EFFECTS OF MATERNAL HYPOTHYROXINEMIA ON THE EXPRESSION OF BIOCHEMICAL FUNCTIONS IN THE CNS OF THE ADULT ~~PROGENY~~ RAT

Thesis submitted to the Faculty of Science at the University of London for the degree of Doctor of Philosophy

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1991

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Dedicated to my faithful wife Fatemah and to my children.
Thank you all for your prayer, encouragement and support.

"He, who teaches me a word (of wisdom), renders me his servant".

Imam Ali ebne' Abu Talib

ACKNOWLEDGEMENTS

I wish to express my deep sense of gratitude to Professor R P Ekins for suggesting the project and his encouragement through all phases of this thesis. I would like to thank most sincerely Dr A K Sinha for his help, his continously guidance and important advice throughout this work. At the same time, my special thanks go to Dr M Pickard for his excellent advice and proof-reading. My thanks also go to Dr M Hubank for his helpful criticism.

I would like to thank all members of the Department of Molecular Endocrinology, in particular Mr A O'shea for his technical assictance in gel electrophoresis, Dr P R Edwards, Dr Zaidan Al Mazidi, Mr T Jowett, Mr M Khaled Ms M Sgherzi, Mr M Ahmad and Mrs J Sloley for her help during typing of this thesis.

I should like to thank the Goverment of The Islamic Republic of Iran / Ministry of Science and Higher Education and the Ministry of Health and Medical Education for offering me a grant to finance this work.

ABSTRACT

The importance of thyroid hormones in the development and maintenance of normal CNS functions, has long been well established. Iodine-deficient areas are scattered all over the world. Maternal hypothyroxinemia in early pregnancy, as a consequence of iodine deficiency, is associated with an increased incidence of neurological cretinism in the offspring. This condition is irreversible and is manifested in severe cases by mental retardation, deaf-mutism, diplegia of the inferior extremities, gait disorders and sometimes strabismus and stunted growth. In the adult, these conditions cannot be corrected by a normal thyroid state or replacement therapy, suggesting the existence of specific phases in early brain development during which correct exposure to thyroid hormones is of critical importance, and there is a possible fetal dependance on maternal thyroxine. However, the putative biochemical disorders associated with latter have not been studied in detail. The aim of the study was thus to evaluate the biochemical changes in different brain regions due to maternal hypothyroxinemia in an animal model.

Seven month old (adult) progeny born to normal and partially thyroidectomised rat dams were used. Total protein, protein profiles of cytosolic and particulate fractions, neuronal cell marker enzymes, myelin metabolic enzymes (oligodendroglial cell markers), and certain lipids were studied. Body weight, brain weight and protein concentrations of brain regions in experimental progeny were apparently unchanged. On the other hand protein profiles of both cytosolic and particulate fractions demonstrated significant alterations in some brain regions. Cytosolic protein bands of 25 kD and 38 kD were significantly changed in medulla, while a 31 kD protein band was absent in this brain region. A 97 kD and a 89 kD cytosolic protein bands were also changed respectively in cerebral cortex and paleocortex of TX dam progeny. A 79 kD protein band was the only particulate protein which significantly increased in medulla in the experimenrtal progeny.

Significant changes were observed in myelin metabolic enzymes in specific brain regions of experimental progeny, e.g. CNPase activity was decreased in medulla by 37% ($p<0.05$) and in midbrain by 32% ($p<0.05$), and 5'-nucleotidase activity was decreased significantly in the same regions by 33% ($p<0.05$) and 35% ($p<0.05$), respectively. In paleocortex, oleate esterase activity (a myelin metabolic enzyme) was

significantly increased by 39% ($p<0.05$), whereas β-D glucuronidase activity (a neuronal marker) was significantly decreased by 30% ($p<0.05$), N-acetyl-β-D galactosaminidase activity (a general glial marker) was unchanged and the activity of sphingomyelinase was also unchanged in all brain regions studied. No change was observed in the concentration of cholesterol (a major component of the myelin lipid) in any brain region but other two specific myelin lipids, cerebroside and sulphatide demonstrated significant changes in experimental progeny. The former was decreased in medulla and the latter also decreased in the midbrain and paleocortex of progeny. Changes in myelin metabolic enzymes and lipids have suggested a deficit in myelin metabolism of adult progeny from partially thyroidectomised dam.

In conclusion, these findings indicate that the effects of maternal hypothyroxinemia are brain region-specific. These effects are also irreversible, since they are observed in adult progeny and persist despite a normal thyroid state. It is well known that glial cell proliferation and acquisition in the rat occurs postnatally, when the thyroid is functionally active. It is argued that the thyroid hormone environment of the fetus, before the onset of an independent fetal hypothalamic-pituitary-thyroid axis may be of critical importance for the expression of glial functions after birth. It is suggested that maternal hypothyroxinemia in early pregnancy may cause irreversible brain region-specific biochemical changes in adult progeny and thyroid hormones in early pregnancy may have an epigenic effect on the function of the adult CNS.

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AMA	antimicrosomal antibodies
ATA	antithyroglobulin antibodies
cAMP	cyclic adenosine monophoshate
CB	cerebellum
CC	cerebral cortex
CH	congenital hypothyroidism
CNPase	2', 3'-cyclic nucleotide 3'-phosphohydrolase
CNS	central nervous system
CST	cerebroside sulfotransferase
D	Dalton
D-I	(5- or 5'-) deiodinase
D-II	5'-deiodinase (outer or phenolic ring)
D-III	5-deiodinase (inner or tyrosyl ring)
DIT	diiodothyronine
DTT	dithiothreitol
EAE	experimental allergic encephalomyelitis
EEG	electroencephalogram
EGL	external granular layer
FT3	free triiodothyronine
FT4	free thyroxine
GABA	γ-aminobutyric acid
GFAP	glial fibrillary acidic protein
GH	growth hormone
h	hour
hCG	human chorionic gonadotrophin
IDD	iodine deficiency disorders
IQ	intellectual quotient
KD	kilodalton
MAG	myelin associated glycoprotein
MAP	microtubule associated protein
MB	midbrain

MBP	myelin basic protein
MC	myxoedematous cretinism
ME	medulla
MIT	mono-iodothyronine
MMI	methylemercaptoimidazol
Mr	molecular weight
mRNA	messenger RNA
MS	multiple sclerosis
4-MU	4-methyumbellifrone
N-CAM	neural cell adhesion molecule
NS	not significant
OL	oligodendrocyte
PAHO	pan American health organization
PAPS	3'-phosphoadenosine-5'-phosphosulphate
PC	paleocortex
Pi	inorganic phosphate
PLP	proteolipid protein
PNS	peripheral nervous system
PTU	propylthiouracil
RIA	radiimmunoassay
rT3	reverse T3
T2	3, 3'-diiodothyronine
T3	3, 3', 5-triiodothyronine
T4	thyroxine
TBG	thyroxine binding globulin
TBII	TSH-binding inhibitor immunoglobulin
TCA	trichloroacetic acid
TETRAC	tetraiodothyro-acetic acid
Tg	thyroglobulin
TH	thyroid hormone
THNR	thyroid hormone nuclear receptors
TNH	transient neonatal hypothyroidism
TRH	thyrotropin releasing hormone
TRIAC	triiodothyro-acetic acid

Tris	tris (hydroxymethyl)-methylamine
TSH	thyroid stimulating hormone
TX	thyroidectomised dam
V-erb-A	avian erythroblastosis virus
WHO	world health organization

CHAPTER 1 : INTRODUCTION

1.1 Historical Review

The thyroid gland derives its name from the Greek word "*Thyrus*", meaning shield-like and was so named by Wharton in 1656. However, references to goitre can be found in various manuscripts dating from the first and second millenia BC.

The first successful thyroidectomy on record was performed on a goitrous subject by Albucasis in Spain, in about 952 AD. He called the tumour "elephant of the throat and noted that it commonly occurs in women and is of two kinds: congenital and acquired (Albucasis, in.\. Spink and Lewis 1973).

Paracelsus recognized cretinism in 1567 and noted its relation with goitre (Cranefield 1962). An excellent clinical description of cretinism was given by Felix Plattner, a Swiss physician, who visited the valleys and examined cretins in 1562. His report was published in 1602 (Cranefield 1962, Konig 1981). Norris described one small focus of endemic cretinism in England, in the village of Chiselborough; transience was an interesting feature of this endemia (Trotter 1960).

Curling (1850) and Fagge (1871) described cases of hypothyroidism outside endemic goitre regions and introduced the term "sporadic cretinism" (Trotter 1964, Kaplan 1989). The term "myxoedema" for adult hypothyroidism was introduced by Ord (Ord 1878). In 1883, Kocher (the father of thyroid surgery) and the Reverdins independently correlated the symptoms of cretinism with those of a patient in whom a total thyroidectomy had been performed. Bircher, performed the first epidemiological study of cretinism in the same year (Konig 1981, Kaplan 1989).

In 1890, Horsley suggested that human myxoedema could be treated by grafting of thyroid tissue (Horsley 1890), and in 1891 Murray treated a myxoedematous patient with twice weekly injections of a sheep thyroid extract (Murray 1891). Fox and Mackenzie showed in 1892 that oral administration of thyroid extract had the same effects (Fox 1892, Mackenzie 1892). The first successful transplantation of the thyroid was reported by Payr in 1906. He transplanted a portion of the gland from a woman into her myxoedematous daughter's spleen with successful results (Kaplan 1989). In 1908 McCarrison first classified cretinism into 2 types: myxoedematous and neurological. He also noted the presence of mixed types (McCarrison 1908).

The work of Coindet (1820) had shown that potassium iodide or tincture of iodine were useful in the treatment of goitre. However, it was not until 1914 that Kendall first purified an iodine-containing substance from thyroid extracts (Kendall 1919). The

structure of thyroxine (T_4) was determined as 3, 3', 5, 5'-tetraiodothyronine by Harington and Barger in 1927 and triiodothyronine (T_3) was discovered in thyroid extract and plasma by Gross and Pitt-Rivers and also by Roche *et al.* in 1952 (Harington and Barger 1927, Gross and Pitt-Rivers 1952). Four years later, the existence of biologically inactive compounds: 3, 3' 5'-triiodothyronine (reverse T_3 or rT_3) and 3, 3'-diiodothyronine (T_2) in thyroid tissue and plasma of rat was reported by Roche *et al.* (1956).

1.2 The Thyroid Gland

1.2.1 Anatomical Consideration

The thyroid gland is highly vascular and is located in the lower neck, positioned between the fifth cervical and the first thoracic vertebrae. Its right and left lobes are extended along the sides of the larynx, reaching the level of the middle of the thyroid cartilage. The two lobes are connected with a narrow median isthmus (cited in Gray's anatomy 1989, Kaplan 1989). A pyramidal lobe exists in 30% of thyroid glands, it projects from the isthmus near the left lobe (Ekholm 1989). The gland is enclosed by two connective tissue capsules, the outer one attaches the thyroid gland to the trachea. From the inner capsule, trabeculae of collagen fibers extend into the gland, dividing it into varying sizes and shapes of lobules. The lobules are composed of follicles. Usually the gland is bigger in the female than in male, and it enlarges during the second half of menstruation and pregnancy.

The follicle (or acinus) is the basic functional unit of the thyroid gland. It is composed of a single layer of epithelial cells enclosing a cavity, the lumen, which is filled with a viscous solution (colloid) containing thyroglobulin. Its diameter ranges from 15 to 900 μm (Ekholm 1989, O'Riordan *et al* 1985). Follicular epithelial cells are commonly squamous to cuboidal in the euthyroid state. Cells are flattened in hypoactive folicules whereas they become tall and columnar in hyperactive follicles.

The follicle is surrounded by a basement membrane; lying between this membrane and the epithelial cells are the parafollicular C-cells. These are bigger than the epithelial cells and secret calcitonin (Fujita 1988). Scanning electron microscope studies have shown that each follicle is encapsulated by a basket-like capillary network which is independent of adjacent networks. Nevertheless a few anastomoses or common capillaries are sometimes seen between follicles. This suggests that each follicle

functions somewhat independently of the others, and all cells in a follicle function synchronously. Blood vessels are localized in the interfollicular connective tissue. A delicate meshwork of reticular fibers and an extensive network of capillaries and nerve fibers surround the follicles. Endothelial cells of the capillaries are fenestrated and secreted hormones are believed to enter the capillary lumen through these fenestrae; the number and density of which change according to the functional state and secretory activity of the gland (Fujita 1988, O'Riordan *et al* 1985).

1.2.2 Nerve and Blood Supply

The major arteries of the thyroid gland are the paired superior thyroid arteries (arising from the external carotid arteries) and the paired inferior thyroid arteries (arising from the thyrocervical trunk of the subclavian arteries). A fifth artery, the *thyroida ima,* is sometimes present and arises from the arc of the aorta. It enters the thyroid at the midline (Kaplan 1989).

Superior, middle and inferior thyroid veins carry the outflow from the thyroid gland. The first two end in the internal jugular vein, and the third enters the left brachiocephalic vein. Lymph vessels are found in the interlobular connective tissue (cited in Gray's anatomy 1989). It has been estimated that the thyroid blood flow ranges from 4-6 ml/min/g of tissue, which is nearly twice that of the kidney (O'Riordan *et al* 1985).

The thyroid is innervated by sympathetic, cholinergic and peptidergic fibers. Sympathetic fibers arise from the cervical ganglia and enter with the blood vessels, terminating at both endothelial vessel cells and follicular cells. It has been reported that sympathetic nerves may stimulate the thyroid epithelial cells to secrete thyroxine (Melander *et al* 1974, Kaplan 1989). Parasympathetic fibres, deriving from the vagus, enter the thyroid with branches of the superior laryngeal and recurrent laryngeal nerves.

1.2.3 Embryology and Histology

Thyroid development begins at the third gestational week in the human as a midline thickening and out-pouching of the endodermal floor of the pharyngeal cavity. This primordium eventually forms a sac-like diverticulum which at about one month, is a solid mass of cells. By the 6th to 7th week, it is bilobed and, as the embryo elongates, it descends in the neck but remains connected to its origin by a narrow canal, called the thyroglossal duct. By the second month, the duct normally undergoes fragmentation

and disappears, leaving a pit called the foramen caecum at the junction of the middle and posterior third of the tongue.

Cells in the lower portion of the duct differentiate into thyroid tissue. By the 7th week, cells are grouped into clusters, and at about the 11th week, a central lumen appears in each cluster, surrounded by a single layer of cells (O'Riordan *et al* 1985). The lower portion of the 4th pharyngeal pouch fuses when it comes into contact with the thyroid gland forming the C cells.

The human fetal thyroid gland starts to secrete hormone at around the beginning of the fourth gestational month (70-80 days). However the fetal thyroid does not respond to pituitary-secreted thyrotropin until the 22nd week (Fisher & Klein 1981).

1.3 Biochemistry of the Thyroid Hormones

In quantitative terms the most important hormone secreted by the thyroid is thyroxine (T_4). This hormone is generally regarded as a pro-hormone, its biological potency being only one tenth of the more active compound 3, 5, 3'-triiodothyronine (T_3). The majority of plasma T_3 and rT_3 are derived from the peripheral deiodination of T_4. The structures of important iodothyronines, which are composed of two iodinated rings (inner or tyrosyl ring and outer or phenolic ring) with an alanine side chain, are shown in Figure 1.

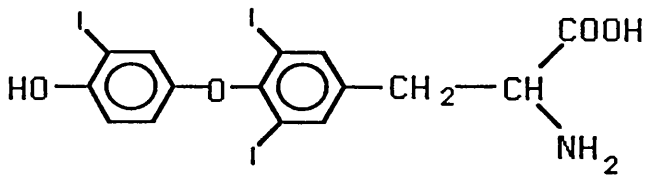
1.4 Thyroid Hormone Synthesis

Iodine is absorbed from the gastrointestinal tract in the form of inorganic iodide, concentrated in the thyroid and transformed through a series of metabolic steps into the thyroid hormones. These metabolic events can be divided into: 1) active transport of iodide, 2) oxidation of iodide and iodination of thyroglobulin, 3) coupling of iodotyrosines in the thyroglobulin molecule, 4) hydrolysis of thyroglobulin, release of thyroid hormones and iodothyronines, and secretion of thyroid hormones from the follicular cell into the blood capillaries or lymph vessels.

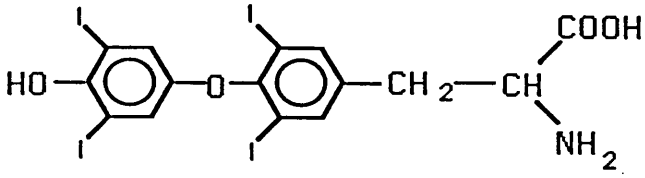
1.4.1 Iodide Transport Mechanism

The first step in the synthesis and storage of thyroid hormones is the concentration of iodide in the follicular cell, catalysed by the iodide pump (or iodide trap)(Wolff 1964,

Figure 1 : Structure of the Thyroid Hormones



3,5,3' - Triiodothyronine (T3)



Thyroxine (T4)

1972, Halmi 1961 , Bastomasky1974). Iodide taken up into the thyroid from the blood is bound in the organic form within seconds, therefore, generally less than 1% of the total glandular iodine is in the form of inorganic iodide (Ekholm 1990). The iodide pump is located in the basal plasma membrane. Iodide is pumped from the extracellular space into the follicular cell against electrical and chemical gradients, and then rapidly diffuses into the follicular lumen (Wolff 1983). It was demonstrated that iodide accumulation by sheep thyroid slices is abolished in media without Na^+ and decreased in media lacking K^+, and Wolff and Halmi (1963) showed that a ouabain-sensitive Na^+, K^+-ATPase is involved in iodide transport.

Thyroid iodide uptake is regulated by both TSH-dependent and TSH-independent mechanisms, with TSH affecting both iodide influx and efflux. Stimulation of iodide influx requires a relatively low TSH concentration, is cAMP-mediated and protein synthesis-dependent. Stimulation of iodide efflux, requires much higher TSH concentrations, is cAMP independent but Ca^{2+}-mediated.

Besides the thyroid gland, the gastric mucosa, the salivary and mammary glands, the choroid plexus and the placenta also take up iodide. Iodide concentrated by the mammary gland is converted into protein-bound monoiodotyrosine and diiodotyrosine, but not into iodothyronines. Iodotyrosines are secreted in the milk and are an important source of iodine for the infant (Greer 1990).

1.4.2 Oxidation of Iodide and Thyroglobulin Iodination

Iodide must first be converted to a higher oxidative state before it can be incorporated into thyroglobulin. Amongst biological oxidising agents, H_2O_2 is one of the most potent (Ekholm 1981). Peroxide is synthesized at the apical plasma membrane, with only NADPH and oxygen being necessary for its production (Virion *et al.* 1984). The enzyme involved in this process is thought to be a NADPH oxidase system. TSH can stimulate H_2O_2 generation, albeit at high concentrations, and Ca^{2+} is required for this process. This mechanism is cAMP-independent and is mediated via the phosphatidylinositol pathway.

Thyroid peroxidase (TPO) catalyses the oxidation of iodide to a higher oxidative state in the presence of H_2O_2. Both I^0 and I^2 are produced at neutral pH. The peroxidase also takes part in the binding of oxidized iodide to tyrosyl residues of thyroglobulin and in the coupling of iodotyrosines (Ekholm 1981).

TPO and the microsomal antigen which is involved in the autoimmune destruction

of the thyroid gland in several thyroid diseases, especially Hashimoto's thyroiditis and Grave's disease, are closely related (Czarnoka *et al*1985, Portman *et al.* 1985) The study of Libert et al (1987) has indicated that the microsomal antigen and TPO have an identical molecular structure. TPO is located in the luminal colloid, apical plasma membrane, rough endoplasmic reticulum, Golgi apparatus, and subapical secretory granules (Fujita 1988). Recently, the use of monoclonal antibodies has allowed the immunopurification of human TPO with a molecular weight (Mr) close to 100 KD (Czarnocka *et al* 1985). TSH has been demonstrated to stimulate rat TPO activity *in vivo* (Nagataki 1973, Yamamota and DeGroot 1974). This enhancement in activity is proposed to be due to a stimulatory effect of TSH on TPO biosynthesis.

A) Thyroglobulin the specific hormone forming protein

Thyroglobulin (Tg) plays an essential role in the homeostasis of the thyroid hormones and modulates hormone synthesis in relation to iodine availability. The formation of Tg allows thyroid hormone storage in a form not directly active in an extracellular space (follicular lumen). It is a very large dimeric glycoprotein, having a Mr of 660 KD and a sedimentation constant of 19S (Robbins and Rall 1960). Under conditions which do not break covalent bonds (such as raising pH, reducing the ionic strength, etc), it dissociates into equal-sized subunits of Mr 330 KD, to which two types of oligosaccharide unit (A and B), are attached (Ekholm 1990).

The iodine content of Tg is dependent on iodine intake and the species under examination. For the human, values of 0.1-1.1% (approx. 5 to 55 atoms of iodine/mole of Tg) have been reported (Taurog 1979), with a normal average physiological level of approximately 25 atoms/mole of Tg. Although the molecule has 130 tyrosyl residues, no more than 3-4 iodothyronines are formed, even in well iodinated Tg (Lissitzkey 1984). At low iodine levels, T_3 is synthesized in preference to T_4, and in cases of iodide excess, the T_4/T_3 ratio increases (Azizi 1984).

Regulation of Tg synthesis is under TSH control. Normal TSH levels induce near-maximal expression of the Tg gene. TSH controls transcription by direct interaction with the thyrocyte TSH receptors, with cAMP as the mediator (Van Heuverswyn *et al* 1985)

B) Iodination of Tyrosyl Residues in Tg

The tyrosyl residues of the Tg molecule are iodinated in the follicular lumen, especially

at the cell-colloid interface. TPO binds and oxidizes iodide into its active form, which is then transferred to the tyrosyl residue of Tg, which is itself bound at another site on TPO. Both mono-iodothyronine (MIT) and di-iodothyronine (DIT) are produced. TSH has a rapid stimulatory effect on Tg iodination which can be seen within minutes (Ahn and Rosenberg 1968). This effect may be due to both accelerated H_2O_2 generation and accelerated exocytosis of Tg (Ekholm 1990).

1.4.3 Coupling

Iodination also initiates formation of iodothyronines within Tg; no specific coupling enzyme is thought to be involved. The coupling reaction comprises 3 steps: i) oxidation of iodotyrosyl residues to an activated form within the protein matrix, catalysed by TPO, ii) coupling of two iodotyrosyl radicals to form a quinolether intermediate within the same Tg molecule, and iii) splitting of the quinolether to form an iodothyronine with a dehydroalanyl residue as a by-product (Gavaret *et al.* 1980).

The iodinated ring is ether-linked to another iodotyrosyl residue within the same Tg molecule, thus forming T_4 or T_3, depending on the identities of the two participating iodotyrosyls (MIT or DIT). Coupling does not occur randomly; T_4 or T_3 is formed in one or more limited domains of the Tg molecule which have unique amino acid sequences (Lissitzky 1984). The iodinated protein is then stored in the follicular lumen until hydrolysis and hormone release.

1.4.4 Hydrolysis of Tg and Hormone Release

The first step in thyroid hormone secretion is the reabsorption of colloid from the lumen into the follicular cell by micropinocytosis and pinocytosis. The apical cell membrane of the follicular cell is covered by microvilli and pseudopods, which function in these processes. Luminal colloid is taken up into the cell in the form of droplets, which have different shapes and sizes (0.5-5 μm in diameter)(Fujita 1988).

Two filamentous structures, the microfilaments and microtubules, present under the apical surface of the cells, are involved in colloid droplet reabsorption. Reabsorped colloid droplets containing Tg eventually link with lysosomes, their membranes fuse together and the lysosomal enzymes catalyse the liberation of T_4, T_3, iodothyronines and various kind of amino acids. Thyroxine and T_3 pass through the cytoplasmic matrix, before release into the connective tissue fluid from where they enter the fenstrated capillary bed. Iodothyronines are deiodinated in follicular cells to release

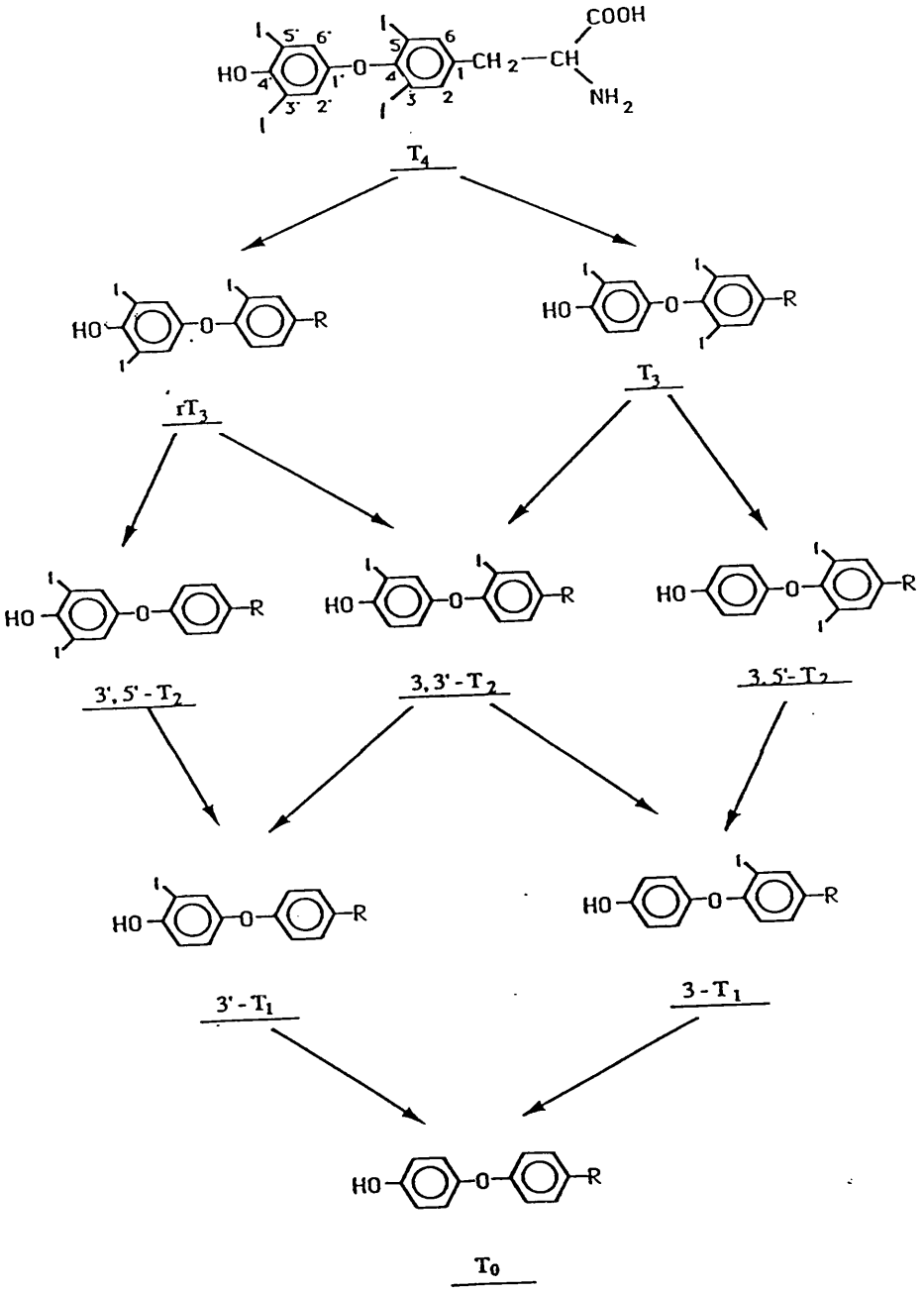
iodide which is reused by the thyroid gland. Amino acids may be partly reused for Tg synthesis and partly released into the extra cellular fluid. Proteolysis of Tg and thyroid hormone secretion are stimulated by TSH and are inhibited by iodide (Azizi 1984, O'Riordan *et al.* 1985, Fujita 1988).

1.5 Thyroid Hormone Metabolism

Deiodination is the most important pathway of iodothyronine metabolism (Figure 2). Nevertheless, nondeiodinative pathways, as minor degradative routes, should also be taken into account in thyroid hormone metabolism, especially in the disposal of T_3 and rT_3 (Burger 1986). Two distinct types of deiodinations are recognized: 5'-deiodination and 5-deiodination. 5'-Deiodination converts T_4 to T_3, and rT_3 to 3,3'-T_2. 5-deiodination, converts T_4 to rT_3, and T_3 to 3,3'-T_2 (Visser 1980). Since T_3 is biologically more potent than T4, 5'- monodeiodination may be regarded as an activating pathway, whereas 5-deiodination can be considered as an inactivating pathway in thyroid hormone metabolism. Studies have shown the existence of at least three different deiodinases enzymes involved in the monodeiodination of iodothyronines. These enzymes are distinguished by their substrate specificity and susceptibility to inhibition by PTU (Visser 1980, Leonard and Visser 1986).

On this basis, deiodinase type I is a nonselective enzyme (the iodine substrate can be situated either on the outer or inner ring) which is inhibited by PTU. Type II deiodinase only catalyzes outer ring deiodination and is a true 5'-deiodinase. Type III enzyme only deiodinates the tyrosyl ring and the substrate is exclusively 5-linked iodine. Both type II and type III enzymes are insensitive to PTU (Leonard and Visser in GH). The deiodinating enzymes have common features, e.g. a requirement for free sulfhdryl group as co-factors, though in different concentrations (Chopra 1978). Dithiothreitol (DTT) is used at various concentrations as the RSH group *in vitro*. For *in vivo* experiments, glutathione (GSH) may be the operational co-factor (Sato *et al.* 1983). It has been demonstrated, in the case of deiodinase type-I, that the proportion of glutathione in the reduced form compared to the oxidized form is crucial for enzyme activity. For a better understanding of deiodination, each enzyme type is discussed in more detail below.

Figure 2 : Sequential Deiodination of Iodothyronines



5- (↙) and 5'- (↘) deiodination. Adapted and redrawn from Visser (1980). Abbreviations as follows: thyroxine (T_4); 3, 5, 3'-triiodothyronine (T_3); 3, 5', 3'-triiodothyronine (rT_3); 3', 5'-diiodothyronine (3', 5'-T_2); 3, 3'-diiodothyronine (3, 3'-T_2); 3, 5-diiodothyronine (3, 5-T_2); 3'-monoiodothyronine (3'-T_1); 3-monoiodothyronine (3-T_1) and thyronine (T_0).

1.5.1 Type I Deiodinase Activity

5'-DI is predominantly localised in the liver, kidney and thyroid; its activity is low in rat eye, lung, pituitary, and cerebral cortex. Deiodination of T_4 to T_3 is increased in the TSH-stimulated thyroid and in thyroid tissue from patients with Grave's disease. The preferred substrate for hepatic deiodinase type I is rT_3: the maximal deiodination rate by rat kidney microsomes is 27-fold higher with rT_3 as substrate as compared with T_4 (Leonard & Rosenberg 1980). Factor(s) controlling whether type I deiodinase exhibits 5- or 5'- deiodinating activity are not yet clear. It has been proposed that in the rat, deiodination of the outer ring is maximal at a pH of 6.4, while inner ring deiodination is most active at pH 8.0 (Mol and Visser 1985). Thus, it was suggested that intracellular pH may be an important factor in determining the preferred substrate. 5'-DI Activity increases in hyperthyroidism (Kaplan and Utiger 1978), and the hepatic content of type I deiodinase in the hypothyroid rat is decreased. In humans, hyperthyroidism slightly reduces the whole body conversion rate of T4 to T3, whereas hypothyroidism increases the whole body T_4 to T_3 conversion rates.

1.5.2 Type II Deiodinase Activity

It has been estimated from several studies that only 20% of daily produced T_3 originates from thyroid secretion, the remainder (80%) being produced by deiodination of T4 in peripheral tissues (Henneman 1986). 5'-DII is thought to play a major role in this peripheral production of T_3. In mammals, 5'-DII is distributed in the central nervous system (CNS) (Visser *et al* 1981, Kaplan and Yaskoski 1980), pituitary gland (Silva 1986), brown adipose tissue (BAT)(Leonard *et al* 1983, Silva and Larsen 1983) and placenta (Kaplan and Shaw 1984).

In the CNS, cellular distribution studies have shown that 5'-DII activity is primarily associated with neurons (Leonard and Larsen 1984), albeit glial cells in culture can be induced to synthesise 5'-DII (Leonard and Galvin 1983). In the pituitary, isolated somatotrophs and lactotrophs contain the highest level of 5'-DII activity, while the thyrotrophs possess the lowest enzyme levels (Ḱoenig *et al* 1984). In human placenta 5'-DII is present in both decidual and trophoblast membranes. At the subcellular level, 5'-DII is found predominantly in the membrane bound fractions (Kaplan and Show 1984).

Insensitivity to PTU, high responsiveness to changes in plasma thyroid hormone

concentrations and a rapid increase in response to thyroidectomy are three main criteria of 5'-DII (Silva 1986). Km values for T_4 and rT_3 are 0.5-2 nM and 1.5-10 nMrespectively, as determined in the presence of 20 mM DTT. Using V max/Km ratio as an indicator of substrate specificity, T_4 is the preferred substrate (5-6 fold difference over rT_3). The enzyme is responsible for the local generation of T_3 from T_4, and is particularly active in brain and pituitary (Leonard and Visser 1986). For instance, the proportion of T_3 produced by local T_4 deiodination in various brain regions is as follows: cerebral cortex 80%, cerebellum 60% and anterior pituitary 50% (Silva 1986).

The enzyme is particularly active in brain and pituitary where it plays an important role in the local production of T_3 throughout life. In neonatal brain, where T_3 production and turnover is a very active processes, more than 85% of the T_3 content of cerebellum and cerebral cortex is produced by local deiodination of T_4. Hypothyroidism at this stage of development is associated with an increase of enzyme activity. It has been shown that the cerebrocortical conversion of T_4 to T_3 is highly regulated by a 5-8 fold increase in 5'-DII activity within 24 h after thyroidectomy, and a single injection of thyroid hormone reduces the elevated level of enzyme activity to normal within 4 h (Leonard *et al* 1984).

5'-DII activity has also been measured in fetal brain. In human cerebral cortex, 5'-DII activity increases up to 22nd week and then decreases by 25th week (Karmarkar *et al.* 1988). In the rat, the enzyme activity increases 4-fold between 17 and 22 days of gestation, with the main increase taking place between 19-21 gestational days. At birth, the cerebral T_3 concentration approaches that of the adult, despite very low circulating T_3 levels. The effects of hypothyroidism on fetal 5'-DII activity are also of interest. Combined maternal and fetal hypothyroidism, induced by giving MMI to mothers on the 12th gestational day, depletes all fetal tissues (including the thyroid) of thyroid hormones and results in a 2-3 fold increase in 5'-DII by gestational days 17/18. Treatment of MMI-treated mothers with T_4 will reverse this increase, indicating that the hypothyroid fetal brain can respond to maternal thyroid hormone (Ruiz de Ona *et al* 1988).

5'-DII activity in brown adipose tissue (BAT) is modulated by thyroid hormone and α1-adrenergic agonists. Hypothyroidism, cold stress, and injection of norepinephrine result in an 8-20 fold increase in activity (Silva and Larsen 1983).

1.5.3 Deiodinase Type III

This is an inner ring deiodinase which serves to deiodinate T_4 and T_3. Its activity has been reported in human and rat placenta and rat CNS (Kaplan &Yakoski 1980). The enzyme is located in the microsomal fraction. Iopanic acid and propranolol have inhibitory effects on human and rat placental 5'-DIII in the presence of 10 mM DTT; however, thyroid status and fasting are without effect on placental 5-deiodinase activity (Braverman 1989b).

1.5.4 Other Pathways of Thyroid Hormone Metabolism

Nondeiodinative routes are minor metabolic pathways of the thyroid hormones. Some 20% of secreted T_4 is disposed of in feces and urine, either in the free form or as conjugates with glucuronide and sulfate. Although these alternative pathways of disposal are essentially degradative, they can regulate the availability of T_3. They are briefly mentioned below:

1) Oxidative deamination, leads to the formation of acetic acid derivatives. 3,3', 5, 5'-Tetraiodothyroacetic acid (TETRAC) is formed from T_4, and 3,3',5-triiodothyroacetic acid (TRIAC) is formed from T_3. TRIAC is of potential biological importance since the nuclear receptor has a high affinity for this derivative.

2) Enzyme-catalyzed conjugation to glucuronide and sulfate occurs in the liver and kidney. Glucuronide conjugates lack biological activity and are excreted into the bile and subsequently the feces. Approximately 10-25% of T_4 is eliminated by this route in human; the fraction being somewhat larger in the case of rat.

3) Cleavage of the ether link. Hydrolysis of the ether bond is only a minor pathway of T4 degradation.

4) Decarboxylation of T_4 to thyroxamine (Visser 1980, Greenspan and Rapaport 1985).

1.6 Molecular Basis of Thyroid Hormone Action

1.6.1 Thyroid Hormone Nuclear Receptors

The nucleus as the primary site of action was first proposed by Tata and his colleagues in 1966 (Tata and Widnell 1966). Substantial progress in understanding the molecular mechanism of thyroid hormone (TH) action became possible following the identification of nuclear binding sites by Oppenheimer in 1972 (Oppenheimer *et al*

1972). There is now general agreement that most of the physiological effects of the TH are initiated by interaction with these nuclear sites. The criteria which have established these sites as nuclear receptors are summerised in Table (1).

Table (1): Receptor Characteristics of TH Nuclear Binding Sites

1	Low capacity, high affinity binding.
2	Occupancy correlates quantitatively with biological response.
3	Presence in all TH-responsive tissues at levels proportional to the TH-sensitivity of the tissue.
4	Iodothyronine affinity parallels biological potency.
5	Prompt response of nuclear-related events (modulation of transcription of TH-sensitive genes) following receptor occupancy.

Adapted from: Oppenheimer *et al.* (1974 & 1987).

The thyroid hormone nuclear receptors (THNR) from the pituitary cell lines GH1 and GC and rat liver have been extensively studied. The receptors are chromatin-associated and can be recovered by extraction with high salt concentrations (0.4 M KCl). Solubility characteristics and susceptibility to enzymatic degradation indicate that they belong to the class of non-histone proteins. The partially purified protein has a molecular weight of approximately 50 KD and a sedimentation coefficient of 3.5 S.

