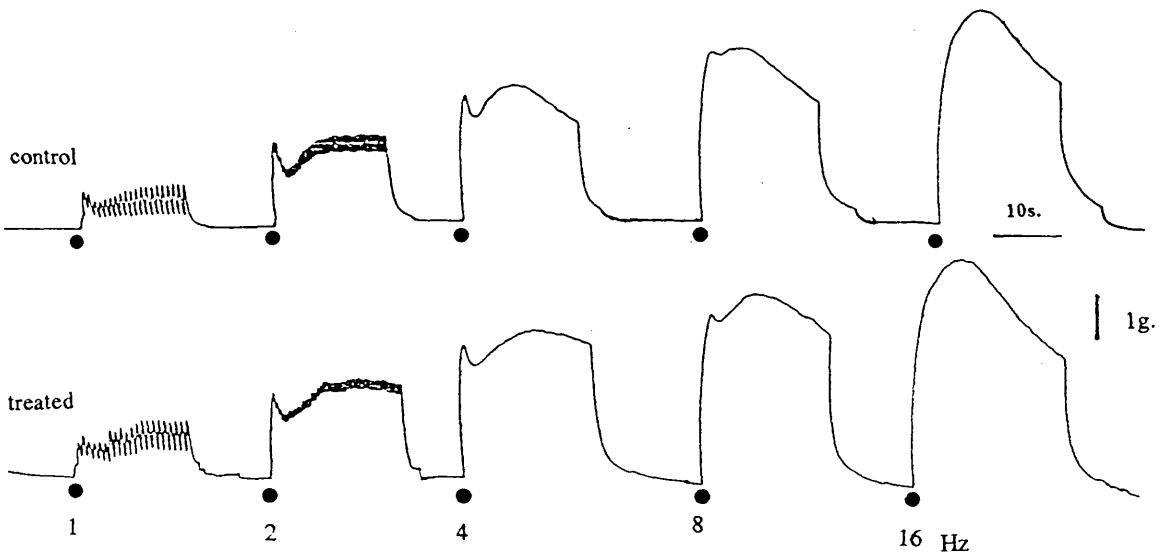
Field stimulation of the intrinsic nerves of the anococcygeus muscle from treated animals produced a frequency-dependent contraction which did not differ significantly from the responses of control tissues (figures 30, 31a).

Figure 30: The effects of porphyrinogenic treatment 1.3 (30 days sucinylacetone, 6 days phenobarbitone, 1 day 4-ethyl DDC) on the five tissue responses, in individual experiments (bottom traces), compared to individual control tissue responses (top traces). The time bars apply during the stimulation periods only and the interstimulus interval in all cases was 3 min. Stimulation frequencies are shown above or below each response. For clarity only the frequencies for one tissue are shown but both tissues received the same stimulation parameters. The top left hand traces show the motor response of the rat anococcygeus muscle to stimulation with 50 pulses and the traces on the top right are the inhibitory response of the anococcygeus muscle to stimulation with 20 pulses. The middle traces show the response of the rat vas deferens to field stimulation with a 20s. train of pulses. The bottom left hand traces show the response of the perfused rat tail artery to field stimulation with 100 pulses. The bottom right hand traces shows the response of the rat hemidiaphragm to phrenic nerve stimulation with 50 pulses. This treatment did not significantly alter the responses of the tissues to nerve stimulation.

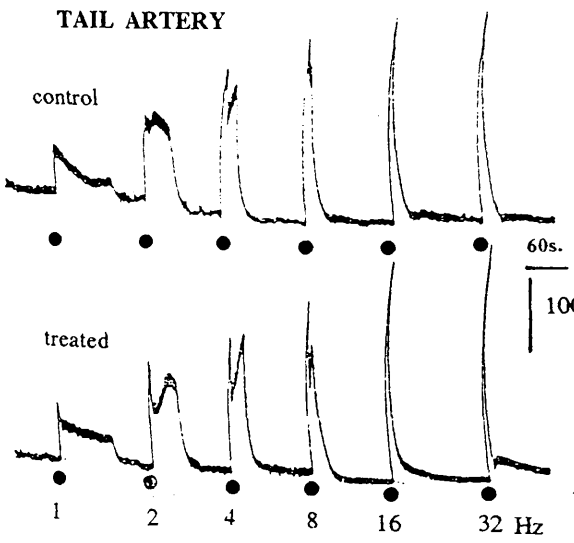
ANOCOCCYGEUS



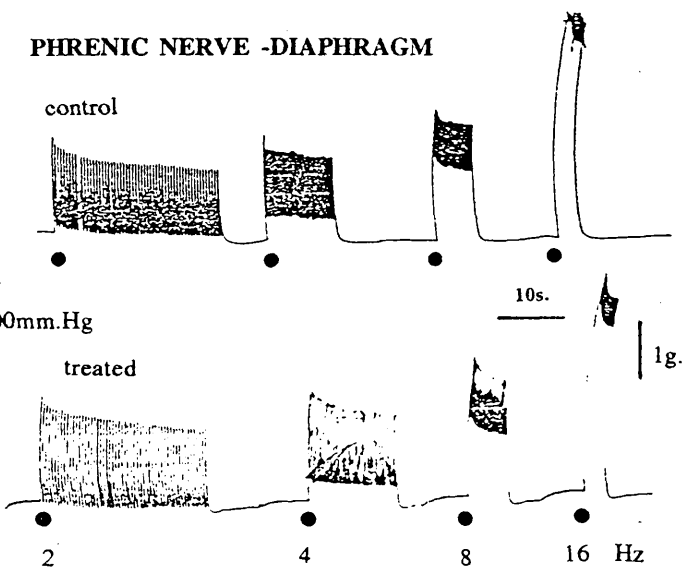
VAS DEFERENS



TAIL ARTERY



PHRENIC NERVE -DIAPHRAGM



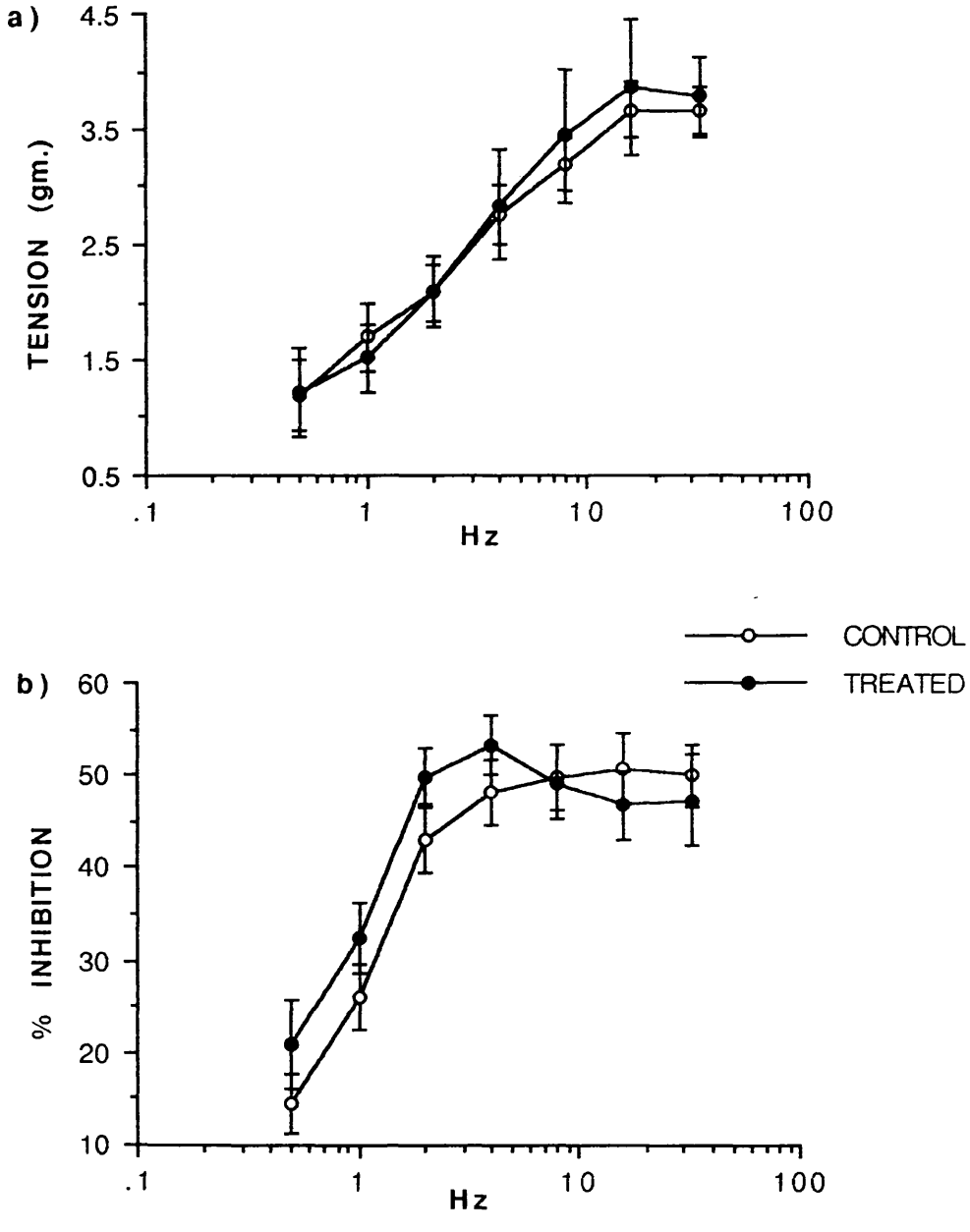


Figure 31: The top graph a) shows the mean (+/- S.E.M.) of the motor response the rat anococcygeus muscle, in gm. tension, to stimulation with 50 pulses at 0.5-32 Hz in tissues from control animals (n=14) and in tissues from animals that had received treatment 1.3 (succinylacetone, phenobarbitone, 4-ethyl DDC (n=6). Graph b) shows the inhibitory response of the anococcygeus muscle to intrinsic nerve stimulation with 20 pulses at 0.5-32 Hz in tissues from control animals (n= 14) and treated animals (n=5). There were no significant difference between control and treated tissue responses.

e. Anococcygeus inhibitory response:

Following contraction with $3x10^{-5}M$ guanethidine, field stimulation of the intrinsic nerves of the anococcygeus muscle from treated animals produced a frequency-dependent inhibitory response which did not significantly differ from the responses elicited in control tissues (figures 30, 31b).

f. Vas deferens motor response:

Electrical stimulation of the intrinsic nerves of the rat vas deferens, from animals treated with this porphyrinogenic drug combination, produced a frequency-dependent contraction of the tissue. Neither component of the biphasic response differed significantly from the responses elicited from control tissues (figures 30, 32a, 32b).

g. Tail artery pressor reponse:

Electrical field stimulation of the perfused rat tail artery isolated from treated animals produced a frequency-dependent pressor response which did not significantly differ from the responses of tissues from control animals (figure 30, 33a).

h. Phrenic nerve diaphragm:

On stimulation of the phrenic nerve the diaphragm contracts in a frequency-dependent manner. The response of tissues isolated from treated animals did not differ significantly from the responses of tissues isolated from control animals (figures 30, 33b).

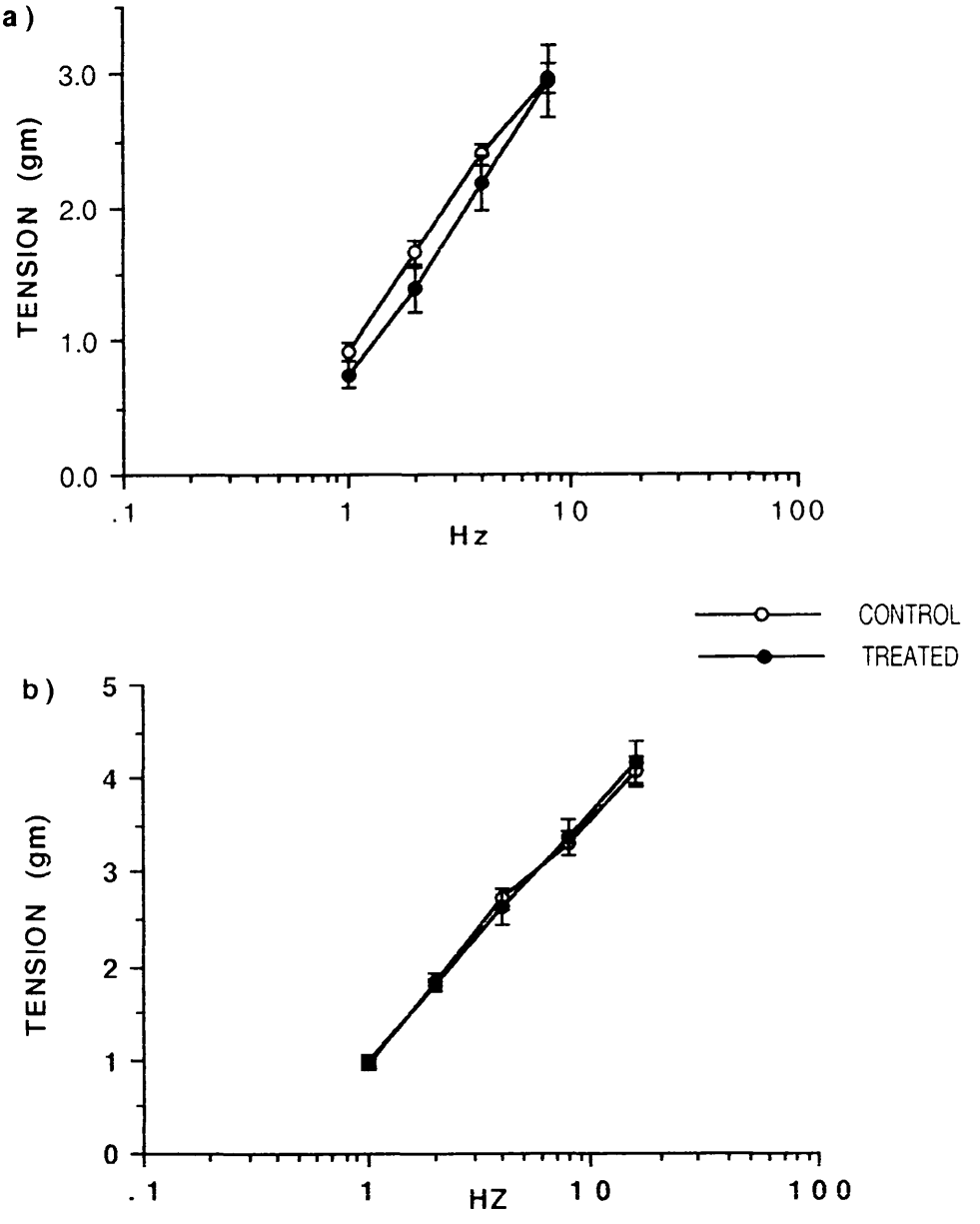


Figure 32: The top graph a) shows the mean (+/- S.E.M.) of the "fast" component of the rat vas deferens motor response, in gm. tension, to stimulation for 20s. at 1-8 Hz in tissues from control animals (n=20) and in tissues from animals that had received treatment 1.3 (succinylacetone, phenobarbitone, 4-ethyl DDC) (n=6) . Graph b) shows the "sustained" component of the rat vas deferens response to intrinsic nerve stimulation with a 20s. train of pulses at 1-16 Hz in tissues from control animals (n= 20) and treated animals (n=6). There were no significant differences between control and treated tissues.

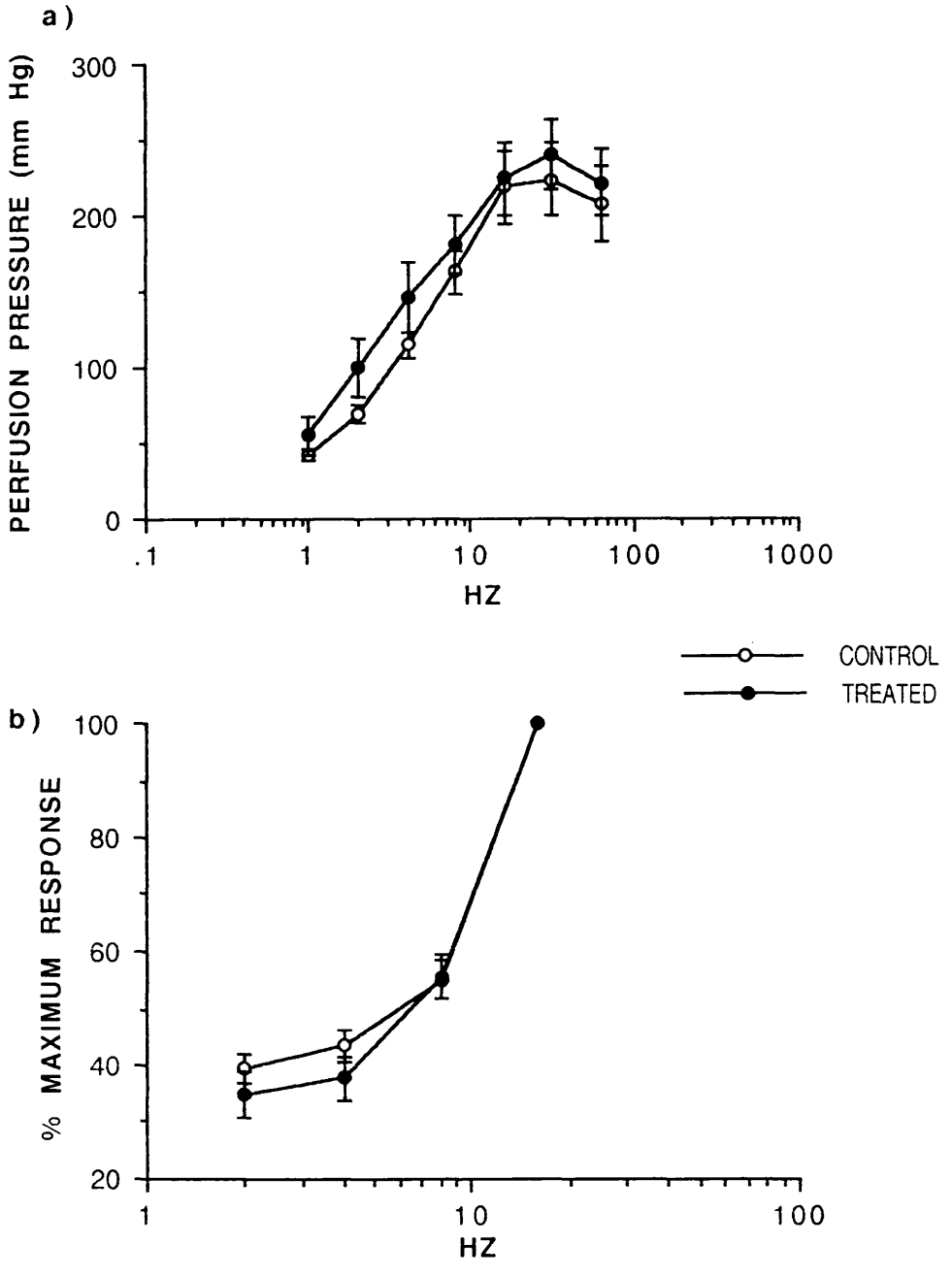


Figure 33: The top graph shows the mean (+/- S.E.M.) pressor response of the perfused rat tail artery to intramural nerve stimulation with 100 pulses at 1-64 Hz in tissues from control animals (n=8) and from animals that had received treatment 1.3 (succinylacetone, phenobarbitone 4+ethyl DDC) (n=6). The bottom trace shows the mean (+/- S.E.M.) of the response of the rat hemidiaphragm to phrenic nerve stimulation as the % of the maximum response achieved at 16 Hz in control animal tissue (n=8) and in tissues from treated animals (n=4). There were no significant differences between control and treated tissues.

3.3.7. The effects of porphyrinogenic treatment 1.4 (30 days succinylacetone; 6 days phenobarbitone; 1 injection of DDC) on the responses of a range of innervated muscle preparations:

3.3.7.1. The effects of agonists:

a. The effect of phenylephrine on the rat anococcygeus muscle:

At a concentration of $3x10^{-6}M$, phenylephrine induced a contraction of the anococcygeus muscle from treated animals of approximately 4.1 +/- 0.35g., which did not differ significantly from the contraction of 3.48 +/- 0.3g that this compound elicits in tissues from control animals (figure 19).

b. The effects of sodium nitroprusside on the responses of the anococcygeus muscle:

When the tone of the anococcygeus muscle was raised with $3x10^{-5}M$ guanethidine, $10^{-7}M$ sodium nitroprusside caused a reduction in the induced tone of approximately 50%. The effects of this compound in tissues from treated animals did not differ significantly from the effect produced in tissues from control animals (figure 20).

c. The effect of phenylephrine on the rat vas deferens muscle:

$3x10^{-5}M$ phenylephrine induces a mean contraction of 1.19 +/- 0.14g. in tissues from treated animals which did not differ significantly from the responses of tissues from control animals (1.30 +/- 0.1g) (figure 19).

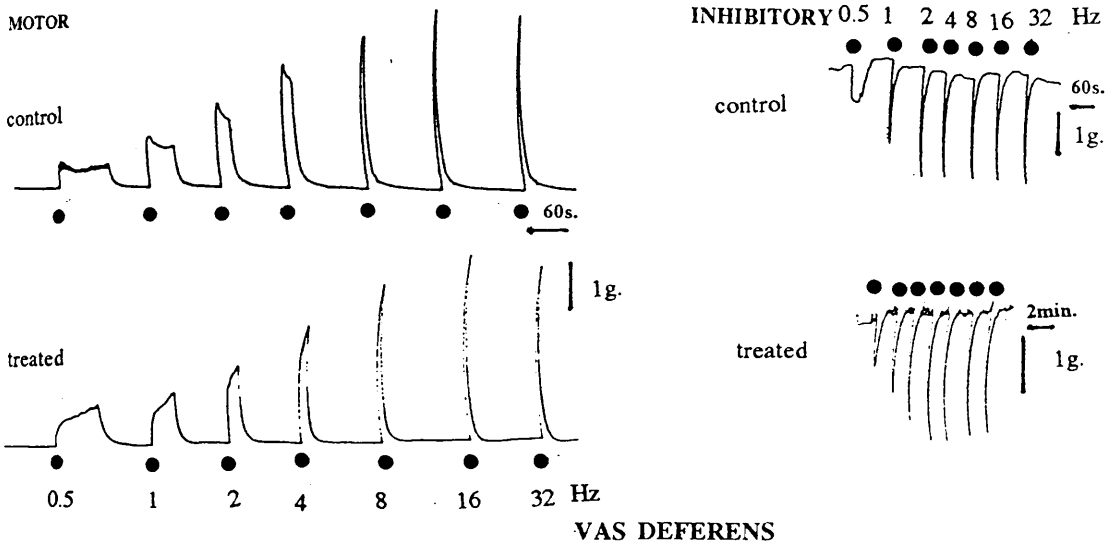
3.3.7.1. Electrical stimulation of the nerve/muscle preparations:

d. Anococcygeus motor response:

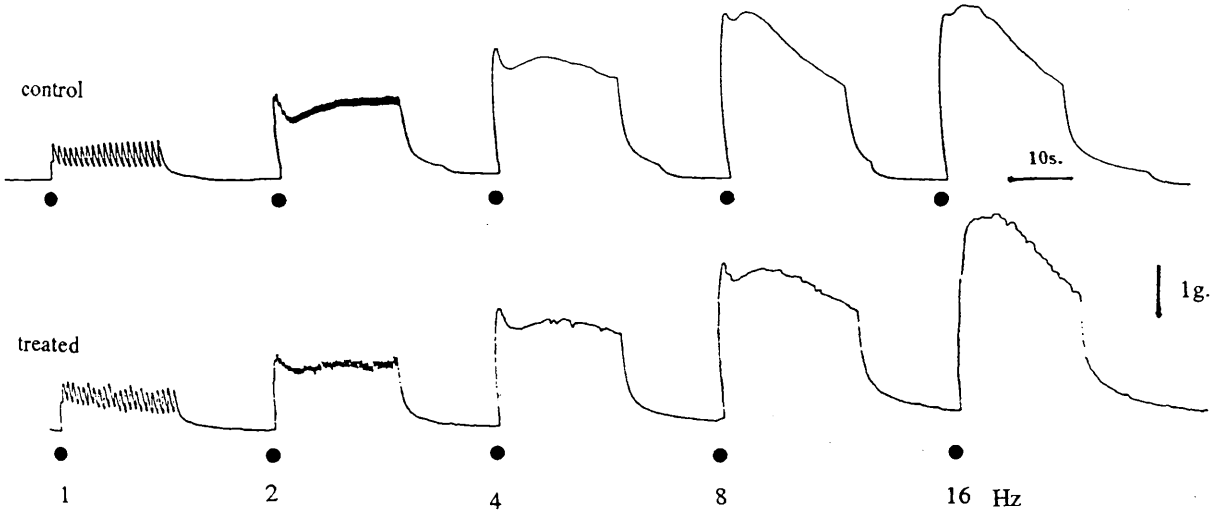
Field stimulation of the intrinsic nerves of the anococcygeus muscle from treated animals produced a frequency-dependent contraction which did not differ significantly from the responses of control tissues (figures 34, 35a).

Figure 34: The effects of porphyrinogenic treatment 1.4 (30 days sucinylacetone, 6 days phenobarbitone, 1 day DDC) on the five tissue responses, in individual experiments (bottom traces), compared to the responses from individual control tissue responses (top traces). The time bars apply during the stimulation periods only and the interstimulus interval in all cases was 3 min. Stimulation frequencies are shown above or below each response. For clarity only the frequencies for one tissue are shown but both control and treated tissues received the same stimulation parameters. The top left hand traces show the motor response of the rat anococcygeus muscle to stimulation with 50 pulses and the traces on the top right are the inhibitory response of the anococcygeus muscle to stimulation with 20 pulses. The middle traces show the response of the rat vas deferens to field stimulation with a 20s. train of pulses. The bottom left hand traces show the response of the perfused rat tail artery to field stimulation with 100 pulses. The bottom right hand traces shows the response of the rat hemidiaphragm to phrenic nerve stimulation with 50 pulses. This treatment did not significantly alter the response of the tissues to nerve stimulation.

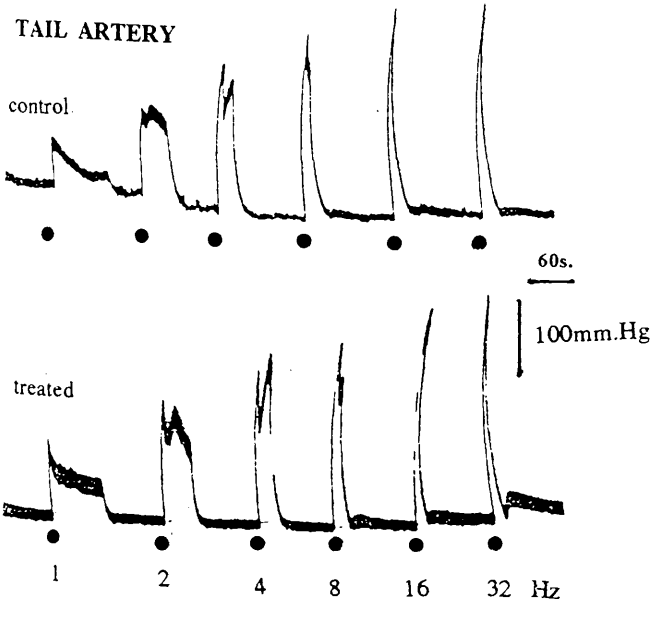
ANOCOCCYGEUS



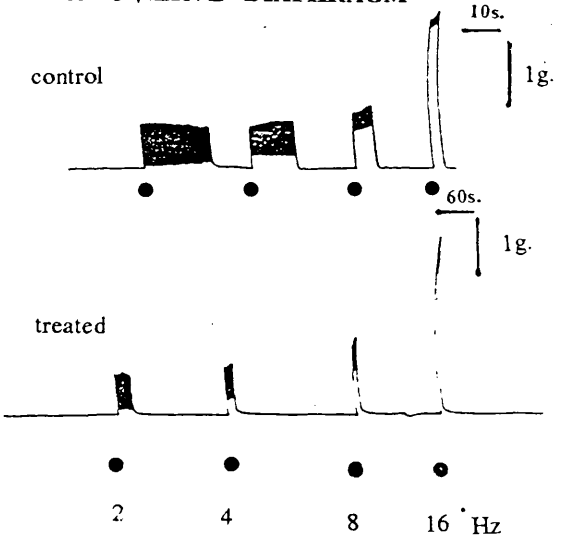
VAS DEFERENS



TAIL ARTERY



PHRENIC NERVE -DIAPHRAGM



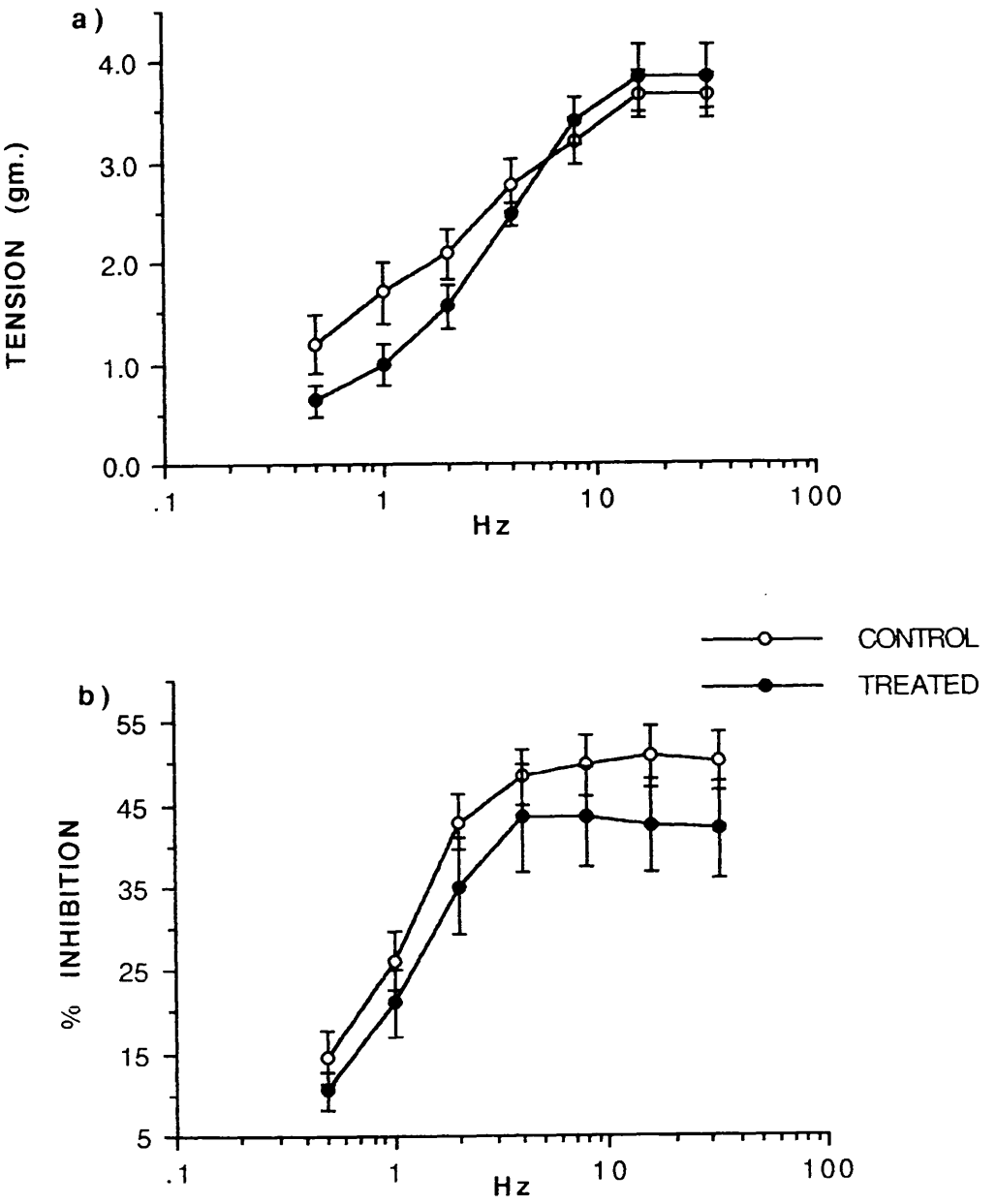


Figure 35: The top graph a) shows the mean (+/- S.E.M.) of the motor response the rat anococoocygeus muscle, in gm. tension, to stimulation with 50 pulses at 0.5-32 Hz in tissues from control animals (n=14) and in tissues from animals that had received treatment 1.4 (succinylacetone, phenobarbitone, DDC) (n=8) . Graph b) shows the inhibitory response of the anococcygeus muscle to intrinsic nerve stimulation with 20 pulses at 0.5-32 Hz in tissues from control animals (n= 14) and treated animals (n=8). There were no significant differences between control and treated responses.

e. Anococcygeus inhibitory response:

Following contraction with $3x10^{-5}M$ guanethidine, field stimulation of the intrinsic nerves of the anococcygeus muscle from treated animals produced a frequency-dependent inhibitory response which did not significantly differ from the responses elicited in control tissues (figures 34, 35b).

f. Vas deferens motor response:

Electrical stimulation of the intrinsic nerves of the rat vas deferens, from animals treated with this porphyrinogenic drug combination, produced a frequency-dependent contraction of the tissue. Neither component of the biphasic response differed significantly from the responses elicited from control tissues (figures 34, 36a, 36b).

g. Tail artery pressor response:

Electrical field stimulation of the perfused rat tail artery isolated from treated animals produced a frequency-dependent pressor response which did not significantly differ from the responses of tissues from control animals (figures 34, 37a).

h. Phrenic nerve diaphragm:

On stimulation of the phrenic nerve the diaphragm contracts in a ferquency-dependent manner. The response of tissues isolated from treated animals did not differ significantly from the responses of tissues isolated from control animals (figures 34, 37b).

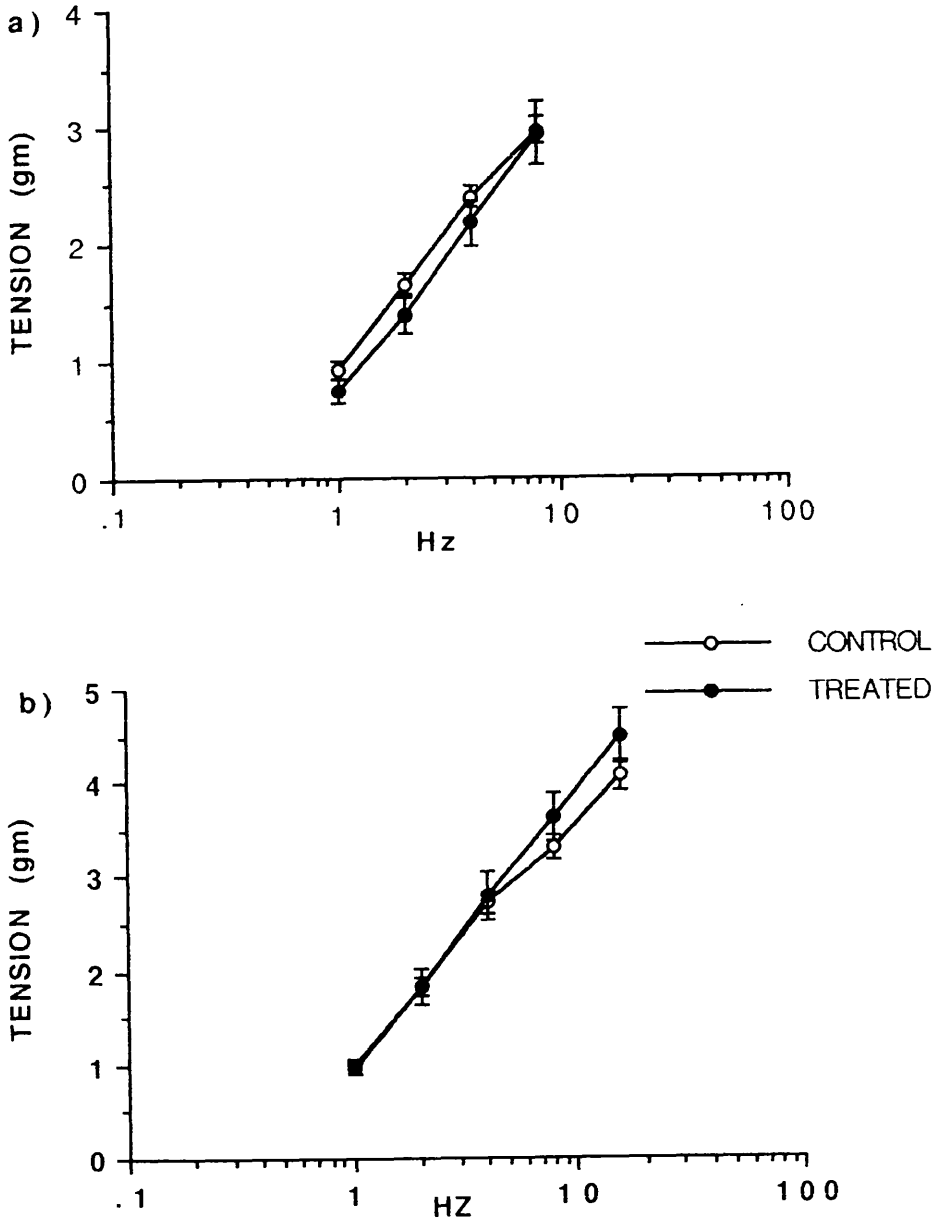


Figure 36: The top graph a) shows the mean (+/- S.E.M.) of the "fast" component of the rat vas deferens motor response , in gm. tension, to stimulation for 20s. at 1-8 Hz in tissues from control animals (n=20) and in tissues from animals that had received treatment 1.4 (n=8). Graph b) shows the "sustained" component of the rat vas deferens response to intrinsic nerve stimulation with a 20s. train of pulses at 1-16 Hz in tissues from control animals (n= 20) and treated animals (n=8). There were no significant differences between control and treated tissue responses.

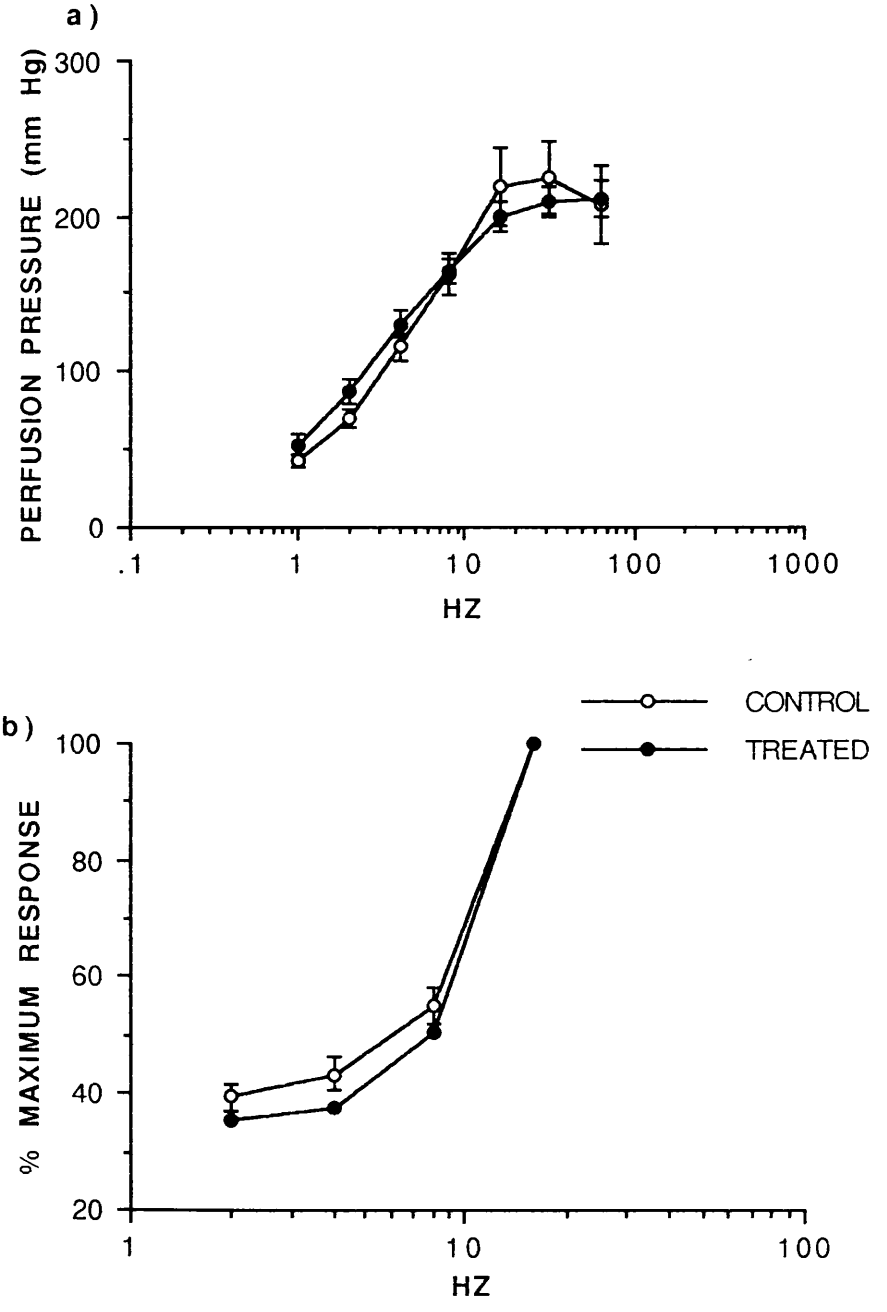


Figure 37: The top graph shows the mean (+/- S.E.M.) pressor response of the perfused rat tail artery to intramural nerve stimulation with 100 pulses at 1-64 Hz in tissues from control animals (n=8) and from animals that had received treatment 1.4 (succinylacetone, phenobarbitone, DDC) (n=7). The bottom trace shows the mean (+/- S.E.M.) of the response of the rat hemidiaphragm to phrenic nerve stimulation as the % of the maximum response achieved at 16 Hz in control animal tissue (n=8) and in tissues from treated animals (n=2). This treatment did not alter the responses of the tissues to nerve stimulation.

3.3.8. The effects of porphyrinogenic treatment 1.5 (30 days succinylacetone; 10 days of 4-ethyl DDC; 4 days phenobarbitone; 1 large dose of 4-ethyl DDC) on the responses of a range of innervated muscle preparations:

3.3.8.1. The effects of agonists:

a. The effect of phenylephrine on the rat anococcygeus muscle:

At a concentration of $3x10^{-6}M$, phenylephrine induced a contraction of the anococcygeus, from treated animals , of approximately 3.17 +/- 0.41g., which did not differ significantly from the contraction of 3.48 +/- 0.3g. that this compound elicits in tissues from control animals (figure 19).

b. The effects of sodium nitroprusside on the responses of the anococcygeus muscle:

When the tone of the anococcygeus muscle was raised with $3x10^{-5}M$ guanethidine, $10^{-7}M$ sodium nitroprusside caused a reduction in the induced tone of approximately 50%. The effects of this compound in tissues from treated animals did not differ significantly from the effect produced in tissues from control animals (figure 20).

c. The effect of phenylephrine on the rat vas deferens muscle:

$3x10^{-5}M$ phenylephrine induced a mean contraction of 1.15 +/- 0.15g. in tissues from treated animals which did not differ significanlty from the responses of tissues from control animals (1.30 +/- 0.1g.) (figure 19).

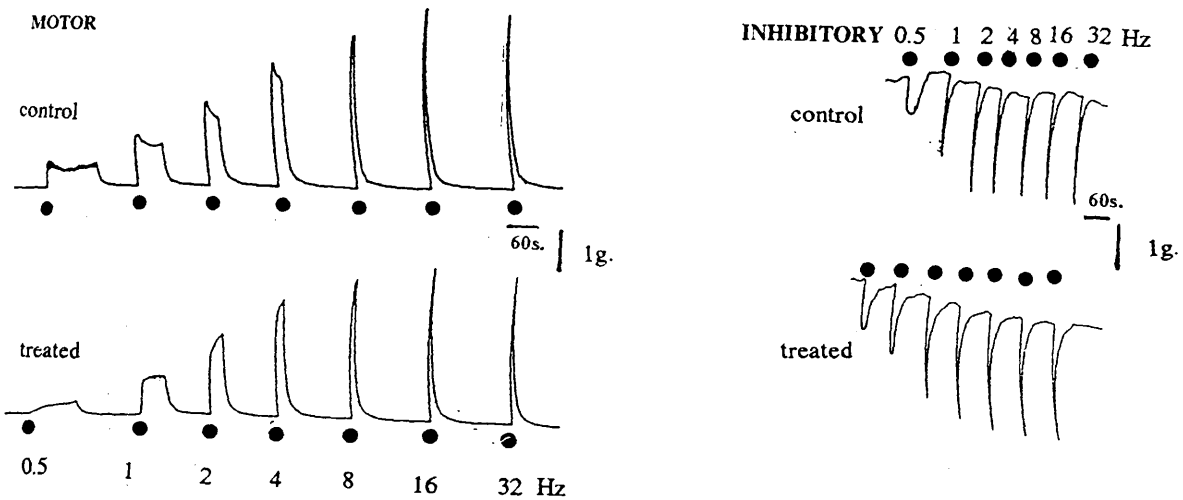
3.3.8.2. Electrical stimulation of the nerve/muscle preparations:

d. Anococcygeus motor response:

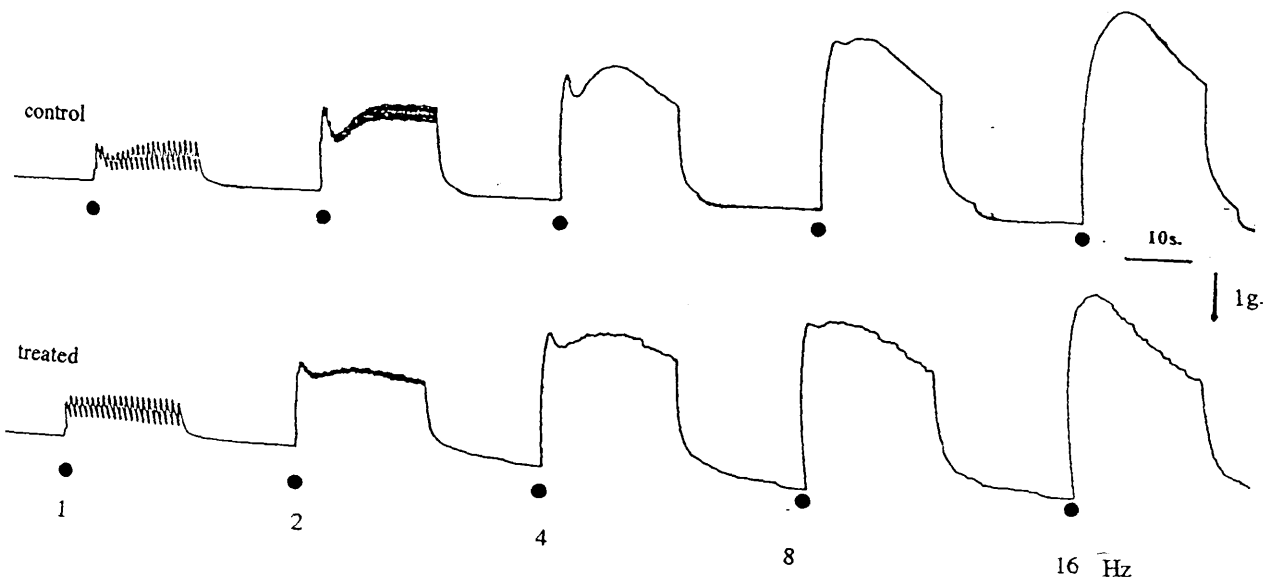
Field stimulation of the intrinsic nerves of the anococcygeus muscle from treated animals produced a frequency-dependent contration which did not differ significantly from the responses of control tissues (figures 38, 39a).

Figure 38: The effects of prophyrinogenic treatment 1.5 (30 days sucinylacetone, 10 days 4-ethyl DDC, 4 days phenobarbitone, 1 day 4-ethyl DDC) on the five tissue responses, in individual experiments (bottom traces), compared to the responses from individual control tissue responses (top traces). The time bars apply during stimulation periods only and the interstimulus interval in all cases was 3 min. Stimulation frequencies are shown above or below each response. For clarity only the frequencies for one tissue are shown but both control and treated tissues received the same stimulation parameters. The top left hand traces show the motor response of the rat anococcygeus muscle to stimulation with 50 pulses and the traces on the top right are the inhibitory response of the anococcygeus muscle to stimulation with 20 pulses. The middle traces show the response of the rat vas deferens to field stimulation with a 20s. train of pulses. The bottom left hand traces show the response of the perfused rat tail artery to field stimulation with 100 pulses. The bottom right hand traces shows the response of the rat hemidiaphragm to phrenic nerve stimulation with 50 pulses. This treatment did not significantly alter the responses of the tissues to nerve stimulation.

