

STUDIES ON THE SYNTHESIS OF RAT
BRAIN SYNAPTIC MEMBRANE PROTEINS

A THESIS SUBMITTED BY
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IN
BIOCHEMISTRY

DEPARTMENT OF BIOCHEMISTRY
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ACKNOWLEDGEMENTS

I wish to express my gratitude to Dr P.W. Beesley for his enthusiasm and guidance throughout the course of this research project. I would also like to thank Professor J.B. Pridham for his interest and support. In addition I am indebted to Mrs G. Anderson, Dr E. Skone and especially Ms C. Spurdon for their patient help with the electron microscopic techniques used in this project. I would also like to thank Ms K. Pengelly for her technical advice and Mr D. Ross for his aid in the production of the photographs presented in this thesis. I would also like to thank the postgraduate students in the Biochemistry Department at Royal Holloway College for their helpful discussions. I am also grateful to Mrs H. Chillingworth who typed this thesis so expertly. Finally I would like to thank my husband, Pat, for his patient support and encouragement.

ABSTRACT

The study of the molecular organisation of specialised synaptic structures has been considerably advanced by the development of methods for the isolation of synaptic subfractions enriched in synaptic plasma membranes (SPM), synaptic junctional complexes (SJC's) and postsynaptic densities (PSD's). The present thesis deals with one important aspect of this organisation, namely the synthesis of SPM proteins and its modulation by selected chemical agents.

SPM, mitochondrial and myelin-enriched fractions of comparable purity to published data have been isolated from adult rat brain. An in vivo method for monitoring incorporation of L-(³⁵S)methionine into the protein of these fractions has been developed. This involved injection of flooding concentrations of L-(³⁵S)methionine into the lateral ventricle via a pre-implanted cannula. Since the precursor specific activity is known and constant for at least 45 minutes the incorporation rates can be reliably measured over this period.

The effects of two anaesthetics, halothane and pentobarbitone, on brain protein synthesis were investigated. Halothane (3%) produced a gross inhibition of protein synthesis in total forebrain and SPM, myelin and mitochondrial fractions. Pentobarbitone produced no such inhibition at doses of 60 or 75 mg/Kg body weight. L-DOPA was also found to inhibit incorporation rates by close to 30% in total forebrain and mitochondrial protein in hyperthermic animals (body temperature 39-40°C). Other subcellular fractions were less affected by the drug.

Thyroid hormone deficiency during brain development impairs brain structure and function including synapse formation. The present study showed that hypothyroidism decreased protein yields in SPM, myelin and mitochondrial fractions and impaired synthesis of SPM and myelin protein. The rates of incorporation into total forebrain and mitochondrial protein were not significantly affected. SDS polyacrylamide gel electrophoresis and fluorographic studies tentatively showed that the relative rates of synthesis of specific polypeptides were altered in thyroid deficient animals.

CONTENTS

Page No.

1. INTRODUCTION	12
1.1 Isolation of Synaptic Subfractions	12
1.1.1. Synaptic Plasma Membrane Fractions	12
1.1.2. Synaptic Junctional Complex Fractions	19
1.1.3. Postsynaptic Density Fractions	22
1.1.4. Purity of Synaptic Subfractions	24
1.1.5. The Heterogeneity of Synaptic Subfractions	24
1.2. Characterisation of Synaptic Subfractions	26
1.2.1. Morphological Analysis	26
1.2.2. Enzymic Analysis	29
1.2.3. Additional Methods for Assessing Purity	32
1.3. The Composition of Synaptic Plasma Membranes	34
1.3.1. Protein Composition	34
1.3.2. Glycoprotein Composition	39
1.3.3. Phosphoproteins	43
1.3.3.1. Protein Phosphorylation Mediated by cyclic AMP	43
1.3.3.2. Protein Phosphorylation Mediated by Calcium Ions	45
1.3.4. Antigenic Components of SPM	47
1.4. Metabolism of Synaptic Proteins	48
1.4.1. Introduction	48
1.4.2. Factors Affecting Brain Protein Synthesis	51
1.4.2.1. Development	51
1.4.2.2. Other Factors	55
Summary	56
2. MATERIALS AND METHODS	58
2.1. Materials	58
2.1.1. Chemicals	58
2.1.2. Surgical Materials	58
2.1.3. Animals	58
2.2. Isolation of Synaptic Plasma Membrane Fractions	59
2.2.1. The Jones and Matus (1974) Isolation Procedure	59
2.2.2. The Cotman and Matthews (1971) Isolation Procedure	59
2.2.3. The Salvaterra and Matthews (1980) Isolation Procedure	60
2.2.4. The Gray and Whittaker (1962) Procedure for isolating Synaptosomes	61

2.3. Characterisation of SPM Fractions	61
2.3.1. Marker Enzyme Assays	61
2.3.2. Protein Determination	63
2.3.3. Electron Microscopy	64
2.3.4. SDS Polyacrylamide Gel Electrophoresis	66
2.3.5. Fluorographic Analysis of Brain Fractions	68
2.4. Techniques Used for the Measurement of <u>In Vivo</u> Brain Protein Synthesis	69
2.4.1. Stereotaxic Techniques	69
2.4.2. Measurement of the Incorporation of L- ^[35S] methionine into Brain Proteins	69
2.4.3. Measurement of the Incorporation of L- ^[U14C] valine into Brain Proteins	70
2.4.4. Determination of the Distribution of L- ^[35S] methionine in the Brain	70
2.4.5. Measurement of TCA-insoluble Radioactivity	71
2.4.6. t.l.c. Analysis of ^[35S] -labelled Amino Acids in Brain Extracts	72
2.5. <u>In Vivo</u> Drug and Thyroid Hormone Deficiency Studies	72
2.5.1. The Effect of Anaesthetics on Brain Protein Synthesis	72
2.5.2. The Effect of L-DOPA on Brain Protein Synthesis	73
2.5.3. The Effect of Thyroid Hormone Deficiency on Brain Protein Synthesis	73
3. RESULTS AND DISCUSSION	74
3.1. Characterisation of Brain Subcellular Fractions	74
3.1.1. Morphological Analysis of Brain Subcellular Fractions	74
3.1.2. Distribution of Marker Enzymes in Brain Subcellular Fractions:	75
3.1.2.1. In Fractions Isolated Using the Jones and Matus Method	75
3.1.2.2. In Fractions Isolated Using the Cotman and Matthews Method	83
3.1.3. SDS Polyacrylamide Gel Electrophoretic Analysis of Brain Subcellular Fractions: Comparison of Fractions Obtained by the Jones and Matus (1974) and Cotman and Matthews (1971) Procedures	85
3.1.4. DISCUSSION	89
3.1.4.1. Comparison of the Purity of Synaptic Fractions with Literature	89

3.1.4.2. Comparison of SPM Fractions Isolated by Both Procedures	91
3.2. Measurement of Total Cerebral Protein Synthesis and Precursor Incorporation into Brain Subcellular Fractions <u>In Vivo</u>	92
3.2.1. Principles Governing the Choice of Method for the Measurement of Cerebral Protein Synthesis Rates <u>In Vivo</u>	93
3.2.2. Measurement of the Rate of Incorporation of L- ^{35}S methionine into Brain Protein Using Gross Intraventricular Injection	95
3.2.3. The Distribution of ^{35}S in Methionine, Cysteine and Cystine Following Intracranial Injection	99
3.2.4. Rates of Incorporation of L- ^{35}S methionine into the TCA-Insoluble Fractions of Gross Brain Areas	99
3.2.5. The Effect of Selected Drugs on the Incorporation of L- ^{35}S methionine into the Proteins of Total Brain Homogenates and Subcellular Fractions <u>In Vivo</u>	104
3.2.5.1. The Effect of Halothane and Pentobarbitone-induced Anaesthesia	104
3.2.5.2. The Effect of L-DOPA	107
3.2.6. DISCUSSION	113
3.2.6.1. The Use of Intraventricular Injections of Flooding Concentrations of L- ^{35}S methionine for Measuring Cerebral Protein Synthesis <u>In Vivo</u>	113
3.2.6.2. The Effect of Selected Drugs on Brain Protein Synthesis <u>In Vivo</u>	116
3.3. The Effects of Hypothyroidism on the Development and Synthesis of Total Brain Protein and SPM and Mitochondrial Polypeptides	120
3.3.1. General Effects of Hypothyroidism	120
3.3.1.1. The Effect on Body and Brain Weights	120
3.3.1.2. The Effect on Protein Yields in Total Homogenates and Subcellular Fractions	123
3.3.2. The Effect of Hypothyroidism on the Incorporation of L- ^{14}C valine into Total Homogenates and Subcellular Fractions <u>In Vivo</u>	127
3.3.3. The Effect of Hypothyroidism on the Amounts of, and Incorporation of L- ^{35}S methionine into, SPM, Mitochondrial and Total Brain Polypeptides <u>In Vivo</u>	129
3.3.4. DISCUSSION	148

SUMMARY
BIBLIOGRAPHY

153
156

LIST OF FIGURES

<u>Figure No.</u>	<u>Page No.</u>
1.1. Isolation of SPM (Cotman and Matthews, 1971)	15
1.2. Isolation of SPM (Jones and Matus, 1974)	16
1.3. Isolation of Synaptic Junctional Complexes	20
1.4. Isolation of Postsynaptic Densities	23
1.5. Protein Phosphorylation at the Synapse	44
3.1.1. Morphology of the Jones and Matus-type Myelin Fraction	77
3.1.2. Morphology of the Myelin-enriched Fraction Isolated Using the Cotman and Matthews Procedure	77
3.1.3. Morphology of the SPM Fraction Prepared by the Jones and Matus Method	78
3.1.4. Morphology of the SPM Fraction Prepared by the Cotman and Matthews Method	78
3.1.5. Morphology of the SPM Fraction Prepared by the Jones and Matus Method and Stained with E-PTA	79
3.1.6. Morphology of the Intermediate Cotman and Matthews Fraction Located at the 32.5-35% (w/w) sucrose Interface	80
3.1.7. Morphology of the Intermediate Cotman and Matthews Fraction Located at the 35-38% (w/w) sucrose Interface	80
3.1.8. Morphology of the Jones and Matus-type Mitochondrial Fraction	81
3.1.9.a) Coomassie Blue Stained 10% SDS Polyacrylamide Gel Comparing Subcellular Fractions Isolated Using Both Procedures	87
3.1.9.b) Densitometric Scans of Electrophoretic Protein Profiles of Myelin, SPM and Mitochondrial-enriched Fractions Isolated Using Both Procedures	86
3.2.1. Time Course of Incorporation of 1mg and 2.5mg of L- ^[35S] methionine into Total Forebrain Protein <u>In Vivo</u>	96
3.2.2. Time Course of Incorporation of L- ^[35S] methionine (2.5mg) into Brain Subcellular Fractions <u>In Vivo</u>	101
3.2.3. Distribution of Perchloric Acid-Soluble ^[35S] Labelled Amino Acids in Brain Extracts Following t.l.c.	102
3.3.1. The Effect of Hypothyroidism on the Body Weight of Developing Rats	121
3.3.2. The Effect of Hypothyroidism on the Weight of Forebrain and Cerebellum in Developing Rats	122

LIST OF TABLES contd.

<u>Figure No.</u>	<u>Page No.</u>
3.3.3.1. Comparison of the Coomassie Brilliant Blue-stained electrophoretic profiles of forebrain protein from hypothyroid and euthyroid neonatal rats.	131
3.3.3.2. Comparison of the incorporation of L-(³⁵ S)methionine into forebrain protein from neonatal hypothyroid and euthyroid rats by fluorographic analysis.	133
3.3.3.3. Comparison of the Coomassie Brilliant Blue-stained electrophoretic profiles of cerebellar protein from neonatal hypothyroid and euthyroid rats.	135
3.3.3.4. Comparison of the incorporation of L-(³⁵ S)methionine into cerebellar protein from neonatal hypothyroid and euthyroid rats.	137
3.3.3.5. Comparison of the Coomassie Brilliant Blue-stained electrophoretic profiles of SPM protein from neonatal hypothyroid and euthyroid rats.	139
3.3.3.6. Comparison of the incorporation of L-(³⁵ S)methionine into SPM protein from neonatal hypothyroid and euthyroid rats.	141
3.3.3.7. Comparison of the Coomassie Brilliant Blue-stained electrophoretic profiles of mitochondrial protein isolated from neonatal hypothyroid and euthyroid rats.	143
3.3.3.8. Comparison of the incorporation of L-(³⁵ S)methionine into mitochondrial protein from neonatal hypothyroid and euthyroid rats.	145

LIST OF TABLES

<u>Table No.</u>	<u>Page No.</u>
1.1. Buoyant Densities of Some Brain Subcellular Fractions	14
1.2. Reported Yields of Protein in Synaptic Subfractions Isolated from Rat Brain	18
1.3. Reported Purity of Synaptic Subfractions	25
1.4. Specificities of Plant Lectins	41
1.5. Molecular Weights of Synaptic Phosphoproteins	46
1.6. Factors which Affect Brain Protein Metabolism	52
1.7. SPM and SJC Polypeptides which Show Development- Related Changes	54
3.1.1. Percentage Distribution of Marker Enzymes and Protein in Brain Subfractions Obtained Using the Jones and Matus Method	76
3.1.2. Percentage Distribution of Marker Enzymes and Protein in Brain Subfractions Obtained Using the Cotman and Matthews Method	84
3.2.1. Trial Comparison of the Rate of Incorporation of L-[³⁵ S]methionine Using Doses of 1mg or 5mg	97
3.2.2.a & b Rates of Incorporation of L-[³⁵ S]methionine into Gross Brain Areas	100
3.2.3. The Effect of Halothane and Pentobarbitone on the Incorporation of L-[³⁵ S]methionine into Total Homogenate and Subcellular Fractions	105
3.2.4. The Effect of Pentobarbitone on the Rate of Incorporation of L-[³⁵ S]methionine into Total Homogenate and Subcellular Fractions	106
3.2.5. The Effect of L-DOPA on the Incorporation of L-[³⁵ S]methionine into Total Homogenate and Subcellular Fractions (Ambient Temperature, 26°C)	108
3.2.6. The Effect of L-DOPA on the Incorporation of L-[³⁵ S]methionine into Total Homogenate and Subcellular Fractions (Ambient Temperature, 30°C) 1.	109
3.2.7. The Effect of L-DOPA on the Incorporation of L-[³⁵ S]methionine into Total Homogenate and Subcellular Fractions (Ambient Temperature, 30°C) 2.	110
3.2.8. The Effect of L-DOPA on the Incorporation of L-[³⁵ S]methionine into Total Homogenate and Subcellular Fractions (Ambient Temperature, 30°C) 3.	111

LIST OF TABLES contd.

- 3.3.1. Protein Yields in Homogenates and Subcellular Fractions
Isolated from Hypothyroid and Euthyroid Rats (mg protein/brain)
- 3.3.2. Protein Yields in Homogenates and Subcellular Fractions
Isolated from Hypothyroid and Euthyroid Rats (mg protein/
g tissue wet weight)
- 3.3.3. Protein Yields in the SPM Fraction Isolated from Hypothyroid
and Euthyroid Rats (μg protein/ μg protein in the Forebrain
Homogenate)
- 3.3.4. The Effect of Hypothyroidism on the Incorporation of
L-[U¹⁴C] valine into Homogenates and Subcellular Fractions
Isolated from Hypothyroid and Euthyroid Rats

ABBREVIATIONS

AChE	acetylcholinesterase
CSF	cerebrospinal fluid
DMSO	dimethyl sulphoxide
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis(β -aminoethyl ether)N,N'-tetraacetic acid
E-PTA	ethanolic phosphotungstic acid
GABA	γ -aminobutyric acid
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid
[Na ⁺ , K ⁺] ATPase	sodium, potassium dependent, ouabain-sensitive ATPase
PMS	phenazine methosulphate
PPO	2,5-diphenyloxazole
PSD	postsynaptic density
PTU	6-n-propyl-2-thiouracil
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SJC	synaptic junctional complex
SPM	synaptic plasma membrane
TCA	trichloroacetic acid
TEMED	N,N,N,N'-tetramethylethylenediamine
Tris	2-amino-2-hydroxymethylpropane-1,3-diol

1. INTRODUCTION

The central nervous system is made up of a vast network of neurones each of which interacts at many specific sites with other neurones. These sites of interaction are synaptic junctions. Thus the synaptic region, although constituting only a small area of the neurone, is of great importance.

In the early 1960's Gray and Whittaker (1962) and De Robertis et al. (1962) independently found that particles which structurally resembled nerve endings in intact tissue were present in crude mitochondrial preparations. Their identity was confirmed by extensive electron micrographic and enzyme distribution studies. These nerve ending particles were named synaptosomes.

Since the development of methods for their isolation synaptosomes have provided an invaluable tool for the study of synaptic function. Subsequently methods have been established for isolation of synaptic subfractions such as the synaptic plasma membrane (SPM), synaptic junctional complex (SJC) and postsynaptic density (PSD).

The synaptic plasma membrane is intimately involved in both regulating the release and actions of neurotransmitters and in the formation and maintenance of synaptic connections. Little, however, is known of the synthesis of SPM proteins or of their function. The aim of the work presented in this thesis, therefore, has been to examine the synthesis of SPM proteins and some factors which may affect this.

1.1 Isolation of Synaptic Subfractions

1.1.1 Synaptic Plasma Membrane Fractions

Several procedures for the isolation of SPM from mammalian brain have been published (Cotman and Matthews, 1971; Morgan et al. 1971; Gurd, et al., 1974; Jones and Matus, 1974). These methods involve the initial isolation of a crude mitochondrial fraction by differential centrifugation. A synaptosomal fraction may then be obtained by density gradient centrifugation. This is followed by a period of hypotonic lysis to release the synaptosomal contents and

density gradient centrifugation to obtain a fraction enriched in synaptic membranes. (Figs. 1.1 and 1.2).

Subsequent treatment of this fraction with detergents yields junctional or postsynaptic density fractions (Figs. 1.3 and 1.4). The exact procedures, however, differ between individual methods.

The starting material for the isolation of synaptic membrane fractions is usually a 10% (w/v) homogenate of brain tissue in either a 0.32 M or 10% (w/w) sucrose solution (homogeniser clearance 0.25 mm). The homogenate is subjected to differential centrifugation to produce a crude mitochondrial fraction which may be used as the parent fraction for isolation of SPM (Jones and Matus, 1974). Alternatively a synaptosome-enriched fraction can be isolated from the crude mitochondrial fraction by discontinuous density gradient centrifugation. (Morgan et al., 1971; Cotman and Matthews, 1971; Gurd et al., 1974). The aim of this is to minimise the contamination of subsequent fractions by non-synaptosomal components.

The similarity in buoyant densities of synaptosomes and potential contaminants makes adequate resolution on this basis difficult (Table 1.1). Although most microsomes are removed during differential centrifugation there is some residual microsomal contamination in the crude mitochondrial fraction. Gurd et al. (1974) reported a reduction in the microsomal contamination in this fraction from more than 50% to less than 15%. This was achieved by washing the crude mitochondrial pellet three times. Levitan et al. (1972) and Tamir et al. (1974) claim to have achieved similar results by reducing the centrifugation time and force in the preparation of the P₂ fraction.

Cotman (1972) suggested that the use of Ficoll gradients, which maintain an isotonic environment, should maximise the difference in buoyant density between components. Morgan et al. (1972) reported that synaptosomes prepared in this way had lower proportions of myelin, glial and axonal fragments than similar fractions prepared on sucrose gradients. They did, however, observe higher levels of mitochondrial contamination which is difficult to explain in view of Cotman's suggestion that resolution of components may be improved under these conditions.

TABLE 1.1

Buoyant densities of some brain subcellular fractions

<u>Fraction</u>	<u>Buoyant density in sucrose (g/ml)</u>	<u>Reference</u>
Synaptosomes	1.13-1.17	Whittaker, 1968
Microsomes	1.13-1.182	Cotman <u>et al.</u> , 1971
Mitochondria	1.15-1.187	" " "
Myelin	1.10	Autilio <u>et al.</u> , 1964
Glial membranes	1.121-1.167	Cotman <u>et al.</u> , 1971

FIG. 1.1

Isolation of SPM

(Cotman and Matthews, 1971)

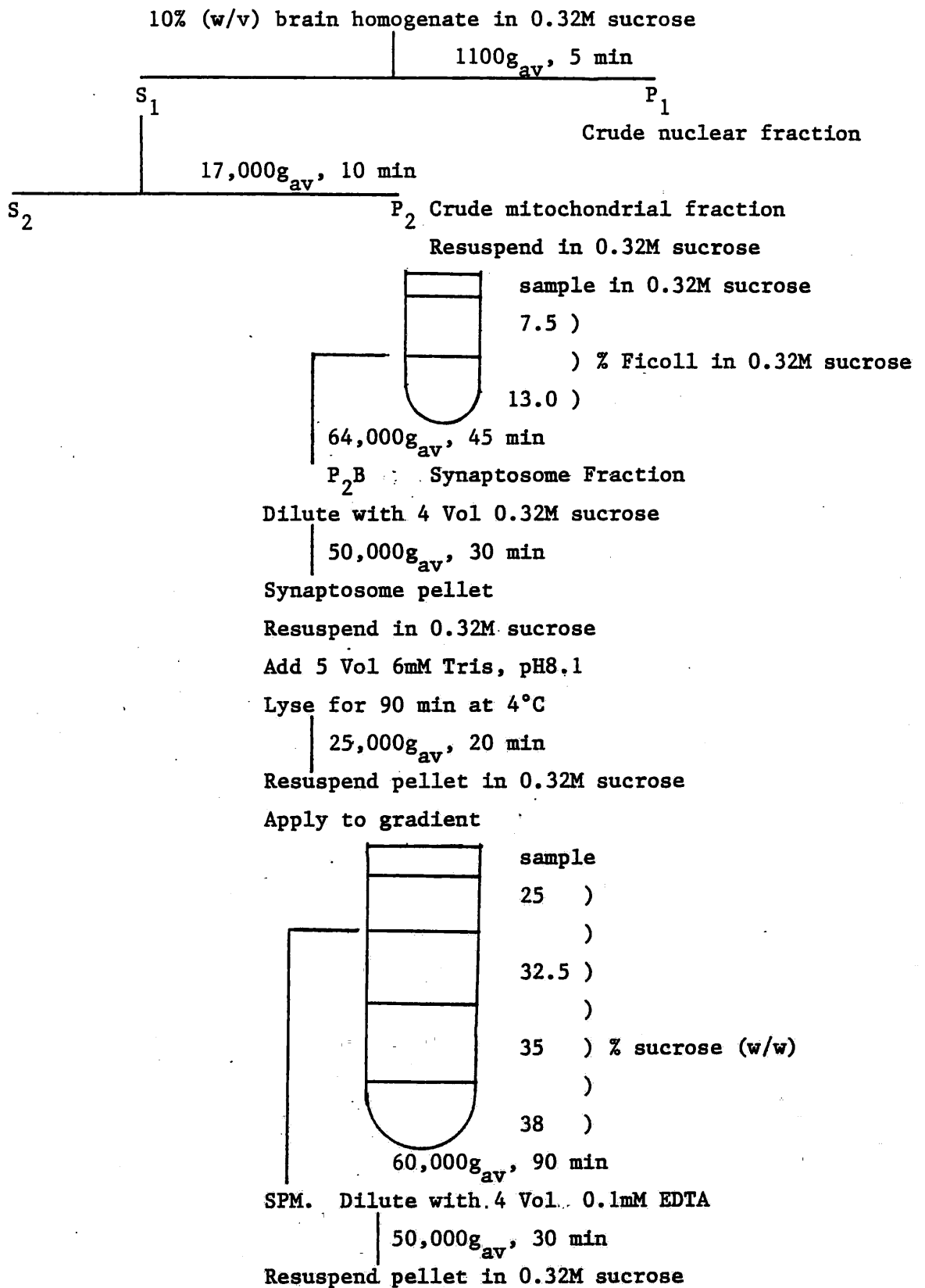
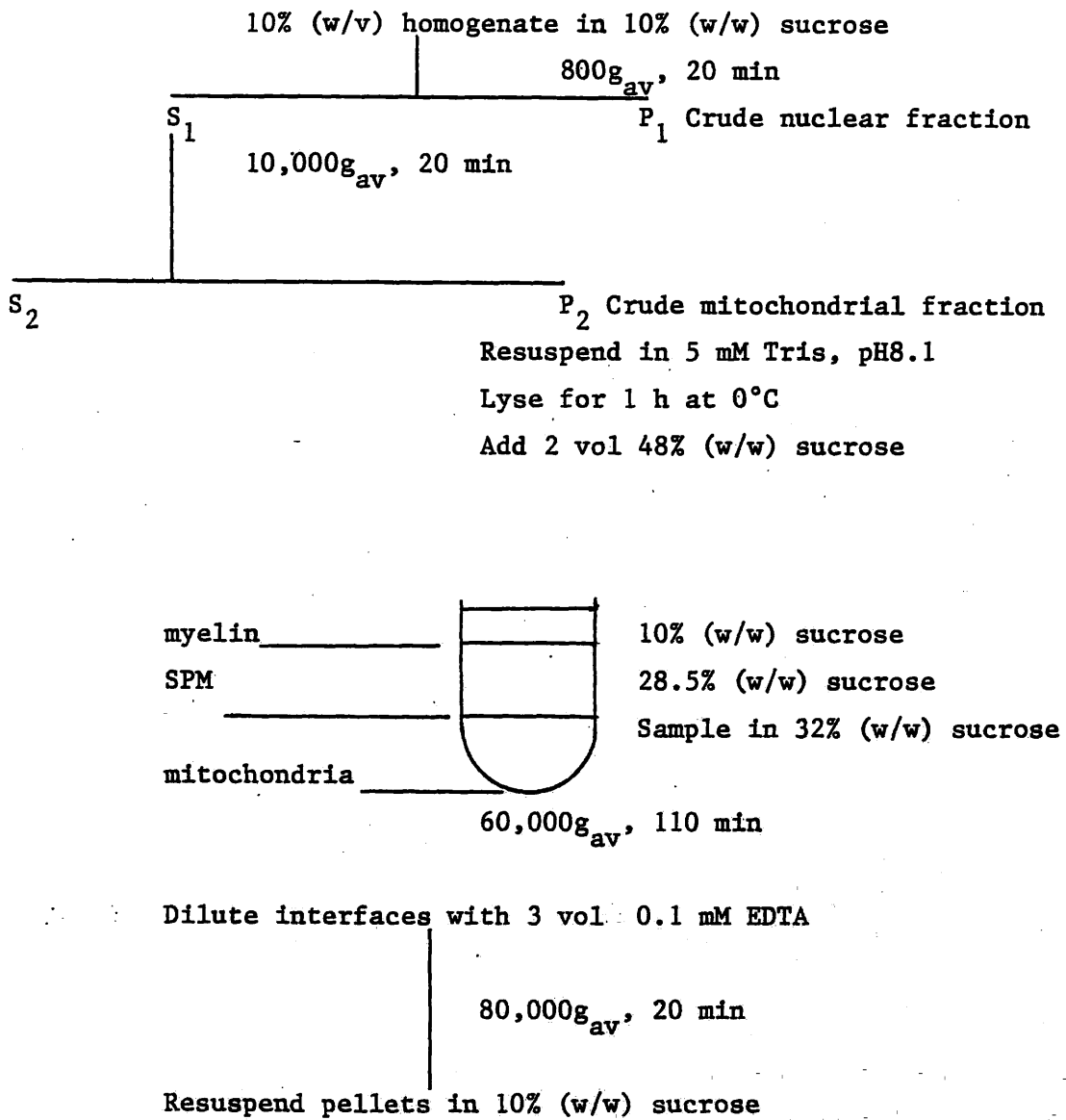


FIG. 1.2

Isolation of SPM

(Jones and Matus, 1974)



In addition to possibly improving the resolution from contaminants synaptosomes prepared on Ficoll gradients are more susceptible to lysis which is an important factor in the preparation of SPM (Morgan et al., 1972). Sucrose has also been reported to cause non-specific association between membranes (Day et al., 1971) which, again, would be minimized by the use of Ficoll gradients.

Tamir et al. (1974) claim to have improved their yield of synaptosomes by using a linear gradient (10-18%) of sodium diatrizoate. They attribute this to the improved separation of synaptosomes from mitochondria in this medium.

Recent evidence using monoclonal antibodies has suggested that even in synaptosome-enriched fractions only 20% of synaptosomes bind a putative neurone-specific antibody (Reichardt and Matthew, 1982). These workers suggest that this may indicate high levels of contamination even in relatively 'purified' synaptosome fractions. Once the parent fraction has been isolated it is necessary to lyse the synaptosomes in order to release the synaptoplasm and synaptic organelles. This is achieved by homogenisation in a hypotonic alkaline buffer such as 5-6 mM Tris, pH8.1 (Cotman and Matthews, 1971; Jones and Matus, 1974), 5 mM HEPES, pH8.4 or 1 mM phosphate/0.1 mM EDTA, pH7.5 (Gurd et al., 1974). The pH of the lysis medium is critical. By examining the distribution of acetylcholinesterase, $(\text{Na}^+, \text{K}^+)\text{ATPase}$ and cytochrome c oxidase on a continuous sucrose gradient Cotman and Matthews (1971) demonstrated that only at pH values above 8.0 was there adequate separation of the membrane marker enzymes from the mitochondrial marker cytochrome c oxidase.

Lysis is usually carried out at 0°C for periods varying from 30 minutes (Jones and Matus, 1974) to 90 minutes (Cotman and Matthews, 1971; Mena et al., 1980). The particulate fraction may then be pelleted prior to application to the gradient (Cotman and Matthews, 1971; Gurd et al., 1974).

Some workers have attempted to minimise mitochondrial contamination by including a procedure which selectively increases the density of mitochondria, thereby enabling better resolution from synaptic membranes. This can be achieved by incubating the lysed synaptosomal fraction with the dye iodinitrotetrazolium violet (INT) which results in the succinate

TABLE 1.2

Reported yields of protein in synaptic subfractions isolated from rat brain

	<u>Yield</u> (mg protein/g tissue)	<u>Reference</u>
<u>SPM</u>	0.12 ¹	Morgan <u>et al.</u> , 1971
	1.5-2.0	Cotman and Matthews, 1971
	3.0	Cotman and Taylor, 1972
	5.0 ¹	Levitan <u>et al.</u> , 1972
	2.5-3.3	Davis and Bloom 1973
	0.2-0.25	Gurd <u>et al.</u> , 1974
	1.0	Jones and Matus, 1974
	3.0	Cotman <u>et al.</u> , 1974
	2.75	Therien and Mushynski, 1976
	0.3-0.5 ²	Smith and Loh, 1977
	2.9	Nieto-Sampedro <u>et al.</u> , 1981
<u>SJC</u>	0.10-0.15	Cotman and Taylor, 1972
	0.15-0.20	Davis and Bloom, 1973
	0.012-0.004	Therien and Mushynski, 1976
	0.242 ³	Webster and Klingman, 1979
	0.46	Nieto-Sampedro <u>et al.</u> , 1982a
<u>PSD</u>	0.025	Cotman <u>et al.</u> , 1974
	0.100-0.150	Cohen <u>et al.</u> , 1977
	0.078	Gurd <u>et al.</u> , 1982
	Triton X-100	6.8 ⁴)
	Na ⁺ cholate	4.8)
	Na ⁺ deoxycholate	1.7) Matus and Taff-Jones, 1978
	N-lauroyl sarcosinate	0.4)

1. Yield reported as mg protein/brain.
2. SPM isolated from mouse brain.
3. SJC's isolated from chick forebrain.
4. Yield reported as mg protein/100mg SPM protein.

dehydrogenase catalysed deposition of the reduced dye, formazan, within the mitochondria. (Cotman and Taylor, 1972; Davis and Bloom, 1973; Nieto-Sampedro et al., 1981a). Using 1 mg INT/g brain tissue (wet weight) Nieto-Sampedro et al. (1981a) found that the mitochondrial contamination of the SPM fraction was 10-13% of the SPM protein.

The lysed fraction is then applied to a discontinuous sucrose gradient of typical composition 0.6M, 0.8M, 1.0M, 1.3M sucrose (Gurd et al., 1974) or 25, 32.5, 35, 38% (w/w) sucrose (Cotman and Matthews, 1971). The gradients are then centrifuged at 53-60,000_{gav} for 1.5-2 hours in a swing-out rotor.

Jones and Matus (1974) used a sedimentation-floatation gradient for isolation of SPM in which the lysed crude mitochondrial fraction was mixed with a high concentration sucrose solution and formed the bottom step (32% (w/w) sucrose) of a three step gradient (overlaid with 28% and 10% (w/w) sucrose solutions). Salvaterra and Matthews (1980) adapted this technique for use with a fixed angle rotor. They reported that this reduced the centrifugation time from 110 minutes to about 35 minutes and also increased the yield of SPM although they did not quote value for this.

After centrifugation the fractions can be collected from the gradient either by aspiration or by use of a tube cutter. The fractions are then diluted and pelleted. Cotman and Matthews (1971) recommended using a 0.1 mM EDTA solution for this step.

The yield of SPM protein obtained reflects the method of isolation used. Those schemes entailing many steps generally produce lower yields of SPM protein (e.g. Morgan et al., 1971; Gurd et al., 1974). Most workers, however, have reported yields of SPM protein as being between 1 and 5 mg/g tissue (wet weight). (See Table 1.2)

1.1.2 Synaptic Junctional Complex Fractions

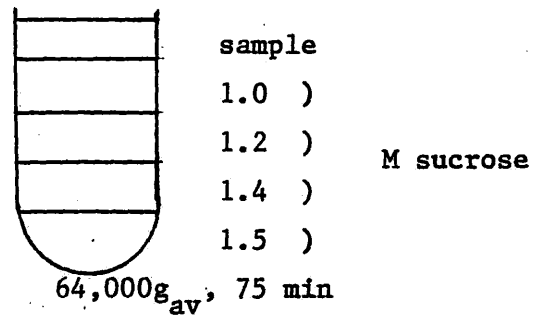
Fiszer and De Robertis (1967) demonstrated that it is possible to solubilize the extra-junctional membrane by treating SPM fractions with Triton X-100 (0.1%, v/v) leaving the area of junctional contact intact.

FIG. 1.3

Isolation of synaptic junctional complexes

(Cotman and Taylor, 1972)

SPM (4 mg/ml) in
2 mM Bicine, pH7.5
Add solution of Triton X-100 (4 mg/ml),
2 mM EDTA, 2 mM Bicine, pH7.5
Incubate at 4°C or room temperature for 10 min
Apply to gradient



Collect interfaces and dilute with water
Pellet at $100,000g_{av}$, 30 min.

Electron microscopic evidence showed that the pre- and postsynaptic densities and their associated membranes survived the detergent treatment.

A considerable amount of work has been carried out to establish routine methods for the isolation of synaptic junctional fractions (Cotman et al., 1971b; Cotman and Taylor, 1972; Davis and Bloom, 1973; Therien and Mushynski, 1976; Webster and Klingman, 1979; Nieto-Sampedro et al., 1982a). (See Fig. 1.3) Generally the isolated SPM fraction is subjected to solubilization in a Triton X-100 solution at either a Triton X-100: protein ratio of 1:1 or 2:1 (w/w) (Cotman and Taylor, 1972) or 0.2-0.4% Triton X-100 (Davis and Bloom, 1973; Webster and Klingman, 1979). The detergent is usually buffered with a low concentration of Tris 3mM at pH7.2-7.5 (Cotman et al., 1971b). Solubilization is conducted at 0-4°C or room temperature (Cotman and Taylor, 1972) and the solubilized fraction is either applied directly to a discontinuous sucrose gradient of composition 1.0M, 1.2M, 1.4M, 1.5M sucrose (+50µM CaCl₂, pH7.0, Cotman and Taylor, 1972) or pelleted to give the detergent insoluble fraction (Davis and Bloom, 1973).

All of these workers have noted the improvement in structural integrity of the junctions when calcium is included in the isolation media. Addition of CaCl₂ to the Triton X-100 solution, however, results in a significant decrease in the amount of protein solubilized - which is reduced from about 60% to around 35% (Davis and Bloom, 1973; Cotman and Taylor, 1972). If CaCl₂ is added at the required concentrations (3-5mM) only at the solubilization step the particulate fractions have been observed to clump during density gradient centrifugation (Davis and Bloom, 1973). This, therefore, gives poor resolution of the fractions. This effect was noted at concentrations of CaCl₂ as low as 100µM. They found, however, that inclusion of CaCl₂ (50µM) in all of the solutions used to isolate the parent SPM fraction maintained the junctional structure but did not cause clumping of the SJC fractions during their isolation. Inclusion of EDTA (1mM) in the detergent solution was found to have no adverse effect on junctional structure but prevented clumping of the particulate fraction. Davis and Bloom (1973), therefore, recommended that inclusion of CaCl₂ in all isolation media used in the preparation of SPM protected the junctional structure but that if it was included in the detergent solution the resolution of SJC's from contaminants was poor.

Therien and Mushynski (1976) developed an alternative method for isolating SJC's in which the SPM fraction was homogenised in a biphasic system consisting of Freon 113 and 0.2% (v/v) Triton X-100. The aqueous Triton X-100 phase was shown to contain about 25% of the total SPM protein. This fraction was subsequently pelleted and resuspended in a solution of Triton X-100, dextran sulphate and sucrose (to give final concentrations of 0.05, 0.02% (v/v) and 1.2M respectively). The resultant suspension was then centrifuged on a discontinuous gradient of composition 2.0, 1.5, 1.4 and 1.3M sucrose. The SJC fraction was located at the 1.5-2.0M sucrose interface. By adding Triton X-100 and dextran sulphate to the gradient Therien and Mushynski found that they could minimise the interactions between SJC's and other membranes reported by Davis and Bloom (1973).

Isolated SJC fractions are composed predominantly of postsynaptic structures plus fragments of associated presynaptic membranes. The number of intact synaptic junctions, i.e. where both pre- and postsynaptic structures are present, are relatively few. (See section 1.2.1) Therien and Mushynski's method produces an SJC fraction with a relatively high proportion of intact SJC's which retain their presynaptic dense projections. These structures are usually lost during the isolation of synaptic subfractions. The yield of SJC protein in this method is, however, lower than that in fractions produced by other methods. The yields of SJC protein reported in the literature are given in Table 1.2.

1.1.3 Postsynaptic Density Fractions

The postsynaptic density can be isolated by application of more rigorous solubilization conditions. A range of detergents have been employed for this purpose and include N-lauroyl sarcosinate (NLS), sodium deoxycholate, high concentrations of Triton X-100 and a combination of polyethylene glycol, dextran T500 and 1-0-n-octyl- β -D-glucosidase (Cotman et al., 1974; Matus and Taff-Jones, 1978; Cohen et al., 1977; Gurd et al., 1982). The morphological and biochemical characteristics of the resultant fraction depend on the isolation conditions employed (Matus and Taff-Jones, 1978). PSD fractions isolated using Triton X-100 contain a higher percentage of contaminating membranes than fractions produced using the other detergents but the PSD itself retains its characteristic

FIG. 1.4

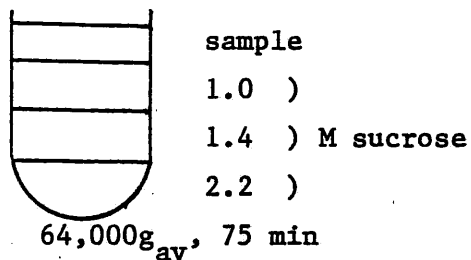
Isolation of postsynaptic densities

1. Cotman et al., 1974
2. Cohen et al., 1977

1. SPM fraction

Extract with N-lauroyl sarcosinate at 0.5 or 3.9% (w/v) for 6-10 min at 4°C.

Apply to gradient



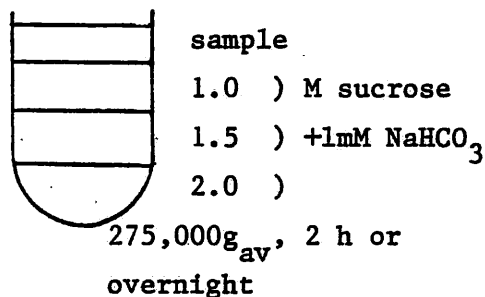
Dilute interface 1:3 with 0.1 mM EDTA and pellet at 78,000g_{av} for 20 min

2. SPM fraction

Extract with equal volume of 1% (v/v) Triton X-100 for 15 min, 0-4°C.

48,000g_{av}, 20 min

Resuspend pellet in 0.32M sucrose containing 1mM NaHCO₃
Apply to gradient



Dilute interface with distilled water and pellet at 275,000g_{av} for 1 h

structure as seen in situ (Matus and Walters, 1975). Sodium deoxycholate and NLS solubilize a greater proportion of the PSD protein and yield a fraction in which the PSD's have an open lattice-like structure (Matus and Taff-Jones, 1978). The procedures used for the isolation of PSD fractions are similar to those used for SJC's (Fig. 1.4). The SPM fraction is incubated with the detergent solution for 10 minutes at 0-4°C or 37°C followed either by direct centrifugation to give the detergent-insoluble fraction (designated by some workers as the PSD fraction) (Cohen et al., 1977; Matus and Taff-Jones, 1978) or by discontinuous sucrose density gradient centrifugation (Cohen et al., 1977, 'long procedure'). Gurd et al. (1982) developed a novel system in which a PSD fraction of high purity could be rapidly isolated from SPM. In this system the SPM fraction is homogenised in polyethylene glycol, dextran T500 and 1-0-n-octyl- β -D-glucoside which is then centrifuged at 2250 _g for 7.5 minutes. During centrifugation the polyethylene glycol and dextran T500 phases separate and the PSD fraction bands at the interface. These workers found that by using the mild, neutral detergent n-octyl-glucoside the activity of a PSD protein kinase is preserved. (Gurd, unpublished observations reported in Gurd, 1982). The reported yields of PSD protein are given in Table 1.2

1.1.4 Purity of Synaptic Subfractions

The reported purities of the synaptic subfractions are given in Table 1.3. The methods for assessing the purity of fractions are fully discussed in section 1.2. The purity of the fraction depends on the isolation method used. The use of the classical procedures of differential and density gradient centrifugation for the isolation of synaptic subfractions do not, unfortunately, yield fractions of extremely high purity. This is an inevitable consequence of the similarity in buoyant densities of the synaptic membranes and potential contaminants. It is possible that with the advent of monoclonal antibody techniques methods for the production of highly purified subfractions will be developed. A method for isolating synaptic vesicles using monoclonal antibodies has been developed by Reichardt and Matthew, (1982).

