LIPID PEROXIDATION IN TISSUES AND NEURAL FRACTIONS DURING EXPERIMENTAL VITAMIN E DEFICIENCY AND IN PATIENTS WITH FRIEDREICH'S ATAXIA

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A thesis submitted for the degree of Doctor of Philosophy

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ABSTRACT

A severe deficiency of vitamin E in man results in a characteristic neurological syndrome which is very similar to that seen in patients with Friedreich's ataxia (FA). The mechanism(s) of action of vitamin E in neurological tissues is unknown although it is generally accepted to act as a lipid soluble antioxidant. The purposes of this study were to examine lipid peroxidation in (a) the vitamin E deficient rat and (b) autopsy material from patients with FA.

A fractionation procedure using brainstem was validated to isolate myelin, an axolemma-enriched fraction (AEF) and a fraction containing the axoplasmic membranes and organelles (M/O). Endogenous lipid peroxidation was assessed by measuring thiobarbituric acid reactive substances (TBARS), free, bound and total malondialdehyde (MDA) and aliphatic aldehydes. Susceptibility to in-vitro free radical stress was also investigated using the copper sulphate / hydrogen peroxide generating system and measuring free MDA.

Endogenous lipid peroxidation was investigated in vitamin E deficient and control rats after 21 and 56 weeks. After 56 weeks concentrations of TBARS in the deficient animals were increased in all tissues except for peripheral nerve. Increases were less in neural than non-neural tissues ($< 35\%$ and 2 to 4-fold respectively). Bound MDA was elevated in brain and spinal cord from 56 week deficient animals 2 to 5-fold, but although free MDA was also increased in brain there was no increase in the cord. Aliphatic aldehydes showed the greatest increases ($>$ 6-fold). Lesser changes in lipid peroxidation were seen after 21 weeks of deficiency. These results, therefore, provide evidence of increased lipid peroxidation during vitamin E deficiency.

When tissues and fractions from 20 and 60 week old rats were stressed in-vitro using copper sulphate / hydrogen peroxide the following observations were made (a) the deficient samples in contrast to control material did not exhibit a lag phase and (b) the following order of susceptibility to peroxidation was noted: brain $>>$ spinal cord $>$ nerve; M/O $>>$ AEF $>$ myelin. The latter result is consistent with the neuropathology seen in vitamin E deficiency where a primary axonopathy leads to secondary demyelination.

In studies of autopsy material from patients with FA, vitamin E concentrations in neural tissues were not significantly different from control values. Studies of lipid peroxidation failed to show any significant differences between patients and controls. The results would, therefore, seem to rule out an antioxidant defect in the aetiology of this disease.

ACKNOWLEDGEMENTS

First and foremost I would like to thank Dr. David Muller, my supervisor, for his guidance, continued support and encouragement throughout this study. I am also grateful to Professor Dame June Lloyd for making available to me the research facilities of the Department of Child Health in the Institute of Child Health.

I would also like to thank the following: Dr. Patrick McCarthy, Department of Biochemistry, Guy's Hospital Medical School for help and advice on setting up the HPLC system for MDA analysis and use of methodology for the estimation of aliphatic aldehydes; Dr. Susan Chamberlain, Department of Biochemistry, St.Mary's Hospital Medical School, for Friedreich's ataxia post mortem tissues and Dr. F.R. Wells, The Parkinson's Disease Brain Bank, Institute of Neurology for control post mortem tissues; Mrs. Virpi Smith, Department of Histochemistry, Institute of Neurology, and Dr. Rosalind King, Department of Neurological Science, Royal Free Hospital for electron microscopic analysis of neural fractions; Mr. Paul Biggs and the staff of the animal unit of the Institute of Child Health for their help and cooperation.

A special thanks to The Friedreich's Ataxia Group for funding the major part of this project, and also to Hoffmann-La Roche (Basle) and Merchant Taylors' for additional support.

I would like to thank the members of the department for their "kind" words of encouragement. Finally, and most sincerely, I would like to thank Kathy Stenson, my adopted mother, for nutritional support during periods of moderate weight loss.

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CHAPTER 1

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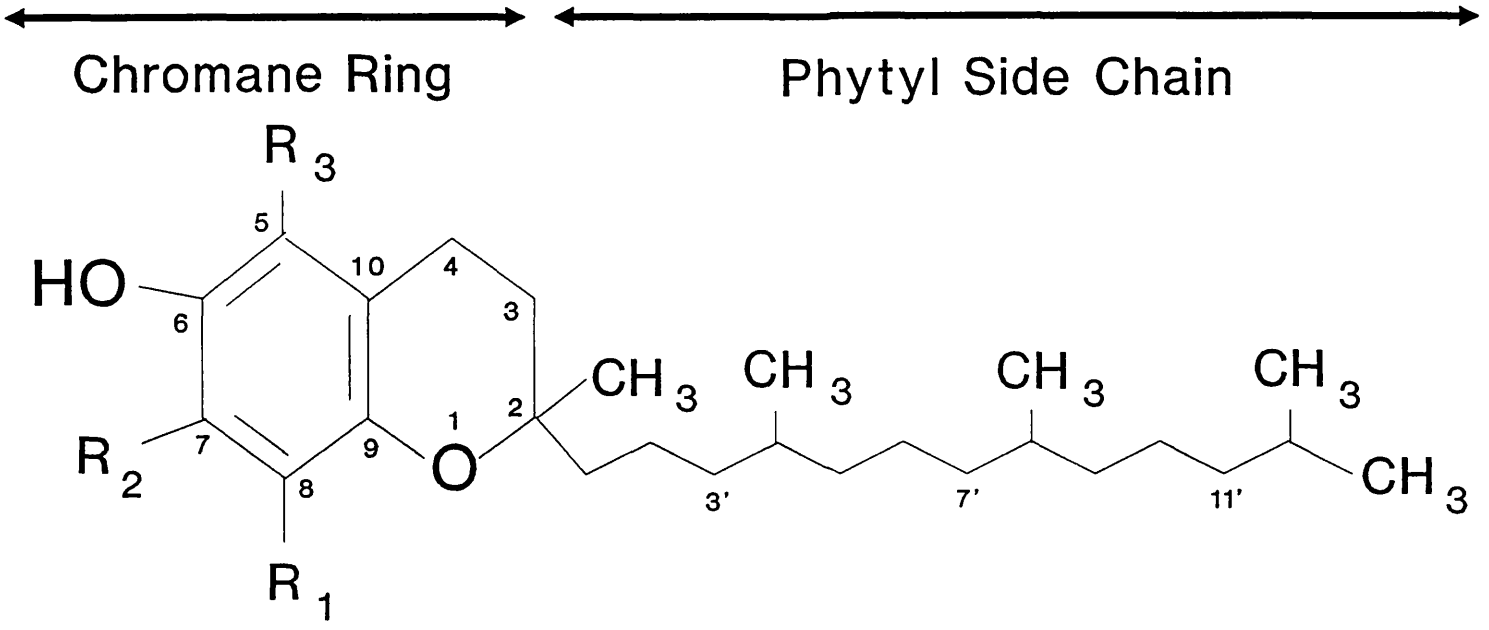
1.1 History of Vitamin E

Evans and Bishop in 1922 described the existence of an unknown fat soluble dietary factor, a deficiency of which resulted in fetal death and resorption in the laboratory rat. These observations were made from studies on rats maintained on semipurified diets known to lack this factor. However, supplementation with fresh lettuce known to be a good source of the unidentified dietary factor, prevented the reproductive defects. Sure in 1924 was the first to propose the name vitamin E, the identities of vitamins A, B, C and D having already been established. In 1936 Evans isolated the vitamin from wheat germ oil, for which they proposed the name α-tocopherol and provided the correct chemical formula of $C_{29}H_{50}O_2$. Two years later Ferholz (1938) established the structural formula of α-tocopherol.

To date eight tocopherols have been isolated from various vegetable oils, all of which are closely related derivatives of 2-methyl-2 (4,8,12-trimethyldecyl)-chroma-6-ol, or tocol. The eight compounds are grouped into two classes which differ in the saturation of the phytyl side chain. Those containing a saturated side chain are referred to as tocopherols, while those with a phytyl chain containing three double bonds (i.e. between carbons 3'-4', 7'-8' and 11'-12') are termed tocotrienols. The four compounds in each group are designated alpha, beta, gamma and delta, and differ in the number and position of the methyl groups on the chromane ring. The structure of the tocopherols are given in fig 1.

Fig 1. Structure Of The Tocopherols



Tocopherol	Chromane -CH_3 Groups		
	R_1	R_2	R_3
α	R_1	R_2	R_3
β	R_1		R_3
γ		R_2	R_3
δ			R_3

1.2 Early Animal Studies of Vitamin E

After the initial recognition of the involvement of vitamin E deficiency in reproductive defects in the laboratory rat, the following years saw the publication of numerous reports of the effects of feeding semipurified diets similar to those used by Evans and Bishop on different animal species. Many of these studies appeared to contradict one another and it was only some years later that an explanation for many results became apparent. Pappenheimer and Goettch (1931) using semipurified diets showed that chicks developed "nutritional encephalomalacia" while guinea pigs and rabbits given a similar diet developed nutritional muscular dystrophy (Goettsh and Pappenheimer 1931) as did ducklings (Pappenheimer and Goettsh 1934). Vitamin E deficiency was not specifically implicated until 1940 when Pappenheimer showed that the duckling myopathy could be prevented by oral administration of α-tocopherol. Among numerous other studies, Ringsted in 1935 described paralysis in adult vitamin E deficient rats while Martin and Moore (1939) reported uterine pigmentation, also in the vitamin E deficient rat. Acute or chronic necrotizing myopathy is now regarded as the most common feature of vitamin E deficiency (reviewed by Wasserman and Taylor 1972).

1.3 Vitamin E in Man

Despite the numerous studies carried out into the effects of experimental vitamin E deficiency in different animal species, the role of vitamin E in human nutrition was a source of much controversy. Many highly sceptical claims were made such as vitamin E having the ability to put zest into ones sex life or even, as reported in the American press, to protect against the effects of ionizing radiation generated by thermonuclear bombs. Such claims only increased scepticism towards vitamin E and delayed its general acceptance as a compound having a genuine nutritional role in man. Over the past 25 years or so, however, evidence has accumulated for vitamin E having a possible pharmacological role in a number of disorders of the newborn and

an established role in the maintenance of normal neurological structure and function.

The average diet is rich in vitamin E with good sources being nuts, seeds, oils, fruit, vegetables and dairy products. A recent Department of Health report on dietary reference values (DRVs) of various nutrients concluded that since vitamin E requirements depend on the polyunsaturated fatty acid intake, which varies widely, it was impossible to set a DRV of practical value and that there was little merit in giving more than ranges of acceptable intake (1990). A survey by Gregory et al (1990) of 1,629 UK adult subjects found that the 2.5 and 97.5 centiles of intakes of vitamin E from their 7-day weighted dietary records were 3.5 and 19.5 (median 9.3) mg α-tocopherol equivalents/d for men and 2.5 and 15.2 (median 6.7) mg /d for women.

1.3.1 Vitamin E in the Newborn

Some evidence of a role for vitamin E in human nutrition became available in the late 1950's and 1960's. Both Oppenheimer (1956) and Blanc et al (1958) demonstrated that infants with cystic fibrosis had abnormally low serum vitamin E levels associated with focal necrosis of striated muscle and widespread deposition of ceroid in smooth muscle. However, the first and for some period of time, the only condition attributable to a deficiency of vitamin E was a clinical syndrome in premature infants first reported by Hassan et al (1966). This disorder consisted of skin lesions, oedema and haemolytic anaemia which was associated with a deficiency of vitamin E. Administration of oral preparations of vitamin E were found to result in the disappearance of these problems. These findings were confirmed by a number of groups including Oski and Barness (1967) and Ritchie et al (1968). Subsequently, numerous studies of the prophylactic use of vitamin E in the prevention of anaemia in the neonate (reviewed by Bell and Filer 1981) have produced conflicting reports. The reason for the variation in the findings of

different groups may have been due to variables besides vitamin E intake, such as the dietary content of polyunsaturated fatty acids (PUFA) and the ability of the infant to absorb vitamin E. However, with the introduction of vitamin E into all infant milk formulas at a vitamin : PUFA ratio of greater than 0.6mg/g, haemolytic anaemia is now rare so that additional vitamin E supplementation is not necessary for its prevention in premature infants.

Pharmacological doses of vitamin E have been suggested as potentially beneficial in the treatment of a number of other disorders associated with the neonate, namely, bronchopulmonary dysplasia, retrolental fibroplasia and intraventricular haemorrhage.

The pathological changes in the lung of premature infants maintained on artificial ventilation and oxygen, (as part of the management of respiratory distress syndrome), was reported by Ehrenkranz et al (1978) to be preventable using intramuscular vitamin E. However the same group using a double blind randomised trial failed to confirm their earlier findings (Ehrenkranz et al 1979).

Another condition seen in premature infants treated with prolonged ventilation is retrolental fibroplasia, also referred to as retinopathy of prematurity (ROP), which was first described by Terry in 1942. The incidence of this condition dramatically decreased during the 1960's after the discovery of its association with uncontrolled oxygen administration. However this problem has re-emerged in recent years with the increased survival rates of very low-birthweight infants. Owens and Owens (1948) were the first to suggest that prophylactic vitamin E could reduce the severity and incidence of this eye disorder. Since that time numerous studies have been described investigating the role vitamin E supplementation in preventing ROP. The results have however been conflicting. A review of seven published double blind,

randomised controlled trials of the effect of vitamin E supplementation on retrolental fibroplasia by Law et al (1990) concluded that there was no statistically significant reduction in the incidence of the disease in those patients given vitamin E supplements.

The most prevalent finding in the brains of premature babies who die within the first week of life is intraventricular haemorrhage and is probably one of the most important lesions responsible for handicap in surviving infants. Chiswick et al in 1983 reported that vitamin E supplementation reduced the incidence of intraventricular haemorrhage in premature infants under 32 weeks of gestation. Sinha et al (1987) postulated that vitamin E may trap free radicals that are generated during ischaemic injury of the subependymal layer and so limiting the extent of the damage. All four randomised trials of the effect of vitamin E therapy on this condition, reviewed by Law et al (1990), reported a reduction in treated cases. Taken together, the four studies gave approximately a 50% reduction which was highly significant ($p < 0.001$).

Of the three disorders occurring in premature infants and neonates described above, the most convincing evidence for a beneficial effect of pharmacological doses of vitamin E was seen in the case of intraventricular haemorrhage. However, even in this disorder, Law et al (1990) calculated that since major neurological damage associated with intraventricular haemorrhage alone affects approximately 3% of all very low-birthweight infants, vitamin E therapy would only reduce the incidence by about 1.5%. It is questionable whether in the light of this slight reduction in the incidence of intraventricular haemorrhage prophylactic vitamin E should be given to all premature infants because of recent reports of possible toxicity associated with vitamin E supplementation. Both Finer et al (1984) and Johnson et al (1985) who administered oral and intramuscular vitamin E preparations respectively, reported an increased incidence of necrotizing enterocolitis, with the latter

group also observing a greater frequency of sepsis in the treated group. Johnson et al (1985) suggested that the supranormal serum concentrations of vitamin E (i.e. 50 mg/l) achieved in their study resulted in excess antioxidant capacity. This was postulated to interfere with the normal oxygen-dependent antimicrobial defence mechanisms and consequently in an increased susceptibility to infection. Since, in the case of intraventricular haemorrhage, serum concentrations of greater than 30 mg/l are regarded as therapeutic, the margin for error may be small. The decision to administer high concentrations of vitamin E in the treatment of the neonatal disorders discussed above has therefore to be weighed against any potential toxic effects.

1.3.2 Vitamin E and Neurological Function

Over the last few years it has become increasingly evident that vitamin E has an important role in the maintenance of normal neurological structure and function. Evidence for this role for α-tocopherol comes from four main sources. From cases of abetalipoproteinemia, patients with other chronic disorders of fat absorption, a familial isolated deficiency of vitamin E without fat malabsorption, and finally comparison of the neuropathological findings in vitamin E deficient man and experimental animals. Each of these will now be considered in turn.

1.3.2.1 Abetalipoproteinemia

Patients suffering from abetalipoproteinemia (formerly known as the Bassen and Kornzweig syndrome) provided the first indication of a neurological role for vitamin E in man. This is a rare inborn error of lipoprotein metabolism inherited in an autosomal recessive manner. Three groups in 1960 independently demonstrated the complete absence of beta (low density) lipoprotein from the plasma of such patients (Lamy et al 1960, Mabry et al 1960, Salt et al 1960) and it was subsequently shown ten years later that apolipoprotein (apo) B, which is an essential component of chylomicrons,

very low density lipoprotein (VLDL), as well as low density lipoprotein (LDL) was undetectable in the plasma of these patients (Gotto et al 1971). As a result of the lack of apo B all these lipoproteins are absent in this condition and consequently the major route of fat absorption from the small intestine (via chylomicrons) is blocked, resulting in steatorrhoea from birth. Since the fat soluble vitamins also depend on this same route for absorption there is a concomitant impairment in their absorption from the gut (Herbert et al 1978). Although plasma vitamin E concentrations tend to be reduced in many fat malabsorptive conditions, the severest deficiency occurs in abetalipoproteinemia where the vitamin is undetectable from birth (Kayden et al 1965, Muller at al 1974). Other features of abetalipoproteinemia include acanthocytosis (spiky red cells), also present from birth, and a severe and progressive ataxic neuropathy and retinopathy which usually develop during the second decade of life. The clinical features of this neuropathy include ataxia, areflexia and loss of position sense (proprioception). There have been no reports to date of spontaneous improvement in the neurological condition of these patients. However, a number of groups have shown that treating these patients with large oral doses of α-tocopherol, if administered early enough, prevented the development of the neurological syndrome, or in already established cases prevented further deterioration or even improved their neurological status (Muller et al 1977, Azizi et al 1978, Muller and Lloyd 1982). Because of the undetectable levels of vitamin E in the serum of patients with abetalipoproteinemia, they provide an ideal model for studying vitamin E deficiency in man.

1.3.2.2 Other Chronic Disorders of Fat Absorption.

Additional evidence comes from a number of conditions in which there is a chronic fat malabsorption associated with a severe deficiency of vitamin E. After abetalipoproteinemia, the most pronounced deficiency occurs when the bile salt concentration in the intestinal lumen is reduced, e.g. biliary

atresia (Muller et al 1974). Vitamin E is highly hydrophobic and requires an adequate supply of bile salts to facilitate solubilization and absorption (Harries and Muller 1971). The majority of the published reports relating a severe deficiency of vitamin E to a neurological syndrome have come from children with cholestatic liver disease (e.g. Rosenblum et al 1981, Elias et al 1981, Sokol et al 1983). The neurological features of these patients are very similar to those seen in abetalipoproteinemia (Muller et al 1983). In cases of very low bile salt levels, oral preparations of tocopherol will not be absorbed, necessitating the use of intramuscular injections. Guggenheim et al (1983) described improvement in the neurological condition of three children suffering from chronic cholestasis employing this treatment. Sokol et al (1985) reported that the neurological function in three symptomatic children with chronic cholestasis below the age of 3, normalised after 18 to 32 months of vitamin E therapy. However, restitution of neurological function was more limited in 9 symtomatic children aged between 5 and 17.5 years of age. Vitamin E deficiency associated with cystic fibrosis has also been reported to result in a neurological disorder (e.g. Umetsu et al 1980, Elias et al 1981). Patients who have undergone multiple ileal resections, i.e. have large amounts of the small bowel surgically removed, e.g. Crohn's disease (Howard et al 1982), have also been shown to develop a severe deficiency of vitamin E. These patients may develop neurological problems similar to those previously described (Harding et al 1982, Nakajima et al 1987) which have also been shown to be alleviated using appropriate vitamin E therapy (Harding et al 1982).

1.3.2.3 Familial Isolated Vitamin E Deficiency

To date 9 patients worldwide have been described who have very low serum vitamin E levels and exhibit similar neurological abnormalities to those seen in abetalipoproteinemia and other chronic fat malabsorption conditions. Whereas in the latter two situations the deficiency is secondary to chronic and severe fat malabsorption, there is no evidence of a generalised defect in fat absorption in these 9 patients (Burck et al 1981, Laplantet al 1984, Harding et al 1985, Krendel et al 1987, Yokoto et al 1987, Kohlschutteret al 1988, Sokol et al 1988). Treatment of these cases with vitamin E therapy was shown to either prevent deterioration in their neurological condition or actually improve neurological status. For example Burck et al (1981) reported that a boy of 11 who had a rapidly progressing ataxia of gait and muscle weakness, regained the ability to walk and to feed and dress himself unaided following vitamin E supplementation.

When these patients are given vitamin E supplements their plasma vitamin E concentrations increase from almost undetectable to normal or above on the same time scale as seen for control subjects, suggesting that absorption from the gut is normal. Thereafter however, there is a rapid decline in serum levels compared with the controls. Kohschutter et al (1988) suggested that this rapid fall could be due to increased metabolism of tocopherol. Traber et al (1990) using deuterated tocopherol confirmed that absorption of tocopherol was normal but suggested that the deficiency was due to a lack of a liver tocopherol binding protein that incorporates α-tocopherol into nascent VLDL.

Whatever the causative factor(s) in the aetiology of this selective deficiency, the fact that these patients develop the characteristic neurological syndrome in the absence of any generalised fat malabsorption strengthens the causal link between vitamin E deficiency and the observed neuropathology.

1.3.2.4 Comparative Neuropathological Studies

The final piece of evidence for a role for vitamin E in neurological structure and function comes from comparative neuropathological studies. The characteristic neuropathological features in vitamin E deficient humans (Sung et al 1980, Rosenblum et al 1981) are very similar to those observed in experimental vitamin E deficient rats (Einarson 1952, Machlin 1977, Towfighy 1981, Bradly 1986) and monkeys (Nelson 1981). Degenerate and dystrophic axons are found in the posterior column sensory relay nuclei, particularly the gracile nuclei of rat and man while the cuneate nuclei are more affected in the monkey. There is also a selective loss of large calibre myelinated sensory axons from the posterior columns of the spinal cord, the damage being most pronounced in the rostral segments.

In the peripheral nervous system there is also a selective loss of large calibre myelinated sensory axons being confined to the most distal segments of axons in peripheral nerves e.g. ulnar (Rosenblum et al 1981) and sural (Wichman et al 1985). Severe degeneration has also been reported to occur at axonal endings in muscle spindles, i.e. end plates and cutaneous sensory capsules of the hind paw of vitamin E deficient rats. Electron microscopic analysis reveals the presence of disorganized neurofilaments, tubulovesicular bodies and abnormal vesicles in affected axons. These observations are indicative of a distal or dying back neuropathy, i.e. those segments most distal from the neuronal cell body are the first and most severely affected. In addition there is some evidence that the neuropathology is due to a primary axonopathy with secondary demyelination (Nelson et al 1981, Thomas et al 1984, Wichman et al 1985).

Observations like those in the preceding sections have meant that vitamin E is now recognised as having an important role to play in the maintenance of normal neurological function and integrity. Although the exact

mode of action of vitamin E in neurological tissues has not yet been established it is assumed to act as a lipid soluble antioxidant. The following sections will therefore deal with oxygen toxicity, lipid peroxidation and the likely biological actions of vitamin E.

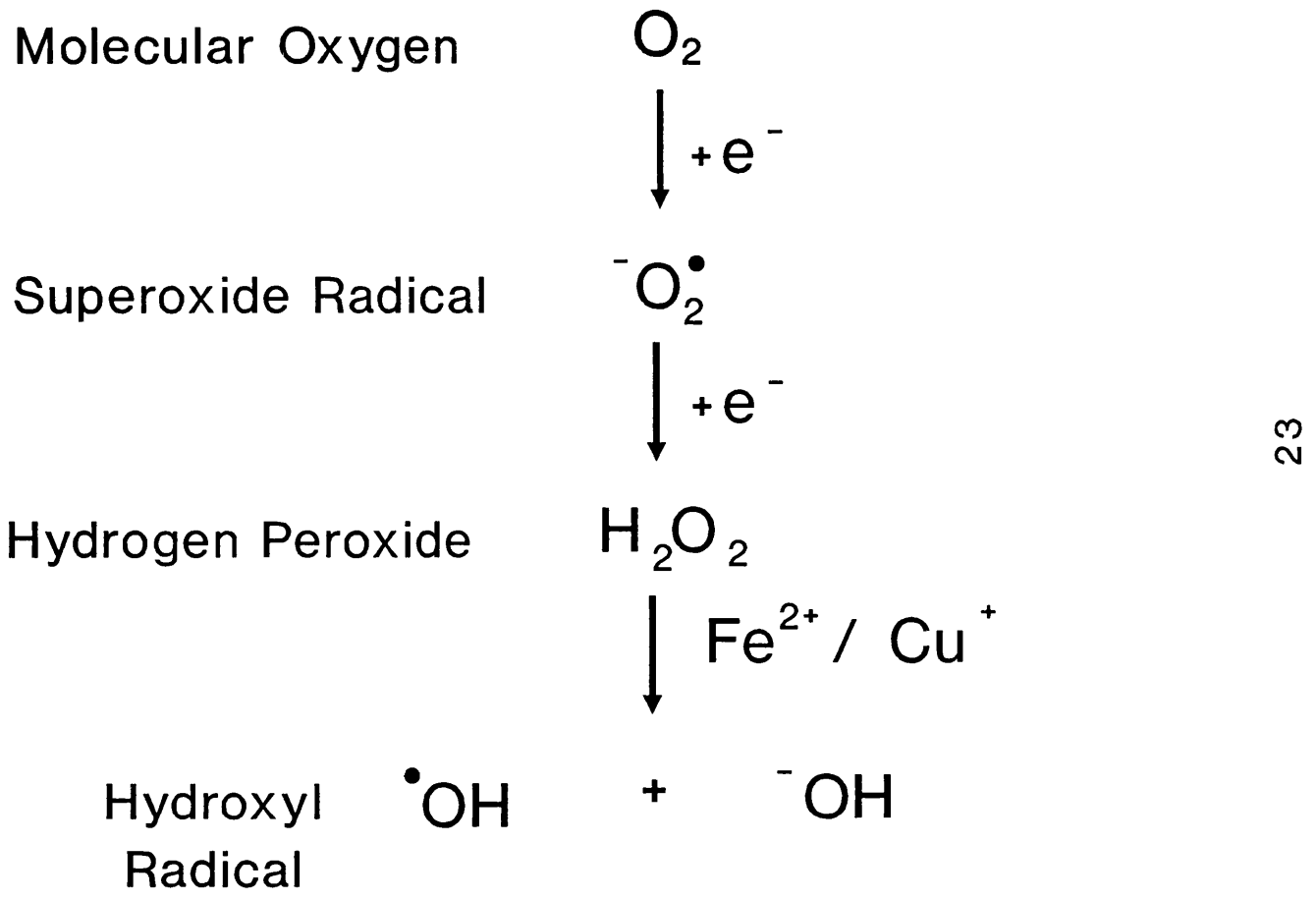
1.4 Oxygen, Oxygen Toxicity and Free Radicals.

Despite the complete dependence of numerous life forms on oxygen for their survival, it must not be forgotten that oxygen has many toxic effects. Oxygen at concentrations greater than those present in normal air are known to damage various organisms and plots of the logarithm of survival time against that of oxygen pressure show an approximately linear inverse relationship (e.g. Krieg and Hoffman et al 1986).

In 1954 Gershman and Gilbert proposed that many of the damaging effects of oxygen could result from the formation of oxygen derived free radicals. Halliwell and Gutteridge (1989) have defined a free radical as "any species capable of independent existence that contains one or more unpaired electrons". Free radicals have long been known in the food and plastics industries. However, as recently as the 1960's a speculative article in the Lancet provoked an eminent correspondent to state that "free radical reactions are inherently incompatible with organized life" (Dormandy 1989). Free radicals are now, however, being implicated in an increasing number of human disease processes, e.g. see review by Halliwell and Gutteridge (1989).

Many free radicals are highly reactive and may react with numerous different compounds in an indiscriminate manner. Molecular oxygen, although containing two unpaired electrons is relatively stable and non reactive due to electronic configuration restrictions (Halliwell and Gutteridge 1984). The toxic effects of oxygen result from other oxygen radicals, the relationship between these is shown in fig 2.

FIG 2. RELATIONSHIP BETWEEN OXYGEN FREE RADICALS



A simple mixture of H_2O_2 and reduced copper or iron leads to the formation of the hydroxyl radical ($\cdot OH$) and a hydroxide ion (reaction 1)

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{-}OH + \cdot OH \quad (1)$$

Reaction (1) is referred to as the Fenton reaction, after the chemist who first "observed" it. The hydroxyl radical is very unstable and reacts with extremely high rate constants with almost every type of molecule found in living cells, e.g. lipids, proteins, sugars and DNA, either by hydrogen abstraction, addition or electron transfer. It is so reactive that it will react with the first molecule with which it comes into contact after its formation. In addition to the initial formation of $\cdot OH$, other reactions are also possible;

$$\cdot OH + H_2O_2 \rightarrow H_2O + H^+ + \cdot O_2^- \quad (2)$$

$$\cdot O_2^- + Fe^{3+} \rightarrow Fe^{2+} + O_2 \quad (3)$$

$$\cdot OH + Fe^{2+} \rightarrow Fe^{3+} + {}^{-}OH \quad (4)$$

Thus a whole series of radical reactions are possible with a single mixture of H_2O_2 and a reduced transition metal.

The main question regarding free radical processes in living systems is whether the reactions shown above which take place in-vitro, also occur in-vivo, i.e. are the various precursors of free radical formation such as H_2O_2 and transition metals freely available in living cells? The superoxide radical ($\cdot O_2^-$) is known to be produced in-vivo by a number of systems including the

"respiratory burst" of phagocytic cells when they come into contact with foreign particles or immune complexes (Babior 1978). Another major source of $\cdot O_2^-$ is the activity of mitochondrial and microsomal electron transport chains as some components, such as ubiquinone, leak electrons directly to O_2 forming $\cdot O_2^-$. In aqueous solution $\cdot O_2^-$ is converted to H_2O_2 by a reaction known as "dismutation" i.e.

$$2 \cdot O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (5)$$

Because of this reaction, systems that produce $\cdot O_2^-$ also tend to form H_2O_2. Some enzymes such as L-amino acid oxidase and monoamine oxidase release H_2O_2 directly. Sient et al (1980) have shown that H_2O_2 is produced in-vivo by rat brain and the lens of the human eye has been shown to contain micromolar concentrations of H_2O_2 (Bhuyan and Bhuyan 1977). Haber and Weiss postulated in 1934 that $\cdot O_2^-$ could react with H_2O_2 to form an hydroxyl radical, i.e.

$$H_2O_2 + \cdot O_2^- \rightarrow {}^-OH + \cdot OH + O_2 \qquad (6)$$

This reaction (6) has become known as the Haber-Weiss reaction. It was subsequently shown however, that this reaction was thermodynamically unfavourable under physiological conditions. However, formation of $\cdot OH$ can be accounted for if the reaction is catalysed by traces of transition metal ions which transfer electrons between $\cdot O_2^-$ and H_2O_2 giving the overall reaction (6)

above. Alternatively,the hydroxyl radical could be generated if H_2O_2 comes into direct contact with a reduced transition metal (see reaction 1).

Another important point regarding generation of free radicals in-vivo is the availability of transition metals particularly iron and copper. Although virtually all of the body's iron is tightly bound by various proteins including haemoglobin (which binds approximately 2/3) and ferritin, some can also be chelated by low molecular weight compounds such as ATP or citrate. Iron in this form is capable of participating in a Fenton reaction. Although free iron is undetectable in serum (Gutteridge et al 1981), low molecular weight chelates of iron have been detected in human synovial fluid and cerebrospinal fluid at micromolar concentrations (Halliwell and Gutteridge 1984).

Likewise, copper which readily binds to protein would not appear to be capable of participating in free radical generation reactions. However, although bound, it would be accessible to H_2O_2 and capable of generating $\cdot OH$ which would react immediately in a site-specific manner with the binding component, resulting in the formation of $\cdot OH$ which is undetectable in free solution as suggested by Samuni et al (1981).

It must not, however, be assumed that all free radical processes are detrimental as they are essential to many normal biological processes such as the "respiratory burst" of macrophages employed in bacterial killing and the involvement of the superoxide radical in the action of indoleamine dioxygenase which catalyses cleavage of the indole rings of tryptophan, tryptamine and serotonin. Usually any free radical "leakage" from these processes can be efficiently removed before causing any damage by primary antioxidants (see section 1.6). It is only when their production or elimination is not tightly controlled that they become a highly destructive force.

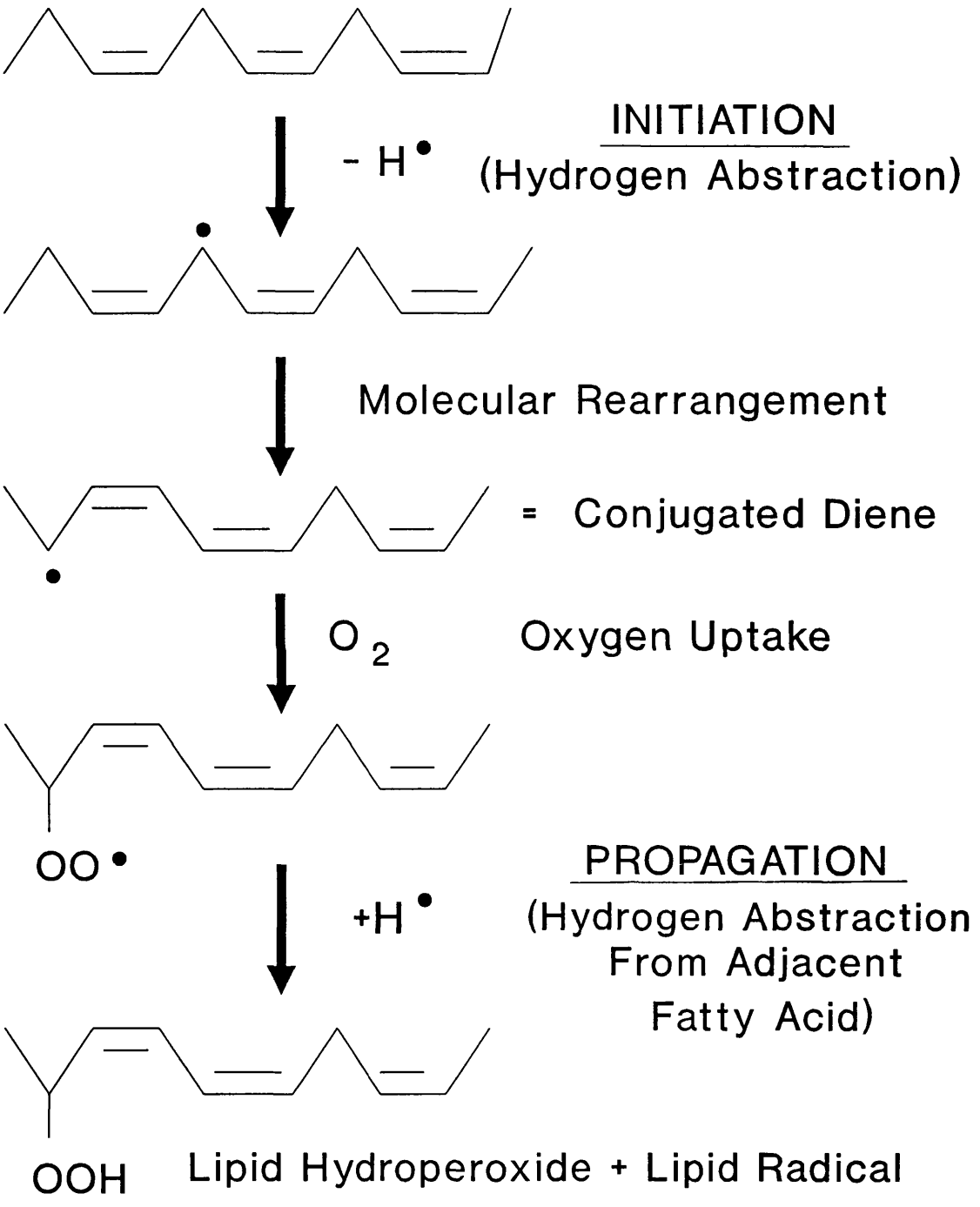
1.4.1 Lipid Peroxidation.

Because of the lipid soluble nature of vitamin E and the postulated involvement of peroxidation of neural membranes in the aetiology of the neuropathology associated with vitamin E deficiency, this section will describe the lipid peroxidation process. Lipid peroxidation can be considered as a special case of free radical damage where the stress is targeted at the polyunsaturated fatty acids located within the various cellular membranes. Lipid peroxidation can be broadly defined as the oxidative deterioration of polyunsaturated lipids, i.e. lipids containing more than two carbon-carbon double bonds. The sequence of reactions of lipid peroxidation are well recognised (Halliwell and Gutteridge 1985) and comprise three distinct stages, namely initiation, propagation and termination (fig 3).

Briefly, lipid peroxidation is initiated by the attack of any species that has sufficient energy to abstract a hydrogen atom from a methylene ($-CH_2-$) group adjacent to a carbon-carbon double bond on the fatty acid side chain. This results in the formation of a carbon centred free radical which in the presence of molecular oxygen will "stabilize" itself by forming a peroxy radical. This marks the end of the initiation stage.

The peroxy radical which is still highly reactive can abstract a hydrogen atom from an adjacent fatty acid side chain to form a "stable" lipid hydroperoxide, abbreviated to lipid peroxide,and another free radical. This is referred to as the propagation stage. This secondary radical can then lead to further peroxidation. Thus the peroxidative process follows a chain reaction.

FIG 3. LIPID PEROXIDATION PROCESS



The third stage of lipid peroxidation is termination where either two radicals combine to form a stable end product, or some compound donates a hydrogen atom to the reactive radical again producing a non- radical product and therefore preventing further peroxidation. The latter mode of termination will be discussed under antioxidants (section 1.6). Pure lipid peroxides are relatively stable at physiological temperatures, however, in the presence of transition metals, they will decompose to either a peroxy or alkoxy radical depending on the oxidation state of the metal (reactions 7 and 8). In both cases the radicals formed can stimulate lipid peroxidation.

$$L\text{-}OOH + Fe^{2+} \rightarrow Fe^{3+} + {}^{-}OH + L\text{-}O^{\cdot} \quad \text{Alkoxy radical (7)}$$

$$L\text{-}OOH + Fe^{3+} \rightarrow Fe^{2+} + {}^{+}H + L\text{-}OO^{\cdot} \quad \text{Peroxy radical (8)}$$

Decomposition of lipid peroxides leads to the formation of a complex mixture of peroxidation products. The postulated mechanisms for the formation of some of these products in addition to their toxic effects are discussed in chapter 3.

Halliwell and Gutteridge (1989) emphasised the importance in distinguishing between actual initiation and stimulation of lipid peroxidation. They proposed that the term "first-chain initiation" be applied to the initial abstraction of hydrogen. The abstraction of hydrogen atoms by peroxy radicals during the propagation stage is then referred to as "stimulation" of lipid peroxidation so as to prevent any confusion with "first-chain initiation"

In principle when studying lipid peroxidation related damage two mechanisms should be considered, (i) deterioration of membrane structure and function due to peroxidation of the membrane fatty acids and (ii) the ultimate damaging

process is actually due to reaction of one or more of the non radical products, e.g. aliphatic aldehydes, with some critical target(s).

Peroxidative stress is known to result in loss of membrane structure and integrity. Lipid peroxidation of membrane lipids is known to lead to an increase in the order and "viscosity"of the membrane bilayer, alters the thermotropic phase behaviour and decreases electrical resistance (Richter 1987). Scott et al (1989) showed that when a renal epithelial cell line was exposed to oxidative stress, i.e. H_2O_2, there was first a loss of plasma membrane potential, whereas changes in cellular size and eventually membrane permeability occurred relatively late in the overall process. Although these alterations took place more quickly at higher concentrations of H_2O_2, the same sequence of events was maintained. It is possible that progressive damage to the plasma membrane results in sequential abnormalities in permeability to small ions and hence decreasing membrane potential, with later increases in permeability to larger molecules.

In addition to damage to the lipid moiety of the membrane, free radical stress can also lead to concomitant peroxidation of proteins within, and associated with the membrane. For example, in-vitro peroxidation of a myelin fraction isolated from rat brain resulted in the loss of protein banding on SDS-polyacrylamide gels with an accumulation of protein at the origin due to cross-linking (Konat and Wiggins 1985).

As opposed to direct free radical attack on the various membrane components, it is possible that secondary damage caused by lipid breakdown products may be at least as equally important. The non radical-toxic products of lipid peroxidation, particularly the hydroxyalkenals have been the focus of much attention over the past decade. As discussed in greater detail in chapter 3, hydroxyalkenals are strongly electrophilic and readily react with the

sulphydryl anion (RS^-) of low molecular thiols (glutathione, cysteine) and the SH groups of enzymes and proteins. 4-Hydroxynonenal has been demonstrated to have numerous adverse effects including inhibition of calcium sequestration activity of rat liver microsomes (Benedetti 1984), and to interfere with the proliferation of Ehrlich ascites tumour cells by inhibiting DNA-polymerase (Wawra et al 1986). Compared to the highly reactive oxygen radicals, the non-radical lipid peroxidation products are relatively stable, and since it has been shown by Poli et al (1985) that peroxidation products can escape from isolated hepatocytes, it is possible that the original site of the free radical attack could lead to "peroxidative" damage some distance away.

When studying lipid peroxidation it is important to recognise that simply because there is an observed increase in the level of a certain peroxidation product associated with a disease or condition, that this does not automatically mean that lipid peroxidation is the causative factor in that condition. The observed lipid peroxidation may simply be a secondary consequence of the disease rather than actually leading to the observed changes. Increased lipid peroxidation can also occur as a consequence of tissue injury and breakdown. To prove a causal relationship it must be shown that the peroxidation either precedes or is concomitant with the cell damage and that prevention of the peroxidation by antioxidants also prevents the cell damage.

Finally, in a paper entitled "In praise of peroxidation", Dormandy (1988) argued that lipid peroxidation has received too much bad press, whereas in fact it may actually have an important survival role. He suggested that lipid peroxidation may be involved in the cell's "self-destruct" mechanism which removes old or injured cells. Dormandy postulated that the greatly diminished peroxidisability of malignant cells could be a result of a failure of this mechanism, allowing cancer cells to survive and multiply.

1.4.2 Lipid Peroxidation Products and Methods for Their Estimation.

Until relatively recently the only compounds definitely characterized as being peroxidation products in biological systems were malondialdehyde (MDA), ethane and pentane (Esterbauer 1985). However, with improvements in methodology, particularly high performance liquid chromatography (HPLC), it became increasingly clear that lipid peroxidation leads to the formation of a highly complex mixture of lipid degradation products. Since the overall process is oxidative, most of the compounds formed will contain some oxygen function such as aldehyde, keto, epoxy, hydroxy or carbonyl groups. For example, stressing rat liver microsomes with ADP-iron and an NADPH-generating system leads to the formation of four different 4-hydroxyalkenals, six different 2-alkenals and 5 n-alkanals and a mixture of other products including MDA (Esterbauer et al 1982). The precise mixture of peroxidation products generated in-vitro will depend on a number of factors including the free radical generating system employed and the fatty acid profile of the sample being stressed.

Because of the different stages in the lipid peroxidation process and the large number of different products formed, numerous methods are available to follow the process. Methods such as measurement of oxygen uptake will reflect the overall process, whereas estimation of the loss of fatty acids and the production of conjugated dienes provide data for specific steps.

When studying lipid peroxidation it is important to be aware of what exactly is being measured and not to draw too many conclusions form any one result. In addition, where possible it is advisable to employ two or more different methods, since no single technique by itself can be considered to give an accurate assessment of lipid peroxidation. The products of lipid peroxidation and methods of assessing lipid peroxidation will be discussed in greater detail in chapter 3.

1.5 Antioxidants.

Halliwell and Gutteridge (1989) defined an antioxidant as "any substance that when present at low concentrations compared to those of an oxidizible substrate, significantly delays or inhibits oxidation of that substrate". Therefore in the case of lipid peroxidation an antioxidant can be considered as any species that either prevents the initiation of the peroxidative process, interrupts the process once it has started or converts the peroxidation products to a stable non reactive form.

The first class of antioxidants, i.e. primary or preventative, act by preventing the initiation of lipid peroxidation by inhibiting the formation of highly reactive free radical species by removing their precursors or mopping up the free radicals themselves should they be formed. Included in this group are the enzymes catalase and glutathione peroxidase, both of which remove H_2O_2. Catalases usually contain a haem (Fe(III)-protoporphyin) group and catalyse the overall reaction shown in equation (9);

$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad (9)$$

Glutathione peroxidase catalyses the overall reaction below (10), where the reducing equivalents are provided by reduced glutathione, GSH;

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O \quad (10)$$

Oxidised glutathione (GSSG) is reduced back to GSH by glutathione reductase (reaction 11), with the NADPH being generated by the hexose monophosphate shunt;

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+ \quad (11)$$

Whereas catalase is specific for H_2O_2, glutathione peroxidase will reduce other peroxides besides H_2O_2.

Superoxide dismutases (SOD) acts by increasing the rate of dismutation of the superoxide radical as shown in reaction 5. Other compounds that can be included in the primary group of antioxidants are those that bind transition metals e.g. ferritin (iron) and ceruloplasmin (copper), and therefore prevent their reaction with H_2O_2, $\cdot O_2^-$ or lipid peroxides. Other compounds such as mannitol, formate and thiourea can react directly with $^{\bullet}OH$.

The second group of antioxidants interrupt peroxidation once it has started and are referred to as chain-breaking or secondary antioxidants. Examples of chain breaking antioxidants include vitamin E (see section 1.6.1), urate and ascorbate. Ascorbate has been shown to be an effective antioxidant in human blood plasma with detectable lipid peroxidation only commencing after ascorbate had been completely consumed (Frei et al 1989).

There are also a number of enzymes that act on the products of lipid peroxidation, and thereby prevent secondary damage. Particularly important in this class of antioxidants is the enzyme glutathione peroxidase. As stated previously, this enzyme can reduce H_2O_2 and other peroxides including lipid peroxides which are thus prevented from decomposing to form alkoxy and peroxy radicals and causing additional peroxidation. Certain glutathione-S-transferases have been shown to inactivate various toxic compounds including 4-hydroxyalkenals by conjugating them with reduced glutathione, e.g., the acidic glutathione transferase in rat heart (Ishikawa et al 1986). Hepatocytes possess a powerful enzymatic detoxification system which rapidly metabolizes alkanals, 2-alkenals and 4-hydroxyalkenals to the corresponding alcohols (Esterbauer et al 1985). Enzymes that repair the damage caused by peroxidative stress can also be included in this group of enzymes. These

include DNA repair, removal and replacement of damaged membrane lipids (Buttriss and Diplock 1988) and the increased turnover of peroxidatively damaged protein by specific proteolytic enzymes (Salo et al 1988).

1.5.2 Vitamin E (α-Tocopherol) as an Antioxidant.

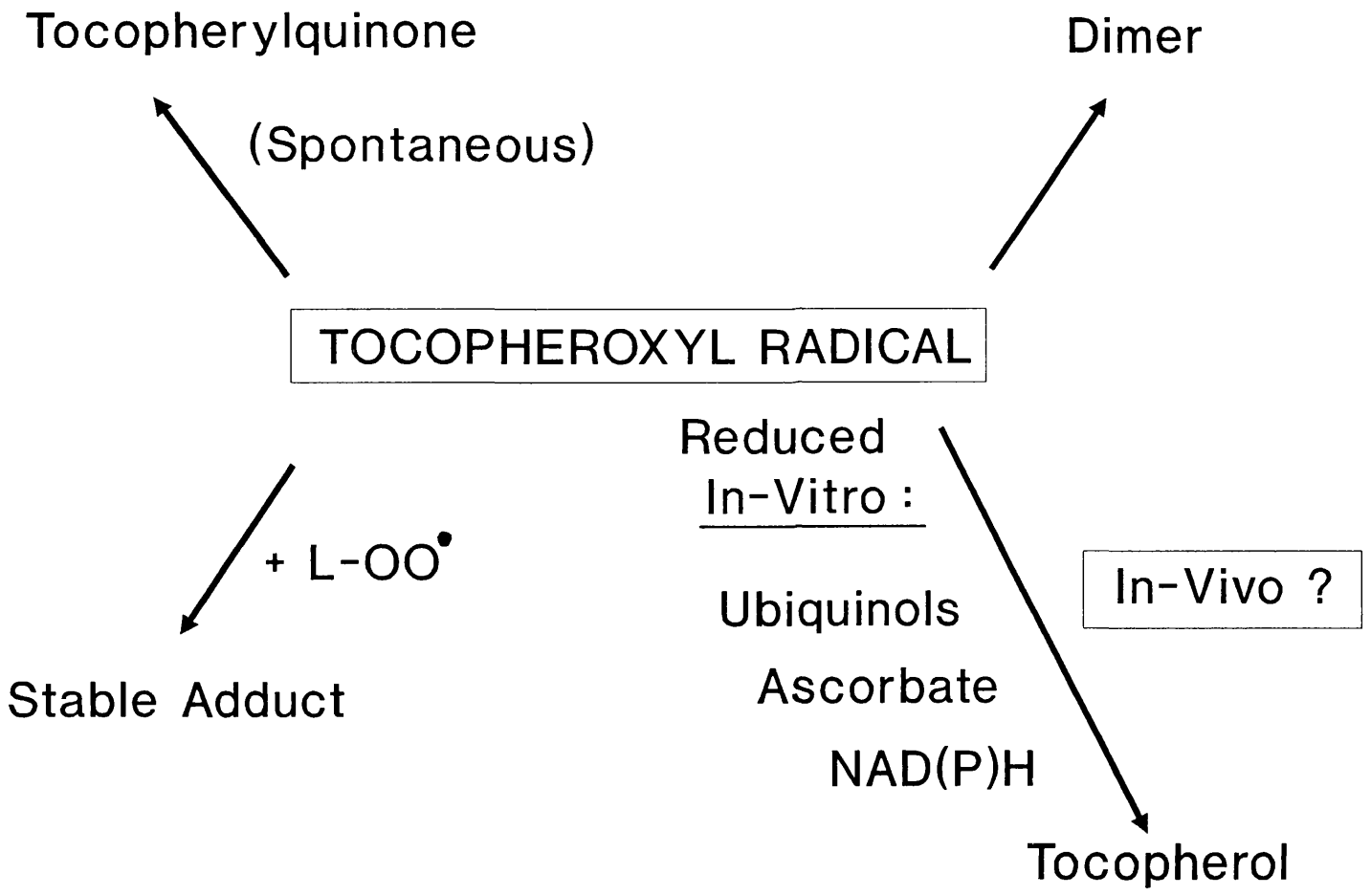
Vitamin E is included in the chain-breaking or secondary group of antioxidants. In-vitro, and probably in-vivo, vitamin E is thought to act as a lipid soluble antioxidant. It has been shown that α-tocopherol accounts for most, if not all, of the lipid soluble antioxidant in human blood (Burton et al 1983) as well as in various rat tissues and tumours (Cheeseman et al 1988). Although vitamin E can prevent peroxidation by reacting directly with the attacking free radical species and both quench and react with singlet oxygen, the major mode of action in biological membranes is assumed to be the scavenging of lipid peroxy and alkoxy radicals. Tocopherol (T-OH) is able to donate the labile hydrogen atom of its phenol group to the lipid radical i.e.

$$L\text{-}OO^{\cdot} + T\text{-}OH \rightarrow L\text{-}OOH + T\text{-}O^{\cdot} \qquad (12)$$

$$L\text{-}O^{\cdot} + T\text{-}OH \rightarrow L\text{-}OH + T\text{-}O^{\cdot} \qquad (13)$$

Although a tocopheroxyl radical ($T\text{-}O^{\cdot}$) is formed, it is not sufficiently reactive to abstract a hydrogen atom from membrane lipids since the radical is resonance stabilised by delocalizing the unpaired electron over the aromatic ring structure. Therefore vitamin E is a very effective radical trap. The tocopheroxyl radical can undergo a number of reactions (see fig 4). Firstly the tocopheroxyl radical can react with a second lipid radical (equation 14), probably by reaction of the peroxyl radical with position 9 of the chromane ring (Porter and Wagner 1986) i.e.,

Fig. 4 Possible Reactions of the Tocopheroxyl Radical



$$L\text{-}O^{\cdot} + T\text{-}O^{\bullet} \rightarrow \text{Inert products} \qquad (14).$$

The vitamin E radical can also spontaneously rearrange to form α-tocopherylquinone or alternatively form a dimer.

A number of mechanisms have also been suggested for the conversion of the tocopheroxyl radical back to tocopherol. Thus a number of in-vitro studies have demonstrated a synergistic effect on the inhibition of lipid peroxidation by vitamins E and C (e.g. Niki et al 1985), where ascorbate is thought to reduce the tocopheroxyl radical back to the active tocopherol form. However, evidence for a similar effect existing in-vivo remains unconvincing. Chen and Thacker (1985) investigated the effect of varying the vitamin E and/or vitamin C intake of rats on the biochemical changes associated with vitamin E deficiency, and concluded that vitamin C may partially spare the degradative metabolism of vitamin E only when vitamin E is present at low concentrations. In a recent study of a similar type, Burton et al (1990) using deuterated tocopherol in the guinea pig concluded that any "sparing" action of vitamin C on vitamin E in-vivo was of negligible importance in comparison with the normal metabolic processes which consume vitamin E.

A number of recent reports have provided evidence for NAD(P)H having a role in the regeneration of tocopherol from the tocopheroxyl radical in mitochondrial (Maguire 1989) and microsomal (Kagan et al 1990) membranes. It was shown that the in-vitro NAD(P)H-dependent decrease of the ESR signal of the analogues of the tocopheroxyl radical was due to their recycling by NAD(P)H-supported electron transport in these membranes. In addition the same group have also produced evidence that the antioxidant activity of ubiquinols results from their ability to reduce the tocopheroxyl radical, whereas their direct radical scavenging capacity appeared to be negligible in comparison to that of tocopherol (Kagan et al 1990a).

Reduced glutathione has been shown in-vitro to spare vitamin E. However, this is most likely due to an indirect effect as opposed to interacting directly with the tocopheroxyl radical, i.e. glutathione is thought to spare vitamin E by inhibiting processes that initiate lipid peroxidation in the aqueous phase (Barclay 1988). In addition glutathione is involved in the reduction of lipid peroxides via glutathione peroxidase and so may affect vitamin E consumption by decreasing peroxide-mediated oxidation of tocopherol.

Serbinova et al (1991) have recently provided evidence for α-tocotrienol having a much greater antioxidant activity than α-tocopherol. They found that there was a considerable discrepancy between the relative in-vitro antioxidant activities of these two forms of vitamin E with the conventional bioassays of their activity, and that it was necessary to compare the two forms of vitamin E under conditions that were important for their antioxidant function. For example α-tocotrienol manifests only 30% of the activity of α-tocopherol in rat resorption-gestation tests (Leth and Sondergaard 1977) whereas α-tocotrienol exhibited 40-60 times higher in-vitro antioxidant activity against iron / ascorbate and iron-NADPH induced lipid peroxidation in rat liver microsomal membranes. They suggested that the greater potency of α-tocotrienol was due to (i) its greater recycling efficiency from the chromanoxyl radical, (ii) its more uniform distribution within the membrane and (iii) the stronger disordering of membrane lipids, facilitating the interaction with lipid radicals. However, because of the greater membrane disordering effect associated with α-tocotrienol it is possible that the tocopherol form of vitamin E represents a balance between a relatively effective lipid soluble antioxidant activity and the ability to increase membrane stability and order.

Finally, Dean and Cheeseman (1987) have shown that vitamin E can also protect membrane proteins from free radical damage. In a study of the fragmentation of monoamine oxidase in submitochondrial particles following free radical stress, they demonstrated that the damage to the protein was due to attack by lipid peroxides formed within the membrane, i.e. protein modification was dependent on concomitant lipid peroxidation. Therefore vitamin E, by preventing lipid peroxidation, also protected against protein degradation.

1.6 Vitamin E as a Membrane Stabiliser.

In addition to vitamin E functioning as a lipid soluble antioxidant, it is thought that tocopherol also plays a role in the stabilisation of the membrane by way of its physicochemical interaction with other components of the membrane. Diplock and Lucy in 1973 hypothesised that the tocopherol molecule is anchored within the membrane by its phytyl side chain closely associating with the arachidonic acid fatty acid side chains of the membrane lipids. The methyl groups of the phytyl side chain of tocopherol were thought to interact with the cis double bonds "pockets" of arachidonic acid within the hydrophobic core of the membrane, with the functional hydroxyl group of the chromane ring positioned at the membrane surface. They subsequently added to this model by suggesting that vitamin E may interact on a dynamic basis with more than one polyunsaturated phospholipid as opposed to a strict 1:1 molar ratio between the two and concluded that "it is not impossible that α-tocopherol may have both antioxidant and structural functions in biological membranes in-vivo" (Maggio et al 1977). This hypothesis has however been criticized since it has been calculated that at most there is only approximately one molecule of α-tocopherol per 500 arachidonic acid molecules in the membrane.

At present it is thought that vitamin E is not homogeneously distributed within the membrane but is preferentially partitioned into the most fluid domains (Gomez-Fernandez et al 1989), which will cause α-tocopherol to be associated with the most unsaturated fatty acyl chains. It is also known that α-tocopherol can protect membranes from the damaging effects of phospholipases, especially phospholipase A by interacting with the phospholipase hydrolysis products. Kagan (1989) demonstrated that tocopherol can offset the membrane disordering effects of free fatty acids and lysophospholipids on phospholipid bilayers by forming complexes with them within the membrane. In-vivo vitamin E deficiency caused an increase in the sensitivity of the membrane preparations (synaptosomes, rat liver microsomes and skeletal muscle sacroplasmic reticulum) to these hydrolysis products which could be overcome by enriching the diet with vitamin E or adding exogenous α-tocopherol in-vitro.

1.7 Friedreich's Ataxia - An Antioxidant Defect ?

Friedreich's ataxia (FA) is an autosomal recessive neurological condition which exhibits very similar clinical and neuropathological features to those seen in chronic and severe vitamin E deficiency in man. Despite being first described almost 130 years ago, no consistent underlying metabolic defect has so far been identified. Because of the similarities between FA and vitamin E deficiency, it was possible that they shared a common or similar aetiology. Although serum concentrations of vitamin E have been shown by Muller et al (1987) to be normal in patients with FA, a relative deficiency at the cellular level could not be ruled out. This could result, for example, from a selective defect of cellular incorporation or utilization of the vitamin resulting in reduced antioxidant capacity.

1.8 Aims of the Present Study.

Because of the known consequences of a chronic and severe deficiency of vitamin E on neurological function and integrity, and uncertainty as to why neurological tissues should be particularly susceptible to damage during deficiency, it was of interest to investigate lipid peroxidation of neural tissues and fractions during experimental vitamin E deficiency in the rat.

Firstly, various tissues (both neural and non-neural) from the vitamin E deficient and control rats were examined for evidence of in-vivo lipid peroxidation to see whether a deficiency of vitamin E resulted in increased endogenous lipid peroxidation.

Secondly, the effect of vitamin E deficiency on the susceptibility to in-vitro peroxidative stress was studied in neural and non-neural tissues, and also in different neural membrane fractions isolated from myelinated axons. In the case of the neural fractions it was hoped that the results might provide some indication as to which regions of the neuron would be most susceptible to a deficiency of vitamin E and whether they agreed with the known neuropathology.

Finally, studies similar to those carried out on vitamin E deficient rats were also performed on post mortem tissues from patients with Friedreich's ataxia and controls to examine the possibility of an antioxidant defect in the aetiology of this disease.

CHAPTER 2.

ISOLATION OF NEURAL FRACTIONS FROM RAT BRAINSTEM

2.1 Introduction

2.2 Overall structure of the nervous system

2.2.1 General structure of the neuron

2.2.2 The neuron and vitamin E deficiency

2.3 Isolation of neural fractions from rat brainstem

2.4 Assessment of purity of the isolated neural fractions

2.4.1 Protein assay

2.4.2 5'-Nucleotidase assay

2.4.2.1 $ZnSO_4$ - $Ba(OH)_2$ Precipitation Procedure

2.4.3 Acetylcholinesterase assay

2.4.4 2',3'-Cyclic nucleotide 3'-phosphodiesterase assay

2.4.5 Marker enzymes specific activities and recoveries

2.4.6 Purity assessment using electron microscopy

2.5 Discussion

2.1 Introduction

A severe and chronic deficiency of vitamin E results in a characteristic neurological syndrome (Muller et al 1983) where the main neuropathological feature is a selective loss of large calibre myelinated axons (Nelson 1983). This chapter provides a description of the neuron and a detailed description of the fractionation procedure developed and validated to isolate neural fractions from these cells, or more specifically, from their myelinated axons. These fractions were to be investigated in studies of in-vitro lipid peroxidation (chapter 7) in order to gain some idea of the relative susceptibilities of the different components of the myelinated axon to peroxidative stress and to examine what effect a deficiency of vitamin E would have on the peroxidisibility of these fractions.

2.2 Overall Structure of the Nervous System

The nervous system is arbitrarily divided into the central nervous system (CNS) comprising the brain and spinal cord, and the peripheral nervous system (PNS) which is made up of the 12 pairs of cranial nerves and all the other nerves of the body. There are basically two classes of cells in the nervous system. Firstly, nerve cells or neurons, responsible for the conduction of electrical impulses down the nerve, and secondly the neuroglial cells or glia which have important ancillary functions. For example, the astrocytes whose functions include holding together the delicate neurons and helping to create the blood-brain barrier. An extensive capillary network supplies this complex neuronal-glial system.

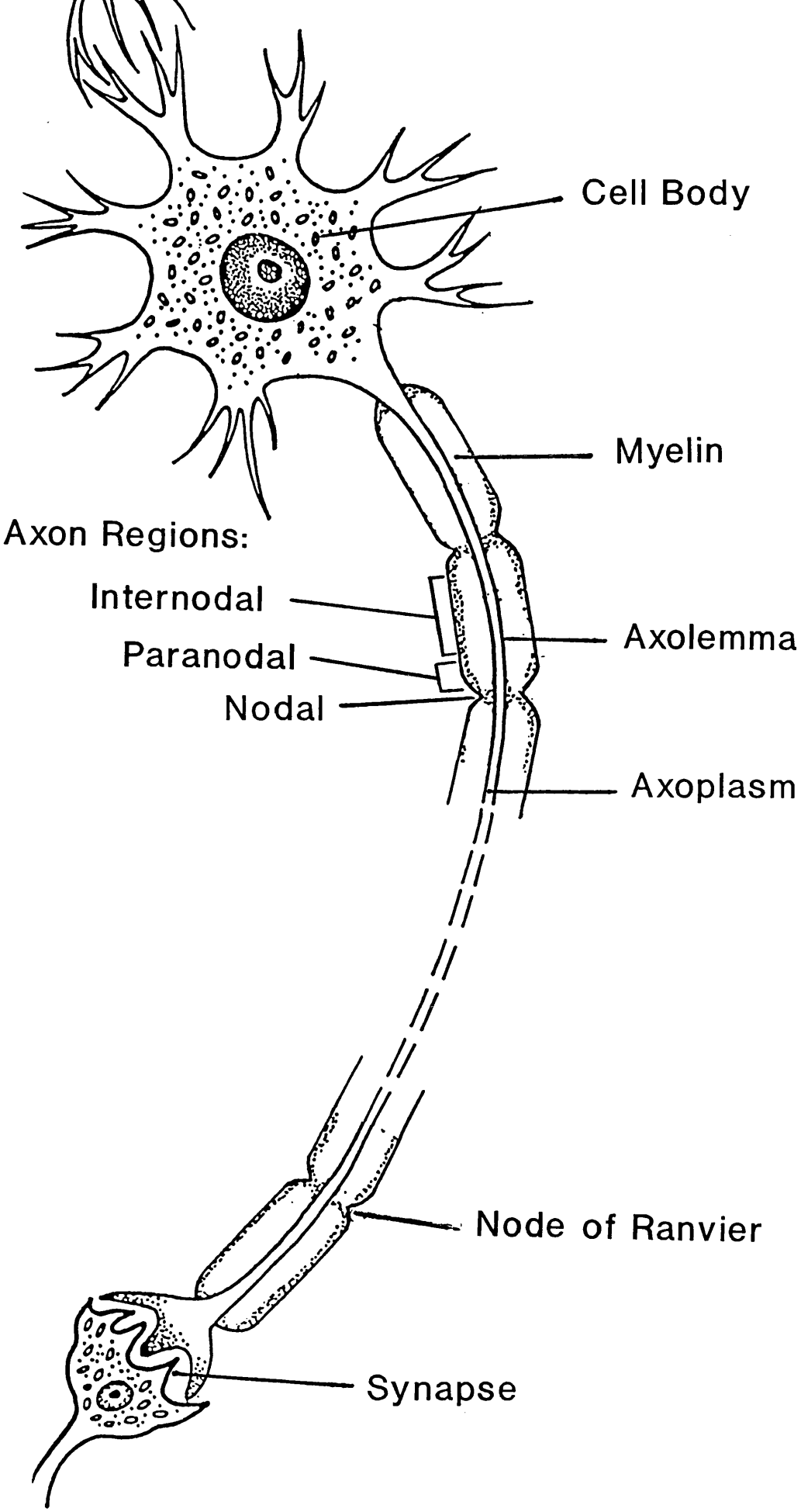
Functionally and structurally there are many types of neurons. A motor or efferent neuron is one that transmits impulses to muscles and/or glands, whereas a sensory or afferent nerve cell propagates a sensory impulse. The main properties that distinguish neurons from other cell types are their specialisation for the conduction of impulses, their great diversity of size and

shape, and the their reduced ability to replicate.

2.2.1 General Structure of the Neuron.

A simple diagrammatic representation of a typical neuron is shown in fig 1. The neuron is composed of three main sections, namely the cell body, the axonal process and the synaptic region. The cell body which contains the nucleus is the metabolic centre of the neuron. Neurons are amongst the most actively synthesising cells as evidenced by the abundant Nissle substance, i.e. rough endoplasmic reticulum (ER) surrounded by "clouds" of free polyribosomes. The cytoplasm of the cell body, referred to as the perikaryon, is also rich in other organelles including mitochondria, smooth ER, Golgi apparatus, lysosomes and multivesicular bodies which are thought to be lysosomal in origin. Also present are the three cytoskeletal fibrillar polymer proteins, i.e., microtubules, microfilaments and neurofilaments. Projecting from the cell body are numerous short processes, the dendrites, which synapse with other neurons and conduct impulses towards the cell body. From the cell body a single long axonal process transmits the electrical signal away and out to the dendrites of other neurons or to muscles or glands. The contents of the axonal cytoplasm, the axoplasm, are enclosed within the axonal plasma membrane which is referred to as the axolemma. The axoplasm includes scattered mitochondria, patches of smooth ER and tubulovesicular profiles (probably derived from smooth ER).

The axoplasm also has an extensive cytoskeletal system with the microtubules and neurofilaments arranged longitudinally and the microfilaments arranged both longitudinally and circumferentially to the axon. These three fibrillar proteins account for 20% by weight of the total protein content of the axoplasm and are thought to be cross linked by various filament associated proteins. The image of the axoplasm that emerges is of a dense 3-dimensional network of fibrillar proteins giving the axoplasm a gel like consistency.


Cell Body
Myelin
Axon Regions:
Internodal
Paranodal
Nodal
Axolemma
Axoplasm
Node of Ranvier
Synapse

The axonal plasma membrane is surrounded by a lipid rich multilamellar myelin sheath which is not formed by the neuron itself but laid down spirally by specialised glial cells, namely the oligodendrocytes in the CNS and by the Schwann cells in the PNS. The nodes of Ranvier indicate those points at which regions formed by different glial cells adjoin. The axonal plasma membrane at the node of Ranvier has no myelin covering and is referred to as the nodal axolemma. On either side of the node are the paranodal regions where the myelin sheath begins and may even form close contact with the axolemma. The internodal region lies between the two paranodal regions at the end of each segment of myelin. The terminal portions of the axon enlarge to form the synaptic region.