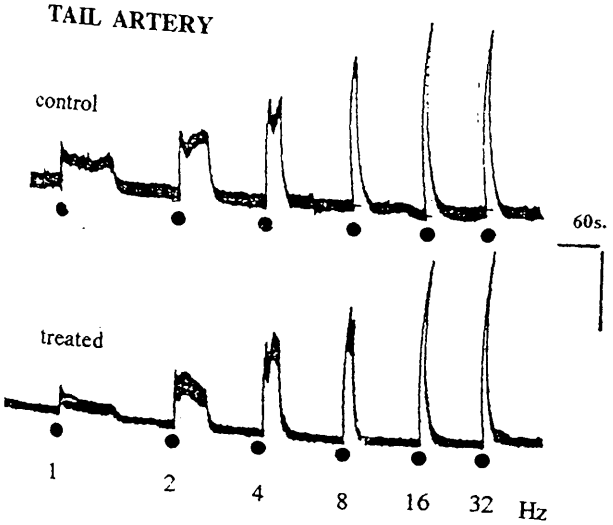
ANOCOCCYGEUS



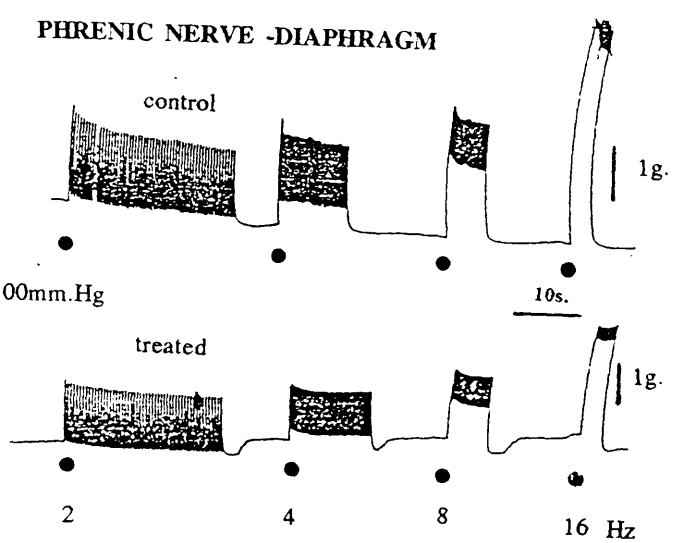
VAS DEFERENS



TAIL ARTERY



PHRENIC NERVE -DIAPHRAGM



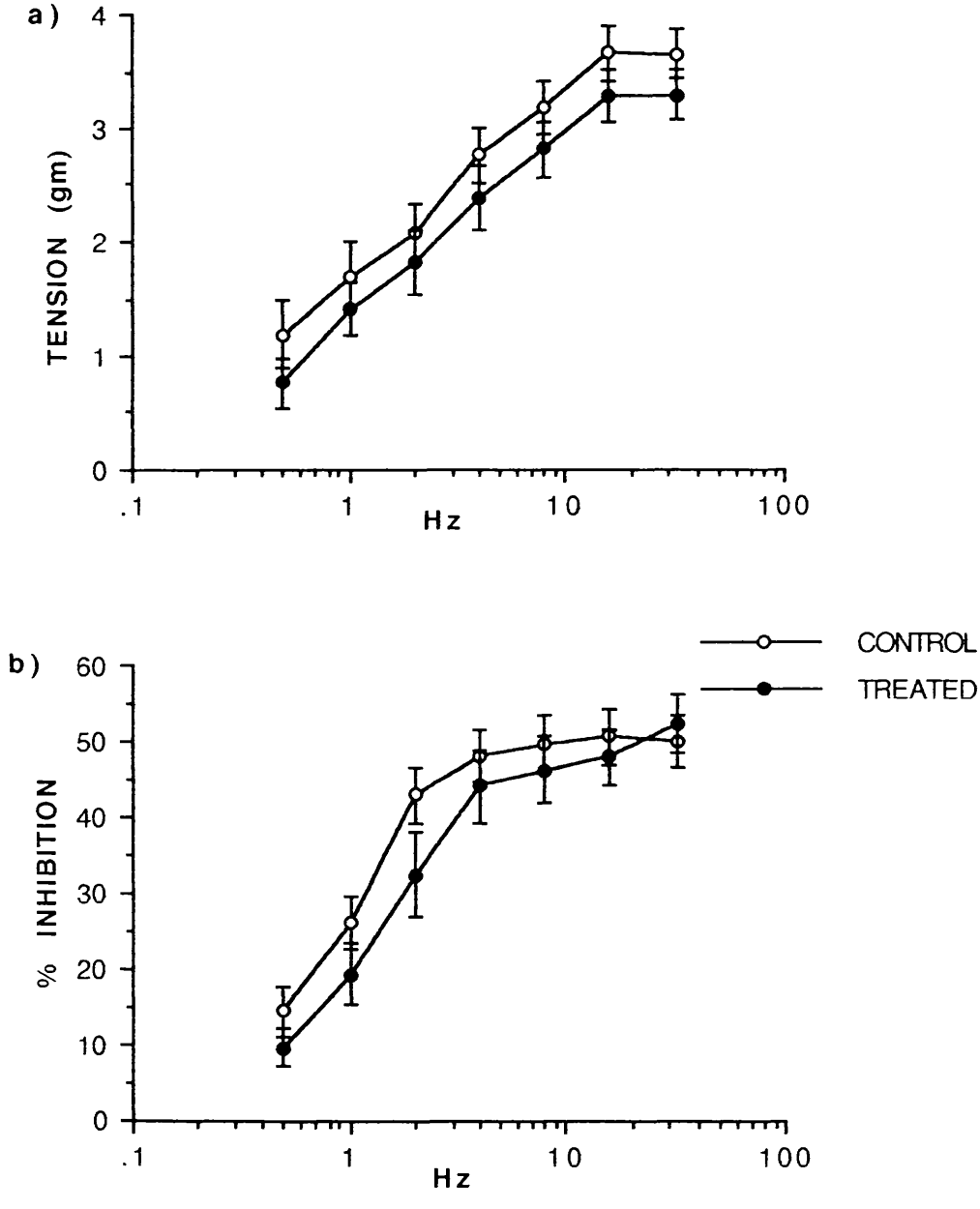


Figure 39: The top graph a) shows the mean (+/- S.E.M.) of the motor response the rat anococoocygeus muscle, in gm. tension, to stimulation with 50 pulses at 0.5-32 Hz in tissues from control animals (n=14) and in tissues from animals that had received treatment 2.5 (succinylacetone, 4-ethyl DDC, phenobarbitone, 4-ethyl DDC) (n=8). Graph b) shows the inhibitory response of the anococcygeus muscle to intrinsic nerve stimulation with 20 pulses at 0.5-32 Hz in tissues from control animals (n= 14) and treated animals (n=8). There were no significant differences between treated and control tissue responses.

e. Anococcygeus inhibitory response:

Following contraction with $3x10^{-5}M$ guanethidine, field stimulation of the intrinsic nerves of the anococcygeus muscle from treated animals produced a frequency-dependent inhibitory response which did not significantly differ from the responses elicited in control tissues (figures 38, 39b).

f. Vas deferens motor response:

Electrical stimulation of the intrinsic nerves of the rat vas deferens, from animals treated with this porphyrinogenic drug combination, produced a frequency-dependent contraction of the tissue. Neither component of the biphasic response differed significantly from the responses elicited from control tissues (figure 38, 40a, 40b).

g. Tail artery pressor response:

Electrical field stimulation of the perfused rat tail artery isolated from treated animals produced a frequency-dependent pressor response which did not significantly differ from the responses of tissues from control animals (figures 39a, 41a).

h. Phrenic nerve diaphragm:

On stimulation of the phrenic nerve the diaphragm contracts in a ferquency-dependent manner. The response of tissues isolated from treated animals did not differ significantly from the responses of tissues isolated from control animals (figures 39, 41b).

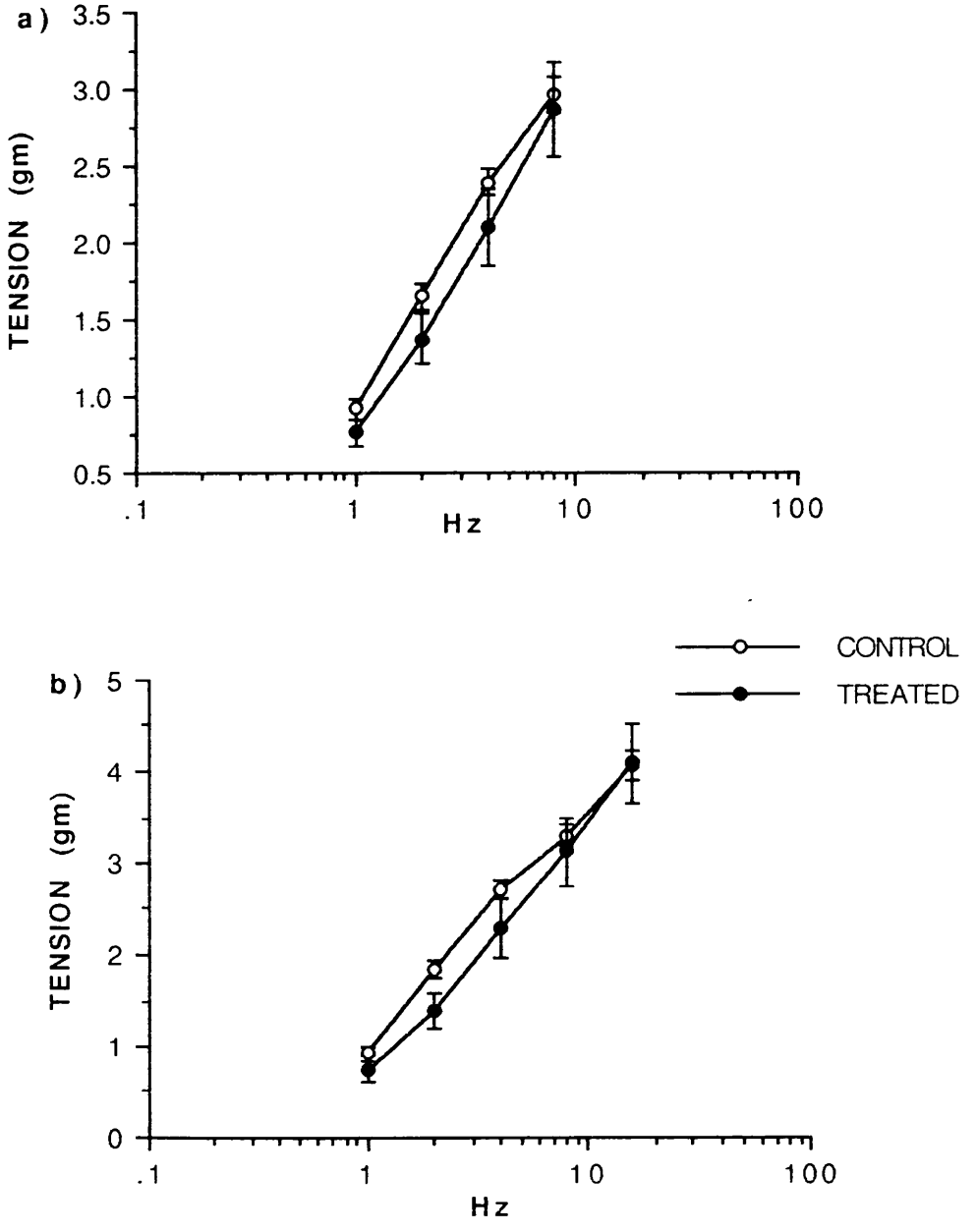


Figure 40: The top graph a) shows the mean (+/- S.E.M.) of the "Fast" component of the rat vas deferens motor response , in gm. tension, to stimulation for 20s. at 1-8 Hz in tissues from control animals (n=20) and in tissues from animals that had received treatment 1.5 (succinylacetone, 4-ethyl DDC, phenobarbitone, 4-ethyl DDC) (n=8). Graph b) shows the "sustained" component of the rat vas deferens response to intrinsic nerve stimulation with a 20s. train of pulses at 1-16 Hz in tissues from control animals (n= 20) and treated animals (n=8). There were no significant differences.

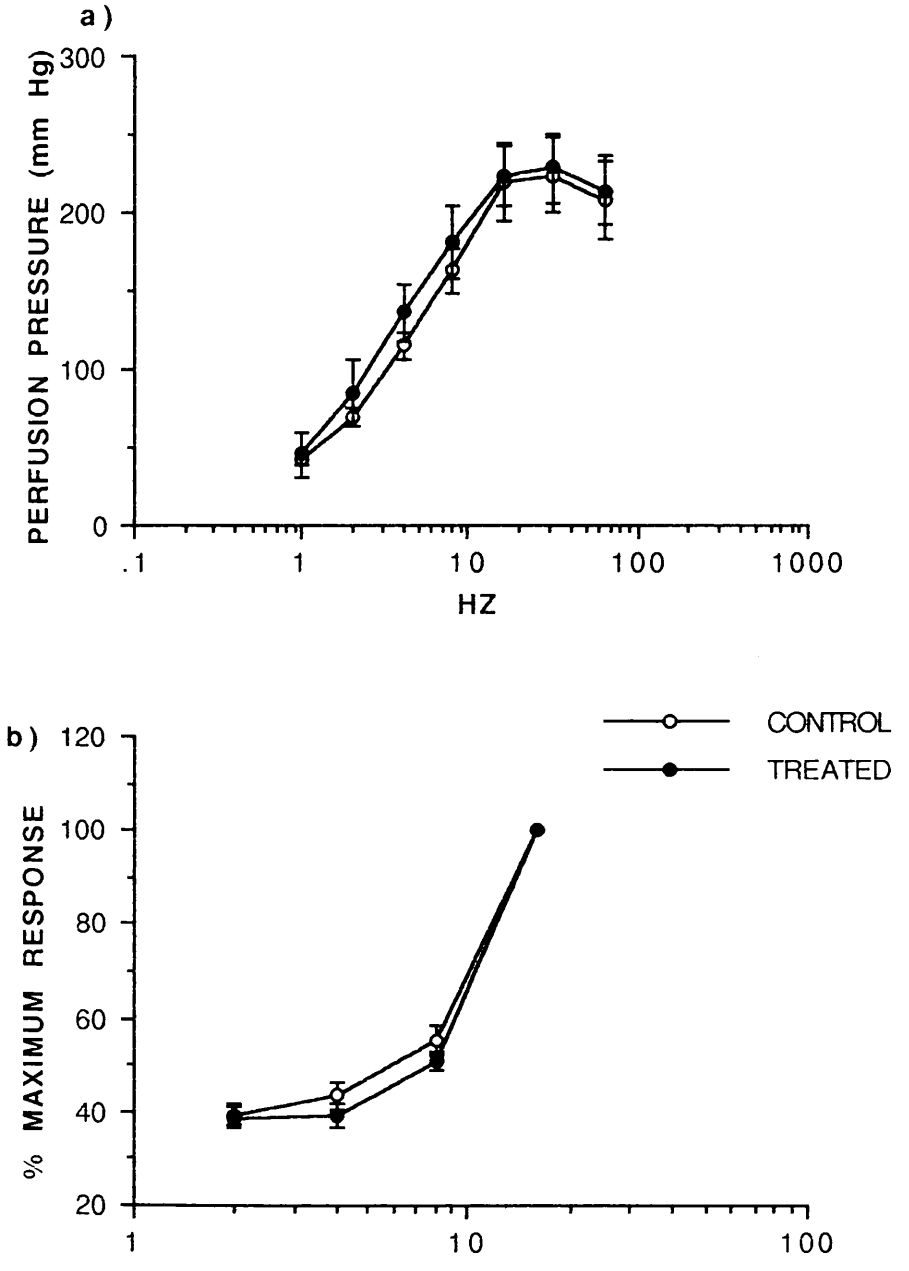


Figure 41: The top graph shows the mean (+/- S.E.M.) pressor response of the perfused rat tail artery to intramural nerve stimulation with 100 pulses at 1-64 Hz in tissues from control animals (n=8) and from animals that had received treatment 1.5 (succinylacetone, 4-ethyl DDC, phenobarbitone, 4-ethyl DDC) (n=5). The bottom trace shows the mean (+/- S.E.M.) of the response of the rat hemidiaphragm to phrenic nerve stimulation as the % of the maximum response acheived at 16 Hz in control animal tissue (n=8) and in tissues from treated animals (n=6). There were no significant differences.

3.3.9. The effects of porphyrinogenic treatment 1.6 (30 days succinylacetone; 10 days DDC; 4 days phenobarbitone; 1 large dose of DDC) on the responses of a range of innervated muscle preparations:

3.3.9.1. The effects of agonists:

a. The effect of phenylephrine on the rat anococcygeus muscle:

At a concentration of $3x10^{-6}M$, phenylephrine induced a contraction of the anococcygeus, from treated animals, of approximately 3.16 +/- 0.54g., which did not differ significantly from the contraction of 3.48 +/- 0.3g. that this compound elicits in tissues from control animals (figure 19).

b. The effects of sodium nitroprusside on the responses of the anococcygeus muscle:

When the tone of the anococcygeus muscle was raised with $3x10^{-5}M$ guanethidine $10^{-7}M$ sodium nitroprusside caused a reduction in the induced tone of approximately 50%. The effects of this compound in tissues from treated animals did not differ significantly from the effect produced in tissues from control animals (figure 20).

c. The effect of phenylephrine on the rat vas deferens muscle:

$3x10^{-5}M$ phenylephrine induced a mean contraction of 1.28 +/- 0.17g. in tissues from treated animals which did not differ significanlty from the responses of tissues from control animals (figure 19).

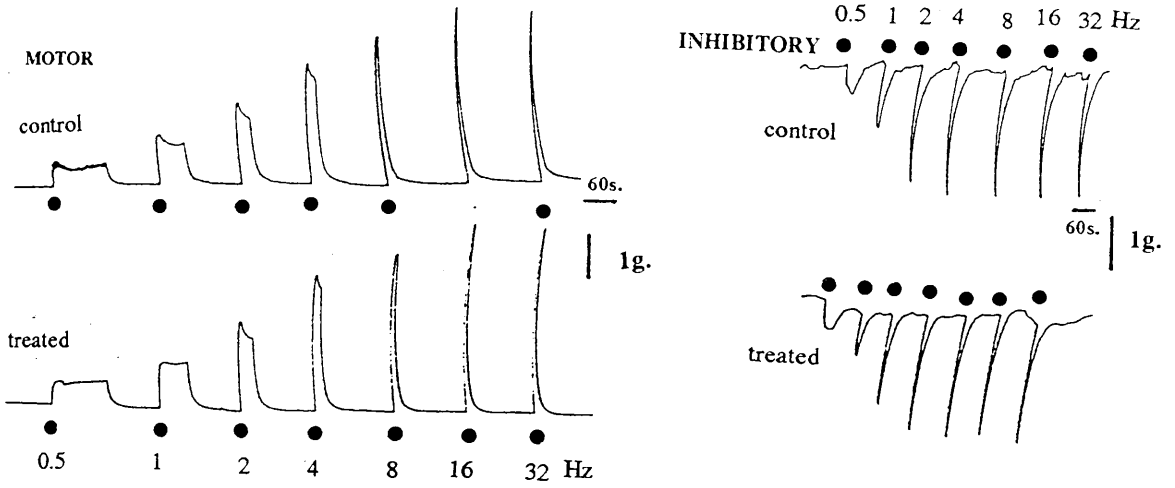
3.3.9.2. Electrical stimulation of the nerve/muscle preparations:

d. Anococcygeus motor response:

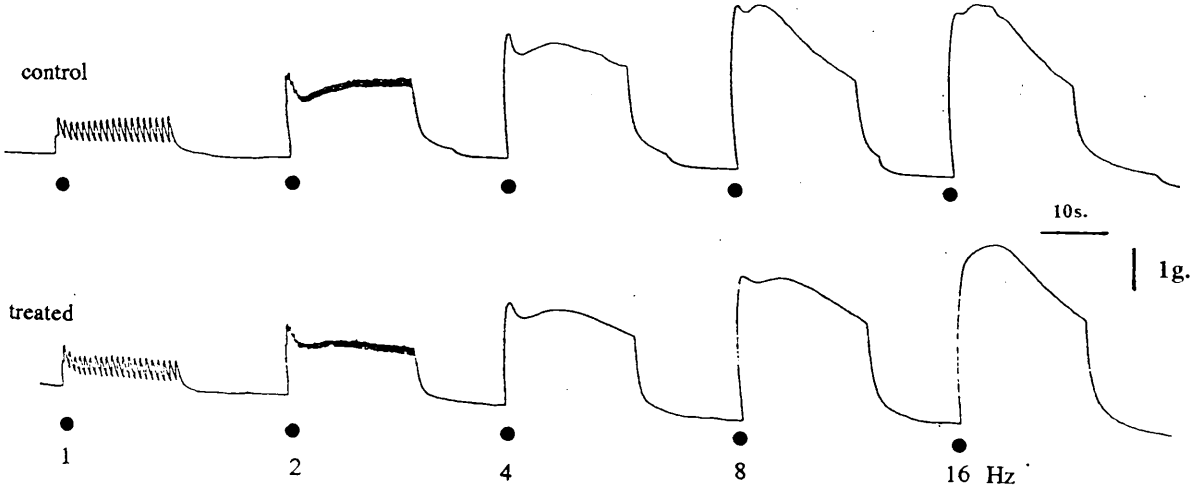
Field stimulation of the intrinsic nerves of the anococcygeus muscle from treated animals produced a frequency-dependent contraction which did not differ significantly from the responses of control tissues (figures 42, 43a).

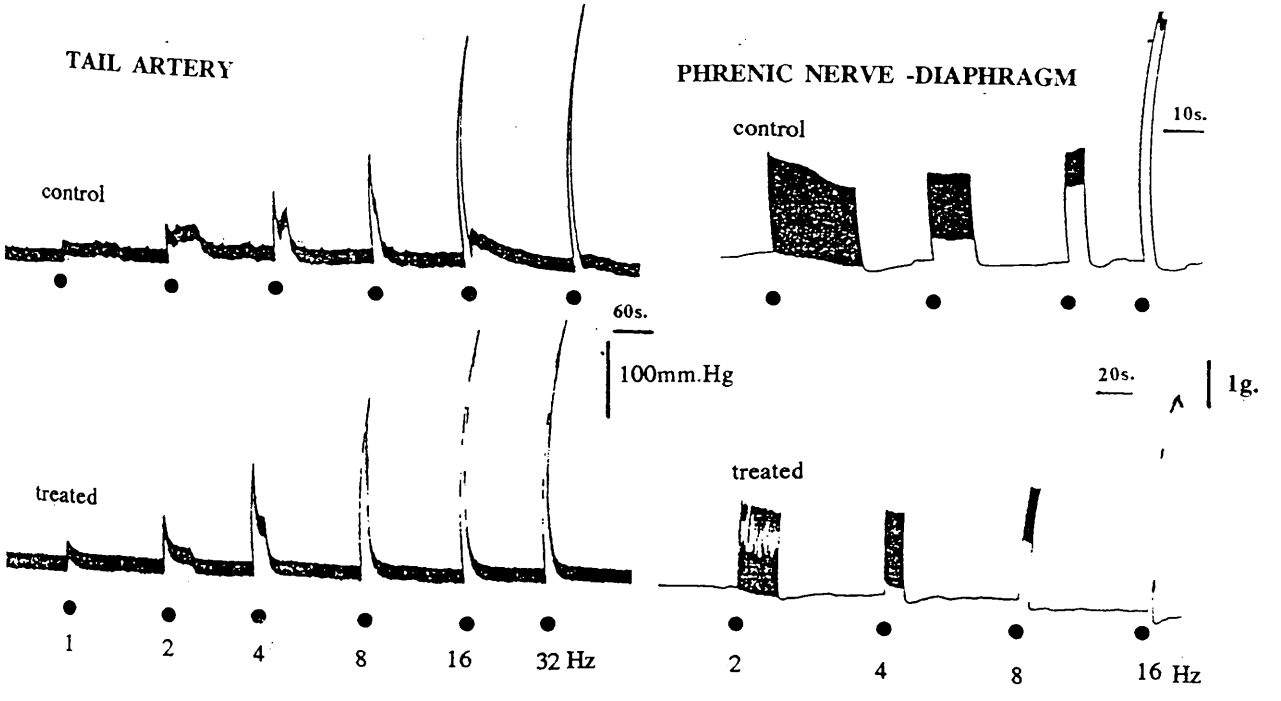
Figure 42: The effects of porphyrinogenic treatment 1.6 (30 days sucinylacetone, 10 days DDC, 4 days phenobarbitone, 1 day DDC) on the five tissue responses, in individual experiments (bottom traces), compared to the responses from individual control tissue responses (top traces). The time bars apply to stimulation periods only and the interstimulus interval in all cases was 3 min. Stimulation frequencies are shown above or below each response. For clarity only the frequencies for one tissue are shown but both tissues received the same stimulation parameters. The top left hand traces show the motor response of the rat anococcygeus muscle to stimulation with 50 pulses and the traces on the top right are the inhibitory response of the anococcygeus muscle to stimulation with 20 pulses. The middle traces show the response of the rat vas deferens to field stimulation with a 20s. train of pulses. The bottom left hand traces show the response of the perfused rat tail artery to field stimulation with 100 pulses. The bottom right hand traces shows the response of the rat hemidiaphragm to phrenic nerve stimulation with 50 pulses. This treatment did not significantly alter the responses of the tissues to nerve stimulation.

ANOCOCCYGEUS



VAS DEFERENS





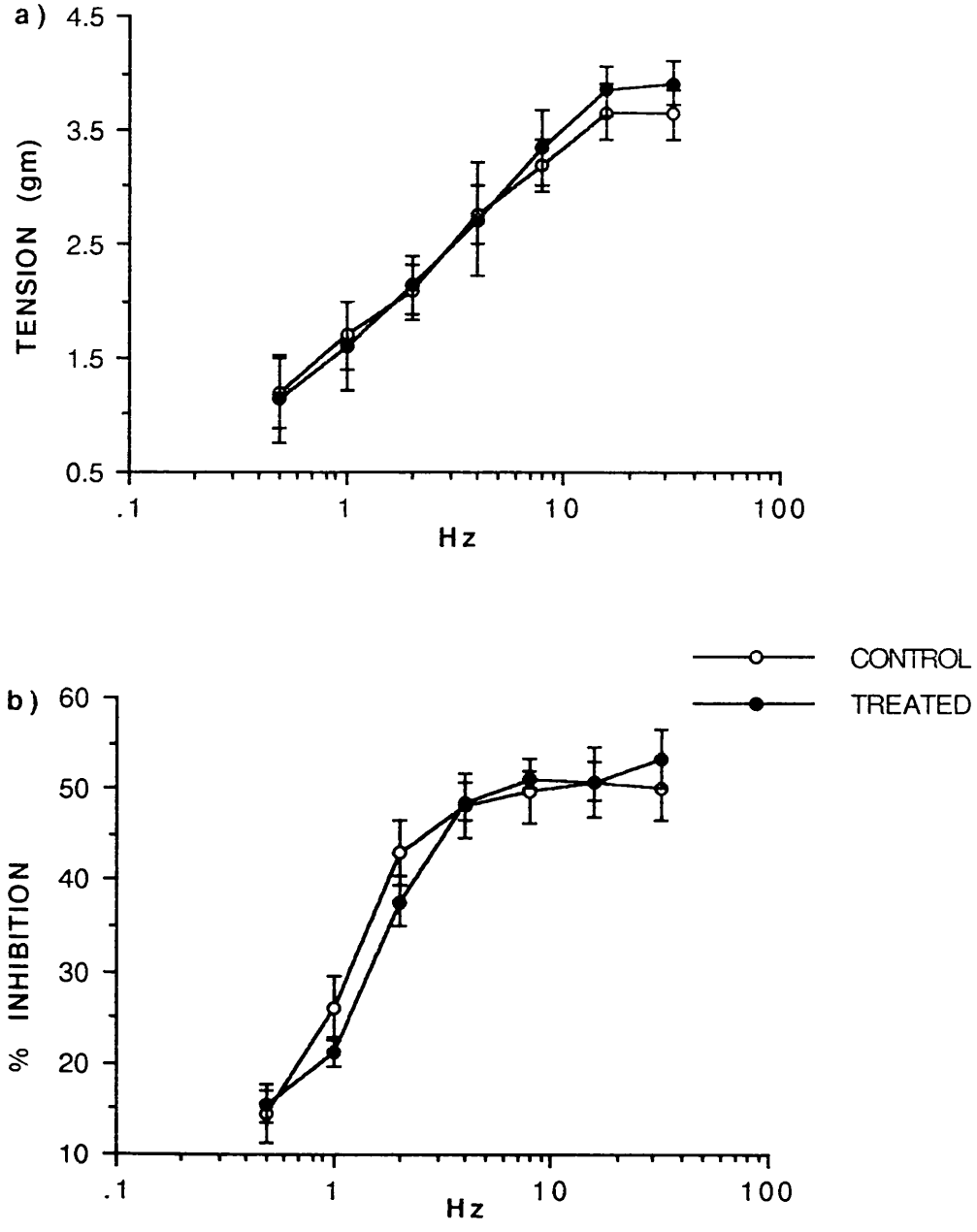


Figure 43: The top graph a) shows the mean (+/- S.E.M.) of the motor response the rat anococoocygeus muscle, in gm. tension, to stimulation with 50 pulses at 0.5-32 Hz in tissues from control animals (n=14) and in tissues from animals that had received treatment 1.6 (succinylacetone, DDC, phenobarbitone, DDC) (n=4) . Graph b) shows the inhibitory response of the anococcygeus muscle to intrinsic nerve stimulation with 20 pulses at 0.5-32 Hz in tissues from control animals (n= 14) and treated animals (n=4). There were no significant differences between control and treated tissue responses to nerve stimulation.

e. Anococcygeus inhibitory response:

Following contraction with $3x10^{-5}M$ guanethidine, field stimulation of the intrinsic nerves of the anococcygeus muscle from treated animals produced a frequency-dependent inhibitory response which did not significantly differ from the responses elicited in control tissues (figures 42, 43b).

f. Vas deferens motor response:

Electrical stimulation of the intrinsic nerves of the rat vas deferens, from animals treated with this porphyrinogenic drug combination, produced a frequency-dependent contraction of the tissue. Neither component of the biphasic response differed significantly from the responses elicited from control tissues (figures 42, 44a, 44b).

g. Tail artery pressor response:

Electrical field stimulation of the perfused rat tail artery isolated from treated animals produced a frequency-dependent pressor response which did not significantly differ from the responses of tissues from control animals (figures 42, 45a).

h. Phrenic nerve diaphragm:

On stimulation of the phrenic nerve the diaphragm contracts in a frequency-dependent manner. The response of tissues isolated from treated animals did not differ significantly from the responses of tissues isolated from control animals (figures 42, 45b).

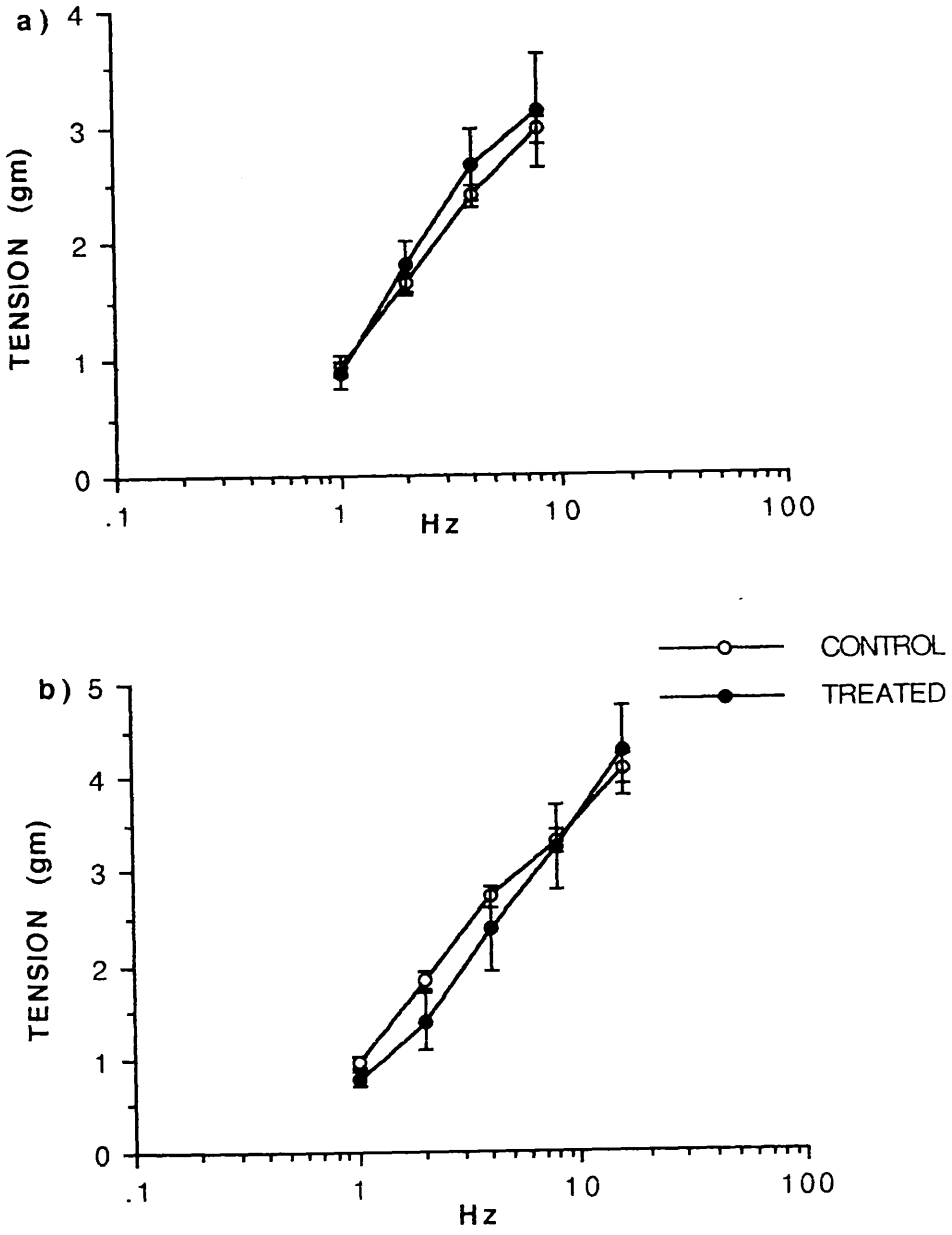


Figure 44: The top graph a) shows the mean (+/- S.E.M.) of the "fast" component of the rat vas deferens motor response, in gm. tension, to stimulation for 20s. at 1-8 Hz in tissues from control animals (n=20) and in tissues from animals that had received treatment 1.6 (succinylacetone, DDC, phenobarbitone, DDC) (n=4) . Graph b) shows the "sustained" component of the rat vas deferens response to intrinsic nerve stimulation with a 20s. train of pulses at 1-16 Hz in tissues from control animals (n= 20) and treated animals (n=4). There were no significant differences.

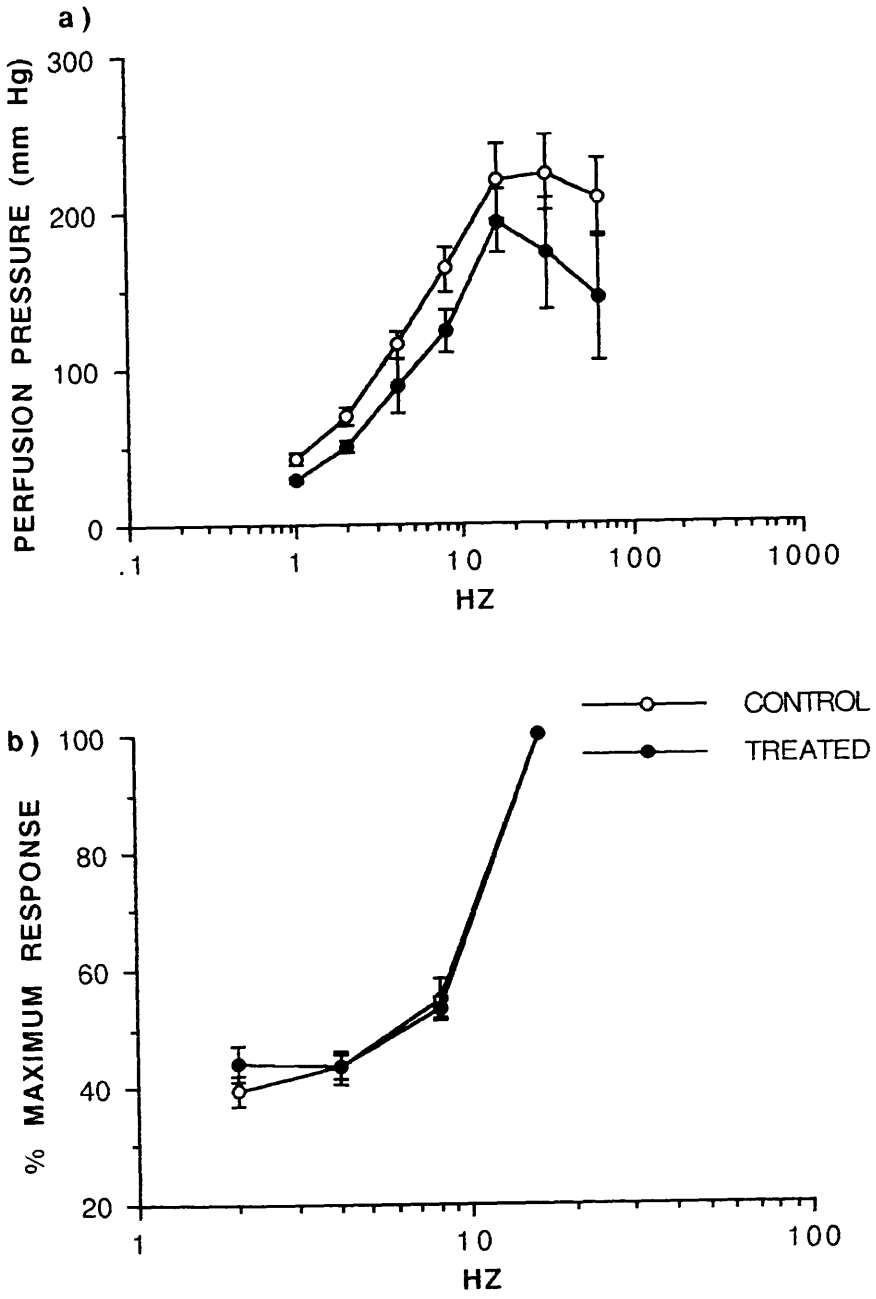


Figure 45: The top graph shows the mean (+/- S.E.M.) pressor response of the perfused rat tail artery to intramural nerve stimulation with 100 pulses at 1-64 Hz in tissues from control animals (n=8) and from animals that had received treatment 1.6 (succinylacetone, DDC, phenobarbitone, DDC) (n=3). The bottom trace shows the mean (+/- S.E.M.) of the response of the rat hemidiaphragm to phrenic nerve stimulation as the % of the maximum response acheived at 16 Hz in control animal tissue (n=8) and in tissues from treated animals (n=3). There were no significant differences between control and treated tissue responses.

3.3.10. The effects of porphyrinogenic treatment 1.7 (14 days of lead tetraacetate) on the responses of a range of innervated muscle preparations:

3.3.10.1. The effects of agonists:

a. The effect of phenylephrine on the rat anococcygeus muscle:

At a concentration of $3x10^{-6}M$, phenylephrine induced a contraction of the anococcygeus, from treated animals , of 3.28 +/- 0.32g., which did not differ significantly from the contraction of 3.48 +/- 0.3g. that this compound elicited in tissues from control animals (figure 19).

A one way ANOVA compairing the effects of this dose of phenylephrine in all treatments determined that there were no significant differences amongst groups (p=0.2793)

b. The effects of sodium nitroprusside on the responses of the anococcygeus muscle:

When the tone of the anococcygeus muscle was raised with $3x10^{-5}M$ guanethidine $10^{-7}M$ sodium nitroprusside caused a reduction in the induced tone of approximately 50%. The effects of this compound in tissues from treated animals did not differ significantly from the effect produced in tissues from control animals (figure 20).

A one way ANOVA compairing the effects of this dose of sodium nitroprusside in all treatments determined that there were no significant differences amongst groups (p=0.6048)

c. The effect of phenylephrine on the rat vas deferens muscle:

$3x10^{-5}M$ phenylephrine induced a mean contraction of 1.35 +/- 0.15g. in tissues from treated animals which did not differ significanlty from the responses of tissues from control animals (1.30 +/- 0.1g.) (figure 19).

A one way ANOVA compairing the effects of this dose of phenylephrine in all treatments determined that there were no significant differences amongst groups (p=0.4948)

d. Anococcygeus motor response:

Field stimulation of the intrinsic nerves of the anococcygeus muscle from treated animals produced a frequency-dependent contraction which did not differ significantly from the responses of control tissues (figures 46, 47a).

3.3.10.2. Electrical stimulation of the nerve/muscle preparations:

e. Anococcygeus inhibitory response:

Following contraction with $3x10^{-5}M$ guanethidine, field stimulation of the intrinsic nerves of the anococcygeus muscle from treated animals produced a frequency-dependent inhibitory response which did not significantly differ from the responses elicited in control tissues (figures 46, 47b).

f. Vas deferens motor response:

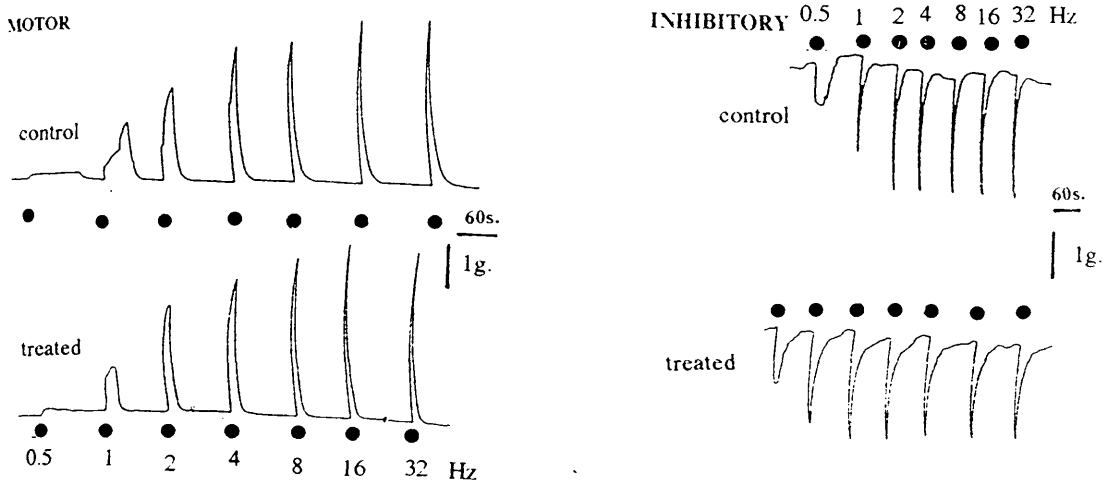
Electrical stimulation of the intrinsic nerves of the rat vas deferens, from animals treated with this porphyrinogenic drug combination, produced a frequency-dependent contraction of the tissue. The "fast" component of the biphasic response did not differ significantly from the responses elicited from control tissues (figures 46, 48a, 48b). The "sustained" noradrenergically-mediated component of treated tissues was significantly lower at the 4Hz stimulation frequency ($p<0.05$), whereas the responses at all other frequencies were unaltered.

g. Tail artery pressor response:

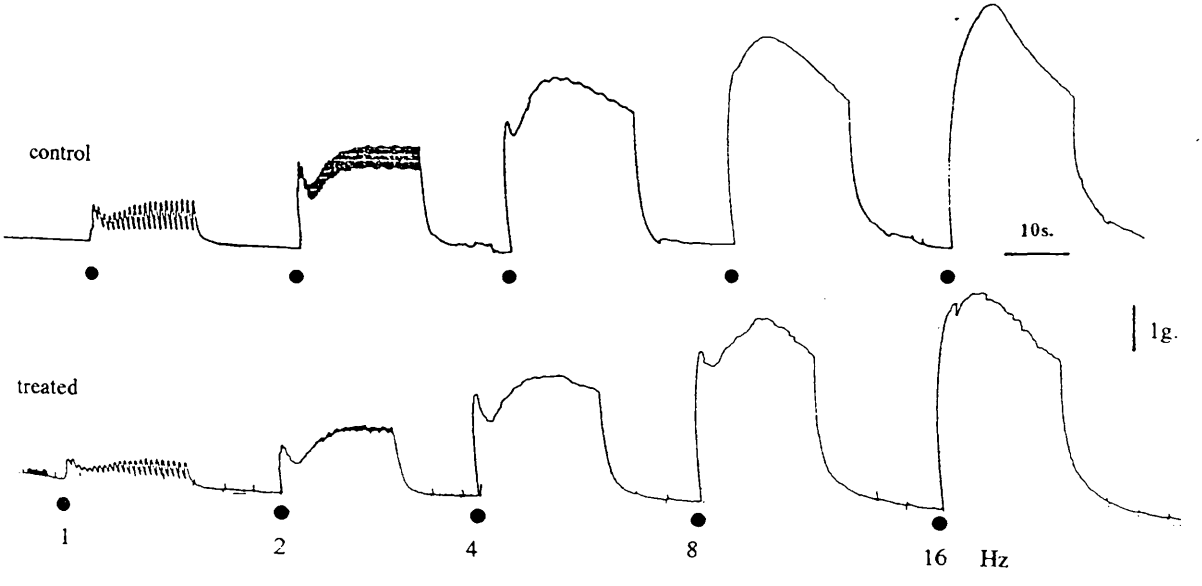
Electrical field stimulation of the perfused rat tail artery isolated from treated animals produced a frequency-dependent pressor response which did not significantly differ from the responses of tissues from control animals (figures 46, 49a).

Figure 46: The effects of porphyrinogenic treatment 1.7 (14 days of lead tetraacetate) on the five tissue responses, in individual experiments (bottom traces), compared to the responses from individual control tissue responses (top traces). The time bars apply during stimulation periods only and the interstimulus interval in all cases was 3 min. Stimulation frequencies are shown above or below each response. For clarity frequencies are shown for one tissue only but both control and treated tissues received the same stimulation parameters. The top left hand traces show the motor response of the rat anococcygeus muscle to stimulation with 50 pulses and the traces on the top right are the inhibitory response of the anococcygeus muscle to stimulation with 20 pulses. The middle traces show the response of the rat vas deferens to field stimulation with a 20s. train of pulses. The bottom left hand traces show the response of the perfused rat tail artery to field stimulation with 100 pulses. The bottom right hand traces shows the response of the rat hemidiaphragm to phrenic nerve stimulation with 50 pulses. There was a reduction in the noradrenergically-mediated component of the vas deferens, at a frequencyof 4 Hz. No other differences were observed in the responses of the tissues to nerve stimulation.

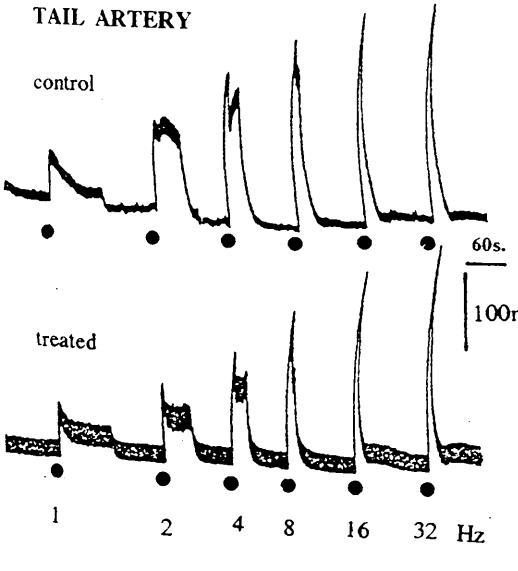
ANOCOCCYGEUS



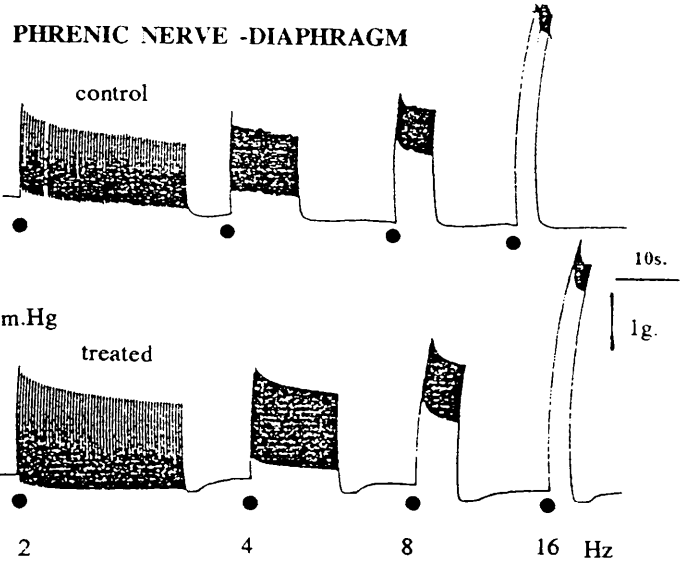
VAS DEFERENS



TAIL ARTERY



PHRENIC NERVE -DIAPHRAGM



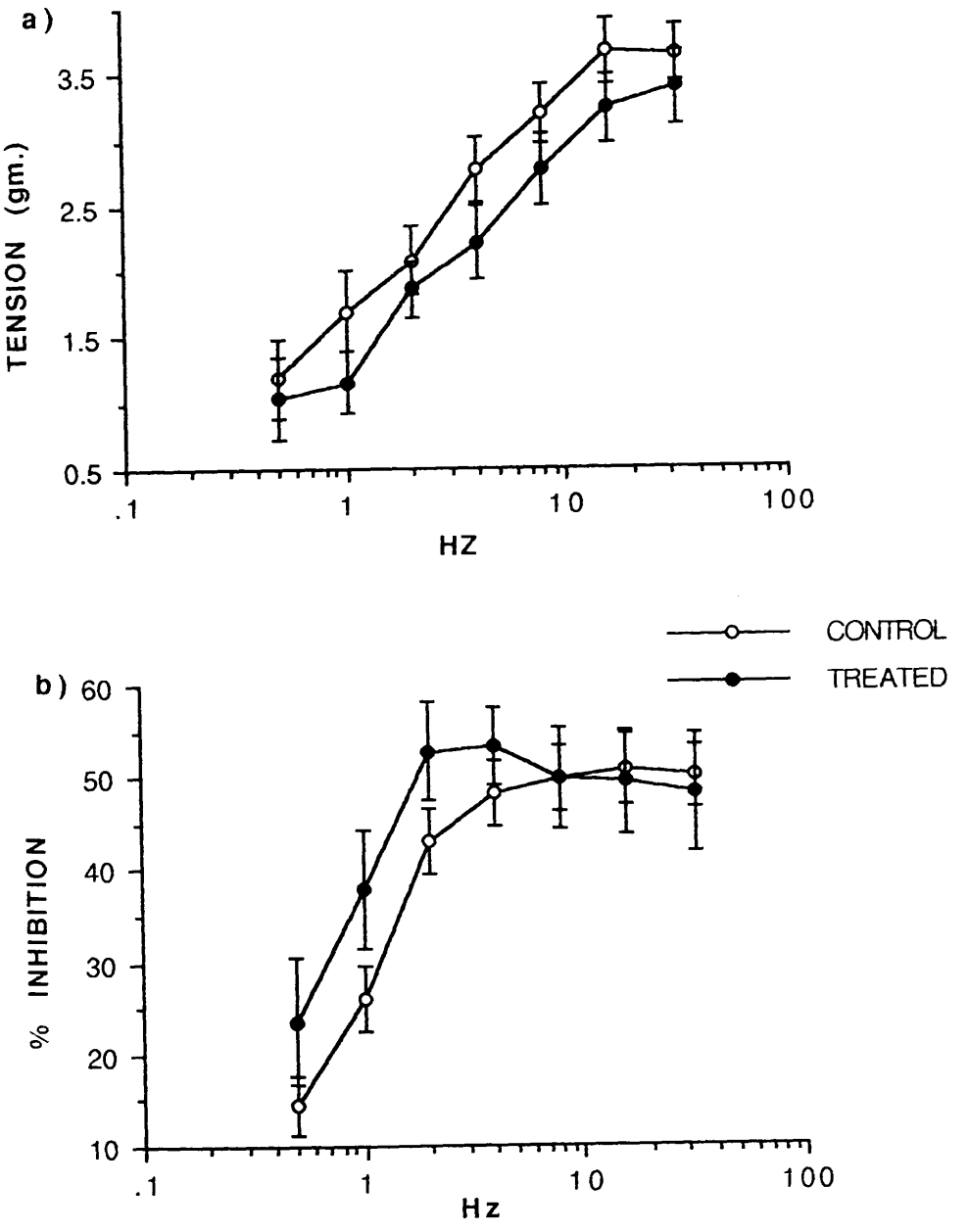


Figure 47: The top graph a) shows the mean (+/- S.E.M.) of the motor response the rat anococoocygeus muscle, in gm. tension, to stimulation with 50 pulses at 0.5-32 Hz in tissues from control animals (n=14) and in tissues from animals that had received treatment 1.7 (lead tetraacetate 14 days 50μM./day) (n=8) . Graph b) shows the inhibitory response of the anococcygeus muscle to intrinsic nerve stimulation with 20 pulses at 0.5-32 Hz in tissues from control animals (n= 14) and treated animals (n=8). There were no significant differences between control and treated tissue responses to nerve stimulation.