1.1.5 The Heterogeneity of Synaptic Subfractions

The synaptic plasma membrane fraction comprises portions of the pre- and

TABLE 1.3

Reported purity of synaptic subfractions

SPM

<u>% purity</u>	<u>Basis of estimate</u>	<u>Reference</u>
over 85	chemical and enzymatic	Morgan <u>et al.</u> , 1971
85-90	enzymatic	Levitan <u>et al.</u> , 1972
80	morphological	Jones and Matus, 1974
85-90	enzymatic	Gurd <u>et al.</u> , 1974
85-90	enzymatic	Smith and Loh, 1977

SJC

<u>% purity</u>	<u>Extraction medium</u>	<u>Basis of estimate</u>	<u>Reference</u>
65	0.5% Triton X-100	morphology	Cotman and Taylor, 1972
50	0.2% Triton X-100	"	Davis and Bloom, 1973
65	0.2% Triton X-100/ Freon 113	"	Therien and Mushynski, 1976
75	2:1 Triton X-100:protein	"	Nieto-Sampedro <u>et al.</u> , 1982a

PSD

>85	3% NLS	morphology	Cotman <u>et al.</u> , 1974
>90	0.5% Triton X-100	"	Cohen <u>et al.</u> , 1977

postsynaptic membranes and the electron dense bodies associated with them. Thus, even at the simplest level, SPM is a heterogeneous fraction consisting of membranes and proteinaceous structures derived from two cells. Synaptic subfractions appear to be derived, at least primarily, from Type I (or asymmetric synapses) rather than Type II (or symmetric synapses). This classification of synapses was first made by Gray (1959) who later correlated Type I synapses with excitatory transmission and Type II synapses with inhibitory transmission (Gray, 1969). This selectivity may, however, be due to difficulty in identifying Type II synapses since the presynaptic dense projections are usually lost during the isolation procedure. Matus and Walters (1976) identified both types of synapses in synaptosome fractions thus it is feasible that the apparent selectivity of the isolation procedures is merely indicative of the difficulty in identification of Type II synapses.

In addition to this structural variety of synapses there is also functional heterogeneity. Synapses derived from nerve tracts utilising different neurotransmitters have been shown to vary in buoyant density. De Robertis et al. (1962; 1963) isolated two distinct populations of synaptosomes of which the lighter fraction was enriched in cholinergic terminals whilst the more dense fraction appeared to contain non-cholinergic nerve endings.

More recently it has been shown to be possible to select a specific population of synaptosomes using antisera against transmitter-related enzymes. Docherty et al. (1982) were able to specifically lyse a cholinergic subpopulation of synaptosomes by incubation with an antiserum against choline acetyltransferase. Subsequently this group has also been able to specifically lyse GABAergic synaptosomes using an antiserum against glutamate decarboxylase (Docherty et al., 1983). The possibility of selecting specific subpopulations of synapses in this way may offer a means of limiting the innate heterogeneity of synaptic plasma membrane fractions.

1.2 Characterisation of Synaptic Subfractions

1.2.1 Morphological Analysis

Synaptic subfractions can be characterised in a number of ways to

ascertain both the content and degree of purity of the fractions. By examining a fraction morphologically it is possible to determine whether the component of interest is present (assuming that it is morphologically identifiable) and is structurally preserved and whether the fraction is significantly contaminated (again assuming that any contaminants are unambiguously identifiable).

The SPM fraction is typified by the presence of vesicular profiles 0.5-1.2 μ m in diameter (Jones and Matus, 1974) with their characteristic synaptic densities. Some profiles contain residual synaptic vesicles adhering to the presynaptic membrane. Some profiles are, inevitably, sectioned in a plane which does not include the synaptic densities which makes definite identification of these structures difficult since other non-junctional membranes (e.g. axonal and dendritic) have a similar appearance. Jones and Matus (1974) reported other contaminants to be "sparsely distributed amorphous dense bodies" and synaptic mitochondria still enclosed within some nerve endings. Cotman and Matthews (1971) also found small electron-dense bodies in their SPM fractions and suggested that, on the basis of a similarity in morphology, these might be lysosomes.

SJC and PSD fractions have been almost exclusively characterised by morphological methods since enzymic analysis is unreliable due to the effects of the detergents used in the isolation procedures.

SJC fractions have been reported to consist predominantly of synaptic complexes with prominent PSD's which may be enclosed within a small vesicle of postsynaptic membrane (Cotman and Taylor, 1972). Several groups have applied quantitative electron microscope methods to the analysis of SJC and PSD fractions and have found that only a small proportion of structures in SJC fractions are intact synaptic complexes, i.e. with both pre- and postsynaptic elements present. Nieto-Sampedro et al. (1982b) estimated that in their SJC fraction only 6-31% of identifiable structures were intact SJC's although a minimum of 85% were PSD's or PSD plus a fragment of postsynaptic membrane. Most groups have found the presynaptic dense projections to be severely damaged or missing (Cotman and Taylor, 1972; Davis and Bloom, 1973; Matus et al., 1975). Therien and Mushynski (1976), however, found that a much greater proportion of identifiable structures in their SJC fraction were intact

complexes and estimated that about 65% of the total occupied area was attributable to junctional structures. In addition many of the complexes retained their presynaptic dense projections which, in a few cases, appeared to be attached to the PSD although no intervening membrane remained. Contaminants have usually been reported to be membranes or dense bodies of uncertain origin (Therien and Mushynski, 1976; Nieto-Sampedro et al., 1982a).

Postsynaptic density fractions are usually of a higher degree of purity than SJC fractions (See Table 1.5). The morphology of the PSD fraction depends on the isolation method used. In fractions isolated using Triton X-100 (0.5% or 3%, v/v) or low concentrations of deoxycholate (1-2%, w/v) the appearance of the PSD closely resembles that seen in situ and consists of particles 10-20nm in diameter (Matus and Walters, 1975; Cohen et al., 1977; Matus and Taff-Jones, 1978). PSD fractions prepared using N-lauroyl sarcosinate (0.5% or 3.9%, w/v) or higher concentrations of deoxycholate (3%, w/v) remove a higher proportion of the adhering membrane (Cotman et al., 1974; Matus and Taff-Jones, 1978). At this concentration deoxycholate produces PSD-like structures of lower electron density than those produced using Triton X-100 and appear to have a more 'open' structure which prompted Matus and Walters (1975) to name them postsynaptic junctional lattices. Matus and Taff-Jones (1978) suggested that these arose due to differential removal of intrinsic PSD proteins by deoxycholate. This fraction resembles the "loosened" structures produced by various chemical treatments of PSD's which have also been reported to remove certain PSD proteins (Blomberg et al., 1977).

The appearance of PSD's produced by the less rigorous treatments has been reported as being either bars or crescents with an overall diameter of 150-350 nm and 30-40nm thick (Cotman et al., 1974; Cohen et al., 1977; Matus and Taff-Jones, 1978; Gurd et al., 1982; Nieto-Sampedro et al., 1982a). Some PSD's, however, have been found to have a central perforation giving them a "doughnut-like" appearance. These PSD's have diameters at the higher end of the range (350-370 nm) (Cohen et al., 1977; Cohen and Siekevitz, 1978; Nieto-Sampedro et al., 1982b).

The PSD appears to consist of dense-staining aggregates 20-30 nm in diameter which are connected, in some preparations, by filaments of diameter 6-9 nm (Carlin et al., 1980). Cohen et al. (1977) observed

diffuse material extending up to 100 nm from the cytoplasmic face of the PSD with occasional subsynaptic bodies being connected to the PSD via filaments.

Regional differences in PSD morphology have been reported in dog brain where cerebellar PSD's appeared to have no central perforations and were thinner than cerebral cortex PSD's (Carlin *et al.*, 1980). These workers suggested that these PSD's may arise from Type II synapses since this type predominates in the cerebellum.

Contaminants of PSD fractions have been reported to be membranes, microtubules or amorphous material of uncertain origin (Cotman *et al.*, 1974; Cohen *et al.*, 1977; Gurd *et al.*, 1982; Nieto-Sampedro *et al.*, 1982a). On the basis of quantitative electron microscopic studies these workers have estimated that PSD fractions are contaminated 10-15% by these components.

Thus, the morphology (and degree of contamination) of the SJC and PSD fractions depends on the solubilization conditions employed and the brain region used as the source of tissue.

1.2.2 Enzymic Analysis

In order to obtain an estimate of the degree of contamination of the various synaptic subfractions it is necessary to complement the morphological data with marker enzyme distribution analysis.

Determination of the degree of purity of synaptic subfractions on the basis of enzymic analysis has been hampered by the lack of specific marker enzymes for the junctional region of the neuronal membrane. Thus negative marker enzymes, i.e. those specific to putative contaminants and positive marker enzymes, i.e. those at least substantially located in the synaptic plasma membrane, have been used in characterisation studies.

The enzymes commonly used as positive markers for SPM are $(\text{Na}^+, \text{K}^+)\text{ATPase}$, 5'-nucleotidase and acetylcholinesterase (AChE). Enrichment of these enzymes in the SPM fraction over the homogenate is generally considered to be a good indication of SPM being the predominant component in that fraction.

Neuronal $(\text{Na}^+, \text{K}^+)$ ATPase is usually measured by its sensitivity to ouabain which distinguishes it from other ATPase activities which may be present. Cotman and Matthews (1971) found that the activity of $(\text{Na}^+, \text{K}^+)$ ATPase was enriched 5-fold in their SPM fraction over the homogenate. Morgan et al. (1971) and Gurd et al. (1974), however, obtained a 10-15 fold enrichment of this enzyme in their SPM fractions. Mahadik et al. (1978) suggested that this might be due to the removal of an inhibitor of the enzyme by the repeated washings of the fractions in these procedures. Schaffer et al. (1974), however, demonstrated the presence of a soluble, dialyzable inhibitor of $(\text{Na}^+, \text{K}^+)$ ATPase which may be co-extracted with SPM. The inhibiting effect was able to be negated by the use of chelators such as EDTA and EGTA.

$(\text{Na}^+, \text{K}^+)$ ATPase, however, is also thought to be present in glial membranes. Jones and Matus (1974) found that 48% of the total $(\text{Na}^+, \text{K}^+)$ ATPase activity recovered from their sucrose density gradient was present in the myelin fraction and 44% in the SPM fraction. If the crude mitochondrial fraction was subjected to sonication in addition to hypotonic shock 91% of the total activity was found in the SPM fraction. Thus excessively high levels of $(\text{Na}^+, \text{K}^+)$ ATPase activity may indicate glial contamination which, in turn, depends on the isolation conditions used.

5'-nucleotidase is also frequently used as a marker for SPM although this has been more extensively characterised as a plasma membrane marker in liver fractions (Evans and Gurd, 1973). Cotman and Matthews (1971) found that 35% of the total activity of this enzyme was associated with the myelin fraction and 42% with the SPM fraction which they suggested was an indication that this enzyme was not primarily associated with SPM. Henn and Hamberger (1976) have reported finding high activity of 5'-nucleotidase associated with glial membranes, whilst Kreutzberg et al. (1978) suggested that this enzyme is almost exclusively associated with glial membranes.

Cotman et al., (1971a) demonstrated that a subfraction of cultured glial cells sediment at the same isopycnic density as synaptosomes. Using a mixed fraction containing (^{14}C)leucine labelled cultured glial cells and a whole rabbit brain homogenate Henn et al. (1976) found that in the synaptosome fraction isolated from this mixture at least 40% of the material was of glial origin.

Thus high levels of activity of either $(\text{Na}^+, \text{K}^+)\text{ATPase}$ or 5'-nucleotidase in synaptic subfractions may indicate significant contamination by glial membranes which have no morphologically characteristic features.

Acetylcholinesterase also appears to be associated with low buoyant density membrane fragments (Cotman and Matthews, 1971). Shute and Lewis (1966) found this enzyme to be associated with axonal, dendritic and microsomal membranes. Cotman and Matthews (1971) estimated that about 44% of the total activity of this enzyme recovered from the gradient was present in the SPM fraction. Jones and Matus (1974), however, found about 78% of the total activity in the SPM fractions. Since AChE is found in a range of membranes which transport water or ions including erythrocyte and platelet membranes (for extensive discussion see Silver, 1974) this enzyme is a poor marker for SPM.

In addition to assaying for positive markers of synaptic membranes it is possibly more fruitful to assess markers known to be specific to putative contaminants.

Mitochondrial contamination is usually estimated by assaying for cytochrome c oxidase or succinate dehydrogenase both of which are exclusively located on the inner mitochondrial membrane. Only low levels of activity of these enzymes are usually found in the SPM fraction e.g. 6% (Cotman and Matthews, 1971), 9% (Jones and Matus, 1974) and less than 3% (Morgan et al., 1971) using cytochrome c oxidase. Morgan et al. (1971), however, suggested that the outer mitochondrial membrane may be damaged during the lysis step of the isolation procedure. They based this suggestion on the observation of high levels of monoamine oxidase (MAO) activity present in their SPM fraction. These workers also suggested that MAO in brain tissue might not be confined to the outer mitochondrial membrane since estimation of the levels of NADH:cytochrome c reductase, which is also present in the outer mitochondrial membrane, did not correspond well with the MAO data. NADH:cytochrome c reductase is also found in microsomal membranes although microsomal contamination is usually monitored by assaying rotenone-insensitive NADPH:cytochrome c reductase. Using the latter enzyme the levels of contamination of SPM by microsomal membranes has been estimated to be less than 10% (Morgan et al., 1971), 3% (Levitan et al., 1972) and 10-15% (Gurd et al., 1974).

Myelin is another major contaminant of SPM fractions. This can be detected by assaying for 2',3'-cyclic nucleotide 3'-phosphohydrolase (Kurihara and Tsukada, 1967). Using this enzyme Jones and Matus (1974) estimated that 5% of the total activity recovered from the gradient was present in the SPM fraction. Tamir et al. (1976) reported a very low activity of this enzyme in their SPM preparation and estimated the myelin contamination to be less than 0.5%. They attributed this to careful trimming of the cortex prior to homogenisation to remove as much white matter as possible and also to the use of sodium diatrizoate gradients instead of sucrose. Levitan et al., (1972) estimated myelin contamination of their SPM fraction by adding radiolabelled myelin to the preparation prior to homogenisation. Subsequently they determined the specific activity of the membrane fraction and estimated that myelin protein accounted for 1-2% of the total protein in the SPM fraction. In a similar way glial and axonal membrane contamination was estimated to be 2-3% each and microsomal and mitochondrial contamination to be 3-5% each.

Cotman and Matthews (1971) estimated contamination of SPM fractions by lysosomes by assaying for acid phosphatase and β -N-acetylglucosaminidase. They found that about 44% of the total recovered activity of these enzymes was present in the SPM fraction. These workers suggested that lysosomal contamination of the SPM fraction probably accounted for only a very small proportion of the protein since purified lysosomes have been shown to have a specific activity 100 times that of the homogenate (Sawant et al., 1969). They also suggested that these enzymes may be loosely associated with SPM since some lysosomal enzymes have been found in plasma membranes. (Fleischer and Fleischer, 1969). Gurd et al., (1974) estimated that lysosomal contamination of their SPM fraction was less than 10% on the basis of the distribution of these enzymes.

1.2.3 Additional Methods for Assessing Purity

To assess the purity of any fraction accurately the ideal marker should be specific to that fraction. Protein species which satisfy this criterion for synaptic fractions are the neurotransmitter receptors. These are most easily assayed by radioligand binding studies. (See review by Mahler, 1977). Salvaterra and Matthews (1980) determined the

relative enrichment of muscarinic and nicotinic cholinergic receptors by measuring the specific binding of (^3H) quinuclidinyl benzilate and (^{125}I) bungarotoxin in their SPM fraction. They found a 2-4 fold enrichment of these receptors in their SPM fraction.

It is also possible to compare the electrophoretic protein profiles of the isolated SPM fraction with those of known contaminants. By critical comparison of the relative mobilities and abundance of polypeptides on SDS polyacrylamide gels it is possible to detect the presence of contaminating material.

Myelin, for example, has a very characteristic profile with highly enriched polypeptide bands corresponding to the basic proteins of apparent molecular weights 14, 17, 18 and 21K, the DM-20 protein, 20.5K, the proteolipid protein, 24K and the Wolfgram proteins, 51, 54 and 62K (molecular weights taken from Waehnelde, 1978). Thus contamination of the SPM fraction by myelin is readily identifiable especially in the lower molecular weight regions of the gel.

The mitochondrial fraction also has a distinctive electrophoretic protein profile and shows enrichment of polypeptides of 50 and 51K over SPM fractions (Mena et al., 1980). Other major mitochondrial proteins have molecular weights 46.5, 45.5, 42.5, 34, 32.5, 31 and 29.5K. (Mena et al., 1980). These low molecular weight polypeptides are particularly distinctive and are useful indicators of mitochondrial contamination of SPM fractions.

SPM fractions contain four major polypeptides of molecular weights 99, 55, 52 and 43K (Matus, 1978). The identities of these are thought to be a subunit of (Na^+ , K^+)ATPase, tubulin, a unique tubulin-like PSD protein and actin respectively (Mahler, 1977).

Microsomes show a very similar electrophoretic protein profile to SPM (Gurd et al., 1974; Mena et al., 1980). The latter found, however, that SPM fractions appeared to be enriched in a polypeptide of 32K.

Wang and Mahler (1976) compared the electrophoretic profiles of SPM fractions isolated by the methods of Gurd et al. (1974) and Jones and

Matus (1974). They found that although the overall patterns were similar and showed the four major and twelve minor polypeptide bands there appeared to be more polypeptides present in the Jones and Matus preparation. Since these were in the low molecular weight region of the gel they suggested that these may be mitochondrial or myelin proteins.

Interpretation of electrophoretic profiles may be difficult where samples have similar profiles. For example, in all of the fractions described above, the 50-60K region is extremely complex and contains a number of major polypeptides of similar molecular weights. This can be overcome, to some extent, by judicious use of gels of different acrylamide concentrations which improve the resolution in the chosen area of the gel.

Polypeptides are usually located by staining with Coomassie Brilliant Blue. Many workers have estimated the relative amounts of polypeptides present on the basis of their staining intensities. Coomassie Brilliant Blue staining is, however, only semi-quantitative. Phillips and Slater (1975) showed that actin binds less dye than does serum albumin. Bray and Thomas (1975) estimated that actin showed only 67% of the staining intensity of the same amount of serum albumin. It has also been demonstrated that Coomassie Brilliant Blue staining of proteins deviates from Beer's law at high protein concentrations (Chrambrach et al., 1967).

Matus et al. (1980)^b used antisera against glial and neuronal filaments to detect filament antigens in SDS polyacrylamide gels of synaptic subfractions. Other workers have used 2-dimensional electrophoresis to identify proteins in synaptic subfractions following trypsin digestion (Kelly and Cotman, 1978). Thus, electrophoresis is a powerful tool for the analysis and characterisation of synaptic subfractions.

1.3 The Composition of Synaptic Plasma Membranes

1.3.1 Protein Composition

Most work on the composition of SPM has centred on the protein components in order to define the significance of these constituents with regard to the structure and function of the synapse.

The SPM fraction consists of 50-70 polypeptides when separated by SDS polyacrylamide gel electrophoresis (Banker et al., 1972; Mahadik et al., 1976; Wang and Mahler, 1976; Mahler, 1977) of which about 20 are major. Wang and Mahler (1976) estimated that eleven polypeptides of molecular weights, 250, 225, 175, 137, 97, 68, 54, 44, 37 and 33K accounted for 93% of the total SPM protein with the 97, 54, 44 and 37K polypeptides constituting more than 60%. All SPM fractions appear to be particularly enriched in polypeptides of 54 and 45K (Kelly and Cotman, 1977).

Synaptic junctional complex fractions have similar electrophoretic profiles to SPM but the relative intensities of some polypeptide bands differ from equivalent bands in the SPM fraction (Kelly and Cotman, 1977). These workers found that, although no single SJC polypeptide accounted for more than 10-15% of the total, eight polypeptides accounted for more than 5% each. SJC polypeptides which were markedly enriched over SPM fractions had molecular weights of 280, 275, 150, 63, 55, 52, 45 and 32K (Kelly and Cotman, 1977). Therien and Mushynski (1976) reported that the 97K polypeptide was enriched in SJC fractions.

The PSD fraction, by comparison, has a simpler electrophoretic protein profile although the number of polypeptides present varies depending on the extraction procedure. The major polypeptide has a molecular weight of 52K and has been estimated to account for 45% of the total protein in this fraction (Banker et al., 1974; using NLS extraction). These workers also found significant quantities of polypeptides of 180, 86, 55, 45, 28 and 26K while Gurd et al. (1982) found polypeptides of 180, 130, 110, 94, 65 and 60K to be enriched in their PSD fraction. Kelly and Cotman (1977) estimated that the 55K polypeptide accounted for 14% of the total protein while Banker et al. (1974) estimated that the 97K polypeptide constituted 17% of the total protein.

The identities of the synaptic proteins have been sought in order to gain some insight into the working of the synapse at the molecular level. A number of SPM proteins have now been identified.

* The exact value for the molecular weight varies slightly according to the gel system used. Thus the same protein may be reported to have different molecular weights by different workers.

The presence of intermediate filaments and microtubules in electron micrographs of the synaptic region (Westrum and Gray, 1976; Yen et al., 1977) prompted workers to examine synaptic subfractions for the presence of fibrous proteins characteristic of the cytoskeleton. In all synaptic subfractions there are major polypeptides (molecular weights 56, 54 and 45K*) with similar electrophoretic mobilities to those reported for certain fibrous proteins, namely α and β tubulin and actin (Kelly and Cotman, 1977). The polypeptides of 56 and 54K have been shown to comigrate, in SDS polyacrylamide gels, with purified β and α tubulin respectively. (Feit et al., 1977; Kelly and Cotman, 1977). SJC and PSD fractions have been reported to contain both α and β tubulin which are identical to their cytoplasmic form as demonstrated by 2-dimensional electrophoresis and tryptic peptide analysis (Kelly and Cotman, 1978). These workers estimated that these proteins constitute about 9% of SJC and 14% of PSD total protein. Blomberg et al. (1977), however, found that tubulin was only a minor component of PSD's isolated from dog cortex. This prompted them to suggest that tubulin might be loosely linked to the PSD structure. This seems unlikely since tubulin was not removed from the PSD under the more severe extraction conditions employed by Kelly and Cotman (1977). Babitch (1981) reported that tubulin could only be removed from SPM fractions under conditions where the membrane was completely destroyed and interpreted this as indicating that tubulin was an integral SPM protein. Carlin et al. (1982) demonstrated that detergent treatment of the PSD did not reduce the amount of tubulin in this fraction. This group, however, observed that the PSD became increasingly enriched in tubulin in the postmortem period. Thus the variable amounts of tubulin reported by different groups may be a consequence of the isolation conditions. It is now thought that tubulin is not a major component of synaptic subfractions.

The major PSD polypeptide (mPSDp) has a molecular weight of 52K and migrates ahead of purified tubulin (Kelly and Cotman, 1977). Tryptic peptide analysis demonstrated that this protein was similar to tubulin (Feit et al., 1977) but unrelated to glial fibrillary acidic protein which has a similar molecular weight (Mena et al., 1981).

Extensive characterisation of the mPSDp now seems to suggest that this protein is unique to the synapse (Kelly and Cotman, 1978). Tryptic peptide maps of proteins of similar molecular weights from rat brain

cytosol, rat liver plasma membranes and chick lens gap junctions proved to be dissimilar to that of the mPSDp (Kelly and Montgomery, 1982). These workers, however, did find a 52K polypeptide in a microsomal subfraction (P_3B_2) which resembled the mPSDp in all respects. De Blas and Mahler (1978) reported that this fraction was enriched in neurotransmitter receptors and concluded that this fraction contained postsynaptic membranes minus the PSD. Mena et al. (1981) also observed a mPSDp-like protein in a similar microsomal subfraction but, unlike Kelly and Montgomery (1982), could find no electron micrographic evidence of the presence of junctional structures in this fraction.

Carlin et al. (1980) found that cerebellar PSD's contained relatively low amounts of the mPSDp but possessed an apparently unique protein of 74K. Kelly and Montgomery (1982) also observed a reduction of about 20-fold in the amount of mPSDp in cerebellar PSD's while SJC fractions prepared from rat forebrain minus the cerebral cortices contained about half the amount of mPSDp compared with SJC's from the cortex alone. Both groups suggested that these deficits might reflect the higher proportion of Type II synapses in the cerebellum and the cerebral structures underlying the cortex. This is difficult to reconcile with the suggestion of Kelly and Montgomery (1982) that the microsomal SJC-like fraction possibly represented Type II synapses since this fraction contained significant amounts of the mPSDp. Thus more work is necessary to define the role of the mPSDp within different types of synapses.

The mPDSp appears to be linked to the PSD via intermolecular disulphide bonds since electrophoretic profiles of unreduced PDS's lack both this and a 55K polypeptide (Kelly and Cotman, 1976). These workers suggested that, on this basis, disulphide bonds may play an important role in the regulation of synaptic function at the level of junctional structure.

The 45K polypeptide present in all synaptic subfractions has been found to comigrate with actin (Kelly and Cotman, 1977). Two dimensional electrophoresis has shown that β and γ -actin are present in these fractions in approximately equal amounts but not the α -actin characteristic of muscle cells (Kelly and Cotman, 1978). Matus et al., (1982) used anti-actin antibodies to demonstrate that in intact tissue actin is concentrated in the PSD although there is significant

immunohistochemical staining of the surrounding cytoplasm particularly within dendritic spines. Blomberg et al. (1977) found that actin could be selectively removed from the PSD by deoxycholate/KCl, deoxycholate/dithiothreitol and deoxycholate/parachloromercuribenzoate. All of these treatments caused an 'opening up' of the structure leaving a lattice-like entity similar to that derived using high concentrations of deoxycholate (Matus and Taff-Jones, 1978). Blomberg et al. (1977) suggested that the PSD was composed of aggregates (10-20 nm in diameter) of the mPSDp since this was resistant to all of these chemical treatments and that these aggregates might be bundles of filaments seen in cross-section. Electron micrographs appeared to show that these aggregates were connected by narrow filaments composed primarily of actin since these were removed on treatment with the above reagents.

Beach et al. (1981) produced a monospecific antibody against chicken gizzard myosin to which SPM and SJC fractions reacted positively although PSD and myelin fractions showed no reaction. These workers showed that the tryptic peptide fingerprints of the putative SJC myosin was indistinguishable from purified brain myosin but distinct from purified smooth and skeletal muscle myosins. It was also demonstrated that the presence of myosin in these fractions was not due to adherence of the molecule to the junctional apparatus during isolation since addition of (¹²⁵I) labelled myosin during fractionation was not subsequently found in the subfractions.

The presence of these proteins may indicate the existence of an actomyosin complex with functional activity at the synapse. Indeed, Berl et al. (1973) demonstrated that actomyosin-like proteins were concentrated in synaptosomal fractions. These workers postulated that these contractile proteins might be involved in transmitter release from the presynaptic terminal (Berl and Nicklas, 1975). It is possible that other myofibrillar proteins are present at the nerve ending. Blomberg et al. (1977) examined the electrophoretic profiles of mixtures of PSD's and myofibrillar proteins. Eight proteins were found to have equivalent electrophoretic mobilities (molecular weights 110, 100, 59, 45, 31, 26, 18 and 17K although the 110, 59 and 17K polypeptides were thought to be contaminants). No myofibrillar protein, however, has been definitively identified in synaptic subfractions. The 18K polypeptide was suggested

by Blomberg et al. (1977) to be similar to one of the troponin subunits - the calcium binding protein troponin C. This was subsequently demonstrated to be calmodulin (Grab et al., 1979). Its presence in the PSD was confirmed by immunohistochemistry and radioimmunoassay (Lin et al., 1980; Wood et al., 1980) (for further discussion of calmodulin see section 1.3.3.)

Recent evidence suggested that a high molecular weight doublet observed in SDS polyacrylamide gels is fodrin - a spectrin-like protein which forms a 'lining' on the cytoplasmic face of plasma membranes (Carlin et al., 1983; Kakiuchi et al., 1982; Sobue et al., 1982). Fodrin comprises two polypeptides of 250 and 240K which are prominent in SPM, SJC and PSD fractions. Fodrin has been reported to interact with actin in vitro (Levine and Willard, 1981). These workers suggested that fodrin might have a role in linking submembranous structures to the membrane (Levine et al., 1981). Thus fibrous proteins appear to have an important role in synaptic structure and function.

1.3.2 Glycoprotein Composition

Synaptic subfractions have been found to contain a number of glycoproteins (Kelly and Cotman, 1977; Rostas et al., 1977). This fact has generated considerable interest since glycoproteins have been implicated in cell-cell recognition and adhesion (see review by Barondes, 1975). Although there is no firm evidence to support the supposition several workers have tentatively suggested that the junctional glycoproteins might play an important part in the formation and maintenance of synaptic contacts (Barondes, 1976). Matus (1980) suggested that this could be achieved by either trans-synaptic linkage of oligosaccharides from the opposing membranes or by a lectin-glycoprotein type bonding. The former possibility would need to be supported by demonstration of the existence of glycosyltransferases in the region of synaptic junction. While Goodrum et al. (1979) have found receptors for glycosyltransferase activity at the synaptic junction other workers have been unable to demonstrate the presence of these enzymes in this area (Raghupathy et al., 1972; Reith et al., 1972). The alternative suggestion of a lectin-like interaction is possible since lectins have been reported to be present in brain tissue (see review by Simpson et al., 1978). Matus, however, was unable to

find any lectin activity in either soluble or particulate fractions from rat brain (Banner and Matus - unpublished observations reported in Matus, 1980).

While several groups have examined the sugar composition of synaptic subfractions there is little agreement as to the relative proportions of the sugars present (Margolis et al., 1975; Churchill et al., 1976). The most abundant sugars are N-acetylglucosamine, N-acetylgalactosamine, mannose, galactose, fucose and sialic acid.

SJC's and PSD's contain relatively low amounts of sialic acid although PSD's show high levels of mannose (Churchill et al., 1976; Gurd, unpublished observations reported in Gurd, 1982). Both SJC's and PSD's have been found to contain high levels of glucose (Churchill et al., 1976) which is an unusual component of glycoproteins. These workers suggested that this may be due to the presence of residual sucrose from the isolation media.

Histochemical examination of the synapse has demonstrated the presence of high concentrations of lectin receptors in the junctional region (Cotman and Taylor, 1974; Kelly et al., 1976). Receptors for Con A and Ricinus communis lectin (see Table 1.4) have been identified on the postsynaptic membrane where those overlying the PSD show restricted lateral mobility and are unable to aggregate in the presence of lectin (Cotman and Taylor, 1974; Kelly et al., 1976; Matus and Walters, 1976).

Subsequent studies in which the binding of radiolabelled lectins to synaptic subfractions was examined following electrophoresis showed that while SPM fractions contained 10-12 Con A binding proteins (Zanetta et al., 1975; Gurd, 1977b) SJC fractions contained only 3 major classes (molecular weights 180, 130 and 110K) (Gurd, 1977a, b). Kelly and Cotman (1977) identified an additional junctional glycoprotein of 95K and designated these glycoprotein components I-IV respectively. These workers estimated that these 4 components accounted for 53% of the total Con A binding activity of SJC fractions. No binding components were found in the 45-55K region in SJC fractions although considerable Con A binding was found in this region in SPM fractions. Kelly and Cotman (1977) attributed this to the presence of extrajunctional glycoproteins in SPM fractions. In addition, they found no Con A binding activity in

TABLE 1.4

Specificities of plant lectins

<u>Lectin</u>	<u>Sugar specificity</u>
Concanavalin A (Con A)	D-mannose, D-glucose
Wheat germ agglutinin (WGA)	N'-acetyl-D-glucosamine, sialic acid
<u>Lens culinaris</u> phytohaemagglutinin (LCH)	D-mannose, D-glucose
<u>Ricinus communis</u> agglutinin (RCA)	D-galactose, N-acetyl-D- galactosamine
<u>Lotus tetragonolobus</u> lectin (fucose binding protein)	Fucose

the PSD fraction and concluded that all of the binding sites must, therefore, reside in the junctional membranes. This is supported by earlier histochemical studies which indicated that synaptic glycoproteins are located on the external surface of synaptic membranes in Type I synapses (Wang and Mahler, 1976; Chiu and Babitch, 1978) although Con A binding sites have been observed on the cytoplasmic face of postsynaptic junctional membranes in Type II synapses (Matus and Walters, 1976). Gurd (1977a, 1982), however, has reported the presence of the three major classes of Con A binding proteins in PSD fractions prepared under a variety of isolation conditions. The major junctional Con A binding proteins appear to be highly conserved since their presence has been demonstrated in different brain areas (Rostas et al., 1979) and a range of species including mammals (Blomberg, et al., 1977; Gurd, 1977a; Kelly and Cotman, 1977; Rostas et al., 1979; Freedman et al., 1980) and amphibia, fish and reptiles (Nieto-Sampedro et al., 1982a). In addition, the tryptic peptide maps of the three higher weight Con A receptors are very similar (Mena and Cotman, 1982). These glycoproteins appear to be unique to the synaptic junction and have not been detected in extrajunctional membranes, synaptic vesicles, axolemma, microsomes or myelin (Gurd, 1980).

The binding of Con A to these junctional glycoproteins is sensitive to α -mannosidase and endoglycosidase H indicating the the lectin binds primarily to mannose-rich oligosaccharides (Gurd, 1980; Gurd and Fu, 1982). These workers have demonstrated that the major proportion of Con A binding activity is attributable to two classes of mannose-rich oligosaccharides containing 5 and 9 mannose residues (Gurd and Fu, 1982).

In addition to Con A receptors synaptic junctional fractions (both SJC's and PSD's) are capable of binding wheat germ agglutinin, Ricinus communis agglutinin, Lens culinaris phytohaemagglutinin and the fucose specific lectin isolated from Lotus tetragonolobus. This indicates that a wide range of oligosaccharide structures are present at the synaptic junction (Gurd, 1977a, 1979, 1980) and has led Gurd and his co-workers to suggest that glycoproteins may play an important role in synaptic function.

1.3.3 Phosphoproteins

Protein phosphorylation is thought to be involved in a number of regulatory systems including those at the synapse (Rubin and Rosen, 1975; Greengard, 1978a). There is a considerable body of literature on this topic much of which is outside the scope of this review. Only synaptic phosphoproteins, therefore, will be discussed.

1.3.3.1 Protein Phosphorylation Mediated by Cyclic AMP

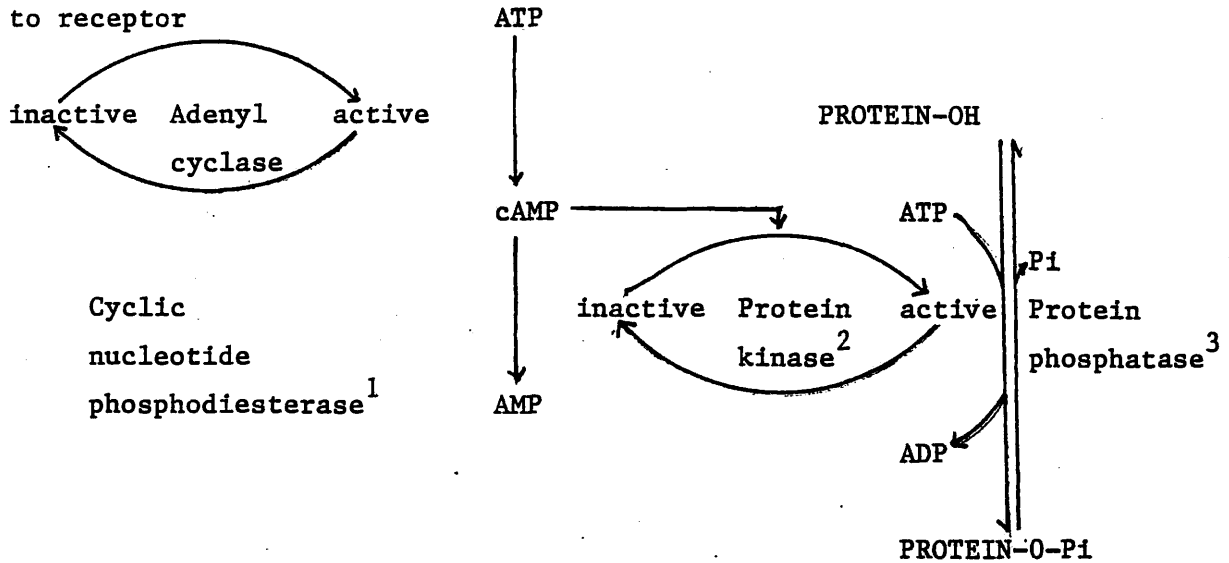
On the basis of the substantial body of evidence built up during the 1970's (see review by Greengard, 1979) Greengard and his co-workers have suggested that cyclic AMP (cAMP) - mediated protein phosphorylation may have a key role in neurotransmission. These workers suggested that cAMP may play a 'second messenger' role in an analogous way to its role in the physiological response of target cells to hormonal action. Greengard has demonstrated that binding of certain neurotransmitters to their receptors results in activation of adenylyl cyclase and thence to increased levels of cAMP (Greengard, 1978b). This led Greengard to suggest that cAMP-sensitive protein kinases present at the synaptic junction catalyse the phosphorylation of certain membrane proteins following neurotransmitter-receptor binding, which in turn regulates some essential property of the junctional membranes e.g. permeability. This hypothesis has been widely reviewed in recent years (Bloom, 1975; Daly, 1977; Nathanson, 1977; Greengard, 1978a; Berridge, 1979) and is represented diagrammatically in Fig. 1.5.

The presence of cAMP-sensitive protein kinases in SJC and PSD fractions has been demonstrated by several workers (De Blas *et al.*, 1979; Kelly *et al.*, 1979; Ng and Matus, 1979a; Carlin *et al.*, 1980). These enzymes catalyse the phosphorylation of endogenous substrates in the presence of ATP. Termination of the effects of cAMP and protein kinase activity requires the conversion of cAMP to AMP by a cyclic nucleotide phosphodiesterase (CNP) and the dephosphorylation of the substrate by a protein phosphatase. Both enzymes have been detected in synaptic subfractions. CNP activity has been detected in isolated PSD's (Cotman *et al.*, 1974) and SJC's (Therien and Mushynski, 1979b). Protein phosphatase activity has been detected in PSD fractions (Ng and Matus, 1979a) but not SJC's (Therien and Mushynski, 1979a).

FIG. 1.5

Protein phosphorylation at the synapse

Binding of transmitter
to receptor



1. Cyclic nucleotide phosphodiesterase has been located in SJC and PSD fractions (Cotman et al., 1974; Therien and Mushynski, 1979b) and has been shown to be activated by Ca^{2+} /calmodulin (Grab et al., 1980).

2. A protein kinase activity has been reported in SJC and PSD fractions (Kelly et al., 1979; Carlin et al., 1980). Activation of this enzyme is thought to occur by binding of cAMP to the regulatory subunit thereby enabling the phosphorylation reaction to be carried out by the catalytic subunit (Walter et al., 1978). Protein kinase activation by Ca^{2+} /calmodulin has also been reported (Grab et al., 1980).

3. Protein phosphatase activity has been reported in PSD fractions where it has been estimated to be enriched 2-3 times over SPM fractions (Ng and Matus, 1979). Therien and Mushynski (1979), however, could detect no activity in their SJC fraction.

A number of junctional proteins have been found to undergo phosphorylation in vitro of which a number are phosphorylated in a cAMP-dependent manner (De Blas et al., 1979; Kelly et al., 1979; Ng and Matus, 1979a; Berman et al., 1980) (see Table 1.3). All of these groups reported cAMP-dependent phosphorylation of proteins in the molecular weight regions 85-75K and 56-54K which correspond to Greengard's proteins 1 and 11 respectively. Protein 1 appears to be unique to nervous tissue and is concentrated in SPM and synaptic vesicle fractions (Greengard, 1978b).

Berman et al. (1980), however, found that these two major classes of phosphoproteins did not appear to undergo phosphorylation in vivo where the predominant phosphorylated proteins were of molecular weight 70, 51 and 44K. Other work seems to indicate that the pattern of phosphorylation seen in in vitro experiments may be greatly influenced by the experimental conditions employed (Ng and Matus, 1979b; Weller, 1979; Matus et al., 1980a). For example, if low ratios of ATP to membrane protein are used substrate depletion may influence the results due to hydrolysis of ATP by intrinsic ATPases (Matus et al., 1980a). In addition, Ng and Matus (1979b) were unable to obtain complete dephosphorylation of the endogenous substrates under the standard conditions used in in vitro experiments.

Thus, at present, the results of in vitro phosphorylation experiments are difficult to interpret in the context of synaptic physiology. The results of in vivo experiments are also problematic in view of the long duration of phosphorylation of the innate substrates.

1.3.3.2 Protein Phosphorylation Mediated by Calcium Ions

The depolarisation-induced influx of Ca^{2+} ions into the neurone has been found to regulate various neurotransmitter-associated processes e.g. the rate of neurotransmitter synthesis (Patrick and Barchas, 1974; Morgenroth, et al., 1975) and phosphorylation of specific endogenous substrates (Krueger et al., 1977).

A number of SPM proteins have been found to undergo Ca^{2+} -stimulated phosphorylation in the presence of calmodulin (see Table 1.5) (Schulman and Greengard, 1978; De Blas et al., 1979; DeLorenzo, 1982).