2.2.2 The Neuron and Vitamin E Deficiency.

Although neurons vary greatly in size and shape, many nerves contain neurons with very long axons. For example the axon of a motor neuron in the spinal cord that innervates a toe muscle of a six foot man has a length of about 5,000 cell body diameters. Schwartz (1980) has likened this to a football representing the cell body resulting in an axonal process equivalent to 15 football fields. The neuron's nucleus and the machinery for the synthesis and assembly of proteins and membranes are restricted to the perikaryon and so the cell body is responsible for the maintenance of a potentially huge volume of axoplasm and axolemma. The proteins and membranes produced in the perikaryon are moved down the axon by an energy dependent process known as axonal transport. In fact it is thought that the vast majority of the cell's energy is consumed in maintaining the various components of the axon, whereas the amount expended on the actual signalling process is probably trivial by comparison. Although some transported materials such as synaptic vesicles will only be used at the axon terminals, others will be consumed or incorporated as they are moved down the axon. It has been estimated by Gillette et al (1981) that probably only about 5% of the material leaving the

cell body actually reaches the terminal regions. This system leaves the neuron susceptible to any condition that reduces the supply of a particular nutrient to the neuron or interferes with the transport of that nutrient within the neuron itself.

From the foregoing discussion it is not surprising that the greatest degenerative changes seen during vitamin E deficiency are in the primary sensory neurons since these nerve cells are known to have the longest axonal processes. In addition the damage is most pronounced in the rostral segments of the dorsal columns of the spinal cord and in the terminal portions of the peripheral nerves, ie. those segments most distal from the cell body. This is described as of a distal or "dying-back" neuropathy. Evidence suggests that the accompanying demyelination seen in vitamin E deficiency is secondary to damage to the axon, i.e. the neuropathology is consistent with a primary axonopathy followed by secondary demyelination (Wichman et al 1985, Thomas et al 1984).

The mechanism resulting in the observed neuropathology is unknown, although studies of Nelson (1987) suggested that it is related to the antioxidant function of vitamin E rather than its postulated effect on membrane order and packing. He found that supplementing the diet of rats maintained on a vitamin E deficient diet with synthetic antioxidants prevented the development of the neuropathy. Nelson also postulated that the primary site of oxidative damage is the axolemma which then leads to demyelination. Goss-Sampson et al (1988) suggested that the distal axonopathy might result from a defect in axonal transport. Southam et al (1991) hypothesised that the mitochondria and smooth ER were the initial sites of free radical attack, with peroxidative damage to the former organelle resulting in a reduction in the axon's capacity to generate energy. This in turn would produce a defect in the energy requiring axonal transport process.

Because of the extreme susceptibility of nerve cells, it was decided to study the effect of vitamin E deficiency on different neural fractions. The objective was not to establish the sequence of events leading to the observed neuropathology but to see which neural components of the myelinated axon were most susceptible to peroxidative stress and the effect vitamin E had on these membranes.

2.3 Isolation of Neural Fractions from Rat Brainstem.

Three neural fractions, i.e. myelin, a fraction enriched in axolemma and a fraction containing the axoplasmic membranes and organelles were isolated from rat brainstem using a modification of the method described by DeVries (1981). The fractionation procedure was based on the following principles; (i) isolation of a myelinated axon fraction from a dilute starting whole homogenate by repeated flotation in a buffered salt-sucrose medium. Because of their lipid rich myelin sheath myelinated axons have a lower density than the other tissue components so that they will float to the top of the tube when spun in a dense sucrose solution with the remaining material being pelleted, (ii) subjecting the myelinated axons to a hypotonic osmotic shock which results in the myelin sheath swelling, vesiculating and breaking away from the axolemma, (iii) separation of the different fractions on discontinuous density gradients, taking advantage of the differences in their lipid compositions and hence densities and (iv) additional purification to eliminate cross contamination. Note that the method only isolates components that form the myelinated axon portion of the neuron and should not include the cell body or synaptic regions.

Reagents:

TES buffer; [N-tris (hydroxymethyl) methyl-2-amino-ethanesulphonic acid], 0.1M, pH 7.5

EGTA; [ethyleneglycol-bis-(ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid], 0.1M, pH 7.5. EGTA (3.8g) was added to 50ml H_2O and dissolved by adding 25ml 1M NaOH. The pH was adjusted to 7.5 with 1M HCl and brought up to 100ml with H_2O.

NaCl; 1.5M (stock).

All solutions were made up in ultrapure water (Milli-RO / Milli-Q Water System purified to 18 Megohms) and were stable at 4°C for one week. Sucrose solutions (reagent grade) were made up either on the day of use or on the previous day and stored at 4°C. Concentrations were as described in the fractionation procedure.

Fractionation Procedure

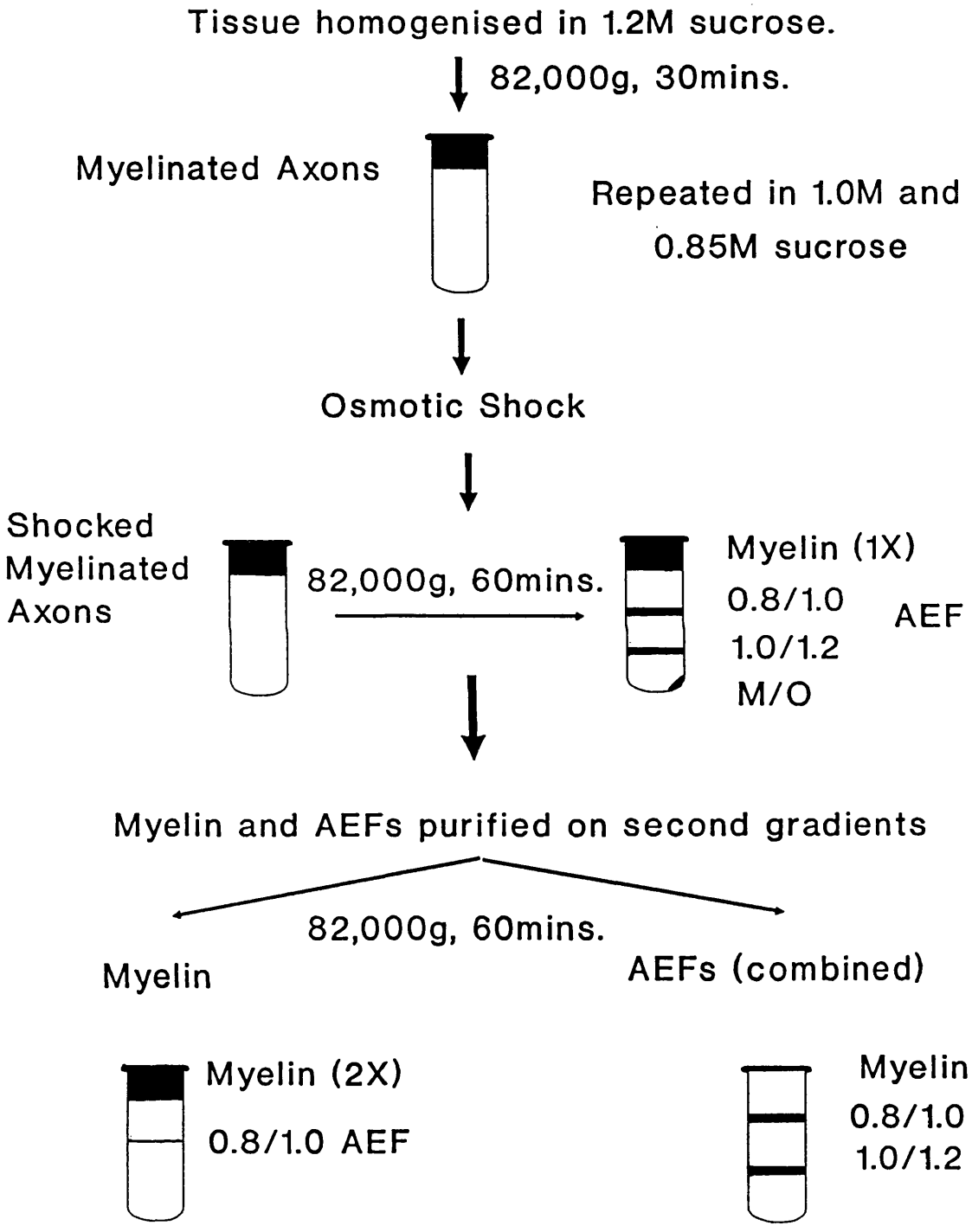
All sample handling was carried out on ice in a cold room while all centrifugations were carried out at 4°C and 82,000g using an MSE Prepspin 65 ultracentrifuge. A summary of the procedure is presented in fig 2.

(1). The starting tissue was 3 brainstems or sufficient tissue to give 25ml of a 2.5% (wet wt./vol.) whole homogenate.

(2). Tissues were chopped into a fine "mince". 100mg of this mince was homogenised in 4ml of 40mM trizma buffer, pH 7.4 and stored at -70°C. This fraction was taken as the whole homogenate.

(3). The remainder was homogenised in 25ml of 1.2M sucrose using a motor driven glass-teflon potter homogeniser (Fluid Equipment Co. Ltd.) at a medium setting, with 8 strokes of a loose pestle and 4 strokes of a tight pestle. This sucrose solution, and all sucrose solutions used up to step 5 of the procedure, contained 0.15M NaCl and 10mM TES, pH 7.4. The homogenate was added to a 25ml polypropylene tube and spun for 30 min.

Fig.2 Brainstem Fractionation Procedure



(4). The resulting floating layer of myelinated axons was compact enough to remove in one piece with a pre-wetted flame-sealed pasteur pipette and transferred to a loose potter homogeniser and thoroughly resuspended in 25ml of 1.0M sucrose. The sample was then centrifuged for a further 30 min.

(5). Step 4 was repeated using 0.85M sucrose.

(6). **Osmotic shock:** The myelinated axon fraction was homogenised in 15ml of 10mM TES, pH 7.4 with 10 strokes of a tight pestle at a high rpm setting, diluted to 25ml with TES and spun for 30 min to give a pellet of shocked myelinated axons.

(7). **Fractionation of shocked myelinated axons (SMA);** All gradient sucrose solutions contained 1mM EGTA and 1mM TES, pH 7.5. The SMA pellet was resuspended in 12ml of 0.75M sucrose and divided evenly between three tubes containing discontinuous density gradients constructed as follows; 2ml of 1.2M, 3ml of 1.0M, 3ml of 0.8M sucrose and 4ml of the SMA fraction in 0.75M sucrose. The gradients were then spun for 60 min. This resulted in a myelin band situated at the top of the gradient with two axolemma bands located in the middle. Small pellets (see below) were present in each of the three tubes.

(8). A tube slicer was used to cut between the myelin and the axolemma-enriched fractions (AEFs) so that the myelin fraction could be aspirated off the gradient. The two axolemma bands from each of the three gradients were aspirated off and combined.

(9). **Additional purification steps**

The three pellets were combined and resuspended in 12ml of 10mM TES, pH 7.5 and spun for 30 min. The pellet was resuspended in 1.5ml of 40mM trizma buffer, pH 7.4 and stored at -70°C. This fraction was referred to as the axoplasmic membranes and organelles fraction, i.e. M/O.

(10). The combined myelin fractions from step 7, termed 1x-myelin since they were prepared from a single density gradient, were diluted at least twice with 10mM TES, pH 7.5 and spun for 45 min.

(11). The myelin pellet was resuspended in 11.5ml of 0.75M sucrose and respun in three tubes as in step 7 to give 2x-myelin.
(12). The myelin bands were combined and pelleted in 10mM TES, resuspended in 5ml of 40mM trizma, pH 7.5 and stored at -70°C.
(13).The axolemma bands from step 7 were combined with any additional lower density axolemma (0.8/1.0) that may have been produced when 1x-myelin was purified on a second gradient (step 10). The fraction was pelleted in 10mM TES by spinning for 45 min, and resuspended in 5mls of 1.0M sucrose. A single gradient was prepared comprising 4ml 1.2M sucrose, 5ml sucrose containing the axolemma-enriched material and overlayed with 3ml of 0.8M sucrose. The gradient was spun for 60 min.
(14). The fractions of similar density were aspirated off the gradient, combined and pelleted in 10mM TES. The AEF pellet was resuspended in 1.5ml 40mM TES, pH 7.5 and stored at -70°C.

2.4 Assessment of the Purity of the Isolated Neural Fractions.

Two methods were employed to assess the purity of the different fractions, namely enrichment of marker enzyme and electron microscopy (EM). The following sections will deal with enzyme enrichment while EM results are presented in section 2.4.6.

Since no protein or enzyme unique to the axolemma has been described to date, the putative plasma membrane markers 5'-nucleotidase and acetylcholinesterase were used to determine the purity of this fraction. DeVries (1981), whose fractionation procedure was employed, used these two enzymes, in addition to Na^+,K^+-activated ATPase, to characterise AEF isolated from rat brainstem. Myelin purity was assessed by measuring the activity of the enzyme 2',3'-cyclic nucleotide 3'-phosphodiesterase. A marker enzyme assay was not established for the axoplasmic membranes and organelles fraction (M/O) since initially I was only interested in studying the

myelin and axolemma-enriched fractions. However, the activities of the other markers were estimated in the M/O fraction which gave information regarding its contamination with myelin and AEF. Electron microscopic examination was carried out on all three neural fractions.

2.4.1 Protein Estimation

The activities of the marker enzymes were related to protein concentrations which were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce, UK) which was based on the method of Smith et al (1985).

Procedure:

All assays were carried out in duplicate. 0.1ml of sample was mixed with 2ml of working reagent as directed by the kit and incubated at 37°C for 30 min. The absorbances of the assay mixtures were read at 562nm against a reagent blank. The assay (in duplicate) was linear up to at least 40μg bovine serum albumin (BSA) (fig 3).

2.4.2 5'-Nucleotidase Assay

5'-Nucleotidase was assayed by a modification of the method of Seymour and Peters (1977). The assay involved the hydrolysis of radiolabelled adenosine monophosphate (AMP) followed by separation of the labelled adenosine from the unhydrolysed substrate. Unhydrolysed AMP was removed from the solution using $ZnSO_4$ / $Ba(OH)_2$ where the AMP was adsorbed onto the barium sulphate precipitate.

Reagents:

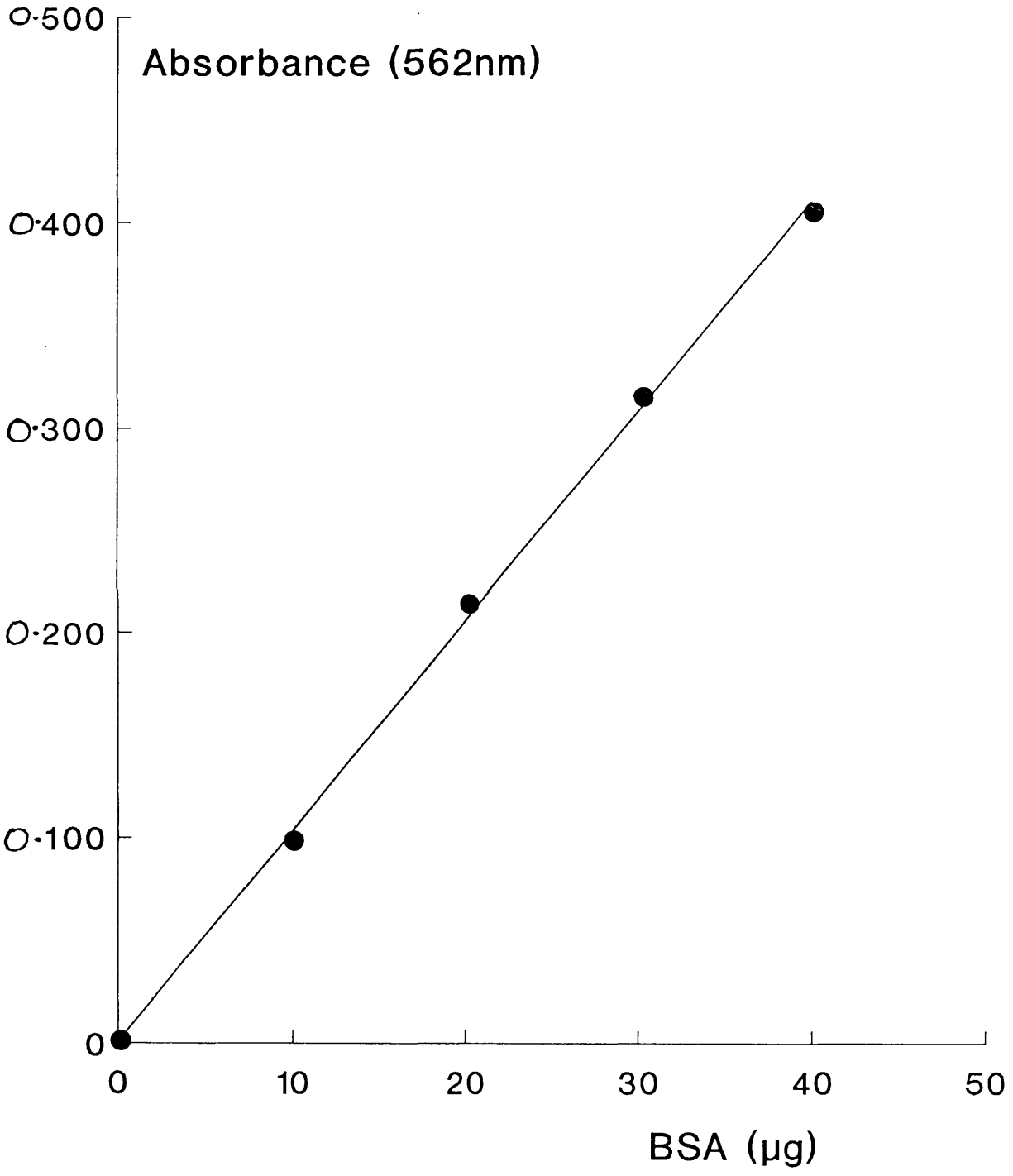
30% Trichloroacetic acid (TCA).

10M NaOH.

0.15M $ZnSO_4$.

$Ba(OH)_2$; 8g were added to 100ml of H_2O and heated to almost boiling point

Fig 3. Bovine Serum Albumin (BSA) Standard Curve for BCA Protein Assay



with continuous stirring. The saturated solution was left standing overnight at room temperature and filtered before use.

The substrate solution was freshly prepared in 250ml 60mM piperazine buffer, pH 9.0 and contained 0.12mM AMP, 5μl [2-^{3}H]-AMP (Amersham Int., 1mCi/ml), 24mM $MgCl_2$ and 12mM β-glycerophosphate. The latter compound acts as a substrate diverter for alkaline phosphatase so as to eliminate non-specific hydrolysis of AMP.

Procedure:

0.1ml of sample was mixed with 0.5ml of the substrate solution and incubated at 37°C for 60 min.

The reaction was stopped by adding 0.12ml 30% TCA and vortex mixing.

The pH was adjusted with 1 drop 10M NaOH.

0.2ml of the $ZnSO_4$ and $Ba(OH)_2$ reagents were added, the mixture vortex mixed for 30sec and then spun for 10 min at 3000rpm in an Heraeus RF minifuge.

Second aliquots of $ZnSO_4$ and $Ba(OH)_2$ were added without disturbing the first precipitate and respun for 10 mins.

500μl of the supernatant was counted in 10ml scintillant (Optiphase, Fisons UK) on an LKB Wallac Rackbeta liquid scintillation counter using external standardisation for quench correction.

2.4.2.1 $ZnSO_4$ - Ba(OH)$_2$ Precipitation Procedure

The zinc sulphate - barium hydroxide procedure as described by Seymour and Peters (1977) was found to be inefficient and non-reproducible in removing unhydrolysed AMP from the assay mixture. The procedure of Seymour and Peters involved removing AMP from solution with two additions of equal volumes of 0.25M $ZnSO_4$ and $Ba(OH)_2$ essentially as described in the assay procedure above. Two modifications were made to achieve reproducible and efficient precipitation. Firstly, after the reaction was stopped with TCA, the

pH of the mixture was adjusted to >12 by adding 1 drop of 10M NaOH so as to ensure that the phosphate group was in the doubly ionized state, i.e. $-PO_3^{2-}$ and would therefore adsorb to the Ba^{2+} - Zn^{2-} precipitation complex. Although adjusting the pH greatly improved the removal of AMP, the results were still inconsistent. The published method stated that 0.25M solutions of both reagents were used. A solution of $Ba(OH)_2$ at this concentration could not however be made even after heating to boiling point - with approximately 30% remaining undissolved. There was therefore an imbalance between the concentration of the two reagents. The $Ba(OH)_2$ reagent was made by adding sufficient solid in 100ml H_2O to give a 0.25M "solution", heated to almost boiling point, left standing at room temperature overnight and filtered before use. The effect of varying the concentration of $ZnSO_4$ on the precipitation efficiency was then studied. The results are given in table 1. The best results were obtained with 0.15M and 0.10M $ZnSO_4$ which were probably closest to the actual concentration of $Ba(OH)_2$. 0.15M $ZnSO_4$ was therefore used routinely in this assay.

Table 1: Effect of varying $ZnSO_4$ concentration on the efficiency of removal of AMP from solution (n=3).

$ZnSO_4$ Concentration (mol/l)	Precipitation Efficiency (%)
0.25	84
0.20	74
0.15	98
0.10	96
0.05	81

Validation of Assay;

When the activity of rat brainstem whole homogenate (3μg protein) was followed with time (in duplicate) the assay was found to be linear up to at least 120 min or approximately 10,000dpm (fig 4). Similarly, when increasing amounts of sample were assayed (in duplicate) for a fixed time (60 min), the assay was linear up to 6μg protein or up to approximately 10,000dpm (fig 5).

2.4.3 Acetylcholinesterase Assay.

Acetylcholinesterase (AChEase) was assayed by a modification of the method of Tucek (1974) which measured the release of [1-^{14}C]-acetate from [1-^{14}C]-acetylcholine chloride (306μCi/mg, Amersham Int.).

Reagents:

(A) Sample buffer containing 25mM sodium phosphate, pH 7.5 and 0.5% Triton X-100.

(B) Assay buffer containing 20mM sodium phosphate, pH 7.4, 0.25% Triton X-100 and 0.1mM ethopropazine (pseudocholinesterase inhibitor).

(C) 5μCi/ml ^{14}C-acetylcholine in 30mM unlabelled acetylcholine chloride.

(D) 2mM eserine salicyclate (cholinesterase inhibitor).

(E) 15mg/ml sodium tetraphenylboron in 3-heptanone.

Procedure:

The sample was suitably diluted with the sample buffer (A), vortex mixed for 30 sec and centrifuged at 3000rpm in an Heraeus RF minifuge to give clear supernatant.

20μl of the supernatant, containing the extracted AChEase, was mixed with 90μl of assay buffer (B) and 10μl of substrate (C) and incubated at 37°C for 60 min.

The reaction was stopped by adding 250μl solution (D).

Unhydrolysed acetylcholine was extracted by vortex mixing for 15 sec with

Fig 4. 5'-Nucleotidase Activity Versus Time

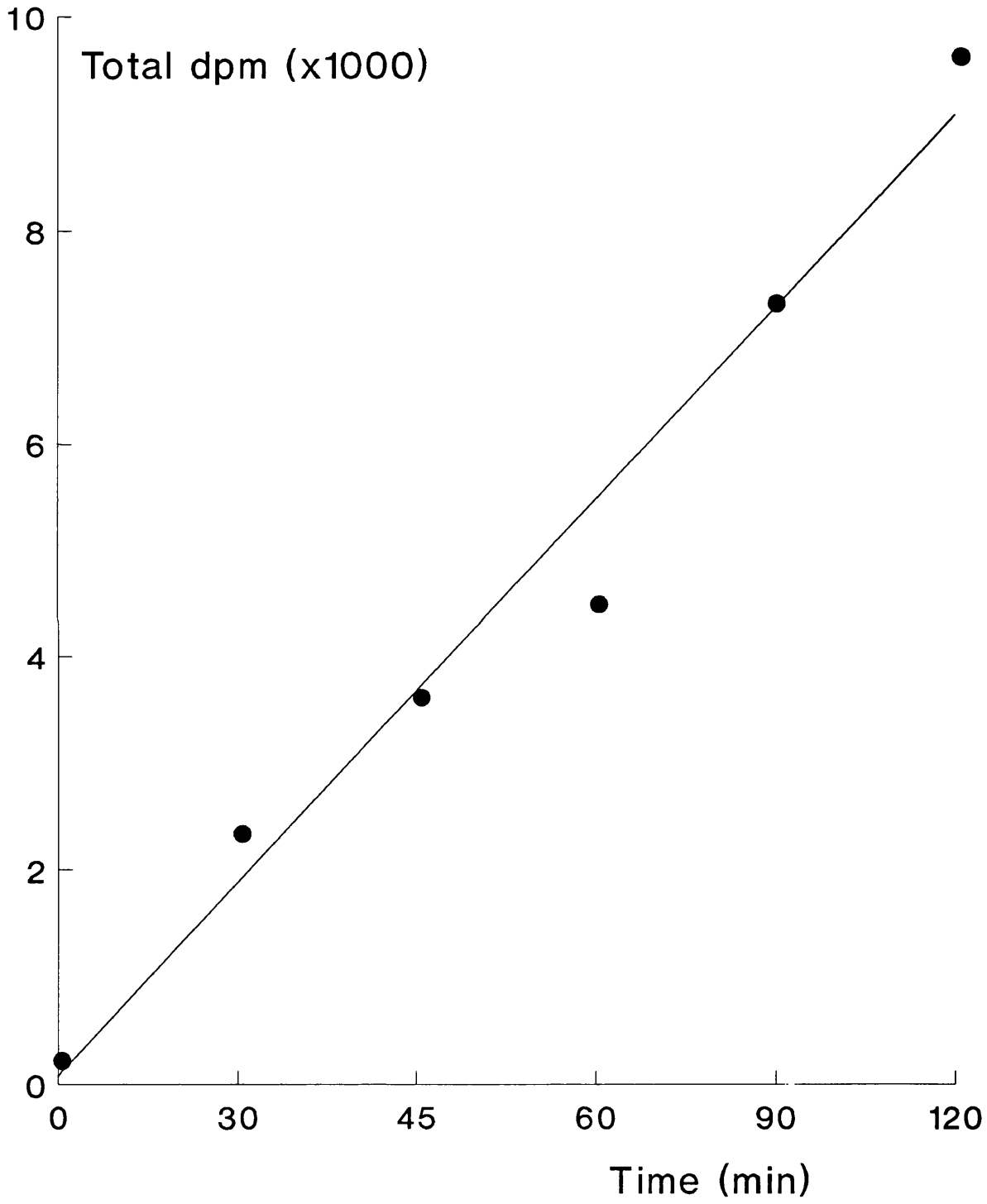
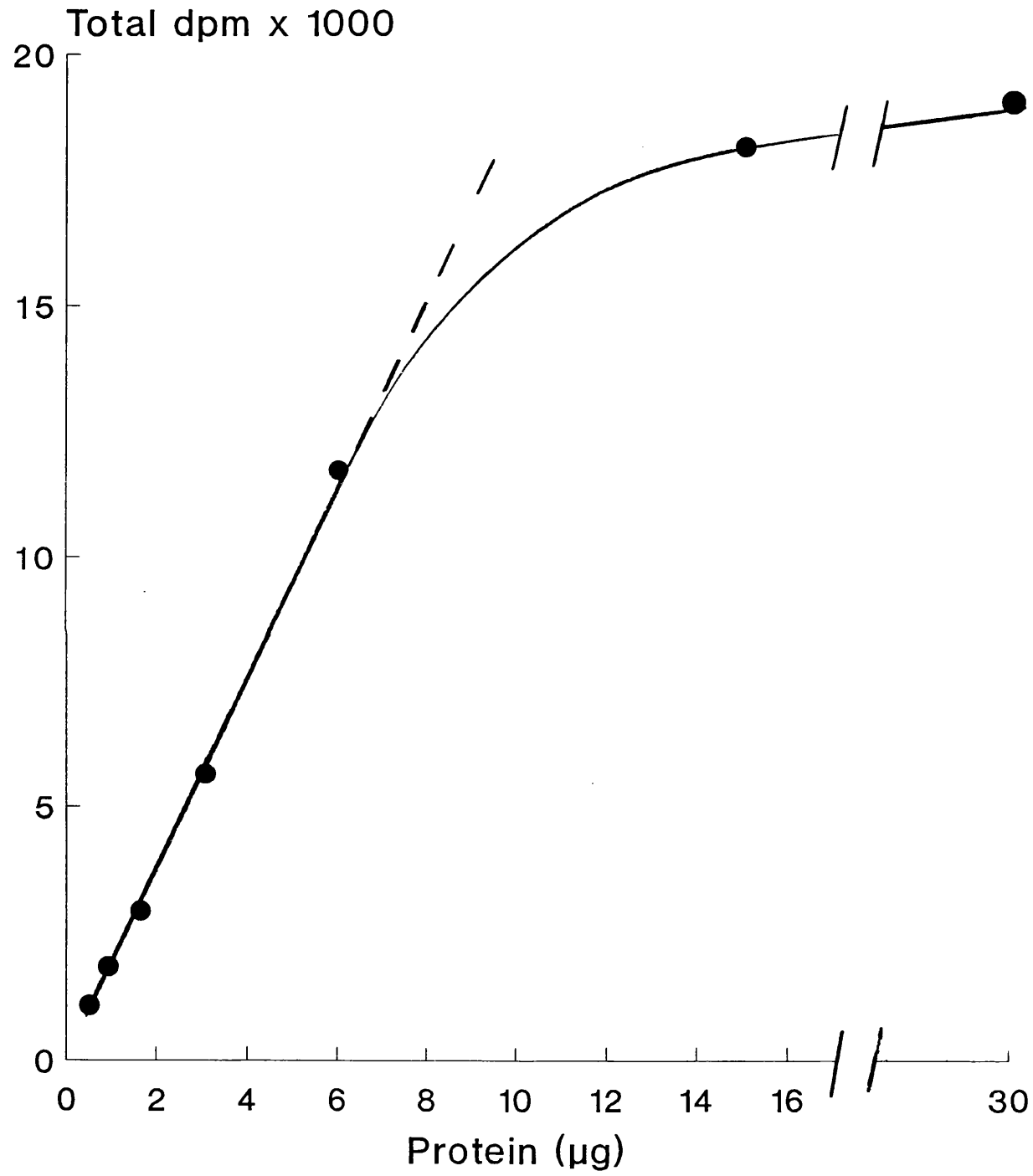


Fig 5. 5'-Nucleotidase Activity versus Protein Concentration



400μl of solution (E), spinning at 3000rpm for 5 min and discarding the supernatant. A second extraction was carried out as above.
The radioactivity in a 250μl aliquot of the remaining aqueous phase was counted in 10ml scintillant (Optiphase, Fisons UK) on an LKB Wallac Rackbeta liquid scintillation counter using external standardisation for quench correction.

Validation of assay

The assay was carried out in duplicate using whole homogenate of rat brainstem (8μg protein) and found to be linear up to 90mins or 20,000dpm (fig 6). When increasing amounts of sample were assayed in duplicate for 60 min the assay was linear up to approximately 6μg protein or approximately 16,000dpm (fig 7).

2.4.4 2',3'-Cyclic Nucleotide 3'-Phosphodiesterase Assay.

2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) was used to assess the purity of the isolated myelin fraction. There now exists unequivocal evidence supported by numerous studies that CNPase is primarily located in myelinated regions of the CNS, and in particular the oligodendrocytes that produce myelin (Sprinkle 1989). Although first described in the 1950's, the physiological role of this enzyme remains unknown. In-vitro, however, CNPase catalyses the hydrolysis of 2',3'-cyclic nucleotides to yield nucleoside 2'-phosphates.

CNPase was assayed using a modification of the method of Weissbarth et al (1980). The scheme of the assay is shown in fig 8 and involves the hydrolysis of a synthetic nucleotide, cyclic-NADP, with coupled enzyme conversion to yield NADPH which is estimated fluorimetrically. The rate-limiting step is catalysed by CNPase so that the amount of NADPH formed was directly related to the activity of CNPase.

Fig 6. Acetylcholinesterase Activity Versus Time

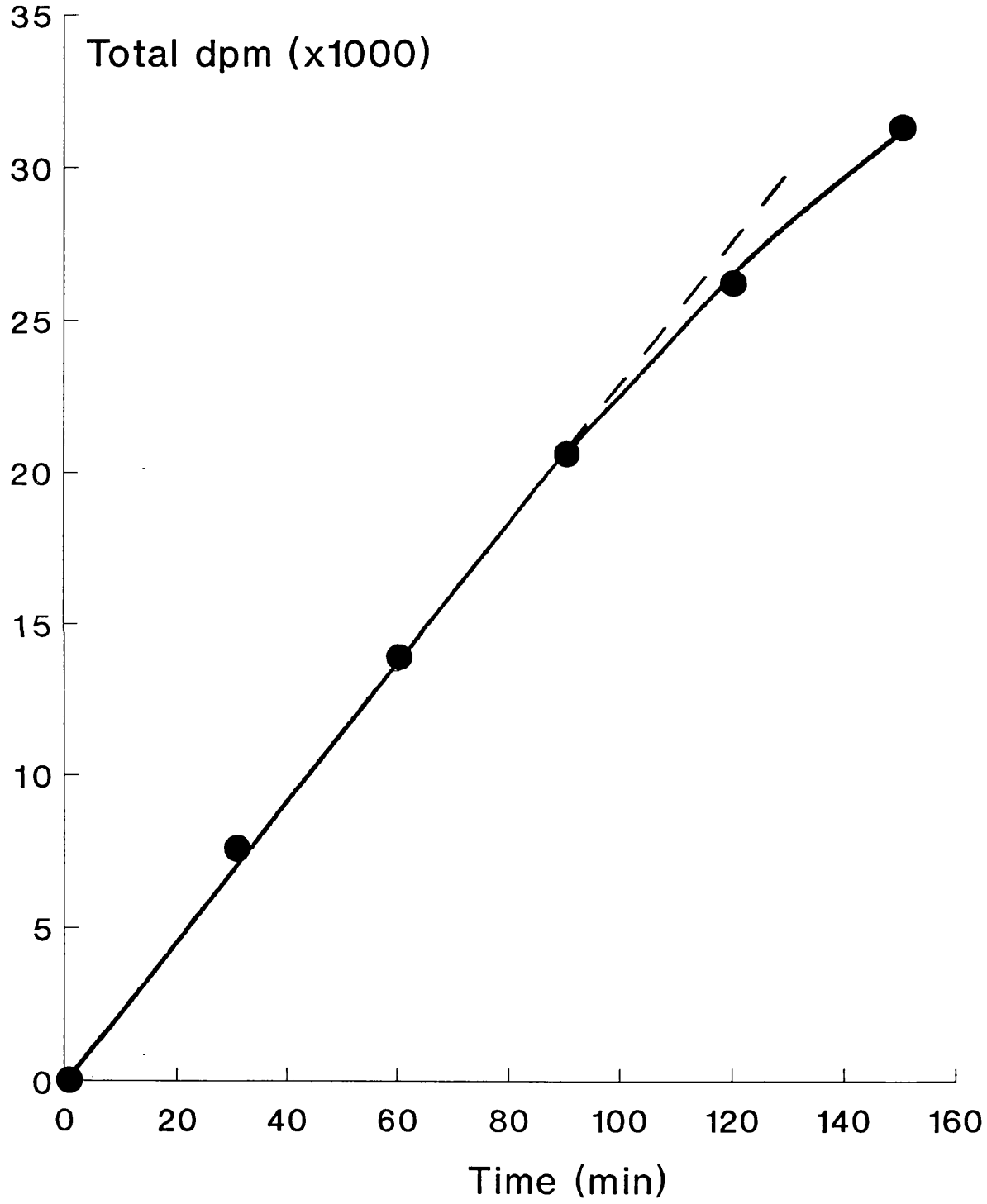


Fig 7. Acetylcholinesterase Activity Versus Protein Concentration

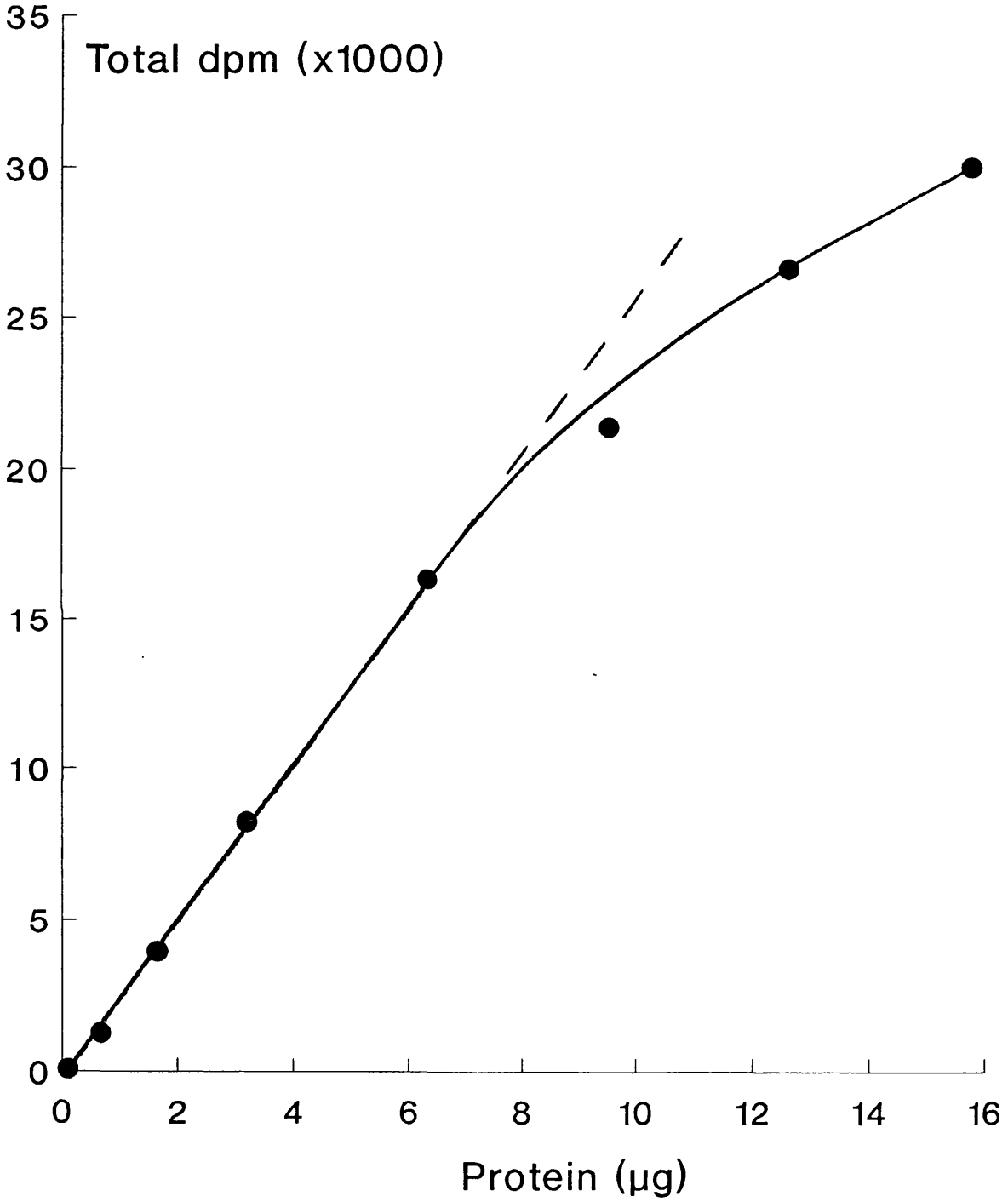
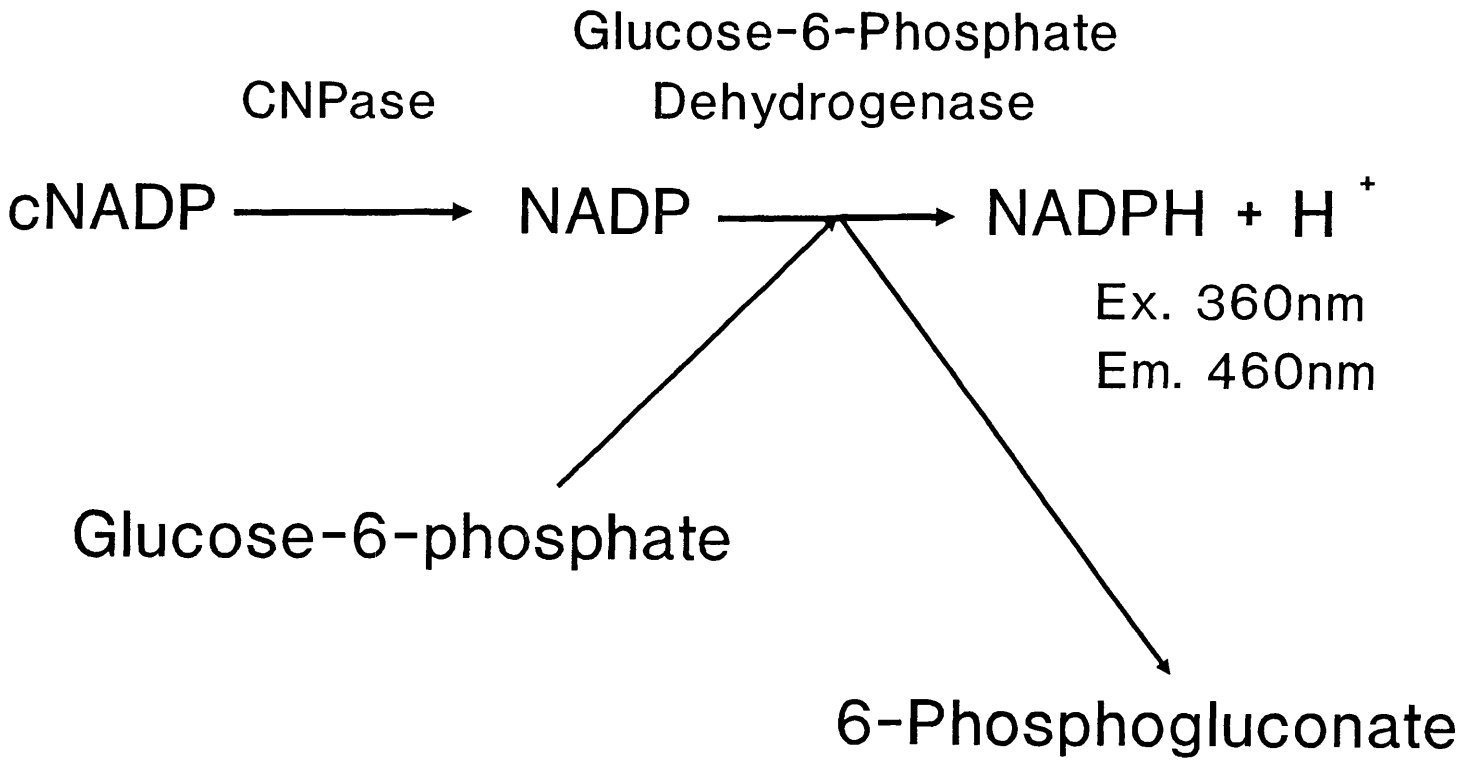


Fig 8. Coupled Enzyme Assay System For CNPase



Procedure:

An aliquot of sample was suitably diluted in 1%-Triton X-100 and gently mixed. 20μl of diluted sample was incubated at 25°C for 60 min with 600μl assay buffer which contained 0.2M MES, [2(N-morpholino)ethanesulphonic acid], pH 6.0. 1mM 2',3'-cyclic NADP, 1.5mM glucose-6-phosphate, 30mM $MgCl_2$, 1mM EDTA, 0.03% BSA and 20μg/ml glucose-6-phosphate dehydrogenase (140U/mg, Boehringer Mannheim).

The reaction was stopped by the addition of 2.5ml 50mM sodium bicarbonate, pH 10.6.

The fluorescence of the mixture was measured using a Perkin Elmer LS-3 fluorescence spectrometer (excitation 360nm and emission 460nm). The concentrations of NADPH were determined by including a set of NADP standards which were incubated with the assay buffer in the same run as the actual samples.

In the method adopted from Weissbarth (1980) the tissue was homogenised in 1% Triton X-100. However, in this study the tissue could not be homogenised in detergent as a number of different assays had to be performed on the whole homogenate from the brainstem fractionation, and Triton interfered with some of the assays. In addition it was difficult to homogenise tissue using a potter homogeniser when the buffer contained 1% X-100 as the homogenate became excessively frothy and difficult to handle. However, as shown in table 2, simply diluting an aliquot of the tissue sample that had been previously homogenised in sucrose with Triton X-100, yielded the same specific activity compared to homogenising the tissue directly in detergent.

Table 2 Effect of sample preparation procedure on CNPase Activity

Sample Preparation	Specific Activity (μmol/hr/mg protein)
Sucrose homogenate diluted with sucrose	6
Sucrose homogenate diluted with Triton X-100	199
Tissue homogenised directly in Triton X-100	209

In table 2 above sucrose homogenate means that the tissue sample was homogenised in a sucrose buffer containing no Triton X-100. For each of the three sample preparation procedures listed the final protein concentration in the samples assayed was the same. The above data agrees with the membrane-associated nature of CNPase since diluting a sucrose homogenate with sucrose buffer resulted in a greatly reduced enzyme activity compared to treating the sample with detergent.

Validation of assay

The NADP standards (reduced to NADPH by the assay system), had a linear range up to 40 nmoles (fig 9). The assay (in duplicate) was linear over 45 min for 37ng protein of rat brainstem homogenate (fig 10). Varying the amount of protein assayed for a fixed period of 60 min showed the assay to be linear up to approximately 25ng of protein (fig 11). This was therefore a sensitive assay of CNPase activities. Note that a different fluorimeter was used to monitor

Fig 9. NADP Standard Curve For CNPase Assay

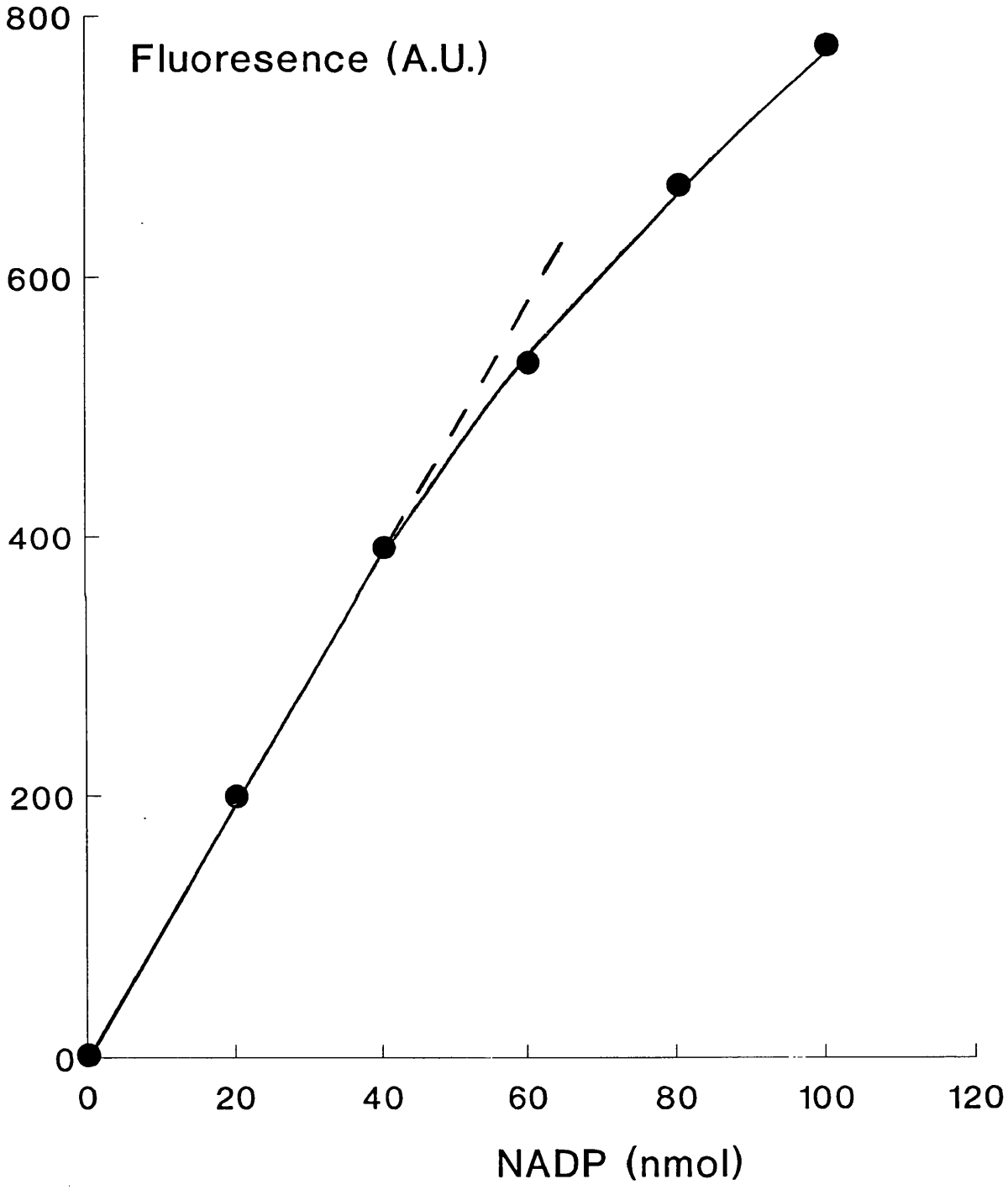


Fig 10. CNPase Activity versus Time

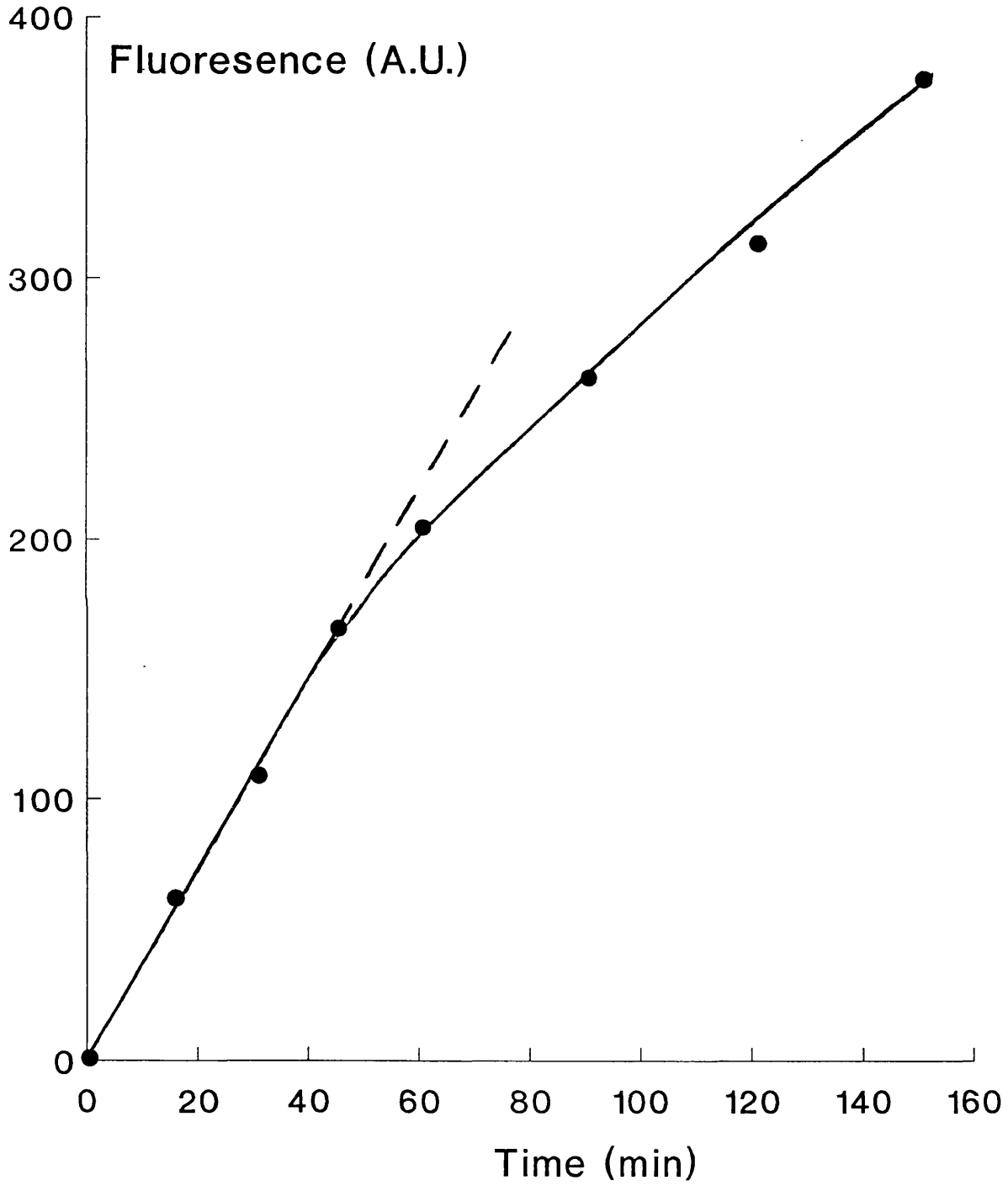
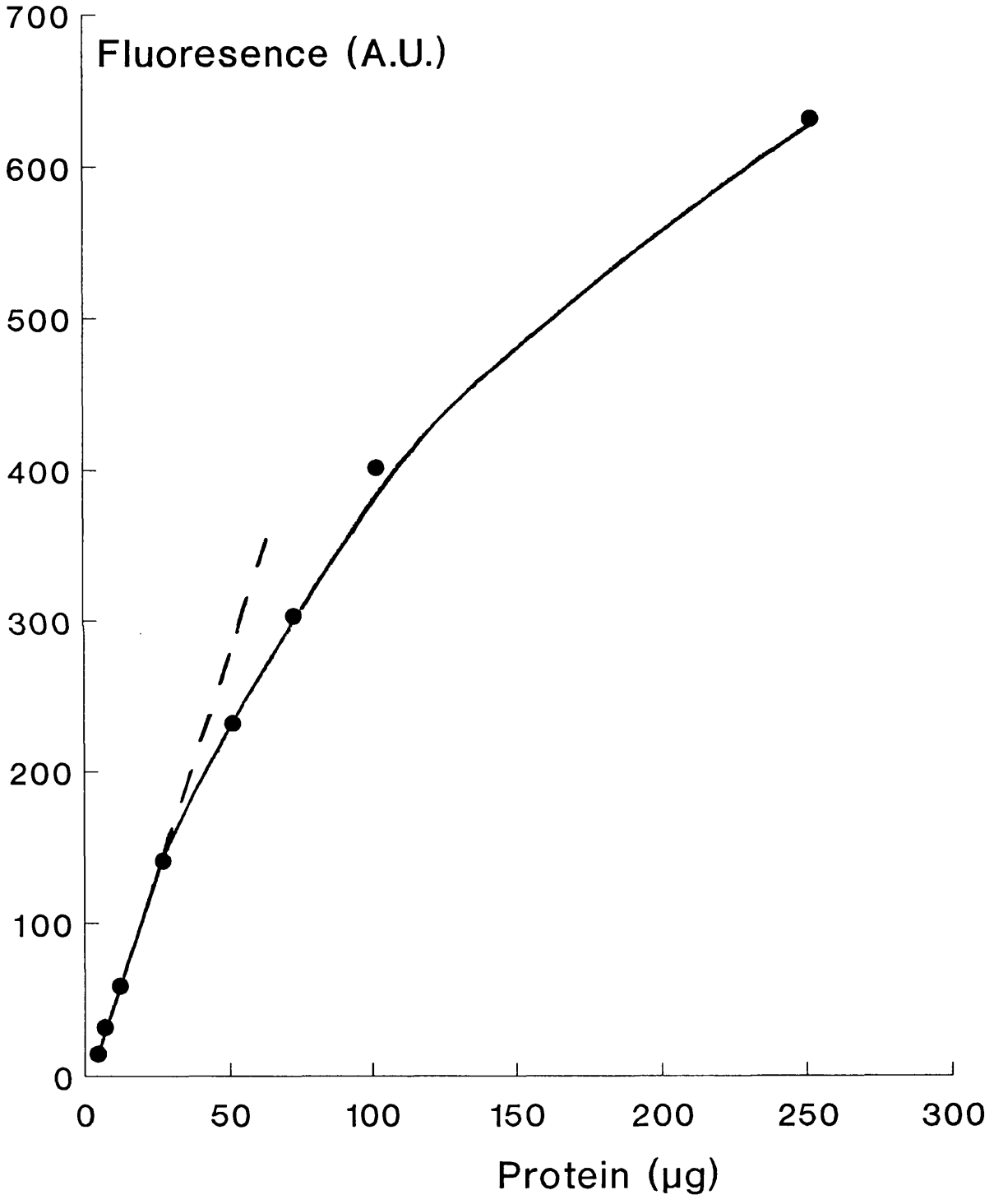


Fig 11. CNPase Activity versus Protein Concentration



fluorescence for the NADP standard curve plot (fig 9) than that used in the other two validation studies. This could account for the fact that linearity was achieved up to 400 arbitrary units (AU) for the standards and only < 200AU for the validation studies with the tissue.

2.4.5 Marker Enzymes - Specific Activities and Recoveries.

The specific activities of the different marker enzymes in the isolated neural fractions were determined. The total recoveries of the enzyme activities and protein were also determined by measuring activities in all the fractions and also the combined pellets and solutions under the floating myelinated axons, i.e. tissue components other than the myelinated axons. Although the two AEFs were combined and treated as a single fraction for peroxidation studies, the enzyme activities and protein concentrations were assayed in both fractions. Three rat brainstem fractionations were carried out using tissues that had been dissected out the previous day and stored overnight at -70°C. After each fractionation the marker enzymes were assayed in the order of 5'-nucleotidase, CNPase and AChEase within five days (unless otherwise stated) of isolating the fractions The results for these assays are presented in table 3 where the "remainder" fraction refers to the combined pellets and solutions under the myelinated axons in the density gradients. Only two sets of data for acetylcholinesterase are included since one of the AChEase assays was carried out two weeks post isolation. By comparison with the other two fractionations it appeared that the extended period between isolating and assaying the fractions had resulted in loss of AChEase activity and this data was therefore excluded.

Activity of the myelin marker enzyme, CNPase, was enriched by a factor of 2.7 in the myelin fraction compared to the whole homogenate. The RSA values for 5'-nucleotidase and AChEase in myelin were both < 1.

(mg) and total recoveries (%) following fractionation of brainstem (n=3).

Fraction	5'Nucleotid.	AChEase	CNPase	Protein
W. Homog.	3.2[1] (1.0)[2] 3.2,2.7,3.7[3]	3.6 (1.0) 3.6, 3.6	464 (1.0) 494,486,412	72 78,64,74
2x-Myelin	1.3 (0.4) 1.7,0.7,1.5	2.1 (0.6) 2.4, 1.8	1262 (2.7) 1360,1127 1299	11.3 11,11,12
0.8/1.0 AEF	3.2 (1.0) 3.9,2.1,3.5	7.0 (1.9) 7.5, 6.4	1343 (2.9) 1423, 1223 1384	0.24 0.36, 0.1, 0.27
1.0/1.2 AEF	3.6 (1.1) 3.2,4.5,3.0	4.2 (1.2) 4.2, 4.2	517 (1.1) 666,527,358	0.28 0.42, 0.25, 0.3
M/O	1.6 (0.5) 1.5,2.3,1.1	1.5 (0.4) 0.3, 2.6	103 (0.2) 145,83,82	0.57 0.52, 0.45, 0.7
Remainder	3.5 (1.1) 3.1,3.0,4.4	3.7 (1.0) 3.5, 3.9	254 (0.5) 301,251,210	49.7 49,55,46
Recoveries (%)	85 73,100,81	88 98, 77	83 79,84,86	88 78,105,80

1. Mean specific activity
2. Specific activity relative to whole homogenate
3. Specific activities of individual preparations

5'-nucleotidase showed no enrichment in the AEFs (1.0 and 1.1), whereas AChEase exhibited a doubling of RSA in the 0.8/1.0 AEF with only a slight increase in the more dense AEF. CNPase exhibited no enrichment in the 1.0/1.2 AEF (1.1), whereas in the 0.8/1.0 AEF it gave an RSA value of 2.9.

Although a maker enzyme assay was not developed for the M/O fraction, the specific activities of the three marker enzymes for the AEF and myelin fractions were all 50% or less than that in the homogenate.

From the data in table 3 it can be seen that there was a good agreement between the total recoveries of the three marker enzymes with percentages varying between 83 and 88%.

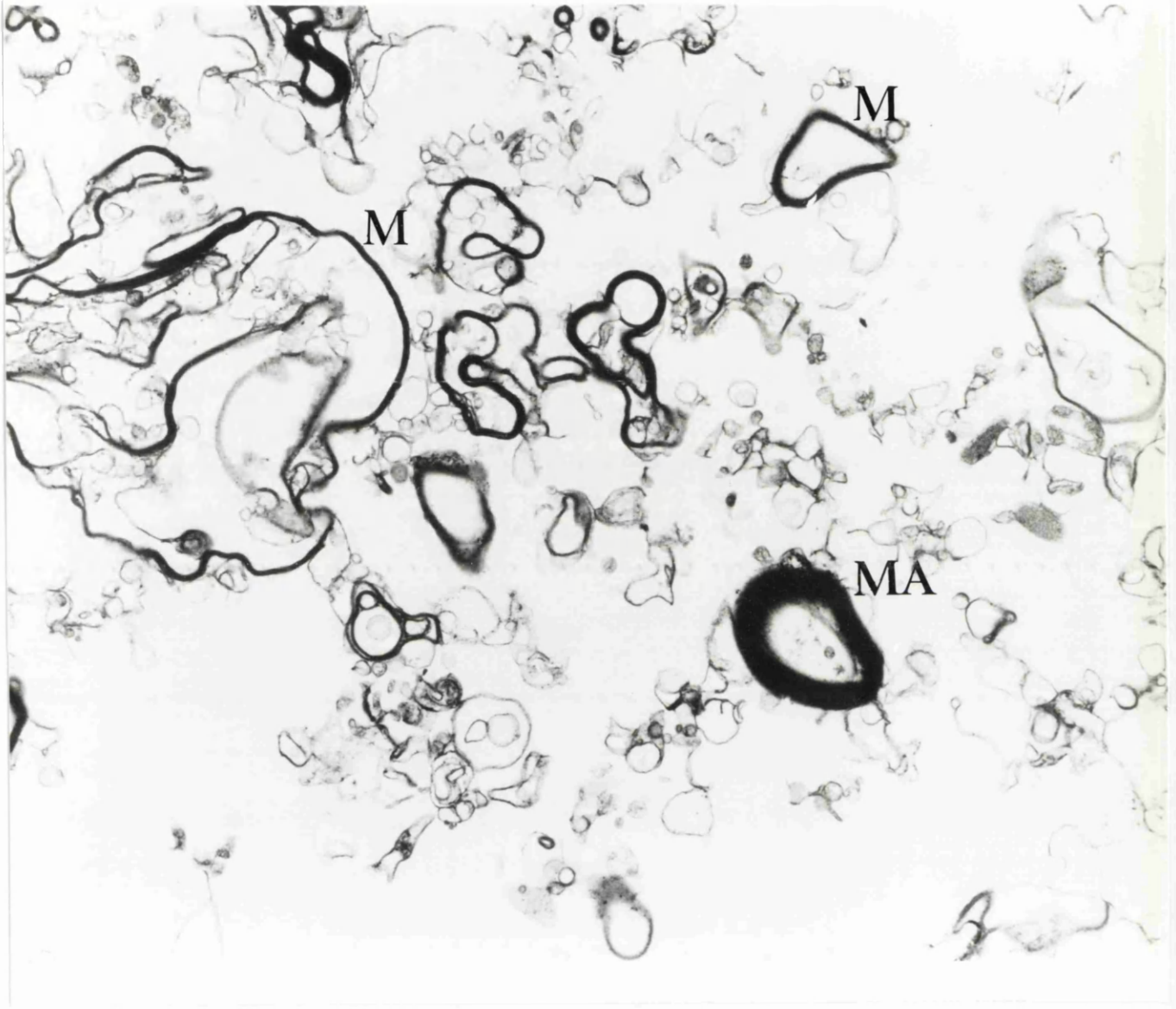
A relatively large proportion of the total starting protein was recovered in the myelin fraction, i.e. approximately 16%, whereas only small amounts of protein (< 1%) were recovered in the other neural fractions.

2.4.6 Purity Assessment Using Electron Microscopy.

Electron microscopic analysis of the 2x-myelin fraction and of both the AEFs were carried out by Mrs. Virpi Smith of the Dept. of Histochemistry, Institute of Neurology, London. These three fractions were obtained from the same fractionation whereas the M/O fraction was isolated in a separate fractionation and was analysed by Dr. Rosalind King of the Dept. of Neurological Science, Royal Free Hospital, London. The micrographs shown were typical of the overall appearance of the isolated fractions. Magnifications were as stated on the micrographs.

The myelin fraction (fig 12) contained large amounts of dark highly refractile areas which are characteristic of the multilamellar myelin sheath. The thickness of the sheath appeared to be variable with that towards the centre of

FIG 12. ELECTRON MICROGRAPH
OF 2x-MYELIN FRACTION (x20,000)



M = MYELIN MA = MYELINATED AXON

the micrograph possibly representing an undisrupted myelinated axon. There was also significant amounts of smaller unilamellar vesicles.

Neither of the AEFs gave a very homogenous profile, although unilamellar vesicles were the predominant feature in both fractions. There was however, greater myelin contamination in the less dense fraction (fig 13) compared to the 1.0/1.2 AEF (fig 14) as revealed by the presence of the typical multilamellar membranes.

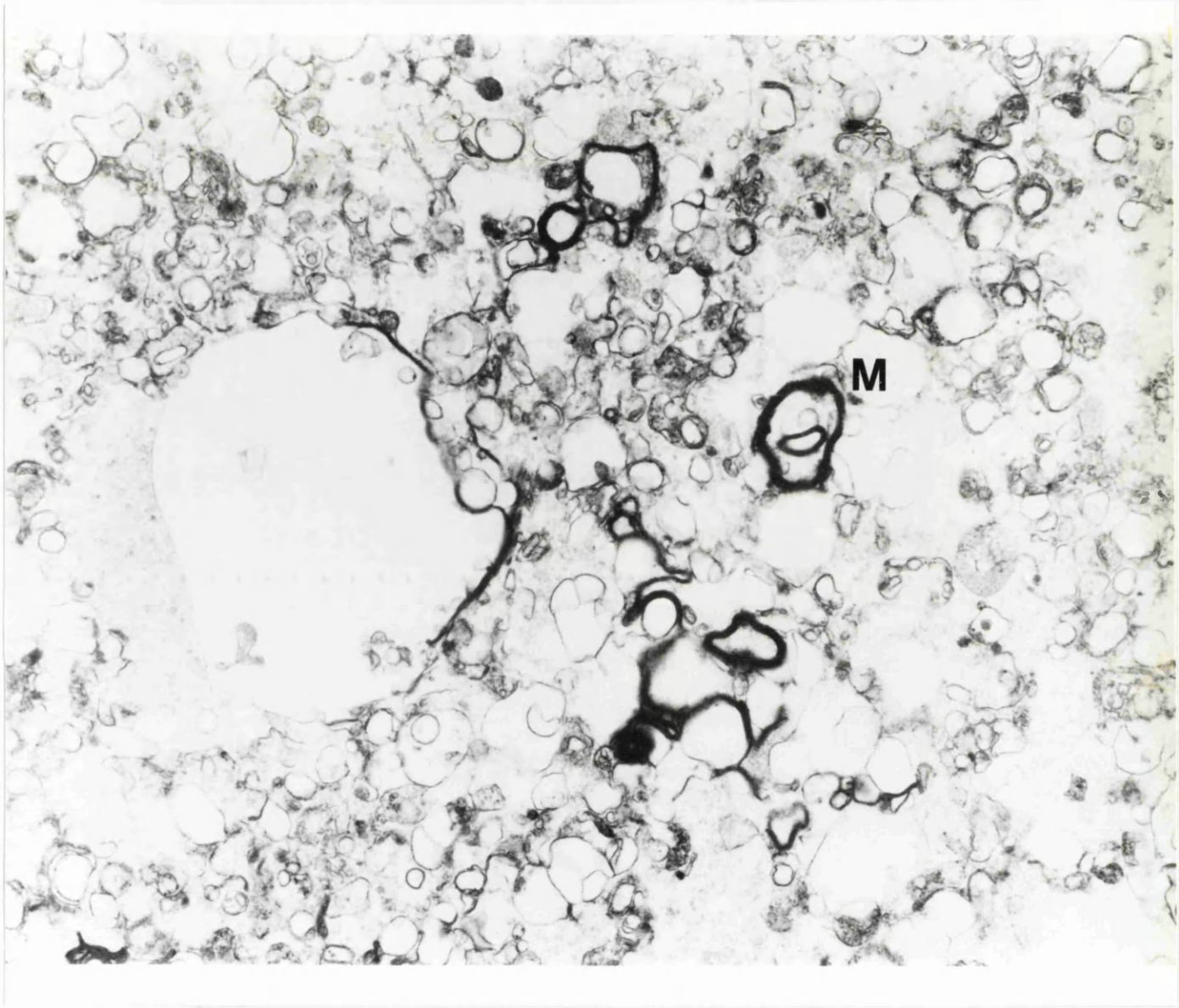
Although analysis of the M/O fraction showed it to be highly enriched in mitochondria (fig 15) there was also considerable amounts of other membraneous material and myelin.