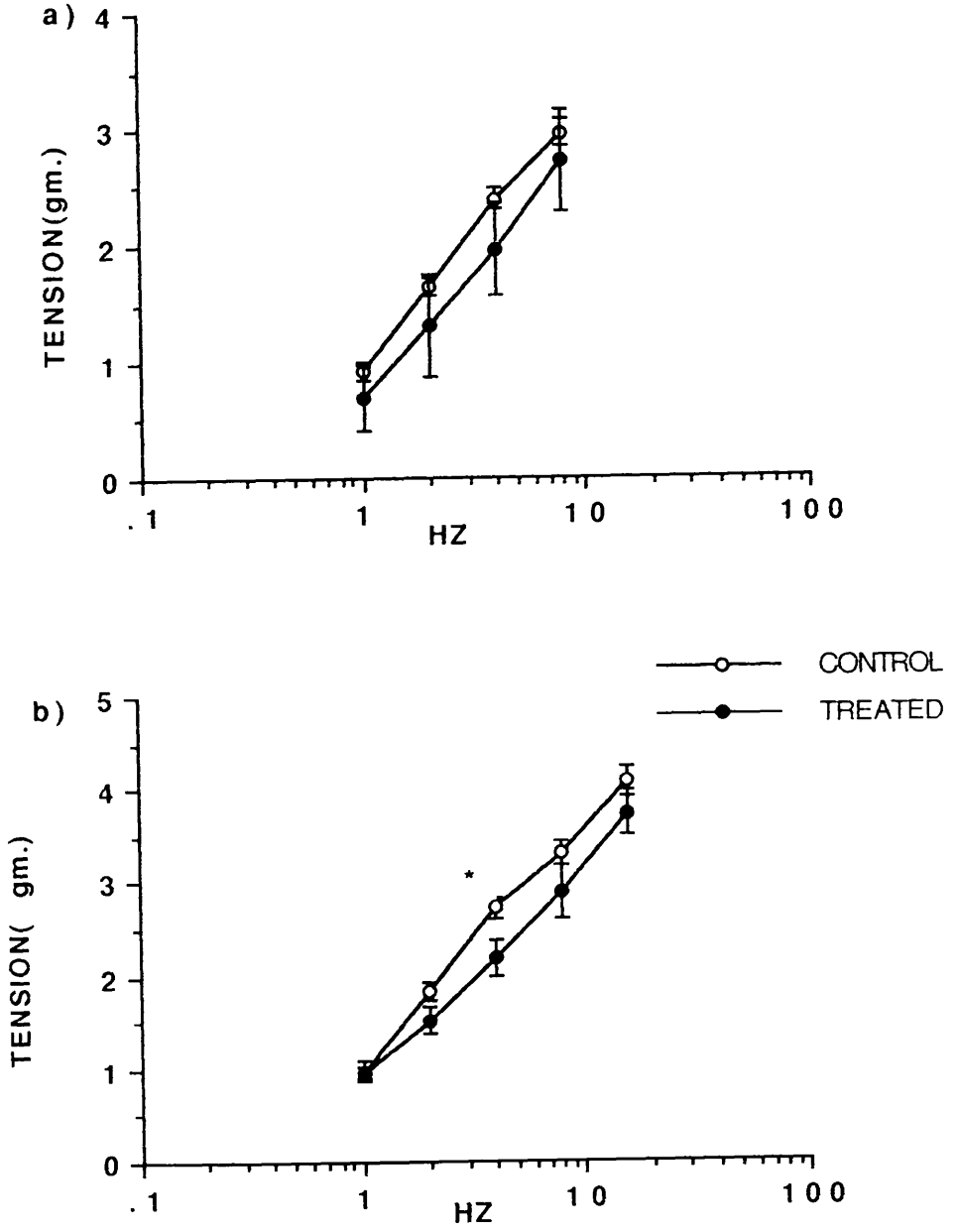


Figure 48: The top graph a) shows the mean (+/- S.E.M.) of the "fast" component of the rat vas deferens motor response , in gm. tension, to stimulation for 20s. at 1-8 Hz in tissues from control animals (n=20) and in tissues from animals that had received treatment 1.7 (14 days lead tetraacetate 50μM.) (n=8). Graph b) shows the "sustained" component of the rat vas deferens response to intrinsic nerve stimulation with a 20s. train of pulses at 1-16 Hz in tissues from control animals (n=20) and treated animals (n=8). At a stimulation frequency of 4 Hz treated tissue responses were significantly lower than control responses (* $p<0.05$). There were no other significant differences.

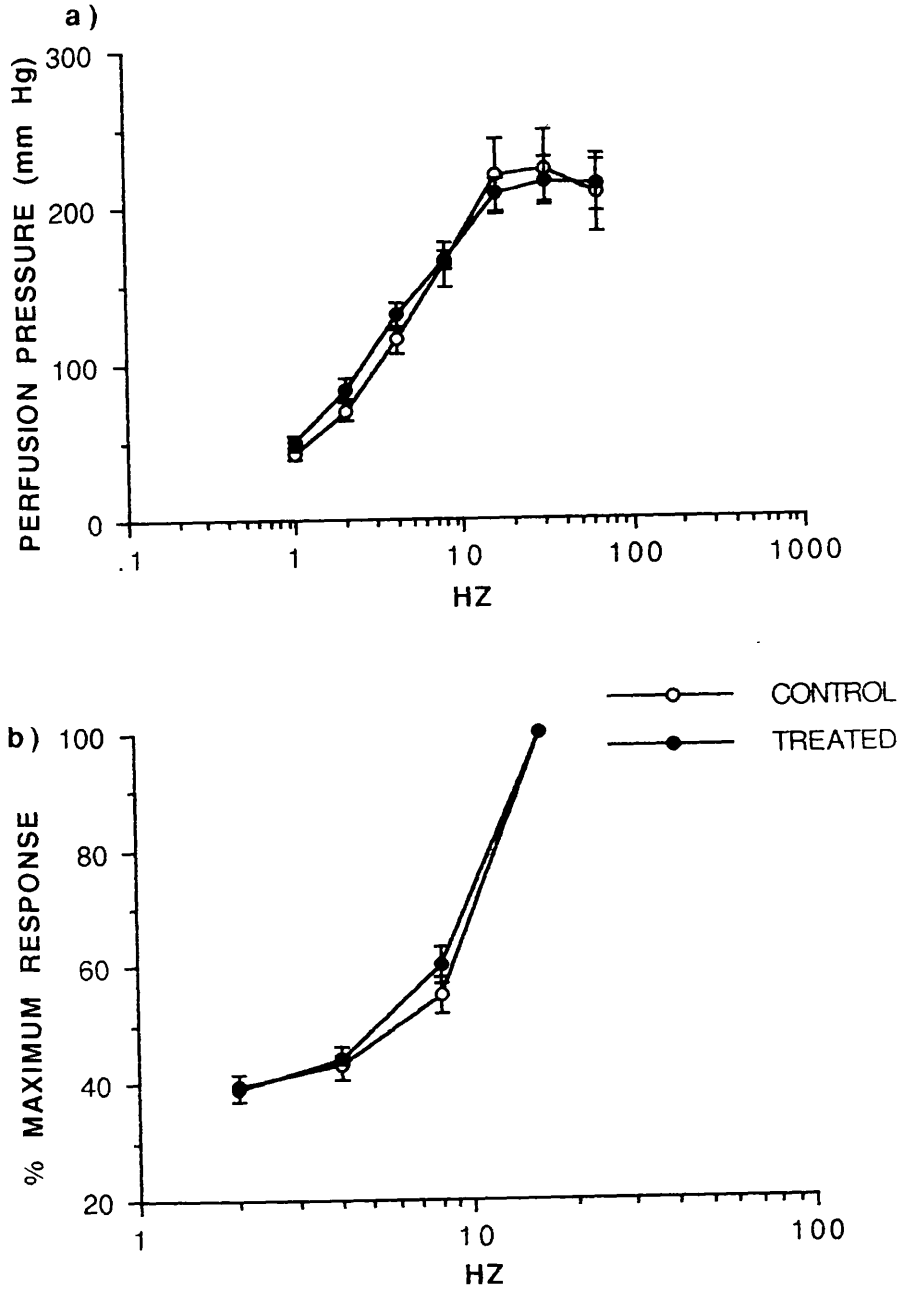


Figure 49: The top graph shows the mean (+/- S.E.M.) pressor response of the perfused rat tail artery to intramural nerve stimulation with 100 pulses at 1-64 Hz in tissues from control animals (n=8) and from animals that had been treated for 14 days with 50μMoles of lead acetate (n=10). The bottom trace shows the mean +/- S.E.M. of the response of the rat hemidiaphragm to phrenic nerve stimulation as the % of the maximum response acheived at 16 Hz in control animal tissue (n=8) and in tissues from treated animals (n=5). There were no significant differences.

8. **Phrenic nerve diaphragm:**

On stimulation of the phrenic nerve the diaphragm contracted in a frequency-dependent manner. The response of tissues isolated from treated animals did not differ significantly from the responses of tissues isolated from control animals (figures 46, 49b).

3.3.11. The effects of porphyrinogenic treatment on the responses of the isolated mouse vas deferens.

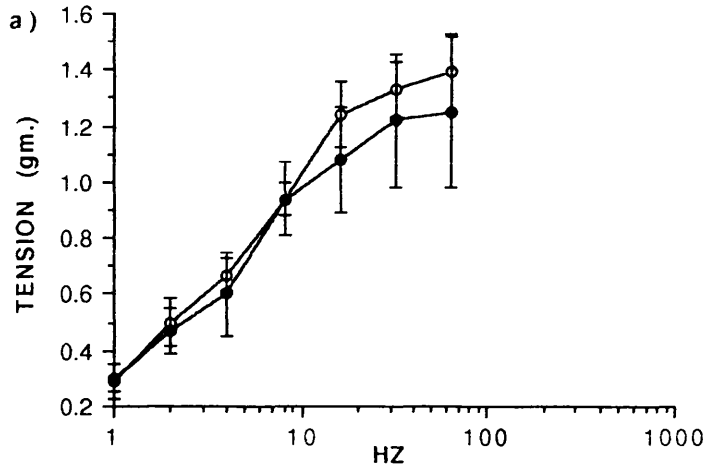
The three treatments (succinylacetone + either AIA, 4-ethyl DDC or DDC) all caused a marked peritonitis and two animals from the AIA treated group died.

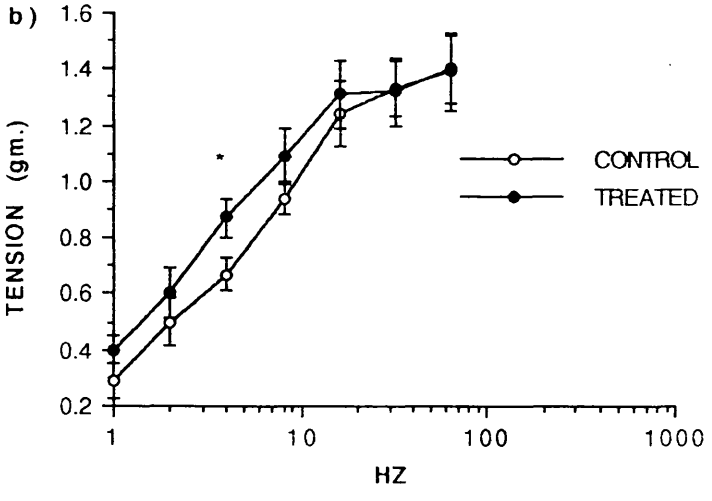
During the equilibration period, following the set up of the tissues, the initial tension of 0.5g. placed on the tisssue decayed to approximately 50-70% of this initial value. Stimulation of the intrinsic nerves of the tissue with a 20s. train of 1-32Hz, 0.5ms. duration, supramaximal voltage, induced a frequency-dependent motor response. The maximum contraction caused by field stimulation of the tissues from treated animals did not differ significantly from the responses of tissues from control mice (figure 50), except that of succinylacetone and 4-ethyl DDC treatment where the mean response of treated tissues at 4Hz were significantly greater than that of control tissues.

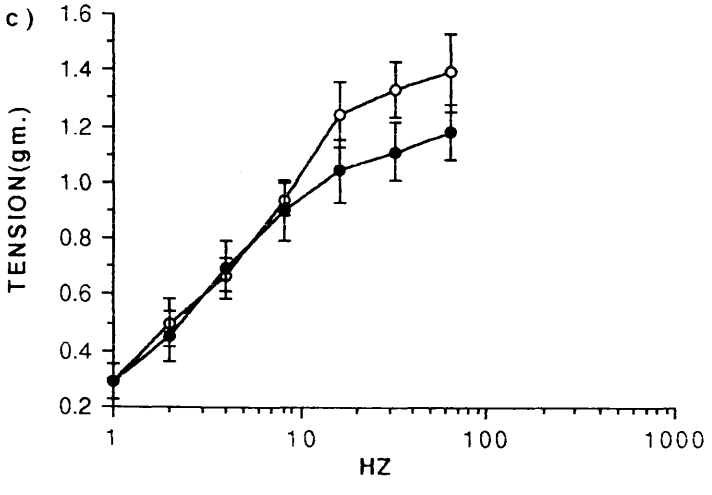
Summary:

In this section the effects of various combinations of porphyric drugs, given for different periods of time, have been examined on a wide range of isolated nerve/muscle preparations from two species. None of these combinations or treatment periods produced any overall change in the tissue responses, either to drug or nerve stimulation. There was, therefore, no experimental evidence of a neuropathy or that an animal model of human porphyria had been produced, in spite of the significant rises in ALA urinary concentrations.

Figure 50: The effects of 6 days treatment with **a)** succinylacetone (40mg/kg) +DDC (25mg/kg) (n=6); **b)** succinylacetone(40mg/kg)+ 4-ethyl DDC (25mg/kg) (n=6); **c)** succinylacetone (40mg/kg) +AIA (200mg/kg) (n=4) on the mean +/- S.E.M. contraction of the mouse vas deferens to field stimulation in control animal tissue (n=6) and in tissues from treated animals. At a stimulation frequency of 4 Hz the response of the vas deferens of animals treated with succinylacetone and 4-ethyl DDC was significantly greater that that of control tissue responses (*$p<0.05$).


a)
TENSION (gm.)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
1
10
100
1000
HZ


b)
TENSION (gm.)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
*
CONTROL
TREATED
1
10
100
1000
HZ


c)
TENSION(gm.)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
1
10
100
1000
HZ

CHAPTER 3

THE EFFECTS OF PORPHYRINOGENIC DRUGS ON NERVE AND MUSCLE.

DISCUSSION.

The symptoms of acute porphyria are indicative of a central, autonomic and somatic neuropathy (Goldberg 1959; Yeung Laiwah et.al. 1985; Mustajoki and Seppalainen 1975). The results of the previous group of experiments (chapter 2) provide no evidence that the haem precursors ALA and PBG are neurotoxic when examined on a range of innervated muscle preparations. The experiments reported in the present chapter examine the alternative thesis that the neurological symptoms of acute porphyria are the consequence of a haem deficient environment. Several lines of evidence suggest that porphyric patients have a deficit in haem-dependent functions such as cytochrome P-450 mediated drug metabolism (Song et.al. 1974; Anderson et.al. 1976) and muscle cytochrome oxidase activity (Goldberg et.al. 1985). Additionally, exogenously administered haem therapy alleviates some of the symptoms of acute porphyria (Tenhunen 1987) and haem arginate improves the abnormal antipyrine clearance in porphyric patients, suggesting a functional restoration of the haemoprotein, cytochrome P-450 (Tokola et.al. 1988). Although it may be argued that the therapeutic value of exogenous haem is due to its ability to reduce the levels of the haem precursors ALA and PBG, the lack of any convincing evidence that these haem precursors are neurotoxic would suggest that the beneficial actions of haem are mediated via reconstitution of haemoproteins essential for neural function.

The mitochondrial respiratory cytochromes are undoubtedly the most important haemoprotein for neural function as they are the mediators of energy production necessary for many cellular processes. Due to the unusually elongated morphology of neurones the function of these cells is dependent on the transport of newly synthesized materials from the cell body to the axon terminals and the transport of metabolites in the opposite direction. This axonal transport occurs at both fast and slow rates depending upon the materials that are being transported. Slow anterograde transport which occurs at a rate of 1-2mm/day transports structural proteins such as neurofilaments and neurotubules. Transport of mitochondrial enzymes by a fast anterograde process occurs at a rate of 50mm./day whereas vesicular transport of glycoproteins, lipid, neurotransmitters and Na^+/K^+ ATPase proceeds at a rate of 400mm/day. Metabolites from the

periphery of the cells are transported in vesicular bodies, secondary lysosomes, at a rate of 200mm./day (Jakobsen et.al. 1986). Ochs and Hollingsworth (1971) demonstrated that fast anterograde axonal transport of neurotransmitters and materials necessary for axonal repair is dependent on oxidative phosphorylation. When fast axonal transport was disrupted by induced hypoglycaemia in rats (Sidenius and Jakobsen 1983) Wallerian degeneration of axons resulted. Wallarian degeneration of neuronal tissue has been reported on post mortem histological examination of acute porphyric patients (Anzil and Dozic 1978) If the energy producing capacity of the mitochrondria was compromised by a cytochrome deficiency this type of transport may also lead to the development of a neuropathy. The main aim of this group of experiments was by reducing haem production with porphyrinogenic compounds, which disrupt haem synthesis to reduce respiratory cytochrome levels and hence energy production with an ensuing neuropathy development.

Decreased energy production is not the only way in which a reduction in haem may adversely affect the function of the neuromuscular preparations examined in this group of experiments. ATP produced as the high energy product of oxidative phosphorylation is a postulated co-transmitter in the neurogenically-mediated responses of the rat vas deferens, tail artery and the diaphragm (Swedin 1971; Sneddon and Westfall 1984; Sneddon and Burnstock 1984; Silinsky and Hubbard 1973) A reduction in oxidative phosphorylation resulting from haem deficiency would be manifest as a reduction in these partially ATP-mediated responses. This should have been especially easy to detect in the vas deferens where ATP alone is responsible for an easily defined component of the response.

The functioning of the haemoprotein guanylate cyclase is also of importance in this group of experiments. The cytosolic isoenzyme of guanylate cyclase is a haemoprotein and is the putative "second messenger" that mediates the NANC inhibitory responses of the rat anococcygeus. Guanylate cyclase catalyses the conversion of the guanine nucleotide, guanosine triphosphate to cyclic guanosine monophosphate (cyclic GMP). Cyclic GMP then activates a protein kinase which mediates the phosphorylation of the myosin light chain kinase (MLCK) weakening its binding of calmodulin, the Ca^{2+} receptor

of the contractile protein. This impaired binding reduces the amount of myosin that can be phosphorylated and therefore, induces relaxation (Drazin et. al. 1983;1986). Cyclic GMP also mediates Ca^{2+} extrusion from the muscle cell contributing to muscle relaxation (Itoh et.al.1985). The overall effect is, therefore, a reduced intracellular Ca^{2+} store and subsequent relaxation.

Nitric oxide (NO) and substances which release NO, glycerine trinitrate, sodium nitroprusside, sodium azide, hydroxylamine and intrinsic NANC nerve stimulation activate guanylate cyclase (Waldman and Murad 1987). Porphyrins in their free form or as haemoproteins exert a profound effect on the regulation of guanylate cyclase. Activation of the purified enzyme by sodium azide or hydroxylamine requires either its haem moiety intact or an exogenous source of haem such as catalase or cytochrome c (Murad et.al. 1978a). Haem also inhibits the activation of guanylate cyclase by NO, by acting as a binding sink for NO (Murad et.al 1978b). The haem moiety of guanylate cyclase is important as it is the haem-NO complex which is thought to regulate the activity of guanylate cyclase. (Craven 1978;1979) Sodium nitroprusside does not activate haem deficient guanylate cyclase (Gerzer et.al. 1981a;1981b;1982). The haem deficient enzyme can, however, be functionally reconstituted by exogenous haem. The iron deficient precursor of haem protoporphyrin 1X also activates guanylate cyclase and this stimulation of enzyme activity is as potent as that induced by nitro compounds in the presence of haem (Ignarro et. al. 1982). Wolin et.al. 1982 suggested that protoporphyrin 1X competes with haem for the binding site on the guanylate cyclase molecule and suggests that when NO binds to the haem moiety the Fe^{2+} is pulled out of the plane of the tetrapyrrole nucleus producing a protoporphyrin 1X-type molecule. These authors suggest that it is the formation of the protoporphyrin 1X-type molecule that is important in guanylate cyclase activation.

There are three main ways, therefore, that haem depletion could alter the function of the neuromuscular preparations examined in this section of the study.

All treatments employed in the experiments reported in this chapter, caused a significant increase in the urinary excretion of the haem

precursor ALA (figure 16). This increased ALA excretion was taken as an indication of a reduction in the free haem pool, which regulates the synthesis and activity of the rate limiting enzyme of the pathway ALAS. The porphyrinogenic treatments employed can be broadly divided into short, medium and long term. The short term treatment consisted of 3 days succinylacetone administration and did not alter the responses of any of the preparations examined to either agonists or nerve stimulation (figures 18-24). Therefore, although there was evidence of reduced haem availability in the animals, as a consequence of the treatment, there was no functional deficit in any of the haemoproteins necessary for normal nerve and muscle responses. This three day treatment period was probably too short to reduce the respiratory cytochromes, which have a half-life of 6 days, to a level where a compromise is exerted on neuronal activity either by a decreased energy production for enzyme synthesis and transport or for the production of ATP used as a transmitter. Sodium nitroprusside and neuronally-mediated activation of guanylate cyclase were both unaltered by this short term treatment (figures 20, 22b), indicating that haem synthesis was not reduced to levels at which guanylate cyclase became haem deficient.

Medium term treatment consisted of 9 days of a combination of succinylacetone, AIA and phenobarbitone and provided a greater assault on the haem biosynthetic pathway. This period of treatment covered 1.5 half-lives of the respiratory cytochromes. Again, however, there was no compromise in any of the responses of the tissues examined to agonist or nerve stimulation. The inability of this combination of porphyrinogenic drugs to elicit a neuropathy could be due to the failure of these drugs to alter haem synthesis in neural tissue. AIA exerts its effects as a result of its destruction of the haemprotein cytochrome P-450 during metabolism by a mechanism-based process. Most of the body's cytochrome P-450 is contained within the hepatocytes although cytochrome P-450 is present in the other tissues including the brain. There is no evidence, to date, that peripheral neurones contain cytochrome P-450. AIA may, therefore, be incapable of exerting its porphyrinogenic action on the function of these neuromuscular preparations either due to its inability to enter neural tissue or to a lack, in neurones, of the

haemoprotein necessary for AIA metabolism. Systemically administered AIA does not alter brain ALAS whereas it induces the hepatic enzyme (Paternitti et.al. 1978). Phenobarbitone is a lipophylic compound and as such should be capable of crossing neuronal membranes, and indeed since its main therapeutic effect is on brain function it is clear that it can cross the blood brain barrier. Nevertheless, it is unable to alter the activity of haem synthesizing enzymes in the brain while doing so in hepatic tissue (De Matteis and Ray 1982). There is no conclusive evidence, therefore, that these two compounds can alter neuronal haem synthesis. Succinylacetone, on the other hand, does induce activity of the rate limiting enzyme, ALAS, in the brain when administered intracerebroventricularly (De Matteis and Ray 1982). The full porphyrinogenic impact of this combination of compounds may not be exerted on neuronal tissue and could account for their inability to induce a peripheral neuropathy.

The four long term treatment groups employed in this study were designed to produce a model of an acute porphyric crisis, where the genetic defect in haem synthesis produces a situation where although the patient's haem level has not reached a critical low it is hovering near this level. A further assault on haem availability, whether it be due to stress, hormones or drugs, is enough to place a further strain on an already compromised system and the body's haem content falls to a level where clinical symptoms of neuropathy are manifest. In the studies reported here, succinylacetone, although not inhibiting the pathway at the level of defect in acute intermittent porphyria, PBG deaminase, does causes an enzymatic lesion early in the pathway at the stage immediately prior to PBG deaminase. The initial treatment of 30 days succinylacetone produced an increase in urinary ALA excretion throughout the period of treatment (these results are discussed more fully in chapter 4) and therefore produces a prolonged period of haem synthesis disruption. The further assaults on haem synthesis by the 4 long term treatment groups were provided by phenobarbitone and either DDC, or its more potent porphyrinogenic analogue 4-ethyl DDC (Ortiz de Montellano et.al. 1981c). In addition to phenobarbitone's ability to increase synthesis of the haemoprotein cytochrome P-450 it also increases the quantity of ferrochelatase inhibitory metabolite, an N-alkylated protoporphyrin, from the

metabolism of these dihydrocollidines. In the case of 4-ethyl DDC phenobarbitone also increases the proportion of the most active inhibitory isomer of the alkylated protoporphyrin, the N_A isomer (De Matteis et. al. 1983). In the last two treatments the increased period of DDC and 4-ethyl DDC treatment, although using a smaller concentration, provided inhibition at this last important step in the pathway for a further period of 14 days following succinylacetone treatment. This last impact on the haem pathway provided by the dihydrocollidine in the final two long term groups, therefore, spanned a period of more than two half-lives of the respiratory cytochromes. Even these long term treatments consisting of a combination of powerful porphyrinogenic compounds were unable to alter the functional capacity of the neuromuscular preparations examined (figures 30-45). A more effective treatment would have been to precede the low doses of dihydrocollidine with the phenobarbitone augmenting the drugs' effects for a longer period. The low doses of the dihydrocollidines were probably still susceptible to interaction with phenobarbitone as, on sacrifice of the animals, it was observed that the dihydrocollidine had precipitated out in the abdominal cavity and was therefore still available for uptake into the circulation for some time after injection. Both DDC and 4-ethyl DDC are known mediators of ferrochelatase inhibition and in doing so cause an accumulation of protoporphyrin 1X (Marks et. al. 1987). In a state of haem depletion and protoporphyrin 1X excess this latter compound could replace haem as the cofactor in the guanylate cyclase molecule. This would create increased basal activity of guanylate cyclase and could consequently antagonise the electrically-stimulated motor responses of the anococcygeus muscle and its response to agonists. This, however, was not the case, which indicates that in the peripheral nerves an environment did not exist which would promote protoporphyrin 1X activation of guanylate cyclase. It is possible that the porphyrinogenic dihydrocollidine compounds were not effective in inhibiting ferrochelatase in neural tissue and the evidence of reduced haem availability (increased ALA excretion) was a product of liver activity and not of nervous tissue.

Mice are more susceptible to the porphyrinogenic effects of the dihydrocollidines (Tephly et.al. 1979; De Matteis et.al. 1973) although

they are less sensitive than rats to the effects of AIA (De Matteis et.al. 1973). Three groups of mice were treated with succinylacetone for 6 days in addition to AIA(200mg/kg), DDC (25mg/kg) or 4-ethyl DDC (25mg/kg). All treatments caused the development of abdominal adhesions and 2 of the AIA group died before sacrifice. However, even in these ill animals, the response of the vas deferens to field stimulation did not differ significantly from the responses of control animal tissue to nerve stimulation. Porphyrinogenic treatment in mice similarly produced no evidence of porphyric neuropathy. Here again, mouse nervous tissue may be refractory to the porphyrinogenic effects of these compounds.

The porphyrinogenic drugs employed in this group of experiments were, therefore, incapable of reducing neuronal respiratory cytochromes to a level where their deficiency would cause a neuronal malfunction. There is the possibility that some of these compounds are not effective in neural tissue as AIA, DDC and 4-ethyl DDC require to be metabolized by cytochrome P-450 before they can exert their porphyrinogenic action. All three compounds destroy cytochrome P-450 while the latter two additionally produce an N-alkylated protoporphyrin 1X metabolite that inhibits ferrochelatse. Even if these compounds are metabolized in the nervous tissue they may be incapable of blocking the haem enzymes in this tissue. Brain ALAS is refractory to systemically administered AIA, DDC and phenobarbitone, compounds that cause an induction of the hepatic enzyme (De Matteis and Ray 1982; Paternitti et. al.1978). The existence of different isoenzymes of ALAS in erythropoietic and hepatic tissue (Bishop 1990) may be indicative of differential tissue control mechanisms and, therefore, drugs that exert porphyrinogenic activities in hepatic tissues may not do so in other tissues.

Kappas et.al. in 1989 suggested the possible existence of a mitochondrial, a cytosolic and a microsomal regulatory free haem pools. Due to the proximity, on the inner mitochondrial membrane, of the respiratory cytochromes and ferrochelatase, these haemoproteins may have priority to the limited amount of newly synthesized haem, following porphyrinogenic treatment. The increase in urinary excretion of ALA is probably the reaction to a decrease in the

cytosolic free haem pool whereas the mitochondrial haem availability may be uncompromised.

The effects of lead tetraacetate (50μM./day for 14 days) were examined on the responses of a similar range of rat tissues. This concentration of lead is larger than the dose employed by Goldberg et.al. in 1985 (10μM./kg) which resulted in a decreased ALAD, coproporphyrinogen oxidase and ferrochelatase activity in rats after 14 days treatment. The authors also measured a decrease in microsomal cytochrome P-450 content and increased hepatic ALAS activity. A small but significant increase in urinary ALA excretion was also observed in this study (figure 16). Although at a stimulation frequency of 4 Hz there was a significant difference in the noradrenergically-mediated response of the vas deferens from treated animals when compared to control animal tissue (figure 48b), the overall results did not provide convincing evidence of a neuropathy.

Although Schmid et.al. 1955 reported evidence of a neuropathy in rabbits treated with AIA other researchers have failed to observe any sign of neurological malfunction following porphyrinogenic treatment with AIA (Goldberg 1953; Goldberg and Rimington 1955) or DDC (Haeger-Aronsen 1961). The function of nerve and muscle may have a large reserve capacity following a reduction in respiratory cytochromes. McAllister et.al. 1990 observed that depleting cytochrome oxidase content by 45% using the mitochondrial protein inhibitor, chloramphenicol, does not compromise the extent to which skeletal muscle can contract. It may be necessary to deplete the respiratory cytochromes by more than 50% of their normal value, with these porphyrinogenic compounds, before a neuropathy is observed.

There was, therefore, no evidence, from the group of experiments reported in this chapter, that the various combinations of porphyrinogenic drugs employed for different periods were capable of producing a peripheral neuropathy. This suggests that the drugs did not reduce neural respiratory cytochromes or at least below the level critical for the maintenance of normal function.

The next group of experiments will examine the extent to which the mitochondrial cytochromes are reduced, following porphyrinogenic drug treatment in both hepatic and neural tissue.

CHAPTER 4

THE EFFECTS OF PORPHYRINOGENIC DRUGS ON HAEMOPROTEINS.

INTRODUCTION.

The porphyrinogenic agents, used in chapter 3 of this study, have been shown to cause a reduction in haemoprotein content or function Succinylacetone reduces cytochrome P-450 content in cultured chick embryo hepatocytes (Sassa and Kappas 1983). In cultured rat bone marrow cells (Beru et.al. 1983), human erythroleukemia cells (Bottomley et. al. 1985) and rabbit reticulocytes (Ponka et.al. 1982) succinylacetone decreases ^{59}Fe incorporation into haem. AIA reduces rat liver cytochrome P-450 content (Farrel and Correia 1981, Bornheim et.al. 1985; De Matteis 1971; Ortiz de Montellano and Mico 1981; Ortiz de Montellano et.al. 1983). A loss of microsomal cytochrome P-450 was seen in rat liver and in chick embryo hepatocytes following treatment with 4-ethyl DDC (Ortiz de Montellano et. al. 1981b; Mackie and Marks 1989). 4-Ethyl DDC additionally decreased cytochrome P-450-dependent peroxidative function in hamster olfactory epithelium, although direct measurement of the haemoprotein itself was not possible (Reed et.al. 1988). Indirect evidence exists regarding N-methyl protoporphyrin's ability to decrease tissue haem content. De Matteis and Marks in 1983 demonstrated that N-methyl protoporphyrin causes an increase in ALAS in cultured chick hepatocytes and suggest that this increase is a consequence of a reduction of haem synthesis by the alkylated porphyrin. Lead poisoning causes a reduction in cytochrome P-450 dependent liver metabolism (Meredith et.al. 1977). The experiments reported in the previous chapter provide indirect evidence that these compounds reduce the regulatory free haem pool, by their ability to cause an increase in ALA production and excretion, a process brought about by the lifting of haem's negative feed-back on ALAS production and activity. There was no evidence, however, from this last group of experiments that this disturbance of haem availability in any way depleted essential haemoproteins to levels where a deficit in nerve/muscle function developed. This poses the question as to where and to what extent a deficiency in haemoproteins occurred as a result of treatment with these chemicals. Sassa and Granick (1970) proposed that apoproteins have either high or low affinity for their haem moiety. The low affinity haemoproteins such as cytochrome P-450 and tryptophan pyrrolase provide an indication of the size of the regulatory free haem pool. The haem content of these compounds can

fluctuate widely without any obvious serious effect on bodily function. The high affinity haemoproteins such as catalase, haemoglobin and the respiratory cytochromes do not fluctuate in their haem content as readily as the low affinity haemopoteins and may be a more sensitive measure of overall haem depletion. The mitochondrial cytochromes, in particular, are extremely important to neuronal function and a depletion in the haem content of these proteins could lead to a compromise in energy production in the neurones and subsequently in neuronal function.

The half-lives of the haemoproteins are an important factor to be considered when using biosynthesis inhibition as a means of depleting haem, as the time taken to reduce the functional haemoprotein will be dependent on their life span. Catalase with a half-life of 2 days will be depleted more readily than the respiratory cytochromes, with a half-life 6 days or rat haemoglobin with a life span of 60 days. This would only be the case, however, if other factors such as relative affinity and local availability were identical. The time span of porphyrinogenic treatment would have to be related to the half-lives of the compounds. In this study, therefore, treatment regimes have to take into account the differential life span of the haemoproteins measured and all treatments covered at least two half-lives of the respiratory cytochromes and at least 12 of hepatic catalase. For practical reasons of time, treatments were not long enough to cover at least one half life of the erythrocyte.

Following treatments with porphyrinogenic agents the content or activity of specific haemoproteins was examined in hepatic, erythrocytic and neural tissue for evidence of a differential haem deficiency.

CHAPTER 4

THE EFFECTS OF PORPHYRINOGENIC DRUGS ON HAEMOPROTEINS.

METHODS.

4.2 Haemoglobin, respiratory cytochromes and catalase were measured in liver, erythrocytes and brain of rats following porphyrinogenic drug treatment. In erythrocytes only haemoglobin and catalase are present and the brain contains no catalase.

4.2.1. Drugs:

Sodium dithionite (Riedel, Hannover); sucrose (May and Baker, England); N-methyl Protoporphyrin (Porphyrin products, Utah, U.S.A.); Heparin, Na salt; deoxycholic acid, Na salt; cholic acid, Na salt; [tris(hydroxymethyl)aminomethane hydrochloride](TRIS-HCl); Triton X-R100; Lead acetate; propylene glycol; Lowry's Reagent; Folin and Ciocalteu's Reagent; Drabkin's Reagent; bovine serum albumin. Unless stated, all chemicals were obtained from Sigma Chemical Co.Ltd., England.

Unless specified, male Wistar rats of the same weight and strain as in chapter 3 above were used in this group of experiments. All injections were intraperitoneal, unless stated otherwise. Succinylacetone was administered at 1ml/kg., in distilled water, whereas 4-ethyl DDC and DMSO was administered in a volume of 0.5ml/kg.

4.2.2 Treatment:

GROUPS:

2.1) Animals received 30 daily injection of succinylacetone at a dose of 20mg./kg. Two of these animals spent the first 25 days of treatment in metabolic cages and the urinary ALA and total porphyrin excretion were determined daily over this period.

2.2) Animals received 30 days of succinylacetone 20mg./kg. This was followed by 10 days of 4-ethyl DDC (25mg./kg) , 4 days of phenobarbitone (80mg./kg.) and a final dose of 300mg./kg. of 4-ethyl DDC. Succinylacetone was continued throughout the treatment.

2.3) 14 days of 4-ethyl DDC (100ml/kg.) was administered to animals in this group.

2.4) N-methyl protoporphyrin 800nmoles/day was injected in a volume of 0.1ml. into the tail vein, for 14 days. The compound was flushed through with 0.2ml. of saline.

2.5) Lead acetate (1gm./litre) was given in the drinking water of nursing rat mothers whose pups were 5 days old. Lead treatment was started at this period of development to inhibit haem synthesis enzymes at a critical stage in respiratory cytochrome synthesis. Lead administration, by this means, was continued after weaning until the animals were used at approximately 250gm. (6-8 weeks old) Only male animals were used in the experiments

2.6)10 day old male rats were injected with a single dose of 4-ethyl DDC (100mg/kg in 0.1ml of propylene glycol). The animals were sacrificed at 20 days of age. Again, treatment at this developmental stage was aimed at blocking the brain respiratory cytochrome surge which occurs at this time.

Controls consisted of water fed controls for the lead group, saline injections, by the same route for the N-methyl protoporphyrin group; propylene glycol for the 4-ethyl DDC treatment in 10 day old animals; 14 days DMSO for 4-ethyl DDC and the long term treatment combination.

Animals were killed by CO_2 asphyxiation.

4.2.3. WHOLE ANIMAL PERFUSION:

As the presence of haemoglobin would interfere with subsequent spectral absorption measurements of other haemoproteins, preliminary experiments were carried out to reduce tissue haemoglobin content. After killing by CO_2 asphyxiation rats were perfused with heparinised saline to clear haemoglobin from the tissue vasculature. The thoracic cavity was opened and the apex of the heart cut away. A large plastic cannula was inserted into the left

ventricle, pushed gently up into the aortic arch and secured in place by a ligature. The animals were perfused with heparinised saline (90 units/ml) at a rate of 85mls/min. The perfusate leaving the circulation via the right ventricle quickly ran clear, free of blood and the liver was seen to change colour uniformly. A histological examination was carried out on liver and brain tissue samples following perfusion with either 1 or 2 litres of fluid for evidence of erythrocytes in the microcirculation. Additional evidence of remaining haemoglobin was sought spectrophotometrically. Following homoginisation in 0.44M sucrose, a sample of a 600g supernatant was diluted 1:1 with Drabkin's reagent and assayed for haemoglobin content (Haemoglobin assay described later). The levels of haemoglobin in both tissues were less than the threshold sensitivity of the assay employed.

4.2.4. Dissection:

The animals were killed, a sample of blood taken directly from the heart by cardiac puncture, added to a tube containing lithium heparin coated pellets and placed on ice. The whole animal was then perfused with 1 litre of ice cold heparinised saline as described above. The liver and brain were removed, placed on ice and prepared for tissue haemoprotein measurement.

4.2.5. HAEMOGLOBIN ASSAY.

a) Principle:

In the presence of potassium ferricyanide at alkaline pH, haemoglobin is oxidised to methaemoglobin. The latter reacts with potassium cyanide to form cyanmethaemoglobin which possesses a maximum absorption at 540nm. The colour intensity, at this wavelength and at the standard concentrations used in the assay, is linear and is proportional to the total haemoglobin content (figures 51a, 52d).

b) Assay procedure:

Reagents:

i) Drabkin's reagent contains 100 parts, sodium bicarbonate: 20 parts potassium ferricyanide: 5 parts potassium cyanide in 1 litre of distilled water.

ii) Lyophylised human methaemoglobin standard containing 18gm. methaemoglobin/dl. was used in this assay.

iii) Standards were freshly prepared each day at the time of assay containing 0, 6, 12 and 18 gm. methaemoglobin /dl. in Drabkin's reagent. Samples were prepared by adding 20µl. of whole blood to 5ml. of Drabkin's reagent, washing the pipette 3 or 4 times with the solution. The standards and sample tubes were allowed to stand for at least 15 min. at room temperature. The spectral absorption of the samples was determined at 540 nm. on a Shimadzu UV-visible recording Spectrophotometer against the constructed standard curve (figure 51a).

4.2.6. TISSUE PREPARATION AND CATALASE ASSAY.

4.2.6.1. Tissue preparation.

a) Erythrocyte:

Erythrocyte lysates were prepared by the method of Aebi (1984). The sample of blood taken by cardiac puncture was centrifuged at 300g on an MSE "Minor" bench centrifuge and the plasma and buffy coat removed by suction. The erythrocytes were washed three times in isotonic saline and lysed by adding four parts by volume of distilled water. The haemoglobin content of the lysate was determined from a sample by the Drabkin's method described above.

b) Liver:

Liver tissue was prepared for catalase measurement by the method of Cohen et. al. (1970).

Reagents: i. Isotonic buffer:

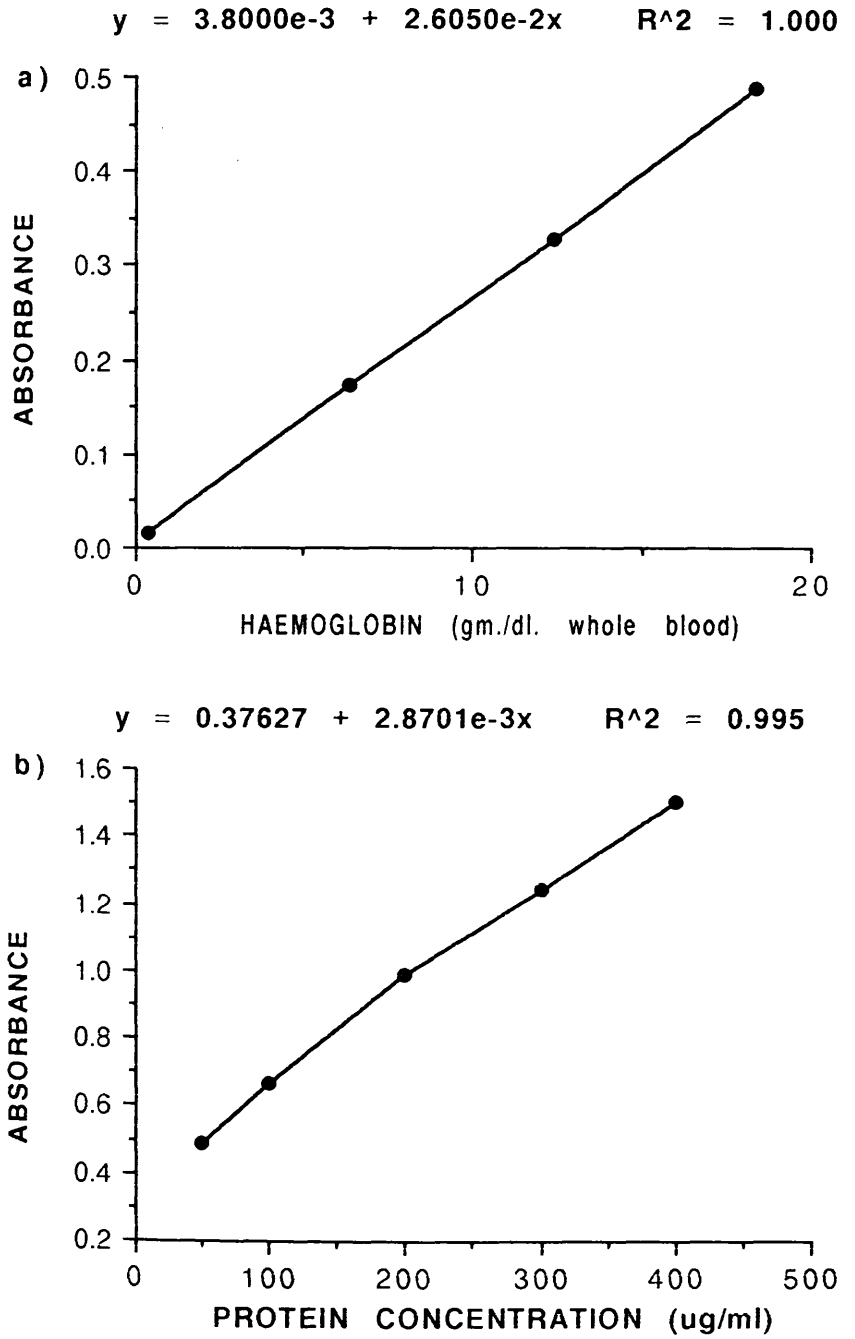


Figure 51: Graph a) shows a typical calibration curve for the absorbance of haemoglobin at 540nm. assayed by the Drabkin method. The regression equation used for calculation of the haemoglobin content is shown above the graph. The lower graph b) shows a typical calibration curve for the absorbance of protein (Lowry Method) at 750nm. The regression equation used to calculate protein concentration is shown above the graph.

stock: NaCl-180g; Na_2HPO_4-27.3g; NaH_2PO_4-4.86g made up in 2 litres of distilled water, pH 7.4.

This stock solution was diluted 9ml. in 100ml. of distilled water to give an isotonic buffer solution.

ii. 10% Triton X-R 100 in isotonic buffer. Triton X-R 100 does not absorb in the UV wavelength and is therefore suitable for this assay.

iii. Absolute ethanol.

2g. of liver was homogenised in 10 volumes of isotonic buffer with 8 strokes of a glass/Teflon homogeniser at 1000 r.p.m. The homogenate was centrifuged at 600g for 10min. and the supernatant decanted. An aliquot was incubated for 30 min. in an iced water bath with absolute alcohol at a final concentration of 0.17M. Triton X-R 100 was then added from the 10% stock to give a final concentration of 1%. A 100µl aliquot of this supernatant was frozen for subsequent protein determination.

4.2.6.2. ASSAY:

Catalase activity was measured by the decomposition of hydrogen peroxide, whose reduction can be followed spectrophotometrically at 240nm (Aebi 1984). Quartz cuvettes are necessary at this wavelength.

Reagents: i. Phosphate Buffer:

solution (a) 6.81g of KH2PO4 in 1l. of distilled water.

solution (b) 8.9g of $NaHPO4.2H_2O$ in 1l. of distilled water.

The assay buffer was made by mixing the two reagents in the proportion of a:b :: 1:1.5

ii. Hydrogen peroxide (30mM) in phosphate assay buffer.

A 500 fold dilution of the erythrocyte lysate and a 100 fold dilution of the liver supernatant were made. Catalase activity was measured in these solutions.

Both sample and reference cuvettes contained 2ml. of the sample solution. 1ml. of buffer was added to the reference cuvette and a steady baseline obtained. 1ml. of hydrogen peroxide solution was added to the sample cuvette, stirred quickly and thoroughly with a plastic paddle and the decrease in absorbance followed for 1-2min. This reaction demonstrates linear kinetics for the first 15s. (Aebi 1984) and this was used to determine the activity of the catalase enzyme in both tissues (figures 52b, 52e).

4.2.7. RESPIRATORY CYTOCHROME MEASUREMENT.

4.2.7.1. TISSUE PREPARATION:

The brain was halved sagittaly and duplicate samples of both brain and liver (2g) were processed. Both liver and brain were homogenised in ice cold sucrose (0.44M). This denser homogenising medium was shown by Lovtrup and Zelander (1962) to be a better medium for isolation of brain mitochondria due to the high myelin content of this tissue. The tissues were placed in a cold glass homogenising tube and a 10% homogenate prepared by 8 passes of a Teflon pestle at 1000 r.p.m. The homogenate was spun at 600g. on a Damon IEC centrifuge for 10min. The supernatant was decanted and the P1 pellet discarded. The supernatant was centrifuged at 10,000g. for 15min. on a Beckman Ultacentrifuge to produce a P2 mitochondrial pellet. The supernatant was discarded, the pellet resuspended by gentle hand homogenisation in ice cold sucrose solution and centrifuged again at 6,500g for 15min. This last process was repeated twice to give the final washed mitochondrial pellet. This final mitochondrial pellet was resuspended in 3ml. of a 1:1 solution of 0.44 M sucrose and 0.1M Tris HCl buffer pH 8.0 A 100µl. aliquot of the mitochondrial suspension was frozen for subsequent protein determination.

The mitochondrial suspension was clarified by the addition of 20% sodium cholate and 10% deoxycholate. 100µl. of each were added to the liver mitochondrial preparation whereas 200µl. were required for the more turbid brain mitochondrial sample. To obtain a clarified brain sample a short 5min. spin at 500g. on a MSE "Minor" bench centrifuge pelleted the heaver myelin contaminant leaving the solubilised mitochondrial membrane in solution. The clarified mitochondrial membrane samples were transfered into fresh sample tubes.

4.2.7.2. RESPIRATORY CYTOCHROME ASSAY:

a) Principle:

From the oxidised-reduced difference spectra the differences in absorbance at the following wavelength pairs can be determined:- 1. 550-535 nm. (cytochrome c); 2. 554-540nm. (cytochrome c_1); 3. 563-577nm. (cytochrome b); 4. 605-630nm. (cytochrome $a+a_3$ or cytochrome oxidase (cox)). To calculate the concentration of each cytochrome 4 simultaneous equations with 4 unknowns were solved. The principle applied here is that the change in ΔA from one wavelength to another in the difference spectrum (reduced - oxidised) of the mitochondria equals the sum of the products of appropriate extinction coefficients and the 4 (unknown) cytochrome concentrations. Applying this principle at the four pairs of wavelengths produces the 4 simultaneous equations with the 4 unknowns. The extinction coefficients employed by Vanneste (1966) were taken from Van Gelder and Slater (1962) for cytochrome c, Williams (1964) for cytochrome c_1, Zaugg and Rieske (1962) for cytochrome b and Van Gelder and Slater (1963) for cytochromes $a+a_3$.

From these data a set of extinction coefficients of the cytochromes at the 4 wavelength pairs were produced.

Wavelength pairs (nm.)	*cytochromes* ($mM^{-}.cm^{-1}$) c	c_1	b	$a+a_3$	*symbol for change in* ΔA
550-535	25.1	10.3	-6.22	0.69	α 15
554-540	7.78	18.8	5.08	1.03	α 25
563-577	-1.39	0.91	28.5	-0.36	α 35
605-630	-0.26	-0.59	0	13.1	α 45

The equations produced are, therefore :-

$$25.1\ C1 + 10.3\ C2 - 6.22\ C3 + 0.69\ C4 = \alpha 15$$

$$7.78\ C1 + 18.8\ C2 + 5.08\ C3 + 1.03\ C4 = \alpha 25$$

$$-1.39\ C1 + 0.91\ C2 + 28.5\ C3 - 0.36\ C4 = \alpha 35$$

$$-0.26\ C1 - 0.59\ C2 + 0\ C3 + 13.1\ C4 = \alpha 45$$

Where C1= cytochrome c concentration.