Thyroid hormone nuclear receptors have been reported in all vertebrate species which have detectable levels of T_3 (Oppenheimer and Schwartz 1986). The affinities of the receptor for a variety of iodothyronine analogues are similar in most tissues studied. By giving the affinity of the receptor for T_3 the value of 1, the values for other analogues are as follows:

TRIAC (3) > L-T_3 (1) > L-T_4 (0.1) = TETRAC (0.1) > L-rT_3 (0.01).

These relative affinities have been reported from binding studies with rat brain, liver and kidney, as well as a variety of cell lines in culture (Samuels *et al* 1989a & Samuels *et al* 1989b). Similar relative affinities have been reported for neuronal and glial THNR of both neonatal and adult rat brain (Hubank *et al* 1990).

Tissues containing high levels of THNR are: liver, anterior pituitary, kidney and

heart. Lung is considered to have moderate levels of the receptor, whereas low levels are found in testis and spleen (Oppenheimer *et al* 1974). Although the adult brain is often considered to posses low to moderate levels of the receptors, more recent work has demonstrated both regional and cell-specific distribution of the receptors; receptor concentrations in neuronal nuclei being greater than those of glial nuclei (Gullo *et al* 1987a and Gullo *et al* 1987b).

The ontogenesis of brain THNR has been investigated in a variety of species, including rat (Schwartz and Oppenheimer 1978, Perez-Castillo *et al* 1985, Hubank *et al* 1990) and human (Bernal and Pekonen 1984). In the rat, THNR can be detected in whole rat embryos as early as 13 days of gestation, and in brain from the 14th gestational day. The concentration of receptors increases 3-fold up to day 17, when a plateau is reached. After birth, levels then increase further, reaching a peak at postnatal day 6 (Perez-Castillo *et al* 1985, Schwartz and Oppenheimer 1978).

In the human, THNR have been reported in brain from 9-10 week old embryos. Receptor concentrations increase 10-fold by week 16. Both T_4 and T_3 are present in human fetal brain at 10 weeks; T_3 being produced locally from T_4 deiodination (plasma T_3 is not detectable until the 30th gestational week). Sufficient amounts of T_3 are present at this early stage to saturate some 25% of nuclear receptor sites, indicating that the TH can influence brain development throughout the critical period of active neuroblast multiplication (10-20 weeks) (Ferriro *et al.* 1988, Bernal and Pekonen 1984).

The use of a photoaffinity label derivative of L-[^{125}I]T_3, N-2-diazo-3,3,3-trifluoropropionyl-L-T_3, combined with SDS gel electrophoresis has led to identification of two forms of the THNR: an abundant 47 KD form and a less abundant 57 KD species. These two forms have similar synthetic rates, but different half-lives (2h for the 57 KD species and 6h for the 47 KD species). Several possibilities have been forwarded to explain these findings including the presence of a precursor protein (57 KD species), and posttranslational modification (phosphorylation) of the 47 KD species resulting in a product with aberrant migration in SDS-gels. However, following more recent studies employing molecular biological techniques, it is increasingly accepted that several different THNR proteins may be expressed in mammalian cells.

To date, at least two different THNR genes (c-erb-Aα and c-erb-Aβ) have been identified. They are related to the avian erythroblastosis virus oncogene product, v-erb-A, which is involved in the transformation of fibroblasts and erythroblasts to neoplastic

phenotypes *in vitro* (Weinberger *et al* 1986, Sap *et al* 1986). The THNR genes are localized on different chromosomes; a human homologue of the v-erb-A gene was identified on human chromosome 17 and probably 3 (Samuels *et al* 1989 & Spurr *et al* 1984). Differential splicing of each gene transcript results in at least 4 different products: $\alpha 1$, $\alpha 2$, $\beta 1$ and $\beta 2$ isoforms. However, the $\alpha 2$ isoform cannot be considered a THNR since it is unable to bind TH.

It is now recognized that the THNR's belong to a superfamily of nuclear receptor proteins, members of which are specific for progestins, oestrogens, androgens, glucocorticoids, retinoids and vitamin D. Based on the work of Krust *et al.*, the various receptors are thought to comprise at least five domains (A/B, C, D, E and F), each with a specific function as discussed below (Krust *et al* 1986).

The C region is the DNA binding domain, and this shows a high degree of homology amongst the various THNR subtypes. The C domain is rich in cysteine residues which are involved in the formation of the zinc fingers which participate in DNA binding. The E region of the receptor is the ligand binding domain, and has been suggested to influence the DNA binding activity of the receptor. The A/B region is the most variable part of the receptor and has a role in the regulation of transcription. The D domain is also variable and thought to act as a hinge region, influencing intramolecular interactions. Region F is associated with the hormone binding activity (Goldberg *et al* 1989).

The actual mechanism of TH-mediated regulation of gene expression is exceedingly complex. The THNR bind hormone at the ligand binding domain which is located in the carboxyl portion of the protein. The TH-receptor complex then binds through the zinc fingers to specific DNA sequence elements termed "hormone response elements" which are located in the 5'-flanking region of thyroid hormone responsive genes. The hormone response elements belong to the general class of cis-acting genetic elements, known as enhancers. This may result in the stimulation or inhibition of transcription of the relevant mRNAs (DeGroot 1989).

The rat growth hormone (rGH) gene contains four TH-response elements, located between positions -237 and +1 nucleotides (Lavin *et al* 1989). Thyroid hormone-responsive genes may contain sequences which are thought to be involved in mediating cell-specific expression, eg - 137 to - 107 and - 95 to - 65 in rGH.Nevertheless, the mechanisms of cell-specific expression of certain genes by TH is unclear and needs further investigation (Samuels *et al* 1989).

In the cell, TH may act at both transcriptional and posttranscriptional levels, as in the case of S_{14} mRNA expression in rat liver cells. TH administration causes an enhancement of transcription and a major increase in the steady state concentration of the mRNA (stabilizing the mRNA) (Samuels *et al* 1989). Both *in vivo* and *in vitro* experiments demonstrate that carbohydrates have a synergistic effect with T_3 in this response while insulin is without effect (Oppenheimer et al 1987).

1.6.2 THNR in Hypothyroidism

In hypothyroidism THNR concentrations increase in both neonatal, and adult rat brain (Valcana & Timiras 1978, Hamada & Yoshimasa 1983). Activity of 5'-DII is increased during hypothyroidism, thereby increasing the rate of conversion of thyroxine to T_3. This mechanism is active in the fetus from the 17th day of gestation (the earliest time studied) (Obregon et al 1989). Thus, both the increase in THNR concentration and in T3 production from T_4 may serve to compensate for a hypothyroid state in the brain.

1.6.3 Thyroid Hormone Extranuclear Receptors

In addition to nuclear receptors, a variety of extranuclear binding sites have been identified for the thyroid hormones, located in the plasma membrane, cytosol and the mitochondrion.

Plasma membrane binding sites have been reported in many cell types and tissues of different species, such as: rat liver and kidney, human placenta, rat thymocytes and in rat pituitary GH3 cells in culture (Gharbi-Chihi & Torresani 1981). Two distinct sites have been identified, one of high-affinity, low-capacity binding and the other of high-capacity low-affinity TH binding (Segal and Ingbar 1986). Since these binding sites may be important in determining the intracellular TH concentration, they may play a role in the cell response to TH. Uptake of glucose, amino acids and nucleosides may also be influenced directly by membrane binding sites (Segal and Ingbar 1986).

Cytosolic thyroid hormone binding sites have also been reported in different tissues, for instance, rat heart, rat kidney (Nishii *et al* 1989) and human carcinoma cell lines (Obata *et al* 1989). Although the function is unclear, cytosolic binding sites may act to regulate thyroid hormone availability to cellular organelles, including the nucleus.

The presence of specific thyroid hormone binding sites located on the inner membrane of the mitochondrion was first demonstrated by Sterling and his colleagues. They observed that injection of hypothyroid rats with thyroid hormone immediately

increased oxygen consumption and ATP synthesis in liver mitochondria, independent of protein synthesis (Sterling *et al* 1980). Mitochondrial binding sites are present in neonatal rat brain up to 12 days but cannot be detected in brains of older neonatal (14 and 17 days) or adult rats. It was proposed by Segal and Ingbar that TH directly act on mitochondria and increase oxidation and oxidative phosphorylation (Segal and Ingbar 1986).

1.7 Biological Effects of the Thyroid Hormones

A variety of clinical studies and biochemical investigations in human and experimental animals have revealed that many of the body systems are under TH control. Nevertheless, different tissues exhibit different TH dependencies. The spleen, testis and adult brain are often considered to be TH-insensitive tissues, whereas the liver, kidney and skeleton are among the most sensitive. This latter category of TH-responsive tissue are considered briefly bellow. TH effects on the developing brain are considered in more detail in the next section because of this relevance to the proposed study. In order to understand the various defects that are related to thyroid hormones, it is useful to bear in mind the more important effects of TH on the organism. Thyroid hormones exert a range of effects on different aspects of cellular growth and development; they affect calorigenesis, protein synthesis, cell growth and maturation; they regulate amino acid, carbohydrate and electrolyte transport into the cells, affect carbohydrate and lipid metabolism, neurotransmission, and finally interact with other hormones and specific drugs (Burgi and Konig 1988).

1.7.1 The Peripheral Nervous System (PNS)

A delay in the maturation of neuromuscular synapses in the heart and iris as well as the diaphragm have been reported in hypothyroidism (Timiras 1988,1989). Alterations in the action of the autonomic nervous system (e.g. low body temperature, constipation and dryness of skin) have been recognized as one of the first symptoms of congenital hypothyroidism (Letarte & Franchi 1983). On the other hand, hyperthyroidism causes motor hyperactivity, tachycardia, excessive sweating and dyspnea (cited in Nelson 1987). In sensory organs, varying degrees of deafness are common both in cretinism and congenital hypothyroidism (Stanbury 1972, Trotter 1960). Deafness is thought to be due to the impairment of hair cells in the organ of Corti, which is susceptible to TH

during its maturation. In the hypothyroid rat, the maturation of medial efferent fibres to outer hair cells is delayed, and efferent fibres and postsynaptic terminals are impaired. Dysgenesis of the cochlear nerve may also be present (Pujol and Uziel 1988).

1.8 The Influence of Thyroid Hormones on the Brain

The role of TH in the development of the nervous system has been recognized for a long time. This relationship in the human, in situations like hypothyroidism and cretinism, has been established for more than a century. Animal studies have also demonstrated that TH deficiency during critical periods of development significantly impairs the morphological, biochemical, functional and behavioral maturation of the CNS.

Before considering the effects of TH on the developing brain, it is first essential to review normal brain development. Many studies suggest that during the period of the "growth spurt" the brain is particularly vulnerable to environmental modifications. Nevertheless, the period preceding the brain growth spurt is also vulnerable to environmental factors, of which TH deficiency is the most important (Dobbing and Smart 1974, Morreale de Escobar *et al* 1983).

1.8.1 Brain Development

The gross shape of the human brain is determined during the first trimester of pregnancy. The initial stimulus to form the CNS occurs during gastrulation. Induction results in the formation of the neural plate at approximately 2-3 weeks of gestation. This step is followed by neurulation, when a closed cylinder of cells (neural tube) is formed from the neural plate at 3-6 weeks after implantation (Lou 1982). The germinal matrix that surrounds the neural tube is called the neuroepithelium. From the neural tube, several vesicles (extensions) are formed: the embryonic myelencephalon, rhombencephalon, mesencephalon, diencephalon and telencephalon (Altman & Bayer 1988).

From the primary neuroepithelium, the secondary germinal matrices are derived. Both the primary neuroepithelium and/or the secondary germinal matrices are the sites of cell proliferation. Cells that leave these germinal layers lose their proliferative capacity. For instance, in this way neuroblasts, the proliferative precursor cells, change

into immature or differentiating neurons (neurogenesis). Immature neural cells migrate from their sites of origin to their final location. This phase is called migration. The fourth phase in the development of neural cells in CNS is the maturation of settled cells, and this includes the growth of axons and dendrites as well as synaptogenesis.

Neural development as a whole, in addition to general growth related process, includes specific characteristic events, such as synaptogenesis, development of electrical activity, neurotransmitter formation and myelinogenesis. Every small region in brain follows an accurate planned developmental sequence. The timing of specific events (i.e. differentiation of neurons), may differ for each region of the brain. However, the timing of one event (as well as its sequential maturation) in a specific region may affect the development of other events in other regions (Prestige 1974).

The most active phase of neuroblast multiplication in the human forebrain is between 10-18 weeks of gestation, when neuronal cell numbers approach adult levels. Active neuroblast multiplication, coincides with the commencement of the brain growth spurt, which is the phase of rapid increase in brain weight. This active phase precedes the increase in the circulating T_4 level (Dobbing and Smart 1974, Altman and Bayer 1988). Abnormal circumstances may thus affect the fetus at this early stage of development. The number of neurons in the brain (with the exception of the cerebellum) do not increase after midgestation; however, the growth and development of axonal and dendritic processes contribute to the increase in brain weight.

With respect to the glia, astrocyte progenitor cells can be detected as early as 10 weeks of gestation, at least in the optic nerve of human fetus (Fedoroff & Doucette 1988). Fibrous astrocytes in the brain stem have been detected at the 14th week of gestation (Stagard & Mollgard 1989). Progenitors of oligodendroglial cells have also been reported in early pregnancy, during the first half of the brain growth spurt. The increase in brain weight is due to extensive multiplication of the glial cell population as well as deposition of protein and lipids on the neurons and glia. According to Dobbing and Smart, the brain growth spurt starts at about midgestation in human and is ended by about 3-4 postnatal years. In rats, it begins at birth and is over by 25 postnatal days (Dobbing and Smart 1974).

In the human, total brain weight and total numbers of glia increase rapidly during the first 6 months of postnatal life (though, most of the cerebellar growth spurt is postnatal). Glial cells significantly increase in brain weight by increasing their numbers, as well as by differentiation (Stagard and Mollgard 1989). Myelin formation in the

CNS varies with the region. In general, myelination of spinal cord matures first during the prenatal period and that of higher cortical areas later, extending into the period of adolescence. Myelination in brain proceeds rapidly from birth to 6 months and remains in a steady state of increase until about 10 years. It then slows down but still continues until 15 to 20 years of life (Einstein & Adams 1988).

In the human, cerebellum growth and maturation start much later than those of the forebrain. The growth spurt in the cerebellum takes place in a short time; thus the rate of cerebellar growth is very high. The cerebellum is therefore highly vulnerable to insults during the early postnatal period. For example, neonatal hypothyroidism results in a cerebellum that is smaller than normal, with undifferentiated neurons, decreased synaptic density and an excess of glial cells (Smith 1981). In rat cerebellum, only 3% of the final cell number are present at birth, and over 90% of the microneurons in the cerebellar cortex originate after birth. In the adult rat cerebellar cortex, three layers are seen: molecular, Purkinje and granule cell layers. Purkinje cells are the largest neurons in the cerebellar cortex, and provide the only efferent output of the cerebellum (via the cerebellar subcortical nuclei and the lateral vestibular nucleus). They are formed prenatally, and arise in to primary germinal zone and migrate to a subcortical position a few days after birth (Smith 1981).

The cerebellar cortex has three populations of smaller neurons. The most numerous are the granule cells, over 50% of which are formed during the second and early third week. Basket cells are formed during the end of the first week, and stellate cells during the beginning of the second week (Altman & Bayer 1988). The dendrites of the granule cells establish contacts with the afferent (mossy) fibers, and their axons split into T shapes, forming the parallel fibers, and contact with Purkinje cells to make the "wiring pattern" of the adult rat cerebellum. Basket cell perikarya are located in the molecular layer, their axons wrap around the dendrites of the Purkinje cells. The stellate cells are located in the upper part of the molecular layer, the shape of their axon is variable but they preferentially contact the secondary branches of Purkinje cell dendrites (Altman and Bayer 1988).

1.8.2 Effects of Thyroid Hormones on the Developing Brain

The role of TH in the development and maturation of the CNS has been extensively studied by the employment of different animal models, organ and tissue culture systems

and by clinical observations (Timiras 1988, Morreale de Escobar *et al* 1986). Nevertheless, a few reports from studies of the human fetus (Liu *et al* 1989) and adult cretins (Yuqin *et al* 1988) are available in the literature

A Morphological Effects

Hypothyroidism during development results in a spectrum of morphological defects in the brains of all vertebrate species so far studied. The frequency of neural lesions such as open neuropore and microencephaly are increased in rat embryos cultured in hypothyroid serum (Harakawa *et al* 1989). Generally a reduction in whole brain weight is observed, concurrent with a decrease in cell number and cell size (Nunez 1984b, Mano *et al* 1989). Hypoplasticity of the neuropil is apparent in cerebral cortex, cerebellum and hippocampus. The number and length of dendrites are reduced, and the degree of branching is limited. Axonal development is affected, in particular the axonal length is reduced, with consequent impairments in inter-neuronal connections, wiring patterns and electrical activity (Nunez 1984b, Smith 1981). Hypothyroidism also results in impaired synaptogenesis in all regions of the brain, either directly, or as a consequence of the hypoplastic neuropil (Timiras 1988). Thyroid hormones may contribute to reductions in neurotransmitter levels and their metabolic enzymes. The specificity of receptors may alter in a region-specific manner; for instance, TH may cause an increase in muscarinic receptors in the cortex, but not in the cerebellum (Nunez 1984b).

Balazs *et al.* reported that in the rat, thyroidectomy at birth does not affect the number of cells in cortical areas; however cell size is reduced (Balazs *et al* 1968). This finding is consistent with the fact that the majority of forebrain neurogenesis is completed by birth, but it appears that the smaller cells are densely packed in the thyroidectomized rats. The length and branching of dendrites of pyramidal neurons, the density of axon terminals and the number of dendritic spines are also reduced in hypothyroid animals. In brief, there is hypoplasia of the neuropil (Potter *et al* 1982, Timiras 1988). Thyroid hormones enlarge cell bodies of proliferating cells, but shrink those of other cells, like Mauthner's neurons which normally regress (Timiras 1989). Each group of neurons responds to the endocrine signals in a distinctive fashion.

The differentiation of apical spines in pyramidal cells from the visual and auditory cortices has been employed as the bases of quantitative studies of the effects of hypothyroidism on the development of cerebral cortex in rats (Ruiz-Marcos *et al*

1983). Thyroidectomy impaires the normal development of spines; the increase in the total number of spines along the apical shafts that occurs between 10 and 30 days is reduced following thyroidectomy. In another report, after thyroidectomy at day 10, there was no further increase in spines after 25 days of age, and spines remained at 60% of the level of the normal control (Morreale de Escobar *et al* 1983). These results demonstrate that thyroidectomy in the neonatal rat produces a general derangement of connectivity of primary areas of the cerebral cortex, with those shafts that are located in the superficial layers of the cortex are more severely affected. Thyroxine replacement therapy can alleviate this damage, if started before day 12. If treatment was delayed until 20 days, then damage was irreversible. Thus, a critical period for TH action on the pyramidal cell exists in rat between approximately 10-20 postnatal days (Morreale de Escobar *et al* 1986).

The effects of thyroid hormones on cerebellar development have been extensively investigated and adequately reviewed in recent years (see Legrand 1967, Balazs *et al* 1968, Smith 1981, Morreale de Escobar *et al* 1983, Dussult 1987, Lauder 1989 and Dussaut 1989). Thyroidectomy at birth results in a marked reduction in both cerebellar weight and the migration of the cells from the external granular layer (EGL) at 14 days (Balazs *et al* 1968). Ramification of the Purkinjie cells is also reduced (Legrand 1967). In hypothyroidism, the EGL proliferates for a longer period of time, the effect occurring at the level of mitosis (inhibition of cleavage). The rate of granule cell migration through the molecular layer and the growth of parallel fibers are also significantly reduced by hypothyroidism (Lauder 1989).

Hypothyroidism also affects the density and total number of synapses. Synapses of parallel fibers with the Purkinje cell are reduced and there is an increased rate of cell death in the internal granular layer. In contrast, hyperthyroidism leads to an earlier disappearance of the EGL, partly due to accelerated cell proliferation, parallel fiber growth and synaptogenesis in the molecular layer. Ultimately both hypo- and hyperthyroidism lead to significant reductions in the total number of synapses in the cerebellum (Lauder 1989). The number of Purkinjie cells (the only efferent neurons of the cerebellar cortex) is not reduced by hypothyroidism, but their maturation is permanently affected. Arborization and dendritic spine formation are markedly reduced. Thus, permanent hypoplasia with morphological changes are evident in the cerebellum (Morreale de Escobar *et al* 1983, Dussalt 1989).

The hippocampus, like the cerebellum, undergoes a large part of its development

after birth. In the normal hippocampus, the volume of the cell bodies and the number of branching points on the apical and basal dendrites of pyramidal cells increase between 6 to 10 postnatal days in the rat. Hypothyroidism causes impairment of dendritic arborization of both granule and pyramidal cells (Rami et al 1986). In the case of pyramidal cell dendrites, the damage is more severe for those that receive input from granule cell axons (the mossy fibers). Interestingly, those cells which are more affected by TH deficiency are more sensitive to T4 treatment (Rami *et al* 1986). Hyperthyroidism also affects the growth of mossy fibers and results in inappropriate synapse formation, with consequent functional and behavioral impairments (Lauder 1989)

B Biochemical Effects of Thyroid Hormones in the CNS

Thyroid hormones control a large number of biochemical systems in the developing brain. Alterations in DNA and RNA contents have been observed in thyroidectomized rats. The DNA concentration (per/g wet weight) in the developing brain is highest at the first week and then gradually decreases, reflecting a reduction in the neuronal cell density due to the development of neuropil. This process is slowed in thyroidectomized neonates (Geel and Timiras 1967, Balazs *et al* 1968). In normal animals, the cellular RNA content rises sharply in the first week of postnatal life and the concentration of protein in the brain also rises during the first 30 days. Neonatal thyroidectomy results in a severe reduction in both RNA and protein concentrations in developing brain. The amount of protein and the RNA/DNA ratio are also significantly reduced both in cerebral cortex and cerebellum as a consequence of neonatal thyroidectomy (Balazs *et al* 1968). This effect on protein synthesis is most probably mediated at both the transcriptional and translational levels in varying degrees. However, alterations in the supply of amino acids (resulting from alterations in membrane permeability/blood-brain barrier exchange events) may play a role (Geel *et al* 1967).

Amino acid metabolism is generally decreased in hypothyroid rat brain as demonstrated by incorporation of [^{14}C]leucine into protein and lipids (Balazs et al 1973). Changes in amino acid metabolism may be involved in the impact of hypothyroidism on neurotransmitters (Timiras 1988). Certain free amino acids are precursors for the neurotransmitter systems; tyrosine in the case of catecholamines and tryptophan in the case of serotonin. On the other hand, glutamate, GABA, and glycine all play direct roles in neurotransmission. Neurotransmitters are thought to play a direct

role in neurogenesis, since their presence has been demonstrated during embryogenesis prior to the onset of synaptogenesis or neurotranmission, in both simple (sea urchin) and complex (rodents) organisms. Also, pharmacological agents that affect the synthesis, uptake, receptor interaction or metabolism of neurotransmitters produce defects in embryological development and neurogenesis (Lauder 1988). A trophic role has been suggested for certain neurotransmitters e.g GABA (Lauder 1988) and acetylcholine (Clos *et al* 1989). Indeed, the neurotransmitters are thought to play important roles in developmental processes such as: neural tube formation, cell proliferation and the onset of neuronal differentiation in the neural tube, postnatal cell proliferation, postnatal neuro- and gliogenesis, and finally axonal growth and synaptogenesis. Consequently, changes in neurotransmitter metabolism due to an altered thyroid state, may indirectly impinge upon such critical developmental processes.

In general, the establishment of neurotransmitter systems is retarded by hypothyroidism. The TH are thought to influence neurotransmission at several levels, including: the number and structure of synaptosomes, the activity of the metabolic (synthetic and catabolic) enzymes and the number and binding affinities of their receptors (Timiras 1988). TH deficiency has been reported to decrease the number of catecholamine (β-adrenergic), muscarinic acetylcholine and GABA receptors in neonatal brain (Smith *et al* 1980). A reduction in the enzyme activities related to neurotransmitter metabolism has also been reported. For instance, GABA transaminase, acetylcholine esterase, glutamate decarboxylase and monoamine oxidase activities are reduced by hypothyroidism (Balazs 1968, Vaccarei et al 1983). In a recent report, hypothyroidism in the rat has been shown to reduce the total choline acetyl transferase activity, whereas total acethylcholine esterase activity is unaffected (Clos *et al* 1989).

Thyroid hormone also affects the general metabolic enzymes. The activities of glucose-6-phosphate dehydrogenase, succinic dehydrogenase, aspartate aminotransferase, and neuronal acid phosphatase have all been shown to decrease in hypothyroidism. Hypothyroidism decreases Na^+, K^+-ATPase activity but not Mg^{2+}-ATPase activity. Alanine aminotransferase and lactate dehydrogenase activities do not change in hypothyroidism, whilst total acid phosphatase activity increases. Energy metabolism including glucose uptake and oxygen consumption are also significantly reduced in hypothyroidism, particularly in the cerebral cortex (Ford and Cramer 1977,

Timiras 1988).

Thyroid hormones play a major role in the control of myelination (both "maturational" and "growth" process of myelination) (Rosman & Malone 1977). Under hypothyroid conditions, most brain regions (including cerebellum) show a reduction in the level of myelin constituents such as cerebroside, sulfatide, phospholipid and cholesterol with associated deficits in myelin metabolic enzymes (Balazs *et al* 1969). Early postnatal hypothyroidism is associated with a delay in myelinogenesis which may be due to a reduced numbers and function of oligodendroglial cells. However, at later ages, the oligodendrocytes attain the expected concentration and finally the myelin formation capability. Nevertheless, temporary changes in myelinogenesis may bring about a desynchronisation in the normal pattern of development.

Thyroid hormones also control the formation of the cytoskeleton. Microtubules, microfilaments and the intermediary filaments are the three major components of the cytoskeleton. Microtubules which are composed of β tubulin and several microtubule associated proteins (MAPs), are present in the linear structure of the axon and the dendrite (Nunez 1984a). Tubulin units aggregate in a long tube in an assembly and disassembly equilibrium. Hypothyroidism slows the rate of assembly, and treatment with thyroid hormones restores the normal rate of assembly and microtubule stabilization. The composition and activity of MAPs change markedly during brain development and TH show clear effects on these changes (Nunez 1984a, Timiras 1988).

C Functional and Behavioral Effects of Thyroid Hormones

Deficits in synaptogenesis, neurotransmitter system development and myelinogenesis lead to alterations in the development of electrical activity. This is reflected in the human by EEG abnormalities and neurological defects, such as the persistence of infantile reflexes, late acquisition and hypofunctional reflexes, deafness, speech disorders and several defects in neuromuscular co-ordination (Timiras 1989). Hypothyroidism results in the late maturation of many inhibitory control systems and thus contributes to behavioral abnormalities including decreased stress response, motor activity, exploration and learning difficulties.

In congenitally hypothyroid children detected by newborn screening, the impairment of several cognitive functions has been reported. Perceptual-motor, visuospatial, and language functions were most affected. Although hypothyroid

children performed within the normal range, their scores were significantly lower than that of the control group (Rovet *et al* 1987). Deficits in co-ordination, fine manipulation and verbal scores were more frequent in congenitally hypothyroid children. The frequency of impairments was related to the prenatal onset of hypothyroidism as well as to the intensity of thyroid deficit at the time of diagnosis (Rochccioli *et al* 1989, Farriaux *et al* 1989). In animal experiments, the severity of neurological dysfunctions are related to the intensity of deprivation (or excess) of TH during critical period of brain development. In the neonatal rat, PTU-induced hypothyroidism delays evoked cochlear electrical activity, which can be prevented by thyroxine therapy only during a critical period extending from 3 days before birth to 5-10 postnatal days (Dussault & Ruel 1987). In the human, this critical period for treatment of congenital hypothyroidism occurs between postnatal week two to four. Thyroxine replacement therapy, even in this critical period, may not be totally successful due to varying degrees of severity of TH deficiency and different ages of prenatal onset (Timiras 1989). Hypothyroidism may also affect the acquisition of a behavioral response. PTU-induced hypothyroidism in the rat results in severe reduction in the acquisition of immobile responses. However, it can be reversed completely 5-10 minutes after thyroxine administration at physiological doses (Jefferys and Funder 1989). In another report, it was shown that the offspring of thyroidectomized rat dams had significantly increased hyper activity; and a lack of memory to traverse the maze (Hendrich *et al* 1984).

In summary, it appears that thyroid hormones exert influences upon a wide range of interrelated processes during brain development. Critical periods for TH effects are region and probably cell type-specific, and it is likely that the thyroid hormones affect most stages of brain development.

1.9 Clinical Considerations

Thyroid hormone deficiency or excess can cause a spectrum of diseases in human and other mammals, with different clinical manifestations, affecting different stages of life, from the fetus to the adult. Cretinism and thyrotoxicosis are two extreme ends of this spectrum; others include stillbirth, spontaneous abortion, sporadic and endemic goitre and congenital hypothyroidism.

1.9.1 Goitre

Goitre is an enlargement of thyroid gland, it can be classified in different ways. Goitre is sporadic or endemic, toxic or non-toxic and nodular or diffuse. Goitrous subjects may be euthyroid, hypothyroid or hyperthyroid according to the functional state of their thyroid (cited in Behrman *et al* 1987, Thomas and Williams 1988). Goitre with its associated disorders is still a public health problem. Some 5% of the total world population are affected by goitre. Sporadic goitre is associated with autoimmune thyroiditis, hypothyroidism, hyperthyroidism, and possibly with thyroid carcinoma.

TSH is the main factor stimulating the growth of the thyroid epithelial cells (goitre producing effect). Other growth factors such as IGF-I (which modulates TSH induced growth) (Williams *et al* 1987), epidermal growth factor and fibroblast growth factor may also stimulate thyroid growth directly (Roger and Dumont 1984).

The following goitre grading system was recommended by the WHO-PAHO, which classifies 4 grads:

Grade 0	no palpable or visible goitre, even with the neck in extended position.
Grade 1	subjects with a palpable goitre.
A	goitre detected only by palpation.
B	goitre palpable and visible in extended neck.
Grade 2	visible with the neck in normal position.
Grade 3	very large goitre visible from a considerable distance.

A) Endemic Goiter

Endemic goitre is defined by the WHO recommendations as: more than 10% prevalence of goiter in a defined geographical area in the whole population or in 6-12 year old children. In iodine deficient regions, the level of urinary iodine excretion is an excellent indicator of plasma iodine concentration and thyroidal iodide uptake, especially in the absence of factors such as thiocyanate. The daily iodine required by the adult is in the range of 100-200 μg (50 μg/day in infant), but this requirement rises in pregnancy and during lactation Several endemias have been reported throughout the world and goitre is still a major public health problem for all ages from fetus to adult (Liu *et al* 1989, Lopez *et al* 1990, Hetzel 1990). Liu *et al* found goitre in 43.3% of the fetuses in an endemic region in China before iodine supplementation. In a similar report from the province of Andalucia in Spain, 34% of school children were found to be goitrous and

in another report from Boyer-Ahmad province in Iran it was demonstrated that 95% of females and 87% of males over 6 years of age were goitrous (Kimiagar *et al* 1989).

1.9.2 Endemic Cretinism

Cretinism was first described by European authors in the 17th and 18th centuries, the most comprehensive report being given by Fodree (1800). In those early reports, cretinism was found to be localised in strict geographical areas (mountainous valleys) and most inhabitants had goitre (Trotter 1960, Konig 1981). Cretinism can be classified into sporadic cretinism, which will be described later, and endemic cretinism. The condition of endemic cretinism, as defined by the Pan American Health Organization (PAHO), consists of three major features:

Epidemiology: endemic cretinism is associated with severe iodine deficiency and endemic goitre.

Clinical manifestation: endemic cretinism comprises mental retardation together with either a predominant neurological syndrome (consisting of deaf-mutism and characteristic disorders of stance and gait), or a predominant hypothyroidism with stunted growth. It is noteworthy that in some regions one form may predominate, whereas in other areas a mixed type may be seen.

Prevention: endemic cretinism can be prevented by iodine supplementation of mothers especially before conception or within the first trimester of pregnancy (Delange *et al* 1981, Pharoah and Connolly 1989).

A) Epidemiology

Iodine deficiency as the main etiopathological factor in association with endemic cretinism, is now recognized as a major international health problem (Hetzel 1989). Iodine deficient areas are scattered throughout the world, especially in Asia, Africa, South America and certain countries in western Europe including: Germany, Austria, France, The Netherlands, Switzerland and Spain. In these European countries, although iodine deficiency still persists, endemic cretinism no longer exists (Picardet 1990). Iodine deficient regions with endemic goiter and endemic cretinism were recently reported in Turkey and the Middle East (Kologlu 1990).

In all, approximately one billion people live in iodine deficient areas, and are therefore at risk from iodine deficiency disorders (IDD). China has more than 300

million at risk, India 200 million and Africa at least 100 million (Hetzel 1990). In a report from Asia, Hetzel estimated that approximately 280 million people are at risk from IDD, with 100 million suffering from goitre and up to 16 million mental defectives. Of the latter, 4 million can be classified as cretins (Hetzel 1990).

B) Clinical Features

Endemic cretinism was originally classified into types: neurological and myxoedematous by McCarrison, following his work in the Chitral and Gilgit valleys in the Himalayas (McCarrison 1908). His clinical description of neurological cretinism was somewhat complete; consisting of deaf-mutism, mental retardation, a congenital diplegia and a spastic rigidity, especially affecting the lower limbs, with a characteristic gait. Nystagmus and strabismus were evident in some cases in his report (McCarrison 1908).

Neurological cretinism is the most common type in all parts of the world: Alpean Europe, South America, Papua New Guinea, Indonesia, China and India. In contrast, the myxoedematous type predominates in Zaire (Hetzel 1986). This may be due to the high intake of cassava (contains goitrogens) or selenium deficiency (Goyens *et al* 1987). A mixed type that displays features of both neurological and myxoedematous cretinism has also been reported (Boyages *et al* 1988, Chaouki *et al* 1988). Besides these a subclinical type of cretin in various endemias in China has also been reported (Ma *et al* 1989)

C) Neurological Cretinism

This type of cretinism is prevalent in most iodine-deficient endemias. Neurological cretinism is characterised by mental retardation, deaf-mutism, disorders of gait and stance and strabismus with absence of clinical hypothyroidism (Trotter 1960, 64, Delange 1981, Boyages *et al* 1988, Chaouki *et al* 1988).

Mental deficiency is present in all neurological cretins and it appears that intellectual deficits are generalized. Most areas of mental function such as: language, reasoning, visuomotor integration, visuospatial and motor planning are affected, with the exception of memory which is relatively well spared in moderate neurological cretinism (Delong 1989, Ma *et al* 1989). In very severe neurological cretins, a kind of autism is evident and very poor social responsiveness and motivated activity have been reported.

A characteristic gait and stance has been noted for cretins by different investigators.

Most obvious features of this are broadening of the base and knock-knees. Legs are hold apart from each other and the knees and hips partially flexed. Spastic dipligia sometimes has been used to describe the gait disorders. Internal rotation of the hip, flat and everted feet and flexed knees have also been reported (Pharoah 1972, Halpern 1989). The upper limbs are less affected and muscle tone is increased in different muscle groups. In infants, head control is poor and in children the stance is not upright. Truncal rigidity, extended and immobile fingers are prevalent in cretins.

Deaf-mutism is frequent in almost all cretins. In a report from an iodine-deficient endemia in China, 95% of cretins had different degrees of hearing defects in association with deficits in speech (Ma *et al* 1989). Abnormalities of the middle ear including hypertrophic bone changes of promontorium, deformation of the ossicles, distortions of the oval and round windows and thickening of the mucous membrane of the tympanic cavity have been reported (Koenig and Neiger 1972). Impairement of vestibular function is also frequent in neurological cretins. In the human the formation of the cochlea of the inner ear, occurs between 10-18 weeks of gestation when it is particularly vulnerable to thyroid hormone deficit. Nonetheless, no data with regard to defects of the inner ear in neurological cretinism are available.

Strabismus occurs frequently in neurological cretins. It has been usually reported in more severely affected patients (Pharoah 1972). Usually it is an internal squint and may alternate between both eyes. In a recent report, 47% of neurological cretins in an iodine-deficient endemic area in China were reported to have strabismus (Halpern 1989).

It has been demonstrated that correction of iodine deficiency in female resident in iodine-deficient regions can prevent neurological cretinism in their offspring, providing that treatment is started before conception or during early pregnancy. This observation was indicating to a direct role of elemental iodine *per se* on brain development of fetus, and it was suggested that iodine itself and not thyroid hormone is involved in the pathogenesis of neurological cretinism (Pharoah 1971). More recent work has demonstrated that maternal hypothyroxinemia is frequent in endemic areas and it has been associated with an increased incidence of morbidity and mortality in offspring. Maternal hypothyroxinemia has also been correlated with neurological cretinism, since this type of cretinism results from hypothyroxinemia during the first half of gestation before the onset of independent fetal thyroid function, when the only source of TH for the fetus is the mother (Delong 1989, Pharoah and Connolly 1989, Chaouki *et al*

1988).

D) Myxoedematous Cretinism (MC)

Myxoedematous cretinism is predominant in central Africa (Zaire) and the western part of China. The main features are mental retardation and hypothyroidism with its consequences (especially stunted growth). Deaf-mutism and goitre are also prevalent. In Zaire, the prevalence of goitre was reported as 55% of the total population, with 3% of the population being cretins. Of the cretins studied, 97% (175 out of 181) were of the myxoedematous type (Delange *et al* 1981).

Clinical symptoms of the myxoedematous type have been reported as: thickened scaly skin, soft ductile cartilages of the nose and ears, loss of the lateral third of the eyebrow, coarse and dry hair and bradycardia, occasionally with hypotension and drowsiness. In a report from China, saddle nose was often present and the goitre frequency was 12%. In comparison with neurological cretins, myxoedematous and mixed type cretins are of a shorter height. Sexual development is delayed in both sexes. Testicular volume was less than the normal average for age in 6 out of 20 hypothyroid male cretins. Bone maturation delay (chronological age minus radiological age) is evident in myxoedematos cretins and positively correlates with serum TSH concentrations (Boyages *et al* 1988, Goyens *et al* 1987). Myxoedematous cretinism is more frequent in female than in male subjects.