TABLE 1.5

Molecular weights of synaptic phosphoproteins

Enzyme dependence: cAMP				Ca ²⁺ /Calmodulin		
Fraction: SPM	SJC	PSD	PSL	SPM	SJC	PSD
300 ¹	300 ¹					
220 ³		220 ³	220 ³			230 ⁵
210 ²						180 ⁵
180 ³		180 ³	180 ³	155 ¹	155 ¹	155 ¹
160 ^{2,3}		160 ³	160 ³			150 ⁵
140 ³		140 ³	140 ³			105 ⁵
100 ³		100 ³	100 ³	100 ¹		100 ⁵
85 ²	85 ²					70 ⁵
82 ²	82 ²					62 ⁵
80 ^{1,4}	80 ¹	80 ¹				58 ⁵
75 ¹	78 ²	75 ¹				51 ⁵
74 ³	75 ¹	74 ³		48 ¹	48 ¹	48 ¹
68 ³		68 ³	68 ³			45 ⁵
66 ¹	66 ¹	66 ¹				
59 ³		59 ³	59 ³			
56 ³		56 ³	56 ³			
55 ^{2,4}	55 ²					
54 ¹	54 ¹	54 ¹				
51 ³		51 ³	51 ³			
50 ¹	50 ¹					
47 ³						

Values are expressed as molecular weight x10⁻³

1. De Blas et al., 1979
2. Kelly and Cotman, 1979
3. Ng and Matus, 1979
4. Berman et al., 1980
5. Grab et al., 1980

PSL = postsynaptic junctional lattice (Ng and Matus, 1979)

Calmodulin has been identified in association with the postsynaptic membrane (Wood et al., 1980) and the PSD (Grab et al., 1979; Grab et al., 1980) where a calmodulin-activated protein kinase activity (Grab et al., 1981b) and a number of calmodulin-binding proteins (Carlin et al., 1981) have been located. The major calmodulin-binding protein has been reported to have a molecular weight of 51K and appears to be identical to the major phosphorylated protein. Another calmodulin-binding protein present in PSD's and which undergoes Ca^{2+} -dependent phosphorylation has been identified recently as fodrin, a spectrin-like molecule of molecular weight 230-240K (Kakiuchi et al., 1982; Sobue et al., 1982; Carlin et al., 1983).

The function of this Ca^{2+} -dependent phosphorylation system at the synapse is uncertain. DeLorenzo has suggested that this may be involved in neurotransmitter release (DeLorenzo, 1982). A postsynaptic role may also exist in view of the presence of the necessary components in PSD fractions (see review by Grab et al., 1980).

1.3.4 Antigenic Components of SPM

Immunochemical techniques have been applied to the study of SPM in an attempt to further characterise this fraction at a more quantitative level than that allowed by SDS gel electrophoresis.

The most extensively characterised SPM antigens are those designated synaptin, D_1 , D_2 and D_3 by Bock and collaborators (see review by Bock, 1978). D_1 , D_2 and D_3 appear to be specific to and enriched in SPM fractions while synaptin is present in both SPM and synaptic vesicle fractions (Bock and Jørgensen, 1975) but is also found in chromaffin granule membranes of the adrenal medulla (Bock and Helle, 1977).

Topographically D_1 and D_2 have been found to be localized on the exterior of synaptic membranes while D_3 and synaptin have been located on the cytoplasmic face of the membrane and, in the case of synaptin, on the outside of synaptic vesicles (Jørgensen, 1976) and chromaffin granules (Bock and Helle, 1977).

All of these antigens are proteins with molecular weights D_1 , 50 and 116K (two polypeptides); D_2 , 139K and D_3 , 141, 34.4 and 23.5K (three polypeptides) (Jørgensen, 1977). Synaptin is a glycoprotein of 45K.

Mahadik and co-workers found six SPM components reacted with their antisera (Mahadik et al., 1981) although all six antigens were also present in rough and smooth microsomal fractions and synaptic vesicles. These antigens have molecular weights of 66, 64, 63, 62, 58 and 56K.

Three protein components of PSD's have been shown to have antigenic activities (molecular weights 95, 82 and 72K). (Nieto-Sampedro et al., 1981). The 95K antigen appeared to be specific to the PSD and was found to be present in PSD fractions isolated from lower vertebrates (Nieto-Sampedro et al., 1982a). These workers suggested that this antigen may be useful as a marker for PSD's in subcellular fractionation and developmental studies.

The production of monoclonal antibodies against nervous tissue antigens promises to be an extremely powerful tool in the investigation of synaptic composition. Several workers have reported the production of monoclonal antibodies against synaptic structures (MacPherson and Kleine, 1978; De Blas et al., 1981; Hawkes et al., 1982; Reichardt and Matthew, 1982; Stoughton et al., 1983), although the specificity of these antibodies has not been definitely established. Clearly monoclonal antibodies will prove to be useful in both analysis and isolation of synaptic plasma membranes.

1.4 Metabolism of Synaptic Proteins

1.4.1 The metabolism of nervous system proteins is of particular interest in view of its probable involvement in neuronal function and pathology and in specific nervous system functions such as information processing. It is possible that alterations in the metabolism of synaptic proteins may affect synaptic function. Very little information is available, however, on the synthesis and turnover of synaptosomal and SPM proteins.

The majority, if not all, brain proteins are in a dynamic state and subject to continuous turnover. In experiments in which rats were fed

radioactive amino acids during development Lajtha and Toth (1966) showed that, at most, 3% of total proteins in adult brain were metabolically stable, the remainder being replaced at an average rate of 0.7% per hour (Lajtha and Dunlop, 1981). The average rate is thought to comprise two elements of which the first is a small rapidly turned over fraction with a half-life of 10 hours and represents about 3.5% of the total protein. The larger element (about 96% of the total protein) is more stable with a half-life of 10 days (Lajtha et al., 1979).

The SPM fraction also contains two classes of protein distinguishable by their difference in turnover rate. In vivo studies have shown that one group of proteins undergoes rapid turnover which is typified by a rapid decline in radiolabel between 4 and 16 hours after injection and a more slowly degraded component (Gurd, 1978). Using tritiated fucose Langley and Kennedy (1977) found a similar pattern to be true of synaptic glycoproteins. On SDS polyacrylamide gel electrophoresis of these fucosylated SPM proteins these workers observed that the decline in labelling of 123 and 85K components was particularly rapid although a 31K component appeared to be stable. It is possible, however, that these results reflect the metabolism of the carbohydrate moieties rather than of the whole protein.

Using intraventricular injection of tritiated leucine Von Hungen et al. (1968) found that the half-lives of total brain protein, SPM, synaptic vesicle and mitochondrial proteins were about 20-21 days. This tended to indicate that brain proteins were more stable than liver proteins whose average half-lives have been reported to be 5-6 days.

Ramirez et al. (1972) reported that their SPM fraction incorporated radiolabelled amino acids and that this incorporation was inhibited by chloramphenicol but not by cycloheximide. Other in vitro studies using intact synaptosomes have indicated that there may indeed be a protein synthetic system in the nerve terminal (Morgan and Austin, 1968; Gambetti et al., 1972; Jones, et al., 1975; Wedege et al., 1977; Boyar et al., 1981). Most workers have reported inhibition of this protein synthesis by cycloheximide indicating that an extramitochondrial protein synthetic system exists in the nerve terminal. Wedege et al. (1977) observed that the cycloheximide-sensitive system was stimulated under depolarising conditions.

The existence of a synaptosomal protein synthesizing system is controversial since ribosomes have not been observed in electron micrographs of the nerve ending (Palay et al., 1968). Autoradiographic studies, however, have shown that tritiated amino acids are incorporated into synaptosomes as well as ribosome containing particles (Cotman and Taylor, 1971). Several workers have been unable to demonstrate extramitochondrial protein synthesis in synaptosomal fractions (Gordon and Deanin, 1968; Deanin and Gordon, 1973; Hernandez, 1974).

It is thought, therefore, that most, if not all, SPM proteins are produced in the cell body and delivered to the nerve terminal by fast axoplasmic transport. The presynaptic membrane proteins and membrane-associated enzymes such as adenylyl cyclase and acetylcholinesterase appear to be transported by the most rapid component of axoplasmic transport (see review by Schwartz, 1979). Similar rapid transport of membrane proteins produced in the cell body has been found in dendrites (Kreutzberg et al., 1973). All proteins appear to pass through the Golgi apparatus prior to transport since studies using monensin (a sodium ionophore which has been reported to specifically damage this organelle) showed that transport of proteins was decreased although protein synthesis was not impaired (Hammerschlag et al., 1982). Tsukita and Ishikawa (1980) suggested that a membrane system might be involved in axonal transport of SPM proteins so that these proteins are continuously located in a membrane environment. They proposed that membrane proteins are transferred in a series of vesicles down the axon. This process appears to be calcium-dependent (Hammerschlag, 1980).

These studies concur with the findings of investigations using other tissues (see reviews by Rothman, 1980 and Sabatini et al., 1982). There is now considerable evidence which indicates that membrane proteins are synthesized on membrane bound polysomes and simultaneously inserted into the adjacent membrane (Sabatini et al., 1982). Wickner (1979), however, has suggested that membrane proteins may be released into the cytoplasm in a conformation compatible with the aqueous environment but that on reaching their target membrane, refold to expose their hydrophobic regions, thus enabling them to penetrate the membrane. There is evidence that most mitochondrial and chloroplast proteins are coded for by nuclear DNA and are synthesized on cytoplasmic

ribosomes (Schatz and Mason, 1974; Chua and Schmidt, 1979). Matsuura et al. (1981) demonstrated that newly synthesized cytochrome c (apoprotein) contains an internal sequence of amino acids which specifically recognises the mitochondrial membrane as its target. The holocytochrome c does not recognise mitochondrial membranes since the recognition sequence is sequestered by the folding of the molecule following addition of the haem moiety.

Much work still remains to be done before the mechanisms of SPM synthesis are fully elucidated. In addition, nothing is known of the synthesis and construction of the insoluble protein dense bodies associated with the synaptic membrane.

Very little is known of the degradation of plasma^a membranes and their proteins. In general membrane proteins have been found to undergo extensive turnover at heterogeneous rates with degradation being largely intracellular (see review by Schimke, 1973). In the case of SPM local breakdown of synaptic proteins is thought to play an important role in degradation (see review by Droz, 1973) although the means by which SPM proteins are removed from the membrane is unknown.

1.4.2 Factors Affecting Brain Protein Synthesis

There is a considerable body of literature pertaining to factors which affect brain protein metabolism of which only a small proportion refers specifically to SPM proteins. A number of these factors are listed in Table 1.6. It is possible to suppose that many of the factors which affect brain protein synthesis generally will also affect the synthesis and turnover of SPM proteins. In a few instances factors which have a specific effect on synaptic proteins has been studied and these are now discussed.

1.4.2.1 Development

The rat is born in a relatively immature state and in the perinatal period there are substantial changes in the brain. The period of maximal synaptogenesis occurs between the second and fourth weeks after birth at which time the majority of nerve endings are formed. A number of synapses are present at birth and control essential functions but

TABLE 1.6

Factors which affect brain protein metabolism

<u>Factor</u>	<u>Effect on protein synthesis</u>	<u>Reference</u>
Physical: Hypothermia	Inhibition	Schain and Watanabe, 1971
Hyperthermia	Inhibition	Millan <u>et al.</u> , 1979
Chemical: LSD)	Inhibition which parallels the increase in body temperature	Heikkila <u>et al.</u> , 1979
L-DOPA)		
D-amphetamine)	Effect due to polysome disaggregation	Moskowitz <u>et al.</u> , 1977
Barbiturates		
Alcohol (chronic)	Inhibition	Satake <u>et al.</u> , 1974
	Decline in synthesis rate	Wasterlain <u>et al.</u> , 1977
Biochemical:		
Hypothyroidism	Inhibition <u>in vivo</u>	Gonzales and Geel, 1978
Hyperthyroidism	Possible stimulation	Cook and Kiernan, 1976
Pathological:		
Ischaemia	Inhibition	Cooper <u>et al.</u> , 1977
Electroshock seizures	Inhibition	Dunn and Berget, 1977
Intracerebral puncture	Transient inhibition	Dunn, 1975
Malnutrition	Inhibition	Banay-Schwartz <u>et al.</u> , 1979

probably on constitute about 10% of those present in the adult brain (Bloom, 1972). During synaptogenesis it is probable that there are alterations in composition of the growth cone membrane to that of the mature synaptic membrane (for reviews see Bloom, 1972; Pfenninger and Rees, 1976).

The yield of protein in SPM fractions increases from about 0.5 mg/g brain (wet weight) at birth to about 4 mg/g at 90 days old with a roughly linear increase occurring over the first 20 days after birth (Kelly and Cotman, 1981). During the period of postnatal brain development the protein composition of the SPM fraction also changes (Jones and Matus, 1975). A number of these development-related changes are given in Table 1.7. Of particular interest are the changes in glycoprotein composition during this period since these are implicated in cell-cell recognition (see section 1.3.2). De Silva et al. (1979) demonstrated that the binding of the lectins Con A and WGA increased significantly between 5 and 17 days postpartum. The major Con A receptors in the SJC fraction were also shown to increase substantially over this period.

The synapse-specific antigens synaptin, D₁ and D₃ rise during postnatal development to reach a constant level at about 40 days after birth while the level of D₂ initially increases but decreases after the brain growth spurt (Jaquet et al., 1976). These workers suggested that this pattern paralleled the rate of synapse formation. More recently it has been found that D₂ cross-reacts with antibodies to the cell adhesion molecule isolated from chick embryo neural tissue which implies that D₂ might be involved in cell-cell recognition during synaptogenesis (Jørgensen et al., 1980).

The major development-related increase has been reported to be in the amount of the major PSD protein (Fu et al., 1981; Kelly and Cotman, 1981) which increases 20 fold during the 3rd and 4th weeks of life. Both groups also reported a small decrease in the amounts of tubulin and actin in SPM fractions during development.

The rates of synthesis of individual proteins have not been measured. Fu et al. (1981), however, showed that the absolute incorporation of (³H)fucose into synaptic proteins decreased during postnatal

TABLE 1.7

SPM and SJC polypeptides which show development-related changes

<u>Polypeptides which show increases</u>		<u>Polypeptides which show decreases</u>	
<u>SPM</u>	<u>SJC</u>	<u>SPM</u>	<u>SJC</u>
	250	270	270
220	220		130
	190		120
180*	180*(ConAR)		87
160*			85
150-140*	150-140*	73	
	130*(Con AR)		60
	125	59	
110*	110*(Con AR)	56)	
95	95	55*) Tubulin	
88-70		54)	
65*	65*	45 (Actin)	
	62		43
	53	12	
52*(mPSDp)	52*(mPSDp)	10	
43		9	
39			

Values are given as molecular weight x 10⁻³.

* Fu et al., 1981; remainder Kelly and Cotman, 1981.

Tentative identifications are given in parenthesis.

Con AR = Con A receptor protein.

development. The specific activity of the homogenate decreased about 4 fold between 5 and 28 days postpartum although incorporation into SPM decreased by only 2-2.5 fold. This may, however, reflect alterations in glycosylation rather than protein synthesis.

Thus there are substantial changes in SPM protein metabolism during development with major changes occurring at the time of synaptogenesis.

1.4.2.2. Other Factors

The immature brain is capable of various plastic changes in response to external influences. This is apparent in the corresponding alterations in the synthesis of the synaptic junctional proteins.

When the rats reared in the dark are first exposed to light the incorporation of L-(³H) fucose and L-(³H)lysine into SPM proteins increases during the first hour. After three hours, however, the incorporation of L-(³H)lysine is depressed by 79% (Burgoyne and Rose, 1980). Burgoyne et al. (1981) demonstrated that the increased synthesis of SPM proteins was associated with polypeptides of molecular weights 100, 71, 44 and 38K with the 44K species being tentatively identified as actin.

Prenatal hypoxia has been reported to result in decreased incorporation of (¹⁴C) valine into SPM proteins (Gross et al., 1981).

LSD-induced hyperthermia has been shown to cause a 35-45% inhibition in both total brain and SPM protein synthesis (Freedman et al., 1981). The SPM fraction, however, showed a specific increase in a polypeptide of 75K. This increase appeared to be dependent on the hyperthermic effect of the drug. Hyperthermia induced by raising the ambient temperature resulted in increased levels of 75 and 95K polypeptides which were thought to be 'heat shock' proteins.

Repeated seizures have also been found to cause reduction in cell size and number during development (Wasterlain and Plum, 1973).

Electrically-induced seizures have been reported to result in decreased cerebral protein synthesis (Wasterlain, 1974). Jørgensen and Bolwig, (1979) found that in rats subjected to regular electroconvulsive shocks

the levels of the synaptic membrane-specific antigens, synaptin and D₂, were increased in both the forebrain and occipital cortex.

During the period of postnatal development the brain is extremely susceptible to insult which may, in turn, result in long term impairment of brain function. Some pathological conditions are known to result in reduced numbers of synapses although it is not known whether the overall process of synapse formation is retarded. For example, administration of testosterone to female rats early in development results in reduced numbers of synapses in the pre-optic area (Raisman and Field, 1973).

Thyroid hormone deficiency during brain development causes gross effects typified by reduced brain weight and water content, reduced cell numbers and cell size, decreased branching of dendrites and decreased density of axons and dendrites (see reviews by Sokoloff, 1971; Balazs *et al.*, 1977). Synapse formation has been found to be impaired as indicated by morphological studies and developmental profiles of transmission-related enzymes such as choline acetyltransferase (Nicolson and Altman, 1972; Kalaria *et al.*, 1981). In view of the asynchronous development of the brain those areas, such as the cerebellum, which are relatively immature at birth are more susceptible to thyroid hormone deficiency than areas, such as the cerebral cortex, which are more mature at birth.

It has been reported that cerebral protein synthesis in vivo is depressed in neonatal hypothyroid rats (Geel *et al.*, 1967; Balazs and Gaitonde, 1968), although this has not been substantiated in vitro using brain slices (Valcana and Eberhardt, 1977) or cell-free systems (Andrews and Tata, 1971). Lindholm (1982) has recently reported that protein synthesis in perikarya isolated from the brains of two week old hypothyroid rats was inhibited. Jarlstedt and Norstrom (1972) found that thyroid hormone deficiency in the developing rat results in inhibition of total synaptosomal and mitochondrial protein synthesis in vivo. The effect of hypothyroidism on total and individual SPM proteins is not known, however.

Summary

The considerable amount of work carried out on synaptic fractions in

recent years is now yielding much information about the nature of the synapse. Many of the major synaptic structural proteins have now been identified and the interactions between them have been elucidated to some degree. Very little is known, however, of the metabolism of synaptic proteins and specifically of those proteins present in the synaptic plasma membrane. Clearly when the metabolism of the constituents is elucidated the importance of the synaptic membrane proteins to the functioning of the nervous system will become apparent.

The aim of this project has been, therefore, to determine the rates of synthesis of SPM proteins and to investigate certain conditions (using drugs and chemically-induced hormone deficiency) in which the synthesis of synaptic proteins would be expected to be grossly affected.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Radiochemicals were obtained from Amersham International, Bucks. The high molecular weight standards kit for SDS polyacrylamide gel electrophoresis was obtained from Bio-Rad Laboratories Ltd., Watford, Herts. Acrylamide and N,N'-methylenebisacrylamide (specially purified for electrophoresis) were obtained from BDH Ltd., Eastleigh, Hants. Halothane (Fluothane) was obtained from Arnold Veterinary Products, Reading, Berks, pentobarbitone (Sagatal) from May and Baker Ltd., Dagenham, Essex and L-DOPA and 6-n-propyl-2-thiouracil from Sigma Chemical Co. Ltd., Poole, Dorset. Spurr's embedding resin was obtained from Taab Laboratories, Reading, Berks and X-Omat S X-ray film from Kodak Ltd., Hemel Hempstead, Herts. All other chemicals were routinely of ANALAR grade.

2.1.2 Surgical Materials

The materials used in surgery and implantation of cannulae were as follows: number 3 round burr drill bits (Busch and Co. Ltd., Germany); brass screws $\frac{5}{32}$ " IOBA, cheese head (Walter Spencer Components Ltd., Birmingham); 9 mm Clay Adams wound clips (Horwell Ltd., London); Howmedica Simplex Rapide dental cement (G. H. Bloore, Havant, Hants) and cannulae with perspex bodies 6 mm long, 3 mm in diameter with hypodermic tubes (19 SWG) 5 mm long protruding from the base from Harvard Bioscience, Edenbridge, Kent. The general construction of these cannulae was as described by Hayden et al. (1966) as modified by Goff et al. (1975).

2.1.3 Animals

All rats used were of the Wistar strain and were bred in the departmental animal house. The rats were fed ad libitum on PRD pellets (Labsure, Poole, Dorset) except those used in the thyroid deficiency studies which were fed on the powdered rat diet 41B (Labsure, Poole, Dorset) mixed with 0.3% (w/w) 6-n-propyl-2-thiouracil. The light regime

in the animal house was 12 h dark/12 h light. The age and sex of the rats used is indicated, where appropriate, in the text.

2.2 Isolation of Synaptic Plasma Membrane Fractions

2.2.1 The Jones and Matus (1974) isolation procedure (Fig. 1.2)

Rats were killed by cervical dislocation, their brains removed and quickly rinsed twice in ice-cold 0.9% NaCl. The forebrain was then homogenised in 10% (w/w) sucrose buffered to pH7.4 with 5 mM HEPES and containing 50 μ M CaCl₂ (all subsequent sucrose solutions were similarly buffered) to give a 10% (w/v) homogenate. Where appropriate cerebella were similarly homogenised. Homogenisation was effected by 8 passes at 400 rpm in a glass-teflon homogeniser of clearance 0.25 mm. All subsequent manipulations were carried out at ice temperature, unless otherwise stated.

The homogenate was centrifuged at 1000g_{av} for 10 min to remove cell debris and nuclei. The resultant supernatant was then centrifuged at 10,000g_{av} for 20 min to produce the crude mitochondrial pellet. This was washed once and then resuspended in 5mM Tris, pH 8.1 (2.5 ml/brain) using a tight-fitting hand-held homogeniser (clearance 0.15 mm). Lysis was carried out at 4°C for 60 min. After lysis had been allowed to occur two volumes of 48% (w/w) sucrose, buffered as before, were added to the lysate. This was successively overlaid with an equal volume of 28.5% (w/w) and 5 ml 10% (w/w) buffered sucrose. The gradients were centrifuged at 60,000g_{av} for 2h in a 6x38 ml MSE swing-out rotor. After centrifugation the interfaces were removed with a Pasteur pipette, diluted with 5 volumes of 10% (w/w) buffered sucrose and pelleted at 80,000g_{av} for 20 min. The pellets were resuspended in 10% (w/w) buffered sucrose and stored at 4°C until the enzyme assays were completed and then stored frozen at -70°C.

2.2.2 The Cotman and Matthews (1971) isolation procedure (Fig. 1.1)

A 20% (w/v) forebrain homogenate was prepared in 0.32 M sucrose buffered with 5 mM Tris to pH7.4 and containing 50 μ M CaCl₂ (as were all subsequent sucrose solutions) under the same conditions as for the

previous procedure. The resulting homogenate was diluted to 10% (w/v) and centrifuged at $1000g_{av}$ for 5 min. The supernatant was centrifuged at $17,000g_{av}$ for 10 min to yield the crude mitochondrial pellet. This pellet was resuspended in 0.32M buffered sucrose (1.5 ml/brain) and applied (5 ml resuspension/gradient) to a Ficoll-sucrose gradient of composition 13% and 7.5% Ficoll in 0.32M buffered sucrose (15 ml of each). The gradients were centrifuged at $64,000g_{av}$ for 90 min. The synaptosome fraction was removed from the interface between the 13% and 7.5% Ficoll steps, diluted with 4 vol. of 0.32M buffered sucrose and pelleted at $50,000g_{av}$ for 30 min. The pellet was resuspended in 0.32M buffered sucrose (0.5 ml/brain) to which 5 vol. of 6 mM Tris, pH8.1 were added and was allowed to lyse at 4°C for 90 min. The lysate was centrifuged at $25,000g_{av}$ for 20 min after which the pellet was resuspended in 0.32M buffered sucrose (0.6 ml/brain) and 0.2 ml of this was applied to discontinuous sucrose gradients comprising the following steps: 38%, 35%, 32.5% and 25% (w/w) buffered sucrose (7.5 ml each). The gradients were centrifuged at $60,000g_{av}$ for 90 min in a 6x38 ml MSE swing-out rotor. The interfaces were collected, diluted with 3.5 vol. of 0.1 mM EDTA and centrifuged at $50,000g_{av}$ for 30 min. The pellets were resuspended in 0.32M sucrose.

Ficoll used for this procedure was purified by dissolving it in a small amount of redistilled water and precipitating it with 95% ethanol. The precipitated Ficoll was then freeze-dried and stored at -20°C until used. This procedure reduces the salt concentration in commercial Ficoll and enables full use to be made of its negligible osmotic pressure.

2.2.3 The Salvaterra and Matthews (1980) isolation procedure

This is a modification of the Jones and Matus method designed so that a fixed angle rotor instead of a swing-out rotor can be used for the gradient step.

Two vol. of 48% (w/w) sucrose (buffered as in section 2.2.1) were added to the lysed crude mitochondrial fraction (12.5 ml/brain). This was overlaid with 8 ml of 28.5% (w/w) buffered sucrose and 4 ml of 10% (w/w) buffered sucrose. The gradients were centrifuged in a Beckman Type 30

rotor at 30,000 rpm, $\omega^2 t = 1.8 \times 10^{10}$ rad²/sec (approximately 85,000g_{av} for 30 min) and allowed to decelerate without application of the brake. The interfaces were removed and diluted with 5 vol. of 0.1 mM EDTA and pelleted at 80,000g_{av} for 20 min. The pellets were resuspended in 10% (w/w) buffered sucrose.

The advantage of this method is that up to 12 brains can be processed separately compared with only 6 when a swing-out rotor is used.

2.2.4 The Gray and Whittaker (1962) procedure for isolating synaptosomes

A 10% (w/v) forebrain homogenate was prepared as in section 2.2.2. The homogenate was centrifuged at 1000g_{av} for 10 min. The pellet was washed once (1000g_{av}, 10 min.) and the supernatant added to the initial supernatant which was then centrifuged at 17000g_{av} for 55 min. to yield the crude mitochondrial pellet. This pellet was washed once (17000g_{av}, 55 min.) and then resuspended in 0.32M sucrose (2 ml/g tissue) and 5 ml was layered on top of a two step discontinuous sucrose gradient of composition 0.8M and 1.2M sucrose (15 ml each). The gradients were centrifuged at 53000g_{av} for 2h in a 6 x 38 ml MSE swing-out rotor. The synaptosome fraction was removed from the 0.8M/1.2M sucrose interface, diluted with an equal volume of distilled, deionised water and centrifuged at 80000g_{av} for 60 min. The pellets were resuspended in 0.32M sucrose.

All of the solutions used in these three procedures were prepared not more than 24 h before use and were stored at 0-4°C.

2.3 Characterisation of SPM Fractions

2.3.1 Marker Enzyme Assays

2.3.1.1 (Na⁺,K⁺)activated,ouabain-sensitive ATPase ((Na⁺,K⁺)ATPase)

This enzyme was assayed by the method of Mahler and Cotman (1970). The samples were preincubated in assay buffer (50 mM Tris, 100 mM NaCl, 30 mM KCl, 3 mM MgCl₂, 0.75 mM EGTA, pH7.5) in the presence or absence of

0.2 mM ouabain for 5 min at 37°C. The reaction was started by addition of 50µl of 60 mM disodium ATP (vanadium-free). The reaction was allowed to proceed for 10 min and was then stopped by placing the reaction mixtures on ice and adding 5 ml of 0.2% (w/v) ammonium molybdate in 0.2 M H₂SO₄ to them. The colour was developed by adding 0.2 ml of Fiske-SubbaRow reducing agent (see below). After 10 min the absorbance at 680 nm was measured and the amount of phosphate released calculated from a phosphate standard curve (range 0-80 µg phosphate).

Fiske-SubbaRow reducing agent

0.5 g 1-amino-2-naphthol-4-sulphonic acid
30.0 g sodium bisulphite
6.0 g sodium sulphite

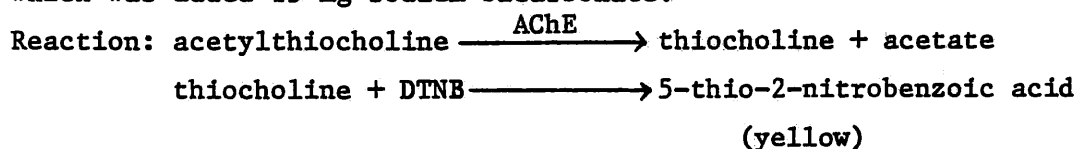
Made up to 250 ml with distilled, deionised water, filtered and stored at 0-4°C in a dark glass bottle.

2.3.1.2 Acetylcholinesterase

This enzyme was assayed by the method of Ellman et al. (1961).

Assay mixture: 2.6 ml 0.1 M sodium phosphate buffer, pH8.0
0.1 ml 0.01 M dithiobisnitrobenzoic acid* (DTNB)
0.4 ml enzyme solution

*The substrate is more stable when prepared in the following way: 39.6 mg DTNB was dissolved in 10 ml sodium phosphate buffer, pH7.0 (0.1 M) to which was added 15 mg sodium bicarbonate.



The absorbance at 412 nm was recorded to establish the background rate. The reaction was started by addition of 20µl of 0.075 M acetylthiocholine iodide.

The extinction coefficient of the yellow anion is 1.36×10^4 .

2.3.1.3 Succinate PMS reductase

This enzyme was assayed by the method of Duncan and Mackler (1966).

Assay mixture: 0.01 ml 10% (w/v) phenazine methosulphate (PMS)
0.10 ml 0.01% (w/v) dichlorophenol indophenol
0.01 ml 1.0 M sodium succinate

0.10 ml 10 mM potassium cyanide
0.20 ml 0.2 M potassium phosphate buffer, pH7.5
0.55 ml distilled, deionised water

The reaction was started by the addition of 10 μ l of the sample. The rate of reaction was measured by following the decrease in absorbance at 600 nm at a temperature of 38°C.

The extinction coefficient of dichlorophenol indophenol is 1.61×10^4 .

2.3.2 Protein Determination

Protein concentrations were measured by the method of Lowry et al. (1951) using crystalline bovine serum albumin as the standard. Since brain protein is difficult to redissolve after precipitation with trichloroacetic acid (TCA) the following procedure was adopted. 0.1 ml of sample, diluted, if necessary, with distilled, deionised water, was precipitated by addition of 0.25 ml of 10% (w/v) TCA.

The precipitate was pelleted in a Mechanika Precyzyjna microcentrifuge (6 min). The supernatant was removed by syringe and 0.5 ml of 0.2 M NaOH was added to the pellet which was dispersed by vortex mixing and allowed to stand overnight at 4°C. 0.5 ml of distilled, deionised water were added and the solution was thoroughly mixed. Aliquots were assayed for protein content.

Stock solutions:

- A 1% (w/v) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$
- B 2.12% (w/v) potassium, sodium tartrate. $5\text{H}_2\text{O}$
- C 4 g NaOH + 20g Na_2CO_3 in 1 l distilled, deionised water
- D A + B 1:1 (v/v))
- E C + D 50:1 (v/v)) Directly before use

5 ml of solution E were added to 1 ml of protein solution. This was mixed thoroughly and allowed to stand for 10 min. 0.5 ml Folin-Ciocalteu reagent (diluted 1:1 with distilled, deionised water) was added to the mixture which was then allowed to stand for 30 min. The absorbance at 750 nm was measured and the protein concentration calculated from a bovine serum albumin standard curve (range 0-200 μ g).

2.3.3 Electron Microscopy

2.3.3.1 Procedure for osmium tetroxide/uranyl acetate staining of membrane fractions

Membrane fractions were centrifuged at $80,000g_{av}$ for 20 min. The pellets were fixed by addition of 4% (v/v) glutaraldehyde in Millonig's phosphate buffer (see below) for 30 min at 0-4°C. The fixed samples were washed 3 x 30 min in Millonig's buffer and then stained with 1% (w/v) osmium tetroxide dissolved in Millonig's buffer for 2 h at 4°C. This was followed by 3 washes (10 min each) in Millonig's buffer and 1 wash (10 min) in Kellenberger's buffer (see below). The samples were then stained with 2% (v/v) uranyl acetate dissolved in Kellenberger's buffer overnight at 4°C. The stained samples were washed 3 x 10 min in Kellenberger's buffer and dehydrated through graded alcohols as follows: 30%, 50%, 70%, 90% (15 min each), followed by 2 x 30 min washes in 95% ethanol.

The samples were then transferred to ethanol:propylene oxide (1:1, v/v) for 30 min. This was decanted and replaced with fresh propylene oxide for a further 30 min. The samples were left overnight in a mixture of propylene oxide:Spurr's resin (1:1, v/v), passed through 2 x 2 h changes of fresh resin and then transferred to fresh resin and cured in a vacuum oven for 24 h at 60°C.

2.3.3.2 Procedure for ethanolic-phosphotungstate staining of membrane fractions

Pelleted fractions were fixed with 5% (v/v) glutaraldehyde as before and then washed 3 x 10 min in Millonig's buffer. The samples were dehydrated by passing through graded alcohols as before and then stained with 1% (w/v) phosphotungstic acid dissolved in absolute ethanol containing 5 drops of 95% ethanol per 10 ml solution. Staining was allowed to proceed for 4 h after which the fractions were quickly rinsed with propylene oxide:ethanol (1:1, v/v) followed by a brief rinse with propylene oxide. These rinses were necessary since mixing of ethanolic phosphotungstate and propylene oxide for longer than 3 min may result in a explosive exothermic reaction (Bloom and Aghajanian, 1968). The

samples were subsequently washed 2 x 15 min in propylene oxide then immersed in propylene oxide:Spurr's resin (1:1, v/v) overnight and embedded in resin as before.

Samples processed in both ways were cut on an LKB ultramicrotome into thin sections (500-700 Å, pale gold/silver). All sections were stained on their grids with lead citrate for 3 min to enhance the contrast. The grids were then examined on a Zeiss EM 9a microscope.

Millonig's Phosphate Buffer

Stock solutions: A. 2.26% (w/v) sodium dihydrogen phosphate
 B. 2.52% (w/v) sodium hydroxide
 C. 5.40% (w/v) glucose
 D. 41.5 ml A + 8.5 ml B.
Buffer:- 45 ml D + 5 ml C.

Kellenberger's Buffer

Veronal-acetate buffer:- 2.94 g sodium barbitone
 1.94 g sodium acetate
 3.40 g sodium chloride
 Made up to 100 ml with distilled,
 deionised water.
Kellenberger's buffer:- 5.0 ml veronal-acetate buffer
 13.0 ml distilled, deionised water
 7.0 ml 0.1M HCl
 0.25 ml 1M calcium chloride

Adjust to pH6.0 with dilute HCl.

Lead Citrate Solution

0.2115g of lead citrate was shaken with 30 ml of carbon dioxide-free, millipore-filtered water to form a milky suspension. 0.8 ml of 1M NaOH (freshly made up in carbon dioxide-free water) was added to the suspension which was then made up to a final volume of 50 ml. When the suspension had cleared the solution was stored in a polythene bottle in an airtight container containing potassium hydroxide pellets.

Spurr's Embedding Resin

ERL 4206	10.0 g
DER 736	8.0 g
NSA	26.0 g
S-1	0.4 g

2.3.4 SDS Polyacrylamide Gel Electrophoresis

The SDS polyacrylamide gel electrophoresis system used was basically that developed by Laemmli (1970).

The separating gel consisted of 9.8% (w/v) acrylamide, 0.3% (w/v) bisacrylamide, 4.54% (w/v) Tris, 0.074% (w/v) EDTA (disodium salt), 0.1% (w/v) SDS, 0.03% (w/v) ammonium persulphate and 0.05% (v/v) TEMED. The gel mixture was degassed using a water pump. Slab gels of dimensions 120mm x 160mm x 1mm were cast, overlaid with n-butanol and allowed to polymerize overnight. The following day the butanol was removed by blotting the gel surface with filter paper. The stacking gel of composition 4.8% (w/v) acrylamide, 0.15% (w/v) bisacrylamide, 1.5% (w/v) Tris, 0.074% (w/v) EDTA (disodium salt), 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulphate and 0.05% (v/v) TEMED was applied and allowed to polymerise for at least 1 h. Samples were prepared by heating protein solutions with a buffer of composition 0.76% (w/v) Tris, 12.5% (v/v) glycerol, 1.25% (w/v) SDS, 1.25% (v/v) 2-mercaptoethanol and 0.0025% (w/v) bromophenol blue for 4 min at 100°C. Solubilized samples were applied to the gels (100 µg/track) by syringe through the electrode buffer (composition: 0.05 M Tris, 0.384 M glycine, 2 mM EDTA (disodium salt), 10% (w/v) SDS, pH 8.3). Electrophoresis was carried out at 20 mA per gel for 1 h to allow entry of proteins into the stacking gel. The current was then increased to 40 mA per gel until electrophoresis was complete as judged by the migration of bromophenol blue marker dye. Gels were stained in 50% (w/v) TCA containing 0.2% (w/v) Coomassie Brilliant Blue R for 30 min at 60°C and then destained in propan-2-ol: glacial acetic acid:water, (12.5:7.5:80, by volume).

The protein standards used were myosin (200 K), β -galactosidase (116.5 K), phosphorylase B (94 K), bovine serum albumin (68 K), ovalbumin (43 K), pepsin (34.7 K) and trypsinogen (24 K), molecular weights are given in parenthesis.

Stock solutions: 30% acrylamide) w/v in distilled, deionised
0.9% bisacrylamide) water
1.5 M Tris-HCl, pH8.8
0.5 M Tris-HCl, pH6.8
10% (w/v) SDS
0.2 M EDTA (disodium salt)
10% (w/v) ammonium persulphate
0.1% (w/v) bromophenol blue

Composition of Gels

Composition	Volume (ml)	
	10% separating gel	5% stacking gel
Distilled, deionised water	18.0	5.6
Acrylamide solution	14.7	1.6
1.5 M Tris-HCl, pH8.8	11.25	-
0.5 M Tris-HCl, pH6.8	-	2.5
0.2 M EDTA	0.45	0.1
10% SDS	0.45	0.1
10% ammonium persulphate	0.15	0.1
TEMED	22.5 μ l	5 μ l

Composition of Sample Buffer

Component	Volume (ml)
Distilled, deionised water	7.05
0.5 M Tris-HCl, pH6.8	1.5
Glycerol	1.5
10% SDS	1.5
2-mercaptoethanol	0.15
0.1% bromophenol blue	0.3

2.3.5 Fluorographic Analysis of Brain Fractions

The incorporation of L-(³⁵S)methionine into cerebral polypeptides was determined by fluorography following the electrophoresis of brain fractions on SDS polyacrylamide gels. Fluorography was carried out as described by Bonner and Laskey (1974) and later modified by Laskey and Mills (1975). The procedure was as follows:-

The gel was initially soaked in 20 times its volume of dimethyl sulphoxide (DMSO) for 30 min then transferred to fresh DMSO for a further 30 min. After dehydration the gel was soaked in 4 volumes of DMSO containing 22.2% (w/v) PPO for 3 h. Following impregnation with PPO the gel was placed in 20 volumes of water for at least 1 h.

The gel was then dried under vacuum on to Whatman No. 3 filter paper using a Bio-Rad gel drier. The dried gel was placed in contact with Kodak X-Omat S film which had previously been preflashed according to the recommendations of Laskey and Mills (1975). The film and gel were then wrapped in black paper and placed between two glass plates which were covered with aluminium foil. The exposure was carried out for 4-6 weeks at -70°C. Following exposure (4-6 weeks depending on radioactivity loading which was usually about 10,000 dpm per track). The X-ray film was developed using Kodak DX80 developer and FX40 fixer.

2.4 Techniques Used for the Measurement of in vivo Brain Protein Synthesis

2.4.1 Stereotaxic techniques

Rats were initially anaesthetised in a plastic box, maintained at 37°C by partial immersion in a heated water bath, using 4-6% halothane at a flow rate of 1 l/min. The unconscious animal was transferred to a Kopf small animal stereotaxic frame. The anaesthetic was administered through a face mask at a concentration of 2% and a flow rate of 0.6 l/min.

The skull was exposed, the bregma located and the co-ordinates noted. In rats weighing 250-350 g the lateral ventricle is located 0.9 mm posterior and 2.5 mm lateral to the bregma. A hole was drilled at this position using a number 3 round burr dental drill bit. A second hole was drilled 4-5 mm distant from the first and a brass screw was secured to the skull with a nut. The cannula was then placed in the first hole and fixed to the skull and screw with dental cement. The wound was sutured using steel clips. The animal was then injected intramuscularly with benzyl penicillin (20,000 units) and locally around the wound with xylocaine.

All surgical instruments were sterilised in 0.5% hibitane for 30 min and rinsed in sterile 0.9% NaCl before use.

Rats were allowed to recover for 5 days during which time they were injected once with 50µl sterile saline to accustom the animals to the injection procedure and, therefore, reduce handling stress.

2.4.2 Measurement of the Incorporation of L-(³⁵S)methionine into Brain Proteins

2.4.2.1 Adult animals (gross injection)

Rats were injected intraventricularly with 2.5 mg L-(³⁵S)methionine (20 or 30 µCi, injection volume 50 µl) via pre-implanted cannulae. Following the injection the rats were marked and returned to their cage where they had free access to food and water. After the incorporation

period the animals were killed by cervical dislocation. Synaptic subfractions were isolated from the forebrain by the Jones and Matus (1974) or Salvaterra and Matthews (1980) method.