C2 = cytochrome c_1 concentration

C3 = cytochrome b concentration

C4 = cytochromes $a+a_3$ concentration.

By using matrix algebra these equations are simplified to a secondary set of equations :-

$$C1 + 0.41\ C2 - 0.25\ C3 + 0.0275\ C4 = \beta 15$$

$$C2 + 0.45\ C3 + 0.0523\ C4 = \beta 25$$

$$C3 - 0.0144\ C4 = \beta 35$$

$$C4 = \beta 45$$

These set of equations can then be easily solved by substitution of the constants with their respective values :-

$$\beta 15 = \alpha 15/25.1$$

$$\beta 25 = \alpha 25 - 7.78\ \beta 15/15.6$$

$$\beta 35 = \alpha 35 + 1.39\ \beta 15 - 1.48\ \beta 25/27.5$$

$$\beta 45 = \alpha 45 + 0.26\ \beta 15 + 0.48\ \beta 25 - 0.153\ \beta 35/13.13$$

Therefore,

$C4 = \beta 45$ = cytochrome $a+a_3$ concentration in umoles

$C3 = \beta 35 + 0.0144\ C4$ = cytochrome b concentration.

$C2 = \beta 25 - 0.0523\ C4 - 0.45\ C3$ = cytochrome c_1 concentration.

$C1 = \beta 15 - 0.0275\ C4 = 0.25\ C3 - 0.41\ C2$ = cytochrome c concentration.

b) Assay:

The clarified mitochondrial solution was aerated by vortexing. 1ml. samples were added to both a reference and sample cuvette. A baseline difference spectra was obtained on the scanning mode of the spectrophotometer between 650 and 500nm. 5mg. of sodium dithionite was added to the sample cuvette and mixed thoroughly with a plastic paddle. The oxidised-reduced difference spectra was

recorded 2min. later and the cytochrome content determined by the method of Vanneste (1966) (figures 52a, 52g).

4.2.8. PROTEIN ASSAY:

a) Principle:

An alkaline cupric tartrate reagent (Lowry Reagent) complexes with the peptide bonds of proteins to form a purple-blue colour when reacted with the phenol reagent, Folin's Ciocalteu's Reagent. This colour has a maximum absorption at 750nm. and is proportional to the protein content of the sample.

b) Assay:

Liver homogenate and mitochondrial protein was measured by the method of Lowry (1951) using bovine serum albumin as standard. Standards were prepared in deionised water to a total volume of 1ml. at concentrations of 0, 50, 100, 200, 300, and 400μg./ml.

Samples were prepared by adding a small measured quantity of the homogenate or mitochondrial suspension to test tubes and made up to a total volume of 1ml. with deionised water. 1ml. of Lowry's reagent was added to each tube and vortexed immediately. The standards and samples were allowed to stand at room temperature for 20min. following which 0.5ml. of the Folin and Ciocalteu's Reagent was added to each tube with rapid and immediate mixing. The absorbance of the standards and samples were determined at 750nm. 30min. afterwards. A calibration curve was constructed for each assay (figure 51b) and the sample protein concentration determined from this.

4.2.9 Urinary ALA and Total Porphyrin (Uroporphyrin and Coproporpphyrin) were determined by the methods described by Moore 1983. These were carried out at the Western Infirmary, Glasgow, Scotland.

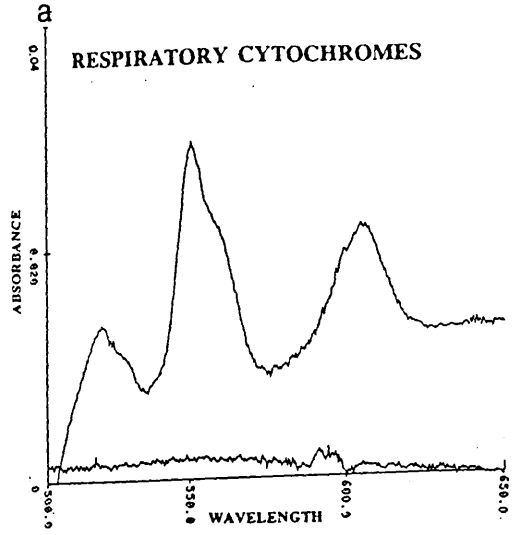
Figure 52: Shows a) an oxidised-reduced difference spectra of liver mitochondrial cytochromes. The baseline is the difference spectra between the reference and sample cuvettes prior to the reduction of the contents of the sample cuvette contents with sodiun dithionite. Graph b) shows the reduction in absorption of hydrogen peroxide, with time in the presence of liver homogenate containing catalase. Graph c) demonstrates the inhibition of catalase's hydrogen peroxide decomposing capacity by 1mM. sodium azide. Graph d) shows the linear relationship in absorption of increasing concentrations of haemoglobin at 540nm. Graph e) shows shows the reduction in absorption of hydrogen peroxide in the presence of an erythrocyte lysate containing catalase. Graph f) demonstrates the inhibition of catalase's hydrogen peroxide decomposing capacity by 1mM. sodium azide. Graph g) shows an oxidised-reduced difference spectra of brain mitochondrial cytochromes. The baseline is the difference spectra between the reference and sample cuvettes prior to the reduction of the contents of the sample cuvette contents with sodium dithionite.

HAEMOPROTEIN MEASUREMENT

LIVER

a

RESPIRATORY CYTOCHROMES



b CATALASE ACTIVITY

c

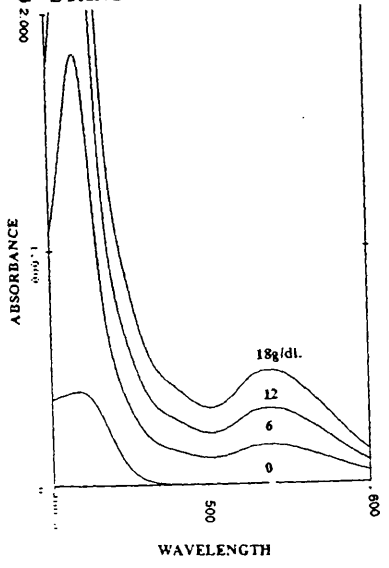
15s.

ABS.005

IN THE PRESENCE OF
1mM. SODIUM AZIDE

BLOOD

d CYANOMETHAEMOGLOBIN



CATALASE ACTIVITY

e

f

15s.

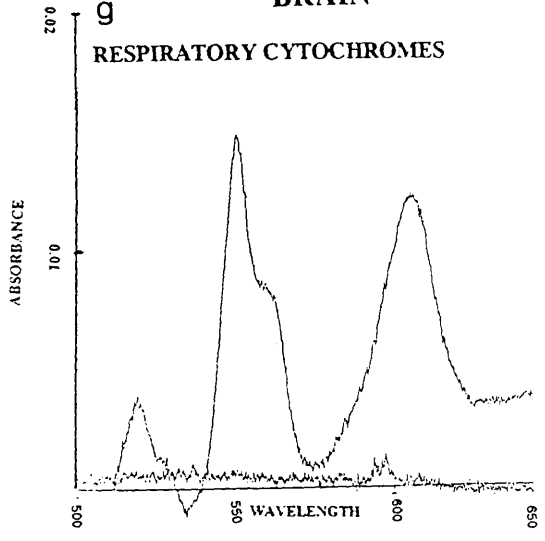
ABS.0·05

IN THE PRESENCE OF
1mM. SODIUM AZIDE

BRAIN

g

RESPIRATORY CYTOCHROMES



4.2.10. Ferrochelatase Assay, was carried out by the method of Houston et.al. (1988) by Dr. T. Houston, Western Infirmary, Glasgow, Scotland.

CHAPTER 4

THE EFFECTS OF PORPHYRINOGENIC DRUGS ON HAEMOPROTEINS.

RESULTS.

CHAPTER 4 PORPHYRINOGENIC TREATMENTS.

Succinylacetone injections were administered in a volume of 1ml/kg. in distilled water vehicle. Phenobarbitone was dissolved in 0.1M NaOH and brought to pH 9 with 0.1MHCl and was also administered in a volume of 1ml/kg. 4-ethyl DDC was administered in a volume of 0.5ml/kg. in DMSO. N-methyl protoporphyrin was dissolved in saline at pH 9.0 and brought back to a pH of 7.4 with 0.1M HCl. N-methyl protoporphyrin injections were administered intravenously via the tail vein.

TREATMENT 2.1: Rats received succinylacetone at a dose of 20mg/kg. daily for 30days.

TREATMENT 2.2: 30 days of succinylacetone (20mg/kg.) followed by 10 days administration of 4-ethyl DDC (25mg/kg.) then 4 days of phenobarbitone (80mg/kg.) with a further 300mg/kg of 4-ethyl DDC 24 hours prior to killing. Succinylacetone was continued throughout the treatment period.

TREATMENT 2.3: 14 days of 4-ethyl DDC (100mg/kg.).

TREATMENT 2.4: 14 days of N-methyl protoporphyrin (0.8 μMole in a volume of 0.1ml) administered via the tail vein.

TREATMENT 2.5: Lead acetate was administered in the drinking water firstly to nursing mothers when the pups were 5 days old and then to the pups themselves after weaning at a concentration of 1g./ litre of drinking water. This treatment continued until the animals were used at a weight of 200-300g.

TREATMENT 2.6: 10 day old male rats received a single intraperitoneal injection of 4-ethyl DDC in propylene glycol in a volume of 0.1ml. Control animals received the same volume of propylene glycol.

Figure 52 shows typical spectrographic traces of the haemoproteins measured in the three tissues.

4.3.1. The effects of 30 days succinylacetone (treatment 2.1) on rat urinary ALA levels:

Two rats received daily intraperitoneal injections of succinylacetone (20mg/kg.). This treatment induced a marked increase in urinary ALA and total porphyrin excretion which was maintained for the first 25 days of treatment (figure 53). These results confirm that long term treatment with succinylacetone, at this dose, is capable of sustaining a reduction in the haem biosynthetic pathway.

4.3.2. The effects of perfusing the whole animal with saline on the content of haemoglobin in the microcirculation:

Perfusion of the whole animal with 1 litre of heparinised saline was sufficient to clear the liver and brain of red cell haemoglobin, which could possibly confound respiratory cytochrome measurement, as measured both by the biochemical haemoglobin assay of a 1:1 dilution of the S1 supernatant following a 600g. spin (figure 54) and by histological examination of the tissues (plate 1). Erythrocytes could be detected by histological examination of non-perfused tissue (plate 1)and Hbwas detected spectrophotometrically at a concentration of around 6g./dl. (figure 54). There may be some spectral interference of other cytochromes with the measurement of haemoglobin. Although there was absorbance at 540nm in the perfused liver sample, there was no peak similar to that produced by cyanomethaemoglobin, suggesting that the tissue was haemoglobin-free. Haemoblobin could not be detected in either perfused or non-perfused brain tissue, by this method (figure 54) although there was histological evidence of erythrocytes in the brain microcirculation (plate 1).

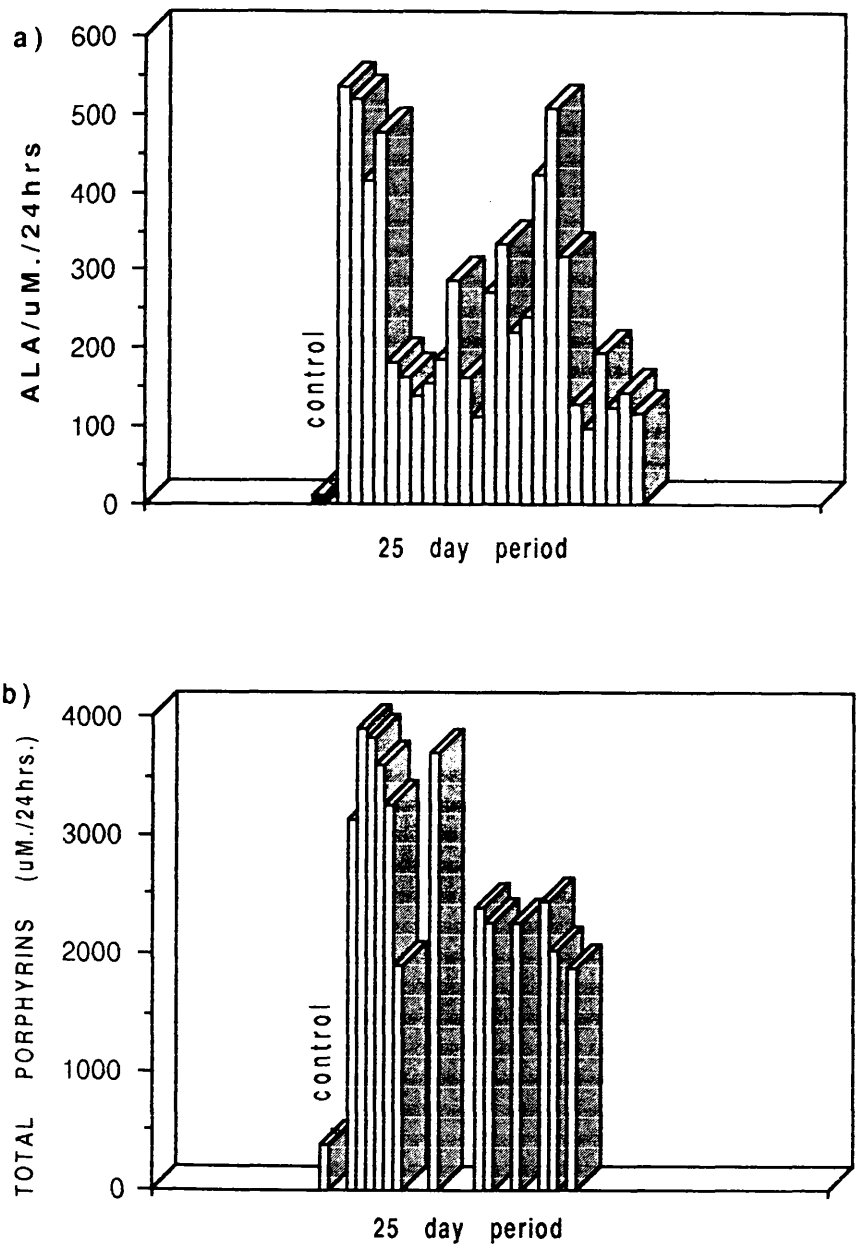
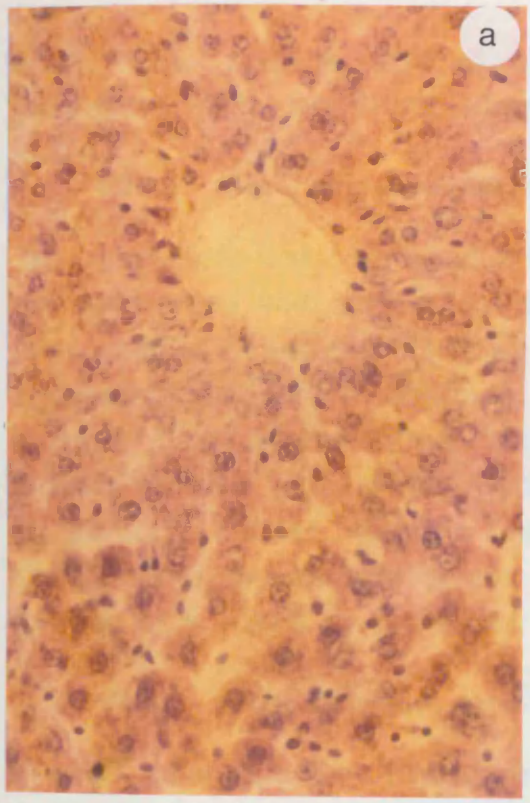


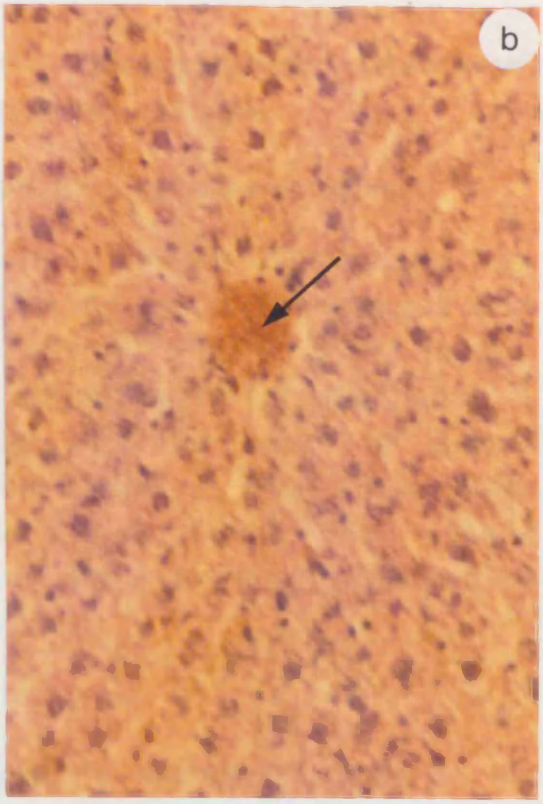
Figure 53: Graph a) shows the mean urinary excretion of ALA (μMoles/l/day), of two animals, on the first 25 days of daily succinylacetone administration (20mg/kg). Graph b) shows the urinary total porphyrin excretion (μMoles/l/day) of the same two animals on selected days over the same 25 day period.

Figure 54: Shows spectrographic traces of liver and brain tissues, examined for the presence of haemoglobin by the Drabkin's method. Haemoglobin could be detected in liver tissue, at 540nm., from animals that had not been perfused with saline, whereas no haemoglobin peak was observed in liver from an animal which had been perfused with 1 liter of heparinised saline solution. Haemoglobin could not be detected, by this method, in either perfused or non-perfused animal tissue.

ABSORBANCE
0
0·75
1·5
400·0
500·0
600·0
WAVELENGTH(nm)
LIVER
NON-PERFUSED
1 L. PERFUSATE
BRAIN

Plate 1: Shows histological sections (haematoxylin and eosin stained) (x 440 magnification) of a) liver from an animal perfused with 1 litre of heparinised saline. There is no evidence of erythrocytes in the tissue. b) In non-perfused animal liver erythrocytes (arrow) can be clearly seen in a central vein. c) In a perfused animal brain there is no histological evidence of erythrocytes in the microcirculation. d) In non-perfused animal brain tissue erythrocytes (arrow) can be seen in the blood vessels.


a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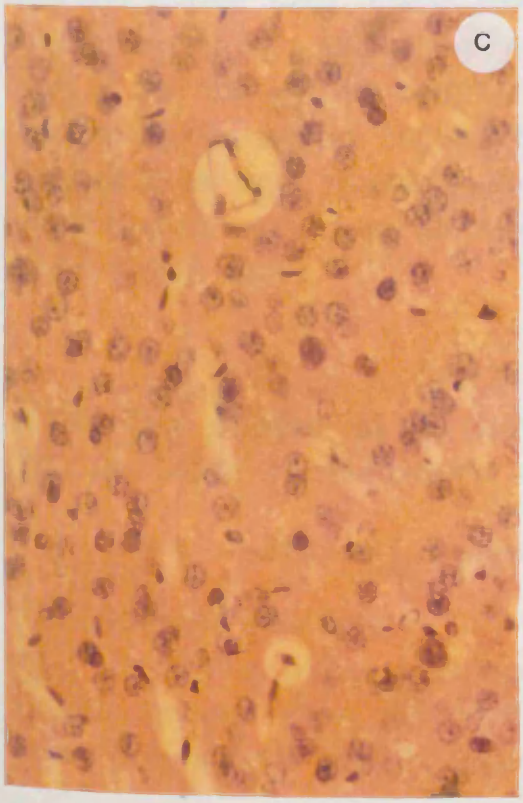
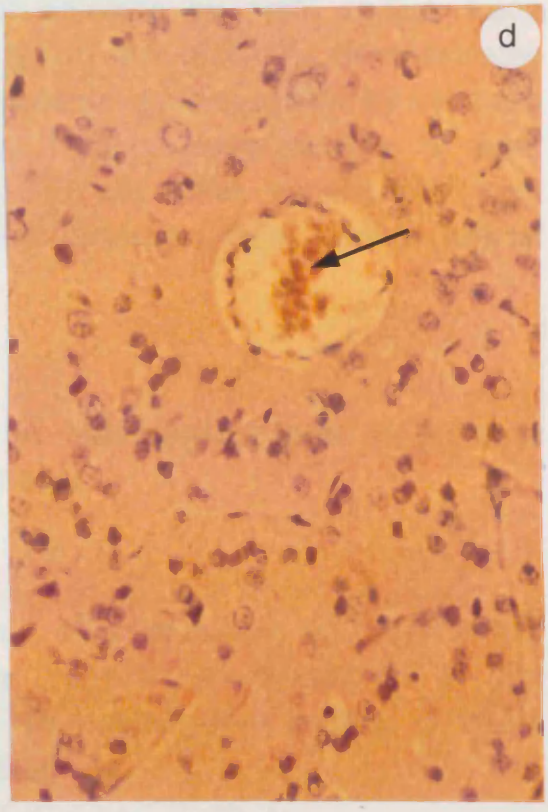

c


d

4.3.3. The effects of 30 days succinylacetone treatment (treatment 2.1) on liver haemoprotein content:

4.3.3.1. Liver mitochondrial respiratory cytochrome content:

The respiratory cytochrome content of solubilised mitochondria from animals treated with succinylacetone for 30 days was measured from their oxidised-reduced difference spectra (figure 55a). Neither cytochrome oxidase, cytochrome b, cytochrome c nor cytochrome c_1 content from mitochondria isolated from the livers of treated animals differed significantly from the content of these cytochromes in control animal hepatic mitochondria (figure 55a). These mitochondrial cytochrome levels were similar to those measured by (Horrum et.al. 1985) by the same method.

4.3.3.2. Liver catalase activity:

The UV absorption of hydrogen peroxide decreases rapidly in the presence of tissue homogenates which contain catalase and is proportional to the activity of the enzyme over the first 15s. of reaction (figure 52b). Inactivation of catalase by 1mM sodiun azide which reacts with the haem moiety of the enzyme, demonstrates that, in this assay, only hydrogen peroxide decomposition by catalase was being measured (figure 52c). The catalase activity of liver homogenates from treated animals did not differ significantly from the enzyme's activity in homogenates prepared from control animal livers (figure 55b).

4.3.4. The effects of 30 days of succinylacetone (treatment 2.1) on blood haemoprotein content:

4.3.4.1. Haemoglobin content:

The treatment employed in this group of animals did not significantly alter the whole blood haemoglobin content, as measured by the Drabkin method, when compared to the haemoglobin content of blood taken from control animals (figure 56a).

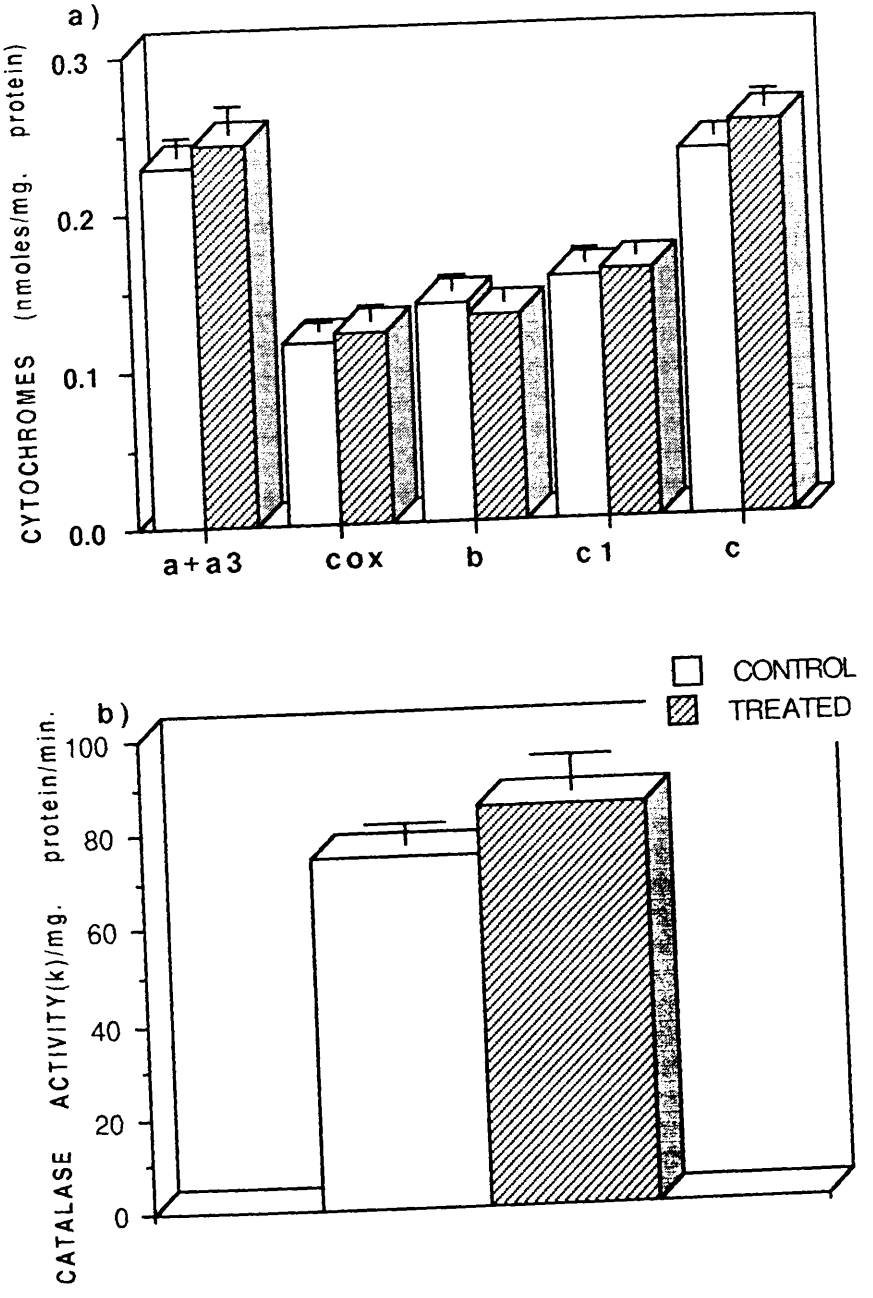


Figure 55 : Graph a) shows the mean +/- S.E.M. of liver mitochondrial cytochrome oxidase (cox), b, c_1 and c from control tissues (n=6) and from hepatic mitochondria isolated from animals that had received porphyrinogenic treatment 2.1 (30 days of succinylacetone) (n=6). Graph b) shows the mean +/- S.E.M. of the catalase activity of liver homogenate of control animals (n=6) and of livers of animals that had received treatment 2.1 (n=6). There were no significant differences.

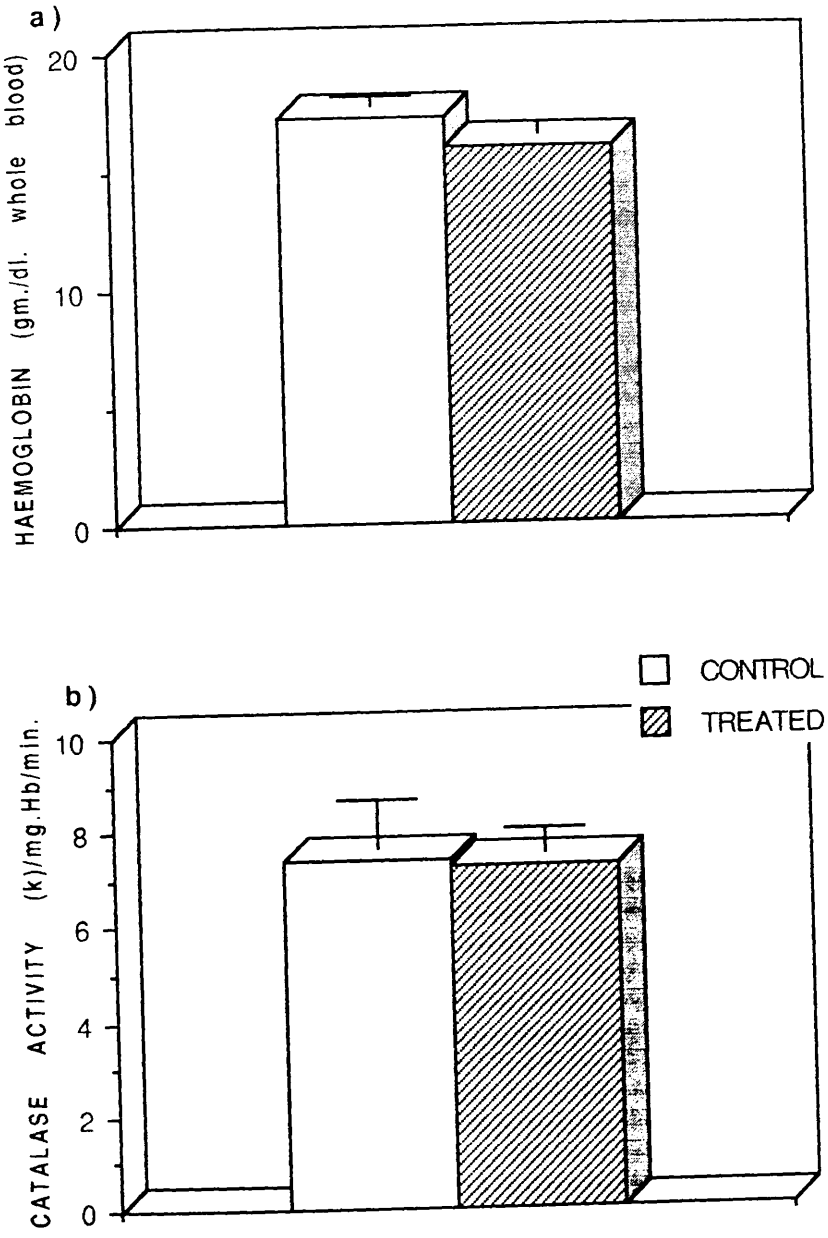


Figure 56: Graph a) shows the mean +/- S.E.M. of the whole blood haemoglobin concentration from control animals (n=6) and from animals that had received porphyrinogenic treatment 2.1 (30 days succinylacetone) (n=6). Graph b) shows the mean +/- S.E.M. of the catalase activity in erythrocyte lysates from control animals (n=6) and in erythrocyte lysates from animals that had received treatment 2.1 (n=6). There were no significant differences.

4.3.4.2. Erythrocyte catalase activity:

The activity of catalase from lysed erythrocytes was measured by its capacity to decompose hydrogen peroxide. Catalase alone was measured by this assay as 1mM sodium azide, which inhibits the activity of the haem moiety of the enzyme completely abolished the lysates ability to decompose hydrogen peroxide (figure 52f). The catalase activity of erythrocyte lysates prepared from the blood of treated animals did not significantly differ from lysates prepared from control animal blood (figure 56b).

4.3.5. Brain mitochondrial respiratory cytochrome content:

Brain cytochrmes were measured from their oxidised-reduced difference spectra at the appropriate wavelengths. Although brain mitochondrial suspensions were more turbid than liver mitochondrial suspensions prominent difference spectra were always obtained by the method used in this study (figure 52g). The brain mitochondrial content of respiratory cytochromes oxidase, b, c and c_1 of treated animals did not differ significantly from the content of these cytochromes in mitochondria prepared form brains of control animals (figure 57).

4.3.6. The effects of porphyrinogenic drug treatment 2.2 (30 days succinylacetone;10 days 4-ethyl DDC, 10mg./kg.; 4 days phenobarbitone; 1 large dose of 4-ethyl DDC) on liver haemoprotein content:

The livers of these animals showed a dark green pigmentation which was also observed in the mitochondrial pellet. This green pigment has been widely reported by others, in the liver of treated animals (Tephly et.al. 1979; DeMatteis et.al. 1980a).

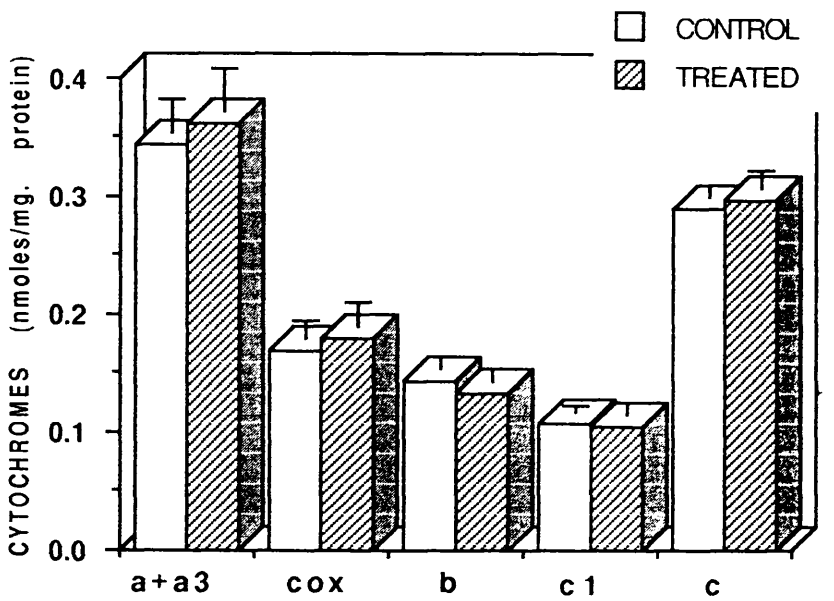


Figure 57: The mean +/- S.E.M. of brain mitochondrial cytochrome oxidase (cox or $a+a_3$) and cytochromes b, c and c_1 from control tissues (n=6) and from brain mitochondria isolated from animals that had received porphyrinogenic treatment 2.1(30 days succinylacetone) (n=6). There were no significant differences.

4.3.6.1. Liver mitochondrial respiratory cytochrome content:

This porphyrinogenic drug treatment caused a significant reduction in the content of hepatic respiratory cytochromes oxidase, b, c and c_1 ($p<0.05$ in all cases) when compared with liver cytochromes from control animals (figure 58a).

4.3.6.2. Liver catalase activity:

This treatment produced a marked decrease in hepatic catalase activity of liver homogenates when compared to the activity in homogenates prepared from control animal livers ($p<0.005$) (figure 58b). A reduction in hepatic catalase activity was also recorded by Ginsberg et.al. (1963) and Haeger-Aronsen (1962) following DDC treatment.

4.3.7. The effects of porphyrinogenic drug treatment 2.2 (30 days succinylacetone;10 days 4-ethyl DDC; 4 days phenobarbitone; one large dose of 4-ethyl DDC) on blood haemoprotein content:

4.3.7.1. Haemoglobin content:

Treatment of animals with porphyrinogenic drug combination did not significantly alter the whole blood haemoglobin content when compared with control animal blood (figure 59a).

4.3.7.2. Erythrocyte catalase activity:

The erythrocyte catalase activity of treated animals did not differ significantly from the activity of this enzyme in control animal erythrocytes (figure 59b). Schmid et.al. (1955) similarly found that porphyrinogenic drugs, which reduce liver catalase activity are ineffective in reducing the activity of the erythocytic enzyme.

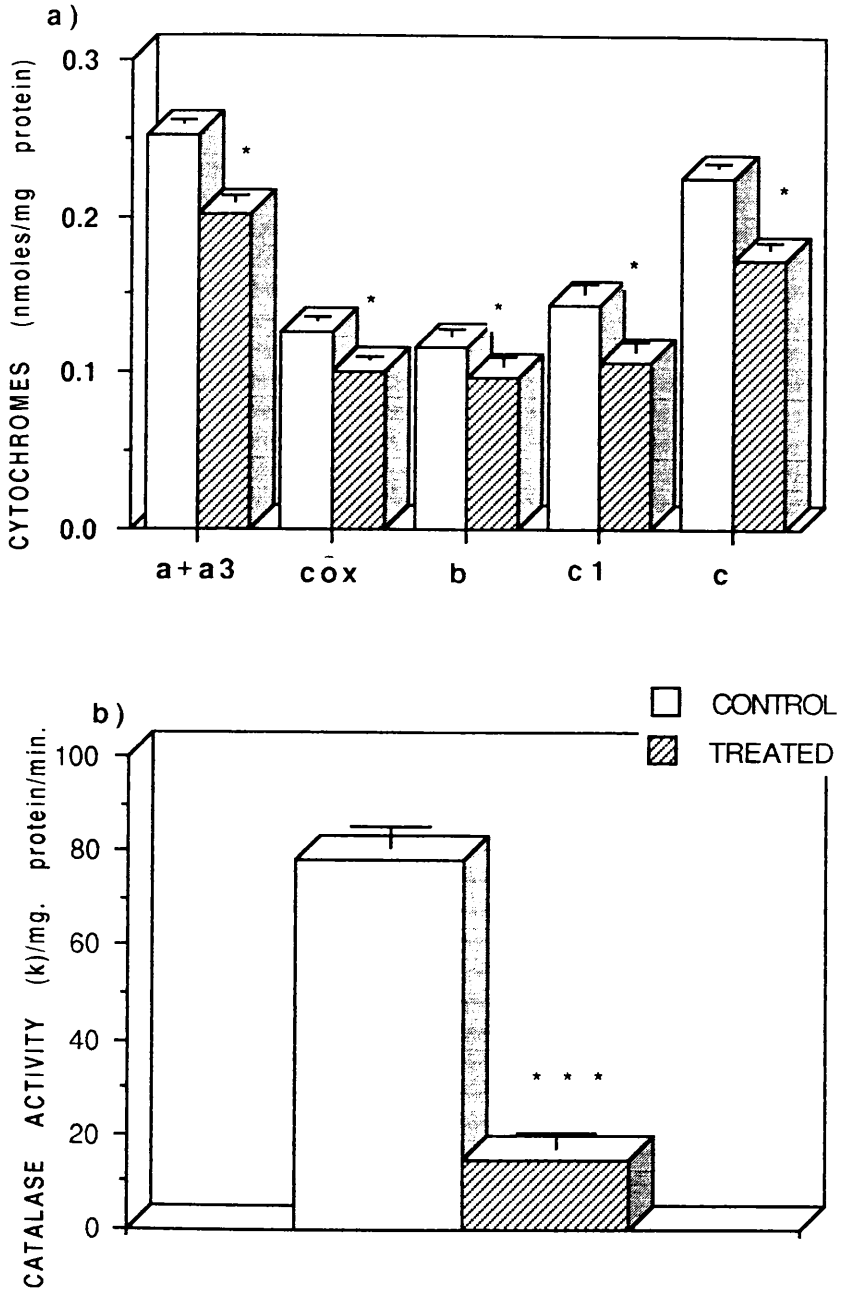


Figure 58: The effect of porphyrinogenic treatment 2.2 (succinylacetone, 4-ethyl DDC, phenobarbitone, 4-ethyl DDC) on the content and activity of hepatic haemoproteins:
Graph a) is the mean +/- S.E.M. of liver mitochondrial cytochrome oxidase (cox or $a+a_3$) and cytochromes b, c and c_1 from control tissues (n=8) and from hepatic mitochondria isolated from treated animals (n=5). Graph b) shows the mean +/- S.E.M. of the catalase activity of liver homogenate from control animals (n=8) and from livers of treated animals (n=5). (* $p<0.05$; ***$p<0.001$)

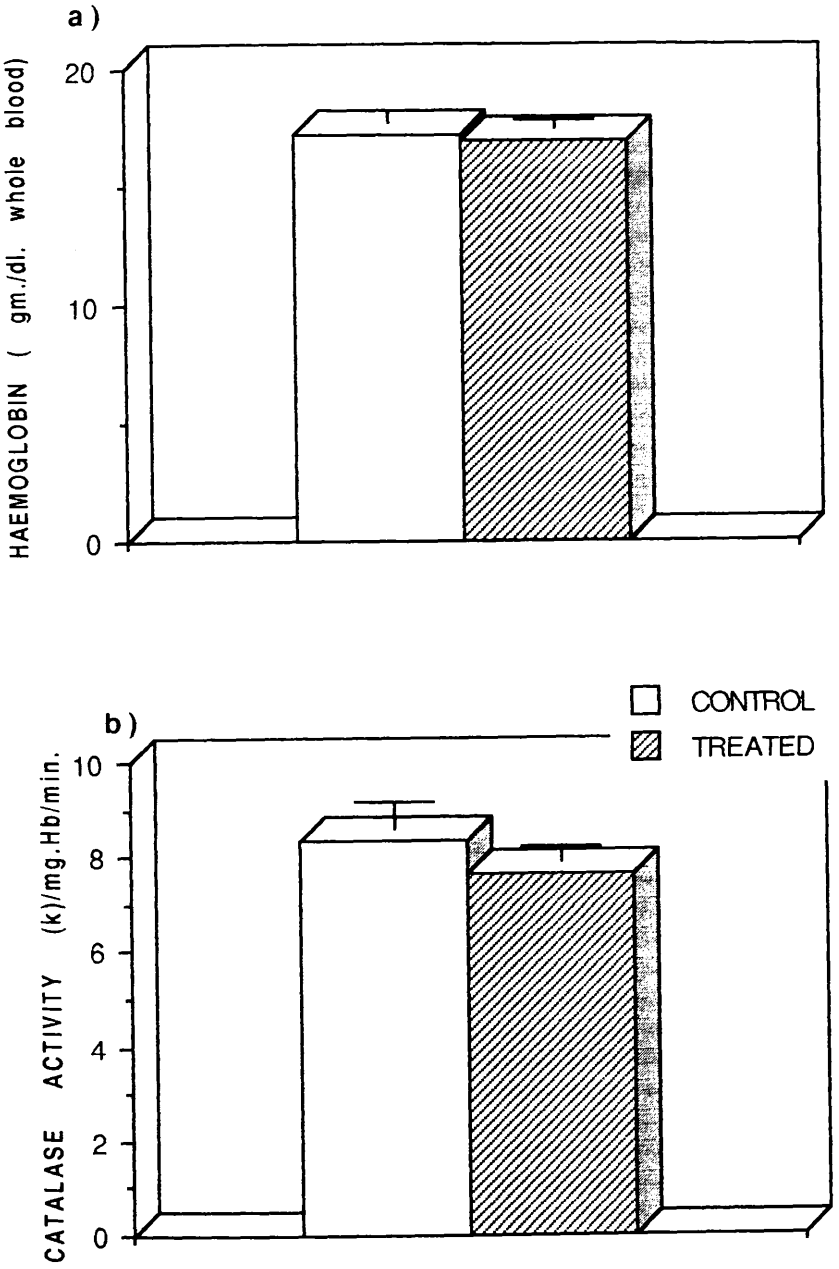


Figure 59: The effects of porphyrinogenic treatment 2.2 (succinylacetone, 4-ethyl DDC, phenobarbitone, 4-ethyl DDC) on the content and activity of erythrocyte haemopoteins:
Graph a) is the mean +/- S.E.M. of the whole blood haemoglobin concentration from control animals (n=8) and from treated animals (n=5). Graph b) shows the mean +/- S.E.M. of the catalase activity of erythrocyte lysates of control animals (n=8) and of erythrocyte lysates from treated animals (n=5). There were no significant differences.

4.3.8. The effects of porphyrinogenic drug treatment 2.2 (30 days succinylacetone;10 days 4-ethyl DDC; 4 days phenobarbitone; one large dose of 4-ethyl DDC) on brain mitochondrial cytochrome content:

The content of all brain mitochondrial respiratory cytochromes from treated animals were equivalent to those of control animals (figure 60).

4.3.9. The effects of porphyrinogenic drug treatment 2.3 (14 days 4-ethyl DDC) on liver haemoprotein content:

This treatment was employed in an attempt to increase the haemoprotein reductions caused by the last group of porphyrinogenic drugs. As succinylacetone alone did not alter any haemoprotein measured, in any of the tissues examined whereas the addition of 4-ethyl DDC and phenobarbitone caused a reducton in hepatic haemoproteins, 4-ethyl DDC was considered the most likley candidate for mediation of these reductions.

The livers of these animals, like those of the previous treatment group, showed a dark green pigmentation which was also observed in the mitochondrial pellet. The livers and mitochondrial pellet from these animals were, however, much darker than in the previous group. Histological examination of hepatic tissue from animals from this group showed a wide spread fluorescence (plate 2), indicative of the presence of porphyrins (probably both the alkylated protoporphyrin, metabolite of 4-ethyl DDC and protoporphyrin 1X). Electron micrographs of mitochondrial pellets revealed the presence of a dark lysosomal-type material (plate 3). This is most probably iron accumulation in the lysosomes. These last two observations are congruent with the proposal that 4-ethyl DDC is converted into an alkylated protoporphyrin within the liver cells and accumulation of iron and protoporphyrin 1X, substrates of ferrochelatase, provides indirect evidence of inhibition of ferrochelatase. The absence of this darkly stained fraction in control liver (plate 3) and treated brain

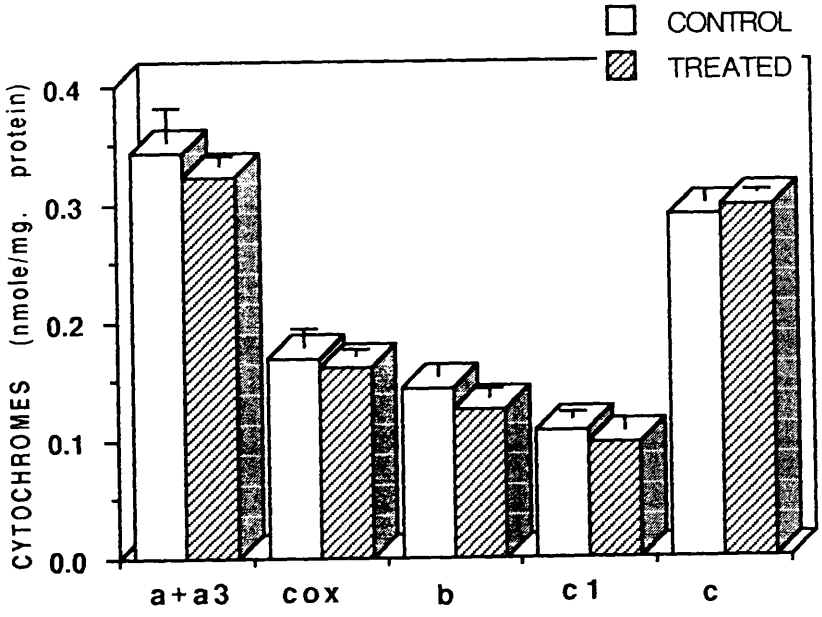
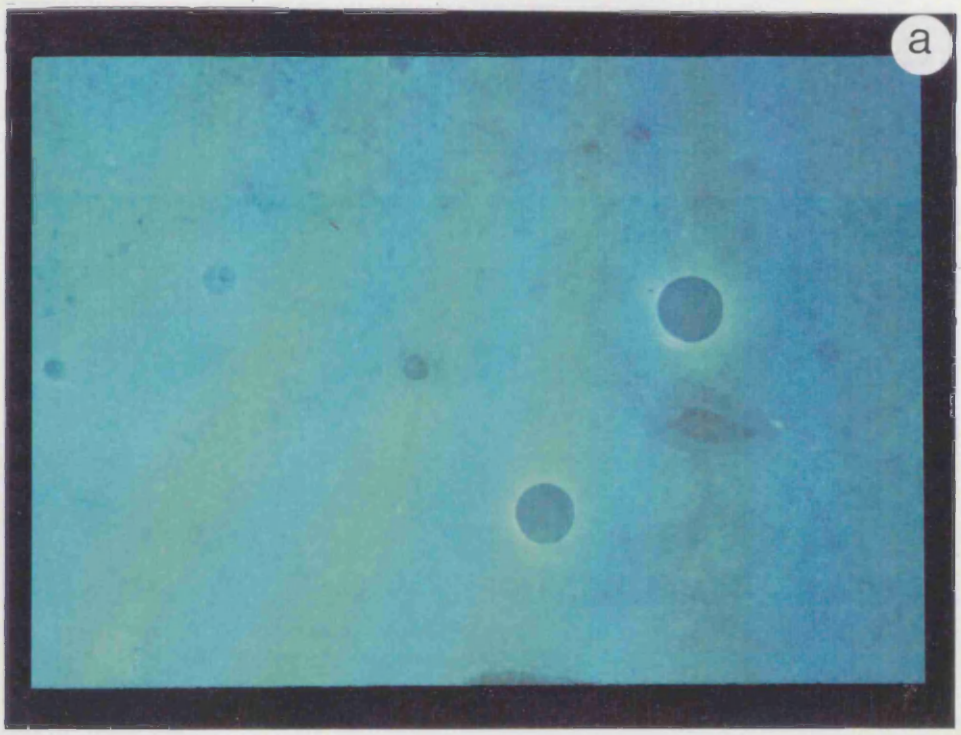


Figure 60: The mean +/- S.E.M. of brain mitochondrial cytochrome oxidase (cox or $a+a_3$) and cytochromes b, c_1 and c from control tissues (n=7) and from brain mitochondria isolated from animals that had received porphyrinogenic treatment 2.2 (succinylacetone, 4-ethyl DDC, phenobarbitone, 4-ethyl DDC) (n=5). There were no significant differences.