E) Mixed Type Cretinism

Mental retardation, neurological abnormalities and several symptoms of hypothyroidism are prevalent in mixed cretinism. It has been suggested that endemic cretinism is a syndrome with a spectrum of neurological and myxoedematous signs, with typical neurological cretinism at one extremity of this spectrum and typical myxoedematous cretinism at the other. The majority of cretins fall somewhere between these two extremities (Ma *et al* 1989, Boyages *et al* 1988).

F) Subcretinism

Subcretins are generally shorter in height than normal, their school performance is poor, their IQ is subnormal and hearing difficulties are sometimes observed. The main criteria for subcretinism are:

a) subclinical mental retardation and mild psychomotor defect.

b) subclinical hearing impairment.

c) mild stunted growth.

d) chemical hypothyroidism (only detectable by hormone assays).

It was reported that in a severe endemic area in China, the incidence of cretinism was 21% and that of subcretinism 41% (Ma *et al* 1989)

G) Iodine Supplementation

It was demonstrated by Marine and Kimple in 1921 that iodine prophylaxis could benefit endemic goitre. Prophylaxis with iodinated salt was commenced in Switzerland and the USA. The incidence of deaf-mutism in Switzerland started to fall after iodine prophylaxis from a level of 1.2-1.7 per 1000 births between 1915 and 1922 to 0.4 per 1000 birth by 1925. By that time only 23% of the salt used had been iodized (Trotter 1960). Goitre prevalence declined from 55% to 6.1% in school children within 4 years after iodine supplementation of water supply to 13000 inhabitants in north eastern Sicily. In a recent report, the prevalence of goitre in school children in Finland has been shown to be decreased from over 30% in the 1940s to around 1% by the end of the 1970s as a result of a salt iodization programme. Goitre in newborns has now been eradicated in Finland (Lamberg *et al* 1990) . Kochupillai and Mahajan demonstrated that in an iodine deficient area of India, iodized salt is more effective than iodized oil in the prevention of neonatal hypothyroidism (Kochupillai and Mahajan 1990).

A single injection of iodized oil for supplementation of iodine was first established by McCullogh in New Guinea in 1957. Following trials in the highlands of Papua New Guinea, South America and Africa, it was demonstrated that 2-4 ml of iodized oil could correct iodine deficiency for a period of 3-5 years, resulting in the prevention of goitre prevalence, cretinism and other IDD (Pharoah *et al* 1971, Eastman *et al* 1988). Iodized oil administration to pregnant women before conception or during the first trimester of gestation can prevent endemic cretinism, endemic goitre and endemic neonatal hypothyroidism in children. Children of treated mothers have better school performance and perform better in tests of neuromotor function (Fierro-Benitez *et al* 1986, 1989, Connolly *et al* 1989).

The effect of iodized oil on the correction of hypothyroidism in children and adults seems to be time dependent. Vanderpas *et al.* reported that iodine supplementation could restore a biochemical euthyroid state in cretins, only in children less than 4 years old. In some older children, hypothyroidism can also be corrected but many do not

respond to the replacement therapy (Vanderpas *et al* 1986). This finding was confirmed later by Boyages *et al.*, who showed that iodine supplementation cannot reverse thyroid hormone deficiency or its sequelae in adolescents and adults (14-52 year) with the myxoedematous condition. They suggested that iodized oil appears not to be beneficial and should be used with caution beyond the 4th year of life in myxoedematous cretins (Boyages et al 1990). Mothers who had iodized oil injection one year before delivery secrete adequate amount of iodine into the milk, and the iodine intake of their breast fed infants appears to be adequate for 18-24 months without further iodine supplementation (Kochupillai 1986, Chaouki *et al.*1990)

1.9.3 Hypothyroidism

According to Foley, hypothyroidism was first documented in a lecture by Paracelsus in 1527. In 1871, Hinton Fagge precisely defined the characteristic features of non-goitrous hypothyroidism. Hypothyroidism results from a deficiency in TH production. Hypothyroidism may be due to a defect in the thyroid gland itself (primary hypothyroidism), the result of a reduction of pituitary TSH (secondary hypothyroidism), or a concequence of a defect in hypothalamic TRH (tertiary hypothyroidism).

A) Neonatal Hypothyroidism (Sporadic Congenital Hypothyroidism)

The incidence of congenital hypothyroidism in developed countries, detected by mass screening programmes is: 1/7000 in Japan, 1/4250 in North America, 1/3910 in Australia and 1/3600 in Europe (Delange et al 1980) with an average of 1/3000 - 1/4000 (Delange 1988).

Congenital hypothyroidism (CH) is twice as common in females as in males (Dussalt and Glorieux). The familial occurrence of CH and occasional occurrence in twins or siblings, its association with an elevated frequency of HLA types AW24 or BW44 and a low incidence of CH in black children may suggest a genetic defect (Delange 1988). This and the possible seasonal variations in the incidence of the disorder suggests that CH is a syndrome of multifactorial etiopathology including genetic and environmental factors. Developmental defects of the thyroid (athyroid or thyroid dysgenesis) are the most common causes of CH. Autoimmune factors acting alone or in combination with environmental factors, could affect the function and

probably the ontogenesis of the thyroid gland. It was demonstrated that a majority of mothers of hypothyroid infants, developed an antibody which acts specifically on the fetal thyroid gland, either affecting its growth or function (Dussalt 1989). Antithyroglobulin antibodies (ATA) and antimicrosomal antibodies (AMA) were reported to have a higher frequency in CH infants than in the controls.

Clinical symptoms in CH infants are rare or non-specific during the first few weeks of life (Foley 1983). The main clinical findings during the first week are: large posterior fontanel, retardation of skeletal maturation and growth length, prolonged icterus, abdominal distension, large tongue, skin mottling, muscle hypotonia and goitre (Foley 1983, Delange 1988, Beck-Peccoz and Medri 1988). Feeding difficulties, lack of interest, somnolence, failure to gain weight and decreased stooling are seen during the first month. Temperature is subnormal, the skin may be cold and edema of extermitis and genitalia may be present. Screening programs for CH were proposed by Dussault and colleages in 1973. In general, TSH levels > 25 μU/ ml or TT_4 < 5 μg/dl are suggestive of CH (Beck-Peccoz and Medri 1988).

Treatment of CH infants is a pediatric emergency, because of the harmful effects of TH deficiency to the neonatal brain. Replacement thyroxine therapy must be initiated within the first few days of life. Sodium-L-thyroxine is the drug of choice, given orally. The dose of L-T_4 is shown in Table (2). Serum total and free T_4 should be checked and kept at the upper level for age but hyperthyroxinemia should be avoided.

Table (2): Dose of L-Thyroxine

	Dose L-T_4	
Age	(μg/day)	(μg/kg/day)
0 - 6 months	25 - 50	8 - 10
6 - 12 months	50 - 75	6 - 8
1 - 5 years	75 - 100	5 - 6
6 - 12 years	100 - 150	4 - 5
> 12 years	100 - 200	2 - 3

(From Foley 1983)

The main aim of early treatment of CH is to avoid mental retardation. With an

adequate therapy, infant with CH will reach mean IQ values similar to control groups but significant impairment of fine motor-coordination and fine manipulative skills still remain (Rochiccioly *et al* 1989)

B) Transient Neonatal Hypothyroidism (TNH)

In this syndrome, serum TSH is elevated and serum total and free T_4 are decreased for several weeks after birth. Thyroid function becomes normal spontaneously or after a short period of therapy. Antithyroid therapy of the mother during pregnancy, iodine overload, iodine deficiency and immunological factors such as TSH-binding inhibitor immunoglobulins (TBII) are involved in the pathogenesis of TNH. Neonates are more sensitive to the antithyroid effects of iodine deficiency than adults. The main causes of TNH in western countries are iodine excess or moderate iodine deficiency (Delonge 1989a, 1989b and Delange *et al* 1983)

C) Acquired Hypothyroidism (Juvenile and Adult Hypothyroidism)

The development of hypothyroidism in a euthyroid subject may be due to several causes. Major causes of hypothyroidism are: autoimmune thyroiditis which is often recognized during the postpartum period, radioiodine therapy (or any drug with high content of iodine) and external radiotherapy, biosynthetic defects in thyroid hormonogenesis, antithyroid drugs, TSH deficiency, TRH deficiency and generalised resistance to thyroid hormones.

1.10 Maternal to Fetal Transfer of Thyroid Hormones

1.10.1 Theoretical consideration

Approximately 99.97% of total T_4 is bound to the plasma proteins (0.03% is free), whereas in the case of T_3, 99.7% is protein-bound (0.3% being free). Although the plasma concentration of TBG is less than that of the other two binding proteins, nevertheless, it carries some 75% of plasma T_4 due to its high affinity. Approximately 8% of plasma T_4 is carried by TBPA, whereas albumin carries some 16% (albumin has a much lower affinity for the thyroid hormones relative to the specific binding proteins but it is present at much high concentrations in the blood) (Azizi 1984, Robbins and Bartalena 1986). Although the role of the binding proteins is at present poorly understood, it is generally accepted that it is the free hormone fraction which is

physiologically important (free hormone hypothesis).

A variety of roles have been proposed for the specific-hormone binding proteins, for instance it has been suggested that they may act as a buffering system or reserver, they may minimise excessive loss of hormone by glomerular filtration, or they may protect sensitive tissues from excessive hormone concentrations. With respect to the latter, it has been suggested that the 2-3 fold increase in TBG seen in human pregnancy may serve to restrict the transport of maternal thyroid hormones to the developing fetus (Osorio and Myant 1960).

In an attempt to gain a better understanding of the role of the specific binding proteins in thyroid hormone delivery to target tissues, Ekins has produced a physicochemical model. Although the analysis is mathematically complex, and will not be considered in detail here, it nevertheless predicts that in those tissues with high hormonal demand, an increase in the serum binding protein may serve to enhance hormone delivery to such tissues. Furthermore, in the case of TBG which carries both T_4 and T_3, an increase in this protein will result in the preferential delivery of the T_4 relative to T_3 to tissues with a high hormone demand (Ekins 1985, Ekins et al 1989, Ekins 1990). The question then arises as to the purpose of the pregnancy-induced increase in TBG. It is feasible that this may serve to redirect T_4 to the feto-placental unit, providing that this tissue has a high demand for the hormone.

1.10.2 Practical Evidence

It has been the accepted view amongst endocrinologists for many years that the TH do not cross the placenta in significant amounts (Fisher *et al*, 1977, Fisher 1986), The major criticisms of the early experimental work underlying such a view is that i) experiments were largely conducted late in pregnancy, after the onset of independent fetal thyroid function, and it is not necessarily correct to extrapolate such findings to the early phase of development, and ii) that when transfer was observed it was often simply disregarded as either minimal or insignificant. However, a large body of evidence is now accumulating in favor of significant transplacental passage of maternal thyroid hormones.

The evidence has derived largely from animal models, in particular the rat. Radiotracer studies have shown that the transfer of maternal T_4 to the fetus is relatively high at gestational days 9-10 and then subsequently falls (Woods *et al* 1984). In order to determine the possible physiological relevance of this transferred T_4, concentrations

of radioactivity in various maternal tissues and fetus were compared. At 9-10 gestational days, the concentration in the fetal iodothyronine fraction was comparable with that in maternal heart, brain and ovary, whereas at later stages of gestation, fetal values were comparable with only those of maternal brain. These findings indicate that in at least early gestation, the supply of maternal T_4 is sufficient to influence fetal development.

Another group, again working with the rat, has employed a different approach - direct measurement of the thyroid hormones in extracts of fetuses and fetal tissues (Obregon *et al* 1984, Obregon *et al* 1989). They found T_4 and T_3 in embryonic tissues from the earliest time studied (4 days after uterine implantation). Concentrations of T_4 in the embryotrophoblast fell from gestational days of 10-12, in agreement with the findings of Woods *et al*, and then embryonic levels remained fairly constant until 18 days of gestation. Concentrations then increased coincident with the onset of independent fetal thyroid function.

In embryotrophoblasts and embryos from thyroidectomized rat dams, both T_4 and T_3 were undetectable until after the onset of fetal thyroid function, confirming that the transferred hormone was of maternal origin. Total fetal extrathyroidal T_4 and T_3 were reduced even after the onset of fetal thyroid function, suggesting that the transfer of maternal hormone to the fetus may make an important contribution to fetal thyroid hormone homeostasis, even after the establishment of independent fetal thyroid function. Indeed the use of radiolabled T_4 and a steady state approach has recently confirmed this possibility (Morreale de Escobar *et al* 1990).

Other investigators have demonstrated the presence of thyroid hormone nuclear receptors in the rat fetus early in pregnancy. They have been detected at 13 gestational days in whole rat embryo and at 14 gestational days in brain, indicating that the fetus (and fetal brain in particular) has the capacity to respond to transferred maternal T_4 (Bernal and Pekonen 1984)

Turning to the evidence from human studies. Recent work by Vulsma and coworkers has demonstrated the presence of T_4 in cord serum from congenitally hypothyroid neonates. Since the subjects were affected by a total organification defect, they were completely unable to synthesis T_4 themselves. The only possible origin of this T_4 was the mother (Vulsma *et al* 1989). In neonates with thyroid agenesis, the levels and disappearance kinetics of serum T_4 were the same as those for the neonates with total organification defects, also indicating that the source of this TH was the

mother. These results indicate that substantial maternal to fetal transfer of T_4 can occur in the human late in pregnancy, at least when the fetal thyroid function is inadequate (Larsen 1989).

Bernal's group has identified the presence of T_4, T_3 and TH nuclear receptors in a variety of tissues (including brain) from 10 week old human fetuses (Bernal and Pekonen 1984). Levels of these receptors then increase 10 fold up to the 16th week, coincident with fetal thyroid development and neuroblast multiplication. In a later study, they calculated the saturation of the brain nuclear receptor as approximately 25% at 10-13 weeks of gestation, indicating a possible influence of the TH on brain development from around the 9th to 10th week (Ferreiro *et al* 1988). Morover, this group has unpublished data demonstrating the presence of the nuclear T_3 receptor and T_4 in human embryo as early as 7 weeks of gestation (cited in Morreale de Escobar *et al* 1985).

Pharaoh *et al.* working in an iodine-deficient endemia in Papua-New-Guinea, found that iodine-replacement therapy (iodized oil) could reduce the incidence of neurological cretinism in offspring (Pharaoh *et al* 1971). However, for a completely successful treatment, therapy had to be initiated very early in pregnancy or before conception. Since the human fetal thyroid does not achieve synthesis of TH until approximately 12th week of gestation, they interpreted these findings as a role for elemental iodine *per se* in brain development. To date no such role has been identified (Pharoah *et al* 1981, Pharoah *et al* 1984).

A more likely interpretation of these data is that maternal thyroid hormone synthesis was compromised as a consequence of iodine deficiency, and iodine replacement restoring TH levels to near normal. Indeed, measurement of TH levels in women from such endemias has shown that although total and free T_3 are approximately normal (or even raised), both total and free T_4 are reduced. Further work by Pharoah's group has shown a correlation between the severity of the impairments of cognitive and motor function in such children and maternal thyroxine levels.

A reports from an iodine-replete environment (North America) have shown decreased IQ, and motor deficits, in children born to hypothyroxinemic mothers (Man and Jones 1969, Man *et al* 1971, Man and Serunian 1976). The offspring themselves were euthyroid, suggesting that the deficits were attributed to the lack of maternal T_4 (and thus placental transfer). Furthermore, there is some evidence that maternal hyperthyroxinemia is associated with an increased incidence of schizophrenia in the

offspring (Delong 1989).

Taken together, these animal and human studies indicate that, in the words of Morreal de Escobar *et al*: "we believe it can no longer be stated that the mammalian placenta is virtually impermeable to thyroid hormones, either early or late in gestation" (Morreale de Escobar *et al* 1990).

1.11 Myelin in the Central Nervous System

In higher vertebrate, brain and spinal cord are composed of both grey and white regions. In man more, more than 40% of a cross section through the brain may consist of white matter. The white matter is composed of axons ensheated in a fatty white coat called myelin (Morell and Norton 1980, cited in Berne and Levy 1983). Rudolf Virchow, a German pathologist introduced the word "myelin" in 1864. This name was drived from the Greek "*myelos*", meaning marrow. The term myelin reflects Virchow's observation that it comprises the core of the brain.

In the CNS, myelin sheath is produced by oligodendrocytes (OL). The myelin sheath is a modified plasma membrane which is wrapped in a compact multilammelar fashion around the nerve axon. It insulates axons from each other, but its main function is to facilitate axonal conduction. The velocity of an impulse in a fiber without myelin is proportionate to the square root of its diameter, while the conduction velocity in a myelinated fiber is proportionate to its diameter. Thus, an advantage of myelination is saving of energy and space in higher vertebrates (Morell and Norton 1980, Morell *et al* 1989) Myelin compositions and and the effects of TH on metabolism of myelin will be discused in more detail in chapter 3.

1.12 Rationale

In iodine deficient areas throughout the world, the effects of iodine deficiency on the development of the nervous system has been reported to extend from fetal life through the neonatal period and into childhood. Neurological cretinism, characterised by mental retardation, deaf-mutism, a characteristic gait and stance disorder and strabismus is frequent in children born to mothers in iodine deficient regions. Administration of iodine to these mothers, can only prevent the neurological damage to the offspring if the treatment is started before conception (Pharoah et al 1971). Thus, the fetal damage may

occur very early in pregnancy before the establishment of independent fetal thyroid function. In mothers from the iodine deficient regions, concentrations of serum thyroxine are in many cases low but those of T_3 are normal or slightly raised. Furthermore maternal T_4 concentrations correlate with the degree of impairment of cognitive and motor function in affected progeny. Such findings, taken together with an increasing body of evidence (see maternal to fetal transfer of TH) in favour of transplacental passage of T_4 from mother to fetus, suggest that maternal T_4 may play an important role in brain development. The purpose of this study was to investigate the possible consequences of maternal hypothyroxinemia *in utero* on the brain biochemistry of progeny.

For ethical reasons and inaccessibility to human material, a hypothyroxinemic animal model was chosen for this study. The rat was selected as a reliable model, since it is similar to the human with respect to the timing of the functional and anatomical development of the thyroid gland in the fetus, the onset of thyroid hormone secretion and the presence of thyroid hormone nuclear receptors in brain tissue before birth. The type of placentation in rat is also close to that of human. The use of the rat also allows considerable and control of environmental and nutritional variables which are important in a study of this kind.

The biochemistry of five different brain regions of adult progeny from hypothyroxinemic dams were studied with the emphasis on neuronal cell markers, oligodendroglial cell markers and myelin metabolic markers. Since in neurological cretinism it has been reported that the deficits are present in specific brain regions such as cerebral cortex and basal ganglia (paleocortex), as well as myelin sheath (Ma *et al* 1986, Delong 1989).

CHAPTER 2 : MATERIALS AND METHODS

2.1 Animal Model

Partially thyroidectomized, parathyroid-spared (TX) female Sprague Dawley rats, and normal (N) females, were maintained under normal animal house conditions at 22° C constant temperature on a 12 hour light/dark cycle. They received food and water *ad libitum* and iodine intake was the same for both TX and normal groups at 36 μg/day.

After thyroidectomy and prior to mating, blood was taken from a representative number of TX dams for the assessment of plasma T_4 and T_3. Before mating, serum concentrations of total thyroxine (T_4) and 3, 5, 3'-triiodothyronine (T_3) were measured for both groups by in-house radioimmunoassay. The animals were mated with normal males and were allowed to give birth. The litter size was reduced to 6 for both groups and the animals were allowed to grow until 7 month of age, when they were used for experiments. Both progenies of normal and thyroidectomized dams were kept under normal animal house conditions, where a calculated amount of iodide was added to their food.

Normal and TX dam progeny were taken from the animal house just prior to sacrificing and were killed by concussion following by cervical dislocation. The animal's chest was opened quickly, and while the heart was beating, cardiac blood was collected for hormone assays. Blood was collected into heparinized tubes, and after gently shaking it was centrifuged at 2000 rpm for 10 minutes; the supernatant was collected in marked plastic tubes, and stored at - 20 °C for serum T_4 and T_3 assays.

2.2 Tissue Preparation

Brains were removed, weighed and then transfered to a petri dish on ice for dissection. Each brain was dissected into: cerebellum, medulla, midbrain, cerebral cortex and paleocortex (see bellow). Blood vessels and meninges were removed from each region before weighing, then tissue was either stored at -20° C or homogenized the same day. Brain regions, were homogenized in 0.32 M sucrose to yield a 10% (w/v) homogenate. Homogenization was carried out in a hand held all glass homogenizer (0.1 mm clearance) by 25 up and down strokes, while the homogenizer was standing on ice. The homogenate was then divided into 1 ml aliquots and stored at -20° C for subsequent biochemical determination.

2.2.1.Dissection of Brain Regions

a) Cerebellum

The cerebellum was firstly removed by carefully cutting the cerebellar peduncles which connect this structure to the superior surface of the pons. The whole cerebellum was used for this study; this includes the cerebellar hemispheres and vermis.

b) Medulla

The brain stem area from below the cerebellar peduncles (lower part of the pons) to the level of the foramen magnum was designated as the medulla (see Fig A, page 64). This region mainly contains the vagus nucleus, some cranial nerve centers and the fibres from the extrapyramidal tracts.

c) Midbrain

After removal of the medulla, the midbrain was obtained by cutting lower edge of the infundibulum (taking care to avoid any cerebral cortical tissues). This gross region primarily contains: the optic tectum, the reticular formation of the mesencephalon, the rubrospinal tract, the nucleus of medial geniculate body, the lateral geniculate body, the auditory perception areas, the tegmentum, the red nucleus, the substantia nigra and the upper parts of the pons.

d) Paleocortex

The brain tissue remaining after disection of the midbrain comprises the cerebral cortex, the paleocortex and the olfactory system. After cutting the olfactory peduncles, the cortical flaps were folded out and the remaining structures were designated as the paleocortex (old cortex) for this study. This region contains the thalamus, the hypothalamus, the globus pallidus, the basal ganglia, parts of the corpus callosum, hippocampus and the reticular formation.

e) Cerebral cortex

Finally, remaining white matter from the undersides of the cortical flaps were scraped off and discarded. The remaining cortical flaps were designated as cerebral cortex. This region contains the sensory cortex (precentral gyrus), the motor cortex, the auditory cortex (parietal cortex), the visual cortex (occipital cortex), speech and cognition centers, and the median, lateral and central gyri.

Figure A

ME Medulla
MB Midbrain
IF Infundibulum
OT Optic tract
CH Cerebral hemisphere
OL Olfactory bulb

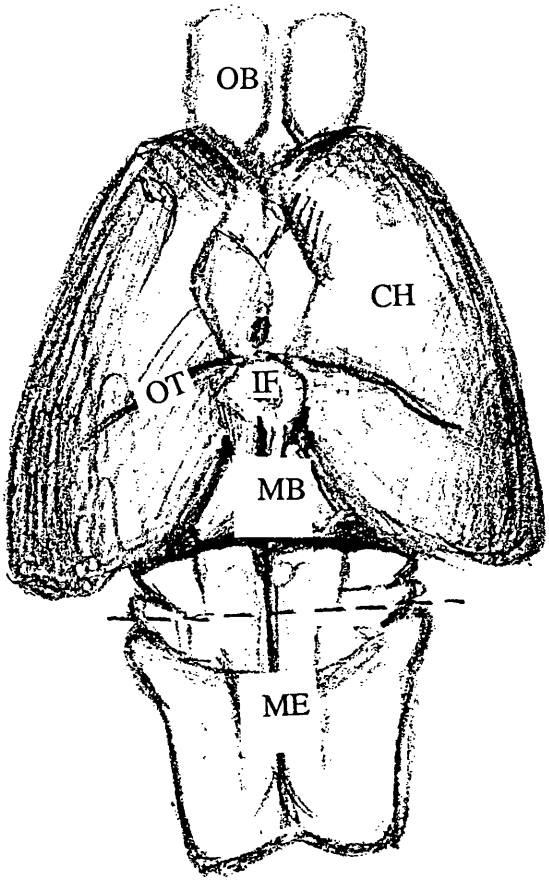


Figure B

CF Cortical flap
PC Paleocortex

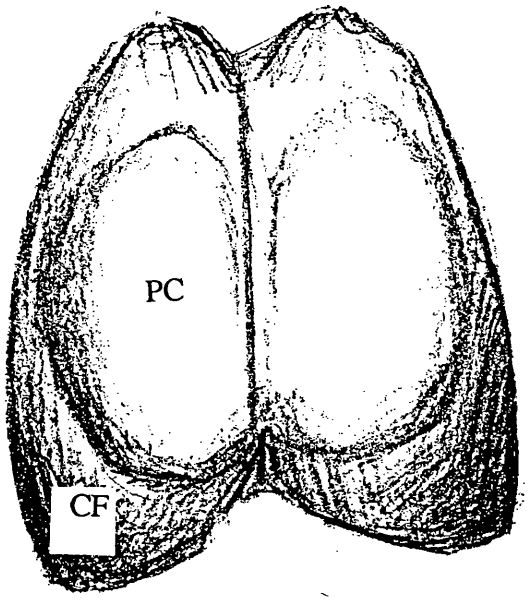


Figure A (ventral surface) and Figure B (cerebral hemisphers) of rat brain.
Gross anatomical regions used in this investigation are designated in the figures A and B.

2.3 Biochemical Analysis

2.3.1 Total Protein Determination

A) Principle

The method used for protein estimation was that of Lowry *et al.* (1951). Alkaline copper sulfate reacts with compounds possessing two or more peptide bonds to produce a violet-coloured complex. The maximum colour results after the reduction of the phenol reagent at pH 10 by the copper-protein complex. The intensity of the colour produced is proportional to the number of peptide bonds present in the protein.

B) Solutions

Solution A : 2% (w/v) sodium carbonate in 0.1 M sodium hydroxide.

Solution B : 0.5% (w/v) copper sulfate ($CuSO_4$, 5 H_2O), in 1% (w/v) sodium potassium tartrate.

Solution C : A mixture of solutions A and B in a ratio of 50 : 1.

Solution D : Folin and Ciocalteau's phenol reagent diluted 1 : 1 with distilled water.

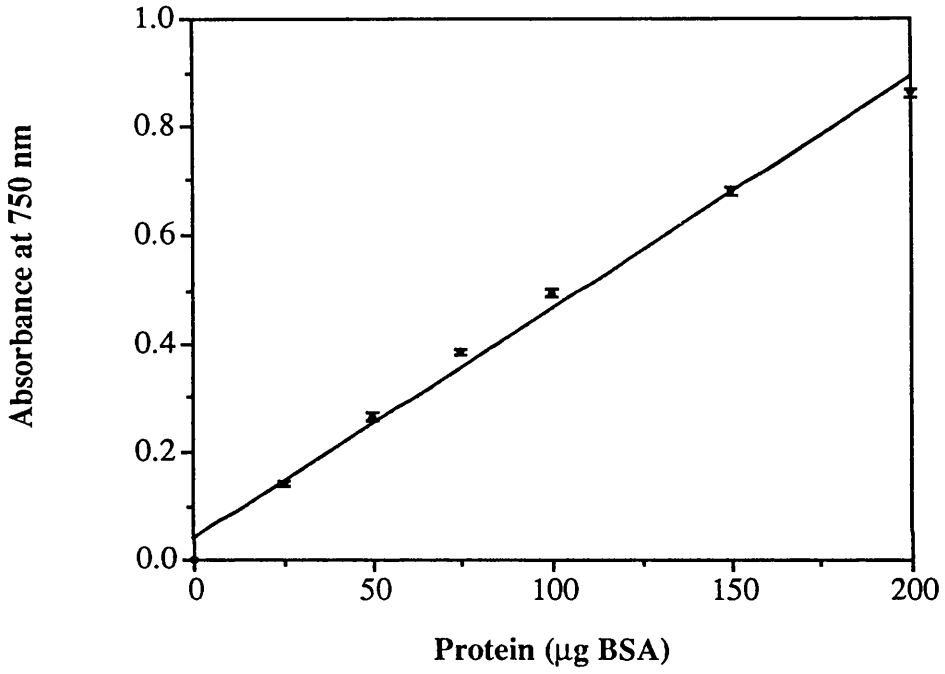
Stock Standard Solution: Bovine serum albumin (BSA) was dissolved in distilled water at a concentration of 1 mg/ml.

C) Method

Aliquots of the standard protein (0-250 µg) were dispensed in duplicate into test tubes and the volume brought to 400 µl, with distilled water. Aliquots of diluted tissue homogenate were also dispensed in duplicate into similar test tubes, and treated exactly as standard, through all steps .

Solution C (3 ml) was added to each tube, and after mixing, the tubes were allowed to stand at room temperature for 15-20 min. Solution D (0.3 ml) was added and the tubes were mixed throughly. The tubes were then left at room temperature for 30 minutes. The intensity of the blue-colour produced was measured against a reagent blank at 750 nm in a Pye Unicam spectrophotometer. A standard curve was constructed (Figure 3) and the values of the unknowns read from the curve. Results were expressed as mg protein/g tissue wet weight.

Figure 3 : Protein Standard Curve



Each point represents the mean ± SEM of at least 8 separate measurements in duplicate, as described in Materials and Method. Intra assay and inter assay variations were less than 5% and 7% (at all deses) respectively.

2.3.2 Determination of Inorganic Phosphate

A) Principle

The method used was based on that of Martin and Doty (1949) with the modification of Cammer *et al.* (1980). Inorganic phosphate reacts with ammonium molybdate yielding phosphomolybdic acid, the latter is then extracted by butanol/benzene. Reduction of molybdenum in the phosphomolybdate compound gives rise to a blue colour which is measured spectrophotometrically at a wavelength of 730 nm.

B) Solutions

Solution A : Isobutanol/benzene, 1 : 1 (v/v).

Solution B : 2.5 M Sulphuric acid.

Solution C : 10% (w/v) Ammonium molybdate in distilled water.

Solution D : 87% Ethanol, containing 0.3 M sulphuric acid and 0.006% (w/v) stannous chloride ($SnCl_2$).

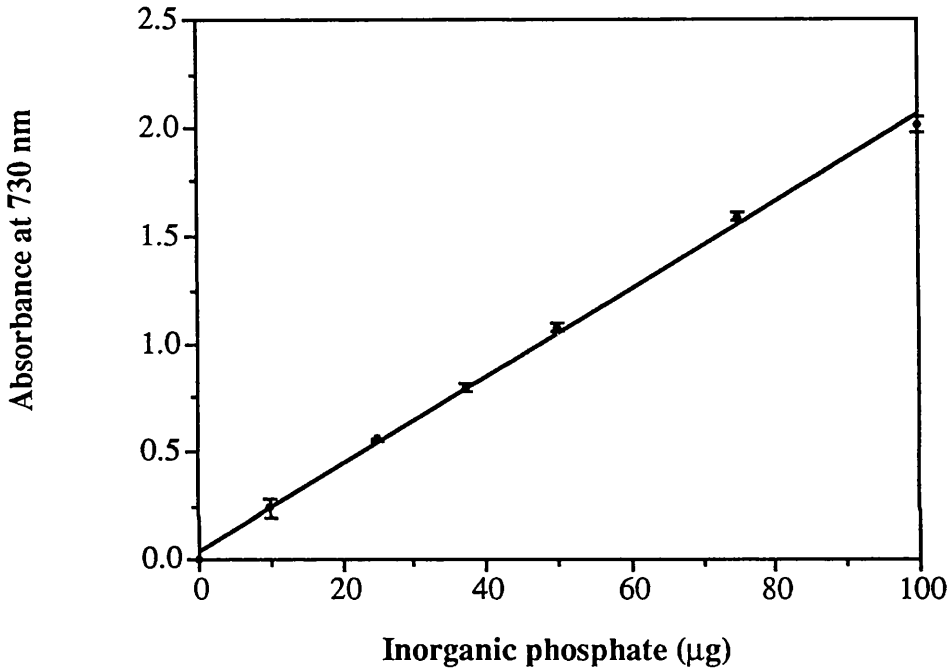
Solution E : 10% (w/v) stannus chloride in concentrated HCl.

Standard Phosphate Solution: Disodium hydrogen phosphate (0.5 mg/ml) in distilled water.

C) Method

Samples and standard (0-100 µg) were dispensed in quadruplicate into glass tubes and the volume brought to 200 µl with water. To each tube, 300 µl of solution B and 2 ml of solution A were added, followed by the addition of 0.2 ml solution C. After vortex mixing, the reaction mixtures were centrifuged (500 rpm, for 15 minutes at 4 °C). A portion (1 ml) of the organic upper phase was transferred to clean tubes, 2.5 ml solution D was added and, after through mixing, 20 µl of solution E was added. All tubes were then shaken vigorously and the blue colour was read in a spectrophotometer against a reagent blank at 730 nm. Tissue concentrations of inorganic phosphate were determined from standard curves (Figure 4).

Figure 4 : Inorganic Phosphate Standard Curve



Each point represents the mean ± SEM of 10 separate measurements in quadruplicate. Intra and inter assays variations were less than 5% and 6.6% (for all doses) respectively.

2.3.3 An Introduction to Enzyme Kinetics

Enzymes are biological catalysts which affect the rate of reactions. They are involved in many metabolic processes; therefore, a study of enzyme kinetics is essential for a better understanding of enzymic reactions.

In typical enzyme catalysed reaction, enzyme reacts with substrate to form an enzyme-substrate complex which then dissociates to yield enzyme and substrate or enzyme and product. Based on this assumption and others: i) that the rate of back reaction from product to substrate is negligible, and ii) that the concentration of free substrate is unchanged by formation of E-S complex, the following basic equation for enzyme kinetics can be derived:

$v = Vmax\ s / Km + s$ Michaelis-Menten equation

Where: v is the velocity of the reaction, Vmax is the maximum velocity, s is the substrate concentration and Km is the Michaelis constant.

Both Vmax and Km are very important parameters. Vmax represents the maximum reaction velocity that can be attained for a given amount of enzyme under optimum conditions, ie when the total amount of enzyme is saturated with substrate. Km represents an inverse measure of affinity of enzyme for its substrate; thus, in relative forms, a low Km signifies that the enzyme has a high affinity for the substrate and a high Km means that a higher concentration of substrate is required to saturate the enzyme. Km can also be defined as the concentration of substrate which gives a reaction velocity of half maximum (s = Km, v = 1/2 Vmax). Km is a constant characteristic of the enzyme itself and Km values for most enzymes are in the range 10^{-1}-10^{-6} M.

Vmax and Km can be determined graphically. At a fixed enzyme concentration, a graph of velocity against substrate concentration is hyperbolic, and at a fixed substrate concentration a graph of v against enzyme concentration is a straight line (so long as the substrate concentration is not rate-limiting). Thus, at a fixed substrate concentration the velocity will be directly proportional to the enzyme concentration. For practical purposes, a double-reciprocal plot (Lineweaver-Burk graph) is more convenient. This is a graph of 1/v against 1/s which gives a straight line, with slope Km/Vmax, its intercept on the y-axis equal to 1/Vmax and its intercept on the x-axis equal to -1/Km.

When measuring enzyme activity, it is important to select an enzyme concentration

When measuring enzyme activity, it is important to select an enzyme concentration which is proportional to the reaction rate and to ensure that the time chosen for assay falls within the linear portion of the reaction rate versus time plot (Dixon and Webb 1979, The Open University 1977).

For all the enzymes studied here, the following steps were taken to optimize the assay procedure. Saturating amounts of substrate were used, and the reaction was performed at the pH optimum of the enzyme. The relationship between reaction rate and the enzyme (protein) concentration was determined to ensure that the reaction rate was proportional to the protein concentration employed in the assay. Finally, using this selected protein concentration, the relationship between reaction rate and assay time was determined to ensure a constant reaction rate during the period of measurement.

2.3.4 2', 3'-Cyclic Nucleotide 3'-Phosphohydrolase (CNPase)(EC 3.1.4.37) Assay.

A) Principle

The method employed is that described by Prohaska *et al.* (1973). CNPase is a myelin-associated enzyme (Kurihara *et al* 1969), with preferential localization in oligodendroglial cells (Zanetta *et al* 1972). CNPase catalyses the conversion of 2', 3'-cyclic adenosine monophosphate to 2'-AMP. This is further hydrolysed by alkaline phosphatase, resulting in the liberation of inorganic phosphate, which can be determined with molybdate reagent.

B) Solutions

A) 100 mM Tris-HCl buffer, pH 9.0 containing 21 mM magnesium chloride and 2 U/ml alkaline phosphatase.

B) 30 mM Tris-HCl buffer, pH 7.5 containing 0.5% (w/v) deoxycholic acid.

C) 10.5 mM 2', 3'-cAMP in 90 mM Tris-maleate buffer, pH 6.2.

C) Method

Stock homogenates were first treated with an equal volume of solution B for 20 minutes at 4 °C to solubilise of the enzyme activity. Solubelised homogenate was then diluted to a protein concentration of 0.25-0.5 mg/ml. Aliquots (40 µl) of approximately 10-20 µg protein/assay tube were incubated with 2', 3'-cAMP (7.5 mM) in a final volume of 200 µl, in a shaking water bath at 37 °C, for 30 minutes. Reactions were

terminated by immersing the rack in a boiling water bath for 5 minutes.

A second incubation step was conducted to liberate inorganic phosphate from the product (2'-AMP). After cooling on the bench, 0.1 ml of solution A was added to each tube and incubated for another 30 minutes at 37 °C. This reaction was terminated by the addition of 0.2 ml of 30% TCA. The liberated inorganic phosphate was determined as described above (section 2.3.2). The assays were carried out in quadruplicate in glass tubes, with no homogenate in blank tubes. CNPase activity was expressed as μmol inorganic phosphate liberated/mg protein/hour.

A Km of 5-6 mM has been reported for CNPase (Prohaska *et al* 1973). All methods for CNPase assay operate at a fixed substrate rather than a saturating substrate. An enzyme concentration course (5-50 μg tissue protein) was determined under these conditions and the velocity was found to be linear up to 25 μg protein. With a fixed amount of protein (20 μg), the time course (5-90 min) was linear up to 60 min. Thus, the protein concentration and assay time selected for routine assay fell within the linear portions of the appropriate standardization curves.

2.3.5 5'-Nucleotidase (EC 3.1.3.5) Assay

A) Principle

Nucleotides are degraded by 5'-nucleotidase to nucleoside and inorganic phosphate. Enzyme activity was determined by measurement of inorganic phosphate (Pi) released from AMP, using the procedure of Cammer, Sirota, Zimmerman and Norton (1980).

