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THE SUBCELLULAR DISTRIBUTION OF PALMITOYL COENZYME A SYNTHETASE IN RAT BRAIN

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A Dissertation submitted to the Faculty of Science,
University of the Witwatersrand, Johannesburg,
for the Degree of Master of Science.

Johannesburg, 1981

ABSTRACT

Unique among natural membranes, myelin has a very high lipid content and in the mature animal, a very slow metabolic turnover rate. Following reports in the literature (Pande and Mead, 1968; Cantrill, R.C., Ph.D. Thesis, 1978) of a putative plasma membrane location for the fatty-acid activating enzyme, palmitcyl coenzyme A synthetase, the possibility arises that this enzyme might, to some extent, be responsible for the reduced fatty acid metabolism of myelin and oligodendrocyte cytosol.

The aim of this study is to investigate the sub-cellular distribution of palmitoyl coenzyme A synthetase in the mature rat brain, in particular to determine whether any enzyme activity is associated with the myelin or myelin-related fractions.

The assay of marker enzymes specific for the different brain sub-cellular fractions, myelin, mitochondria, microsomes, cytosol, provides an estimate of the degree of contamination of myelin by other fractions. Thus the contribution of contaminating membranes, especially mitochondria and microsomes, to any activity of palmitoyl coenzyme A synthetase found in the myelin fraction can be established. Activity in excess of that due to impurities may then be considered of myelin or plasma membrane origin.

The results to be presented show that i) contamination of myelin by microsomal membranes is very high when high ionic strength homogenizing buffers are employed, ii) all activity of palmitoyl coenzyme A synthetase found in myelin can be attributed to microsomal contamination and iii) the presence of phosphate in the homogenizing medium enhances the activity of the enzyme in rat brain.

I hereby declare
that this dissertation has not been submitted to any other University and that the results contained herein were obtained by me while a student at the University of the Witwatersrand.

E J L Kerr

ACKNOWLEDGEMENTS

I wish to thank my supervisors, Professor Nerina Savage and Dr Richard Cantrill for their constant help and encouragement throughout the duration of this project.

I am grateful to Mrs E Kenedi for the electron micrographs, Mrs C Bladergroen for typing this manuscript, and my husband, Brian, for support.

This work was supported by a Senior Bursary from the University of the Witwatersrand.

C O N T E N T S

		Page
	Title Page	i
	Abstract	ii
	Declaration	iv
	Acknowledgements	v
	Table of Contents	vi
	List of Abbreviations	x
	List of Tables	xi
	List of Figures	xiii
1.	INTRODUCTION	1
1.1	Myelin	2
1.1.1	Origin of Central Nervous System Myelin	4
1.1.2	Synthesis of Central Nervous System Myelin	5
1.1.3	Morphology of Central Nervous System Myelin	7
1.1.3.1	Fatty Acids of the Myelin Membrane	10
1.1.4	Metabolism of Central Nervous System Myelin	14
1.1.4.1	Enzymes Associated with the Myelin Sheath	22
1.2	Palmitoyl Coemzyme A Synthetase E.C. 6.2.1.3	24
1.2.1	Subcellular Location of Palmitoyl Coenzyme A Synthetase	26
1.2.2	Factors Affecting Palmitoyl Coenzyme A Synthetase Activity	26

C O N T E N T S (Continued)

		Page
1.3	Brai.. Subcellular Fractionation	28
1.3.1	Marker Enzymes in Brain Subcellular Fractions	30
2.	MATERIALS A.'D METHODS	
2.1	Materials	31
2.1.1	Radiochemicals	31
2.1.2	Other Chemicals	31
2.1.3	Animals	32
2.2	Methods	32
2.2.1	Preparation of Brain Subcellular Fractions	32
2.2.2	Sucrose Density Gradient Centrifugation	35
2.2.3	Preparation of Palmitoyl Coenzyme A Carnitine Palmitoyl Transferase E.C. 2.3.1.2	36
2.2.4	Preparation of Potassium Palmitate Solution	36
2.2.5	Preparation of Palmitoyl-D,L-Carnitine	37
2.2.6	Determination of Protein	37
2.2.7	Determination of Radioactivity	37
2.2.8	Determination of Inorganic Phosphorus	37
2.3	Assay of Enzyme Activity	38
2.3.1	Palmitoyl Coenzyme A Carnitine Palmitoyl Transferase E.C. 2.3.1.2	38
2.3.2	Palmitoyl Coenzyme A Synthetase E.C. 6.2.1.3	38

C O N T E N T S (Continued)

		Page
2.3.3	NADPH Cytochrome C Reductase E.C. 1.6.2.3	39
2.3.4	Ferrocytochrome C Oxidase E.C. 1.9.3.1	39
2.3.5	Acetylcholinesterase E.C. 3.1.1.7	39
2.3.6	2',3'-Cyclic Nucleotide 3'-Phosphohydrolase E.C. 3.1.4.37	40
2.3.7	5'-Mononucleotidase E.C. 3.1.3.5	40
2.3.8	Lactate Dehydrogenase E.C. 1.1.1.27	40
3.	RESULTS	
3.1	Subcellular Fractionation	41
3.2	Marker Enzyme Distribution in Subcellular Fractions	43
3.2.1	NADPH Cytochrome C Reductase Distribution E.C. 1.6.2.3	44
3.2.2	Ferrocytochrome C Oxidase Distribution E.C. 1.9.3.1	45
3.2.3	Acetylcholinesterase Distribution E.C. 3.1.1.7	46
3.2.4	2',3'-Cyclic Nucleotide 3'-Phosphohydrolase Distribution E.C. 3.1.4.37	47
3.2.5	5'-Mononucleotidase Distribution E.C. 3.1.3.5	47
3.2.6	Lactate Dehydrogenase Distribution E.C. 1.1.1.27	48
3.2.7	Summary of Marker Enzyme Results	49
3.3	Distribution of Palmitoyl Coenzyme A Synthetase E.C. 6.2.1.3	49

C O N T E N T S (Continued)

		Page
	Legend to Tables 1 - 14	51
4.	DISCUSSION	67
4.1	Discussion of Subcellular Fractionation	67
4.2	Discussion of Marker Enzyme Distribution	70
4.2.1	NADPH Cytochrome C Reductase E.C. 1.6.2.3	70
4.2.2	Ferrocytochrome C Oxidase E.C. 1.9.3.1	71
4.2.3	Acetylcholinesterase E.C. 3.1.1.7	72
4.2.4	2',3'-Cyclic Nucleotide 3'-Phosphohydrolase E.C. 3.1.4.37	74
4.2.5	5'-Mononucleotidase E.C. 3.1.3.5	75
4.2.6	Lactate Dehydrogenase E.C. 1.1.1.27	76
4.3	Palmitoyl Coenzyme A Synthetase E.C. 6.2.1.3	78
4.4	Conclusions	84
	References	85

A B B R E V I A T I O N S

5'-AMP	5'-adenosine mono phosphate
2'-3'-c AMP	2',3'-cyclic adenosine mono phosphate
ATP	adenosine triphosphate
CoA	coenzyme A
NADPH	reduced nicotinamide adenine dinucleotide phosphate
Tris	tris (hydroxymethyl) methylamine
2',3'-CNP	2',3'-cyclic nucleotide 3'-phosphohydrolase
NP40	Nonidet P 40

L I S T O F T A B L E S

		Page
Table 1	Distribution of Protein Throughout Fractions Prepared From Adult Rat Brain in Sucrose	53
Table 2	Distribution of Protein Throughout Fractions Prepared From Adult Rat Brain in Sucrose/Phosphate	54
Table 3	Distribution of NADPH Cytochrome C Reductase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose	55
Table 4	Distribution of Ferrocytochrome C Oxidase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose	56
Table 5	Distribution of Acetylcholinesterase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose	57
Table 6	Distribution of 2',3'-Cyclic Nucleotide 3'-Phosphohydrolase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose	58
Table 7	Distribution of 5'-Mononucleotidase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose	59
Table 8	Distribution of Lactate Dehydrogenase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose	60
Table 9	Distribution of Palmitoyl Coenzyme A Synthetase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose	61
Table 10	Distribution of Palmitoyl Coenzyme A Synthetase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose/Phosphate	62

L I S T O F T A B L E S

		Page
Table 1	Distribution of Protein Throughout Fractions Prepared From Adult Rat Brain in Sucrose	53
Table 2	Distribution of Protein Throughout Fractions Prepared From Adult Rat Brain in Sucrose/Phosphate	54
Table 3	Distribution of NADPH Cytochrome C Reductase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose	55
Table 4	Distribution of Ferrocytochrome C Oxidase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose	56
Table 5	Distribution of Acetylcholinesterase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose	57
Table 6	Distribution of 2',3'-Cyclic Nucleotide 3'-Phosphohydrolase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose	58
Table 7	Distribution of 5'-Mononucleotidase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose	59
Table 8	Distribution of Lactate Dehydrogenase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose	60
Table 9	Distribution of Palmitoyl Coenzyme A Synthetase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose	61
Table 10	Distribution of Palmitoyl Coenzyme A Synthetase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose/Phosphate	62

L I S T O F T A B L E S

		Page
Table 1	Distribution of Protein Throughout Fractions Prepared From Adult Rat Brain in Sucrose	53
Table 2	Distribution of Protein Throughout Fractions Prepared From Adult Rat Brain in Sucrose/Phosphate	54
Table 3	Distribution of NADPH Cytochrome C Reductase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose	55
Table 4	Distribution of Ferrocytochrome C Oxidase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose	56
Table 5	Distribution of Acetylcholinesterase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose	57
Table 6	Distribution of 2',3'-Cyclic Nucleotide 3'-Phosphohydrolase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose	58
Table 7	Distribution of 5'-Mononucleotidase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose	59
Table 8	Distribution of Lactate Dehydrogenase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose	60
Table 9	Distribution of Palmitoyl Coenzyme A Synthetase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose	61
Table 10	Distribution of Palmitoyl Coenzyme A Synthetase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose/Phosphate	62

L I S T O F T A B L E S (Continued)

		Page
Table 11	Distribution of NADPH Cytochrome C Reductase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose/Phosphate	63
Table 12	NADPH Cytochrome C Reductase and Palmitoyl Coenzyme A Synthetase Activities in Rat Liver Microsomes in Sucrose	63
Table 13A	Ratio of NADPH Cytochrome C Reductase Activity to Palmitoyl Coenzyme A Synthetase Activity in Myelin Fractions (F11 and F21) and F3 Prepared from Adult Rat Brain in Sucrose	64
Table 13B	Ratio of 5'-Mononucleotidase Activity to Palmitoyl Coenzyme A Synthetase Activity in Myelin Fractions (F11 and F21) and F3 Prepared from Adult Rat Brain in Sucrose	64
Table 13C	Ratio of NADPH Cytochrome C Reductase Activity to Palmitoyl Coenzyme A Synthetase Activity in Myelin Fractions (F11 and F21) and F3 Prepared from Adult Rat Brain in Sucrose/Phosphate	64
Table 14A	Ratio of NADPH Cytochrome C Reductase Activity to Palmitoyl Coenzyme A Synthetase Activity in Myelin Fractions (F11 and F21) and F3 Prepared from Adult Rat Brain in Sucrose	65
Table 14B	Ratio of 5'-Mononucleotidase Activity to Palmitoyl Coenzyme A Synthetase Activity in Myelin Fractions (F11 and F21) and F3 Prepared from Adult Rat Brain in Sucrose	65
Table 14C	Ratio of NADPH Cytochrome C Reductase Activity to Palmitoyl Coenzyme A Synthetase Activity in Myelin Fractions (F11 and F21) and F3 Prepared from Adult Rat Brain in Sucrose/Phosphate	65

L I S T O F F I G U R E S

		Page
Figure 1	The Role of Palmitoyl CoA Synthetase in Fatty Acid Metabolism in Brain	25
Figure 2	Scheme of Subcellular Fractionation	34
Figure 3	Electron Micrograph of F11 Myelin	66
Figure 4	Electron Micrograph of F21 Myelin	66

1. INTRODUCTION

The study of fatty acid metabolism is of particular relevance in myelin since lipid comprises approximately 75% of its dry weight. It is important to have normal fatty acid metabolism in order that there is normal development and maintenance of myelin membrane structure. In contrast with other cellular membranes myelin is relatively stable. C 18 fatty acids predominate in other sphingomyelins, whereas in myelin C 24:0 and C 24:1 are the major fatty acids (Norton, 1977). Their presence increases the transition temperature of myelin sphingolipids thus enhancing the stability of the membrane (Chapman, 1973).

The high concentrations in myelin of saturated and mono-unsaturated fatty acids, which are not susceptible to attack by lipoxygenase and peroxidase, help to maintain membrane integrity (Bourre et al., 1977). Ethanolamine plasmalogens may have a possible role in preserving myelin membrane stability by competitively inhibiting the hydrolysis of diacyl lipids by phospholipase A (Rumsby, in press).

Long-term studies (Bowen et al., 1974) have shown that there is only a very slow exchange between circulating fatty acids and myelin lipids. Since membranes are completely permeable to free fatty acids, although not to their coenzyme A esters, it is possible that activation of fatty acids by palmitoyl coenzyme A synchetase is a cellular mechanism whereby myelin and oligodendrocyte

cytoplasmic fatty acids are retained, and only slowly exchanged with circulating fatty acids. Some enzymes, notably 2',3'-cyclic nucleotide 3' phosphohydrolase, leucine amino peptidase, and a cholesterol ester hydrolase have been demonstrated in myelin.

This study was undertaken to determine whether palmitoyl coenzyme A synthetase was present in myelin or myelin-related fraction of rat brain, in the light of reports of a possible plasma membrane location for the enzyme. This was postulated by Pande and Mead in 1968 to account for the difference in activity between the whole homogenate and that recovered from microsomes and mitochondria. Subsequently, Cantrill (Ph.D. Thesis, 1978) reported some residual activity of palmitoyl coenzyme A synthetase associated with mature brain myelin and considered that this activity might be attributable to plasma membrane. The degree of contamination of the myelin by other subcellular components such as microsomes and mitochondria was assessed using marker enzyme assays.

1.1 Myelin

Myelin forms a sheath consisting of several layers around nerve axons. Ranvier, in 1878, ascribed two functions to myelin. The first was its role in protecting the axon-cylinder from compression. He stated that because of its liquid, or almost liquid, nature, pressure exerted on the myelin sheath would be uniformly distributed throughout the sheath, thus reducing the constricting effects on the axon-cylinder.

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The second role was that of an insulator. He made the analogy of nerve impulse transmissions with the transmission of electricity and speculated that the transmission of impulses might be more effective if each nerve was insulated (Ranvier, 1878).

The effect of the insulating property of the myelin sheath is to reduce the nerve-fibre diameter required to achieve high conduction velocity (Rogart and Ritchie, 1977). Demyelination of nerve fibres in disease conditions, notably multiple sclerosis, results in reduced conduction velocity, the extent of which is directly related to the degree of demyelination (Halliday and McDonald, 1977).

In the central nervous system the myelin sheath is formed by the wrapping of a process from the oligodendroglial cell around an axon. One oligodendrocyte may extrude up to fifty such processes, each of which may form a myelin internode (Peters and Vaughn, 1970). It can be appreciated that damage to a few oligodendroglial cells would have adverse effects on many myelin internodes.

Myelin is normally very stable (O'Brien, 1965) but recent studies have indicated that it is not completely inert metabolically (Benjamins and Smith, 1977) as demonstrated by its capacity to regenerate.