Plate 2: Shows a fluorescence photograph of a histological section of liver from a control animal (x 110 magnification). b) shows a similar photograph of a liver section from an animal treated for 14 days with 4-ethyl DDC (100mg/kg.) (x 110 magnification). The autofluorescence seen in the treated animal tissue is most likely due to accumulating protoporphyrin and N-ethyl protoporphyrin (the 4-ethyl DDC metabolite). This fluorescence histological examination confirms that the effects of 4-ethyl DDC are widespread in the liver, as all hepatocytes fluoresce.


a

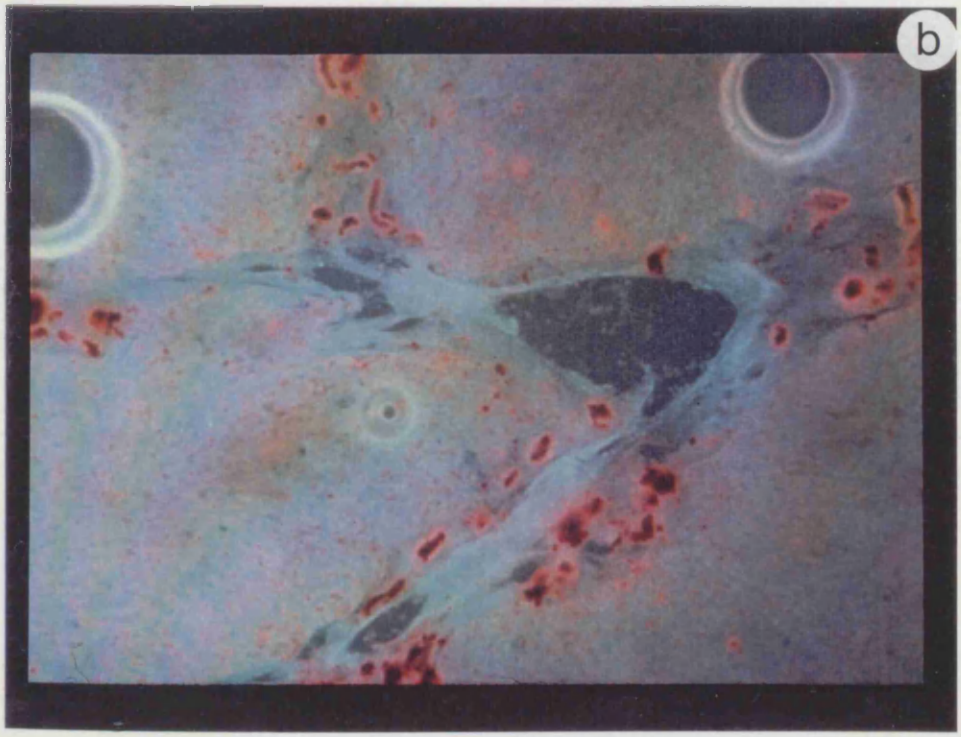
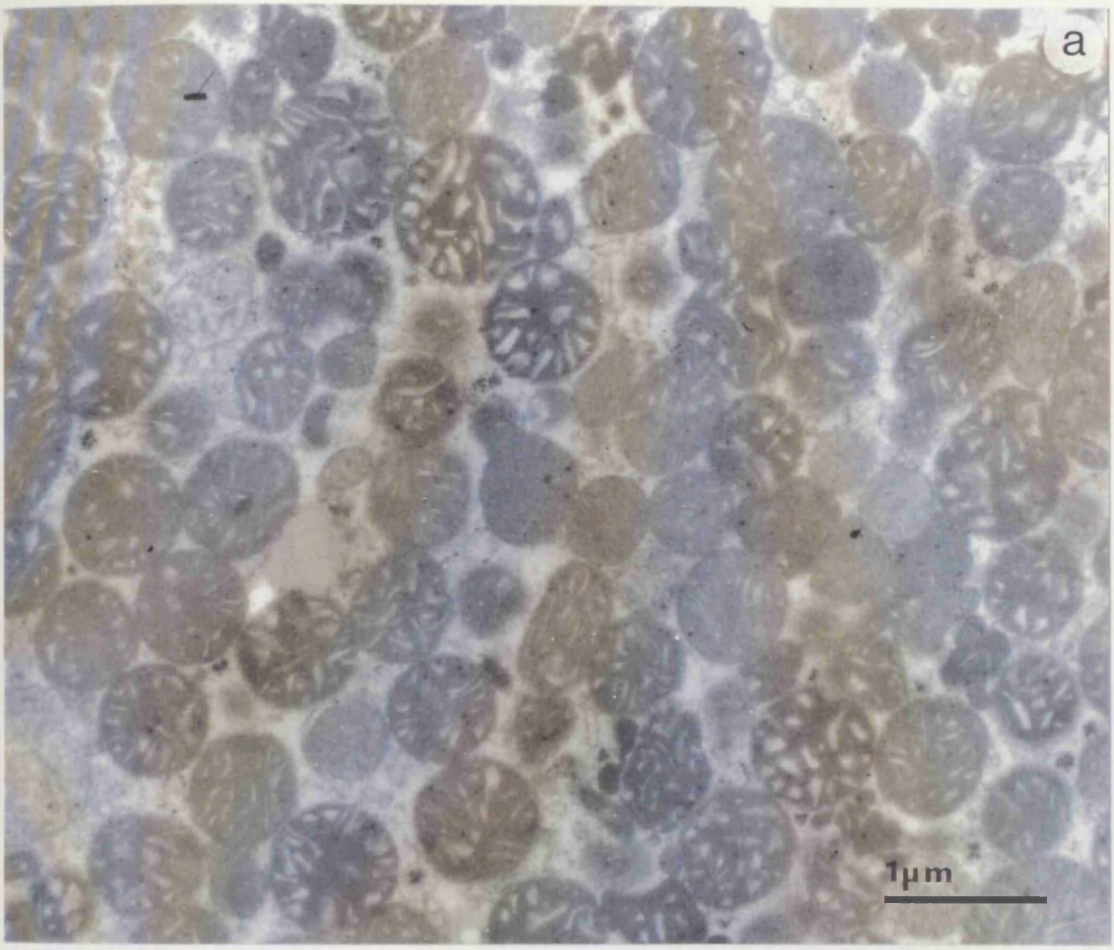
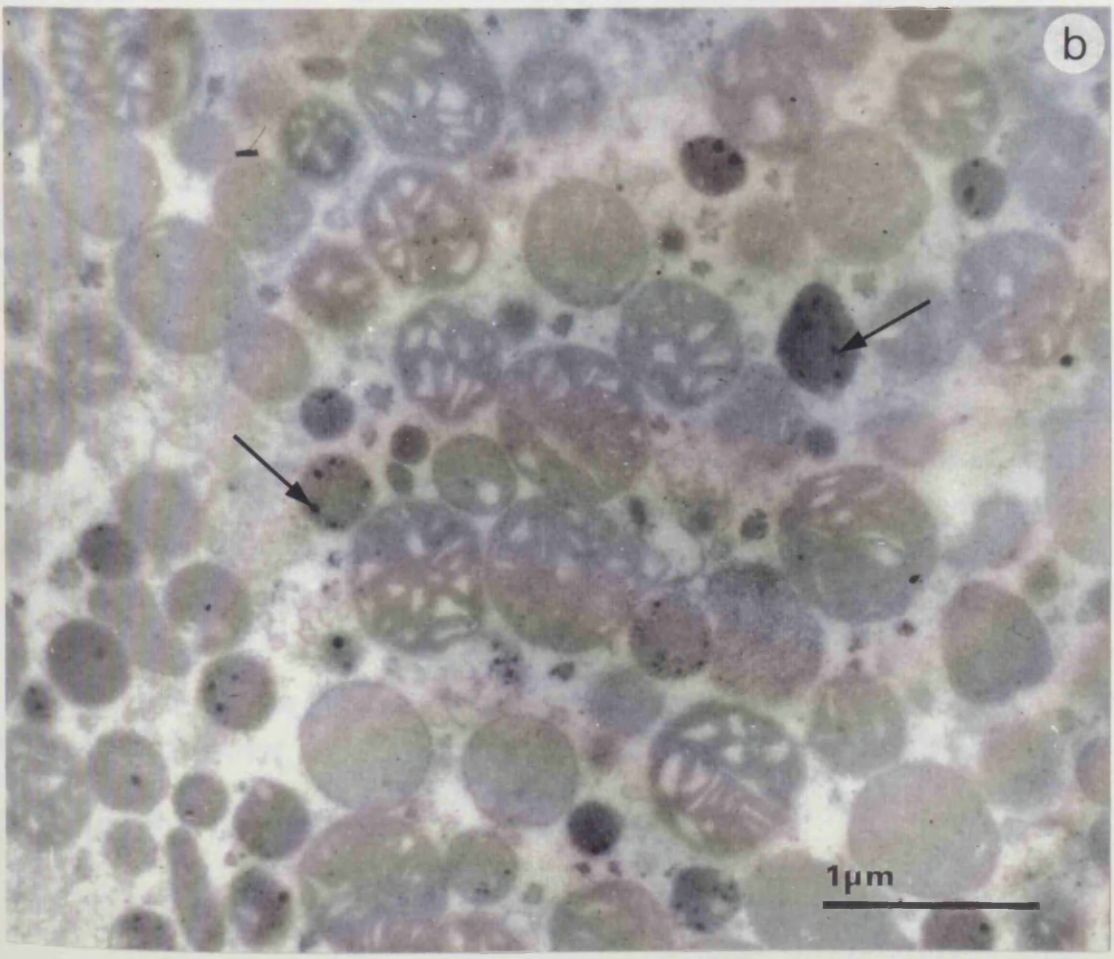

b

Plate 3: a) An electron micrograph of a mitochondrial pellet isolated from a control animal liver. Intact mitochondria can be clearly seen. b) Is an electron micrograph of liver mitochondria isolated from an animal that had received 14 days administration of 4-ethyl DDC (100mg./kg.). Again, intact mitochondria can be clearly identified. Dark deposits can be seen in lysosomal-like bodies (arrows). These dark deposits are probably iron accumulating as a result of ferrochelatase inhibition. There is no evidence of these dark deposits in control liver mitochondrial pellets.


a
1µm


b
1µm

mitochondrial pellets (plate 4) indicates that 4-ethyl DDC may not be effective in gaining access to the brain.

50% of the animals in this treatment group developed a red colouration around the tips of their ears which progressed to a degeneration of the tissue with encrustation (plate 5). There was no histological evidence of iron accumulation in the area of this damage. The lesions which developed on the ears of these animals bear a close resemblence to the skin lesions of patients suffering from porphyria cutanea tarda, and like this phenomena are most probably due to the action of light on overproduced porphyrins accumulating in the skin.

4.3.9.1. Liver mitochondrial respiratory cytochrome content:

This porphyrinogenic drug treatment caused a significant reduction in the content of hepatic respiratory cytochromes oxidase, b, c ($p<0.05$) when compared with liver cytochromes from control animals (figure 61a). Cytochrome c_1 content, although reduced, was not significantly altered by this treatment. In half of the liver mitochondrial samples the oxidised-reduced spectra showed an unusual dip between 650nm and 575nm (figure 62). This trough in the difference spectra of the hepatic respiratory cytochromes is probably due to the oxidised-reduced difference spectra of the alkylated protoporphyrin as N-methyl protoporphyrin itself, in tris buffer demonstrates a difference spectra which has a nadir between these same wavelengths (figure 62).

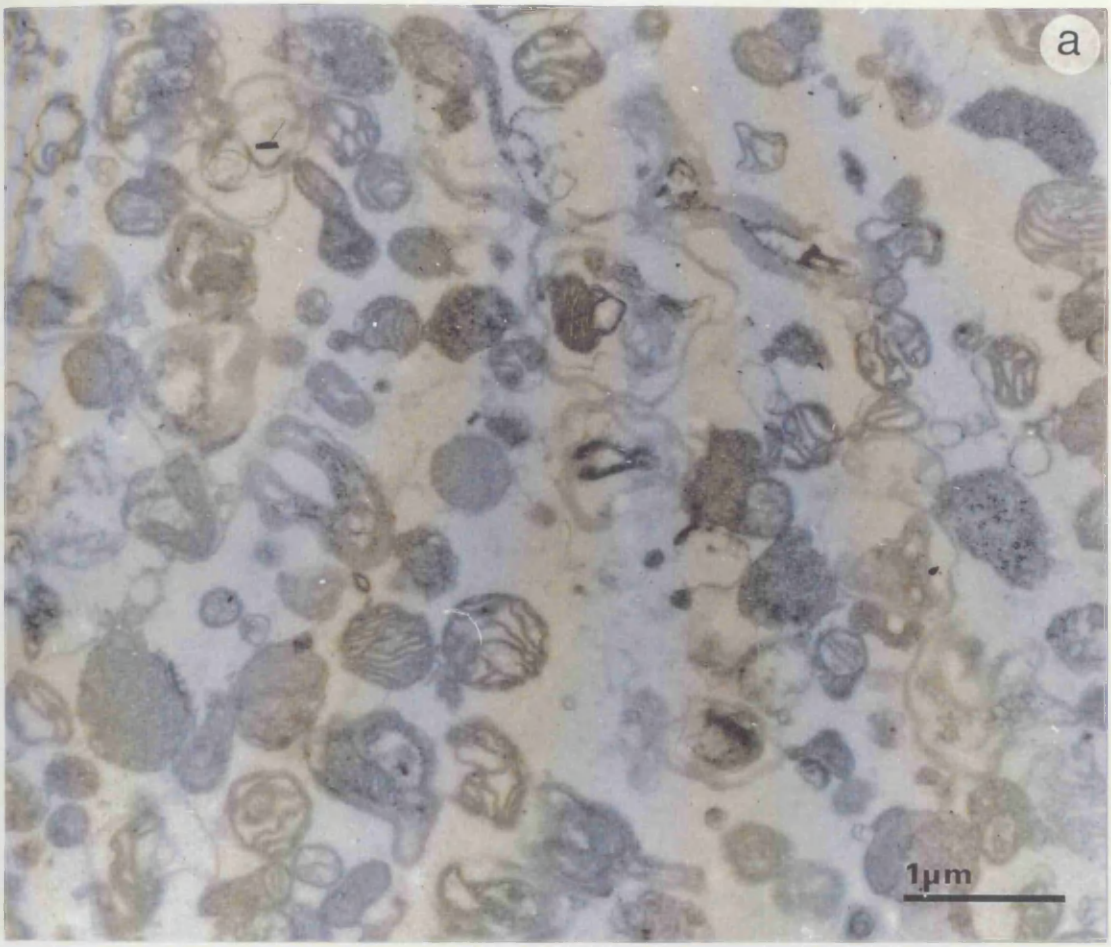
4.3.9.2. Liver catalase activity:

This treatment, like the previous treatment, also produced a marked decrease in hepatic catalase activity of liver homogenates when compared to the activity in homogenates prepared from control animal livers ($p<0.001$) (figure 61b).

4.3.10. The effects of porphyrinogenic drug treatment 2.3 (14 days 4-ethyl DDC) on blood haemoprotein content:

4.3.10.1. Haemoglobin content:

Plate 4: Electron micrographs of a) a control animal brain mitochondrial pellet and b) a treated animal brain mitochondrial pellet. Mitochondria can be clearly seen in both tissues and there is no evidence of dark lysosomal bodies, in the treated animal mitochondria.


a
1µm

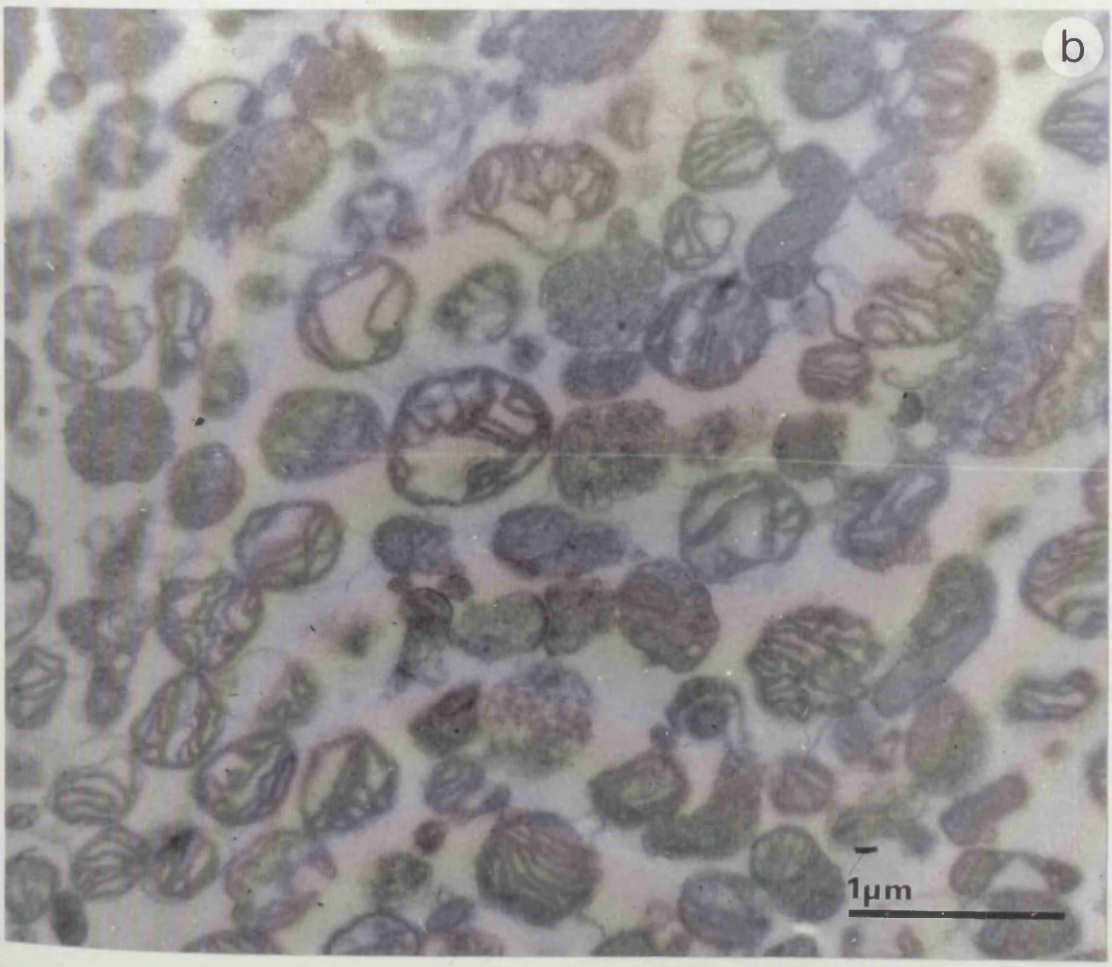
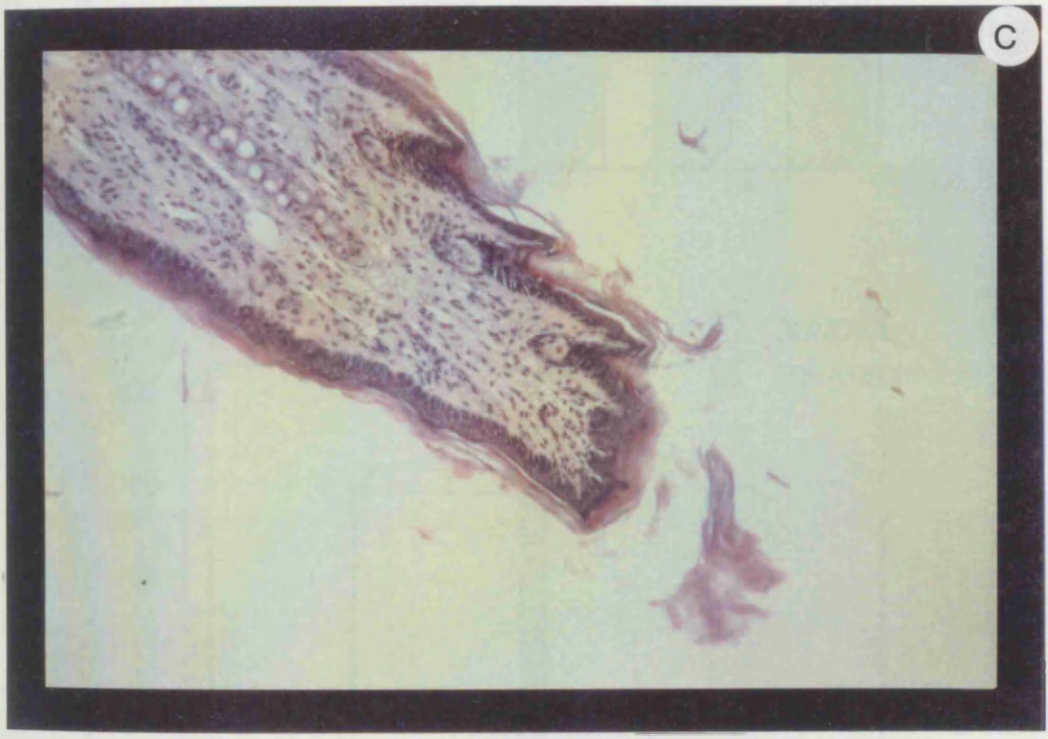
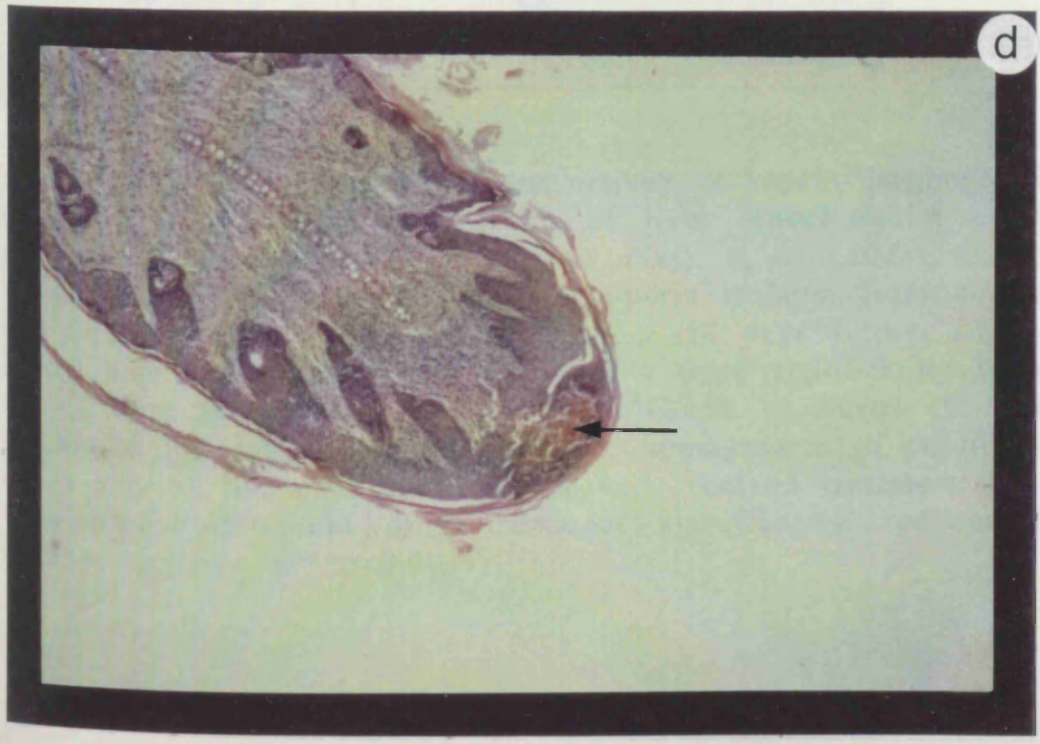

b
1µm

Plate 5: Shows a) A red colouration which developed arround the tips of the ears of a rat treated for 14 days with 4-ethyl DDC. b) A later stage in ear damage following 4-ethyl DDC treatment, when the ear tissue begins to degenerate. c) A haematoxylin and eosin stained histological section (x 140 magnification) of a control animal ear and d) an haematoxylin and eosin stained histological section (x 35 mgnification) of an ear from a treated animal, showing subcutaneous accumulation of a substance which could be protoporphyrin (arrow).


a


b


c


d

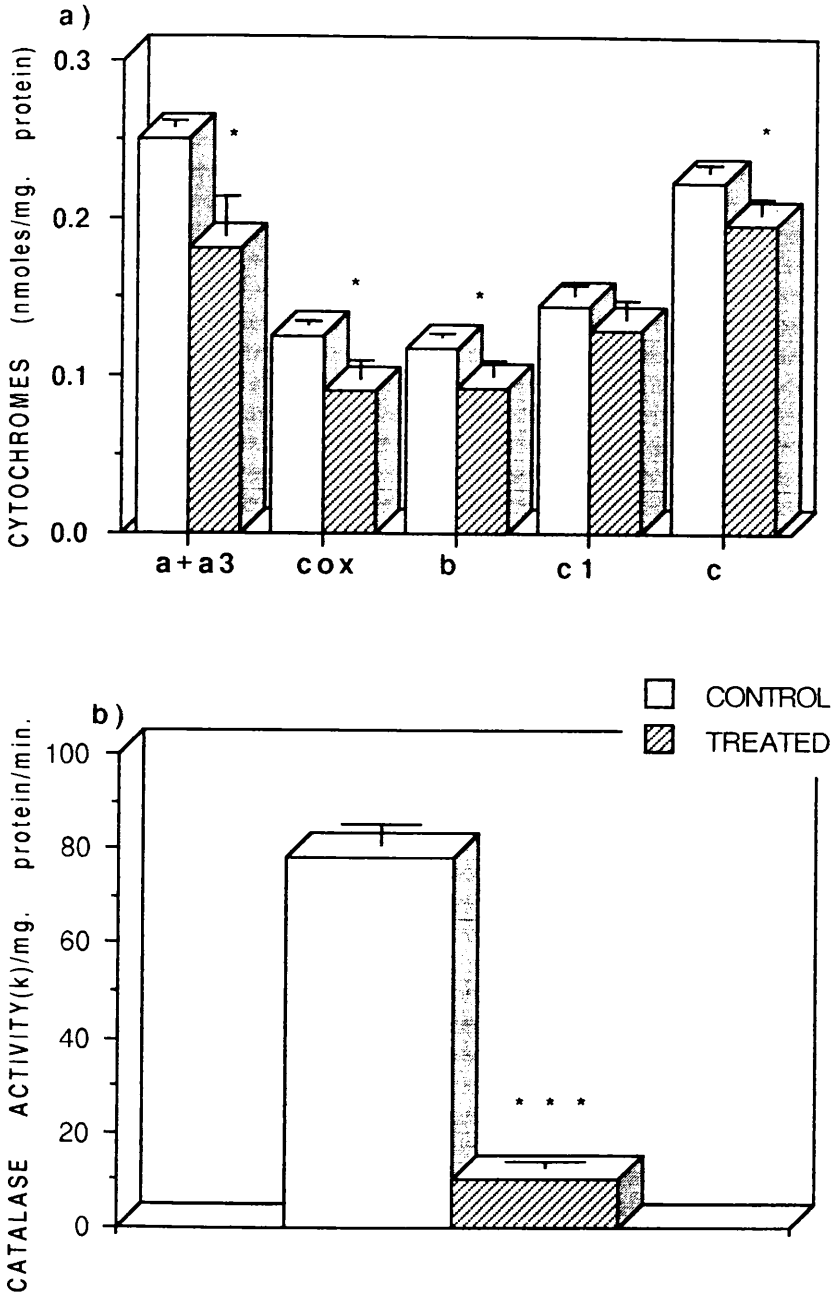
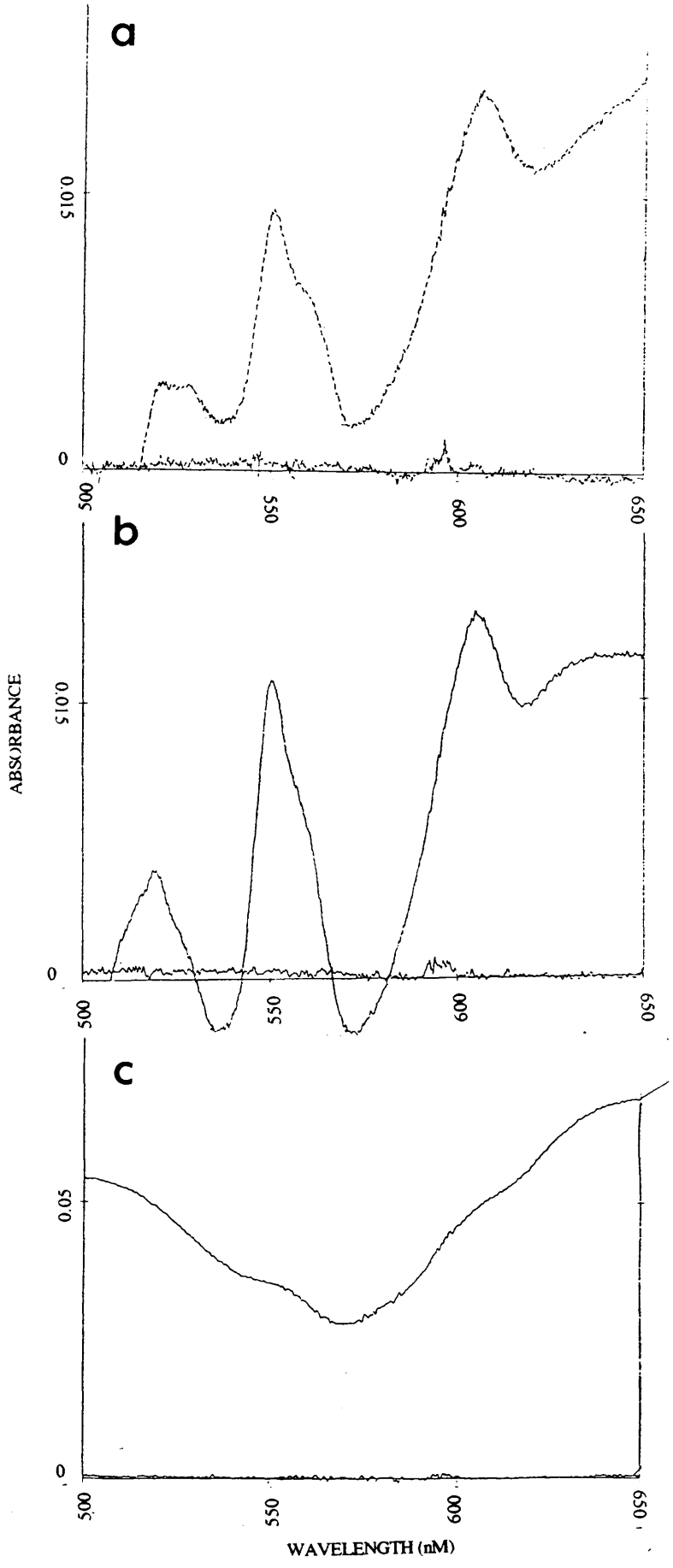


Figure 61: Shows the content and activity of hepatic haemoproteins: Graph a) is the mean +/- S.E.M. of liver mitochondrial cytochrome content of cytochrome oxidase (cox or $a+a_3$), b, c_1 and c from control tissues (n=8) and from hepatic mitochondria isolated from animals that had received porphyrinogenic treatment 2.3 (14 days 4-ethyl DDC) (n=6). Cytochrome oxidase, cytochrome b and c were significantly reduced in treated liver mitochondria (* $p<0.05$). Graph b) shows the mean +/- S.E.M. of the catalase activity of liver homogenates of control animals (n=8) and of livers of animals that had received treatment 2.3 (n=6). This porpphyrinogenic drug treatment significantly reduced hepatic catalase activity (***$p<0.001$).

Figure 62: The effect, in a single experiment, on the hepatic oxidised-reduced absorbance spectra of a) a mitochondrial sample from an animal treated with14 days of intraperitoneally administered 4-ethyl DDC and b) 14 days of intravenous administration of N-methyl protoporpyrin. c) the effects of sodium dithionite on a sample of N-methyl protoporphyrin, in Tris buffer. In both tissue cases there is an uncharacteristic trough in the difference spectra at 570nm, which is not detectable in control spectra (figure 52a). This trough is however, present in a dithionite treated sample of N-methyl protoporphyrin (trace c).


a
0.015
0
500
550
600
650
b
0.015
0
500
550
600
650
ABSORBANCE
c
0.05
0
500
550
600
650
WAVELENGTH (nM)

Treatment of animals with this porphyrinogenic drug combination did not significantly alter the whole blood haemoglobin content when compared with control animal blood (figure 63a).

4.3.10.2. Erythrocyte catalase activity:

The erythrocyte catalase activity of treated animals did not differ significantly from the activity of this enzyme in control animal erythrocytes (figure 63b).

4.3.11. The effects of porphyrinogenic drug treatment 2.3 (14 days 4-ethyl DDC) on brain mitochondrial cytochrome content:

The content of all brain mitochondrial respiratory cytochromes from treated animals did not differ significantly from the cytochrome content of brains from control animals (figure 64).

4.3.12. The effects of porphyrinogenic drug treatment 2.4 (14 days intravenous N-methyl protoporphyrin) on liver haemoprotein content:

Following the first four or five days of N-methyl protoporphyrin administration into the tail vein it became progressively more difficult due to both venous collapse and the alkylated protoporphyrin leaking into the perivascular tissue, to achieve successful intravenous administration on every injection. Although the livers of these animals did not appear to be excessively pigmented the mitochondrial pellet did show a high degree of green colouration. In 50% of the liver mitochondrial samples in this treatment group, as was the case with the previous treatment group, a dip was observed in the difference spectra (figure 62) probably due to the spectral interference of N-methyl protoporphyrin.

4.3.12.1. Liver mitochondrial respiratory cytochrome content:

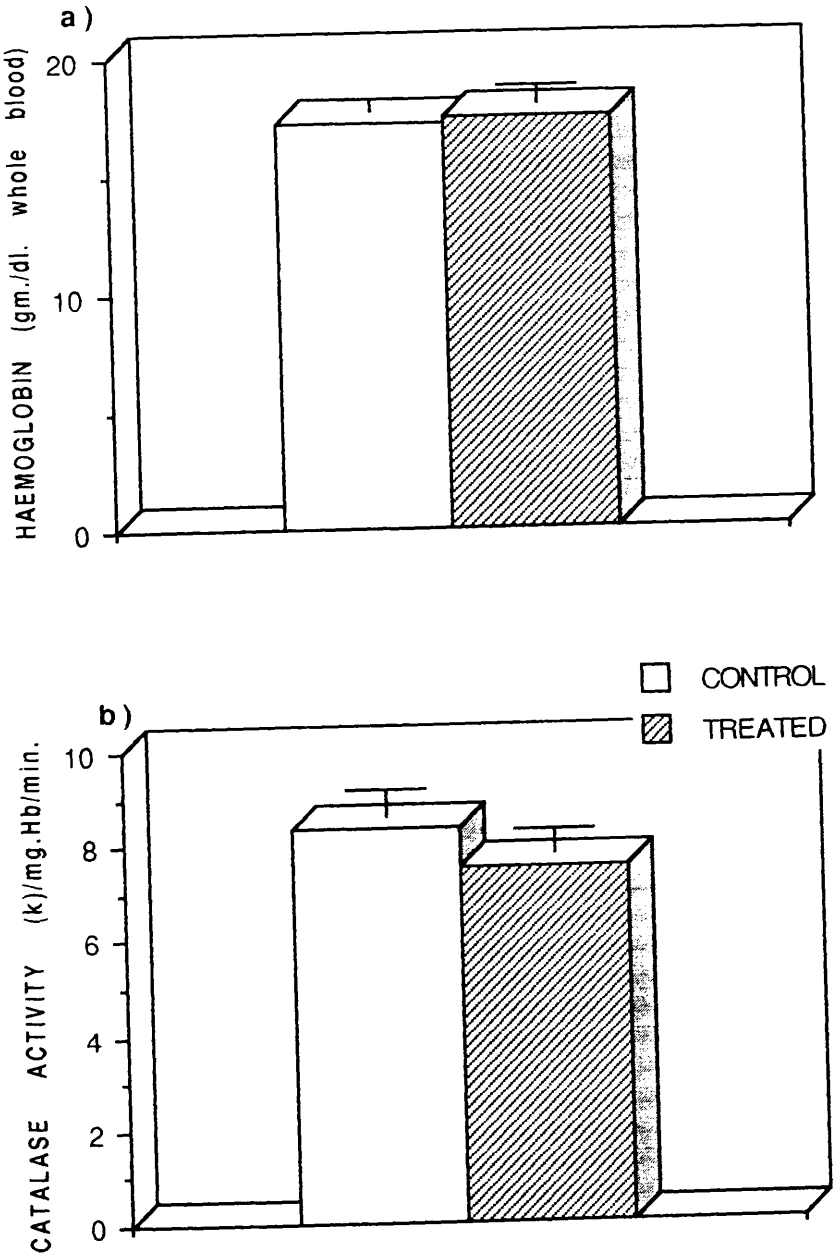


Figure 63: Shows the content and activity of erythrocyte haemoproteins: graph a) is the mean +/- S.E.M. of the whole blood haemoglobin concentration from control animals (n=8) and from animals that had received porphyrinogenic treatment 2.3 (14 days 4-ethyl DDC) (n=6). Graph b) shows the mean +/- S.E.M. of the catalase activity of erythrocyte lysates of control animals (n=8) and of erythrocyte lysates from animals that had received treatment 2.3 (n=6). There were no significant differences.

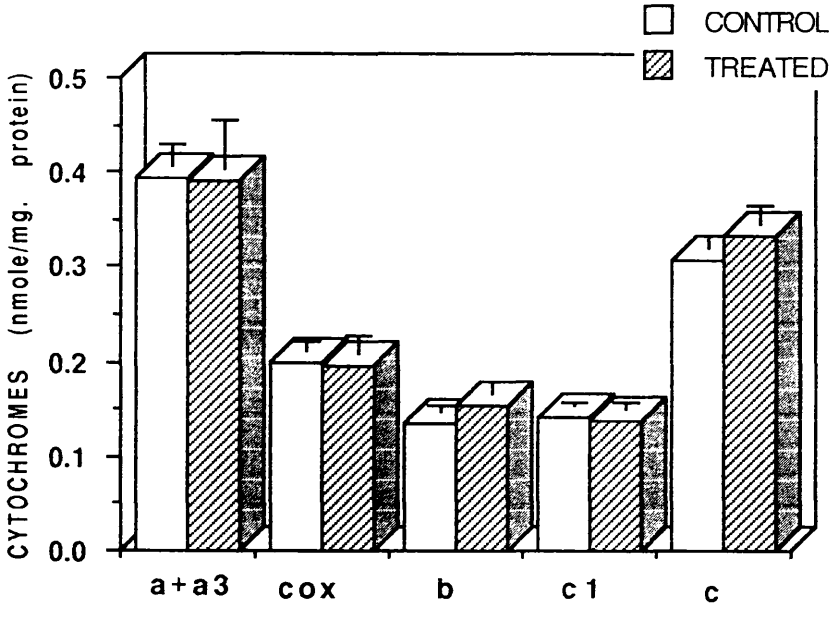


Figure 64: The mean +/- S.E.M. of brain mitochondrial cytochrome content of cytochrome oxidase (cox or $a+a_3$), b, c_1 and c from control tissues (n=6) and from brain mitochondria isolated from animals that had received porphyrinogenic treatment 2.3 (14 days 4-ethyl DDC) (n=6). There were no significant differences.

This porphyrinogenic drug treatment caused a significant reduction in the content of hepatic respiratory cytochromes oxidase only ($p<0.05$) when compared with liver cytochrome from control animals. All other respiratory cytochromes were unaltered by this treatment (figure 65a).

4.3.12.2. Liver catalase activity:

N-methyl protoporphyrin treatment produced a significant decrease in the catalase activity of liver homogenates when compared to the activity in homogenates prepared from control animal livers ($p<0.05$) (figure 65b).

4.3.13. The effects of porphyrinogenic drug treatment 2.4 (14 days intravenous N-methyl protoporphyrin) on blood haemoprotein content:

4.3.13.1. Haemoglobin content:

Treatment of animals with porphyrinogenic drug combination did not significantly alter the whole blood haemoglobin content when compared with control animal blood (figure 66a).

4.3.13.2. Erythrocyte catalase activity:

The erythrocyte catalase activity of treated animals did not differ significantly from the activity of this enzyme in control animal erythrocytes (figure 66b).

4.3.14. The effects of porphyrinogenic drug treatment 2.4 (14 days intravenous N-methyl protoporphyrin) on brain mitochondrial cytochrome content:

The content of all brain mitochondrial respiratory cytochromes from treated animals did not differ significantly from the cytochrome content of brains from control animals (figure 67).

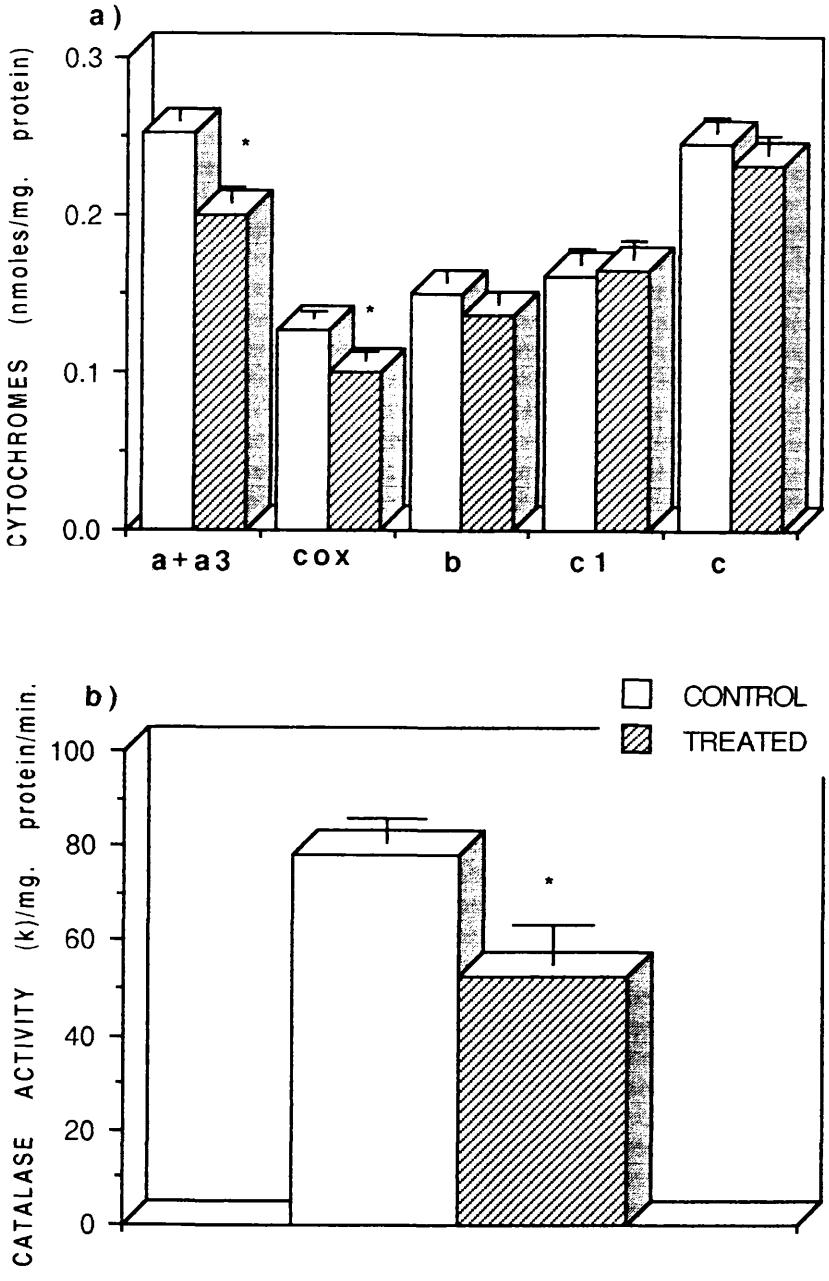


Figure 65: Shows the content and activity of hepatic haemoproteins: graph a) is the mean +/- S.E.M. of liver mitochondrial cytochrome content of cytochrome oxidase (cox or $a+a_3$), b, c_1 and c from control tissues (n=6) and from hepatic mitochondria isolated from animals that had received porphyrinogenic treatment 2.4 (14 days i.v. N-methyl protoporphyrin) (n=5). Cytochrome oxidase was the only respiratory component that was significantly reduced (* $p<0.05$). Graph b) shows the mean +/- S.E.M. of the catalase activity of liver homogenates of control animals (n=6) and of livers of animals that had received treatment 2.4 (n=5). Hepatic catalase activity was significantly reduced in treated animals (* $p<0.05$).

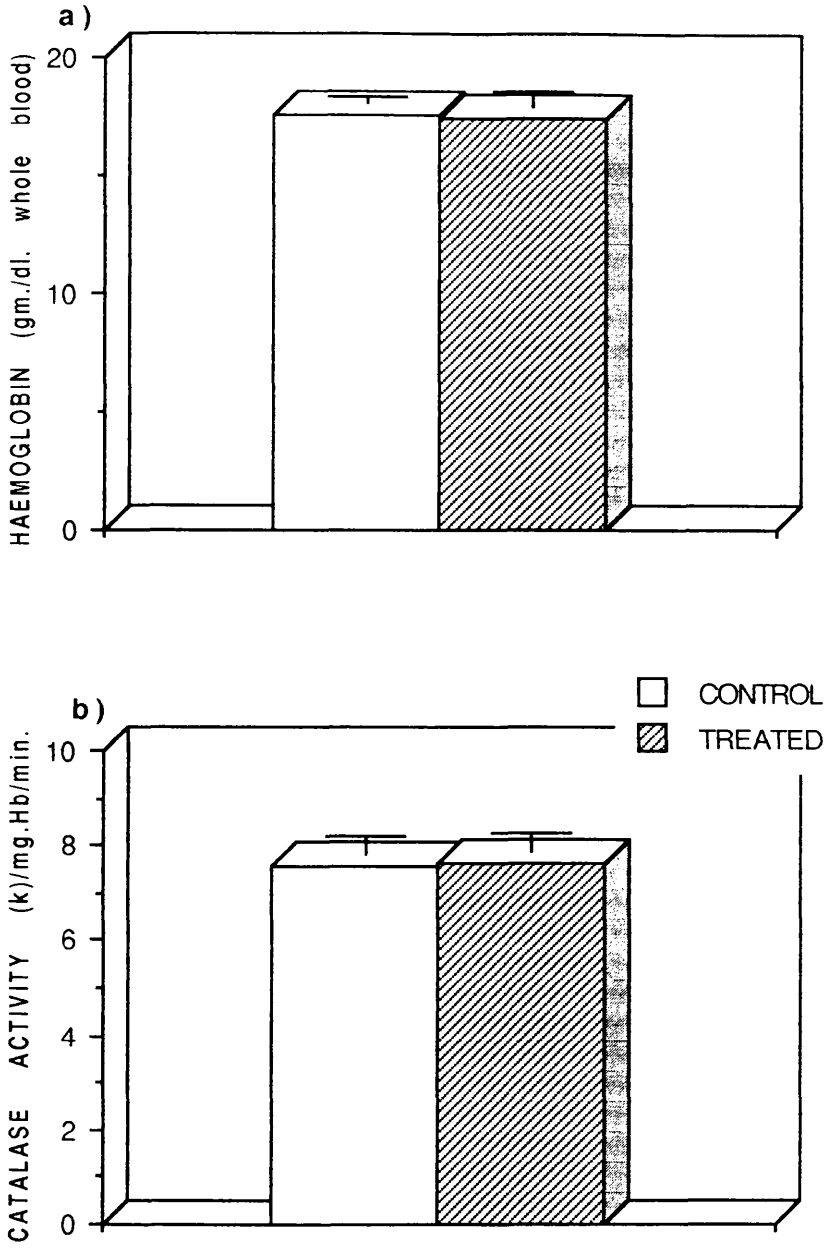


Figure 66: Shows the content and activity of erythrocyte haemoproteins: graph a) is the mean +/- S.E.M. of the whole blood haemoglobin concentration from control animals (n=6) and from animals that had received porphyrinogenic treatment 2.4 (14 days i.v. N-methyl protoporphyrin) (n=5). Graph b) shows the mean +/- S.E.M. of the catalase activity of erythrocyte lysates of control animals (n=6) and of erythrocyte lysates from animals that had received treatment 2.4 (n=5). There were no significant differences.

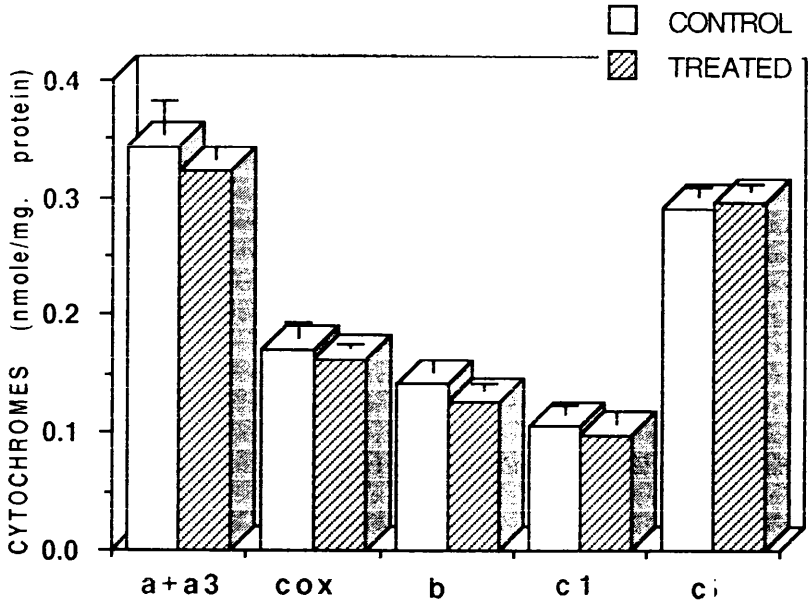


Figure 67: The mean +/- S.E.M. of brain mitochondrial cytochrome content of cytochrome oxidase (cox or $a+a_3$), b, c_1 and c from control tissues (n=6) and from brain mitochondria isolated from animals that had received porphyrinogenic treatment 2.4 (14 days i.v. N-methyl protoporphyrin) (n=5). There were no significant differences.

4.3.15. The effects of porphyrinogenic drug treatment 2.5 (oral lead administration) on liver haemoprotein content:

Following long term lead acetate treatment the animals did not demonstrate any obvious abnormalities.

4.3.15.1. Liver mitochondrial respiratory cytochrome content:

Oral lead administration failed to cause any changes in the hepatic respiratory cytochrome content when compared to control animals (figure 68a).

4.3.15.2. Liver catalase activity:

Hepatic catalase activity demonstrated a small but significant increase in liver homogenates from lead fed rats when compared to homogenates from control animals (figure 68b).

4.3.16. The effects of porphyrinogenic drug treatment 2.5 (oral lead administration) on blood haemoprotein content:

4.3.16.1. Haemoglobin content:

Lead administration caused a significant decrease in the haemoglobin content of whole blood ($p<0.05$) (figure 69a).

4.3.16.2. Erythrocyte catalase activity:

The erythrocyte catalase activity per mg. of haemoglobin in lead treated animals did not differ significantly from the activity of this enzyme in control animal erythrocytes (figure 69b). If, however, haemoglobin concentration decreased as a result of a fall in red cell content the over all activity of catalase per volume of whole blood must also decrease.