2.4.2.2 Young animals (trace injection)

In the thyroid deficiency studies the hypothyroid rats were too small to implant with cannulae with routine success. It was, therefore, necessary to inject the tracer amino acid directly through the skull into the lateral ventricles. A needle with a stop was used so that the correct depth could be gauged. The accuracy of placement using this technique was tested using the dye Pontamine sky blue. It was found that small rats could be successfully injected in this way. The younger animals (1 and 2 weeks old) were injected while conscious. Older animals were lightly anaesthetised with halothane before injection. The body temperatures of all hypothyroid and 1 and 2 week old control animals were maintained by housing them in a warm box. The rats were injected with either 50 μ l L-(³⁵S)methionine (1-3 weeks old) or 100 μ Ci L-(³⁵S)methionine (specific activity 143 mCi/mmol) (4 weeks old). The injection volume in all cases was 20 μ l and the incorporation period was 2 h. The rats were killed cervical dislocation and synaptic subfractions were isolated by the method of Salvaterra and Matthews (1980).

2.4.3 Measurement of the Incorporation of L-(U¹⁴C)valine into Brain Proteins

Hypothyroid and control rats were injected intraperitoneally with L-valine at 15 μ mol/g body weight as a solution containing 0.5 mmol/ml and 9.5 μ Ci L-(U¹⁴C)valine/ml. This quantity has been shown to be sufficient to flood the valine pool for 2 h incorporation periods (Dunlop *et al.*, 1975). Animals were killed and fractions isolated as above.

2.4.4 Determination of the Distribution of L-(³⁵S)methionine in the Brain

The brain was divided into left (injected), and right (non-injected) hemispheres and cerebellum. These were homogenised separately and the incorporation of L-(³⁵S)methionine into each area was determined.

2.4.5 Measurement of TCA-insoluble Radioactivity

The TCA-insoluble radioactivity in the homogenates and subcellular fractions was measured by the filter paper disc method of Mans and Novelli (1961).

Brain fractions were sonicated for 5 sec at maximum amplitude in 10% (w/v) sucrose (buffered with 5 mM HEPES, pH7.4) and then kept on ice. Aliquots were applied to Whatman cellulose filter paper discs (3 mm thick, 2.4 cm diameter) and dried at 60°C. In "flooding" experiments 100 µl aliquots were used while in non-flooding experiments aliquots of 20 µl and 40 µl of homogenates and subcellular fractions respectively were used.

The discs were immersed in ice-cold 10% (w/v) TCA for 5 min and then transferred to 5% (w/v) TCA at 90°C for 30 min. The discs were rinsed in ice-cold 5% (w/v) TCA (5 min) and then placed in ethanol:ether (3:1, v/v) at 37°C for 30 min. This was followed by a 5 min rinse in the ethanol:ether mixture at room temperature. The discs were dried and placed in scintillation vials to each of which were added 100 µl of distilled, deionised water and 500 µl of tissue solubiliser (NCS). Digestion was carried out at 37°C overnight or for 2 h at 50°C. After cooling the vials 20 µl of glacial acetic acid were added to each followed by 10 ml of toluene/PPO (5 g/l).

In early experiments the samples were counted on a Packard Tri-Carb liquid scintillation counter (Model 3390). Counting efficiencies were determined using the external standards channels ratio method. A quench correction curve was produced by adding increasing concentrations of chloroform to 10 ml aliquots of toluene/PPO scintillation cocktail which contained n-(1,2(n)-³H)hexadecane or n-(1-¹⁴C)hexadecane as internal standards.

In the majority of experiments, however, samples were counted on a Beckman LS 7500 liquid scintillation counter using the automatic H-factor correction to yield d.p.m. values.

2.4.6 t.l.c. Analysis of ^{35}S Labelled Amino Acids in Brain Extracts

In order to determine the distribution of ^{35}S in sulphur-containing amino acids soluble brain extracts were subjected to t.l.c. in the same system as that used by Amersham International for analysis of L-(^{35}S)methionine.

Samples were prepared by adding 100 μl of 0.3M perchloric acid to 100 μl of brain homogenate. The precipitate was pelleted in a microcentrifuge (6 min). The supernatant was decanted and its pH raised above 1 by dropwise addition of 0.5 M potassium carbonate. 25 μl of the resultant solution was then applied to cellulose t.l.c. plates (thickness 0.1 mm) as a 5 cm band. This was necessary to obtain sufficient radioactivity for scintillation counting but without overloading the plate. Solutions of standard amino acids (L-methionine, L-cysteine and L-cystine) were prepared by dissolving them at a concentration of 5 mg/100 ml in 10% (v/v) aqueous propan-2-ol. 5 μl of each of these standards was applied over 0.5 cm. The plates were run in the following solvent system: butan-1-ol: glacial acetic acid: water (120:30:50, by volume). Following chromatography the sections of the plates containing the amino acid standards were cut off and stained with ninhydrin. The area in which the sample had run was not stained but 0.5 cm bands were scraped off from the origin to the solvent front into separate scintillation vials to which were added 0.5 ml NCS and 0.1 ml water. Digestion and scintillation counting were carried out as in section 2.4.5.

2.5 In vivo Drug and Thyroid Hormone-Deficiency Studies

2.5.1 The Effect of Anaesthetics on Brain Protein Synthesis

2.5.1.1 Halothane

Experimental rats were anaesthetised with halothane (3%) for 20 min prior to intraventricular injection, via pre-implanted cannulae, of flooding concentrations of L-(^{35}S)methionine (2.5 mg L-(^{35}S)methionine, 20 or 30 μCi in an injection volume of 50 μl). These rats remained under anaesthesia during the incorporation period (30 min). Control rats were intraventricularly injected with a solution of the same composition as given above.

The anaesthetised rats were kept in a warm box to maintain their body temperatures during the course of the experiment. The rectal temperature of the anaesthetised rats was recorded at death.

2.5.1.2 Pentobarbitone

Rats were injected intraperitoneally with pentobarbitone (60 or 75 mg/Kg body weight). The tail-flick and eye-blink reflex activities were checked to determine whether the animals were unconscious. The rats were intraventricularly injected as above 15 min after administration of the anaesthetic. Control animals were treated as described in section 2.5.1.1.

2.5.2 The Effect of L-DOPA on Brain Protein Synthesis

Rats were housed at 30-32°C for 45-60 min before the start of the experiment. The experimental rats were injected intraperitoneally with L-DOPA (500 mg/Kg body weight). The drug was injected as a suspension 50 mg/ml in 0.05 M HCl + 0.2 (v/v) Tween 80. Control rats were injected with the appropriate volume of vehicle.

After 45 min all rats were injected, via pre-implanted cannulae, with 2.5 mg L-(³⁵S)methionine (20 µCi, injection volume 50 µl). Following a 30 min incorporation period the animals were killed by cervical dislocation. Synaptic fractions were isolated by the method of Jones and Matus (1974) or Salvaterra and Matthews (1980). Synaptosomes were isolated by the method of Gray and Whittaker (1962).

2.5.3 The Effect of Thyroid Hormone Deficiency on Brain Protein Synthesis

Hypothyroidism was induced by addition of 6-n-propyl-2-thiouracil to the maternal diet four days after the birth of the litter. This antithyroid agent was added to powdered rat food (see section 2.1.3) at 3% (w.w). 100 g of this diet was fed per day. The young rats were killed at 1, 2, 3 or 4 weeks old and synaptic subfractions were isolated by the method of Salvaterra and Matthews (1980).

3. RESULTS AND DISCUSSION

3.1 Characterization of Brain Subcellular Fractions

Since the principal aim of the present study was to examine the in vivo synthesis and turnover of proteins present in the synaptic membrane it was necessary to select an isolation method which produced SPM fractions of an adequate purity for this purpose. Thus, SPM fractions isolated by the rapid method of Jones and Matus (1974) and by the longer procedure of Cotman and Matthews (1971) were characterised and compared by electron microscopy, marker enzyme analysis and SDS polyacrylamide gel electrophoresis.

3.1.1 Morphological Analysis of Brain Subcellular Fractions

The myelin fraction prepared using the Jones and Matus procedure contained large membranous profiles typical of the myelin lamellae (Fig. 3.1.1) and was similar in morphology to the fraction obtained by these workers. Some of the profiles contained residual material possibly of axonal or dendritic origin. The myelin-enriched fraction obtained using the Cotman and Matthews procedure contained a variety of membranous profiles of which only a small proportion were characteristic of myelin. Many profiles were bounded by only a single membrane and contained residual material. The identity of these profiles is uncertain (Fig. 3.1.2).

The SPM fractions obtained using both procedures contained mainly empty membranous profiles (Figs. 3.1.3 and 3.1.4). These were consistent in size and shape with the SPM profiles obtained by Jones and Matus (1974). Detailed interpretation of these fractions was hindered by the presence of numerous small, crystalline structures apparently adhering to the membranes. This artefact was observed in all SPM fractions examined. Ideally the reason for this artefact would have been sought but in view of the limited electron microscopic facilities available and the comparative nature of these studies this was not possible. Since the other subcellular fractions had morphological compositions consistent with those reported in the literature it was felt that it was feasible to assume that the SPM fractions were also consistent.

The diameters of the profiles in Figs. 3.1.3 and 3.1.4 were 0.738 ± 0.047 and $0.793 \pm 0.079 \mu\text{m} \pm \text{S.E.M.}$ ($N=18$ and $N=16$) respectively. These values compared well with those ($0.5-1.2 \mu\text{m}$) reported by Jones and Matus (1974).

It has been reported that an ethanolic solution of phosphotungstic acid preferentially stains the synaptic junctional apparatus (Bloom and Aghajanian, 1968). An SPM fraction isolated using the Jones and Matus procedure prepared in this way revealed numerous electron dense bar-shaped structures against a grey background (Fig. 3.1.5). The appearance of these structures was similar to that reported by Bloom and Aghajanian (1968) and was consistent with them being the protein densities associated with the nerve terminal membrane.

The fractions obtained from the 32.5-35% and 35-38% interfaces of the Cotman and Matthews-type sucrose gradient contained mixtures of membranous profiles and mitochondria with the latter predominating in the fraction of higher buoyant density (Figs. 3.1.6 and 3.1.7).

The mitochondrial fraction obtained using the Jones and Matus procedure contained readily identifiable mitochondria with the majority showing a high level of structural preservation (Fig. 3.1.8). The corresponding Cotman and Matthews-type fraction was poorly embedded and could not, therefore, be examined.

3.1.2 Distribution of Marker Enzymes in Brain Subcellular Fractions

3.1.2.1 In Fractions Isolated Using the Jones and Matus Procedure

The distribution of the plasma marker enzymes $(\text{Na}^+, \text{K}^+)\text{ATPase}$ and acetylcholinesterase and the mitochondrial marker succinate PMS reductase in SPM, myelin and mitochondrial-enriched fractions was determined. The plasma membrane markers were concentrated in the SPM fraction while succinate PMS reductase was found principally in the mitochondrial fraction (Table 3.1.1). $(\text{Na}^+, \text{K}^+)\text{ATPase}$ and acetylcholinesterase, while following a similar pattern to that reported by Jones and Matus (1974), did, however, differ slightly in some fractions (Table 3.1.1). It was found that the percentage of the total λ covered $(\text{Na}^+, \text{K}^+)\text{ATPase}$ and acetylcholinesterase activities present in the myelin fraction were lower (80% and 67% of the Jones and Matus' values respectively) than those

TABLE 3.1.1

Percentage Distribution of Marker Enzymes and Protein in Brain Subfractions
Obtained Using the Jones and Matus Method

Fraction	Marker Enzyme			
	Protein (N=4)	(Na ⁺ ,K ⁺)ATPase (N=4)	AChE (N=4)	Succinate PMS reductase* (N=2)
Myelin	16.6±6.4	9.7±3.6 (48.8)	6.1±2.5 (18.7)	5.0 (8.3)
SPM	23.1±4.2	68.4±4.0 (44.0)	72.2±7.1 (78.2)	10.4 (8.9)
Mitochondria	60.3±2.9	24.4±7.5 (7.2)	23.3±8.9 (3.5)	84.8 (83.8)

The values are expressed as the percentage of the total activity recovered from all three fractions ± S.E.M. The values in parentheses are those of Jones and Matus (1974).

N = number of isolations.

* Jones and Matus used cytochrome c oxidase as the mitochondrial marker.

Fig. 3.1.1. Morphology of the Jones and Matus-type myelin fraction.
The profiles are typical of myelin lamellae (ML).
Some profiles contain residual material (R).
Magnification x 7000.

Fig. 3.1.2. Morphology of the myelin-enriched fraction isolated
using the Cotman and Matthews procedure. A variety
of membranous profiles are present of which only a
few show the morphology characteristic of myelin (ML).
Some profiles contain vesicular material (V), possibly
of synaptic origin. Magnification x 7000.

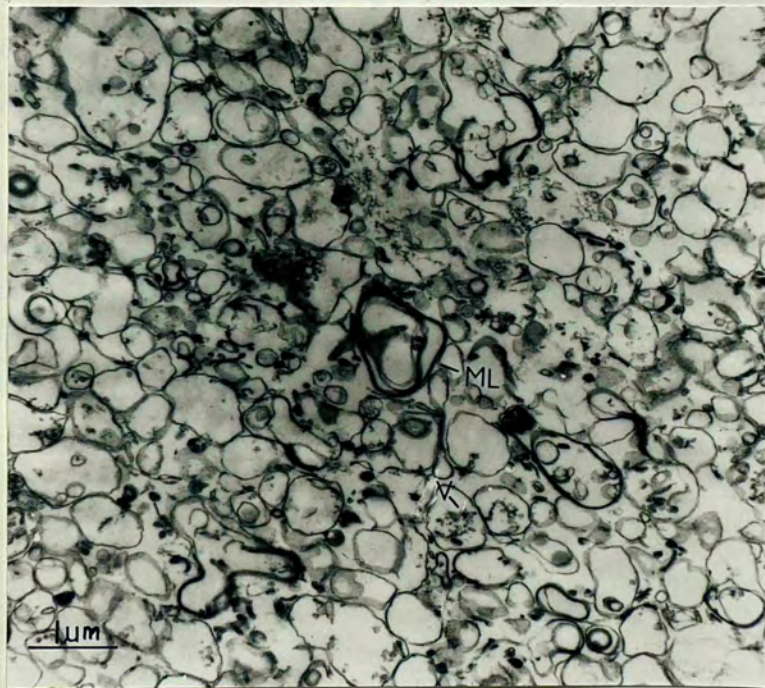
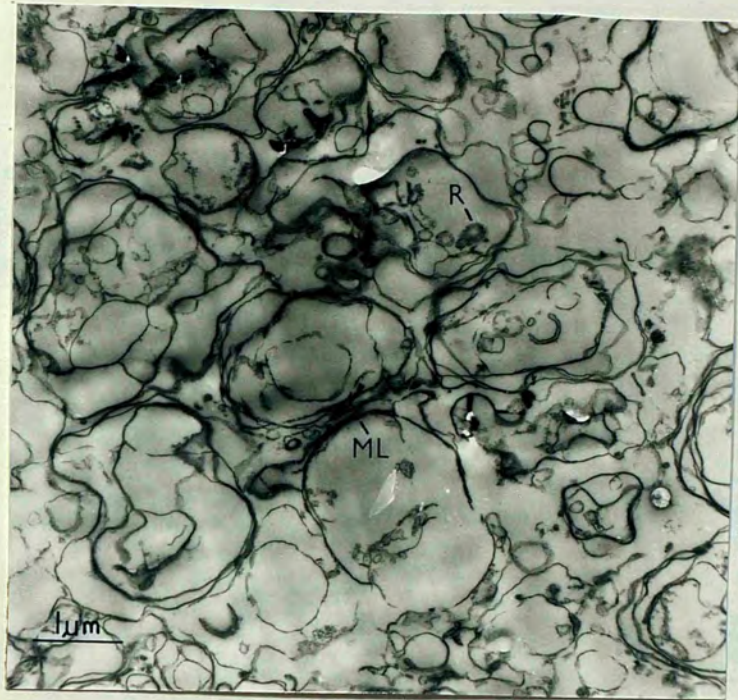


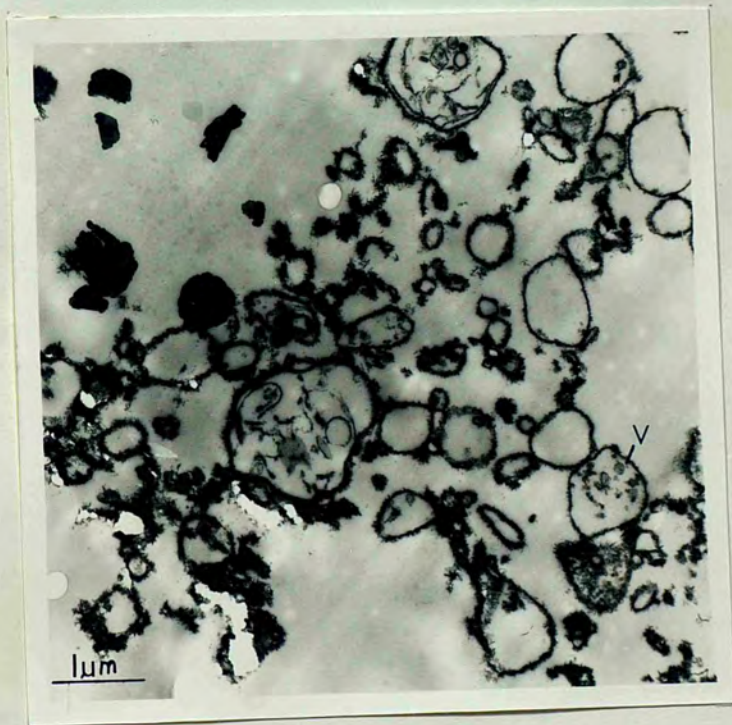
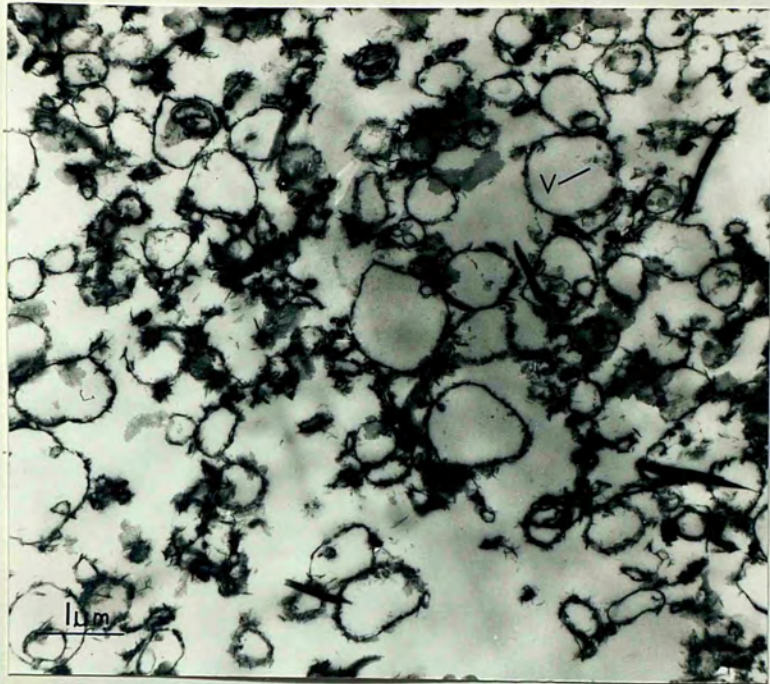


Fig. 3.1.3. Morphology of the SPM fraction prepared by the Jones and Matus method. A number of membranous profiles are present some of which contain residual vesicular material (V). Magnification $\times 7000$



Fig. 3.1.4. Morphology of the SPM fraction prepared by the Cotman and Matthews method. This fraction consists mainly of empty membranous profiles although some appear to contain residual vesicular material (V). Magnification $\times 7000$.





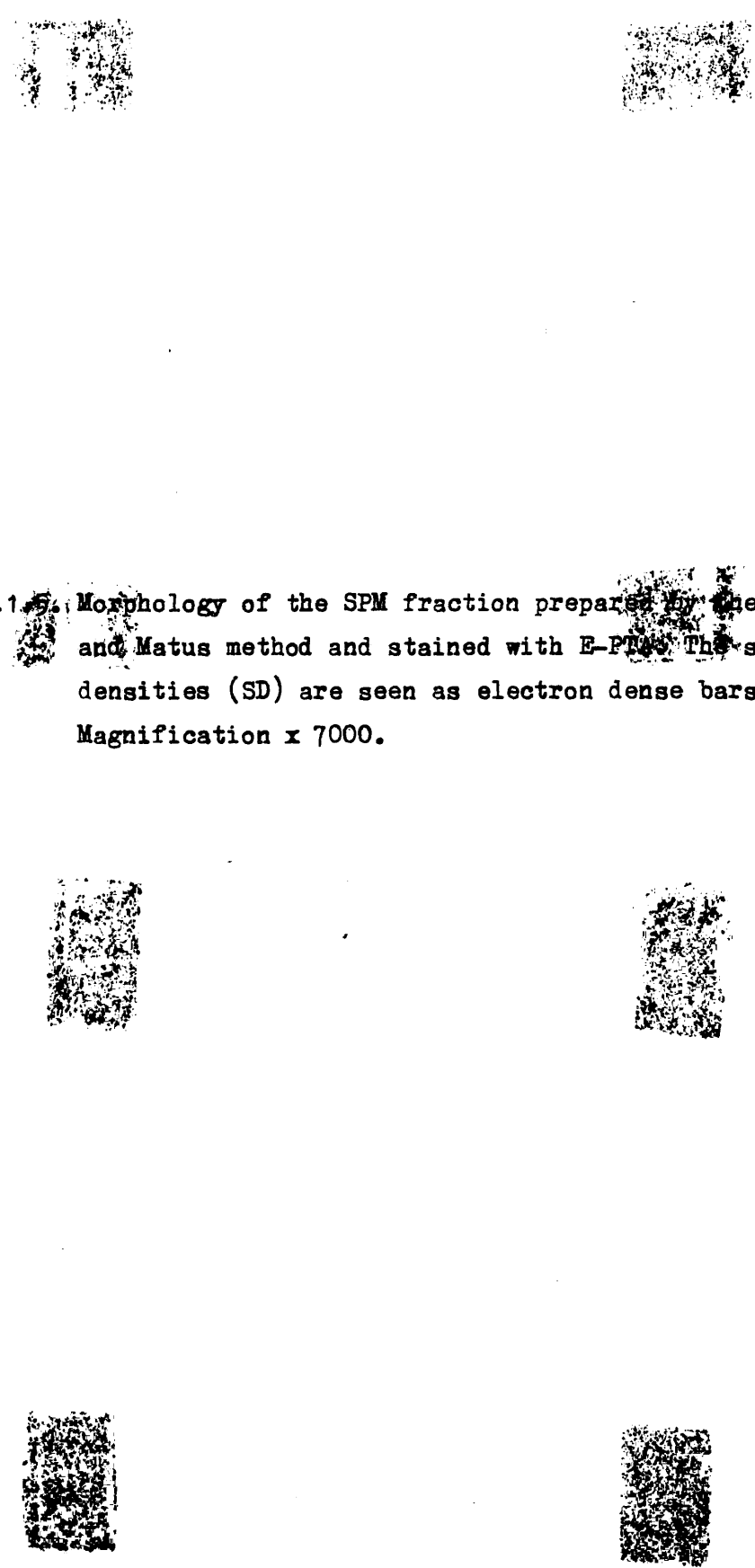
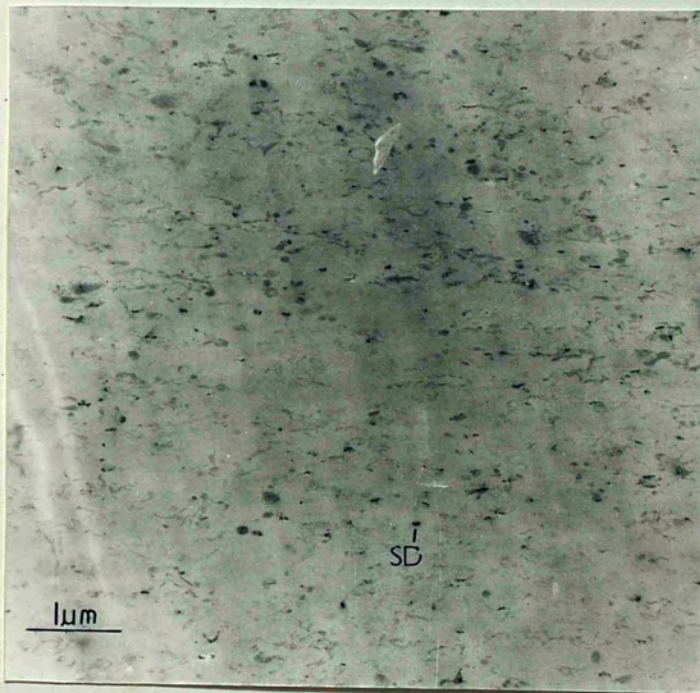


Fig. 3.1.5. Morphology of the SPM fraction prepared by the Jones and Matus method and stained with E-PTA. The synaptic densities (SD) are seen as electron dense bars. Magnification x 7000.



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Fig. 3.1.6. Morphology of the intermediate Cotman and Matthews fraction located at the 32.5-35% (w/w) sucrose interface. This fraction contains a variety of membranous profiles including some mitochondria (Mt). Magnification x 4000.

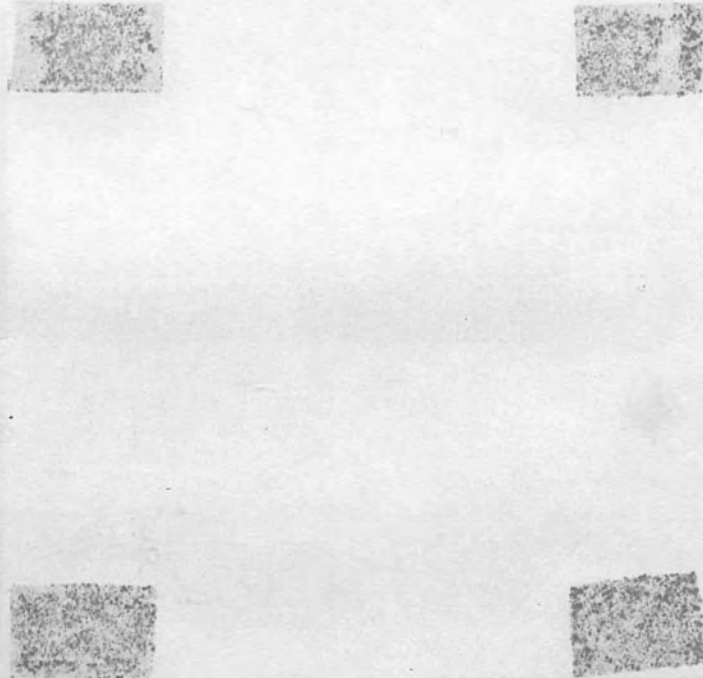
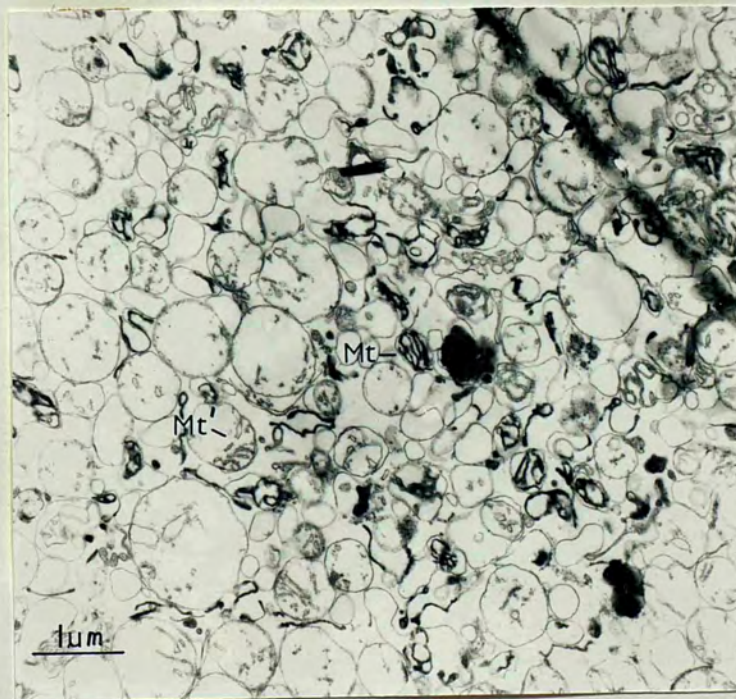


Fig. 3.1.7. Morphology of the intermediate Cotman and Matthews fraction located at the 35-38% (w/w) sucrose interface. This fraction contains a mixture of empty membranous profiles and mitochondria (Mt) some of which appear to be damaged (Mt'). Magnification x 7000.



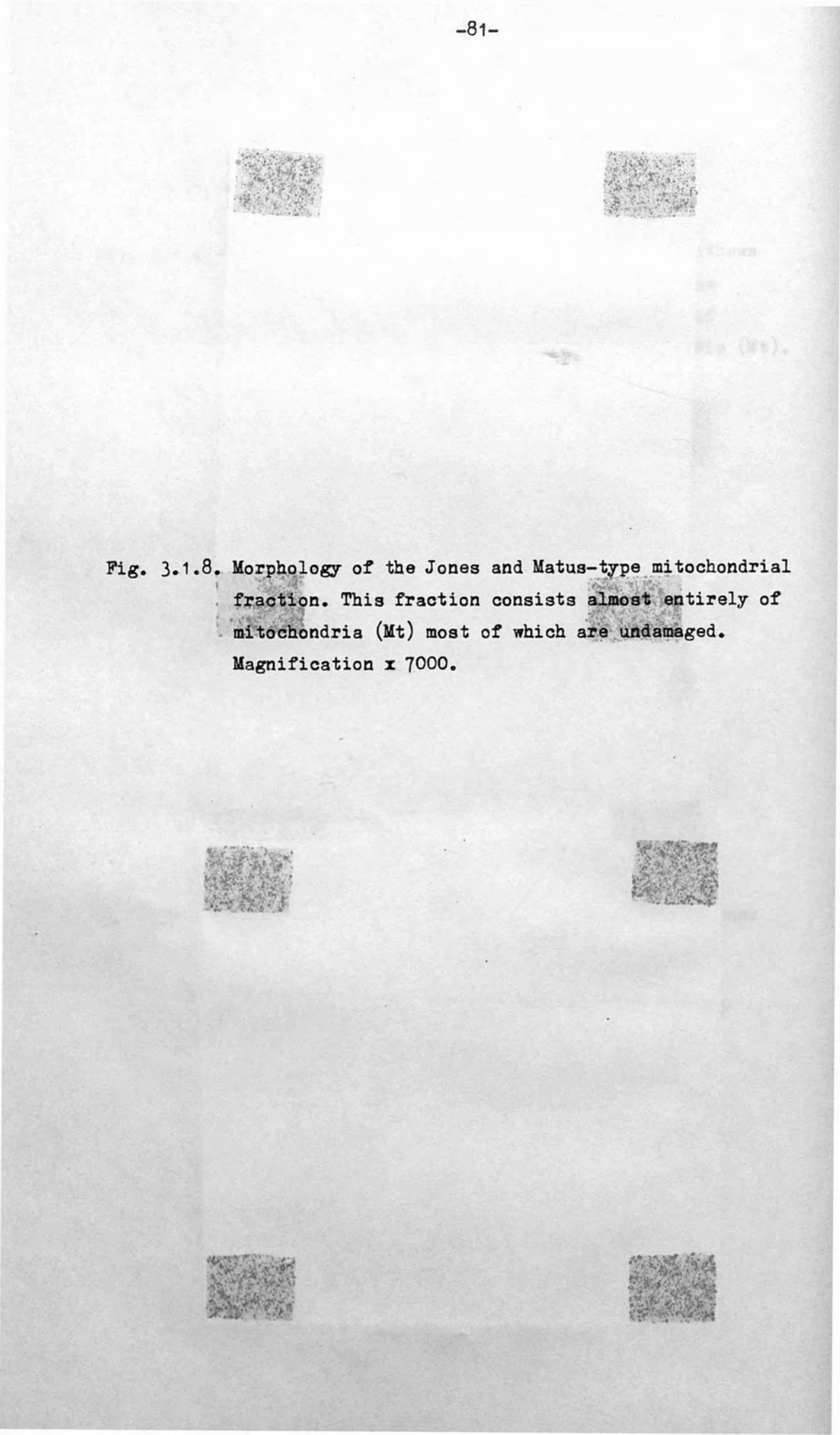
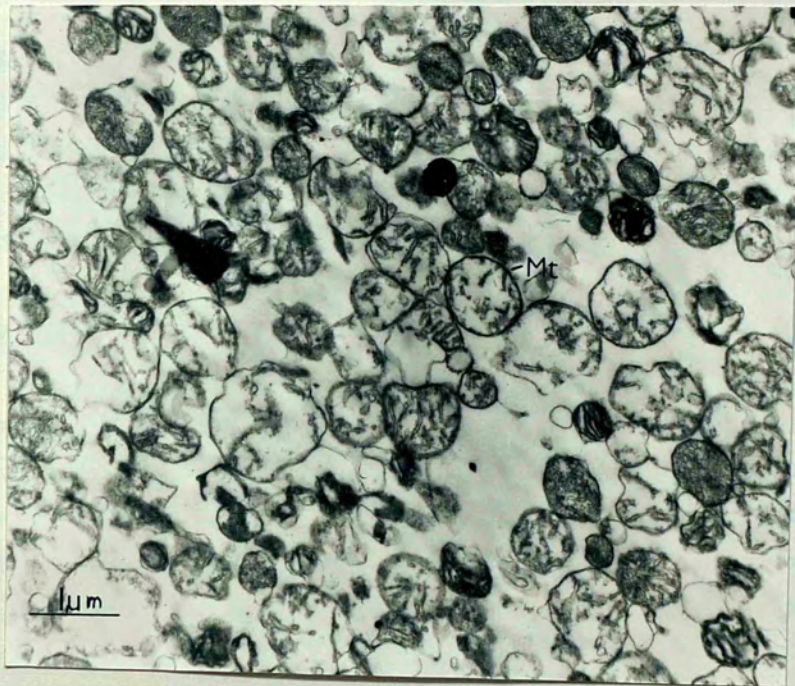


Fig. 3.1.8. Morphology of the Jones and Matus-type mitochondrial fraction. This fraction consists almost entirely of mitochondria (Mt) most of which are undamaged. Magnification x 7000.



reported by Jones and Matus (1974). The percentage of these enzymes in the mitochondrial fraction was higher than reported by these authors. While the percentage of acetylcholinesterase in the SPM fraction was found to correspond well with the literature value the percentage of $(\text{Na}^+, \text{K}^+)\text{ATPase}$ was 55% higher. It is impossible to determine whether these differences are statistically significant since no standard errors or deviations were given by these workers.

The distribution of the mitochondrial marker enzyme corresponded very closely to that obtained by Jones and Matus using cytochrome c oxidase. The yield of protein in the SPM fraction was 1.36 ± 0.20 mg protein/g tissue (wet weight) \pm S.E.M. which compared well with the value of 1.0 mg protein/g tissue reported by Jones and Matus (1974).

3.1.2.2 In Fractions Isolated Using the Cotman and Matthews Procedure

The plasma membrane marker enzymes were located primarily in the SPM fraction (located at the 25-32.5% (w/w) sucrose interface), while the mitochondrial marker was found to be concentrated in those fractions which, on the basis of electron microscopic examination contained the highest proportions of mitochondria (i.e. the fraction located at the 35-38% (w/w) sucrose interface and the pellet) (Table 3.1.2). $(\text{Na}^+, \text{K}^+)\text{ATPase}$ was enriched 3.32 ± 1.2 times (\pm S.E.M., N=3) and acetylcholinesterase 3.05 times (N=2) in the SPM fraction over the homogenate.

The distribution of protein in the fractions obtained from the sucrose gradient was similar to Cotman and Matthews' data. The largest difference, although not statistically significant, was in the slightly lower percentage of protein present in the myelin-enriched fraction (10.5 ± 4.7 compared with 18.5 ± 1.4 obtained by Cotman and Matthews).

The overall pattern of enzyme distribution was similar to that reported by Cotman and Matthews. A lower percentage of $(\text{Na}^+, \text{K}^+)\text{ATPase}$ and acetylcholinesterase, however, was found in the myelin-enriched fraction (56.8 and 57.2% of the Cotman and Matthews values respectively). Conversely, the mitochondrial fraction contained higher levels of these enzymes (397% and 844% respectively) although the acetylcholinesterase value did show a large standard error of the mean. None of these

TABLE 3.1.2

Percentage Distribution of Marker Enzymes and Protein in Brain Subfractions
Obtained Using the Cotman and Matthews Method

Fraction	Marker Enzyme			
	Protein (N=5)	(Na ⁺ ,K ⁺)ATPase (N=5)	AChE (N=4)	Succinate PMS reductase* (N=3)
1	10.5±4.7 (18.5±1.4)**	10.3±3.3 (18.0±4.5)	19.7±7.4 (39.6±6.7)	3.0±0.4 (0.6±0.1)
2	31.7±2.0 (31.8±1.7)	51.3±5.3 (53.0±3.9)	48.9±4.7 (43.7±6.9)	11.8±6.6 (6.2±2.4)
3	20.8±3.9 (18.0±2.3)	17.5±4.4 (13.0±4.9)	11.1±2.6 (10.5±1.1)	12.2±2.2 (19.3±5.5)
4	22.0±4.7 (20.3±2.7)	8.9±1.6 (13.1±3.0)	9.0±4.8 (4.4±1.1)	27.9±10.7 (43.1±3.5)
5	15.0±3.0 (11.5±4.9)	11.9±4.9 (3.0±1.1)	15.2±12.8 (1.8±0.1)	31.9±10.2 (30.4±7.6)

The composition of the fractions was as follows:-

1. predominantly myelin; 2. SPM; 3. membrane fragments + mitochondria;
4. mitochondria + membrane fragments; 5. mitochondria.

The values are expressed as in Table 3.1.1.

* Cotman and Matthews used cytochrome c oxidase as the mitochondrial marker.

** Cotman and Matthews' values

differences were found to be statistically significant using Student's t test. The distribution of the mitochondrial marker, succinate PMS reductase, was similar to that obtained by Cotman and Matthews using cytochrome c oxidase. The percentage of the former enzyme located in the SPM fraction was higher than reported ($11.8 \pm 6.6\%$ compared with $6.2 \pm 2.4\%$) by Cotman and Matthews but similar to that obtained using the Jones and Matus procedure (10.4%). The differences in distribution of the mitochondrial marker compared with the data of Cotman and Matthews were not statistically significant.

The yield of protein in the SPM fraction was 2.38 ± 0.38 mg protein/g tissue (wet weight) which compared well with that of 1.5-2.0 mg/g tissue reported by Cotman and Matthews (1971).

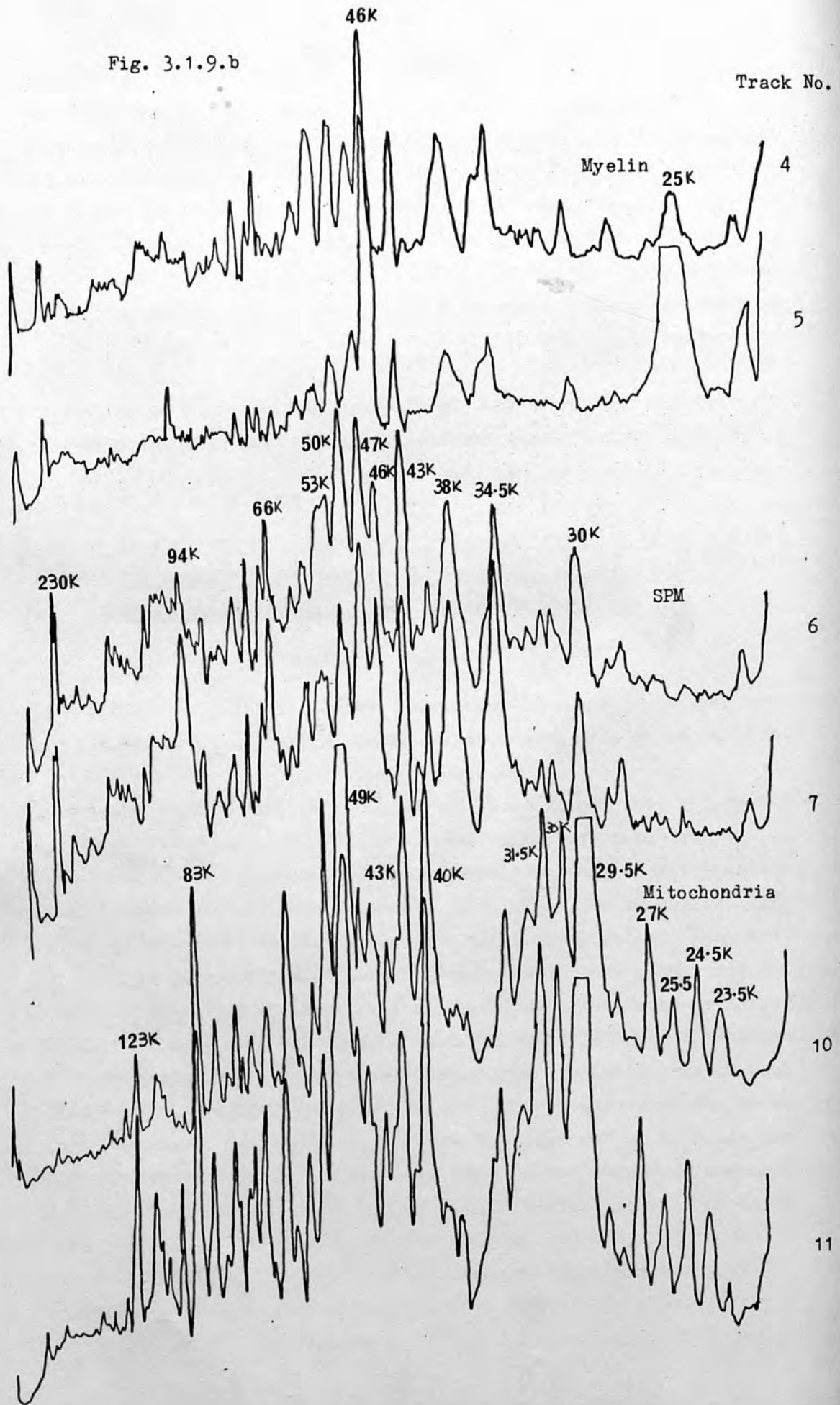
3.1.3 SDS Polyacrylamide Gel Electrophoretic Analysis of Brain Subcellular Fractions: Comparison of Fractions Obtained by the Jones and Matus (1974) and Cotman and Matthews (1971) Procedures

A comparison of the electrophoretic protein profiles of fractions obtained using both procedures is shown in Figs. 3.1.9.a and 3.1.9.b.