1.1.1 Origin of Central Nervous System Myelin

The oligodendroglial cell is responsible for the synthesis of central nervous system myelin (Bunge et al., 1962). The plasma membrane of the oligodendrocyte is extruded to form a process which is continuous with the myelin membrane. Mixed fibres in the central nervous system tissue have an average internodal length of 250 µm (Matthews and Duncan, 1971). It is of interest to note the remarkable synthetic capabilities of the oligodendrocyte, which has a diameter of 10-15 µm. Norton and Poduslo (1973b) have calculated that there may be fewer than 2×10^7 oligodendroglia per brain, the dry weight of each cell body being approximately 50×10^{-12} g. Since in the brain of a twenty-day old rat, 3,5 mg of myelin are deposited in one day, each cell is synthesizing about 175×10^{-12} g, that is, roughly three times its own dry weight, of myelin in one day.

Although myelin is continuous with the oligodendroglial plasma membrane (Peters, 1964: Bunge and Glass, 1965; Hirano, 1968) there are distinct differences in their lipid and protein compositions (Poduslo, 1975). In bovine oligodendrocytes the galactosyl ceramide constitutes less than 6% by weight of total lipids in comparison with about 22% in myelin. Glucosylceramide, on the other hand, comprises 0,8% of total lipids compared to only 0,09% of myelin total lipids. Sphingomyelin is 7,7% by weight of total myelin lipid and 4,4% of total oligodendrocyte lipid and Norton, 1979).

It would appear that cerebrosides are essential in the formation of mature myelin (Davison and Cuzner, 1977). Carruthers and Carey (1979), found Wolfgram protein, proteolipid protein and myelin basic protein in oligodendroglial plasma membrane. 2',3'-cyclic nucleotide 3'-phosphohydrolase was also found (Poduslo and Norton, 1972; Volpe et al., 1975) but was of lower specific activity than the enzyme in myelin (Carruthers and Carey, 1979). Plasmalogenase was present in oligodendrocytes (Dorman et al., 1977a; Carruthers and Carey, 1979).

1.1.2 Synthesis of Central Nervous System Myelin

It is known that before myelination commences the tissue has to attain a state conducive to the onset of myelination. Axons will only begin to be myelinated after they have reached a diameter of about 1 μm. The continuous flow of lipid vesicles and protein into the surface membrane of the oligodendrocyte is the driving force for myelination (Rumsby, 1978).

It is not yet known what factor or factors initiate myelination, but from studies on mutant mice which have demyelinating syndromes of different genotype, it seems that myelin formation is affected by genes on several different chromosomes. At myelination, there is a massive synthesis of lipids and proteins, which are most likely transferred to the oligodendrocyte plasma membrane according to the principles established by Palade (1975), for newly synthesized proteins to be transported from the

endoplasmic reticulum and Golgi apparatus to the surface membrane. In the pancreatic exocrine cell Jamieson and Palade (1968) found that transport of protein from the endoplasmic reticulum via its transitional elements was ATP-dependent. Protein left the transitional elements inside Golgi vesicles in which it was transported to condensing vacuoles. It is likely that some similar pathway occurs in the oligodendroglia (Rumsby, 1978). Phospholipid exchange proteins may also supply certain lipids to the sites of fusion on the cytoplasmic side of the plasma membrane (Wirtz, 1974). It is envisaged that lipids and proteins are incorporated into the oligodendroglial plasma membrane as vesicles which subsequently fuse with the membrane in such a way that the new membrane components are correctly oriented (Rothman and Lenard, 1977).

There is probably some kind of recognition between an axon of the appropriate diameter and an oligodendroglial process contacting it. The growing myelin membrane spreads lengthwise along the axon towards the nodes, and simultaneously commences to wrap around the axon. Initially, the membrane is loosely wrapped round the axon. The multilamellar system is formed as more vesicles flow into the oligodendrocyte process, thereby promoting its extension around the axon. The membrane will tighten as more lamellae are formed and as the axon diameter increases during growth.

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Hirano and Dembitzer (1967) postulated that adjacent lamellae could slide over one another thus enabling the sheath to expand to accommodate increases in axon diameter. For such a phenomenon to occur the bulk of the lipids of both the myelin sheath and the axolemma would have to be in a liquid-crystalline state. It is probable that this is the case for myelin lipids (Lee, 1975).

Finally, in myelination there must be some factor which is released to control the extent of thickening of the sheath. At present, this is unknown.

1.1.3 Morphology of Central Nervous System Myelin

The compact myelin sheath consists of from 5 to 20 lamellae surrounding axons of diameter greater than about 1 μm. The internodal length is directly related to the axon diameter. Central nervous system myelin consists of a repeating double bilayer arrangement, each bilayer having a width of 4,7 nm. The bilayers are separated by intermembrane fluid layers approximately 3 nm wide. The two surfaces of each bilayer are probably asymmetric, since different staining intensities are shown by the external surface apposition (intraperiod dense line) and the cytoplasmic surface apposition (major dense line) in electron micrographs. The dimension of 4,7 nm for bilayer width is too small to accommodate the long fatty-acyl chains of the sphingolipids in a rigid, extended all-trans form (Lee, 1975); however, if the majority of the myelin lipids exist in a liquid-crystalline state, the acyl

chains will have a less ordered structure and consequently the chain length will be shorter (Lee, 1975).

Fluidity in the lipid phase of myelin appears dependent on a large extent of hydration and on the presence of cholesterol. Cholesterol reacts selectively with sphingomyelin rather than glycerophospholipids, because, it is believed, the hydroxyl group of cholesterol is bound by hydrogen bonds to water molecules at the lipid bilayer surface. Sphingolipids have more associated water molecules at their hydrophobic-hydrophilic boundary than do glycerophospholipids. According to Pascher (1976) the hydrogen bond donor and acceptor properties of sphingolipids enable the formation of lateral networks of hydrogen bonds within the plane of the membrane.

Such bonding would increase membrane stability and reduce its permeability, the latter effect being relevant to the insulating properties of myelin. The additional stability afforded to the membrane might account partly for the slow turnover rates of its lipid and protein constituents which have been observed (Rumsby, 1978). Lipid comprises about 75% of the dry weight of purified myelin. The cholesterol:phospholipid:galactocerebroside ratio is approximately 2:2:1 in the central nervous system of different vertebrate species (Cuzner et al., 1965).

Myelin also contains proteins, including the Wolfgram protein, proteolipid protein, and basic protein which is specific to myelin. The basic protein of myelin is located at the cytoplasmic apposition.

Further evidence for this localization is provided by the observation that in the Shiverer mutant mouse there is no main dense line in compact myelin and basic protein is absent (Rumsby, 1978).

Myelin basic protein in its isolated, uncomplexed form, is susceptible to proteolysis by virtue of its open, ordered structure (Rumsby, 1978). In vivo, hydrophobic interaction between N-terminal sequences of the protein and lipid protects the protein against proteolysis (London et al., 1973). There may be ionic interaction with lipid at sites along the C-terminal section of the protein. Palmer and Dawson (1969) showed myelin basic protein complexed with triphosphoinositide, sulphatide and other anionic phospholipids in a biphasic system and bonding was dissociated at high ionic strength. London and Vossenberg (1973) have shown that acidic lipids and sulphatide protect myelin basic protein in the region of amino acid residues 20-113 from hydrolysis by trypsin.

The proteolipid protein is situated in the lipid phase of the bilayer and may be slightly exposed at the external apposition (Poduslo and Braun, 1975). It complexes with cholesterol, cerebrosides and phospholipids. Acidic phospholipids and sulphatides are thought to be of significance in ionic reactions with basic residues. Sabri et al., (1974) and Agrawal et al., (1976) have shown that the proteolipid protein is metabolically stable with a half-life of 188 days in the adult rat.

Infrared absorption spectra, proton magnetic resonance spectra and electron spin resonance data all indicate that apolar groups of lipids in myelin are in a fluid state, resulting from apolar associations between membrane lipids and proteins (Braun, 1977). Rumsby (1978) suggested that the cerebroside mixed with other lipids ensures an overall liquid crystalline state in the myelin membrane system.

It is now widely thought that myelin is a continuum of membranes of different lipid:protein ratios and a number of myelin fractions of different densities has been obtained by sucrose gradient centrifugation. It has been suggested that these fractions may represent either different areas of myelin sheath or different stages in maturation or differentiation of myelin (Benjamins et al., 1973, 1976; Matthieu et al., 1973; Agrawal et al., 1974; Waehneldt, 1978).

1.1.3.1 Fatty Acids of the Myelin Membrane

The fatty acids of myelin phosphoglycerides generally have greater amounts of monounsaturated, and lower amounts of saturated and polyunsaturated fatty acids than do those of grey matter (Norton, 1977). In the ethanolamine plasmalogens the acyl groups on the C-2 position of the phosphoglycerides are usually unsaturated, the major fatty acids being 18:1, 20:1, 20:4 and 22:4. Docosahexaenoic acid (22:6), which is the metabolic end-product of linolenic acid, is enriched in ethanolamine and serine phosphoglycerides (Kanoh, 1969, 1970; Trewhella and

Collins, 1969), however, it is much more prevelant in grey matter than in myelin. The majority of brain sphingolipid fatty acids are C24, with some C18, C22 and C26 (O'Brien and Rouser, 1964). Sphingomyelin does not contain α-hydroxylated fatty acids. Myelin sphingomyelin has large amounts of 24:0, 24:1 and 18:0, in contrast to sphingomyelin of other membranes where 18:0 is the principal fatty acid (Norton, 1977). The presence of the 24-carbon fatty acids increases the transition temperature of the sphingolipids thus hindering their exchange in a membrane system (Chapman, 1973). Saturated and mono-unsaturated fatty acids are not susceptible to attack by lipoxygenase or peroxidase and their high concentrations in myelin therefore help preserve the integrity of the membrane (Bourre et al., 1977).

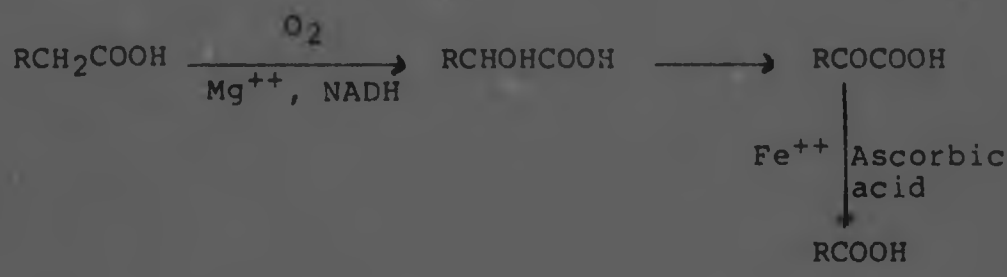
Cerebroside and sulphatide contain 24:0 and 24:1, and 24h:0 and 24h:1 (h = hydroxy) as their major fatty acids. 24:1 is greater in amount than 24:0 but 24h:0 is higher than 24h:1 (Norton, 1977).

Choline phosphoglycerides in myelin contain mainly 16:0, 18:0 and 18:1 fatty acids with few polyunsaturated fatty acids. Similarly, serine phosphoglycerides contain mostly 18:0 and 18:1 fatty acids with some 20:4 acid and traces of other polyunsaturated fatty acids.

The brain is capable of taking up both non-essential (Dopeshwarkar and Mead, 1969; Gozlan-Devilliere et al., 1976) and essential (Mohrhauer and Holman, 1953; Galli et al., 1972; Sun, 1972) fatty acids from the circulation.

Long-chain fatty acids can undergo elongation by brain microsomes by a malonyl CoA dependent process (Aeberhard and Menkes, 1968; Bourre et al., 1970; Brophy and Vance, 1975).

Fulco and Mead (1961), Mead and Hare (1971) and Hoshi and Kishimoto (1973) have described a reaction sequence for the one-carbon degradation of long-chain fatty acids via α-hydroxylation in the microsomal fraction of brain. The steps are as follows:



This enzyme system is most active for C22-C26 fatty acids (Hoshi and Kishimoto, 1973), although lignoceric acid (C24) is mainly degraded via β-oxidation (Seidel et al., 1975). Deficiency of phytanic acid alpha-oxidase in Refsum's disease causes central and peripheral nervous system demyelination (Herndon et al., 1969).

Fatty acids may be desaturated by the brain microsomal desaturase system, as in the production of γ-linolenoyl-CoA from linoleoyl-CoA. The former can be subsequently elongated to eicosatrienoyl-CoA (C20), and again desaturated to yield arachidonyl-CoA.

In 1959 Fulco and Mead proved that eicosatrienoic acid may be derived from oleic acid (18:1) through an enzyme reaction sequence apparently identical to that for arachidonate formation from linoleic acid.

Exchange of the fatty acyl moieties of various phospholipids, separately from the rest of the phospholipid molecule, has been described (Van den Bosch et al., 1968; Hill and Lands, 1970). Such exchange processes may contribute to the different kinetics of incorporation of radioactive precursors found in different molecular species.

The fatty acid composition of the myelin membrane alters with age. For example, in primate myelin (Sun and Samorajski, 1973) there is an increase in the proportion of 18:1 and 20:1 fatty acids in ethanolamine phosphoglycerides, and a corresponding decrease in the proportion of polyenoic fatty acids, with age. These age-related alterations in acyl group composition are not found in mouse brain (Sun and Samorajski, 1972). Cerebroside and cerebrotide in infant (human) white matter showed predominantly C16 and C18 acids with reduced amounts of odd-chain and hydroxy acids (O'Brien et al., 1964; Kishimoto and Radin, 1959) compared to the adult. The odd-chain and hydroxy fatty acids therefore accumulate in cerebroside and sulphatide with age.

Alterations or deficiencies in diet cause distortions of the normal fatty acid composition of the myelin membrane. In a study of rats maintained on a

fat-deficient diet from one week before birth until one year of age, Galli et al., (1971) observed an increase in trienes, and a very gradual increase of pentaenes, in myelin ethanolamine phosphoglycerides, with a corresponding decrease in tetraenes and, later, of hexaenes (Galli et al., 1971; Sun, 1972). In controls, the ratio of trienes to tetraenes was 1:20, and in one-year old fat deficient rats the ratio was 19,5:10 (ibid). Some reversal of this ratio occurred after the rats were returned to a fat-containing diet, which indicated that these polyenoic fatty acids were undergoing continuous turnover (Benjamins and Smith, 1977).

Bass et al., (1970) deprived neonatal rats of food for three weeks and found that even at 120 days of age cerebroside in myelin was still greatly reduced. Chase et al., (1967) found that the decreased rate of sulphatide synthesis caused by starvation of neonatal rats could only be corrected if re-feeding was commenced by ten days of age. In linoleic acid deficiency in mice, Allman and Gibson (1965) noted accumulation of eicosatrienoic acid.

1.1.4 Metabolism of Central Nervous System Myelin

Myelin that is first deposited in the brain has a different composition from that of the adult (Horrocks, 1968a; Cuzner and Davison, 1968; Dalal and Einstein, 1969; Norton and Poduslo, 1973b). Myelin galactolipids increase by approximately 50% and cholinephosphoglyceride decreases by about 50% in weight as the rat matures (ibid).

Polysialogangliosides decrease and the monosialoganglioside GM_1 increases to 90% of the total gangliosides, although the total ganglioside sialic acid remains constant (Suzuki et al., 1967). There is apparently a continual increase in the amount of myelin in the brains of mice and rats throughout their lifetimes (Horrocks, 1973). Norton and Poduslo (1973b) have shown that the amount of myelin per rat brain is a linear function of the logarithm of age at least up to 14 months, and that myelin deposition appears to be almost solely responsible for the continued increase in brain weight after about 100 days of age. For the rat, this linear function is exactly parallelled by the increase of cerebroside in whole brain.

It was formerly believed that myelin was metabolically inert and that, once deposited, its lipid composition was invariant. These hypotheses have been modified substantially in the light of more recent results (Davison, 1972).