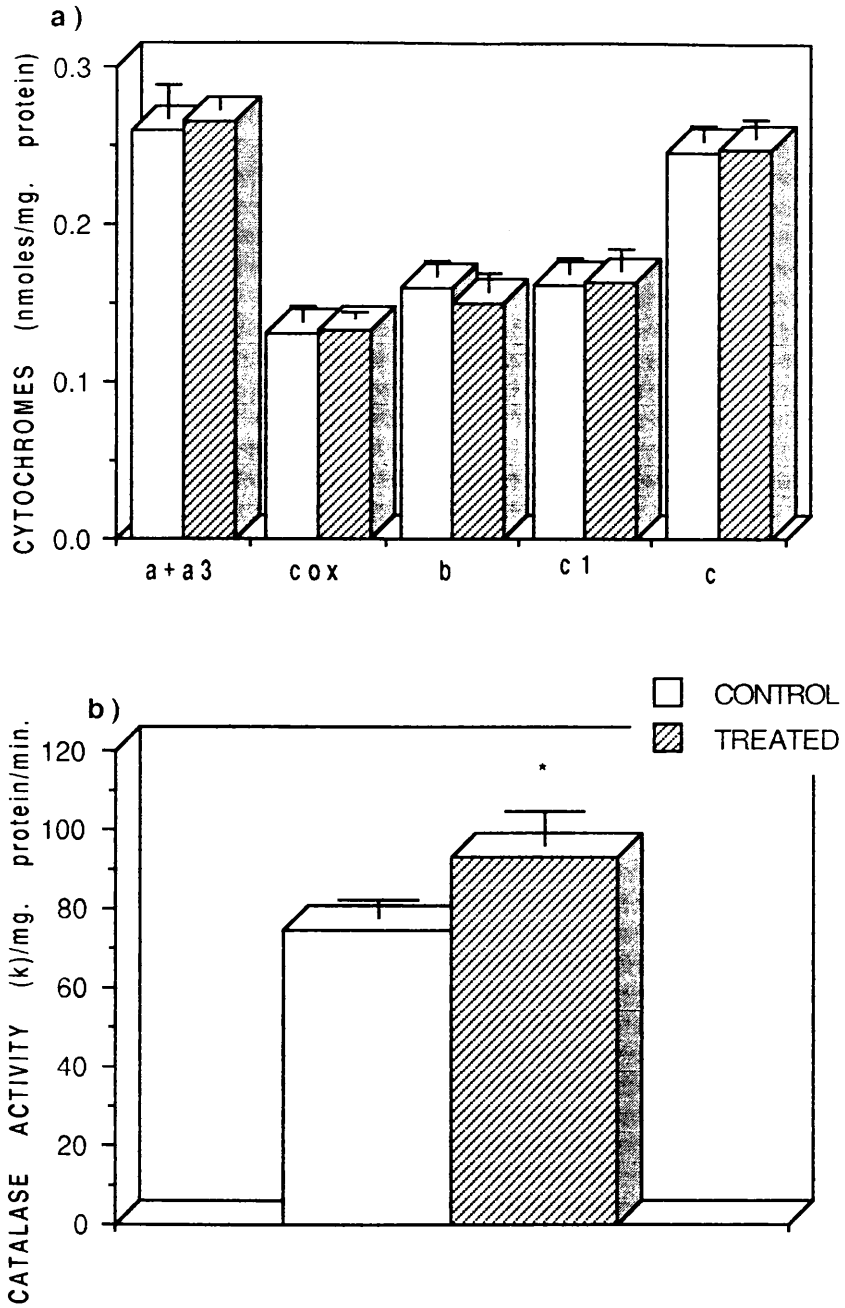


Figure 68: Shows the content and activity of hepatic haemoproteins: graph a) is the mean +/- S.E.M. of liver mitochondrial cytochrome content of cytochrome oxidase (cox or $a+a_3$), b, c_1 and c from control tissues (n=6) and from hepatic mitochondria isolated from animals that had received porphyrinogenic treatment 2.5 (lead acetate in their drinking water) (n=7). Graph b) shows the mean +/- S.E.M. of the catalase activity of liver homogenates of control animals (n=6) and of livers of animals that had received treatment 2.5 (n=7). Lead treatment caused a significant increase in hepatic catalase activity (* $p<0.05$).

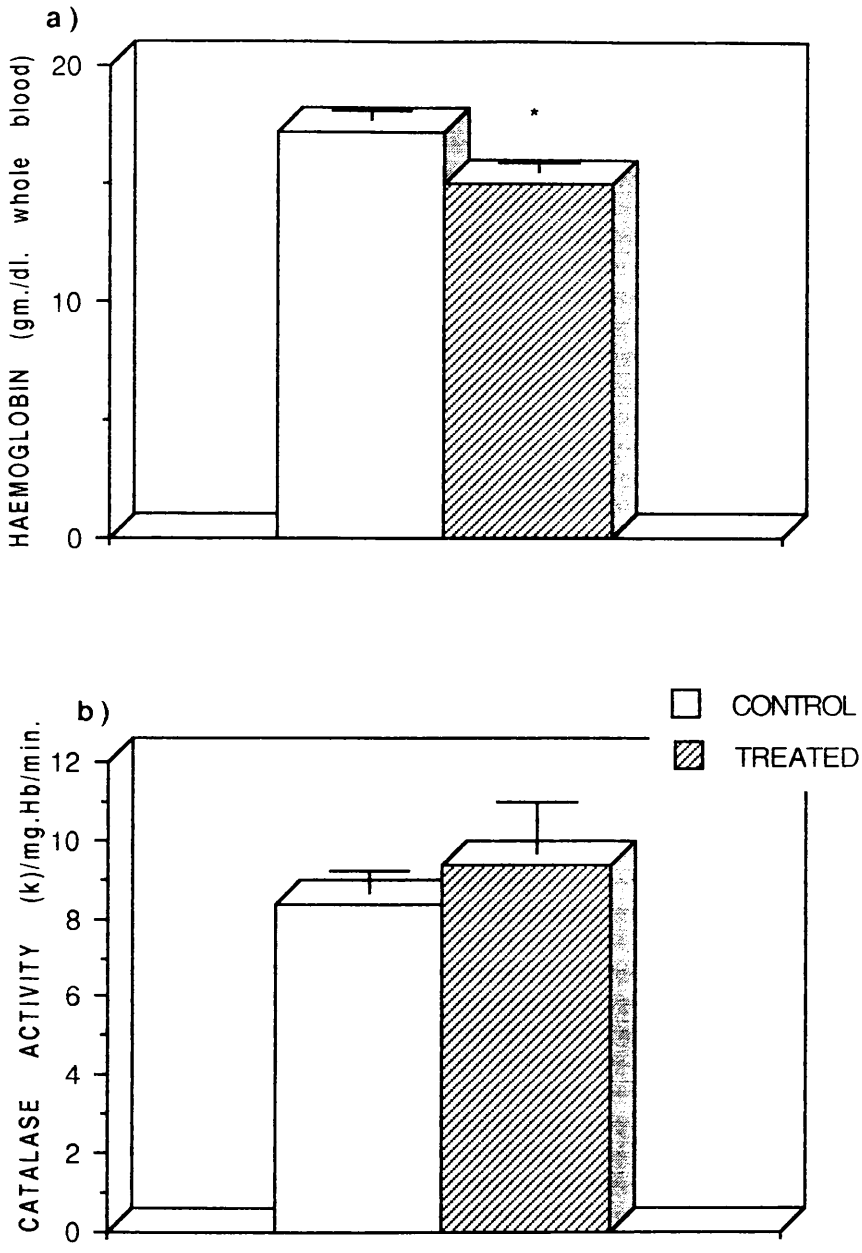


Figure 69: Shows the content and activity of erythrocyte haemoproteins: Graph a) is the mean +/- S.E.M. of the whole blood haemoglobin concentration from control animals (n=6) and from animals that had received porphyrinogenic treatment 2.5 (lead acetate in drinking water) (n=10). Lead treatment caused a significant fall in whole blood haemoglobin content (*$p<0.05$). Graph b) shows the mean +/- S.E.M. of the catalase activity of erythrocyte lysates of control animals (n=6) and of erythrocyte lysates from animals that had received treatment 2.5 (n=5). Catalase activity was unaltered by this treatment.

4.3.17. The effects of porphyrinogenic drug treatment 2.5 (oral lead administration) on brain mitochondrial cytochrome content:

The content of all brain mitochondrial respiratory cytochromes from treated animals did not differ significantly from the cytochrome content of brains from control animals (figure 70).

4.3.18. The effects of treatment 2.6 (4-ethyl DDC) administration on haemoprotein content of neonatal rats:

4.3.18.1. Hepatic respiratory cytochromes:

The hepatic mitochondrial respiratory cytochrome content in 20 day old animals was unaffected by either 4-ethyl DDC or propylene glycol vehicle when administered when 10 days old (figure 71a). The presence of a pigmented mitochondrial pellet and a dip in the liver respiratory cytochrome difference spectra (similar to that seen with 4-ethyl DDC and N-methyl protoporphyrin treatment) demonstrates that 10-20 day old rats can metabolise the dihydropyridine to the alkylated protoporphyrin.

4.3.18.2. Brain respiratory cytochromes:

None of the brain respiratory cytochromes was significantly altered by either 4-ethyl DDC or propylene glycol vehicle when administered 10 days prior to measurement (figure 71b).

4.3.19. The effects of treatment 2.7 (6 days of 4-ethyl DDC) on liver and brain ferrochelatase activity:

Animals were treated for 6 days with intraperitoneal 4-ethyl DDC at a daily concentration of 100mg/kg. The activity of hepatic ferrochelatase was measure in 2 treated and 2 control animals and the brain enzyme was measured in 1 treated animal and 1 control animal. 4-Ethyl DDC treatment profoundly inhibited hepatic

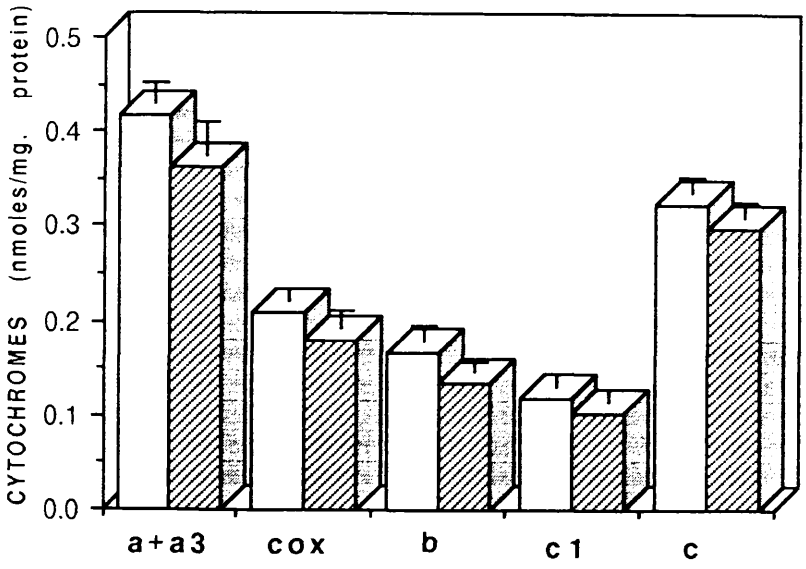


Figure 70: The mean +/- S.E.M. of brain mitochondrial cytochrome content of cytochrome oxidase (cox or $a+a_3$), b, c_1 and c from control tissues (n=6) and from brain mitochondria isolated from animals that had received porphyrinogenic treatment 2.5 (lead acetate in drinking water) (n=5). There were no significant differences.

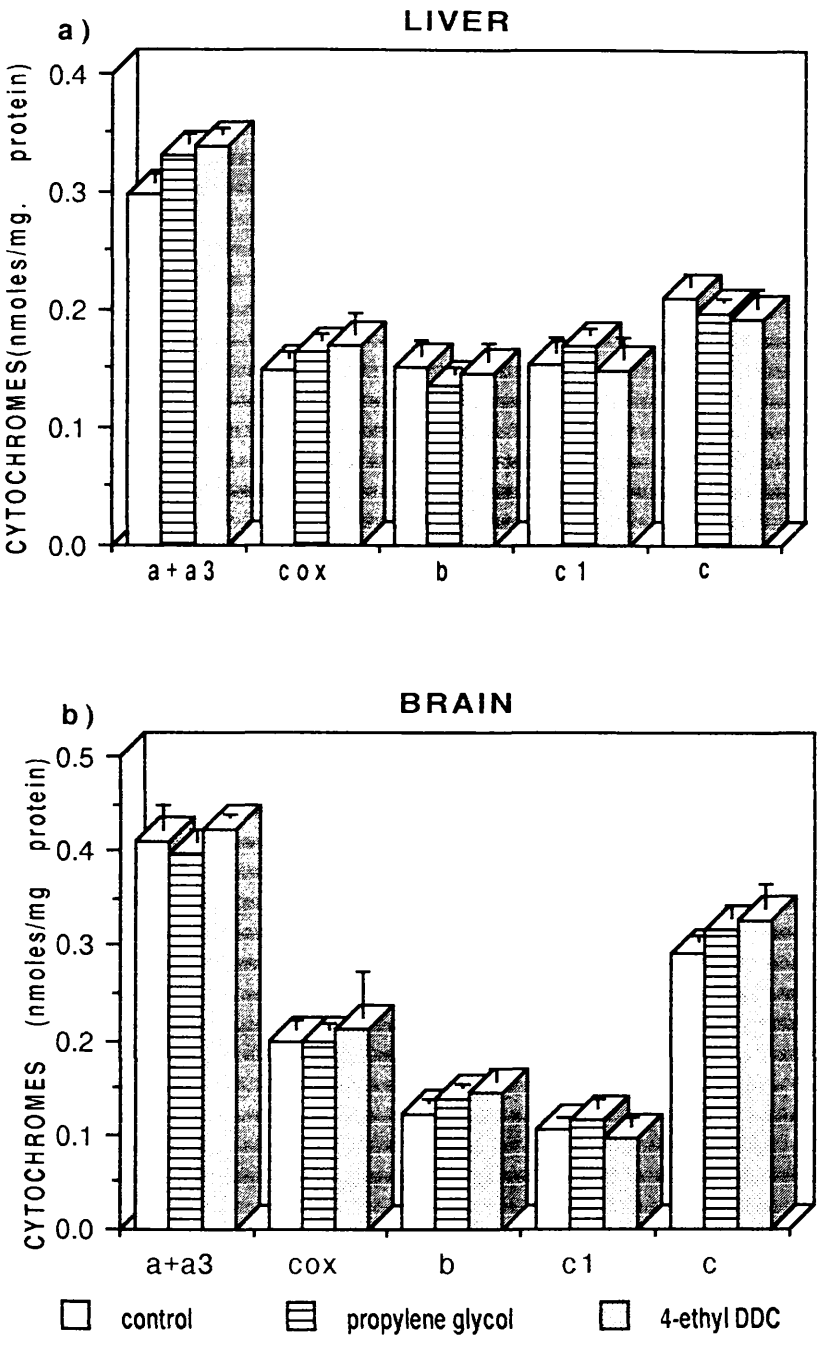


Figure 71: Graph a) shows the liver mitochondrial content of cytochrome oxidase (cox, a+a3), b, c_1 and c of 20 day old rats following 4-ethyl DDC (100mg/kg) at 10 days old,(n=6) ; propylene glycol vehicle (n=5) ; untreated control animals (n=6). Graph b) shows the content of the respiratory cytochromes in the brains of the same animals. 4-Ethyl DDC did not significantly alter the mitochondrial cytochrome content of either tissue.

ferrochelatase activity but was incapable of inhibiting the brain enzyme (figure 72).

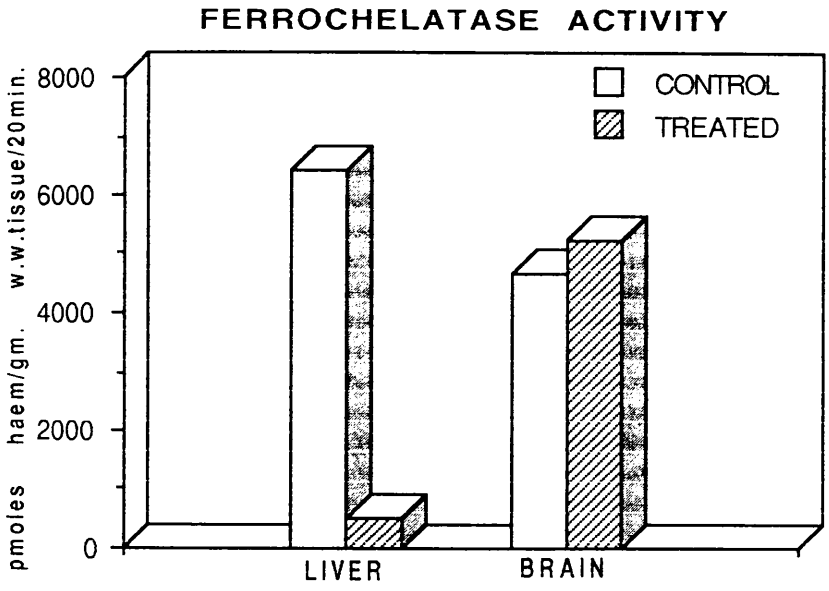


Figure 72: The activity of mitochondrial ferrochelatase in liver and brain as measured by the amount of ^{59}Fe haem synthesized by 1gm. of tissue in 20min. In these limited number of samples, treatment with 4-ethyl DDC over a period of 6 days, caused a profound drop in the activity of the hepatic enzyme whereas brain ferrochelatase activity was unaltered by this treatment.

CHAPTER 4

THE EFFECTS OF PORPHYRINOGENIC DRUGS ON HAEMOPROTEINS.

DISCUSSION.

Haemoglobin in the microcirculation interferes with the measurement of mitochondrial respiratory cytochromes (Bull et.al. 1979). The whole animal perfusion procedure, carried out in these experiments, completely clears the tissues of haemoglobin prior to mitochondria isolation, protecting the measurement of the respiratory cytochromes against any confusing spectrographic interaction of haemoglobin. Despite brain tissue being less accessible to many compounds than peripheral neural tissue, the physiological processes of both of these tissues are similar and chemicals that alter central neural function should also alter peripheral neural function. As the quantity of peripheral neural tissue, in particular the sympathetic ganglia, yielded insufficient material for mitochondrial biochemical study, the whole brain was used to evaluate the ability of porphyrinogenic agents to alter neural haemoprotein content.

The porphyrinogenic treatments employed in the previous section of this study (chapter 3) disrupt haem biosynthesis. This was manifest as an increase in the production and excretion of the haem precursor ALA (figure 16), presumably the consequence of an increase in the rate limiting enzyme of the pathway ALAS. Although these compounds exert their effects at different levels in the haem biosynthetic pathway, their ability to increase production of ALA is postulated to be due to a similar mechanism. All are proposed to reduce the regulatory free haem pool and release the rate-limiting enzyme ALAS from end product inhibition. This derepression facilitates increased ALA synthase production and activity.

The results of the previous section indicate, indirectly, that although haem synthesis is disrupted and haem availability has been decreased, the function of haemoproteins, essential for normal peripheral neuronal function, has not been compromised. The present set of experiments were designed to measure directly the effects of porphyrinogenic agents on tissue haemoprotein content and function. If the hypothesis that porphyric neuropathy is due to a state of haem deficiency, it is essential to produce a model in which haem production is decreased.

The long term treatments employed in this study were designed to inhibit the haem pathway for a period long enough to cover as many half-lives of the measured haemoproteins as practically possible (hepatic catalase 2 days; respiratory cytochromes 5-6 days; haemoglobin 60 days.

Succinylacetone was chosen because it inhibits haem synthesis close to the site of the enzymatic defect of acute intermittent porphyria and the level at which disruption occurs in hereditary tyrosinaemia and hereditary ALAD deficiency. Additionally, succinylacetone inhibits ALAD activity and haem production in a range of tissues; rat brain (De Matteis and Ray1982); rat bone marrow cells (Beru et.al.1983); rat haemoglobin (Tschudy et.al. 1982); rabbit reticulocytes (Ponka et.al. 1982); avian, murine and bovine hepatocytes (Sassa and Kappas 1983); human erythrocytes (Sassa and Kappas 1983).

Over the course of 30 days treatment with 20mg./kg. of succinylacetone, daily, urinary excretion of the haem precursor ALA was elevated (figure 53) and although there was considerable variation in the level of ALA excreted this increase was maintained throughout the treatment period. The level of total porphyrins excreted during this period of treatment was also increased, when compared to total porphyrin excretion by normal control animals. Succinylacetone could be causing increased porphyrin synthesis as a result of enhanced substrate (ALA) availability. This could be due either to a build up of ALA as a direct consequence of ALAD inhibition, to derepression of ALAS by a reduced free haem pool or to direct induction of ALAS by succinylacetone. The later hypothesis would be incongruous with the reduction in haem production caused by succinylacetone (Sassa and Kappas 1983; Beru et.al. 1983; Bottomley et. al. 1985; Ponka et.al. 1982). Graham (1991), however, reported that haem arginate could not totally reverse the induction of ALAS caused by 3 days treatment with succinylacetone at 20mg/kg, a similar concentration to that employed in this study. Partial reversal only of succinylacetone's effects, by exogenous haem, was also reported by Ebert et.al. 1979; 1981 and by Beaumont 1984. These observations imply that ALAS induction, by succinylacetone, is not

due solely to the derepression of the enzyme by reduced haem but that there is another factor at play in succinylacetone-induced increase in ALAS activity. Direct induction of ALAS by succinylacetone would account for the increased porphyrin production and the lack of decreased in haemoproteins, observed in this study. Additionally, De Matteis and Ray (1982) calculated that even when ALAD activity is inhibited, by succinylacetone, by 90% in the cerebellum there would still be enough enzymatic capacity to metabolize 200pmoles of ALA /min/g. of cerebellum, which is in excess of the normal capacity of cerebellar ALAS to produce ALA (53pmoles of ALAS /min/g. tissue). Similarly, in liver and erythroid tissue the activity of ALAS is only 2% that of ALAD (Kappas et.al. 1989). Additionally, the activity of ALAD is in great excess of that of the next enzyme in the haem biosynthetic pathway, PBGD (0.25%, Kappas et.al. 1989), an enzyme with low enzymatic activity. A considerable inhibition of the former enzyme could, therefore, be tolerated before a serious compromise was exerted on haem synthesis capability. There is no doubt, however, that succinylacetone does disrupt haem metabolism by inhibiting at least one enzyme in the haem synthesis pathway, in many tissues, and as such is a useful experimental tool with which to study experimental porphyria. Therefore, consistent with the observation, in this study, that succinylacetone does not reduce total porphyrin production, none of the haemoproteins measured in liver, blood or brain were reduced (figures 55,56,57). The rise in total porphyrins caused by succinylacetone treatment in these animals may, on the other hand, be expected to result in an increase in tissue haemoprotein content. The observation that no increase is observed in tissue content of any haemoprotein is most probably due to haem induction of its degradative enzyme haem oxygenase (Tenhunen et.al. 1970; Smith 1990), which would prevent increased haemoprotein production.

4-ethyl DDC induces an increase in ALAS, firstly, as a result of its capacity to destroy the haemoprotein cytochrome P-450. The residual cytochrome P-450 apoprotein utilises haem from the regulatory pool resulting in a shrinkage of this pool with a resultant derepression of the rate limiting enzyme ALAS. Secondly, the product of 4-ethyl DDC's metabolism, N-ethyl protoprophyrin, inhibits ferrochelatase.

Phenobarbitone enhances both the quantity of alkyl protoporphyrin produced and the fraction of the most potent isomer, the N_A isomer (Riddick et. al 1989; De Matteis et. al. 1983). Phenobarbitone itself induces production of the haemoprotein cytochrome P-450 (Waxman and Walsh 1982) creating an environment where increased haem synthesis is required to replenish haem consumed in cytochrome P-450 synthesis and to replace the haemoprotein destroyed by the dihydrocollidine. Additionally, phenobarbitone inhibits the haem biosynthetic pathway at the level of uroporphyrinogen decarboxylase with a concomitant accumulation of uroporphyrin, heptacarboxilic acid porphyrin and coproporphyrin (DeVerneiul et.al. 1983 ; Marks et.al.1987).

The second porphyrinogenic treatment was designed to cause a relatively long period of haem synthesis inhibition, initially by blocking ALAD, followed by further assaults on the pathway both by 4-ethyl DDC and phenobarbitone. It is perhaps not surprising that no neuropathy was produced, by the treatments employed in the experiments reported in chapter 3, as there was no decline in neural respiratory cytochrome content even after long term porphyrinogenic drug treatment, in spite of a significant reduction in hepatic levels. The explanation of this failure to act in the brain is unclear. It could be that the drugs are unable to gain access to the brain because of the blood brain barrier. Alternatively, the most effective of these drugs, 4-ethyl DDC, is dependent upon cytochrome P-450 metabolism for its ferrochelatase inhibitory action and the levels of this haemoprotein are low in the brain compared to the liver. It would have been interesting to have measured the levels of respiratory cytochromes in peripheral nerve cells, where the blood brain barrier is deficient (e.g. hexamethonium, a highly charged drug gains easy access to ganglia). An attempt to get such measurements failed because the total weight of both complete sympathetic chains, in the rat, was too low. One tissue in which changes might have been expected was the anococcygeus muscle, in response to either nitrates or NANC nerve stimulation. Guanylate cyclase, a haemoprotein, is an essential component in this response and there is no barrier to drug access and yet the response to NANC nerve stimulation was not reduced by this

combination of drugs. Again, a deficiency of cytochrome P-450, within muscle cells, could explain this failure.

This second treatment regime caused a reduction in all the hepatic haemoproteins measured. Liver catalase was markedly reduced in treated animals when compared to the enzyme activity in control animals. The catalase molecule has a short half-life of 2 days and contains 4 haem moieties. The activity of this haemoprotein, therefore, provides a fairly rapid index of haem depletion. The 4-methyl analogue of 4-ethyl DDC reduces hepatic catalase activity in rats and rabbits (Ginsberg et.al. 1963; Haeger-Aronsen 1962) and the porphyrinogenic agent, allylisopropylacetyl carbamide (Sedormid) was also potent in reducing hepatic catalase activity in rabbits and rats within 3 days of drug administration (Schmid et.al.1955). Sedormid was, however, ineffective in altering erythrocyte catalase activity even after prolonged drug administration of up to 42 days. The reduction in hepatic catalase activity, observed by Schmid and his colleagues, was due to inhibition of catalase synthesis as a reduction in the incorporation of radioactive glycine into catalase was concomitant with its decreased activity. It is unlikely that the reduction in hepatic catalase activity, observed in this study, is due to gross toxicity of the liver as other hepatic functions, in particular the mitochondrial ALAS activity, is in great excess of its normal function.

Similar to the effects of Sedormid, erythrocyte catalase activity, in this study, remained unaltered by the combination of porphyrinogenic drugs. It is well documented that the half-life of hepatic catalase is 2 days. There has been very little study of the half-life of erythrocyte catalase. Erythrocyte catalase content peaks during reticulocyte development (Denton et.al. 1975). The mature erythrocyte does not contain mitochondria nor does it contain the full complement of haem biosynthetic enzymes, including the inner membrane-bound mitochondrial enzyme ferrochelatase (Kappas et.al. 1989). The major haemoprotein of the erythrocyte, haemoglobin, is not synthesized de novo during the life time of the cell. As the mature erythrocyte is incapable of synthesizing haem it is unlikely that the half-life of erythrocytic catalase is 2 days. The half-life of blood catalase is most likely similar to that of haemoglobin in that it

is linked to the life of the erythrocyte itself, 60 days in the rat. It is, therefore, probable that the effective period of haem synthesis inhibition brought about by this treatment was not long enough to produce an appreciable reduction in either erythrocyte catalase activity or whole blood haemoglobin content of animals receiving this treatment. The possibility that the decomposition of hydrogen peroxide in erythrocyte lysates was due to the activity of glutathione peroxide and not catalase, and for this reason was unaltered by depletion of haem, was ruled out by the observation that azide abolished peroxidative activity. Glutathione peroxidase, a selenium containing enzyme, would be unaffected by the addition of sodium azide (Mills 1958).

The inability of the porphyrinogenic drugs, used in this study, to alter erythrocyte catalase activity, while at the same time reducing the activity of this enzyme in the liver, could also be due either to the inability of the active metabolite of 4-ethyl DDC, N-ethyl protoporphyrin, to leave the hepatocyte or that this ferrochelatase inhibitory agent does not enter reticulocytes.

This treatment, which is effective in reducing hepatic mitochondrial respiratory cytochromes, is incapable of altering brain respiratory cytochromes. Several possibilities may account for this observation. Following metabolism in the hepatocytes, by cytochrome P-450, the N-ethyl protoporphyrin may not leave the liver cells. There is a strong possibility that 4-ethyl DDC itself does not cross the blood brain barrier. If this substance does, however, gain access to the brain the low levels of cytochrome P-450, about 3% of that in the liver (Percy and Shanley 1979), may not produce enough of the active alkylated protoporphyrin metabolite to significantly impair ferrochelatase activity, to the extent that haem production is reduced. DDC metabolism does occur in other tissues with the destruction of cytochrome P-450. Reed and his co-workers in 1988 reported that hamster olfactory epithelial cells are capable of metabolizing DDC and although the direct measurement of cytochrome-P450 was not possible, due to the low levels of this compound in this tissue, cytochrome P-450 dependent-processes were reduced by DDC administration. The isoenzyme type present in cells is extremely important in the ability of the cytochrome to

metabolize the dihydrocollidine. β-Naphthoflavone and phenobarbital-inducible isoenzymes are more potent than the 3 methylcholanthrene-inducible type in their ability to metabolize 4-ethyl DDC, to the active ferrochelatase inhibitory substance (Riddick 1989; De Matteis et.al. 1983). A large proportion of brain cytochrome P-450 is an oestrogen-metabolising isoenzyme (Paul et.al 1977), and may not readily oxidise 4-ethyl DDC. Local reductions in haem synthesis may be missed by the large scale measurement of whole brain respiratory cytochromes. The hypothalamus is an important site for many brain functions, particularly hormonal secretions. Neurotransmitters, impinging on the hypothalamus, control the secretion of releasing and inhibitory factors, into the median eminence blood vessels, which in turn regulate adenohypophyseal secretion. Acute porphyric episodes are associated with hormonal changes, especially during the oestrus cycle, at a time when there could be a strain put upon hypothalamic haemoproteins. The neurohypophysis may also be affected, during acute porphyric attacks, as there is ample evidence indicating inappropriate ADH secretion. Although regional differences have been found in the activity of the rate limiting enzyme, ALAS, in the brain, with the highest levels in the cerebellum, followed by the cortex and hypothalamus, the total porphyrin and haem content of various brain regions do not differ greatly (Maines 1980).

From the evidence obtained from the first two groups of experiments, in this section, it appears that the 30 days treatment with succinylacetone was not a major contributory factor to the reduction in the hepatic haemoproteins observed in the second experiment. 4-ethyl DDC is thought to be the major cause of haem synthesis inhibition. The next group of experiments sought to exploit this finding and in an attempt to increase this haem depletory effect, the dose of 4-ethyl DDC was increased to 100mg/kg for 14 days, making the 4-ethyl DDC treatment period the same as that in the previous experiment. This treatment, with increased 4-ethyl DDC, produced a similar level of hepatic haem reduction as that seen with the lower doses of 4-ethyl DDC and phenobarbitone (figures 56a, 58a). This could be because maximum inhibition of haem was already reached with the lower doses and only a time factor would alter the degree of reduction observed. It is likely that the phenobarbitone, in the second

group of experiments, played a role in the depletion of respiratory cytochromes and catalase activity. Phenobarbitone effects would be exerted for the last 6 days of treatment, 3 half-lives of the hepatic catalase enzyme and 1 half-life of the respiratory cytochromes. Treatment with 4-ethyl DDC for 14 days, was also incapable of reducing either erythrocyte or brain haemoprotein content. The same arguments put forward for the inability of the long term drug combination to reduce these levels, also holds for this treatment regime.

Measurement of liver and brain ferrochelatase activity, in a small number of animals treated for 6 days with 4-ethyl DDC, shows that although the hepatic enzyme is profoundly inhibited by this treatment, the brain enzyme remains unaltered. This observation explains the unaltered brain haemoprotein content in treated animals. Either the active metabolite of the 4-ethyl DDC is not entering the brain or brain ferrochelatase is refractory to inhibition by N-ethyl protoporphyrin. Brain ferrochelatase activity measured in this study was equivalent to that in hepatic tissue. This is not in keeping with the measurements of brain ferrochelatase activity in other studies where the activity of the brain enzyme has been shown to be very much lower than that in hepatic tissue (Percy and Shanley 1979; Barnes et. al. 1971). Ferrochelatase activity is expressed in a variety of units in different studies. However, when the results for hepatic enzyme activity obtained in the present experiments were corrected for approximate activity per mg. of mitochondrial protein the results gives the same activity as that measured in rat liver by Percy and Shanley (1979), and about 1/10th. that measured by De Matteis et.al. 1973 and Barnes et.al. 1971, using deuteroporphyrin or mesoporphyrin and Co^{2+} as substrates. The method used in this study (Houston et.al. 1988 and in preparation) is a sensitive method using physiological substrates (which may account for the lower measured levels of enzyme activity) and employing both HPLC separation of haem and radiochemical assay for the detection of radioactive iron incorporation into haem. The method, therefore, provides a sensitive measurement of ferrochelatase activity in both liver and brain tissue.

To overcome the possibility that the N-ethyl protoporphyrin metabolite of 4-ethyl DDC was not leaving the hepatocyte,

commercially obtained N-methyl protoporphyrin was injected directly into the systemic circulation for a period of 14 days. Although multiple injection difficulties arose, N-methyl protoporphyrin was gaining access, at least to hepatic tissue, as evidenced by pigmentation of the mitochondrial fraction. This treatment, although less effective than the previous two treatment regimes, did alter some hepatic haemoproteins. Only cytochrome oxidase ($a+a_3$) content and catalase activity were significantly reduced (figures 65a, 65b). The porphyrinogenic impact of the N-methyl protoporphyrin is not expected to be as profound as that of 4-ethyl DDC as the latter exerts a dual effect on haem availability, both destroying cytochrome P-450 and inhibition of ferrochelatase. It could be argued that the reduction in hepatic catalase activity, by 4-ethyl DDC, is due to dihydrocollidine toxicity or to increased catalase utilisation and breakdown during the decomposition of hydrogen peroxide produced during cytochrome P-450's 1 electron metabolism of the 4-ethyl DDC. However, in this experiment, the observation that N-methyl protoporphyrin, administered alone, significantly reduces hepatic haemoproteins indicates that its ferrochelatase inhibitory effect is the cause of at least some of the reductions in liver haemoproteins. Again, as with previous experiments in this group of studies, N-methyl protoporphyrin was incapable of reducing either blood or brain haemoproteins. Even though the alkylated protoporphyrin has direct access to the systemic circulation, blood haemoprotein are unaltered after 14 days exposure. Erythrocytes can accumulate protoporphyrin from an exogenously administered source (Nakao et.al. 1966) of 200μg/day for 4-6 days. It would not be unreasonable to assume, therefore, that the concentration of the alkylated protoporphyrin, although structurally slightly different from protoporphyrin, used in this study (1μmole/ day) should be able to gain access to erythrocytes. Additionally, De Matteis and Rimington (1963) found that systemically administered DDC caused a rise in erythrocyte protoporphyrin in mice, indicating that DDC either inhibits reticulocyte ferrochelatase, causing a rise in red cell protoporphyrin, or that blood born protoporphyrin, of hepatic origin, can enter the red blood cell. This chemical's inability to alter blood haemoproteins is probably due to the long half-lives of the erythrocytic constituents. The experimental results also indicate

that the alkylated metabolite of the dihydrocollidine DDC either does not enter the brain or does not inhibit brain ferrochelatase.

The livers of animals treated with 4-ethyl DDC were extremely dark and on homogenisation gritty. The mitochondrial pellet and the post mitochondrial supernatant were also extremely dark. This last observation, is in keeping with the concept that the alkylated porphyrin is produced on the hepatic microsomes and transported into the mitochondria, where it binds to the ferrochelatase enzyme on the inner mitochondrial membrane. N-methyl protoporphyrin, on the other hand by-passes microsomal metabolism and, as would be expected, no appreciable pigmentation was observed in the post-mitochondrial supernatant. Fluorescence histological examination of a liver from an animal treated for 14 days with 100mg/kg. of 4-ethyl DDC shows a wide spread fluorescence, caused by accumulating protoporphyrin and N-methyl protoporphyrin which gives the dark pigmentation to the liver. Electron microscopy of liver mitochondrial pellets shows dark accumulations in lysosomal-like bodies (plate 3). This is, probably, accumulating iron, which is probably the cause of the gritty texture and appearance of the livers. Iron accumulation was also observed in the livers of porphyria cutanae tarda patients. Grossman et.al. (1979) observed siderosis in 80 % of the 40 patients they examined. Pigmentation of brain tissue was never observed in any of the systemically administered treatment groups neither in the intact tissue nor in the mitochondrial pellet (plate 4). 4 out of 8 animals, treated for 14 days with 4-ethyl DDC, developed red deposits around the periphery of the ear lobes which developed into scarring of the tissues (plate 5). This observation closely resembles the porphyrin deposition and subsequent skin lesioning seen in patients with porphyria cutanea tarda where an enzymatic defect in the haem pathway, occurring at the level of uroporphyrinogen decarboxylase, causes accumulation of porphyrins in the liver, blood and subcutaneous tissue. Fluorescence microscopy was not carried out on this tissue but would have determined whether the skin deposits may have been porphyrins. Iron staining in the tissue proved negative but this is not surprising as accumulating iron would be deposited in the liver and not in the skin.

There is a neonatal surge in haem synthesizing enzymes, which coincides with an increase in brain mitochondrial cytochrome content (De Matteis et.al.1981a; Chepelinsky and Arnaiz 1970; Bull et.al. 1979). A single injection of 4-ethyl DDC was given at the time of this surge, in an attempt to prevent this increase in haem synthesis. For practical reasons only one injection was administered to 10 days old neonates. By 20 days of age the brain mitochondrial cytochrome content has reached adult status. 4-Ethyl DDC failed to prevent the neonatal increase in either brain or liver mitochondrial cytochrome content. The drug was metabolized in the neonate liver. Indirect evidence of this metabolism comes from the dark pigmentation of the hepatic mitochondrial pellet. At this stage, therefore, the neonates have sufficient hepatic cytochrome P-450 to metabolize the dihydrocollidine. 4-ethyl DDC treatment of these neonates was incapable of altering either brain or hepatic mitochondrial respiratory cytochromes. It is possible that in these animals the initial rise in haem synthesis had occurred before drug administration. Cytochrome content of both hepatic and brain mitochondria were comparable to the levels measured in adult tissues.

Lead possesses the porphyrinogenic properties of inhibiting haem biosynthetic enzymes. A reduction in cytochrome P-450-dependent mechanisms have been widely observed (Alvares et.al. 1972; Meredith et. al. 1977; Goldberg et.al. 1978) suggesting that lead may reduce haemoprotein production. In the last group of experiments in this study lead was administered to neonate rats via their dams drinking water. This early treatment (beginning at 5 days of age) was, also, designed to overlap with the early period of increased production of brain mitochondrial cytochromes. This long term lead poisoning did not inhibit hepatic or central respiratory cytochrome content. The morphology of mitochondria are altered by lead poisoning (Fowler et.al. 1987). However, the results reported here indicate that even if mitochondrial morphology is altered by lead treatment the haemoprotein content of mitochondrial is not reduced. The measurements carried out, in the present study, give no indication of mitochondrial function processes, which would be altered by morphological changes. The reduction in cytochrome oxidase activity

in muscle biopsies from lead exposed subjects reported by Goldberg and his colleagues in 1985, may be due to mitochondrial structural changes, rather than an alteration in content. Brain mitochondrial function was altered by lead feeding in suckling rats (Holtzman and Shen Hsu 1976). The time taken for these deficits to occur was, however, less than 2 days. This period was too short for the effects of lead to be mediated via haem depletion, as the half-lives of the mitochondrial cytochromes are approximately 6 days and these effects are more likely due to morphological changes. Consistent with the results of this study are those reported by Bull et.al. in 1979 who showed that although lead delayed cerebral respiratory cytochrome development, in 10-15 day old rats, this deficit was reversed by the time the animals were 30 days of age.

Erythrocyte catalase activity, expressed per mg. of haemoglobin was unaltered by lead exposure. The major blood haemoprotein, haemoglobin, was, however, significantly reduced, which could be taken as a reduction in erythrocyte catalase activity in a given volume of whole blood. The reduction in haemoglobin, seen following exposure to lead, was most probably due to a combination lead's haemolytic effect, which would reduce the half-life of the red cell, and a reduced haem synthesis due biosynthetic enzyme inhibition (Moore and Goldberg 1985). However, a lack of decreased haem synthesis in the liver is supported by the observation that the activity of hepatic catalase, which has the shortest half-life of the haemoproteins measured was increased as a result of this treatment. If haemoprotein depletion was the cause of the decreased haemoglobin content hepatic catalase activity would have been compromised first. Toxic compounds, such as ethyl-chlorophenoxy-isobutyrate have been shown to cause hepatomegaly and as part of this process hepatic catalase activity is increased (Hess et. al. 1965). This type of process may also account for the significant increase in hepatic catalase activity measured following lead exposure in this study, although hepatomegaly has not been reported in either human lead poisoning or experimental lead toxicity.

In 50% of both 14 days 4-ethyl DDC and N-methyl protoporphyrin treatment the dip in the oxidised-reduced difference spectra. This phenomenon is probably due to the effects of sodium dithionite on the

alkylated protoporphyrin itself, as evidenced by the effects of this reducing agent on the spectra of N-methyl protoporphyrin alone. It is surprising, however, that this same alteration in difference spectra was not observed with the long term combination with lower concentrations of 4-ethyl DDC and phenobarbitone, as the same degree of hepatic haemoprotein inhibition suggests a similar level of ferrochelatase inhibitory activity. It is possible that ferrochelatase inhibition by the alkylated porphyrin was not the prominent inhibitory effect and phenobarbitone has a more important role to play in haem inhibition. In this case, low levels of N-ethyl protoporphyrin would not greatly interfere with the cytochrome difference spectra. The distortion of the difference spectra, seen in these two groups, could confound the absolute measurement of the respiratory cytochromes. If this were the case, an apparent decrease may be measured for cytochrome oxidase, but by the same token, an apparent increase would be observed with the other cytochromes. Overall, however, even with these distortions the reduction in at least cytochromes b, c and c_1 are true and the reduction in the second treatment group are also valid.

In this study, dimethyl sulfoxide was chosen as the vehicle for the dihydocollidines and AIA. As multiple injections were carried out, this chemical was considered the best choice of vehicle. It is well known that DMSO induces haem biosynthetic enzymes in erythropoietic tissue. ALAD, PBGD, protoporphyrinogen oxidase and ferrochelatase have all been shown to increase following DMSO addition to cultured erythropoietic cells (Sassa 1983). However, in hepatic cells DMSO is a potent inducer of haem oxygenase, the rate limiting step of haem degradation. The volume of DMSO employed, in this study, would give a relatively low tissue concentration. Additionally, in this study 4-ethyl DDC caused a profound inhibition of hepatic ferrochelatase when DMSO was used as the vehicle. Other studies, where DMSO was used as the vehicle for dihydrocollidine, similarly showed that this compound did not compromise the ability of DDC to inhibit ferrochelatase or prevent the decrease in haemoprotein content (De Matteis et.al. 1980a; Ortiz de Montellano et.al. 1981a 1981b). Succinylacetone completely prevented the DMSO-mediated induction of PBGD observed by Beaumont et.al (1984) and,

therefore, the presence of succinylacetone should also offset any stimulatory effects of DMSO in this study.

CHAPTER 5

THE EFFECTS OF PORPHYRINOGENIC DRUGS ON MITOCHONDRIAL FUNCTION.

INTRODUCTION.

The results of the experiments reported in chapter 4 demonstrate that the porphyrinogenic chemicals employed in this study exert a differential effect on tissue haemoproteins. Although hepatic catalase activity is markedly reduced by some agents, erythrocyte catalase is unaltered. A similar differential effect is observed between hepatic and neural respiratory cytochromes, where hepatic mitochondrial cytochromes were significantly decreased whereas brain mitochondrial cytochromes were unaffected.

ATP is formed as a consequence of electron transfer from energy rich sources to oxygen in a sequence of redox steps. As the electrons move down an electrochemical gradient protons are pumped into the intramitochondrial space producing a proton motive force across the inner membrane. When these protons flow back through a protein complex, ATP synthase, they drive the synthesis of ATP (figure 73) (Mitchell 1961;1979). Reduced nicotine adenine dinucleotide phosphate (NADPH) is the reducing equivalent produced from mitochondrial oxidative phosphorylation substrates glutamate and malate. Electrons from NADPH are transfered to an oxidised flavin mononucleotide (FMN) of Complex 1 and in turn the electrons are passed to a non-haem iron-sulphur compound of Complex 1. Protons are pumped across the membrane at this site. The electron shuttle Ubiquinone (Q) then accepts the electrons transfering them to cytochrome b of Complex 111. Via another iron-sulphur centre, electrons are transfered to the cytochrome c_1 of Complex 111. The soluble cytochrome c shuttles electrons to Complex 1V, cytochrome oxidase. Protons are pumped at both Complex 111 and Complex 1V. Cytochrome oxidase mediates the reduction of molecular oxygen to water. Succinate can act as a substrate for mitochondrial oxidative phosphorylation, donating its electrons to an oxidised flavin adenine dinucleotide (FAD), which are in turn transfered via an iron-sulphur centre to ubiquinone and then to the cytochrome b of complex 111. The protons, which have been pumped across the mitochondrial membrane into the intermitochondrial space, as a result of this respiratory process, are channelled back into the inner mitochondrial space via Complex V, ATP synthase. This flow back of protons through ATP synthase drive the production of ATP, from ADP and phosphate (figure 73). The function of the inner membrane is, therefore, energy

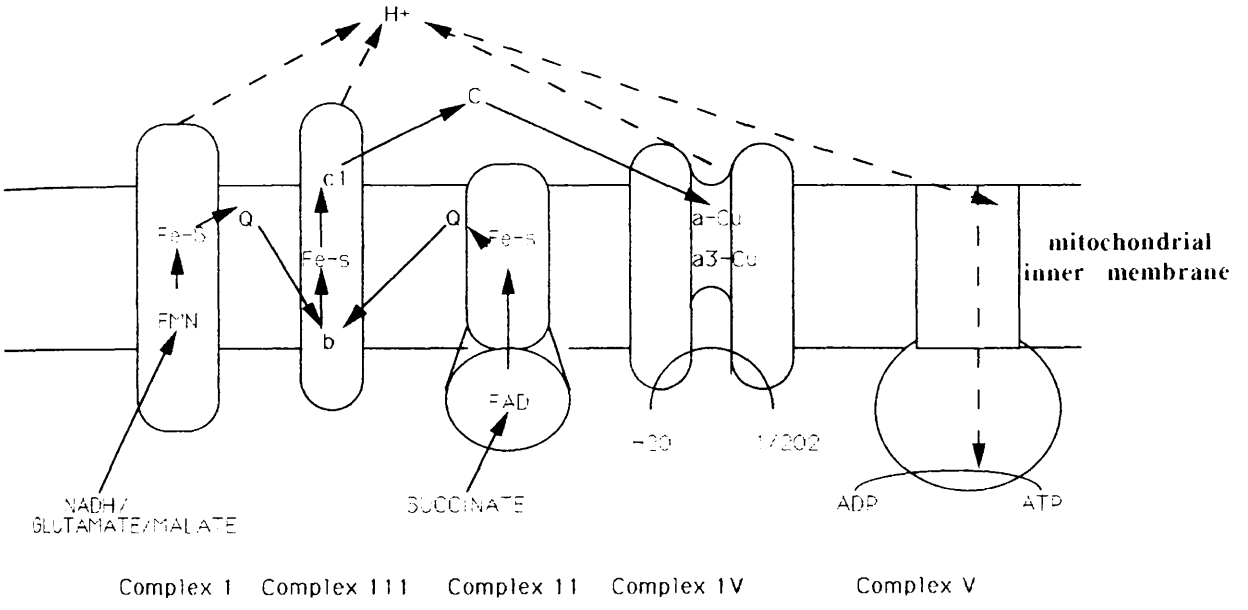


Figure 73: Shows the sequence of components of the inner mitochondrial membrane, necessary for the process of oxidative phosphorylation.

transduction. A defect in any of the four complexes of the electron transport chain, Complex I (NADH-ubiquinone reductase), Complex II (succinate-ubiquinone reductase), Complex III (ubiquinone cytochrome c oxidoreductase or bc_1 complex) or Complex IV could lead to mitochondrial malfunction.

The fourth group of experiments examines the effect of cytochrome depletion on mitochondrial function. The porphyrinogenic drugs used in chapter 4 experiments did not reduce brain mitochondrial cytochromes while doing so in liver tissue. This could be due to a failure of these drugs to cross the blood brain barrier. If the practical problem of access could be overcome, it is important to determine whether a reduction in brain respiratory cytochromes would lead to a compromise in mitochondrial function and subsequent energy production. Although the respiratory cytochromes will not be measured directly, a reduction in these should be manifest as a deficit in mitochondrial function.

Reduced mitochondrial cytochrome content exists in some of the mitochondrial encephalomyopathies and an animal model of this type should provide an insight into the aetiology of porphyric neuropathy which may also be the result of a mitochondrial cytochrome deficiency.

Firstly, this group of experiments examines the effects of compounds, which are known to reduce hepatic mitochondrial haemoprotein content (chapter 4), on several parameters of mitochondrial function. The supposition that the inability of these compounds to cross the blood brain barrier was the cause of their failure to reduce brain respiratory cytochromes was examined by the study of brain mitochondrial function following central administration of N-methyl protoporphyrin 1X. Additionally, as N-methyl protoporphyrin 1X may interfere with with the spectrographic measurement of mitochondrial respiratory cytochromes, mitochondrial function experiments by-pass this difficulty and provide a further functional measurement of haem depletion.

CHAPTER 5

THE EFFECTS OF PORPHYRINOGENIC DRUGS ON MITOCHONDRIAL FUNCTION.

METHODS.

The function of liver and brain mitochondria from animals treated with porphyrinogenic was studied in this section.

5.2.1. Drugs:

Hypnovel (Roche); Hypnorm (Janssen Pharmaceuticals, England); D-mannitol; glutamic acid, monosodium salt; malic acid, disodium salt; (3-[N-morpholino]propane sulfonic acid) sodium salt (MOPS); ethylene glycol-bis β-aminoethyl ether, (EGTA); adenosine 5'-diphosphate, sodium salt; bovine serum albumin (fatty acid free). All chemicals were obtained from Sigma Co.Ltd., unless stated otherwise.

Unless specified, male Wistar rats of the same weight and strain as above were used in this group of experiments.

5.2.2. Construction of jugular catheter:

Catheters were made from a 10cm. length of Tygon polythene,internal diameter 0.5mm, external diameter 1.25mm. (Norton Plastics, Ohio, U.S.A.) and a 3cm. length of Portex polythene tubing, internal diameter 0.5mm., external diameter 0.8mm. (Portex Ltd., Kent, England). One cm. at the end of the Tygon tubing was enlarged by stretching it over a No. 21 gauge hypodermic needle (the needle point was filed down) and heating it against a warm light bulb. The tubing was allowed to cool before removing the needle. The Portex polythene tubing was inserted into the widened end of Tygon tubing, heated to form a shrink fit and cut straight across at the tip (a bevelled tip being more likely to either pierce the vein or to cause a blockage against the vessel wall). The tip of the polythene tubing was held against a hot light bulb to melt away any sharp edges. A 1cm. plug was made from stainless steel wire (0.7mm diameter) which was rounded at both ends to prevent damage to the catheters.