2.5 Discussion

The fractionation procedure described above provided a reproducible method for the isolation of different components of the myelinated axon from rat brainstem. It must be remembered, however, that the axolemma fraction can only be refereed to as an "enriched" fraction whereas the M/O fraction appears to be a heterogenous mixture of membranes and organelles which requires additional characterisation or subfractionation. The method was, nonetheless, adequate as a basis for preliminary studies of in-vitro peroxidation of neural fractions during vitamin E deficiency.

Two axolemma-enriched fractions (AEF) were seen when the shocked myelinated axons were fractionated on the discontinuous density gradient. They are termed 0.8/1.0 and 1.0/1.2 AEF, which refer to their positions at the interphases on the gradient. DeVries (1981) showed that while the specific activity of the positive and negative enzyme markers were similar in both fractions, the less dense fraction was 60% lipid whereas the other was 50% lipid. The former has been shown to have greater myelin contamination and a

FIG 13. ELECTRON MICROGRAPH OF 0.8/1.0 AXOLEMMA-ENRICHED FRACTION (x20,000)



M = MYELIN

FIG 14. ELECTRON MICROGRAPH OF 1.0/1.2 AXOLEMMA-ENRICHED FRACTION (x20,000)

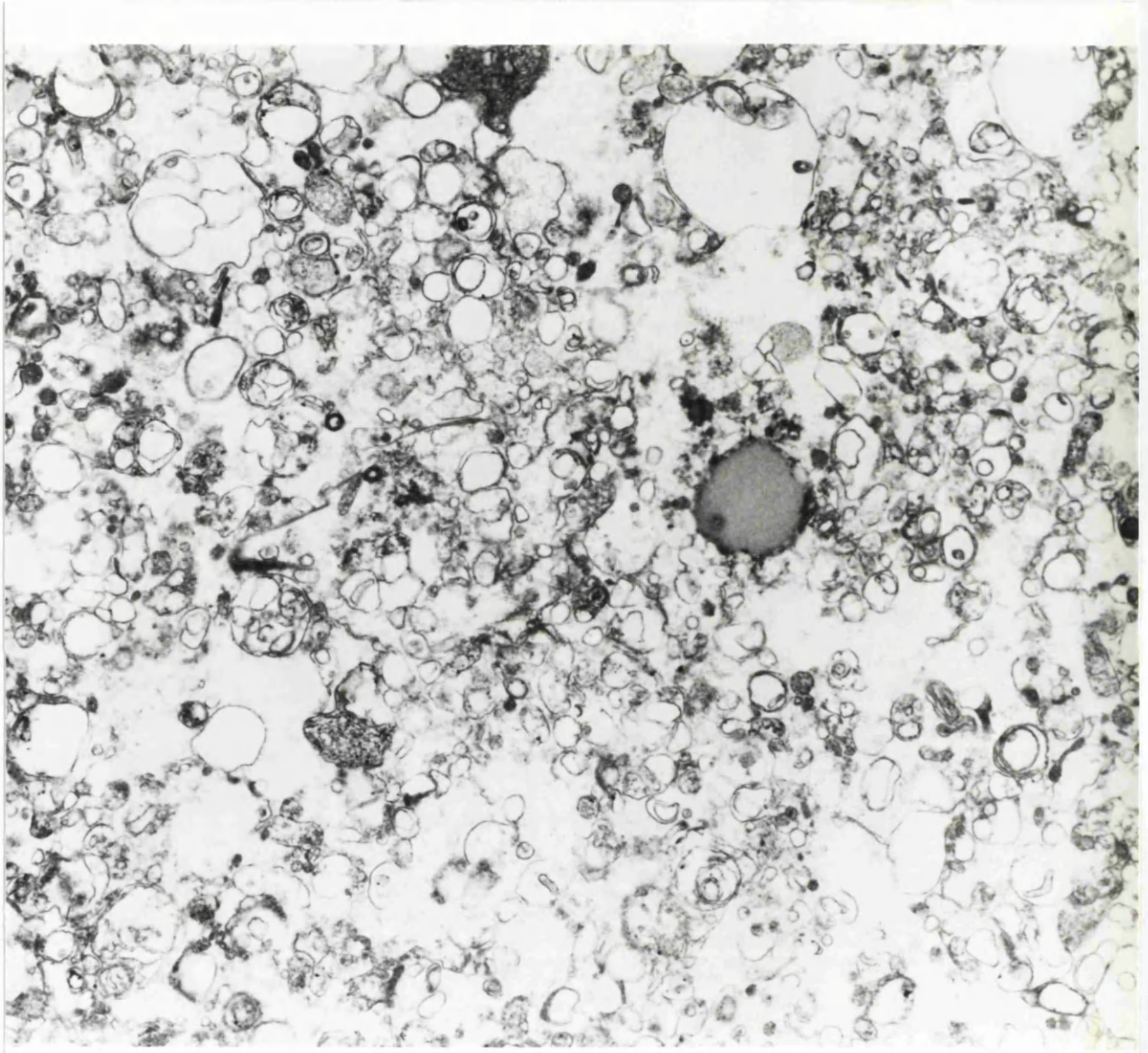
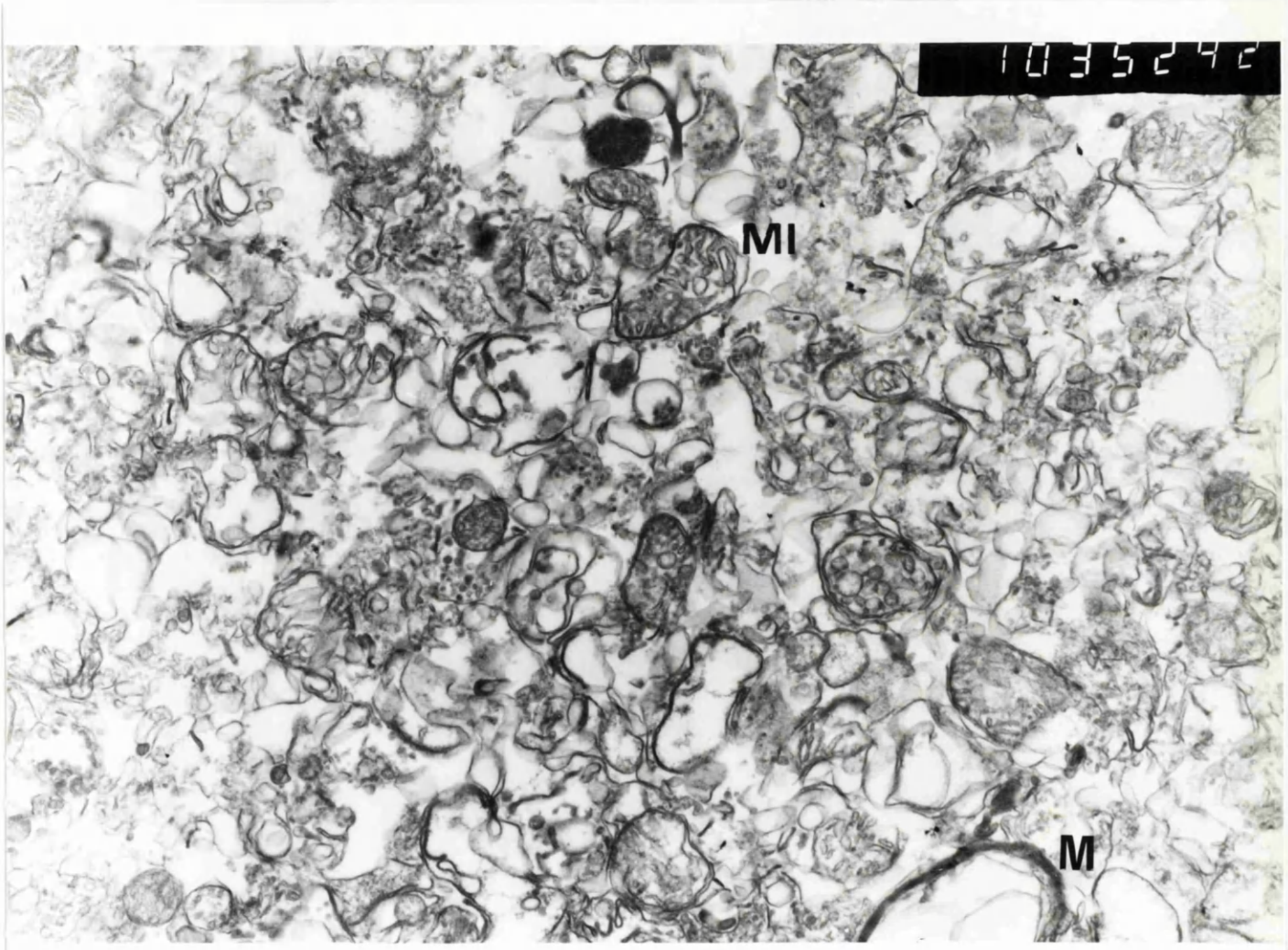


FIG 15. ELECTRON MICROGRAPH OF AXOPLASMIC MEMBRANES AND ORGANELLES (M/O) FRACTION (x24,000)



M = MYELIN MI = MITOCHONDRIA

p25 Babior (p265).

p22 Gerhan ref missing from ref list (Stick in

p33 error with equation 11 (GSSG).

p34 OH • missing

p36 L-OO • missing

p44 Referred (not refereed) - large pore 3rd Lene

p54 Units of absorb

p56 PO_3^{2-} not $2+$ and

p88 Correct MDA etc. ??

p120 eluting washes 25 min

p126 all rac (click on).

lipid composition that was intermediate between that of myelin and the more dense AEF (DeVries and Zmachinski 1980, DeVries et al 1981a).

There are two possible explanations for the origins of these distinct fractions. The first is that the less dense, as suggested by its higher myelin contamination, has myelin fragments attached to it. However, subjecting this AEF to further osmotic shock did not produce any additional myelin (DeVries 1981). A second possible explanation comes from the fact that protein composition and density varies along the length of the axolemma with nodal axolemma having a greater density of intramembraneous particles compared to para- and internodal axolemma (Schnapp and Mugani 1978) as well as a higher concentration of sodium channels (Ritchie and Rogart 1977). It is therefore possible that the more dense fraction contains a greater proportion of dense nodal axolemma compared to the other AEF. Evidence for this comes from the fact that the more dense fraction exhibited a greater binding of tetrodotoxin (DeVries et al 1978) and saxitoxin (DeVries et al 1981a) than the 0.8/1.0 AEF.

In addition to studies of the variation of the activity of the marker enzymes with time and protein concentration, further validation of the enzyme assays is provided by the good recoveries of activity during brainstem fractionation (table 3). The mean total recovery for all three marker enzymes was $>80\%$ which correlated with a mean total protein recovery of 88%.

Activities of the two axolemma marker enzymes (5'-nucleotidase and AChEase) were only slightly enriched in both the AEFs with the highest relative specific activity (RSA) being that of AChEase in the 0.8/1.0 fraction, which was 1.9. DeVries (1981) assayed the more dense AEF isolated from rat brainstem and obtained enrichments for 5'-nucleotidase and AChEase of 4.2 and 2.5 respectively. He also found similar increases in specific activities

in 1.0/1.2 AEF isolated from human post mortem white matter.

In the case of AChEase it was possible that the low RSA of the present study could be explained by the loss of activity during storage. Although formal stability studies were not carried out, when AChEase was assayed two weeks after being isolated, the specific activities were much lower than those obtained when assayed within 5 days. It is possible that even a five day interval may have resulted in loss of activity. DeVries (1982) found that the specific activities of AChEase in fractions isolated from bovine and human tissue samples where some degree of autolysis had occurred, were approximately 10-fold lower than for AEF prepared from fresh starting tissues. However Alberghina et al (1988) who assayed AChEase in an AEF isolated from rabbit brainstem reported an enrichment of only 1.1.

There is also some evidence that 5'-nucleotidase may lose activity during storage. Cammer et al (1980) found that there was a 30 to 40% loss of activity in isolated myelin that had been stored either as a frozen pellet or a suspension, whereas when lyophilised before freezing there was no reduction in specific activity compared with freshly isolated membrane. Activity in the whole homogenate was, however, unaffected by storage. Alberghina et al (1988) reported a 5.8-fold enrichment in activity of 5'-nucleotidase despite only a minor elevation in AChEase levels. In the present study both enzymes had the greatest activities in the two AEFs while both the myelin and M/O fraction had RSAs < 1.

The specific activities of the AEF markers in the myelin sample were in agreement with those of DeVries (1981) for rat myelin with RSAs of less than 1 for both enzymes. However, caution should be used when employing 5'-nucleotidase as an AEF marker since it appears that the enzyme is also intrinsic to myelin. Biochemical studies on isolated myelin have shown that

CNS myelin from at least three species is quite rich in this enzyme i.e. rat (Cammer et al 1980), bovine (Farooq et al 1981) and mice (Cammer and Zimmerman 1983). For example Cammer et al have argued that myelin is a major locus of this enzyme with 24% of the activity in rat whole brain homogenate being recovered in the isolated myelin with a 2 to 3-fold increase in specific activity. From the data in table 3, assuming a 60% yield of myelin, it can be seen that approximately 10% of the total enzyme activity was present in the myelin fraction. This value is in agreement with a recovery of 5 to 15% of most myelin-associated enzymes (Norton 1980).

CNPase, which was assayed as a marker for myelin, was enriched 2.7-fold in the appropriate fraction compared with the starting homogenate which compares favourably with other published results including those of DeVries (1981) who reported a 2.2-fold enrichment for rat myelin. This was obtained for 1x-myelin, whereas my result was for 2x-myelin. I found that purifying myelin that had been isolated using a single density gradient (1x), on a second gradient (2x) not only reduced the activity of 5'-nucleotidase by 50%, but also CNPase by 25%. This is similar to the findings of DeVries. Thus if 1x-myelin had been assayed an even greater increase in the RSA of CNPase would have been expected.

The most convincing evidence for the localisation of a particular enzyme in an organelle is if most of the activity in the starting homogenate is recovered in the isolated fraction. Norton and Poduslo (1973) found that in general, isolation procedures tend to be only 60% efficient and it can therefore be estimated that approximately 19mg of the starting protein was accounted for by the myelin fraction. This means that it can be calculated from the data in table 3 approximately 70% of the CNPase activity of the starting homogenate was present in the myelin component. This provides convincing evidence for the myelin-associated nature of the enzyme.

Although CNPase is firmly established as a myelin-associated enzyme, its specific activity was increased by 2.9-fold in the less dense AEF. DeVries (1981) demonstrated that the 1.0/1.2 fraction was enriched in CNPase by 60% compared with the starting homogenate. The 0.8/1.0 AEF was not assayed. These results can be explained from a consideration of the structure of the myelin sheath. The true definition of myelin should be morphological and not chemical or operational. From the morphological viewpoint, myelin is a very heterogeneous structure comprising areas of compact multilamellar membranes, unit membranes in areas such as the lateral loops where the myelin coating lies opposite the axolemma in the paranodal regions, and specialised segments of the myelin that form actual contact with the neuronal plasma membrane. All these different membranes cannot be isolated intact and most myelin isolation procedures are designed to exclude pockets of cytoplasm and unit membranes so that only the condensed membrane systems are isolated. Recent studies point to CNPase having an asymmetric distribution in the oligodendroglia. High activities of the enzyme have been found to be associated with the inner loop, the outer tongue process and the paranodal loops, i.e. single membrane fragments and segments of relatively few lamellae, while there is thought to be a near absence of CNPase in mature, compact myelin (Trapp et al 1988). Waeneldt et al (1977) described a fraction isolated from a myelin preparation (rat forebrain) by gentle centrifugation that exhibited a CNPase specific activity 2-fold greater than that of the myelin fraction. Electron microscopic analysis revealed that, in contrast to the multilamellar myelin, this fraction consisted of small vesicular profiles of a mixture of single membranes and some triple layered structures.

The distribution characteristics of CNPase described above may explain the high activities found in the axolemma-enriched fractions. The single membrane fragments and those of only a few lamellae have a higher density than the bulk of the myelin so that when the shocked myelinated axons are

fractionated on a discontinuous density gradient some of these membranes may migrate down the gradient to higher density positions compared to the myelin fraction which is retained in the lowest density position at the top of the gradient. Because of the high levels of CNPase associated with these membranes it is feasible that they contaminated the AEFs, particularly the less dense fraction, resulting in increased specific activities compared to the whole homogenate. Although the AEFs are purified on second gradients, the high density myelin membranes may have a density so similar to the axolemma fractions that they cannot be separated on a discontinuous gradient. It would probably require a continuous density gradient to remove them. An alternative explanation is that the lateral loops, one of the regions of the myelin sheath where CNPase is concentrated, are known to form junctional complexes (zonulae occludentes) with the neuronal plasma membrane (Morell 1984). These complexes may be sufficiently strong to withstand the fractionation conditions, including osmotic shock, so that some membranes with high CNPase activity remain attached to the axolemma.

Regardless of whether or not some of the high density myelin membrane fragments do in fact co-migrate with the AEFs, a certain degree of myelin contamination is known to occur in the AEFs particularly in the less dense fraction. The greater CNPase activity in the 0.8/1.0 fraction compared to the other AEF correlates with a higher content of myelin basic protein and a lipid composition intermediate between that of 1.0/1.2 AEF and myelin (DeVries 1981). These results were also in agreement with the EM studies which revealed evidence of greater myelin contamination in the less dense fraction.

Myelin has been shown to account for 7.5 to 10% of the total protein of an adult rat brain (Morell 1984a). From the experimental data presented here for rat brainstem fractionation 15.7% of the total protein was recovered in the isolated myelin. However, assuming a 60% efficiency in the extraction of

myelin it can be estimated that approximately 26% of the total starting protein can be accounted for by myelin. This high value agrees with the fact that the brainstem is a heavily myelinated brain region accommodating numerous nerve pathways between the spinal cord and the rest of the brain. Compared to myelin, very small amounts of material were recovered in the other neural fractions.

The results from the EM analysis of the myelin and AEF fractions were similar to the findings of DeVries (1981), the M/O fraction had an unexpected appearance. Although the pellet was enriched in mitochondria there were also substantial amounts of myelin as evidenced by their characteristic multilamellar membranes. As stated previously, whereas typical myelin membrane profiles were observed in the less dense AEF, they did not appear to be present in the 1.0/1.2 fraction. Therefore these compact myelin membranes would not have been expected to migrate below the 0.8M sucrose layer of the gradient. The M/O sample used for the EM studies was isolated from a different preparation from the other fractions which were obtained in a single fractionation. It was therefore possible that this fractionation was not representative. In the majority of fractionations the M/O pellet was extremely small, i.e. a thin film, with a dark appearance, whereas the pellet submitted for EM analysis was relatively large with a creamy colour suggesting that myelin may have been present.

In conclusion, although with the exception of myelin, the fractionation procedure described in this chapter did not produce pure fractions, they were satisfactory for the purposes of an initial investigation of lipid peroxidation of different components of the myelinated axon. The method was reproducible both in terms of the yields of each fraction and the specific activities of the marker enzymes in these fractions. The fractionation procedure could form the basis of a more selective procedure should such additional studies be required.

CHAPTER 3

METHODS OF ASSESSING LIPID PEROXIDATION

3.1 Introduction

3.2 Malondialdehyde

3.2.1 MDA as an index of lipid peroxidation

3.3 Measurement of MDA

3.4 The thiobarbituric acid test

3.4.1 Fluorimetric determination of thiobarbituric acid reactive substances

3.5 Measurement of MDA using high performance liquid chromatography

3.5.1 Estimation of free MDA

3.5.2 Estimation of bound MDA

3.5.2.1 Is the assay actually measuring bound MDA ?

3.5.2.2 Sucrose interference in bound MDA assay

3.6 Non-MDA aliphatic aldehydes

3.6.1 Aliphatic aldehydes as an index of lipid peroxidation

3.6.2 Measurement of aliphatic aldehydes

3.6.3 HPLC method for the estimation of aliphatic aldehydes following derivatisation with 1,3-cyclohexanedione

3.7 Discussion

3.1 Introduction

Numerous methods are available to assess the lipid peroxidation process, each measuring a different substrate or product and each assessing a different stage of the overall process. In addition a number of techniques may be available to quantitate the same compound. In general methods can be divided into non-invasive and invasive. The main procedure in the former category is the measurement of exhaled alkanes, i.e. ethane and pentane derived from w-3 and w-6 polyunsaturated fatty acids (PUFA) respectively. This technique, which was developed by Riely and coworkers (1974), has the advantage of being highly sensitive and since it is non-invasive repeated samples can be obtained from the same animal or subject. Lemoyne et al (1987,) used this method to demonstrate a significant negative correlation between breath pentane and vitamin E status in human subjects.

The vast majority of methods used to assess lipid peroxidation are of the invasive type and require biological fluids and tissues. Methods such as the estimation of conjugated dienes by UV-absorption and oxygen uptake will give information on the early stages of the peroxidative process (see fig 1, chapter 1). Esterbauer et al (1989) demonstrated that in the case of human low density lipoproteins, the measurement of conjugated dienes could be used to continuously assess in-vitro lipid peroxidation. Information on the intermediate stages can be obtained by assaying for lipid peroxides by a number of different methods including gas chromatography-mass spectrometry (van Kuijk et al 1990). The thiobarbituric acid assay has been suggested by some groups as a good method for the estimation of the lipid peroxide content in a sample (see section 2.4). The breakdown of these peroxides yields a large number of secondary products. Estimation of these compounds which include malondialdehyde (MDA) and other aliphatic aldehydes, provide data for the late stages of lipid peroxidation. This chapter will provide a discussion of the

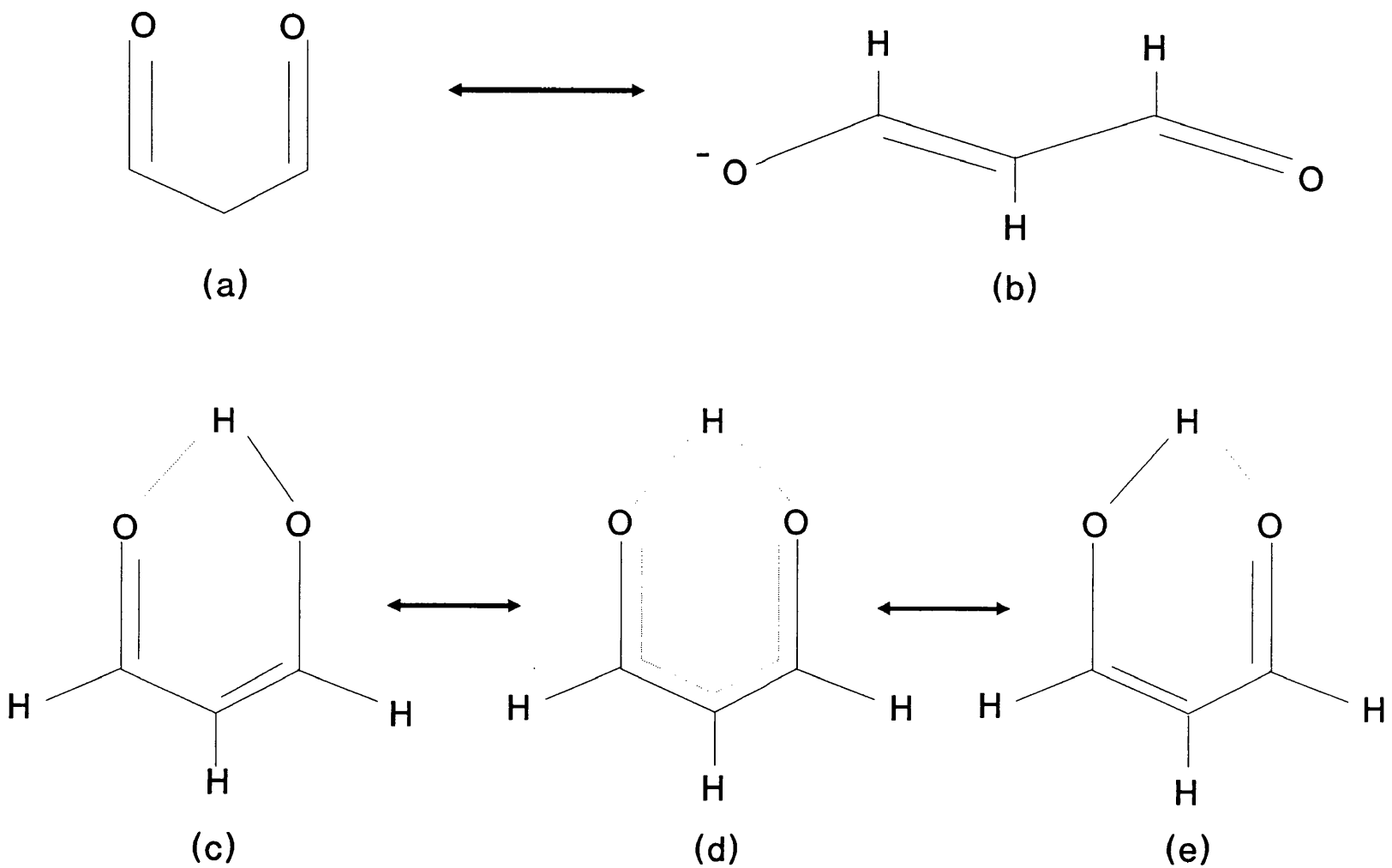
various methods available to measure these aldehydic products and the actual techniques employed during the course of this project to assess lipid peroxidation.

3.2 Malondialdehyde

Malondialdehyde or malonaldehyde (MDA) is the most extensively studied of all lipid peroxidation products and the most frequently quoted as an index of lipid peroxidation. MDA is a volatile, low molecular weight ($C_3H_4O_2$, formula wt. = 72.07), 3-carbon compound containing two carbonyl groups, i.e. a dialdehyde (fig 1 a). In solution MDA is entirely enolised and rapidly alternates between two intramolecularly hydrogen-bonded asymmetric forms (fig 1, structures c and e) via a resonance stabilised intermediate (structure d). In organic solvents the s-cis-enol forms are favoured (c and e) where the acidic proton forms a hydrogen bridge between the two oxygens. MDA is a moderately weak acid (pk_a = 4.46) and in aqueous solution at pH > 7.0 will exist as the conjugate base, i.e. enolate anion (s-trans-conformation, b).

The uncharged enol forms of MDA (c and e) are much more reactive than the conjugate base, particularly towards nucleophiles. In addition to undergoing aldol type self condensation reactions, MDA can also readily react with nucleophiles upon heating at low pH to give a variety of condensation products which forms the basis of the thiobarbituric acid (TBA) test for assessing lipid peroxidation. However, all these reactions lack selectivity in that no substrate to date reacts with MDA to the exclusion of other low molecular weight aldehydes. In general, MDA tends to form pigmented condensation products. However, in some cases fluorescent products exhibiting distinct excitation and emission spectra are formed (Kikugawa 1986). A few of these adducts, most notably $(TBA)_2$-MDA, are both coloured (red) and fluoresce (Nair and Turner 1984, Yagi 1976). Whereas the

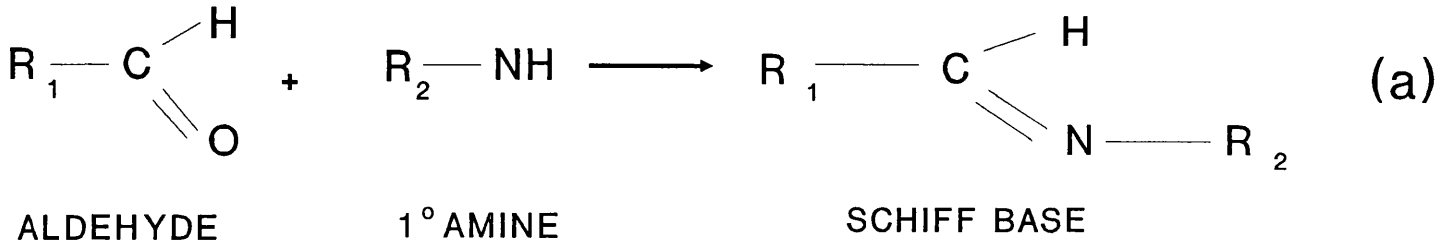
FIG 1. Structural Chemistry of Malondialdehyde (MDA)

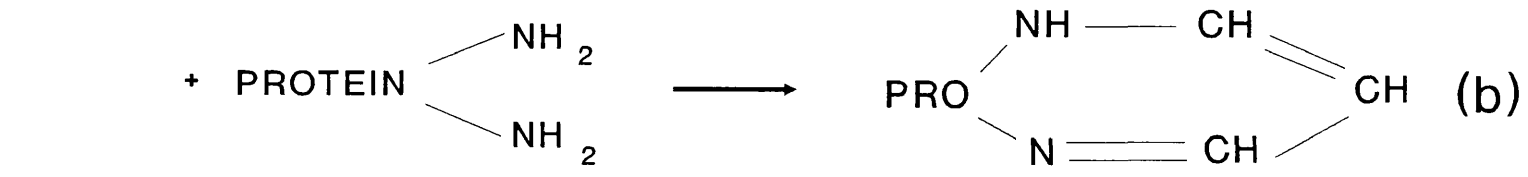


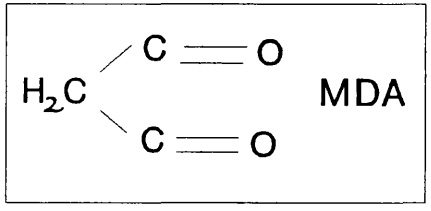
production of MDA-derived pigments usually requires elevated temperatures of 80 - 100°C and a low pH, some fluorescent derivatives can be formed under physiological conditions (pH 7.0, 37°C) in aqueous solution (Kikgawa and Beppu 1984).

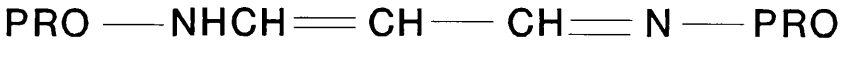
The fluorescent MDA-derived products seen in biological systems generally result from MDA being readily reactive, in a non-selective manner, with biomolecules containing primary amine groups, e.g. proteins (Kikgawa and Beppu 1987), nucleic acids (Nair et al 1984) and amino phospholipids (Kikgawa et al 1981). These reactions result in the formation of products known as Schiff bases having the characteristic conjugated N-C=C-C=N fluouromorphic system, i.e. two amines combining with a single MDA molecule via a non-fluorescent 1:1 intermediate (fig 2 a). Tappel (1973) postulated that this is the mechanism whereby MDA causes both the intra- and intermolecular cross-linking of proteins (Fig 2 b and c) and the formation of lipofuscin pigment in-vivo. However Kikugawa and Beppu (1987) argued that these fluorescent pigments are more likely to be due to the formation of 1,4-dihydropyridine-3,5-dicarbaldehydes and that other non-MDA aldehydic products could also participate in the formation of lipofuscin. Their results were based on the reaction of MDA under physiological conditions, whereas Tappel employed relatively strong acid conditions. The ability of MDA to cross-link different biomolecules may contribute to its toxicity. The mutagenic/carcinogenic properties associated with this aldehyde could be explained by the previously mentioned ability to condense with nucleic acid bases. Modification of the apoprotein B moiety of low density lipoproteins by MDA and other aldehydic lipid peroxidation products has been postulated to be responsible for the increased uptake of these lipoproteins by sub-endothelium monocytes and hence a potentially important role in atherosclerosis (Steinberg et al 1989)

Fig. 2 Reaction of MDA with Primary Amino Groups









The exposure of a variety of different compounds to ionising radiation or oxidative stress have been reported to result in the production of MDA. A number of non-lipid sources of MDA have been suggested which include amino acids and carbohydrates (Gutteridge 1981) and bile pigments (Gutteridge and Tickner 1978). In most of these cases, however, MDA formation has been indirectly inferred and not unequivocally demonstrated. One exception is the formation of MDA as a secondary product of oxidative stress to DNA (i.e. the 2-deoxyribose moieties), as a result of radiolytic (Cheeseman et al 1988a) or bleomycin-iron induced (Giloni et al 1981) generation of oxygen free radicals. No MDA per se is formed under physiological conditions (37°C, pH 7.4) but is formed during the subsequent TBA test via the decomposition of primary base propenal products. At present there is no evidence that MDA represents a primary oxidative product of any non-lipid compound.

Although MDA is produced as a by-product of the action of thromboxane synthase during prostaglandin biosynthesis, the major source of MDA in biological systems is thought to be the oxidative decomposition of PUFA containing 3 or more double bonds. Furthermore, MDA is produced in greater quantities than the other aldehydic products following in-vitro peroxidation. For example, stressing liver microsomal suspensions with iron-ADP resulted in the formation of 60 to 70 nmol/mg protein of MDA, whereas only 4 to 6 nmol of 4-hydroxynonenal (HNE), one of the other major aldehydes, was produced (Esterbauer et al 1986). However, the underlying lipid transformations and the various factors influencing these transformations are poorly understood with the mechanistic proposals on MDA formation remaining largely inferential.

Two postulated mechanisms for the formation of MDA are shown in fig 3. Dahle et al (1962) were one of the first to propose a mechanism whereby lipid peroxy radicals with a double bond beta-gamma to the peroxy bearing carbon cyclise to oxybridged radicals which resolve into peroxides having a 5-membered ring system. The subsequent decomposition of these compounds was suggested to yield MDA as well as other fragmentation products (scheme A, fig 3). Pryor et al (1976) also put forward a hypothesis for MDA formation. Again the pathway depended on an oxybridge radical, but diverged from Dahle's theory by suggesting the formation of prostaglandin-like endoperoxides. Cleavage across the bicyclic peroxide was postulated to yield MDA (scheme B). The studies of Porter and Funck (1975) provided supporting evidence for the postulate of Pryor et al demonstrating that hydroperoxides with a double bond beta-gamma to the peroxy bearing carbon underwent cyclisation to form prostaglandin-like endoperoxides upon hydrogen abstraction. It must be remembered that these mechanistic studies were carried under non-physiological conditions employing acid hydrolysis or thermal decomposition to study MDA formation. It is possible that peroxide decomposition in-vivo proceeds via a different pathway than those proposed by these two groups.

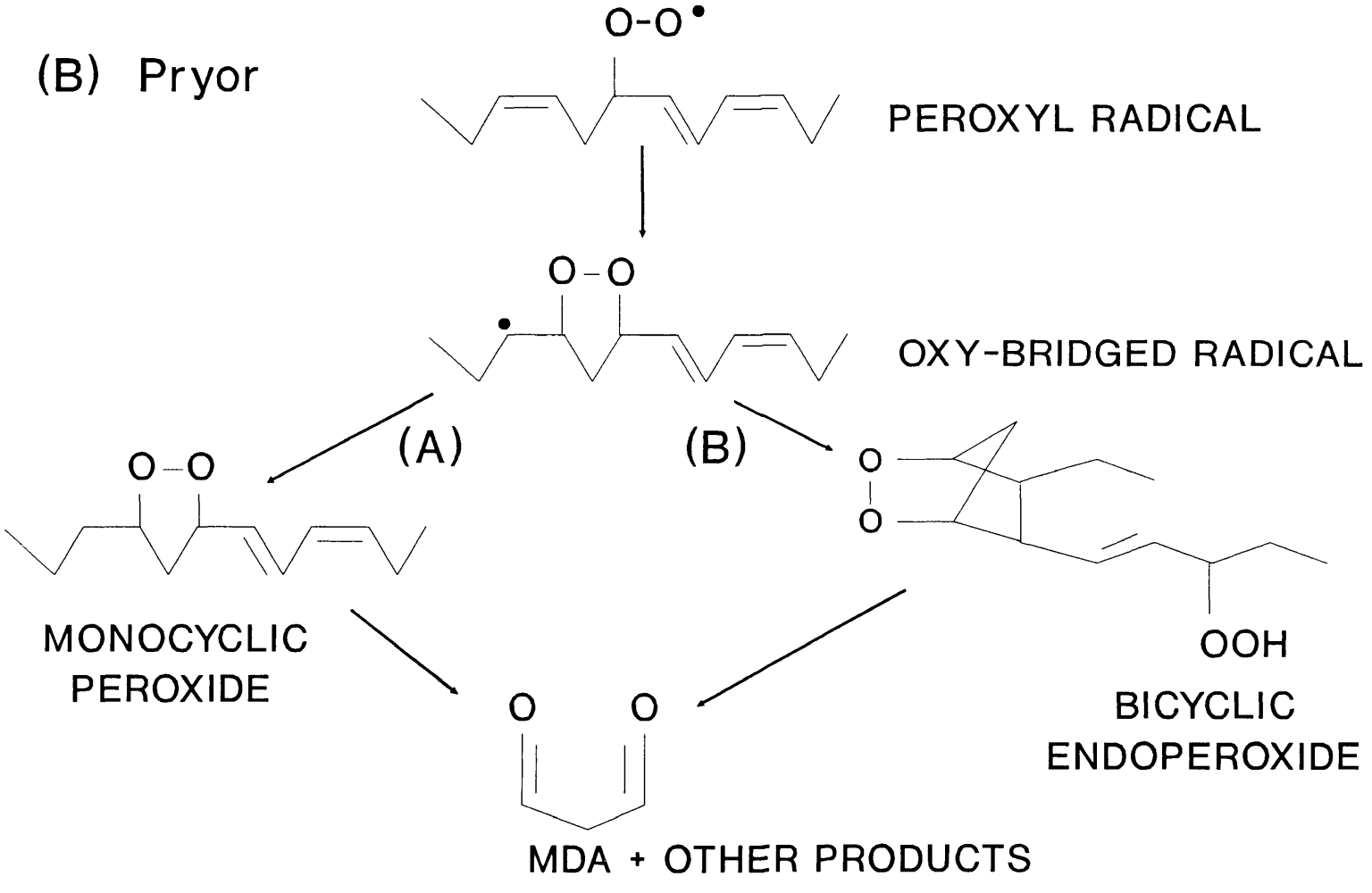
3.2.1 MDA as an Index of Lipid Peroxidation

Despite the widespread use of MDA concentrations as an index of lipid peroxidation there are a number of limitations associated with its use which must be taken into account. Even under ideal experimental, sample and analytical conditions for lipid-derived MDA formation and detection, the utility of MDA as a quantitative index of lipid peroxidation is limited by the non-exclusive, indirect and inefficient nature of MDA formation from lipid oxidation products. MDA is only one of a very complex mixture of lipid peroxide decomposition products, with numerous other aldehydes being formed (Esterbauer et al 1982). MDA can only be formed from PUFA

Fig. 3 Postulated Mechanisms of MDA Formation

(A) Dahle

(B) Pryor



containing 3 or more double bonds (Pryor 1976) and only certain peroxidation products, including 5-membered hydroperoxy epidioxides and 1,3-dihydroperoxides, are capable of breaking down to yield MDA (Frankel et al 1984). In addition the yield of MDA from these precursors either under physiological conditions favouring PUFA oxidation (Janero and Burghardt 1989) or hydrolytic conditions favouring product decomposition (Frankel et al 1984), is invariably low, i.e. 5% or less. Other limitations when using MDA are that the amount of MDA produced varies with the sample under study, being dependent on the degree of unsaturation of the fatty acids and peroxidative stress employed. MDA is a reactive compound and as previously discussed can form condensation products with various biomolecules. Furthermore, MDA is susceptible to catabolic processes in-vivo. Siu and Draper (1982) demonstrated that approximately 70% of an oral dose of radiolabelled MDA was expired by rats as CO_2 within 12 hr, suggesting a relatively rapid endogenous removal system. Several different aldehyde dehydrogenases are known to be capable of reducing MDA and other aldehydic products to their corresponding alcohols implying an important physiological role for these enzymes in detoxifying cytotoxic aldehydes formed as a consequence of lipid peroxidation (Sladek et al 1989). Finally, since MDA is only produced from the peroxidation of fatty acids with more than 2 double bonds its measurement does not fully reflect the extent of PUFA oxidation. For example, linoleic acid which has 2 double bonds and does not breakdown to MDA, is in many instances the major PUFA.

3.3 Measurement of MDA

The analytic techniques available to estimate MDA can be divided into two major classes, the "direct" methods where MDA itself is the analyte, and the "derivative" procedures where MDA is converted to a derivative with properties that can be exploited in its detection and/or separation.

Of the direct methods, separation of MDA by high performance liquid chromatography (HPLC) coupled to UV detection is the most widely used procedure. Methods using ion-pairing (Bull and Marnett 1985, Janero and Bughardt 1988) and aminophase (Esterbauer and Slater 1981, Largilliere and Melancon 1988) take advantage of the negative charge of the enolate anion, whereas the method of Csallany et al (1984) exploits the low molecular weight of the aldehyde using size exclusion columns. Because of the large number of UV absorbing compounds present samples some sample cleanup may be necessary prior to analysis on the HPLC system. One useful method is ultracentrifugation where the sample is passed through an ultrafiltration membrane to remove compounds with a molecular weight greater than 300 (Csallany et al 1987). However, this procedure cannot be used with concentrated samples, e.g. tissue homogenates.

Chromatographic conditions, e.g. flow rate or mobile phase composition, may have to be varied for different samples to allow for adequate resolution of MDA from other closely eluting compounds, e.g. ascorbate (see section 3.5.1).

Derivatisation methods measure MDA indirectly as a fluorescent (e.g. Yagi 1976), UV-absorbing (e.g. Ekstrom et al 1988) or a volatile (e.g. Frankel et al 1984) product. These techniques take advantage of the reactivity of MDA towards nucleophiles and in general tend to give good yields. To date however no derivatising agent specific for MDA has been described so that non-MDA related products can interfere with subsequent analysis. This necessitates separation of the true MDA, with HPLC being favoured if spectrophotometric detection is being used.

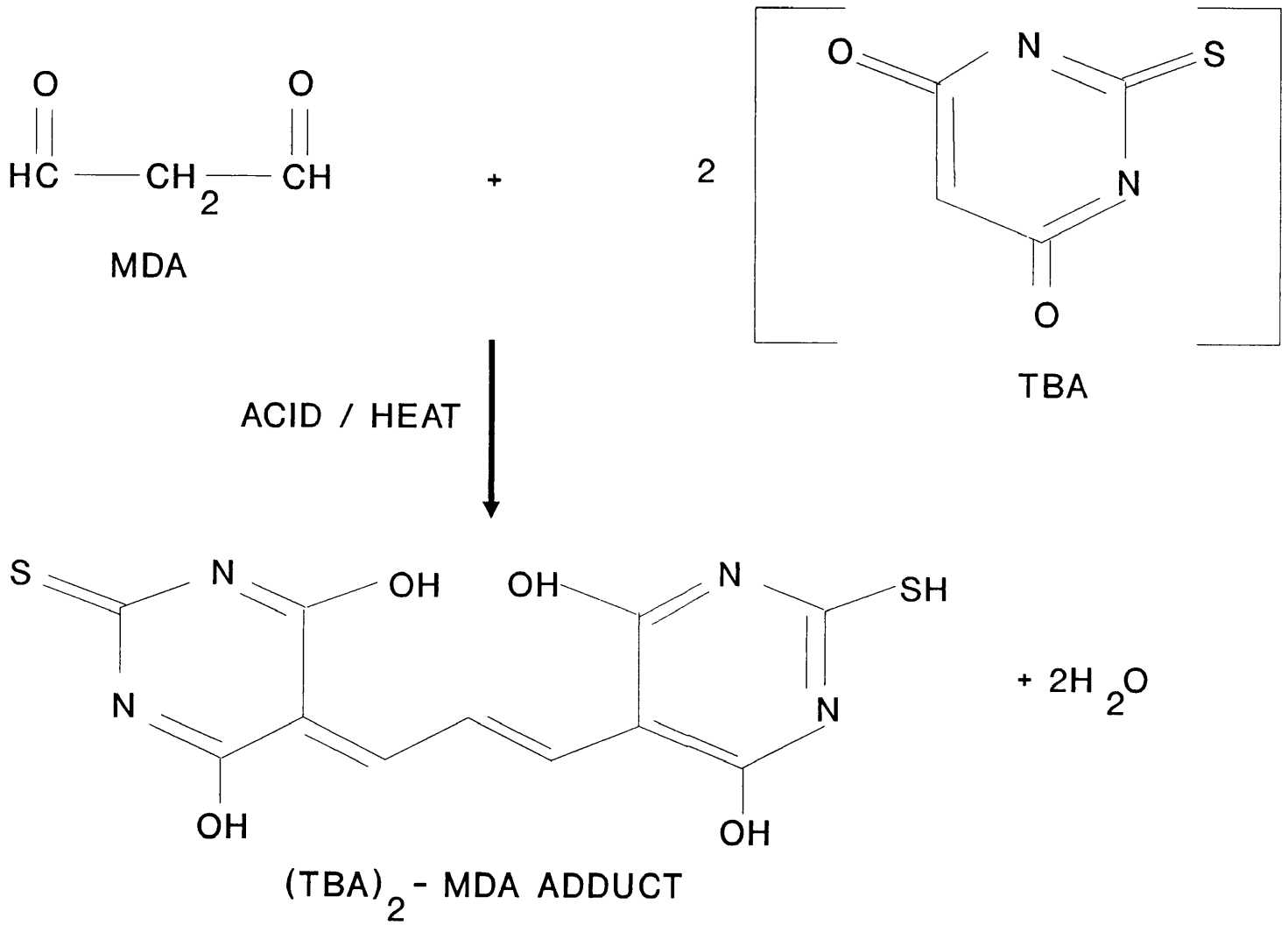
In addition, the derivatisation reactions usually demand prolonged exposure of the sample to harsh non-physiological conditions (e.g acid, heat, organic solvent) in order to achieve good yields. These treatments may result in peroxidative damage to the intact PUFA as well as decomposition of lipid peroxides. The assay conditions will also probably preclude discrimination between free and bound MDA since any aldehyde present in the bound form would probably be liberated during incubation.

3.4 The Thiobarbituric Acid Test

The thiobarbituric acid (TBA) reaction is one of the "derivative" methods of MDA estimation. It was first introduced into biology by Kohn and Liversedge in 1944 who noted that aerobic oxidation of a brain homogenate generated a compound which exhibited high reactivity with TBA under acid conditions. It was later suggested by Sinnhuber et al (1958) that the characteristic colour reaction given by oxidised lipids and TBA was a product of the reaction of two molecules of TBA with 1 molecule of MDA, i.e. $(TBA)_2$ - MDA (Fig 4). This has since been confirmed by nuclear magnetic resonance (NMR) techniques (Nair and Turner 1984). Basically the TBA test consists of heating the sample with TBA at a low pH to form a red/pink colour complex.

The advantages of this method are that it is sensitive, cheap and easy to perform and as a result is probably the most widely used single assay for measuring lipid peroxidation. Despite this, there are numerous drawbacks and misconceptions associated with the test. The simplicity of the assay has led to many different versions being used and hence comparison of results between different laboratories is extremely difficult since the value obtained is known to be dependent on the assay conditions used. No TBA test is at present suitable for all applications. Substantial changes will occur in the sample during the assay since it is subjected to a severe chemical reaction. An important point often not realised or ignored by many contemporary users of

Fig. 4 Formation of $(TBA)_2$-MDA Adduct



the method is that very little free MDA is present in biological samples and that most of the MDA is formed from the breakdown of lipid peroxides during the acid heating stage of the assay. Therefore the TBA test is said to "release" MDA from precursor molecules. Indeed the value of this method is that the peroxidative process beginning in the reaction mixture is effectively amplified in the assay itself, and thereby making it a very sensitive method. Thus it is important to recognise that this assay measures "MDA-equivalents" or thiobarbituric acid reactive substances (TBARS) and not MDA per se.

Besides the limitations regarding the use of MDA in general as an index of lipid peroxidation, additional considerations have to be taken into account when MDA is estimated via its TBA derivative. It is now clear that a number of important observations make the relationship between MDA derived from lipid peroxidation products and its reactivity in a TBA test less than direct. These include the effects of varying assay conditions and reagents on the test response, the non-specificity of TBA towards MDA, the fact that other lipid degradation products other than MDA react with TBA and also the inability of spectrophotometry alone to distinguish the authentic $(TBA)_2$-MDA adduct from similar TBA adducts.

Various factors have been shown to influence the TBARS value obtained for a particular sample. Since TBARS are formed from the thermal/acid catalysed decomposition of lipid peroxides and MDA precursors, the presence of transition metals will effect the test result. In fact contaminating levels of transition metals in the assay reagents are sufficient to stimulate the decomposition of peroxides without initiating PUFA peroxidation during the test (Janero and Burghardt 1989). Additionally, Asakawa and Matsushita (1980), demonstrated that added transition metals could lead to peroxidation of previously unaffected PUFA in the sample during the assay. These problems can be prevented or overcome by passing the assay reagents through

metal chelating resins before use. Asakawa and Matsushita (1980) recommended the inclusion of transition metals (e.g. Fe^{3+}) to support peroxide decomposition and so achieve maximal concentrations of TBARS, and the addition of antioxidants to prevent peroxidation of any co-existing unoxidised PUFA.

Whereas the reactivity of MDA itself is virtually unaffected by either the specific acid used or the pH, the reactivity of peroxidised lipids has been found to be dependent on the absolute pH and the acid used (Asakawa and Matsushita 1980). This may be a result of the different tendencies of lipid peroxidation products to decompose to TBARS. The choice of buffer can also affect the TBA test since both citrate and phosphate buffers inhibit colour formation by chelating metals.

The main drawback of the TBA assay is the non-specificity of TBA for MDA. The reactivity of TBA towards MDA under the conditions of the test reflects the nucleophilicity of the acid and the susceptibility of the carbonyl groups of MDA to nucleophilic attack at low pH. The same conditions render most carbonyl compounds (aldehydes, ketones) susceptible to attack from TBA. Consequently there is no chemical or mechanistic specificity towards MDA. Due to the complex mixture of products resulting from lipid peroxidation, including both saturated (alkanals) and unsaturated (alkenals) aldehydes, many other TBA adducts are also likely to be formed during the TBA test besides that with MDA. In addition some of these aldehydes are capable of forming complexes which are spectrophotometrically indistinguishable from authentic $(TBA)_2$-MDA (Kosugi et al 1989). Therefore the various monofunctional aldehydes have the potential to interfere in a number of ways with the estimation of MDA when using the TBA assay to assess lipid peroxidation. Firstly the absorption spectra of both yellow (ϵ_{max} = 495nm) and orange (ϵ_{max} = 552nm) pigments are broad and overlap the red MDA product.

A more direct effect comes from those aldehydes that form an adduct indistinguishable from that of MDA. Kosugi et al (1989), who studied the effect of lipid peroxidation products generated from different samples on the TBA test response, found that red pigmented TBA adducts were produced that were indistinguishable from that formed from authentic MDA itself under a number of conditions. These TBA adducts had identical absorption and fluorescent spectra and chromatographic profiles (HPLC) to $(TBA)_2$-MDA. In addition the TBA test response (red pigment) of, for example oxidised methyl linoleate and erythrocyte ghost lipids, were greater by a factor of 10 than those expected from the values obtained for MDA using direct chemical analysis, i.e. by the Hantzsch method where a 1,4-dimethyl-1, 4-dihydropyridine-3, 5-dicarbaldehyde derivative of MDA was determined by HPLC. The total aldehyde contents of the oxidised samples, as determined by the dinitrophenylhydrazine (see section 3.6.3) and dimedone (derivatisation with 5,5-dimethyl-1,3-cyclohexanedione) methods were also much higher than the corresponding MDA value estimated by the Hantzsch procedure. In addition Kossugi et al demonstrated a synergistic enhancement of red pigment formation between alkenals and alkadienals, and the marked additional enhancement provided by organic hydroperoxides under the conditions of the TBA test employed. They found that while the reaction of each alkenal and alkadienal individually yielded 0.2-0.5% and 5-10% respectively, yields of the red adduct were greatly increased (by $>30\%$) when both were present or in the presence of hydroperoxides.

Certain non-lipid compounds including sugars (particularly sucrose), amino acids and DNA are known to give a positive TBA response and are thus an additional source of potential interference (Gutteridge 1980).

It should not be concluded from the foregoing discussion that the TBA test is totally without use as a means of assessing lipid peroxidation. For example Esterbauer et al (1982) demonstrated that in liver microsomal suspensions in which peroxidation was stimulated by ADP-iron there was a close correlation between free MDA concentrations as determined using a direct HPLC method and those obtained using the TBA assay. One possible explanation for the favourable agreement seen in this particular case was that prior to the TBA test, aliquots of the reaction mixture were treated with 10% trichloroacetic acid (TCA) so as to precipitate out most of the potential MDA precursors, e.g. oxidised lipids or MDA bound to protein. Esterbauer also found that 98% of the MDA produced in this system was released into the aqueous phase, whereas approximately 60% of the other aldehydes produced remained associated with the hydrophobic lipid membranes. In addition any TBA-positive compounds remaining in the TCA treated supernatant produced only weak colour formation in the subsequent TBA test. For example the αß-unsaturated aldehydes, 4-hydroxynonenal and 4,5-dihydroxydecanal form TBA adducts with molar absorption coefficients which were at least 450-fold less than that for MDA. It was suggested, therefore, that these aldehydes would have to be present at a 100-fold excess over MDA in this system to have a significant ($< 10\%$) effect on the TBA result. Esterbauer (1985) stated that the critical point is that on a molar basis the yield of the chromophore is about 3 orders of magnitude greater with MDA, and estimated that in a system containing equimolar concentrations of MDA and other TBA reactive aldehydes, MDA would account for 99.9% of the total colour formation.

A second system which exhibited a close correlation between MDA formation as estimated by a direct HPLC method and the corresponding TBARS value was the study of the peroxidation of purified cardiac membrane phospholipids (as liposomes) carried out by Janero and Burgardt (1988). The explanation for the good agreement was due to the negligible amounts of non-lipid derived,

non-MDA TBARS produced in this particular system.

Problems will however occur under less ideal conditions, e.g. when using tissue homogenates and prolonged incubation times, and also the presence of high concentrations of transition metals. An example of this was provided by Kosugi et al (1989) who followed the production of MDA and TBARS in rat liver microsomes following in-vitro peroxidation with iron-ADP/ascorbate. In contrast to Esterbauer they found that the estimation of MDA using the TBA test gave a 3-fold greater value than that expected from the direct determination of MDA using the Hantzch method. These conflicting results probably reflect the differences in the biological materials studied and the particular TBA test employed.

3.4.1 Fluorimetric Determination of Thiobarbituric Acid Reactive Substances

Thiobarbituric acid reactive substances (TBARS) were assayed by a modification of the fluorimetric method described by Yagi (1976).
1ml sample in 40mM trizma buffer, pH 7.4, was mixed with 0.2ml 7% SDS, 0.3ml 10% phosphotungstic acid and 1ml 0.67% thiobarbituric acid. The TBA reagent was prepared by adding the required amount of solid in ultrapure water, heated to almost boiling point with continuous stirring, allowed to cool and filtered before use. The samples were then incubated at 95°C for 1 hour. The tubes were then cooled to room temperature and the TBARS extracted by mixing with 5ml n-butanol and spinning for 10 min at 3000rpm. The resulting upper phase was aspirated off and its fluorescence measured in a Perkin Elmer LS-3 fluorescence spectrometer (excitation 515nm and emission 553nm).

MDA Standard; Concentrations of TBARS in the samples were determined from a set of MDA standards that were treated in the same manner as the samples. 16.5μl of 1,1,3,3-tetramethoxypropane (TMP) which forms MDA on acid hydrolysis, was diluted to 1L with dH_2O. An aliquot of this was diluted 1/50 with 40mM trizma buffer, pH 7.4, such that 1ml contains 2nmol. A set of working standards, i.e. 0, 0.5, 1.0, 1.5 and 2.0 nmol MDA/ml, (made by appropriately diluting the 2nmol/ml solution) was assayed with each batch of samples.

The assay was linear up to 2 nmole/ml of MDA standard (fig 5) and up to at least 1mg protein of whole homogenate from control rat cortex (fig 6). The sensitivity limit of the assay was approximately 0.1 nmol/ml.

3.5 Measurement of MDA Using High Performance Liquid Chromatography

Because of the problems associated with the TBA assay and in particular its lack of specificity, it is important that if MDA is to be used as an index of lipid peroxidation that a more specific method is also employed. Development of HPLC techniques has allowed the direct determination of MDA and to date at least three chromatographic methods have been described. The method of Esterbauer et al (1984) uses a weak ion-exchange system, separation on an ion pairing basis is used in the method of Bull and Marnett (1985), while MDA is separated by size exclusion chromatography in the method of Csallany et al (1984). Initially I gained experience of the method of Esterbauer at Guy's Hospital Medical School in collaboration with Dr. Patrick McCarthy. I subsequently set up my own system at the Institute of Child Health. This method was then used to measure free, bound and total MDA.

Fig 5. MDA Standard Curve for TBA Assay

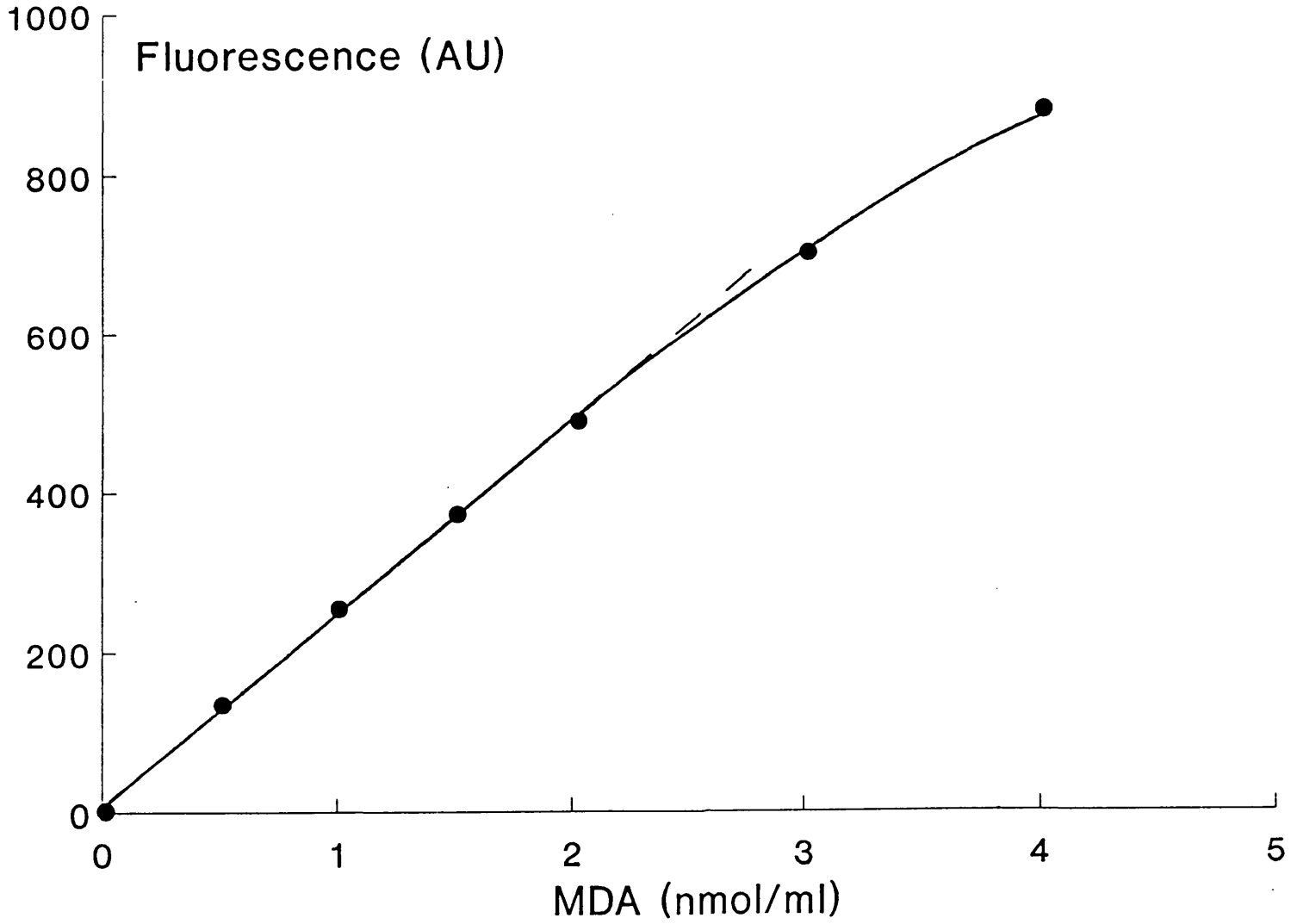
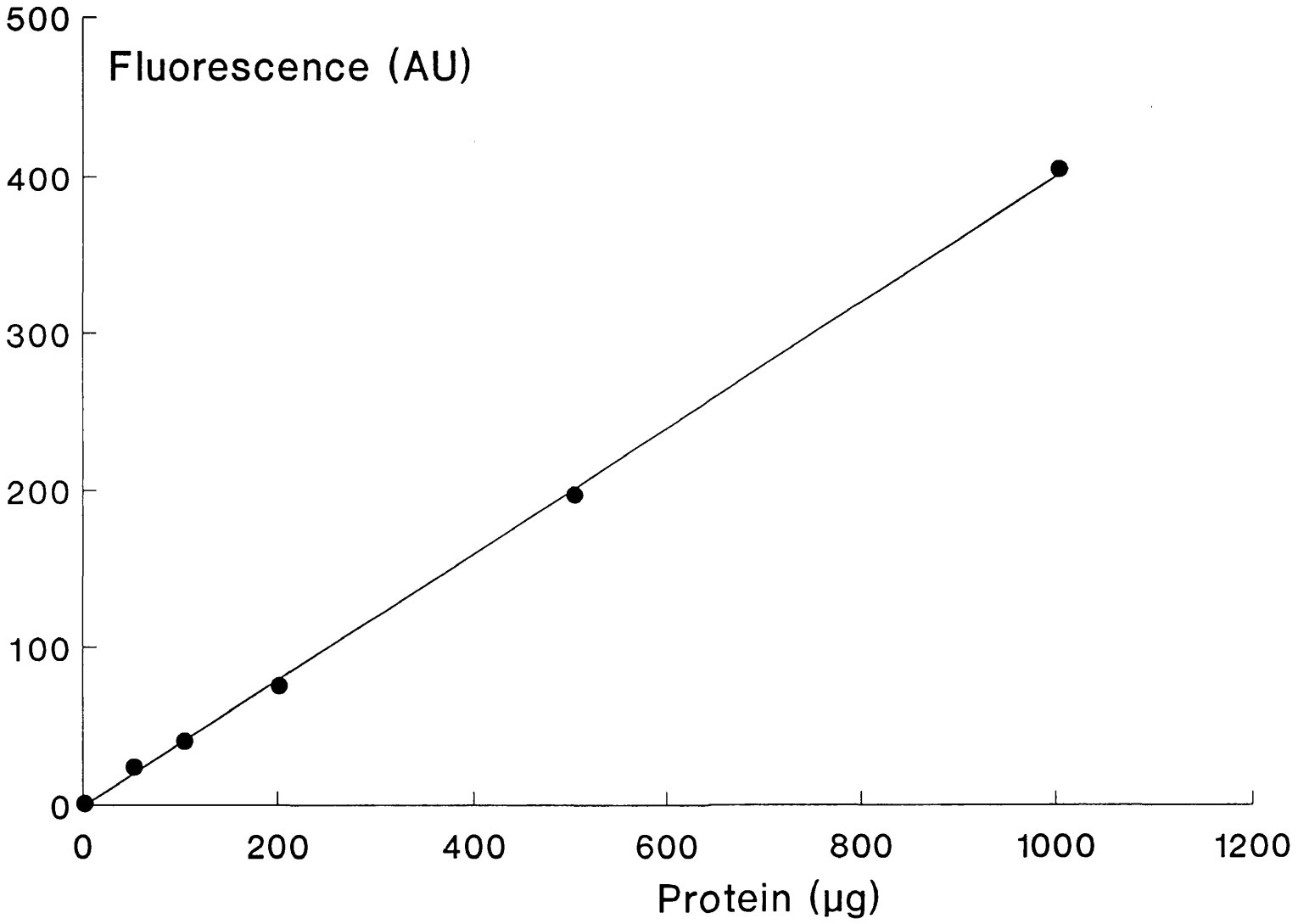


Fig 6. Relationship Between Protein Concentration (Rat Cortex) and Fluorescence (Endogenous TBARS)



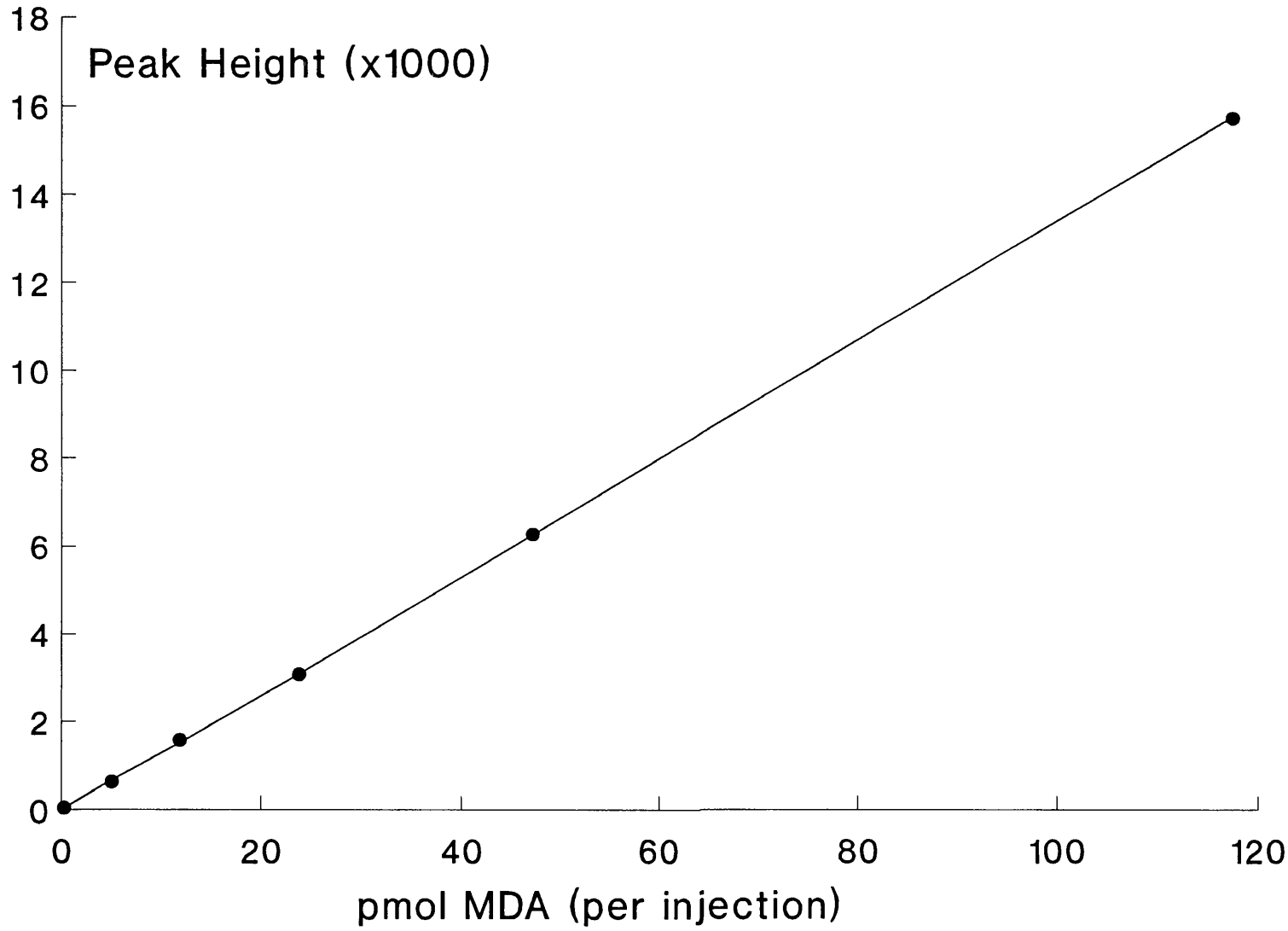
3.5.1 Estimation of Free MDA

The HPLC system I developed at the Institute of Child Health for measurement of MDA was a modification of the method of Esterbauer et al and comprised the following components; separations were carried out on a Spherisorb S5NH column (25cm x 4.6mm, Hichrom UK); mobile phase was 0.03M trizma, pH 7.0 / acetonitrile (21:79 v/v) at a flow rate of 1.4 ml/min (Perkin Elmer Binary LC Pump 250). The detector (LDC Analytical SpectroMonitor 3100) was set at a wavelength of 267nm. The injection volume was 20μl. Chromatograms were recorded on a Spectra Physics SP4100 computing integrator.