Considerable data have accumulated indicating that in general, lipids of the adult nervous system do not undergo dynamic metabolism as is evident for most body constituents. Cuzner et al., (1965) Davison (1970), and Smith (1967) injected a wide range of radioactively-labelled precursors into young animals and the specific activities of labelled components in whole brain or in isolated subfractions were measured at intervals up to one year. At a long period after injection almost no radioactivity was lost from total lipid or total protein

fractions, or from phospholipids, sulphatide, cholesterol or proteolipid protein. Cuzner et al., (1965) interpreted these results as evidence that myelin is stable and is metabolized as a unit.

Conflicting results were obtained in some short-term studies, for example that by Horrocks, (1973), where the fate of ^{14}C-ethanolamine was followed from 1 to 7 days after its injection by the ventricular route into mice aged 150 days. He calculated a half-life of 3 days for ethanolaminephosphoglyceride in myelin. Ansell and Spanner (1968), injecting ^{14}C-ethanolamine intracranially into adult rats found a half-life of 21 days having followed the label for 63 days after injection. Jungalwala and Dawson (1971) found a similar half-life of 20 days after following the fate of the label for 120 days after intracranial injection into adult rats.

August et al., (1961) found that radioactivity was readily incorporated into the apparently inert myelin of adult animals, and Eichberg and Dawson (1965) showed that the polyphosphoinositides of myelin were quite active metabolically. Jungalwala and Dawson (1971) examined incorporation of ^{32} P-phosphate, U-^{14}C-glycerol, and ^{14}C-ethanolamine into microsomal and highly purified myelin fractions of adult rat brain. By 20 days after intracerebral injection the reduced specific activities of phospholipids in both fractions were similar, despite initial higher specific activities in microsomal phospholipids. They found half-lives of 6 days and 27

days, of the fast and slow components respectively, for glycerol, 6 days for the fast component of phosphate and 20 days for the half-life of ethanolamine, in myelin. Smith and Eng, (1965) showed that different myelin lipids turned over at different rates, lecithin had a half-life of 2 months, cerebrosides of more than one year.

The evidence for the metabolic stability of myelin and the long half-lives of its components comes mainly from long-term studies and, conversely, results indicating short half-lives come from shorter-term experiments.

Disappearance of labelled molecules from myelin appears to follow an exponential curve with rapid decay soon after maximum labelling is attained, followed by an ever- decreasing rate of decay. The nearer the time of maximal labelling and the shorter the time interval investigated then the shorter the half-life which is calculated (Kabara, 1973; Jungalwala, 1974a and 1974b).

In contrast with the results obtained by Jungalwala and Dawson (1971) and Horrocks (1973) for the half-life of ethanolaminephosphoglyceride, Smith and Eng (1965) found a half-life of 6 months injecting ^{14}C-glucose into adult rats. A possible explanation for the discrepancy may be that the ethanolamine moiety may exchange rapidly, separately from the rest of the phospholipid molecule, or re-utilization of the label from glucose into fatty acids may result in long apparent half-lives (Benjamins and Smith, 1977). Smith and Eng (1965) injected young animals with ^{14}C-acetate to label primarily nonpolar regions of

lipids. The half-lives of individual lipids were determined between 2 months and 2 years after injection. Cerebroside, sulphatide, sphingomyelin, cholesterol and ethanolaminephosphoglycerides were very stable with half-lives varying between 7 months and one year. Cholinephosphoglyceride and inositolphosphoglyceride had half-lives of 2 months and 5 weeks respectively. Similar results were obtained when ^{14}C-glucose was injected into adult rats (Smith, 1968) but turnover was measured from one day to 60 days after injection. Even with the reservations that acetate and glucose are extensively re-cycled, and that ^{14}C-glucose labels more than one part of a given molecule, the results show differences in the long-term metabolism of several phospholipids. The stability of the galactolipids agrees with the results of Davison (1970), when ^{35}S-sulphate was used to label cerebroside sulphate. Smith and Eng (1965) postulated that more stable lipids were complexed with cholesterol in the membrane, while cholinephosphoglyceride and inositolphosphoglyceride were more available to exchange with non-membrane-bound lipids.

From long-term studies of myelin metabolism, three observations may be made: i) some of the newly-synthesized molecules incorporated into myelin have apparent half-lives which are comparable with those of molecules in other membranes, while other newly-synthesized molecules exhibit long-term metabolic stability; ii) various lipids and proteins of myelin seem to leave the membrane at different rates, indicating that myelin is not degraded in

units during normal turnover, but that each individual component may leave the membrane independently of the others; iii) some of the components labelled during myelination appear metabolically more stable than the same components labelled in the adult. The validity of these conclusions depends on the assumption that the rate of disappearance of radioactivity from myelin represents the true half-life of the molecule in the membrane. It is known that in several studies long half-lives have been calculated, partly owing to re-utilization of certain precursors and perhaps partly to the unique anatomical and metabolic properties of myelin itself. The three properties which emerged from the long-term investigation await experimental confirmation using precursors presented as a pulse and known to undergo minimal re-cycling (Benjamins and Smith, 1977).

Exposure of the membrane components to enzymes occurs only in the cytoplasmic loops which may not be in equilibrium with larger pools in the oligodendrocyte cell body. The limited exchange which could occur may constitute long-term myelin turnover. There is considerable incorporation of newly-synthesized molecules into myelin even in adults, and metabolic studies indicate that a portion of the added material in both young and adult animals undergoes fairly rapid turnover. The metabolically stable portion may not be accessible to exchange or degradation, or it may be recycled within the myelin membrane. Synthesis and exchange of materials may

occur in the cytoplasmic inclusions, and in the Schmidt-Lantermann incisures. Bretscher (1973) found that choline phosphoglyceride was enriched on the external surface of the erythrocyte membrane and ethanolamine phosphoglyceride was enriched on the internal surface. The distribution of these two phospholipids in myelin is not known. Differences in their half-lives may reflect different membrane locations for choline- and ethanolaminephosphoglycerides (Benjamins and Smith, 1977).

If the carbohydrate moieties of cerebroside and sulphatide were located on the external surface of the membrane, their acyl groups would be less exposed to enzymes involved in lipid metabolism and this might help to explain the long half-lives of these molecules (Benjamins and Smith, 1977).

Miller and Dawson (1972) showed that guinea-pig brain contained a non-dialysable, heat-labile, macro-molecular factor in the cytosol which was capable of exchanging phospholipids between microsomes and mitochondria. Harvey et al., (1973) found brain lipoproteins, capable of catalyzing the exchange of cholinephosphoglyceride and inositolphosphoglyceride between mitochondrial and microsomal membranes, in the soluble fraction of bovine brain. Benjamins and McKhann (1973b) demonstrated the presence of lipoproteins binding cholinephosphoglyceride and ethanolaminphosphoglyceride in the supernatant fraction of rat brain. Sulphatide synthesized in vitro is transferred to myelin from the microsomal fraction and the

exchange is enhanced by the presence of a supernatant fraction from brain (Herschkowitz et al., 1968).

The chief site of phospholipid synthesis in brain subcellular fractions is the microsomes. Myelin has little, if any, ability to synthesize phospholipid (Miller and Dawson, 1972a). Therefore myelin synthesis must involve considerable transfer of phospholipids between microsomal membranes and myelin. Anionic lipids, such as inositolphosphoglyceride, in membranes inhibit phospholipid exchange by binding the exchange protein (Machida and Ohnishi, 1978). This inhibition can be overcome by the presence of polyvalent cations (Wirtz et al., 1976). Eichberg and Dawson (1965) have suggested that, in isolated myelin, lipids are present as Ca^{++} or Mg^{++} salts, therefore anionic phospholipid inhibition would not account for the low exchange of phospholipid between myelin and other membranes found in vitro.

The multilamellar structure of myelin may limit its capacity for phospholipid exchange (Brammer, 1978). Barkusov et al., (1974) have shown that in sonicated cholinephosphoglyceride liposomes only those phospholipids in the outer face of the bilayer are readily exchangeable. Wirtz et al., (1976) showed that phospholipid exchange between mitochondria and unsonicated liposomes was slower than that between mitochondria and sonicated liposomes, which have a greater total surface area.

Carey and Foster (1977) have suggested that only

15-20% of the cholinephosphoglyceride in myelin is accessible to the phospholipid exchange protein in brain supernatant.

1.1.4.1 Enzymes Associated with the Myelin Sheath

Leucine aminopeptidase was recovered in myelin by Adams and Glenner, (1962) and confirmed by Banik and Davison (1969) although the latter authors found most activity in the cytosol fraction. Marks (1972) showed that an aminopeptidase hydrolysing the leucine-glycine-glycine sequence increases in myelin with increasing purification. Riekkinen and Rumsby (1969) and Rumsby et al., (1970, 1973) and Frey et al., (1971) found a small amount of non-specific esterase activity at low salt concentration; when salts were present, high values of mitochondrial and lysosomal enzymes were also found (Mitzen et al., 1974). These two enzymes are not myelin-specific.

Kurihara and Tsukada (1967) first demonstrated 2',3'-cyclic nucleotide 3'-phosphohydrolase (2',3'-CNP) in myelin. Its increase in brain and spinal cord parallels myelination (Kurihara and Tsukada, 1968). Olafson et al., (1969) proved that it is a myelin enzyme. These authors found that isolated myelin contains approximately 60% of whole brain activity (Olafson et al., 1969; Kurihara et al., 1971). 2',3'-CNP activity is also found in erythrocyte membranes (Sudo et al., 1972). Activity is low in peripheral nervous system myelin and in peripheral nerve (Braun and Barchi, 1972; London, 1972; Uyemura et

al., 1972). These factors have suggested that the enzyme is widely distributed in mammalian plasma membranes even if in co centrations lower than in myelin.

The function of the enzyme is unknown - no 2',3'-cyclic nucleotides or 2'-nucleotides appear to be present in the free nucleotide pools of those tissues with 2',3'-CNP activity. No other phosphodiester bonds in phosphorylated sugars, lipids or inositols are hydrolysed by the enzyme. The enzyme has no effect on phosphomonoesters. The possibility of a protein- or lipid-linked cyclic nucleotide serving as a substrate nas not been fully explored (Norton, 1977).

The taurocholate-activated cholesterol ester hydrolase with a pH optimum of 7,2 appears to be localized in myelin; it constitutes 70-80% of whole brain activity (Eto and Suzuki, 1971, 1973). It also has no known function in myelin. Costantino-Ceccarini and Suzuki (1975) have presented evidence that an appreciable proportion of UDP-galactose:ceramide galactosyl transferase activity is present in myelin.

Myelin contains cAMP-dependent and -independent protein kinases which phosphorylate myelin basic protein *in vitro* (Miyamoto and Kakiuchi, 1974; Carnegie *et al.*, 1974; Steck and Appel, 1974; Miyamoto, 1975). Basic protein phosphorylation *in vivo* can be demonstrated by injecting labelled phosphate into rat brain (Miyamoto *et al.*, 1974; Miyamoto and Kakiuchi, 1974; Steck and Appel, 1974). However, there is less than 1mol of phosphate per

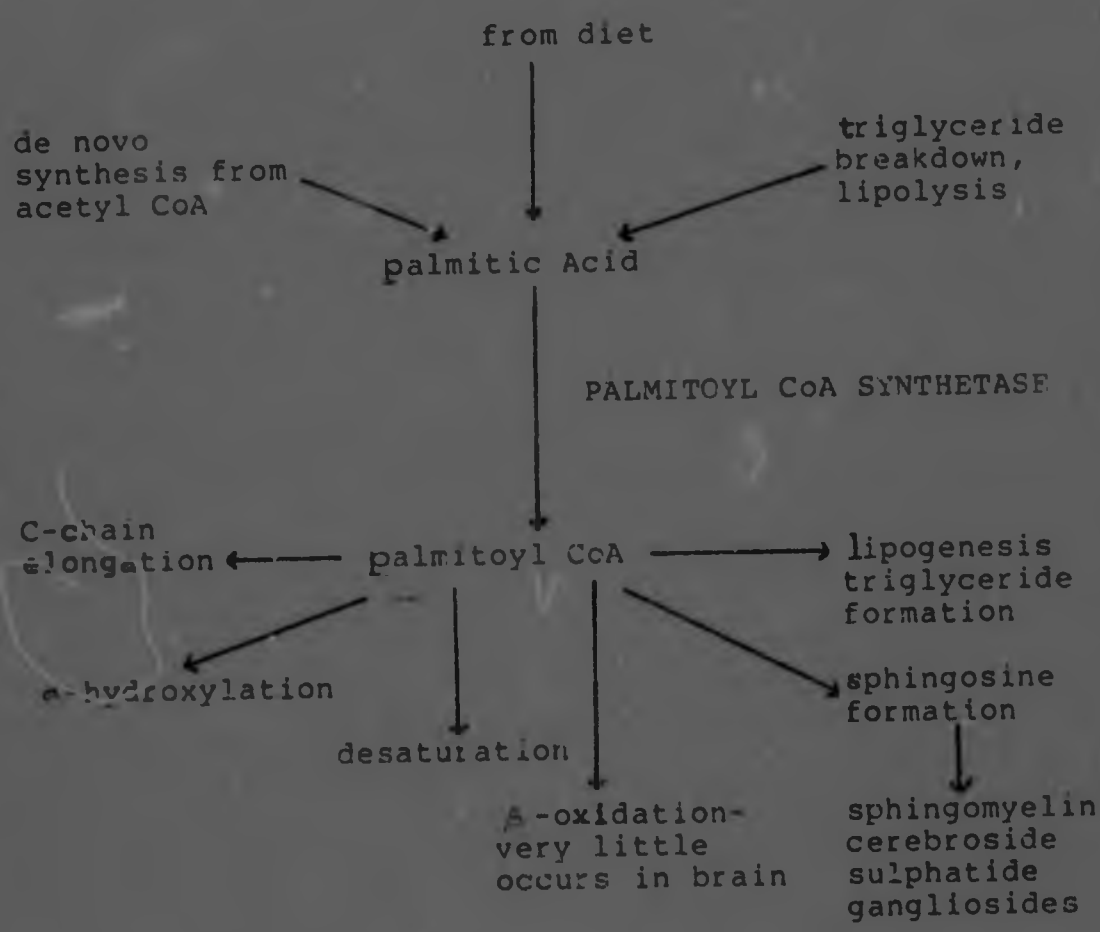
mol of basic protein *in vivo*, or per mol of basic protein phosphorylated *in vitro* by endogenous kinase. Exogenous kinases can phosphorylate basic protein much more extensively. There is also an endogenous phosphoprotein phosphatase in myelin which acts on the phosphorylated basic protein (Miyamoto and Kakiuchi, 1975). It is not known whether the kinase and the phosphatase are myelin specific.

1.2 Palmitoyl CoA Synthetase E.C. 6.2.1.3

In all tissues the enzyme palmitoyl CoA synthetase supplies long chain acyl-CoA esters for i) synthesis of triglycerides and phospholipids; ii) elongation of fatty acids and iii) β-oxidation and desaturation (Fig. 1). In the adult animal, the rate of *in vivo* fatty acid oxidation is low in brain mitochondria, as measured by the oxidation of radioactive fatty acid to $^{14}CO_2$ (D'Adamo, 1971; Carey, 1975). The role of palmitoyl CoA synthetase in brain mitochondria is unclear, since although the mitochondria are capable of fatty acid oxidation *in vitro*, the measured respiratory quotient of almost 1 for brain tissue would implicate carbohydrate as opposed to lipid as the major energy source. Cantrill (1978) suggested that during myelination the enzyme might be involved in acetyl CoA-dependent fatty acid elongation in mitochondria, by activating fatty acids to the CoA form prior to elongation.