Implantation of jugular vein catheter:

The catheter was filled with heparinised saline solution (150 International units/ml.) and the end plugged. The rat was anaesthetised with a mixture of 1 part Hypnovel:1 part water/1 part

Hypnorm: 1 part water. The final mixture was injected intaperitoneally in a volume of 3ml./kg. The animal was shaved at both the front and the back of the neck and a mid-line incision made on the ventral aspect of the neck. The jugular vein was identified and freed from surrounding connective tissue by blunt dissection. Two lengths of fine thread were passed beneath the vein. The thread most distal from the heart was tied and gentle traction exerted on this tie by artery forceps. The second thread was tied in a loose single knot. An small incision was made in the side of the vein between the thread and the tie. The catheter was inserted into the vein and pushed gently towards the heart. The thread was doubly knotted ensuring that the whole of the polythene tubing lay within the vessel. The strands of the second thread were tied around the catheter for supplementary security. A further thread was sewn into the underlying muscle and tied securely around the catheter. A large hollow needle was inserted through the skin at the back of the neck at the right hand side and brought through the subcutaneous tissue emerging at the perimeter of the ventral cut. The catheter was passed to the back of the neck via this needle where it was secured by a double suture and cut to a length which made it impossible for the rat to chew. The catheter was left fairly loose to avoid dislodgement. The ventral incision was sutured after liberal spraying with Polybacterin antibiotic.

5.2.3. CENTRAL ADMINISTRATION OF N-METHYL PROTOPORPHYRIN.

5.2.3.1. Osmotic pumps:

Alzet osmotic pumps (Alza Corporation, Palo Alto, California, U.S.A.) were employed for central administration of N-methyl protoporphyrin. The alkyl protoporphyrin was dissolved in saline pH 9.0 (with 0.1M NaOH) and brought to a pH 7.4 with 0.1M HCl. The porphyrin was sterilised by passing it through a 0.2µm. pyrogen free filter. The Alzet osmotic pumps used in this procedure had a reservoir volume of 200µl., a diameter of 0.7cm and a length of 3cm. This pump delivers 0.5µl. of solution/hr. over a period of 14 days. The

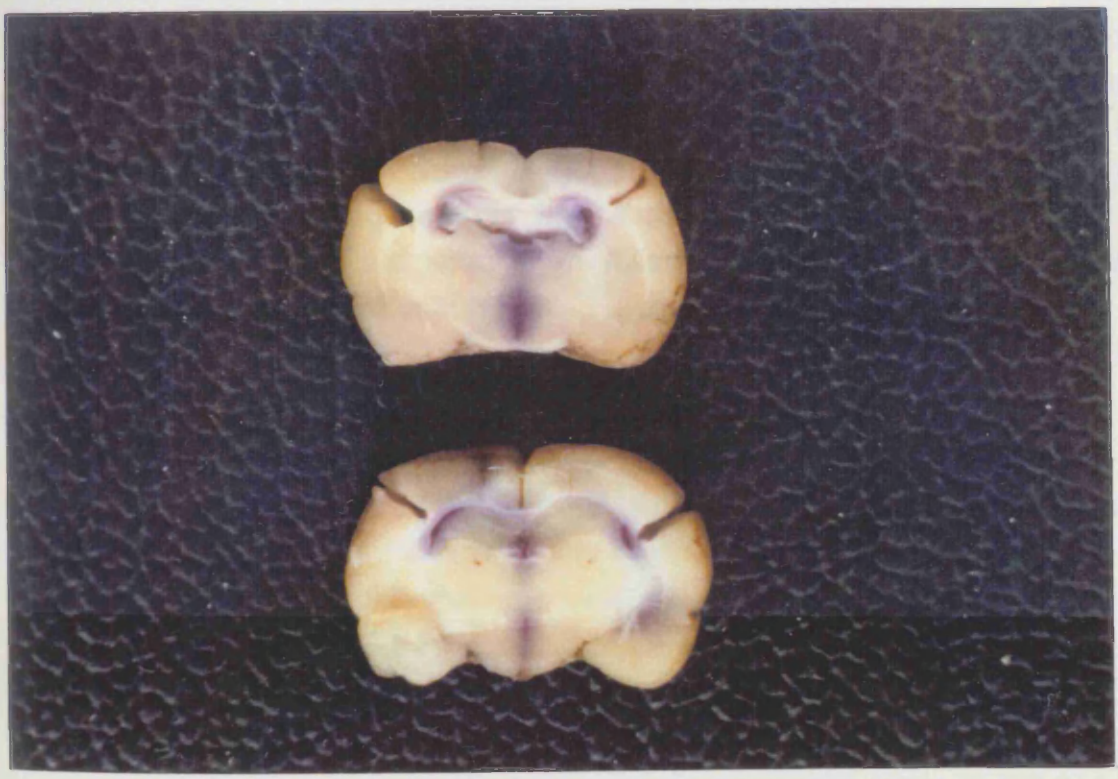
empty pump and its flow moderator were weighed. A 1ml. syringe attached to a 25 gauge blunt-ended needle was filled with the solution, care being taken to ensure that the solution was free of air bubbles. With the flow moderator removed, the pump was held in an upright position and the filling needle inserted into the pump until it could go no further. The plunger of the syringe was the pushed slowly until the solution appeared at the outside of the pump. The syringe needle was removed, excess fluid wiped off the pump and the flow moderator inserted slowly into the pump. The filled pump was reweighed to determine that it is filled correctly and free of air.

One arm of an L-shaped cannula was attached to a 9cm. length of Tygon tubing (internal diameter 0.5mm, external diameter 1.25mm) and filled with the porphyrin solution. The free arm of the L-shaped cannula was a stainless steel tube (0.43mm x 0.3mm). The other end of the Tygon tubing was attached to the end of the flow moderator. This procedure resulted in bubble-free filling of the pump, connecting tubing and L-shaped cannula assembly. The filled pump and cannula were placed into a sterile 50ml. capped tube containing sterile saline and incubated at 37^0C overnight to prime the pump.

5.2.3.2. Insertion of central cannula:

The rats were anaesthetised with the mixture already described. The animal was anaesthetised as described above, the ear bars inserted into the aural canals and the animal placed in a rat stereotaxic frame (Tren Wells. Inc. U.S.A.). The fur over the head was shaved, washed with 70% alcohol and a midline incision made through the scalp to the cranium. The skin was eased back from the skull and the bregma identified. A haemostat was used to make a subcutaneous pocket extending caudally about 6cm from the incision. The fluid-filled pump was passed, round end first, into the pocket. The positioning of the cannula was then determined stereotaxically from a stereotaxic atlas of the rat brain (Pellegrino et.al.1981) using the bregma as the zero reference point. A small hole was drilled through the cranium over the target area and the cannula placed in position. Pilot studies injecting cresyl violet dye by this method confirmed the injection site was correct for injection into the ventricular system (plate 6). The cannula length enabled it to reach the 3rd. ventricle

Plate 6: A photograph (x 2.5 magnification) of a bisected brain, preserved in formal saline, after injection of cresyl violet into the third ventricle. The dye is carried in the CSF away from the site of injection. This confirms that the injection co-ordinates employed for delivery of N-methyl protoporphyrin delivered the drug into the ventricular system.



when placed in position. A small cranial screw (2mm x 0.8mm) was inserted into but not through the skull close to the cannula. Both cannula and screw were secured with quick drying dental cement (De Trey). The dental cement consists of an acrylic resin powder and a methyl methacrylate monomer liquid. The most successful method for achieving a quick and secure fixture was to apply some polymer powder over the site followed by dropwise addition of the monomer liquid. This procedure was repeated several times until the whole site was adequately covered with cement. The screw secured the cannula and cement to the scull. The cement was left to dry to the touch, about 5min. before the skin was sutured enclosing the whole structure. The procedure took approximately 20min. and the animal began to recover from the anaesthetic in about 30min. N-methylprotoporphyrin from the osmotic pump was administered through the indwelling cannula for 14 days. The skin healed rapidly in this time. The animals were then killed and the brains removed for preparation of the mitochondria.

5.2.4. Porphyrinogenic Treatment:

Rats of the same age and weight as described above were employed in this part of the study. Four groups with different drug regimes were used.

GROUPS:

1) Animals received intraperitoneal injections of 20mg/kg of succinylacetone for 30 days followed by 10 days of 25mg/kg of 4-ethyl DDC then 4 days of phenobarbitone (80mg/kg) and a final dose of 4-ethyl DDC (300mg/kg). Succinylacetone was administered throughout the treatment period.

2) 4-Ethyl DDC at a concentration of 100mg/kg was administered intraperitoneally for 14 days.

3) N-methyl protoporphyrin was administered via an indwelling jugular catheter at a daily dose of 1μmole for a period of 14 days.

4) N-methyl protoporphyrin was administered into the third ventricle at a total daily concentration of 50nmoles, for a period of 14 days, at a rate of 0.5µl/hr.

Animals were killed by a sharp blow to the head and bled except in the jugular and central cannulation group where the animal were killed by CO_2 asphyxiation.

5.2.5. MITOCHONDRIAL FUNCTION:

1) Tissue preparation:

a) Mitochondrial isolation medium:

the medium used for mitochondrial isolation contained:-

0.225M mannitol
0.07M sucrose
0.4mM EGTA
2mM MOPS, pH 7.2

b) Mitochondrial preparation:

The animals were killed and the brains and livers removed. The tissues were washed three times in ice cold isolation medium, minced finely with scissors, transfered to a glass homogenising tube and homogenised in 10 volumes of medium at 1000 r.p.m. with 8 passes of a Teflon pestle. The homogenate was centrifuged at 600g. for 10min to remove nuclei and cell debris. From here onwards the homogenising medium, described above, contained 1% BSA. The supernatant was decanted and spun at 6,500g. for 15min. on an MSE centrifuge to produce the P2 mitochondrial pellet.. The pellet was washed twice more with 7min. spins at 6,500g. intervening. The final mitochondrial pellet was resuspended by mild hand homogenisation in 2ml. of isolation medium.

2. ASSAYS:

a) Mitochondrial assay medium:

Mitochondrial function assay medium contained:-

0.22M mannitol
0.05M sucrose
10mM NaH_2PO_4
20mM MOPS, pH 7.2

Other assay reagents:

i) 1M glutamate/malate (sodium salts) dissolved in 0.05M Tris HCl and brought to pH 7.4.

ii) 100mM adenosine diphosphate (ADP) dissolved in 0.05M Tris buffer and brought to pH 6.8

iii) 5mg./ml. Bovine serum albumin (BSA) (fatty acid free).

b) equipment:

Oxygen electrode:

The oxygen electrode measures changes in oxygen concentration in a solution. The principle is that if a potential difference (0.6 volts) is applied from an external source across the platinum-Ag/KCl electrode system such that the platinum electrode is negative with respect to the silver electrode, a current will flow only if oxygen is present in the surrounding medium. Under these circumstances oxygen is electrolytically reduced to water at the surface of the platinum electrode and the current which flows is directly proportional to the concentration of oxygen in the solution.

Calibration of oxygen electrode:

Distiled water at 25°C contains 260µM/litre of dissolved oxygen (see Slater 1967).

1. 3ml. of distiled water, left to equilibrated with air at 25°C, was added to the reaction chamber of the oxygen electrode assembly and a few crystals of sodium dithionite added. The oxygen concentration in

the chamber quickly falls to zero and the output of the electrode falls to a position on the pen recorder chart which is set as zero oxygen concentration.

2. The electrode chamber was washed several times with distiled water to remove all of the sodium dithionite.

3. A 3ml. sample of the air equilibrated distiled water was added to the reaction chamber and the level reached on the chart corresponds to 260μM of oxygen. This calibration provides two known oxygen concentrations, 0 and 260μM from which other concentrations can be determined.

c) Measurement of Respiratory control ratios (RCR) and Phosphate:oxygen (P:O) ratios:

1. **P:O ratios** are a measure the efficiency of the phosphorylation capacity of the mitochondria calculated as the quantity of phosphate esterified per atom of oxygen consumed.

These ratios were calculated as the amount of ATP produced (measured indirectly as the amount of ADP consumed) divided by the quantity of oxygen consumed.

The assay buffer was equilibrated with air at 25^0C before the start of the experiment. 3.3ml. of assay buffer and 0.2ml. of 5mg./ml. BSA were added to the reaction chamber. A measured volume of between 0.2-0.6ml. of mitochondrial suspension was added before inserting the lid to seal the assembly. Subsequent additions to the chamber were made via the injection port using a Hamilton micro-syringe. Respiration was followed for approximately 2min. to obtain a baseline. 30μl. of 1M glutamate/malate solution was added and again, respiration was followed for a further 2 min. 10μl. of ADP was then added, which stimulated oxygen consumption. The rate of fall of the oxygen content was followed until the ADP was consumed and respiration returned to a low base rate. More ADP was added in 10

and 30µl aliquots, respectively, until the system became anaerobic (figure 74).

2. Respiratory control ratio is a measure of the control the product of oxidative phosphorylation, ATP, has on its own synthesis and is an index of efficiency of function of the ATP production machinery. When mitochondrial membranes are damaged two phenomena occur to alter the respiratory control ratio: 1. hydrogen ions leak across the damaged membrane depleting the proton potential across the membrane and thereby accelerating electron transfer to return the proton motive force; 2. Protons may be pumped from the matrix to the intra mitochondrial space by the hydrolysis of ATP. This last phenomenon is particularly important when damaged membranes are present in the mitochondrial preparations, as a proton motive force is required to drive ATP synthesis and in the absence of this driving force ATP is hydrolysed to ADP and phosphate. This means that ATP is no longer controlling respiration as it is being hydrolysed as fast as it is being produced.

RCRs were calculated as the rate of oxygen consumption in the presence of substrates, glutamate/malate and ADP (state 3 respiration) divided by the rate of oxygen consumption when all of the ADP is converted to ATP, but glutamate/malate is still present (state 4 respiration).

ANALYSIS OF RESULTS.

Where appropriate, results are expressed as the mean±standard error of the mean (S.E.M.) of n (number of observations). Student's t-test or Mann Whitney U test were used to test for significance between means. A p value of <0.05 was taken as the level of significance.

CHAPTER 5

THE EFFECTS OF PORPHYRINOGENIC DRUGS ON MITOCHONDRIAL FUNCTION.

RESULTS.

CHAPTER 5 PORPHYRINOGENIC TREATMENTS.

Succinylacetone injections were administered in a volume of 1ml/kg. in distilled water vehicle. Phenobarbitone was dissolved in 0.1M NaOH and brought to pH 9 with 0.1MHCl and was also administered in a volume of 1ml/kg. 4-ethyl DDC was administered in a volume of 0.5ml/kg. in DMSO. N-methyl protoporphyrin was dissolved in saline at pH 9.0 and brought back to a pH of 7.4 with 0.1M HCl. N-methyl protoporphyrin injections were administered intravenously via the jugular vein or via an indwelling intaventricular cannula.

TREATMENT 3.1: 30 days of succinylacetone (20mg/kg.) followed by 10 days administration of 4-ethyl DDC (25mg/kg.) then 4 days of phenobarbitone (80mg/kg.) with a further 300mg/kg of 4-ethyl DDC 24 hours prior to killing.

TREATMENT 3.2: 14 days of 4-ethyl DDC (100mg/kg.).

TREATMENT 3.3: 14 days of N-methyl protoporphyrin (1μMole) in a volume of 0.1ml) administered via thejugular vein by means of an indwelling catheter.

TREATMENT 3.4: 14 days of N-methyl protoporphyrin (50nMoles/day) into the 3rd. ventricle via an indwelling cannula attached to an osmotic mini-pump.

5.3.1. The effects of porphyrinogenic drug treatment 3.1 (30 days succinylacetone; 10 days 4-ethyl DDC; 4 days phenobarbitone; 1 large dose of 4-ethyl DDC) on mitochondrial respiratory chain function:

1. Liver mitochondria:

Intact liver mitochondria at a concentration of approximately 1.5mg/ml. (in the absence of glutamate/malate and ADP) did not consume any appreciable amount of oxygen (State 1 respiration) (figure 74). Nor on addition of 30µl of a 1M solution of glutamate/malate was there any appreciable increase in oxygen consumption. The further addition of 1 uMole of adenosine diphosphate (ADP), however, produced a marked stimulation of mitochondrial oxygen utilisation (State 3 respiration). After some time the increased rate of O_2 consumption slowed, almost, to that which existed before the addition of of ADP, presumably as a consequence of the consumption of the added ADP (State 4 respiration). One further addition of 1µMole of ADP and one of 3µMoles were possible before anaerobia was reached (figure 74).

a) The P:O ratio is measured from the amount of ADP consumed (in these experiments 1µMole) divided by the amount of oxygen consumed, in µatoms, from the time of ADP addition to the point where state 3 respiration ceases. In these experiments the P:O ratio of mitochondria isolated from animals that received porphyrinogenic drug treatment, 3.1, did not significantly differ from the P:O ratios of mitochondria isolated from control animals (figure 75a).

b) The Respiratory Control Ratio (RCR), (the ratio of state 3:state 4 respiration) of mitochondria isolated from the livers of animals that received this porphyrinogenic drug treatment were significantly lower than the RCR ratios of hepatic mitochondria from control animals ($p<0.05$) (figure 75b). This was manifest as a steeper state 4 respiration slope. Additionally, the oxygen consumption of the mitochondria prior to the addition of exogenous ADP was steeper than that of control mitochondria.

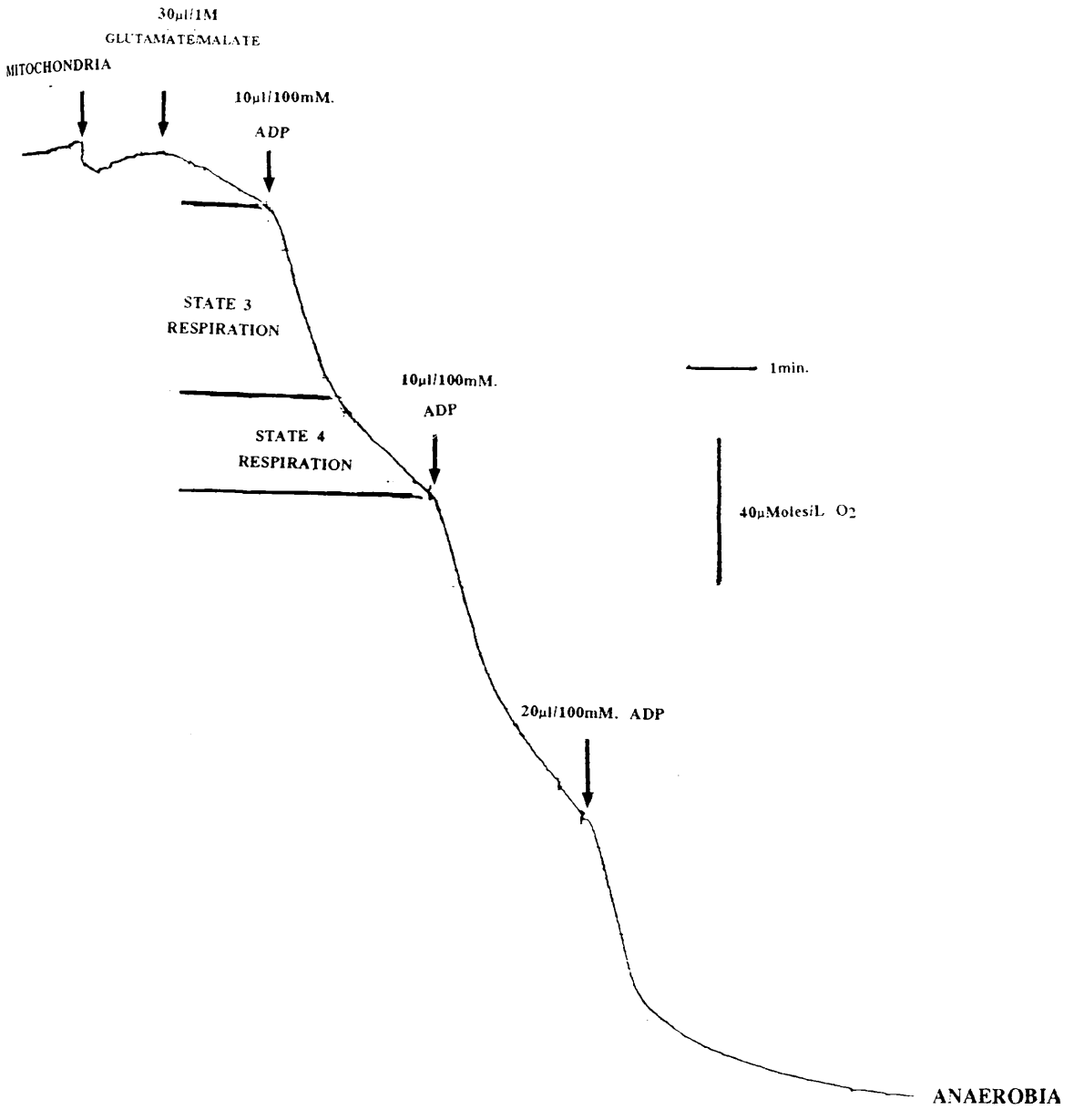
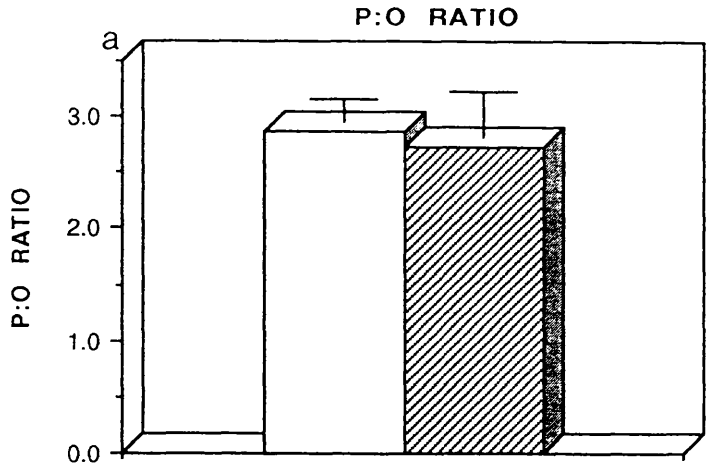
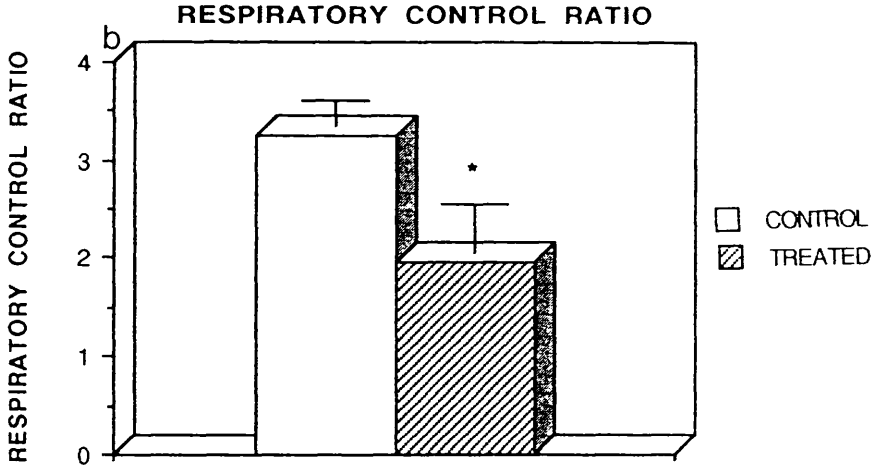


Figure 7 4 : Shows a trace of the ADP-stimulated oxygen consumption of isolated liver mitochondria with glutamate and malate as substrates, as measured by the Clarke electrode.

Figure 75: Shows 3 liver mitchondrial function parameters:
Graph a) shows the mean (+/- S.E.M.) P:O ratios (phosphate esterified /oxygen consumed ratios) from mitochondria isolated from control animal liver (n=5) and from liver mitochondria from animals that had received porphyrinogenic treatment 3.1 (30 days succinylacetone, 4-ethyl DDC, phenobarbitone, 4-ethyl DDC) (n=6). Graph b) shows the mean (+/- S.E.M.) respiratory control ratio (RCR, state 3 respiration/state 4 respiration) in mitochondria from control animal liver (n=5) and from mitochondria from treated animal liver (n=6). Graph c) shows the mean (+/- S.E.M.) oxygen consumption during ADP stimulation of respiration in control liver mitochondria (n=5) and in treated liver mitochondria (n=6). Respiratory Control Ratios in treated animals were significantly lower in treated animal liver mitochondrial than corresponding control liver mitochondria (*$p<0.05$).


P:O RATIO
a
P:O RATIO
3.0
2.0
1.0
0.0


RESPIRATORY CONTROL RATIO
b
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4
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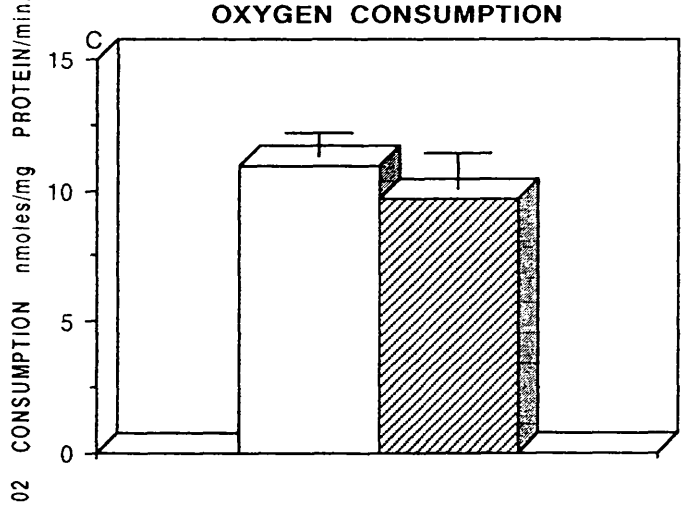

OXYGEN CONSUMPTION
c
02 CONSUMPTION nmoles/mg PROTEIN/min.
15
10
5
0

c) On addition of excess ADP, the oxygen consumption of hepatic mitochondria, of treated animals, did not differ significantly from the oxygen consumption of ADP-stimulated mitochondria from control animals (figure 75c).

2. Brain mitochondrial function:

The P:O ratio of brain mitochondria were similar to those of liver mitochondria. The RCR of brain mitochondria were, however, lower than those of liver. This is probably due to the fact that mitochondria from both tissues were isolated by a method which is normally used to isolate liver mitochondria. As can be seen from the electron micrographs of mitochondrial pellets, brain mitochondrial pellets contained more membrane contaminents than the corresponding hepatic mitochondrial pellet (plates 3, 4). Brain mitochondria isolated by the method of Lovtrup and Zelander (1962) contained less membrane contamination (plate 7). For practical reasons, however, mitochondria from both liver and brain were isolated by the liver method. Additionally, in these experiments the function of liver mitochondria was important as it was in these mitochondria that a cytochrome deficiency had been induced.

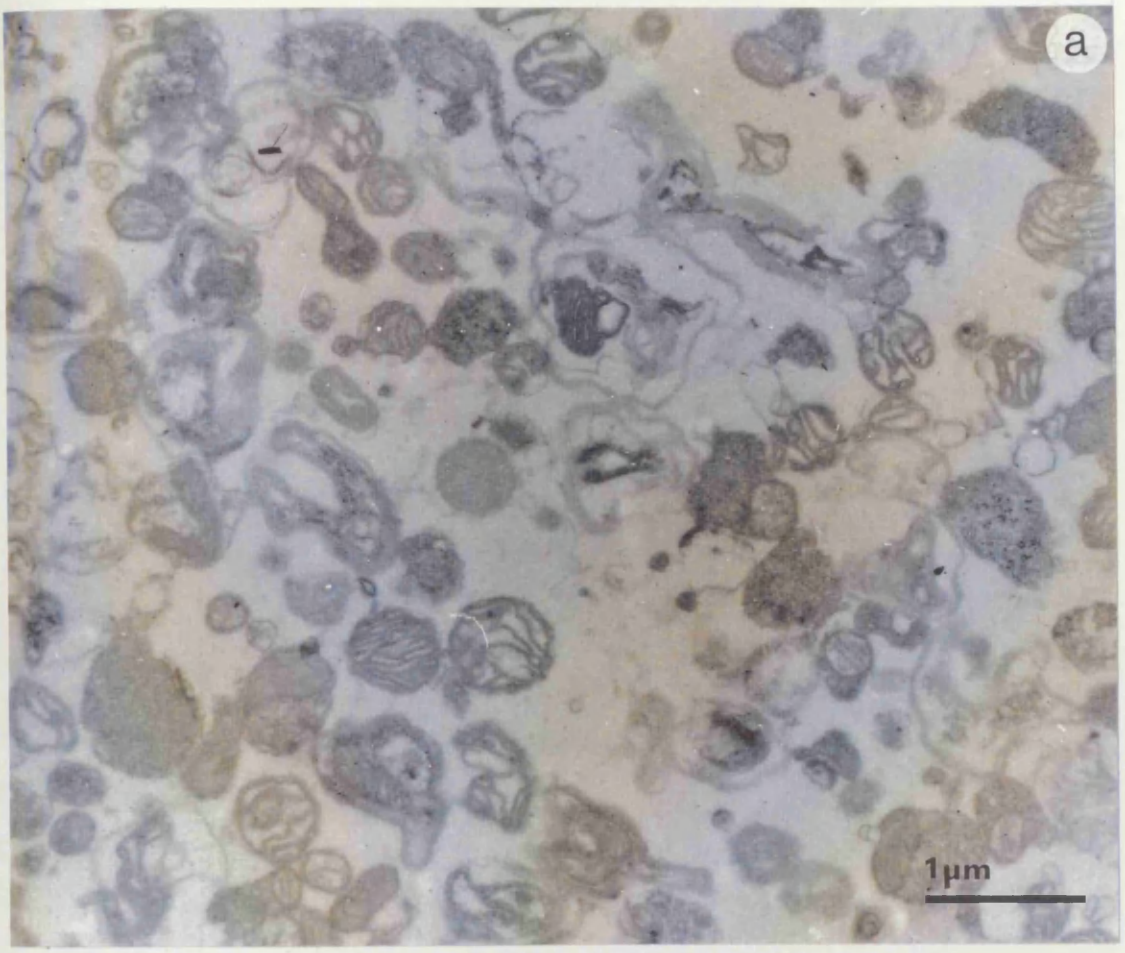
In brain mitochondria isolated from animals that had received this drug combination neither the P:O ratio, the RCR nor the oxygen consumption of stimulated mitochondria was significantly different form those of brain mitochondria isolated from control animals (figure 76).

5.3.2. The effects of porphyrinogenic drug treatment 3.2 (14 days 4-ethyl DDC (100mg/kg.)) on mitochondrial respiratory chain function:

1. Liver mitochondria:

a) The P:O ratio of mitochondria isolated from animals that received porphyrinogenic drug treatment 3.2 did not significantly differ from the P:O ratios of mitochondria isolated from control animals (figure 77a).

Plate 7: Shows electron micrographs of a) brain mitochondria isolated by the method employed in chapter 5 of this thesis, for measurement of mitochondrial function and b) brain mitochondria isolated by the method of Lovtrup and Zelander (1962), employed in chapter 4 of this study. A comparison of mitochondria prepared by the two methods shows that the method of Lovtrup and Zelander produced more intact mitochondria than the method employed in chapter 5 of this thesis.


a
1µm

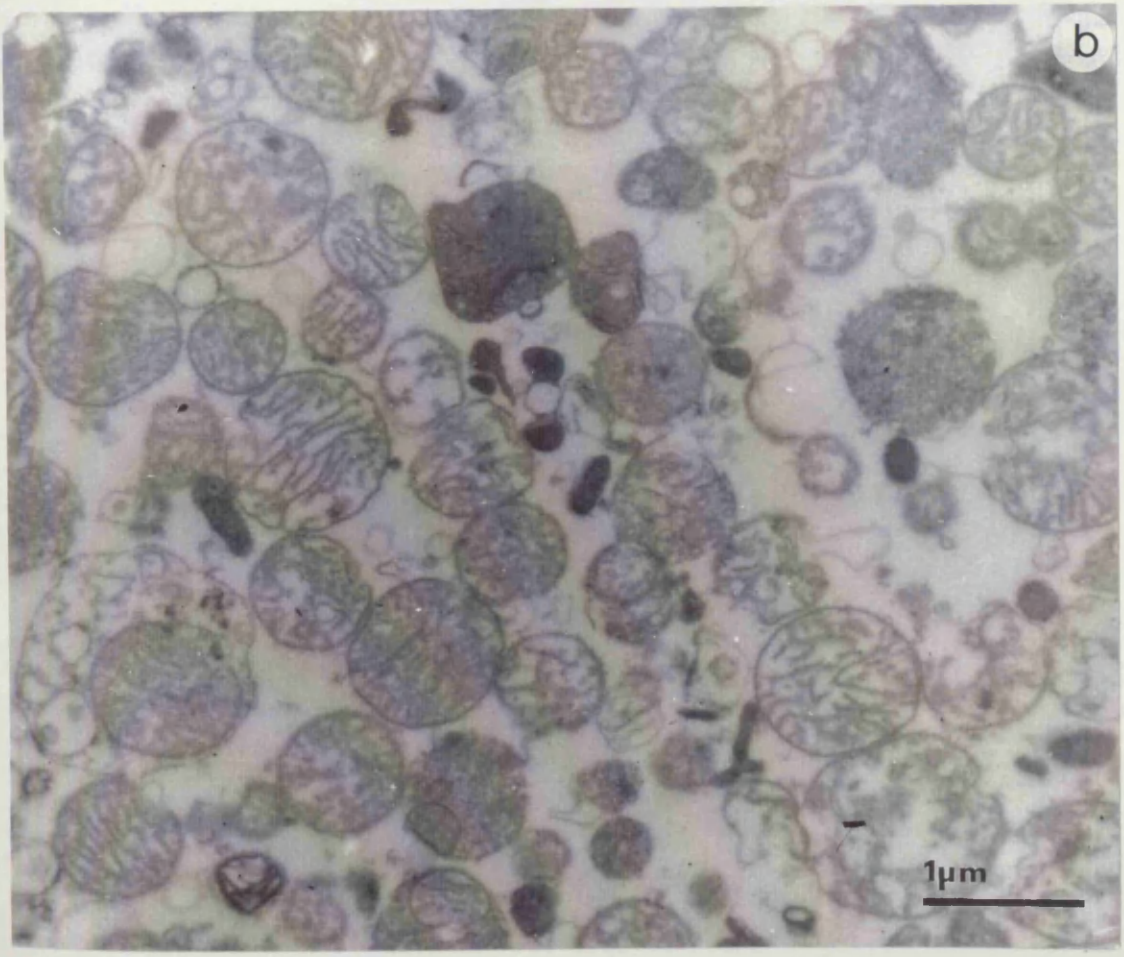

b
1µm

Figure 76: Shows 3 brain mitchondrial function parameters: Graph a) shows the mean +/- S.E.M. P:O ratios (phosphate esterified /oxygen consumed ratios) from mitochondria isolated from control animal brain (n=5) and from brain mitochondria from animals that had received porphyrinogenic treatment 3.1 (30 days succinylacetone, 4-ethyl DDC, phenobarbitone, 4-ethyl DDC) (n=6). Graph b) shows the mean +/- S.E.M. respiratory control ratio (RCR, state 3 respiration/state 4 respiration) in mitochondria from control animal brain (n=5) and from mitochondria from treated animal brain (n=6). Graph c) shows the mean +/- S.E.M. oxygen consumption during ATP stimulation of respiration in control brain mitochondria (n=5) and in treated brain mitochondria (n=6). There were no significant differences in the function of mitochondria from treated animals when compared to control brain mitochondria.

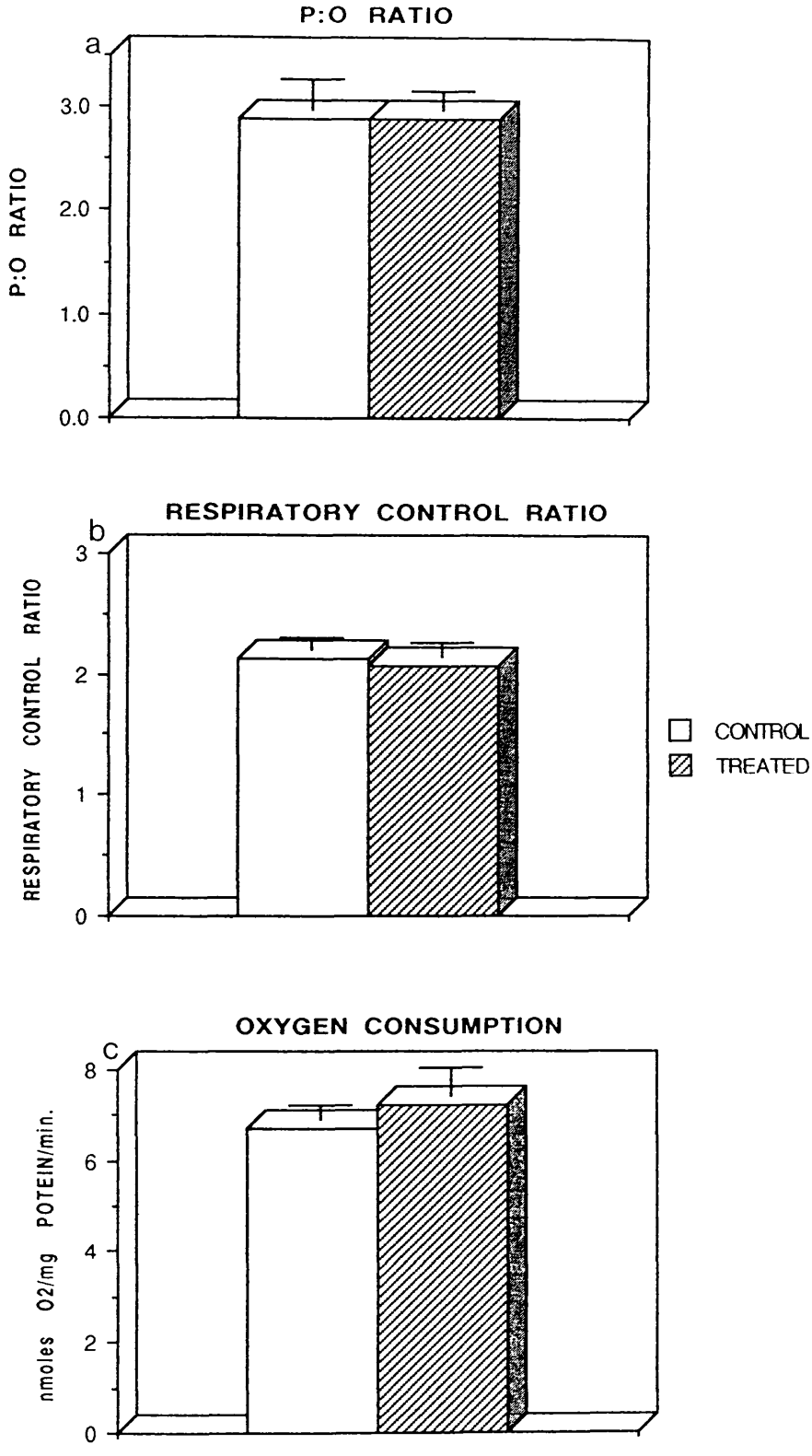
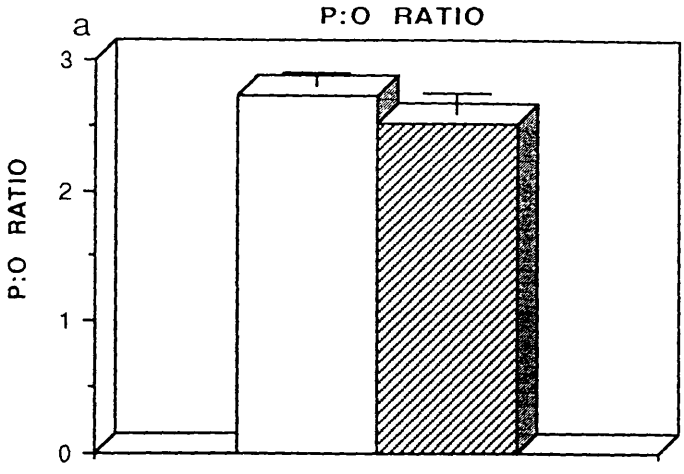
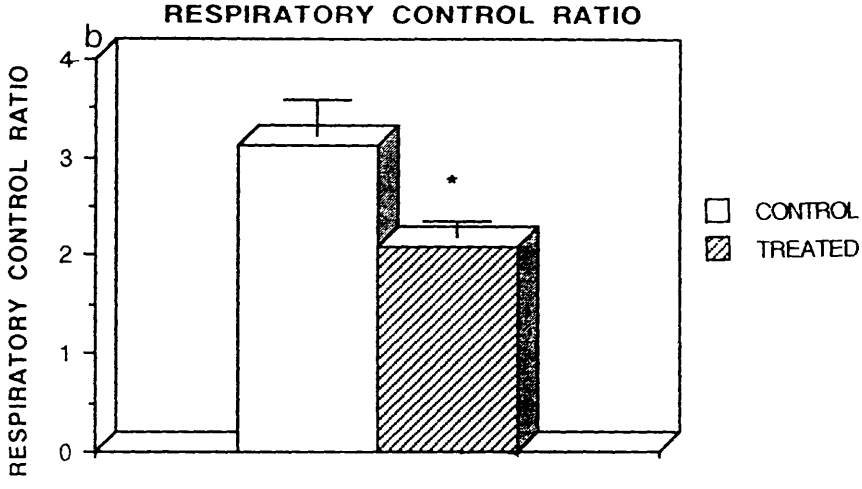
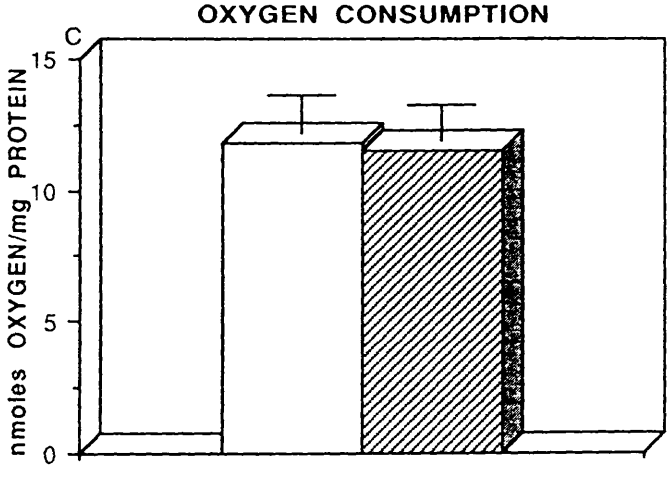

P:O RATIO
a
P:O RATIO
3.0
2.0
1.0
0.0
RESPIRATORY CONTROL RATIO
b
RESPIRATORY CONTROL RATIO
3
2
1
0
CONTROL
TREATED
OXYGEN CONSUMPTION
c
nmoles O2/mg POTEIN/min.
8
6
4
2
0

Figure 77: Shows 3 liver mitchondrial function parameters: Graph a) shows the mean (+/- S.E.M.) P:O ratios (phosphate esterified /oxygen consumed ratios) from mitochondria isolated from control animal liver (n=6) and from liver mitochondria from animals that had received porphyrinogenic treatment 3.2 (14 days 4-ethyl DDC) (n=6). Graph b) shows the mean (+/- S.E.M.) respiratory control ratio (RCR, state 3 respiration/state 4 respiration) in mitochondria from control animal liver (n=6) and from mitochondria from treated animal liver (n=6). Graph c) shows the mean (+/- S.E.M.) oxygen consumption during ATP stimulation of respiration in control liver mitochondria (n=6) and in treated liver mitochondria (n=6). Respiratory Control Ratios of treated animal hepatic mitochondria were significantly lower than the corresponding control animal liver mitochondria (*$p<0.05$).


a
P:O RATIO
P:O RATIO
3
2
1
0


b
RESPIRATORY CONTROL RATIO
RESPIRATORY CONTROL RATIO
4
3
2
1
0
*
CONTROL
TREATED


c
OXYGEN CONSUMPTION
nmoles OXYGEN/mg PROTEIN
15
10
5
0

b) The RCR ratios of hepatic mitochondria from treated animals were significantly lower than those of control animal liver mitochondria ($p<0.05$) (figure 77b). This was manifest as a steeper state 4 respiration slope. The oxygen consumption of the mitochondria prior to the addition of exogenous ADP was also steeper than that of control mitochondria.

c) The oxygen consumption of hepatic mitochondria, on addition of excess ADP, of treated animals did not differ significantly from the oxygen consumption of ADP-stimulated mitochondria from control animals (figure 77c).

2. Brain mitochondrial function:

In animals that had received this drug combination the P:O ratio was not significantly different form those of brain mitochondria isolated from control animals (figure 78a). Although both the RCR and the oxygen consumption of these mitochondria were reduced in mitochondria from treated animals these did not differ significantly from control brain mitochondria (figures 78b, 78c).

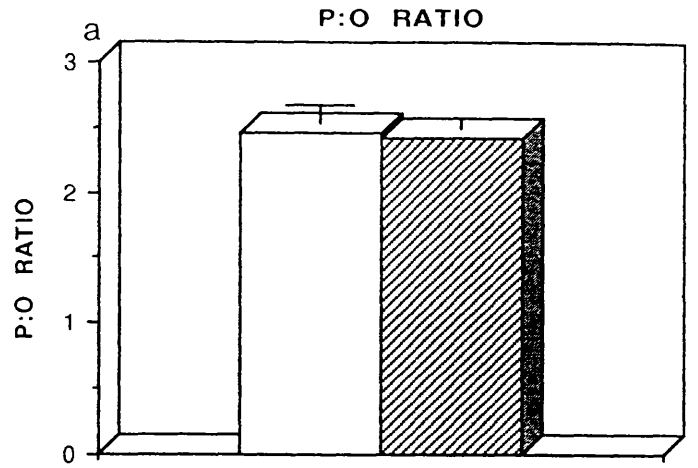
5.3.3. The effects of porphyrinogenic drug treatment 3.3 (14 days of N-methyl protoporphyrin, 1μM/day) administered via the jugular vein) on mitochondrial respiratory chain function:

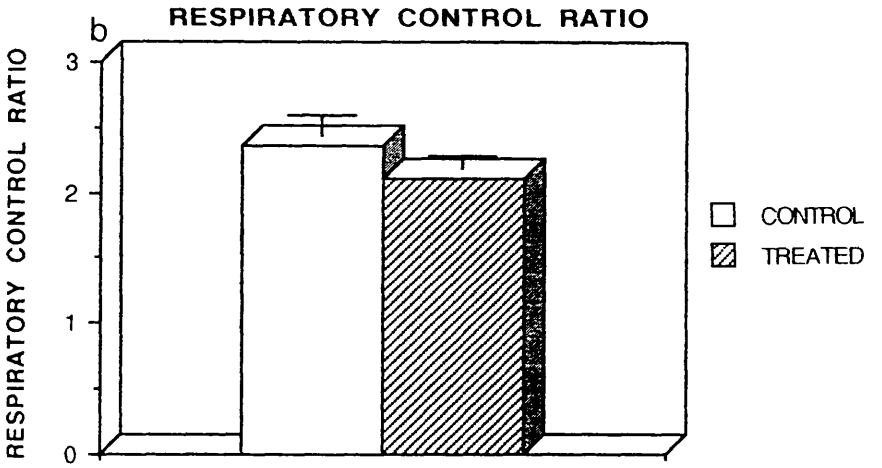
The indwelling jugular cannula in this group of treated animals remained patent for the duration of the experiment and allowed intravenous administration of the N-methyl protoporphyrin over the 14 days of treatment. The livers from these animals were not as pigmented as those receiving 4-ethyl DDC treatment. The mitochondrial pellet was, however, a very dark green colour, similar to that seen in the livers of animals treated with 4-ethyl DDC.

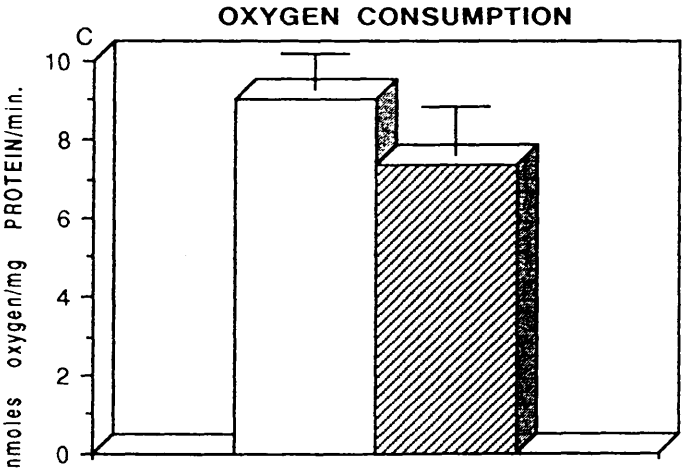
1. Liver mitochondria:

a) The P:O ratio of mitochondria isolated from animals that received porphyrinogenic drug treatment 3.3 did not significantly differ from

Figure 78: Shows 3 brain mitchondrial function parameters: Graph a) shows the mean (+/- S.E.M.) P:O ratios (phosphate esterified /oxygen consumed ratios) from mitochondria isolated from control animal brain (n=6) and from brain mitochondria from animals that had received porphyrinogenic treatment 3.2 (n=6). Graph b) shows the mean (+/- S.E.M.) respiratory control ratio (RCR, state 3 respiration/state 4 respiration) in mitochondria from control animal brain (n=6) and from mitochondria from treated animal brain (n=6). Graph c) shows the mean (+/- S.E.M.) oxygen consumption during ATP stimulation of respiration in control brain mitochondria (n=6) and in treated brain mitochondria (n=6). There were no significant changes in any brain mitochondrial functional parameters as a result of this porphyrinogenic drug treatment.


a
P:O RATIO
P:O RATIO
3
2
1
0


b
RESPIRATORY CONTROL RATIO
RESPIRATORY CONTROL RATIO
3
2
1
0
CONTROL
TREATED


c
OXYGEN CONSUMPTION
nmoles oxygen/mg PROTEIN/min.
10
8
6
4
2
0

the P:O ratios of mitochondria isolated from control animals (figure 79a).

b) Surprisingly, however, the RCR ratios of hepatic mitochondria from treated animals were not significantly different from those of control animal liver mitochondria (figure 79b). This outcome was, therefore, not in keeping with the actions of 4-ethyl DDC on the RCR of hepatic mitochondria, effects proposed to be mediated via the actions of N-ethyl protoporphyrin, an analogue of N-methyl protoporphyrin.

c) The oxygen consumption of hepatic mitochondria, on addition of excess ADP, of treated animals was lower than the oxygen consumption of ADP-stimulated mitochondria from control animals (figure 79c). However, this did not reach significance.

2. Brain mitochondrial function:

In animals that had received this drug combination, neither the P:O ratio, the RCR nor the oxygen consumption of stimulated mitochondria from brain tissue was significantly different form these parameters in brain mitochondria isolated from control animals (figure 80).