B) Solutions

A) 200 mM Tris-HCl, pH 7.5.

B) 100 mM Mg Cl_2.

C) 100 mM 5'-Adenosine monophosphate.

C) Procedure

Homogenate (50 μl or approx. 0.4 mg tissue protein) was incubated in a final volume of 0.4 ml, containing 10 mM AMP and 10 mM Mg Cl_2 in buffer. Tubes were incubated in a 37 °C shaking water bath for 1 hour. The reaction was terminated by addition of 0.3 ml of 2.5 M sulphuric acid. Liberated inorganic phosphate was then determined by reaction with molybdate as described above (section 2.3.2). 5'-

Nucleotidase activity was expressed as μmol inorganic phosphate liberated/mg protein/hour. Assays were carried out in quadruplicate with appropriate reagent blanks. The apparent Km for this enzyme has been reported to be 11 μM (Segal and Brenner 1960). A saturating concentration of substrate (10 mM), was chosen for assay and concentration course (0.25-1.5 mg tissue protein) was prepared. The velocity was found to be linear up to 1 mg protein for assay. A time course (10-120 min) with 0.4 mg protein demonstrated that the velocity was linear up to 90 min.

2.3.6 β-D-Glucuronidase (EC 3.2.1.31) Assay

A) Principle

The procedure was adapted from Sinha and Rose (1972). β–D-Glucuronidase (β-D-glucuronide glucuronohydrolase) is a lysosomal enzyme which takes part in the degradation of the polysaccharides, such as chondroitin sulfate, that contain glucuronide residues. The enzyme is thought to be predominantly localised in neuronal cell bodies. The substrate used for the enzyme assay was the synthetic 4-methylumbelliferyl glucuronide. The product, 4 -methylumbelliferone (4-MU) is highly fluorescent at alkaline pH. The fluorescence produced was then read fluorometrically.

B) Solutions

A) 4-Methylumbelliferyl glucuronide (0.47 mM) in 100 mM acetate buffer, pH 4.5.
B) Glycine/NaOH buffer (0.5M), pH 10.4.

C) Procedure

Stock homogenate was diluted to a concentration of 1%, and was frozen and thawed three times prior to use to obviate latency (Sinha and Rose 1972). Approximately 200-250 μg of tissue protein (200 μl of 1% homogenate) was incubated in a final volume of 2 ml, containing 50 mM substrate at 37° C in a water bath for 15 minutes. The reactions were terminated by addition of 3 ml of glycine/NaOH buffer, pH 10.4.

Assay tubes were cooled on the bench and, after addition of homogenate to reagent blanks, were read in a fluorimeter (Perkin-Elmer LS-5), at 360 nm excitation and 444 nm emission wavelengths. Blank values were subtracted and the enzyme specific activity was calculated and expressed as μmol 4-methylumbelliferone liberated/mg protein/h.

a Km of 14 μM has been reported (Robins et al 1968). Thus a saturating concentration of substrate was used for routine assay. An enzyme-concentration course (50-300 μg tissue protein) revealed that the velocity was linear throughout. With 200 μg protein, a time course of 5-90 min was set up and found to be linear in this range.

2.3.7 N-Acetyl β-D-Galactosaminidase (EC 3 2 1 53) Assay

A) Principle

N-acetyl β-D-galactosaminidase is a lysosomal enzyme. The activity of this acid hydrolase enzyme was measured in homogenates of different brain regions of normal and TX dam progeny. The enzyme catalyses the hydrolesis of the β-N-acetylgalactosaminide moieties from glycoconjugates. The substrate used for this assay was methylumbelliferyl-galactosaminide, yielding 4-methylumbelliferone, which is maximally fluorescent at pH 10.4 (Hirsch *et al* 1977). This alkaline pH is provided by a glycine/NaOH buffer. The fluorescence was then read at 360 nm excitation and 444 nm emission wavelengths.

B) Solutions

A) 4-Methylumbelliferyl β-D galactosaminide (0.87 mM) in 100 mM citrate buffer, pH 4.5.

B) 0.5 M glycine/NaOH buffer, pH 10.4.

C) Method

Homogenates diluted and were frozen and thawed three times. Aliquots (200 μl or 200-250 μg of protein) were incubated with 1 ml solution A, in a final volume of 2 ml. Assay tubes (in quadruplicate and two blanks) were incubated at 37 °C for 15 minutes. Reactions were terminated by the addition of 3 ml solution B. Homogenate was added to blank tubes and after cooling on the bench, the fluorescence was read as described for the β-D-glucuronidase assay. After subtraction of blank values, enzyme specific activity was calculated and expressed as μmol 4-methylumbelliferone liberated/mg protein/h

In a preliminary experiment, both an enzyme-concentration course and a time-course were set up for this enzyme, exactly as described for β-D-glucuronidase assay. Both were found to be linear in the assay range.

were found to be linear in the assay range.

2.3.8 Acid Lipase Esterase (Oleate Esterase) Assay

A) Principle

Oleate esterase is a myelin metabolic enzyme which is mainly located within oligodendrocytes and myelin. 4-Methylumbelliferyl oleate was used as a substrate for this enzyme, and the liberated 4-methylumbelliferone was read fluorometrically as described for the sugar hydrolases (sections: 2.3.5 & 2.3.6).

B) Solutions

A) 4-Methylumbelliferyl oleate (10 mM), in methylselosolve.

B) Citrate-phosphate buffer (0.1 M) pH 6, containing 0.1% (v/v) Triton-X 100.

C) Phosphate buffer (0.1 M), pH 7.0.

D) Working solution: a mixture of solutions A & B, in a ratio of 1:25.

E) Tissue Homogenate: stock homogenate was diluted with 0.32 M sucrose to yield a 5% (v/v) homogenate.

C) Procedure

Assays were set up in quadruplicate with two extra tubes as blanks. Assay mixtures (final volume 160 µl) contained: solution D 150 µl (0.375 mM substrate), and homogenate 10 µl (50 µg protein). After incubation at 37 °C for 30 minutes, reactions were terminated by the addition of 3 ml solution C. Homogenate was added to blanks and the liberated 4-methylumbelliferone was determined as described for β-D-glucuronidase (section 2.3.5). A standard of substrate (4-methyl umbelliferone) was prepared in phosphate buffer, pH 7.0. The assays were read, and after subtraction of blanks, the values were calculated in comparison with the standard. The activity of the enzyme was expressed as µmol 4-MU liberated/mg protein/h. This procedure is a modification of the method described by Hirsch *et al* (1977).

A Km value of 3.3 µM has been reported (Jack and Kircher 1967). Thus, a saturating concentration of substrate was used in this assay. In both enzyme-concentration (10-100 µg tissue protein) and time (10-90 min) courses the reaction rate was found to be linear over ranges employed.

2.3.9 Sphingomyelinase (EC 3.1.4.12) Assay.

Sphingomyelinase (sphingomyelin phosphodiesterase) catalyses the hydrolysis of sphingomyelin to ceramide and phosphorylcholine. In mammalian tissues, four different sphingomyelinase activities have been reported; 1) a lysosomal enzyme with a pH optimum at 5; 2) a neutral magnesium-dependent activity, with pH optima at 7.4 (human brain) and 7 (rat brain) (Yamaguchi *et al* 1978); 3) a neutral magnesium-independent enzyme which is bound to myelin in rat brain and; 4) a neutral Mn^{2+}, Mg^{2+}-activated membrane-bound enzyme located in the bovine adrenal medulla (Bartolf *et al* 1986).

A) Principle

Sphingomyelinase catalyses the release of methyl-phosphorylcholine and cereamide from sphingomyeline. The activity of the enzyme is measured after extraction of phosphorylcholin. The method was adapted from Yamaguchi *et al* (1978).

B) Procedure

Homogenate was diluted to 1% (v/v) which was used for the assay. Aliquots (50 µl or 40-80 µg protein) were then incubated with 60 nmol sphingomyelin (approximately 10000 cpm) (Amersham UK and Sigma, England), and 0.05% (w/v) Triton-X 100 in 0.1 M sodium acetate buffer, pH 5.0 or 0.1 M Tris/maleate buffer, pH 7.0 (final volume of 0.2 ml). Assays were set up in quadruplicate glass tubes (while racks were standing on ice) and blanks were without homogenate. The reaction mixtures were incubated in a shaking water bath, at 37 °C either for 30 minutes (pH 7.0) or 1 h (pH 5.0).

The reactions were terminated by the addition of 1 ml of chloroform/methanol/HCl (1000/1000/6, v/v/v), followed by 0.3 ml of 1 M HCl-0.1 M EGTA. After through mixing, all tubes were centrifuged at 2500 rpm, for 15 min and 0.4 ml of the upper phase was added to 4 ml of scintillation liquid (Ecoscint A). Radioactivity was counted in a LKB beta counter and all counts were quench corrected. Sphingomyelinase specific activity was expressed as µmol liberated phosphorylcholine/mg protein/h. The procedure is based on the methods of Yamaguchi *et al* (1978) and Vanha-Perttula (1988).

Km's of 61 µM and 190 µM have been reported for acidic and neutral

sphingomyelinase respectively (Carre *et al* 1989). In this assay, a near saturating substrate concentration (300 μM) was used and in a preliminary set of experiments the time course (5-90 min) was found to be linear up to 90 min and 60 min for acidic and neutral sphingomyelinase respectively. An enzyme-concentration course (10-200 μg tissue protein) was linear up to 100 μg tissue protein for both enzymes.

2.4 Myelin Lipid Assays

In this part, the aim of study was to determine the contents of the myelin lipids cerebroside, sulfatide and cholesterol in different brain regions of normal and exprimental progeny.

2.4.1 Homogenization of brain tissue

The different brain regions were homogenized in 19 volume (w/v) of chloroform/methanol (2:1 v/v), in a hand-held homogenizer. Homogenates were centrifuged at 1900 g for 15 min at 4° C. The upper aqueous phase was discarded and the supernatant transferred to a glass vial. The pellets were suspended and washed with chloroform/methanol (2:1), and processed as before. Supernatants were collected and combined. A final wash was conducted with chloroform//methanol (7:1) saturated with ammonia and, after centrifugation, the supernatants were combined. The combined supernatants were divided into aliquots for cholesterol, cerebroside and sulfatide assays. All extracts were evaporated under nitrogen at room temperature. The procedure is that described by Walravens and Chase (1969).

2.4.2 Cholesterol Measurement

A) Principle

Cholesterol was first extracted with ethanol, then reacted with a ferric chloride-sulfuric acid reagent. The resulting product has a peak absorbance at 560 nm. The method was adapted from Francy and Amador (1968).

B) Reagents

A) Colour reagent: Ferric chloride (1 mg/ml, w/v) in ethyl acetate.

B) Concentrated sulphuric acid.

C) Cholesterol standard: 2 mg/ml in ethanol.

C) Method

Dried lipid extracts were dissolved in 0.5-1 ml ethanol. Then 0.1 ml of cholesterol extract was added to 1.9 ml of ethanol (0.1 ml water for reagent blank). After thorough mixing and centrifugation at 2000 rpm for 5 min, 0.5 ml of supernatent was taken and placed into a glass vial. Solution A (2 ml) was added, followed by 2 ml H_2 SO_4 while the glass vial was continuously swirled. The assay vials were left for 15 minutes at room temperature. A standard cholesterol solution (0-200 μg) was prepared and treated through all steps as unknown assay samples.

The brown colour produced was read at 560 nm, against the reagent blank and a standard curve was plotted. The amount of cholesterol in the test sample was then read from the standard curve (Fig.5). Results were expressed as mg cholesterol/g wet weight of tissue.

2.4.3 Determination of Cerebroside and Sulphatide

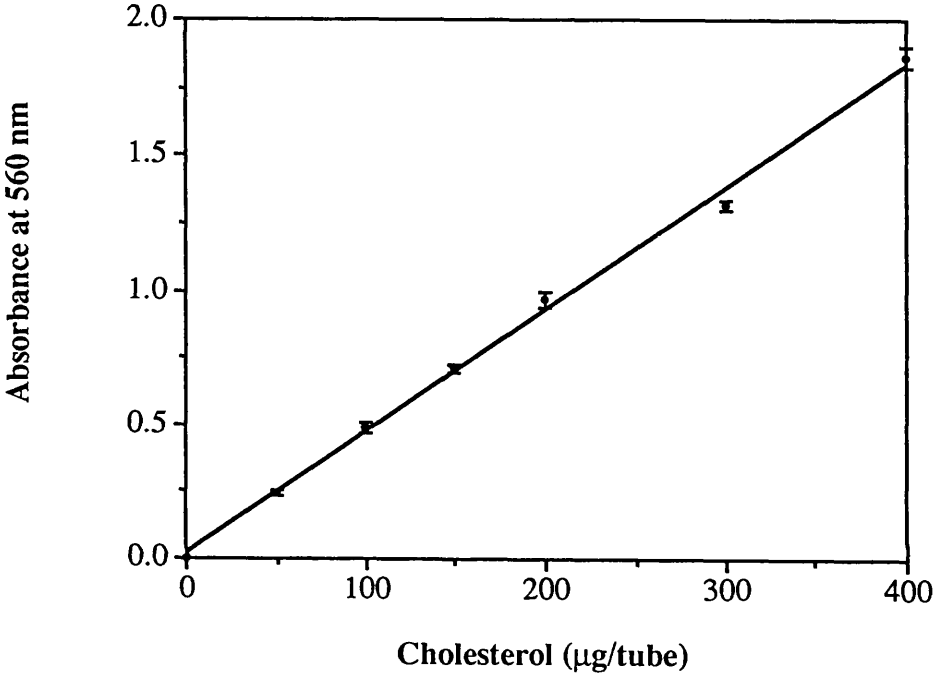
Brain and other neural tissues contain very high concentration of glycosphingolipids, most of which are cerebroside and sulphatide. Cerebroside and sulphatide occur as constituents of biological membranes, especially the myelin sheath. In this study, thin-layer chromatography (TLC) was used for the separation of cerebroside and sulfatide according to the method of Kundu (1981).

A) Materials

1) TLC developing tank: this is a glass chamber with 29 cm length, 10 cm width and 21.5 cm hight. Usually a glass cover for the tank is necessary during the running of the chromatogram.

2) Thin-Layer Plates: the most commonly used adsorbents are silica gel G and silica gel HR; due to the presence of Ca^{2+}, the former gives a better separation of gangliosides (Kundu 1981). Thin-layer plates are commercially available, and because of their convenience, the superior resolving properties and reproducibility, the commercial plates are commonly used in laboratories. One which was used in this study was a 20 by 20 cm aluminium sheet silica gel 60 with layer thickness of 0.2 mm (Merck).

Figure 5 : Cholesterol Standard Curve



Each point represents the mean ± SEM of at least 5 separate measurements in triplicate. Intra assay and inter assay variations were less than 7% and 8% respectively.

B) TLC solvent

Different solvent system have been employed successfully by investigators. A mixture of chloroform/methanol/acetone/acetic acid/water (5:1:2:1:0:5 by volume) was used throughout the study (Walrawen & Chase 1969).

C) Procedure

Nitrogen-dried extracts of different brain regions, were re-extracted in appropriate volumes (0.5 or 1 ml) of chloroform/methanol/water (2:1:0.1, v/v/v). Trace amounts of cerebroside and sulphatide were also dissolved in the same solvent and kept on ice for loading on to TLC plates.

D) Preparation of Thin-Layer Plates

Silica gel G plates were heated in an oven for 1 hour at 110 °C prior to sample application. Then a 0.5 cm width of gel was scraped off in left, right and top edges of the plate. A straight line was drawn gently along the width of the plate, 1 inch from the bottom edge. Samples were applied as a series of microdroplets, in thin bands using a Hamilton syringe, whilst the tip of the needle was continuously in touch with the gel plate. Cerebroside and sulphatide standards were also applied at the left and right hand sides of the sample. For standards usually 1-2 µl aliquots and for samples 50-100 µl aliquots were applied. The plates were dried with a stream of hot air just before development.

E) Chromatography

The solvent system of Walraven and Chase (1969) was used. Tanks were equilibrated for at least 3 hours before chromatography. The chromatography was allowed to proceed until the solvent had reached 1-2 cm below the upper edge (usually 3-3.5 hours). The plate was then removed from the tank and dried in an oven or by a hair dryer for 5-10 minutes.

F) Detection of Cerebroside and Sulphatide

Specific detection tests are available for each lipid group. The orcinol test is specifically used for glycolipids and yields a pinkish violet colour with neutral glycosphingolipids and gangliosides.

The reagent is composed of 0.2% (w/v) orcinol in concentrated sulphuric acid-

water (3:1, v/v). This solution should be stored in an amber bottle in refrigerator. Dried chromatograms were sprayed with orcinol solution until damp and then the plates were heated with hair dryer for 10-15 minutes. Within this time, cerebroside and sulfatide bands were completely visible. The bands have a pink violet colour against a white-yellow background of the plates. Cerebroside migrates faster than sulphatide in the solvent system (Figure 6). Developed plates were stored over silica gel granules in a desiccator before the next step.

The bands corresponding to crebroside and sulphatide were cut out and the silica gel scraped into glass tubes using a spatula. Lipid was eluted from the gel with chloroform/methanol/water (2:1:0.1) whilst mixing with a spatula. The tubes were then centrifuged (2500 rpm for 15-20 min) and the supernatant was transferred to clean glass tubes. The pellets were rinsed and mixed with solvent as before, and the process was repeated 2-3 times until the pellets were colourless. Supernatants were combined and evaporated with oxygen free nitrogen at room temperature.

G) Measurement of the Galactose Content of Cerebroside and Sulphatide

The galactose content of each fraction, was determined by the sulphuric acid-orcinol reaction (Svennerholm 1956).

1) Principle

In this method the sugars are converted to furfural derivatives, which are then reacted with a phenol. The yellow colour produced can be determined colorimetrically.

2) Reagents

Orcinol reagent: 0.2% (w/v) orcinol in concentrated sulphuric acid.

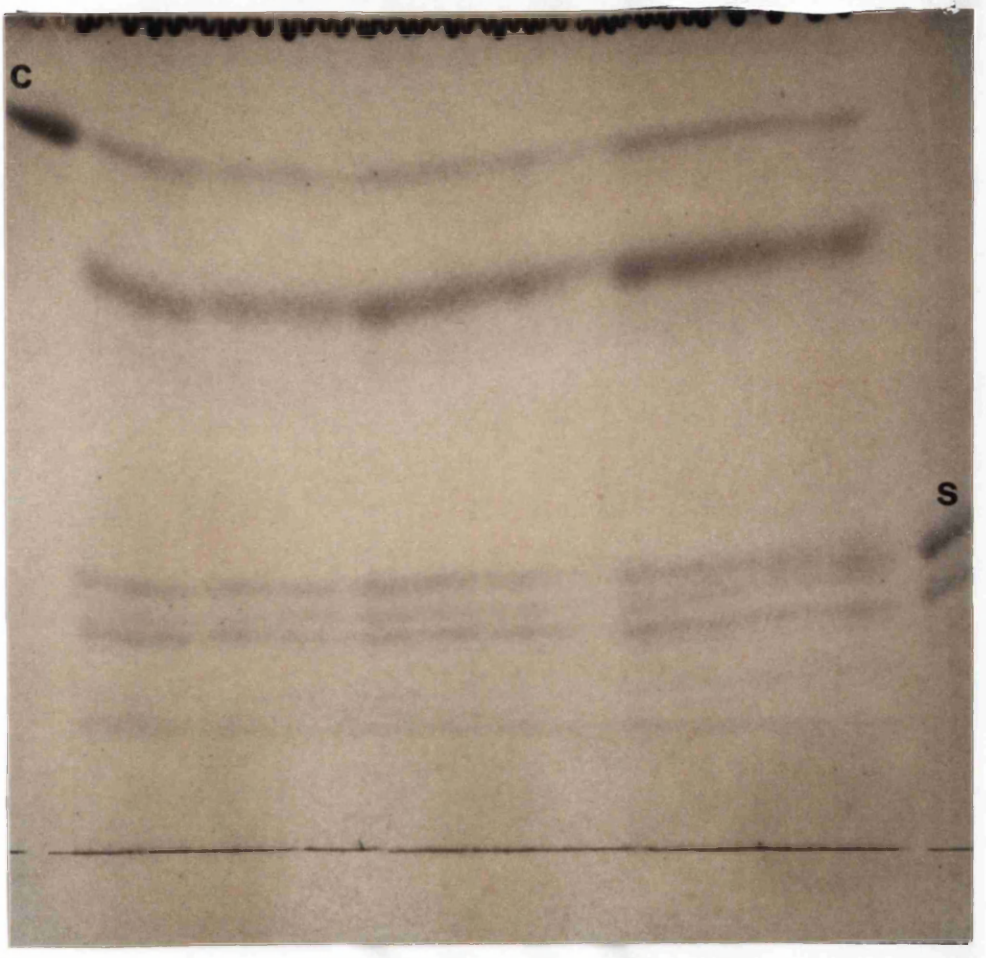
Blank reagent: Concentrated sulphuric acid.

Galactose standard: Galactose 1 mg/ml in distilled water.

3) Procedure

The residues of the cerebroside and sulphatide extracts were dissolved in 1 ml ethanol. To 0.5 ml of lipid, 2 ml of 1.5 M sulphuric acid was added. Tubes were stoppered with marbles and the samples hydrolysed at 100 °C for 2 hours. After cooling with running water, the hydrolysate was transferred to a 10 ml cylinder and the hydrolysis tubes were rinsed 3 times with 1-1.5 ml distilled water. and transferred to the same cylinder. The final volume was adjusted to 8 ml.

Figure 6 : Thin-Layer Chromatography of Cerebroside and Sulphatide.



Cerebroside and sulphatide were separated as described in Materials and Methods. C &S indicate the corresponding bands for cerebroside and sulphatide respectively.

Hydrolysate (2 ml) was pipetted into triplicate test tubes on ice. After 15 minutes, 4 ml of orcinol reagent was added to two of the assay tubes and 4 ml concentrated H_2SO_4 to the blank. The tubes were thoroughly mixed and the rack was placed in an 80° C water bath for 20 minutes. The reaction mixture was chilled on an ice tray and then read at 505 nm in the spectrophotometer.

Galactose standards of 0-75 µg/tube, were run through all steps. A standard curve was plotted (Figure 7) and the galactose content of lipids determined from this standard curve after subtraction of the reagent blank. The cerebroside content was calculated by multiplying the galactose value by 4.6 to express as mg/g wet tissue and the sulfatide value was expressed in terms of µmol/g wet tissue.

2.5 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Gel electrophoresis of proteins has been widely used for both quantitative and qualitative studies of proteins. SDS-PAGE was employed in the present study to evaluate the effects of maternal hypothyroxinemia on protein profiles of different brain regions of experimental and normal progeny. The method was adapted from Weber and Osborn (1969).

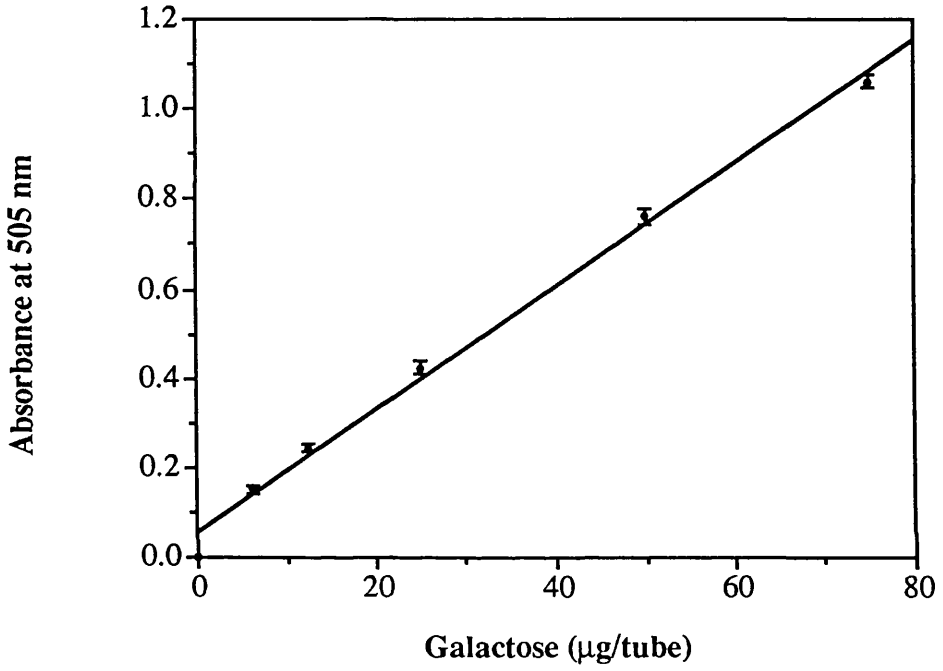
2.5.1 Principle

The intrinsic charge of polypeptide in comparison with SDS is negligible, so different SDS-polypeptide complexes have identical charges. Therefore, the migration of proteins on a SDS-gel is related to molecular weights of polypeptides. The gel acts as a porous media and its molecular sieving effects cause a slower migration rate for the proteins with higher molecular weight in comparison to ones of smaller molecular weights (Hames 1990).

2.5.2 Preparation of Proteins

Fresh homogenates (10% in 0.32 M sucrose) of the various brain regions were centrifuged at 104,000 g by ultracentrifugation (MSE superspeed 50) for 30 minutes at 4° C to separate cytosolic and particulate fractions. Apart from membranes, the latter fraction also contains various organelles (nuclei, mitochondria and membranes). Pellets

Figure 7 : Galactose Standard Curve



Each point represents the mean ± SEM of 6 experiments in duplicate. Intra assay variations were less than 5% but inter assay variations (at levels up to 50 µg) were less than 10% (3.2-9.8%).

were dissolved in 0.01 M phosphate buffer pH 7.1, containing 1% (w/v) SDS and the concentration of protein in both fractions was determined by the method of Lowry *et al.* (1951).

Appropriate amounts of soluble proteins (usually 560 µg) were pipetted into test tubes and the water evaporated. Usually the amount of insoluble protein was enough; sometimes however it was necessary to concentrate fractions and protein measurement was repeated.

2.5.3 Sample Treatment

Both soluble and particulate fractions were treated in a protein:SDS ratio of 1:4, with a sample buffer containing: 5% (w/v) SDS, 1% (v/v) 2-mercaptoethanol and 10% glycerol in 0.1 M phosphate buffer (sodium salt), pH 7.1. The treated samples were incubated at 37° C for 2 hours prior to loading on the slab gel. Proteins were reduced by mercaptoethanol and they were also denatured by heating in boiling water for 2 minutes. To mark the gel front, 3-5 µl of a dye (8% (w/v) of bromophenol blue in water) was added just prior to running. The final concentration of proteins were 70 µg/10µl of the gel slot.

2.5.4 Gel Preparation

A) Materials

1) Slot former

2) Rubber gaskette

3) Thin & thick glass templates

4) Fifty ml syringe

B) Solutions

1) Sodium phosphate buffer 0.2 M, pH 7.1, containing 0.4% (w/v) SDS.

2) Acrylamide solution: 30% (w/v) acrylamide, and 0.8% (w/v) NN'-methylenebisacrylamide. Stock solution was stored in a dark bottle at 4 °C.

3) Ammonium persulphate, 150 mg/10 ml in distilled water (prepared just before use).

4) TEMED solution.

5) Protein marker: a mixture of seven different protein each coloured and purified

separately. These proteins are: myosin (200 KD), phosphorylase b (97.4 KD), BSA (69 KD), ovalbumin (46 KD), carbonic anhydrase (30 KD), trypsin inhibitor (21.5 KD) and lysozyme (14.3 KD) with a concentration of approximately 1 mg/ml of each protein (according to the maufacturer).

C) Preparation of Template

Rubber gasket and slot former (LKB, with 20 projections on each side for 5 & 10 µl slot), were soaked in Ilford wetting agent (approximately 3 ml in 250 ml distilled water), for 1 hour. Thin and thick glass templates, were soaked in detergent for the same time. The glass templates were rinsed with water, sprayed with acetone, and left on the bench to dry, whilst the slot former and rubber gasket were removed and left on the bench to dry.

The thick glass template acts as a support for the thin one. The rubber is a framework, lined around the thin template in a rectangular shape. The slot former covers the rubber and glass template, then all were fixed with clips.

The polyacrylamide gel is made by polymerization of acrylamide monomer. In this reaction N, N'-methylenebisacrylamide (bisacrylamide) is the cross-linker and ammonium persulphate serves as the catalyst. Tetramethylethylendiamine (TEMED) acts as the accelerator of the polymerization proccess.

According to the procedure described by Weber & Osborn (1969), polyacrylamide gels were prepared in phosphate buffer (solution A). 8% Acrylamide gels were prepared with 2.6% cross-linker solution (distilled water: buffer A: solution B, 12.1: 33: 17.6 v/v). Because of the inhibitory effects of oxygen on polymerization the mixture was degassed for 30-60 minutes, then ammonium persulfate and TEMED were quickly added (3.2 ml and 0.1 ml respectively). The gel solution was then quickly poured into the gel template and all bubbles were removed. After 45-60 minutes, the set gel was transferred to a humidified chamber and stored overnight.

2.5.5 Electrophoresis

The gel was prerun at 150 mA for 30 minutes, after which 10 µl of SDS treated samples (70 µg tissue protein were applied to each slot, and 10 µl molecular weight marker (Rainbow^{TM} code RPN 756, Amersham) in the middle slot (number 10). Samples were applied in duplicate. The gel was then run at constant current of 20 mA

for first 10 minutes and then at 150 mA, for 4-5 hours. The gel was removed quickly and fixed overnight in a fixing solution containing: 11.4% (w/v) TCA, 3.4% (w/v) sulphosalysylic acid (SSA) in 30% (v/v) methanol.

2.5.6 Gel Staining and Destaining

The gel was washed in several changes of water for at least 30 min before staining. After washing, it was stained with a Comassie brilliant blue (G 250) solution (0.25%) containing: 45.4% (v/v) methanol and 9.2% (v/v) acetic acid for 1-1.5 h. The gel was then destained with ethanol:acetic acid:water (3:1:6 by volume). The destaining solution was changed several times until the gel background was completely colourless (Figure 8). Gels were then transferred to a 7% (v/v) acetic acid solution.

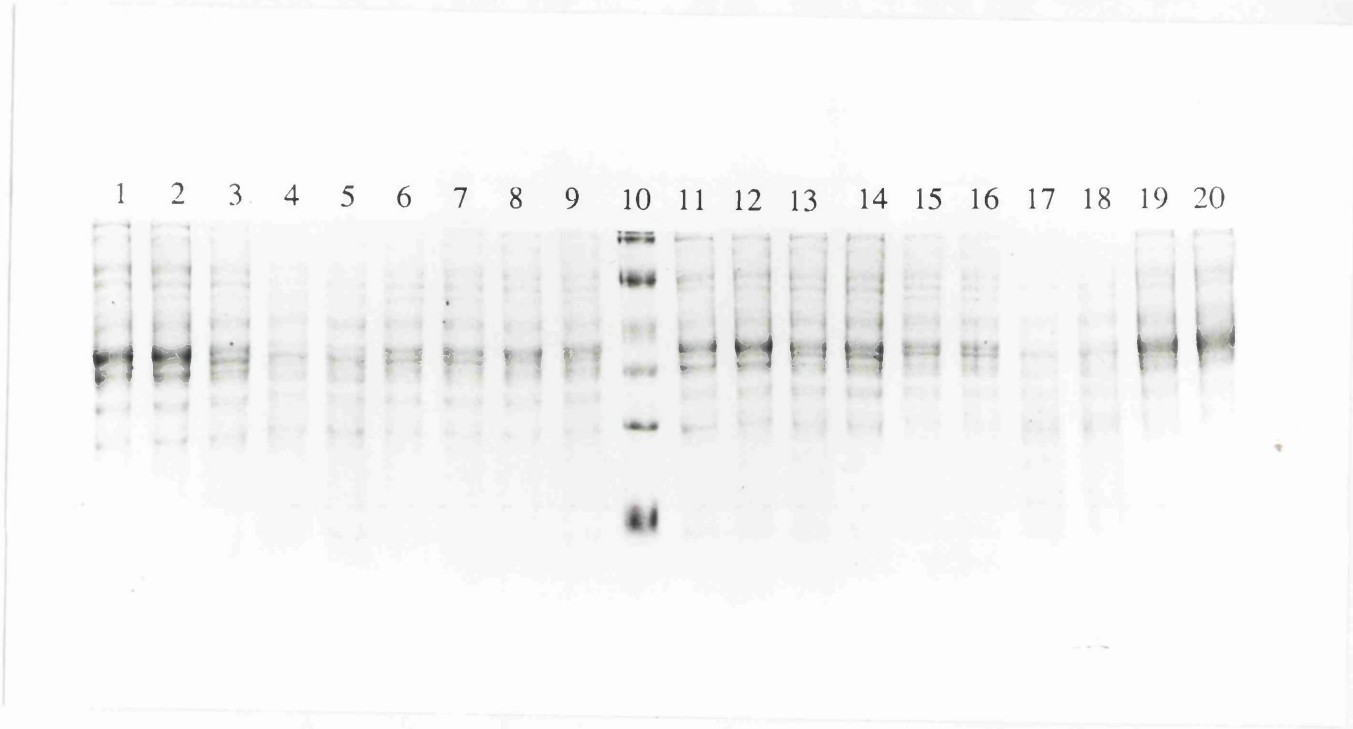
2.5.7 Densitometry

Gels were sectioned into lanes which were then loaded into a Pye-Unicam densitometer, and scanned at a wavelength of 540 nm, at a scanning speed of 0.4 mm/s. Before scanning the machine was zeroed using a blank portion (usually the beginning) of the lane. Each protein band on the gel results in a peak on the graph paper. As Figure 8 shows, many protein bands can be seen in the gel, but only certain of these bands were sufficiently intense to be scanned by the densitometer, from which only those within the linear range of the molecular weight standard curve were selectively calculated. The area under the curve for each peak was calculated, as was the total area under the curve for all peaks (absolute value for each peak and for total amount of protein). Then these values were represented as arbitrary units for each protein band.

2.5.8 Standard Protein Marker

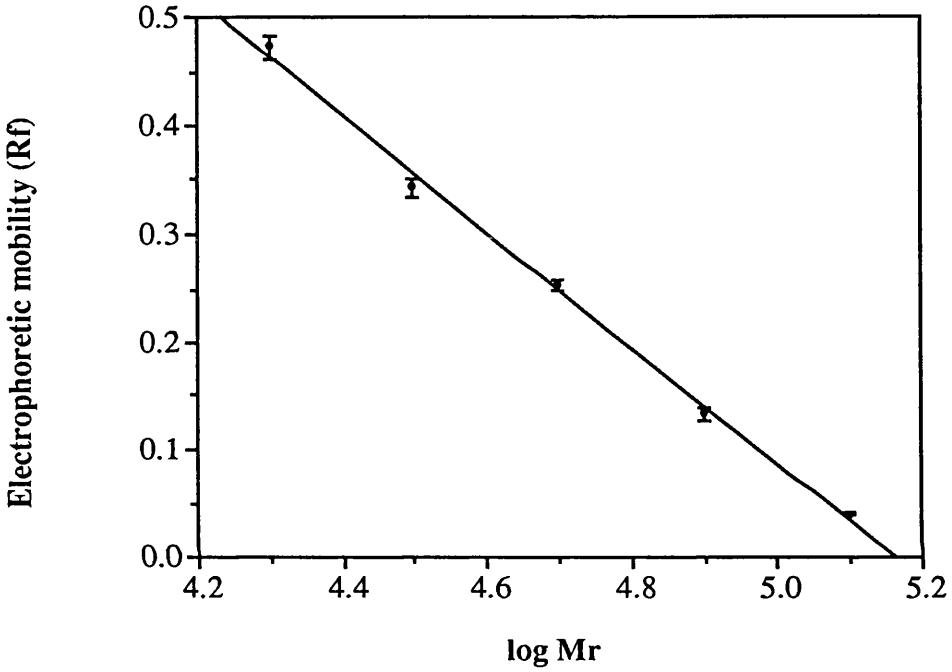
As described above, a standard protein marker which composed of seven separate proteins with a range of molecular weight from 14,300 to 200,000 was run in each experiment. After densitometry the true distance migrated by each single protein was calculated. Distances were calculated from the beginning of the gel to the middle of the peak for each band and to the end of the peak for dye. A calibration standard curve with the $\log_{10}$ molecular weight against relative mobility (R_f) was plotted (Fig 9). Relative

Figure 8 : SDS-PAGE Run of Proteins from Brain Regions of Adult Progeny.



Separation of different protein bands (cytosolic fraction) on SDS-polyacrylamide slab gel. Homogenates from five different brain regions of normal progeny (1-9) and partially thyroidectomised dam progeny (11-20) were loaded on the gel and was run as described in Materials and Methods. Number 10 shows the Rainbow^{TM} protein marker.

Figure 9 : SDS-PAGE Molecular weight calibration curve.



Electrophoretic mobility (Rf) was determined for a range of standard molecular weight proteins (200 KD-14.3 KD) as described in Materials and Methods, and the Rf was plotted against log Mr. The linear part of the graph (97.4-21.5) is shown here.

molecular weights of different bands in sample proteins were calculated from this curve.

2.6 Liquid Scintillation Counting

Samples were added to scintillation vials, containing 4 ml of a scintillation fluid, resistent to excessive quenching (Ecoscint A, National Diagnostic, Aylesbury, Bucks).