FIGURE 1

The Role of Palmitoyl CoA Synthetase in Fatty Acid Metabolism in Brain



1.2.1 Subcellular Location of Palmitoyl CoA Synthetase

Palmitoyl CoA synthetase was first demonstrated in guinea-pig and rat liver microsomes by Kornberg and Pricer (1953). Subsequently, Yates et al., (1966) and Farstad et al., (1967) found that the enzyme was present in liver mitochondria also. Pande and Mead (1968) recovered about 60% of the total activity of rat liver in their cell debris plus nuclei fraction; on further purification of this fraction the activity was found to be associated with the cell-membrane rich non-nuclear fraction. Cantrill (1978) found that up to 79% of the total rat brain activity of the enzyme was present in his crude F1 fraction (plasma membrane, nuclei and heavy myelin fraction); however, up to 60% of this activity was attributable to microsomal and mitochondrial contamination. Nevertheless, about 30% of the total activity remained associated with the myelin fraction and could not be removed by repeated density gradient centrifugation. He suggested that this activity could be associated with a membrane fraction of similar bouyant density to myelin.

1.2.2 Factors Affecting Palmitoyl CoA Synthetase Activity - Stimulators of Activity

The presence of phospholipid, namely cholinephosphoglyceride and ethanolaminephosphoglyceride, in the microsomes was found to enhance palmitoyl CoA synthetase activity (Pande and Mead, 1968). Bar-Tana et

al., (1971), also found cholinephosphoglyceride to be stimulatory and suggested that this was a result of its detergent-like effects on the enzyme system.

Pande and Mead (1968) found that, up to a concentration of 0,04M, phosphate did not stimulate palmitoyl CoA synthetase activity in rat liver microsomes, in contrast to the effect of phosphate on the adipose-tissue enzyme. Cantrill and Carey (1975) showed that phosphate present in the homogenizing medium increased brain palmitoyl CoA synthetase activity.

Relatively high salt concentrations in the assay system have been reported to enhance activity (Farstad, 1968; Aas, 1970; Pande, 1972; Groot and Hülsmann, 1973; Tanaka et al., 1979).

There have been contradictory reports about the presence of a stimulating factor in the supernatant fraction. Farstad et al., (1967) found that the addition of cytosol to the assay system markedly enhanced activity of palmitoyl CoA synthetase and proposed the existence of a kinase in the supernatant fraction which phosphorylated the enzyme to an active form (Farstad, 1968). However, Aas (1971a) obtained conflicting results with the addition of supernatant.

ATP has a stabilizing effect on palmitoyl CoA synthetase (Farstad, 1968; Aas, 1972; Pande, 1972; Sanchez et al., 1973), which Groot et al., (1976) thought might be explained as a protection against endogenous proteolysis. Rose et al., (1979) have demonstrated that uncomplexed ATP

stabilising palmitoyl CoA synthetase against ageing denaturation. ATP protects 3 out of 6 available thiol groups of the enzyme protein against sulphydryl reagents such as 5,5' dithiobis (2-nitrobenzoic acid) (unpublished data, Ross et al., 1970). Addition of Mg^{++} substantially reduces this effect.

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Inhibitors of Palmitoyl CoA Synthetase

Adenosine triphosphatases, lipases and enzymes which destroy CoASH interfere with palmitoyl CoA synthetase activity (Skrede, 1973). In the coupled assay system of the enzyme using carnitine palmitoyl transferase, acyl carnitine hydrolase also interferes with the determination of enzyme activity.

The binding of fatty acids to proteins or lipids in the assay system strongly interferes with Km determinations (Groot et al., 1976).

Triton X-100 has been reported to inhibit microsomal palmitoyl CoA synthetase at a concentration of 0,2% (Pande and Mead, 1968), whereas Bar-Tana et al., (1971) found that at a concentration of 0,1%, Triton X-100 was not inhibitory. Aas (1971) also found no inhibition at 0,1% concentration.

1.3 Brain Subcellular Fractionation

Central nervous tissue is very heterogeneous with respect to cell types, of which the chief are neuronal and glial cells. When gentle homogenization is used on brain

tissue, synaptosome formation occurs. The material, mitochondria and cytosol, occluded in the synaptosomes is best released by hypo-osmotic shock (Whittaker et al., 1964), freezing and thawing being much less effective (Johnson and Whittaker, 1963). Sonication of brain tissue causes the formation of membrane fragments so small that they vesiculate spontaneously with consequent occlusion of material. Because of the greater complexity of nervous tissue, the fractions yielded by the differential centrifugation scheme developed for liver are more heterogeneous. These initial fractions may subsequently be separated by density gradient centrifugation.

As regards myelin, the strength of homogenization determines the size of the myelin vesicles which will be formed, which ultimately determines the distribution of myelin between the mitochondrial and nuclear fractions. If ions are present in the original homogenizing medium and mild conditions are used, the myelin sheath is preserved on the axon and the "crude myelin layer" will consist of myelinated axons (Marchbanks, 1975). Microsomal membranes appear to be a major contaminant of myelin (Marchbanks, 1975), as is axoplasma trapped during homogenization. Media of high ionic strength should not be used as this promotes coacervation of the particles which are then difficult to separate on the gradient (Marchbanks, 1975).

1.3.1 Marker Enzymes in Brain Subcellular Fractions

In order to assess the relative purity of any subcellular fraction, the biochemical and morphological characteristics need to be determined. The most satisfactory evaluation of biochemical "purity" is to obtain all subcellular fractions and assay each one for the purpose of a specific enzyme activity. In this way, and by employing a variety of enzyme markers, each specific for different fractions, the percentage recoveries of enzyme activity and enzyme distribution are obtained. By using an enzyme known to be localized primarily in one fraction the extent of its contamination of other subcellular fractions can be assessed. Once the nature and degree of contamination of the myelin fraction is known, this can be considered in any estimation of palmitoyl coenzyme A synthetase activity.

The biochemical analyses are best correlated with morphological evidence. Electron micrographs of myelin fractions provide visual confirmation of biochemical data.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Radiochemicals

D L-[Methyl-^{3}H] carnitine hydrochloride (1.7 Ci/ mmol specific activity) was purchased from The Radiochemical Centre, Amersham, Bucks. [^{3}H] Coenzyme A was purchased from New England Nuclear, Boston, Mass. U.S.A.

2.1.2 Other Chemicals

DL carnitine HCl, 5,5'-dithiobis-(2-nitrobenzoic acid), NADPH tetrasodium salt, type III, butyrylthiocholine-iodide Grade II, Coenzyme A from yeast Grade 1-S sodium salt and βNAD from yeast Grade III were purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A. Alkaline phosphatase from calf intestine, pyruvate kinase, fructose-1,6-diphosphate, phosphoenol pyruvate, NADH disodium salt Grade II, adenosine-2',3'-monophosphate (cyclic), adenosine-5'-diphosphate disodium salt, acetylthiocholine iodide, and cytochrome C from horse heart, salt free, were purchased from Boehringer Mannheim GmbH, Mannheim, West Germany. Bovine albumin fraction V was obtained from Miles Laboratories Inc., U.S.A. Scintillant was Aquagel I from Chemlab (Pty) Limited, Johannesburg, R.S.A. Sephadex G-25 (coarse) was obtained from Pharmacia, Uppsala, Sweden. All other chemicals were of Analar (or Aristar) grade and were purchased from British Drug Houses. Poole,

U.K., and Merck, Darnstadt, West Germany. Ovalbumin was prepared from egg white. Ferrocytochrome C was prepared by adding a few grains of solid sodium dithionite to a solution of cytochrome C, followed by column filtration of the mixture through Sephadex G-25 (coarse). Millipore GSWP 025 filters were purchased from Millipore Corporation, Bedford, Mass, U.S.A. Nonidet P40 (NP 40) was a gift from Shell, Johannesburg, R.S.A. Calf liver was supplied gratis by the City Deep Abattoir, Johannesburg, R.S.A.

2.1.3 Animals

These were adult Wistar rats of over 170 gm in weight from the Physiology Department Animal House, University of Witwatersrand Medical School.

2.2 Methods

2.2.1 Preparation of Brain Subcellular Fractions

Animals were killed by cervical dislocation. The whole brain was rapidly removed into cold homogenizing medium, either 0,3M Sucrose or 0,3M sucrose/0,25M potassium phosphate, pH 6,8. The brain was weighed and homogenized in 6 volumes (w:v) of homogenizing medium using 15 up and down passes of a motor-driven Potter-Elvehjem homogenizer with a teflon pestle, clearance 0,1 mm at 1200 rev. per min.

The scheme of subcellular fractionation used (Fig. 2) was a modification (Lapetina et al., 1969) of the method of Gray and Whittaker (1962), which gives all subcellular

fractions. In the Lapetina modification, cytosol, mitochondria and microsomes trapped in the first slow-spin pellet are released by washing four times for 8000 gmin. With this fractionation scheme the following subcellular fractions were obtained:

from the nuclear spin - nuclei, cell debris and large myelin fragments (F1)

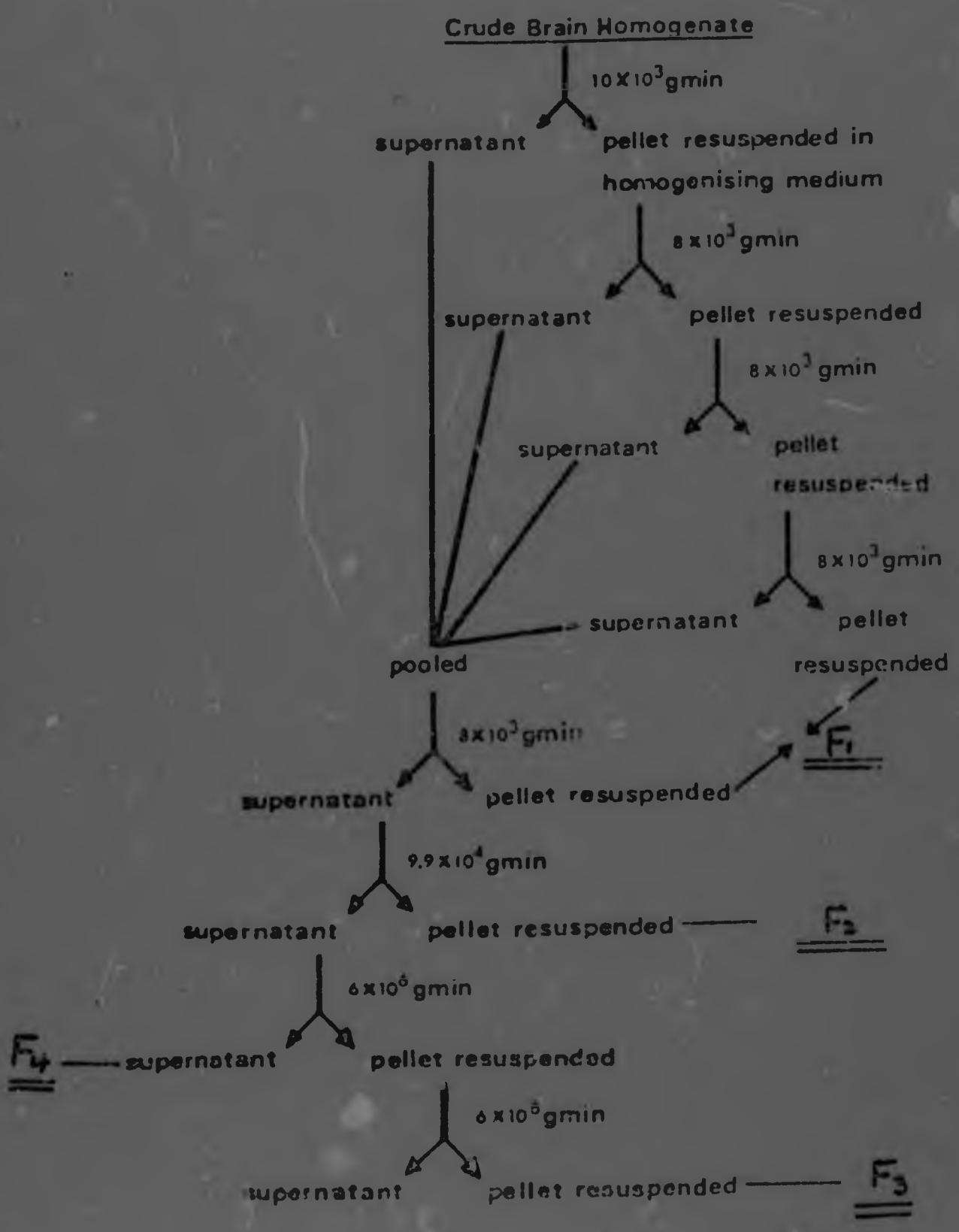
from the mitochondrial spin - mitochondria, small myelin fragments, large membrane fragments, lysosomes and "synaptosomes" (F2)

from the post-mitochondrial spin - endoplasmic reticulum (microsomes), ribosomes, vesicles and small membrane fragments (F3)

- cytosol (F4)

FIGURE 2 Scheme of Sub-cellular Fractionation

(from Cantrill, 1978)



Fractions F1 and F2 were obtained using the MSE HS 25 or the Beckmann J-21 and L5-65B centrifuges; either the Beckmann L5-65B or the MSE HS 50 centrifuge was used for the preparation of the F3 and F4 fractions. The F1 and F2 fractions were subjected to hypo-osmotic shock by suspending the pelleted fractions in ten volumes of distilled water for one hour. All operations in the preparation of subcellular fractions were performed on ice and refrigerated centrifuges were used.

2.2.2 Sucrose Density Gradient Centrifugation

Linear density gradients of either sucrose or sucrose/phosphate solutions were formed using a Gilson 3-way peristaltic pump. The gradients were formed at room temperature using 0,5M sucrose as the initial solution and 1,5M sucrose as the limiting solution, or, in the case of sucrose/phosphate, 0,5M sucrose containing 0,25M potassium phosphate pH 6,8 and 1,5M sucrose containing 0,25M potassium phosphate pH 6,8 as the respective solutions. The gradients were of 12 ml volume and were spun in swing-out rotors in the Beckman L5-65 or MSE HS 25 centrifuges (Rotors SW 27,1 and 6x15ml respectively). The gradients were left to equilibrate for 1-2 hours before use. The pellets of the F1 and F2 osmotically-shocked fractions were resuspended in 0,3M sucrose or 0,3M sucrose/0,25M potassium phosphate pH 6,8 to a concentration of about 100-120 mg protein per ml, and 1 ml of resuspended fraction was loaded on to a gradient.

The gradients were centrifuged at $4,5x10^6$ g min.

After centrifugation the bands were removed by Pasteur pipette. When bands were close together cross-contamination was unavoidable. The fractions from the gradients were diluted to a sucrose concentration of approximately 0,3M and stored at -20°C. Generally, three bands were obtained from both the F1 and F2 gradients and the bands were numbered from the top of the gradient thus, F11, F12, etc. The supernatants from the osmotic shocking were designated F1S and F2S respectively.

2.2.3 Preparation of Palmitoyl Coenzyme A Carnitine Palmitoyl Transferase E.C. 2.3.1.2

This enzyme was prepared from calf liver according to the method of Norum (1964). The resuspended washed mitochondrial pellet (concentration approximately 25mg/ml) was purified by the method of Sanchez et al., (1973).

2.2.4 Preparation of Potassium Palmitate Solution

Sufficient palmitic acid to yield a final potassium palmitate solution of 2mM was dissolved using 2mM potassium hydroxide and NP 40 to give a final concentration of 1%. The pH of the potassium palmitate solution was 8,0; it was stored at 4°C and warmed to 30°C before use.

2.2.5 Preparation of Palmitoyl-D,L-Carnitine

This was prepared from palmitoyl chloride and D,L carnitine. Palmitoyl chloride was prepared by refluxing 500 mg of palmitic acid with 15 ml re-distilled thionyl chloride for 30 min at 77°C.