An MDA standard of known concentration was prepared form 1,1,3,3-tetramethoxypropane (TMP) by acid hydrolysis. 200μl of TMP was diluted to 100 ml with 1% (v/v) sulphuric acid and left standing at room temperature for two hours. An aliquot of this solution was diluted 1:100 with sulphuric acid (1%). This was termed the stock MDA solution. The MDA concentration in the stock was determined by carrying out a UV scan between 400 and 190nm against an acid blank (ϵ = 13750 at 245nm). The stock MDA, which was stable for at least one week at 4°C, was diluted 1:100 in 0.1M trizma pH 7.4 / acetonitrile (50:50 v/v). This was used as the running standard and was prepared fresh daily. 20μl of the running standard contained on average 23 pmol MDA. The assay was linear up to at least 120 pmole/20μl injection volume at 0.02 arbitrary units full scale (AUFS), see fig 7.

Preparation of test samples for estimation of free MDA simply involved mixing an aliquot of the sample with an equal volume of acetonitrile, mixing vigorously for thirty seconds on a vortex mixer and spinning in an eppendorf minifuge for 5 min. The supernatant was aspirated off and kept on ice. 20μl was then injected onto the column. This treatment deproteinises the sample

Fig 7. MDA Standard Curve for HPLC Method



and protects the HPLC column from damage. This treatment was also capable of terminating lipid peroxidation in samples when studying in-vitro peroxidation systems, e.g. peroxidation of rat brain homogenate using $CuSO_4$ / H_2O_2 (see chapter 6).

The principal modification from the method of Esterbauer et al (1984) was that whereas Esterbauer employed a mobile phase of 9:1 (v/v) tris buffer / acetonitrile at 1.0 ml/min, I found it necessary to use a 21:79 (v/v) composition with a flow rate of 1.4 ml/min. The main reason for this was to resolve the small amount of free MDA present in the sample from the high concentrations of ascorbate present in brain tissue (fig 8).

3.5.2 Measurement of Bound MDA

As discussed in section 3.2, MDA is capable of binding to various biomolecules including proteins and DNA. It was therefore decided to develop a method to release and measure bound MDA which could then be used as an index of lipid peroxidation in the fractions of nerves, since any free MDA present initially, i.e. in the whole homogenate, would be removed by the fractionation procedure. In the case of the whole homogenate, the assay for bound MDA actually measures the total MDA present. The concentration of bound MDA can be determined by subtracting the value obtained for the free form from the total. The method used to assay bound MDA was a modification of the alkaline hydrolysis system of Lee and Csallany (1987) who studied free and bound MDA levels in liver during vitamin E deficiency in the rat.

Reagents

Saturated NaOH solution (filtered). Diluted 1:4 in dH_2O.

Concentrated HCl diluted 1:4 in dH_2O.

Promethazine (aq. soluble antioxidant) 0.5mg/ml in 40 mM Trizma, pH 7.4.

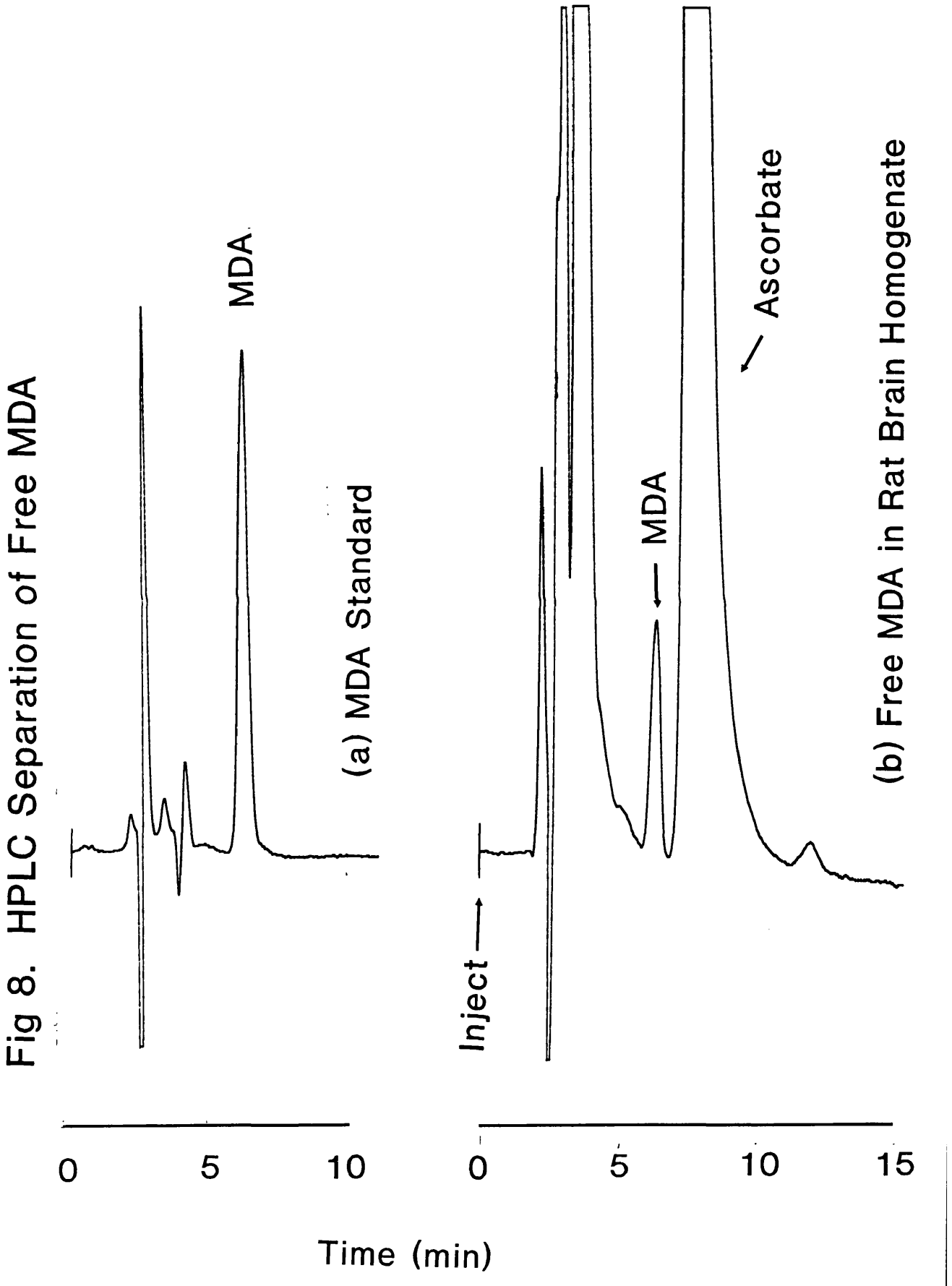


Fig 8. HPLC Separation of Free MDA

Procedure

80μl of a 2.5% (wt/vol) homogenate was mixed with 10μl promethazine and 12μl NaOH and incubated at 60°C for 70 minutes. After cooling to room temperature the hydrolysed samples were neutralized with 17μl HCl. 120μl of acetonitrile was added and the mixtures mixed thoroughly for 30 sec. The samples were then spun in an eppendorf minifuge for 5 min. The supernatants were aspirated and kept on ice in the dark (promethazine is light sensitive). 20μl of the supernatant was analysed on the same HPLC system as used to measure free MDA except that the composition of mobile phase was 17:83 (v/v). The flow rate was unchanged at 1.4 ml/min.

3.5.2.1 Is the Assay Actually Measuring Bound MDA ?

Since the method used to estimate bound MDA involved relatively strong assay conditions, i.e. heating the samples at 60°C for 70 min under alkaline conditions, it was possible that what was actually being measured was not authentic bound MDA, but MDA produced during the assay from peroxidation of PUFA, or from preformed lipid peroxides as occurs in the TBA test. However, a number of observations appeared to exclude these possibilities.

(i) Incubation of a sample with promethazine alone (and no NaOH) at 60°C for 70 min did not result in any increase in free MDA compared to time zero. Although bound MDA was not determined, Esterbauer (1982) demonstrated that almost all of the MDA generated during in-vitro peroxidation was released in the free form. Incubation of the same sample in the absence of both promethazine and NaOH resulted in 13-fold higher MDA levels after a 60 min incubation. Therefore, these results suggest that promethazine is acting as an antioxidant and preventing peroxidation in the sample.

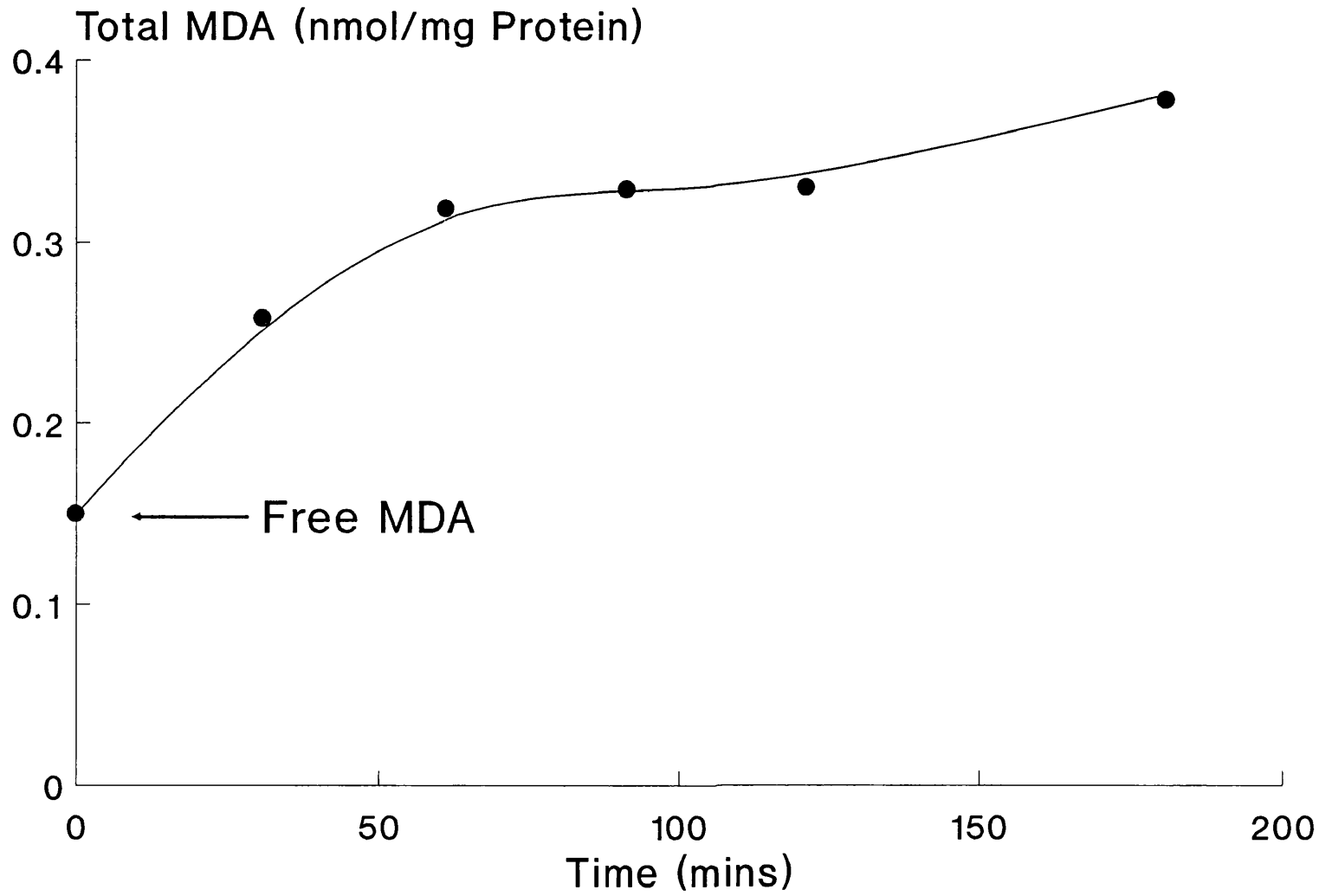
(ii) From a representative plot of bound MDA released with time (fig 9) it can be seen that maximal release is achieved within approximately 60 min with no additional increase up to 120 min. If the increases in MDA were due to in-vitro peroxidation, much larger amounts of MDA would be expected since as stated above incubating the same sample without promethazine and without NaOH resulted in much higher MDA levels. Moreover, the plot would not be expected to plateau at 60 min but continue to show a linear increase. The increase in MDA seen at 180 min is probably due to promethazine breakdown resulting in some sample peroxidation. This hydrolysis profile was reproducible.

(iii) The final piece of evidence that authentic MDA was being measured by this assay comes from the following experiment. To check that MDA released during the initial linear 60 min was not due to promethazine breakdown and hence loss of antioxidant protection, promethazine was incubated with NaOH alone at 60°C for 60 min. This pretreated promethazine was found to have retained its antioxidant capacity. Firstly, it was still capable of completely preventing peroxidation when the tissue sample was incubated without NaOH. Secondly, when used in the hydrolysis system the same MDA value was obtained as when untreated antioxidant was employed.

3.5.2.2 Sucrose Interference in Assay for Total MDA

Originally when using the alkaline hydrolysis system to measure bound MDA, the samples were made up in 0.32M sucrose. It was found however that when sucrose was incubated with promethazine and NaOH, or NaOH alone, and subsequently run on HPLC, that a peak was seen with the same retention time as the MDA standard. Spiking the sample with MDA produced a single peak on the chromatogram. It is probable therefore that MDA was formed from the breakdown of sucrose during the assay since it is known that an authentic $(TBA)_2$ - MDA adduct can be formed in the TBA test from compounds

Fig 9. Effect of Incubation Time on Release of Bound MDA



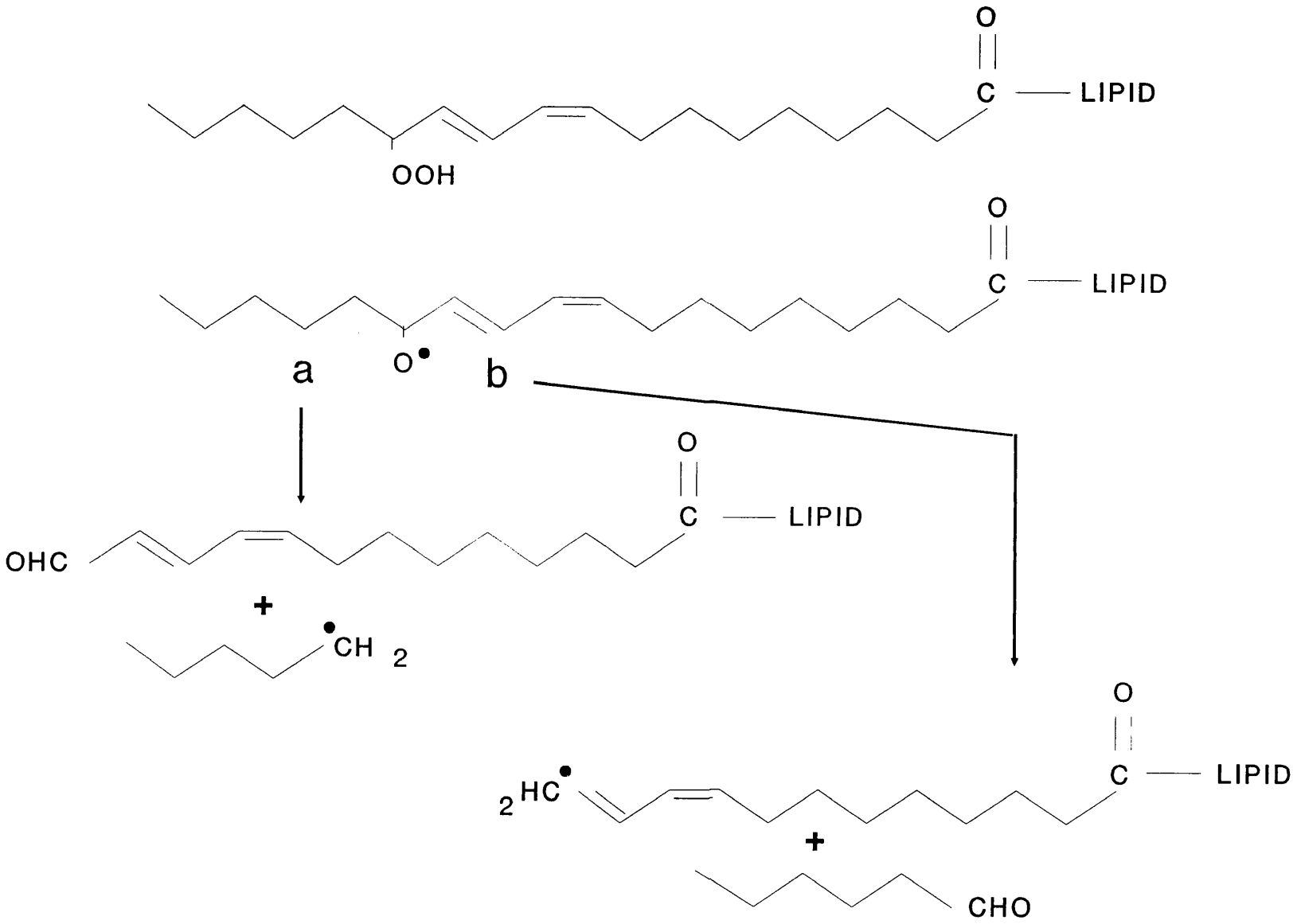
including sugars (Gutteridge and Halliwell 1990). In addition, the "bound" MDA values obtained from tissue samples in sucrose were much higher than that which would be expected from the sum of the bound MDA in the tissue and the MDA formed from the sucrose. It is possible that breakdown products of sucrose and tissue sample could combine to form MDA. Evidence for this comes from the studies of Knight et al (1988) who showed that certain compounds, including unsaturated aldehydes could react with TBA in the presence of sucrose to produce relatively large amounts of $(TBA)_2$ - MDA compared to the total formed from the aldehydes and sucrose separately. Sucrose should therefore be avoided if one intends measuring bound MDA. If this cannot be done, for example when using sucrose gradients during tissue fractionation, any sucrose should be removed by resuspension and pelleting in at least 25ml of trizma buffer before performing the assay.

3.6 Non-MDA Aliphatic Aldehydes

As previously discussed numerous carbonyl containing compounds besides MDA are formed from the decomposition of lipid peroxides. Esterbauer (1985) reported an increase in 31 compounds after subjecting rat liver microsomes to oxidative stress. Of these the identity of five n-alkanals, six 2-alkenals, four 4-hydroxyalkenals and eight other products including MDA were confirmed by mass spectrometry.

The principal mechanism for the formation of aliphatic aldehydes during lipid peroxidation is thought to be via the decomposition of lipid alkoxy radicals (fig 10). Scission of the C-C bonds on either side of the oxygen bearing carbon occurs spontaneously, and results in the formation of two types of aldehydes, one where the carbonyl function remains with the parent fatty acid (scheme a, fig 10) and a second (scheme b) where the products are derived from the methyl terminus of the molecule (Frankel 1982). Other mechanisms,

Fig 10. Formation of Aldehydes from 13-Hydroperoxy Linoleic Acid



however, are also thought to contribute to the complex mixture of aldehydic products. These include dismutation of hydroperoxides and the reversible aldol condensation of hydroxyl groups on adjacent carbon atoms (Esterbauer 1982). Although a good correlation has been found between the extent of lipid peroxidation and the amount of phospholipid-bound aldehyde, e.g. rat liver microsomes following in-vitro intoxication with CCl_4, $CBrCl_3$ or iron-NADPH (Fulceri et al 1990), the majority of studies have estimated free aldehydes. It is noteworthy, however, that Fulceri et al also postulated the existence of a detoxifying system capable of removing these bound aldehydes in-vivo in rat liver.

The spectrum of the non-radical aldehydic lipid peroxidation products goes from the less reactive ones such as the n-alkanals to the highly reactive αß-unsaturated aldehydes, particularly the 4-hydroxyalkenals. This latter group have been the focus of increasing attention over the past decade or so. The pathological importance of hydroxyalkenals was first demonstrated in 1980 by Benedetti et al who found that 4-hydroxy-2-trans-nonenal (HNE) was the major diffusible toxic product generated by rat liver microsomes during peroxidation. The credibility of these products as having genuine in-vivo toxicity was enhanced by a group who were investigating an area which was completely unrelated to free radicals and lipid peroxidation. Segall and coworkers (1985) showed that 4-hydroxyhexenal was the ultimate toxic agent responsible for the detrimental effects seen following the ingestion of pyrrolizidine alkaloid e.g. senecionine, by livestock and humans. These alkaloids which are found in various plants are metabolised in the liver to generate the reactive aldehyde.

Hydroxyalkenals are strongly electrophilic and like alkylating agents, readily react with nucleophilic groups. Although capable of reacting with amino groups, the sulphydryl anion (RS^-) of low molecular weight thiols

(e.g. glutathione and cysteine) and the SH groups of enzymes and proteins are the main targets of these aldehydes since these chemical groups are the strongest nucleophiles in biological systems. Therefore the majority of the reported deleterious effects of hydroxyalkenals have been explained in terms of attack on essential SH groups. For example HNE was demonstrated by Hauptlorenz et al (1985) to affect the proliferation of cultered Ehrlich ascites tumour cells by inhibiting DNA synthesis and it was subsequently shown that the alpha and beta DNA-polymerases were the sites of HNE attack (Wawar et al 1986). Although, HNE is inhibitory when incubated at high concentrations with adenylate cyclase (10^{-5} M and higher), at lower concentrations (micromolar or less) it was found to exhibit a strong stimulatory effect on the enzyme (Paradisi et al 1985). A recent study by Rossi et al (1990) on the effect of different biogenic 4-hydroxyalkenals on phosphatidylinositol-4,5-bisphosphate-phospholipase C (PL-C) is of interest for a number of reasons. Three of the hydroxyalkenals examined stimulated PL-C activity in liver membranes i.e. hydroxyhexenal, HNE and hydroxyoctenal (HOE), over relatively wide concentration ranges. More importantly the results demonstrated that HNE is not the only, nor the most active lipid peroxidation product since 10^{-11}M HOE yielded similar stimulation of PL-C as 10^{-6}M HNE. Also of interest was that pre-incubation of the membranes with a 10,000-fold excess of reduced glutathione failed to prevent the stimulation produced by HNE indicating that in this case at least the reaction with SH groups did not appear to be responsible for the observed effects.

Compared to the highly reactive oxygen radicals these non-radical lipid breakdown products are relatively stable and since it has been shown that peroxidation products can escape from isolated hepatocytes (Poli et al 1985), it is possible that the original site of free radical attack could lead to peroxidative damage some distance away.

The main question regarding the demonstrated detrimental effects of the hydroxyalkenals under in-vitro conditions is whether the concentrations required to produce these effects can be achieved in-vivo considering the known metabolism of these compounds (see section 3.6.1). Many of the in-vitro experiments where the effect of exogenously added HNE was studied required that the aldehyde be added at concentrations of 100μM and above. These concentrations cannot be generated when liver microsomal suspensions or hepatocytes are peroxidised in-vitro. However, the lipophilic nature of HNE means that if formed from membrane lipid peroxidation, the aldehyde is more likely to remain associated with the membrane than escape into the surrounding aqueous phase. It is possible that HNE could reach very high local concentrations within the membrane which are sufficient to elicit detrimental effects. Benedetti et al (1984) suggested that the local concentration of total aldehydes within the membrane of peroxidised microsomes could achieve levels of 100mM and Koster et al (1985) suggested that concentrations of HNE in membranes could reach 4.5mM. Regardless of the postulated existence of high intramembraneous concentrations of HNE, certain effects of HNE including activation of PL-C (described above) have been shown occur at very low levels of the aldehyde. Moreover, micromolar concentrations of endogenous HNE have recently been demonstrated in the liver and plasma of the rat and exhibited an increase during experimental vitamin E deficiency (Yoshino et al 1986). Gas chromatography-mass spectrometry has been used to demonstrate elevated HNE levels in the retina of vitamin E deficient rats and dogs (Dratz et al 1989) and in tissues of dogs with neuronal-retinal ceroidosis (Siakotos et al 1988).

3.6.1 Aliphatic Aldehydes as an Index of Lipid Peroxidation

Aliphatic aldehydes, like MDA, represent end-products of lipid peroxidation and are therefore also dependent for their formation on the decomposition of lipid peroxides and the various factors which may effect this decomposition. Moreover, the fatty acid profile of the sample under investigation will determine the composition of the mixture of aldehydic products formed, e.g. 4-HNE is only derived from the peroxidation of ω-6 PUFAs including linoleic, gamma-linolenic and arachidonic acid (Esterbauer et al 1986), 4-hydroxyhexenal is related to ω-3 PUFA oxidation, particularly docosahexaenoic acid (van Kuijk et al 1990a).

In common with MDA some of these peroxidation products are reactive substances and are also subject to different metabolic processes. These characteristics may affect their availability as indices of lipid peroxidation. This point is illustrated by the studies of Yoshino et al (1986) who found that the recovery of hexanal added in-vitro to a liver homogenate prior to analysis was time dependent with 59% recovered immediately after addition and only 22% after 2 min, whereas the recovery of hexanal added to plasma was unchanged over the same time period. Alin et al (1985) showed that 4-hydroxyalkenals were good substrates for rat liver cytosolic glutathione transferases, enhancing the conjugation of these peroxidation products with glutathione by a factor of 300-600 compared to that which can occur spontaneously. Therefore a failure to detect an increase in an aldehydic lipid peroxidation product may simply reflect the efficiency of their metabolism/degradation by detoxification systems.

3.6.2 Measurement of Aliphatic Aldehydes

Because of the greater toxicity and range of detrimental effects associated with hydroxyalkenals compared to MDA, measurement of these compounds is probably more informative, particularly when assessing in-vivo peroxidation. However, methodology for the estimation of aliphatic aldehydes requires extensive sample preparation and is time consuming in comparison to the direct measurement of MDA by HPLC. Even after sample pretreatment, separation of the compounds may still be difficult because of the complex mixture of aldehydic products involved.

The method most widely used to determine aldehydes in biological samples is to derivatise them with 2,4-dinitrophenylhydrazine (DNPH) to convert the aldehydes to the corresponding DNPH derivatives. This has a number of advantages. Firstly, the derivatives are more stable and less volatile compared to most free aldehydes which helps prevent loss during subsequent sample work-up. The derivatives also have a strong absorption (ϵ = 2500 - 2800M^{-1} at 360 - 380nm) which aids in detection on either thin layer chromatography (TLC) or HPLC. After the initial derivatisation of the sample, additional steps including solvent extraction, separation on TLC plates, and elution are necessary before the sample can be run on the HPLC system. In a study by Esterbauer et al (1982) of the aldehydic products formed during the in-vitro peroxidation of liver microsomes (ADP-Fe^{2+}), highly complex chromatograms were still seen despite the extensive sample work-up, making peak identification difficult. Another method (Yoshino et al 1986) has been described where a number of different aldehydes can be analysed in a single run after derivatisation with 1,3-cyclohexanedione using HPLC and fluorescence detection. This method will be dealt with in the next section.

Caution must be exercised when estimating aliphatic aldehydes using derivatisation techniques since they frequently involve subjecting the sample to harsh incubation conditions in order to achieve maximal yields. Kosugi et al (1989) demonstrated that a pure lipid peroxide preparation appeared to contain surprisingly high levels of total aldehydes determined by the DNPH method indicating that aldehydes were liberated from the hydroperoxide itself during the derivatisation stage of the assay.

3.6.3 HPLC Method for the Estimation of Aliphatic Aldehydes Following Derivatisation with 1,3-cyclohexanedione.

The aliphatic aldehydes propanal, butanal, pentanal, hexanal and HNE were estimated using a modification of the method of Yoshino et al (1986) developed by Dr. Patrick McCarthy of Guy's Hospital Medical School, London. This method involved HPLC separation of decahydroacridine derivatives of the aldehydes formed by their reaction with 1,3-cyclohexanedione. Since fluorescence detection was employed, this procedure was more sensitive than the previously described DNPH method which uses UV absorption, i.e. a sensitivity limit of about 0.1 pmol compared to 1 pmol per injected sample.

Reagents

The 1,3-cyclohexanedione (CHD) reagent was prepared as follows; 10g of ammonium sulphate, 0.25g CHD and 5ml of glacial acetic acid were mixed in 70ml dH_2O and the pH adjusted to 5.0. The volume was brought up to 100ml.

Contaminating aldehydes were removed from HPLC grade methanol by reacting overnight with DNPH and distilled before use.

Procedure

Samples: 200μl of a 2.5% (wt./vol.) homogenate was mixed with 200μl of purified methanol and 800μl of the CHD reagent. The mixtures were then incubated at 60°C for 60 min and cooled to room temperature. The derivatives were extracted with 5ml $CHCl_3$ by vortex mixing for 1 min followed by spinning at 3000rpm for 10 min. The upper phase was discarded while the remainder was dried under nitrogen. The extract was redissolved in 200μl of tetrahydrofuran (THF) / dH_2O (26:74 v/v). 20μl was injected onto the HPLC system.

Aldehyde Standards: Stock solutions of propanal, butanal, pentanal and hexanal (Aldrich UK) were made up to a concentration of 0.8mg/ml in purified methanol. HNE, made up to a similar concentration, was a gift from Dr. Esterbauer, Institute of Biochemistry, University of Graz, Austria. 125μl of each aldehyde solution was mixed with an equal volume of dH_2O and derivatised with 500μl of CHD as described above for the samples. The $CHCl_3$ extracts were redissolved in 5ml methanol. 100μl of each derivative was added to a single tube, dried down under nitrogen and redissolved in 10ml THF / dH_2O (26:74 v/v). This was used as the running standard mixture, 20μl of which contained 40 to 70 pmole of each of the different aldehydes. The HNE standard was made up and run separately from the other standards.

HPLC System: Separations were performed on a 5μ Hypersil ODS column (150cm x 4.6mm, Hichrom UK) with fluorescence detection, Ex. 380nm and Em. 445nm. Resolution was achieved using a linear gradient mobile phase system run at a rate of 1 ml/min;

0 min: 30% methanol/26% THF

9 min: 26% THF

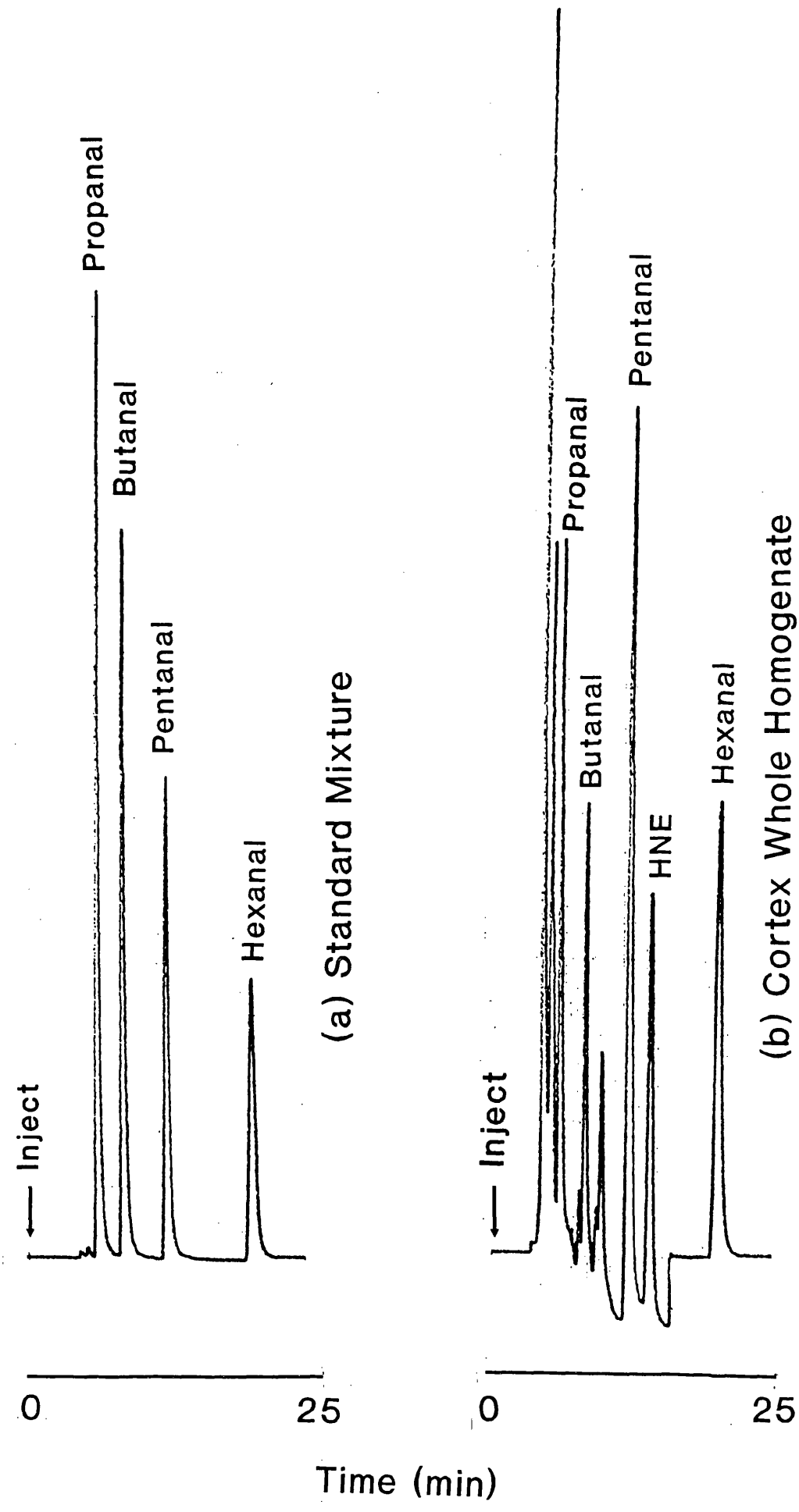
A number of modifications were made to the original method of Yoshino et al (1986). Firstly, in the original method plasma samples and liver homogenates were centrifuged following the addition of the reagents and only the supernatants were used for analysis, whereas when I assayed rat brain tissue the complete unspun homogenate was used. Secondly, chloroform extraction was used for sample clean up following derivatisation as opposed to purification on Sep-pak C_{18} columns. The main difference in the HPLC system was that a greatly simplified gradient was used compared to that of Yoshino. On the present system a single step linear gradient over 9 min was used with all the peaks eluting within 20 min, whereas in the original procedure separations were carried out using a 3 step gradient with a total running time of greater than 50 min. It must be pointed out however that only 5 aldehydes were assayed on our system, whereas Yoshino's group studied an additional 5 compounds. The aldehydes eluted from the column in the following order; propanal, butanal, pentanal, HNE and hexanal, with retention times of 6.0, 8.3, 12.2, 14.0 and 19.8 min respectively (fig 11). This was in agreement with the elution order obtained by Yoshino's group.

3.7 Discussion

The most direct approach for the assessment of lipid peroxidation is the estimation of the primary (hydroperoxide) products. In practice this is difficult due to the fleeting nature and instability of these species. Consequently, assessment of the peroxidative process has relied largely on indirect methods, that is measurement of secondary or end-products derived from the metabolism or decomposition of these lipid peroxides.

The three methods described in this chapter all measured aliphatic end products of lipid peroxidation. The TBA test, despite its limitations, can still be regarded as a useful method for estimation of lipid peroxidation. The best way of confirming the validity of the TBA assay in a given system is to

Fig 11. HPLC Separation of Decahydroacridine Derivatives of Aliphatic Aldehydes



compare the results with those obtained by direct measurement of MDA by HPLC.

The HPLC method for MDA has the advantages of specificity and being more sensitive than the TBA test. Only minor sample pretreatment is required to measure free MDA. In addition the HPLC method has the advantage that it can be used to measure both free and bound MDA. The measurement of bound MDA is useful when studying cellular fractions since any free MDA would be lost during the fractionation process. Bound MDA may also be a useful index of lipid peroxidation in complex systems, e.g. tissue homogenate, where the free form may be present at very low concentrations and most of the aldehyde is present in the bound form.

Measurement of aliphatic aldehydes other than MDA using the cyclohexanedione method, although requiring greater sample preparation, has the advantage of assaying a number of different peroxidation products including HNE in a single run. In fact measurement of hydroxyalkenals, particularly HNE, may be more appropriate for in-vivo studies considering the numerous toxic effects associated with these products.

As Halliwell and Gutteridge (1989) pointed out, MDA and the other aldehydes are all "end products" of lipid peroxidation and arise from peroxide decomposition which can often be controlled by the availability of transition metal complexes rather than by the rate of peroxide formation. In addition, it must also be remembered that all methods give a result at a single instant in time which reflects a balance between the rate of formation and removal or metabolism of that compound. Failure to see an increase in a particular parameter may, therefore, reflect equal rates of removal and production. Alternatively failure to see an increase may be due to the prevention of lipid peroxidation by endogenous antioxidants.

The reason that so many different methods have been used to assess lipid peroxidation reflects the fact that no one method is truly satisfactory for all purposes. Thus the choice of assay is of great importance. The convenience of the method is an important factor but should not be the sole consideration in designing the experimental approach to be employed. Whatever method is chosen, one should think clearly what is being measured and how it relates to the overall lipid peroxidation process. If possible two or more methods should be used simultaneously.

CHAPTER 4.

EXPERIMENTAL ANIMALS, DIET AND VITAMIN E CONCENTRATIONS

4.1 Introduction

4.2 Animals and maintenance

4.3 Experimental diet

4.4 Animal dissection

4.5 Estimation of tissue vitamin E concentrations

4.5.1 Tissue and serum vitamin E concentrations

4.6 Discussion

4.1 Introduction

This chapter provides a description of the experimental rats, their diets and the tissues removed for analysis. Vitamin E concentrations were determined in a number of tissues in order to compare the concentrations with previous data from this department using the same experimental model.

4.2 Animals and Maintenance

Specific pathogen free male Wistar rats, aged 21 to 23 days (Charles Rivers, UK) were divided into two groups and placed on either a vitamin E deficient or control diet and allowed access to diet and water ad libitum. After 4 days any unused diet was discarded and replaced with fresh diet. Rats were housed in sets of five in grid-bottomed cages to prevent copraphagia, and in a controlled environment (i.e. constant temperature and humidity). The rats were isolated from other groups of rats and animal species so as to reduce the risk of opportunistic or cross infections.

4.3 Experimental Diet

Synthetic rat diet, with or without added vitamin E, was obtained from Dyets Inc. (Pennsylvania, USA). The composition is given in table 1. This diet had been successfully employed in this department in a number of previous studies on the effects of experimental vitamin E deficiency in the rat. Two age groups of rats were examined in my studies, i.e. 20-21 weeks and 55-60 weeks old. Animals in the latter group were those remaining from a previous study where the controls had been fed a diet containing 100mg of tocopheryl acetate/kg diet. These rats were analysed first in order to assess the potential of a longitudinal study, i.e. to see whether the methods developed could detect differences between deficient and controls rats when tissue vitamin E concentrations were very low or undetectable. Rats in the younger age group were set up specifically for this project with the controls maintained on a diet containing 40mg/kg tocopheryl acetate. A lower control diet concentration of

Table 1. Composition of Dyets tocopherol deficient diet.

INGREDIENT	Concentration (g/kg diet)
Vitamin free casein	200.0
Dextrose	653.2
Tocopherol stripped lard (stabilised with 0.02% BHA)	100.0
Choline Bitartrate	1.8
Salt mix 200650	40.0
Vitamin mix 302362	5.0

The vitamin E sufficient (control) diet contained the same ingredients as listed above but also contained either 40 or 100mg tocopheryl acetate/kg diet* (see text).

*All-rac-α-tocopherol

vitamin E was selected for the 20-21 week group of rats as this was considered more physiological compared to the earlier diet. The vitamin E deficient diets were the same for both age groups.

4.4 Animal Dissection

Rats were killed by an intraperitoneal injection of 60mg sodium pentobarbitol (Sagatal, May and Baker UK). They were immediately dissected with the following tissues being removed and stored at -70°C; brain, cervical (C 1-8), thoracic (T 1-13) and lumbar (L 1-6) spinal cord segments, sural nerve, heart, muscle (gastrocnemius from lower limb) and liver. Brain was further dissected into six distinct anatomical regions, (i.e. cortex, striatum, midbrain, hypothalamus, cerebellum and brainstem), by a similar method to that described by Glowinski and Iverson (1966). Blood samples were obtained by cardiac puncture. The animals were not perfused prior to removal of tissues.

4.5 Estimation of Tissue Vitamin E Concentrations

Tissue vitamin E concentrations were determined in a number of tissues at 20 and 55 weeks and compared with results obtained from previous studies in this department that used the same diet. As stated above, the older age group of control rats had been maintained on 100mg tocopherol acetate/kg diet, whereas the younger group were fed 40mg/kg diet. It was therefore necessary to check whether altering the vitamin E content of the control diet resulted in reduced tissue concentrations since this could potentially affect the results of the various studies of lipid peroxidation.

Vitamin E (α-tocopherol) was determined by high performance liquid chromatography (HPLC) with fluorimetric detection by a modification (Metcalfe et al 1984) of the method of Buttriss and Diplock (1983). This method had been extensively used and validated in this department for estimation of routine plasma samples and experimental tissues. For tissues,

approximately 100mg was homogenised in 1ml 75% ethanol at 4°C using a polytron disintegrator (Kinematica GmBH) until a consistent homogenate was formed (30 sec). Hydrophobic species were extracted into 1ml of cold HPLC grade hexane (Fisons UK) by vortex mixing for 1 min. The samples were then centrifuged for 1min in an Heraeus minifuge. 10 to 100μl of the hexane phase, depending on whether control or deficient tissues were being assayed, was injected onto a 25cm, 5μm silica direct phase column with an internal diameter of 4.9mm (Jones Chromatography Ltd) and eluted with hexane containing 1% methanol (1.2 ml/min). Any water present was removed from all solvents with 4 angstrom molecular sieves. Fluorescence was monitored with a Perkin-Elmer LS1 fluorimeter using an excitation cut off filter at 280nm and a 310nm emission filter. Unknown concentrations of α-tocopherol were determined by a direct comparison with an external standard, i.e. 5μM DL-α-tocopherol (Sigma, UK) in hexane, and expressed in relation to total lipid content.

In order to estimate the concentration of total lipid the remainder of the hexane / 75% ethanol mixture was dried down in a vacuum oven followed by an extraction with chloroform : methanol (2:1) after Folch et al (1957). Total lipid concentrations of these extracts were determined by the hydroxamic acid method of Snyder and Stevens (1958) using glycerol trioleate as a standard.

4.5.1 Tissue and Serum Vitamin E concentrations

Alpha-tocopherol concentrations were estimated in control and deficient cortex, liver and muscle of 20 and 55 week old rats (n=3). Serum levels were measured in the older age group only (n=5). Tissue concentrations of α-tocopherol were related to total lipid concentrations (μg/g lipid) with serum concentrations expressed as nmol/ml. Results for 20 and 55 week samples are presented in table 2a and b respectively.

Table 2a. 26 week α-tocopherol concentrations (μg/g lipid)

SAMPLE	CONTROL mean + range	DEFICIENT mean + range	DEFICIENT AS % OF CONTROL	
			EXPT.[a]	REF.[b]
Cortex n=3	615 (478 - 793)	84 (79 - 87)	13.7	10.7
Liver n=3	737 (674 - 829)	8.0 (5.0 - 10.2)	1.1	3.4
Muscle n=3	733 (473 - 1013)	37 (42 - 59)	4.6	N.A.[c]

Table 2b. 55 week α-tocopherol concentrations (μg/g lipid)

Cortex n=3	480 (381 - 646)	15.7 (12 - 19)	3.3	2.0
Liver n=3	958 (709 - 1237)	N.D.[d]	0.0	0.0
Muscle n=3	752 (520 - 938)	15.6 (11.7 - 17.7)	2.1	N.A.
Serum n=5	36.6[e] ± 10.0[f]	N.D.	0.0	0.0

(a) vitamin E concentrations from the present study, (b) reference data of Goss-Sampson et al (1988), (c) not assayed, (d) non-detectable, (e) units = nmol/ml and (f) 1 standard deviation.

At 20 weeks concentrations of α-tocopherol in the deficient tissues were 13.7, 1.1 and 4.6% of control values in cortex, liver and muscle respectively. By 55 weeks, whereas vitamin E was undetectable in both deficient serum and liver, deficient cortex and muscle still retained 3.3 and 2.1% of control levels respectively. The control rats maintained on the higher content of tocopherol, i.e. the older age group, did not in general have greater tissue concentrations of α-tocopherol. Results of this study were very comparable to those previously reported from this department by Goss-Sampson et al (1988).

4.6 Discussion

There were no significant differences between the concentrations of α-tocopherol in the control animals of the two age groups. These results were obtained despite the older animals having being maintained on a diet containing 2.5-fold more α-tocopherol. A greater difference in dietary vitamin E levels is probably required to achieve significant differences in tissue levels. In agreement with this Vatassery et al (1988) found that increasing the basal dietary concentration of vitamin E by 20-fold resulted in increases in brain tissue concentrations of only 40%.

As previously observed when using this animal model, vitamin E is selectively retained by neural tissues compared to non-neural tissues. For example, at 20 weeks, whereas deficient liver contained only 1.1% of control levels of vitamin E, deficient cortex still retained 13.7%. By 55 weeks α-tocopherol was undetectable in deficient liver with 3.3% of control levels remaining in deficient cortex. These results are in general agreement with those of Vatassery et al (1984) who studied the loss of vitamin E in mice fed vitamin E deficient diets, and with those of Goss-Sampson et al (1988) of this department who employed the same animal model as used in this project. In addition Ingold et al (1987) found that the half-life of deuterated α-tocopherol was higher in the neural tissues of control rats than non-neural tissues. For

example, the half-life in brain was 29 days compared to 10 days in liver. In contrast to other non-neural tissues, muscle seemed to have a greater capacity for retaining vitamin E with 4.6 and 2.1% of control concentrations being present in deficient samples at 20 and 55 weeks respectively.

Because of the similarity of my results with those previously obtained in this department it was possible to correlate my lipid peroxidation studies with other studies in vitamin E deficiency, e.g. axonal transport. In addition, the tissue vitamin E concentrations obtained by Goss-Sampson et al (1988) will be referred to when discussing my studies since the only tissues where vitamin E concentrations were determined in this project were those described above.

CHAPTER 5.

ASSESSMENT OF ENDOGENOUS LIPID PEROXIDATION IN THE VITAMIN E DEFICIENT RAT

5.1 Introduction

5.2 Sample preparation

5.3 Endogenous TBARS

5.4 Endogenous MDA

5.4.1 Free MDA

5.4.2 Bound MDA

5.4.3 Total MDA

5.4.4 Comparison of TBARS and total MDA concentrations

5.5 Aliphatic aldehydes

5.6 Discussion

5.1 Introduction

Endogenous concentrations of lipid peroxidation products were measured in 21 and 56 week vitamin E deficient rats and compared with age matched controls to investigate whether vitamin E deficiency resulted in increased levels of lipid peroxidation in-vivo.

Lipid peroxidation products were estimated in both neural and non-neural tissues. Emphasis was placed on neural regions because of the particular susceptibility of these tissues to a deficiency of vitamin E. Thiobarbituric acid reactive substances (TBARS) and free, bound and total malondialdehyde (MDA) concentrations were determined in 6 brain regions, 3 spinal cord segments, peripheral nerve (sural) and in liver, muscle (gastrocnemius from hind limb) and heart. Aliphatic aldehydes were estimated in 52 week brain cortex only.

5.2 Sample Preparation

Three each of control and vitamin E deficient animals were sacrificed at each time point using a barbiturate (Saggital) overdose. Tissues were removed immediately, sub-dissected if necessary (on ice) and stored at -70°C until analysis. Whole brain was divided into six distinct anatomical regions, i.e. cortex, striatum, midbrain, hypothalamus, cerebellum and brainstem. The spinal cord was removed in 3 segments, i.e. cervical, thorax and lumbar.

Approximately 2.5% (wet wt./vol) homogenates of all tissues were made up in 40mM trizma buffer, pH 7.4, divided into aliquots and stored at -70°C. Brain, spinal cord, nerve and liver samples were homogenised using a hand-operated glass/glass potter homogeniser. Prior to homogenisation, the epineurial (outermost) sheath of the sural nerve samples was removed and the remainder of the tissue homogenised as for brain and cord samples. This pretreatment was necessary since this outer nerve layer is rich in collagen and

hence highly resistant to homogenisation. Muscle and heart tissues were finely chopped on a chilled glass plate and then homogenised using a motor driven Polytron disintegrator (Kinematica GmBH) which combines strong shearing forces with sonication.

Concentrations of all the lipid peroxidation products were related to protein concentrations in the sample as determined by the Pierce BCA protein assay kit (see chapter 2).

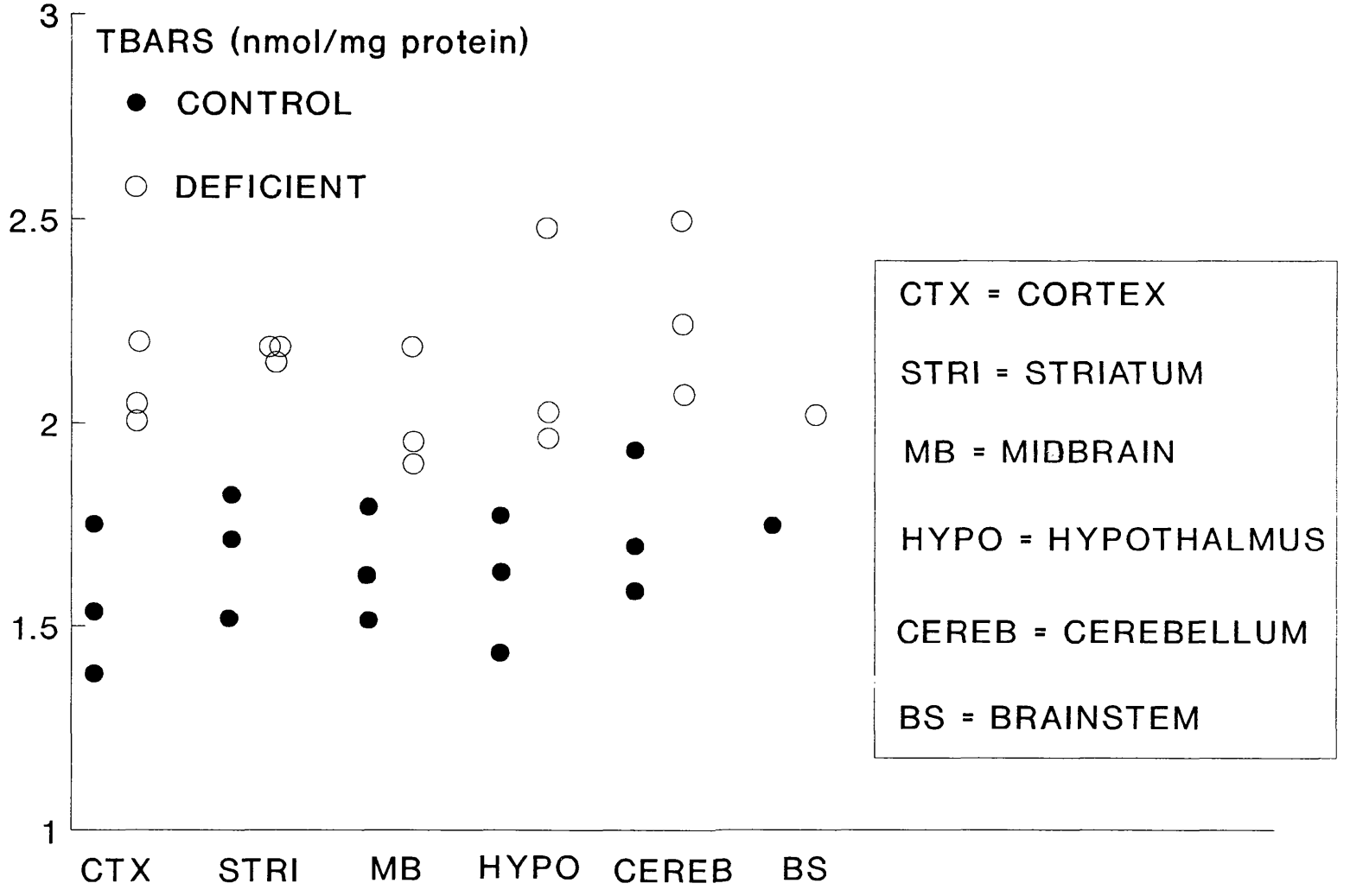
5.3 Endogenous TBARS

Endogenous concentrations of TBARS were determined using a modification of the microfluorimertic method of Yagi (1976) as described in chapter 2. TBARS were measured in all samples at 56 weeks whereas at 21 weeks, only the non-neural tissues were assayed, i.e. liver, muscle and heart. The reason for this was the fact that results were actually obtained for the 56 week stage first and as the differences obtained between control and vitamin E deficient neural tissues were so small after 56 weeks, it was considered highly unlikely that there would be significant differences after 21 weeks of deficiency. Note that in relation to my results, the term "significant" is defined as there being no overlap of data from control and deficient groups. Actual statistical analysis was not possible due to the small number of samples assayed (n=3).

Neural Tissues

The endogenous concentrations of TBARS in 56 week control and deficient brain regions, namely, cortex, striatum, midbrain, hypothalamus, cerebellum and brainstem are plotted in fig 1. Note that the three brainstem tissue samples were combined for fractionation purposes (see chapter 2) so that the single data point on the graph for this tissue represents the mean concentration of three combined tissues. There was a small but significant increase in mean TBARS levels in all vitamin E deficient brain regions of between 15 and

FIG 1. ENDOGENOUS TBARS IN 56 WEEK CONTROL AND DEFICIENT RAT BRAIN REGIONS (n=3)



34%. The concentrations were similar in all control samples as was the case for deficient regions.

The deficient spinal cord tissues also exhibited small but significant mean increases compared to controls, i.e. 19, 17 and 15% in cervical, thoracic and lumbar segments respectively. Three separate cord segments were dissected out from each rat and assayed individually. The concentrations in the three sections were similar and therefore for the sake of clarity, only the mean of the three different regions from each rat are plotted in fig. 2. Similarly, the data points shown for the brain are the mean of the six different regions assayed. From fig. 2 it can be seen that there was no significant difference between control and deficient nerve samples. Concentrations of TBARS were similar in all control neural tissues.

Non-Neural Tissues

Concentrations of endogenous TBARS at 56 weeks were significantly increased in all three deficient tissues, i.e. mean increases of 3.6-fold in liver, 2.1-fold in muscle and by a factor of 3.5 in deficient heart (Fig 2). Measurement of endogenous TBARS in 21 week liver, muscle and heart, however, did not reveal a significant difference between vitamin E deficient tissues and controls (Fig 3).

5.4 Endogenous MDA

Endogenous concentrations of free, bound and total MDA were determined in all tissues at both 21 and 56 weeks.

5.4.1 Free MDA

Free MDA was determined using a modification of the selective HPLC system described by Esterbauer et al (1984) as presented in chapter 3.

FIG 2. ENDOGENOUS TBARS IN 56 WEEK CONTROL AND DEFICIENT NEURAL AND NON-NEURAL TISSUES (n=3)

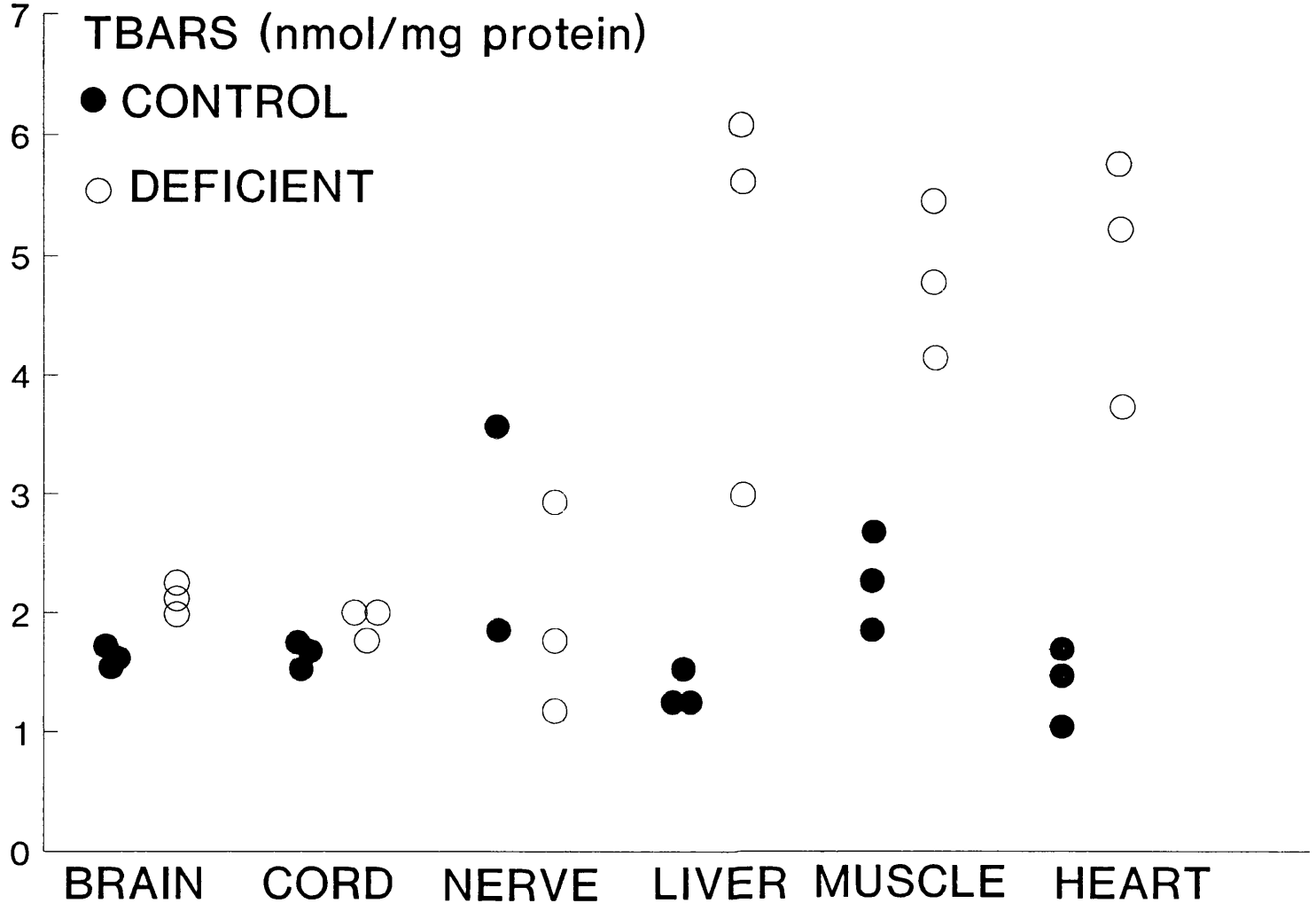
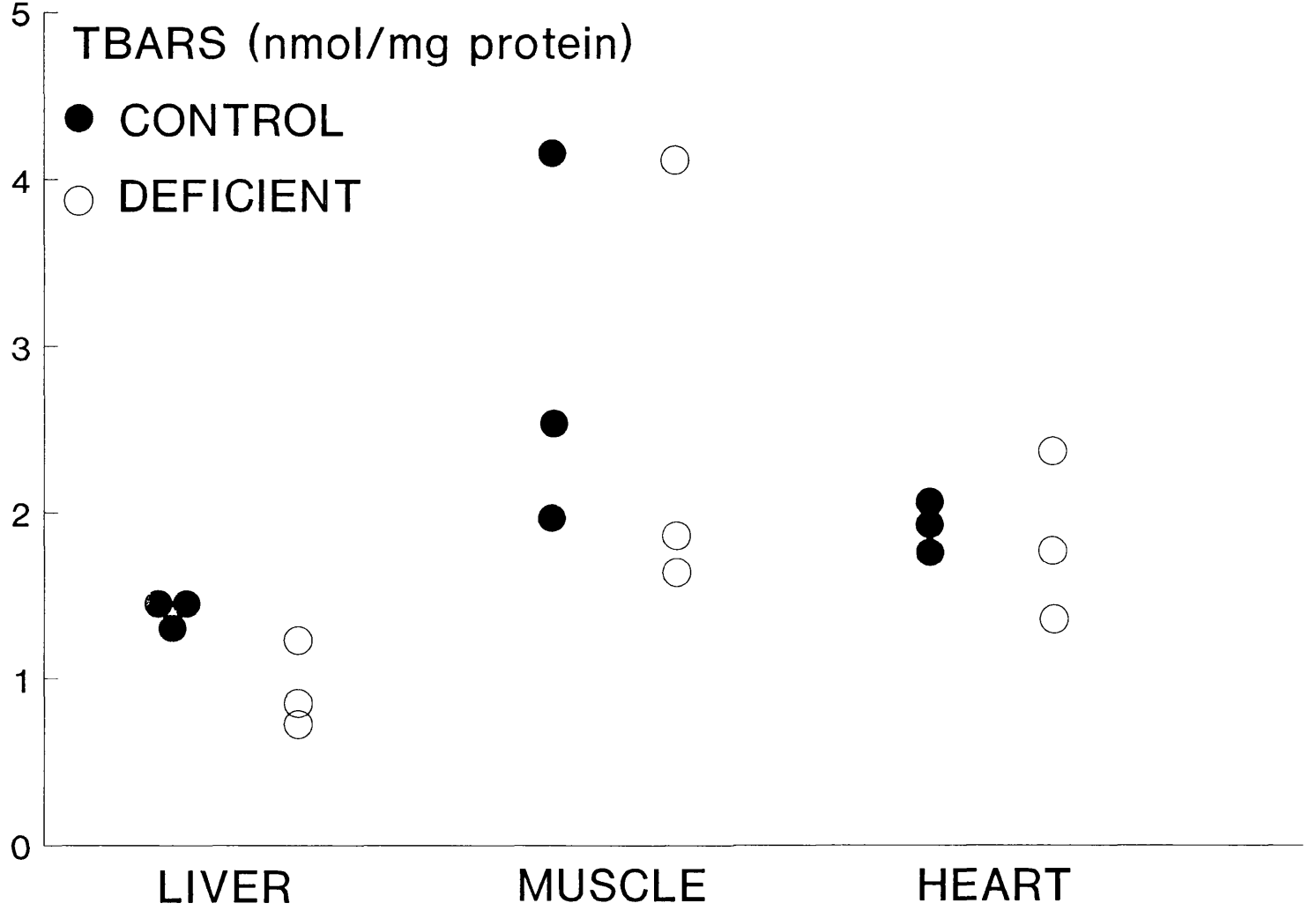


FIG 3. ENDOGENOUS TBARS IN 21 WEEK CONTROL AND DEFICIENT NON-NEURAL TISSUES (n=3)



Neural Tissues

Concentrations of free MDA in 21 and 56 week brain regions are shown in figs 4 and 5 respectively. At 21 weeks the mean concentration in deficient regions was 1.6 to 2.1-fold higher compared to controls. However, with the exception of striatum, and possibly brainstem, there was overlap between the concentrations of free MDA found in the control and deficient brain regions. At 56 weeks, however, significant increases of between 2.5 (cerebellum) and 5.5-fold (hypothalamus) were found in all deficient brain regions. There was no apparent difference between free MDA levels in the various control regions. Similar concentrations were also seen in the different vitamin E deficient regions. Control concentrations were similar in the two age groups.

Whereas free MDA was elevated in some deficient brain regions at 21 weeks and in all samples at 56 weeks, there was no difference between control and deficient concentrations in both spinal cord and nerve at both 21 weeks (fig 6) and 56 week (fig 7). Levels in control brain, cord and nerve were similar.