The myelin fraction isolated by the Jones and Matus procedure contained relatively few polypeptide bands (track 5). By comparison, the myelin-enriched fraction isolated by the longer procedure contained more polypeptides of which ten were present in substantial amounts (track 4). This more complex polypeptide composition of this fraction is in agreement with the morphological finding that the Cotman and Matthews'-type myelin-enriched fraction contains a high proportion of membranous material of non-myelin origin. The most prominent polypeptide band in this fraction corresponded to the 46K species observed in the Jones and Matus-type myelin fraction. Only a faint band, however, corresponded to the major 25K species in the latter fraction. The mitochondrial profiles of fractions obtained by the two methods were found to be very similar (tracks 10 and 11). The two most prominent mitochondrial proteins had molecular weights of 49 and 29.5K. Additional major polypeptides had molecular weights of 123, 83, 43, 40, 31.5, 30, 27, 25.5, 24.5 and 23.5K. The four lowest molecular weight species formed a distinctive pattern in the low molecular weight region of the

Fig. 3.1.9.b

Track No.



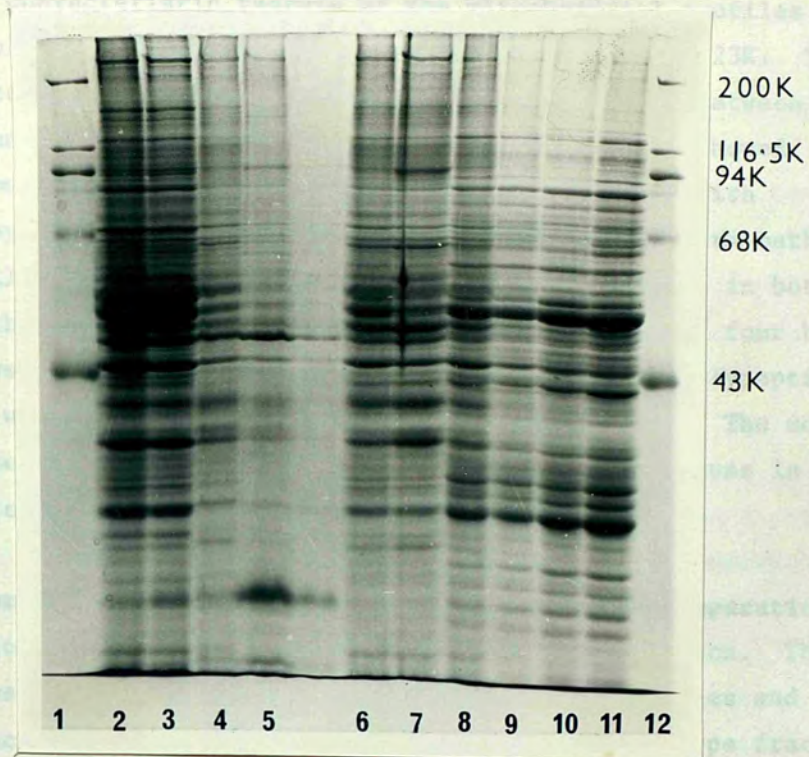


Fig. 3.1.9. a) Coomassie blue stained 10% SDS polyacrylamide gel comparing subcellular fractions isolated using both procedures.

Tracks contain;

1. protein standards
- 2,3. brain homogenates
4. Cotman and Matthews myelin-enriched fraction
5. Jones and Matus myelin fraction
6. Cotman and Matthews SPM fraction
7. Jones and Matus SPM fraction
8. Cotman and Matthews 32.5-35% (w/w) sucrose fraction
9. Cotman and Matthews 35-38% (w/w) sucrose fraction
10. Cotman and Matthews mitochondrial fraction
11. Jones and Matis mitochondrial fraction
12. protein standards

Each track contained 100 μ g protein

Fig. 3.1.9. b) Densitometric scans of electrophoretic protein profiles of myelin, SPM and mitochondrial-enriched fractions isolated using both procedures.

gel. Another characteristic feature of the mitochondrial profiles was the lack of polypeptides of molecular weights in excess of 123K. The Cotman and Matthews-type fractions which were intermediate between the SPM fraction and the mitochondrial pellet (tracks 8 and 9) showed increasing amounts of the major mitochondrial polypeptides with increasing buoyant density. The SPM fractions prepared by both methods were very similar with all polypeptide species being present in both profiles. Both fractions showed a characteristic pattern of four bands of molecular weights 53, 50, 47 and 46K. Other major SPM polypeptides had molecular weights of 230, 94, 66, 43, 38, 34.5 and 30K. The most notable difference between the SPM electrophoretic profiles was in the amount of protein present in the 94K band.

This was a prominent species in the Jones and Matus-type preparation but only a minor component in the Cotman and Matthews SPM fraction. The 53 and 50K species were present in lower proportions in the Jones and Matus-type fraction compared with the Cotman and Matthews-type fraction.

No band was present in either SPM fraction which corresponded to the major 25K myelin polypeptide. The presence of a band corresponding to the 46K myelin polypeptide could not be positively ascertained since this would have been located in the most complex region of the SPM profiles. The major mitochondrial polypeptides did not appear to be represented in either SPM fraction and no bands corresponded to the characteristic low molecular weight mitochondrial species.

Both myelin fractions, by comparison, appeared to contain bands which corresponded in molecular weight to principal components of the SPM fraction, particularly the 53, 50, 47, 46, 38 and 34.5K species. Traces of the 230 and 94K polypeptides were also observed in both myelin fractions. Relatively more of all of these species was present, however, in the Cotman and Matthews-type myelin-enriched fraction.

The intermediate Cotman and Matthews-type fractions (tracks 8 and 9) also contained small amounts of polypeptides which corresponded to major SPM species, particularly the 230, 47 and 38K polypeptides.

3.1.4 DISCUSSION

3.1.4.1 Comparison of the Purity of Synaptic Fractions with Literature

On the basis of these characterisation studies it would appear that the SPM fractions isolated by both methods are comparable in purity and composition to those reported in the literature. While the overall pattern of marker enzyme distribution compared well with that reported by Jones and Matus (1974) and Cotman and Matthews (1971) some differences were observed. Comparison of the distribution of enzymes in Jones and Matus-type fractions with the literature was difficult, however, since these authors did not quote any standard deviations or standard errors of the mean in their results. By comparison with the figures quoted by Cotman and Matthews (1971), however, the S.E.M. values in the present study were similar to those reported by these authors. Although some differences were observed in the percentage distribution of marker enzymes were observed between the present study and that of Cotman and Matthews none were found to be statistically significant using Student's t test. This suggests that although individual S.E.M. values were relatively small there is, nevertheless, a substantial degree of variability in the isolation procedures. While every effort was made to standardise the routine procedures involved in the isolation of these SPM fractions it is inevitable that various steps in the preparative procedures are susceptible to substantial systematic errors inherent in the methods used, for example, in the removal of fractions from the sucrose gradients. This, in itself, may account for the differences in percentage distribution that were observed.

In the brain subfractions obtained by the two procedures it was found that smaller percentages of the plasma membrane markers were observed in the myelin fractions and higher percentages in the mitochondrial fractions than were reported in the literature. It is unlikely that this was due to malfunctioning of the sucrose gradients since the morphology, as judged by electron microscopy, and the electrophoretic protein profiles of the isolated fractions compared well with the literature (Cotman and Matthews, 1971; Jones and Matus, 1974; Blomberg et al., 1977; Mena et al., 1980). In addition, the enrichment of the plasma membranes marker enzymes in the SPM fractions over the homogenates were similar to those reported by other workers (Cotman and Matthews, 1971;

Morgan et al., 1971; Gurd et al., 1974). Furthermore the distribution of protein and the mitochondrial marker enzyme succinate PMS reductase on the sucrose gradients was consistent with that reported by the respective authors.

It is possible that the low percentage of plasma membrane markers in the myelin fractions is a consequence of the lower yield of protein in this fraction. In the present study a lower yield of protein (57% of the Cotman and Matthews' value) was routinely obtained in this fraction although this was not statistically significant. This deficit may, therefore, have distorted the percentage distribution of the plasma membrane markers in the remaining fractions.

The higher levels of plasma membrane markers in the mitochondrial fractions, it may be argued, might be due to the presence of unlysed or partially lysed synaptosomes or to non-specific association of membranes containing these enzymes with mitochondria due to the effect of sucrose as described by Day et al., (1971). While neither of these possibilities can be entirely ruled out both are unlikely since the electron microscopic evidence suggests that neither synaptosomes or significant amounts of non-mitochondrial membranes were present in the mitochondrial fraction.

It was observed that the Jones and Matus-type SPM fraction contained a higher percentage of $(\text{Na}^+, \text{K}^+)\text{ATPase}$ than was reported by these workers. Jones and Matus (1974) suggested that high levels of this enzyme might indicate glial contamination of SPM since sonication of the lysate resulted in a 46% increase of this marker in the SPM fraction. These workers suggested that this was due to the release of glial membranes, which contain $(\text{Na}^+, \text{K}^+)\text{ATPase}$ (see section 1.2.2), from myelin by this procedure. While the possibility that the present SPM fraction was contaminated by glial membranes cannot be ruled out it is unlikely that this contamination is substantial since brain tissue was homogenised under the same conditions as those used for the Cotman and Matthews procedure. The SPM fraction isolated using the latter procedure showed a percentage distribution of plasma membrane markers very close to that reported by Cotman and Matthews. In addition, morphological examination of the Jones and Matus-type myelin fraction showed that this was not significantly more damaged than the corresponding fraction obtained by these workers.

In order to determine the percentage purity of SPM fractions a number of workers have isolated highly purified fractions of putative contaminants e.g. microsomes, mitochondria, etc. and compared the specific activity of marker enzymes in these fractions with the specific activity of these enzymes in SPM fractions (Levitan et al., 1972; Gurd et al., 1974). This was not done in the present study since the aim was to compare the distribution of marker enzymes with those reported in the literature. It is feasible to assume that if the percentage distribution of marker enzymes is similar to literature values that the percentage contamination is similarly comparable. In early papers the levels of contamination of SPM were generally agreed to be between 10 and 15% (see section 1.1.4). These percentages were based, however, only on measured contaminants and failed to take into account axonal and dendritic etc. membranes which could not be distinguished from SPM using the available techniques. It is now thought that the 'purity' of SPM fractions may be much lower than original estimates. Based on quantitative electron microscopic evidence the purity of SPM fractions may be as low as 55-65% (Gurd, 1982). Thus these fractions should be referred to as SPM-enriched fractions.

Thus the distribution of marker enzymes in SPM, myelin and mitochondrial-enriched fractions isolated by these two procedures is comparable to those obtained by these authors except for minor, non-significant differences.

3.1.4.2 Comparison of SPM Fractions Isolated by Both Procedures

The SPM fractions isolated by both methods were similar in their marker enzyme constitution, morphology and electrophoretic protein profiles. The latter were used to compare the protein composition of both SPM fractions. The electrophoretic profiles of the SPM fractions were virtually indistinguishable and closely resembled published profiles (Blomberg et al., 1977; Matus et al., 1980b; Mena et al., 1980; Nieto-Sampedro et al., 1981a). The prominent SPM polypeptides reported by Jones (1976) and Walters (1976) were present in both fractions. The principal SPM polypeptides were shown to have apparent molecular weights of 53, 50, 47, 45, 43, 38, 34.5 and 30K. Although there was a slight discrepancy in the measured molecular weights (Carlin et al., 1982, have suggested that this may be due to variations in batches of SDS) it is thought that the 53 and 50K polypeptides may be β and α tubulin, the

47K band may be the major PSD protein and the 43K band actin (see section 1.3.1). The identity of the 45K band is not known but has been observed in all published profiles of SPM fractions. Some of the major myelin proteins, the Wolfgram proteins, have molecular weights in this region but it is unlikely that the 47 or 45K bands are due to myelin contamination since the other major myelin proteins, the proteolipid and basic proteins, were not detected in either SPM fraction.

The Jones and Matus-type SPM fraction contained a prominent polypeptide of 94K which has been reported to be a major component of SPM electrophoretic profiles (Blomberg et al., 1977; Matus et al., 1980b; Burgoyne et al., 1981). It has been suggested that this SPM band may correspond to the major subunit of $(Na^+, K^+)ATPase$ (Morgan et al., 1973).

Of the remaining SPM polypeptides a 230K species has been reported to be fodrin (Kakiuchi et al., 1982; Sobue et al., 1982; Carlin et al., 1983). Jones (1976) tentatively suggested that, on the basis of a similarity in molecular weights, a 28K polypeptide (which corresponds to the 30K band in the present study) might be glucose-6-phosphatase.

Neither SPM fraction appears to be significantly contaminated by either myelin or mitochondrial polypeptides which suggests that contamination from these sources is minimal and is unlikely to be in excess of the values quoted by other workers.

Thus the SPM fraction produced by the rapid method of Jones and Matus does not seem to be substantially different in composition from that produced by the longer procedure. There does not appear, therefore, to be any merit in using the Cotman and Matthews method in preference to the more rapid method. The Jones and Matus method has now been adopted by a number of workers (De Blas and Mahler, 1976; Burgoyne and Rose, 1980; Sorensen and Mahler, 1981; 1983) and is now, therefore, considered to be of use in the study of functional components of the synaptic membrane.

3.2 Measurement of Total Cerebral Protein Synthesis and Precursor Incorporation into Brain Subcellular Fractions In Vivo

While a considerable amount of work has been carried out in recent years to determine the rate of synthesis of brain proteins very little is known

about the synthesis of SPM proteins and their incorporation into the synaptic plasma membrane. The aim of the present study has been, firstly, to establish a method for measuring the in vivo incorporation of radiolabelled precursor into SPM proteins and other subfractions of the brain and, secondly, to examine the effects of selected drugs and thyroid hormone deficiency on this incorporation in vivo.

3.2.1 Principles Governing the Choice of Method for the Measurement of Cerebral Protein Synthesis Rates In Vivo

Overall rates of protein synthesis are usually determined by measuring the accumulation of radioactivity in protein at a measured interval after administration of radiolabelled precursor. The rate of accumulation is an index of the rate of protein synthesis but only approximates closely to the absolute rate of synthesis if certain conditions are fulfilled. To determine the absolute rate of synthesis it is necessary that the precursor specific activity in the pool used for protein synthesis is known during the incorporation period or can be calculated.

Frequently trace amounts of precursor have been used in the estimation of cerebral protein synthesis rates. This method, however, is subject to many problems stemming from the difficulty in determination of the precursor specific activity in the tissue and uncertainty as to the precursor pool used for protein synthesis. These problems have been discussed extensively elsewhere and, therefore, will not be examined in detail here (for reviews see: Oja, 1973; Lajtha and Dunlop, 1976; Dunlop et al., 1977; Dunn, 1977; Waterlow et al., 1978; Rannels et al., 1982). Since the precursor specific activity varies during the incorporation period when trace amounts are used it is necessary to measure the time-integrated specific activity in order to obtain an estimate of the rate of protein synthesis (Heath and Barton, 1973). This procedure is lengthy and requires large numbers of animals and substantial amounts of labelled precursor and is, therefore, rarely attempted.

In order to overcome the problems associated with using trace quantities of labelled precursor a number of methods have been developed in which the precursor specific activity is maintained at a constant level during the incorporation period. This can be achieved by repeated injections (Austin et al., 1972) implantation of pellets (Lajtha and Dunlop, 1976)

and continuous infusion of precursor (Garlick and Marshall, 1972; Seta et al., 1973). These methods are still subject to uncertainty as to whether the precursor specific activity in the protein synthetic pool is close to the mean specific activity of the free precursor in the tissue. In addition, the incorporation periods used in these methods tend to be long (several hours) which may, therefore, lead to errors in calculating the rate of protein synthesis due to reutilisation of labelled amino acid.

An alternative method is available, however, in which high concentrations of precursor are administered in a single injection in order to swamp the in vivo pools (Dunlop et al., 1975). In using this method it is assumed that over short incorporation periods (less than 1 h) all pools are labelled to the same specific activity which is close to that of the injected precursor. This method is the simplest of those designed to maintain the precursor specific activity and, since all precursor pools are of approximately the same specific activity, overcomes the problem of definition of the protein synthetic pool. All methods for measuring rates of protein synthesis assume, however, that the specific activity of the precursor in the tissue is close to the specific activity of the relevant amino acyl tRNA which is the ultimate precursor in the protein synthetic pool.

Single amino acid precursors are most commonly used in studies of protein synthesis. Ideally the precursor should be an essential amino acid with a small cerebral free pool and subject to only minimal metabolism in the brain. If the precursor is to be administered peripherally it should have a high uptake index into the brain. The route of administration may be either peripheral (sub-cutaneous, intraperitoneal or intravenous) or intracranial. The choices of route of administration and precursor are interdependent and are governed by the aim of the experiment.

In order to measure rates of incorporation of radiolabelled precursor into SPM proteins it is necessary to be able to achieve a sufficiently high precursor specific activity in the brain for measurable amounts of radioactivity to be attained in SPM protein. This can best be achieved by intracranial injection of flooding levels of labelled precursor. Gaitonde and Richter (1956) used gross injections of L-(³⁵S)methionine to measure cerebral protein synthesis rates. These workers administered the

precursor by either intracisternal or subarachnoid injection under ether anaesthesia. It has been reported, however, that ether anaesthesia inhibits cerebral protein synthesis (Schotman et al., 1977). The effect of anaesthesia can be avoided^{ed} by injecting animals while conscious via a previously implanted cannula. Hence a method where flooding levels of L-(³⁵S)methionine were injected via pre-implanted intraventricular cannulae was developed to measure total cerebral protein synthesis rates and precursor incorporation into brain subcellular fractions in vivo.

3.2.2 Measurement of the Rate of Incorporation of L-(³⁵S)methionine into Brain Protein Using Gross Intraventricular Injection

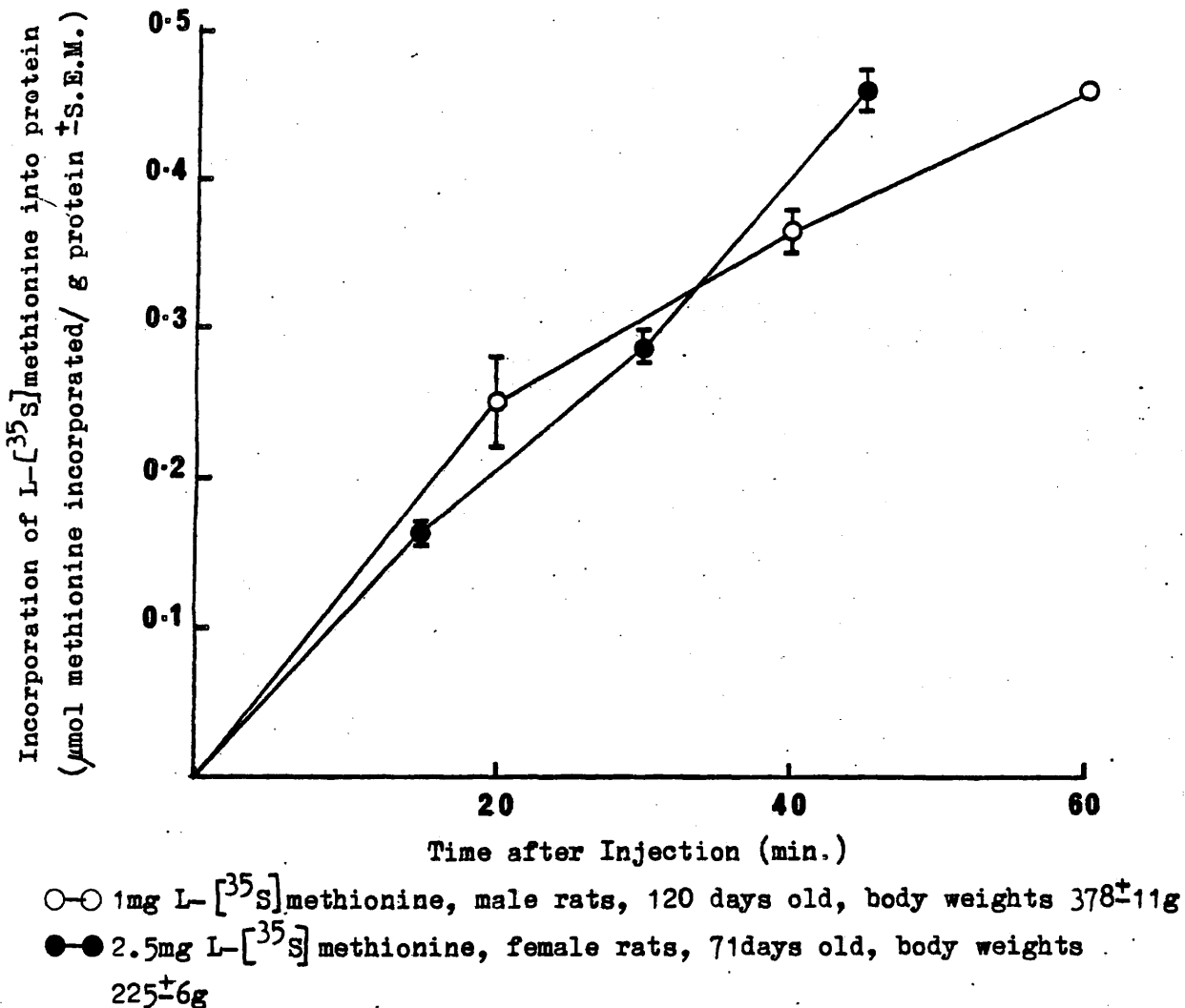
Rats were injected intraventricularly initially with 1 mg L-(³⁵S)methionine since this was reported by Gaitonde and Richter (1956) to be sufficient to flood the brain methionine pools. It was found, however, that the incorporation of precursor into brain protein over 1 h, using this concentration, was not linear. In addition, the calculated rates of incorporation at 20, 40 and 60 min after injection steadily decreased from 0.75 to 0.46 $\mu\text{mol L-(}^{35}\text{S)methionine incorporated/g protein/h}$ (Fig. 3.2.1). This indicated that the precursor specific activity during the incorporation period was decreasing and, therefore, that the methionine pools were not flooded.

A trial study was made, therefore, to compare the incorporation rates measured in animals injected with either 1 mg or 5 mg L-(³⁵S)methionine (Table 3.2.1).

The higher value represented the maximum amount of L-methionine able to be dissolved in 100 μl . There was found to be a large difference in the measured rates of incorporation of L-(³⁵S)methionine into brain protein at 40 min after administration with the rate at 5 mg being considerably higher than at 1 mg.

The larger injection volumes used in this study, however, resulted in adverse reactions in the rats. Animals appeared to lose their balance and become disorientated for 2-3 min after injection although they recovered quite quickly. This effect was probably a consequence of the increased intracranial pressure following the injection of such a large volume of liquid. The maximum feasible injection volume, based on the

Fig. 3.2.1. Time Course of Incorporation of 1mg and 2.5mg of L-[³⁵S]methionine into Total Forebrain Protein In Vivo



Conscious rats were intraventricularly injected via preimplanted cannulae with 1 or 2.5mg L-[³⁵S]methionine (30 μ Ci, injection volume, 50 μ l). Each time point is the mean of values obtained from 4 rats (2.5mg time course) or 3 rats (1.0mg time course) with the exception of the 60min point where N=2. The animals were killed by cervical dislocation after the appropriate incorporation period. The rates of incorporation at each time point were as follows; 20 min, 0.75[±]0.08; 40 min, 0.55[±]0.02; 60 min, 0.46; for the 1mg time course: 15 min, 0.65[±]0.03; 30 min, 0.57[±]0.03; 45 min, 0.62[±]0.02 μ mol methionine incorporated/ g protein/h[±]S.E.M. for the 2.5mg time course

TABLE 3.2.1

Trial Comparison of the Rate of Incorporation of L-(³⁵S)methionine Using
Doses of 1 mg or 5 mg

Dose of methionine	Rate of incorporation (μmol methionine incorporated/ g protein/h \pm S.E.M.)
1 mg (N = 3)	0.47 \pm 0.04
5 mg (N = 2)	0.96, 0.78

N = number of rats.

Female rats (100 days old, body weights 393 \pm 18 g) were intraventricularly injected with either 1 or 5 mg L-(³⁵S)methionine (10 μCi , injection volume, 50 μl).

The animals were killed by cervical dislocation after a 40 min incorporation period.

absence of any discernible stress in the animals, was found to 50 μ l. It was necessary, therefore, to determine whether it was possible to flood the brain methionine pools with the maximum amount of L-methionine able to be dissolved in this volume. Trial studies showed this amount to be 2.5 mg L-methionine. Using this concentration of precursor the incorporation of L-(³⁵S)methionine into the total forebrain TCA-insoluble fraction was examined (a representative graph is given in Fig. 3.2.1) at the incorporation periods of up to 45 min. In addition, the rates of incorporation at 15, 30 and 45 min were constant which implied that, at this concentration, the precursor specific activity was constant during the incorporation period.

The rate of incorporation of L-(³⁵S)methionine into total forebrain protein under these conditions was found to be 0.64 μ mol methionine incorporated/g protein/h (this figure is a representative value based on 3 separate time course observations). Using intraperitoneal flooding injections of L-(U¹⁴C)valine King (1980) obtained a value of 2.23 \pm 0.08 μ mol valine incorporated/g protein/h for the rate of cerebral protein synthesis. To compare the value obtained in the present study with that obtained by King the value must be corrected for the different abundances of methionine and valine in the brain. Lajtha and Toth (1974) estimated that, in rat brain, methionine accounted for 2.5% (μ moles/100 μ moles amino acid residues) and valine constituted 8.6%. Thus by correcting the rate obtained in the present study for comparison with valine a value of 2.2 μ mol valine incorporated/g protein/h is obtained. Thus the rate of incorporation of L-(³⁵S)methionine into brain protein obtained using intraventricular injection is comparable to values for the rate of cerebral protein synthesis reported by other workers.

The incorporation of L-(³⁵S)methionine into brain subcellular fractions was also examined (Fig. 3.2.2). It was found that the rate of incorporation of precursor into mitochondrial protein was close to that of total forebrain protein (e.g. 0.571 \pm 0.03 and 0.513 \pm 0.006 μ mol methionine incorporated/g protein/h \pm S.E.M. into forebrain and mitochondrial proteins respectively at 30 min after administration) and followed a similar pattern. Incorporation of labelled methionine into the proteins of the SPM and myelin fractions, however, was much lower (e.g. 61.3% \pm 2.03 and 38.4 \pm 1.20 of the forebrain values at 30 min after administration (\pm S.E.M.)). In all of the fractions the rates of

incorporation remained constant over incorporation periods of up to 45 min.

3.2.3 The Distribution of ^{35}S in Methionine, Cysteine and Cystine Following Intracranial Injection

TLC analysis of perchloric acid extracts of forebrain homogenates showed that the major proportion of the label was found in a band corresponding in mobility to the methionine standard (Rf 0.57 ± 0.008 (\pm S.E.M.)) (Fig. 3.2.3). The radioactivity in this band accounted for 70-80% of the total recovered from the t.l.c. plate. In the presence of oxidising acids (e.g. performic or perchloric acid) cystine and cysteine are oxidised to cysteic acid (Lehninger, 1976). This amino acid derivative was found to migrate close to the origin (Rf 0.08 ± 0.003 (\pm S.E.M.)). The percentage of ^{35}S in this band was difficult to quantitate since it was not substantially greater than the background activity found between the origin and the methionine band. Estimations of the radioactivity due to cysteic acid, however, were found to be never in excess of 10% of the total radioactivity recovered from the plate. Other labelled metabolites of methionine were not measured.

3.2.4 Rates of Incorporation of L-(^{35}S)Methionine into the TCA-Insoluble Fractions of Gross Brain Areas

The rates of incorporation of L-(^{35}S)methionine into the protein of the left and right cerebral hemispheres (injected and non-injected sides respectively) and the cerebellum were compared to determine whether the distribution of labelled precursor was uniform throughout the brain. A small deficit (6.4%) was observed in the rate of incorporation of precursor into the protein of the right cerebral hemisphere compared with the left (injected) hemisphere but this was not found to be statistically significant using Student's t test (Table 3.2.2.a). This finding was corroborated by further experiments. The largest deficit observed was of 10.45% (Table 3.2.2.b) although this, again, was not statistically significant.

TABLE 3.2.2.a

Rates of Incorporation of L-(³⁵S)methionine into Gross Brain Areas

Brain Area	Rate of incorporation (umol methionine incorporated/g protein/h ± S.E.M.)	Percentage difference
Left hemisphere (N=5)	0.78±0.03	-
Right hemisphere (N=4)	0.73±0.05	-6.4
Cerebellum (N=5)	0.80±0.04	+2.6

TABLE 3.2.2.b

Brain Area	Rate of incorporation (umol methionine incorporated/g protein/h ± S.E.M.)	Percentage difference
Left hemisphere (N=6)	0.72±0.03	-
Right hemisphere (N=6)	0.65±0.05	-10.45
Cerebellum (N=6)	0.71±0.03	-6.4

N = number of rats.

Conscious rats (78 day old male rats, body weights 205 ± 5 g,

Table 3.2.2.a; 83 day old male rats, body weights, 217 ± 9 g

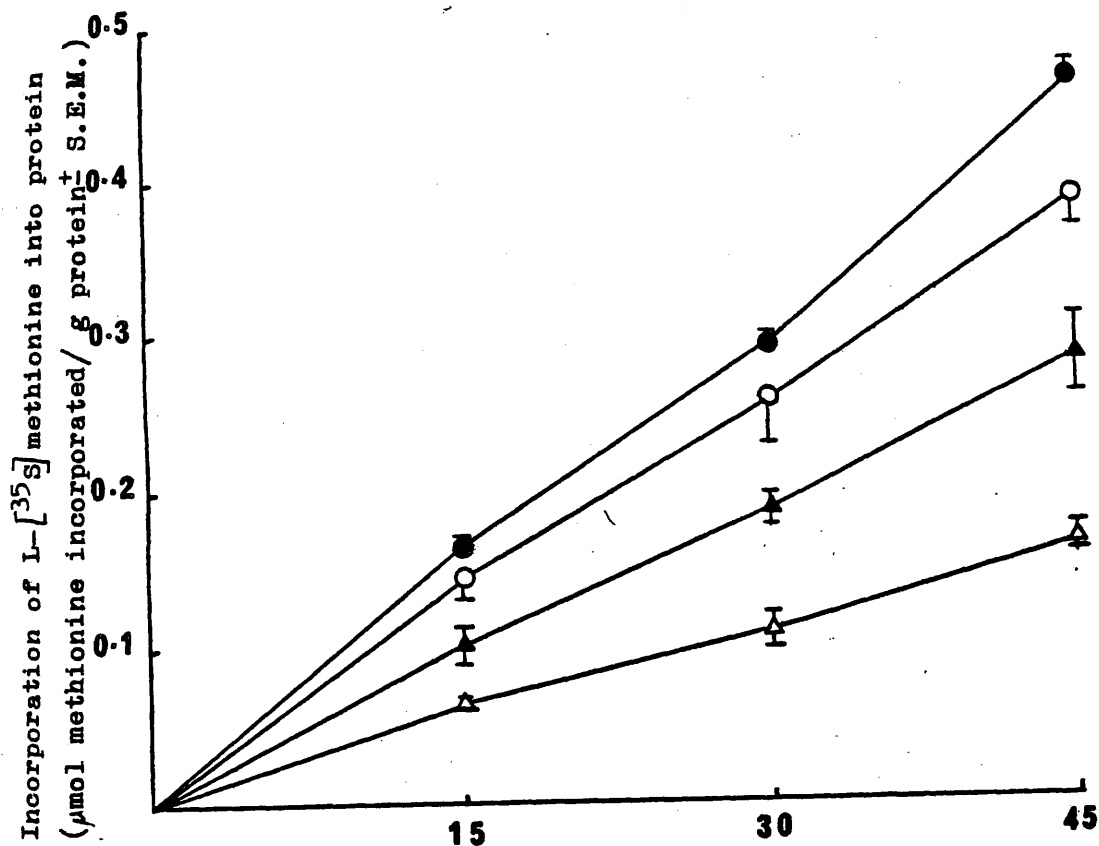
Table 3.2.2.b) were injected into the left lateral ventricle via

preimplanted cannulae with 2.5 mg L-(³⁵S)methionine (20 µCi, injection

volume 50 µl). The animals were killed by cervical dislocation after a 30 minute incorporation period.

None of the differences were statistically significant using Student's t test.

Fig. 3.2.2. Time Course of Incorporation of L-³⁵S methionine(2.5mg) into Brain Subcellular Fractions In Vivo



Female rats (71 days old, body weights 225 ± 6 g) were intraventricularly injected with 2.5mg L-³⁵S methionine (20 μ Ci, injection volume 50 μ l). Animals were killed by cervical dislocation after the appropriate incorporation periods and subcellular fractions were isolated by the method of Jones and Matus (1974).

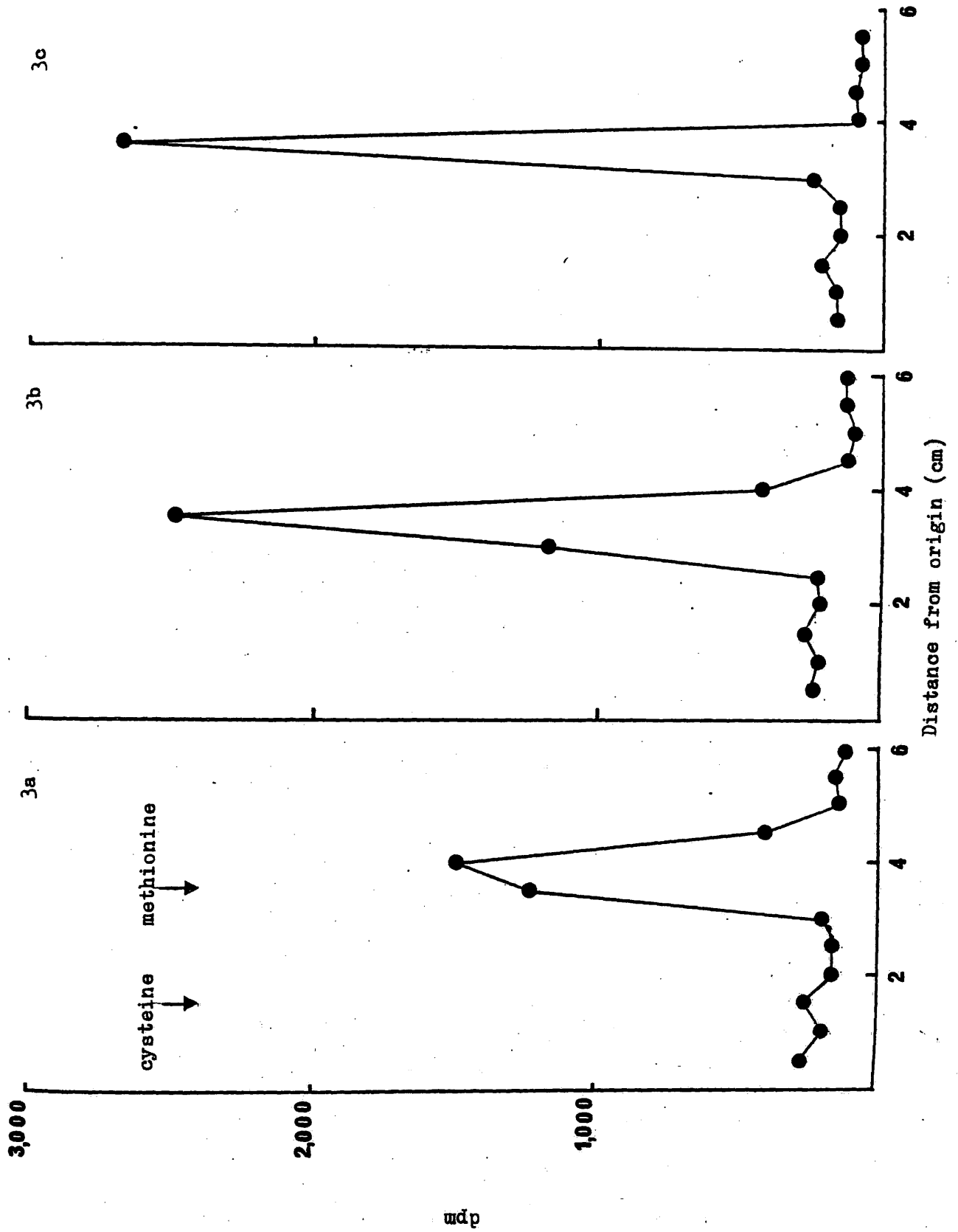
●—● Forebrain homogenate, △—△ myelin, ▲—▲ SPM,
○—○ mitochondria.

Incorp. period (min)	Rate of incorporation (μ mol methionine incorporated/ g protein/ h \pm S.E.M.)		
	15	30	45
Forebrain homogenate	0.65 \pm 0.03	0.57 \pm 0.03	0.62 \pm 0.02
Myelin	0.26 \pm 0.07	0.22 \pm 0.02	0.22 \pm 0.01
SPM	0.38 \pm 0.05	0.37 \pm 0.02	0.38 \pm 0.03
Mitochondria	0.58 \pm 0.06	0.51 \pm 0.006	0.52 \pm 0.03

Fig. 3.2.3. Distribution of Perchloric acid-soluble ³⁵S labelled
Amino Acids in Brain Extracts Following t.l.c.

The perchloric acid extracts of brain homogenates were run on cellulose t.l.c. plates in the solvent system n-butanol: glacial acetic acid: water (12: 3: 5, by volume). After chromatography 0.5cm bands were scraped off the plate and the distribution of radioactivity determined by scintillation counting.

- 3a 15min after injection
- 3b 30min after injection
- 3c 45min after injection



3.2.5 The Effect of Selected Drugs on the Incorporation of L-(³⁵S)methionine into the Proteins of Total Brain Homogenates and Subcellular Fractions In Vivo

A variety of drug treatments have been reported to affect brain protein synthesis (see review by Lajtha and Dunlop, 1981). In many instances, however, drug effects have been studied using trace amounts of labelled precursor and, therefore, it is difficult to assess whether the observed effect is real or an artifact of, for example, precursor availability (see section 3.2.1) or other factors, e.g. alterations in the transport of precursor across the blood-brain barrier. In addition, a substantial number of drugs produce their effect on cerebral protein synthesis due to alterations in body temperature which, in some studies, has not been strictly monitored.

It was decided, therefore, that the effects of selected drugs on brain protein synthesis would be examined using intraventricular injection of flooding concentrations of L-(³⁵S)methionine as described in section 3.2.2.

3.2.5.1 The Effect of Halothane and Pentobarbitone-induced Anaesthesia

Halothane and pentobarbitone are frequently used in the anaesthetisation of small animals. It is, therefore, of interest to determine whether these drugs have any effect on cerebral protein synthesis since, in many studies, this has been examined in anaesthetised animals.

Halothane was found to cause a reduction in the rate of incorporation of L-(³⁵S)methionine into brain protein in young rats (35-40 days old). This depression was observed in both forebrain and cerebellar homogenates and also in brain subcellular fractions (representative results are given in Table 3.2.3). In all of the fractions, except the myelin fraction, the rate of incorporation was reduced by around 40%. The myelin fraction was more severely affected and the rate of incorporation of labelled precursor into myelin protein was reduced by almost 70%. All of these reductions were found to be statistically significant using Student's t test.

TABLE 3.2.3

The Effect of Halothane and Pentobarbitone on the Rate of Incorporation of L-(³⁵S)methionine into Total Homogenate and Subcellular Fractions

Fraction	Rate of incorporation (μmol methionine incorporated/g protein/h \pm S.E.M.)				
	Conscious (N=3)	+ Pentobarbitone (N=4)	+ Halothane (N=4)	Percentage inhibition	
				Pentobarbitone	Halothane
Homogenates:					
Left hemisphere	0.82 \pm 0.05	0.82 \pm 0.05	0.49 \pm 0.07	0	40.2*
Right hemisphere	0.86 \pm 0.09	0.88 \pm 0.08	0.50 \pm 0.07	+2.3	41.9*
Cerebellum	0.88 \pm 0.02	0.76 \pm 0.07	0.45 \pm 0.04	13.6	48.9*
Fractions:					
Myelin	0.22 \pm 0.03	0.22 \pm 0.02	0.07 \pm 0.02	0	68.2*
SPM	0.49 \pm 0.03	0.51 \pm 0.05	0.30 \pm 0.03	+4.1	38.8**
Mitochondria	0.82 \pm 0.06	0.79 \pm 0.07	0.47 \pm 0.05	3.7	42.7**

N = Number of rats.

Male rats (37 days old, body weights 192.5 \pm 13 g) were anaesthetised with either halothane (3%) or pentobarbitone (60 mg/kg body weight, i.p.). After 20 min the rats were injected intraventricularly with 2.5 mg L-(³⁵S)methionine (20 μCi , injection volume 50 μl) via pre-implanted cannulae. The control rats were injected in the same way while conscious. After a 30 min incorporation period the animals were killed by cervical dislocation and subcellular fractions prepared by the method of Jones and Matus (1974). All anaesthetised rats were kept in warm boxes during the experiment.

* p<0.02; ** p<0.01.

The percentage inhibitions due to pentobarbitone anaesthesia were not statistically significant using Student's t test.