The excess unreacted $SOCl_2$ was blown off with dry N_2 until a constant weight was reached. The palmitoyl chloride thus obtained was used to prepare palmitoyl carnitine using the method of Bhomer and Bremer (1968). The palmitoyl carnitine was subjected to thin layer chromatography and melting point determination to establish purity.

2.2.6 Determination of Protein

Protein was determined according to the method of Lowry et al., (1951) using bovine albumin fraction V as standard.

2.2.7 Determination of Radioactivity

Radioactive solutions (200 µl volume) of ammonium sulphate-saturated isobutanol or Millipore GSWP filters were counted in 10 ml of Aquagel I in a Tricarb AAA liquid scintillation spectrometer. A quench curve was generated using sealed standards of increasing chloroform concentration and the external standard ratio.

2.2.8 Determination of Inorganic Phosphorus

This was done in one of three ways, i) using the

enzymatic assay of Scopes, (1971) which is accurate to 1μmol of phosphate, ii) employing the method of Chen et al., (1956) which is more sensitive, detecting down to 0,05 μmol of phosphate, iii) by the micromethod of Berenblum and Chain (1938) as modified by Martin and Doty (1949), also accurate to 0,05 μmol of phosphate.

2.3 Assay of Enzyme Activity

2.3.1 Palmitoyl Coenzyme A Carnitine Palmitoyl Transferase E.C. 2.3.1.2

This enzyme was assayed by the method of Solberg (1972). The palmitoyl carnitine was extracted using ammonium sulphate-saturated, H_2O-saturated, isobutanol. The enzyme exhibited the same activity over the pH range 7,5 - 7,8. The enzyme catalyses the following exchange reaction:

Palmitoyl carnitine + CoASH + ^{3}H-carnitine $\longleftrightarrow$

palmitoyl CoA + carnitine + ^{3}H-carnitine $\longleftrightarrow$

palmitoyl ^{3}H-carnitine + CoASH + carnitine

2.3.2 Palmitoyl Coenzyme A Synthetase E.C. 6.2.1.3

The first method employed to assay this enzyme was that of Farstad et al., (1967). The product of this enzyme reaction, palmitoyl CoA, is exchanged by palmitoyl CoA carnitine palmitoyl transferase, to give palmitoyl carnitine which undergoes the same exchange involving ^{3}H-carnitine shown earlier to yield palmitoyl

^{3}H-carnitine. The use of the non-ionic detergent NP 40 was found to facilitate solubilization of the potassium palmitate substrate and to yield enhanced enzyme activity.

The second method of assaying this enzyme was that of Murphy and Spence (1980) using [^{3}H(G)] coenzyme A in a modification of Polokoff and Bell's Millipore filtration assay (1975). NP 40 was used instead of Triton WR-1339.

The reaction catalysed by palmitoyl CoA synthetase is as follows:

$$\text{palmitic acid} + \text{CoASH} + \text{ATP} \xrightarrow{Mg^{++}} \text{palmitoyl CoA} + \text{AMP} + \text{PPi}$$

2.3.3 NADPH Cytochrome C Reductase E.C. 1.6.2.3

This enzyme was used as a microsomal marker and was assayed using the method of Sottocasa et al., (1967) as modified by McMurray and Dawson (1969) who used the non-ionic detergent Nonidet P40 (NP 40) to solubilize the membranes. The assay was performed on fresh brain fractions.

2.3.4 Ferrocytochrome C Oxidase E.C. 1.9.3.1

This enzyme was a mitochondrial marker. The assay method was that of Rascati and Parsons (1979), and only fresh brain fractions were assayed.

2.3.5 Acetylcholinesterase E.C. 3.1.1.7

This enzyme was employed as a synaptosomal membrane marker using butyrylthiocholine iodide as control

substrate. The method used was that of Ellman et al., (1961).

2.3.6 2',3'-Cyclic Nucleotide 3'-Phosphohydrolase E.C.3.1.4.37

This enzyme is specific to myelin. The activity was determined by the method of i) Olafson et al , (1969) which is a modification of the method of Drummond et al., (1962), or ii) Prohaska et al., (1973). Inorganic phosphate was determined as described above.

2.3.7 5'-Mononucleotidase E.C. 3.1.3.5

The method of Campbell (1962), was used. The enzyme is located in the microsomal fraction but is also used as a cell-membrane marker in sub-cellular fractionation.

2.3.8 Lactate Dehydrogenase E.C. 1.1.1.27

The enzyme was employed as a cytosolic marker and was assayed by the method of Bergmeyer and Bernt (1974). Determinations were performed on fresh material.

3. RESULTS

The results are presented in three sections. The first section gives the protein distribution throughout the subcellular fractions prepared. In the second section the distributions of the various marker enzyme assays are given. The third section examines the distribution of palmitoyl coenzyme A synthetase activity in sucrose and sucrose/phosphate brain fractions.

3.1 Subcellular Fractionation

The method of subcellular fractionation used (Gray and Whittaker, 1962; Lapetina et al., 1969) yielded the following major fractions:

- from the nuclear spin - nuclei, cell debris, and large myelin fragments - designated the F1 fraction;
- from the mitochondrial spin - mitochondria, small myelin fragments, large membrane fragments, lysosomes and "synaptosomes" - designated the F2 fraction;
- from the post-mitochondrial spin - (i) endoplasmic reticulum (microsomes), ribosomes, vesicles and small membrane fragments - designated the F3 fraction; and (ii) cytosol (supernatant) - designated the F4 fraction.

The F1 and F2 fractions were subjected to hypo-osmotic shock followed by density gradient centrifugation, and from each three subfractions were obtained as follows:

from the F1 gradient - an upper band, fraction F11
- a lower band, fraction F12
- a pellet, fraction F13

The supernatant from the osmotically shocked F1 fraction was designated F1S. Similarly, the F2 fraction yielded an upper and a lower band and a pellet, which were designated F21, F22 and F23 respectively. The supernatant from the hypo-osmotic shock of fraction F2 was denoted by F2S.

The F11 and F21 fractions are the heavy and light myelin fractions, respectively. The F12 fraction contained plasma membrane fragments and some myelin. The F23 pellet was nuclei. F22 was mitochondria and plasma membranes, possibly synaptic membranes, and F23 contained mitochondria. On the F2 gradient, there was always a diffuse opaque layer above the lower band, which was removed together with the lower band. This opaque layer may have been synaptosomes.

In brains homogenized in sucrose/phosphate, the pattern of bands on the gradients was similar, except that there was in addition a diffuse opaque layer above the lower band of the F1 gradient. This diffuse layer was removed with the lower band.

It was observed that brains homogenized in sucrose/phospate gave larger F1 pellets and smaller F2 and F3 pellets, than brains homogenized in sucrose. The protein distributions were similarly altered. Thus, the percentages of total protein in the F1 fractions from brains homogenized in sucrose/phosphate and in sucrose, respectively, were 47,8% and 28,7%. The F2 of brains homogenized in sucrose/phosphate contained 13% of the

total protein, in contrast to sucrose-homogenized F2 which had 37,4% of the total protein. The microsomal fraction (F3) had 1,7% of the total protein in sucrose/phosphate-homogenized brains and 4,6% in sucrose-homogenized brains. The gradient subfractions also had different protein distributions. In sucrose/phosphate brains, the F1S fraction constituted 59,5% of the F1 protein, whereas in sucrose brains the F1S was 39,9% of the F1 protein. The F21 fraction had 11,6% of the F2 protein in sucrose/phosphate brains and 24,0% of the F2 protein in sucrose brains. These results are listed in Tables 1 and 2 on pages 53 and 54, respectively.

3.2 Marker Enzyme Distribution in Subcellular Fractions

The subcellular fractions were assayed for various marker enzyme activities in order to assess the degree of contamination of the myelin fractions F11 and F21 by other subcellular components. NADPH cytochrome C reductase was employed as a microsomal marker. Ferrocytochrome C oxidase was a mitochondrial marker. Acetylcholinesterase was used as a marker for synaptosomes. 2', 3'-cyclic nucleotide 3'-phosphohydrolase was a myelin marker. 5'-mononucleotidase was used as a plasma membrane and microsomal marker. Lactate dehydrogenase served as a marker for cytosol.

All enzyme assays were performed using the optimum protein concentrations for each fraction.

3.2.1 NADPH Cytochrome C Reductase Distribution (E.C. 1.6.2.3)

This assay was performed on freshly-prepared brain and liver fractions because freezing for 18 hours at -20°C was found to cause about 80% reduction in activity. The use of Nonidet P40 enhanced activity (Miller and Dawson, 1972) approximately two-fold (Sottocasa et al., 1967). The specific activity of the F3 fraction in sucrose-homogenized brains was 101,7 n mol per min per mg protein. In sucrose/phosphate-homogenized brains the specific activity was 96,2 nmol per min per mg protein. In rat liver microsomes the specific activity was 232,9 nmol per min per mg protein. The distribution of activity throughout fractions prepared from brains homogenized in sucrose is shown in Table 3 on page 55. Table 11 on page 63 gives the distribution in fractions prepared from brains homogenized in sucrose/phosphate. The value for the F3 (microsomal) activity in liver is given in Table 12 on page 63. 43,5% of the total F1 activity was present in F11 in sucrose-homogenized brains, and 30,7% of the F1 activity was present in the F11 in sucrose/phosphate- homogenized brains. 59,6% of the F2 activity was in the F21 fraction in sucrose brains, and in sucrose/phosphate brains, 14,1% of the F2 activity was in F21. The F1 activity in sucrose/phosphate brains was 48,5% of the total activity, in contrast to sucrose brains, where it was 36,2%. The F2 activity in sucrose/phosphate brains was 16% of the total, and in sucrose brains it was 31,5%. The F3 activity in

sucrose/phosphate brains was 4,9% of the total activity and in sucrose brains it was 12,6%.

3.2.2 Ferrocytochrome C Oxidase Distribution (E.C. 1.9.3.1)

As freezing at -20°C for 18 hours caused a 90% loss in activity of this enzyme, assays were always performed on fresh material. The yields from the density gradient fractions were low and therefore two experiments were done to determine whether osmotic shock had a detrimental effect on enzyme activity. The crude F2 fraction was fractionated on sucrose density gradient without prior osmotic shocking. The fractions obtained from these gradients were designated F21NOS, F22NOS and F23NOS and corresponded to the upper and lower bands, and pellet, respectively, of the the gradients from non-osmotically shocked F2 fractions. The results are shown in Table 4 on page 56. The total gradient recovery of fraction F2 was 117,2% with the non-osmotically shocked F2, compared with 55,9% recovery from the osmotically shocked F2 gradient. Thus it appears that hypo-osmotic shock reduces the activity of this enzyme by about 50% according to the results obtained for two experiments. The highest specific activity is in the F23 fraction, 1 µmol per min per mg. The highest specific activity in the non-osmotically shocked F2 subfractions was in F22NOS, 0,8 µmol per min per mg. It is evident that this is a less pure mitochondrial fraction than F23. F11 contained 20,3% of the F1 activity, while F21 contained 10,8% of the F2

activity. There was 33,5% of the F1 activity in the F1S. The presence of this mitochondrial activity could be ascribed to the fact that, after hypo-osmotic shock, the F1 fraction, suspended in distilled water, was centrifuged at 8×10^3 g min, that is, the usual nuclear spin, which would not pellet mitochondria. In contrast, there was no detectable enzyme activity in the F2S, which was to be expected, since the osmotically shocked F2 fraction was spun at $9,9 \times 10^4$ g min, that is, a mitochondrial spin.

3.2.3 Distribution of Acetylcholinesterase (E.C. 3.1.1.7.)

This enzyme was estimated by subtracting the activity with butyrylthiocholiniodide as substrate from the total cholinesterase activity with acetylthiocholiniodide as substrate. The results are shown in Table 5 on page 57. The F2 fraction had 32,3% of the total activity. The highest specific activities of gradient fractions were in fractions F22 and F23 with values of 0,134 and 0,177 µmol per min per mg protein, respectively. These fractions contained mitochondria and possibly synaptosomes, although synaptosome formation is not promoted by the subcellular fractionation scheme employed. The homogenizer clearance was 0,11 mm, which is less than half the optimal clearance (0,25 mm) for synaptosome formation (Marchbanks, 1975). Further, hypo-osmotic shock disrupts synaptosomes. The highest specific activity was in the microsomal fraction, F3, 0,263 µmol per min per mg protein. It has been suggested that <u>in vivo</u> acetylcholinesterase is soluble in the

terminal cytoplasm but that at low ionic strength it binds somewhat non-specifically to membranes (Marchbanks, 1975). This would perhaps account for the activity found in the F3 fraction. The F11 and F21 myelin fractions had 4,1% and 4,6% of the activities of the F1 and F2 fractions respectively.

3.2.4 Distribution of 2', 3'-Cyclic Nucleotide 3'-Phosphohydrolase (E.C. 3.1.4.37)

The subcellular distribution of this enzyme is shown in Table 6 on page 58. The two myelin fractions, F11 and F21 had the highest specific activities, with values of 31 and 38 µmol per min per mg protein, respectively. The F3 fraction contained 1,2%, and the F4 fraction 3,4% of the total activity. No activity was detected in the F13 (nuclei) fraction. 44,9% of the F1 activity was in F1S. This loss of myelin fragments to the supernatant after osmotic shocking must be considered when estimating palmitoyl coenzyme A synthetase activity in the F11 fraction. The F22 had 73,7% of the F2 activity and the F2S had 10,4% of the F2 activity. Fraction F12 had 11,9% of the F1 activity, whereas fraction F22 had only 1,8% of the F2 activity. The F23 fraction had 0,1% of the F2 activity.

3.2.5 Distribution of 5'-Mononucleotidase (E.C. 3.1.3.5)

The highest specific activity of this enzyme was found in the microsomal fraction and in the F21 myelin fraction, with values of 70 and 72 nmol per min per mg

protein, respecti ly. These results indicated microsomal and plasma membrane contamination of the F21 myelin fraction. The F4 (cytosol) fraction contained 24,7% of the total activity, which suggested that not all the microsomal membranes had been pelleted by the 6×10^6 gmin spin. The F11 and F12 contained respectively 16,3% and 10,8% of the F1 activity while the F13 contained 5,2%. 34% of the F1 activity was in the F1S. The F21 had 41,0% of the F2 activity, and the F2S fraction had 7,6% of the F2 activity. The recovery of this enzyme from the F2 gradient was 51,2%, which was rather low. The results are presented in Table 7 on page 59.

3.2.6 Lactate Dehydrogenase Distribution (E.C. 1.1.1.27)

This enzyme was assayed using fresh brain fractions. The F4 fraction had a specific activity of 5,099 µmol per min per mg protein. The F3 fraction had only 1,3% of the total activity and the F4 fraction had 25,6% of the total activity. 29,2% of the F1 activity was in the F11 myelin fraction and 38,2% of the F1 activity was in F1S. In the F2 subfractions, 33,9% of F2 activity was found in F21 and 31,4% in F2S. It was interesting that subjecting the F1 fraction to hypo-osmotic shock did not release more of the enzyme activity into the supernatant, F1S. The subcellular distribution of this enzyme is given in Table 8 on page 60.

3.2.7 Summary of Marker Enzyme Results

The various marker enzyme results indicated that the F11 and F21 myelin fractions were contaminated with other subcellular fractions. Microsomes appeared to be a major contaminant of F11 and F21. Mitochondrial contamination of F11 was indicated by the ferrocytochrome C oxidase activity. Acetylcholinesterase activity was low in F11 and F21, therefore synaptic membranes did not appear to contaminate myelin. Cytosolic contamination was high, judging from the results of the lactate dehydrogenase assay.