Non-Neural Tissues

Results for the endogenous levels of free MDA in liver, muscle and heart at 21 and 56 weeks are presented in figs 6 and 7 respectively. At both time points there was no difference between control and deficient liver. Deficient muscle on the other hand exhibited mean increases of 1.8 and 3.3-fold and deficient heart increases of 6 and 6.3-fold at 21 and 56 weeks respectively. Control 21 week muscle appeared to contain increased levels of endogenous free MDA compared to the other two non-neural tissues, whereas similar levels were present in all three tissues at 56 weeks. There were no significant differences between control concentrations at 21 and 56 weeks, i.e. 21 week concentrations (mean + range) were 0.32 (0.25-0.43), 1.50 (0.92-1.90) and 0.57 (0.52-0.61) in liver, muscle and heart respectively with corresponding 56 week values of 0.41 (0.25-0.52), 1.01 (0.77-1.24) and 0.75 (0.36-0.99)

FIG 4. ENDOGENOUS FREE MDA IN 21 WEEK CONTROL AND DEFICIENT RAT BRAIN REGIONS (n=3)

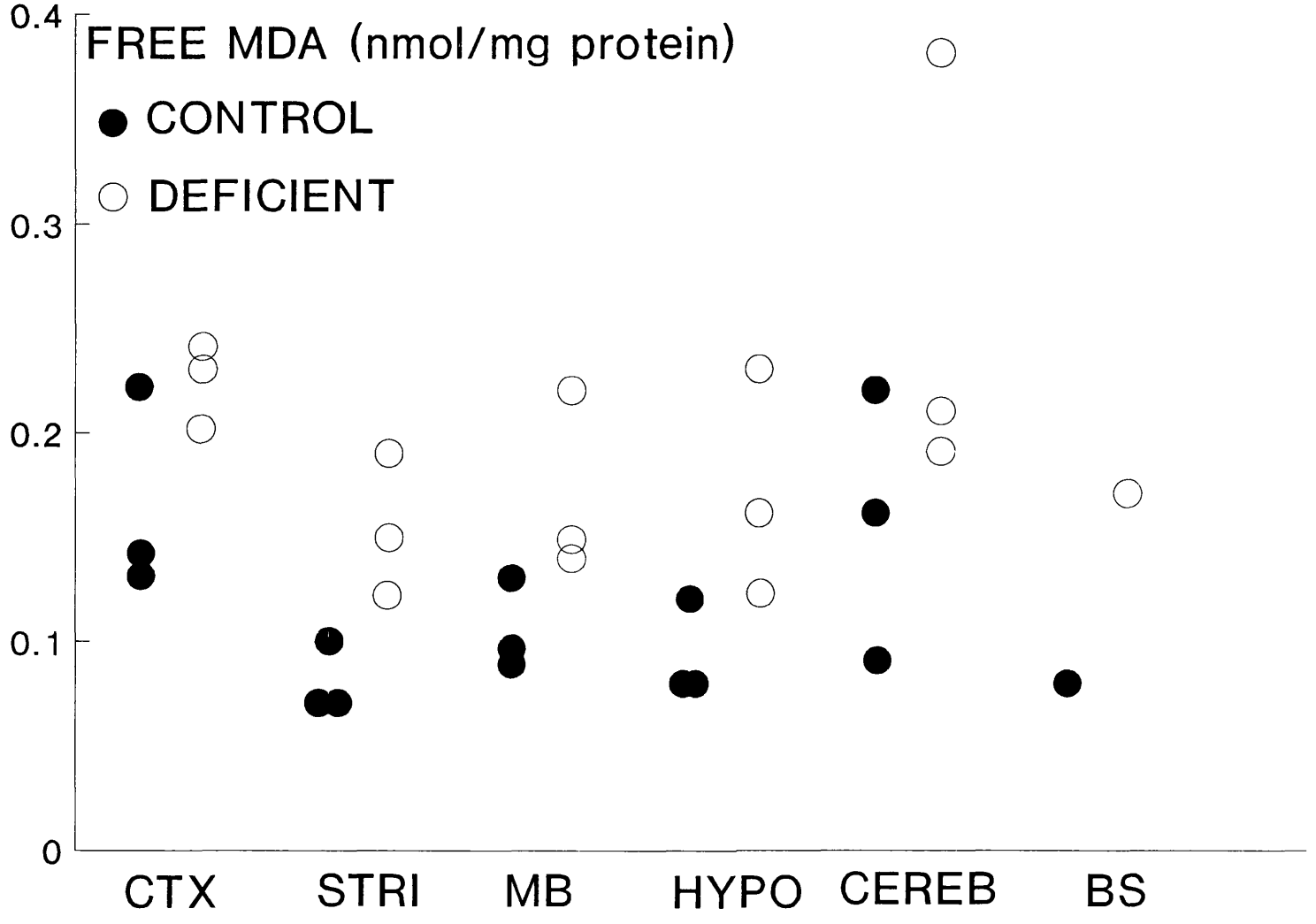


FIG 5. ENDOGENOUS FREE MDA IN 56 WEEK CONTROL AND DEFICIENT RAT BRAIN REGIONS (n=3)

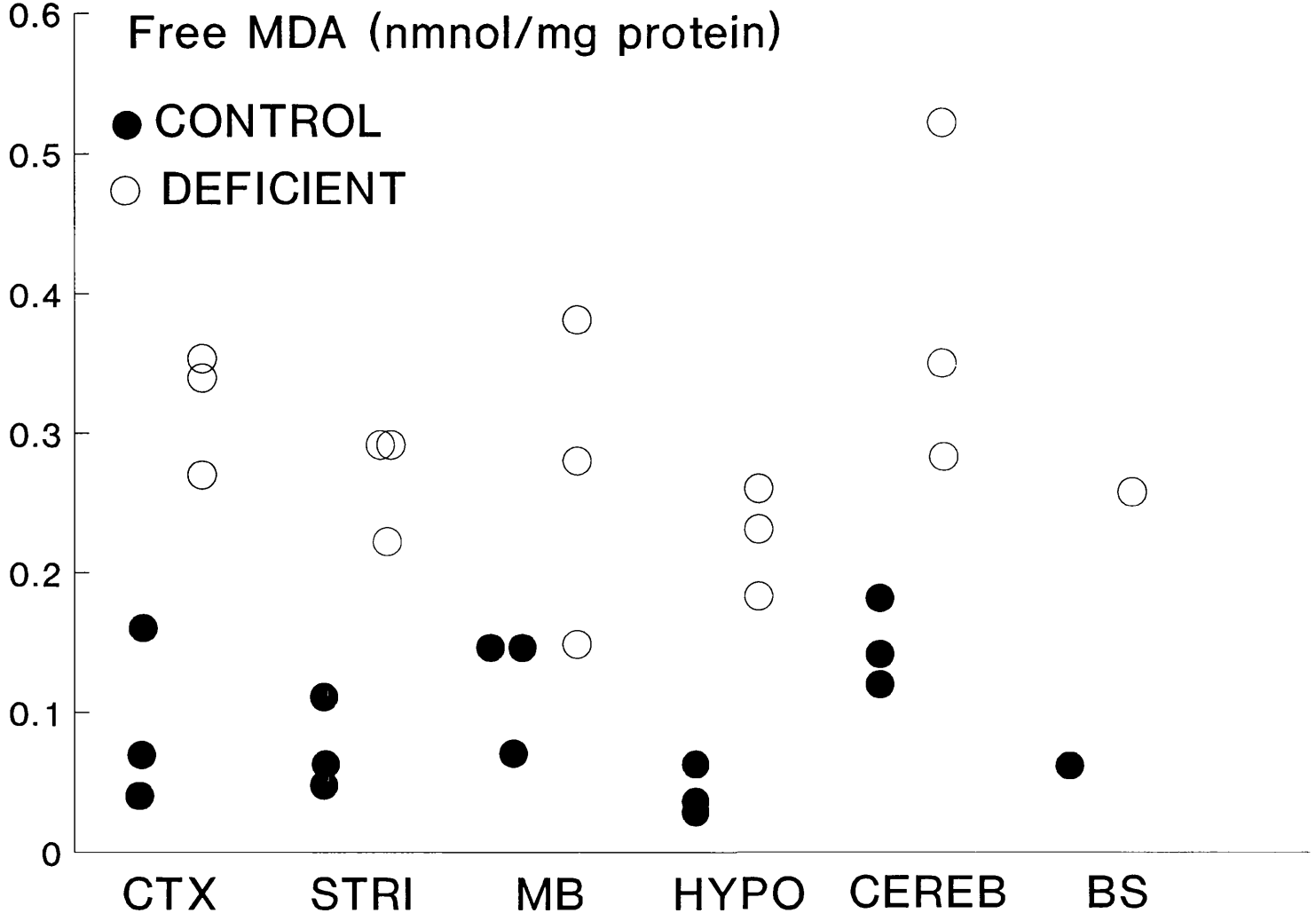


FIG 6. ENDOGENOUS FREE MDA IN 21 WEEK CONTROL AND DEFICIENT NEURAL AND NON-NEURAL TISSUES (n=3)

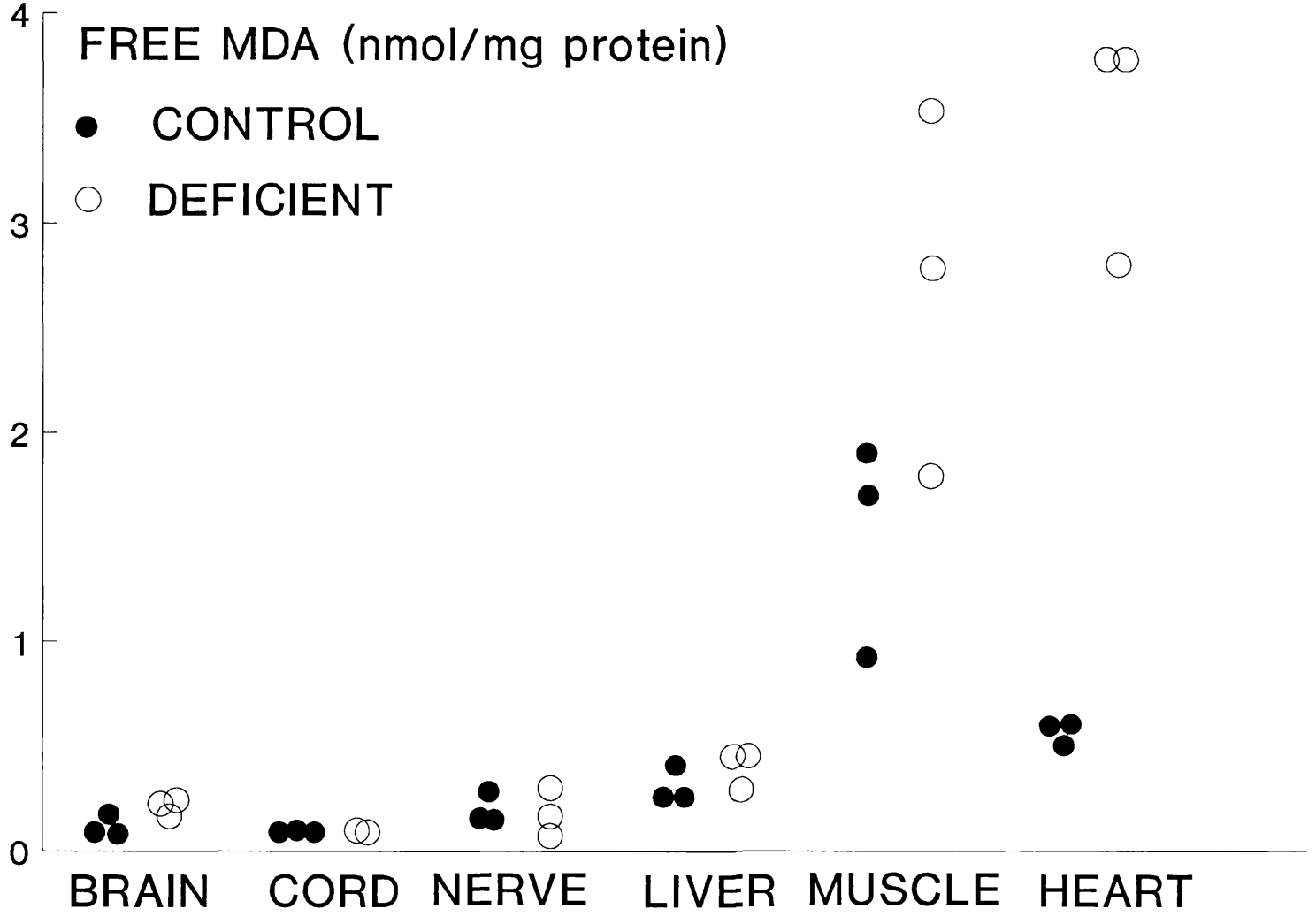
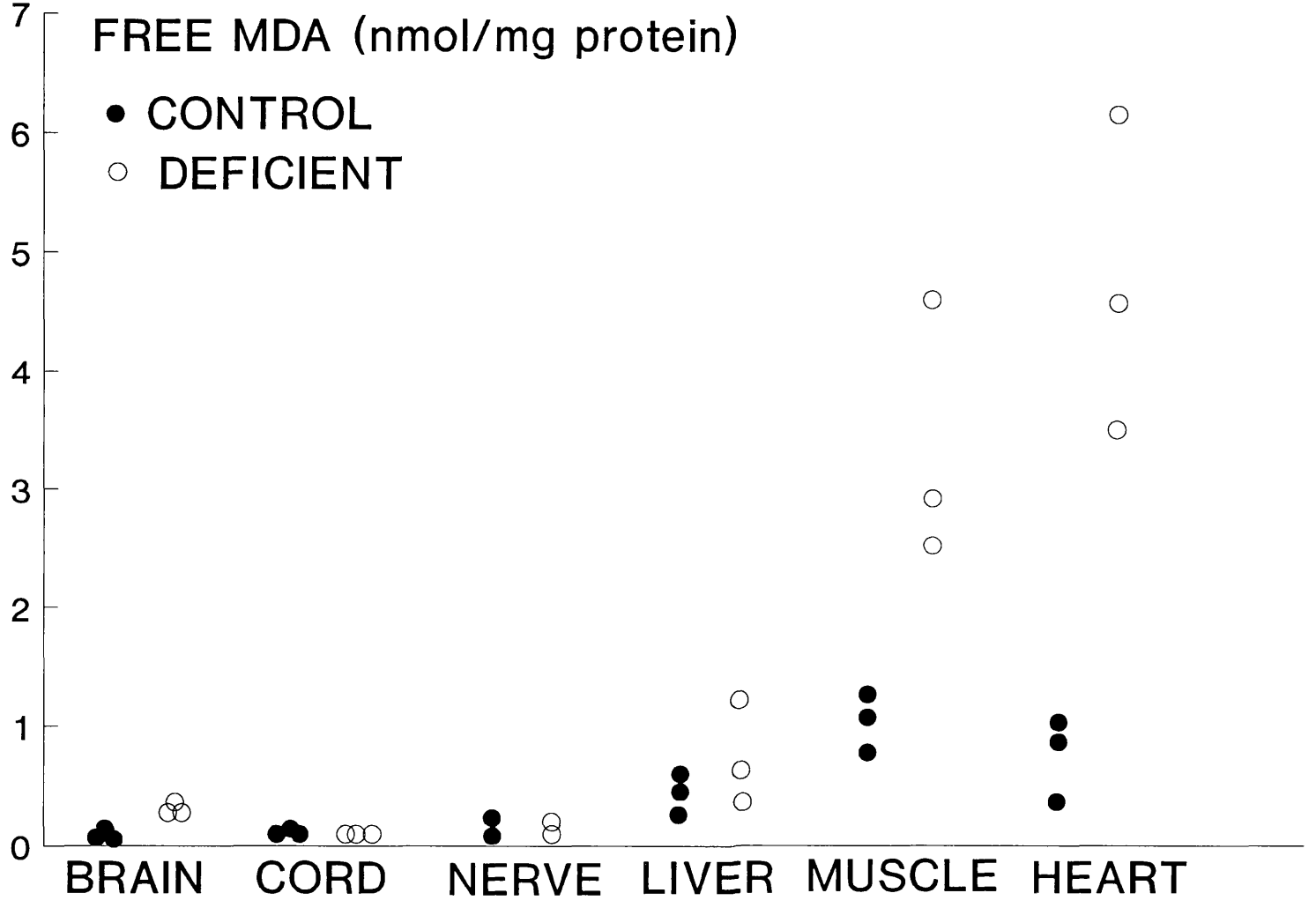


FIG 7. ENDOGENOUS FREE MDA IN 56 WEEK CONTROL AND DEFICIENT NEURAL AND NON-NEURAL TISSUES (n=3)



respectively.

5.4.2 Bound MDA

Bound MDA was determined using a modification of the alkaline hydrolysis method of Lee and Csallany (1987) as described and validated in chapter 3. In the case of whole homogenates, the assay directly measures the total MDA (free + bound) present in the sample so that the amount of the aldehyde present in the bound form was calculated by subtracting the value for the free form from the total MDA.

Neural Tissues

Endogenous bound MDA concentrations in brain regions at 21 and 56 weeks are plotted in figs 8 and 9 respectively. There was insufficient vitamin E deficient brainstem available at 56 weeks to measure bound MDA. The value for the corresponding control tissue was included so as to compare it with the other brain areas. At 21 weeks there was no significant difference between deficient samples and controls in any of the 6 regions examined despite increases in free MDA found in some regions. At 56 weeks, however, all deficient regions exhibited mean increases of between 1.9 (striatum) and 5.1-fold (cerebellum) compared to control brain regions. With the possible exception of cerebellum at 21 weeks and brainstem at 56 weeks concentrations appeared to be similar in all control regions. In addition there was no significant difference between control levels of bound MDA at the two time points examined.

As with the brain regions there were no significant differences between control and deficient concentrations in cord and nerve samples at the early time point (fig 10). However, at 56 weeks (fig 11) all three deficient cord segments had 2.7 to 3.9-fold elevated levels of bound MDA compared to controls, while mean concentrations in peripheral nerve were increased by a

FIG 8. ENDOGENOUS BOUND MDA IN 21 WEEK CONTROL AND DEFICIENT RAT BRAIN REGIONS (n=3)

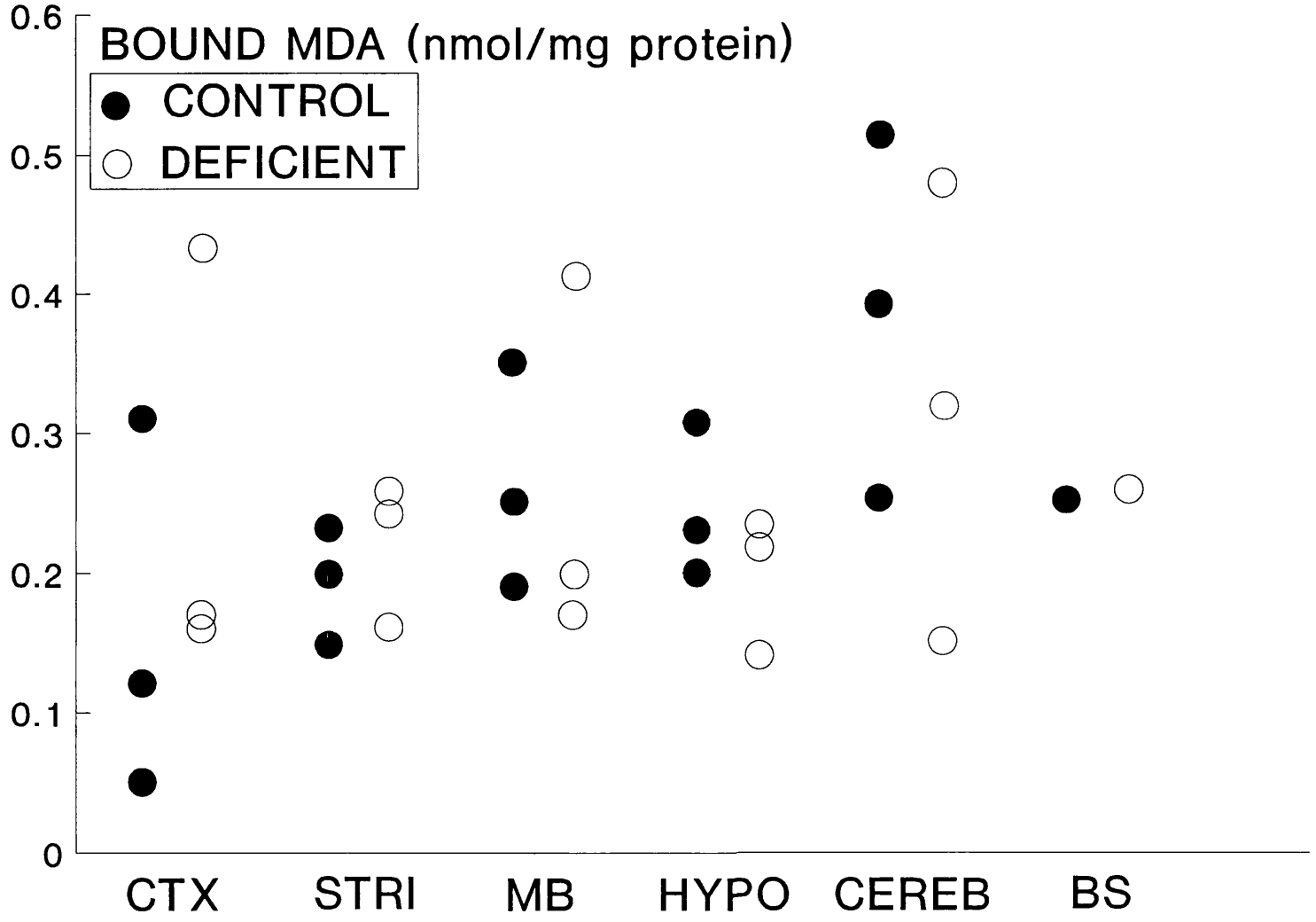


FIG 9. ENDOGENOUS BOUND MDA IN 56 WEEK CONTROL AND DEFICIENT RAT BRAIN REGIONS (n=3)

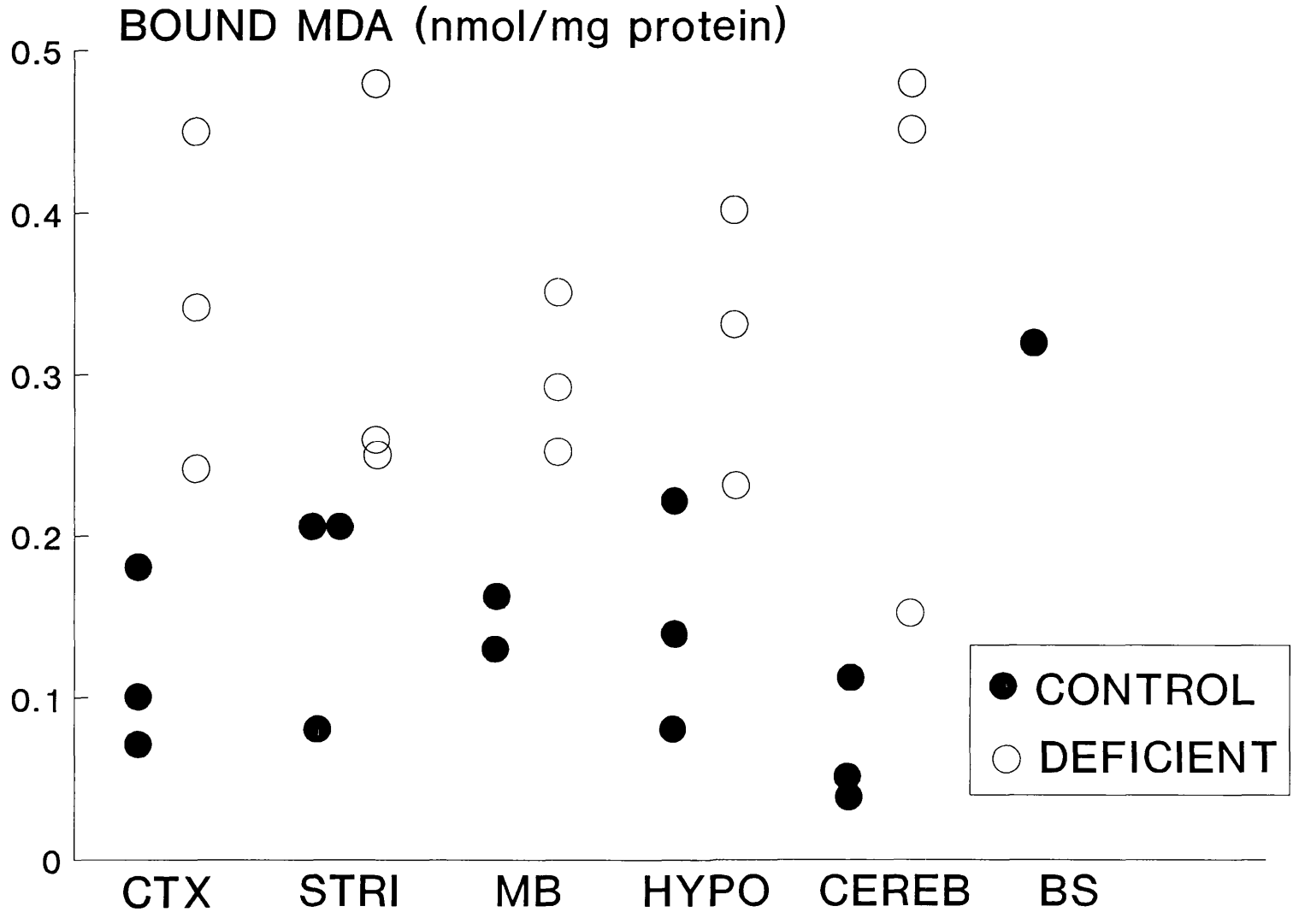


FIG 10. ENDOGENOUS BOUND MDA IN 21 WEEK CONTROL AND DEFICIENT NEURAL AND NON-NEURAL TISSUES (n=3)

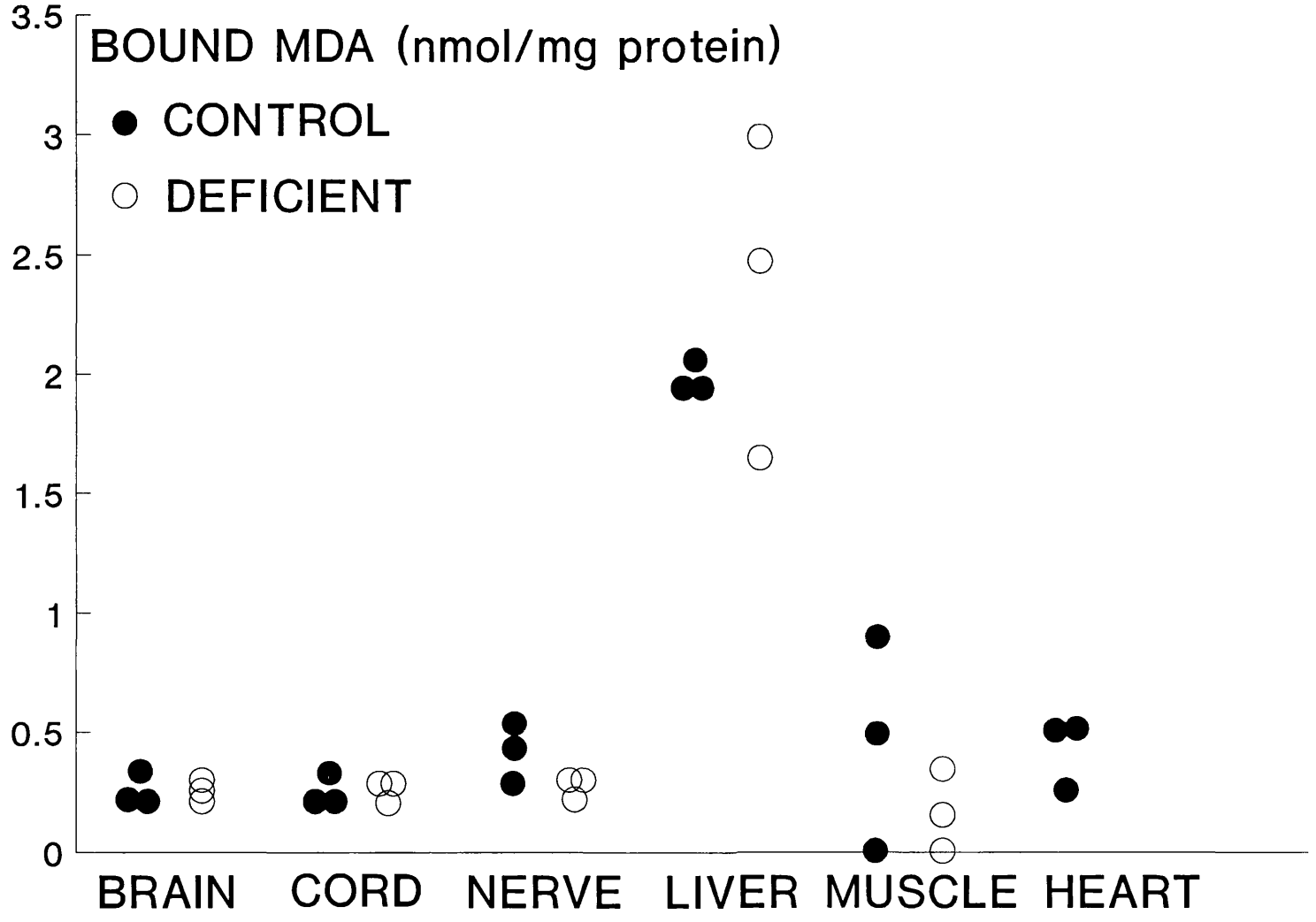
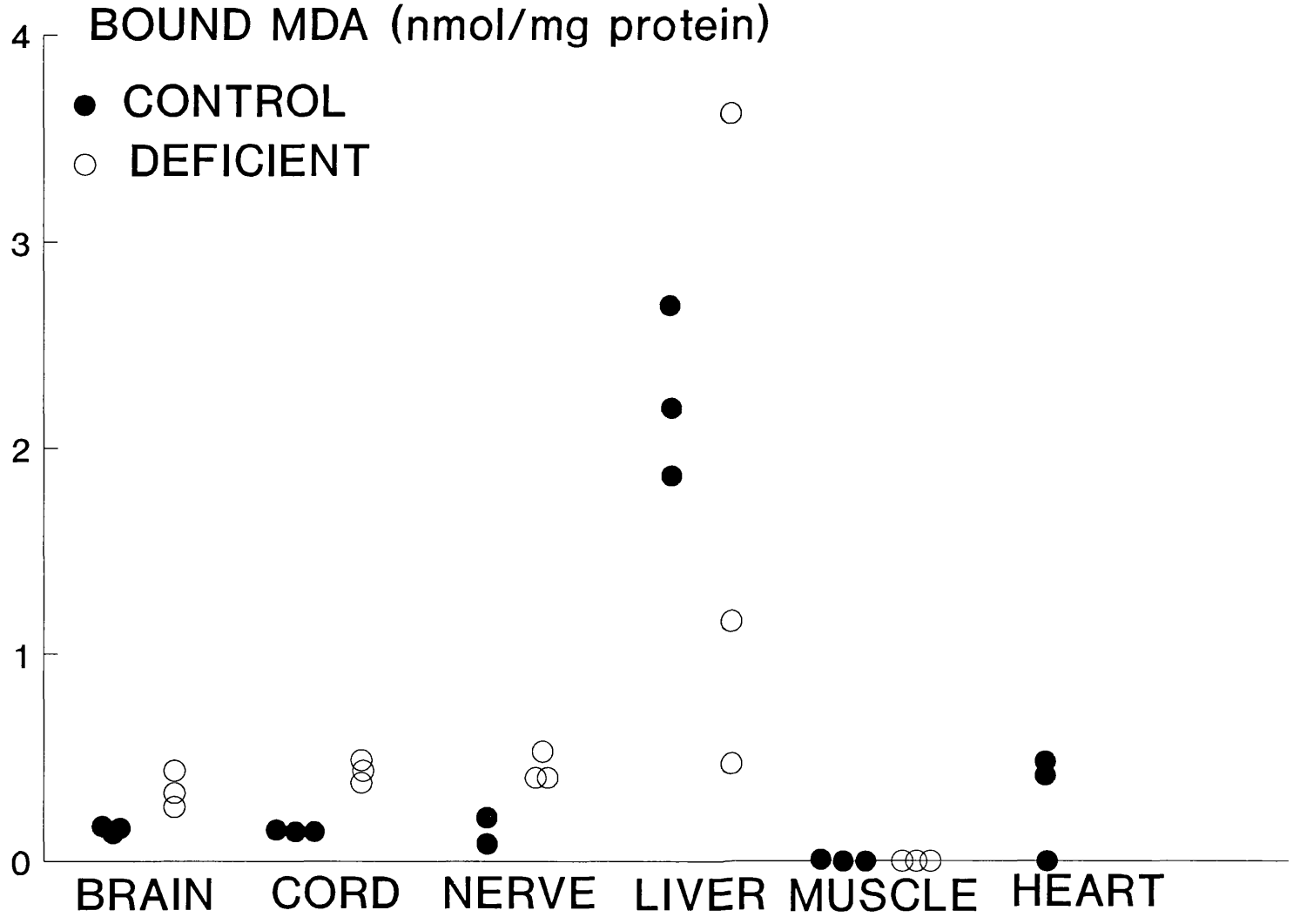


FIG 11. ENDOGENOUS BOUND MDA IN 56 WEEK CONTROL AND DEFICIENT NEURAL AND NON-NEURAL TISSUES (n=3)



factor of 3.1. These increases were observed despite no differences in free MDA in these tissues at 56 weeks. The concentrations in all control and deficient neural tissues were similar at 21 weeks. At 56 weeks there was no significant difference between levels in the various control neural tissues or between the various deficient tissues. In addition, there was no difference in the concentrations in the controls between the two age groups.

Non-Neural Tissues

The results for endogenous bound MDA concentrations in 21 and 56 week non-neural tissues are presented in figs 10 and 11 respectively. There was no difference between deficient and control levels in liver at both time points, although, deficient values did exhibit a large degree of scatter. In the case of muscle, bound MDA could only be detected in two out of three samples for both control and deficient rats at 21 weeks. At this time point concentrations appeared to be increased in controls compared to deficient samples. At 56 weeks bound MDA was undetectable in all three control and deficient muscle samples. Although the presence of bound MDA could be demonstrated in control heart samples at both 21 and 56 weeks, subjecting deficient heart tissue homogenates to the alkaline hydrolysis system to release any bound MDA present, actually resulted in lower "total" MDA values than the corresponding free concentrations. Therefore only the data for control samples was included in figs 10 and 11. There was no significant difference between levels in control liver at the two time points as was the case for control heart concentrations.

5.4.3 Total MDA

As stated in section 5.4.2, the total MDA concentration is obtained after subjecting samples to alkaline hydrolysis. This section presents the results for total MDA in neural and non-neural tissues.

Neural Tissues

The concentrations of total MDA in 21 and 56 week brain regions are presented in figs 12 and 13 respectively. At the earlier time point, with the possible exception of striatum and brainstem, there did not appear to be a significant difference between control and deficient samples. By 56 weeks, however, all deficient regions, (no data for deficient brainstem), exhibited greater mean concentrations compared to controls of between 2.2 and 3.1-fold. Slightly higher endogenous levels were found in 21 week cerebellum compared to the other control regions whereas at 56 weeks there was no difference between the various regions.

Total MDA concentrations were not elevated in 21 week deficient cord and nerve tissues compared to controls (fig 14) and in fact there appeared to be higher levels present in control nerve compared to the corresponding deficient samples. In contrast, by 56 weeks increased levels were found in all deficient neural tissues with concentrations being increased by a factor of 2.3 in cervical, 2.8 in thoracic and 2.2 in lumbar spinal cord and 2 in sural nerve. At 21 weeks similar control concentrations were found in brain and cord with higher levels in peripheral nerve. Deficient total MDA values were similar in all neural tissues. In the older age group there were no significant differences between the different control neural homogenates nor were there any significant differences in the vitamin E deficient tissues.

Non-Neural Tissues

At 21 weeks there did not appear to be a significant difference between control and deficient liver and muscle (fig 14). Due to the apparent instability of MDA in deficient heart homogenates during alkaline hydrolysis no data was available for this particular sample. Deficient liver at 56 weeks again did not exhibit a difference compared to controls (fig 15). However, total MDA was significantly increased in deficient muscle by a factor of 3.4. This

FIG 12. ENDOGENOUS TOTAL MDA IN 21 WEEK CONTROL AND DEFICIENT RAT BRAIN REGIONS (n=3)

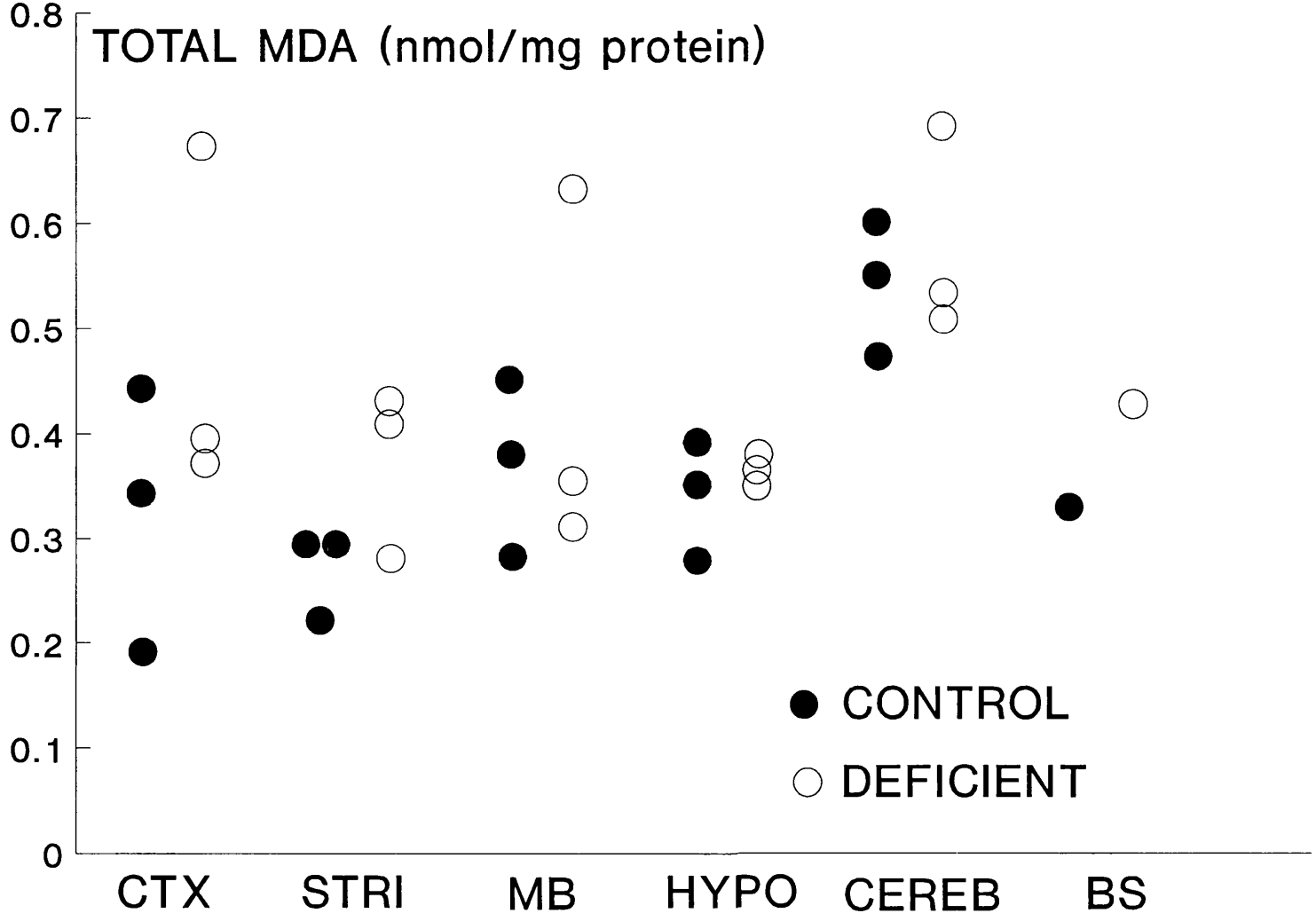


FIG 13. ENDOGENOUS TOTAL MDA IN 56 WEEK CONTROL AND DEFICIENT RAT BRAIN REGIONS (n=3)

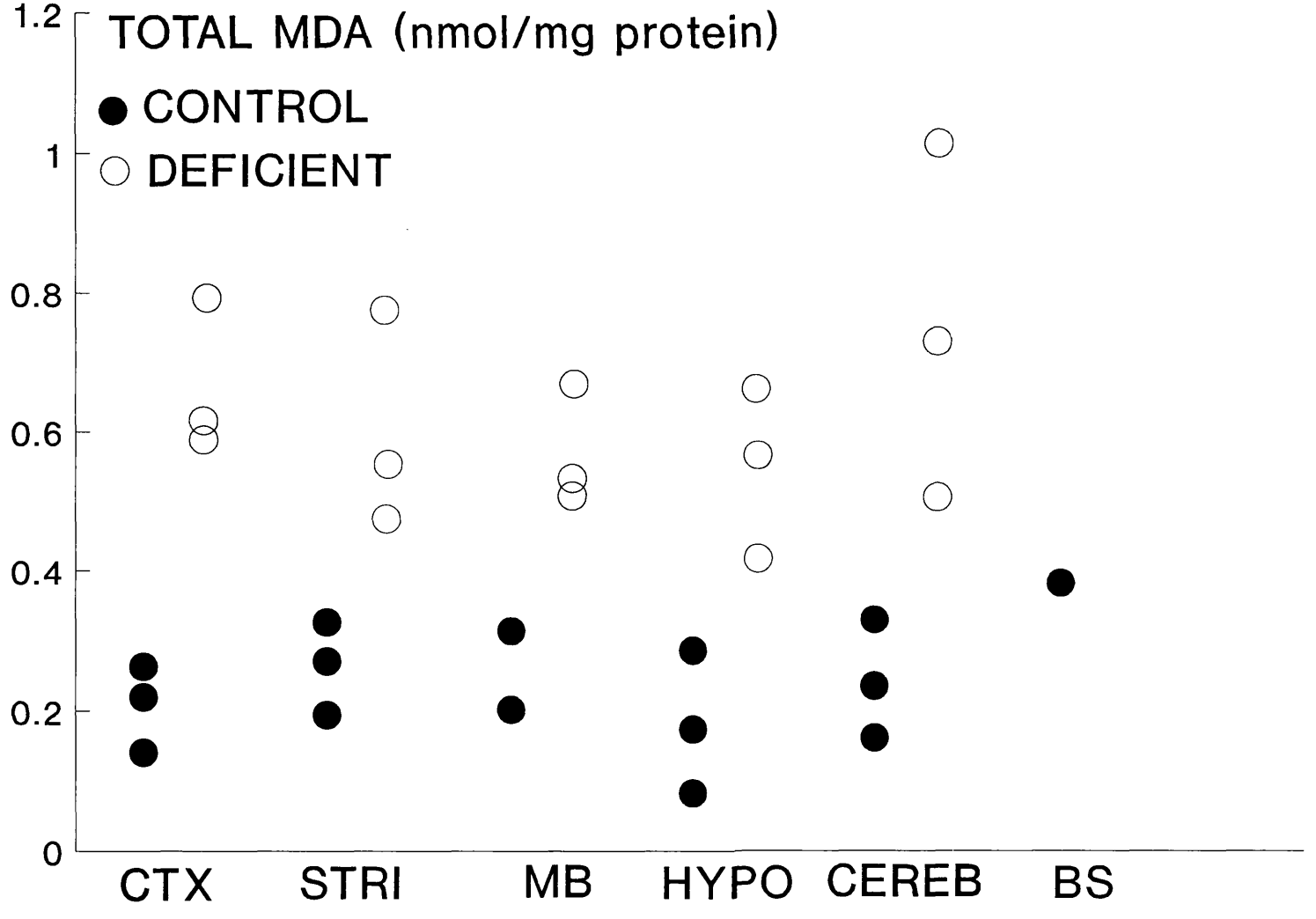


FIG 14. ENDOGENOUS TOTAL MDA IN 21 WEEK CONTROL AND DEFICIENT NEURAL AND NON-NEURAL TISSUES (n=3)

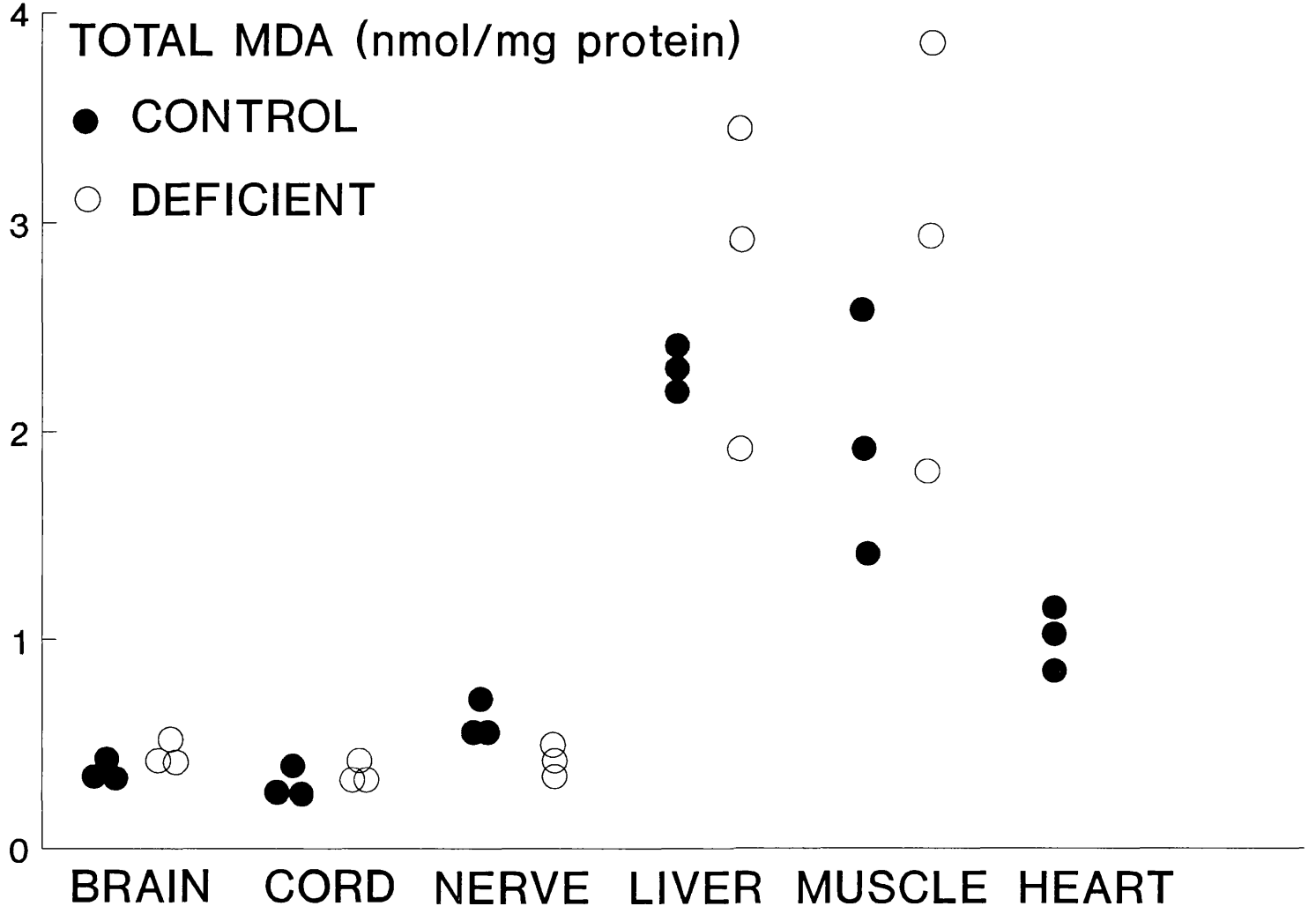
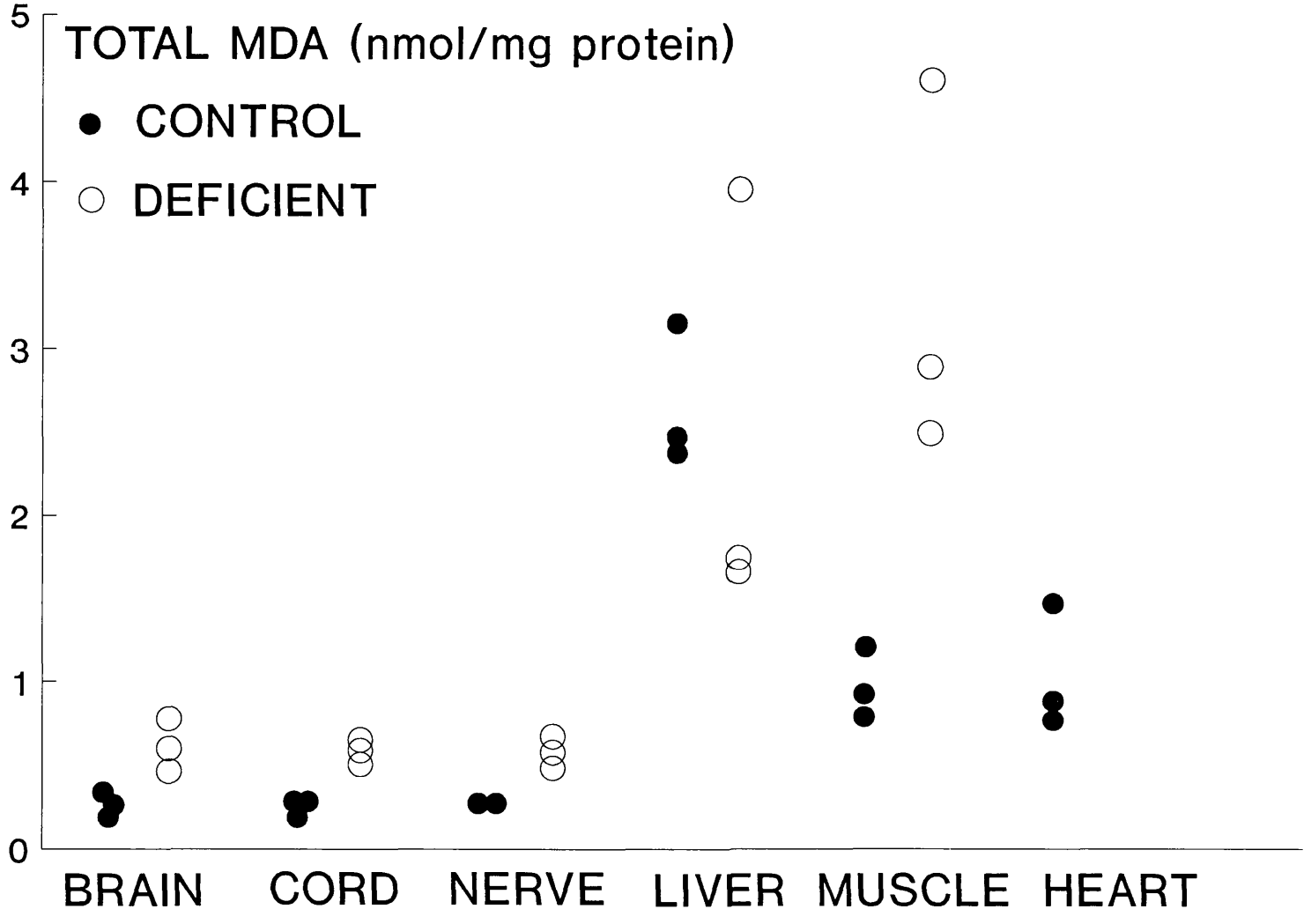


FIG 15. ENDOGENOUS TOTAL MDA IN 56 WEEK CONTROL AND DEFICIENT NEURAL AND NON-NEURAL TISSUES (n=3)



difference resulted largely from an apparent fall in control endogenous levels at 56 weeks compared to 21 weeks with no significant alteration in deficient concentrations during this time period. The explanation for this reduction in total MDA in controls was that whereas bound MDA could be detected at 21 weeks it was undetectable at the later stage. At 21 weeks, concentrations of total MDA in control liver and muscle were similar but greater than those present in control heart. The highest control concentrations at 56 weeks in non-neural samples were found in liver with no difference between muscle and heart. There was no significant change in total MDA in control liver and heart at the two time points.

5.4.4 Comparison of TBARS and Total MDA Concentrations

A comparison was made of total MDA concentrations and the corresponding TBARS values in control (fig 16) and deficient (fig 17) tissues at 56 weeks. For all neural samples, both control and deficient, the TBARS values were significantly higher than total MDA. For control tissues, mean TBARS were 6.6, 7.1 and 9.6-fold greater in brain, spinal cord and peripheral nerve respectively compared to total MDAs. However, in all three deficient neural samples the relative difference was reduced to a factor of 3.4. This was because of the small mean increase in endogenous TBARS in deficient homogenates compared to controls ($< 35\%$), with much larger concomitant increases in MDA concentrations.

For control non-neural tissues, (fig 16), mean TBARS values were 2.3-fold higher in muscle with no significant difference in heart. Surprisingly TBARS were 2-fold lower in control liver compared to total MDA concentrations. In deficient tissues TBARS values were 2 and 1.4-fold greater in liver and muscle respectively although there was some overlap of results. The values quoted for vitamin E deficient heart are actually the free MDA results due to the previously mentioned problems associated with assaying bound MDA in

FIG 16. COMPARISON OF ENDOGENOUS TBARS AND TOTAL MDA IN 56 WEEK CONTROL TISSUES

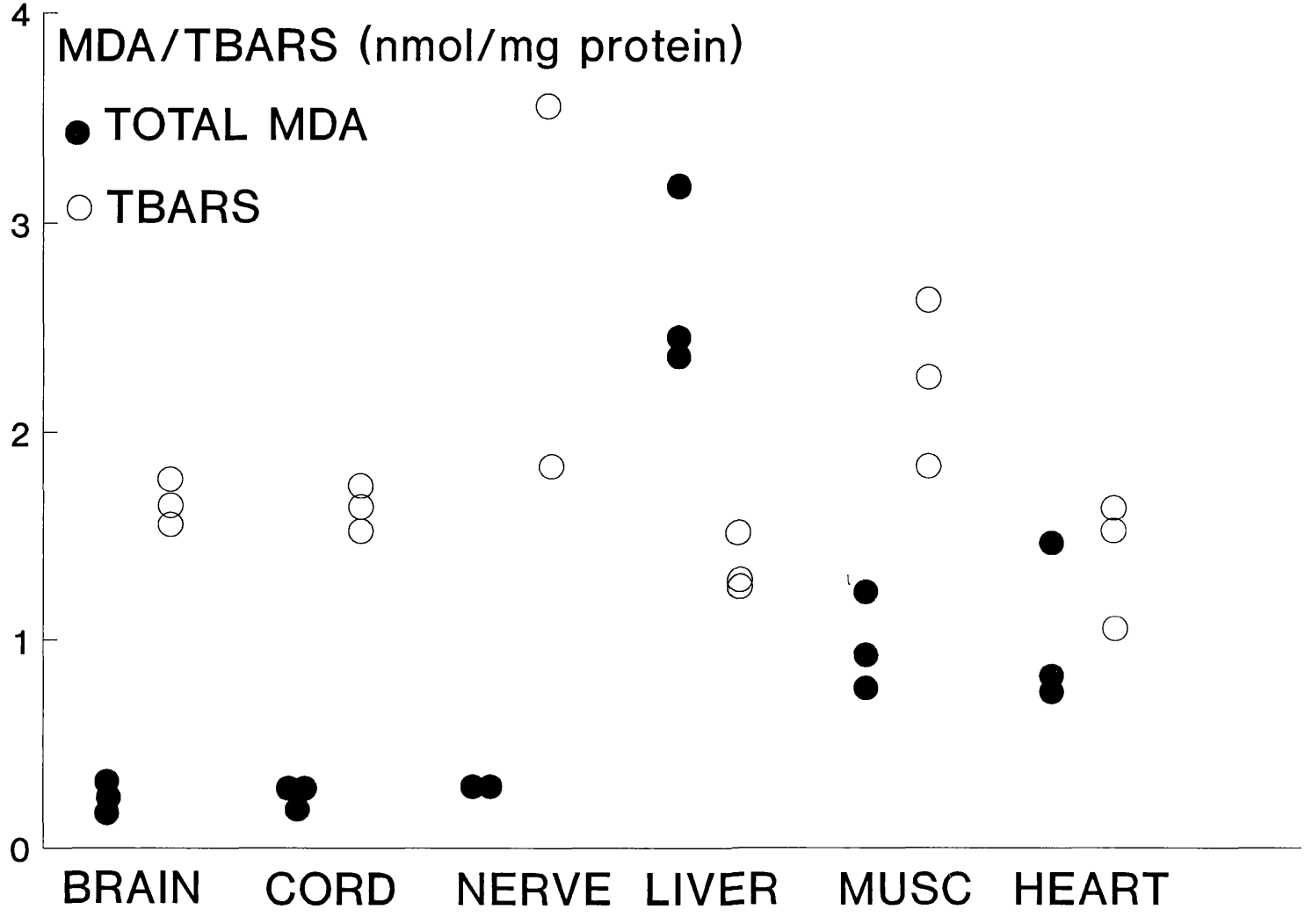
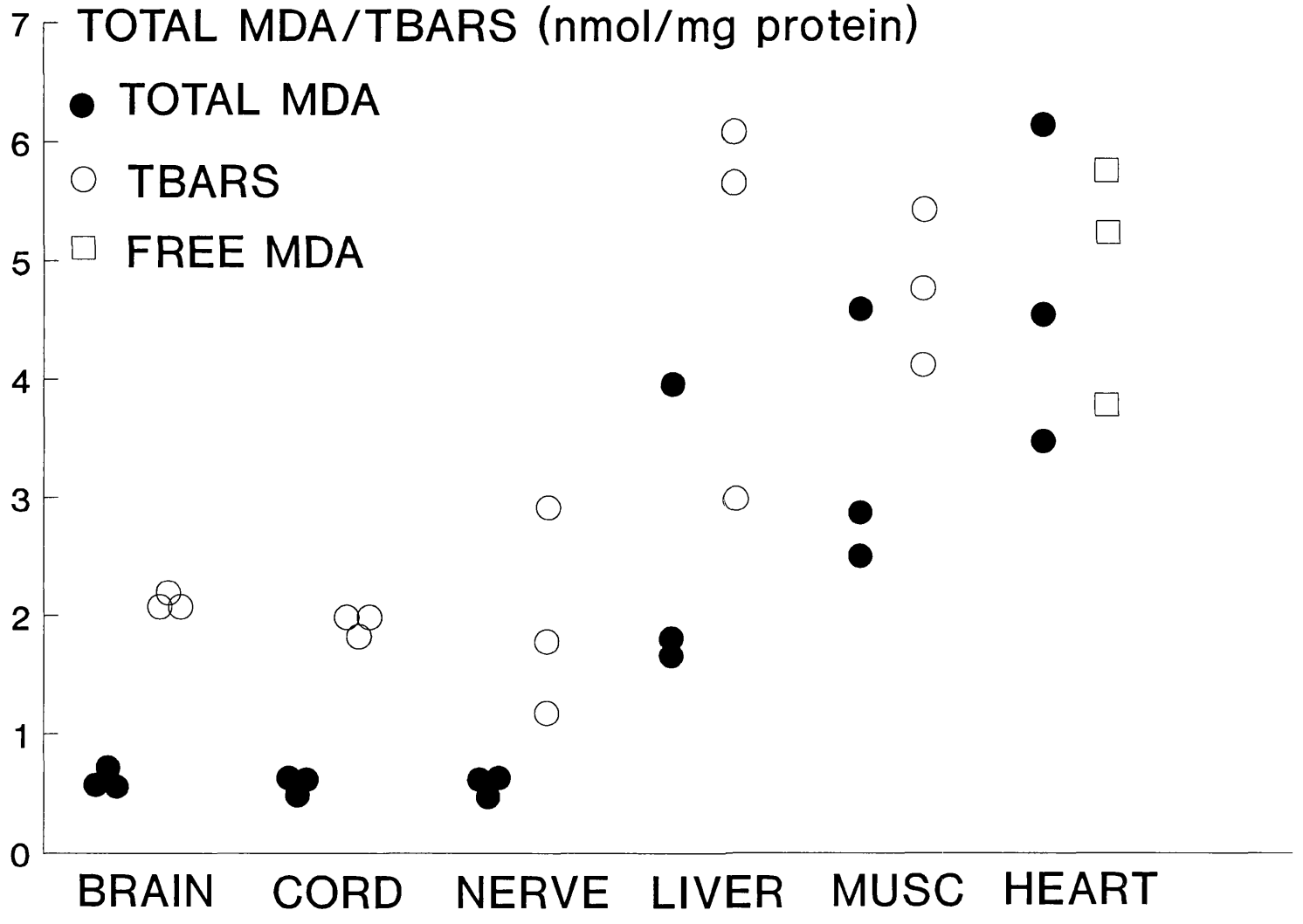


FIG 17. COMPARISON OF ENDOGENOUS TBARS AND TOTAL MDA IN 56 WEEK DEFICIENT TISSUES



these samples.

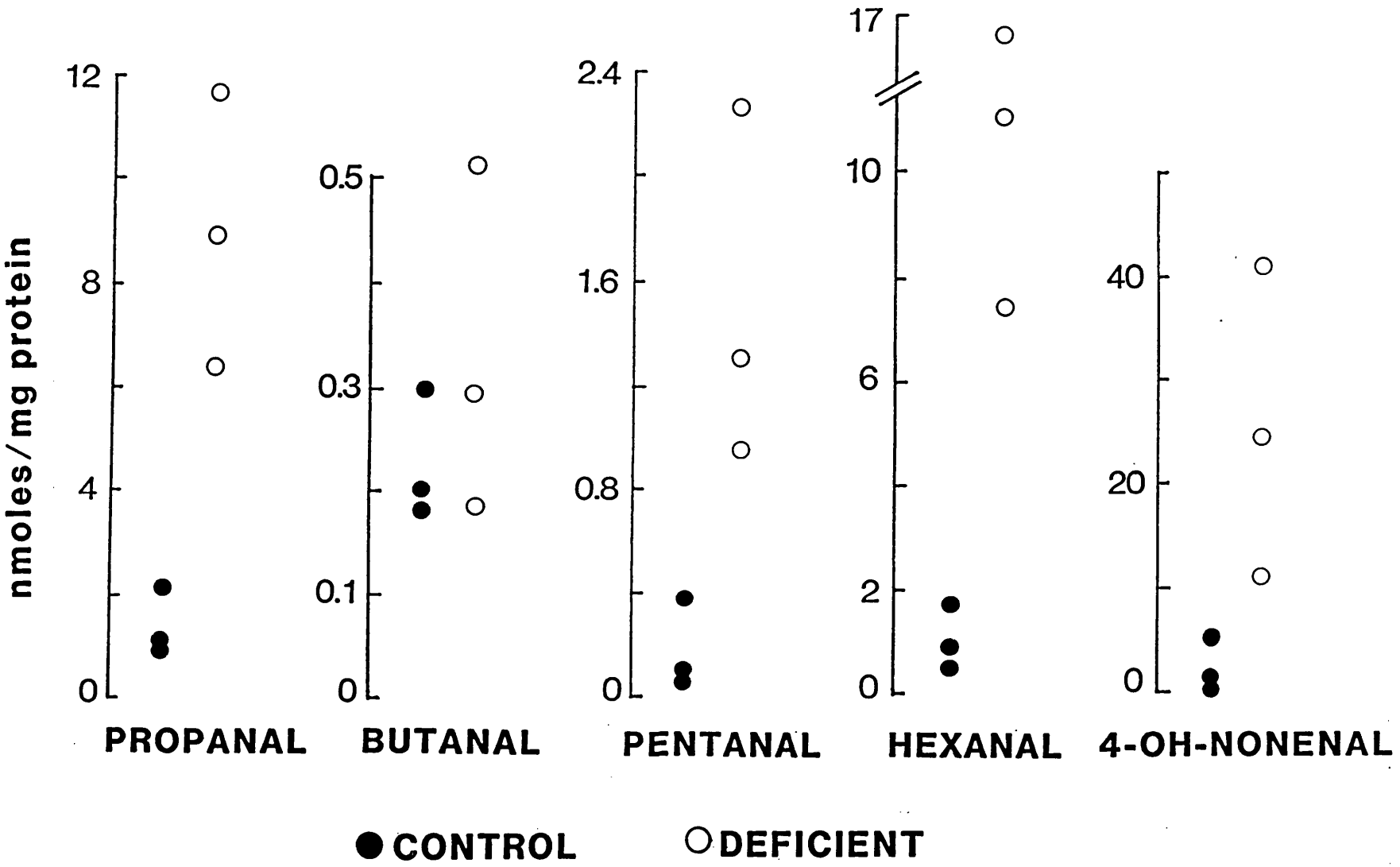
5.5 Aliphatic Aldehydes

Endogenous concentrations of aliphatic aldehydes were measured using a modification of the method of Yoshino et al (1986) as described in chapter 3. This procedure was developed by Dr. Patrick McCarthy of Guy's Hospital Medical School. Propanal, butanal, pentanal, hexanal and 4-hydroxynonenal (4-HNE) were determined in 52 week vitamin E control and deficient cortex (n=3), the results of which are presented in fig 18. With the exception of butanal, aliphatic aldehydes were significantly increased in deficient cortex tissues compared to controls. Mean propanal concentrations were 6.4-fold higher in deficient samples, pentanal was elevated 8.3-fold with hexanal and 4-HNE exhibiting the largest increases of 11.6 and 11.7 respectively. Mean control concentrations decreased in the following order; 4-HNE > propanal > hexanal > > butanal = pentanal. For example hexanal and 4-HNE were present at 4.7 and 9.9-fold higher concentrations compared to butanal. In deficient cortex, concentrations decreased as follows; 4-HNE > hexanal > propanal > > pentanal > butanal. A summary of the findings from the different assays are presented in table 1, where the results are given for deficient tissues in relation to the corresponding controls.

5.6 Discussion

Overall, the results of the concentrations of the different peroxidation products provide evidence for the occurrence of an increased level of in-vivo lipid peroxidation in both neural and non-neural tissues during experimental vitamin E deficiency in the rat. This is therefore in agreement with the generally accepted function of vitamin E in-vivo, i.e. a lipid soluble antioxidant. Peroxidation products were, however, detectable in all control tissues examined, so that lipid peroxidation would appear to be a part of the normal metabolic process of the cell and it is only under conditions of

FIG 18. **CORTEX ENDOGENOUS ALIPHATIC ALDEHYDES (52 wk)**



compared to controls).

PEROXIDATION PRODUCT	OBSERVATIONS
TBARS	
Neural	↑ < 35% at 56 wks
Non-neural	No ↑ at 21 wks, > 2-fold at 56 wks
Free MDA	
Neural	Brain Regions; ↑ mean at 20 wks, significant in all at 56 wks. No ↑ in cord/nerve at both stages.
Non-neural	↑ in muscle/heart at both stages. No ↑ in liver " " "
Bound MDA	
Neural	No ↑ in all tissues at 21 wks. Significant in all at 56 wks
Non-neural	Muscle: Undetectable in both groups at 56 wks. Liver: No ↑ at both stages. Heart: Assay unreliable
Total MDA	
Neural	↑ at 56 wks only
Non-neural	Muscle: 3.4-fold ↑ at 56 wks Liver: No ↑ at both stages Heart: Assay unreliable
Aliphatic Aldehydes	Assayed in 50 wk cortex only. Except for butanal, all ↑ > 6-fold

increased free radical stress or reduced antioxidant capacity that lipid peroxidation may become a serious threat to the cell's viability.

The apparent relative in-vivo susceptibilities of different tissues to a deficiency of vitamin E appeared to depend on the method employed to assess lipid peroxidation. Measurement of TBARS revealed that vitamin E deficiency resulted in an increase of $< 35\%$ in the endogenous concentrations in 56 week deficient rat brain compared to controls and an increase of $< 20\%$ in deficient cord segments, with no difference between control and deficient peripheral nerve. These results were obtained despite endogenous α-tocopherol concentrations of $< 5\%$ in deficient tissues compared to controls (Goss-Sampson et al 1988). Deficient non-neural tissues at the same time point, however, exhibited increases in concentrations of TBARS of between 2 and 3.6-fold, whereas there was no significant difference at the earlier 21 week stage. After 21 weeks deficient liver and muscle had tocopherol concentrations of 1.1 and 4.6% of control values respectively (chapter 4) and by 55 weeks this had reduced to undetectable levels in liver and 2.1% of control levels in muscle. It, therefore, appears that tocopherol levels have to be extremely low or reduced for an extended period before differences will become evident in deficient tissues.

When endogenous lipid peroxidation was assessed by measuring MDA by HPLC, different findings were obtained from those using the TBA test. Estimation of free MDA in the 21 week brain regions revealed mean increases in the deficient animals of between 1.6 and 2.1-fold and by 56 weeks free MDA was significantly elevated in all brain regions by between 2.5 and 5.5-fold. Free MDA also gave different results to TBARS for endogenous lipid peroxidation in non-neural tissues, since at 21 weeks levels of free MDA were elevated 1.8-fold in deficient muscle and by a factor of 6 in heart, although they were unaltered in deficient liver. By 56 weeks, free MDA

concentrations were raised in all deficient non-neural tissues with increases of 1.8, 3.3 and 6.3-fold in liver, muscle and heart respectively. In the cases of spinal cord and peripheral nerve, however, there was no significant differences control and deficient samples at both 21 and 56 weeks.

The measurement of bound MDA in all neural tissues showed significantly increased concentrations in deficient samples compared to controls after 56 weeks, i.e. by a factor of 1.9 to 5.1 in brain regions, by > 2.6-fold in all cord segments and by a factor of 3.1 in nerve. Significant differences in total MDA concentrations were only found in neural tissues at 56 weeks, being elevated in all 3 deficient neural regions. Estimation of bound and total MDA in non-neural tissues appeared to be an unreliable method for the estimation of endogenous lipid peroxidation. For example, at 56 weeks bound MDA was not elevated in deficient liver, was undetectable in both control and deficient muscle homogenates and although detectable in two out of three control heart tissues, the results for bound MDA in deficient heart samples were lower than for free MDA.

There is a poor correlation between total MDA and TBARS in neural tissues in both control and deficient samples. In all neural samples TBARS values were significantly higher than the corresponding total MDA values. In the 56 week control brain, for example, TBARS concentrations were > 6-fold higher than levels of total MDA. Therefore, as discussed in chapter 3, the TBA test is not specific for MDA. For non-neural tissues, however, there was a better correlation. For example, in 56 week deficient tissues there were no significant differences between these two parameters in muscle and heart with TBARS being 2-fold higher than total MDA in liver. Lee and Csallany (1987) found that TBARS were 1.4 and 1.8-fold higher than total MDA values in control and deficient liver tissues respectively while Janero and Burghardt (1989) found increases of 2-fold in control myocardium.

The reason why the difference between TBARS and total MDA in neural tissues is greater than that found in non-neural tissues is unknown. Since an antioxidant was not included in the TBA test it was possible that artifactual lipid peroxidation occurred during the assay, particularly since brain tissue is known to be very susceptible to in-vitro peroxidative stress. However, if this were the case a much greater difference between control and deficient endogenous TBARS concentrations would have been expected. The results would therefore appear to suggest that authentic MDA represents a much lower percentage of the total TBARS in neural compared to non-neural tissues, where in some cases MDA may account for almost all of the TBARS result.

Although, only examined in brain cortex at 52 weeks, the measurement of aliphatic aldehydes appeared to be a promising method of assessing endogenous lipid peroxidation during vitamin E deficiency. The greatest increase in any of the various parameters used to study in-vivo lipid peroxidation was seen in the concentrations of hexanal and 4-HNE which were elevated by a factor of $>$ 11 in deficient animals, with propanal and pentanal exhibiting increases of $>$ 6-fold. The largest increase in any of the other peroxidation products in brain regions was a 5.5-fold increase in free MDA in 56 week deficient hypothalamus. Measurement of these peroxidation products may have proved to be appropriate for the assessment of in-vivo lipid peroxidation after relatively short periods of vitamin E deficiency as suggested from the studies of Yoshino et al (1986). These authors found that after only 5 weeks on a vitamin E deficient diet, the endogenous concentrations of pentanal, hexanal and 4-HNE were significantly increased in liver compared to controls.

The principal disadvantage of the method for the estimation of aliphatic aldehydes was that cyclohexanedione derivatives had to be formed and therefore there was much more sample work-up than, for example, the estimation of free MDA. In addition, since samples were subjected to relatively harsh conditions during derivatisation there remains the possibility of artifactual lipid peroxidation occurring during the assay. The effect, if any, of inclusion of an antioxidant should be investigated.

From the above observations it is clear that the apparent susceptibilities of the various tissues depended on the assay method employed. For example, if the measurement of TBARS had been the sole assay employed to assess in-vivo lipid peroxidation during this study, the conclusion would have been that there was little evidence of neural tissues showing increased lipid peroxidation during deficiency of vitamin E, whereas non-neural tissues exhibited relatively large increases in endogenous lipid peroxidation and would therefore appear to the more susceptible to the damaging effects of lipid peroxidation. When free MDA was used to assess lipid peroxidation brain regions appeared to be equally susceptible to a deficiency of vitamin E compared to non-neural tissues. In addition free MDA concentrations were significantly increased in some deficient tissues compared to controls at 21 weeks, whereas there were no differences in TBARS concentrations in all tissues at the same time point. Hence it is difficult to say whether the data correlated with the known in-vivo susceptibility of neural tissues to a severe deficiency of vitamin E.