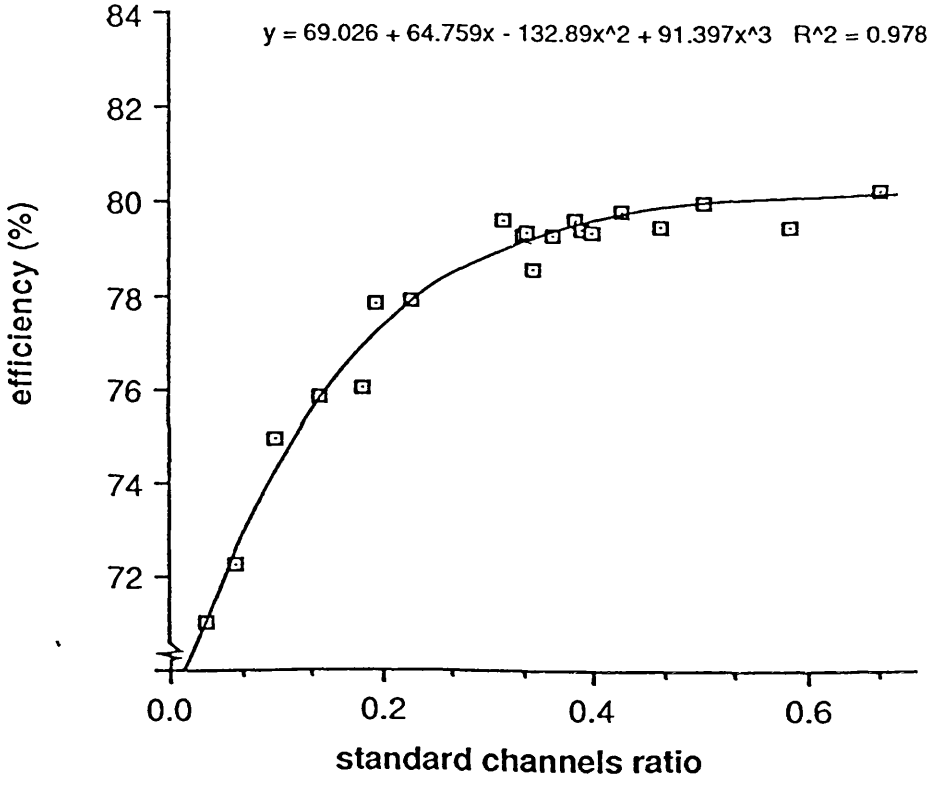
2.6.1 Quench Correction For ^{14}C

Quench correction for ^{14}C, was prepared in the following manner:
To a range of standards, each containing the same amount of radiolabelled [^{14}C]orotic acid, in 3 ml of scintillation fluid (prepared in triplicate), a measured amount of quenching agents were added as follows : water (0.1-1 ml), TCA (0.1-0.5 ml), NCS (0.1-0.5 ml) and chloroform (0.05-0.3 ml). In addition, controls were prepared containing no quenching agent, to determine the counting efficiency of the machine (LKB, Rackbeta) and containing no label, to assess background count. Samples were counted overnight and after blank subtraction, efficiency was plotted against the standard channels ratio to produce a quench curve. Data were converted to dpm by the equation of the curve, drawn by a computer graphic package. All data within the range of quench curve were selected for calculation (Figure 10).

2.7 Expression of Data

Both male and female adult progeny were used in this study. Experimental and normal progeny were grown up under the same condition as mentioned before (this chapter). Differences between two groups were assessed by Student's t-test, and were considered to be significant if probability value (P) was less than 0.05 ($P < 0.05$).

Figure 10 : Quench Curve for ^{14}C Scintillation Counting.



2.8 Materials

Acetic acid glacial, analar grade	MERCK Ltd Broom road, Poole, Dorset, UK.
Acetone, analar grade	MERCK
Acrylamide solution	
Adenosine 2', 3'-cyclic monophosphate	SIGMA
Adenosine 5'-monophosphate	SIGMA Chemical Co. Ltd, Fancy road, Poole, Dorset, UK.
Alkaline phosphatase	SIGMA
Ammonia solution	MERCK
Ammonium molybdate	MERCK
Ammonium persulphate	SIGMA
Benzene, analar grade	MERCK
Bovine serum albumin	SIGMA
Bromophenol blue	SIGMA
Sec-Butanol (isobutanol)	SIGMA
Cerebroside	SIGMA
Chloroform, analar grade	MERCK
Cholesterol	MERCK
Coomassie brilliant blue (G 250)	National diagnostica Somerville New Jersey USA.
Copper sulphate	MERCK
Deoxycholic acid	SIGMA
EcoScint A	National Diagnostics
Ethanol (absolute alcohol), analar grade	Hayman Limited; Witham, Essex CM8 3YE England.
Ethylene Glycol-bis (β-Aminoethyl Ether) N, N, N', N'-Tetra Acetic Acid (EGTA)	SIGMA
Ethylene Glycol Monomethyl Ether (methylcelesolve)	SIGMA

Ferric chloride	MERCK
Folin and Ciocalteau's phenol reagent	SIGMA
Galactose	SIGMA
Glycine	SIGMA
HCl	MERCK
Hamilton syringe	Hamilton Bonaduz AG CH-7 402 Bonaduz Switzerland
Ilford wetting agent	CIBA-GEIGY, UK.
Magnesium chloride	SIGMA
2-Mercaptoethanol	MERCK
Methanol	MERCK
4-Methylumbelliferyl-N-Acetyl -β-D-galactosaminide	SIGMA
4-Methylumbelliferyl-β-D-glucuronide	SIGMA
4-Methylumbelliferyl oleate	SIGMA
Orcinol	SIGMA
Protogel	National diagnostics
RainbowTM code RPN 756	Amersham Amersham International PLC. Amersham Place, Little Chalfont; Amersham, Buckinghamshire UK.
Sodium acetate	MERCK
Sodium carbonate	SIGMA
Sodium citrate	MERCK
Sodium dodecyl sulphate (SDS)	SIGMA
Sodium hydroxide	MERCK
Sodium phosphate	MERCK
Sodium-potassium tartrate	SIGMA
Sphingomyelin	SIGMA
Sphingomyelin [^{14}C-methyl phosphorylcholine]	Amersham
Stannous chloride	MERCK

Sucrose	MERCK
Sulphatide	SIGMA
Sulphuric acid	MERCK
Sulphosalicylic acid (SSA)	SIGMA
Tetra methylethylendiamine (TEMED)	SIGMA
Thin-Layer plates	MERK
Trichloroacetic acid (TCA)	SIGMA
Tris-HCl (Tris[hydroxymethyl] aminomethane)	SIGMA
Tris-Maleate	SIGMA
Triton-X100	SIGMA

CHAPTER 3 : RESULTS

GROSS EFFECTS OF MATERNAL HYPOTHYROXINEMIA ON THE BRAIN BIOCHEMISTRY OF ADULT PROGENY.

3.1 Introduction

The partially thyroidectomised (TX) rat dam has proved a useful model system for studying the effects of maternal hypothyroxinemia on brain development in progeny. To date, the use of this model has demonstrated changes in the brain biochemistry of both developing and adult progeny (Al Mazidi 1989, Hubank 1990).

In developing progeny from hypothyroxinemic dams, brain glycoprotein metabolism is deficient. The amounts of cytosolic glycoprotein are increased in experimental progeny, possibly due to impaired membrane incorporation. Furthermore, investigation of glycoprotein profiles revealed deficiencies in the ontogenesis of particular glycoprotein species. These changes were associated with reductions in the activities of certain sugar hydrolases.

Work with adult progeny has shown selective effects on enzymes associated with several parameters such as energy metabolism, lysosomal function, calcium homeostasis and neurotransmitter turnover (Al Mazidi 1989, Ruiz de Elvira, Sinha, Pickard *et al* 1989). Such studies demonstrated brain region-specific effects of maternal hypothyroxinemia but in certain circumstances such deficits may not be evident in studies of whole brain.

In this investigation, the biochemistry of different brain regions from adult progeny of hypothyroxinemic dams was studied. Initial work was concerned with examination of total protein profiles (SDS-PAGE analysis) from cytosolic and membrane fractions, in order to determine the global effects of maternal hypothyroxinemia on the brain of adult progeny.

In order to determine if maternal hypothyroxinemia resulted in damage to a specific cell population (neurons or glia), the activities of cell marker enzymes were measured. This work demonstrated significant impairments of oligodendroglial cell function. Because of the reported deficits in myelin from human subjects in the iodine-deficient endemias, the final part of these work was concerned with the effects of maternal hypothyroxinemia on myelin metabolism.

3.2 Plasma Thyroid Hormone Levels in Dams and their Progeny

Plasma concentrations of both T_4 and T_3 were measured for both groups of dams and their respective progeny. As Table 3 shows, the thyroidectomised dams were severely

hypothyroxinemic. The level of plasma T_4 was reduced by 70%, and this difference was statistically significant. The plasma T_3 level was reduced in TX dams by only 31%, nevertheless T_3 levels remained within the normal range.

The level of thyroid hormones in progeny of both experimental and normal dams was normal, as shown in Table 3. Thus, maternal hypothyroxinemia during pregnancy was without effect on the thyroid state of the progeny at adulthood, TSH measurement excepted.

Table 3 : Concentrations of Thyroxine (T4) and 3, 5, 3'-Triiodothyronine (T_3) in Plasma From Normal and Partially Thyroidectomised Rat Dams and Their Respective Progeny.

		Concentration (nmol/l)	
	n	T_4	T_3
Dams			
Normal	5	44.7 ± 20.0.	1.22 ± 0.36
Thyroidectomised	5	13.5 ± 8.7*	0.84 ± 0.25
Progeny of:			
Normal dam	6	47.0 ± 12.2	1.15 ± 0.32
Thyroidectomised dam	6	47.3 ± 13	1.11 ± 0.37

Values are mean ± SD.

* $P < 0.02$ compared with normal dam (Student's t-test).

3.3 Body and Brain Weights of Progeny

Previous studies conducted with fetuses and young progeny from thyroidectomised rat dams have demonstrated reductions in brain and body weights (Hubank 1990, Pickard *et al* 1991b). To investigate the effect of maternal hypothyroxinemia on body and brain weights of adult progeny, animals were studied at two different age points (3.5 months and 7 months).

3.3.1 3.5 Months old progeny

In 3.5 months old rats, the body weight of TX dam progeny for all animals (male and female) was slightly higher than controls (by 13%) but the difference was not statistically significant. On the other hand, the body weights of TX dam progeny for both male and female were significantly higher than those of the controls (male: by 8%, $P < 0.05$; female: 25%, $P < 0.005$). No changes were found in brain weights of experimental progeny (male and female) in comparison with controls. Although the brain/body weight ratio (male and female) in experimental progeny was not statistically different from the normal control, this ratio was significantly reduced in experimental group for male and female progeny as can be seen in Table 4.

Table 4 : Effects of Maternal Hypothyroxinemia on Brain weight, Body weight and Brain/Body weight Ratio of 3.5-Months Old Progeny.

	Body weight (g)	Brain weight (g)	Brain/Body weight ratio (%)
Normal dam progeny			
All (10)	341.9 ± 116.4	1.95 ± 0.13	0.637 ± 0.20
Male (5)	457.3 ± 13.8	2.03 ± 0.12	0.445 ± 0.03
Female (5)	226.4 ± 16	1.87 ± 0.08	0.830 ± 0.08
TX dam progeny			
All (10)	387.8 ± 107.9	1.96 ± 0.08	0.545 ± 0.15
Male (5)	492.8 ± 25.9*	1.99 ± 0.09	0.404 ± 0.02**
Female (5)	282.8 ± 23.9***	1.93 ± 0.05	0.687 ± 0.06**

Values are mean ± SD; number of rats are shown in parentheses.

* $P < 0.05$; ** $P < 0.02$ and *** $P < 0.005$.

3.3.2 Seven months old progeny

In seven months old rat progeny, the mean body weight of the experimental group (all animals) was higher than normal controls (by 9%). The body weight of both male and female progeny from TX dam was higher than their respective controls (by 8% and 12% respectively), although none of these differences was statistically significant. As the Table 5 shows, the brain weight of experimental progeny for all animals (male and female) was slightly higher than that of the control group. Again these differences were not statistically significant. In contrast, the brain weight/body weight ratio in normal progeny was higher for all animals, male and female than those of the experimental group.

Table 5 : Effects of Maternal Hypothyroxinemia on Brain weight, Body weight and Brain/Body weight Ratio of 7-Months Old Progeny.

	Body weight (g)	Brain weight (g)	Brain/Body weight ratio (%)
Normal dam progeny			
All (16)	496.8 ± 180.2	2.01 ± 0.25	0.451 ± 0.150
Male (9)	639.7 ± 88	2.11 ± 0.23	0.333 ± 0.049
Female (7)	313.1 ± 28.6	1.88 ± 0.22	0.604 ± 0.070
TX dam progeny			
All (16)	541.9 ± 185.1	2.06 ± 0.23	0.423 ± 0.138
Male (9)	690.8 ± 77.2	2.17 ± 0.25	0.320 ± 0.070
Female (7)	350.4 ± 40.8	1.92 ± 0.10	0.554 ± 0.070

Values are mean ± SD; number of animals are shown in parentheses.

3.4 Total Protein Concentrations in Brain Regions of Progeny

To investigate any gross impairments if any, in protein acquisition due to maternal hypothyroxinemia, protein concentration (mg protein/g wet weight) was determined and compared between progenies from thyroidectomised and normal dams. As Figure 12 shows, no significant differences in total protein content were observed between normal and TX dam progeny, in any of the five brain regions studied. Therefore maternal hypothyroxinemia is without effect on gross protein synthesis and/or acquisition in the brains of progeny. Levels of total protein concentration in brain regions from normal and partially thyroidectomised dam progeny are demonstrated in Figure 11.

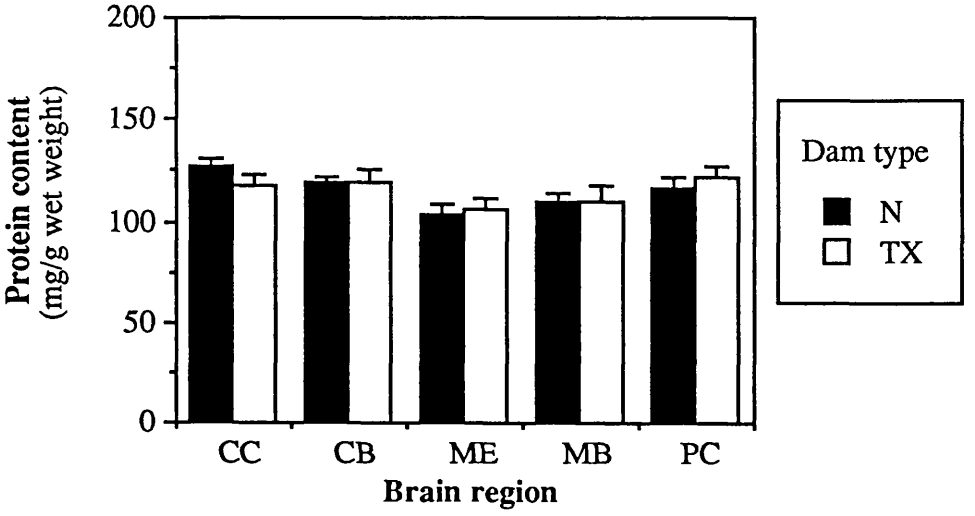
3.5 Effects of Maternal Hypothyroxinemia on Cytosolic and Particulate Protein Profiles

It is well known that the thyroid hormones control the expression of only certain protein products in brain and other tissues, and therefore, it is possible that the production of brain specific proteins may be more susceptible to early hypothyroxinemia than others. In fact this has been previously demonstrated in adult progeny from hypothyroxinemic dams, Thus, the activity of lactate dehydrogenase is reduced in a number of brain regions, whereas that of the succinate dehydrogenase is normal in all brain regions (Al Mazidi 1989).

In order to gain more global information on the protein-specific effects of maternal hypothyroxinemia on brain of progeny, it was decided to analyse protein profiles after separation by SDS-PAGE. Since earlier work with developing progeny had demonstrated differential effects on membrane and cytosolic glycoproteins, and in order to simplify the interpretation of results, brain region homogenates were separated firstly into cytosolic and particulate (membrane-associated and organellar protein) fractions before electrophoresis.

SDS-PAGE resulted in the separation of at least twelve different protein bands (Figure 8). Only those bands of molecular weight 21-97 KD were considered for this analysis, since this corresponded to the linear portion of the calibration curve of relative mobility to molecular weight.

Figure 11 : The effect of maternal hypothyroxinemia on the concentration of total protein in different brain regions from adult progeny.



The brain regions of adult progeny from normal (N) and partially thyroidectomised (TX) rat dams were dissected into: cerebral cortex (CC), cerebellum (CB), medulla (ME), midbrain (MB) and paleocortex (PC). The protein content of tissue homogenates was determined with the Folin reagent, as described in Materials and Methods. Values are the mean ± SEM of at least 8 animals.

3.5.1 Cytosolic Protein

a) Cerebral Cortex

A total of eleven different protein bands (corresponding to molecular weights of 21, 25, 31, 38, 49, 56, 62, 70, 79, 89 and 97 KD) were found for the cytosolic fraction from cerebral cortex (Figure 12). Significant change was only evident for the 97 KD protein band, being increased in TX dam progeny (N = 1.00 ± 0.16, TX = 1.74 ± 0.20, P < 0.02). Although similar increases were observed for the lower molecular weight protein bands in Tx dam progeny, these were not statistically significant.

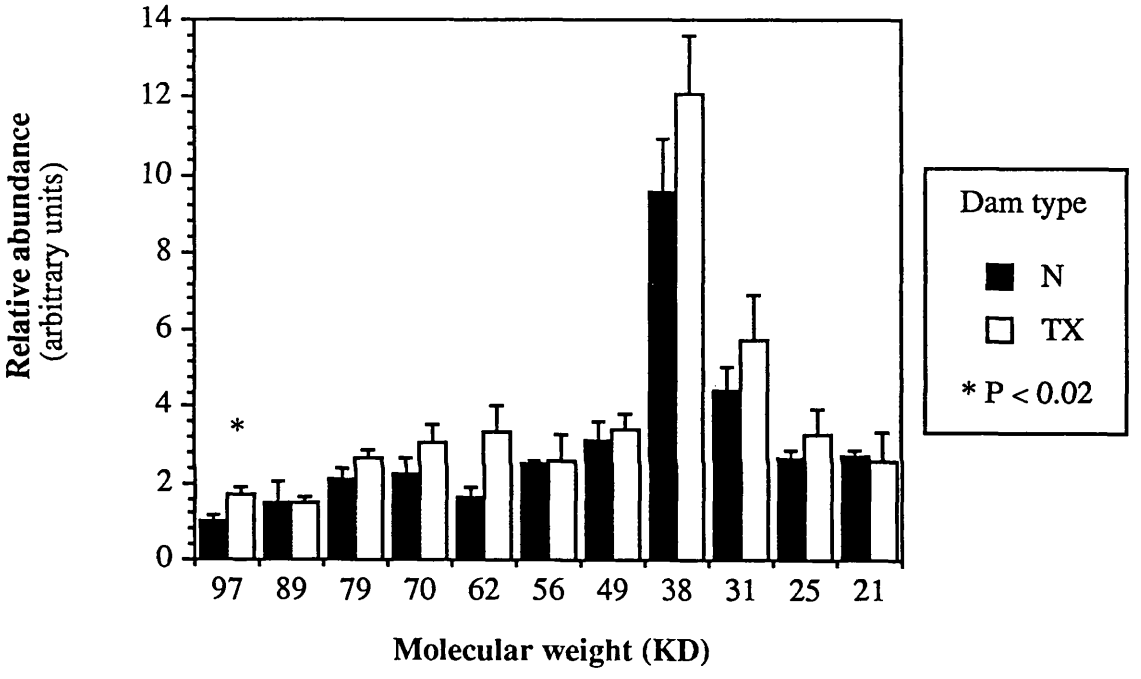
b) Cerebellum

Protein bands of molecular weights 21, 25, 31, 38, 49, 56, 62, 79, 89 and 97 KD were measured in experimental and control progeny. The protein profile of the cytosolic fraction from the cerebellum (Figure 13) was similar to that of the cerebral cortex. Comparison of the cytosolic profiles of the cerebellum from normal and TX dam progeny, revealed a -29% and -20% reduction in the content of the 38 KD and 49 KD protein bands respectively. In contrast, 25 KD, 56 KD, 62 KD and 70 KD were increased by +24%, +39%, +26% and +47% respectively. However these differences were not significant.

c) Medulla

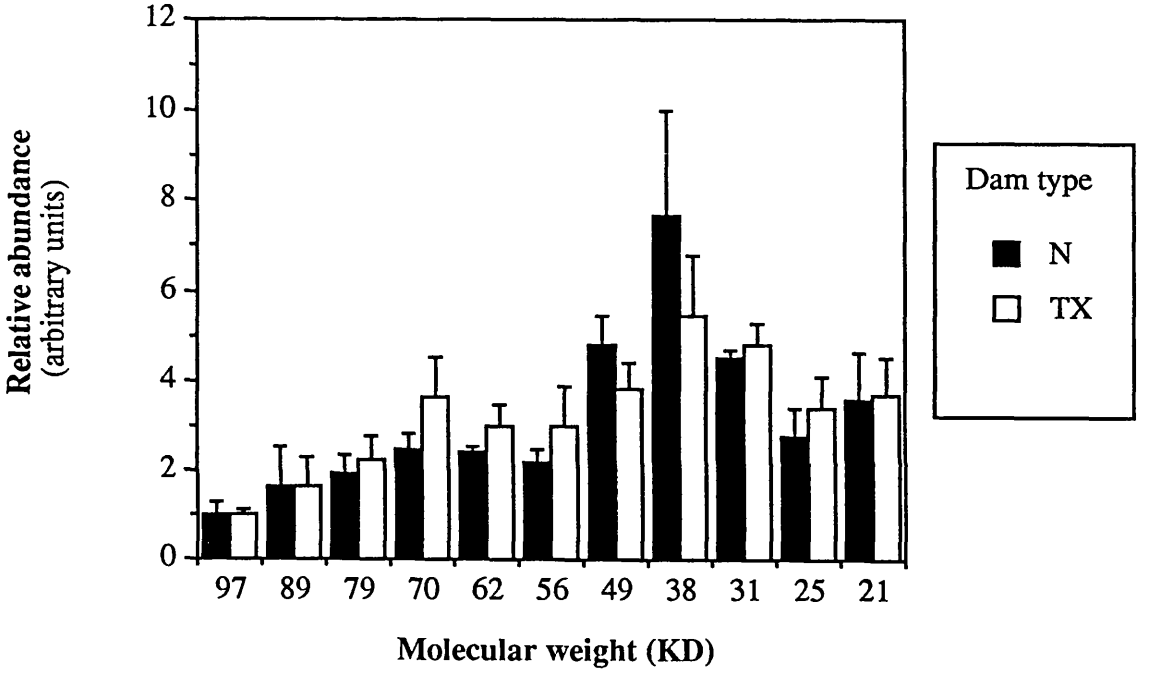
Protein bands of 21, 25, 31, 38, 49, 56, 62, 70, 79, 89 and 97 KD were measured in medulla (Figure 14). In TX dam progeny, the 97 KD protein band was decreased by -32% (though insignificant). A statistically significant deficit was however demonstrated in the 38 KD protein band, which was decreased by -30% in TX dam progeny (N = 9.41 ± 0.53, TX = 6.63 ± 0.74, P < 0.02). In contrast, 25 KD protein band was significantly increased in TX dam progeny (N= 2.24 ± 0.51, TX = 5.08 ± 0.29, P < 0.005). On the other hand a protein band of molecular weight of 31 KD was absent in TX dam progeny (N = 3.35 ± 0.62, n = 5)). Changes in the other protein bands were not significant.

Figure 12 : The effect of maternal hypothyroxinemia on the cytosolic protein profile of cerebral cortex from adult rat progeny.



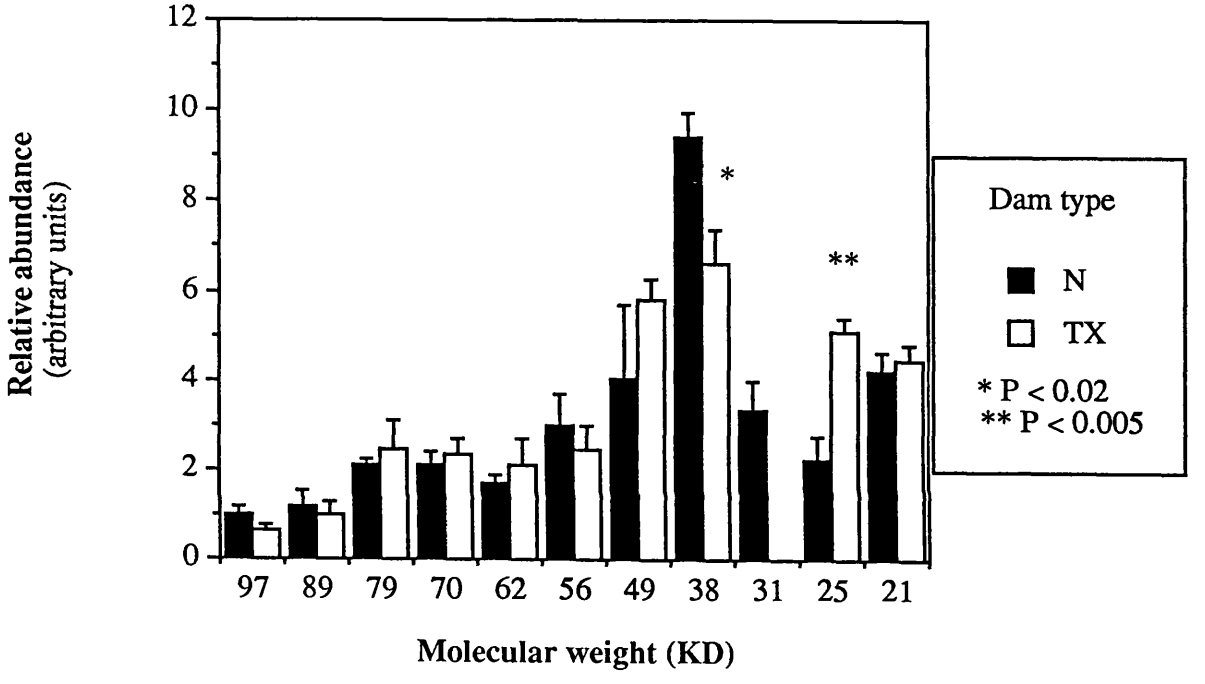
Values are the mean ± SEM of 5-6 separate experiments, except for 79 KD, 56 KD and 62 KD protein bands, where n = 3, 3 and 4 respectively; * P < 0.02.

Figure 13 : The effect of maternal hypothyroxinemia on the cytosolic protein profile of cerebellum from adult rat progeny.



Values are the mean ± SEM of at least 5 separate experiments, except for the 56 KD protein band, where n = 3.

Figure 14 : The effect of maternal hypothyroxinemia on the cytosolic protein profile of medulla from adult progeny.



Values are the mean ± SEM of 5-6 separate experiments, except for 79 KD and 49 KD, where n = 3 and 4 respectively; * P < 0.02 and ** P < 0.005.

d) Midbrain

A total of eleven different molecular weight protein bands (21, 25, 31, 38, 49, 56, 62, 70, 79, 89 and 97 KD) were measured in midbrain (Figure 15). In TX dam progeny, the 49 KD, 62 KD, 79 KD, 89 KD and 97 KD protein bands were increased by +17%, +17%, +37%, +44% and +14% respectively, whereas the protein bands of 21 KD, 25 KD and 56 KD molecular weight were decreased by -16%, -29% and -26% respectively. However none of these changes was statistically significant.

e) Paleocortex

Eleven different molecular weight protein bands were measured in paleocortex like other regions. In the TX dam progeny, the 89 KD protein band was significantly increased by 63% ($N = 0.71 \pm 0.08$, $TX = 1.16 \pm 0.06$, $P < 0.001$). In experimental progeny, protein bands corresponding to 21 KD, 25 KD and 38 KD, 70 KD, 79 KD and 97KD were increased by +19%, +60% and +18%, +19%, +26% and +25% respectively. On the other hand, 31 KD, 56KD and 62 KD protein bands were decreased by -37%, -41% and -62% respectively. Nevertheless, none of these changes was statistically significant (Figure 16).

3.5.2 Particulate (Insoluble) Protein

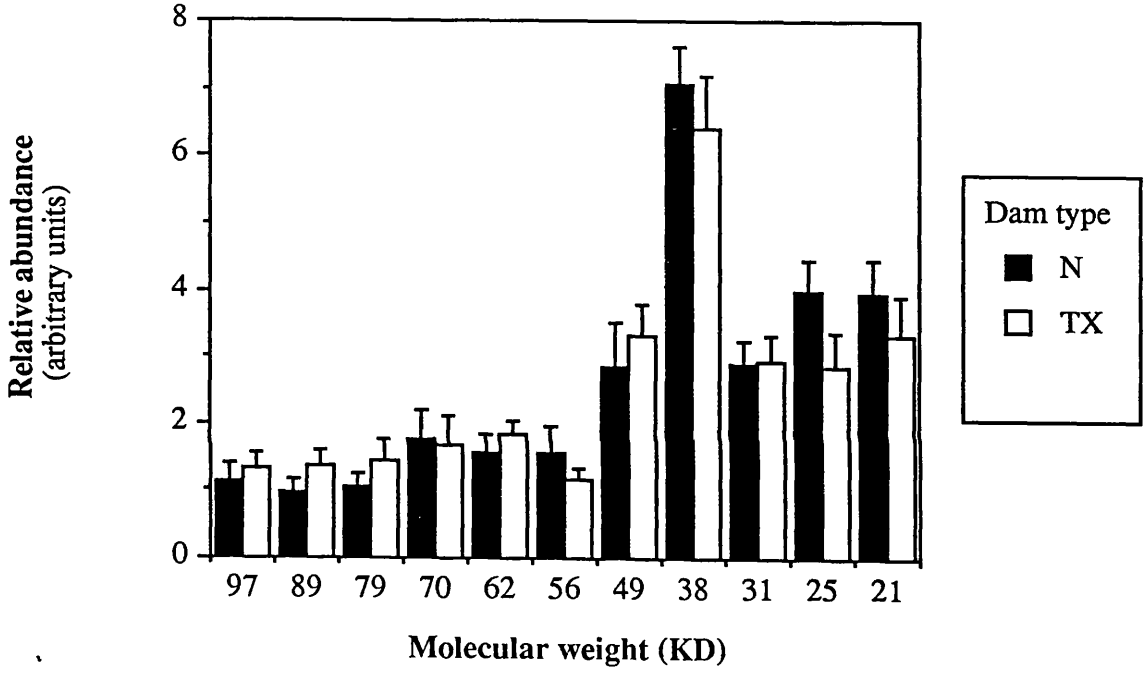
a) Cerebral Cortex

Protein bands corresponding to 21, 25, 31, 38, 49, 56, 62, 70, 79, 89 and 97 KD were measured in this region. Both increases (21 KD, 79 KD and 97 KD) and decreases (31 KD, 38 KD, 70 KD and 89 KD) were observed in protein bands from the particulate fraction of cerebral cortex from TX dam progeny. None of these changes was statistically significant (Figure 17).

b) Cerebellum

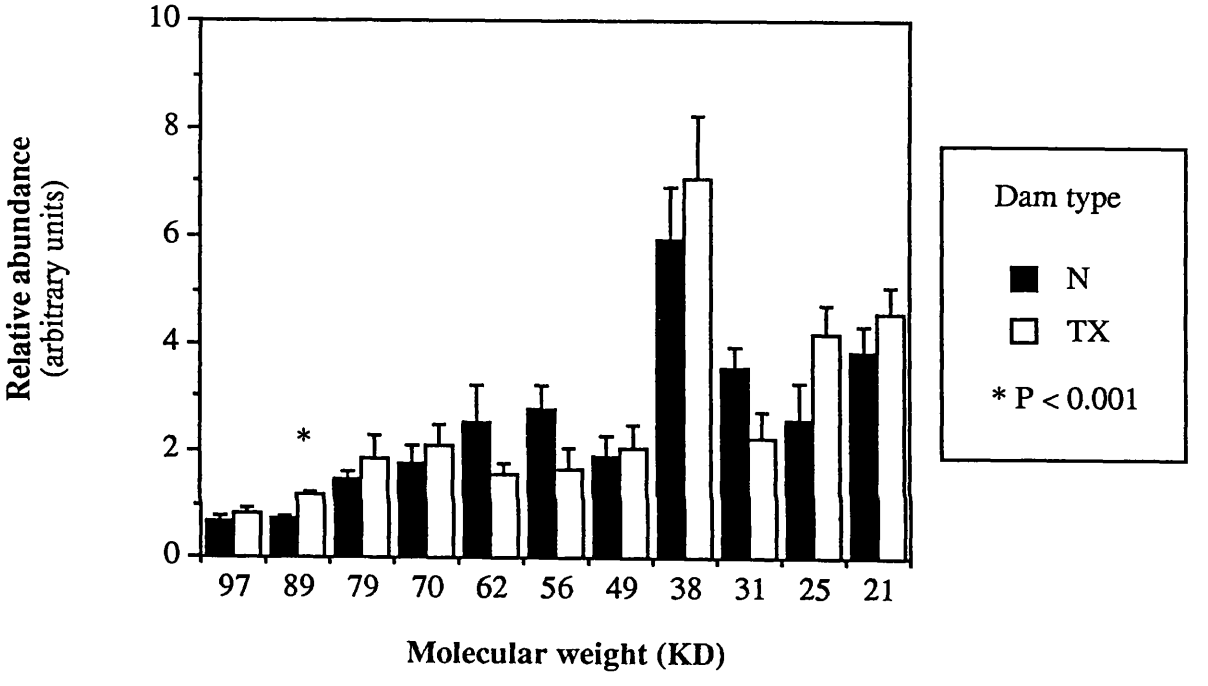
Protein bands of 21 KD, 25 KD, 31 KD, 38 KD, 49 KD, 56 KD, 62 KD, 70 KD, 89 KD and 97 KD molecular weight were analysed in the particulate fraction of cerebellum

Figure 15 : The effect of maternal hypothyroxinemia on the cytosolic protein profile of midbrain from rat adult progeny.



Values are the mean ± SEM of 5-6 separate experiments, except for 31 KD where n = 4.

Figure 16 : The effect of maternal hypothyroxinemia on cytosolic protein profile of paleocortex from adult rat progeny.



Values are the mean ± SEM of at least 5 separate experiments, except for 31 KD where n = 4; * $P < 0.001$.

(Figure 18). The major finding here was an increase in 31 KD (by +33%, NS), 38 KD (by +53%, NS), 49 KD (by +70%, NS), 56 KD (by +23%, NS) and 97 KD (by +28%, NS) protein bands in the TX dam progeny, with a decrease in 21 KD (by -16%, NS) protein band. None of these differences was statistically significant.

c) Medulla

Figure 19 shows the SDS-PAGE analysis of particulate protein fraction in medulla. Like cerebral cortex, protein bands of 21-97 KD molecular weight were measured in this region. The only statistically significant change in this region was that of the 79 KD protein band which increased in TX dam progeny (by +103%, $P < 0.01$).Both increases (25 KD, 31 KD, 70 KD and 89 KD) and decreases (38 KD, 62 KD and 97 KD) were observed in this region. However, these changes were not significant.

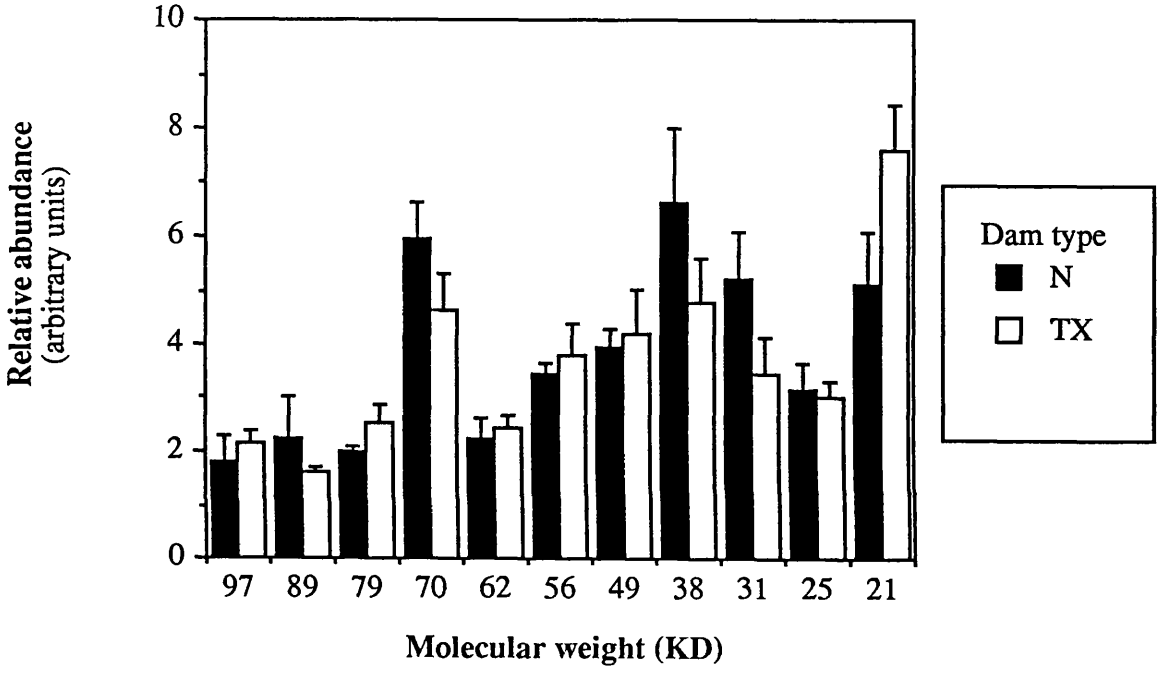
d) Midbrain

The results of SDS-gel analysis of insoluble proteins from midbrain are demonstrated in Figure 20. Different protein bands (21, 25, 31, 38, 49, 62, 70, 79, 89 and 97 KD) molecular weight were measured in this region. 49 KD Protein band was decreased (by -17%, NS) and conversely, 62 KD, 70 KD, 79 KD and 89 KD protein bands were increased (by +51%, +48%, +13% and +65% respectively; all NS). Nevertheless, none of these changes was statistically significant.

e) Paleocortex

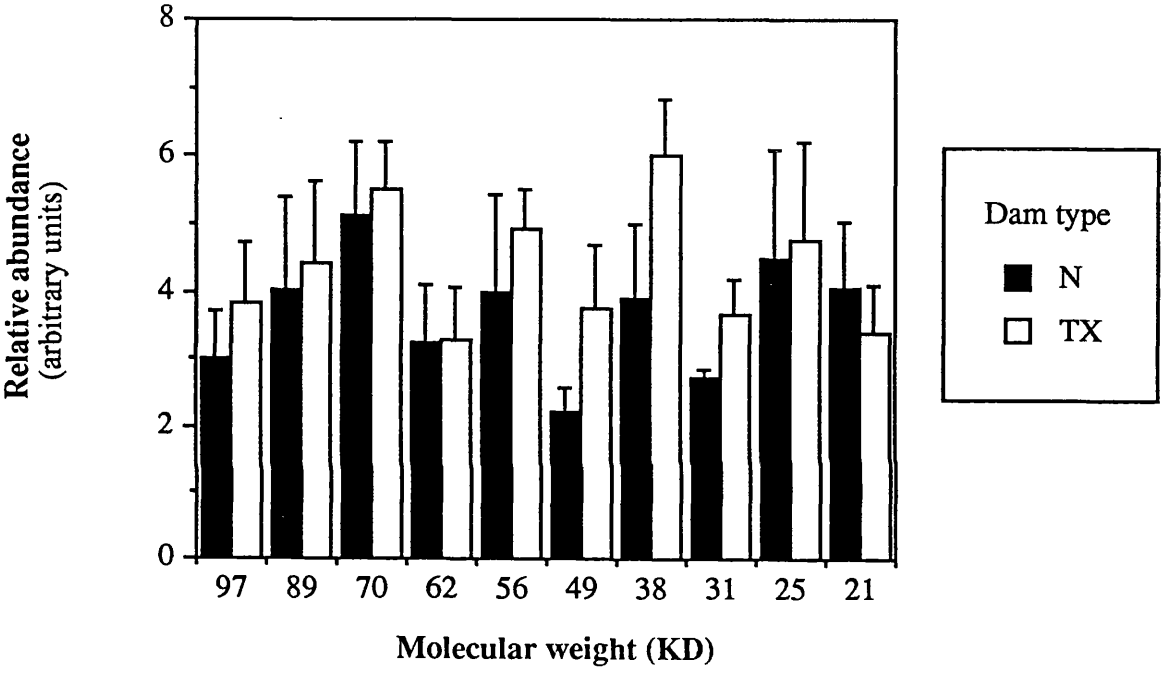
Different protein bands (21, 25, 31, 38, 49, 56, 62, 70, 79, 89 and 97 KD) molecular weights were analysed in paleocortex of both experimental and normal progeny. No statistically significant difference was found at the level of the measured bands. Nevertheless, the 31 KD, 56 KD, 70 KD, 89KD and 97 KD protein bands were increased (by +15%, +69%, +32%, +26% and +19% respectively; all NS). In contrast, 21 KD, 25 KD, 49 KD and 62 KD protein bands were diminished (by -17%, -15%, -12% and -19% respectively; all NS). However, none of these changes was significant (Figure 21).