3.3 Distribution of Palmitoyl Coenzyme A Synthetase (E.C. 6.2.1.3)

The subcellular distribution of this enzyme in brain fractions homogenized in sucrose is shown in Table 9 on page 61. The F3 fraction had a specific activity of 18,9 nmol per min per mg protein. This value is high compared with the activity of 3,9 nmol per min per mg protein for rat brain microsomes (Murphy and Spence, 1980). 13,7% of the total activity was in the F1 fraction and 42,8% in the F2 fraction. The F11 fraction contained 9,5% of the F1 activity. 22,5% of the F2 activity was in F21, which had a specific activity of 7,7 nmol per min per mg protein. Table 10 on page 62 shows the values obtained for the enzyme activity in brains fractionated in sucrose/phosphate. Again, the F3 has the highest specific activity, 33,0 nmol per min per mg protein. This is 1,8

times higher than the F3 value for brains in sucrose. The F1 fraction had 32,3% of the total activity and the F2 fraction had 12,3%. The proportion of the total activity in the F3 was 35,0% of that in sucrose brains, namely 4,3%. The F11 myelin fraction contained 9,0% of the F1 activity, with 39,4% in the F1S fraction. The F2 gradient did not have clear resolution of palmitoyl coenzyme A synthetase activity, with 7,3% in the F21, 20,1% in F22 and 16,2% in F23. There was only 6,2% in the F2S fraction. Table 12 on page 63 shows the value for microsomal palmitoyl coenzyme A synthetase activity in rat liver homogenized in sucrose. The specific activity was 133,4 nmol per min per mg protein.

The addition of phosphate to the sucrose homogenizing medium approximately doubled the total activity in whole brain homogenate. The subcellular distribution of activity also varied in sucrose/phosphate brains.

Tables 13A, B and C on page 64 show the ratios of microsomal marker enzyme activities to palmitoyl coenzyme A synthetase activity in the F11 and F21 myelin fractions. It can be seen that palmitoyl coenzyme A synthetase activity is much lower than NADPH cytochrome C reductase activity and 5'-mononucleotidase activity. This still holds when low recoveries of palmitoyl coenzyme A synthetase and loss of myelin to the supernatant of the osmotically shocked F1 fraction, are considered. (Tables 14A, B and C on page 65 show the amended ratios).

Electron micrographs of myelin are on page 66.

LEGEND TO TABLES 1 - 14

In the measurement of protein content and enzyme activities, all brain fractions are denoted as follows:

W	whole homogenate
1	fraction F1 pellet from the nuclear spin (8×10^3 g min)
2	fraction F2 pellet from the mitochondrial spin ($9{,}9 \times 10^4$ g min)
3	fraction F3 pellet from the post-mitochondrial spin (6×10^6 g min)
4	fraction F4 supernatant from the post-mitochondrial spin (6×10^6 g min)
11	upper band) from the density gradient centrifugation
12	lower band) of fraction F1 (osmotically shocked)
13	pellet)
1S	supernatant from hypotonic lysis of fraction F1
21	upper band) from the density gradient centrifugation
22	lower band) of fraction F2 (osmotically shocked)
23	pellet)
2S	supernatant from hypotonic lysis of fraction F2
21NOS	upper band) from the density gradient centrifugation
22NOS	lower band) of fraction F2 (non-osmotically shocked)
23NOS	pellet)

The results are expressed as the mean + standard error (S.E.), except where only two experiments were performed

and the mean alone is given. Each determination was performed in duplicate. The number of duplicate determinations is shown in parentheses. The total activity of enzymes is expressed either as µmol per min per gm wet weight or as nmol per min per g wet weight. The specific activity of enzymes is expressed either as µmol per min per mg protein or as nmol per min per mg protein. The % recoveries are related to the original fractions.

TABLE 1 : Distribution of Protein Throughout Fractions Prepared From Adult Rat Brain in Sucrose (26)

Fraction	Protein Mg/gm wet weight of brain		Recovery %
W	117,2	± 5,0	
1	33,6	± 2,4	28,7
2	43,8	± 4,4	37,4
3	5,4	± 0,5	4,6
4	23,2	± 2,3	19,8
		TOTAL	90,5
11	7,1	± 0,5	21,1
12	3,6	± 0,2	10,7
13	1,9	± 0,1	5,7
1S	13,4	± 1,4	39,9
		TOTAL	77,4
21	10,5	± 1,1	24,0
22	8,1	± 0,4	18,5
23	0,9	± 0,0	2,1
2S	9,2	± 1,0	21,0
		TOTAL	65,6
21NOS	10,2 (2)		23,3
22NOS	15,9 (2)		36,3
23NOS	3,0 (2)		6,8
		TOTAL	66,4

Average weight of brains = 1,83 ± 0,1 gm

Fractions are as described in the Legend to Tables 1 to 14 on page 51. Results are expressed as mean ± S.E. except for fractions 21NOS - 23NOS inclusive, which are expressed as mean of two determinations. Number of determinations is shown in parentheses.

TABLE 2 : Distribution of Protein Throughout Fractions Prepared From Adult Rat Brain in Sucrose/ Phosphate (5)

Fraction	Protein Mg/gm wet weight of brain			Recovery %
W	116,0	±	5,5	
1	55,5	±	0,3	47,8
2	15,1	±	0,1	13,0
3	2,1	±	0,1	1,8
4	28,3	±	0,2	24,4
			TOTAL	87,0
11	11,5	±	0,1	20,7
12	6,5	±	0,0	11,7
13	0,6	±	0,1	1,1
1S	33,0	±	0,2	59,5
			TOTAL	93,0
21	1,8	±	0,1	11,6
22	3,6	±	0,2	23,6
23	3,2	±	0,3	21,1
2S	4,5	±	0,3	29,7
			TOTAL	86,0

Average weight of brains = 1,82 ± 0,0 gm

Fractions are as described in the Legend to Tables 1 to 14 on page 51. Results are expressed as mean ± S.E. Number of determinations is shown in parentheses.

TABLE 3 : Distribution of NADPH Cytochrome C Reductase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose (8)

Fraction	µmol per min per gm wet weight	nmol per min per mg protein	Recovery %
W	4,354 ± 0,29	37,2	
1	1,576 ± 0,23	46,9	36,2
2	1,372 ± 0,09	31,3	31,5
3	0,349 ± 0,02	101,7	12,6
4	0,259 ± 0,02	11,2	5,9
		TOTAL	86,2
11	0,685 ± 0,03	96,5	43,5
12	0,012 ± 0,00	3,3	0,8
13	0,034 ± 0,01	17,9	2,2
1S	0,798 ± 0,03	59,6	50,6
		TOTAL	97,1
21	0,818 ± 0,02	77,9	59,6
22	0,105 ± 0,04	13,0	7,7
23	0,007 ± 0,00	7,8	0,5
2S	0,420 ± 0,05	45,7	30,6
		TOTAL	98,4
21NOS	0,821 (2)	80,5	59,8
22NOS	0,069 (2)	4,3	5,0
23NOS	0,011 (2)	3,7	0,8
		TOTAL	65,6

Fractions are as described in the Legend to Tables 1 to 14 on p. 51. Results are expressed as mean ± S.E., except for fractions 21NOS - 23NOS inclusive, which are expressed as mean of two individual determinations. Number of determinations is shown in parentheses.

TABLE 4 : Distribution of Ferrocytochrome C Oxidase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose (6)

Fraction	µmol per min per gm wet weight	µmol per min per mg protein	Recovery %
W	26,99 ± 2,13	0,230	
1	6,56 ± 0,50	0,195	24,3
2	15,30 ± 1,97	0,349	56,7
3	0,0	0,0	0,0
4	0,0	0,0	0,0
		TOTAL	81,0
11	1,33 ± 0,18	0,187	20,3
12	0,18 ± 0,01	0,050	2,7
13	0,25 ± 0,02	0,132	3,8
1S	2,20 ± 0,24	0,164	33,5
		TOTAL	60,3
21	1,66 ± 0,19	0,158	10,8
22	6,00 ± 0,32	0,741	39,2
23	0,91 ± 0,05	1,011	5,9
2S	0,0	0,0	0,0
		TOTAL	55,9
21NOS	4,75 (2)	0,466	31,0
22NOS	12,78 (2)	0,804	83,5
23NOS	0,41 (2)	0,137	2,7
		TOTAL	117,2

Fractions are as described in the Legend to Tables 1 to 14 on page 51. Results are expressed as mean ± S.E., except for fractions 21NOS - 23NOS inclusive, which are expressed as mean of two individual determinations. Number of determinations is shown in parentheses.

TABLE 5 : Distribution of Acetylcholinesterase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose (6)

Fraction	µmol per min per gm wet weight	nmol per min per mg protein	Recovery %
W	9,013 ± 0,78	77	
1	1,297 ± 0,20	39	14,4
2	2,908 ± 0,16	66	32,3
3	1,420 ± 0,19	263	15,8
4	0,820 ± 0,04	35	9,1
		TOTAL	71,6
11	0,053 ± 0,01	7	4,1
12	0,103 ± 0,01	29	7,9
13	0,127 ± 0,01	67	9,8
1S	0,661 ± 0,04	49	51,0
		TOTAL	72,8
21	0,135 ± 0,00	13	4,6
22	1,085 ± 0,13	134	37,3
23	0,160 ± 0,00	177	5,5
2S	0,607 ± 0,09	66	20,9
		TOTAL	68,3

Fractions are as described in the Legend to Tables 1 to 14 on page 51. Results are expressed as mean ± S.E. Number of determinations is shown in parentheses.

TABLE 6 : Distribution of 2',3'-Cyclic Nucleotide 3'-Phosphohydrolase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose (7)

Fraction	µmol per min per gm wet weight	µmol per min per mg protein	Recovery %
W	1696,2 ± 203,5	14,47	
1	790,4 ± 133,1	23,52	46,6
2	546,4 ± 140,5	12,47	32,2
3	19,7 ± 2,1	3,65	1,2
4	57,4 ± 2,9	2,47	3,4
		TOTAL	83,4
11	218,3 ± 43,7	30,75	27,6
12	94,2 ± 7,5	26,17	11,9
13	0,0	0,0	0,0
1S	355,2 ± 90,5	26,51	44,9
		TOTAL	84,4
21	402,9 ± 58,7	38,37	73,7
22	9,8 ± 0,1	1,21	1,8
23	0,4 ± 0,0	0,42	0,1
2S	56,7 ± 4,1	6,16	10,4
		TOTAL	86,0

Fractions are as described in the Legend to Tables 1 to 14 on page 51. Results are expressed as mean ± S.E. Number of determinations is shown in parentheses.

TABLE 7 : Distribution of 5'-Mononucleotidase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose (5)

Fraction	µmol per min per gm wet weight	nmol per min per mg protein	Recovery %
W	4,892 ± 0,56	42	
1	1,068 ± 0,26	32	21,7
2	1,840 ± 0,28	42	37,6
3	0,380 ± 0,02	70	7,8
4	1,208 ± 0,05	52	24,7
		TOTAL	91,8
11	0,173 ± 0,01	24	16,3
12	0,115 ± 0,01	32	10,8
13	0,055 ± 0,01	29	5,2
1S	0,360 ± 0,02	2	34,0
		TOTAL	66,3
21	0,755 ± 0,02	72	41,0
22	0,039 ± 0,01	5	2,1
23	0,009 ± 0,00	10	0,5
2S	0,140 ± 0,01	15	7,6
		TOTAL	51,2

Fractions are as described in the Legend to Tables 1 to 14 on page 51. Results are expressed as mean ± S.E. Number of determinations is shown in parentheses.

TABLE 8 : Distribution of Lactate Dehydrogenase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose (5)

Fraction	µmol per min per gm wet weight	µmol per min per mg protein		Recovery %
W	462,9 ± 27,0	3,949		
1	87,9 ± 6,0	2,616		19,0
2	146,5 ± 12,2	3,345		31,6
3	6,1 ± 0,3	1,129		1,3
4	118,3 ± 3,5	5,099		25,6
			TOTAL	77,5
11	25,7 ± 2,2	3,620		29,2
12	3,8 ± 0,3	1,056		4,3
13	4,9 ± 0,3	2 579		5,6
1S	39,6 ± 3,1	2,507		38,2
			TOTAL	77,3
21	49,7 ± 4,6	4,733		33,9
22	2,6 ± 0,2	0,321		1,8
23	0,4 ± 0,1	0,444		0,3
2S	46,0 ± 5,1	5,000		31,4
			TOTAL	67,4

Fractions are as described in the legend to Tables 1 to 14 on page 51. Results are expressed as mean ± S.E. Number of determinations is shown in parentheses.

TABLE 9 : Distribution of Palmitoyl Coenzyme A Synthetase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose (10)

Fraction	nmol per min per gm wet weight			nmol per min per mg protein		Recovery %
W	840,9	±	51,3	7,2		
1	115,6	±	1,4	3,4		13,7
2	360,3	±	0,1	8,2		42,8
3	102,0	±	1,1	18,9		12,1
4	44,9	±	2,5	1,9		5,3
					TOTAL	73,9
11	11,0	±	2,9	1,5		9,5
12	3,1	±	1,7	0,9		2,7
13	2,5	±	0,8	1,3		2,2
1S	56,5	±	2,7	4,2		48,9
					TOTAL	63,3
21	81,1	±	6,3	7,7		22,5
22	34,6	±	2,8	4,3		9,6
23	5,5	±	1,1	6,1		1,5
2S	72,1	±	3,8	7,8		20,0
					TOTAL	53,6

Fractions are as described in the Legend to Tables 1 to 14 on page 51. Results are expressed as mean ± S.E. Number of determinations is shown in parentheses.

TABLE 10 : Distribution of Palmitoyl Coenzyme A Synthetase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose/ Phosphate (5)

Fraction	nmol per min per gm wet weight			nmol per min per mg protein		Recovery %
W	1619,8	±	11,3	14,9		
1	523,7	±	10,2	9,4		32,3
2	198,8	±	4,8	13,2		12,3
3	69,3	±	0,9	33,0		4,3
4	228,9	±	9,2	8,1		14,1
					TOTAL	63,0
11	47,1	±	5,1	4,1		9,0
12	46,6	±	8,3	7,2		8,9
13	2,5	±	0,8	4,2		0,5
1S	206,5	±	12,0	6,3		39,4
					TOTAL	57,8
21	14,5	±	3,8	8,1		7,3
22	41,1	±	8,2	11,4		20,1
23	32,2	±	5,1	10,1		16,2
2S	12,4	±	2,2	2,8		6,2
					TOTAL	49,8

Fractions are as described in the Legend to Tables 1 to 14 on page 51. Results are expressed as mean ± S.E. Number of determinations is shown in parentheses.

TABLE 11 : Distribution of NADPH Cytochrome C Reductase Activity Throughout Fractions Prepared From Adult Rat Brain in Sucrose/Phosphate (2)

Fraction	µmol per min per gm wet weight	nmol per min per mg protein	Recovery %
W	4,130	35,6	
1	2,005	36,1	48,5
2	0,660	43,7	16,0
3	0,202	96,2	4,9
4	0,241	8,5	5,8
		TOTAL	75,2
11	0,615	53,5	30,7
21	0,093	53,1	14,1

Results are expressed as mean of two determinations.

TABLE 12 : NADPH Cytochrome C Reductase and Palmitoyl Coenzyme A Synthetase Activities in Rat Liver Microsomes in Sucrose (2)

NADPH Cytochrome C Reductase

Fraction	µmol per min per g wet weight	nmol per min per mg protein
3	1,836	232,9

Palmitoyl Coenzyme A Synthetase

Fraction	µmol per min per g wet weight	nmol per min per mg protein
3	1,051	133,4

Results are expressed as mean of two determinations.