The results for changes in the endogenous concentrations of TBARS in brain during vitamin E deficiency in the present study are in general agreement with the findings of Meydani et al (1988) who, with the exception of cerebellum, did not find a significant difference between endogenous concentrations of TBARS in control and deficient brain regions after 2 months of deficiency when α-tocopherol levels were reduced on average by 60%. Noda et al (1982)

studied endogenous TBARS in 9 different brain regions of 18 month old rats that had been placed on a vitamin E deficient diet for the last 9 months. They could only find significant increases of $<$ 2-fold in three of these regions. There was no difference in TBARS between control and deficient spinal cord.

Whereas my studies did not reveal any significant regional differences in endogenous TBARS between either the 6 control brain regions, or the 6 deficient brain regions, other studies have demonstrated statistically significant differences in concentrations in different brain regions. For example, Mizuno and Ohta (1986) found that in adult control rats (10-12 weeks) the concentrations of TBARS in the hypothalamus, thalamus and cerebellar vermis were approximately 2-fold greater than those found in the cortex, with the lowest concentration being present in the substantia nigra. In aged rats (16-18 months), however, they found that endogenous concentrations in the cortex had increased compared to the adult rats, whereas levels remained essentially unaltered in the other tissues. This resulted in approximately 1.5-fold higher concentrations in the aged cortex compared to the hypothalamus, thalamus and cerebellum. Interestingly, these workers found that TBARS increased significantly in all areas where superoxide dismutase (SOD) activity exhibited a significant reduction with aging, whereas in those brain regions where there was an increase in activity of SOD the levels of TBARS did not increase with age. Noda et al (1982), however, reported only small regional differences in endogenous TBARS in 4 month rat brain ranging from 210 $\pm$ 42 in the cerebellum to 335 $\pm$ 55 nmol/mg protein in the substantia nigra.

Lee and Csallany (1987) found that TBARS were increased in 43 week vitamin E deficient rat liver by a factor of 3.2 compared to controls when deficient livers still contained approximately 10% of control α-tocopherol levels. This increase is similar to the 3.6-fold increase in mean TBARS

concentration in liver in the present study after 56 weeks of deficiency.

Published data on endogenous concentrations of free MDA measured by selective HPLC in rat tissues during vitamin E deficiency appears to be limited to liver with, as far as I am aware, no previously reported concentrations in neural tissues. Lee and Csallany (1987) found that free MDA was present at a 14-fold greater concentration in deficient liver (43 week) compared to controls, whereas I found this parameter to be increased by a factor of only 1.8 after 56 weeks. Tokarz et al (1988) found that there was approximately a 2-fold increase in concentrations of free MDA in vitamin E deficient liver compared to controls after only 28 days. Therefore the increases in free MDA in the studies of Lee and Csallany and Tokarz et al tended to be greater than those which I found. These differences could possibly be explained by methodological differences. A more complicated sample work up was employed by these groups, who used a polytron to homogenise the tissue sample followed by extraction of MDA by passing the homogenate through ultrafiltration membranes. The use of a polytron may have led to metal ion contamination, and this in addition to the more complicated sample treatment, may have resulted in artifactual lipid peroxidation. Evidence for this is provided by the study of Tokarz's group who demonstrated that inclusion of an antioxidant, i.e. α-tocopherol, at the homogenisation stage resulted in approximately 3-fold lower free MDA concentrations in both control and vitamin E deficient samples.

My results for bound MDA in liver agree with the findings of Lee and Csallany (1987) who could not demonstrate an increase in deficient liver despite 14-fold higher free MDA concentrations. They postulated that this indicated a faster rate of MDA production than the capability to react with various cellular components. They also postulated that vitamin E deficient liver might be more efficient in clearing toxic free MDA by, for example,

increasing the activity of aldehyde dehydrogenases or by excreting it in the urine. Alternatively, the greater free MDA levels in deficient tissues may simply be the result of the fact that more MDA is formed in the deficient tissues as opposed to differences in metabolic capacities.

In conclusion, experimental rats maintained on a vitamin E deficient diet exhibited increased levels of in-vivo lipid peroxidation in both neural and non-neural tissues compared to controls. The different susceptibilities of the tissues to in-vivo lipid peroxidation appeared to be dependent on the particular assay used and therefore additional methods are needed to assess endogenous lipid peroxidation. These observations provide an example of the danger of dependence on a single assay method to assess in-vivo lipid peroxidation. Measurement of aliphatic aldehydes was potentially useful but was not used to study non-neural tissues. It is important to remember, however, that both MDA and aliphatic aldehydes are end-products of the lipid peroxidation process and are formed from the decomposition of lipid peroxides. Hence, the concentration of these products in a particular sample may be influenced by factors capable of causing the break down of these peroxides, e.g. transition metals. Additionally, the concentration of these compounds in a tissue at any time point represents a balance between their production and removal / degradation. Therefore, in general, it is advisable to compare as many methods as practically possible since no one method appears to be applicable to all situations.

CHAPTER 6.

IN-VITRO PEROXIDATION STUDIES OF NEURAL AND NON-NEURAL TISSUES

6.1 Introduction

6.2 Methods of in-vitro lipid peroxidation

6.2.1 The copper sulphate / hydrogen peroxide free radical generating system

6.3 In-vitro lipid peroxidation studies

6.3.1 Method

6.3.2 Brain regions

6.3.3 Comparison of neural and non-neural tissues

6.4 Discussion

6.1 Introduction

In addition to looking for evidence of increased levels of lipid peroxidation occurring in-vivo during experimental vitamin E deficiency as described in the previous chapter, the effect of this deficiency on the susceptibility of these tissues to in-vitro free radical stress was also studied. This allowed for a comparison of the results of the two studies and to examine whether susceptibilities to in-vitro peroxidative stress correlated with the in-vivo situation.

Numerous studies have been published on the relationship between sample vitamin E content and susceptibility to in-vitro peroxidative stress. For example, α-tocopherol incorporated into lecithin liposomes was shown to protect them from iron/ascorbate stimulated lipid peroxidation (Fukuzawa et al 1981). Staats et al (1988) found that mitochondria isolated from the outer zone of control guinea pig adrenal medulla were significantly more resistant to oxidative stress than mitochondria isolated from the inner zone. This finding correlated with a 5-fold greater endogenous concentration of tocopherol in more the resistant organelle fraction. They also demonstrated that addition of exogenous vitamin E inhibited lipid peroxidation in both sets of mitochondria. Vitamin E deficient (21 week) liver whole homogenate was found to produce approximately 6-fold more TBARS compared to controls following in-vitro peroxidative stress (Chen and Thacker 1987).

The in-vitro lipid peroxidation studies in this project were of two types;
(i) peroxidation of whole homogenates of neural and non-neural tissues and
(ii) studies of the in-vitro peroxidation of neural fractions isolated from rat brainstem. This chapter will describe the studies on tissue homogenates while the results from neural fractions will be presented in the next chapter.

6.2 Methods of In-Vitro Lipid Peroxidation

A number of different methods are available to study in-vitro lipid peroxidation. These range from the simplest where samples are incubated in air at 37°C to relatively complex systems such as the microsomal NADPH cytochrome P-450/iron-ADP system. Some peroxidative stress systems are based on the ability of reduced transition metals (TM) to stimulate lipid peroxidation, e.g. Cu^{+} and Fe^{2+}. The TM can be added to the sample directly in the reduced form or with a compound or enzymatic system capable of reducing it to the reactive form. For example Fe^{3+}-ADP can be reduced to Fe^{2+}-ADP by including ascorbate in the incubation mixture. Complexing the iron with ADP increases its solubility and hence effectiveness in stimulating lipid peroxidation. Alternatively in microsomal membrane fractions the iron can be reduced by the P-450 system where reducing equivalents are passed from NADPH to oxidised iron by the P-450 reductase component of the system.

The different methods used in in-vitro peroxidation studies are in general referred to as being either "enzymatic" or "non-enzymatic". The former category includes any method where an enzyme or enzyme system is a component of the system and includes the cytochrome P-450 complex, the xanthine/xanthine oxidase system and the NADH-coenzyme Q reductase complex of the inner mitochondrial membrane. Non-enzymatic methods, as the name suggests, are not dependent on the activity of an enzyme to generate peroxidation. Examples of this group of methods include the iron-ADP/ascorbate and copper sulphate/hydrogen peroxide systems. Halliwell and Gutteridge (1989) have, however, questioned the validity of the use of these terms in describing peroxidation methods and suggested that the term "enzymatic lipid peroxidation" be assigned to describe only the activities of the enzymes cyclooxygenase and lipoxygenase that catalyse specific reactions that have important biological functions. They argue that in those peroxidation

systems generally accepted as being enzymatic, the reduced TM component was the actual peroxidising agent and not the enzyme itself. For example, although the xanthine / xanthine oxidase system reduces iron via the superoxide radical, it is the iron that actually stimulates the peroxidation. Moreover, Halliwell and Gutteridge stressed the importance of these TMs as stimulating lipid peroxidation as opposed to initiating it, i.e. the TM causes peroxidation by decomposing preformed lipid peroxides as distinct from peroxidising previously unoxidised polyunsaturated fatty acids (PUFA).

Systems that generate free radicals directly can also initiate lipid peroxidation. For example, as discussed in chapter 1 (section 1.5) a mixture of a reduced TM and H_2O_2 results in the formation of the highly reactive hydroxyl radical by the Fenton reaction. Additional reactions are also possible including the formation of the superoxide radical ($^{\cdot}O_2$). Defined oxygen free radicals can be generated by radiolysis (e.g. Dean and Cheeseman 1987). The so called azo compounds have become increasingly used in in-vitro peroxidation studies (e.g. Niki et al 1991). These compounds decompose thermally and unimolecularly to yield carbon centred free radicals which react instantaneously with oxygen to form a peroxyl radical. This radical then attacks membrane lipids resulting in peroxidation. Although, the azo compounds are not biologically relevant, they have a number of features that are advantageous in peroxidation studies. In common with radiolysis, the number of free radicals to which a membrane system is exposed can be controlled (and calculated) by varying the temperature of the incubation mixture or by increasing the concentration of the free radical precursor. In addition, selecting the correct compound allows the user to control the site of free radical generation since both hydrophilic and lipophilic forms of these compounds are commercially available.

It is important to remember that the peroxidation profile of a sample or the difference in susceptibilities exhibited by two different samples may depend on the peroxidative system employed. This point was illustrated in a study by Braughler et al (1987) who compared the susceptibilities of brain and liver of aged rats. Brain homogenates incubated at 37°C with 200μM Fe^{2+} for 20 min was 13-fold more susceptible than liver as assessed by the measurement of TBARS. However when the same samples were incubated without exogenous iron, brain was only 60% more susceptible. It is therefore advisable to employ more than one peroxidation method to check whether experimental results are independent of the method chosen or are assay-specific.

6.2.1 The Copper Sulphate / Hydrogen Peroxide Free Radical Generating System

For all in-vitro peroxidation studies described in this project the $CuSO_4$ / H_2O_2 free radical generating system was chosen. This method was originally selected since in the early stages of the project the intention was to study protein peroxidation in addition to lipid peroxidation. Konat and Wiggins (1985) used this system to demonstrate that in-vitro lipid peroxidation of an isolated rat myelin fraction resulted in concomitant peroxidation of proteins within the multilamellar membrane as assessed by the loss of banding patterns on SDS-polyacrylamide gels (SDS-PAGE). Although I was able to reproduce these findings, (data not shown), studies of protein peroxidation were discontinued since SDS-PAGE was not sufficiently sensitive or quantitative to detect possible differences in the susceptibilities of proteins in vitamin E deficient and control membranes.

In general, the $CuSO_4/H_2O_2$ system was used to investigate in-vitro lipid peroxidation in two ways; (i) to study the effect of incubation time on the susceptibility to peroxidative stress by incubating samples with fixed concentrations of $CuSO_4$ and H_2O_2 for increasing periods of time and (ii) to

study the effect of increasing concentrations of free radicals by incubating samples for a fixed time and a fixed concentration of H_2O_2 with increasing concentrations of $CuSO_4$. All studies described in this chapter were of type (i), whereas the in-vitro peroxidation studies on isolated neural fractions as presented in chapter 7 involved the use of both assay modes.

6.3 In-Vitro Lipid Peroxidation Studies

Investigations were carried out using tissue whole homogenates from 21 and 56 week old control and vitamin E deficient rats. The experiments can be divided into two types; (a) investigation of peroxidation of different brain regions and (b) comparison of the susceptibility of neural and non-neural tissues to in-vitro free radical stress. Note that these studies involved comparison of different samples from the same animal. Although control and deficient tissues were assayed separately, both sets of tissues were peroxidised under the same conditions. Inclusion of a quality control (QC) in all assays allowed a comparison of different sets of data. The objective of the experiments presented in this chapter was to investigate which tissue(s) in both vitamin E deficient and control rats were the most susceptible to in-vitro peroxidative stress and how the findings correlated with the results of endogenous lipid peroxidation and the pathology associated with chronic vitamin E deficiency.

6.3.1 Method

For the time course studies a single large peroxidation mixture was incubated at 37°C and aliquots removed in duplicate at fixed time intervals. In general mixtures were incubated for a total of 60 min with a 50 or 100-fold excess of H_2O_2 compared to $CuSO_4$. Tissue homogenates were those used to measure endogenous concentrations of lipid peroxidation products (chapter 5), i.e. 2.5% (wet wt/vol) in 40mM trizma buffer, pH 7.4, (in ultra pure H_2O). $CuSO_4$ and H_2O_2 reagents were prepared in trizma buffer. No attempt was

made to remove contaminating TMs that may have been present. The relatively strong peroxidation conditions used in these assays meant that any contaminating TMs would have had a negligible effect. Lipid peroxidation was followed by measuring the free MDA produced as described in chapter 3. The QC was the whole homogenate of a 52 week deficient rat which was included in all assays.

Procedure

A single assay mixture consisted of 50μl sample and 10μl each of H_2O_2 and $CuSO_4$ reagents. The large scale incubation mixture contained these components in the same ratio. The concentrations of H_2O_2 and $CuSO_2$ quoted for each assay were those in the final incubation mixture. In order to minimise peroxidation prior to actual incubation the following procedure was followed: H_2O_2 was added to, but not mixed with, tissue samples which were kept sitting on ice. $CuSO_4$ was then added and peroxidation mixtures quickly vortex mixed and transferred to a 37°C water bath. This was taken as time zero. Aliquots of the reaction mixture were removed in duplicate at fixed times and immediately added to 0.5ml eppendorf centrifuge tubes containing an equal volume of ice-chilled acetonitrile and vortex mixed for 15 sec. The tubes were then spun in an eppendorf minifuge at maximum speed for 5 min. The clear supernatants were aspirated and kept on ice until estimation of MDA which was carried out within 1 hour of sampling. Handling samples in this manner resulted in the termination of lipid peroxidation since there was no increase in MDA concentrations in samples that were repeatedly analysed for MDA over at least a 3 hour period. Free MDA concentrations were expressed per protein concentration. The data points on the graphs are the mean MDA values of the two aliquots taken at each time point. All peroxidation assays were arranged so that similar amounts of each sample, i.e. protein, was present in different peroxidation mixtures.

6.3.2 Brain Regions

As in the case of the determination of endogenous lipid peroxidation the following brain regions were studied; cortex, striatum, midbrain, hypothalamus, cerebellum and brainstem. The brain regions were incubated at 37°C for increasing periods of time up to 60 min with 20μM $CuSO_4$ and 1mM H_2O_2. Peroxidation mixtures were sampled at time zero and at 10, 30 and 60 min. Approximately 10μg of protein was assayed in all cases. The results for 21 and 56 week control brain regions are plotted in figs 1 and 2 respectively, while those for the corresponding deficient tissues are presented in figs 3 and 4. Because of the large number of samples in these assays only single sets of brain regions were examined.

In general, for all 4 sets of brain regions,(control and deficient at 21 and 56 weeks), cortex, striatum and cerebellum were the most susceptible to free radical stress with the brainstem and hypothalamus tending to exhibit the greatest resistance. For example, peroxidation of 21 week control cerebellum yielded approximately twice as much free MDA as brainstem and hypothalamus. Although, each set of tissues were assayed separately the inclusion of a QC in each experiment allowed a comparison of control and deficient data.

6.3.3. Comparison of Neural and Non-Neural Tissues

The susceptibility of neural and non-neural tissues to in-vitro peroxidative stress was compared by examining the following tissue homogenates; midbrain, thoracic spinal cord, sural nerve, heart and muscle. Tissues from a single rat were assayed from each age group. Midbrain was chosen as a representative sample of brain tissue since it exhibited intermediate susceptibility compared to the other control regions, although in deficient tissues it tended to be relatively resistant to peroxidation. The data for the non-brain tissues was obtained by running all the tissues on the same day,

FIG 1. IN-VITRO PEROXIDATION OF 21 WEEK CONTROL BRAIN REGIONS

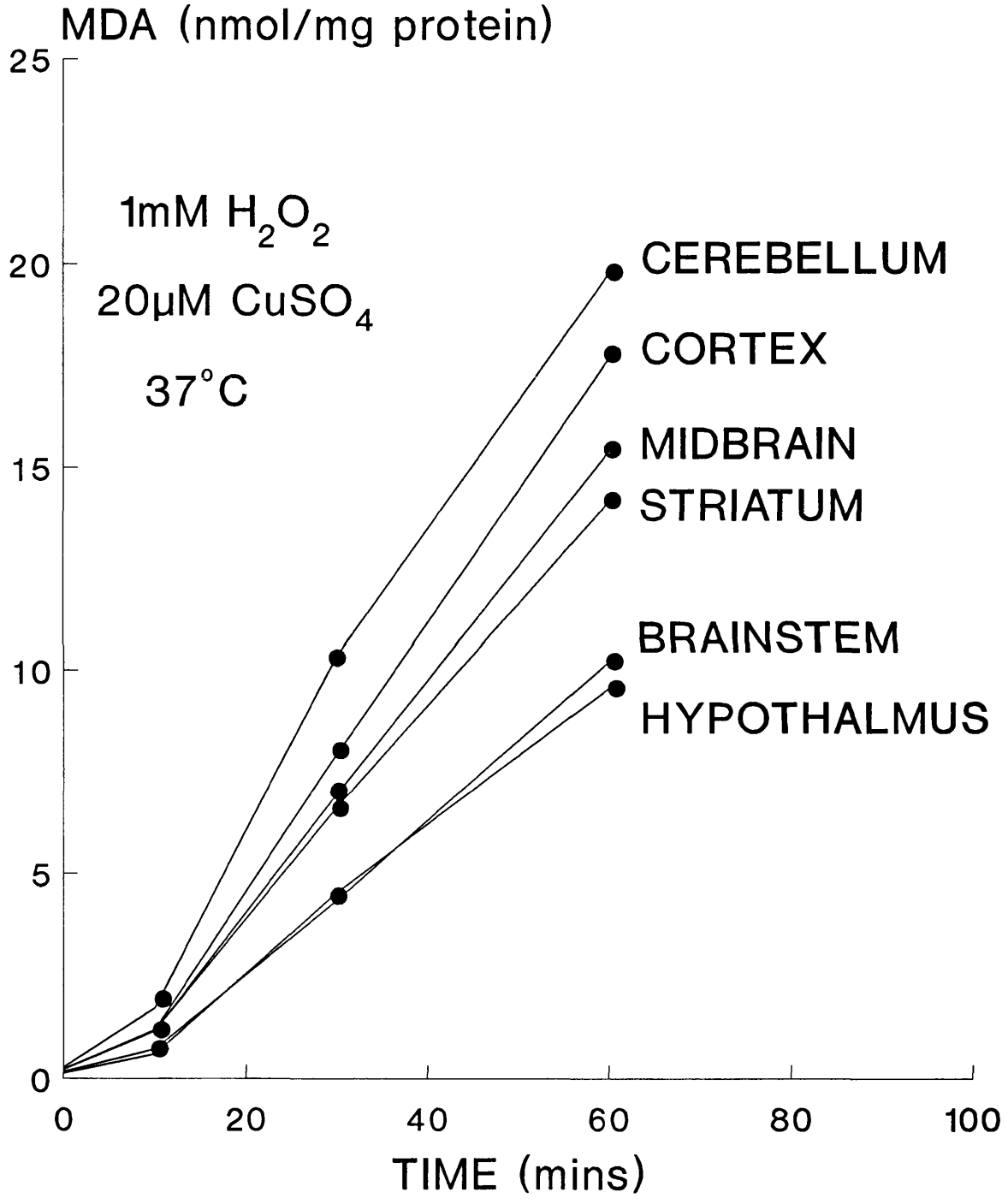


FIG 2. IN-VITRO PEROXIDATION OF 56 WEEK CONTROL BRAIN REGIONS

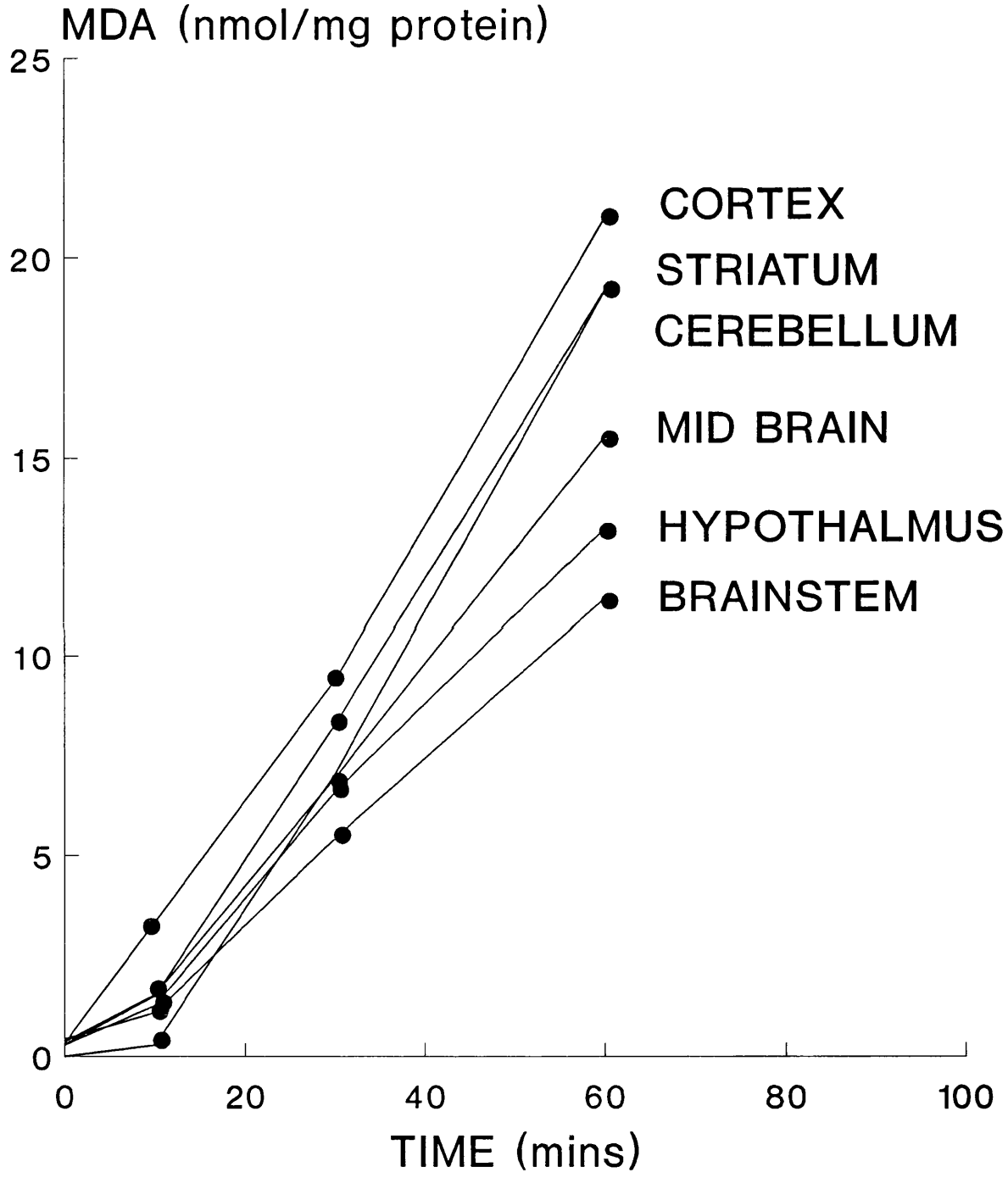


FIG 3. IN-VITRO PEROXIDATION OF 21 WEEK DEFICIENT BRAIN REGIONS

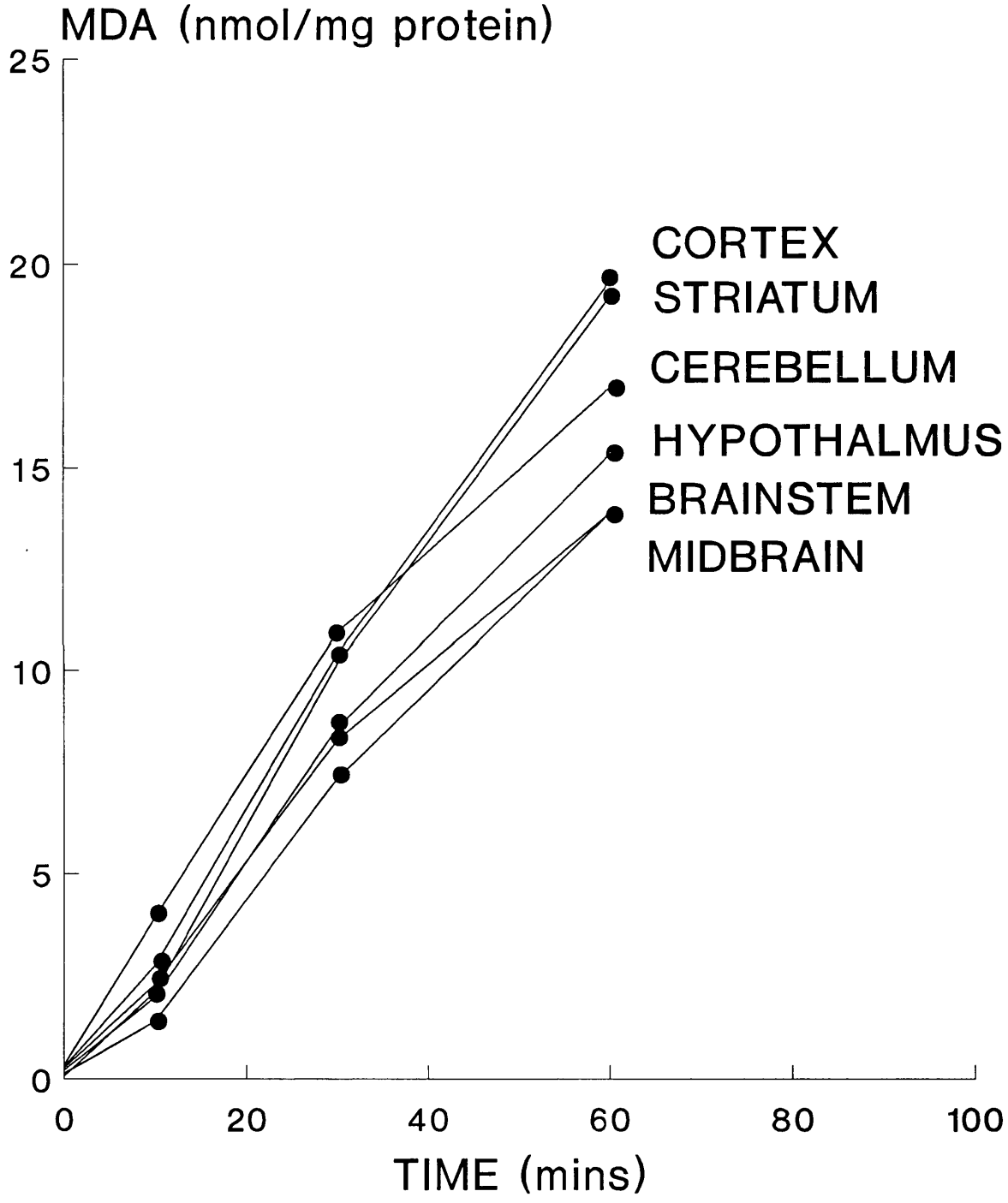
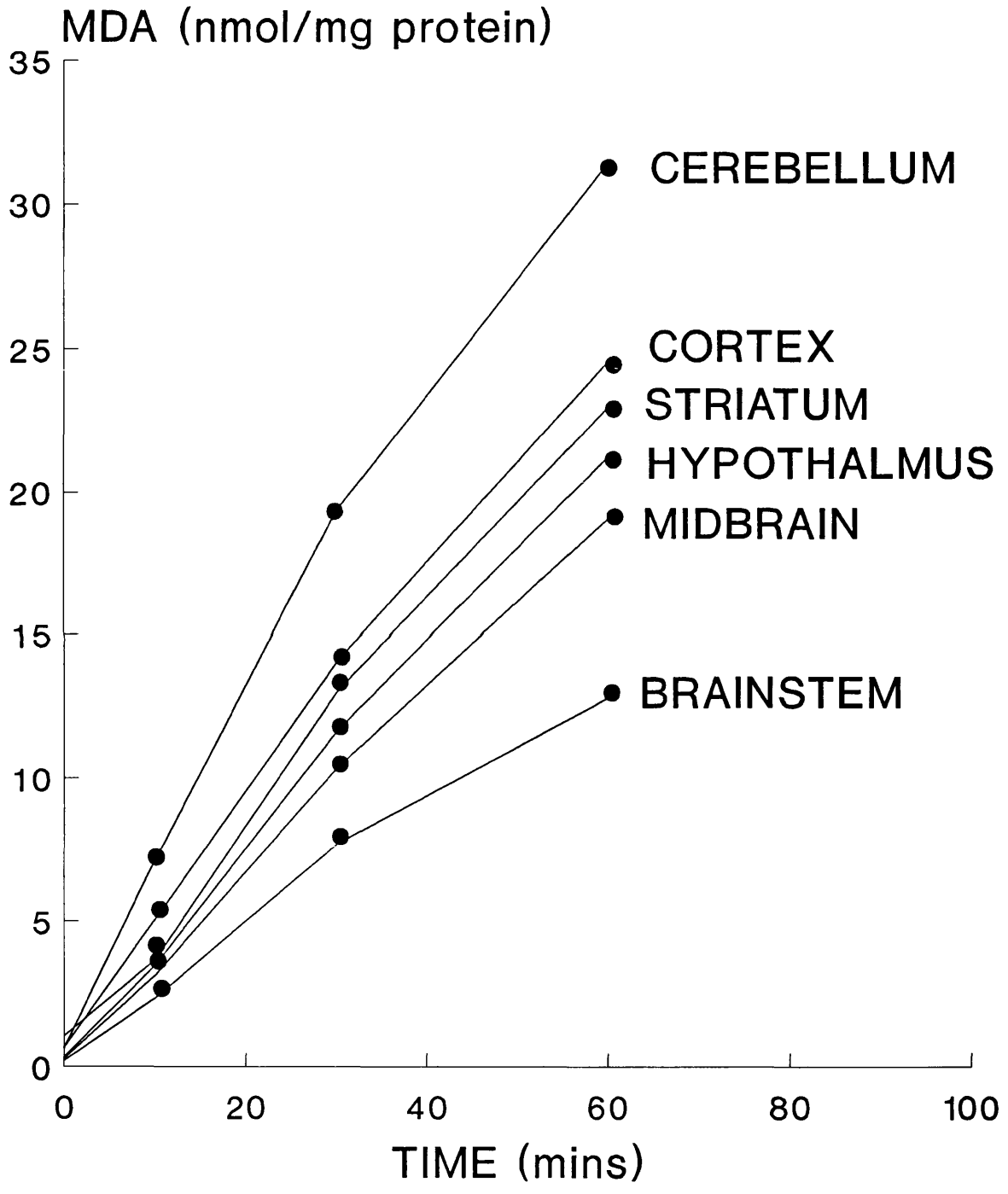


FIG 4. IN-VITRO PEROXIDATION OF 56 WEEK DEFICIENT BRAIN REGIONS



whereas the data for midbrain was obtained from the studies described in the previous section. However, since a QC was included in all assays the midbrain results could be compared with those of the other assays. Data from the liver is not presented graphically since an interfering peak coeluted with the MDA on the chromatogram following HPLC making quantitation of MDA difficult. The liver, however, appeared to be relatively resistant to peroxidative stress compared to brain tissue.

The results for in-vitro peroxidation of control neural and non-neural tissues at 21 and 56 weeks are presented in figs 5 and 6 respectively. The corresponding deficient tissues are shown in figs 7 and 8. The same assay conditions were used as those for the various brain regions. For control tissues at both time points midbrain displayed the greatest susceptibility and generated MDA at a greater rate than the other tissues. Peripheral nerve showed the greatest resistance to free radical stress peroxidising at an extremely slow rate under the particular stress conditions employed. The susceptibilities of the remaining tissues were variable. For neural tissues, however, the following order of susceptibility was consistent;
brain $>>$ spinal cord $>$ peripheral nerve. In deficient animals, although the same pattern was maintained in neural tissues, deficient heart exhibited the greatest sensitivity to peroxidative stress which was even greater than deficient midbrain. A comparison of the susceptibilities of 56 week control and deficient heart is presented in fig 9.

FIG 5. IN-VITRO PEROXIDATION OF 21 WEEK CONTROL NEURAL AND NON-NEURAL TISSUES

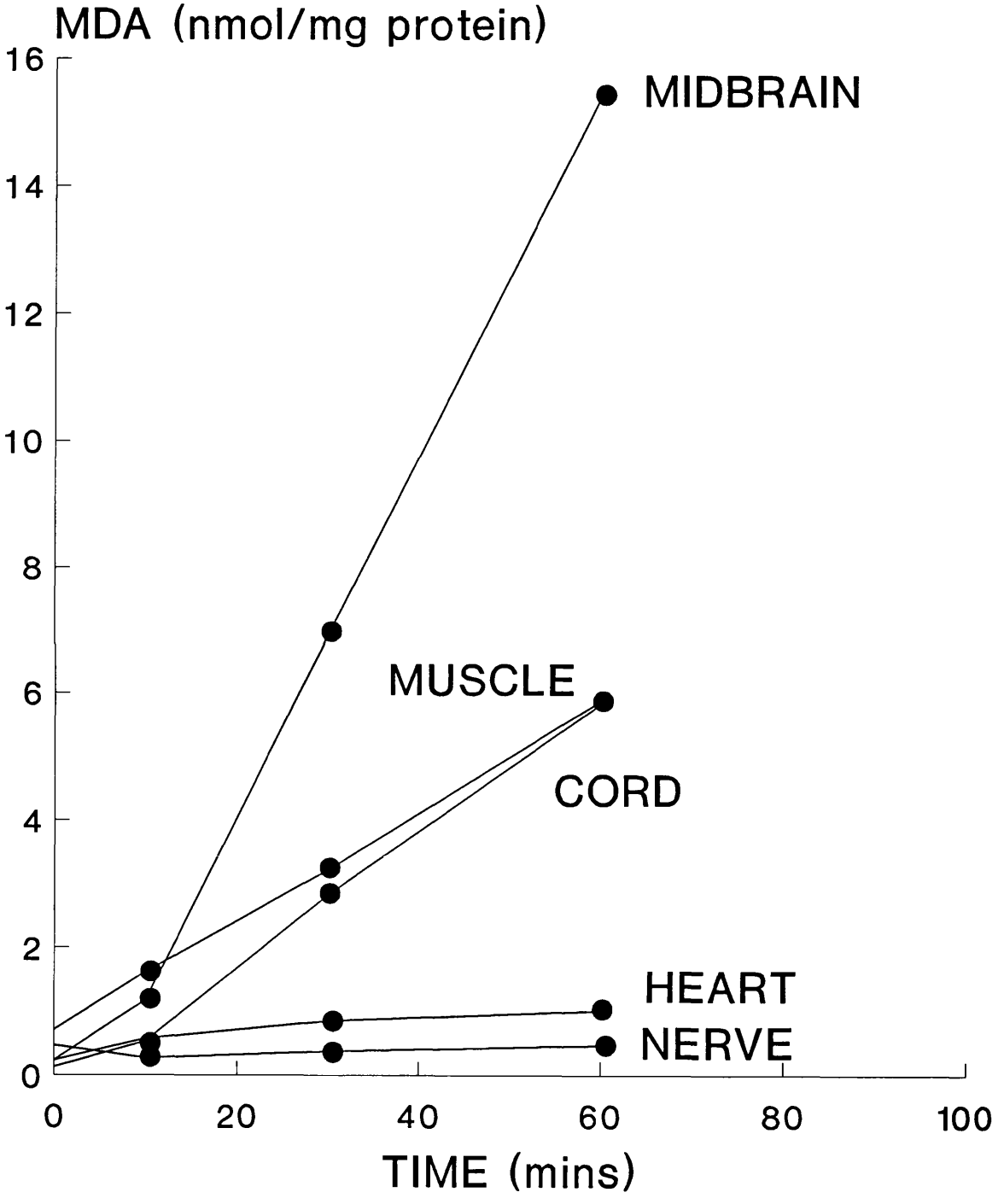


FIG 6. IN-VITRO PEROXIDATION OF 56 WEEK CONTROL NEURAL AND NON-NEURAL TISSUES

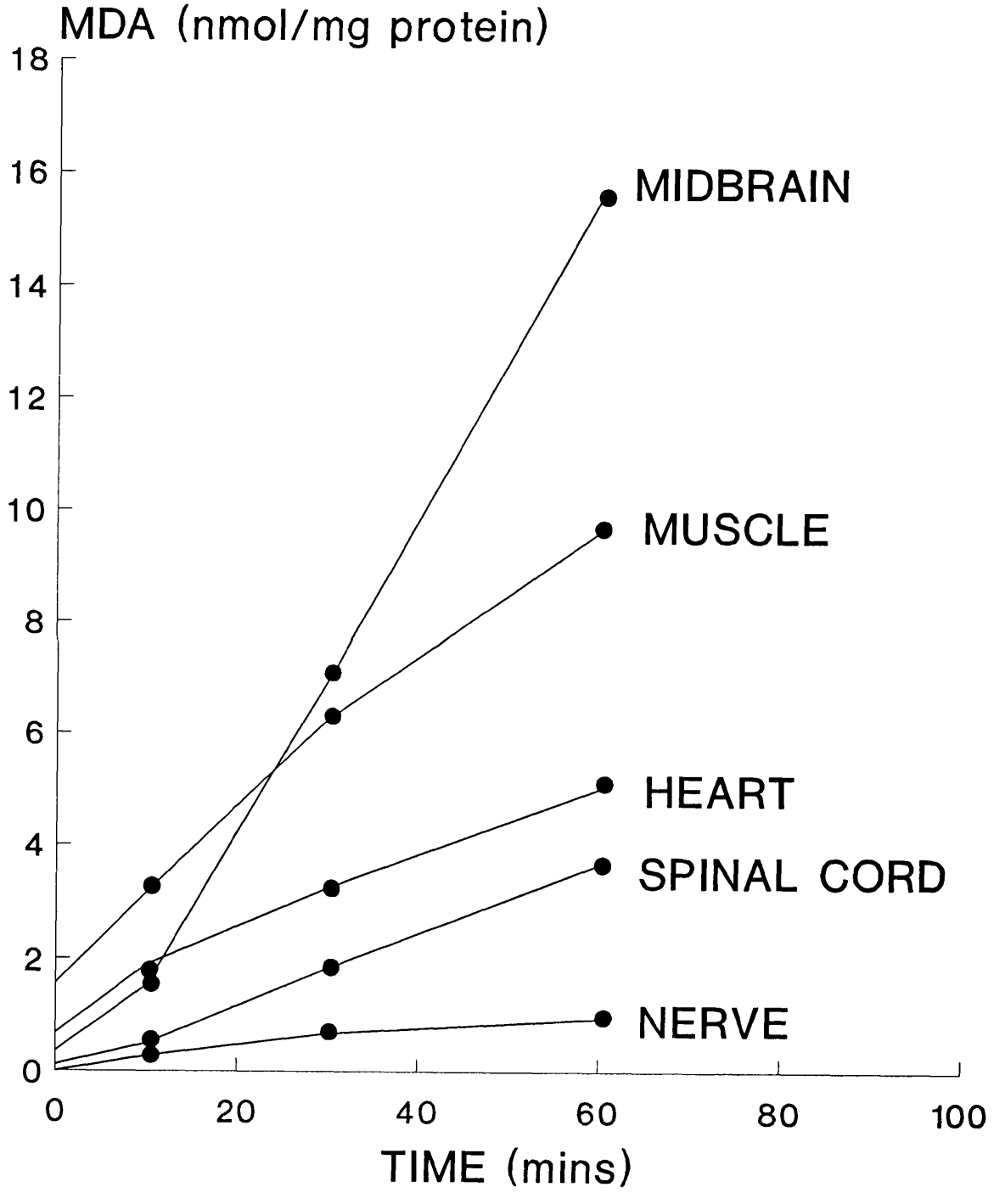


FIG 7. IN-VITRO PEROXIDATION OF 21 WEEK DEFICIENT NEURAL AND NON-NEURAL TISSUES

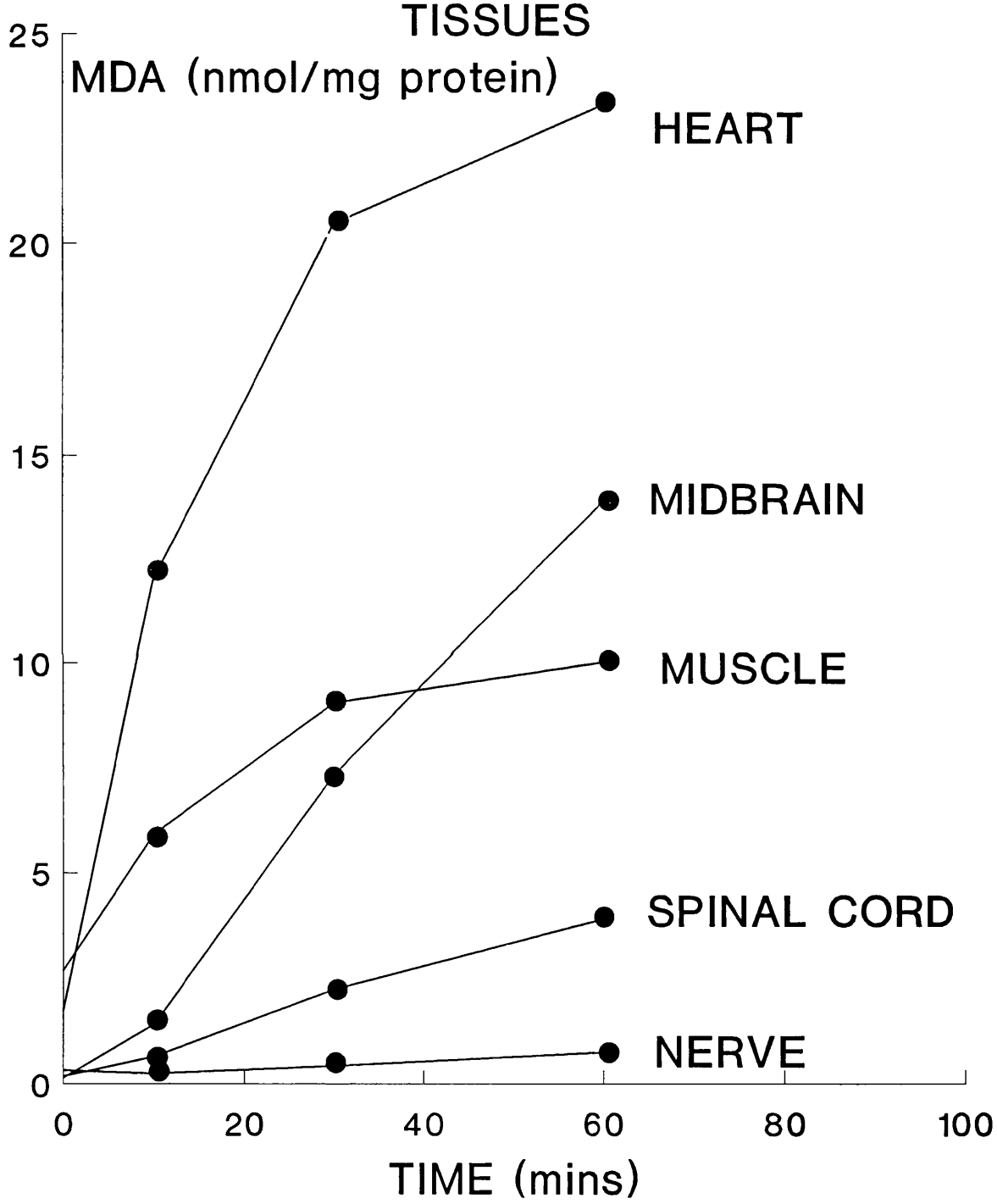


FIG 8. IN-VITRO PEROXIDATION OF 56 WEEK DEFICIENT NEURAL AND NON-NEURAL TISSUES

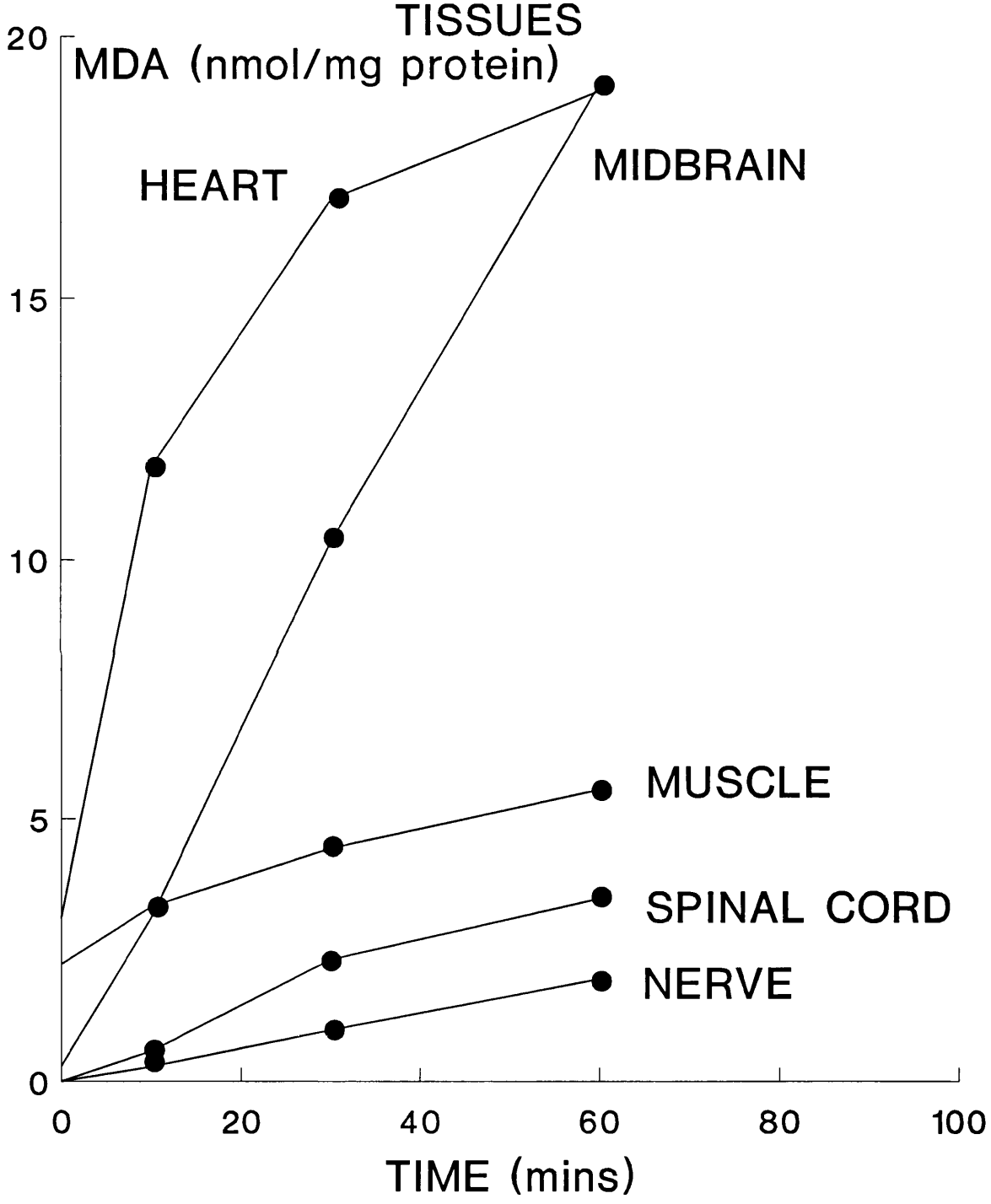
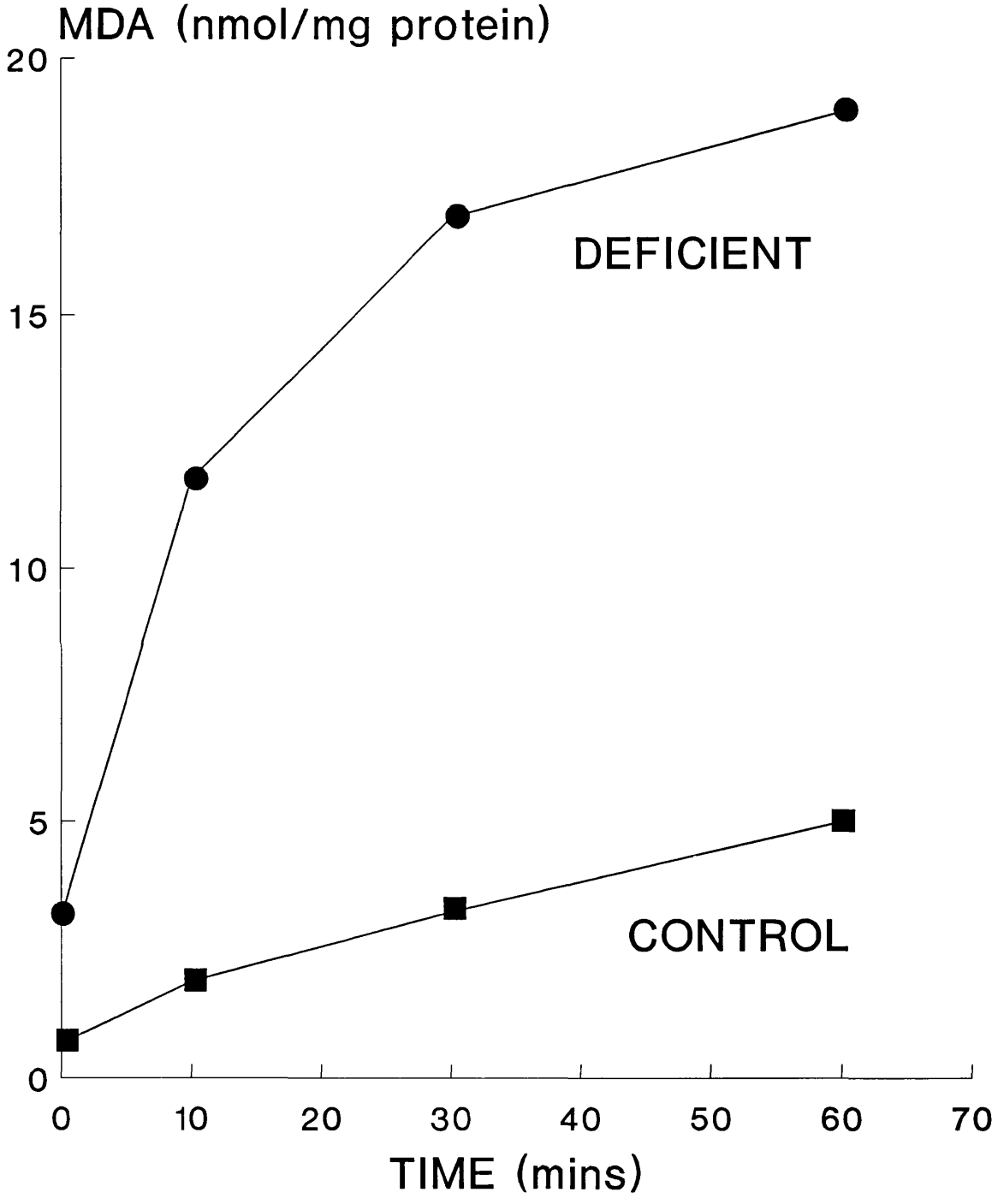


FIG 9. IN-VITRO PEROXIDATION OF 56 WEEK CONTROL AND DEFICIENT HEART



6.4 Discussion

Before discussing the various results presented in this chapter it is important to remember that relatively strong in-vitro peroxidation conditions were employed. Using high concentrations of H_2O_2 and $CuSO_4$ would probably have meant that in some cases relatively small changes in antioxidant capacities were obscured, i.e. there would be no significant difference between the susceptibilities to in-vitro peroxidative stress of a control sample and the corresponding vitamin E deficient sample. In addition such peroxidation conditions would have removed the lag phase from peroxidation profiles due to the rapid consumption of endogenous antioxidants. This latter point will be discussed in greater detail in chapter 7.

Brain Regions

A comparison of the susceptibilities of the 6 brain regions to free radical stress revealed in general, that in both control and deficient sets of tissues at 21 and 56 weeks that the greatest amounts of free MDA were produced in cortex, striatum and cerebellum whereas brainstem and hypothalamus tended to be the most resistant brain regions. The maximum difference was, however, relatively small, i.e. approximately 2-fold. These results agree in part with the findings of Ansari et al (1989) who demonstrated a rostral to caudal decrease in susceptibility to in-vitro peroxidative stress in control brain regions from 3 month old rats. They found after incubation in room air at 37°C for 1 hour the following order of susceptibility as assessed by measurement of TBARS; cortex > basal ganglia > cerebellum > medulla (brainstem). Although essentially the same pattern of susceptibility was seen in 12 month old rats, the quantities of TBARS produced were reduced in all regions. Whereas in the studies of Ansari et al the cerebellum was found to be approximately 25 and 500% more resistant to peroxidative stress than cortex in 3 and 12 month old control rats respectively, I found little difference in the susceptibilities of control cortex and cerebellum. The same was true for the

corresponding deficient tissues. Contrasting results were obtained by Meydani et al (1988) who investigated the in-vitro peroxidation in 4 brain regions of two month old vitamin E deficient and control rats. When control samples were incubated in air at 37°C for 2 hours as used by Ansari et al, the following order of susceptibility was seen as assessed by measuring TBARS; brainstem > cortex = midbrain = cerebellum. The order for the corresponding deficient samples was; cerebellum > cortex > midbrain > brainstem. For both control and deficient brain regions the differences between the most and least peroxidisable were relatively small, i.e. approximately 60 and 70% respectively.

There may be a number of reasons for the variation in findings of different groups in the susceptibility of different brain regions to in-vitro peroxidation. One important factor may be the system employed to produce lipid peroxidation. The studies of Meydani et al (1988) show that the order of susceptibilities in both vitamin E deficient and control rat brain regions was dependent on the type of stress system used. For example, when control samples were incubated with 0.25mM ascorbate at 37°C for 2 hours the following order of susceptibility was observed; cerebellum = brainstem > cortex = midbrain, whereas when stressed with 0.1mM Fe^{2+} a different pattern was produced, i.e. cortex > midbrain = cerebellum > brainstem. Both of these results differ from the results mentioned above when samples were incubated in air only. Moreover, the three systems described by Meydani's group relied upon the breakdown of preformed lipid peroxides in the samples to cause lipid peroxidation. The $CuSO_4/H_2O_2$ system which I used, initiates lipid peroxidation, i.e. attacks previously unoxidised PUFAs via the hydroxyl radical. Hence, different systems and differences in peroxidation mechanisms may explain the differences in the various studies.

Another possible source of variation was the method of estimating lipid peroxidation. I measured free MDA by selective HPLC, whereas TBARS were determined in the other studies. A report by Janero and Burghardt (1988) illustrates the potential for obtaining different results from the same in-vitro peroxidation system by using different methods to monitor the process. They demonstrated that when conjugated dienes were used to assess the in-vitro peroxidation of isolated rat myocardial membranes and liposomes made from the lipids of these membranes, that both began to peroxidise immediately upon incubation. However, when TBARS were used as a measure of lipid peroxidation the liposomes exhibited a lag phase of approximately 20 min before more rapid peroxidation occurred, whereas the membranes did not exhibit an initial lag phase. Alternatively, when free MDA was measured by selective HPLC both samples exhibited lag phases of the same duration.

There are a number of possible explanations for the consistent differences in the susceptibility of different brain regions in both control and deficient rats to in-vitro peroxidative stress in the present study. Since there were no significant differences in the endogenous concentrations of lipid peroxidation products seen in these regions (chapter 5), the order of susceptibilities cannot be readily explained by the in-vivo lipid peroxidation studies.

A number of groups, e.g. Goss-Sampson et al (1988) and Meydani et al (1986 and 1988), have reported a general rostral to caudal decrease in the regional distribution of α-tocopherol in rat brain with highest concentrations in the cortex and lowest in the cerebellum and medulla (part of brainstem). For example, Goss-Sampson et al who employed the same animal model and diet to study vitamin E deficiency as used in this particular project, found that concentrations of vitamin E in 52 week control rat brain were highest in cortex and lowest in cerebellum, i.e. 1032 and 704μg/g lipid respectively.

The corresponding deficient regions also exhibited a rostral to caudal reduction in concentrations during the early stages of deficiency which disappeared after 24 weeks when similar vitamin E concentrations were found in all areas, with no particular region showing evidence of specific loss or retention. In the case of control brain, with the exception of cerebellum, the rostral to caudal increase in resistance to in-vitro peroxidative stress seen at both 21 and 56 weeks did not correlate with vitamin E concentrations which showed a rostral to caudal decrease. In addition, although not determined, the probable similarity of vitamin E levels in deficient regions at both time points would appear to exclude vitamin E concentrations as being the major determinant in differences in susceptibility of the these brain regions to in-vitro free radical stress. Since samples were exposed to a relatively strong peroxidation system it was probable that the antioxidant capacity of the samples was overwhelmed soon after incubation so that other factors, e.g. fatty acid composition, may have been more important in determining tissue susceptibility or resistance.

An examination of the reported specific activities of the various antioxidant enzymes in different rat brain regions does not appear to provide an explanation for the differing susceptibilities. For example, a number of studies have reported a rostral to caudal increase in the levels of glutathione peroxidase (GSHPx), i.e. Brannan et al (1980), Goss-Sampson et al (1988) and Ansari et al (1989), which would correlate with the increases in resistance to peroxidative stress in going from cortex to brainstem. Superoxide dismutase (SOD) activity also tends to be higher in hypothalamus, midbrain and medulla compared to cortex (Thomas et al 1976, Goss-Sampson et al 1988, Ansari et al 1989). In contrast, Ansari's group found that catalase exhibited a decrease in activity in the rostral to caudal direction. The magnitude of the differences of activities of these enzymes in brain regions were, however, relatively small so that individually they would not be expected to have a major effect on the

susceptibility of a particular region to in-vitro peroxidative stress.

Svennerholm (1968) found that the fatty acid profiles of the phospholipids of the gray and white matter of human cerebral cortex were strikingly different with white matter having a much higher concentration of monoenoic acids and lower concentrations of saturated and polyunsaturated fatty acids. For instance, 22:6 accounted for 30 and 23% (weight percentage of total methyl esters) of phosphatidylethanolamine and phosphatidylserine respectively of gray matter while the corresponding values in white matter were only 7.5 and 1.3%. Therefore, the gray matter would be expected to be more susceptible to peroxidative stress. Structurally, the major difference between white and gray matter is that the former is rich in myelinated axons whereas gray matter contains the associated cell bodies of these axons. Hence, white matter will contain a much greater amount of myelin than gray matter. As discussed in greater detail in the next chapter, myelin, compared to other neural membranes is not particularly enriched in PUFAs so that the differences in the percentages of PUFAs between white and grey matter may be the result of the differences in the content of myelin. The white matter of mammalian brain contains 50 to 60% myelin on a dry weight basis (Norton and Autilio 1966), and in the case of whole rat brain it accounts for 20 to 25% of the dry weight and 40% of the total lipid (Norton and Poduslo 1973). The differences in the susceptibilities of various brain regions to in-vitro peroxidative stress seen in this study could be a consequence of differences in the amount of myelin membrane. Both the hypothalamus and brainstem are relatively heavily myelinated regions through which important fibre tracts pass. The relatively high resistance of these two areas to free radical stress compared to other brain regions could result from their high content of myelin and a hence low PUFA content. The actual fatty acid profiles in both control and vitamin E deficient brain regions need to be determined.

There was no major change in the susceptibility of any brain region between the two assay stages, i.e. 21 and 56 weeks. This was true of both experimental groups. The fact that there was not a significant difference between the deficient samples at 21 and 56 weeks despite higher α-tocopherol concentrations at the earlier stage could be explained by the relatively strong peroxidation system used. These conditions would probably not have been subtle enough to differentiate between small differences in vitamin E levels. Other groups have reported conflicting results for the in-vitro peroxidation of rat brain. While Zaleska et al (1984) found no evidence of alterations with age, others have reported an age related decrease in the susceptibility of rat brain to peroxidation, i.e Yoshioka et al (1981), Floyd et al (1984) and Ansari et al (1989). For example, Boehme et al (1977) reported that 540 days old rat brain was 5.5-fold more resistant to peroxidative stress than 1 day old brain. Again the differing results may be due to the differences in the stress and analysis systems used. Alternatively the choice of diet, particularly in relation to its vitamin E and PUFA content, may be an important factor since tissue concentrations of these compounds can be altered by manipulating their dietary levels.

Brain, Spinal Cord and Peripheral Nerve

A comparison of the in-vitro peroxidation of the three neural tissues studied showed that in all cases, i.e. control and deficient at 21 and 56 weeks, that the following order of susceptibility was seen; brain $>>$ spinal cord $>$ nerve. Therefore, in addition to the rostral to caudal decrease in susceptibility in brain regions, there was also a rostral to caudal decrease in susceptibility for the nervous system as a whole. Ansari et al (1989) also showed that cervical spinal cord was more resistant to in-vitro peroxidative stress than all the brain regions examined. In addition, it has been demonstrated that peripheral nerve was more resistant than spinal cord to in-vitro peroxidative stress. This pattern of neural sensitivity to peroxidative stress correlated to

some extent with the results of endogenous concentrations of lipid peroxidation products as measured by TBARS and free MDA.

An examination of the α-tocopherol levels in these tissues does not appear to provide an explanation for the differences in sensitivities. For instance, at 24 weeks the concentrations in deficient midbrain, thoracic cord and sural nerve were 110, 87 and 106 μg/g lipid respectively (Goss-Sampson et al 1988). As suggested, when attempting to explain the differences in the susceptibilities of various brain regions, it is possible that the relative susceptibilities result from differences in myelin and PUFA content. In agreement with this is the fact that the percentage of myelin in these tissues increases in the following order; brain $<$ cord $<$ nerve.

From the above discussion and results it would appear that a deficiency of vitamin E would result in greatest neural damage in the brain with cord and nerve being less affected. However, extensive loss of large calibre myelinated axons is known to occur in both spinal cord and peripheral nerves during chronic vitamin E deficiency. As discussed in the next chapter, the loss of these fibres is thought to result from primary damage to the axonal membranes of the myelinated axon with secondary demyelination. The axonal membranes in comparison to myelin account for a very small amount of the total tissue lipid and protein so that peroxidation of the whole homogenate might not reveal the contributions made by axonal membranes. A comparison of the peroxidation of different neural membranes may therefore be more valid when comparing different neural tissues during vitamin E deficiency.

Neural / Non-Neural Tissues

A comparison of the in-vitro peroxidation of neural and non-neural tissues reveals that in controls, brain (midbrain) exhibits the greatest susceptibility at both 21 and 56 weeks, whereas heart was the most susceptible of the deficient tissues. In all cases sural nerve was the most resistant tissue.

The greater susceptibility of control brain than heart to in-vitro peroxidative stress is in agreement with other studies. Both Boehme et al (1977) in the human and Kornbrust and Mavis (1979) in rat and rabbit found that the heart and lung were more resistant than brain. The much greater resistance of these particular non-neural tissues relative to brain could not be explained in terms of their PUFA content which were similar. However, measurement of vitamin E concentrations revealed much greater levels in lung and heart compared to brain, i.e. 4.3, 1.9 and 0.5 μg vitamin E/mg peroxidisable PUFA respectively.

Further evidence for a correlation between in-vitro susceptibility to peroxidation and concentrations of vitamin E comes from a report by Braughler et al (1987) who investigated the in-vitro peroxidation of rat whole brain and liver of young and aged rats maintained on standard lab rat chow. They demonstrated that the aged livers were approximately 2-fold more resistant compared to the young tissues which correlated with approximately a 3-fold increase in vitamin E liver concentrations. Brain on the other hand exhibited only a 20% decrease in susceptibility during the same time period which correlated with a slight increase of 10% in vitamin E levels. Braughler's group also demonstrated that aged rat brain was approximately 2-fold more susceptible to in-vitro peroxidation with Fe^{2+} than liver which correlated with a 2.7-fold greater concentration of vitamin E in the latter. There was no significant difference between these two tissues in the young rats when tocopherol levels were similar.

The concentrations (mean ± 1SD) of α-tocopherol in control cortex, liver and muscle at 20 weeks were 615±162, 737±81 and 733±271μg/g lipid respectively. The corresponding values at 55 weeks were 480±81, 958±265 and 752±213 μg/g lipid respectively (see chapter 4). Although, concentrations of vitamin E were significantly higher in both 55 week liver and muscle compared to cortex, the comparable levels in cortex and muscle in the younger age group would appear to rule out vitamin E as being the major determinant of the differences in susceptibilities in this particular study. Therefore, my conclusions for neural/non-neural peroxidation are not in agreement with those of both Kornbrust and Mavis, and Braughler et al.

Brain and other neural tissues tend to have lower activities of the various antioxidant enzymes which would correlate with the higher sensitivity of control brain compared to non-neural homogenates. For example, Barja-de-Quirogaet al (1990) in a comparison of liver and brain of 9 month old rats demonstrated that cerebral activities of superoxide dismutase, catalase, total and selenium-dependent glutathione peroxidase were only 48, 1.2, 6.0 and 7.2% of those present in liver. In addition the concentrations of glutathione in different brain regions of 3 month old rats have been shown to be markedly lower than liver levels with > 6-fold lower concentrations in brainstem (Ravindranath 1989).

The order of susceptibility of the vitamin E deficient tissues was essentially the same as found for the controls. However, deficient heart appeared to be extremely sensitive to peroxidative stress, peroxidising at a faster rate than deficient midbrain. The magnitude of the difference between control and deficient heart at both 21 and 56 weeks is much greater than that seen for all the other tissues under the peroxidation conditions employed. This finding correlates with 6.3-fold elevated endogenous free MDA concentrations in deficient heart compared to controls seen at 21 weeks. At the same time point

a few brain regions were the only other tissues to provide evidence of increased endogenous lipid peroxidation during vitamin E deficiency. The approximately 4-fold greater ratio of vitamin E / peroxidisable PUFA of heart microsomes compared to brain as reported by Kornbrust and Mavis (1979) suggests that heart may be enriched in the highly peroxidisable fatty acids so that a reduction in vitamin E content would render this tissue particularly susceptible to peroxidative stress.

A recent study by LeBel et al (1989) provided an important clue as to why neural tissues are so susceptible to a chronic and severe deficiency of vitamin E whereas non-neural tissues remain largely unaffected. To study oxygen radical formation, they utilised the lipophilic probe 2',7'-dichlorofluorescein diacetate (DCFH-DA), which in the presence of oxygen radicals is converted to the highly fluorescent dichlorofluorescein form. When crude mouse cerebral fractions (mitochondria, synaptosomes and myelin fragments) were incubated in air at 37°C for 30min, it was found that 16 week vitamin E deficient fractions generated oxygen free radicals at a 2.5-fold higher rate than controls. Although, basal rates of radical formation were 6-fold higher in liver, there was no difference between deficient and control liver at the same time point. These observations were made despite the fact that vitamin E was almost undetectable in deficient liver, whereas deficient brain regions still retained approximately 60% of control levels. Although, only two tissues were examined, the demonstration of a low basal rate of oxygen radical generation in control brain and an increase in radical production following relatively small reductions in vitamin E levels, confirms the particular susceptibility of neural tissues to a deficiency of vitamin E.