Figure 17 : The effect of maternal hypothyroxinemia on the particulate protein profile of cerebral cortex from adult rat progeny.



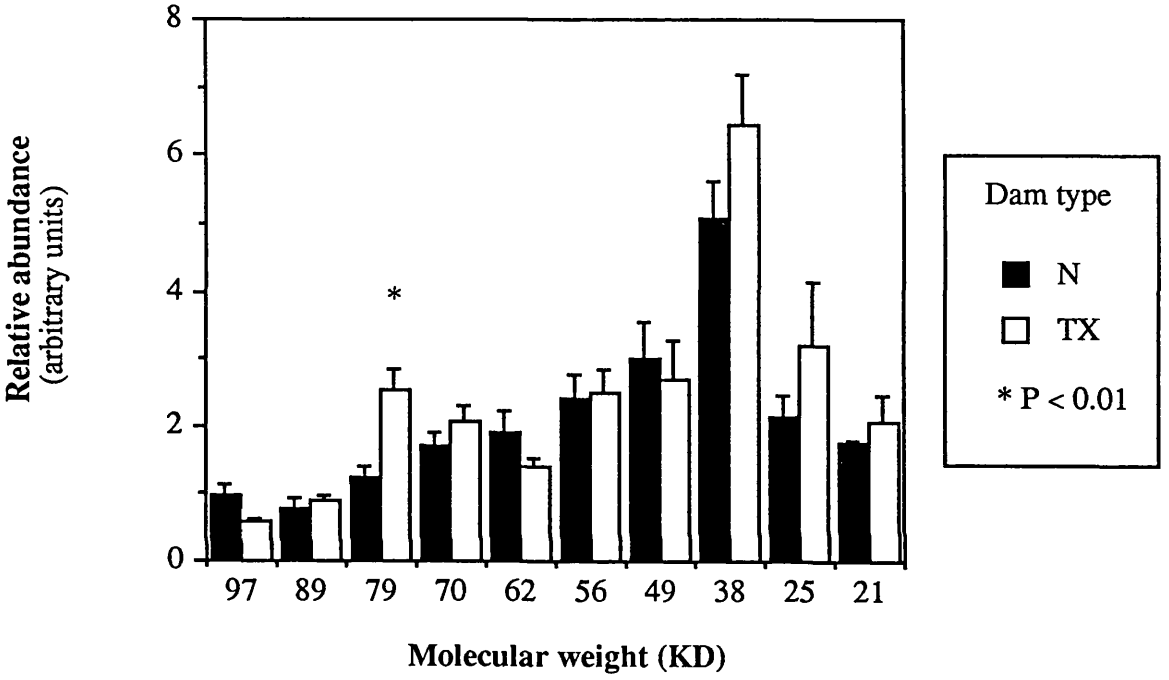
Values are the mean ± SEM of at least 5 animals, except for 89 KD, 25 KD and 21 KD, where n = 4, 3 and 4 respectively.

Figure 18 : The effect of maternal hypothyroxinemia on the particulate protein profile of cerebellum from adult rat progeny.



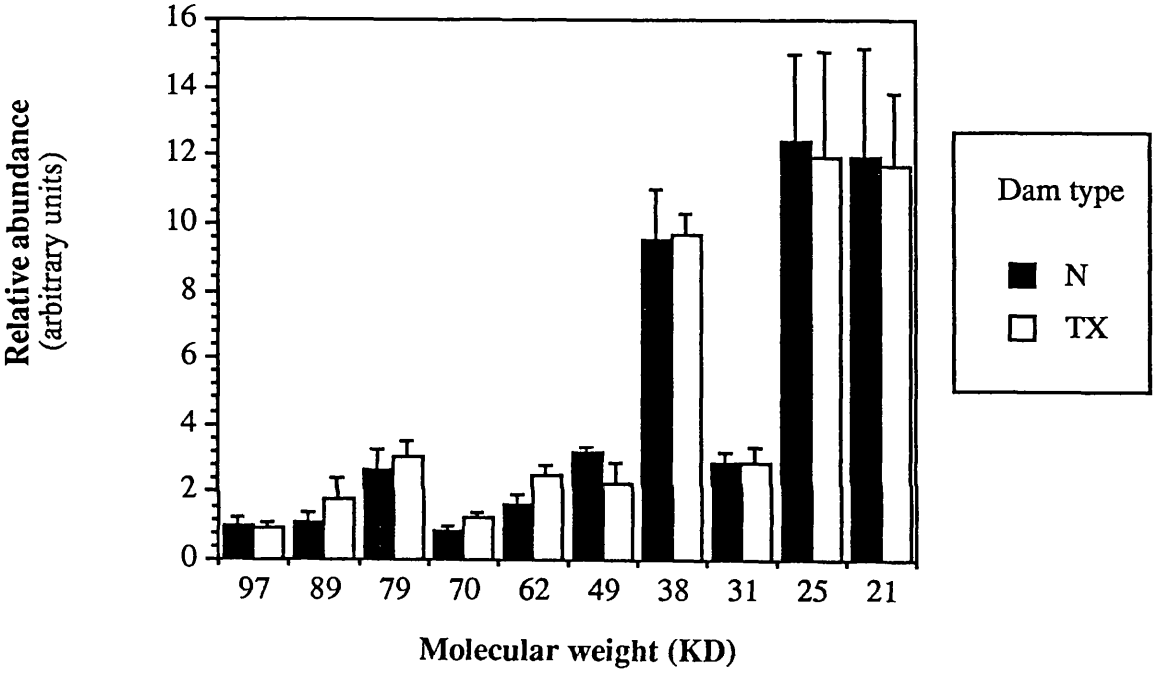
Values are the mean ± SEM of at least 4-5 separate experiments.

Figure 19 : The effect of maternal hypothyroxinemia on particulate protein profile of medulla from adult rat progeny.



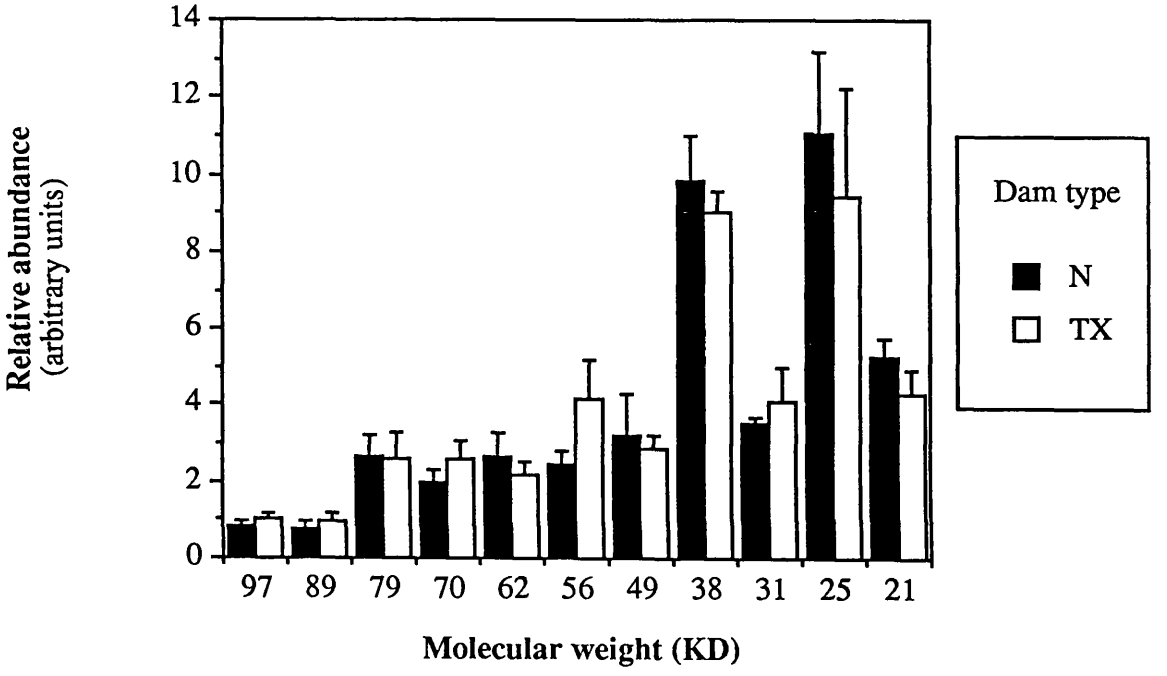
Values are the mean ± SEM of 5-7 separate experiments, excep for 25 KD protein band, where n = 3; * P < 0.01.

Figure 20 : The effect of maternal hypothyroxinemia on the particulate protein profile of midbrain from adult progeny.



Values are mean ± SEM of 5-6 separate experiments, except for 70 KD, 62 KD and 31 KD where n = 3, 4 and 4 respectively.

Figure 21 : The effect of maternal hypothyroxinemia on particulate protein profile of paleocortex from adult rat progeny.



Values are mean ± SEM of at least 5 separate experiments, except for 89 KD 62 KD and 56 KD where n = 4.

A summary of changes in protein profiles of both cytosolic and particulate proteins in different brain regions of the progeny are given in Tables 6 and 7.

Table 6 : Changes in Cytosolic Proteins in Different Brain Regions of Progeny.

	Changes (%)				
Mr	CC	CB	ME	MB	PC
21 KD	-<10	+<10	+<10	-16	+19
25 KD	+22	+24	+126*	-29	+60
31 KD	+30	+<10	absent*	=	-37
38 KD	+26	-29	-30*	-<10	+18
49 KD	+<10	-20	+44	+17	+<10
56 KD	+<10	+39	-18	-26	-41
62 KD	+100	+26	+23	+17	-39
70 KD	+35	+47	+<10	-<10	+19
79 KD	+26	+14	+15	+37	+26
89 KD	-<10	=	-16	+44	+63*
97 KD	+74*	=	-32	+14	+25

(*), (+) and (-) represent: significant difference, increased and decreased changes in Tx dam progeny *vs* control respectively; (=) no changes were found.

Table 7 : Changes in Particulate Proteins in Different Brain Regions of Progeny.

Mr	Changes (%) CC	CB	ME	MB	PC
21 KD	+49	-16	+<10	-<10	-17
25 KD	-<10	+<10	+19	-<10	-15
31 KD	-34	+33	+50	+<10	+15
38 KD	-28	+53	-27	+<10	-<10
49 KD	+<10	+70	-<10	-17	-12
56 KD	+<10	+23	+<10	_	+69
62 KD	+<10	+<10	-27	+51	-19
70 KD	-22	+<10	+20	+48	+32
79 KD	+29	_	+103*	+13	-<10
89 KD	-28	+10	+15	+65	+26
97 KD	+20	+28	-40	-<10	+19

(*), (+) and (-) represent significant difference, increased and decreased changes in TX dam progeny *vs* normal control respectively.

(_) when protein band was not measured.

3.6 Effect of Maternal Hypothyroxinemia on Cell Marker Enzymes in Brain Regions of Progeny.

The brain is a highly heterogenous organ and consists of a number of different cell types e.g. neurons and glia. In the rat model, maternal hypothyroxinemia throughout pregnancy is coincident with early neuroblast proliferation and to a certain extent, neuronal differentiation. It may therefore be expected that the neuronal population in the adult progeny should be affected. Therefore, in order to investigate the brain cell-specific effect of maternal hypothyroxinemia, the activities of a range of cell marker

enzymes were measured in adult brain.

β-D-glucuronidase a lysosomal enzyme is predominantly localized in the neuronal cell bodies and therefore serves as a neuronal marker. In contrast, The lysosomal enzyme, β-D galactosaminidase is predominantly (but not exclusively) localized within the glia, and was used as a general glial cell marker. The glial cell population comprises both astrocytes and oligodendrocytes, and in order to differentiate between these two cell types, the lysosomal enzyme oleate esterase was studied, since this enzyme is localized within myelin and the oligodendrocytes (oligodendroglial cell marker).

3.6.1 β-D-Glucuronidase

The specific activity of β-D-glucuronidase was normal in medulla, midbrain and cerebral cortex from TX dam progeny (Figure 22). A 10% decrease was observed in the specific activity of the enzyme in cerebellum but this was statistically insignificant. In contrast, the specific activity of β-D-glucuronidase in paleocortex from TX dam progeny was statistically decreased (by 30%, $P < 0.05$).

3.6.2 β-D-Galactosaminidase

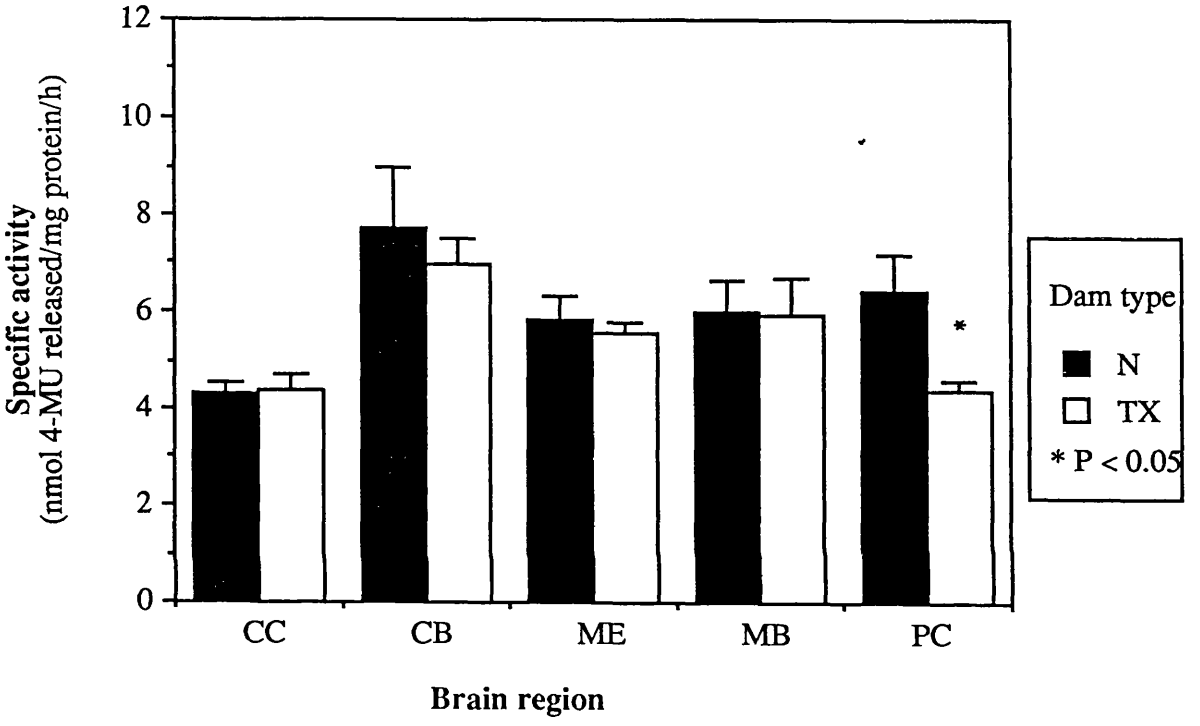
The specific activity of the general glial marker enzyme, β-D-galactosaminidase was unchanged in all five brain regions from TX dam progeny as compared with the control group (Figure 23). However, a small increase (+14%, NS) was observed for cerebral cortex and a slight decrease in paleocortex (-12%, NS) of experimental progeny.

3.6.3 Oleate Esterase

The specific activity of oleate esterase was increased relative to control levels in all brain regions studied with the exception of the cerebellum, where the activity of the enzyme was decreased (by 16%). The activity of oleate esterase was increased in cerebral cortex (by 30%) and in paleocortex (by 39%). However, the only statistically significant change was for the paleocortex (Figure 24).

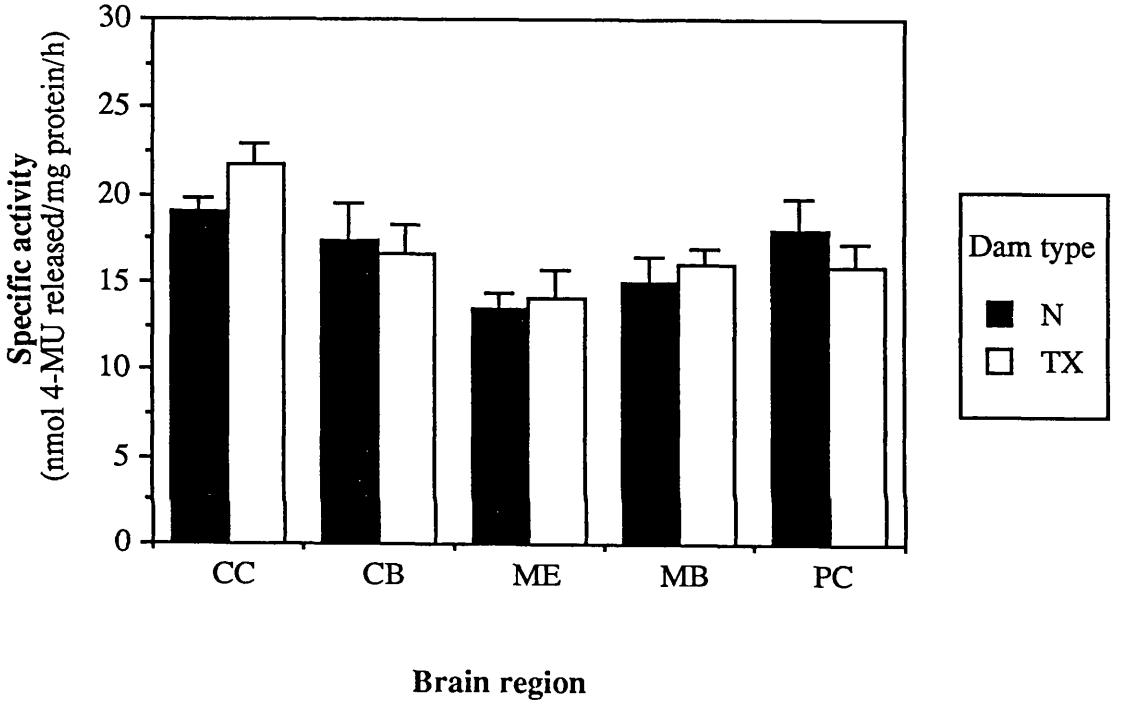
In summary, the neuronal marker enzyme studied was deficient in one region (paleocortex), and the activity of the general glial marker enzyme was unchanged in all

Figure 22 : Effects of maternal hypothyroxinemia on the specific actvity of β-D-glucuronidase in brain regions from adult progeny.



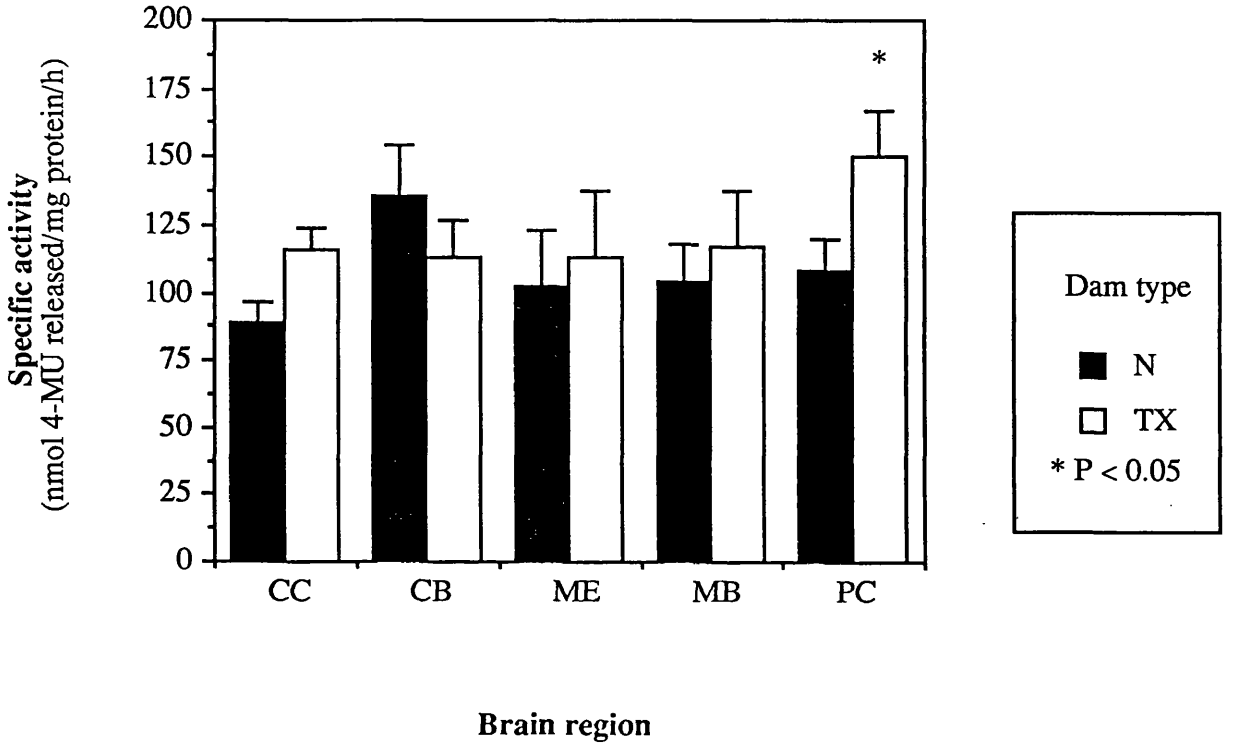
The activity of β–D-glucuronidase was measured in homogenates of cerebral cortex (CC), cerebellum (CB), medulla (ME), midbrain (MB) and paleocortex (PC) from adult progeny of normal (N) and partially thyroidectomised (TX)rat dams, as described in materials and methods. Values are mean specific activity ± SEM of 5-6 animals, except for ME where n = 3.

Figure 23 : Effects of maternal hypothyroxinemia on the specific activity of β-D-galactosaminidase in brain regions from adult progeny.



The activity of β-D-galactosaminidase was measured in homogenates from cerebral cortex (CC), cerebellum (CB), medulla (ME), midbrain (MB) and paleocortex (PC) from adult progeny of both normal (N) and partially thyroidectomised (TX) rat dams, as described in Materials and Methods. Values are mean specific activity ± SEM of at least 6 animals, except ME where n = 4.

Figure 24 : The effect of maternal hypothyroxinemia on the specific activity of oleate esterase in different brain regions from adult rat progeny.



The activity of oleate esterase was measured in homogenates of cerebral cortex (CC), cerebellum (CB), medulla (ME), midbrain (MB) and paleocortex (PC) from adult progeny of both normal (N) and partially thyroidectomised (TX) rat dams, as described in Materials and Methods. Values are the mean specific activity ± SEM of 4 animals.

regions. In contrast, the oligodendroglial cell marker enzyme was higher in all brain regions (with the exception of cerebellum). This increment was statistically significant only in paleocortex.

3.7 Effect of Maternal Hypothyroxinemia on Myelin Metabolism in Brain Regions of Progeny.

The above findings were somewhat surprising, since oligodendroglial cell proliferation and differentiation occur largely in postnatal life in the rat, after the period of insult to the developing fetus. The major function of the oligodendrocytes in the CNS is to produce the myelin sheath. Myelin is a modified plasma membrane which is wrapped in a compact multilammelar fashion around the nerve axon. It insulates axons from each other, but its main function is to facilitate axonal conduction. The velocity of an impulse in a fibre without myelin is proportionate to the square root of its diameter, while the conduction velocity in a myelinated fibre is proportionate to its diameter. Thus, an advantage of myelination is saving of energy and space in higher vertebrates (cited in Berne and Levy 1983, Morell and Norton 1980, Morell *et al* 1989).

It is of interest to note that studies of human populations from the iodine-deficient endemias have shown changes in myelin structure from neurological cretins (Ma *et al* 1986). In view of these findings coupled with dysgenesis of oleate esterase activity, it was decided to investigate myelin metabolism in further detail. Before presenting the results, the compositions of myelin in normal brain are briefly considered here (Table 8).

Table 8 : Major Composition of CNS Myelin in Human and Rat

Substance	Human	Rat
Total protein	30	29.5
Total lipid	70	70.5
Cholesterol	27.7	27.3
Total galactolipid	27.5	31.5
Cerebroside	22.7	23.7
Sulphatide	3.8	7.1
Total phospholipid	43.1	44
Ethanolamine phosphoglycerides	15.6	16.7
Choline phosphoglyceride	11.2	11.3
Inositol phosphoglyceride	0.6	1.2
Serine phosphoglyceride	4.8	7
Sphingomyelin	7.9	3.2
Plasmalogen	12.3	14.1

Adapted from Morell *et al* 1989 & Fedoroff and Doucette 1988.
Total protein and lipids are percent of myelin dry weight and different lipid classes are percent of total lipid (plasmalogens are primarily ethanolamine phosphoglyceride).

Myelin has a high lipid content (some 70%) and a low protein content (approximately 30%) in contrast to most other biological membranes, such as the liver cell membrane, which have a higher ratio of protein:lipid in their structure. In rat brain myelin, phospholipid, glycolipid and cholesterol are the three major types of lipid. Galactolipids (cerebroside and sulphatide) are myelin-specific lipids, and the concentration of cerebroside is directly proportion to the myelin content of brain during development (Morell and Norton 1980, Morell *et al* 1989 & Fedoroff and Doucette 1988)

Two main classes of protein are found in CNS myelin, namely: myelin basic protein and proteolipid protein, which comprise some 60-80% of total myelin protein. A quantitatively minor, but functionally important group has also been recognized in

CNS myelin. Of this latter group, myelin-associated glycoprotein (MAG) and 2', 3',-cyclic nucleotide 3'-phosphohydrolase (CNPase) are important. Other myelin-associated proteins include: 5'-nucleotidase, carbonic anhydrase, cholesterol ester hydrolase, acid phosphatase and sphingomyelinase.

In the CNS, myelinogenesis is region-dependent: Generally the spinal cord myelinates first, then the brain stem and finally the higher cortical areas. In the human, myelination in the PNS and spinal cord starts at about 14th week of gestation and it is increased markedly at 24 weeks. It is assumed that myelination in the spinal cord is complete by the end of second year of life (Fedoroff and Doucette 1988, Morell *et al* 1989). On the other hand, myelination in brain is almost a postnatal event. From birth to 6 months it proceeds rapidly, then slows but remains active until the end of the 20th year of life. In the rat myelination is completely a postnatal event. The onset of myelination is around postnatal day 9 and it is maximal at approximately day 20 (McKhann and Ho 1967, Rosman and Malone 1977). It then decreases but continues until one year (Morell *et al* 1989).

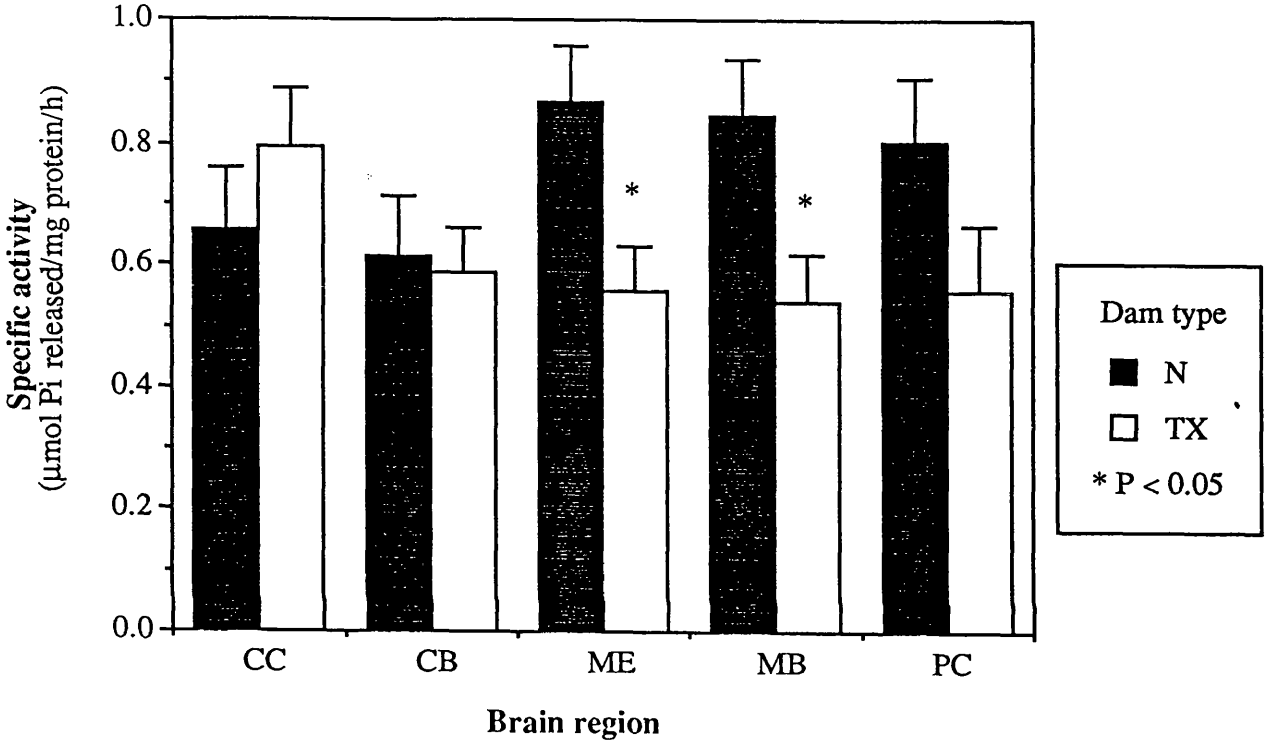
The effects of maternal hypothyroxinemia on selected myelin proteins (enzyme activities) and myelin-lipids in brain regions of adult progeny was investigated and the results are described below.

3.7.1 Effect of Maternal Hypothyroxinemia on Myelin Proteins

3.7.1a 5'-Nucleotidase

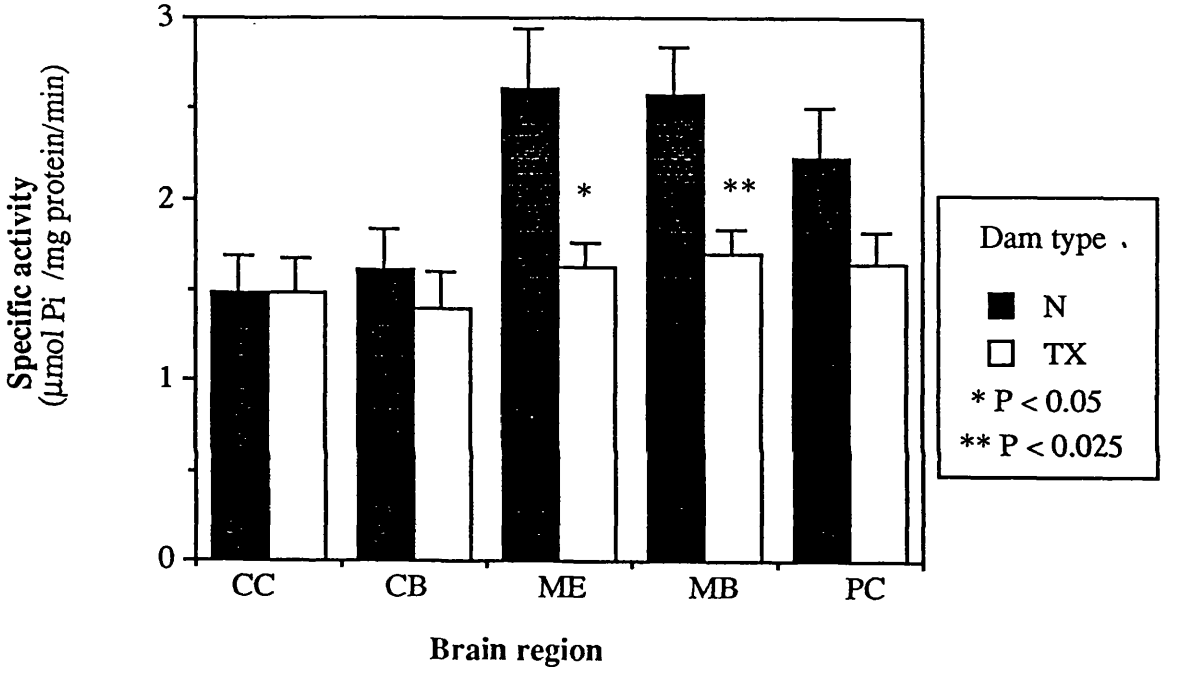
In rat brain, 5'-nucleotidase is localized predominantly in the oligodendrocyte cell population, with high levels of enzyme activity also present in the myelin sheath. It is well known that the activity of this enzyme is under the thyroid hormone control. Hypothyroid neonatal rats (dams were treated by PTU from 12 day of gestation) demonstrated significant reduction of 5'-nucleotidase activity at 21 postnatal day in cerebral cortex, cerebellum, pons-medulla and midbrain (King et al 1983). The specific activity of 5'-nucleotidase in the five brain regions of the adult experimental and control progeny are depicted in Figure 25. As demonstrated in this Figure, in progeny from hypothyroxinemic dams, 5'-nucleotidase specific activity was significantly decreased in the medulla (by 33%, $P < 0.05$) and midbrain (by 35%, $P < 0.05$). A 31% reduction of 5'-nucleotidase activity in paleocortex of TX dam progeny was also observed, although

Figure 25 : Effects of maternal hypothyroxinemia on the specific activity of 5'-nucleotidase in different brain regions from adult progeny.



The activity of 5'-nucleotidase was measured in homogenates of cerebral cortex (CC), cerebellum (CB), medulla (ME), midbrain (MB) and paleocortex (PC) from adult progeny of both normal (N) and partially thyroidectomised (TX) rat dams, as described in Materials and Methods. Values are mean specific activity ± SEM of at least 5 animals.

Figure 26 : Effects of maternal hypothyroxinemia on the specific activity of CNPase in different brain regions from adult progeny.



The activity of CNPase was measured in homogenates of cerebral cortex (CC), cerebellum (CB), medulla (ME), midbrain (MB) and paleocortex (PC) from adult progeny of normal (N) and partially thyroidectomised (TX) rat dams, as described in Materials and Methods. Values are mean specific activity ± SEM of 5-6 animals.

it was not statistically significant.

3.7.1b CNPase

2', 3'-Cyclic nucleotide 3'-phosphohydrolase (CNPase) is a myelin marker enzyme which accounts for approximately 4% (by weight) of myelin protein in the CNS (Vogel and Thompson 1988).The enzyme has been reported to be active in the CNS and its presence in myelin was reported by Kurihara and Tsukada in 1967 (Kurihara and Tsukada 1967). It is not exclusively located in the myelin sheath, but has also been found in oligodendrocytes (Vogel and Thompson 1988, Zanetta *et al* 1972, Prohaska *et al* 1973, Trapp *et al* 1988). CNPase has been reported in plasma membrane of OL and their processes, in compact myelin and in myelin internode (contains cytoplasm of OL). It has also been reported that the perinuclear cytoplasm of OL is the site of CNPase mRNA translation (Trapp *et al* 1988). CNPase activity has been reported in neuronal cell lines and outside the brain too, in a variety of blood cells (erythrocytes, lymphocytes and platelets)(Vogel and Thompson 1988).