TABLE 3.2.4

The Effect of Pentobarbitone on the Rate of Incorporation of L-(³⁵S)methionine into Total Homogenate and Subcellular Fractions

Fraction	Rate of incorporation (μmol methionine incorporated/g protein/h \pm S.E.M.)		
	Conscious (N=5)	+ Pentobarbitone (N=4)	Percentage inhibition
Homogenates:			
Left hemisphere	0.78 \pm 0.03	0.79 \pm 0.07	+1.3
Right hemisphere	0.73 \pm 0.05	0.68 \pm 0.05	6.8
Cerebellum	0.80 \pm 0.04	0.71 \pm 0.03	11.3
Fractions:			
Myelin	0.43 \pm 0.10	0.45 \pm 0.08	+4.7
SPM	0.58 \pm 0.08	0.58 \pm 0.03	0
Mitochondria	0.88 \pm 0.01	0.75 \pm 0.05	14.8

N = number of rats..

Male rats (78 days old, body weights 205 \pm 5 g) were treated as in Table 3.2.3 except that the dose of pentobarbitone was increased to 75 mg/kg body weight.

None of the values were statistically significant.

Pentobarbitone, by comparison, did not appear to significantly alter the rate of incorporation of L-(³⁵S)methionine into brain protein at doses of either 60 or 75 mg/kg body weight (Tables 3.2.3 and 3.2.4) in any of the fractions examined.

The reduction in the incorporation of precursor into brain protein in halothane-anaesthetised rats was not due to any alteration in body temperature since all rats were housed in warm-boxes during the experiments. The rectal temperatures of all rats, both control and anaesthetised, were taken at death and were all close to 37°C.

3.2.5.2 The Effect of L-DOPA

A number of workers have observed that large doses of L-DOPA (500 mg/kg body weight) cause transient disaggregation of brain polysomes and reduce the incorporation of labelled amino acids into brain protein (Weiss et al., 1971; Roel et al., 1974). This effect has been found to be dependent on treated rats becoming hyperthermic on administration of L-DOPA which Moskowitz et al. (1977) have reported to play a permissive role in polysome disaggregation. Work carried out in this laboratory (King, 1980) using either trace levels of L-(3,4-³H)valine or L-(4,5-³H)lysine administered subcutaneously or flooding concentrations of L-(U-¹⁴C)valine administered intraperitoneally failed to show any significant effect of L-DOPA on brain protein synthesis. It was decided, therefore, that the in vivo flooding method described earlier should be applied to the study of the effects of this drug and, therefore, resolve this discrepancy.

Moskowitz et al. (1977) observed that the L-DOPA-related hyperthermia, which was necessary for polysome disaggregation to occur, was only brought about if the treated animals were housed at an ambient temperature of 26°C. Under these conditions the rate of incorporation of L-(³⁵S)methionine into brain protein was not found to be affected by L-DOPA treatment (Table 3.2.5). It was observed, however, that the body temperatures of the L-DOPA treated rats were not significantly elevated and were well below the temperature of 39-40°C reported by Moskowitz et al. (1977) to be necessary for polysome disaggregation. Trial experiments showed that to obtain the requisite degree of hyperthermia L-DOPA treated rates must be housed at an ambient temperature of 30°C for at least 45 min before administration of the drug and

TABLE 3.2.5

The Effect of L-DOPA on the Incorporation of L-(³⁵S)methionine into Total Homogenate and Subcellular Fractions (Ambient Temperature, 26°C)

Fraction	Rate of incorporation (μmol methionine incorporated/g protein/h \pm S.E.M.)	
	Control (N=4)	+ L-DOPA (N=5)
Forebrain homogenate	0.88 \pm 0.07	0.94 \pm 0.06
Myelin	0.46 \pm 0.04	0.54 \pm 0.06
SPM	0.51 \pm 0.05	0.54 \pm 0.04
Mitochondria	1.35 \pm 0.29	1.33 \pm 0.24
Mean body temperature (°C)		
	36.05 \pm 0.26	36.7 \pm 0.30

N = number of rats.

Male rats (34 days old, body weights 140.0 \pm 11.5 g) were maintained at an ambient temperature of 26°C for 1 h prior to injection of L-DOPA (500 mg/kg body weight, i.p., in 0.05 M HCl, 50 mg/ml) or vehicle and throughout the rest of the experiment. After a further 45 min both groups of rats were injected intraventricularly via pre-implanted cannulae with 2.5 mg L-(³⁵S)methionine. After a 30 min incorporation period the rats were killed by cervical dislocation and subcellular fractions were isolated by the method of Jones and Matus (1974).

None of the differences were statistically significant using Student's t test.

TABLE 3.2.6

The Effect of L-DOPA on the Incorporation of L-(³⁵S)methionine into Total Homogenate and Subcellular Fractions (Ambient Temperature, 30°C) 1.

Fraction	Rate of incorporation (μmol methionine incorporated/g protein/h \pm S.E.M.)		Percentage Inhibition
	Control (N=3)	+ L-DOPA (N=3)	
Forebrain homogenate	0.98 \pm 0.10	0.68 \pm 0.11	30.6
Myelin	0.79 \pm 0.10	0.63 \pm 0.18	20.2
Synaptosomes	0.61 \pm 0.08	0.46 \pm 0.09	24.5
Mitochondria	0.92 \pm 0.10	0.62 \pm 0.10	32.6
Mean body temperature (°C)			
	39.9 \pm 0.10	41.5 \pm 0.54	

N = number of rats.

Female rats (45 days old, body weights 165.2 \pm 1.4 g) were maintained at an ambient temperature of 30°C for 1 h prior to injection of L-DOPA (500 mg/kg body weight, i.p., in 0.05 M HCl + 0.2% Tween 80, 50 mg/ml) or vehicle. The rats were subsequently treated as described in Table 3.2.5. Synaptosomes were isolated by the method of Gray and Whittaker (1962). None of the differences were statistically significant using Student's t test.

TABLE 3.2.7

The Effect of L-DOPA on the Incorporation of L-(³⁵S)methionine into Total Homogenate and Subcellular Fractions (ambient temperature, 30°C) 2

Fraction	Rate of incorporation (μmol methionine incorporated/g protein/h \pm S.E.M.)			Percentage inhibition (with regard to room temperature control)
	Control (room temperature) (N=4)	Control (30°C) (N=2)	+ L-DOPA (N=4)	
	Forebrain homogenate	0.90 \pm 0.02	0.95	
Myelin	0.31 \pm 0.01	0.32	0.27 \pm 0.02	13
Synaptosomes	0.48 \pm 0.03	0.53	0.40 \pm 0.04	17
Mitochondria	0.70 \pm 0.03	0.83	0.53 \pm 0.05	24**
Mean body temperature (°C)				
	37.1 \pm 0.12	38.5	39.5 \pm 0.20	

N = number of rats.

Male rats (39 days old, body weights 136.6 \pm 5.1 g) were housed either at an ambient temperature of 30°C or room temperature (23°C) throughout the experiment. The animals were injected as described in Table 3.2.6. Synaptosomes were isolated by the method of Gray and Whittaker (1962).

* p < 0.05; ** p < 0.02

TABLE 3.2.8

The Effect of L-DOPA on the Incorporation of L-(³⁵S)methionine into Total Homogenate and Subcellular Fractions (Ambient Temperature, 30°C) 3.

Fraction	Rate of incorporation (μ mol methionine incorporated/g protein/h \pm S.E.M.)		Percentage Inhibition
	Control (N=6)	+ L-DOPA (N=5)	
Forebrain homogenate	1.11 \pm 0.05	0.79 \pm 0.18	29.3*
Myelin	0.39 \pm 0.05	0.47 \pm 0.19	+20.5
SPM	0.51 \pm 0.07	0.56 \pm 0.17	+ 9.8
Mitochondria	0.84 \pm 0.02	0.55 \pm 0.11	34.0*
Mean body temperature (°C)			
	39.75 \pm 0.21	42.4 \pm 0.43	

N = number of rats.

Female rats (37 days old, body weights 113.5 \pm 3.3 g) were treated as described in Table 3.2.6. SPM fractions were prepared by the method of Jones and Matus (1974).

* p < 0.02

throughout the remainder of the experiment. It is possible that this requirement for a higher ambient temperature during the experimental period is a consequence of the difference in strains since Moskowitz et al. used Sprague-Dawley rats while Wistar rats were used in the present study.

Repetition of the experiment using an ambient temperature of 30°C an inhibition of precursor incorporation of between 20 and 30% was observed in both the homogenates and subcellular fractions (Table 3.2.6). None of these inhibitions, however, were statistically significant. Further experiments produced similar inhibitions of protein synthesis with the deficits in the homogenates and mitochondrial fraction being statistically significant (Table 3.2.7). The deficits in incorporation of precursor into the myelin and synaptosomal proteins, although 13% and 17% respectively, were not statistically significant.

In earlier experiments the control rats had been housed at the same ambient temperature as the L-DOPA treated rats. This resulted in an elevation of body temperature in the control animals although not to as high a degree as that observed in the drug-treated rats (e.g. 39.9°C compared with 41.5°C respectively). It has been reported that hyperthermia results in inhibition of protein synthesis in young rats (Millan et al., 1979) although Lajtha and Dunlop (1981) observed that in young rats protein synthesis proceeded at its maximal rate at 37-39°C. It was of great importance, therefore, to check whether the observed elevation in the body temperature had any effect on precursor incorporation into brain protein. Comparison of control animals housed at a high ambient temperature (30°C) with rats housed at room temperature (20°C) showed that there was no difference in incorporation between these two groups of control rats.

In a further experiment in which SPM fractions were isolated no inhibition in incorporation of precursor into SPM and myelin protein was observed (Table 3.2.8). The depression in the rate of incorporation previously observed in the forebrain homogenate and mitochondrial fractions were found and were, again, statistically significant.

3.2.6 DISCUSSION

3.2.6.1 The Use of Intraventricular Injections of Flooding Concentrations of L-(³⁵S)methionine for Measuring Cerebral Protein Synthesis In Vivo

The time course studies showed that the incorporation of L-(³⁵S)methionine into total brain protein was linear over 45 min after injection and that the rates of incorporation at specified time points within this period were constant. This implies that the precursor specific activity in the brain was also constant during the incorporation period. Comparison of the overall rate of incorporation of precursor into total forebrain protein with the value obtained by King (1980) using L-(U-¹⁴C)valine showed that these values were in close agreement. This strongly suggests that the rate of incorporation of precursor into brain protein measured by this method is close to the absolute rate of protein synthesis.

It is unlikely that the rate of protein synthesis obtained in the present study is subject to serious error due to the reutilisation of labelled precursor since the incorporation periods used were relatively short. Equally, since all cerebral free methionine pools are assumed to be labelled to the same extent under flooding conditions errors due to the heterogeneity of the precursor pools are minimised.

Metabolism of the precursor amino acid which results in the production of other labelled amino acids can prove to be a problem. For example, leucine has been reported to undergo extensive catabolism to glutamate and aspartate (Dunn et al., 1971; Hershkowitz et al., 1975). Both cysteine and cystine are produced from dietary methionine. t.l.c. analysis of perchloric acid extracts of brain tissue indicated that the radioactivity due to both of these amino acids was unlikely to be more than 10% of the total radioactivity. This is not surprising since the catabolism of methionine occurs in the liver. Thus any labelled residues produced from methionine are likely to be greatly diluted in the body and are, therefore, unlikely to contribute significantly to the observed rate of incorporation of ³⁵S-labelled precursor into brain protein.

Similarly errors in the rate of protein synthesis measured by this method due to poor distribution of labelled precursor throughout the brain are probably small since the rates of incorporation measured in different gross brain areas were similar. The primary site of CSF production is in the choroid plexuses of the lateral ventricles from where it circulates via the third and fourth ventricles to the subarachnoid spaces. This circulation is aided by arterial pulsations of choroid plexuses which are transmitted throughout the CSF (Katzman, 1976). Injection of the precursor into the CSF of the lateral ventricles, therefore, maximises the opportunity for its even distribution through the brain.

Dunn (1975) reported that intracerebral injections caused inhibition of brain protein synthesis. These studies, however, were carried out on animals which were anaesthetised with ether and which were operated on on the day of the experiment. Later studies using rats which had been preimplanted with intraventricular cannulae and injected with precursor while conscious showed that protein synthesis was not affected by intracerebral injection, in itself (Schotman et al., 1977).

In the present study every precaution was taken to minimise stress to the rats due to handling, in general, and the injection procedure, in particular. The latter was performed with the aid of an experienced animal technician. Stressful stimuli should be reduced to a minimum since these have been reported to cause an increase in the rate of cerebral protein synthesis. (Jakoubec et al., 1970; Rees et al., 1974; Schotman et al., 1977). The latter workers estimated that the stress due to intracranial injections via preimplanted cannulae was no greater than that due to subcutaneous injections. In the present study the animals did not appear to be unduly stressed following intraventricular injection and their behaviour during the post-injection period was normal.

It might be argued that the injection of gross quantities of precursor may affect the measured rate of protein synthesis. Dunlop et al. (1975), however, were unable to show any significant effect on cerebral protein synthesis following the injection of flooding concentrations of valine. The major advantage of the present study is, that by using the local flooding method, high specific activities of precursor can be achieved in the brain. This, in turn, permits the measurement of the rate of incorporation of precursor into brain subcellular fractions.

The rate of incorporation of L-(³⁵S)methionine into SPM protein was found to be close to 60% of that of total forebrain protein. This is consistent with the finding of Gurd (1978) who, using trace levels of L-(4,5-³H)leucine reported that the specific activity of synaptosomal protein was 40-50% of that of the brain homogenate. The rate of incorporation of precursor into SPM protein cannot be considered to be equivalent to the rate of synthesis of these proteins since the value includes a component due to the speed of axoplasmic transport. The fact that a substantial amount of radioactivity is found in the SPM fraction at 15 min after injection is, perhaps, not surprising in view of the relatively short lengths of axons in the brain (Barondes, 1976). Electron microscopic studies of autoradiograms of mouse brain have demonstrated that labelled protein is found at the synapse within 15 min of injection of precursor (Droz and Barondes, 1969). Gurd (1978) reported that maximal incorporation of L-(4,5-³H)leucine into SPM protein occurred at 2-4 h after administration of the precursor indicating the arrival of proteins synthesized in the cell body and a second, smaller peak of incorporation at 16 h due to the arrival of further protein exported by a slower component of axoplasmic transport. It is possible that the rapid appearance of labelled protein in the SPM fraction may be due, in part, to local synthesis of SPM protein (see section 1.4.1). Due to the complicating factor of axonal transport in the rate of incorporation of L-(³⁵S)methionine into SPM protein it is difficult to determine whether SPM proteins are actually synthesized at a faster or slower rate than the overall rate of cerebral protein synthesis.

The rate of incorporation of precursor into myelin protein is slower than for any of the other fractions. This is consistent with the observation that the half-lives of myelin proteins are longer than those of other brain proteins although the reported values vary widely (Fischer and Morrell, 1974). Hamberger and Blomstrand (1969) estimated that the rate of incorporation of (³H)leucine into a purified 'nerve cell' fraction was three times greater than into a glial fraction isolated from rabbit brain. The lower molecular weight myelin proteins (the basic and proteolipid proteins) are thought to be synthesized more slowly than the higher molecular weight Wolfram proteins (Norton, 1976).

Conversely, the incorporation of precursor into mitochondrial proteins is similar to that of total protein. This is consistent with the findings of Clouet and Richter (1959) that the specific activity of rat brain mitochondria was slightly less than that of the total homogenate 30 min after intracisternal injection of DL-(³⁵S)methionine. Reported values for the turnover of brain mitochondrial proteins vary considerably according to the method used for their measurement. For example, the half-lives of cerebral cortex mitochondrial proteins have been reported as 20.5 days (von Hungen et al., 1968) and 41 days (Rodriguez de Lorez Arnaiz et al., 1971) and, in liver, 4-5 days (Swick et al., 1968).

These overall rates of incorporation are, perhaps, of limited value since they represent the average rates of synthesis of a highly heterogeneous protein population. Thus, it would be of considerable interest to examine the incorporation of labelled precursor into individual SPM proteins.

3.2.6.2 The Effect of Selected Drugs on Brain Protein Synthesis In Vivo

The methodology employed in the present study of the effect of selected drugs has aimed to overcome any inaccuracies due to experimental conditions. Thus rats were preimplanted with cannulae which avoids any effect of surgery and related stress on the measured rate of protein synthesis. In addition, all drug-treated animals were maintained at a strictly controlled temperature. Every effort was made to minimise stress due to handling etc. which may also have affected the rate of protein synthesis (Schotman et al., 1977).

Under these conditions and using young animals (35-40 days old) who are more susceptible to drug treatments the anaesthetic halothane and L-DOPA caused a decrease in the rate of synthesis of total brain protein although the anaesthetic pentobarbitone did not appear to affect protein synthesis.

Halothane reduced the incorporation of precursor into all brain fractions examined although myelin was the most severely affected. Halothane has been reported to cause a reversible inhibition of protein synthesis in lung tissue (Wartell et al., 1981; Rannels et al., 1983). The depression in synthesis was reported by these workers to be equivalent to

10% per 1% increment in halothane concentration. This is consistent with the present results in which a 40% inhibition was observed on administration of 3% halothane. This anaesthetic has been reported to accumulate in brain particulate fractions (Divakaran and Wiggins, 1982). These workers proposed that this was a consequence of the lipophilic nature of halothane. Myelin has been found to accumulate halothane to a much greater extent than any other brain fraction (Cohen et al., 1972; Divakaran and Wiggins, 1982). This is consistent with the observation that the myelin fraction showed that the largest deficit in incorporation of L-(³⁵S)methionine.

Allison and Nunn (1968) suggested that the induction of general anaesthesia is dependent upon the depolymerization of neuronal microtubules. Since most, if not all, SPM proteins are delivered to the nerve ending by rapid axonal transport it might be expected that a large deficit in the incorporation of L-(³⁵S)methionine into this fraction might be observed if the neuronal microtubules were affected by halothane. Since the reduction in incorporation of precursor into the SPM fraction was similar to the overall reduction in the homogenate it is unlikely that halothane significantly affects the axonal microtubules. This is consistent with the findings of Kennedy et al., (1972) who were unable to find any inhibition of axonal transport by halothane in vivo although in vitro 4% halothane caused a 40% reduction in the rate of axonal transport.

General anaesthesia has been reported to cause a general depression of brain metabolism (Abood and Hoss, 1976). The mechanism by which this occurs is not known but may be due to either a reduction in the respiratory rate, reduced cerebral blood flow or by a more specific effect on cerebral metabolism. Since it might be expected that this effect would be observed in both pentobarbitone and halothane anaesthetised rats it is unlikely that the inhibition of protein synthesis in the latter is due to a general depression in brain metabolism. Ether anaesthesia has been reported to result in elevated plasma corticosterone levels and reduced incorporation of L-(³H)leucine into brain protein (Schotman et al., 1977). Whether the inhibition of protein synthesis observed in halothane anaesthetised rats is also mediated by a hormonal mechanism is uncertain but may be an area for further investigation. These results indicate, however, that halothane,

as well as ether, anaesthesia should be avoided if cerebral protein synthesis is to be studied.

Pentobarbitone, by comparison, did not appear to have any significant effect on brain protein synthesis in any of the fractions examined in this study. In studies where trace amounts of precursor have been used the reported effects of barbiturate anaesthetics on cerebral protein synthesis have varied widely. Satake *et al.* (1974) found that amobarbital and phenobarbital both caused inhibition of the incorporation of (¹⁴C)algal protein hydrolysate into isolated perikarya and into the cerebral cortex. Conversely, Hitzemann and Loh (1977) reported that the incorporation of (³H)lysine into the SPM proteins isolated from 'light' synaptosomes i.e. those which band at the interface between 1.0 and 1.1 M sucrose, was enhanced by pentobarbital. It is known that 'light' synaptosomes are enriched in GABAergic nerve endings which are thought to be involved in the mode of action of barbiturate anaesthesia (Willow and Johnston, 1981). The effect reported by Hitzemann and Loh (1977) was relatively small, however, and would be unlikely, therefore, to be observed in the present study which did not differentiate between SPM proteins derived from different synaptosome populations.

The administration of large doses of L-DOPA resulted in reduced incorporation of precursor into the forebrain homogenate, myelin, synaptosomal and mitochondrial protein under conditions where the experimental animals' body temperatures were elevated to at least 39°C. These deficits were only statistically significant in the homogenate and mitochondrial fractions. The lack of significance in the other fractions is probably a consequence of the variation in the results rather than a lack of effect by L-DOPA. Natural variation in the response of individual animals to the drug is inevitable. In the present study it was found that some animals showed only small inhibitions in protein synthesis following L-DOPA treatment. This effect was also observed by King (1980) who suggested that these 'non-responders' might correspond to rats who failed to show the necessary elevation in body temperature. In the present study all animals treated with L-DOPA showed the characteristic behavioural responses to the drug of hyperactivity or stereotypy interspersed with periods of catatonia and extreme sensitivity to acoustic stimuli. In addition there was no correlation between body temperature and poor response to the drug since all rats showed elevated temperatures.

There are, inevitably, several factors contributing to the variability of results in experiments of this nature. Firstly, there is the natural variation in response to both the drug and possibly also to the high ambient temperatures used and, secondly, the variations in subcellular fractions due to small variations in the isolation procedure (as discussed in section 3.1.4). The latter probably explains the lack of significance in the subcellular fractions. Nevertheless in animals which showed a large inhibition in incorporation into the homogenate there was a correspondingly large inhibition in the subcellular fractions. The variation in results can be minimised by using large numbers of rats. In the present investigation, however, this was not possible since only a limited number of brains were able to be fractionated simultaneously.

The results do suggest, however, that the inhibition of incorporation of L-(³⁵S)methionine into total brain protein and mitochondrial protein is real. The value of 20-30% for this inhibition is similar to that obtained by Roel et al. (1974) and King (1980). While it is possible that there is some effect on incorporation into SPM, synaptosomal and myelin proteins further work is needed to clarify this. It should be observed, however, that to obtain an effect on protein synthesis requires very severe treatment of relatively young animals. It was found that the death rate of L-DOPA treated rats during the experimental period was quite high and amounted to about 1 in 6 animals.

In the study of these drug effects it was necessary to use young rats since these are more susceptible to drug treatments. This accounts for the higher rates of protein synthesis in these studies than were observed earlier (using 100 day old rats). The rate of cerebral protein synthesis has been shown to decline with age although this decline is most rapid between birth and 28 days (Dunlop et al., 1977). In a subsequent section (3.1.3) the rate of protein synthesis in young rats was measured using intraperitoneal injection of flooding concentrations of L-(U-¹⁴C)valine. In 31 day old rats it was found that the rate of protein synthesis in the forebrain was 2.95 ± 0.21 μmol valine incorporated/g protein/h. By correcting the value of 0.82 μmol methionine incorporated/g protein/h obtained in the anaesthesia experiments for comparison with valine (as earlier) a figure of 2.82 μmol valine incorporated/g protein/h in these

37 day old rats. Thus the higher rates of synthesis in the anaesthesia studies were consistent with the age of the rats used.

In the L-DOPA experiments, however, the animals were subject to quite severe treatments and it is likely that in addition to the rates of synthesis being higher due to the younger ages of the rats there may also be an increment due to stress due to the high ambient temperatures and the necessity of injecting the drug as a suspension. While these factors can be minimised by careful handling of the rats it is not possible to totally eliminate stressful stimuli which are inherent in the experimental design.

3.3 The Effects of Hypothyroidism on the Development and Synthesis of Total Brain Protein and SPM and Mitochondrial Polypeptides

Thyroid hormone deficiency in young rats is known to produce profound effects on brain structure and function including a reduction in the number of synapses (Balazs et al., 1977). Deficiency of these hormones during the critical period of brain development prevents the normal morphological changes which accompany brain maturation and results in the characteristic anatomical and functional anomalies associated with cretinism. The severity of the effect of hypothyroidism varies between different brain regions and depends on the relative stage of maturity of the region at the onset of hypothyroidism. The overall effect, however, is large enough to be easily studied in gross brain areas.

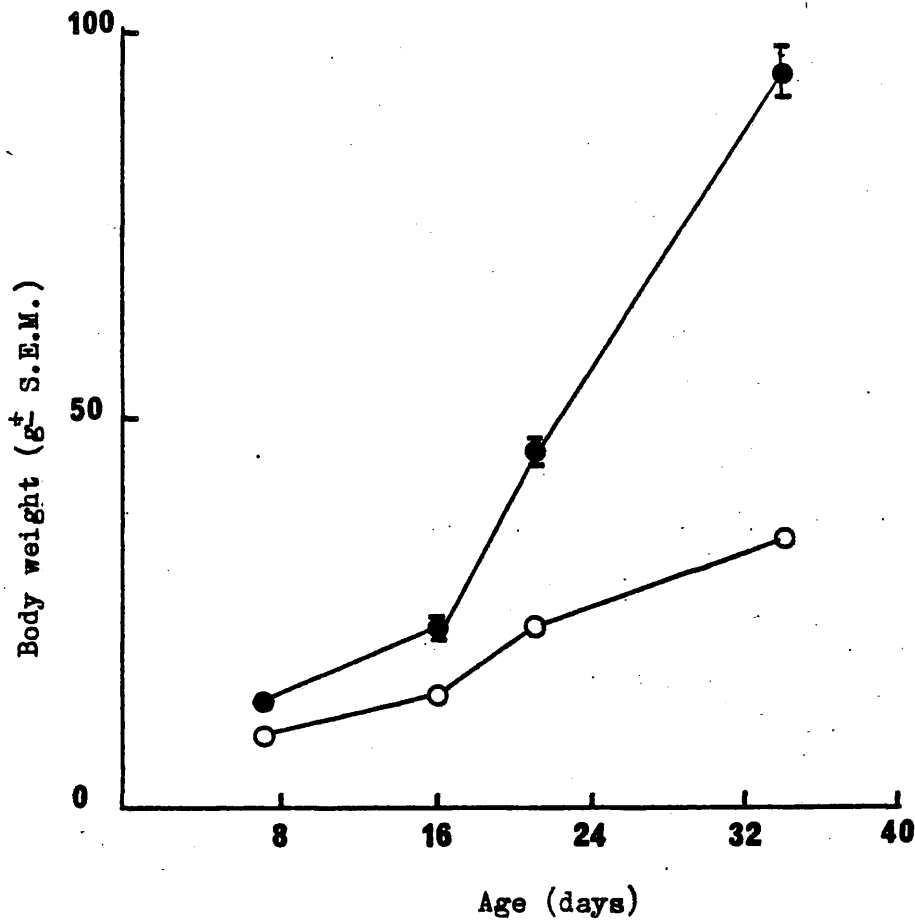
Synaptogenesis has been reported to be severely affected by neonatal thyroid hormone deficiency (Nicholson and Altman, 1972). The aim of this study, therefore, was to determine whether any specific alterations in SPM composition or synthesis occurred in hypothyroid rats.

3.3.1 General Effects of Hypothyroidism

3.3.1.1 The Effect on Body and Brain Weights

The pups of rats fed on a diet containing PTU from 3 days after the birth of the litter and throughout the suckling period showed a variety of symptoms associated with neonatal hypothyroidism. These pups showed retarded development, poor coordination, retention of infantile body shape and features and substantially reduced body weights compared with

Fig. 3.3.1. The Effect of Hypothyroidism on the Body Weight of Developing Rats



N=6 for 8 day old rats

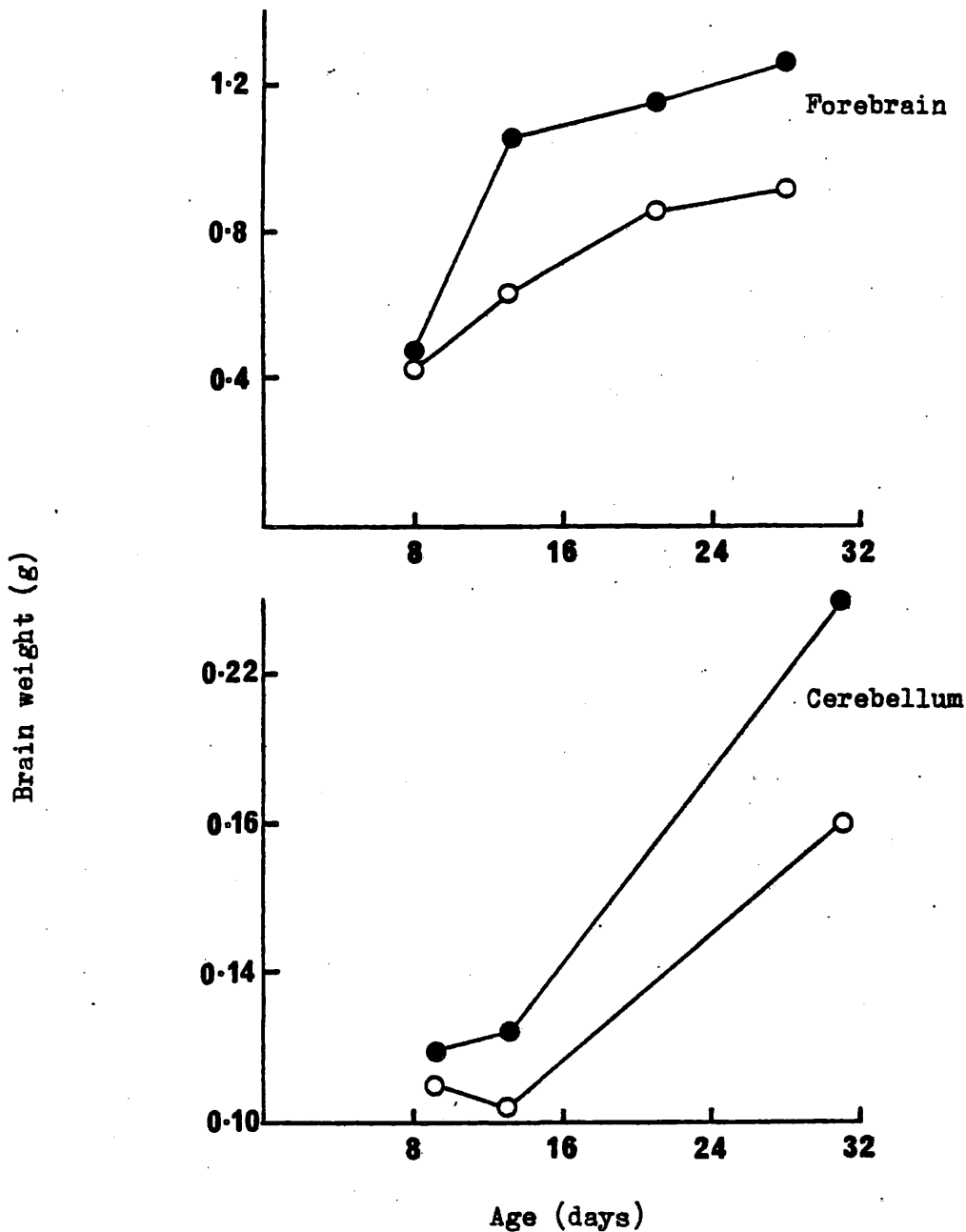
N=4 for the remaining points

Each point represents the mean value for litter mates of the same age. The difference between hypothyroid and euthyroid rats was statistically significant at each age at $p < 0.001$. The S.E.M. values for the hypothyroid rats were 0.20, 0.54^{0.58} and 0.73 at each age respectively.

The number of rats per litter was 9-15.

●—● control, ○—○ hypothyroid.

Fig. 3.3.2. The Effect of Hypothyroidism on the Weight of Forebrain and Cerebellum in Developing Rats.



N=9, N=8, N=4, N=4 at each age respectively (forebrain)

N=6, N=7, N=7 for cerebellum

The S.E.M. values were as follows:-

Forebrain; Control, 0.006, 0.011, 0.008, 0.058;

Hypothyroid, 0.009, 0.012, 0.05, 0.006

Cerebellum; Control, 0.008, 0.005, 0.003;

Hypothyroid, 0.008, 0.005, 0.002.

The p values were as follows:-

Forebrain; 0.001, 0.001, 0.01, 0.001.

Cerebellum; NS, NS, 0.001

control rats (Fig. 3.3.1). The deficit in body weight increased over the first four weeks of life, reaching 64% at 34 days postpartum. This pattern was observed in all of the hypothyroid litters examined and was statistically significant in all age-paired litters.

Deficits in the weights of forebrains and cerebella were observed in hypothyroid rats compared with euthyroid controls (Fig. 3.3.2) although these were not as large as the deficits in body weight. These deficits were observed in at least 4 separate sets of paired litters. The reduction in forebrain and cerebellar weights increased with age and in both regions was over 20% at 4 weeks postpartum. At all of the ages examined the reduction in forebrain weight was statistically significant using Student's t test. The reduction in cerebellar weights at 9 and 13 days postpartum, however, was not statistically significant. This may be a consequence of the difficulty in accurately dissecting all of the underlying material from the cerebellum in such small rats. This was particularly true of the hypothyroid animals whose brains were very soft and, therefore, difficult to dissect.

3.3.1.2 The Effect on Protein Yields in Total Homogenates and Subcellular Fractions

The yields of protein in the forebrain homogenates were reduced on both a mg protein/brain and mg protein/g tissue basis at all four ages (Tables 3.3.1 and 3.3.2). Not all of these deficits were statistically significant, however, but it was observed that on both bases the deficit in the 13 day old rats was significant. The yields of protein in the cerebellar homogenates showed a deficit on a mg protein/brain and mg protein/g tissue basis which was statistically significant at 13 and 34 days postpartum. The deficit in the cerebellar homogenates at 2 weeks postpartum was larger than that in the forebrain (37% and 18.2% respectively, mg protein/brain).

In the SPM fractions it was observed that there was no significant difference in protein yield on either basis. A small deficit of 6-17% was observed in the SPM protein yield on a μg SPM protein/ μg homogenate protein basis although this was not statistically significant (Table 3.3.3).

TABLE 3.3.1

Protein Yields in Homogenates and Subcellular Fractions Isolated from Hypothyroid and Euthyroid rats (mg protein/brain)

Age (days)	Protein yield (mg protein/brain \pm S.E.M.)			
	7	13	21	34
Forebrain				
homogenate:				
Control	27.26 \pm 2.04	62.92 \pm 1.94	73.83 \pm 4.14	106.46 \pm 5.32
Hypothyroid	23.38 \pm 0.93	51.46 \pm 1.49	59.35 \pm 4.59	79.42 \pm 4.14
	(N=9) NS	(N=8) 0.001*	(N=4) NS	(N=4) 0.01
Cerebellar				
homogenate:				
Control	9.60 \pm 0.71	9.66 \pm 0.68	-	22.20 \pm 0.33
Hypothyroid	8.82 \pm 1.36	6.04 \pm 0.05	-	18.97 \pm 0.88
	(N=6) NS	(N=7) 0.01		(N=6) 0.01
Myelin:				
Control	0.31 \pm 0.04	0.39 \pm 0.03	0.81 \pm 0.08	0.72 \pm 0.08
Hypothyroid	0.45 \pm 0.04	0.42 \pm 0.04	0.56 \pm 0.02	0.45 \pm 0.04
	(N=6) 0.05	(N=6) NS	(N=5) 0.02	(N=5) 0.05
SPM:				
Control	0.58 \pm 0.05	1.58 \pm 0.10	3.21 \pm 0.06	1.88 \pm 0.13
Hypothyroid	0.85 \pm 0.03	0.87 \pm 0.04	2.29 \pm 0.05	1.90 \pm 0.25
	(N=6) 0.01	(N=7) NS	(N=6) NS	(N=5) NS
Mitochondria:				
Control	1.70 \pm 0.07	2.53 \pm 0.12	3.71 \pm 0.15	5.30 \pm 0.07
Hypothyroid	2.47 \pm 0.13	2.15 \pm 0.18	2.37 \pm 0.13	3.00 \pm 0.24
	(N=6) 0.001	(N=6) NS	(N=5) 0.01	(N=5) 0.01

N = number of rats.

NS = not statistically significant using Student's t test.

* p values using Student's t test.

All litters contained 9-15 rats.

Subcellular fractions were isolated by the method of Salvaterra and Matthews (1980).

TABLE 3.3.2

Protein Yields in Homogenates and Subcellular Fractions Isolated from Hypothyroid and Euthyroid rats (mg protein/g tissue wet weight)

Age (days)	Protein yield (mg protein/g tissue ± S.E.M.)			
	7	13	21	34
Forebrain				
homogenate:				
Control	57.26±3.86	74.00±0.93	99.52±1.68	89.00±3.31
Hypothyroid	54.88±1.53 (N=9) NS	65.69±0.93 (N=8) 0.001*	85.65±0.89 (N=4) 0.01	82.06±3.03 (N=4) NS
Cerebellar				
homogenate:				
Control	81.34±3.30	78.00±3.25	-	95.45±0.62
Hypothyroid	78.61±7.40 (N=6) NS	59.43±4.13 (N=7) 0.01	-	108.00±3.33 (N=4) 0.01
Myelin:				
Control	0.52±0.05	0.43±0.04	0.82±0.08	0.63±0.07
Hypothyroid	0.67±0.06 (N=6) NS	0.45±0.03 (N=6) NS	0.60±0.03 (N=5) NS	0.52±0.05 (N=5) NS
SPM:				
Control	1.43±0.13	1.53±0.11	2.81±0.07	1.65±0.13
Hypothyroid	1.25±0.06 (N=6) NS	1.37±0.09 (N=5) NS	2.67±0.06 (N=5) NS	2.19±0.29 (N=5) NS
Mitochondria:				
Control	2.81±0.08	2.57±0.13	3.27±0.03	4.43±0.06
Hypothyroid	3.63±0.21 (N=6) NS	2.18±0.18 (N=6) NS	2.59±0.02 (N=5) NS	3.11±0.27 (N=5) NS

N = number of rats.

NS = not statistically significant.

* p values using Student's t test.

Subcellular fractions were isolated by the method of Salvaterra and Matthews (1980).

TABLE 3.3.3

Protein Yield in the SPM Fraction Isolated from Hypothyroid and Euthyroid Rats (μg protein/ μg protein in the Forebrain Homogenate)

Protein yield (μg in SPM/ μg in homogenate \pm S.E.M.)				
Age (days)	9	13	20	31
Control	1.80 \pm 0.20	2.60 \pm 0.10	3.60 \pm 0.40	1.65 \pm 0.10
Hypothyroid	1.68 \pm 0.10	2.39 \pm 0.10	3.00 \pm 0.30	2.20 \pm 0.30
	(N=6) NS	(N=6) NS	(N=5) NS	(N=5) NS

N = number of rats.

NS = not statistically significant.

SPM fractions were isolated by the method of Salvaterra and Matthews, (1980).

On a mg protein/g tissue (wet weight) basis, no significant differences were observed in the myelin and mitochondrial fractions isolated from hypothyroid rats compared with euthyroid controls. On a mg protein/brain basis, however, significant deficits of 30-40% were observed in both fractions at 21 and 34 days postpartum.

3.3.2 The Effect of Hypothyroidism on the Incorporation of L-(U-¹⁴C)valine into Total Homogenates and Subcellular Fractions In Vivo

The rates of incorporation of L-(U-¹⁴C)valine into total brain protein and subcellular fractions were measured using the in vivo flooding method developed by Dunlop et al. (1975). This involved the intraperitoneal injection of flooding concentrations of valine and was adopted since it was found that it was not feasible to carry out the intraventricular cannulation of young rats and particularly the hypothyroid animals with routine success.

In both the control and hypothyroid rats a decline in the rate of incorporation was observed between 9 and 31 days postpartum (Table 3.3.4). In both cases the rate of incorporation was approximately 45% lower at 31 days than 9 days postpartum in the forebrain. A similar decline in the rate of incorporation was observed in all of the fractions examined.

Comparison of the rates of incorporation in the forebrain and cerebellar homogenates showed that while there were small deficits (8-10%) in all but the 31 day old cerebellar homogenates these were only statistically significant in the forebrain at 20 days and in the cerebellum at 13 days after birth.

A similar pattern was observed in the subcellular fractions where deficits were found in hypothyroid animals of up to 20 days old. The SPM fraction showed a statistically significant deficit of 25% in 13 day old rats and was observed in a further two repetitions of this experiment. At 9 and 20 days, while deficits of 11.5% were observed in hypothyroid rats, these were not found to be significant using Student's t test. The rate of incorporation of labelled valine into myelin protein was most

TABLE 3.3.4

The Effect of Hypothyroidism on the Incorporation of L-(U-¹⁴C)valine into Homogenates and Subcellular Fractions Isolated from Hypothyroid and Euthyroid Rats

Age (days)	Rate of incorporation ($\mu\text{mol valine incorporated/g protein/h} \pm \text{S.E.M.}$)			
	9	13	20	31
Forebrain				
homogenate:				
Control	6.35 \pm 0.40	7.12 \pm 0.25	4.42 \pm 0.12	2.95 \pm 0.21
Hypothyroid	5.78 \pm 0.32	6.52 \pm 0.31	3.67 \pm 0.10	2.62 \pm 0.13
	(N=7) NS	(N=6) NS	(N=5) 0.01*	(N=7) NS
Cerebellar				
homogenate:				
Control	6.60 \pm 0.35	6.79 \pm 0.15	-	3.18 \pm 0.25
Hypothyroid	5.95 \pm 0.32	5.78 \pm 0.32	-	3.36 \pm 0.11
	(N=7) NS	(N=6) 0.02		(N=7) NS
Myelin:				
Control	4.88 \pm 0.53	3.67 \pm 0.54	2.95 \pm 0.11	1.35 \pm 0.31
Hypothyroid	3.25 \pm 0.36	1.77 \pm 0.37	2.32 \pm 0.19	2.33 \pm 0.55
	(N=6) 0.05	(N=5) 0.02	(N=5) 0.02	(N=5) NS
SPM:				
Control	4.38 \pm 0.29	4.99 \pm 0.36	3.49 \pm 0.03	1.63 \pm 0.26
Hypothyroid	3.87 \pm 0.33	3.74 \pm 0.31	3.09 \pm 0.33	1.89 \pm 0.15
	(N=6) NS	(N=6) 0.02	(N=5) NS	(N=6) NS
Mitochondria:				
Control	4.35 \pm 0.05	4.85 \pm 0.22	3.59 \pm 0.14	2.35 \pm 0.19
Hypothyroid	4.02 \pm 0.22	4.31 \pm 0.25	3.60 \pm 0.21	2.60 \pm 0.16
	(N=6) NS	(N=6) NS	(N=5) NS	(N=6) NS

N = number of rats.