TABLE 13A : Ratio of NADPH Cytochrome C Reductase Activity to Palmitoyl Coenzyme A Synthetase Activity in Myelin Fractions (F11 and F21) and F3 Prepared from Adult Rat Brain in Sucrose

	11	21	3
NADPH Cytochrome C Reductase / Palmitoyl Coenzyme A Synthetase	62,3	10,1	5,4

TABLE 13B : Ratio of 5'-Mononucleotidase Activity to Palmitoyl Coenzyme A Synthetase Activity in Myelin Fractions (F11 and F21) and F3 Prepared from Adult Rat Brain in Sucrose

	11	21	3
5'-Mononucleotidase / Palmitoyl Coenzyme A Synthetase	15,7	9,3	3,7

TABLE 13C : Ratio of NADPH Cytochrome C Reductase Activity to Palmitoyl Coenzyme A Synthetase Activity in Myelin Fractions (F11 and F21) and F3 Prepared from Adult Rat Brain in Sucrose/Phosphate

	11	21	3
NADPH Cytochrome C Reductase / Palmitoyl Coenzyme A Synthetase	13,1	6,4	2,9

TABLE 14A : Ratio of NADPH Cytochrome C Reductase Activity to Palmitoyl Coenzyme A Synthetase Activity in Myelin Fractions (F11 and F21) and F3 Prepared from Adult Rat Brain in Sucrose

	11	21	3
NADPH Cytochrome C Reductase / Palmitoyl Coenzyme A Synthetase	13,3	4,7	4,7

TABLE 14B : Ratio of 5'-Mononucleotidase Activity to Palmitoyl Coenzyme A Synthetase Activity in Myelin Fractions (F11 and F21) and F3 Prepared from Adult Rat Brain in Sucrose

	11	21	3
5'-Mononucleotidase / Palmitoyl Coenzyme A Synthetase	4,9	8,3	3,0

TABLE 14C : Ratio of NADPH Cytochrome C Reductase Activity to Palmitoyl Coenzyme A Synthetase Activity in Myelin Fractions (F11 and F21) and F3 Prepared from Adult Rat Brain in Sucrose/Phosphate

	11	21	3
NADPH Cytochrome C Reductase / Palmitoyl Coenzyme A Synthetase	10,1	4,0	2,4

Electron Microgaphs of F1 and F2 Myelin

Showing contamination by mitochondrial and plasma membrane fragments.

Figure 3

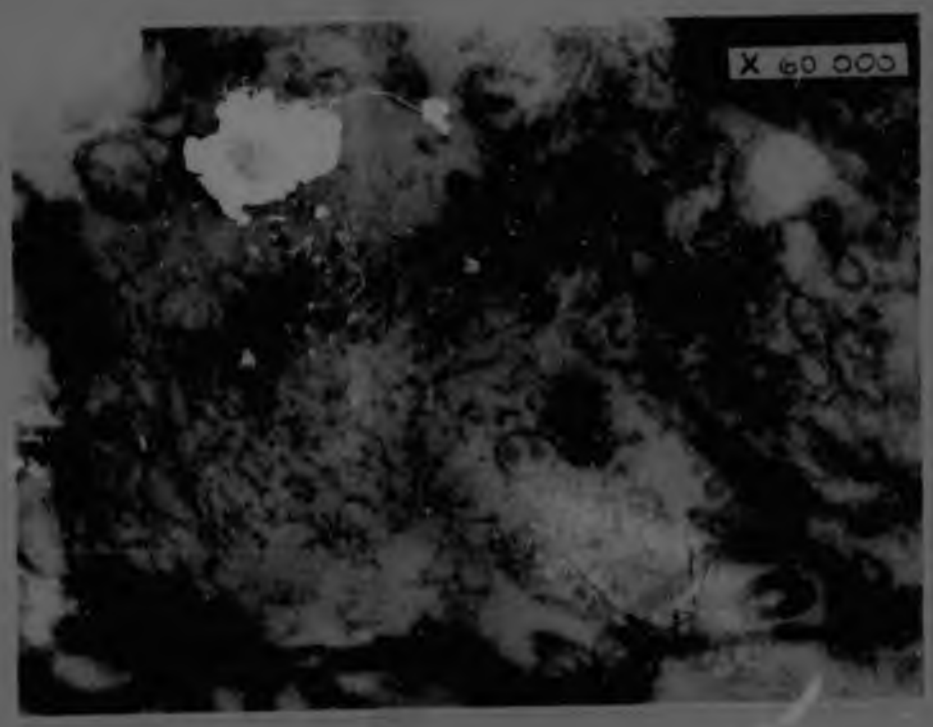
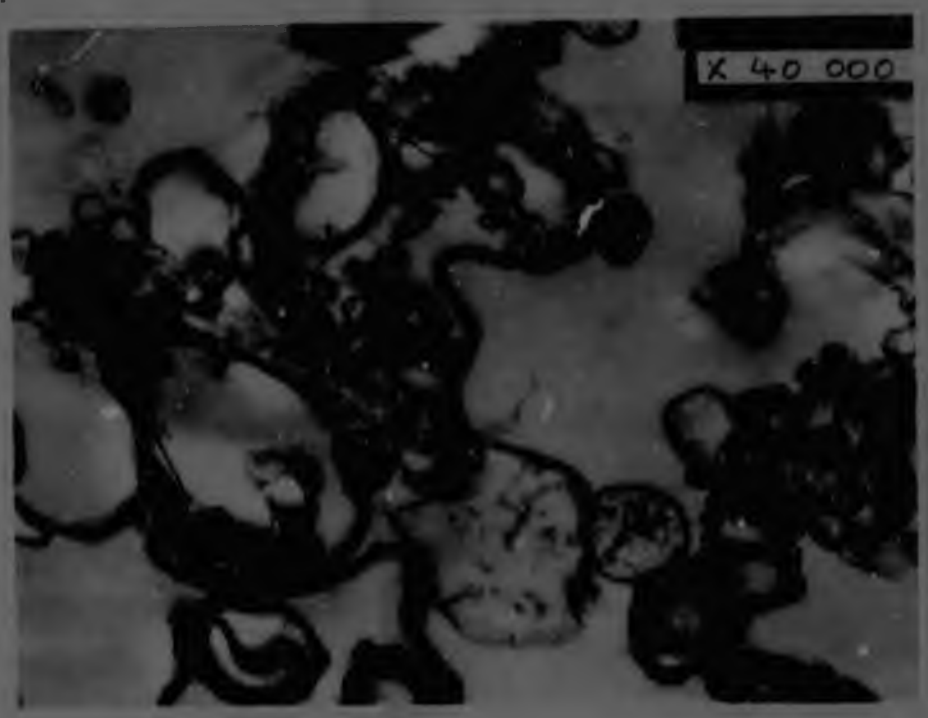


Figure 4



4. DISCUSSION

The discussion is in three parts. The first section is on the subcellular fractionation results, with reference to the protein distribution in the fractions. The second section is on the distribution of the various marker enzymes in the subcellular fractions. The final section is on palmitoyl coenzyme A synthetase distribution in the subcellular fractions.

4.1 Discussion of Subcellular Fractionation

In attempting to isolate as many of the brain subcellular fractions as possible, quality had to be sacrificed to some degree. Because the optimal methods for purification of each subcellular component differ, the preparative procedure was sub-optimal for all components. As the primary interest was in the myelin fractions, the emphasis was on minimizing contamination in these fractions at the expense of other fractions. For example, the radial clearance of the Potter-Elvejhem homogenizer was 0,12 mm, which is optimal for obtaining myelin free of axolemma and axonal fragments. (Aldridge and Johnson, 1959). The radial clearance was therefore not optimal for synaptosome formation for which 0,25 mm clearance is recommended (Marchbanks, 1975). The crude nuclear (F1) and mitochondrial (F2) fractions, which contain myelin, were further purified by hypo-osmotic shock. Omitting this step prior to density gradient subfractionation yielded poorer resolution of subcellular components.

This is shown by the different results for ferrocytochrome C oxidase in osmotically shocked and non-osmotically shocked F2 gradient subfractions. F21NOS contained 31,0% of the F2 ferrocytochrome C oxidase activity and F21 had only 10,8%. Allowing for the different recoveries, 117,2% of the F2NOS gradient and only 55,9% of the F2 gradient, the F21NOS fraction had still about 1,4 times more ferrocytochrome C oxidase activity.

Separation is less clearly defined in continuous, as opposed to discontinuous, gradients. The broad, opague band above the lower F2 band, which was removed with it, might have been synaptosomes or synaptic membrane fragments.

The presence of 0,25M phosphate in the homogenizing medium was observed to cause aggregation of particles. There was poorer resolution of the F2 gradient fractions in sucrose/phosphate brains. The palmitoyl coenzyme A synthetase values for F21, F22 and F23 in sucrose/phosphate were respectively, 7,3%, 20,1% and 16,2% of the total F2 activity. The corresponding values in sucrose brains were 22,5%, 9,6% and 1,5% of the F2 total activity. The higher activity in F21 (sucrose) reflected the much higher activity (42,8%) in the crude sucrose F2 compared with the crude sucrose/phosphate F2 (12,3%) in sucrose/phosphate brains 32,3% of the total activity was

in the F1 in contrast to 13,7% in the sucrose brains. The protein distributions in the sucrose/phosphate and sucrose brains also differed. The respective protein values for F1 and F2 in sucrose brains were 28,7% and 37,4% of the total, whereas in sucrose/phosphate, the same fractions had 47,8% and 13,0% of the total protein.

In sucrose homogenized brains, some microsomal activity (6,6% of the total NADPH cytochrome C reductase activity) was present in the cytosol. Longer centrifugation to pellet the microsomes was deemed unwise in view of the adverse effect on enzyme activity of increased preparation time.

In all the enzyme determinations performed, the F1S fraction contained a higher percentage of the F1 activity than the F2S fraction contained of the F2 activity. This was undoubtedly because of the technique employed in pelleting the osmotically shocked material. For the crude F1 fraction, the material suspended in water was centrifuged at 8×10^3 g min, as for the preparation of the F1 fraction. Thus mitochondria and microsomes released into the supernatant by the hypo-osmotic shock would not be pelleted. The F2 osmotically shocked sample was centrifuged as for a mitochondrial spin, namely at $9,9 \times 10^4$ g min, which would pellet mitochondria and synaptosomes. This would explain the high activity of the oligodendroglial plasma membrane - myelin marker enzyme, 2',3'-cyclic nucleotide 3'-phosphohydrolase (2',3'-CNP) in

the F1S fraction (44,9% of the F1 activity) and the low activity in F2S (10,4% of the F2 activity).

The length of time required to resuspend pellets of sucrose/phosphate brains was greater than for sucrose brains. This may account in part for the lower recoveries of activities of NADPH cytochrome C reductase in sucrose/phosphate brain fractions, despite high protein recoveries. Gradient subfractions, particularly F12, F13, F22 and F23, often required homogenization to resuspend the particles in sucrose/phosphate fractions. This might have caused additional loss of enzyme activity.

Those enzymes which were recovered in greater amount than the corresponding protein, were possibly released from "trapped" positions in membranes, or were exposed on the outer surfaces of membranes by osmotic shocking. This might explain recoveries in excess of 100% from gradients.

4.2 Discussion of Marker Enzyme Distribution

4.2.1 NADPH Cytochrome C Reductase (E.C. 1.6.2.3)

Initially, this enzyme was assayed using fractions which had been stored at -20°C, but such fractions gave low activities, for example, the F3 activity was 9 nmol per min per mg protein. Fresh brain F3 had an activity of 101,7 nmol per min per mg protein. Rat liver microsomes had a specific activity of 44 nmol per min per mg protein (Sottocasa et al., 1967). The higher value in this study may be due to the use of the

non-ionic detergent Nonidet P40, suggested by McMurray and Dawson (1969). Sucrose/phosphate F3 gave a similar NADPH cytochrome C reductase activity, namely 96,2 nmol per min per mg protein. In rat liver microsomes, the specific activity was 232,9 nmol per min per mg protein. The F11 and F21 of sucrose-homogenized brains contained, respectively, 43,5% of the F1, and 59,6% of the F2 activity. In sucrose/phosphate brains, the F11 contained 30,7% of the F1 activity, while the F21 contained 14,1% of the F2 activity.

The F1 activity in sucrose brain was 36,2% of the total activity in sucrose/phosphate brains. These results, coupled with the higher protein value for F1 in sucrose/phosphate as opposed to sucrose, suggest that there was more retention of microsomes in the F1 of brains homogenized in the high ionic strength medium.

4.2.2. Ferrocytochrome C Oxidase (E.C. 1.9.3.1)

This assay was performed according to Rascati and Parson (1979) but with Nonidet P40 at a concentration of 0,025% instead of Tween 80 at a concentration of 0,5%. The specific activity of the enzyme in the "purified" mitochondrial fraction, F23, was 1 µmol per min per mg protein. Two experiments were performed with non-osmotically shocked F2 gradient subfractions to determine whether hypo-osmotic shock adversely affected enzyme activity, since the gradient recoveries were low, 60,3% for the F1 and 55,9% for the F2, and the

mitochondrial specific activity was 25% of the value found for yeast mitochondria (Duncan and Mackler, 1966). The recovery off the non-osmotically shocked gradient was 117,2% (2,1 times greater than the F2 osmotically shocked recovery) which suggested that hypo-osmotic shock did have a detrimental effect on the enzyme activity.

Unfortunately, because of the omission of osmotic shocking, a pure mitochondrial fraction was not obtained and the specific activity of the F22NOS fraction was only 0,8 µmol per min per mg protein. This fraction contained 10% of the NADPH cytochrome C reductase activity, and, in one experiment assaying acetylcholinesterase the highest activity was in the F22NOS fraction (see 4.2.3. for details). Microsomal and mitochondrial contamination may thus contribute to the low specific activity of ferrocytochrome C oxidase in the F22NOS. The F11 fraction had 20,3% of the F1 activity and the F21 fraction had 10,8% of the F2 activity, thereby establishing that there was mitochondrial contamination of both myelin fractions. The F2S fraction exhibited cytochrome C reductase activity in this assay.

4.2.3 Acetylcholinesterase (E.C. 3.1.1.7)

Two factors militated against obtaining a synaptosomal fraction, (i) the radial clearance of the homogenizer was 0,12 mm instead of 0,25 mm (ii) hypo-osmotic shock disrupts synaptosomes.

At high ionic strength, acetylcholinesterase binds,

somewhat non-specifically, to membranes. It is likely that, in vivo, the enzyme, which is completely soluble at high ionic strength, occurs in the terminal cytoplasm (Marchbanks, 1975). Thus it is possible that the relatively strong shear forces used released the enzyme which then bound to membranes in the sucrose homogenate. This would explain the 15,8% activity in the F3 fraction. The F22 fraction had the highest specific activity, 0,134 µmol per min per mg protein. It is likely that this fraction contained microsomes, mitochondria and possibly synaptic membranes, judging from the NADPH cytochrome C reductase, ferrocytochrome C oxidase and acetylcholinesterase activities. The myelin fractions F11 and F21 had 4,1% and 4,6% respectively of the activities of the F1 and F2 fractions, which suggested that either contamination by synaptic membranes was minimal or the enzyme did not bind to myelin membranes (see above).

Acetylcholinesterase activity was determined in one experiment using non-osmotically shocked gradient fractions. The activity in the F22NOS fraction was 3,37 µmol per min per gm wet weight of brain, which corresponded to 116% of the F2 activity. This result, if confirmed by repeated experiments, would have supported the suggestion that acetylcholinesterase was redistributed throughout other membrane fractions as a result of the osmotic shock procedure, thus giving the lower value of 1,085 umol per min per gm wet weight of brain in the F22 fraction.