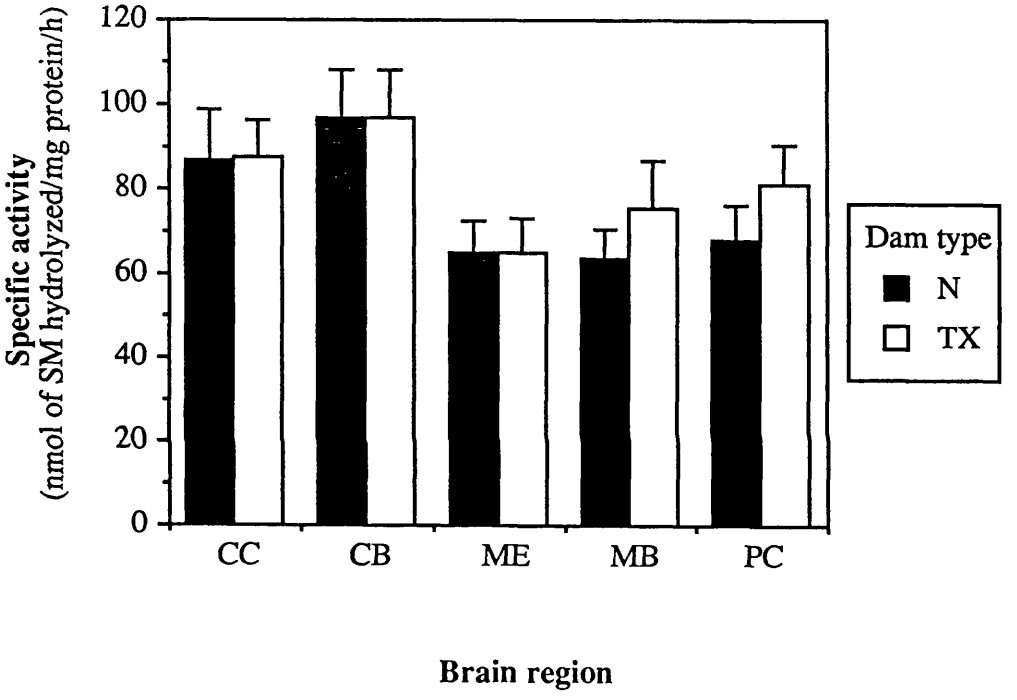
The function of CNPase is not clear. Various proposals have been put forward including: involvement in the synthesis of myelin proteins and phosphorylation of 5'-hydroxyl groups of tRNA. It has also been suggested that CNPase may play a role in the early differentiation of glial cells (Vogel and Thompson 1988, Weissbarth *et al* 1981). Presence of the enzyme in parenchymal organs with important cell membrane interaction has been thought to play a role in cell-cell interactions. Changes in the level of CNPase activity have been reported in several pathological conditions, relating to deficits in myelin metabolism in human and other species.

The effects of maternal hypothyroxinemia on CNPase activity (Figure 26) were brain region-specific. CNPase activity was decreased significantly in medulla (by 37%, $P < 0.05$) and midbrain (by 34%, $P < 0.025$) in TX dam progeny, in comparison with normal control. The activity of CNPase in paleocortex was also decreased in TX dam progeny; nevertheless, it was statistically insignificant (26%, NS). Thus the brain regions were affected in the same manner as 5'-nucleotidase.

3.7.2 Sphingomyelinase

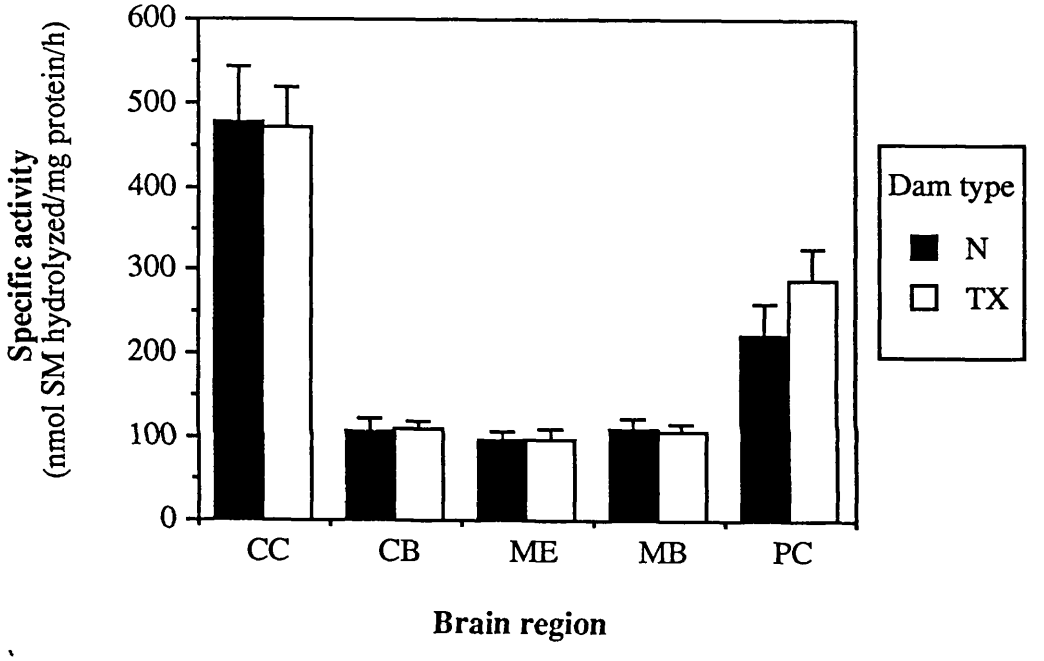
Sphingomyelinase(SMase) catalyses the hydrolysis of sphingomyelin to ceramide and

Figure 27 : Effects of maternal hypothyroxinemia on the specific activity of acidic sphingomyelinase in different brain regions from adult progeny.



The activity of sphingomyelinase was measured in homogenates of cerebral cortex (CC), cerebellum (CB), medulla (ME), midbrain (MB) and paleocortex (PC) from adult progeny of normal (N) and partially thyroidectomised (TX) rat dams, as described in Materials and Methods. Assays were performed at pH 5. Values are mean specific activity ± SEM of at least 6 animals.

Figure 28 : Effects of maternal hypothyroxinemia on the specific activity of neutral sphingomyelinase in different brain regions from adult progeny.



The activity of sphingomyelinase was measured in homogenates of cerebral cortex (CC), cerebellum (CB), medulla (ME), midbrain (MB) and paleocortex (PC) from adult progeny of both normal (N) and partially thyroidectomised (TX) rat dams, as described in Materials and Methods. Assays were performed at pH 7. Values are mean specific activity ± SEM of 6 animals.

phosphorylcholine. Acidic SMase (pH 5) is a lysosomal enzyme and neutral SMase (pH 7) has been associated with rat brain (Yamaguchi *et al* 1978). Activities of sphingomyelinase was determined at both pH 5.0 and pH 7

.

Activity of acidic SMase

Activity of the lysosomal enzyme was determined in whole homogenate of brain regions of experimental and normal progeny. As Figure 27 shows, activity of SMase increased in midbrain and paleocortex of TX dam progeny by 18.9% and 19% respectively. Although, these changes were statistically insignificant, compared with normal control group. No apparent changes were found in medulla, cerebellum and/or cerebral cortex of experimental progeny.

Activity of Neutral SMase

Activity of this enzyme was determined at pH 7.0. Figures 28 & 29 show the specific activity of neutral sphingomyelinas in brain regions of progeny from normal and thyroidectomized dams.

Figure 29 shows the SMase specific activity of experimental progeny as percentage of normal control. It is evident from this Figure that in experimental progeny, SMase specific activity has changed significantly only in paleocortex. Activity of sphingomyelinase was increased in paleocortex of experimental progeny (by approximately 40%, $P < 0.02$). No changes were found in other four regions.

3.7.3 Effects of Maternal Hypothyroxinemia on Myelin Lipids

The content of certain lipids, namely cholesterol, cerebroside and sulfatide were measured in the different brain regions from normal control and experimental progeny. Cholesterol, although a major constituent of myelin, is nevertheless present in all membranous structures. In contrast, both cerebroside and sulphatide are myelin-specific lipids.

a) Cholesterol Concentration

This membrane bound lipid which is highly prevalent in myelin membrane, was

As the results show, the cholesterol concentration was decreased in medulla (by 20%), in cerebellum (by 13.6%) and in cerebral cortex (by approximately 9%), whereas it increased in paleocortex (by 10%) from experimental progeny. Nevertheless, none of these changes was statistically significant.

b) Cerebroside Concentration

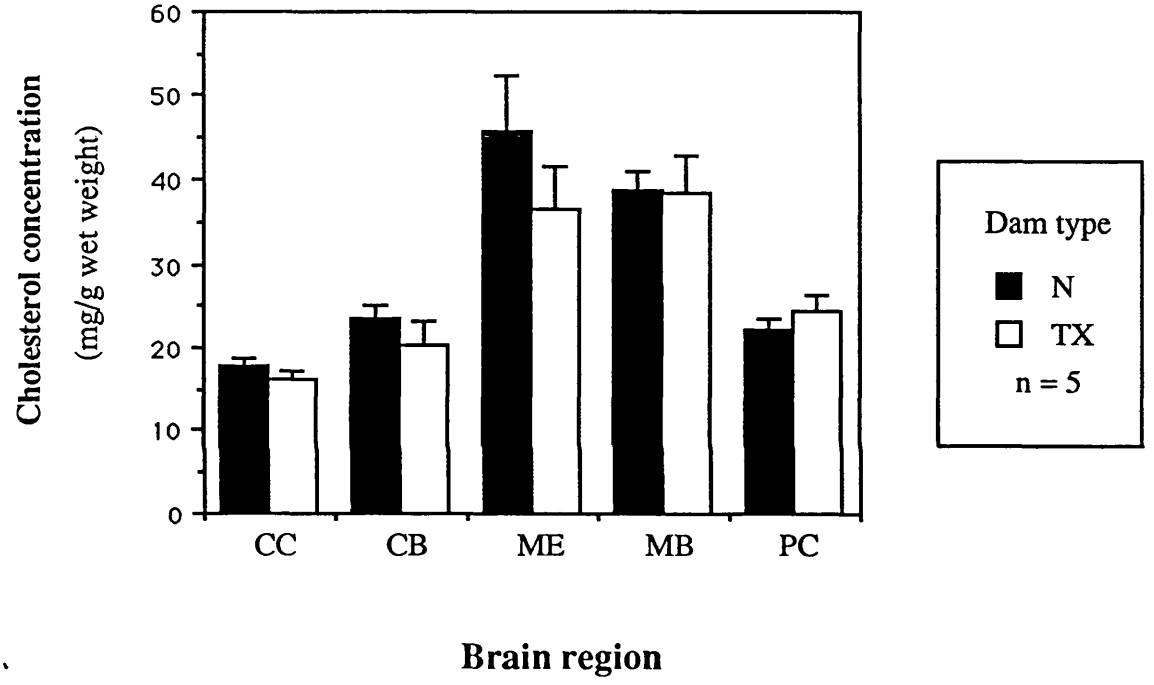
Myelin galactolipids, i.e.cerebroside and galactocerebroside sulphate (sulphatide) are myelin constituents which appear very early during myelination. Thus, they have been used as myelin markers in myelinogenesis studies. Cerebroside is a major myelin lipid; it has been shown only in myelin and oligodendrocytes, thus it is regarded as an excellent myelin marker (Raff *et al* 1978). In the human, it comprises approximately 23% of total myelin lipid. Cerebroside is undetectable in blood and cerebro-spinal fluid (CSF) of normal individuals, but in demyelinating diseases it can be detected in serum by immunocytochemical methods as an index of active demyelination (Thuillier *et al* 1988).

Cerebroside concentration was determined in homogenates of the five different brain regions from thyroidectomized and normal dam progeny. As Figure 31 shows, in TX dam progeny cerebroside concentration was decreased in medulla (by 34%, $P < 0.05$) and in midbrain (by 32.8%), although only the former was statistically significant. On the other hand, cerebroside concentration was increased in paleocortex of experimental progeny (by 22%), though insignificant.

c) Sulphatide Concentration

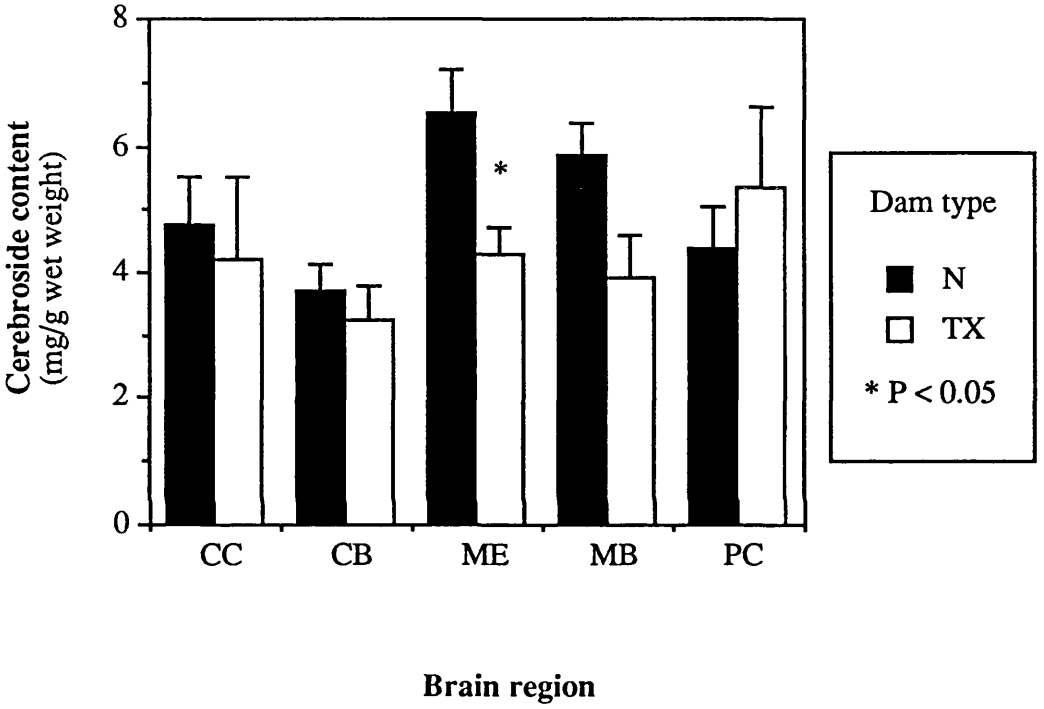
Sulfatide (galactocerebroside sulphate) is synthesized from cerebroside by sulphation of the galactose moiety. The reaction is catalyzed by galactosylceramide sulphotransferase (CST EC 2.8.2.11), and 3'-phosphoadenosine-5'-phosphosulfate (PAPS) serves as sulfate donor. The enzyme is localized in the Golgi apparatus of the oligodendroglial cell (McKhann and Ho 1967, Benjamins and Smith 1984). After synthesis sulphatides are transported to the plasma membrane of the OL, where they are incorporated into the myelin sheath. Once assembled in the myelin sheath, the sulphatide has a very low turnover rate (McKhann and Ho 1967, Morell *et al* 1989). Transport of sulphatide

Figure 29 : Effects of maternal hypothyroxinemia on cholesterol content of different brain regions from adult progeny.



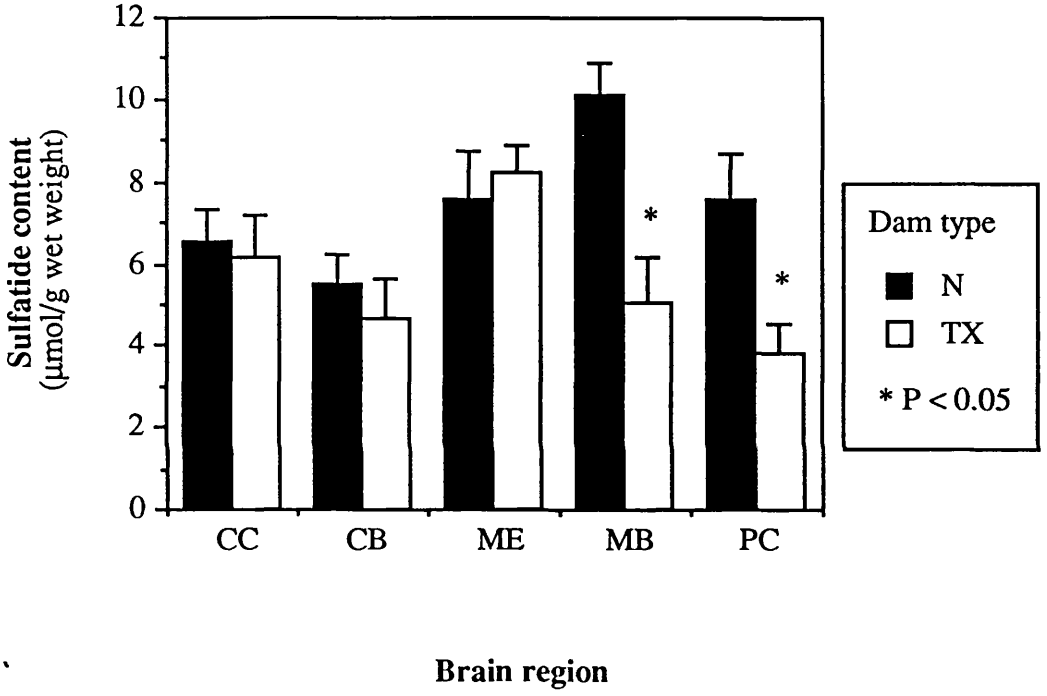
Cholesterol content was measured in homogenates of cerebral cortex (CC), cerebellum (CB), medulla (ME), midbrain (MB) and paleocortex (PC) from progeny of normal (N) and Thyroidectomised (TX) rat dams. Values are mean ± SEM of 5 animals.

Figure 30 : Effects of maternal hypothyroxinemia on the cerebroside content of different brain regions of adult progeny.



The cerebroside content was measured in homogenates of cerebral cortex (CC), cerebellum (CB), medulla (ME), midbrain (MB) and paleocortex (PC) from adult progeny of normal (N) and partially thyroidectomised (TX) rat dams. Values are mean ± SEM of 4 separate experiments.

Figure 31 : Effects of maternal hypothyroxinemia on the sulphatide content of different brain regions from adult progeny.



The sulfatide content was measured in homogenates of cerebral cortex (CC), cerebellum (CB), medulla (ME), midbrain (MB) and paleocortex (PC) from adult progeny of normal (N) and partially thyroidectomised (TX) rat dams, as described in Materials and Methods. Values are the mean ± SEM of 4 different animals, except MB where n = 3.

from the Golgi apparatus to myelin is mediated by microtubules (Benjamins and Smith 1984), and its transport through the oligodendrocyte has been suggested to be coupled with that of PLP, since disruption of sulphatide synthesis can affect PLP transport to the myelin sheath. Sulphatide function in myelin is uncertain. Some proposed roles for sulphatide are: involvement in the transport reaction of Na^+, K^+-ATPase; its association with opiate receptor and finally its role in maintaining the integrity of myelin via electrostatic interaction with MBP (Van der Pal *et al* 1990, Banik *et al* 1974).

Concentration of sulphatide was determined in both experimental and normal progeny. Effects of maternal hypothyroxinemia on sulphatide concentration are demonstrated in Figure 32. As this Figure shows, maternal hypothyroxinemia results in a decreased sulphatide concentration in progeny from TX rat in midbrain (by 50%, $P < 0.05$) and in paleocortex (by approximately 50%, $P < 0.05$). No statistically significant changes were found in other regions of experimental progeny.

In summary, myelin proteins are more affected in medulla and midbrain of TX dam progeny, while the myelin lipids are affected in the same regions as well as paleocortex. Thus, it seems that deficits in myelin metabolism of TX dam progeny are mostly evident in medulla, midbrain and paleocortex.

CHAPTER 4 : DISCUSSION

4.1 Maternal Hypothyroxinemia and Body/Brain Weight of Progeny

It has been known for many years that the thyroid hormones play an important role in the growth and development of vertebrates (Shellabarger 1964). Shortage of thyroid hormones in mammals has been demonstrated to impair the normal growth and development of a number of organs, particularly the brain (Foley 1983, Noguchi *et al* 1982, Hetzel 1989, Ferret-Sena *et al* 1990). The majority of studies on the role of thyroid hormones in brain development in the rat have been conducted largely by thyroidectomy of postnatal progeny. To date, few studies have been concerned with the possible influence of maternal thyroid hormones on fetal brain development, since it has generally been assumed that the placenta is impermeable to these substances. In cosequence of recent studies this assumption is being increasingly questioned. Two independent studies have shown significant transfer of maternal T_4 in early gestation in the rat, and transfer has also been demonstrated in the human, at least late in gestation in cases when fetal thyroid function is impaired (see Introduction).

Thyroid hormone action at the molecular level is primarily mediated via the interaction with nuclear receptors with consequent effects on gene expression (Oppenheimer 1979 & Oppenheimer *et al* 1986a). In early fetal brain, both in human and rat, the presence of thyroid hormone and thyroid hormone nuclear receptors has been reported. In human fetal brain, such receptors have been reported in the 9-10 week-old fetus; at the same time, T_3 has been reported to be available in sufficient quantity to saturate 25% of the receptors (Ferriro *et al* 1988). In the rat, thyroid hormones have been detected in the embryo-trophoblast at 10 days of gestation and in fetal brain at 14 days of gestation (Morreale de Escobar 1986 & Obregon *et al* 1989). Interestingly, thyroid hormone nuclear receptors are also present in rat brain from the 14th gestational day, prior to the establishment of independent fetal thyroid hormone secretion (Perez-Castillo *et al* 1985 & Schwartz and Oppenheimer 1978). Such findings therefore suggested that the transferred maternal T_4 may play an important role in brain development before the onset of independent fetal thyroid function.

Investigation of the role of maternal T_4 in brain development is further indicated by the evidence from studies of human populations living in iodine deficient regions of the world. A diverse spectrum of neurobiological disorders has been observed in children borne in iodine-deficient endemias. Such dysfunctions range from stillbirth, neonatal death, congenital and neonatal hypothyroidism, with deficits in mental and motor

functions, to overt neurological cretinism which is characterized by: mental retardation, deaf-mutism, a characteristic disorder of gait and stance (spastic diplegia) and often strabismus (Pharoah *et al* 1980, Hetzel 1983, Stanbury 1986, Pharoah and Connolly 1989). In such regions, the incidence of neurological cretinism is increased and current data from iodine supplementation studies suggest that the primary insult to the developing fetus occurs early in pregnancy, before the establishment of independent fetal thyroid function (Pharoah *et al* 1971, Obregon *et al* 1984, Woods *et al* 1984 & Ekins *et al* 1989). Mothers of such children are typically hypothyroxinemic (T_3 levels are normal), and maternal T_4 levels have been shown to correlate with the extent of neurological damage (Man *et al* 1971, Pharoah *et al* 1971, Connolly and Pharoah 1989). Nevertheless, such findings should be interpreted with care due to the underlying iodine deficiency.

The aim of the present investigation was to study the effects of maternal thyroid state during pregnancy on brain development in adult rat progeny at 7 months of age which is thought to represent the end point of brain development in this species. The rat dams were typically hypothyroxinemic (T_4 levels were significantly reduced by approximately 70%, whereas T_3 levels were within the normal range) and therefore simulated the low T_4, normal T_3 levels of mothers from iodine deficient regions. The major advantage of this model over other available systems (iodine-restricted diets or maternal thyroidectomy by administration of antithyroid drugs such as PTU or MMI) was that no direct effects on fetal thyroid function was possible.

An adequate iodide supply for normal development and function of fetal thyroid systems was ensured, and the fetuses were therefore hypothyroid only during pregnancy until the onset of independent fetal thyroid hormone synthesis. Both sets of dams and progeny were kept under identical animal house conditions. The daily food intake was similar for normal and TX dams and for both groups of progeny after weaning. Thus, in this animal model, efforts were made to eliminate any possible factor which could affect the experimental progeny, other than maternal hypothyroxinemia *in utero*. Therefore any changes between TX dam progeny and the control group would be attributed to maternal hypothyroxinemia.

It was observed that serum levels of both TT_4 and TT_3 in experimental progeny were normal, indicating that the animals were clinically and metabolically euthyroid. Therefore, maternal hypothyroxinemia was without any effect on the thyroid hormone status of the adult progeny. Although TSH was not measured, it has been reported

interestingly that, in the progeny of animals made experimentally hypothyroid, the TSH level remains permanently elevated, suggesting that an increased level of TSH is required to maintain euthyroid state in the progeny (Porterfield and Hendrich 1981).

Body weights of experimental progeny, both at 3.5 months and 7 months of age, were higher than the normal controls. This difference was significant in 3.5 month old progeny, with the changes being more prominent for female progeny (8% increase for male and 25% increase for female). In 7 month old progeny, body weight was slightly higher in the experimental group (although not statistically significant) and changes in body weight for female progeny were larger than those of the male (12% and 8% respectively). No changes were found in brain weights of the experimental progeny at either of the age points studied. Although the ratio of brain/body weight of 7 months old progeny from TX dams was not different from the control, this was significantly decreased in the 3.5 month old experimental group indicating a relatively higher weight gain for experimental progeny at this age point.

Studies with fetuses from iodine-deficient sheep (Hetzel and Mano 1989) and iodine-deficient or thyroidectomised rat dams (Morreale de Escobar *et al* 1989b) and also fetuses and neonatal progeny from our animal model (Hubank 1990, Pickard *et al* 1991b) have demonstrated reductions in body and brain weights of the progeny, at least at fetal and early postnatal stages of development. However, no such effects on brain and body weights were observed in the present study, in at least 3-4 months old or 7 months old hypothyroxinemic rat dam progeny. In fact body weights of the TX dam progeny were slightly higher than the normal control, suggesting that the early deleterious effects of maternal hypothyroxinemia on somatic growth and brain weight may be ameliorated or even over-compensated in later life. This increased body weight of experimental progeny may be due to increased lipid synthesis, decreased lipid turnover, lower metabolic rate or even an increase in the deposition of proteoglycans as in myxoedematous patients. As the results appear to be sex-related, a link between the increase in body weight and female hormones should be considered, since the increase in body weight of TX dam female progeny was more pronounced than that in the male progeny. Indeed, in the female, the content of subcutaneous tissue (lipid-proteoglycan) is higher than that of the male. The underlying mechanism of this weight gain may be addressed by further work with this animal model.

4.2 Maternal Thyroid Hormones and Protein Profile of Progeny.

Several studies have demonstrated that brain proteins are responsive to thyroid hormone levels and a variety of hypothyroid conditions (e.g. thyroidectomy, hypothyroid mutants or hypothyroidism) can cause substantial alterations in the expression of brain proteins (Shanker *et al* 1987, Silva and Rudas 1990 & Davis and Pieringer 1987). Initial experiments were concerned with the effects of maternal hypothyroxinemia on brain protein content in experimental progeny. No difference was found in total protein content of the various brain regions between normal and thyroidectomised dam progeny, indicating that protein acquisition and turnover were not severely affected as a consequence of maternal hypothyroxinemia, at least at this age point. However in this animal model, it has been demonstrated by Pickard *et al* (1991a) that leucine incorporation into protein was diminished only in cerebral cortex of adult progeny from TX dams *in vitro*, whilst no changes were found in experimental progeny *in vivo* (Pickard *et al* 1991a). Nevertheless, this finding does not preclude the possibility of some deleterious effects of maternal hypothyroxinemia *in utero* on selected protein species; since a decrease in the level of a particular protein in any region may coincide with an increase of another protein in the same region, implying that the change in the level of the first protein is not reflected in the measurement of total protein. Thus the effects of maternal hypothyroxinemia on protein acquisition or turnover may be masked. In order to investigate this possibility, cytosolic and particulate proteins from different brain regions were analysed.

In the cytosolic fraction SDS-gel analysis demonstrated considerable changes in the profile of a number of protein bands in experimental progeny. Although some alteration was evident in almost all measured protein bands, these changes were significant only for some bands indicating that only certain proteins are severely affected in experimental progeny due to maternal hypothyroxinemia in early pregnancy. Selective effects of thyroid hormone deficits on specific proteins have been reported in hyt/hyt mutant mice by Stein *et al* (1990), who demonstrated changes in abundance of mRNAs for tubulin proteins and EGF in this animal model as early as the day of birth. Concentration of microtubule associated proteins (MAP2) in congenital hypothyroid rats (Silva and Peter 1990) and the specific activity of glutamic acid decarboxylase (GAD) enzyme are also selectively controlled by thyroid hormones; indicating that specific cell types are sensitive to thyroid hormones.

Alterations in maternal thyroid hormones during pregnancy appear to be the main factor responsible for the changes in the level of these altered proteins in experimental progeny which may in turn contribute to subsequent abnormal biochemical and behavioral development of these animals. It seems that maternal hypothyroxinemia severely affects some regions e. g. cerebral cortex, paleocortex and medulla, the most deleterious effects being exerted on the latter which is phylogenetically an old part of the brain.

A protein band with molecular weight of approximately 97 KD was increased in cerebral cortex of TX dam progeny. It is worth noting that each protein band comprises a complex of different molecular species (or peptide chains) and this alteration cannot be attributed to a particular protein species. Nevertheless, some functionally important proteins with molecular weights very close to these affected protein bands in rat brain have been recognised; for instance, dynamin is a neuronal protein with Mr of 100 KD (very close to the 97 KD) which is abundant in cytosol and has microtubule-cross linking activity. It has been suggested that dynamin may link vesicles to microtubules in differentiated axons (Scaife and Margolis 1990). A protein band with Mr of 89 KD was also significantly increased in paleocortex of TX dam progeny. As indicated above, maternal hypothyroxinemia appears to exert its most severe effects in the medulla. A 38 KD protein band was significantly reduced in this region (by 30%). This band by molecular weight is very close to neuron specific enolase (NSE). Another protein band, a 31 KD protein was absent in the medulla from experimental progeny, and a 25 KD protein band was also significantly reduced in the same region. Neuron specific enolase is a dimer of two identical subunits of 40 KD. Another soluble protein with Mr of 39 KD is antigen α. It has been proposed that antigen α, 14-3-2 protein (a neuronal cytoplasmic protein) and NSE represent the same protein. It has been reported that the levels of 14-3-2 protein in brain increase with the processing of new information (Zomzely-Neurath 1982). However, behavioral studies have demonstrated significant changes in learning capacity and behavior of the experimental progeny (Sinha *et al* 1991). Whether or not this reduction in 38 KD protein band in medulla can affect the learning ability of the animal may be answered by further work with TX dam progeny. Very close to the 31 KD protein band which was significantly altered in experimental progeny is a cytosolic protein termed phosphatidylinositol-transfer protein which is thought to be involved in the specific transport of phosphatidylinositol to the plasma membrane (Dickeson *et al* 1989). This protein shows a dual specificity, e.g. it also

catalyzes the transport of phosphatidylcholine.

Changes in cytosolic glycoprotein levels for the same neonatal TX dam progeny have been demonstrated for 78 KD and 125 KD glycoprotein species (Hubank 1990). In summary, in adult progeny, it is clear that cytosolic proteins are more affected in medulla (absence of 31 KD, reduction of 38 KD and 25 KD protein bands), cerebral cortex (elevation of 97 KD) and paleocortex(elevation of 89 KD); with no significant changes in cerebellum and midbrain. These findings again emphasizing that the effect of maternal hypothyroxinemia in early pregnancy on brain development of progeny is region-specific.

SDS-PAGE analysis of the insoluble fraction demonstrated that in particulate proteins, as with the cytosolic fraction, changes in different protein bands from the experimental progeny occur, albeit to varying extents. However, the effects are less pronounced. A 97 KD protein band was possibly increased in cerebral cortex and cerebellum (by 20% and 28% respectively) and decreased in medulla (by 40%), although neither of these changes was statistically significant. On the other hand, a 79 KD protein band was significantly increased (103%, $P < 0.01$) in medulla indicating that this phylogenetically ancient brain region is more susceptible to thyroid hormone deficiency during early pregnancy. In rat brain, protein kinase C is a doublet of 80 and 78 KD proteins which being very close to the affected protein band in this investigation. Protein kinase C is a phospholipid- and Ca^{2+}-dependent kinase which is thought to be involved in signal transduction processes in brain (Roth *et al* 1989). In the present study, although different protein bands in brain regions of TX dam progeny showed limited changes in comparison with controls, (with the exception of 79 KD) these changes were not significant, suggesting that the acquisition and turnover of particulate proteins is less affected in the adult experimental progeny as a consequence of maternal hypothyroxinemia during early gestation. Deleterious effects of maternal hypothyroxinemia on protein acquisition *in utero* may be compensated in postnatal life especially at 7 months, as many investigations have shown. A neuron-specific glycoprotein (gp 50), which is a component of the synaptic membrane, has been shown to be reduced in thyroid deficient neonatal rats up to the third postnatal week but eventually becomes normal by the fourth week (Beesly *et al* 1990). Membrane glycoprotein fractions have been demonstrated to diminish from 14 day old TX dam progeny to varying extents, but these effects are less prominent at day 30 (Hubank 1990). In the case of myelin proteins in hypothyroid conditions, a diminution have

been reported up to 24 days of postnatal life, following which protein levels have returned to normal values (Rosman and Malone 1977, Shanker et al 1987).

In the case of particulate proteins, a large number of proteins with different functions could be addressed. These include enzymes such as Na^+, K^+-ATPase, choline acetyl transferase, GABA-transaminase and microtubule-associated proteins which are involved in energy metabolism, neurotransmision and cell architecture respectively. Major particulate proteins are located in the synaptic membrane including synaptin (44 KD) and integral proteins of synaptic vesicles such as synaptophysin (38 KD) and PP^{60csrs} (a protein tyrosine kinase found at high levels in crude synaptic vesicle and crude microsomal fractions). In the particulate fraction of rat brain, the presence of PP^{60csrs} and other protein tyrosine kinase activities together with their substrates have been reported (Hirano et al 1988). It has been suggested that tyrosine kinases are involved in development and maturation of neuronal processes and nerve signal transduction. Another group of functionally important proteins in the particulate fraction are the glycoproteins, which are involved in cell transport and cell surface recognition/adhesion activities. Thus, changes in the levels of either particulate or cytosolic proteins due to maternal hypothyroxinemia may affect a wide range of developmental processes in brain of progeny born to hypothyroxinemic mothers. These alterations could impinge upon a variety of enzyme activities and subsequent functions or upon neural-cell interactions, cell-adhesion and cell-communication. Failure in any of these systems especially those concerned with the establishment of neural connections, may contribute to the symptoms generally associated with neurological cretinism.

Results from the present study show that the effects of maternal hypothyroxinemia on the protein profile are permanent, since changes are still present in 7 month old experimental progeny. However not all brain regions are equally affected. For instance, in the particulate fraction, proteins from cerebellum, midbrain and paleocortex are largely unchanged in experimental progeny. In the cytosolic fraction no changes were evident in cerebellum. Thus, it seems that the irreversible effects of maternal hypothyroxinemia on the brain protein profile of the progeny is confined to certain regions, and that within any particular region, some subcellular proteins are more sensitive to a hypothyroid environment *in utero*.

4.3 Maternal Hypothyroxinemia and Neuronal/Glial Cell Marker Enzymes and Myelin Metabolism.

In order to ascribe a greater degree of specificity to the effects of maternal hypothyroxinemia on the brain of progeny, activities of particular enzymes were measured. The activity of β-D-glucuronidase was studied in both experimental and normal progeny. β-D-Glucuronidase is a lysosomal enzyme which is thought to be present predominantly in the neuronal cell bodies (Sinha and Rose 1972 & Hirsch and Ellen Parks 1975). The activity of this enzyme exhibited a strong regional specificity. In experimental progeny, its activity was significantly decreased in the paleocortex, indicating possible neuronal compromise in this region. Whether neuronal number is reduced, or chemical activities within this cell population (or the neuronal lysosomes) are affected, remains unclear. The lower values for β-D-Glucuronidase may also be due to a reduction in general protein synthesis. It is also possible that change in the activity of this enzyme may be due to a selective dysfunction of the lysosomal particles. It has been demonstrated by Al Mazidi (1989) and Ekins *et al* (1989) that in this animal model, the protein/DNA ratio in paleocortex was lower in TX dam progeny when compared with controls, suggesting a reduction in cell size in this brain region. Thus, the reduction in the activity of β-D-glucuronidase may be due to a smaller cell size in the paleocortex of experimental progeny.

It is not yet clear whether this reduction in enzyme activity associated with maternal hypothyroxinemia is due to a direct effect of thyroid hormone on the expression of synthesis of the enzyme or an indirect effect of thyroid hormone through other mechanisms. Interestingly, it has been reported that a normal supply of GH is required for the maximal induction of β-D-glucuronidase. The induction of this enzyme has also been reported to increase in consequence of an enhancement of the rate of its mRNA synthesis (Paigen 1989). Thyroid hormones regulate GH gene expression and are associated with changes in the level of specific mRNA species and the proteins that they encode. Thus, the reduction in the activity of β-D-glucuronidase in the paleocortex of experimental progeny may be due to a lack of maternal TH in early pregnancy that may cause an irreversible effect on the enzyme activity selectively in paleocortex either independently, or via the expression of growth hormone at mRNA level.

β-D-glucuronidase degrades polysaccharides containing glucuronide residues,

e.g.chondroitin sulfate. In humans, deficiency of this enzyme leads to mucopolysaccharidoses type VII, which are associated with the dysostosis multiplex, characteristic mucopolysaccharidotic facies and psychomotor retardation, all these symptoms being evident within the first year of life (Ayelrod and Garber 1990).

The activity of another lysosomal enzyme N-acetyl-β-D-galactosaminidase, which is preferentially (but not exclusively) localized within the glial cells and serves as a general glial marker (Sinha and Rose 1973), was unaffected in all brain regions in experimental progeny. This again indicates that glial cells in general are not affected by maternal hypothyroxinemia *in utero*.

Three oligodendroglial cell marker enzymes: oleate esterase, 5'-nucleotidase and CNPase were studied. Oleate esterase, a lysosomal enzyme preferentially localised in oligodendrocytes, was increased in the paleocortex of experimental progeny. The observed increase may have been due to a change in the number of glial cells in the affected region, or to an inhibitory effect of TH on expression of genes encoding this enzyme. Indeed, the differentiation of a glial progenitor cell to oligodendrocyte or astrocyte in the optic nerve of rat has been reported to be dependent upon environmental factors (Arenander and Vellis 1989). Thus, the reduced level of maternal thyroxine during pregnancy may contribute to an alteration of the oligodendrocytes in paleocortex of experimental progeny. This alteration may be of critical importance to the metabolic status of the CNS in general and of the affected regions in particular, since neuronal/glial interactions which are important to appropriate development of the CNS might be compromised in this situation. Since the main function of the oligodendrocyte is the production of the myelin sheath, this finding may indicate a derangement in myelin homeostasis. This result is in agreement with the suggestion of Hirsch *et al* (1977) that oleate esterase may play a role in myelin degradation.

Alteration in oleate esterase activity in experimental progeny, the localisation of this enzyme in the myelin sheath (as well as oligodendrocytes), coupled with the fact that deficits in myelin metabolism can affect motor activity and the behaviour of animals, were considerations which led to an investigation in the present study of myelin metabolism in greater detail.