NS = not statistically significant.

* p values using Student's t test.

Animals were injected with L-(U-¹⁴C)valine (15 $\mu\text{mol/g}$ body weight, i.p., 0.5 mmol valine/ml, specific activity 20 $\mu\text{Ci/mmol}$ valine for 1-3 week old rats, 10 $\mu\text{Ci/mmol}$ valine for 4 week old rats). The animals were killed by cervical dislocation 2 h after injection. Subcellular fractions were isolated by the method of Salvaterra and Matthews (1980).

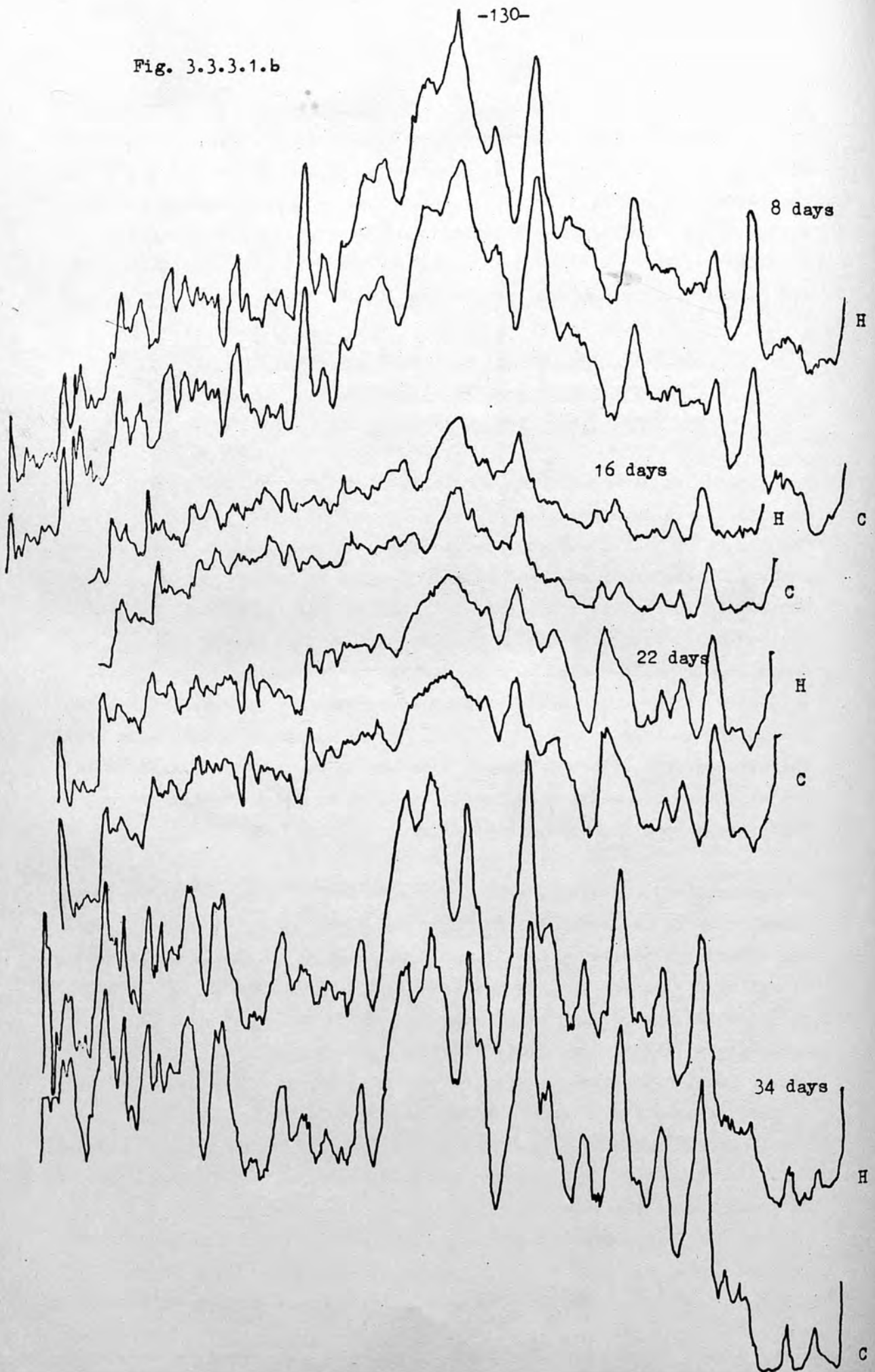
severely affected by thyroid hormone deficiency. The largest deficit (52%) in incorporation was observed at 13 days postpartum. At 9 and 20 days, however, the rate of incorporation of precursor into myelin protein was reduced by 33 and 21% respectively. All of these reductions were statistically significant. The mitochondrial fraction showed small, non-significant reductions in the rate of incorporation of valine at 9 and 13 days postpartum but not at later ages.

3.3.3 The Effect of Hypothyroidism on the Amounts of and the Incorporation of L-(³⁵S)Methionine into SPM, Mitochondrial and Total Brain Polypeptides In Vivo

Homogenates of forebrains and cerebella from both hypothyroid and euthyroid rats were analysed by SDS polyacrylamide gel electrophoresis. The levels of individual polypeptide bands in age-paired hypothyroid and euthyroid samples of the same protein loading were compared by monitoring the relative densities of the Coomassie Brilliant Blue stained bands both by visual inspection of the gels and by densitometry. (It should be observed, however, that on 1-dimensional gels each polypeptide band may comprise a number of polypeptide species of similar molecular weights.) Since in most pairs of tracks some small differences were observed between hypothyroid and euthyroid samples it was necessary to define a criterion by which to judge whether these differences were due to hypothyroidism.

It was decided, therefore, that for any variation between deficient and normal rats to be designated as being due to thyroid hormone deficiency the difference should be consistently present and of similar magnitude in all of the paired samples available for that particular subcellular fraction and age of rat. Routinely this involved a minimum of two separate isolations comprising 4 paired samples for the homogenates and 2 paired samples for the subcellular fractions. Thus variations observed in individual pairs of tracks were discounted. For example, the densitometric scans of 1 week old cerebellar homogenates (Fig. 3.3.3.1.b) show a series of 4 low molecular weight polypeptide bands which appear to be present in lower amounts in the hypothyroid fraction than in the control. This was observed in only 2 out of 4 pairs of samples, however, and was discounted on the criterion described above. Thus, on this basis, no consistent differences induced by thyroid hormone deficiency in

Fig. 3.3.3.1.b



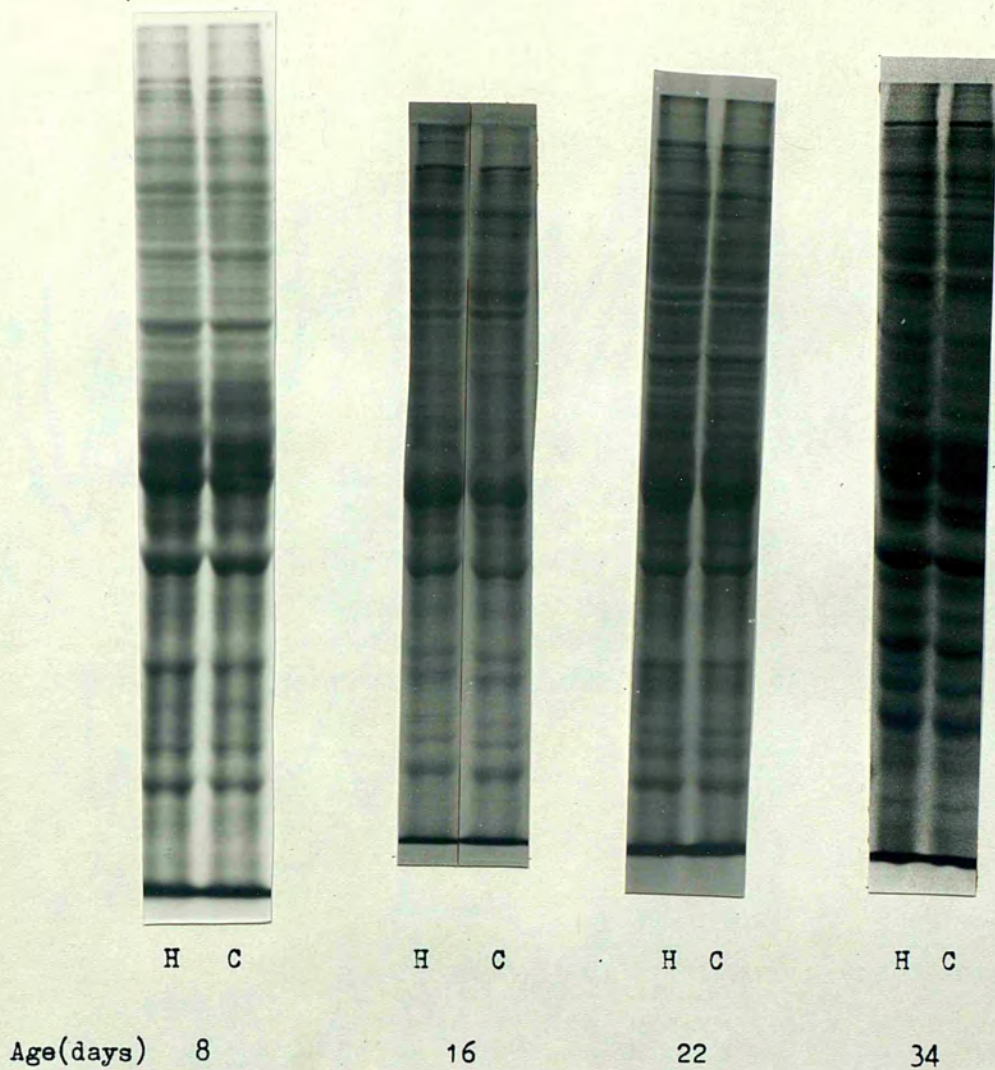


Fig. 3.3.3.1. Comparison of the Coomassie Brilliant Blue-stained electrophoretic profiles of forebrain protein from hypothyroid and euthyroid neonatal rats.

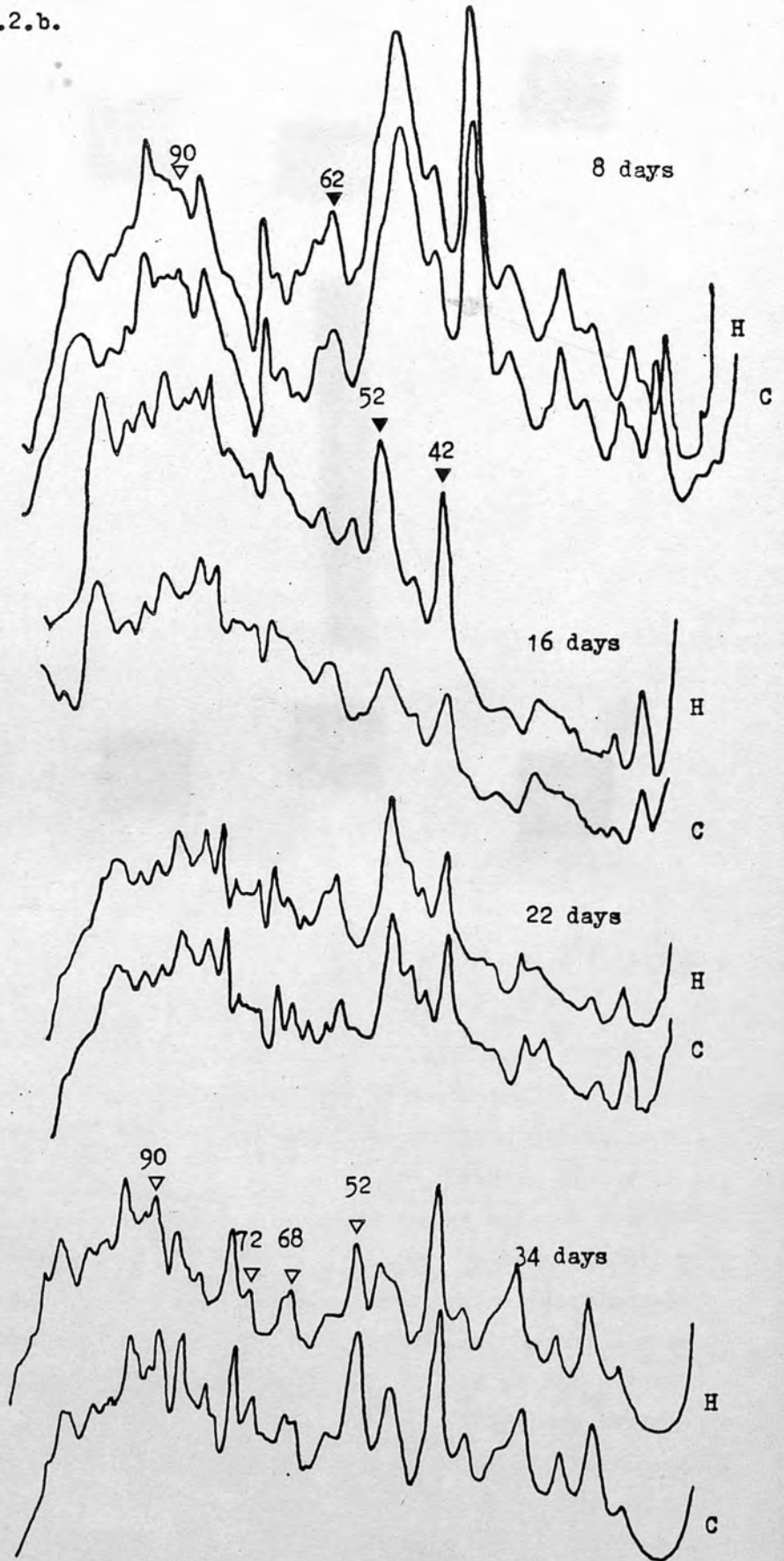
a) Protein samples were separated on 10% SDS polyacrylamide slab gels (100 μ g protein/ track). No consistent differences between hypothyroid and euthyroid samples were observed. (The variation between pairs of samples is an artifact of the photographic processing and is not due to differences between gels which were of similar quality).

b) Densitometric scans of Coomassie Brilliant Blue-stained gels.

H=hypothyroid

C=control

Fig. 3.3.3.2.b.



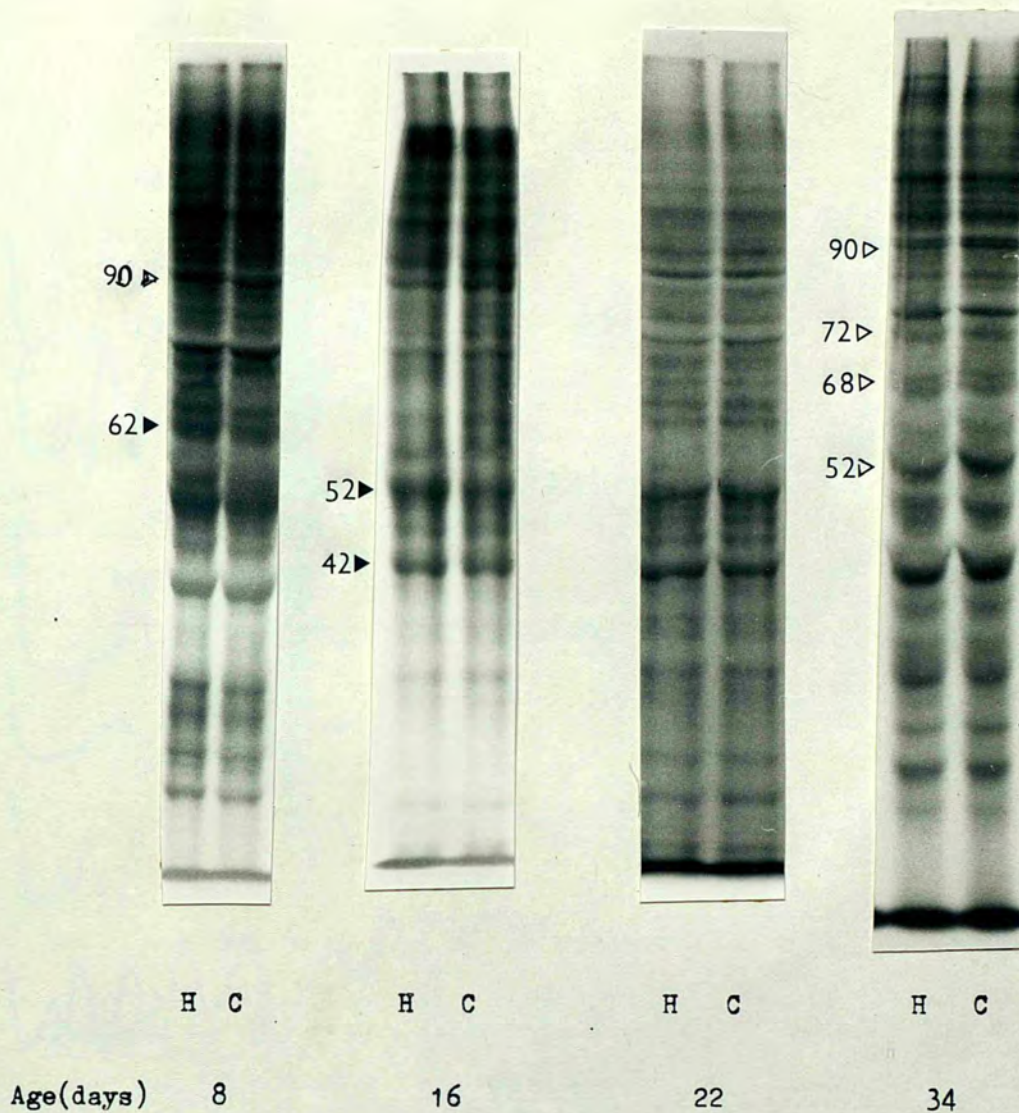


Fig. 3.3.3.2. Comparison of the incorporation of L-(³⁵S) methionine into forebrain protein from neonatal hypothyroid and euthyroid rats by fluorographic analysis.

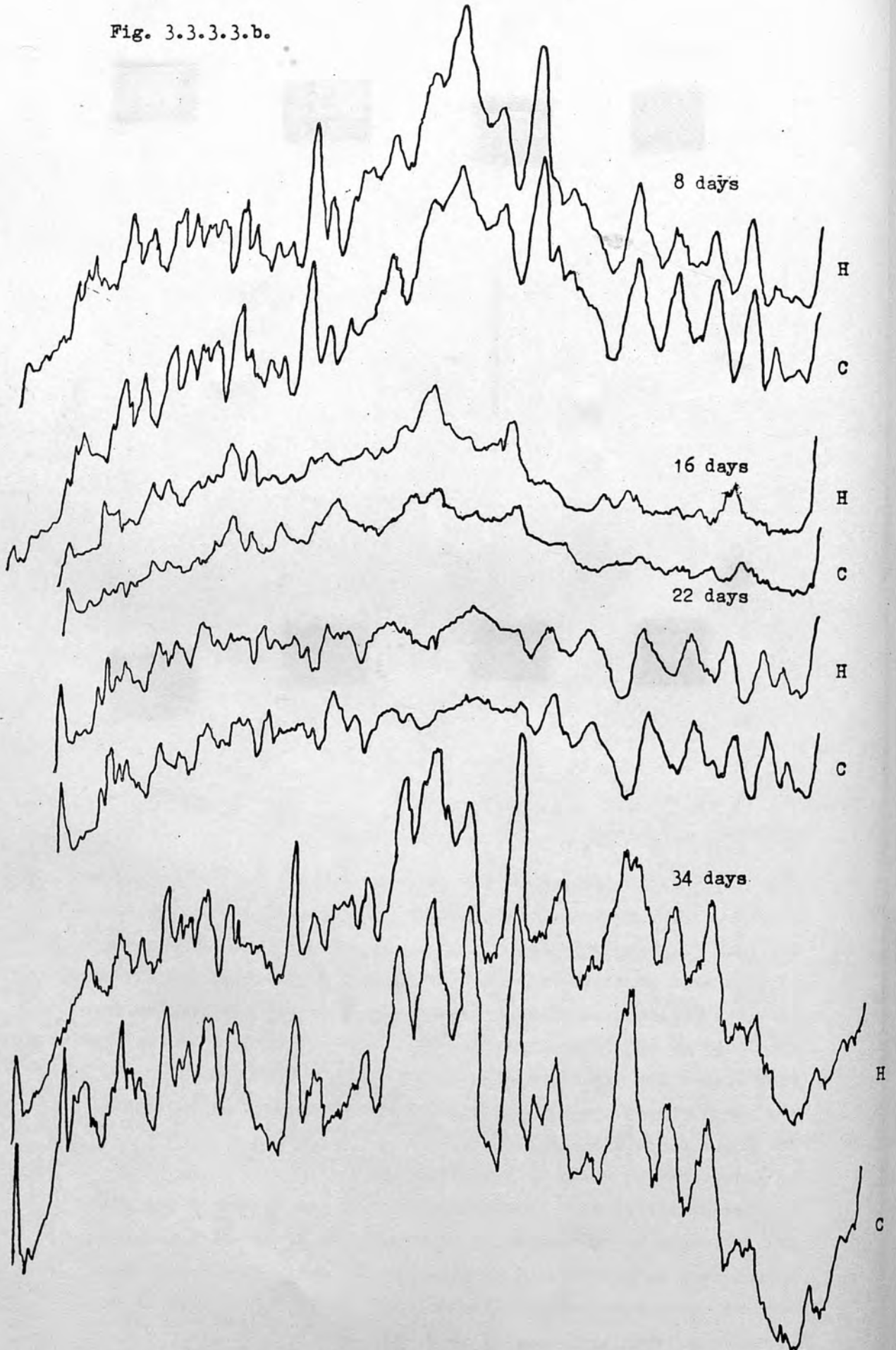
a) Rats were injected as described in section 2.4.2.2. and killed by cervical dislocation after a 2h incorporation period. Samples were separated on SDS polyacrylamide gels. Constant amounts of radioactivity were loaded per track (16,000, 8,000, 5,000 & 6,000dpm at 8, 16, 22 & 34 days respectively). These gels were subsequently processed for fluorographic analysis.

b) Densitometric scans of fluorograms.

Consistent differences between hypothyroid and euthyroid samples are indicated by ▼ where the incorporation of labelled methionine is enhanced in hypothyroid samples and ∇ where incorporation is reduced relative to euthyroid samples.

Molecular weights are given as $\times 10^{-3}$

Fig. 3.3.3.3.b.



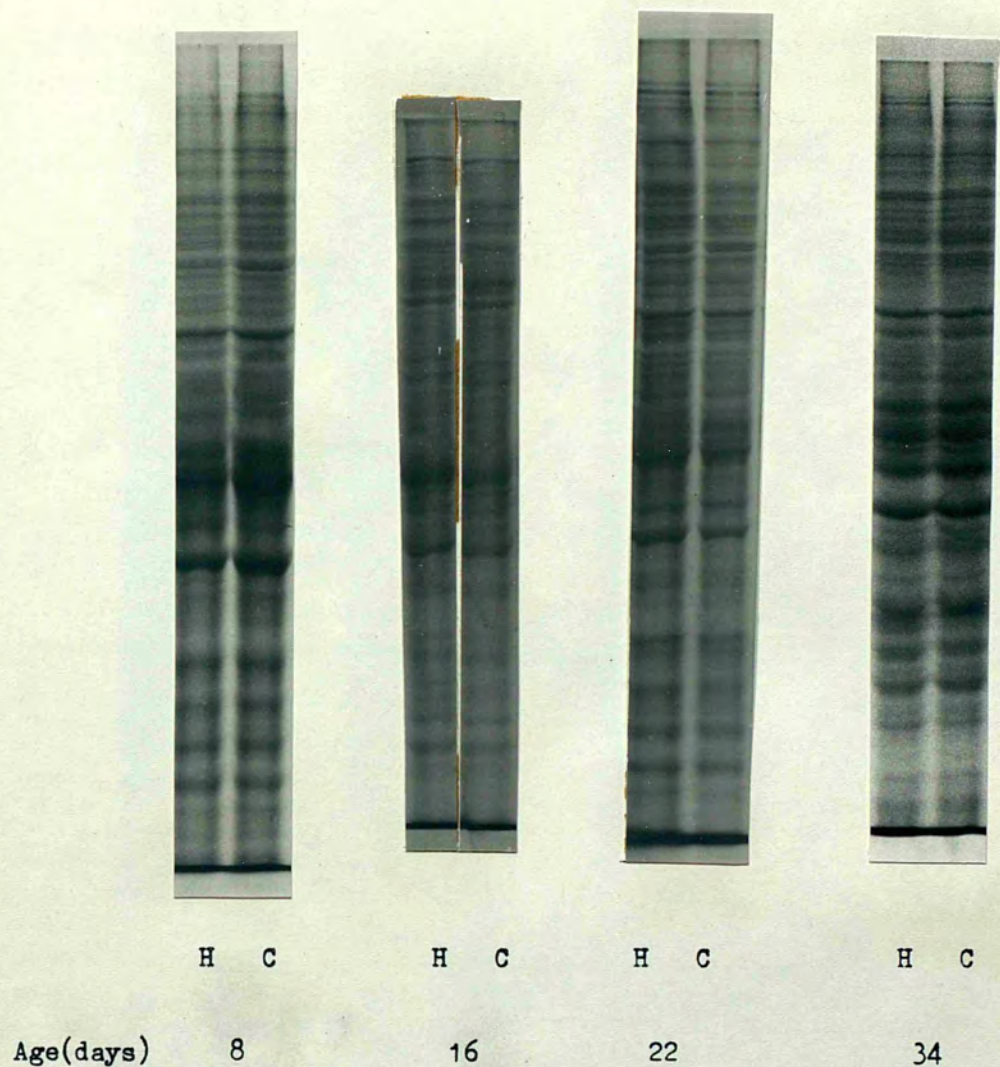


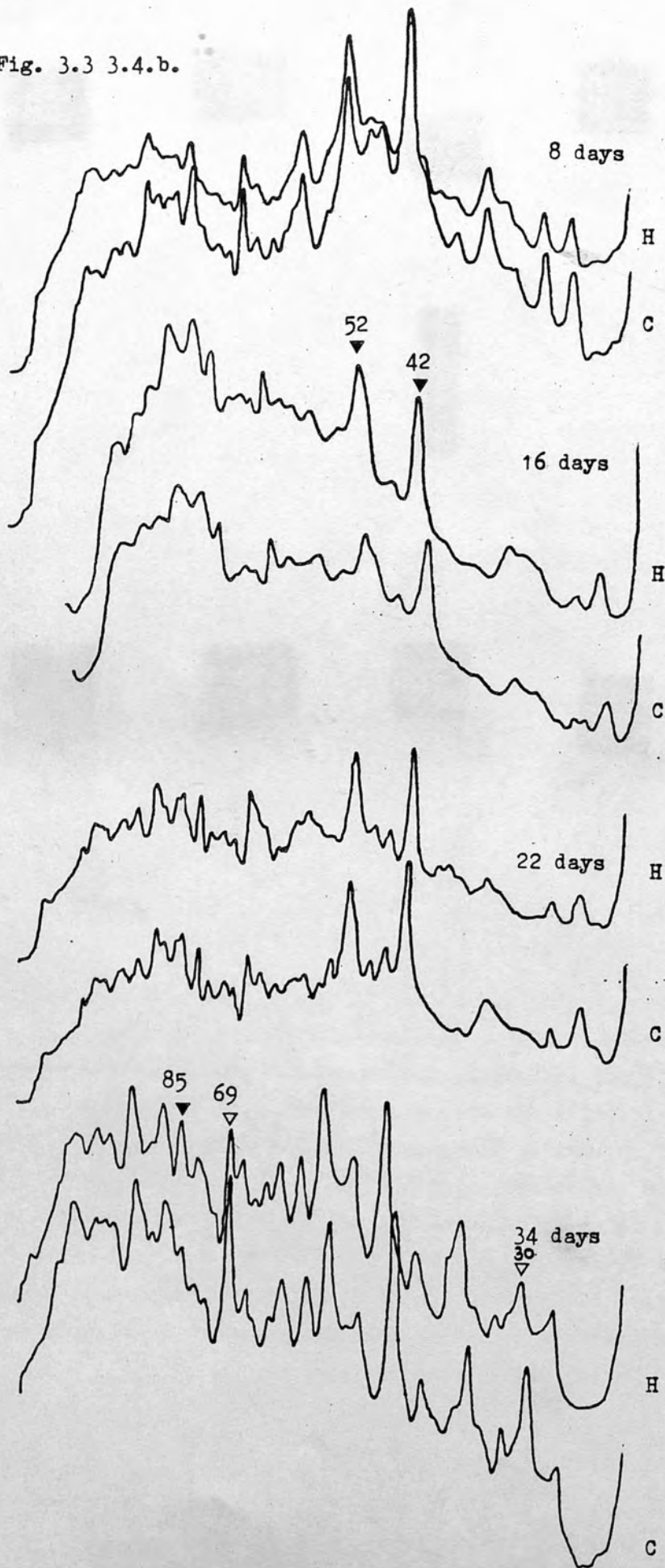
Fig. 3.3.3.3. Comparison of the Coomassie Brilliant Blue-stained electrophoretic profiles of cerebellar protein from neonatal hypothyroid and euthyroid rats.

a) Coomassie Brilliant Blue-stained gels loaded with constant amounts of cerebellar homogenate protein ($100\mu\text{g}/\text{track}$) from both hypothyroid and euthyroid rats.

Densitometric scans of Coomassie Brilliant Blue-stained gels of cerebellar fractions.

No consistent differences in the amounts of individual polypeptide bands were observed at any of the ages examined which could be attributed to thyroid hormone deficiency.

Fig. 3.3 3.4.b.



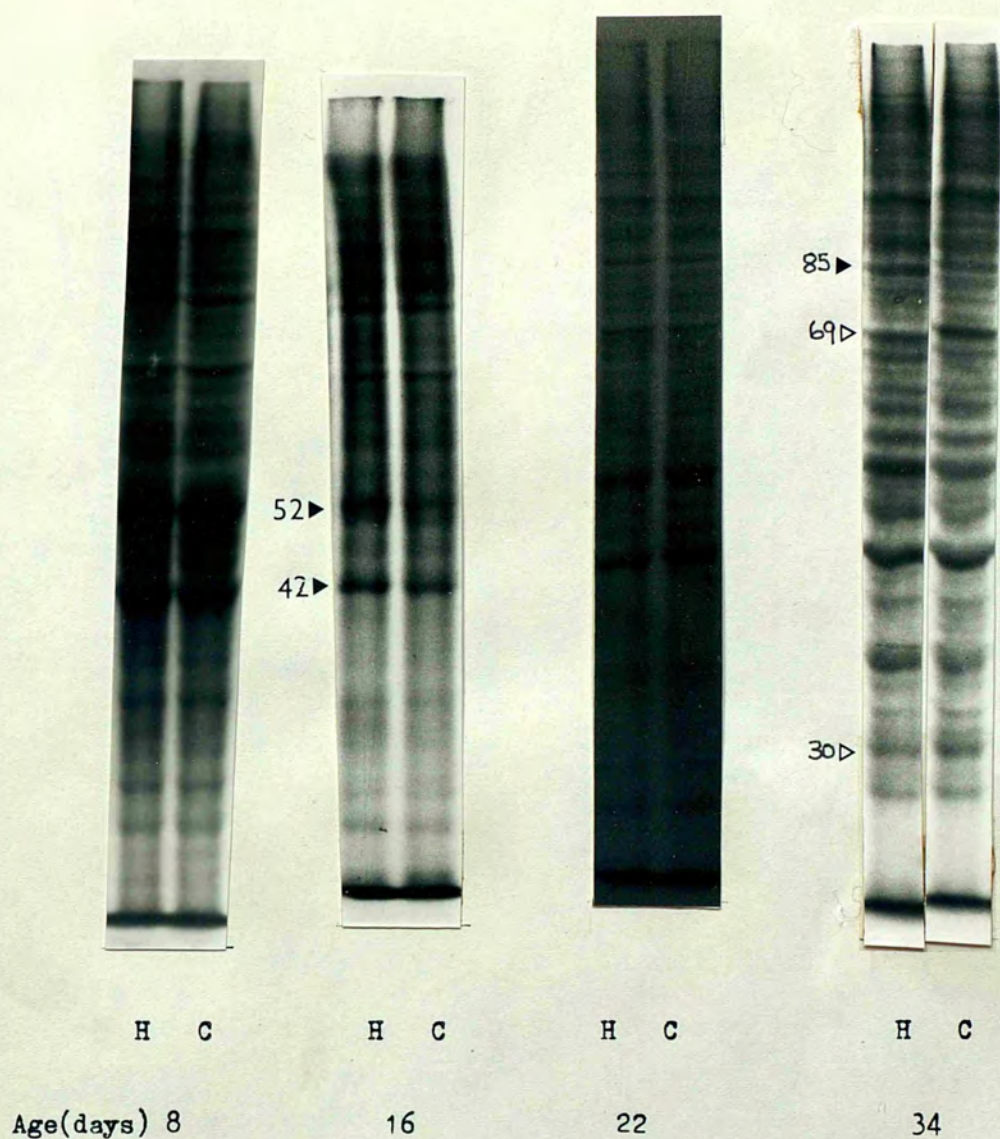
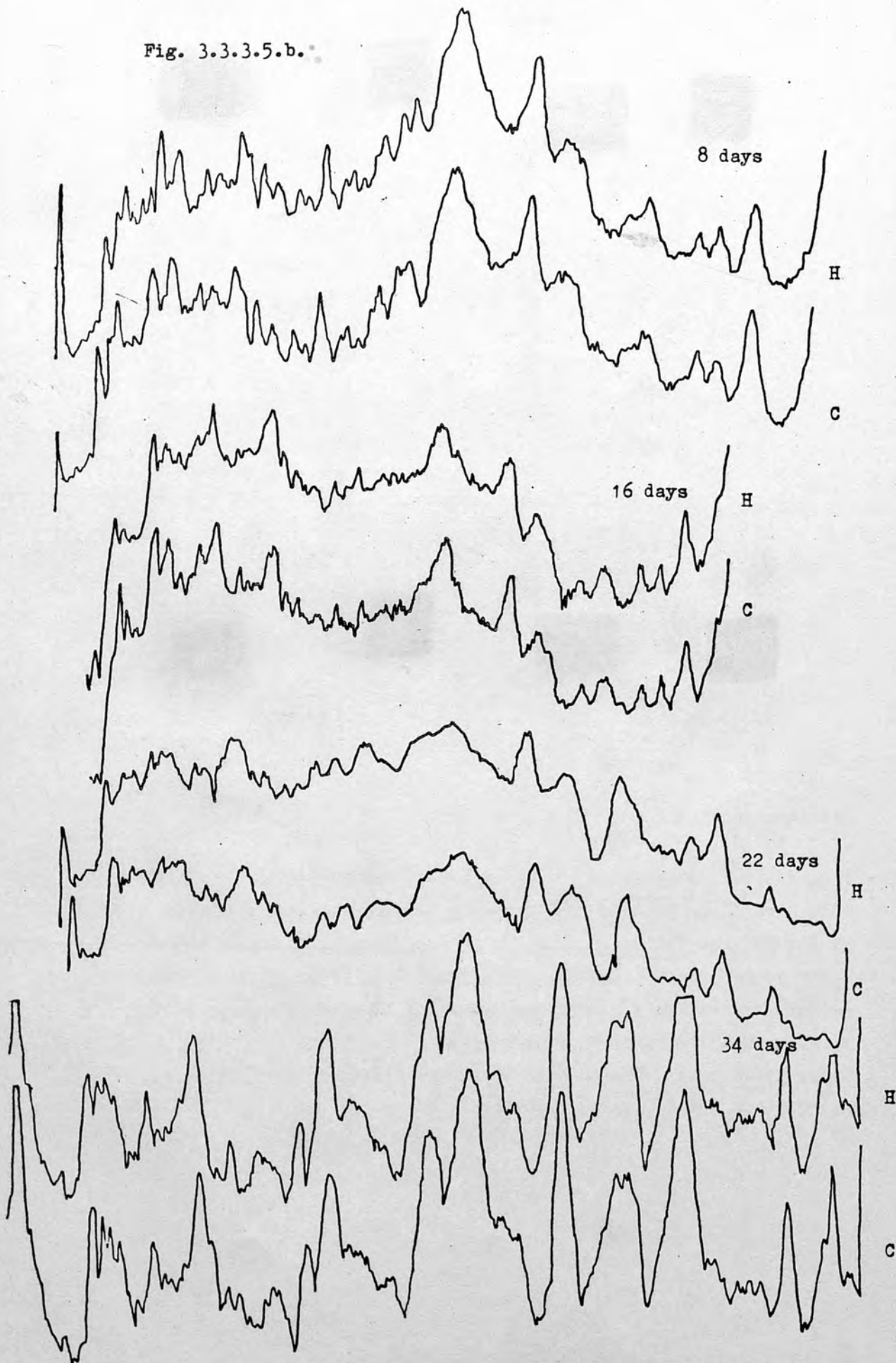


Fig. 3.3.3.4. Comparison of the incorporation of L-(³⁵S) methionine into cerebellar protein from neonatal hypothyroid and euthyroid rats. a) SDS polyacrylamide gels loaded with constant amounts of radioactivity per track (12,000, 6,500, 6,000 & 6,000dpm at 8, 16, 22 & 34 days respectively) were processed for fluorographic analysis. b) Densitometric scans of fluorograms. Polypeptide bands showing possible thyroid hormone deficiency-related alterations are indicated as before.

Fig. 3.3.3.5.b.



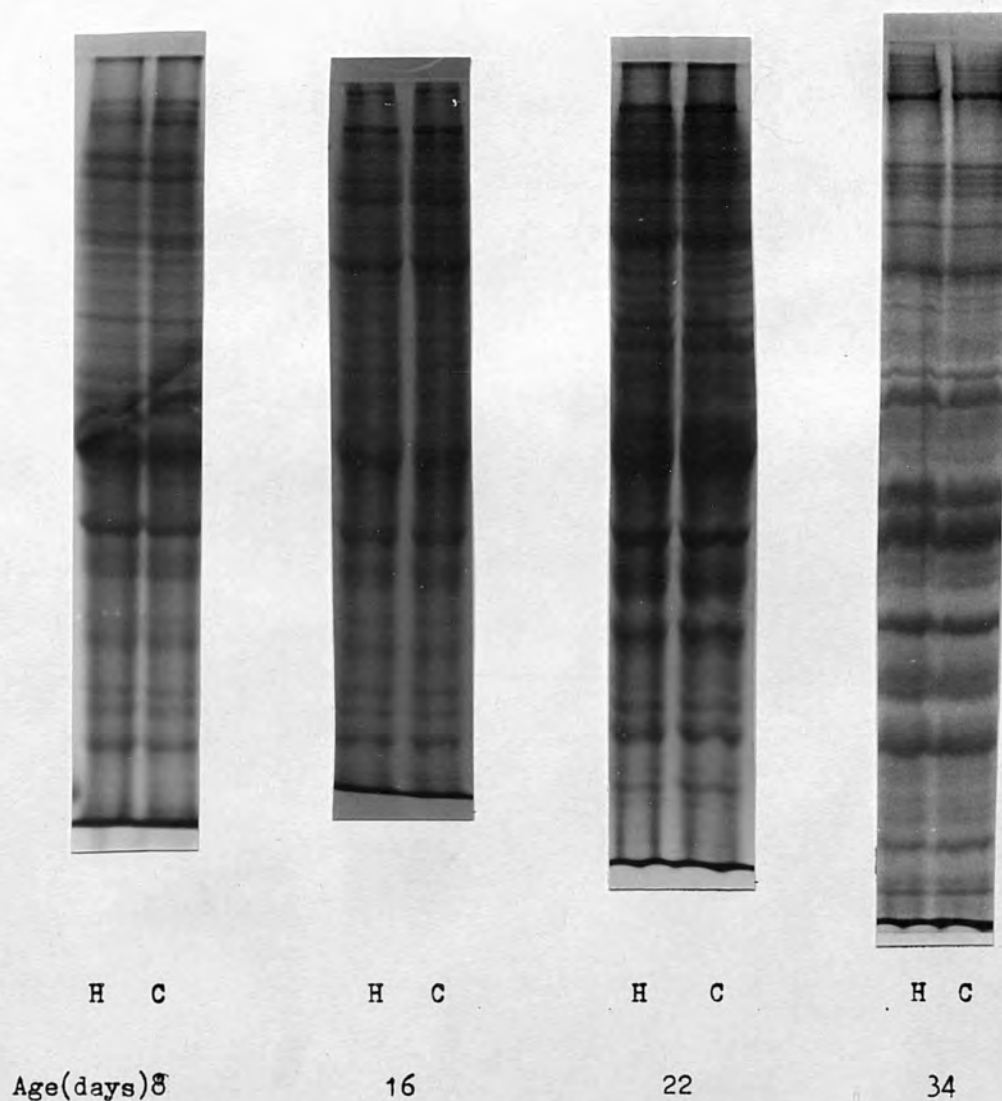


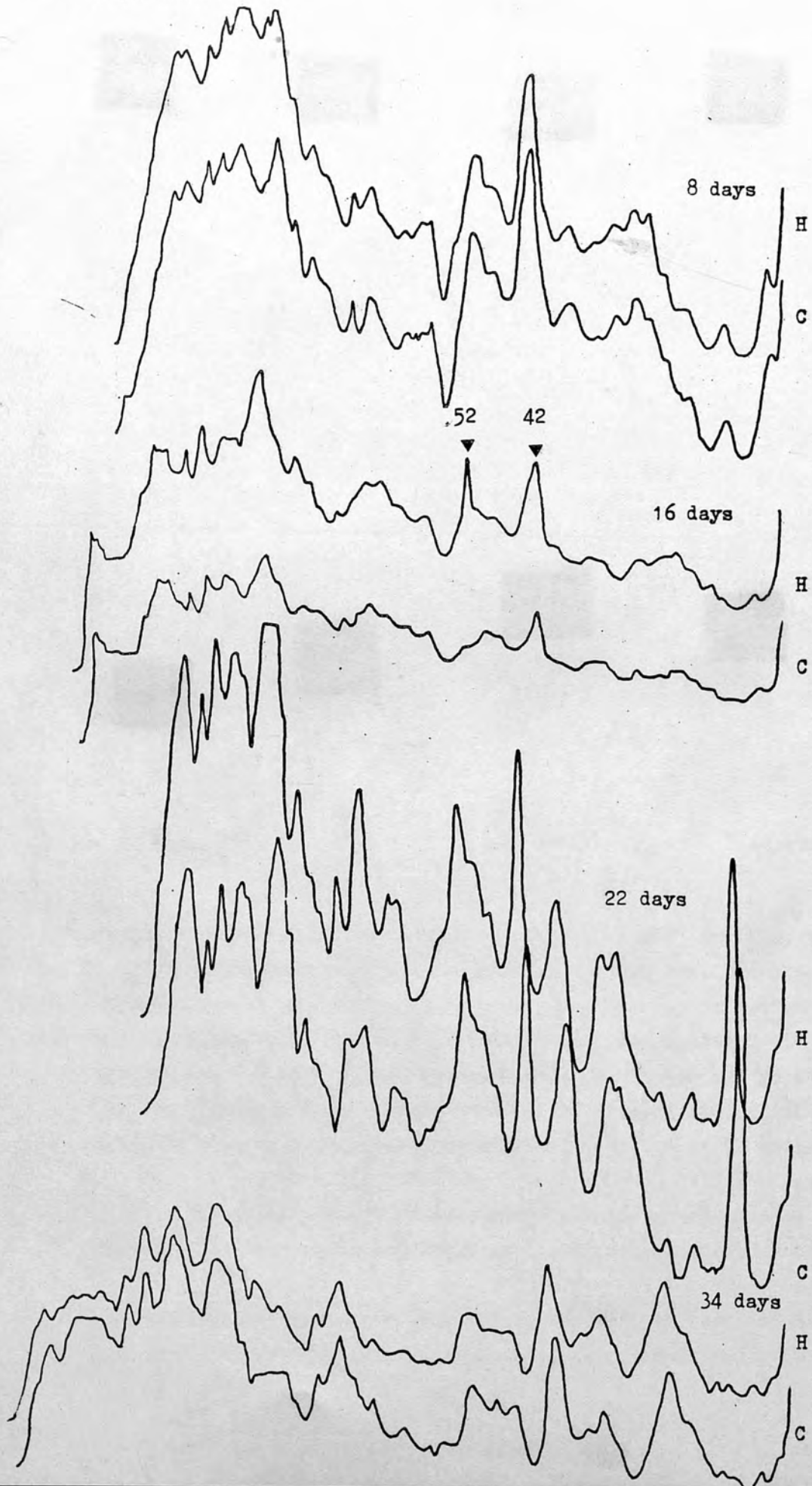
Fig. 3.3.3.5. Comparison of the Coomassie Brilliant Blue-stained electrophoretic profiles of SPM protein from neonatal hypothyroid and euthyroid rats.

a) SPM fractions were isolated from neonatal hypothyroid and euthyroid rats by the method of Salvaterra and Matthews (1980) and subjected to SDS polyacrylamide gel electrophoresis at a constant protein loading of $100\mu\text{g}/\text{track}$ and then stained with Coomassie Brilliant Blue.

b) Densitometric scans of Coomassie Brilliant Blue-stained gels of SPM fractions isolated from hypothyroid and euthyroid neonatal rats.