4.2.4 2', 3'-Cyclic Nucleotide 3'-Phosphohydrolase (E.C. 3.1.4.37)

The results in Table 6 on page 58 were those obtained using the method of Prohaska et al., (1973), except that Nonidet P40 (0,01% concentration) was used instead of 0,4% deoxycholate. 0,01% NP40 was 1,44 times more effective than 0,1% dexycholate, and 2,?? times more effective than 1% dexycholate, in stimulating 2', 3'-CNP activity. (Clapshaw and Seifert, 1980).

The specific activity of the whole brain homogenate was 14,5 µmol per min per mg protein, compared to about 10 umol per min per mg protein for white matter homogenate from mouse brain found by Clapshaw and Seifert (1980). These authors used a radioisotope assay, involving extraction of product, thin layer chromatography, and elution of the chromatographed material, before liquid scintillation counting. These procedures might have introduced sampling errors which could generate lower activity values than a method in which no transfer of reactants occurs (Prohaska et al., 1973).

The myelin F11 and F21 fractions had, respectively, values of specific activity of 30,75 and 38,37 µmol per min per mg protein. The F11 had 27,6% of the F1 activity, with 44,9% in the F1S, a not unexpected finding in view of the hypo-osmotic shock procedure. F21 had 73,7% of the total F2 activity with only 10,4% in the F2S fraction. Again, the $9,9 \times 10^4$ g min spin would have pelleted most of the myelin fragments, leaving very little activity in

the F2S, compared with the F1S fraction. The loss of 45% of enzyme activity to the F1S fraction must be considered when estimating the palmitoyl coenzyme A synthetase activity associated with the F1l fraction. There was minimal 2', 3'-CNP activity in the microsomal (1,2%) and cytosolic (3,4%) fractions.

The function of this enzyme in brain is unknown. Neither 2', 3'-cyclic nucleotides nor 2'-nucleotides have been found in free nucleotide pools of tissues with 2', 3'-CNP activity. Recently, data have been presented indicating that the Wla and Wlb Wolfgram components were predominantly inactive forms of 2', 3'-CNP. (Drummond and Dean, 1980). Sprinkle et al., (1980), have identified the Wl and W2 Wolfgram protein doublet as the oligodendroglial- myelin marker enzyme 2', 3'-CNP. The notations Wl and W2 of Waehneldt and Malotka (1980) correspond to the Wla and Wlb components of the Wolfgram Wl protein described by Nussbaum et al., (1977).

4.2.5 5'-Mononucleotidase (E.C. 3.1.3.5)

This enzyme is used as a microsomal and a plasma membrane marker. The best method of assaying the enzyme activity is by using Ni^{++} ions to selectively inhibit the 5'-mononucleotidase in the controls. The methods using glycerol-2-phosphate are liable to inconsistent errors (Gerlach and Hiby, 1974).

The myelin F21 fraction and the microsomal F3 fraction had 72 and 70 nmol per min per mg protein as values of specific activity. Microsomes and oligodendroglial plasma

membrane must have been responsible for the very high specific activity, with the very low protein content of myelin itself having tended to enhance the value. The 9,9 x 10^4 g min spin of the F2 fraction in distilled water would appear to have pelleted some microsomal membranes along with myelin, mitochondria and synaptic membranes. The F21 had 41,0% of the F2 activity, with 7,6% in the F2S, while the F11 had 16,3% of the F1 activity and 34,0% in the F1S. These values can be compared with the microsomal marker enzyme, NADPH cytochrome C reductase, values. Of the latter enzyme, 43,5% of the F1 activity was in F11 and 50,6% in F1S, with 59,6% of the F2 activity in F21 and 30,6% in F2S.

24% of the total 5'-mononucleotidase activity was in the cytosol indicating that this fraction was still contaminated with other membrane fragments as well as microsomes, as only 5,9% of the NADPH cytochrome C reductase activity was in the F4 fraction.

With a gradient recovery of 51,2%, this enzyme appeared to be susceptible to the longer preparation time of the F2 gradient subfractions in particular. The centrifugation of the osmotically shocked F2 fraction in distilled water at 9,9 x 10^4 g min may affect enzyme activity adversely.

4.2.6 Lactate Dehydrogenase (E.C. 1.1.1.27)

This enzyme was assayed on fresh brain fractions as it is known to decrease in activity with storage (Bergmeyer and Bernt, 1974). The cytosol had a specific

activity of 5,1 µmol per min per mg protein and the F2S fraction 5,0 µmol per min per mg protein. The F11 fraction had 29,2% of the F1 activity with 38,2% in the F1S. The F21 fraction contained 33,9%, and the F2S 31,4%, of the F2 activity. It is surprising that there was so much retention of a soluble enzyme marker in the myelin fractions. These findings emphasized the difficulty of obtaining myelin free from contamination by other subcellular components without repeated washings, which would have diminished enzyme activities.

Leucine amino peptidase (E.C. 3.4.11.2) has been reported to be located in myelin (Adams and Glenner, 1962). Banik and Davison (1969) found 33,2% of the total enzyme activity was in the cytosol. 34,6% of the F2 activity was in their crude myelin fraction (heavy plus light myelin) and 32,4% of the F2 activity was in their synaptosomal fraction. In the light of the results obtained for lactate dehydrogenase activity in the myelin fractions in this study, it is interesting to speculate that the occurrence of activity of leucine amino peptidase (L-leucyl paranitroanilidase) in myelin is in fact due to cytosolic contamination.

From the foregoing results of investigations into marker enzyme distribution it is clear that the F1 myelin fraction (heavy myelin) has microsomal, mitochondrial and cytosolic contamination, as measured by NADPH cytochrome C reductase, ferrocytochrome C oxidase and lactate dehydrogenase activities, respectively. By the same criteria, the F21 myelin fraction has rather more

microsomal contamination than F11 myelin, and also has mitochondrial and cytosolic contamination. This light myelin fraction also has considerable 5'-mononucleotidase activity, indicating that there is contamination by other plasma membranes.

4.3 Palmitoyl Coenzyme A Synthetase (E.C. 6.2.1.3)

Using the method of Farstad et al., (1967), where the controls were incubated in the absence of substrate, there was found to be sufficient endogenous substrate in fresh brain fractions to enable appreciable enzyme activity in the controls. Using the Millipore filter assay, the radioisotope was [H^3]CoASH which effectively swamped the low intracellular pools of CoASH (Polokoff and Bell, 1975). In this study, trichloracetic acid was added to the controls at incubation time 0 min and therefore no enzyme activity could occur, but the enzyme activity using fresh brain fractions was from 25% to 33% of the activity using samples which had been stored for 18 hours at -20°C. Skrede (1973), has stated that enzymes which destroy CoASH interfere with palmitoyl coenzyme A synthetase activity. It is possible that such enzymes are present in the fresh brain fractions, thus destroying some of the radiolabelled CoASH, and giving lowered palmitoyl coenzyme A synthetase values. If such enzymes were themselves destroyed by storage for 18 hours at -20°C, this would explain the increased activity of the frozen and thawed specimens. Farstad et al., (1967) found a

slight increase in enzyme activity with freezing and thawing once.

Table 9 on page 61 gives the enzyme distribution in sucrose-nomogenized brains and Table 10 on page 62 the distribution in sucrose/phosphate-homogenized brains. The highest specific activities were 33,0 nmol per min per mg protein in the microsomal fraction of brains homogenized in sucrose/phosphate, and 18,9 nmol per min per mg protein in the F3 of brains homogenized in sucrose. Brophy and Vance (1976) found a specific activity of 16 nmol per min per mg protein for rat brain microsomes using 0,1% Triton WR 1339 in the assay. The detergent employed in the present assay was Nonidet P40 at 0,05% concentration.

In two experiments using rat liver homogenized in sucrose, the specific activity of the microsomal fraction was 133 nmol per min per mg protein, which compares favourably with 145 nmol per min per mg protein (Pande and Mead, 1968). The corresponding results for total F3 activity were 1,05 and 1,16 µmol per min per mg protein.

Murphy and Spence (1980) obtained a specific activity of about 3,9 nmol per min per mg protein for rat brain microsomal fraction. Several differences in assay method may partly account for this. (i) They used Triton WR 1339 at 0,1% in contrast to Nonidet P40 at 0,05%; (ii) they do not state the pH of their palmitic acid-detergent mixture, which, if low, might have had a deleterious effect on enzyme activity; (iii) the assay was performed at 30°C instead of 37°C, used in this work because it is

preferable to assay at 37°C due to the high activation energy (approximately 20 Kcal per mol) of the enzyme (de Jong, 1971); (iv) they used an extraction procedure for estimation of enzyme activity, which may have introduced sampling error and hence lower values.

The lower recoveries, (53,6% in sucrose and 49,8% in sucrose/phosphate) of enzyme activity in the F2 gradient fractions may partly be due to selective loss of mitochondrial palmitoyl coenzyme A synthetase, wnich is very susceptible to proteolytic attack (Groot et al., 1976). Approximately 90% of enzyme activity in a mitochondrial suspension (in 0,05M phosphate, pH 7.4, 0,002M DTT, and 0,001M EDTA) was lost in 8 hours at 0°C. Microsomes in the same medium had virtually no loss of activity in 24 hours at 0°C (Tanaka et al., 1979).

The low recoveries in sucrose/phosphate-homogenized brains may be attributed in part to the longer time required to re-suspend F1 and F2 pellets and the consequently greater tendency for "frothing". The F12, F13, F22 and F23 fractions required homogenization in order to obtain complete particle suspension, and this could have contributed to further loss of activity. Freezing and thawing resulted in re-aggregation of particles.

The differences in distribution of the enzyme in the crude subcellular fractions in sucrose and sucrose/phosphate homogenates, are caused by the high

ionic strength of the phosphate/sucrose medium, which causes particle coacervation (Marchbanks, 1975). The F1 of sucrose homogenates contained 13,7% of the total activity and the F2 42,8%. In contrast, the F1 and F2 of sucrose/phosphate homogenates contained, respectively, 32,3% and 12,3% of the total activity. The F3 fraction of sucrose homogenates had 12,1% of the total activity while the sucrose/phosphate F3 had 4,3% of the total activity. There was therefore more microsomal retention in the F1 fraction of sucrose/phosphate homogenates; this is substantiated by the results for NADPH cytochrome C reductase, with 48,5% of the total activity in the F1 of sucrose/phosphate homogenates, and 36,2% in the F1 of sucrose homogenates.

The presence of phosphate increased the specific activity of the F3 fraction 1,8 times, and increased the total whole homogenate activity 1,9 times. Phosphate appeared to reduce the resolution obtained from the F2 gradients especially, with 20,1% and 16,2% of F2 activity in the F22, and F23 fractions, respectively. The F11 myelin fraction had 9,0% of the F1 activity, which was similar to the F11 fraction in sucrose, which had 9,5% of the F1 activity.

Table 13A on page 64 shows the ratios of NADPH cytochrome C reductase activity to palmitoyl coenzyme A synthetase activity in the F11, F21 and F3 fractions. It can be seen that the ratio in both the F11 and F21 fractions exceeds the F3 ratio. Table 13B on page 64

compares the 5'-mononucleotidase and palmitoyl coenzyme A sythetase activities in these fractions. Table 13C on pag 64 compares the NADPH cytochrome C reductase and palmitoyl coenzyme A synthetase activities in brains homogenized in sucrose/phosphate.

The respective recoveries of NADPH cytochrome C reductase activity from the sucrose brains were 86,2%, 97,1% and 98,4% for the crude, F1 gradient and F2 gradient fractions. The palmitoyl coenzyme A synthetase recoveries were 75,2%, 63,3% and 53,6% for the crude, F1 gradient and F2 gradient fractions. The 5'-mononucleotidase recoveries for the crude, F1 gradient and F2 gradient fractions were, respectively, 91,8%, 66,3% and 51,2%. The loss of 2',3'-cyclic nucleotide 3'-phosphohydrolase activity to the F1S fraction was 67% and to the F2S fraction 15,0%. Allowing for the different recoveries and the loss of myelin marker enzyme activity, the ratios shown in Tables 13A and 13B on page 64 become those shown in Tables 14A and 14B on page 65.

Unfortunately, the recoveries of NADPH cytochrome C reductase activity from the F1 and F2 sucrose/phosphate gradients are not known as only the F11 and F21 fractions were assayed for this enzyme. Assuming a recovery of 80,0% as a maximum, based on the crude fraction recovery of 75,2%, the ratios in Table 13C on page 64 become those in Table 14C on page 65. As 2',3'-cyclic nucleotide 3'-phosphohydrolase could not be assayed in sucrose/phosphate fractions because of the high phosphate

concentration, no allowance has been made for myelin loss to the F1S and F2S fractions.

However, if the losses were of similar magnitude, considering the very different crude F1 and F2 fractions, the F11 and F21 ratios would not fall below the F3 ratio.

Phosphate undoubtedly protects the enzyme from inactivation but at 0,25M concentration reduces the efficiency of the separation technique. In this regard, it would be interesting to use 0,025M phosphate to determine whether this lower concentration, at which there would perhaps be less particle aggregation, still protects the enzyme activity.

Uncomplexed ATP has been reported to stabilize the enzyme (Farstad, 1968; Aas, 1971, 1972: Pande, 1972, and Sanchez, et al., 1973). Aas (1971) used ATP at 5×10^{-4} M concentration in the sucrose homogenizing medium, but it is difficult to see how the ATP could long maintain its protective effect on the enzyme before it was destroyed by endogenous adenosine triphosphatases. ATP probably exerts its effect on palmitoyl coenzyme A synthetase by protecting against endogenous proteolytic activity. Uncomplexed ATP protects 3 out of 6 available thiol groups on the enzyme against sulphydryl reagents. (Rose et al., 1979). It is possible that 0,25M phosphate protects the enzyme against ageing denaturation by suppressing endogenous adenosine triphosphatase activity and enabling endogenous ATP to protect the thiol groups on the enzyme.

4.4 Conclusions

The aim of the study was to determine the subcellular localization of palmitoyl coenzyme A synthetase in rat brain, the effect of phosphate on the enzyme, and to determine if the activity associated with the oligodendroglial plasma membrane-myelin fraction (Pande and Mead, 1968; Cantrill and Carey, 1975) could be attributed to contamination of that fraction.

The microsomes had the highest specific activity of the enzyme, which agrees with the results of several authors. (Farstad et al., 1967; Pande and Mead, 1968; Cantrill and Carey, 1975; Murphy and Spence, 1980).

Phosphate enhanced enzyme activity approximately two-fold.

From the marker enzyme distributions in the subcellular fractions of rat brain it is evident that no fraction is pure. A pure myelin fraction might have been obtained using white matter only, but this would be difficult with rats. The palmitoyl coenzyme A synthetase activity associated with the light and heavy myelin fractions is probably due to contamination by microsomes, based on the criterion of NADPH cytochrome C reductase activity.

4.4 Conclusions

The aim of the study was to determine the subcellular localization of palmitoyl coenzyme A synthetase in rat brain, the effect of phospnate on the enzyme, and to determine if the activity associated with the oligodendroglial plasma membrane-myelin fraction (Pande and Mead, 1968; Cantrill and Carey, 1975) could be attributed to contamination of that fraction.

The microsomes had the highest specific activity of the enzyme, which agrees with the results of several authors. (Farstad et al., 1967; Pande and Mead, 1968; Cantrill and Carey, 1975; Murphy and Spence, 1980).

Phosphate enhanced enzyme activity approximately two-fold.

From the marker enzyme distributions in the subcellular fractions of rat brain it is evident that no fraction is pure. A pure myelin fraction might have been obtained using white matter only, but this would be difficult with rats. The palmitoyl coenzyme A synthetase activity associated with the light and heavy myelin fractions is probably due to contamination by microsomes, based on the criterion of NADPH cytochrome C reductase activity.

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Author Kerr E J L
Name of thesis The Subcellular distribution of Palmitoyl Coenzyme a synthetase in rat brain 1981

PUBLISHER:
University of the Witwatersrand, Johannesburg
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