In conclusion, the results of the studies presented in this chapter suggested that vitamin E was not the major determinant of differences in the susceptibilities of the various tissues from the same animal, i.e. in both control and vitamin E deficient rats. The lipid composition, particularly the concentration of highly peroxidisable PUFA, may have been more important. It is, therefore, necessary to determine the lipid composition of tissues in this particular animal. In addition, the results should be checked with alternative peroxidation and assessment methods to eliminate the possibility of the results being method specific.

Differences in vitamin E concentrations did appear to explain the differences in susceptibility of a particular tissue from control and deficient animals. Although the assay conditions were not optimised to expose differences between control and deficient tissues of the same type, the deficient tissues were clearly more susceptible. As suggested from in-vitro peroxidation profiles, a deficiency of vitamin E would be expected to result in greater lipid peroxidation in the brain than the spinal cord and peripheral nerve. These results do not correlate with the known neuropathology. However, a study of neural membranes, particularly the axonal membranes, as described in the next chapter, proved to be more informative.

CHAPTER 7

IN-VITRO PEROXIDATION STUDIES OF BRAINSTEM NEURAL FRACTIONS

7.1 Introduction

7.2 In-vitro peroxidation studies

7.3 Isolation of neural fractions

7.3.1 Does peroxidation occur during fractionation ?

7.4 Comparison of vitamin E deficient and control fractions

7.4.1 Brainstem whole homogenate

7.4.2 Myelin

7.4.3 Axolemma-Enriched fraction

7.4.4 Axoplasmic membranes and organelles

7.5 Effect of in-vitro peroxidation on vitamin E concentrations

7.6 Comparison of the susceptibilities of different neural fractions

7.7 Discussion

7.1 Introduction

A chronic and severe deficiency of vitamin E in man, monkey and rat results in a characteristic neurological syndrome where the main neuropathological feature is the selective loss of large calibre myelinated axons. An intriguing question regarding vitamin E deficiency is why neural tissues in general and more specifically, these large myelinated neurons should be particularly susceptible to a lack of this nutrient. The neuropathology associated with vitamin E deficiency is generally accepted as being a primary axonopathy with secondary demyelination. Nelson (1987) suggested that the axonal plasma membrane (axolemma) was the primary site of free radical attack in vitamin E deficiency, while more recently Southam et al (1991) postulated that the membranes of the axoplasmic mitochondria and smooth endoplasmic reticulum membranes may be the first to be affected.

It was, therefore, of relevance and interest to investigate the peroxidation characteristics of membrane fractions isolated from myelinated axons. Neural fractions were isolated from rat brainstem using the fractionation procedure described and validated in chapter 2. With the exception of myelin, very small amounts of material were obtained in the isolated fractions (< 0.5 mg protein) so that it was not possible to determine endogenous concentrations of lipid peroxidation products as for whole tissue homogenates. The lipid peroxidation studies on neural fractions were, therefore, restricted to investigation of in-vitro peroxidation.

The objective of the studies described in this chapter was to gain some insight into the relative susceptibilities of some components of the myelinated axon to in-vitro peroxidative stress and to examine the effect of vitamin E deficiency on the peroxidation profiles of these neural fractions.

7.2 In-Vitro Peroxidation Studies

As with the in-vitro peroxidation studies described for tissue whole homogenates (Chapter 6), the $CuSO_4/H_2O_2$ free radical generating system was used to produce lipid peroxidation in the isolated fractions. The peroxidation system was used in two different modes; (i) to study the effect of incubation time, where neural fractions were incubated at fixed concentrations of $CuSO_4$ and H_2O_2 at 37°C for increasing periods of time and (ii) to study the effect of free radical concentrations, where fractions were incubated for a fixed time and H_2O_2 concentration but with increasing concentrations of $CuSO_4$. In both cases lipid peroxidation was monitored by measuring the production of free MDA as described in chapter 3.

Peroxidation assays of type (i) were used to compare directly the susceptibilities of the same neural fractions from vitamin E deficient and control brainstem in order to investigate what effect, if any, a reduction in the concentration of this antioxidant had on peroxidation characteristics. The effect of in-vitro peroxidation of rat brain whole homogenate was also investigated using this system. The second incubation system was employed to compare the relative susceptibilities of the different fractions isolated from the same starting tissue sample in order to assess which fraction(s) were most susceptible to peroxidative stress both in control and in deficient animals.

7.3 Isolation of Neural Fractions

Myelin, an axolemma-enriched fraction (AEF) and a fraction containing the axoplasmic membranes and organelles (M/O) were isolated from rat brainstem as described in chapter 2. Three brainstems were used for each fractionation. Approximately five days was required to complete the procedure for a control and vitamin E deficient fractionation. Control brainstem was fractionated first and the isolated fractions stored at -70°C. As discussed in chapter 2, two AEFs were isolated in each fractionation. Because of the small amounts of

material in each it was necessary to combine the two bands and treat them as a single fraction. The purity of the different fractions was not assessed on each occasion since almost all of the AEF and M/O fractions were consumed in the in-vitro peroxidation studies. However, all fractionations were comparable to those described in the validation of the method both in terms of the banding patterns of the density gradients and the amounts of material isolated in each of the fractions.

7.3.1 Does Peroxidation Occur During Fractionation ?

Although all fractionation steps were carried out at 4°C the large number of different steps and the total experimental time involved (approximately 16 hrs) meant that the fractions were potentially susceptible to peroxidative damage during the fractionation procedure. The suitability of promethazine (0.5mg/ml) as an antioxidant was tested by incubating it with whole homogenates of 1 year vitamin E deficient and control rat brainstem in air at 37°C for up to 150 min. Promethazine was found to completely prevent peroxidation in both samples as assessed by measuring free and bound MDA (data not shown). Unfortunately when the antioxidant was included in the tissue homogenisation medium and other sucrose solutions the homogenate became excessively frothy and difficult to handle, particularly during the osmotic shock stage. In addition when the shocked myelinated axons were subsequently separated on density gradients there were very low yields of the more dense AEF and the M/O pellet. Also, being an antioxidant, promethazine would have to be removed from the isolated fractions since it would interfere with the subsequent in-vitro peroxidation experiments and also protein assays.

Because of the problems associated with promethazine and the use of antioxidants in general, the fractionation procedure was "mimicked" to assess whether peroxidation was taking place. This was done by homogenising freshly dissected 52 week control and vitamin E deficient brainstems in 10mM

TES, pH 7.4 as opposed to an isotonic buffer. This was done in order that the various cellular components and the different cellular membranes would separate from one another and so reflect to some extent the osmotic shock stage of the fractionation procedure. Brainstem whole homogenate was used for this study since it was not possible to use neural fractions due to the small amounts isolated by fractionation. The homogenate was then pelleted and resuspended in 40mM trizma, pH 7.4 and left standing on ice at 4°C. Peroxidation was assessed by measuring total MDA (free + bound). The results are presented in table 1.

Table 1. Variation of total MDA in brainstem homogenates with time.

Time (hours)	Total MDA (nmol/mg protein)	
	Control	Deficient
0	0.18	0.27
3.5	0.19	0.23
24	0.19	0.27
30	0.18	0.28
48	0.16	0.29

From the data above it can be seen that the concentrations of total MDA did not increase over at least a 48hr period. Although only whole homogenates were examined, the time period over which lipid peroxidation was monitored was 3 times that required to complete the fractionation. It was therefore assumed that little sample peroxidation would occur during isolation of the neural fractions.

7.4 Comparison of Vitamin E Deficient and Control Fractions

The susceptibility of neural fractions isolated from vitamin E deficient and control brainstem were compared at 20 and 60 weeks after starting the animals on the relevant diets. As with all the previous studies, data was obtained for the 60 week animals before the younger animals were examined. As stated above these experiments involved incubating samples at a fixed concentration of $CuSO_4$ and H_2O_2 at 37°C for increasing periods of time.

Method

Incubation mixtures contained sample, $CuSO_4$ and H_2O_2 (at varying concentrations, see below) in a ratio of 5:1:1. 50µl aliquots were removed in duplicate, unless stated otherwise, at fixed time points and assayed for free MDA. Control and deficient incubations contained approximately the same amount of material in terms of protein. The stated concentrations of each reagent in the various graphs refer to the final concentrations in the reaction mixtures. Although only single incubations of control and deficient fractions were compared in each of the peroxidation experiments, three brainstems were used in each fractionation so that the results can be considered to be the mean of these three starting tissues. All results were related to protein concentrations which were determined using the BCA method described in chapter 2.

Procedure

The procedure was essentially as that described for the in-vitro peroxidation of tissue whole homogenates as described in the previous chapter. The $CuSO_4$ and H_2O_2 reagents were both made up in 40mM trizma buffer, pH 7.4 shortly prior to use. The appropriately diluted fraction sample (in trizma buffer), was quickly vortex mixed with the two reagents and a 50µl aliquot immediately removed and transferred to an eppendorf tube containing an equal volume of ice-chilled acetonitrile. This point was taken as time zero. The remainder of

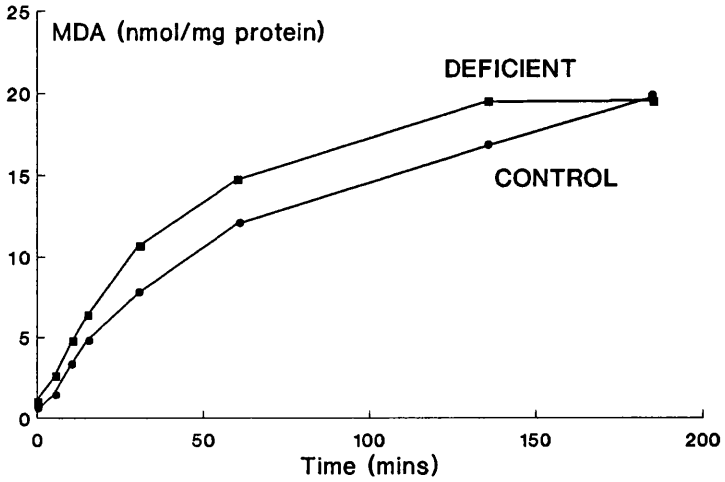
the peroxidation mixture was then placed in a shaking water bath (37°C) and aliquots removed into acetonitrile at fixed time points. The sample/acetonitrile mixture was vigorously vortex mixed for 15 sec and immediately spun for 5 min in an eppendorf minifuge at maximum speed. The resulting supernatant was aspirated off and kept on ice until analysis for free MDA by HPLC as described in chapter 3. As was the case in the studies of whole homogenates this sample treatment was sufficient to terminate further lipid peroxidation.

The peroxidation conditions were arranged so that control fractions exhibited a "lag phase" in their peroxidation profiles, i.e. a period of undetectable or very slow peroxidation prior to a later period of more rapid production of free MDA. Once conditions were standardised for the control fraction, its susceptibility was compared with that of the time matched deficient fraction. It is important to remember that the observation of a lag phase in a particular sample was dependent on the selection of appropriate peroxidation conditions, i.e. the use of too strong a system would not result in a lag phase.

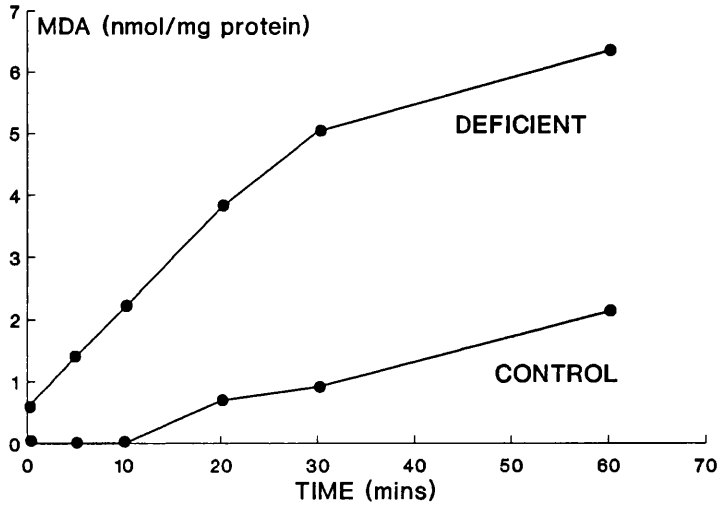
An illustration of the appropriate selection of peroxidation conditions is provided by the peroxidation of 50 week vitamin E deficient and control rat cortex whole homogenate (fig 1 a and b). When samples were incubated with 5mM H_2O_2 and 20μM $CuSO_4$ (Fig 1a) both homogenates began to peroxidise immediately and at similar rates. However, when the same samples were incubated with a milder system, i.e. 0.5mM H_2O_2 and 5μM $CuSO_4$, (Fig 1b) the control cortex exhibited a lag phase of 10 min where MDA was undetectable, whereas the corresponding deficient sample began to peroxidise immediately upon incubation. Once the control tissue began to peroxidise it did so at a lower rate compared to the deficient homogenate, i.e. approximately 2.6 compared to 7.2 nmol MDA/mg protein/hr respectively. In addition to demonstrating a lag phase in the control, use of milder peroxidation conditions also maximises the magnitude of the difference

FIG 1. IN-VITRO PEROXIDATION OF 50 WEEK CORTEX WHOLE HOMOGENATE

(a) 5mM H_2O_2 / 20μM $CuSO_4$



(b) 0.5mM H_2O_2 / 5μM $CuSO_4$



between control and deficient samples. A relatively small difference in susceptibilities was seen between the two with the more concentrated incubation, despite the fact that deficient cortex was approximately 95% depleted of vitamin E.

An example of the danger of relying on single assay time points in order to compare the susceptibilities of different samples is provided by fig 1a. Had samples been analysed at 185 min only, the assumption would have been that there was no significant difference between the two tissues despite the large differences in vitamin E levels.

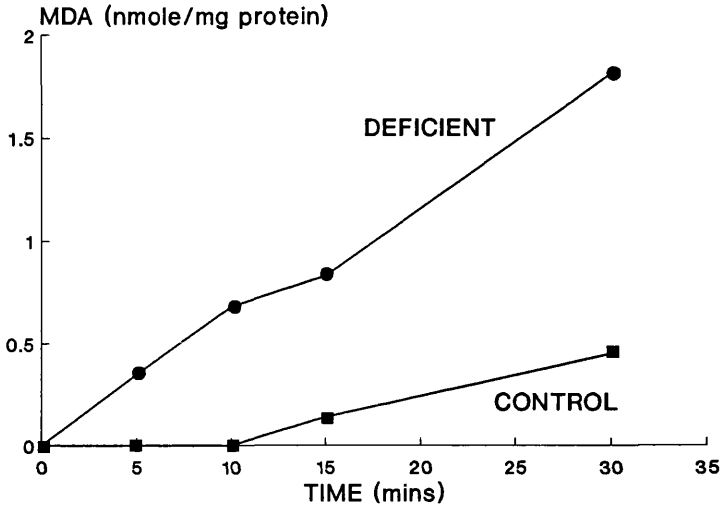
7.4.1 Brainstem Whole Homogenate

The in-vitro peroxidation of 60 and 20 week brainstem whole homogenates are plotted in figs 2a and b respectively. At both time points deficient samples were more susceptible than time matched controls. Using approximately 10μg protein, 0.5mM H_2O_2 and 5μM $CuSO_4$ the 60 week deficient brainstem began to peroxidise immediately, whereas there was a lag phase of at least 10 min in the control. In addition when the control sample did begin to peroxidise it did so a lower rate compared to the deficient, i.e. 1.5 compared to 4.1 nmol MDA/mg protein/hr.

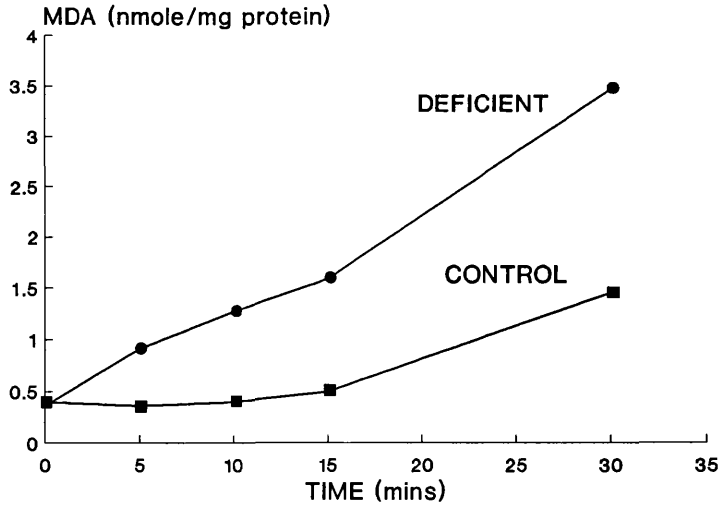
When 20 week homogenates were incubated under the same conditions both control and deficient samples began to peroxidise immediately. In order to demonstrate a lag phase in the control, the concentrations of both reagents had to be reduced by 40%, i.e. 0.3mM H_2O_2 and 3μM $CuSO_4$. Under these milder conditions the control exhibited a lag phase of approximately 10 min with the deficient homogenate still generating free MDA immediately after incubation. After 15 min the control peroxidised at a rate of 3.9nmol MDA /mg protein/hr while the deficient produced MDA at a rate of 5.9. Despite the stronger peroxidation conditions used at 60 weeks and the lower concentration

FIG 2. IN-VITRO PEROXIDATION OF BRAINSTEM WHOLE HOMOGENATE

(a) 60 WEEKS: 0.5mM H_2O_2 / 5μM $CuSO_4$



(b) 20 WEEKS: 0.3mM H_2O_2 / 3μM $CuSO_4$



of vitamin E in the deficient sample compared to the earlier time point, greater amounts of MDA were produced in the 20 week samples during the 30 min incubation.

7.4.2 Myelin

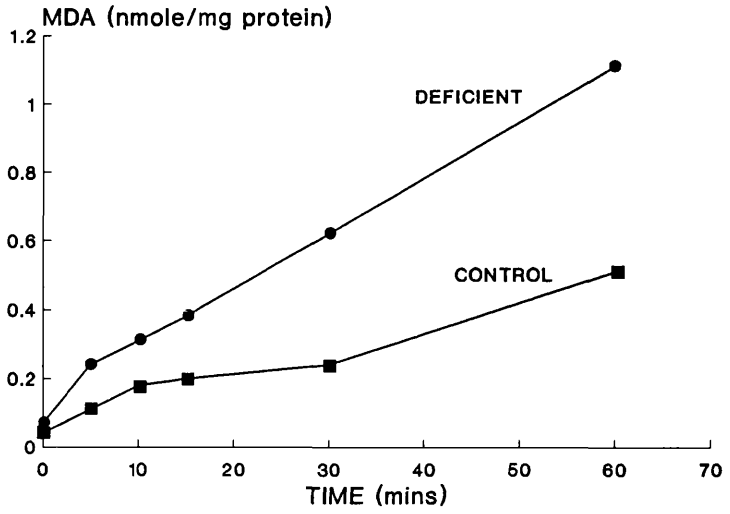
Results for the comparison of the in-vitro lipid peroxidation of control and deficient myelin at 60 and 20 weeks are presented in figs 3a and b respectively. In both cases approximately 20μg protein of each fraction was stressed with 0.5mM H_2O_2 and 15μM $CuSO_4$ for up to 60 min and assayed in duplicate. Under these conditions both control and deficient samples peroxidised immediately on exposure to free radicals at both 20 and 60 weeks. Isolated myelin was found to be relatively resistant to in-vitro peroxidative stress, and although a lag phase could be demonstrated for both control and deficient fractions using milder conditions, the peroxidation rate was very low resulting in very small peaks on the HPLC chromatograms. This made accurate quantitative assessment difficult. In order to obtain reliable results, peroxidation conditions were arranged to obtain measurable peaks on chromatography. As expected, however, vitamin E deficient myelin was clearly more susceptible with 3 and 2.2-fold more MDA/mg protein formed by deficient myelin compared to controls at 20 and 60 weeks respectively after 60 min incubation.

7.4.3 Axolemma-Enriched Fraction

Axolemma-enriched fractions (AEFs) at both 60 (fig 4a) and 20 weeks (fig 4b) were incubated with 0.2mM H_2O_2 and 2μM $CuSO_4$ at 37°C for up to 30 min. Approximately 5μg protein was assayed in each case. Due to the small amounts of material isolated in this fraction only one aliquot was assayed per time point. Under these assay conditions a lag phase was seen in both control peroxidation profiles. In both age groups deficient fractions were more susceptible with 20 week control AEF peroxidising at a rate of 7.8 nmol

FIG 3. IN-VITRO PEROXIDATION OF MYELIN
0.5mM H_2O_2 / 15μM $CuSO_4$

(a) 60 WEEKS



(b) 20 WEEKS

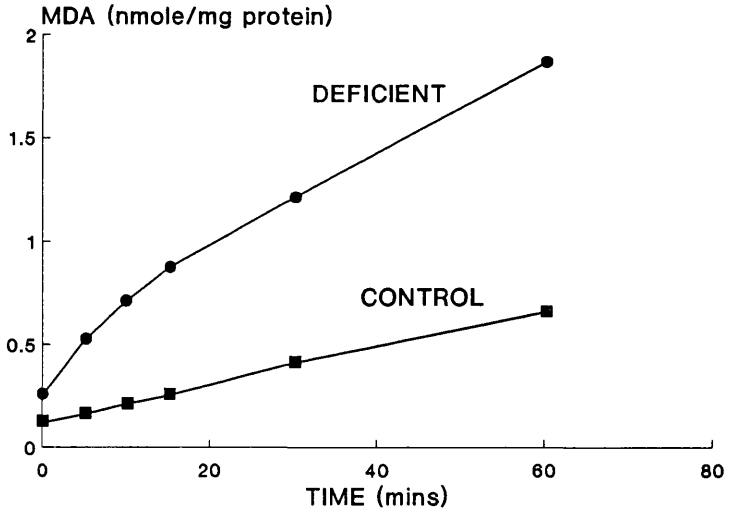
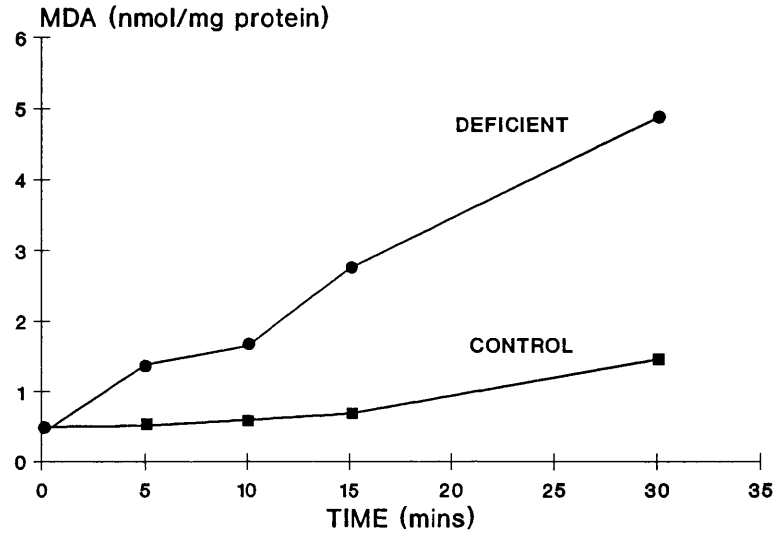
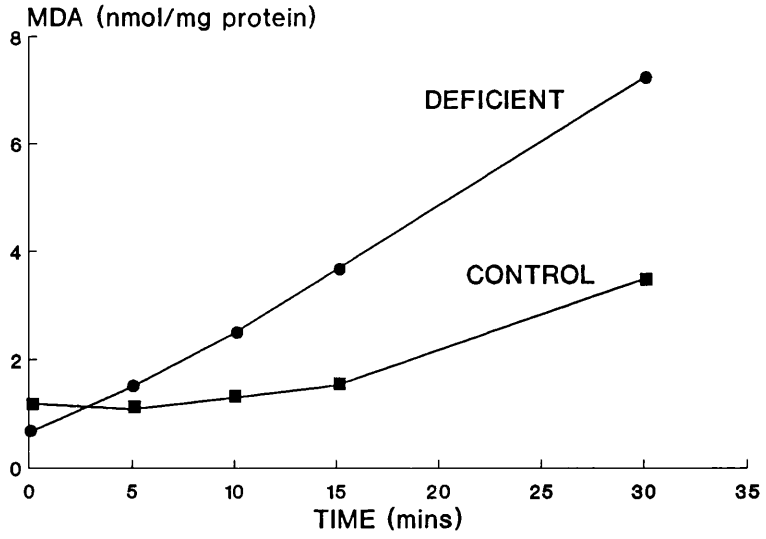


FIG 4. IN-VITRO PEROXIDATION OF AXOLEMMA-ENRICHED FRACTIONS, 0.2mM H_2O_2 / 2μM $CuSO_4$

(a) 60 WEEKS



(b) 20 WEEKS



MDA/mg protein/hr after the lag phase, whereas the deficient AEF peroxidised at a rate of approximately 13.7 nmol MDA/mg protein/hr. Sixty week control and deficient AEF peroxidised at rates of 3.1 and 11nmol MDA/mg protein respectively.

7.4.4. Axoplasmic Membranes and Organelles

As with the AEF assay, only single incubation mixture aliquots were removed for analysis at each time point. Incubation of 60 week fractions containing axoplasmic membranes and organelles (M/O) at 0.2mM H_2O_2 and 2μM $CuSO_4$ (approximately 5μg protein) resulted in a lag phase of 15 min in the control, followed by more rapid lipid peroxidation over the latter half of the incubation, i.e. 18.3nmol MDA/mg protein/hr (fig 5). The corresponding deficient M/O showed no lag phase and peroxidised at approximately a 3-fold faster rate compared to the maximal control rate. When 20 week fractions were exposed to the same conditions as described above both control and deficient M/O began to peroxidise on incubation (fig 6a) with a possible 5 min lag phase in the control. However, both exhibited similar maximum rates resulting in only a 14% difference in the amounts of MDA produced after 30 min peroxidation. Under these peroxidation conditions the maximum 20 week control rate of lipid peroxidation was greater than that at 60 weeks by a factor of 2.5.

Exposing the 20 week fractions to milder conditions, i.e. 15μM H_2O_2 and 0.15uM $CuSO_4$, produced a lag phase of 10 min in the control fraction followed by a rate of peroxidation of 5.7 nmol MDA/mg protein/hr (fig 6b). Although, clearly more susceptible, the deficient M/O fraction appeared to display an initial relatively slow rate of peroxidation over the first 10 min with a higher rate of MDA formation over the final 20 min, i.e. 9.4 and 12.9nmol MDA/mg protein/hr respectively. Both deficient M/O peroxidation rates were greater than either of the control rates.

FIG 5. IN-VITRO PEROXIDATION OF 60 WEEK AXOPLASMIC MEMBRANES AND ORGANELLES

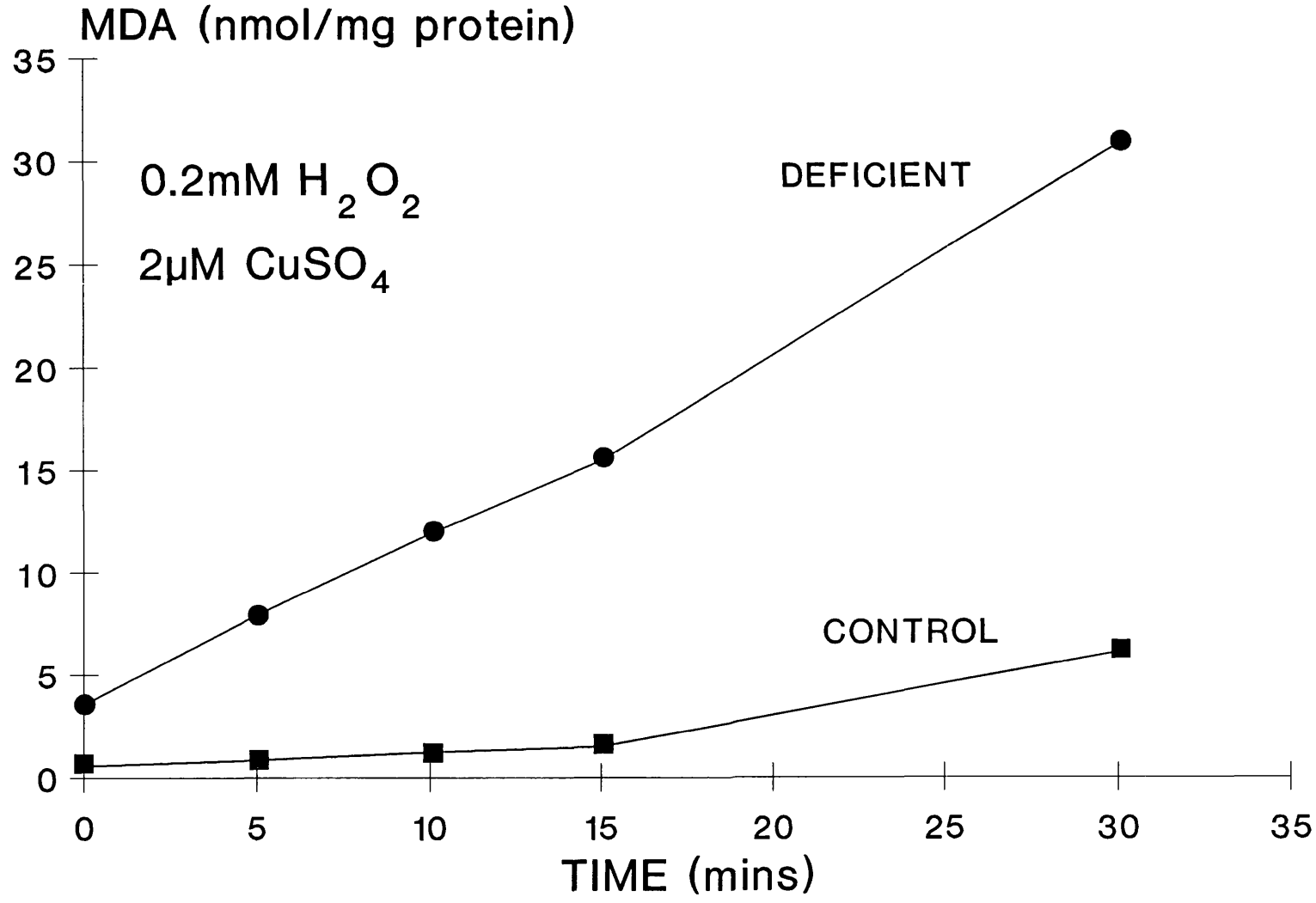
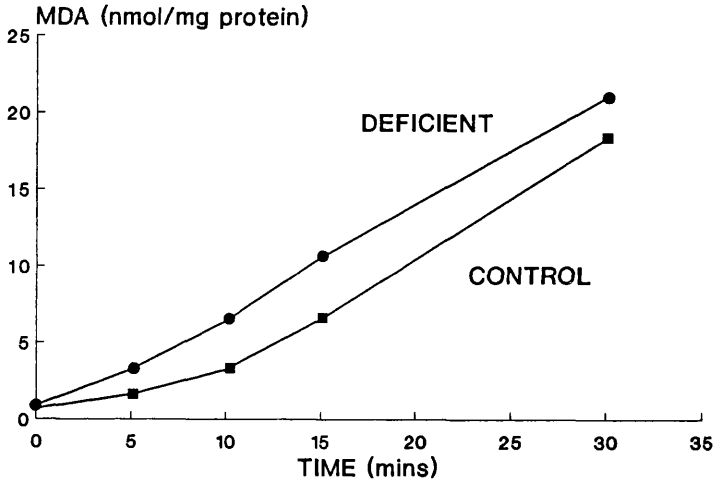
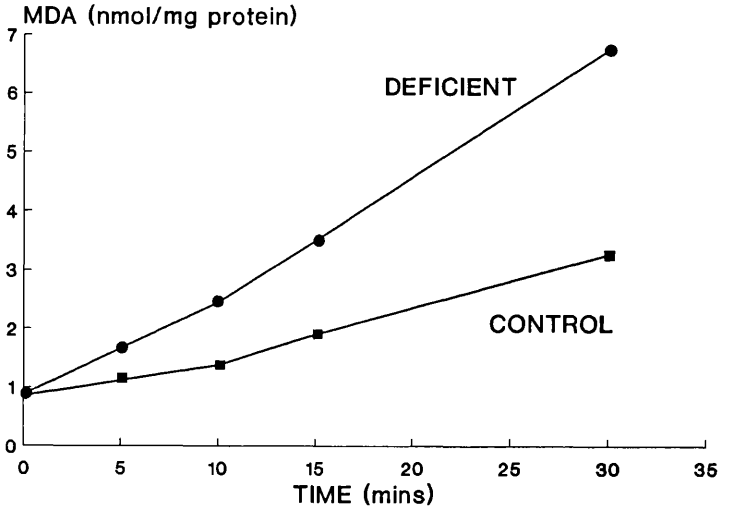


FIG 6. IN-VITRO PEROXIDATION OF 20 WEEK AXOPLASMIC MEMBRANES AND ORGANELLES

(a) 0.2mM H_2O_2 / 2μM $CuSO_4$



(b) 15μM H_2O_2 / 0.15μM $CuSO_4$



7.5 Effect of In-Vitro Peroxidation on Vitamin E Concentrations

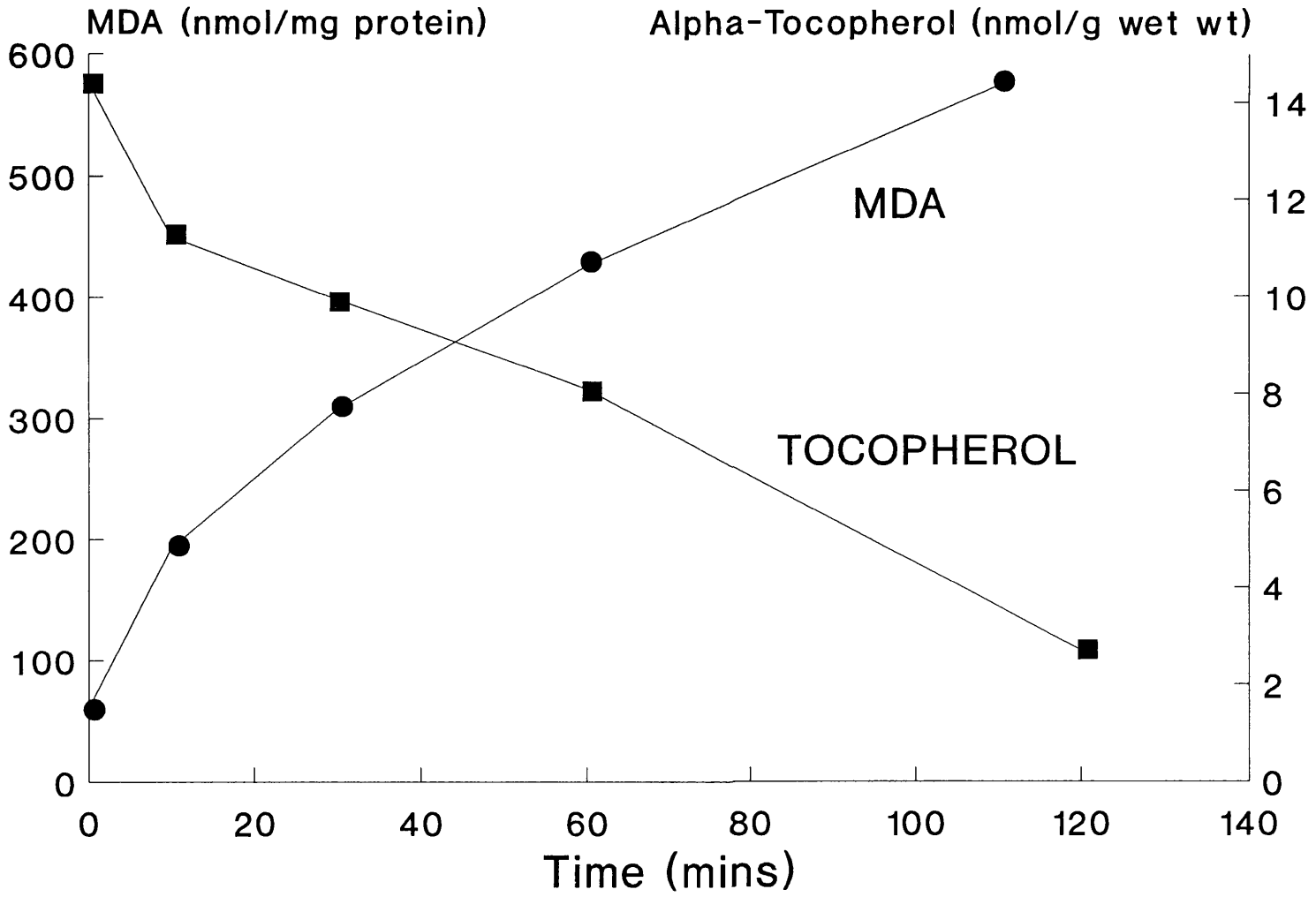
In order to study the effect of in-vitro peroxidation on vitamin E concentrations, control brain whole homogenate was peroxidised with a fixed concentration of H_2O_2 and $CuSO_4$ for increasing periods of time. At fixed intervals, aliquots of the reaction mixture were removed for estimation of both MDA and α-tocopherol concentrations.

Procedure

The peroxidation mixture contained sample [5% (wet wt/vol) in 40mM trizma, pH 7.4], H_2O_2 and $CuSO_4$ in a ratio of 100 : 12.5 : 12.5. The concentrations of $CuSO_4$ and H_2O_2 in the final incubation mixture were 4 and 0.4mM respectively. The reaction was started with the addition of $CuSO_4$ and the mixture was incubated at 37°C in a shaking water bath. To measure the MDA produced a 50μl aliquot was diluted 1/16 with a 50:50 mixture of ice-chilled acetonitrile/dH_2O, vortex mixed for 15 sec and centrifuged. The supernatant was kept on ice until analysis by HPLC. For the estimation of α-tocopherol, 100μl of the incubation mixture was added to a tube containing 400μl of chilled ethanol and immediately vortex mixed for 1 min. Tocopherol was then extracted by the addition of 500μl hexane, vortex mixing for 1 min and spinning for 5 min. The supernatant was aspirated off and kept on ice until analysis by HPLC as described in chapter 4.

The results of following the oxidative stress over 120 min are presented in fig 7. Concentrations of α-tocopherol began to decrease and MDA increase within the first 10 min of incubation. After 60 min approximately 50% of the initial concentration of tocopherol remained in the peroxidised sample with approximately 20% remaining after 120 min. The rate of peroxidation did not increase as vitamin E concentrations dropped but rather decreased with time, exhibiting the highest rate of MDA formation over the first 15 min.

FIG 7. LOSS OF ALPHA-TOCOPHEROL AND PRODUCTION OF MDA DURING IN-VITRO PEROXIDATION OF RAT BRAIN WHOLE HOMOGENATE



7.6 Comparison of the Susceptibilities of Different Neural Fractions

As a consequence of having to vary the peroxidation conditions and use variable amounts of each fraction the results reported above provided some indication as to the relative susceptibilities of the different neural fractions to oxidative stress. However, for a direct comparison it was necessary to assay the different fractions in a single assay. Fractions were therefore isolated from the same starting tissue and incubated for a fixed time period with increasing concentrations of $CuSO_4$ in the peroxidation system. Membrane fractions were exposed to relatively strong peroxidation conditions compared to those used in the time course studies; the latter chosen to demonstrate the presence or absence of a lag phase and to maximise the magnitude of the difference in the susceptibilities of control and deficient fractions.

Method

Using the same isolated neural fractions as employed in the time course studies, approximately 10μg of each fraction was incubated for 30 min at 37°C with 2mM H_2O_2 and either 0, 50, 500 or 1000μM $CuSO_4$. All reagents were made up in 40mM trizma buffer, pH 7.4. Lipid peroxidation was monitored by measuring the free MDA produced. Both myelin and whole homogenate were assayed in duplicate, whereas only a single assay per $CuSO_4$ concentration was used for the other two fractions.

Procedure

Each incubation mixture contained 50μl sample and 10μl of H_2O_2 and $CuSO_4$. Because of the large number of peroxidation mixtures per assay, and in order to minimise the amount of sample peroxidation prior to incubation, the following procedure was followed: $CuSO_4$ was added to, but not mixed with, appropriately diluted neural fractions in eppendorf tubes sitting on ice. H_2O_2 was then quickly added, the tubes vortex mixed and incubated at 37°C which was taken as time zero. The tubes were additionally mixed after 10 and 20

min. After 30 min the peroxidation mixtures were removed from the water bath and immediately vortex mixed with 70ul of ice-chilled acetonitrile. The tubes were then spun at maximum speed for 5 min in an eppendorf minifuge and the resulting supernatant aspirated off and kept on ice until analysis.

Results for the peroxidation of 60 week control and deficient fractions are presented in figs 8 and 9 respectively. For both sets of fractions the following order of susceptibility was seen; M/O $>>$ AEF $>>$ whole homogenate $>$ myelin. For 50μM $CuSO_4$, the amounts of MDA generated were; 30.6, 4.1, 0.7 and 1.2 nmol/mg protein in M/O, AEF, homogenate and myelin respectively. The corresponding deficient fractions generated 31.1, 6.5, 2.5 and 1.9 nmol MDA/mg protein respectively. Since very similar peroxidation profiles were seen for the sets of fractions from both control and deficient animals at 60 weeks despite $<$ 5% of control vitamin E levels being present in the latter, these assays were not repeated with 20 week neural fractions. The control M/O fraction produced a maximum concentration of MDA of 52nmol/mg protein at 500μM $CuSO_4$ but was reduced to 44nmol/mg protein at the higher 1000μM concentration. This characteristic, i.e. under the particular peroxidation conditions chosen was consistently observed in control fractionations. Deficient M/O yielded approximately 30nmol MDA/mg protein at 5μM $CuSO_4$. The maximum amount generated by deficient M/O, however, was approximately 40nmol MDA at 1000μM $CuSO_4$.

FIG 8. IN-VITRO PEROXIDATION OF 60 WEEK NEURAL FRACTIONS FROM CONTROL BRAINSTEM

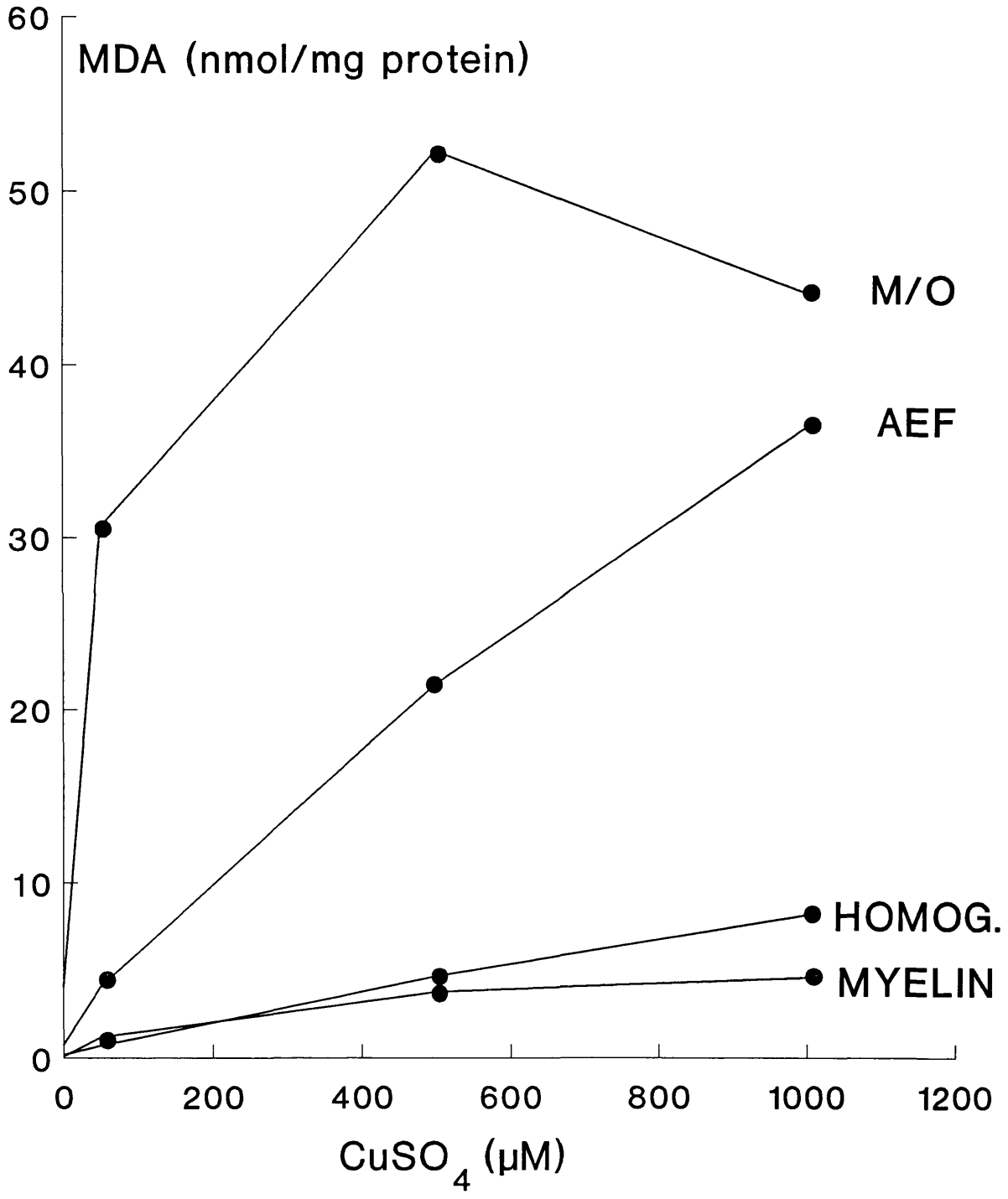
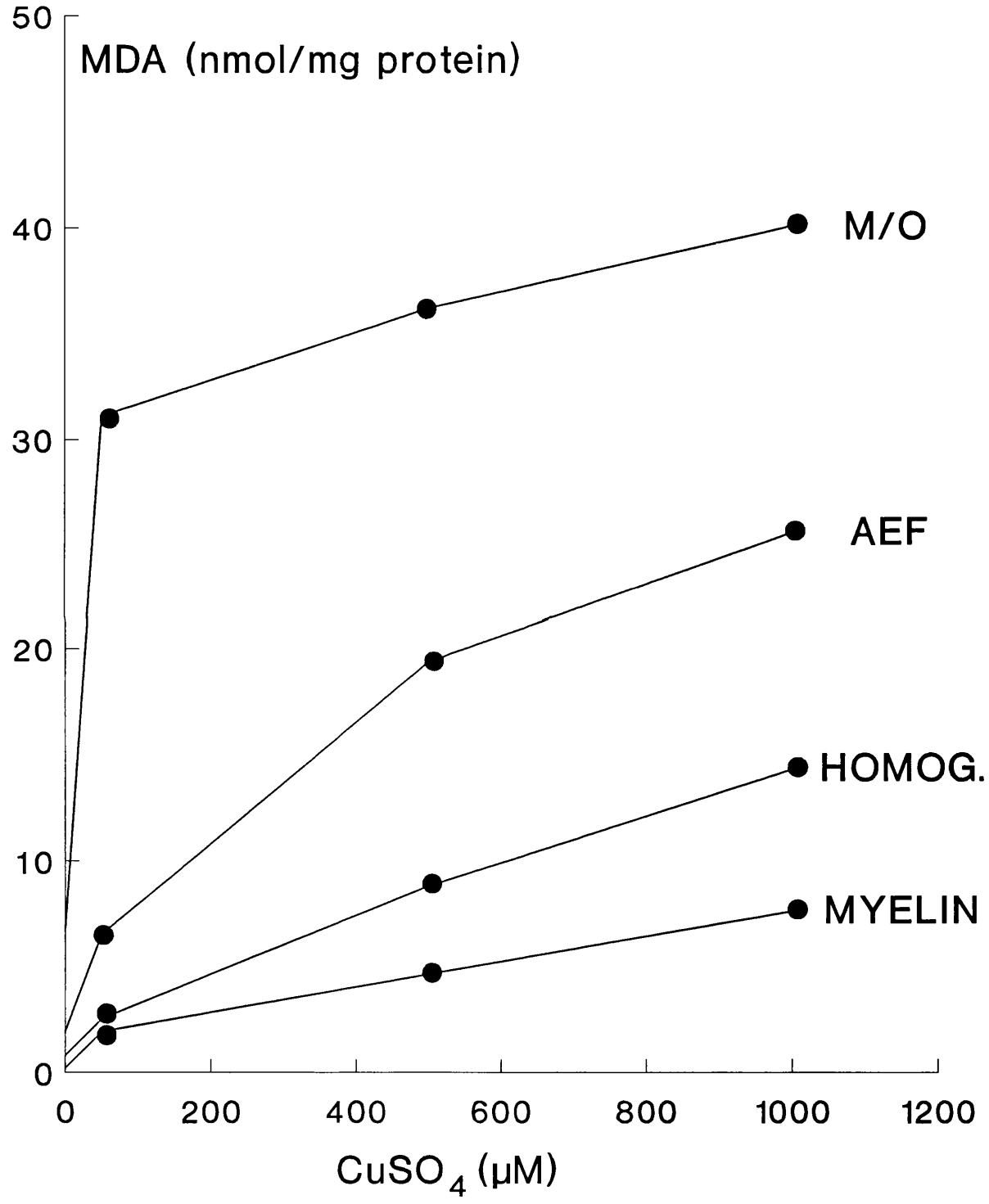


FIG 9. IN-VITRO PEROXIDATION OF 60 WEEK NEURAL FRACTIONS FROM DEFICIENT BRAINSTEM



7.7 Discussion

As with the in-vitro peroxidation studies on tissue whole homogenates, the vitamin E deficient fractions at both 20 and 60 weeks were more susceptible to in-vitro peroxidative stress than time matched controls. The endogenous concentrations of vitamin E were not determined in different fractions due to the small amounts of material available. However, concentrations of α-tocopherol in deficient cortex homogenate in the present study were 13.7 and 3.3 % of control values at 21 and 56 weeks respectively and it can therefore be assumed that levels in the brainstem fractions were reduced to a similar degree.

In order to assess the effect of vitamin E deficiency on the susceptibility of the isolated fractions to in-vitro peroxidative stress, control and deficient samples were incubated with fixed concentrations of H_2O_2 and $CuSO_4$ for increasing periods of time. Whether this meant that the incubated fractions were exposed to a fixed free radical flux during the entire incubation period was not known since it was possible that the free radical generating system could become exhausted after extended periods of time. The strength of the peroxidation conditions used for a particular fraction were arranged so that a lag phase was seen in the control. The peroxidation conditions were not fixed to exclude a lag phase from the deficient peroxidation profile.

With the exception of myelin, control fractions exhibited a lag phase of approximately 10 min and the deficient fractions began to peroxidise immediately. A lag or induction phase has been reported in other in-vitro studies and can be defined as the time between peroxidative challenge and the onset of rapid lipid peroxidation. This later rapid period of peroxidation represents the propagation phase of lipid peroxidation. The most important factor in determining the length of the lag phase appears to be the type and concentrations of antioxidants contained in the system or samples being

peroxidised. A number of studies have shown that peroxidation does not generally take place until the antioxidants have been depleted. For example, Esterbauer et al (1989) who studied the in-vitro peroxidation of human low density lipoprotein (LDL) by continuous monitoring of conjugated dienes, demonstrated that the LDL only began to peroxidise at a slow rate after endogenous vitamin E had been completely consumed and propagation only proceeded following the subsequent loss of ß-carotene and retinyl stearate. The lag phase of control liver microsomes was shown to be extended by the addition of exogenous glutathione (Hill and Burk 1984) or by ascorbate (Wefers and Sies 1988) both of which were explained in terms of regeneration of tocopherol from the tocopheroxyl radical.

The lack of a lag phase in the vitamin E deficient fractions agrees with other studies which demonstrated that the presence or duration of the lag phase was dependent on the content of vitamin E. This is particularly true of isolated membrane fractions where the aqueous antioxidants, e.g. ascorbate and urate, are lost during fractionation so that in the absence of added exogenous antioxidants the main, if not only, antioxidant capacity is provided by vitamin E. Cadenas et al (1984) compared the susceptibility of 14 week vitamin E deficient and control liver microsomal fractions to iron-ADP stress and found that although the deficient fraction exhibited a lag phase, it was shorter than that of the control by a factor of 5.7 which correlated with the 5.4-fold higher concentrations of α-tocopherol in the latter. Dean and Cheeseman (1987) demonstrated that injecting rats in-vivo with α-tocopherol increased the vitamin E content of isolated mitochondrial membranes by a factor of 13 compared to untreated controls and resulted in approximately a 2-fold greater lag phase when stressed with the H_2O_2 / $CuSO_4$ / dithioerythritol free radical generating system. Therefore, in the case of the isolated neural fractions examined in this study the main determinant in peroxidation profiles, at least as regards lag phases, appears to have been the

endogenous concentrations of vitamin E. In the case of the whole homogenate additional factors such as aqueous antioxidants and antioxidant enzymes could in theory at least, have influenced the peroxidation profiles. However, since the two groups of rats received the same diet with the exception of the α-tocopherol content, the only difference between control and deficient was in the levels of vitamin E. It is therefore likely that the observed differences in lipid peroxidation were due to the differences in vitamin E status.

In addition to the absence of a lag phase, vitamin E deficient fractions peroxidised more rapidly than the controls. This finding was in agreement with the results of Cadenas et al (1984) who found that vitamin E deficient rat liver microsomes peroxidised at a higher rate than control microsomes on incubation with iron-ADP and assessed by low-level chemiluminescence. This was in contrast to the findings of Wefers and Sies (1988) who, using the same membrane fractions as that of Cadenas et al, found that control microsomes produced MDA at a 2.2-fold greater rate than the deficient fraction on incubation with iron-ADP and the glucose-6-phosphate dehydrogenase NADPH-regenerating system and monitored as chemiluminescence. Alternatively, Hill and Burk (1984) did not see any significant difference in the peroxidation rates of control and deficient rat liver microsomes on incubation with iron-ADP/ascorbate determined by estimating TBARS. This was despite undetectable α-tocopherol concentrations in the deficient fraction. The variation in the results of different groups may have resulted from the use of different stress systems and/or methods of measuring the lipid peroxidation.

In the case of my in-vitro studies, if the differences in susceptibilities of control and deficient fractions were due solely to differences in their vitamin E concentrations, it would be expected that once the control antioxidant capacity had been consumed during the lag phase, the two fractions would display similar peroxidation rates during the subsequent

propagation phase. One possible explanation for the more rapid peroxidation of the deficient fractions was that this rate represented the sum of the rate of peroxidation of PUFA and the rate of stimulated peroxidation, i.e. decomposition of preformed lipid peroxides. Higher endogenous concentrations of lipid peroxides may have been present in the deficient membranes so that breakdown of these peroxides would enhance the peroxidation rate in these fractions compared to controls.

Control AEF exhibited a lag phase at both 20 and 60 weeks under identical peroxidation conditions, whereas in order to demonstrate a lag phase in 20 week control whole homogenate and M/O fractions it was necessary to use milder peroxidation conditions than those to which the corresponding 60 week fractions had been exposed. Despite the milder free radical stress for the 20 week brainstem sample, approximately 2.5-fold more MDA was formed than from the 60 week homogenate over 60 min. Moreover, 60 week M/O yielded only 2.3-fold more MDA compared to the 20 week fraction despite the markedly stronger peroxidation conditions. Control myelin produced comparable amounts of MDA at the two time points as was the case for AEF. The change in susceptibilities of whole homogenate and M/O agree with other reports of age related decreases in susceptibility to in-vitro peroxidative stress as mentioned in the previous chapter.

These findings contrast with those from the in-vitro peroxidation of tissue whole homogenates, including brainstem, where there was no evidence of age related changes in the susceptibilities between 21 and 56 weeks (chapter 6). The reason for this apparent differences in experimental results was most likely due to the use of relatively strong peroxidation conditions in the whole tissue homogenate studies which would tend to obscure subtle changes in peroxidisability. The reason for the greater susceptibility of the 20 week fractions was unknown but could possibly be related to lower vitamin E levels

or higher concentrations of PUFA. Since only single fractionations were examined, these results should be confirmed with a larger number of samples, and 20 and 60 week fractions should be compared directly in the same assay.

As previously mentioned, a number of reports have demonstrated that the lag phase does not end until essentially all the antioxidant capacity of the sample has been depleted. However, as seen in fig 7, when control rat brain whole homogenate was peroxidised in-vitro α-tocopherol levels decreased at a relatively slow rate, with approximately 20% remaining after 120 min. MDA concentrations steadily increased over this time period despite the presence of vitamin E. McCarthy et al (1989), who studied the in-vitro peroxidation of rat liver microsomes, found that although control fractions exhibited a lag phase of 1 min, approximately 60% of the initial concentration of α-tocopherol was still detectable in the peroxidised sample after 5 min, with 20% remaining at 30 min.

Although the results of McCarthy et al appear to confirm my findings, they disagree with other studies, the majority of which have involved isolated fractions. In the present study, loss of vitamin E during oxidative stress was investigated in a tissue whole homogenate, which is a more complex system. The fact that lipid peroxidation was shown to occur despite the presence of vitamin E does not necessarily contradict vitamin E's generally accepted function as an antioxidant. In whole homogenates vitamin E is known to have a non-homogenous distribution, and although capable of preventing oxidative damage when present in sufficiently high concentrations, it may in theory not be capable of doing so in specific membrane domains where it is present at low levels. In contrast to isolated membrane fractions, a tissue whole homogenate will also contain additional antioxidants which must be taken into consideration. The effect of in-vitro peroxidation on the concentration of vitamin E in the neural fractions was not examined. It is interesting, however,

that both my findings and those of McCarthy et al were obtained using a highly selective method to monitor the peroxidation process, i.e. HPLC of MDA. The other studies employed alternative methods.

A comparison of the susceptibilities of neural fractions isolated from the same brainstem tissue revealed that for both 60 week control and deficient fractions the susceptibility decreased in the following order; M/O >> AEF >> homogenate > myelin. The order of susceptibility agrees in general with the susceptibilities of the different fractions seen in the time course studies, although these cannot be compared directly to each other, since in general different amounts of material were assayed under variable conditions.

The order of susceptibilities of the isolated fractions appears to correlate with published data on the fatty acid composition and vitamin E contents of subcellular fractions and organelles. DeVries, whose fractionation method was adopted, analysed the lipid and fatty acid composition of the same fractions isolated from human post mortem brain white matter (DeVries et al 1981). They found that the unsaturation index, (number of double bonds per 100 fatty acid molecules), in phosphatidyl ethanolamine and choline was significantly higher in the AEF than myelin.

Electron microscopic analysis of the M/O fraction (chapter 2), revealed it to be highly enriched in mitochondria so that this organelle would be expected to have a substantial influence on the overall lipid composition of this fraction. Tyurin et al (1989) who determined the lipid composition of various membranes of rat brain demonstrated that the unsaturation index decreased in the following order; mitochondria = microsomes > synaptosomes > synaptic plasma membrane > myelin. They also found that whereas docosahexaenoic acid (22:6, w-3) accounted for approximately 15% of the total fatty acid in mitochondria, microsomes and synaptosomes, it contributed

only 4% in myelin. Intermediate levels were found in the synaptic plasma membrane fraction.

The above observations suggest that the M/O fraction would be relatively enriched in PUFA compared to the myelin fraction. Hence, the decrease in the order of susceptibility of the neural fractions mirrors the decrease in PUFA concentrations or unsaturation indices. From this it can be postulated that during vitamin E deficiency in-vivo these fractions would also exhibit comparable differences in peroxidisability. Although, vitamin E levels were not measured in these fractions, other studies have found that vitamin E is concentrated in those membranes that are enriched in PUFA. Tyurin et al (1989) determined the vitamin E concentrations in the aforementioned rat brain fractions and found that levels increased in the following order myelin < synaptic plasma membrane < synaptosomes = mitochondria < microsomes. Similar findings were also obtained by Vatassery et al (1984), who examined the distribution of vitamin E in membrane fractions from rat cortex. Therefore the probable order of increase in vitamin E concentrations in the brainstem fractions of this study correlates with the increase in PUFA and susceptibility to peroxidative stress. Therefore, a deficiency of vitamin E might be expected to render the mitochondria (M/O) particularly susceptible to peroxidative stress. This is illustrated by a comparison of the amounts of MDA produced from the peroxidation of deficient 60 week M/O and AEF fractions under the same conditions, i.e. 27.3 and 4.5 nmol MDA/mg protein respectively at 50μM $CuSO_4$.

The neuropathology associated with chronic vitamin E deficiency is generally accepted as being a primary axonopathy with secondary demyelination. As demonstrated by Nelson (1987), the prevention of this neuropathology by supplementing the diet of rats maintained on a vitamin E deficient diet with synthetic antioxidants strongly suggests that the loss of the antioxidant

function of tocopherol was responsible for the pathological changes. Nelson also postulated that the primary site of free radical attack was the neuronal plasma membrane, i.e. the axolemma, which then underwent degenerative changes which caused a secondary demyelination. The much greater susceptibility of the AEF compared to that of the relatively resistant myelin observed in these studies provides some evidence for this hypothesis.

Muller and Goss-sampson (1990), following the demonstration of a reduction in fast axonal transport in vitamin E deficient rats, postulated that the previously reported accumulation of normal and abnormal cytoplasmic organelles in the axon terminals of dystrophic axons was a consequence of a defect in axonal transport. In a recent collaborative study (Southam et al 1991), they postulated that the axoplasmic mitochondria and smooth endoplasmic reticulum (SER) would be the most susceptible to a deficiency of vitamin E. This could result in a defect in axonal transport since the SER is thought to comprise one component of the fast axonal transport system while the mitochondria provide the energy for this process. The order of susceptibilities of the brainstem neural fractions would be consistent with primary damage to the mitochondria (microsomes not examined), followed by damage to the axolemma. The relatively high resistance to in-vitro peroxidative stress of both control and vitamin E deficient myelin despite its lipid rich nature, suggests that the demyelination associated with chronic vitamin E deficiency is the result of degeneration of the axolemma as opposed to disintegration of the myelin sheath itself as a result of lipid peroxidation.

When neural tissues were stressed with an in-vitro free radical system (chapter 6) the following order of susceptibility was seen; brain $>>$ cord $>$ nerve. As suggested in the previous chapter, the myelin content of these three tissues was possibly the major determining factor in their relative susceptibilities. The high resistance of myelin to oxidative stress in this study

agrees with this postulate since the myelin content of these tissues increases in the following order; brain < cord < nerve. However, extensive loss of myelinated axons is known to occur in both the spinal cord and peripheral nerve during chronic vitamin E deficiency. Myelin accounts for a much greater percentage of the total membrane of the myelinated axon compared to either the axolemma or axoplasmic membranes. As a result, when neural whole homogenates are peroxidised in-vitro, the total yield of MDA from M/O and AEF will be very low in comparison to myelin and the whole homogenate. Therefore, when studying the peroxidation of neural tissues in-vitro it is probably more relevant to investigate neural fractions as opposed to whole homogenates.

In conclusion, the results agree with the pathological findings associated with chronic vitamin E deficiency and should be extended to examine subcellular organelles and also intracellular membrane systems in more detail. Additional studies should include the measurement of fatty acid profiles, vitamin E concentrations and endogenous lipid peroxidation in these fractions. Finally, it is important that the various results are confirmed by using alternative in-vitro peroxidation systems and methods to monitor the peroxidation process.

CHAPTER 8.

FRIEDREICH'S ATAXIA - AN ANTIOXIDANT DEFECT ?

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8.6 Discussion

8.1 Introduction

In a series of papers between 1863 and 1877 Nicholaus Friedreich (1863,1863a,1863b,1876,1877) described a distinct neurological syndrome in 9 members of 3 sibships with an age of onset around puberty. Ataxia (loss of muscular coordination) and dysarthria (imperfect articulation of speech) were prominent while sensory loss and muscle weakness were late findings. Today Friedreich's ataxia (FA) is known to be an autosomal recessively inherited neurodegenerative disorder. It is the most common of the inherited ataxias in Europe with an incidence of approximately 1 in 50,000 and a heterozygote frequency of 1 in 110 in the United Kingdom population (Winter et al 1981). Recently Chamberlain et al (1988) narrowed down the location of the gene for FA to chromosome 9. The clinical, neurophysiological and electrophysiological features are very similar to those seen in severe and chronic vitamin E deficiency.

8.1.1 Clinical Features of FA

Clinically, FA displays a diverse range of features. A cooperative study in Canada in the mid 1970s (Geoffroy et al 1976) led to the following features being considered obligatory in the diagnosis of the disease; (i) onset before the end of puberty and never after 20, (ii) ataxia of gait, (iii) progression of ataxia to all extremities within two years preceding examination, (iv) reduction in position and/or vibratory sense in lower limbs, (v) muscle weakness and (vi) deep tendon areflexia in lower limbs. Harding (1981) considered these conditions to be too stringent and could not always be applied in early childhood cases. Following a study of 115 patients with FA from London and the surrounding areas Harding (1981) considered the presence of the following criteria at presentation to be essential for diagnosis; (i) autosomal recessive inheritance, (ii) a progressive ataxia of limbs and gait developing before the age of 25 (usually between 8 and 16) and (iii) the absence of tendon reflexes in the lower limbs. Harding (1988) subsequently

included electrophysiological evidence of an axonal sensory neuropathy in this list of diagnostic conditions. Important secondary clinical features were dysarthria, areflexia, extensor plantar responses, pyramidal weakness and distal loss of proprioception (position sense) and vibration sense. These secondary features are not found in all cases at presentation but are eventually universal.

There is an associated cardiomyopathy thought to be present in greater than two thirds of cases and is responsible for 50% of mortalities. Clinical diabetes occurs in 10 to 20% of patients with FA with an additional proportion exhibiting chemical diabetes following oral glucose loading which produces a hyperinsulinic response (Shapcott et al 1976). Diabetes is also a major cause of death in these patients.

8.1.2 Neuropathology of FA

The neuropathology of FA is characterised by a degeneration of the posterior roots and columns, Clarke's column and the corticospinal (pyramidal) and spinocerebellar tracts (Oppenheimer 1979). There is an extensive degeneration of the large neuron cell bodies in the dorsal root ganglia and of both the centrally and peripherally directed large calibre myelinated axons associated with these cell bodies (e.g. Mc Leod 1971, Ouvrier 1982, Said 1986 and Santoro 1990). Oppenheimer (1979) who examined brain pathology in FA found that lesions were rather more variable than those seen in the spinal cord. For example, the optic nerve could be intact or severely degenerated. Other structures, notably the cuneate nuclei in the brainstem were severely affected in all cases. The findings for primary sensory pathways in the brain confirmed that the lesions are milder in the more rostral parts, the gracile component is always affected to a greater extent than the cuneate and the cuneate lesions are in general worse than the trigeminal. In FA the pyramidal tracts are consistently severely affected in the cord, are sometimes affected in

the medulla but seldom or never at the level of the cerebral peduncles.

Electrophysiological studies show that peripherally FA is characterised by a slight to moderate reduction of sensory conduction velocity along both distal and proximal nerve segments associated with a markedly reduced or absent amplitude of the sensory evoked potentials (e.g. Hughes 1968, Santoro 1982). This abnormality of amplitude in FA is considered to be the most reliable electrophysiological evidence of an underlying axonal loss (Behse 1978). Caruso and colleagues (1983) in a study of sural nerve biopsies from patients with FA, demonstrated that the average amplitude of the sensory response was more than 90% reduced compared to controls. This corresponded with a 29 to 50% reduction in the total number of myelinated fibres and up to a 90% loss of large calibre fibres. Despite this the conduction velocities were 77 to 100% of normal. Abnormalities of peripheral motor conduction have also been described in FA with prolongation of distal latencies being the most consistent feature (Caruso 1983). However, these defects occur to a lesser degree than those of the sensory system.

Centrally, electrophysiological studies exhibit a severe lowering or absence of somatosensory evoked potentials and an increase in central conduction and broadened waveform (Jones et al 1980, Caruso 1987). Recently Vanasse et al (1988) found that in FA patients where a central response could be detected following median nerve stimulation (wrist), the conduction velocity was normal or near normal up to the brainstem but was reduced between the brainstem and cortex.

Although FA is generally accepted as resulting from a distal or "dying-back" neuropathy, with a primary axonopathy resulting in secondary demyelination (Hughes et al 1968, Dyck 1973, Said et al 1986), Santoro and colleagues (1990) have argued against this hypothesis. They demonstrated in a long term

follow up study of 15 patients with FA, (6 to 36 years old at presentation), that there was no deterioration of both peripheral electrophysiological abnormalities and of sural nerve histology between the first and second examinations (mean interval being 4.8 years). The findings were not related to either clinical deterioration during this period nor to the duration of the symptoms, i.e. findings were almost identical whether cases had a 2- or 20-year disease history. Santoro's group agreed with the postulate of Said et al (1986) that the histological abnormalities previously thought to be indicative of degeneration/regeneration processes were actually due to a defective development of specific neurons in FA. In support of this is the fact that although there is a low incidence of degenerating fibres which fitted in well with the slow progression of the disease, it could not explain the extensive loss of large calibre myelinated axons in young children with FA (Ouvrier 1982). The consistency of the electrophysiological and histological findings with time despite a worsening of clinical symptoms, were considered not to be compatible with a dying back process but suggested the progressive involvement of other nervous system structures such as the corticospinal and spinocerebellar pathways.