No consistent differences were observed between individual polypeptide bands which could be attributed to thyroid hormone deficiency.

Fig. 3.3.3.6. b.



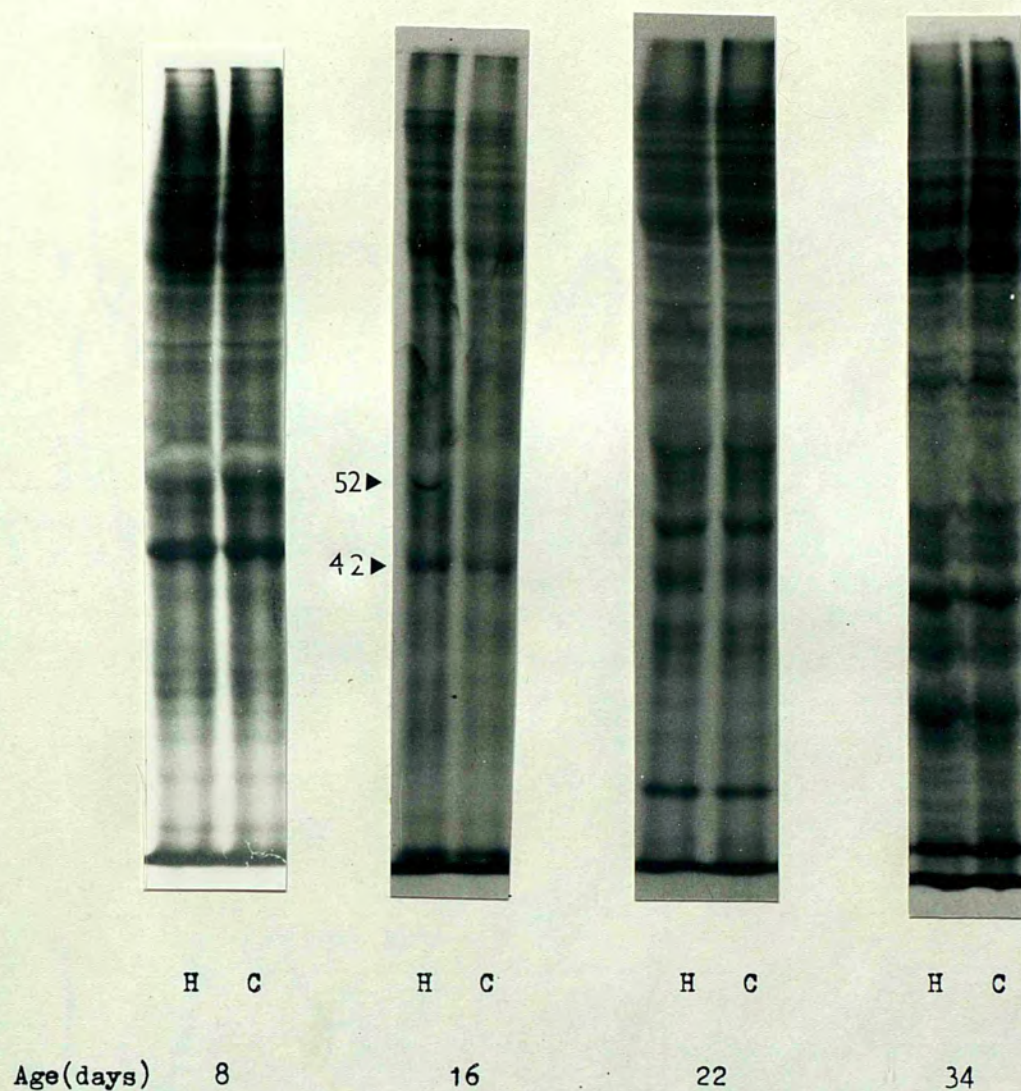
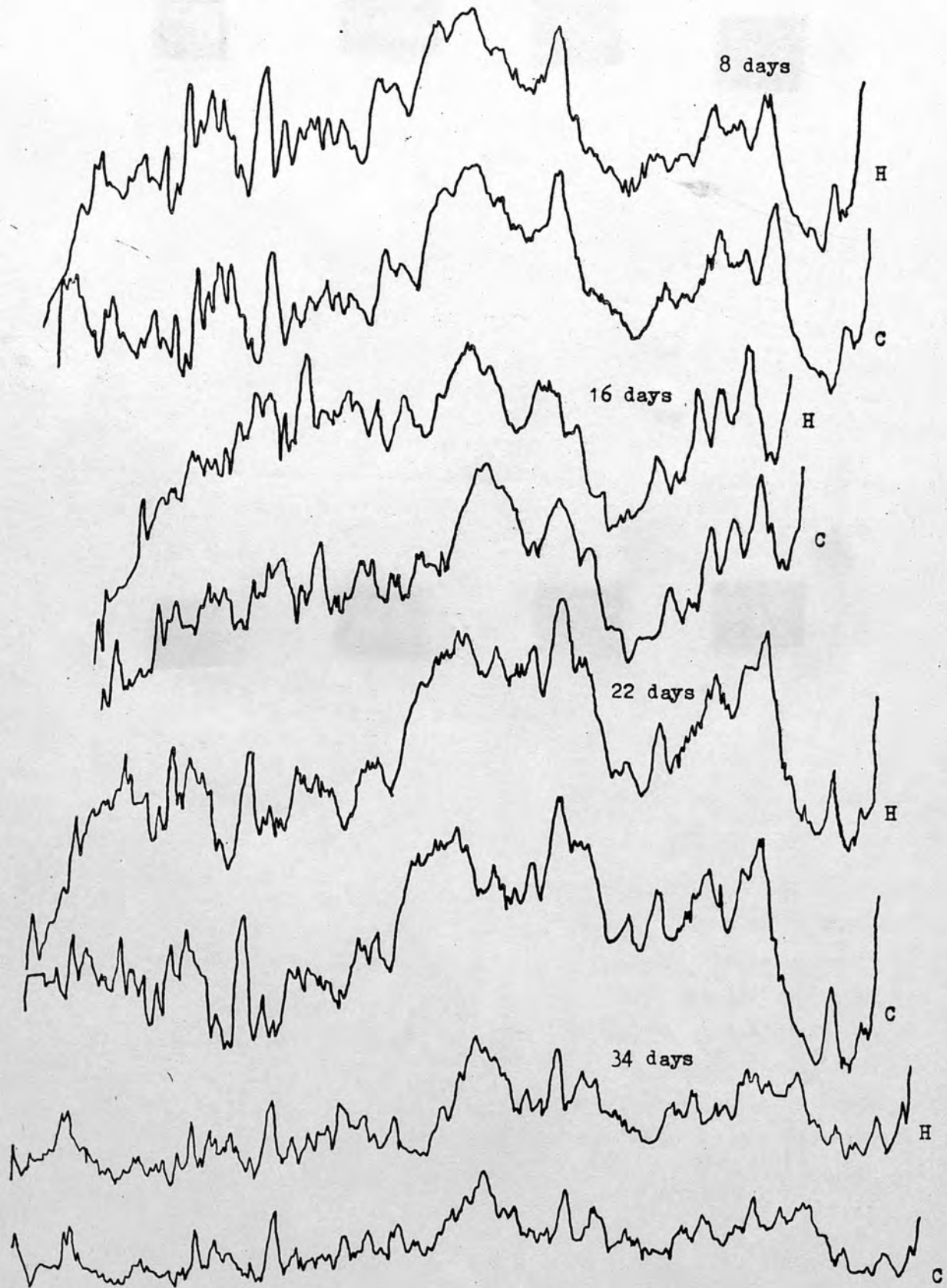


Fig. 3.3.3.6. Comparison of the incorporation of L-(³⁵S) methionine into SPM protein from neonatal hypothyroid and euthyroid rats.
a) Fluorograms of polyacrylamide gels loaded with constant amounts of radioactivity per track (20,000, 6,500, 4,000 & 5,000dpm at 8, 16, 22 & 34 days respectively).
b) Densitometric scans of fluorograms.
Polypeptide bands showing possible thyroid hormone deficiency-related differences are indicated as before.

Fig. 3.3.3.7.b.



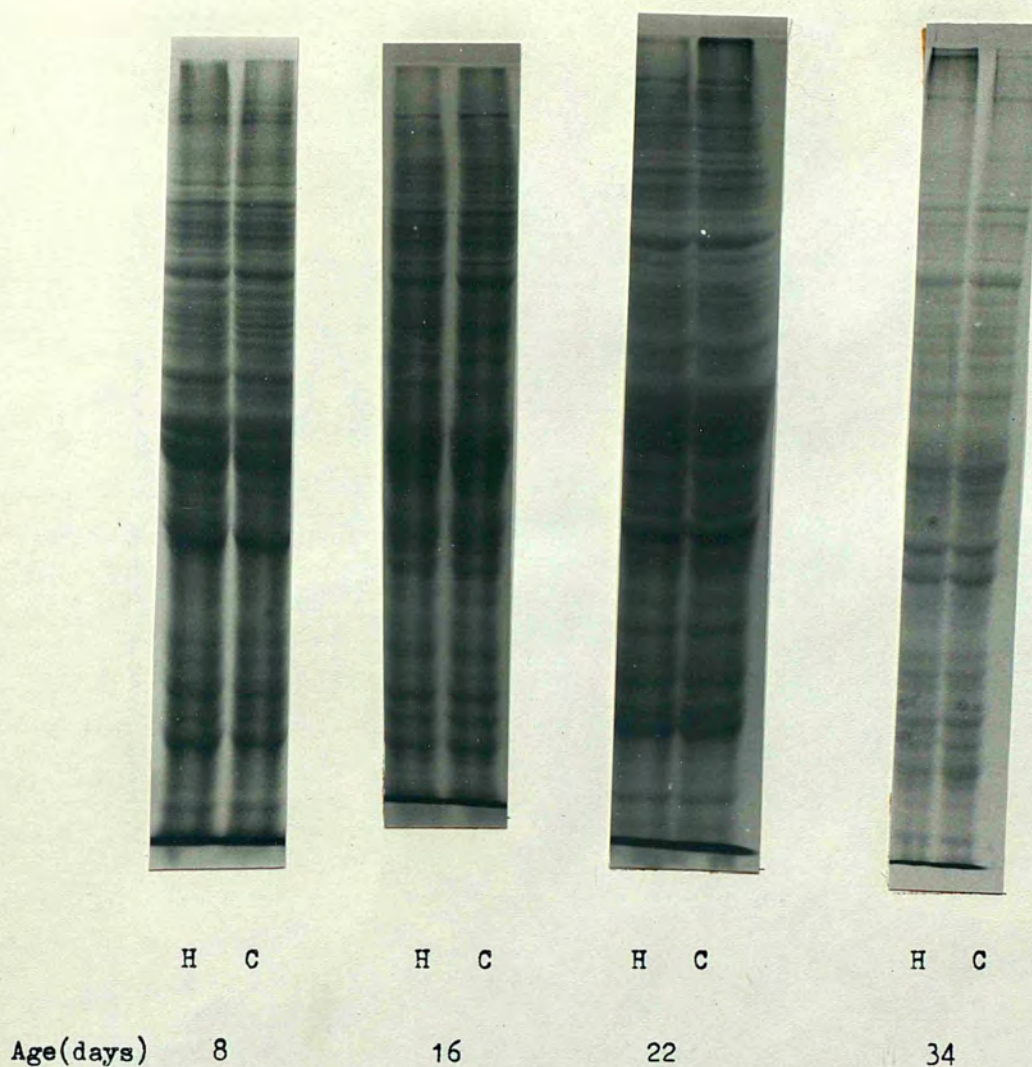


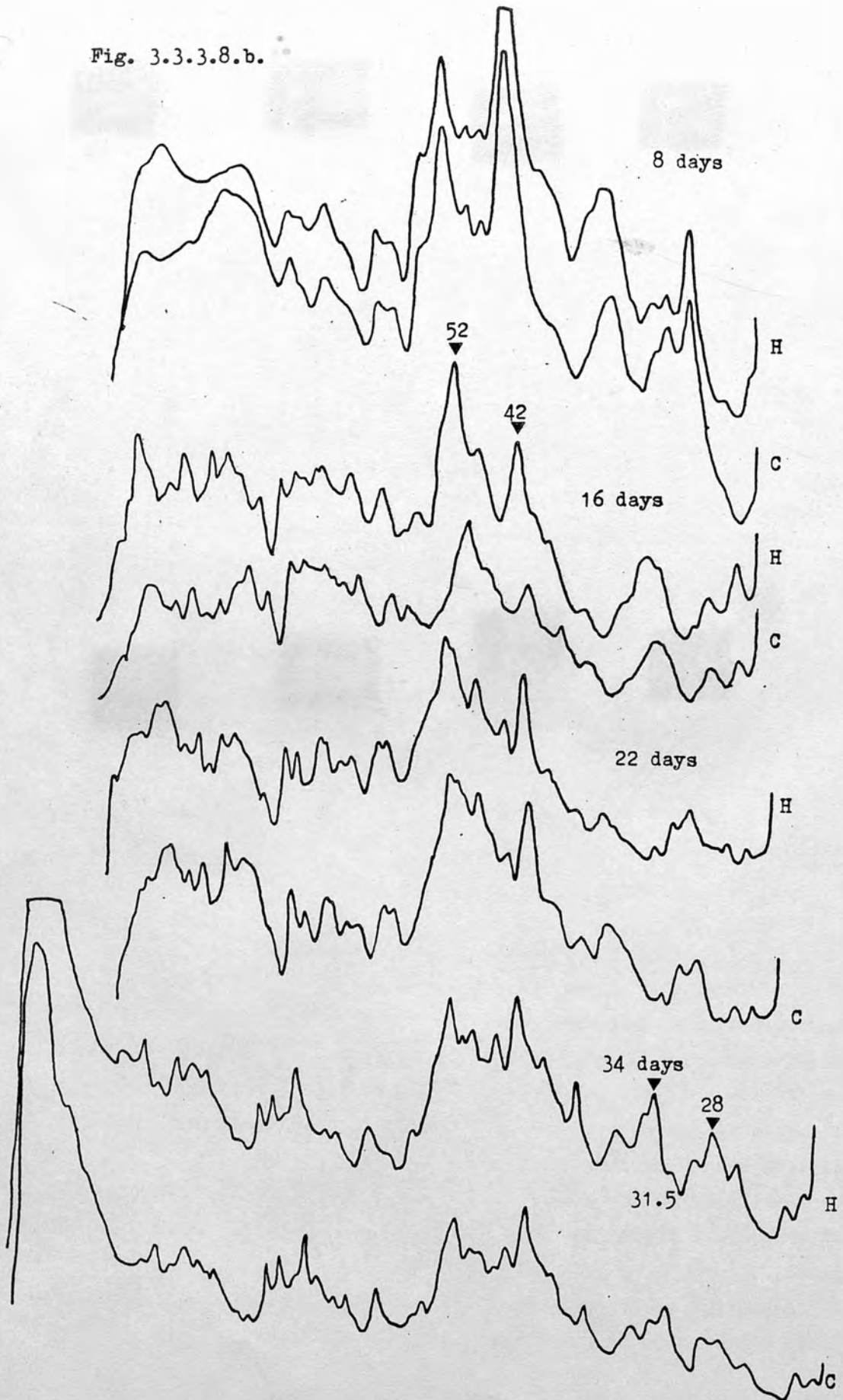
Fig. 3.3.3.7. Comparison of the Coomassie Brilliant Blue-stained electrophoretic profiles of mitochondrial protein isolated from hypothyroid and euthyroid rats.

a) Mitochondrial fractions were isolated by the method of Salvaterra and Matthews (1980) and subjected to SDS polyacrylamide gel electrophoresis at a constant protein loading of $100\mu\text{g}/\text{track}$ and then stained with Coomassie Brilliant Blue.

b) Densitometric scans of Coomassie Brilliant Blue-stained gels of mitochondrial fractions isolated from hypothyroid and euthyroid rats.

No consistent differences were observed between individual polypeptide bands which could be attributed to hypothyroidism.

Fig. 3.3.3.8.b.



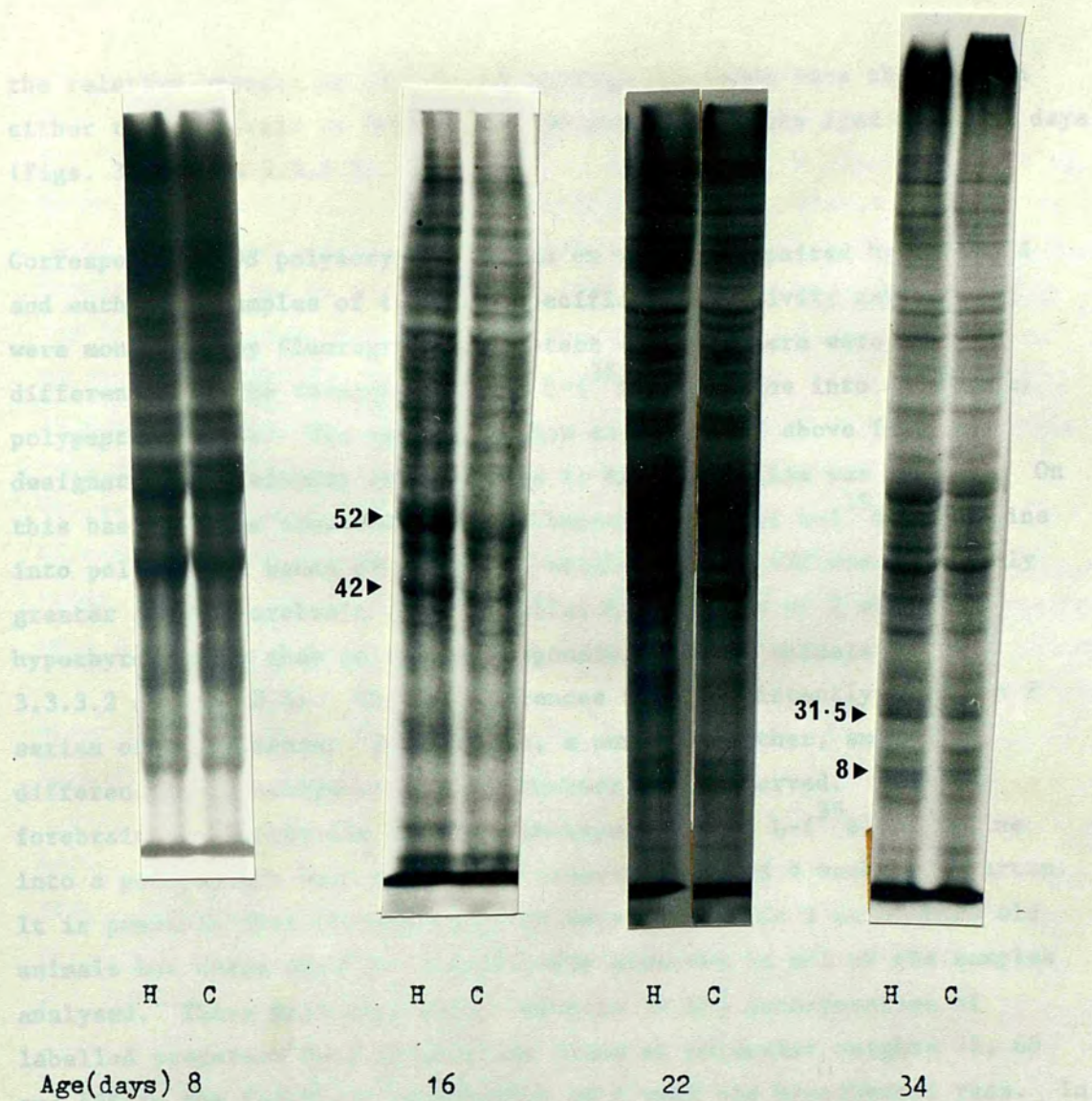


Fig. 3.3.3.8. Comparison of the incorporation of L-(³⁵S) methionine into mitochondrial protein from neonatal hypothyroid and euthyroid rats.

a) Fluorograms of SDS polyacrylamide gels loaded with constant amounts of radioactivity (25,000, 13,500, 5,000 & 6,000dpm at 8, 16, 22 & 34 days respectively).

b) Densitometric scans of fluorograms.

Polypeptide bands showing possible hypothyroidism-related alterations are indicated as before.

the relative amounts of individual polypeptide bands were observed in either the forebrain or cerebellar homogenates of rats aged up to 34 days (Figs. 3.3.3.1 & 3.3.3.3).

Corresponding SDS polyacrylamide gels on which age-paired hypothyroid and euthyroid samples of the same specific radioactivity were loaded were monitored by fluorography to detect whether there were any differences in the incorporation of L-(³⁵S)methionine into individual polypeptide bands. The same criterion as described above for designating differences as being due to hypothyroidism was adopted. On this basis it was observed that the incorporation of L-(³⁵S)methionine into polypeptide bands of molecular weights 52 and 42K was relatively greater in the forebrain and cerebellar homogenates of 2 week old hypothyroid rats than in the corresponding control animals (Figs. 3.3.3.2 and 3.3.3.4). These differences were consistently found in 2 series of experiments. In addition, a number of other, smaller differences in incorporation of precursor were observed. In the forebrain of hypothyroid rats the incorporation of L-(³⁵S)methionine into a polypeptide band of 90K was reduced at 1 and 4 weeks postpartum. It is possible that similar deficits were present in 2 and 3 week old animals but these were not consistently observed in all of the samples analysed. There were also small deficits in the incorporation of labelled precursor into polypeptide bands of molecular weights 72, 68 and 52K in the forebrain homogenates of 4 week old hypothyroid rats. In addition, there were small relative increases in incorporation into polypeptide bands of 62 and 33K in 1 week old hypothyroid animals. In the cerebellar homogenates of 4 week old hypothyroid rats increased incorporation of label into a polypeptide band of 85K was observed while small deficits were found in 69 and 30K polypeptide bands.

Initially it had been intended that subcellular fractions would be isolated from both the forebrain and cerebellum of hypothyroid and euthyroid rats. It was found, however, that the yield of protein in the subcellular fractions isolated from cerebella were very low, even after pooling 4-6 of these. SDS polyacrylamide gel electrophoretic analysis of cerebella-derived fractions was not, therefore, feasible using these procedures. Similarly, the yield of protein in the myelin fractions

obtained from the brains of these young rats was very low. While sufficient myelin could be isolated from each sample to run single gel tracks it was difficult to achieve sufficiently high concentrations of protein and radioactivity in the sample buffer for electrophoresis to allow detailed analysis of this fraction. Since the primary aim of the present study was to monitor the effect of thyroid hormone deficiency on the SPM fraction it was felt that the refinements of the techniques necessary to monitor the myelin fraction also would be prohibitively lengthy. In addition, the effects of hypothyroidism on myelinogenesis have been extensively studied by other workers (Freundl and Van Wynsberghe, 1978; Walters and Morell, 1981). Thus only the SPM and mitochondrial fractions isolated from the forebrain were examined in order to obtain sufficient amounts of protein for analysis 2 forebrains were pooled prior to fractionation.

Assessment of the Coomassie Brilliant Blue staining patterns of hypothyroid and euthyroid SPM fractions showed that there were no consistent differences which could be attributed to thyroid hormone deficiency (Fig. 3.3.3.5). The fluorographic analysis of the SPM fractions showed that at 1, 3 and 4 weeks postpartum there were no obvious differences between hypothyroid and euthyroid fractions (Fig. 3.3.3.6).

In the SPM fractions isolated from 2 week old rats, however, there was an observable increase in the 52 and 42K polypeptide bands in hypothyroid rats. Since this increase was relatively small and partially masked by a flaw in the fluorogram it can only be tentatively suggested that this is a real effect of hypothyroidism. However, since this effect was observed in all of the other fractions examined it seems likely that the parallel enhancement of the labelling of these polypeptide bands in the SPM fraction is due to thyroid hormone deficiency.

Examination of the electrophoretic protein profiles of mitochondria isolated from hypothyroid and euthyroid forebrains showed that there were no significant differences in the levels of individual polypeptide bands (Fig. 3.3.3.7). Fluorographic analysis of mitochondrial profiles (Fig. 3.3.3.8) indicated that the incorporation of labelled methionine

into polypeptide bands of 52 and 42K was, again, enhanced in 2 week old hypothyroid rats. In 1 and 3 week old rats there were no significant differences between hypothyroid and control animals. At 4 weeks postpartum, however, the incorporation of labelled methionine into polypeptide species of 31.5 and 28K was higher in hypothyroid rats than in the corresponding controls.

3.3.4 DISCUSSION

The present investigation of the effects of PTU-induced neonatal hypothyroidism has indicated that this treatment results in reduced body and brain weights, lowered protein yields and depressed rates of protein synthesis. A reduction in body and brain weight due to hypothyroidism has been reported by many workers and the deficits observed in the present study are comparable to those reported in the literature (Geel and Timiras, 1970; Battie and Verity, 1979). Undernutrition which frequently accompanies abnormal hormonal states, has been reported to cause severe retardation of body and brain growth (Balazs, 1977). While this cannot be entirely ruled out in the present study it is unlikely that this contributed significantly to the observed effects since the mother rats fed normally and consumed 70-100 g/day of diet containing PTU.

In addition^{to} the overall decrease in the weight of both forebrain and cerebellum of hypothyroid rats showed a deficit in protein yield on both a mg protein/brain and mg protein/g tissue (wet weight) basis. Although the postnatal increase in protein yield due to the development-related decrease in brain water content (Davis and Himwich, 1973) was observed in both euthyroid and hypothyroid animals this increase was smaller in the deficient rats. These deficits were statistically significant at 2, 3, and 4 weeks postpartum in both the forebrain and cerebellar homogenates. This is consistent with the findings of Geel and Timiras (1970) who reported that the water content of 22 day old hypothyroid rats was significantly higher than euthyroid controls ($82.8 \pm 0.09\%$ in hypothyroid rats compared with $81.9 \pm 0.06\%$ in controls, $p < 0.001$). The protein yields in the subcellular fractions were also reduced in hypothyroid animals although these deficits were not always statistically significant. In addition, the yield of protein in the SPM fraction

obtained from hypothyroid rats showed a slight deficit on a μg protein in this fraction/ μg protein in the forebrain homogenate basis. Although this was not statistically significant it may indicate that, in addition to an overall reduction in protein yield there may also be a relative reduction in the SPM protein yield. This is consistent with the observation reported by Battie and Verity (1979) who found deficits in the yield of protein in synaptosome fractions derived from 7, 14 and 21 day old hypothyroid rats. The deficit at 14 days was statistically significant and was estimated to be about 44%. Similarly Smith *et al.* (1980) reported deficits in the yield of total membrane protein (mg protein/g tissue, wet weight) in 21 and 34 day old rats which were statistically significant although small (12 and 6% respectively).

The lack of statistical significance in many of the results in the present study may be due, in part, to variation in the degree of hypothyroidism achieved by individual rats. Ruiz-Marcos *et al.* (1979) found that, following surgical thyroidectomy, the majority of rats stopped growing and showed a large decrease in pituitary growth hormone. A number of animals, however, continued to grow, although at a much reduced rate compared with controls. This variation is, inevitably, compounded by the variation in the isolation procedure (discussed in section 3.1.4) and, together with the limited number of brains able to be fractionated simultaneously probably accounts for the lack of statistical significance in some of the results. Nevertheless the overall trend and the statistically significant results are consistent with there being a thyroid hormone deficiency-related effect on protein content of the brain.

The effect of thyroid hormone deficiency on cerebral protein synthesis has been subject to much debate in recent years. In a number of studies hypothyroidism has been reported to reduce protein synthesis in the brain (Geel and Timiras, 1970; Jarlstedt and Norstrom, 1972; Lindholm, 1982). It has also been demonstrated that tri-iodothyronine regulates the levels of somatotropin mRNA in cultured rat pituitary cells (Martial *et al.*, 1977). Other workers, however, have been unable to demonstrate any depression of cerebral protein synthesis in hypothyroid animals *in vivo* (Bergner *et al.*, 1981), in cell-free systems (Andrews and Tata, 1971) or in brain slices (Valcana and Eberhardt, 1977). All of these studies, however, measured the rate of incorporation of trace amounts of

precursor into brain protein. It may be argued that by using the trace amounts of precursor, which is subject to methodological difficulties anyway (see section 3.2.1), in a morphologically atypical tissue, such as that found in hypothyroid rats, it is very difficult to extrapolate any effect on the measured rate of incorporation to that on the absolute rate of protein synthesis. It is, for example, possible that the compartmentation of precursor in hypothyroid tissue might be different from that in euthyroid tissue. These difficulties were overcome in the present study by using the in vivo flooding method of Dunlop et al. (1975) to obtain the overall rates of protein synthesis in developing hypothyroid and euthyroid rats.

While the development-related decrease in the rate of cerebral protein synthesis reported by Dunlop et al. (1977) was observed in both hypothyroid and euthyroid rats the rates of protein synthesis were reduced in the hypothyroid animals compared with the controls. Although not all the deficits were statistically significant this, again, suggests that hypothyroidism has an effect on protein synthesis in vivo.

The largest deficit (25%) in the rate of incorporation of precursor into SPM protein was observed at 2 weeks postpartum, was statistically significant and was found in three separate experiments. This deficit in synthesis occurs at a critical period of brain development when neuronal differentiation occurs which is characterised by the proliferation of axonal and dendritic fibres and the subsequent formation of synapses (Bass et al., 1969a; Bloom, 1972). This would indicate, therefore, that normal synaptic development is likely to be severely affected in hypothyroid rats. The myelin fraction also showed large deficits in the rate of incorporation of precursor in hypothyroid rats. The myelin fraction also showed large deficits in the rate of incorporation of precursor in hypothyroid rats. This was the most severely affected subcellular fraction with a 52% statistically significant deficit at 2 weeks after birth. Between 10 and 50 days postpartum neuroglial cells migrate from the subependymal zone into the cerebral cortex where they differentiate. Some cells differentiate to form the oligodendrocytes which are responsible for the myelination of the newly formed axons and dendrites. (Bass et al., 1969b). Hypothyroidism has been reported to impair myelinogenesis in young rats resulting in a permanent deficit in the amount of myelin in the brains

of these animals (Freundl and Van Wynsberghe, 1978; Walters and Morrell, 1981). The results of the present study suggest that this may be at least partly due to a reduction in the synthesis of myelin during the period of its most rapid accumulation.

There did not appear to be any significant effect on the rate of incorporation of precursor into mitochondrial protein due to hypothyroidism although a non-significant deficit (11%) was observed at 2 weeks postpartum.

It might be expected that larger deficits would be found in fractions isolated from cerebellar tissue since this is relatively more immature at birth than the forebrain. Patel et al. (1976) estimated that postnatal cell acquisition in the forebrain accounted for about 50% of the total number of cells in adult forebrain. The major proportion, however, of these cells were glial. In the cerebellum, however, these workers estimated that 97% of cells are formed after birth and the majority of these were neurones.

Examination of the fluorographic profiles of fractions isolated from hypothyroid rats indicated that incorporation of L-(³⁵S)methionine into polypeptides of molecular weights 52 and 42K was higher in 2 week old rats than in euthyroid rats. The similarity in apparent molecular weights, relative abundance and prominence of these polypeptides compared with literature (Feit et al., 1977; Kelly and Cotman, 1978) suggests that these might be tubulin and actin. Both of these proteins have been reported to undergo a development-related decrease (Burgoyne et al., 1981; Fu et al., 1981; Kelly and Cotman, 1981). This decrease appears to be retarded in the brains of hypothyroid rats since the incorporation of precursor into these polypeptides is higher in these rats than in euthyroid controls. By 3 weeks postpartum, this large difference is no longer clearly observable. Examination of the electrophoretic profiles of subcellular fractions of rats of the same age indicate that there is no corresponding increase in the amount of these polypeptides in hypothyroid rats than in controls. Further studies are needed to confirm these findings. For example, alterations in amounts of tubulin could be monitored by colchicine-binding studies.

It was also observed that the incorporation of precursor into a 90K polypeptide in the forebrain homogenate was reduced in 1 and 4 week old hypothyroid rats. Whilst some difference may exist at 2 and 3 weeks this could not be unequivocally demonstrated in the present study. This polypeptide has a similar molecular weight to the major subunit of $(\text{Na}^+, \text{K}^+)\text{ATPase}$. The activity of this enzyme has been reported to be reduced in hypothyroid rats and its developmental increase retarded (Geel and Timiras, 1970; Lo and Edelman, 1976).

The composition of total brain and subcellular fractions isolated from hypothyroid rats, however, was qualitatively and quantitatively very similar to that of euthyroid rats, as was the pattern of incorporation of L- (^{35}S) methionine. This implies that although there are a number of small differences between hypothyroid and euthyroid rats the overall protein composition is not significantly affected but the amount of protein in the brain is reduced by thyroid hormone deficiency.

These results which are in agreement with earlier studies, tend to indicate that brain development in hypothyroid rats is retarded and impaired since those proteins which show development-related alterations do so at a slower rate than that observed in normal rats. The results tend to indicate that structural proteins such as tubulin and actin and plasma membrane markers such as $(\text{Na}^+, \text{K}^+)\text{ATPase}$ are specifically affected. 5'-nucleotidase, which is also located in the plasma membrane, has been reported to show depressed activity in hypothyroid rats (Smith et al., 1980). These findings would tend to agree with the observation that hypothyroid rats have lower amounts of neuropil material i.e. axons and dendrites, and, therefore, have decreased cell surface areas. Nicholson and Altman (1972) and Balazs (1972) suggested that the "ontogenetic clock" is slower in hypothyroid animals. Whether the duration of functional and structural maturation of the brain is sufficiently lengthened in the hypothyroid brain to allow it to approach the normal state cannot be deduced from these relatively short-term experiments but in view of the permanent impairment of mental function this seems likely.

SUMMARY

1. The present study has demonstrated that it is possible to obtain good approximations of the rate of cerebral protein synthesis by using intraventricular injections of flooding concentrations of radiolabelled precursor. Using L-(³⁵S)methionine it was observed that the rate of incorporation of this amino acid was linear over 45 min after injection. In addition, errors due to uneven distribution of precursor or metabolism of methionine to other labelled amino acids were minimal. Thus it was estimated that the rate of synthesis of total brain protein in mature rats (approx. 100 days old) was 0.65 μmol methionine incorporated/g protein/h which was close to values reported by other workers.
2. The use of this local flooding method permits the examination of the incorporation of precursor into subcellular fractions since it is possible to attain relatively high levels of radioactivity in the brain. Although the method developed here provides a value for the rate of incorporation of precursor into SPM protein rather than the rate of SPM protein synthesis it does, however, permit the examination of the effect of various factors on this incorporation.
3. Various drug treatments have been demonstrated to alter the incorporation of precursor into SPM protein. Halothane, a volatile anaesthetic, was shown to depress brain protein synthesis by about 40% and the incorporation of L-(³⁵S)methionine into SPM protein by a similar amount. Conversely pentobarbitone, which is also an anaesthetic, did not appear to have any significant effect on either total or SPM protein synthesis.

The means by which halothane effects this inhibition of cerebral protein synthesis is not known. Ether has been reported to inhibit cerebral protein synthesis via alterations in hormonal levels (Schotman et al., 1977). This may indicate a possible line of investigation of the effect of halothane on brain protein synthesis.

L-DOPA was found to inhibit cerebral protein synthesis by close to 30% under conditions where the animal's body temperature was raised

to at least 39°C. The precursor incorporation into myelin, synaptosomal and SPM proteins appeared to be less affected by L-DOPA. Further experiments are required to verify this reduced effect on the subcellular fractions since this would suggest that the synthesis of synaptic and myelin proteins might be preferentially protected under certain conditions.

4. Propyl thiouracil-induced hypothyroidism was shown to result in reduced body weight, brain weight, protein yields, rate of total protein synthesis and rate of incorporation of radiolabelled amino acid into subcellular fractions. Examination of the incorporation of trace amounts of L-(³⁵S)methionine into individual polypeptides suggested that development-related changes in a number of brain proteins are retarded in hypothyroid rats. This may indicate that the time course of structural and functional maturation of the brain is prolonged in hypothyroid animals and, in addition, may be defective since there appears to be permanent impairment of brain function.

The present results tentatively indicate that the incorporation of precursor into polypeptides of apparent molecular weight 52 and 42K (tentatively identified as tubulin and actin) was enhanced in 2 week old hypothyroid rats compared with control animals. No corresponding increase, however, was found in the amount of these two polypeptides. This, clearly, would be a particularly interesting area for further investigation since these results suggest that there may be a preferential effect of thyroid hormone deficiency on at least some of the major structural proteins of the brain. Possible lines for further investigation are:

- (a) To use protein-specific assays for quantitation of individual structural proteins, e.g. colchicine binding assays for tubulin or quantitative immunological techniques to determine whether hypothyroidism has any effect on the relative amounts of individual proteins.

- (b) To examine the effect of thyroid hormone deficiency on the amounts and synthesis of structural proteins in discrete brain areas. Since different brain areas mature asynchronously it is

possible that areas such as the cerebellum in which the major proportion of neurones develop after birth (in the rat) may show larger effects due to hypothyroidism.

(c) The SPM fraction contains a high proportion of structural proteins. It would, therefore, be of considerable interest to examine the effect of hypothyroidism, in detail, on these proteins. In order to obtain meaningful results it would probably be more useful to examine SPM fractions from different brain areas (see above). This would require a number of refinements to be made to the techniques used for the preparation and examination of SPM proteins. For example, the SPM isolation procedure would need to be adapted for the fractionation of small amounts of tissue.

5. The use of subcellular fractions in experiments of the type carried out in this project is subject to a number of problems. An important difficulty arises from the limited number of observations able to be made, which is dependent upon the number of simultaneous individual fractionations which can be carried out. This is especially important in the determination of the effects of drugs which, ideally, require a large number of observations to be made in order to obtain statistically significant results. Another serious problem is that of contamination of SPM fractions which are now thought to be only 50-70% 'pure'. The combination of these two factors tends to make observations obtained using SPM fractions isolated by traditional methods tentative. It is possible that with the recent rapid development of monoclonal antibody techniques that it will soon be feasible to isolate SPM fractions of high purity. Reichardt and Matthew (1982) reported that they were able to produce a pure synaptic vesicle preparation by incubation of a crude lysed synaptosome fraction with vesicle-specific antibody which was then precipitated after mixing with protein A coupled with acrylamide beads. The development of isolation procedures using these techniques, assuming that suitable monoclonal antibodies can be raised, would clearly permit the preparation of pure fractions and would, possibly, allow more observations to be made.

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