5.3.4. The effects of central porphyrinogenic drug treatment 3.4 (14 days N-methyl protoporphyrin (50nM/day) administered into the third ventricle) on mitochondrial respiratory chain function:

The animals recovered from surgery within 30min. of anaesthetic inducement with no adverse behavioural manifestations except an aversion to being handled which was also the case with control animals. By the end of the treatment period the head wound had healed. Central administration of N-methyl protoporphyrin and saline vehicle was successful in all cases. Although some N-methyl protoporphyrin precipitated out in the ventricle, the compound was widely taken up by the brain as shown by fluorescence histochemistry. Protoporphyrin fluorescence can be identified in the paraventricular tissue (plate 8).

Figure 79: Shows 3 liver mitochondrial function parameters: Graph a) shows the mean (+/- S.E.M.) P:O ratios (phosphate esterified /oxygen consumed ratios) from mitochondria isolated from control animal liver (n=5) and from liver mitochondria from animals that had received porphyrinogenic treatment 3.3 (14 days i.v. N-methyl protoporphyrin) (n=5). Graph b) shows the mean +/- S.E.M. respiratory control ratio (RCR, state 3 respiration/state 4 respiration) in mitochondria from control animal liver (n=5) and from mitochondria from treated animal liver (n=5). Graph c) shows the mean +/- S.E.M. oxygen consumption during ATP stimulation of respiration in control liver mitochondria (n=5) and in treated liver mitochondria (n=5). Although the oxygen consumption of treated lever mitochondria was reduced when compared to control values this did not reach statistical significance.

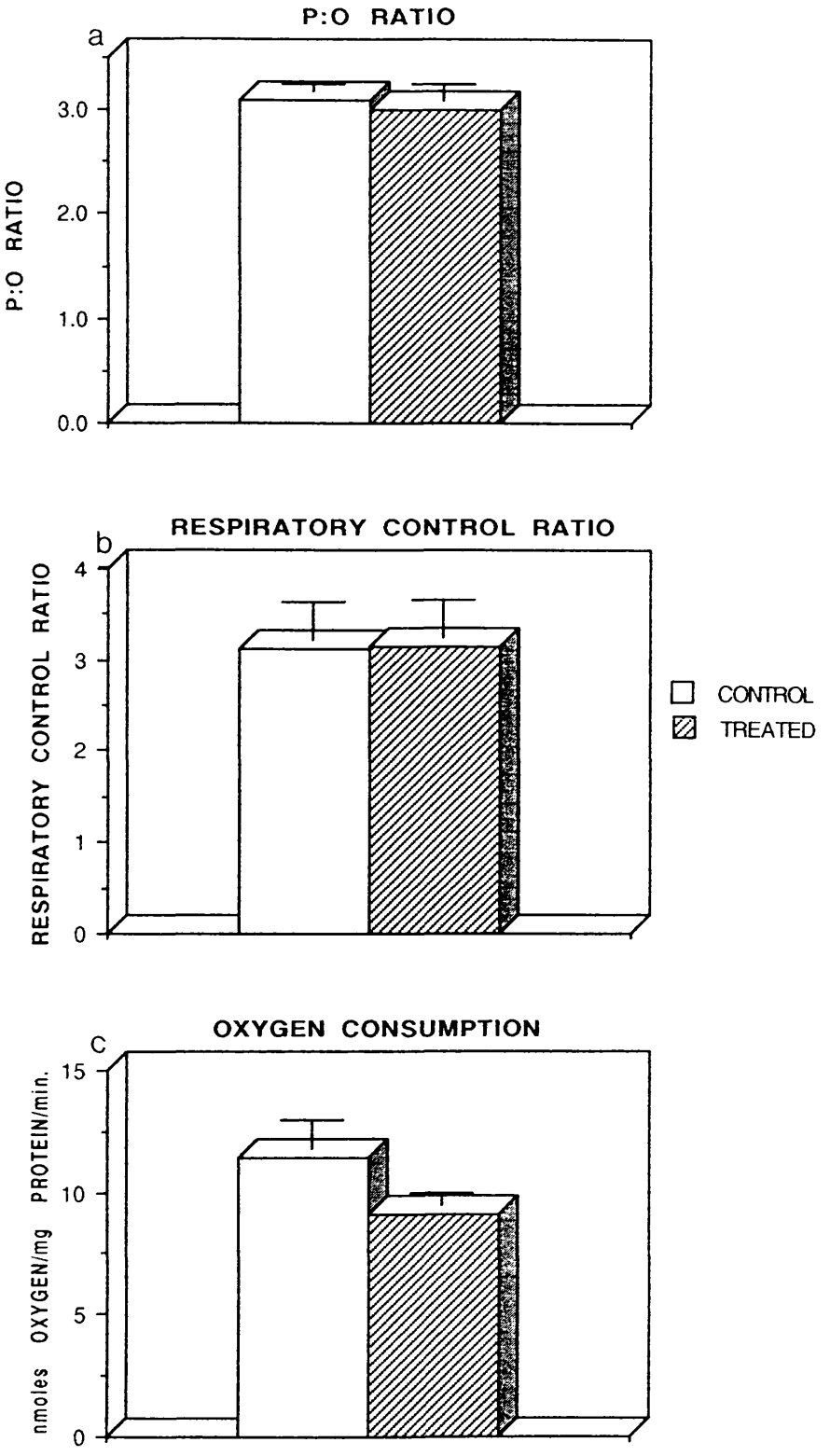

P:O RATIO
a
P:O RATIO
3.0
2.0
1.0
0.0
RESPIRATORY CONTROL RATIO
b
RESPIRATORY CONTROL RATIO
4
3
2
1
0
CONTROL
TREATED
OXYGEN CONSUMPTION
c
nmoles OXYGEN/mg PROTEIN/min.
15
10
5
0

Figure 80: Shows 3 brain mitchondrial function parameters:
Graph a) shows the mean (+/- S.E.M). P:O ratios (phosphate esterified /oxygen consumed ratios) from mitochondria isolated from control animal brain (n=5) and from brain mitochondria from animals that had received porphyrinogenic treatment 3.3 (14 days i.v. N-methyl protoporphyrin) (n=5). Graph b) shows the mean (+/- S.E.M.) respiratory control ratio (RCR, state 3 respiration/state 4 respiration) in mitochondria from control animal brain (n=5) and from mitochondria from treated animal brain (n=5). Graph c) shows the mean (+/- S.E.M.) oxygen consumption during ATP stimulation of respiration in control brain mitochondria (n=5) and in treated brain mitochondria (n=5). There were no significant changes in any brain mitochondrial function parameter measured.

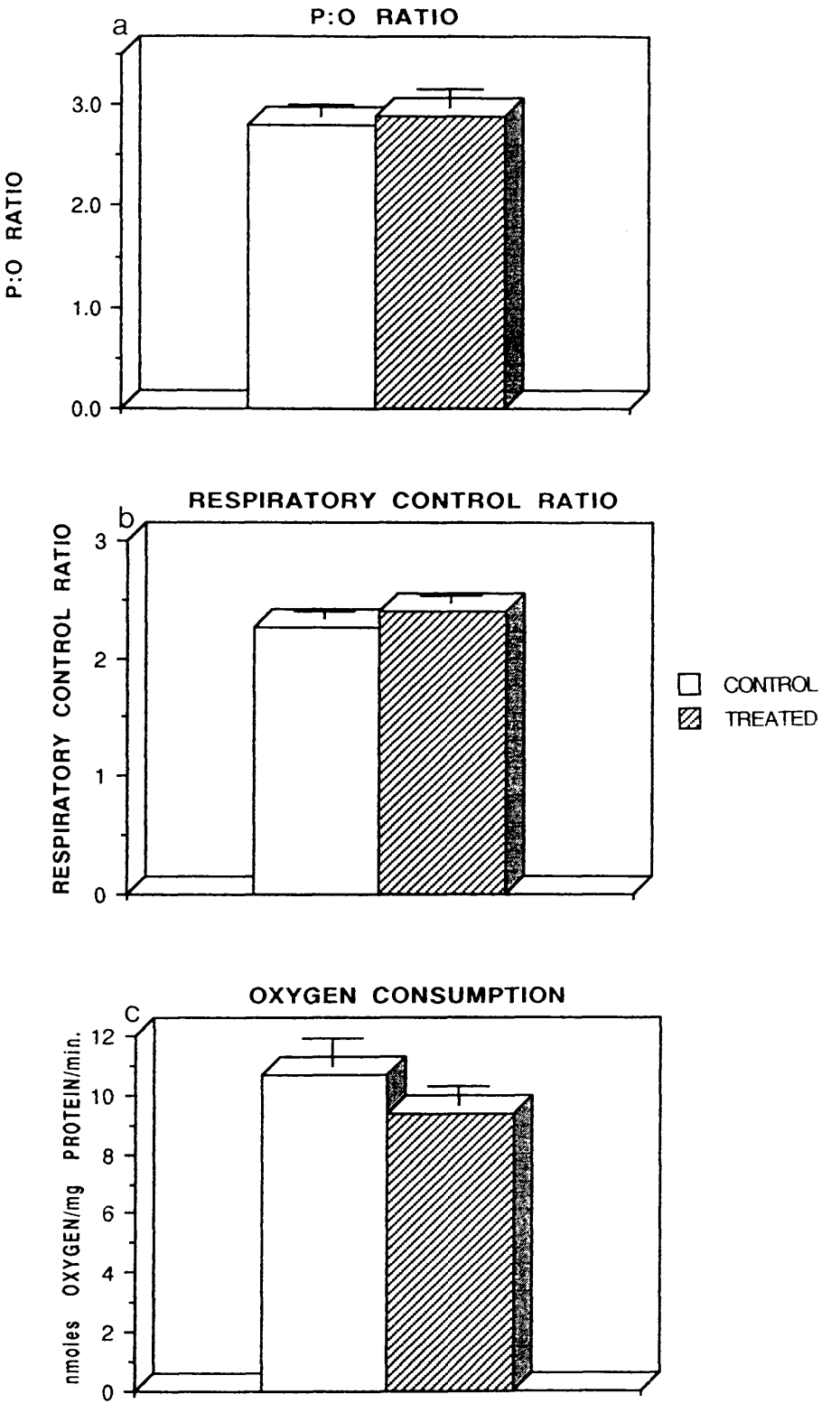
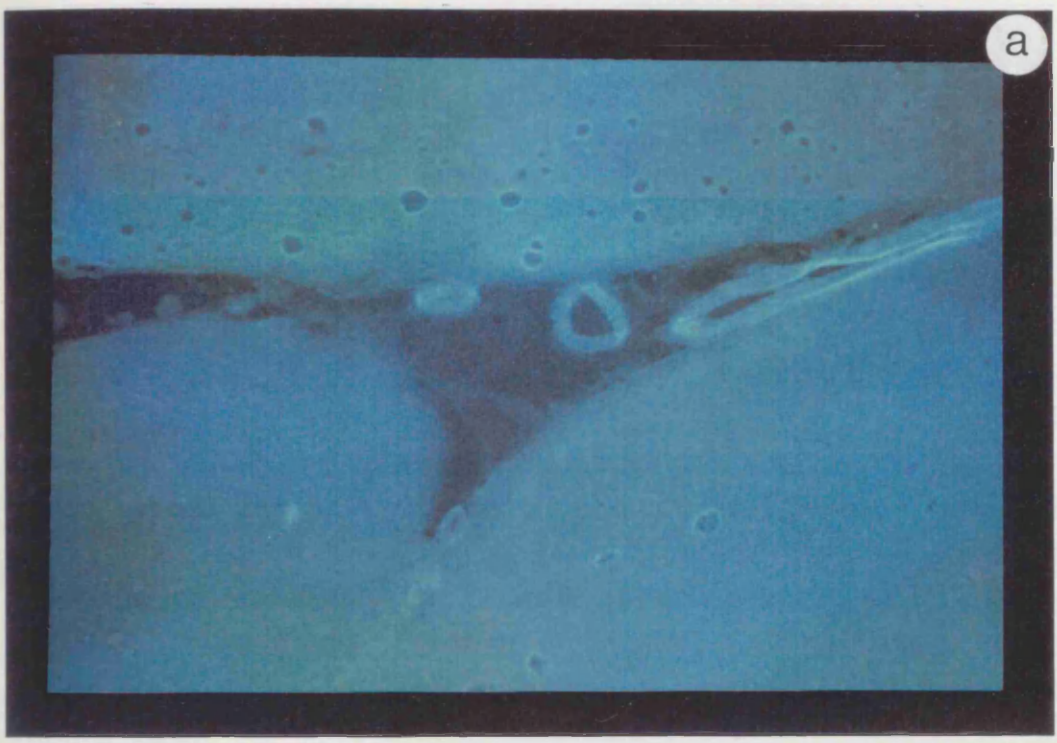
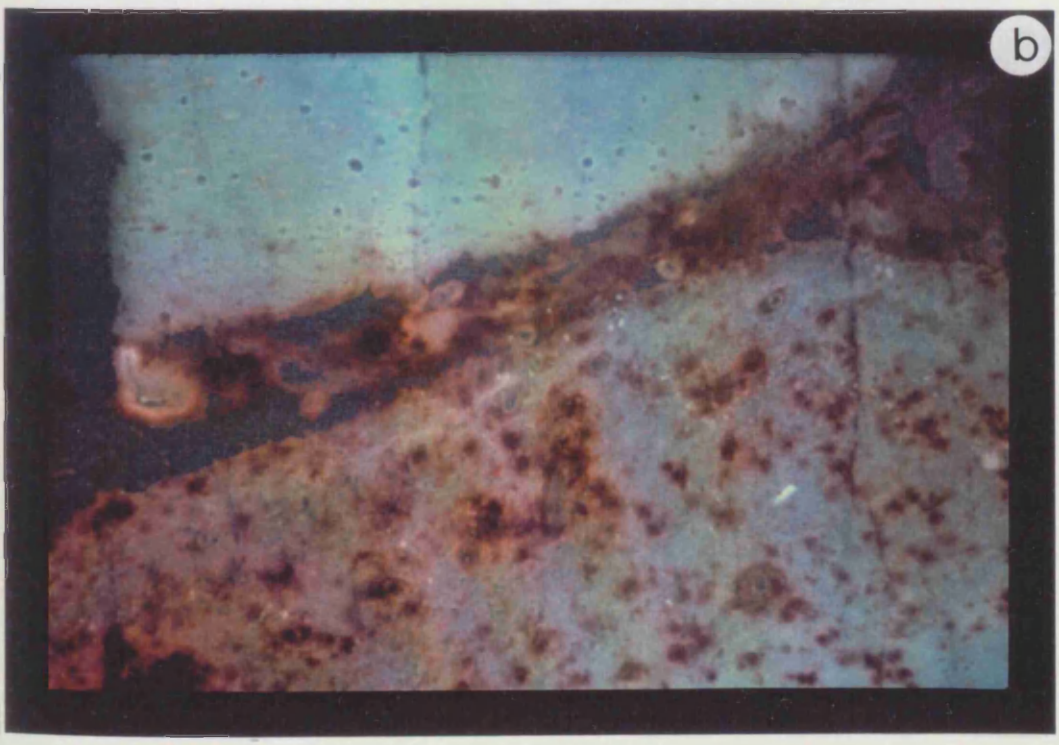

a
P:O RATIO
P:O RATIO
3.0
2.0
1.0
0.0
b
RESPIRATORY CONTROL RATIO
RESPIRATORY CONTROL RATIO
3
2
1
0
CONTROL
TREATED
c
OXYGEN CONSUMPTION
nmoles OXYGEN/mg PROTEIN/min.
12
10
8
6
4
2
0

Plate 8: Fluorescence photographs of histological sections of a) a control brain (x 220 magnification) and b) a brain from a rat, following intraventricular infusion of N-methyl protoporphyrin (x440 magnification). Although the alkylated protoporphyrin precipitated out in the ventricle, photograph b) shows that the alkylated porphyrin had penetrated into the neural tissue.


a


b

1. Liver mitochondria:

a) Neither the P:O ratio, RCR nor oxygen consumption of hepatic of mitochondria isolated from animals that received porphyrinogenic drug treatment 3.4 differed significantly from these parameters in mitochondria isolated from control animals (figure 81).

2. Brain mitochondrial function:

In animals that had received this drug combination neither the P:O ratio nor the oxygen consumption of stimulated mitochondria isolated from brain tissue was significantly different form those of brain mitochondria isolated from control animals (figures 82a, 82c). The RCR of brain mitochondria from treated animals were, however, significantly lower than the RCR's of control brain mitochondria (figure 82b).

Figure 81: Shows 3 liver mitchondrial function parameters:
Graph a) shows the mean (+/- S.E.M.) P:O ratios (phosphate esterified /oxygen consumed ratios) from mitochondria isolated from control animal liver (n=6) and from liver mitochondria from animals that had received porphyrinogenic treatment 3.4 (14 days centrally-administered N-methyl protoporphyrin) (n=6). Graph b) shows the mean (+/- S.E.M.) respiratory control ratio (RCR, state 3 respiration/state 4 respiration) in mitochondria from control animal liver (n=6) and from mitochondria from treated animal liver (n=6). Graph c) shows the mean +/- S.E.M. oxygen consumption during ATP stimulation of respiration in control liver mitochondria (n=6) and in treated liver mitochondria (n=6). This porphyrinogenic treatment caused no significant changes in hepatic mitochondrial function.

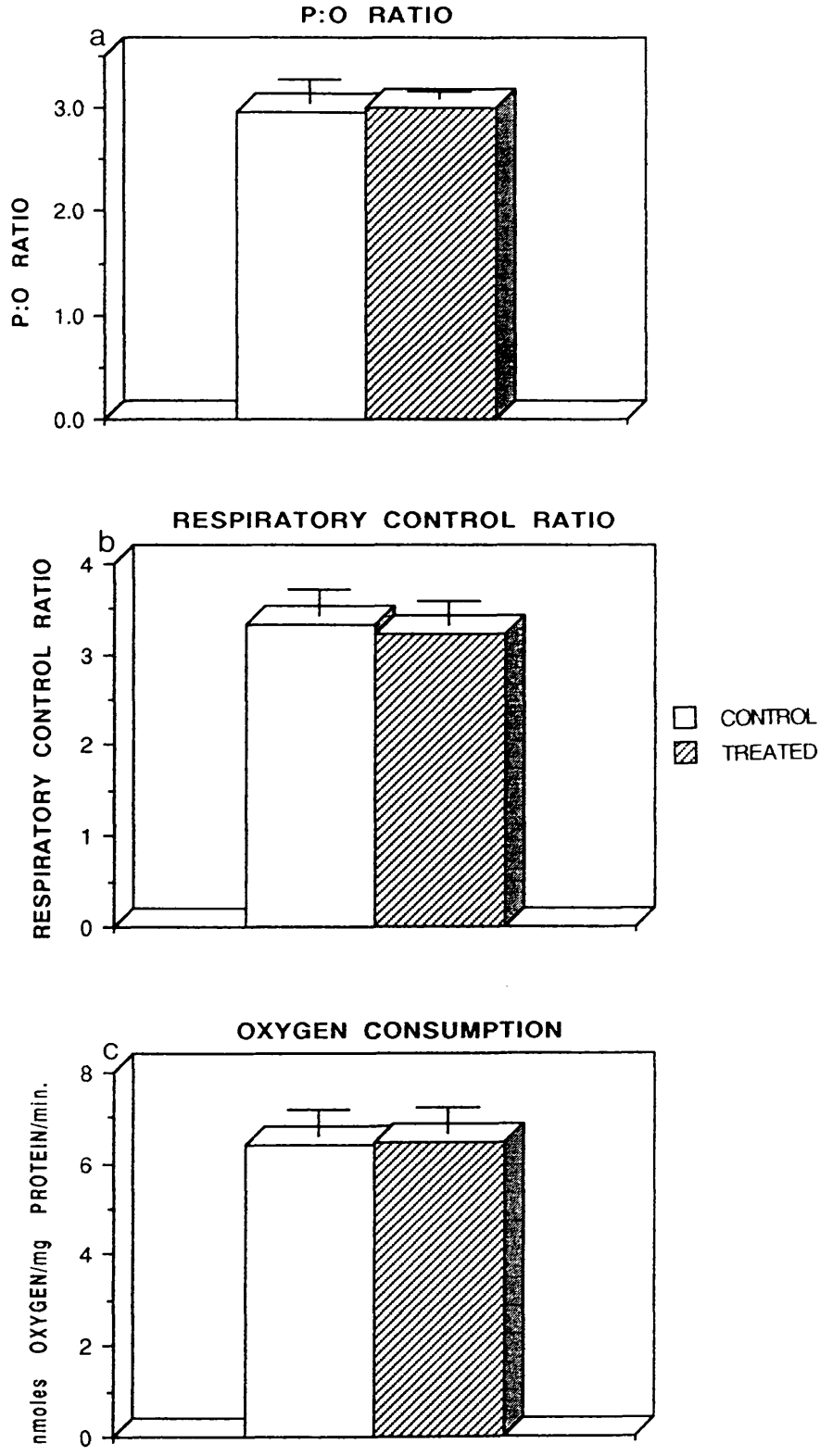
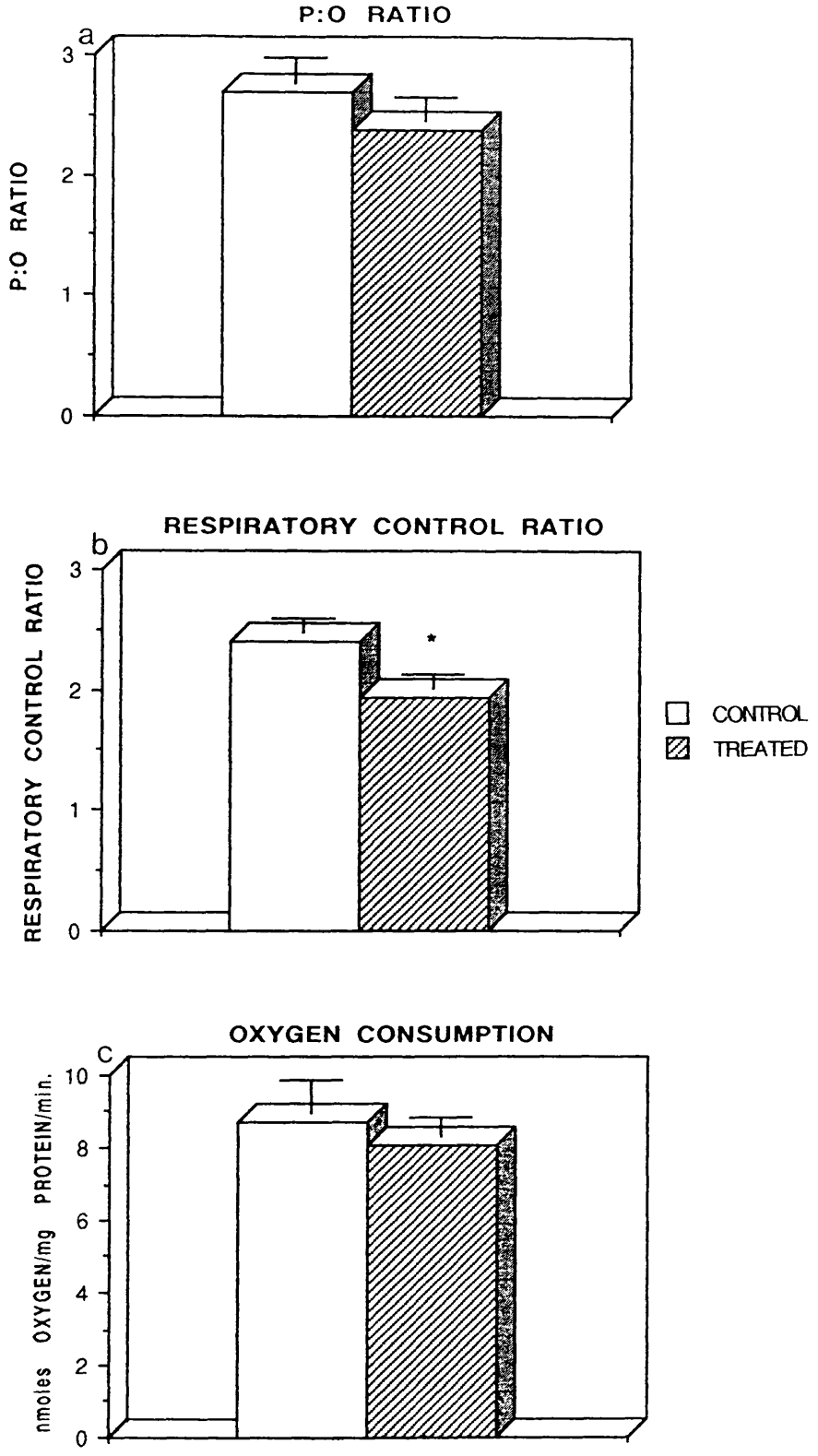

P:O RATIO
a
P:O RATIO
3.0
2.0
1.0
0.0
RESPIRATORY CONTROL RATIO
b
RESPIRATORY CONTROL RATIO
4
3
2
1
0
CONTROL
TREATED
OXYGEN CONSUMPTION
c
nmoles OXYGEN/mg PROTEIN/min.
8
6
4
2
0

Figure 82: Shows 3 brain mitchondrial function parameters:
Graph a) shows the mean (+/- S.E.M.) P:O ratios (phosphate esterified /oxygen consumed ratios) from mitochondria isolated from control animal brain (n=6) and from brain mitochondria from animals that had received porphyrinogenic treatment 3.4 (14 days centrally-administered N-methyl protoporphyrin (n=6). Graph b) shows the mean (+/- S.E.M.) respiratory control ratio (RCR, state 3 respiration/state 4 respiration) in mitochondria from control animal brain (n=6) and from mitochondria from treated animal brain (n=6). Graph c) shows the mean (+/- S.E.M.) oxygen consumption during ATP stimulation of respiration in control brain mitochondria (n=6) and in treated brain mitochondria (n=6). Central administration of N-methyl protoporphyrin caused a significant fall in the Respiratory Control Ratios of brain mitochondria (*$p<0.05$)


P:O RATIO
a
P:O RATIO
3
2
1
0
RESPIRATORY CONTROL RATIO
b
RESPIRATORY CONTROL RATIO
3
2
1
0
*
CONTROL
TREATED
OXYGEN CONSUMPTION
c
nmoles OXYGEN/mg PROTEIN/min.
10
8
6
4
2
0

CHAPTER 5

THE EFFECTS OF PORPHYRINOGENIC DRUGS ON MITOCHONDRIAL FUNCTION.

DISCUSSION.

In the group of diseases classified under the rubric of mitochondrial encephalomyopathies, decreases in the electron transfer components, not surprisingly, lead to altered mitochondrial function with, as a result, major clinical changes such as, neuropathy and muscle weakness. Oxidative phosphorylation is the most important function of mitochondria and probably one of the most vital bodily processes. Oxidative phosphorylation is the process by which the chemical energy entrapped in food is transformed into the high energy compound, ATP, which is then utilised in a myriad of energy requiring functions. The three main parameters used to assess mitochondrial function are Phosphate:Oxygen ratio (P:O ratio), the Respiratory Control Ratio (RCR) and the rate of oxygen consumption during substrate stimulated respiration. The P:O ratio is a measure of the phosphorylating efficiency of the mitochondria and is the quantification of the amount of ATP produced per unit of oxygen consumed. Different substances enter the electron transfer chain at different sites and their oxidation leads to a different but, normally, constant phosphorylation ratio. Some compounds by-pass hydrogen pumping sites and, therefore, have a lower phosphorylating potential even when the oxygen required for their oxidation is the same as that for compounds which enter the process further downstream (figure 73). Normal P:O ratios for glutamate, malate and pyruvate are 3, for succinate 2 and for ascorbate in the presence of an electron shuttle 1. The respiratory control ratio is a measure of the efficiency of the mitochondria at conserving energy at periods when the demand for ATP is low. This parameter is a measure of the rate of oxygen consumption during ADP stimulated oxidative phosphorylation, divided by the rate of oxygen consumption in the absence of ADP and therefore during the absence of ATP formation. When the RCR is low, mitochondria are said to be "uncoupled". Under this uncoupled situation the energy released by the oxidation of substrates in the electron transport chain is dissipated as heat. The rate of oxygen consumption of mitochondria during substrate stimulation is a measure of electron transfer chain efficiency.

Defects have been found in Complex 1 , Complex 111 and Complex 1V in mitochondria of patients suffering from mitochondrial encephalomyopathies (Petty et. al. 1986). The degree of deficit in

these complexes vary as do the severity of symptoms. In a patient with the encephalomyopathy, Menke's disease, there was an almost total lack of spectrographically measured cytochrome oxidase in brain, liver and muscle tissue. This severe form of cytochrome deficiency results in early death. The site of defect in less severe cases can also be detected using mitochondrial function tests in conjunction with spectrographical evidence. Hayes et.al. in 1984 examined the functional properties of mitochondria isolated from a muscle biopsy from a patient diagnosed as encephalomyopathic. The oxygen consumption of glutamate/malate-stimulated mitochondrial respiration was 18% of control values. By employing the artificial electron acceptor tetramethyl-p-phenylene diamine (TMPD) to shunt reducing equivalents from Complex 1 or Complex 11, by-passing Complex 111, to Complex 1V it was shown that the level of defect lay at Complex 111. Oxidised-reduced difference spectra confirmed a lack of cytochromes c_1 and b, although some cytochrome b could be measured independently by actinimycin A reduction. A decrease of approximately 55% in cytochrome b content caused a fall of 30% in both state 3 and state 4 respiration, in glutamate/malate-stimulated skeletal muscle mitochondria, from an encephalomyopathic patient examined by Morgan-Hughes and his colleagues in 1977. A reduction in respiratory cytochromes in the electron transfer chain, therefore, results in a decrease in mitochondrial performance. A loss of tight respiratory control was also seen in the mitochondria of patients when stimulated by glutamate/malate (Hayes et.al. 1984; Morgan-Hughes et.al. 1982). A dual detrimental effect on energy metabolism is, therefore, at play in these circumstances. There is the initial defect in electron chain function due to the reduction in the respiratory cytochrome content. A further inefficiency in energy conservation will result from the dissipation of the inherent substrate energy as heat rather than being channelled into ATP production. Uncoupling of muscle mitochondria was reported by Luft et.al. (1962) in a case of hypermetabolism. This patient had a history of tiredess, muscle weakness, high blood pressure and heart rate and excessive sweating. Muscle mitochondria from the patient were almost totally uncoupled. There are striking similarities in the symptoms of this patient (increased cardiovascular parameters, tiredness, muscle weakness, excessive sweating) to some of the

symptoms manifest by porphyric patients (increased cardiovascular parameters, tiredness, muscle weakness, excessive sweating) (Goldberg et.al. 1959). The examination of mitochondrial function as a possible site of physiological defect in the diseases of acute porphyria would provide valuable information relevant to the aetiology of the clinical symptoms of the disorder.

The treatments employed in the experiments, described in chapter 4 of this thesis, reduced the respiratory cytochromes in hepatic mitochondria. The present group of experiments examined whether this reduction, in electron transport chain components, is accompanied by a defect in the function of these mitochondria. Brain mitochondrial cytochromes were unaltered by all treatments regimes, where drugs were administered systemically, although these cytochromes were reduced in hepatic tissue (chapter 4). The experiments in this chapter were directed at a somewhat different question. Were the cytochromes, whether reduced or not, continuing to function normally? If they were not, particularly if there were a deficit in brain mitochondrial function, this might provide the underlying basis for a neuropathy similar to that of the mitochondrial encephalomyopathies. A particularly important subgroup in the present experiments was group 4, in which the blood brain barrier was circumvented by delivery of N-methyl protoporphyrin directly into the 3rd. ventricle.

Neither in liver nor brain, with any of the treatment groups was there any significant change in either the rate of oxygen consumption or the P:O ratios when treated tissue were compared to control tissue processed at the same time and this, in spite of the fact that treatments (3.1; 3.2; 3.3) are capable of reducing the absolute levels of cytochromes in the liver if not the brain (chapter 4 results). The results of the experiments carried out in this chapter suggest that the P:O ratio is dependent on the ability of the remaining cytochromes to operate the proton pump and the gradient so created to drive ATP synthesis. The fall in cytochrome levels would reduce the rate at which ADP is consumed and also the rate of consumption of oxygen so that the P:O ratio remains constant. Consistent with such an interpretation, although the levels do not reach statistical significance, is the depression of oxygen consumption in all groups,

except where N-methyl protoporphyrin was administered centrally. De Matteis et.al. (1963) failed to show any reduction in hepatic ATP content, following DDC administration, indicating that DDC does not significantly alter the quantity of ATP formed. The one significant change, in the results reported here, is the decline in the RCR of liver mitochondria, following treatments which included 4-ethyl DDC administration and in brain tissue following central administration of N-methyl protoporphyrin. As there is no evidence of a malfunction in electron chain function, in the present studies (normal P:O ratios), it is unlikely that the uncoupling of these mitochondria is related to the change in cytochrome content. Ferrochelatase is inhibited by 4-ethyl DDC and as a result the haem precursor protoporphyrin 1X accumulates (Marks et.al.1987). The alkylated protoporphyrin metabolite of 4-ethyl DDC also accumulates in hepatic tissue following treatment. Protoporphyrin 1X has been implicated as the uncoupling agent in hepatic mitochondria isolated from griseofulvin (a compound with similar action to that of DDC) treated animals (Sandberg and Romslo 1981). Porphyrins are lipophilic compounds and Smith (1990) suggests that, in high concentrations, these compounds can penetrate membranes and could explain the uncoupling phenomenon. Intravenously-administered N-methyl protoporphyrin however, did not result in this loss of RCR in hepatic mitochondria suggesting that N-methyl protoporphyrin and protoporphyrin were not the main cause of the uncoupling, observed in the first two treatments in this section. Although uncoupling was evident in skeletal muscle mitochondria of encephalomyopathic patients (Hayes et.al. 1984; Morgan-Hughes et.al. 1982), there is no evidence to suggest that this uncoupling has a direct causal link with cytochrome deficiency. In porphyric attacks and as a consequence of the porphyrinogenic treatments, employed in this thesis, ALA is overproduced. Hermes-Lima et.al. (1991) have shown that this haem precursor can also cause uncoupling of mitochondria. These authors suggest that reactive oxygen species, O_2^-, H_2O_2 and or OH., produced during autooxidation of ALA lead to disruption of the mitochondrial inner membrane potential causing uncoupling. Support for this theory comes from their observation that this uncoupling effect of ALA was abolished by the addition of catalase and superoxide dismutase. If uncoupling of neuronal mitochondria is at least a contributory factor

in the aetiology of acute porphyric neuropathy, ALA-mediated uncoupling would be of greater importance than protoporphyrin-mediated uncoupling, as protoporphyrin is not an overproduced precursor in the acute porphyrias. Additionally, in the non-acute porphyrias the main sites of porphyrin accumulation is in the liver, erythrocytes and skin and there is possibly no excess porphyrin in neural tissue. However, if ALA has a toxic action on mitochondria, this effect is not manifest as a functional deficit in in vitro nerve/muscle preparations as the results of chapter 2 and 3 experiments failed to show any ALA toxicity.

It is also possible that an alteration in mitochondrial morphology may be a consequence of a loss of inner mitochondrial membrane components (respiratory cytochromes) and this in turn causes a change in respiratory control. Hepatic mitochondria from animals receiving the first three drug treatments, in this chapter, although to different degrees, and none of which reached statistical significance showed reduced glutamate/malate-stimulated oxygen consumption, when compared to that of control liver mitochondria and this taken together with the measurement of reduced cytochromes (chapter 4 results) could cause a morphological change in the mitochondria, with a resultant loss of the proton motive force required to drive ATP synthesis. If the hypothesis that loss of RCR is the result of loss of inner mitochondrial membrane proteins is true, the observation that intravenously-administered N-methyl protoporphyrin did not result in this loss of RCR in hepatic mitochondria may not be totally surprising as N-methyl protoporphyrin (chapter 4) reduced only cytochrome oxidase, to a small, although significant extent.

Although the results of this thesis argue against ALA toxicity in nerve and muscle, if ALA does have a role to play in hepatic mitochondrial function, this could explain the differential effects of porphyrinogenic drug treatment, on RCR, employed in this chapter. Although De Matteis and Marks (1983) demonstrated that N-methyl protoporphyrin does cause a rise in chick hepatic ALAS activity, they also noted the difficulty in observing this induction. The authors measured a rise in ALAS activity 5 hrs after administration, an increase which had subsided by 12hrs. It is possible, therefore, that due to N-methyl protoporphyrin's inability to increase ALA production

for long periods, this treatment did not result in a long term uncoupling of mitochondria.

The brain tissue mitochondria were generally less coupled than hepatic tissue. The method used to isolate all the mitochondria in this section of the study was developed for hepatic tissue and for practical reasons the procedure was used for both brain and liver tissue. The electron micrographs of both liver and brain mitochondria pellets (plates 3, 4) show that the brain mitochondrial fraction contains more membrane contaminents than the corresponding liver fraction. Purer brain mitochondria can, however, be isolated by a different method. Due to the high myelin content of brain tissue Lovtrup and Zelander in 1962 suggested that a denser sucrose solution is better for isolation of these mitochondria. The electron micrograph of a brain mitochondrial pellet, prepared by the method of Lovtrup and Zelander, demonstrates that this procedure does yield purer brain mitochondria (plate 7) and it would have been interesting, if time had permitted to pursue this method of preparation.

N-methyl protoporphyrin, injected directly into the third ventricle, did not produce the same effects in the brain as it did when administered intravenously. Mitochondria isolated from the brains of these animals were significantly uncoupled and there was no evidence of a reduction in their rate of respiration. The histochemical examination of the brain tissue shows clearly that the porphyrin was able to penetrate into the brain tissue (plate 8), despite the fact that the porphyrin precipitated out in the ventricle. Although 50nM./day was injected into the brain, whereas a 20 times greater concentration (1μM) was administered intravenously, the amount of porphyrin getting into the brain tissue following i.c.v. administration could have been much greater than that reaching the hepatocytes, following intravenous administration. In this case the larger concentration of porphyrin in brain cells could be causing the uncoupling. It is also possible that the N-methyl protoporphyrin which precipitated out in the ventricle was acting as an abrasive during initial homogenisation and could have a detrimental affect on mitochondrial coupling. The same problem could also have affected the coupling of the liver mitochondria from animals receiving the first two treatments as the accumulation of iron in the hepatic

tissue, which felt gritty, could be acting as an abrasive agent. Although intravenously administered N-methyl protoporphyrin was readily seen in the mitochondrial pellet, the liver was not as obviously altered as it was following 4-ethyl DDC treatment. This could account for the lack of uncoupling of mitochondria seen in hepatic mitochondria from N-methyl protoporphyrin treatment whereas 4-ethyl DDC administration always caused uncoupling in hepatic mitochondria.

The possibility that abnormally accelerated state 4 respiration, observed in uncoupled mitochondria, in this chapter's results, may be masking a reduction in state 3 respiratory rate may be ruled out if Nicoll's 1982 suggestion that the ADP-stimulated state 3 respiratory rate of mitochondria are maximal and independent of state 4 respiratory rate is true.

The combined results from the experiments reported in chapter 4 and chapter 5 demonstrate that the significant changes in respiratory cytochromes, observed in mitochondria following porphyrinogenic treatment, may not be large enough to elicit major alterations in mitochondrial function, although there were reductions in RCRs, in liver mitochondria from 4-ethyl DDC treated groups and in brain tissue of the centrally administered N-methyl protoporphyrin group. The chemical changes resulting from these treatments, particularly increases in protoporphyrin 1X and or ALA, may be the mediators of the loss of respiratory control seen in these mitochondria. In acute intermittent porphyria, the neurotoxic role of ALA may be mediated via a mitochondrial uncoupling superimposed upon a cytochrome deficient electron transport chain. The experiments carried out in chapter 2 and 3 of this thesis, however, show that, if indeed ALA has a toxic action on mitochondrial function, this effect is not sufficient to offset the function of nerve and muscle.

CHAPTER 6

GENERAL DISCUSSION.

The porphyrias manifest an enzymatic lesion in the haem biosynthetic pathway and the symptoms of the acute form of the disorder are indicative of a central and peripheral neuropathy. The underlying cause of this neuropathy remains obscure. The aim of this thesis was to examine two major hypotheses regarding the basis of the clinical symptoms of acute porphyria. First a neurotoxic role for the haem precursors PBG and, in particular, ALA and second an intermittent state of haem deficit starves the tissue of the metabolic energy necessary for normal function. This haem deficiency causes a reduction in processes such as oxidative phosphorylation, which is dependent upon haemoprotein mediated functions.

Although some researchers have reported that ALA possesses neurotoxic actions in vivo and in vitro, the majority of these studies have employed large mMolar concentrations of ALA, quantities which would not normally occur in porphyric patients, even during the severest of attacks. There was no evidence from the experiments carried out in this study, on a range of isolated rabbit neuromuscular preparations, that the haem precursor ALA was neurotoxic. ALA similarly lacked neuromuscular toxicity when examined on isolated tissues from rats in which haem synthesis had been disrupted by porphyrinogenic agents. Examination of the effects of PBG on rat tissue, also failed to produce evidence of a neurotoxic action. Even in animals treated with porphyrinogenic drugs, in which the threshold for toxicity might have been lowered, no evidence of neuropathy was found.

In an attempt to produce a haem deficient animal model, rats were treated with a variety of compounds, all of which are known to disrupt haem synthesis or availability. All eight treatments employed, significantly increased the urinary excretion of ALA. This provided an indication of reduced haem availability, as the activity of the rate limiting enzyme of the pathway, ALAS, is subject to a negative feedback by the end product of the pathway, haem. A reduction in the free regulatory haem pool causes a derepression of the activity of this enzyme, and accounts for the increased production and excretion of ALA. Although the eight treatments produced evidence of a haem deficiency, all were incapable of

inducing a peripheral neuropathy on a range of in vitro innervated muscle preparations. The examination of these drugs on a different species (mouse) also failed to produce evidence of a haem deficient neuropathy.

The rise in urinary excretion of ALA, in the animals treated with porphyrinogenic agents, although providing indirect evidence of a reduced haem availability did not furnish any information on the possible differential effects of these compounds on the various tissue haemoproteins. Two factors will affect the haem content of the various haemoproteins following a blockade in haem synthesis, the affinity of the particular apoprotein for its haem moiety and the half-life of the particular haemoprotein. Apoproteins, with a high affinity for their haem constituent, such as haemoglobin, myoglobin, catalase and the respiratory cytochromes, will access newly synthesized haem more readily than the low affinity haemoproteins like cytochrome P-450, tryptophan pyrrolase or guanylate cyclase. Additionally, the impact of reduced haem synthesis will be manifest in haemoproteins with short half-lives (tryptophan pyrrolase, cytochrome P-450 and catalase) before compromising haemoproteins with longer half-lives (the respiratory cytochromes and haemoglobin).

Following porphyrinogenic treatment, haemoproteins were measured in the liver, blood and brain. The treatment regimes were designed to cover as many half-lives of the haemoproteins as practically possible. Succinylacetone administration for 30 days failed to reduce any of the haemoproteins examined in the three tissues. In addition to succinylacetone's inhibitory action on ALAD, it is possible that the compound may also induce other enzymes of the haem biosynthetic pathway. Although succinylacetone inhibits an enzyme early on in the pathway it also produced an increase in the total porphyrin production, which indicates that an increased quantity of ALA is being converted into porphyrins. The enzymatic capacity of ALAD is in great excess of that of the next enzyme in the pathway, PBGD, and therefore even when ALAD is profoundly inhibited, enough PBG may still be produced to satisfy the requirements of PBGD. A rise in ALA excretion, concomitant with a rise in increased excretion of total porphyrins, is incongruent with the hypothesis that the increased ALA

is the result of a reduction in haem availability. This would only be true if there was also a block in the pathway at a later stage. The observation of increased porphyrin synthesis, combined with the finding that 30 days of succinylacetone treatment failed to reduce the level of any haemoprotein measured, indicates that this compound may directly increase ALAS activity, causing both a rise in ALA and porphyrin production. Succinylacetone may only be able to reduce haem synthesis at very high concentrations, when the ALAD inhibitory effect overcomes any ALAS inductive action. Hess et.al. (1987) showed that, after prolonged treatment with high concentrations of succinylacetone, haemoglobin concentration, in rats, fell by 20%. The concentration of succinylacetone used in the present studies may, therefore, have been causing an increased haem availability rather than a decrease.

Animal treatment with 4-ethyl DDC for 14 days, produced a small but significant reduction in hepatic respiratory cytochromes and a marked depression in the activity of hepatic catalase. The latter haemoprotein is a low affinity haemoprotein with a fairly rapid turn over (2 days) and would be expected to succumb to a reduction in haem availability, more readily than the high affinity respiratory haemoproteins with a half-life of 6 days. The reduction in hepatic catalase activity, following some of the porphyrinogenic treatments employed in the experiments in this thesis, could account for the reduced mitochondrial RCR due to H_2O_2-induced membrane damage. However, as normal brain tissue contains little catalase, this may be indicative that brain mitochondria produce little H_2O_2 and therefore, H_2O_2 is not the mediator of the reduced RCR in brain mitochondria from animals receiving centrally-administered N-methyl protoporphyrin. The success of 4-ethyl DDC in reducing haemoprotein content is due to the vital site of haem synthesis inhibition. Although the acute porphyria enzymatic lesion is early on in the pathway, in the xenobiotic production of a haem deficient animal model, ferrochelatase is an important site of inhibition, as it is independent of any additional enzymatic actions which the drug may be exerting. N-methyl protoporphyrin 1X which directly inhibits ferrochelatase, while not as effective as 4-ethyl DDC, which both destroys cytochrome P-450 as well as inhibiting ferrochelatase, does show

that inhibition of ferrochelatase alone is capable of reducing the production of, at least one of the final respiratory haem proteins and of catalase. These treatments, which were effective in reducing hepatic haemoprotein content were incapable of reducing either blood or brain haemoproteins. The inability of these compounds to reduce blood haemoproteins, was either due to the long half-lives of the erythrocyte haemoproteins, relative to the treatment period, or to the inability of these compound to alter erythropoietic haem synthesis, which may have a different control mechanism to that of hepatic tissue. Brain tissue was studied as a representative of neuronal tissue. However, the problem of access of drugs to the brain could have been the cause of the inability of someofthese compounds, when administered systemically, to alter brain haemoproteins. Although respiratory cytochrome content was not measured following centrally-administered N-methyl protoporphyrin, the reduction in brain, RCR was similar to that seen in hepatic tissue caused by porphyrinogenic treatments which reduced respiratory cytochrome levels.

As the mitochondrial respiratory cytochromes were reduced by some of these porphyrinogenic treatments, mitochondrial function was examined to determine whether this reduction was also manifest as a functional deficit. Although 4-ethyl DDC treatment caused uncoupling of the hepatic mitochondria there was no evidence that this compound, at the administration parameters used in this study, caused a malfunction in respiratory chain function as both the P:O ratios and oxygen consumption of substrate-stimulated mitochondria were normal. The reduction in cytochromes was probably too small to elicit a significant change in these later two mitochondrial function parameters. The significant uncoupling of hepatic mitochondria could be due to either ALA or protoporphyrin 1X or both. The finding that N-methyl protoporphyrin, when administered intravenously, did not cause uncoupling indicates that 4-ethyl DDC metabolism has an additional role to play in the reduction of haem proteins and deficits in mitochondrial function, seen in chapter 4 and 5 experiments. 4-Ethyl DDC has a dual porphyrinogenic action both in the destruction of a ready formed haemoprotein and due to its inhibition of ferrochelatase. The incongruous finding that intraventricular

administration of N-methyl protoporphyrin caused uncoupling in brain tissue whereas intravenous administration of the same compound did not uncouple hepatic mitochondria may be due to the high concentration of this substance in the brain tissue compared with the amount reaching liver mitochondria following systemic administration. ALA may also be an important candidate for mediation of this uncoupling and whereas ALAS induction is great following 4-ethyl DDC treatment, N-methyl protoporphyrin may not be as potent an inducer. The large amount of this latter compound reaching the brain following central administration may be large enough to increase ALA production to the extent where it influences mitochondrial RCR. Although neither cytochrome content nor nerve/muscle function was measured in this last group of experiments earlier results reported, in this thesis, argue against a major toxic role for ALA in porphyric neuropathy.

A porphyric neuropathy was not produced by the experiments described in this thesis and several factors may have contributed to this failure. The enzymatic lesion in Acute Intermittent Porphyria lies at the level of PBGD. There is no chemical, as yet, that will inhibit this enzyme. In the present study succinylacetone was employed to block the haem biosynthetic pathway at an early stage (ALAD), close to that of the defect in this form of acute porphyria. The early treatment regimes in this study relied heavily on the inhibitory effects of succinylacetone on ALAD. Later evidence, however, indicated that, at the concentrations used, succinylacetone may actually increase haem synthesis. In these experiments ferrochelatase is the most susceptible to inhibition. Ferrochelatase is an enzyme with low enzymatic activity and as the last enzyme in the haem biosynthetic pathway, inhibition at this level ensures decreased haem synthesis. The treatment periods used were also relatively quite short, especially in view of the finding that haem inhibition was probably only achieved during periods of ferrochelatase blockade. Longer and more profound periods of haem synthesis inhibition are required to reduce respiratory cytochrome levels, where a neuropathy may be manifest. Biochemical findings, in patients suffering from mitochondrial encephalomyopathies, suggest that the respiratory cytochrome content of nerve and muscle would

have to be decreased by more than 50%, before there would be a likelihood of producing a neuropathy. The problem of porphyrinogenic drug access to neural tissue will also have to be overcome. Although peripheral neuronal tissue does not possess the same barriers to xenobiotics as brain, it has to be confirmed that, firstly, porphyrinogenic drugs are taken up into nerves and secondly, that the drugs are inhibiting haem synthesis in nervous tissue. Although succinylacetone has been shown to disrupt brain haem synthesis, there is no other evidence, either from this study or from the work of others, to suggest that the porphyrinogenic compounds used in this study reach or inhibit haem synthesis enzymes in neuronal tissue. There was no evidence, from brain mitochondrial function experiments, that intracerebroventricular administration of N-methyl protoporphyrin 1X caused a significant decrease in respiratory cytochromes, although this treatment did alter brain mitochondrial coupling, similar to that found in hepatic tissue of animals treated with drug treatments that did manifest reduced haemoprotein content. This latter evidence suggests that neural tissue is responding in a similar manner to porphyrinogenic treatment, as liver. The experiments carried out, in this study, showed that the levels of respiratory cytochromes could be reduced by systemic porphyrinogenic treatment and, although these were not reduced in neuronal tissue nor were they reduced to an extent which elicited changes in mitochondrial respiration, a longer and more profound central administration treatment could overcome these problems.

From the two aetiological theories examined, in this thesis, there is little evidence to support the hypothesis that porphyric neuropathy is due to a direct action of the haem precursors ALA or PBG on nerve or muscle. ALA could, however, be a contributory factor exacerbating a deficit in mitochondrial function due to a lack of haem production.

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