8.1.3 Biochemical Abnormalities in FA

In 1976 Barbeau described Friedreich's ataxia as a "fascinating puzzle" and despite a renewal of interest in this disease from the mid 1970s, extensive studies have failed to resolve the underlying metabolic defect. By 1982 forty-four separate studies had revealed significant differences between FA and controls. At present four main hypotheses have emerged as possible candidates in the aetiology of FA (Barbeau 1982) none of which are mutually exclusive. These are (i) the "pyruvate hypothesis" where the primary event in FA is proposed to be a defect in the pyruvate dehydrogenase complex or in one of its components or cofactors, (ii) the "lipid membrane hypothesis" where an abnormality of incorporation of linoleic acid into phospholipids

results in a consequent deficiency of this fatty acid in membrane components, particularly the mitochondrial membrane, (iii) the "energy defect hypothesis" based on an enzymatic defect in energy production within the mitochondria and finally (iv) the " taurine hypothesis" where the primary event in FA is related to a defect in the retention of the amino acid taurine.

None of the above abnormalities, however, or indeed the other reported defects have been demonstrated in all FA patients. Moreover, several findings could not be reproduced by independent groups. For example, Blass et al (1974) demonstrated some patients exhibited a reduced activity of the pyruvate dehydrogenase complex and it was subsequently suggested by Kark and Rodriguez-Budelli (1979) that pyruvate intolerance was a consequence of reduced activity of the lipoamide dehydrogenase component of the complex. However, this could not be confirmed by other investigators (Purkiss et al 1981) and Kark and coworkers later retracted their findings (1981). Stumpf et al (1982) demonstrated a 90% reduction in levels of mitochondrial malic enzyme in skin fibroblasts of FA patients and an 80% reduction in heterozygotes (1983). The activities of this enzyme are normally high in the nervous system and in the myocardium so that a deficiency of this enzyme could result in a defect in carbohydrate metabolism in these tissues. Unfortunately, similar studies in patients from the UK (Chamberlain and Lewis 1983, Gray and Kumar 1985) and Canada (Barbeau et al 1984) did not reveal any difference from controls.

Regarding the large number of reported biochemical abnormalities and the conflicting results for some of the investigations in FA, Harding (1988) argued that several of these studies are suspect because of the inappropriate selection of patients and controls. For example, the controls employed were rarely chronically disabled and Hewer and Robinson (1968) noted that disability itself can lead to biochemical abnormalities including glucose

intolerance. Regardless of these potential faults, none of these abnormalities can fully explain the neuropathology seen in FA.

Harding (1981), on the basis of the variation of the clinical symptoms, suggested that FA was genetically heterogenous even when strict diagnostic criteria were employed. However, the results of Chamberlain et al (1988) strongly argue in favour of a single gene locus. Samples for their genetic studies were taken from patients from several geographically isolated populations that were selected using the Geoffroy set of diagnostic conditions.

8.1.4 Does FA Result from an Antioxidant Defect?

The clinical, neurophysiological and neuropathological features of FA are very similar to those seen in chronic vitamin E deficiency suggesting a possible common aetiology in these two conditions. There are, however, important differences between the two. Clinically, proprioceptive loss tends to be less pronounced in FA compared to vitamin E deficiency. Pathologically, (despite the hypothesis of Santoro et al (1990)), although there is thought to be a "dying-back" neuropathy of sensory axons in both conditions, centrally directed axons appear to be preferentially affected in vitamin E deficiency (Nelson 1981), whereas both peripheral and central pathways appear to be equally affected in FA. In addition, neurophysiological studies in vitamin E deficient patients by Harding et al (1985) showed that peripheral sensory nerve conduction might be retained in the presence of delayed somatosensory evoked potentials, i.e. central conduction, whereas Ouvrier et al (1982) found that even in young children with FA there was always a marked loss of the large calibre myelinated axons from the peripheral nerves associated with reduced or absent sensory action potentials. Finally, degeneration of spinocerebellar and corticospinal (pyramidal) tracts occurs to a greater extent in FA (Lamarche et al 1984) than in vitamin E deficiency (Rosenblum et al 1981).

Three studies to date have measured serum vitamin E concentrations in patients with FA. Filla et al (1979) found slightly reduced levels (non-significant) in 15 patients with FA. More recently Eusebi and colleagues (1990) found a small but significant reduction in 29 patients compared to controls, i.e. 18.64 $\pm$ 6.13μM versus 24.4 $\pm$ 6.2μM respectively ($p<0.01$). Muller et al (1987), however, did not see a significant difference between 31 FA cases and both able bodied and disabled controls. Assuming the results of the first two studies to be true, the slight reductions in the concentrations of vitamin E are probably not physiologically significant and would not be sufficient to explain the neuropathology associated with FA. These results, however, do not rule out the possibility of a deficiency of vitamin E at some other level. For example, since tissue vitamin E levels had not previously been determined in patients with FA there is the possibility of a defect in uptake of the vitamin into various tissues. Alternatively, there may be a metabolic abnormality at the cellular level, e.g. increased turnover of vitamin E or the presence of some factor that interferes with its antioxidant function. Another possibility is that there may be a deficiency of some other antioxidant system with functions similar to those of vitamin E.

8.2 Investigation of a Possible Antioxidant Defect in FA

To investigate the possibility of an antioxidant abnormality, particularly of vitamin E, in the aetiology of FA the following studies were carried out in post mortem tissues from FA patients and controls;

(i) measurement of tissue concentrations of vitamin E

(ii) estimation of endogenous levels of lipid peroxidation products, i.e. thiobarbituric acid reactive substances (TBARS) and free, bound and total malondialdehyde (MDA).

(iii) study of the susceptibility of brainstem and associated neural fractions to in-vitro peroxidative stress.

Details of the tissues used in the following studies are listed in table 1. Post mortem tissues from patients with FA were obtained from Dr. Susan Chamberlain, Department of Biochemistry, St. Mary's Hospital Medical School, London. The control samples were supplied by Dr. F.R. Wells from the Parkinson's Disease Brain Bank, Institute of Neurology, London. The tissues from the four patients with FA were the only cases that became available during the course of the study. All four patients died of cardiac complications. All control tissues came from individuals with no history of neurological disease. Although various neural and non-neural tissue samples were available for each of the FA cases, only brainstem, cortex and cerebellum could be obtained from the Parkinson's Disease Brain Bank. Therefore the various investigations centred on these three tissues.

8.3 Vitamin E Concentrations in FA Tissues and Myelin

Concentrations of vitamin E (α-tocopherol) were determined in FA and control brainstem, cortex, cerebellum and myelin (isolated from brainstem, see section 8.5). In addition, vitamin E concentrations were measured in peripheral nerve (sural) samples from three FA patients and compared with published results, since no corresponding control nerve samples were available.

Concentrations were measured using HPLC/fluorimetry as described in chapter 4. In the case of the peripheral nerve samples, the epineurial sheath (outer layer) was removed prior to homogenisation since this structure contains a large amount of collagen and is very resistant to homogenisation. The value quoted for nerve is therefore that present in the remaining components of the sample, i.e. mainly fascicles (axon bundles). To measure

Table 1. Friedreich's ataxia and control post mortem tissues

(a) Friedreich's ataxia tissues

NUMBER	SEX	AGE (yrs)	STORAGE TIME (months)
FA 1	M	24	3
FA 2	F	26	38
FA 3	M	40	36
FA 4	M	53	12

(b) Control tissues

CONTROL 1	M	16	25
CONTROL 2	M	48	16
CONTROL 3	M	46	18
CONTROL 4	M	61	27

vitamin E in myelin fractions, a 250μl aliquot was vortex mixed for 1min with 750μl of 100% ethanol giving a final ethanol concentration of 75% as used to homogenise whole tissues for vitamin E analysis. The diluted myelin sample was then extracted with hexane as previously described. Results were related to wet weight and/or total lipid as determined by the method of Snyder and Stephens (1959) as described in chapter 4.

There was no significant difference between the α-tocopherol concentrations in FA and control brainstem, cortex and cerebellum when related to either wet weight (fig 1a) or total lipid (fig 1b). Myelin (per lipid) levels were also the same in the two groups. The concentrations of vitamin E (mean and range) in the three FA peripheral nerve samples were 0.32 (0.22 - 0.42) mmol/mol lipid.

8.4 Endogenous Lipid Peroxidation in FA.

In order to assess the possibility of an increased level of lipid peroxidation occurring in-vivo in FA, various parameters of lipid peroxidation were determined in FA tissues and myelin and compared to control values. TBARS and free, bound and total MDA concentrations were measured in brainstem, cortex and cerebellum, while TBARS and bound MDA were also measured in the isolated myelin fractions (free MDA is lost during fractionation). All results are reported as nmol/mg protein.

8.4.1 TBARS

Endogenous TBARS were estimated using a modification of the microfluorimetric method of Yagi (1976) as described and validated in chapter 2. There were no significant differences between FA and controls for the three brain regions and myelin (fig 2).

FIG 1a. ALPHA-TOCOPHEROL CONCENTRATIONS IN FA AND CONTROL BRAIN REGIONS (PER WET WEIGHT)

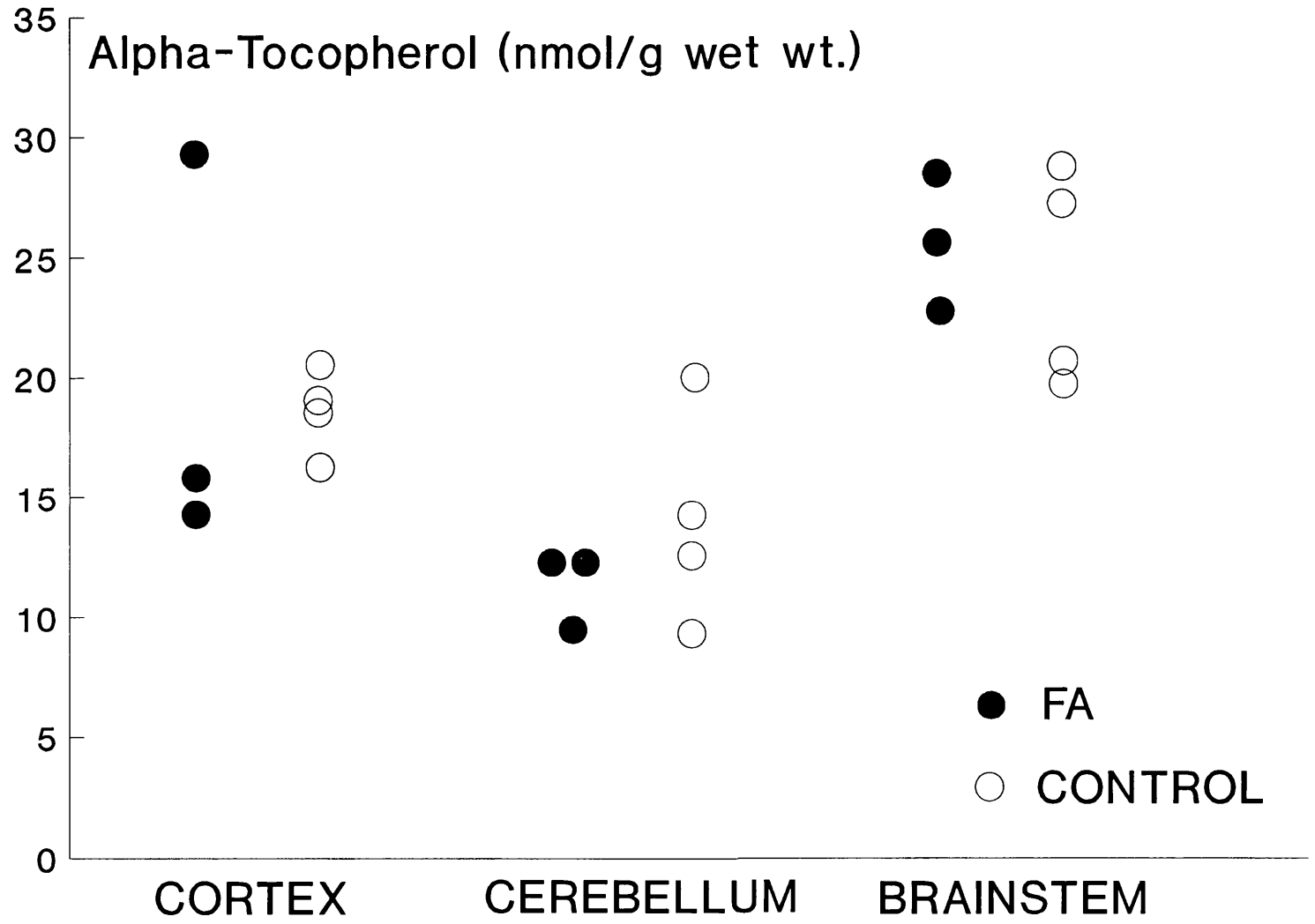


FIG 1b. VITAMIN E CONCENTRATIONS IN FA AND CONTROL BRAIN REGIONS AND MYELIN (PER TOTAL LIPID)

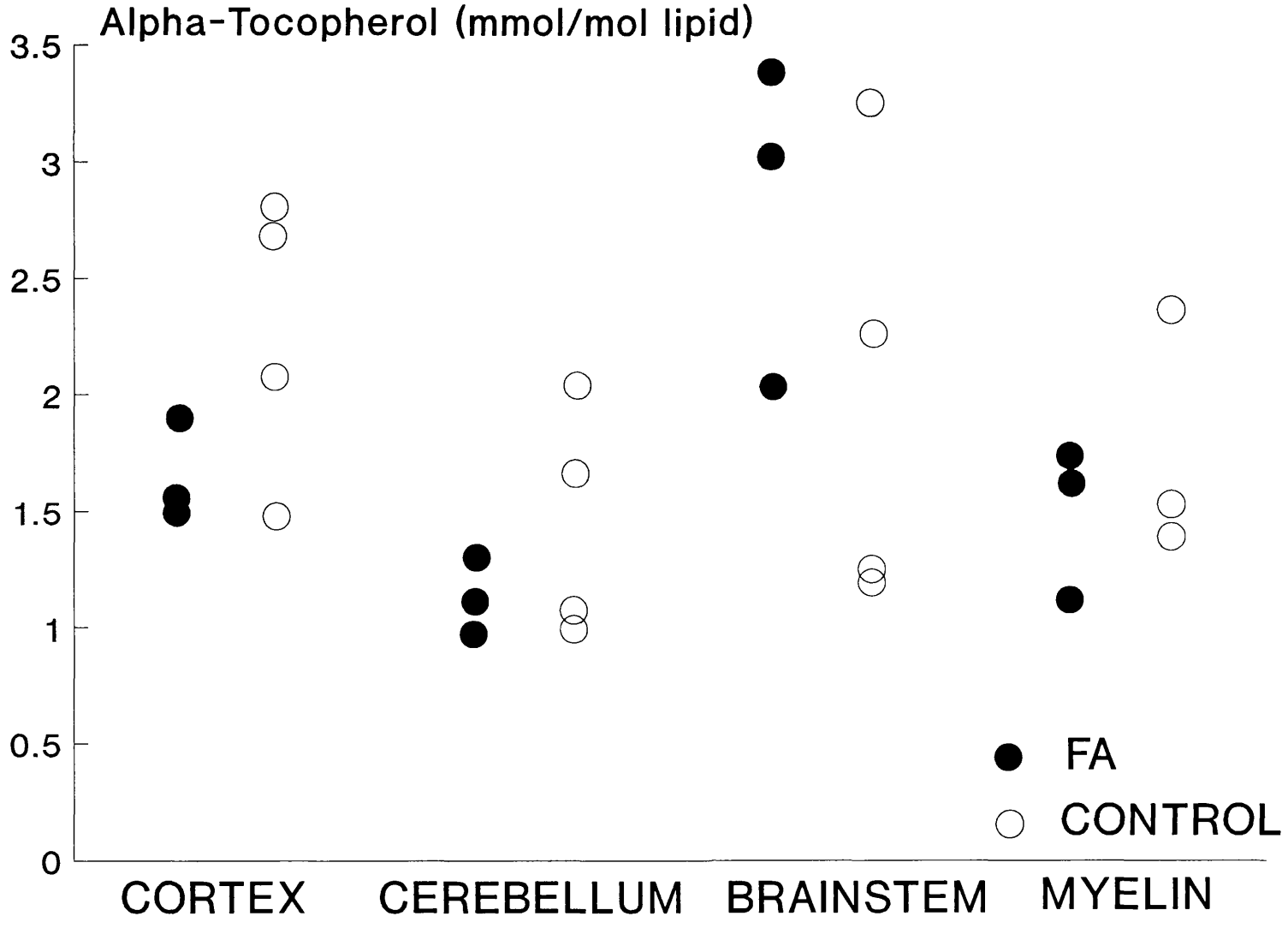
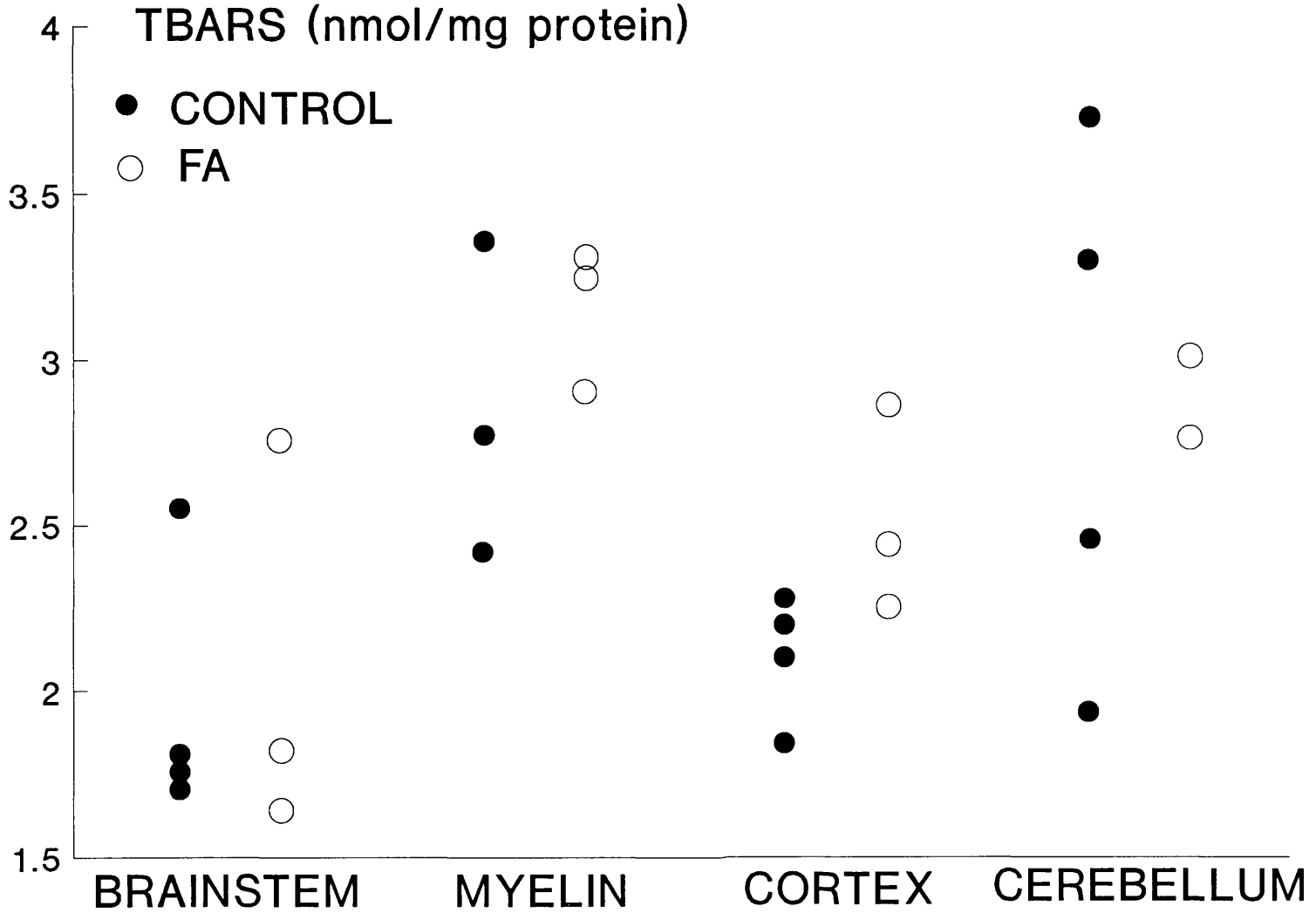


FIG 2. ENDOGENOUS TBARS IN FA AND CONTROL BRAIN REGIONS AND MYELIN



8.4.2 MDA

The following sections will present data on the endogenous concentrations of free, bound and total MDA.

8.4.2.1 Free MDA

Endogenous free MDA was measured using a modification of the direct HPLC method of Esterbauer et al (1984) as detailed in chapter 3. As can be seen from the results presented in fig 3 there were no significant increases in concentrations in the tissues from FA patients compared with controls.

8.4.2.2 Total MDA

Total MDA (free + bound) was estimated using a modification of the alkaline hydrolysis method of Lee and Csallany (1987) as presented in chapter 3. Again there were no significant differences between FA and controls (Fig 4). When total MDA values were compared with the corresponding TBARS values for individual samples, the TBA test in all instances gave a higher mean value than the selective HPLC method. The increases ranged from 7-345% (see fig 5 for controls).

8.4.2.3 Bound MDA

Concentrations of bound MDA were calculated by subtracting the value for the free form from the total concentration. With the possible exception of the myelin fraction bound MDA concentrations were similar in FA compared to controls (fig. 6). Although it appears that levels were significantly elevated in the myelin fractions, the FA sample with the highest concentration was the only sample of all the tissues and fractions examined that had a lower TBARS concentration than total MDA. This therefore suggests that the total MDA result for this myelin sample may have been incorrect.

FIG 3. ENDOGENOUS FREE MDA IN FA AND CONTROL BRAIN REGIONS

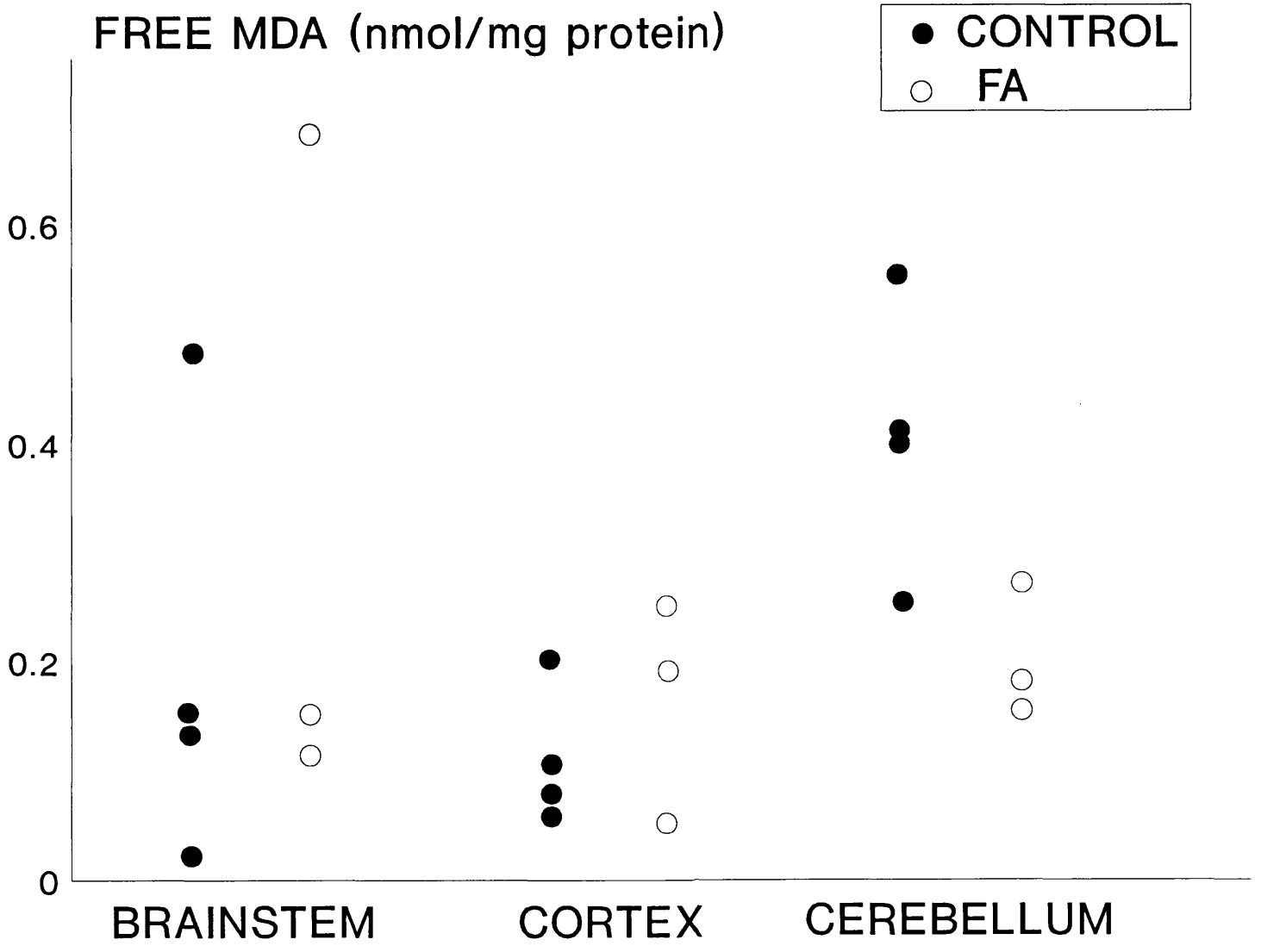


FIG 4. ENDOGENOUS TOTAL MDA IN FA AND CONTROL BRAIN REGIONS

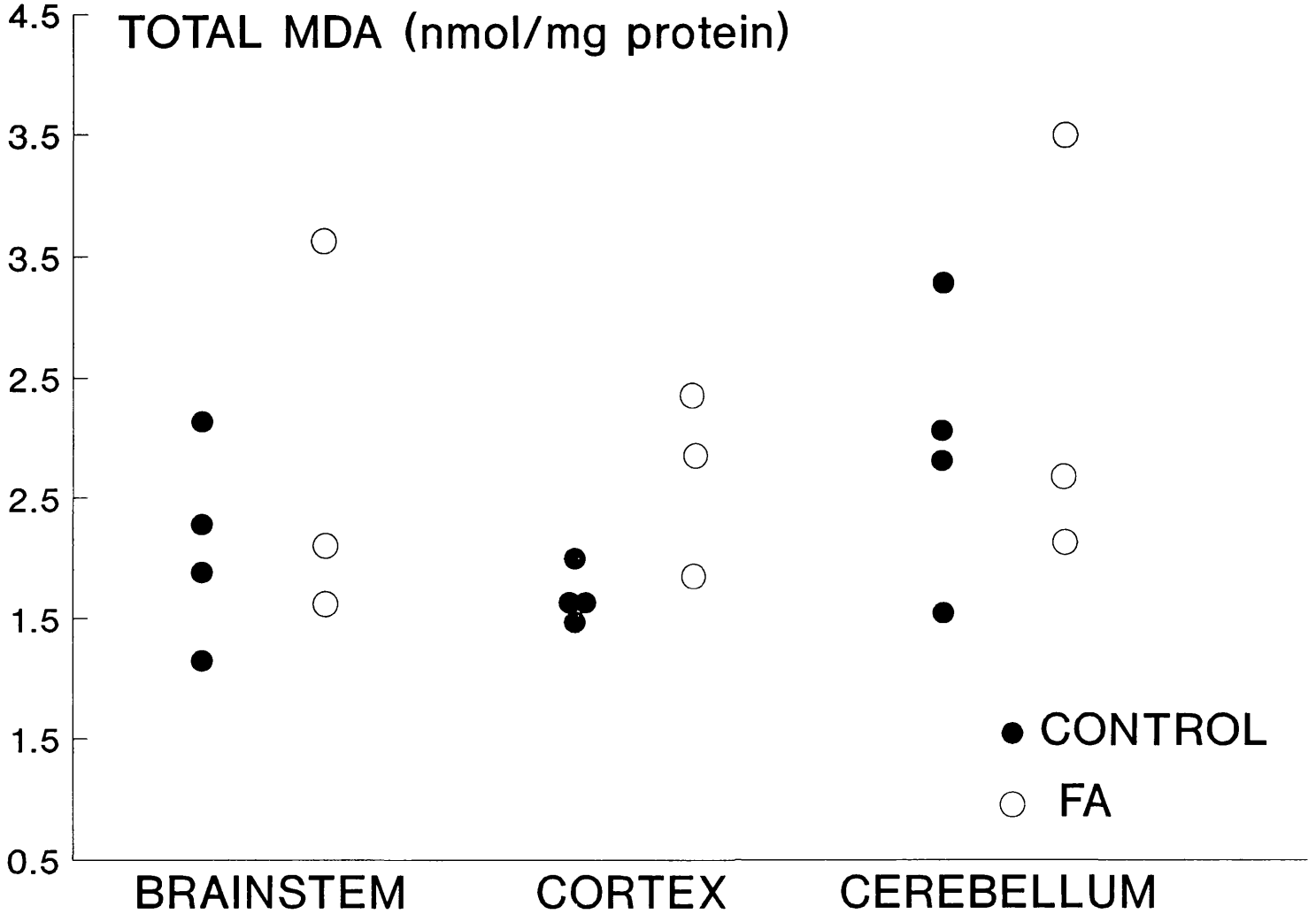


FIG 5. COMPARISON OF ENDOGENOUS TBARS AND TOTAL MDA IN CONTROL BRAIN REGIONS AND MYELIN

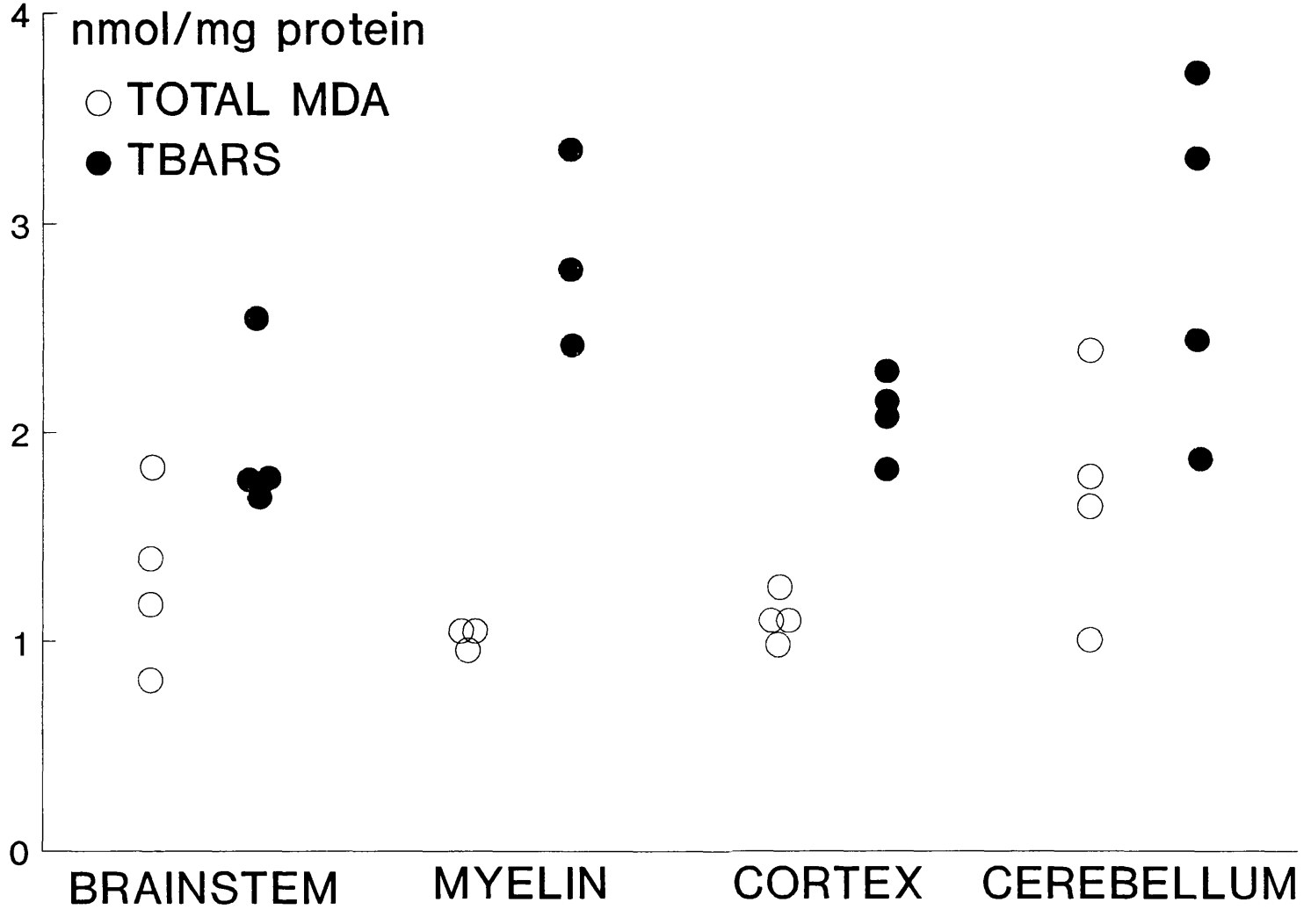
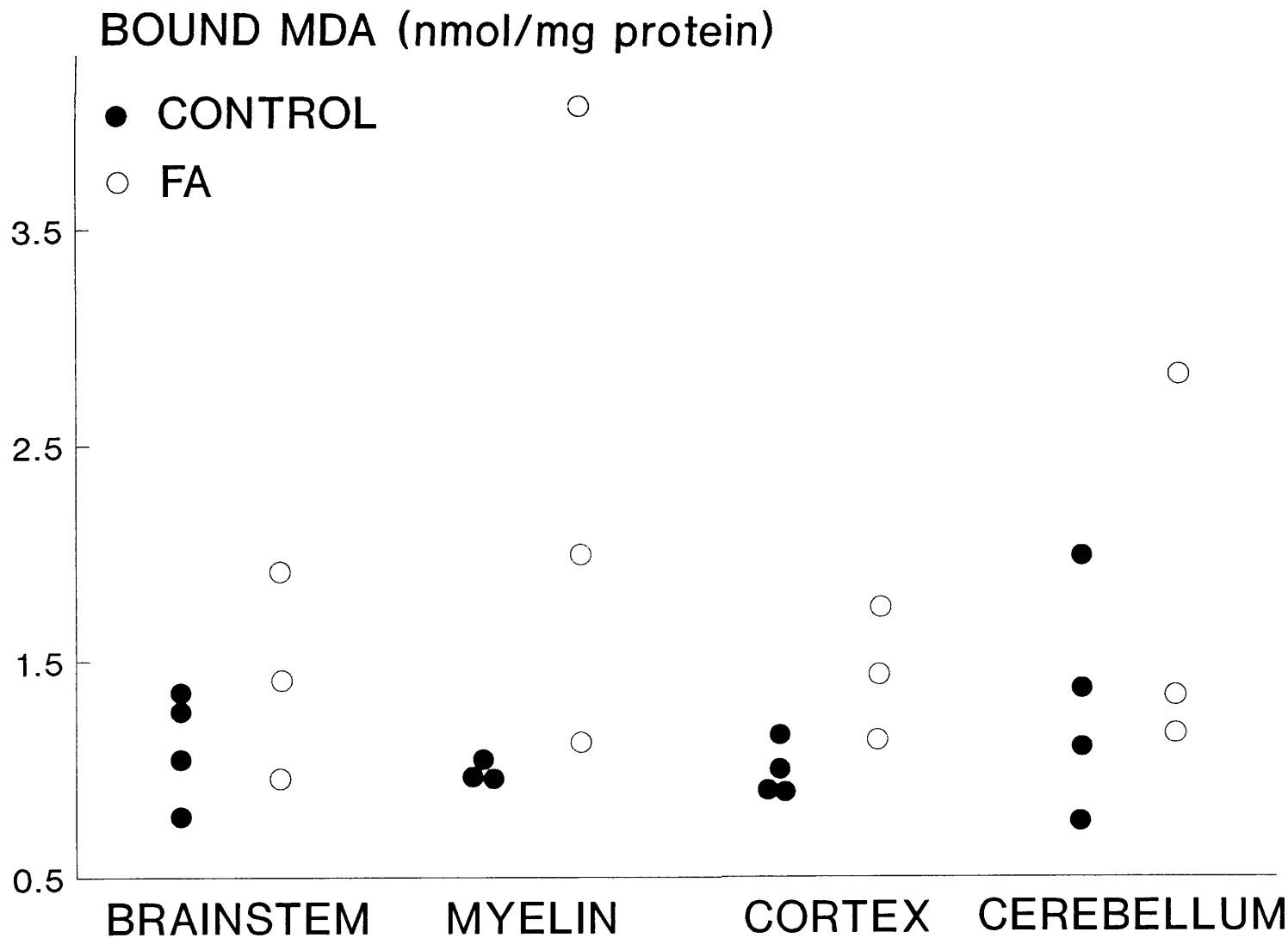


FIG 6. ENDOGENOUS BOUND MDA IN FA AND CONTROL BRAIN REGIONS AND MYELIN



8.5 In-vitro Peroxidation Studies

Neural fractions for in-vitro peroxidation studies were isolated from the brainstem of FA patients and controls using a modification of the method of DeVries (1981) as described and validated in chapter 2. Due to restrictions on the amounts of tissue available, it was not possible to confirm the purity of the fractions obtained using marker enzymes and electron microscopy as was done for preparations from rat tissue. However, DeVries (1981a) demonstrated that the procedure could be successfully applied to the fractionation of human samples. In addition the density gradient profiles of the human tissue fractionations were similar to those seen in the rat, studies both in terms of the locations of the different fractions on the gradients and the relative amounts of material present in each fraction. This implied that the isolated fractions from rat and human tissues were of similar composition.

In-vitro lipid peroxidation studies were of two types. Firstly, a comparison was made of the susceptibility to peroxidative stress of neural fractions from FA and controls in order to investigate whether FA fractions showed a greater susceptibility to peroxidation. Secondly, neural fractions from the same starting tissue were compared in order to determine which fractions were more susceptible to free radical stress, and to compare the results with those obtained from the rat studies.

8.5.1 Comparison of In-Vitro Peroxidation of Neural Fractions from FA and Controls

The susceptibility to peroxidative stress of neural fractions isolated from FA and control brainstem was studied by incubating comparable fractions with the copper sulphate / hydrogen peroxide free radical generating system and measuring free MDA produced by HPLC as described in chapter 6. In all cases samples were incubated with a fixed $CuSO_4$ / H_2O_2 concentration at 37°C for increasing periods of time. Results were related to protein levels

(nmol/mg protein). FA and control fractions were matched as shown in table 1, i.e. FA 1 versus control 1, etc.. The FA fraction and its corresponding control fraction were assayed together with only two fractions assayed per run. A quality control (rat whole brain homogenate) was included in all assays.

In the case of the in-vitro peroxidation studies of neural fractions isolated from vitamin E deficient and controls rats peroxidation conditions were arranged so that the controls exhibited a lag phase (chapter 7). For the FA studies however, peroxidation conditions were chosen so that controls peroxidised at a steady and easily measurable rate. The main objective was to investigate whether the fractions from the FA patients were more susceptible to oxidative stress than controls so that measurement of lag phases was not of importance.

8.5.1.1. Brainstem Whole Homogenate.

Fig 7 shows the results for the peroxidation of separate FA brainstem whole homogenates from three patients with FA and corresponding controls. Samples (approximately 10μg protein) were incubated with 1mM H_2O_2 and 20μM $CuSO_4$ for up to 90 mins. In all cases the FA homogenate appeared to be more susceptible to free radical stress than the control.

8.5.1.2 Myelin

Myelin fractions (approximately 10μg protein) were incubated under the same conditions as used for the homogenate samples, i.e. 1mM H_2O_2 and 20μM $CuSO_4$. From fig 8 it can be seen that there was little difference between FA and controls, although if anything the controls tended to more susceptible.

FIG 7. IN-VITRO PEROXIDATION OF FA AND CONTROL BRAINSTEM WHOLE HOMOGENATE ($1mM\ H_2O_2$, $20\mu M\ CuSO_4$)

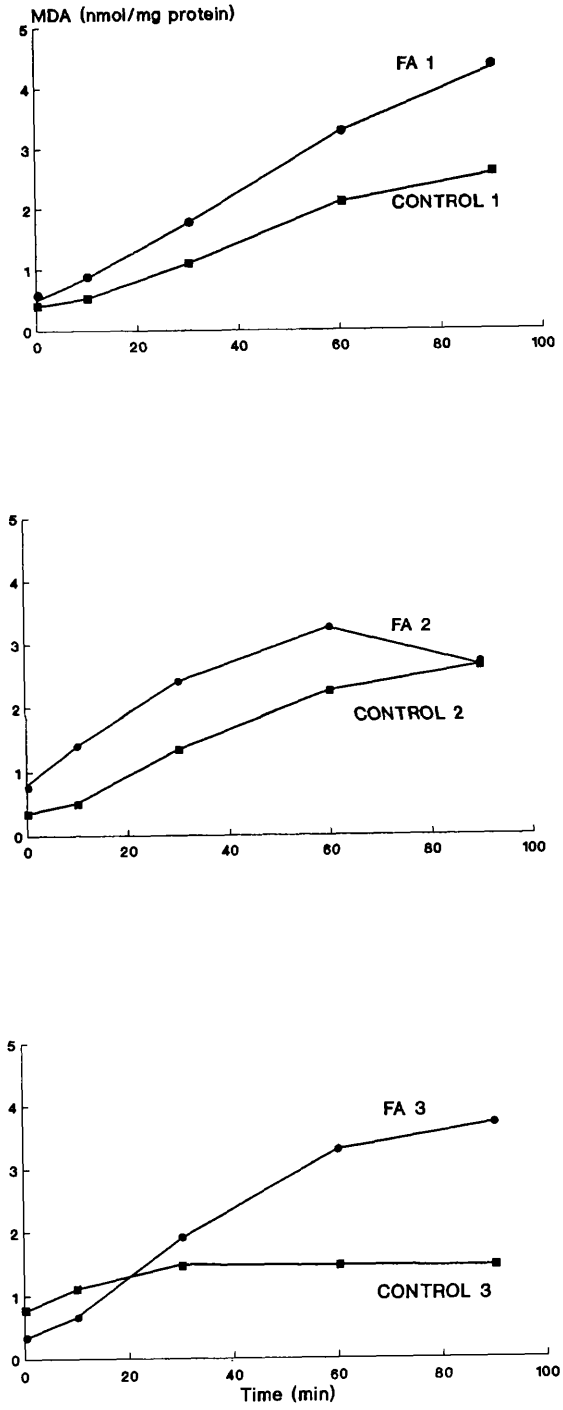
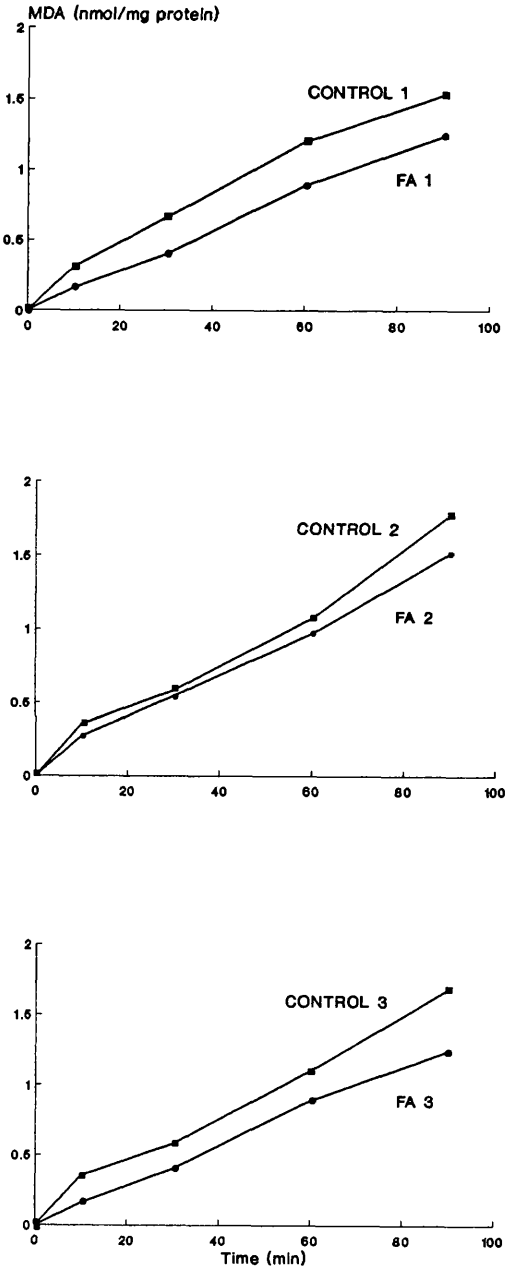


FIG 8. IN-VITRO PEROXIDATION OF FA AND CONTROL MYELIN FRACTIONS ($1mM\ H_2O_2$, $20\mu M\ CuSO_4$)



8.5.1.3 Axolemma-Enriched Fractions

All axolemma-enriched fractions (AEF) were stressed with 0.5mM H_2O_2 and 5μM $CuSO_4$ for up to 90 mins (Fig 9). Approximately 5μg protein was used per assay. As in the in-vitro peroxidation studies of rat brainstem fractions, both the AEF and M/O fractions were incubated under milder conditions than the whole homogenate and myelin fractions. In 2 cases there was little difference between the FA and control samples, but like myelin the controls tended to be exhibit a greater susceptibility to oxidative stress.

8.5.1.4 Axoplasmic Membranes and Organelles

The results for the peroxidation of fractions containing the axoplasmic membranes and organelles (M/O) are plotted in fig 10 where 1 and 3 were incubated with 0.5mM H_2O_2 and 5μM $CuSO_4$, whereas 0.2mM H_2O_2 and 2.5μM $CuSO_4$ was used for number 2. Approximately 5μg protein was incubated per assay. Again the FA fractions did not appear to be consistently different from controls.

In summary the results of the in-vitro peroxidation studies described above showed that whereas the FA brainstem whole homogenate appeared to be more susceptible to peroxidative stress, there was no evidence of an increased susceptibility of the three neural membrane fractions isolated from the same brainstem homogenates.

FIG 9. IN-VITRO PEROXIDATION OF FA AND CONTROL AXOLEMMA-ENRICHED FRACTIONS

(0.5mM H_2O_2, 5μM $CuSO_4$)

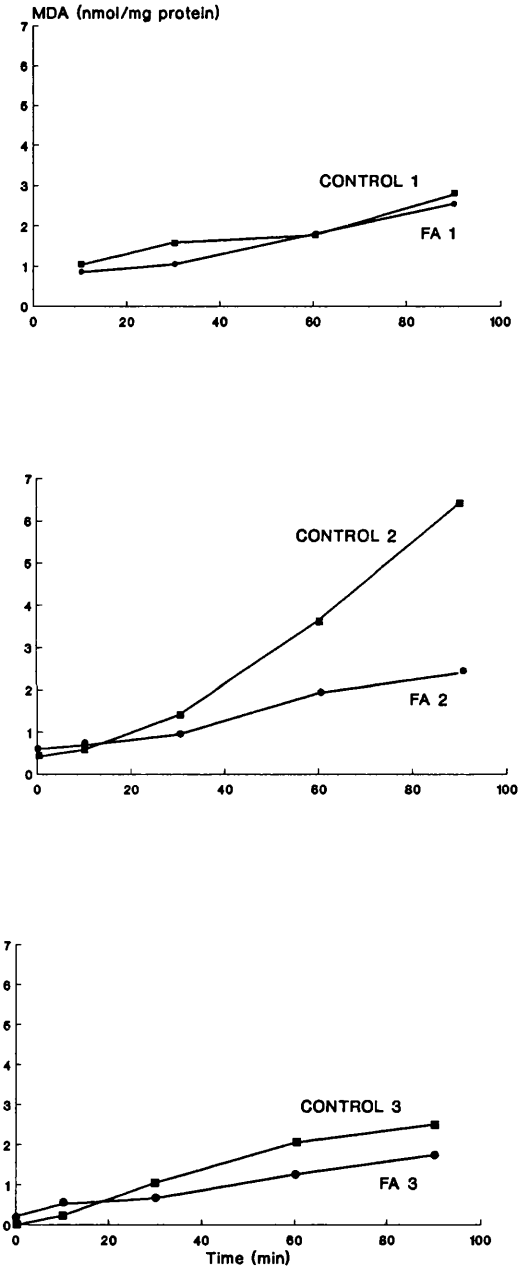
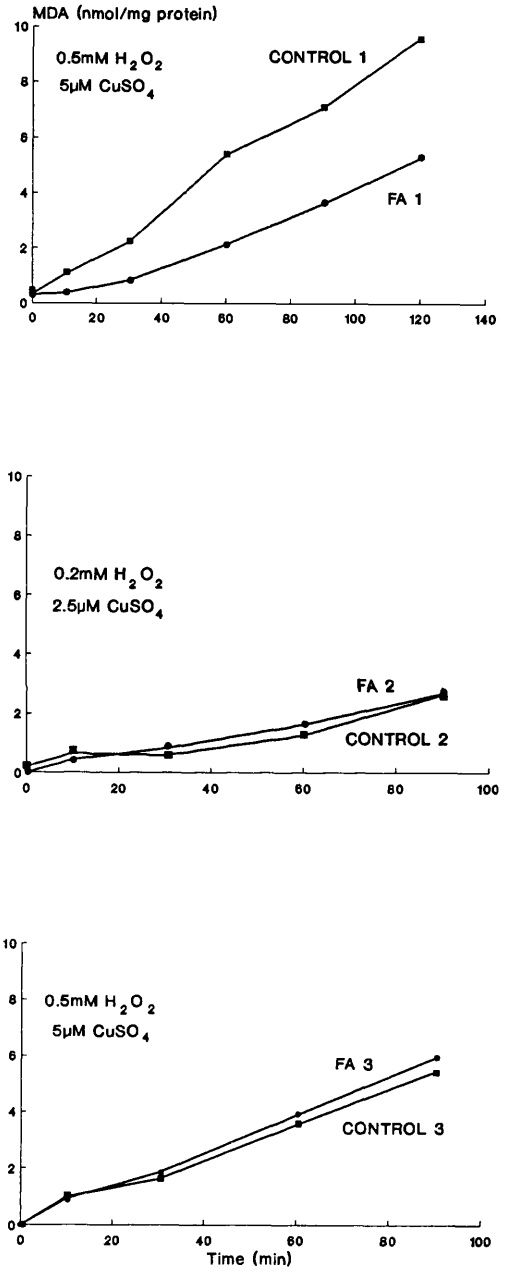


FIG 10. IN-VITRO PEROXIDATION OF FA AND CONTROL AXOPLASMIC MEMBRANES AND ORGANELLES FRACTION



8.5.2 Comparison of Susceptibility of Different Neural Fractions to In-Vitro Peroxidative Stress

Although there was no evidence of increased susceptibility in the myelin, AEF and M/O fractions isolated from FA brainstem compared to controls, the susceptibility of different fractions isolated from the same starting tissue sample were compared in order to see what fraction, if any, would be expected to be the most susceptible under in-vivo conditions of increased oxidative stress or reduced antioxidant capacity. Results could then be compared with those obtained from similar studies in the rat.

For these studies the fractions (approximately 10μg protein) were incubated with 2mM H_2O_2 at 37°C for 30min with increasing concentrations of $CuSO_4$ (0, 50, 500 and 1000μM). Lipid peroxidation was followed by measuring free MDA and results were reported in relation to protein levels (nmol/mg protein). A comparison of the susceptibilities of the different fractions isolated from the brainstem of a patient with FA is presented in fig 11, with data for control in fig 12. The results obtained from both FA and control sets of neural fractions showed that the AEF exhibited the greatest susceptibility to in-vitro peroxidative stress producing twice as much MDA as the other fractions. There was little difference in the susceptibilities of the other fractions and their order of susceptibility differed in the two assays.

FIG 11. IN-VITRO PEROXIDATION OF NEURAL FRACTIONS FROM FA BRAINSTEM

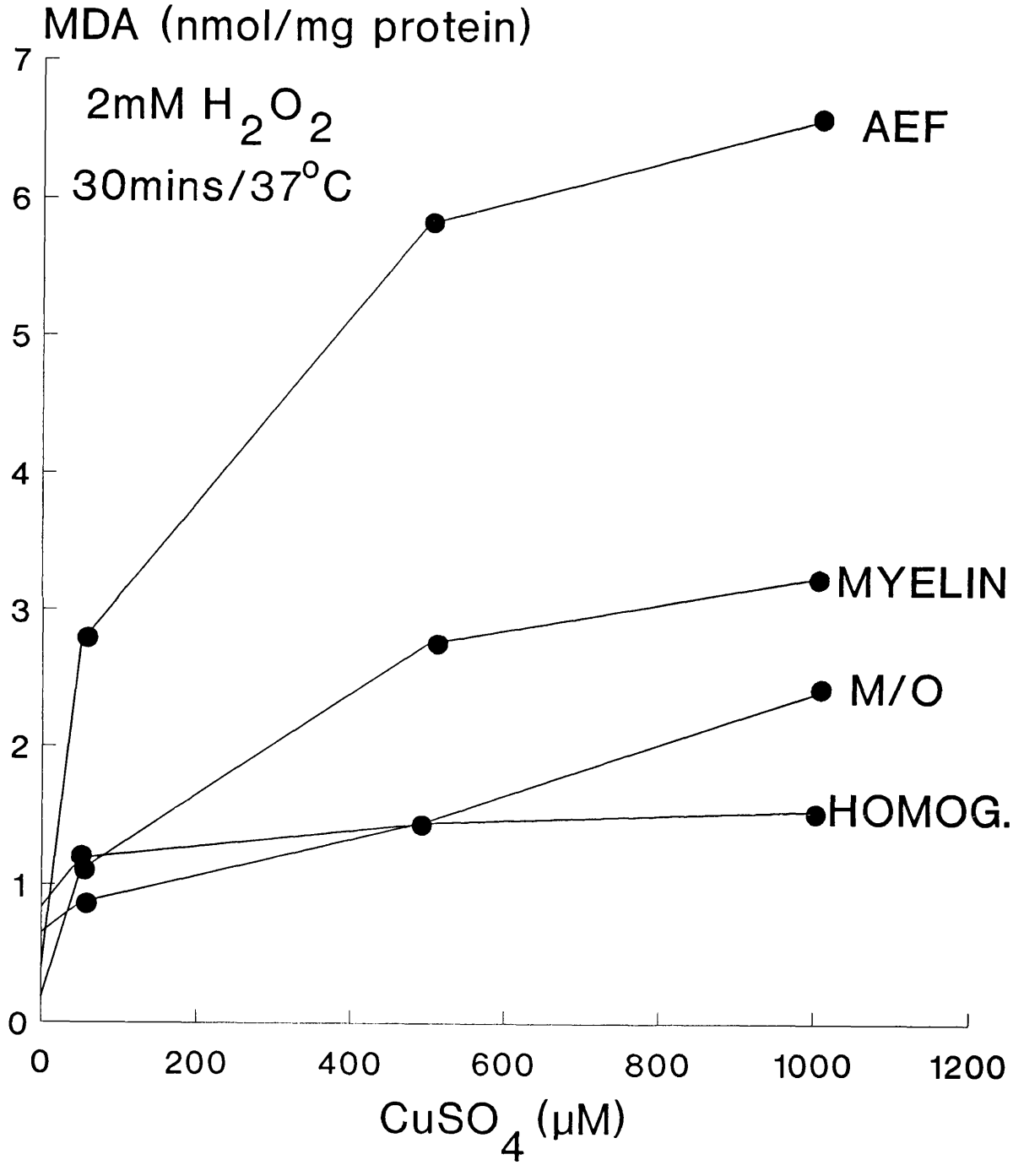
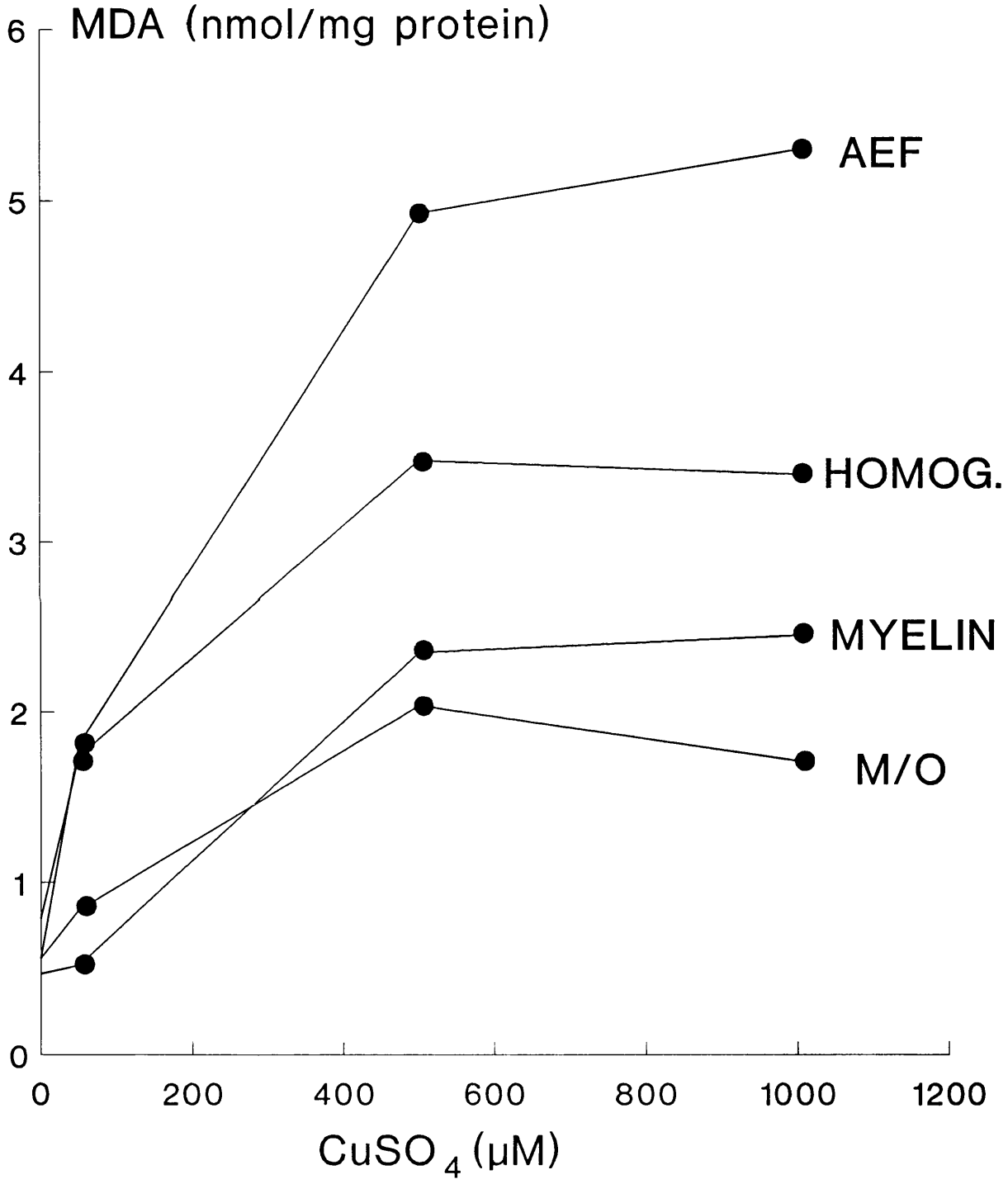


FIG 12. IN-VITRO PEROXIDATION OF NEURAL FRACTIONS FROM CONTROL BRAINSTEM



8.6 Discussion

Although only a small number of cases were investigated the results of the various studies on the post mortem tissue samples from patients with FA and controls appear to rule out an antioxidant defect or increased peroxidative stress in the aetiology of FA.

The concentrations of vitamin E found in the FA tissues were comparable to those in the control tissues. No control peripheral nerve samples were available so that FA nerve concentrations had to be compared with published data. The mean and range of the vitamin E concentrations in FA samples was 0.32 (0.22 - 0.42) mmol/mol lipid. These levels appeared to be at the lower end of the range of concentrations in control sural nerves in a study by Jeffrey et al (1987), i.e. 0.77 (0.29 - 1.28) mmol/mol lipid (n=5). The somewhat reduced concentrations in peripheral nerve in this study could be due to the fact that, in contrast to Jeffrey et al, the outer epineurial sheath was removed and discarded prior to homogenisation. Goss-Sampson (unpublished results) who measured the concentrations of α-tocopherol in three fractions of rat sciatic nerve (n=4), found that highest levels were present in the epineurial sheath and non-myelin pellet, 0.91 (0.85-0.97) and 0.82 (0.76-0.86) μmol/mol lipid respectively, whereas concentrations in the myelin fraction were the lowest, 0.65 (0.52-0.79) μmol/mol lipid. Therefore removal of the epineurial sheath which accounts for a substantial proportion of the total nerve, could result in a lower assay value for "nerve" than if it were included. The similar tissue concentrations of vitamin E (α-tocopherol) in the brain and myelin of patients with FA and controls together with the normal serum levels found in this study appear to exclude an abnormality in tissue uptake of the vitamin.

Despite normal tissue levels of vitamin E, the possibility remained of a defect in its biochemical function and action. However, measurement of endogenous concentrations of different parameters of lipid peroxidation did not reveal a

significant difference between FA and controls. There was, therefore, no evidence of an increased level of lipid peroxidation or reduced antioxidant capacity occurring in-vivo in cases of FA. However, the data cannot be considered conclusive due to the limited number of samples examined. In addition, measurement of alternative peroxidation parameters such as aliphatic aldehydes or lipid peroxides should also be undertaken.

Note that as previously seen and discussed in the case of both control and vitamin E deficient rat neural tissues (chapter 5), the TBA test gave a higher value compared to the selective HPLC method for MDA and so provides further evidence for the non-specificity of the TBA test.

Although no evidence was found for an increase in in-vivo lipid peroxidation it was possible that in-vitro peroxidation studies might reveal differences in the susceptibilities of tissues and brainstem neural fractions from patients with FA compared to controls.

In-vitro peroxidation studies showed, however, that with the possible exception of the brainstem whole homogenate, there was no evidence of increased susceptibility to in-vitro peroxidative stress occuring in FA compared to the controls. If the apparent increased susceptibility of FA brainstem to in-vitro free radical stress is genuine then structures other than the components of the myelinated axons, e.g. astrocytes, could be responsible for this result. The small number of samples used cannot exclude the possibility that the observed differences seen in the brainstem homogenate resulted from experimental variation, particularly as there were no significant differences for the other fractions and in the concentrations of vitamin E and lipid peroxidation products.

A comparison of the susceptibilities of different neural fractions isolated from the same brainstem tissue sample showed that for both FA and control fractions (figs 11 and 12), the AEF was the most susceptible with the remaining fractions generating similar amounts of free MDA. The patterns of susceptibility seen for these two sets of human neural fractions were different to that obtained when the corresponding rat fractions were incubated under identical conditions (chapter 7). The major difference between the results of the rat and human fractions was the susceptibility to free radical stress of the fraction containing the intracellular membranes and organelles (M/O). In the rat this fraction consistently exhibited the greatest susceptibility, whereas in the human brainstem it appeared to be relatively resistant to peroxidative stress. It is possible that the extended storage times for both the FA and control brainstem tissues prior to use, i.e. 38 and 25 months respectively, could have affected the results. There were, however, no significant differences in results obtained from fresh rat brainstem fractions and fractions obtained from tissue that had been stored at -70°C. This was true for both the relative susceptibilities of the different fractions and the amounts of free MDA formed in each fraction.

The greater susceptibility of the AEF compared to that of myelin would fit with the findings of DeVries et al (1981) who studied the lipid compositions of AEFs and myelin isolated from human brain tissue. DeVries and colleagues showed that although the distribution of ethanolamine and choline phospholipid fatty acids (the major phospholipids in these two fractions) were similar in both the more dense AEF and myelin fractions, the unsaturation indices of both phospholipids were significantly higher in the AEF. The M/O fraction was not characterised by DeVries et al and so data was not available on its lipid composition.

Overall, therefore, the results provide evidence of normal lipid peroxidation and antioxidant capacity in FA patients. They would, therefore, appear to remove another postulated metabolic defect from the list of possible candidates involved in the aetiology of FA so that for the moment at least, this disease remains a "fascinating puzzle".

CHAPTER 9.

CONCLUSIONS

Conclusions.

The main objective at the start of this study was to investigate lipid peroxidation in tissues and neural fractions of the rat during experimental vitamin E deficiency. In the case of the neural fractions it was hoped that the studies would provide some idea as to why large calibre myelinated axons were particularly susceptible to a deficiency of vitamin E. In addition similar studies were carried out in post mortem tissues from patients with Friedreich's ataxia to investigate the possibility of an antioxidant defect in the aetiology of this condition.

Measurement of endogenous parameters of lipid peroxidation demonstrated that tissues from vitamin E deficient animals were more susceptible to peroxidative stress than age-matched controls which was in agreement with the generally accepted function of vitamin E in-vivo, i.e. a lipid soluble antioxidant. The apparent relative susceptibilities of the various tissues was dependent on the method employed to assess lipid peroxidation. At 56 weeks the increases in endogenous concentrations of TBARS in deficient non-neural tissues relative to controls were much greater than the corresponding increases seen in deficient neural tissues. At the earlier 21 weeks, however, there were no significant differences in TBARS between deficient and control animals in any of the tissues examined. In contrast to TBARS, comparable differences between deficient and control animals were found in free MDA concentrations in 56 week neural and non-neural tissues, and there were also significant increases in some tissues at 21 weeks. Endogenous lipid peroxidation needs, therefore, to be assessed using additional methods so to obtain a more definite picture of the relative susceptibilities of different tissues to a deficiency of vitamin E.

The effect of vitamin E deficiency on the susceptibility of tissues to in-vitro oxidative stress was investigated by incubating tissue whole homogenates with the $CuSO_4$ / H_2O_2 free radical generating system with the samples being subjected to relatively strong peroxidation conditions. In general, in both control and deficient brains, the cortex, striatum and cerebellum exhibited the greatest susceptibilities, whereas brainstem and hypothalamus tended to be more resistant. The maximum difference in susceptibilities was, however, relatively small, i.e. approximately 2-fold. The following order of susceptibilities was seen for the neural regions; brain >> spinal cord > nerve, suggesting that the brain might be more susceptible to a deficiency of vitamin E than the other two regions. This does not, however, correlate with the known neuropathology of vitamin E deficiency since there is an extensive loss of large calibre myelinated axons in both the spinal cord and nerve. A comparison of neural and non-neural tissues showed brain to be the most susceptible of the control tissues, whereas of the deficient tissues, the heart generated the greatest amounts of free MDA following oxidative stress. In both cases peripheral nerve was the most resistant.

The in-vitro peroxidation of neural fractions isolated from rat brainstem was studied by incubating samples under relatively mild conditions. A comparison of neural fractions from control and deficient animals at both 20 and 60 weeks showed that all deficient fractions were more susceptible than the corresponding controls, with the latter exhibiting a lag phase which was absent from the deficient peroxidation profiles. For neural fractions from both control and vitamin E deficient animals the following order of susceptibility to free radical stress was seen; M/O >> AEF >> myelin. This order was consistent with the neuropathology associated with vitamin E deficiency where a primary axonopathy results in secondary demyelination, with the mitochondria and endoplasmic reticulum possibly being the sites of initial free radical attack. Since the loss of myelinated axons is the major feature of the

neuropathology due to vitamin E deficiency it may be more appropriate when investigating the effects of vitamin E deficiency to study lipid peroxidation in neural fractions rather than examining the whole homogenate. Detailed studies of endogenous lipid peroxidation in neural fractions over time might also give information as to the possible sequence of events and the initial site of oxidative stress during vitamin E deficiency.

Studies of lipid peroxidation on post mortem tissues from patients with Friedreich's ataxia failed to provide any evidence of an antioxidant defect in the aetiology of this disease. There were no significant differences in the tissue concentrations of vitamin E or endogenous lipid peroxidation products, and no consistent differences in the in-vitro susceptibilities of brain regions and neural fractions to peroxidative stress compared to control tissues and fractions.

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