Two oligodendroglia marker enzymes, which also serve as myelin metabolic markers, were analysed. Both 5'-nucleotidase and CNPase were affected in a region-specific manner, being significantly decreased in the medulla and midbrain of the experimental progeny. A small (though not statistically significant) reduction in the

paleocortex was also noted. This pattern of damage differed from that of oleate esterase, and although all three enzymes are localized within the oligodendroglial/myelin sheath, their subcellular localization somewhat differs. In addition to its role in myelin metabolism, 5'-nucleotidase is thought to be involved in the membrane transport of some substrates (Norton 1981). The activity of 5'-nucleotidase in the myelin sheath together with the considerable activities of carbonic anhydrase and Na^+, K^+-ATPase, have been suggested as playing a role in the transport of materials such as adenosine and monovalant cations) across the axon membrane (Morell *et al* 1989). This significant reduction of 5'-nucleotidase in several brain regions of experimental progeny suggests for a deficit in myelin metabolism and or a compromised oligodendrocyte function. In addition this reduction may impinge upon the transport of adenosine and monovalant cations across the axon membrane and consequently alter the physiology of impulse transmission in nerve fibre. Whether or not these alterations are relevant to the abnormal behavior and phsycomotor deficits frequently seen in neurological cretins remains to be examined in further investigations. Chemical-induced hypothyroidism in rat dams from the 12th gestational day has been demonstrated to decrease 5'-nucleotidase activity in several brain regions of neonatal progeny (King *et al* 1983). In this report, although King *et al.* used pups at the 21th day of postnatal age, the values they found for the specific activities of both 5'-nucleotidase and CNPase are comparable with the results of the present study in different brain regions of adult progeny .

Another oligodendroglial cell marker enzyme, CNPase, were found to be decreased in the medulla and midbrain of experimental adult progeny. CNPase as a myelin metabolic enzyme has been extensively studied and the stimulatory effects of thyroid hormones on the activity of this enzyme have been demonstrated by several investigators (Zanetta et al 1972, Prohaska et al 1973, Sarlieve et al 1983, Patel *et al* 1988). Although the specific function of CNPase in myelin is unknown, it has been proposed that CNPase may be involved in the synthesis of myelin proteins, phosphorylation of 5'-hydroxyl groups of tRNA (Vogel and Thompson 1988), differentiation of glial cells in early stages; it may also play a role in cell-cell interaction (Weissbarth *et al* 1981). Reduction of CNPase in a region-dependent manner in experimental progeny strongly suggests that maternal hypothyroxinemia *in utero* can permanently alter myelin metabolism of the progeny and may reduce the number or function of the glial cells in the affected regions. As mentioned earlier, any changes in

glial cells during the neural development may alter the appropriate interaction between neuronal and glial cells and therefore cause inappropriate connectivity of neuronal circuits.

It has been demonstrated that another myelin-metabolic enzyme, arylsulphatase A was also diminished in several brain regions (Al Mazidi 1989, Ekins *et al.* 1989). This enzyme is also associated with the maintenance of myelin homeostasis. In the human, deficits in aryl sulphatase result in metachromatic leucodystrophy which is a demyelinating disease. Al Mazidi's finding is in agreement with the result of this study on alteration in myelin metabolism. Thus, it is reasonable to suggest that myelin turnover is altered in some steps in adult progeny as a consequence of maternal hypothyroxinemia during early pregnancy.

Lysosomal acidic sphingomyelinase specific activity was unchanged in all brain regions of experimental progeny. Although the activity of neutral sphingomyelinase was increased in the paleocortex of this group this alteration was again insignificant Thus this results indicate that maternal hypothyroxinemia is without a significant effect on the activity of sphingomyelinase in the experimental progeny. It may be worth mentioning that Niemann-Pick disease which characterized by abnormal accumulation of sphingomyelin in body tissues, especially in the reticuloendothelial system includes a number of disorders with different clinical features possibly due to differences in sphingomyelinase isoenzyme activities. Severe neurologic disorders are common in type A and C of Niemann-Pick disease. Type A and B are due to a genetic deficiency of lysosomal sphingomyelinase (Axelrod and Garber 1990, Yamaguchi and Suzuki 1977). Type A (acute infantile form) is the most common subtype and is seen in the first month of neonatal life. Infants show hepatosplenomegaly, poor feeding and slow neurologic development. Death occurs within the first few years of life. Type B is a juvenile form without sever neurolologic involvment. The neutral sphingomyelinase is particularly active in the brain (predominantly in the grey matter) (Morell and Norton 1980 & Rao and Spence 1976). Neutral sphingomyelinase is thought to be predominantly associated with the plasma membrane and may be involved in altering the cholesterol content of the membrane, the phosphatidylcholine/sphingomyelin ratio and membrane fluidity. It has also been suggested that the presence of sphingomyelinase in the plasma membrane in neural cells may reflect a role for this enzyme in cell-cell and/or receptor-ligand interactions.

The suggestion of an alteration in myelin homeostasis in experimental progeny was

reinforced by the observation that some substrates associated with myelin metabolism are also compromised in the brain of experimental progeny. Major myelin lipids, namely cholesterol, cerebroside and sulphatide, were measured in brain regions of both TX progeny and normal controls. Cholesterol concentrations remained unchanged in all brain regions investigated. Cholesterol is a general constituent of plasma membrane in the CNS and also a major component of myelin sheath (more than 50% of brain cholesterol is in the myelin sheath). It comprises 4-5% of the fresh weight of cerebral white matter (1% of grey matter) in mammalian brain (cited in McIlwain and Bachelard 1971), and has been used as a reliable marker in study of myelin metabolism. This finding indicates that not all steps of myelin metabolism can be affected in TX progeny due to maternal hypothyroxinemia.

Cerebroside concentrations were found to be significantly decreased in the medulla from experimental progeny. This reduction again supports the postulate of a deficit in myelin metabolism in progeny due to maternal hypothyroxinemia during early pregnancy. It may be argued that the reduced level of maternal thyroxine *in utero* can reduce the activities of enzyme(s) which are involved in the synthesis of cerebroside. In the hypothyroid neonatal rat, it has been demonstrated that the cerebroside concentration is decreased in whole brain homogenate (Walravens and Chase 1969).

Sulphatide, another characteristic and abundant lipid of the myelin sheath, was also significantly decreased in midbrain and paleocortex of experimental progeny. The reduction of sulphatide (and also cerebroside) will impose a degree of alteration in lipid homeostasis in general. These changes also impinge upon myelin metabolism of TX dam progeny, particularly in the affected regions (medulla, midbrain and paleocortex). It may suggest that the compromise of these lipids is likely to manifest itself in neurobehavioural compromise, since the regular myelin synthesis and turnover is essential for normal CNS function (Morrle and Norton 1980). Although the function of sulphatide in myelin membrane is still uncertain, proposed roles for this lipid include: its involvement in the Na^+, K^+-ATPase transport reaction, its association with opiate receptor and with maintaining the integrity of myelin (through electrostatic interaction with MBP) (Van der Pall *et al* 1990). It may also be involved in the transport of PLP through the oligodendrocyte to the myelin sheath (Pasquini *et al* 1989). Reduction of sulphatide in brain regions of experimental progeny shows that early maternal hypothyroxinemia can affect the myelin metabolism. However, since sulphatide is synthesized in the Golgi apparatus of OL and is transported to the myelin membrane, it

is difficult to establish which step(s) in myelin metabolism are affected.

In the intact animal, the availability of cerebroside may be a limiting factor in the synthesis of sulphatide (McKhann and Ho 1967), but this appears not to have been the case in the experimental progeny of the present study, since the concentration of sulphatide in medulla (in which cerebroside concentration was reduced) remained unchanged. Integrity of the PAPS generating system also may affect the synthesis of sulphatide. It has been demonstrated that the formation of PAPS is impaired in hypothyroid rat brain (Walravens and Chase 1969). Whether or not this system is affected in the brain regions of experimental progeny due to maternal hypothyroxinemia is unknown. It has been demonstrated that certain enzymes which are involved in synthesis or degradation of this lipid are affected by TH. Amongst these enzymes, cerebroside sulphotransferase (CST) is a well known enzyme which catalyses the conversion of galactosylceramide (cerebroside) to sulphogalactosylceramide (sulphatide). The activity of this enzyme is more closely related to oligodendrocyte. It has been shown that the activity of CST is regulated by TH at the transcriptional levels (Ferret-Sena *et al* 1990, Fressinaud *et al* 1989). Although the synthesis of CST is regulated by T_3, it is difficult to find any direct correlation between early maternal hypothyroxinemia and CST activity. On the other hand, the effects of maternal hypothyroxinemia during early gestation (before the onset of independent fetal thyroid function) on oligodendroglial progenitor cells cannot be ruled out. Thus, the existence of a developmental window for the TH effect on progenitor glial cells may be the case and the presence of such critical period of time for TH effects in early gestation may influence the myelin metabolism of the offspring in later life.

Before coming to a final conclusion, it seems necessary to consider other factors which may exert important effects on the brain development of progeny and therefore affect the developmental parameters (e.g. chemical parameters) which have been studied in this investigation. For example,the effects of malnutrition of mothers and offspring on development of several organs (including brain) of progeny have been demonstrated by different investigators (Sato *et al* 1985, Yeh 1988, Rosman and Malone 1977).

A nutritional disadvantage in the progeny in the rat model employed in this study is unlikely, since TX dams were apparently eumetabolic and euthyroid (although thyrotropin was not measured, plasma TT_3 was within the normal range), and food intake for hypothyroxinemic dams was similar to that of the control group. Food intake

in both control and experimental progeny after weaning and throughout their life was also similar. Fetal iodine deficiency as a factor can be disregarded, since iodine has a free transplacental passage and the diet used (for dams and progeny) was iodine replete. Also during the suckling period, iodine reaches the pups through the milk. An effect of maternal hypothyroxinemia on thyroid function of the progeny can also be ruled out, since thyroid function of the progeny was normal (at least at the adult stage).

Another possible factor contributing to the damage is placental insufficiency secondary to maternal hypothyroxinemia. The placenta beside its endocrine function, contributes to fetal development by allowing maternal nutrients to pass to the fetus and elimination of the metabolic waste of the fetus into maternal circulation (Jones 1989, Braverman 1989a). To eliminate placental insufficiency as a major contributory factor, studies of the metabolic state of the placenta has been carried out (Hadjzadeh *et al* 1990, Pickard *et al* 1991b). Comparison of placentae from control and TX dams at term, demonstrated no apparent differences in several biochemical parameters (DNA, RNA, inorganic phosphate and protein concentration), glucose metabolism and the activities of a number of enzymes. These data suggest that the placenta is not significantly affected by hypothyroxinemia in partially thyroidectomized animals.

What then may be the causative factor(s) for the observed effects in experimental progeny? It is suggest that, a decrease in normal transplacental passage of maternal T_4 to the fetus during early gestation, before the onset of independent fetal thyroid function (17.5 gestational days in the rat), is the main factor responsible for the biochemical alterations in the progeny of TX dams observed in this study. All brain regions are not affected in the same degree; however, the observed damage was not only confined to those regions which develop largely prenatally in the rat (e.g. medulla and midbrain), but the paleocortex, which is phylogenetically a recently developed brain region, was also affected. These observed effects in the progeny are irreversible, since the age of the rat progeny in this investigation (7 months old) represents the end point of brain development in this species and the alterations observed appear to persist despite the presence of a normal thyroid state. These irreversible effects point to the possible existence of a 'window' or 'windows' for the TH in early brain development. Insufficiency of TH at this critical stage of brain development, due to maternal hypothyroxinemia, cannot be corrected in later stages by subsequent TH supply.

Summary

A rat model of maternal hypothyroxinemia was used in this study to investigate a possible role for maternal thyroxine in the development of the brain of offspring. This model resembles the conditions of women living in iodine-deficient endemias, where several neurological disorders are prevalent. The women in those areas are normal and clinically euthyroid, but they are severely hypothyroxinemic, as were the partially thyroidectomized rat dams observed in this investigation. The dysfunction in children borne in iodine-deficient regions, ranges from subclinical deficits in mental and motor function to the clear symptoms of neurological cretinism. In spite of the previous controversial idea with respect to transplacental passage of the TH, several recent investigations have revealed that the maternal thyroxine cross the placenta especially in early pregnancy. Amongst the pathogenetic factors of neurological cretinism, maternal hypothyroxinemia has recently received specific attention.

The hypothesis that maternal hypothyroxinemia in early pregnancy may cause irreversible effects on brain biochemical functions of progeny which may underlie the neurological and behavioral dysfunctions observed in the progeny, this investigation was started. In general, it has been accepted that the thyroid hormones initiate their effects by combination with specific nuclear receptors, resulting in changes in the level of specific mRNA species and the protein that they encode. Because of the presence of T3 nuclear receptor in fetal brain as early as 14 days of gestation, such a similar mechanisms of TH action is possible.

A range of biochemical factors in different brain regions of adult progeny with several neuronal and oligodendroglial cell marker enzymes, as well as myelin metabolic markers were studied. Significant changes were observed in the protein profiles of some proteins in both cytosolic and particulate fractions. Changes in myelin metabolic enzymes and other myelin metabolic markers (e.g. cerebroside and sulphatide) have revealed a deficit in myelin metabolism of adult progeny from partially thyroidectomized dams.

These changes are region-dependent; however since the age of animals (7 months old) at the time of study represents an end point of brain development in rat, the changes appear to be irreversible in spite of a normal thyroid function of the progeny. These findings support the hypothesis that maternal hypothyroxinemia in early

pregnancy, before the onset of independent fetal thyroid function, is the major factor responsible for the biochemical dysfunction in progeny. Regional deficits in the animal model analogous to the regional dysfunctions observed in cretinism, also makes this model suitable for biochemical studies of the CNS and investigation of behavioral deficits observed in neurological cretinism.

Possible Future Work

As the results of this investigation shows, several biochemical parameters are affected in this animal model. Further studies are required relating to a wide range of biochemical factors. Amongst these, cell type specific markers are important and considerable data could be obtained by the study of such parameters. A complete investigation of purified myelin lipids and proteins could also generate valuable data, since the present investigation showed that, in the progeny from hypothyroxinemic rat dams, myelin metabolism are being affected.

Biochemical studies of neurotransmitters coupled with the study of behaviour in this animal model would also yield informations regarding the biochemical alterations which may underlie those behavioral abnormalities that have been demonstrated in the progeny from hypothyroxinemic dams.

Studies at the molecular level to clarify the interaction between TH and different types of thyroid hormone nuclear receptors should also yield valuable data regarding the regional and cell-type specificity of TH on brain development.

Valuable data could also be obtained by studies of TH effects in utero at the level of glial (oligodendroglial) progenitor cells. Thus, answering many questions which have been raised in regards of the mechanism(s) of TH action on brain development.

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Effect of maternal hypothyroxinaemia in the rat on brain biochemistry in adult progeny

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RECEIVED 26 June 1989

ABSTRACT

The effects of maternal hypothyroxinaemia during pregnancy on subsequent brain biochemistry in progeny was studied. Normal and partially thyroidectomized rat dams were mated and progeny allowed to grow to adulthood. Brain regions (cerebellum, medulla, midbrain, cerebral cortex and paleocortex) were dissected out and the activities of various cell marker enzymes were determined, along with cholesterol contents.

Maternal hypothyroxinaemia was without effect on body weight, brain weight or thyroid status of adult progeny. Oligodendroglial marker enzyme activities were altered in progeny from thyroidectomized dams. 2',3'-Cyclic nucleotide 3'-phosphohydrolase was decreased in the medulla (by 37%) and midbrain (by 32%). 5'-Nucleotidase was also diminished in the same brain regions, by 33% in the medulla and by 35% in the midbrain. In contrast, oleate esterase was increased (by 39%) in the paleocortex. Although these enzymes are putatively involved in myelin metabolism, no changes were observed in the concentration of a major myelin lipid (cholesterol). The activity of β-D-glucuronidase (a general neuronal marker) was decreased (by 30%) in the paleocortex, whereas N-acetyl-β-D-galactosaminidase (a general glial marker) was unchanged in all brain regions.

In summary, maternal hypothyroxinaemia has irreversible effects on brain biochemistry in adult progeny. The damage is parameter-selective and brain region-specific, analogous to the pattern of neurological damage seen in offspring born to hypothyroxinaemic women in iodine-deficient endemias.

Journal of Endocrinology (1990) **124,** 387–396

INTRODUCTION

An increased incidence of neurological disorders is observed in children born in iodine-deficient regions of the world. The dysfunction ranges from subclinical deficits in mental and motor function to overt neurological cretinism, characterized by mental retardation, deaf mutism, spastic diplegia and often strabismus (Pharoah, Delange, Fierro-Benitez & Stanbury, 1980; Hetzel, 1983; Connolly, 1986; Stanbury, 1986). Although administration of iodine to mothers is effective in preventing neurological damage to the offspring, therapy must be initiated before conception to be completely successful (Pharoah, Buttfield & Hetzel, 1971). This implies that the fetal damage occurs very early in pregnancy (most probably in the first trimester), before the establishment of independent fetal thyroid function.

The above findings have often been interpreted in terms of a role for elemental iodine in brain development, independent of its requirement in thyroid hormone synthesis (Pharoah *et al.* 1971, 1980), which remains unproven. Rather, we believe that the damage may result from a decreased fetal supply of maternal thyroxine (T_4) (Ekins, 1985; Ekins, Sinha & Woods, 1986; Ekins, Sinha, Ballabio *et al.* 1989). In mothers from iodine-deficient endemias, the serum concentrations of T_4 (both total and free) are in many cases below the normal range, whereas the serum concentrations of 3,5,3'-tri-iodothyronine (T_3; total and free) are normal or raised (Pharoah, Lawton, Ellis *et al.* 1973; Chopra, Hershman & Hornabrook, 1975). Furthermore, it has previously been demonstrated that there is significant placental transfer of maternal thyroid hormones (in particular T_4) during early pregnancy in the rat (Obregon,

  Printed in Great Britain
0022–0795/90/0124–0387 $02.00/0

Mallol, Pastor *et al.* 1984; Woods, Sinha & Ekins, 1984).

The interpretation of field studies in terms of a fetal requirement for maternal T_4 is compromised by the underlying iodine deficiency; impaired fetal/neonatal thyroid function may contribute to the dysfunction. On the other hand, clinical investigations in iodine-replete environments have reported an association between maternal hypothyroxinaemia and the development of intellectual and fine motor function in the offspring (Man & Serunian, 1976). Despite this evidence, the epistemological relationship between maternal hypothyroxinaemia (as distinct from hypothyroidism) and irreversible neurological dysgenesis in the offspring remains to be proven. In addition, the changes in brain biochemistry which underlie the observed neurological dysfunction are not understood. We therefore initiated studies with a hypothyroxinaemic rat model in order to address these questions.

We have previously reported that maternal hypothyroxinaemia during pregnancy results in brain region-specific changes in DNA, RNA and total protein concentrations in adult rat progeny (Ekins *et al.* 1989). In addition, we have demonstrated effects on selected enzymes associated with parameters such as energy metabolism, calcium homeostasis, neurotransmitter metabolism and lysosomal function (Ekins *et al.* 1989; Ruiz de Elvira, Sinha, Pickard *et al.* 1989). In this communication we have concentrated on brain cell-specific marker enzymes, in particular those associated with the oligodendroglia, which function in myelin metabolism.

MATERIALS AND METHODS

Materials

The following chemicals were obtained from Sigma (Poole, Dorset, U.K.): adenosine 2',3'-cyclic monophosphate (2',3'-cAMP), adenosine 5'-monophosphate (AMP), 4-methylumbelliferyl-N-acetyl-β-D-galactosaminide (4-MU-GalNAc), 4-methylumbelliferyl-β-D-glucuronide (4-MU-GlcU), 4-methylumbelliferyl oleate (4-MU-O), methyl cellosolve, alkaline phosphatase (type III; approximately 300 U/ml), deoxycholic acid and Triton X-100. All other chemicals and solvents were purchased from BDH Ltd (Poole, Dorset, U.K.). Nitrogen gas was from BOC Ltd (London, U.K.).

Animals

Partially thyroidectomized (parathyroid-spared) Sprague–Dawley rat dams were mated with normal males when the total serum concentration of T_4 approximated 10 nmol/l. Appropriate matched control dams were also mated and both pregnancies allowed to continue to term. Offspring were standardized to a litter size of six at birth and allowed to grow up to adulthood (approximately 7 months) before death. Selected age-matched animals were killed by cervical dislocation followed by exsanguination and, when required, samples of blood were collected by cardiac puncture for determination of thyroid hormone. All animals were maintained on an iodine-replete diet and the drinking water of thyroidectomized dams was supplemented with calcium lactate (0·1%, w/v). The daily food intake was similar for both groups of dams and for both groups of progeny after weaning.

Sample preparation

Brain regions were dissected on ice and the tissue samples homogenized in a hand-held homogenizer (0·1 mm clearance) in either 10 vol. ice-cold sucrose (0·25 mol/l; for protein and enzyme activity determinations) or 19 vol. ice-cold chloroform/methanol (2 : 1, v/v; for cholesterol determination). Aliquots of homogenate were stored at −70 °C before enzyme analysis.

Enzyme assays

β-D-Glucuronidase and N-acetyl-β-D-galactosaminidase
β-D-Glucuronidase and N-acetyl-β-D-galactosaminidase were assayed by a procedure adapted from Sinha & Rose (1972). Assays (2 ml final volume, in triplicate) contained 4-MU-GlcU or 4-MU-GalNAc (0·25 mmol/l) and protein (200–250 μg) in sodium citrate buffer (50 mmol/l; pH 4·5). After incubation at 37 °C for 15 min, reactions were terminated by the addition of 3 ml glycine buffer (0·5 mol/l; pH 10·4). Homogenate was added after glycine buffer in blank tubes. Liberated 4-methylumbelliferone (4-MU) was determined fluorimetrically (360 nm excitation and 444 nm emission wavelengths). Activities were expressed as nmol 4-MU released/mg protein per h.

Oleate esterase
The oleate esterase procedure (Hirsch, Wernicke, Myers & Parks, 1977) was slightly different from that for the sugar hydrolases. 4-MU-O was dissolved in methyl cellosolve to yield a 10 mmol/l solution. Assay mixtures (160 μl, in triplicate) contained substrate solution (0·4 mmol/l), protein (50 μg) and Triton X-100 (0·1%, v/v) in citrate-phosphate buffer (0·1 mol/l; pH 6·0). After incubation at 37 °C for 30 min, reactions were terminated by the addition of 3 ml sodium phosphate buffer (0·1 mol/l; pH 7·0). Released 4-MU was determined as described for the sugar hydrolases. Activity was expressed as μmol 4-MU released/mg protein per h.

5'-Nucleotidase

Activity of 5'-nucleotidase was assayed by measurement of inorganic phosphate (P_i) released from AMP (Cammer, Sirota, Zimmerman & Norton, 1980). Assays (0·4 ml, in triplicate) contained AMP (10 mmol/l), $MgCl_2$ (10 mmol/l) and tissue protein (0·5 mg) in Tris–HCl buffer (0·1 mol/l; pH 7·5). Tubes were incubated at 37 °C for 1 h, and the reaction was terminated by the addition of 0·3 ml H_2SO_4 (2·5 mol/l). Released P_i was determined by reaction with molybdate, as described below. Blanks were without homogenate. 5'-Nucleotidase activity was expressed as μmol P_i released/mg protein per h.

2',3'-Cyclic nucleotide 3'-phosphohydrolase (CNPase)

The procedure of Prohaska, Clark & Wells (1973) was followed for assay of CNPase. The product (2'-AMP) released from 2',3'-cAMP was determined as P_i after treatment with alkaline phosphatase. Homogenates were initially treated with an equal volume of 0·5% (w/v) deoxycholate (in 30 mmol Tris–HCl/l; pH 7·5) for 20 min at 4 °C. Solubilized homogenate (approximately 10 μg protein) was then incubated in triplicate with 2',3'-cAMP (5·25 mmol/l) in a final volume of 0·2 ml Tris–maleate buffer (45 mmol/l; pH 6·2). After 30 min at 37 °C, reactions were terminated by placing in a boiling water bath for 5 min.

A second incubation step was conducted to release P_i from any product (2'-AMP) formed. After cooling, 0·1 ml phosphatase solution (2 U alkaline phosphatase/ml and 21 mmol $MgCl_2$/l in 0·1 mol Tris–HCl/l; pH 9·0) were added, and the tubes were incubated for 30 min at 37 °C. This second reaction was terminated by the addition of 0·2 ml 30% (w/v) trichloroacetic acid and released P_i was determined (see below). Blanks were without homogenate. CNPase activity was expressed as μmol P_i released/mg protein per h.

Determination of P_i

The method described by Cammer *et al.* (1980) was used to determine P_i in 5'-nucleotidase and CNPase reaction mixtures. Samples (0·2 ml aliquots) were further acidified with 0·3 ml H_2SO_4 (2·5 mol/l), then 2 ml isobutanol/benzene (1 : 1, v/v) were added, followed by 0·2 ml ammonium molybdate (10%, w/v). After thorough mixing, tubes were centrifuged (300 *g* for 10 min) to separate the layers.

An aliquot (1 ml) of the organic upper phase was transferred to a clean glass tube, 2·5 ml of a dilute stannous chloride solution (0·006% (w/v) $SnCl_2$, 87% (v/v) ethanol and 0·3 mol H_2SO_4/l) were added and the tubes mixed. To develop the colour, 20 μl of a concentrated stannous chloride solution (10% (w/v) in concentrated HCl) were added and the tubes were shaken vigorously. The absorbance at 730 nm was immediately read against a reagent blank and the P_i content was calculated from a standard curve (0–0·6 μmol P_i; Na_2HPO_4 as standard).

Determination of cholesterol

Brain homogenates in chloroform/methanol (2 : 1, v/v) were centrifuged (1910 *g* for 15 min at 4 °C). The pellet was re-extracted four times with chloroform/methanol (2 : 1, v/v) and finally once with chloroform/methanol (7 : 1, v/v; ammonia-saturated). Supernatants were combined and dried under nitrogen at room temperature. Dried extracts were taken up in ethanol (2 ml) and the cholesterol content determined by the procedure of Franey & Amador (1968). Colour reagent (2 ml; 0·1% (w/v) ferric chloride in ethyl acetate) was added to 0·5 ml ethanol extract, followed by 2 ml concentrated H_2SO_4 (added whilst mixing). After cooling, the absorbance at 560 nm was read. Reagent blank tubes contained ethanol in place of extract and cholesterol contents were determined from a standard curve (0–0·3 mg cholesterol).

Determination of thyroid hormone

Total plasma T_3 (TT_3) and T_4 (TT_4) contents were determined by 'in house' solid-phase radioimmunoassay procedures.

Determination of protein

Protein was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951).

Statistical analysis

Statistical significance of the results was determined by paired Student's *t*-test.

RESULTS

Thyroid status of the animals

Analysis of plasma samples revealed a significant ($P < 0·02$) difference in concentration of TT_4 between control and partially thyroidectomized dams (Table 1); the mean value for the experimental dams was only 30·2% of the normal value. The plasma concentration of TT_3 of thyroidectomized dams was also reduced (68·9% of the normal value; Table 1); this difference was, however, statistically insignificant. Thus the partially thyroidectomized dams were severely hypothyroxinaemic, but T_3 values were within the control range. On the other hand, there was no obvious difference in plasma concentrations of TT_4 or TT_3 between adult progeny from thyroidectomized and control dams (Table 1). Thus maternal hypothyroxinaemia was without effect on the thyroid status of adult progeny.

TABLE 1. Concentrations of thyroxine (T_4) and 3,5,3′-triiodothyronine (T_3) in plasma from normal and partially thyroidectomized rat dams and their respective progeny. Values are means ± S.D.; numbers of animals are shown in parentheses

	T_4 (nmol/l)	T_3 (nmol/l)
Dams		
Normal (5)	44·7±20·3	1·22±0·36
Thyroidectomized (5)	13·5±8·7*	0·84±0·25
Progeny		
Normal dam (6)	47·0±12·2	1·15±0·32
Thyroidectomized dam (6)	47·3±13·0	1·11±0·37

*$P<0·02$ compared with normal dams (Student's t-test).

Brain and body weights of adult progeny

Although the body weights of the progeny from hypothyroxinaemic dams were generally higher (8% for males, 12% for females) than those of the control progeny (Table 2), this difference was not statistically significant. Maternal hypothyroxinaemia was without any effect on the brain weight of adult progeny (Table 2).

Activities of β-D-glucuronidase and N-acetyl-β-D-galactosaminidase

β-D-Glucuronidase and N-acetyl-β-D-galactosaminidase are both lysosomal enzymes. The former enzyme is thought to be present predominantly in the neuronal cell bodies (Sinha & Rose, 1972), whereas the latter is preferentially (but not exclusively) localized within the glial cells (Sinha & Rose, 1973).

The activity of β-D-glucuronidase was found to be significantly decreased (by 30%) in the paleocortex of adult progeny from hypothyroxinaemic dams, but other brain regions were unaffected (Fig. 1). No changes were evident in N-acetyl-β-D-galactosaminidase activities in all brain regions of experimental progeny (Fig. 2).

Activities of 5′-nucleotidase and CNPase

5′-Nucleotidase (Cammer *et al.* 1980) and CNPase (Vogel & Thompson, 1988) are preferentially produced by the oligodendroglial cell population in the central nervous system (CNS). High levels of activity are present in the myelin sheath and associated with the oligodendroglial cell membrane.

In adult progeny from hypothyroxinaemic dams, 5′-nucleotidase activity was significantly decreased in the medulla (by 33%) and midbrain (by 35%; Fig. 3). CNPase activity was likewise affected, being decreased by 37% in the medulla and by 32% in the midbrain (Fig. 4). The reductions in activities of 5′-nucleotidase (31%) and CNPase (26%) in the paleocortex, although comparable with changes in the medulla and midbrain, were not statistically significant (Figs 3 and 4).

Activity of oleate esterase

Oleate esterase is also a myelin metabolic enzyme which is mainly localized within the oligodendrocytes. However, it differs from the above two nucleotidases in that it is primarily found within the oligodendroglial cell bodies (Hirsch *et al.* 1977).

The enzyme activity was increased by 30% in the cerebral cortex and by 39% in the paleocortex of experimental progeny (Fig. 5); however, only the latter was statistically significant. A slight (though insignificant) decrease (16%) was observed in the cerebellum.

Cholesterol concentration

Cholesterol is a general constituent of plasma membranes in the CNS and is a major component of the myelin lipid (Norton, 1981). Despite the observed changes in the three myelin metabolic enzymes studied, the concentration of this lipid was unchanged in all brain regions of the experimental progeny (Fig. 6).

DISCUSSION

Previous studies conducted with fetuses and young progeny from thyroidectomized rat dams have demonstrated reductions in brain and body weights (Morreale de Escobar, Pastor, Obregon & Escobar del Rey, 1985; Hubank, Ballabio, Sinha *et al.* 1986), whereas no such effects were apparent in the adult progeny. The deleterious effects of an altered thyroid hormone environment *in utero* on gross brain and somatic growth may therefore have been compensated for by the normal thyroid state of the progeny in later life.

Despite no obvious effects of maternal hypothyroxinaemia on gross brain weight in adult progeny, a variety of biochemical parameters were found to be altered. The changes were brain region-specific and the activities of both neuronal and oligodendroglial cell marker enzymes were affected. However, there was no consistent pattern to the damage. For example, activities of 5′-nucleotidase and CNPase were decreased in the medulla and midbrain, whereas oleate esterase activity was increased in the paleocortex. Since all these enzymes are products of the oligodendrocyte, it is unlikely that maternal hypothyroxinaemia results in permanent changes in the numbers of a given brain cell population. Rather, specific cell functions appear to be selectively altered. This conclusion is reinforced by the observation that a general glial marker enzyme, N-acetyl-β-D-galactosaminidase, is unaffected throughout all brain regions.

TABLE 2. Body weight, brain weight and brain : body weight ratio of progeny of normal and partially thyroidectomized rat dams. Values are means ± S.D.; numbers of animals are shown in parentheses

	Body weight (g)	Brain weight (g)	Brain : body weight ratio (%)
Normal dam progeny			
All (16)	496·8 ± 180·2	2·01 ± 0·25	0·451 ± 0·150
Male (9)	639·7 ± 88·0	2·11 ± 0·23	0·333 ± 0·049
Female (7)	313·1 ± 28·6	1·88 ± 0·22	0·604 ± 0·070
Thyroidectomized dam progeny			
All (16)	541·9 ± 185·1	2·06 ± 0·23	0·423 ± 0·138
Male (9)	690·8 ± 77·2	2·17 ± 0·25	0·320 ± 0·070
Female (7)	350·4 ± 40·8	1·92 ± 0·10	0·554 ± 0·070

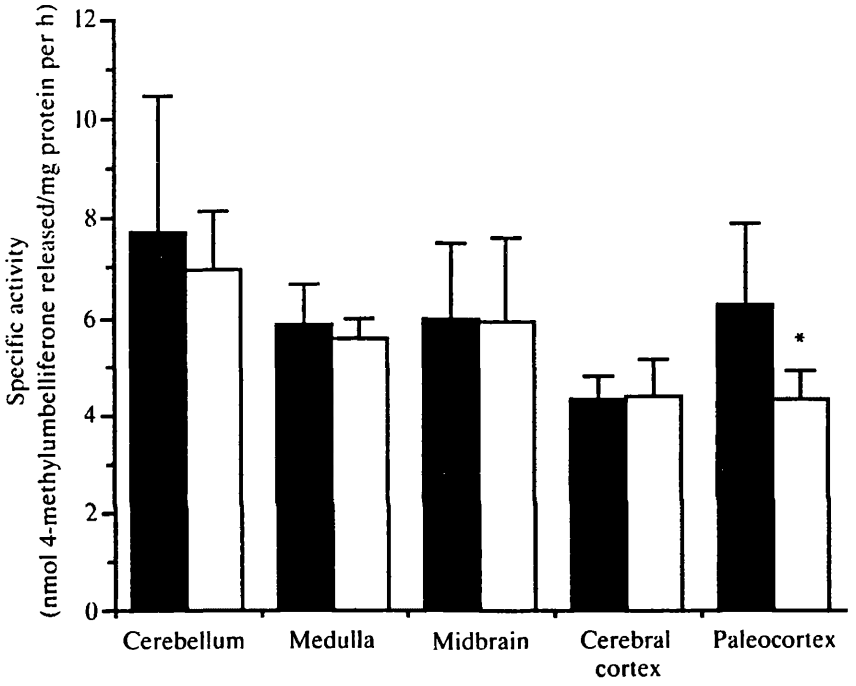

FIGURE 1. Activity of β-D-glucuronidase in brain regions from progeny of normal dams (solid bars) and partially thyroidectomized dams (open bars). Values are means ± S.D. (n = a minimum of three animals). *$P < 0·05$ compared with progeny of normal dams (Student's t-test).

A major function of the oligodendroglia is the production of the CNS myelin sheath, which serves to insulate the axons of neurones and thereby facilitate conduction (Norton, 1981). Indeed, all the oligodendroglial markers studied here have putative involvement in myelin metabolism. 5'-Nucleotidase is thought to be involved in the membrane transport of adenosine (Norton, 1981) and oleate esterase may play a role in myelin degradation (Hirsch *et al.* 1977). Although the function of myelin CNPase is at present unclear, a structural role for this protein is unlikely (Vogel & Thompson, 1988). In addition, we have previously reported a diminution in activity of another myelin-metabolic enzyme, arylsulphatase A, in several brain regions of adult progeny (Ekins *et al.* 1989). Thus it is feasible that myelin turnover is altered in adult progeny as a consequence of maternal hypothyroxinaemia during pregnancy. Not all steps of myelin metabolism can be affected, however, since concentrations of brain cholesterol were unchanged in the experimental group.

We believe that a decrease in the normal transplacental passage of maternal T_4 in early pregnancy, before the onset of independent fetal thyroid function, is the major factor responsible for the biochemical dysfunction. Nutritional disadvantage of the progeny is unlikely, since the hypothyroxinaemic rat dams were apparently euthyroid and eumetabolic (although measurements of thyrotrophin were not performed, plasma concentrations of TT_3 were within the normal range), and food intake was similar to that in control dams. Food intake in control and experimental

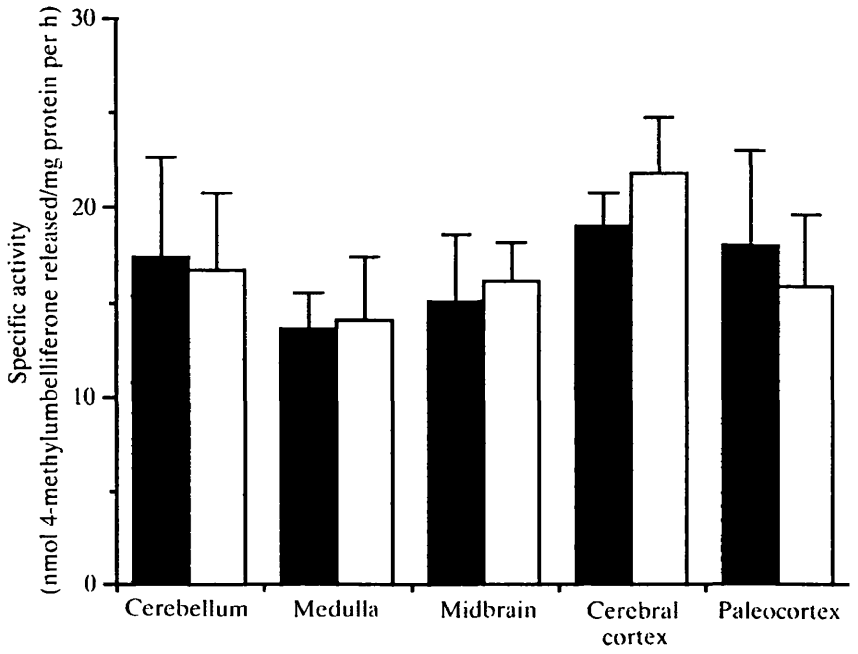

FIGURE 2. Activity of N-acetyl-β-D-galactosaminidase in brain regions from progeny of normal dams (solid bars) and partially thyroidectomized dams (open bars). Values are means ± S.D. (n = a minimum of four animals).

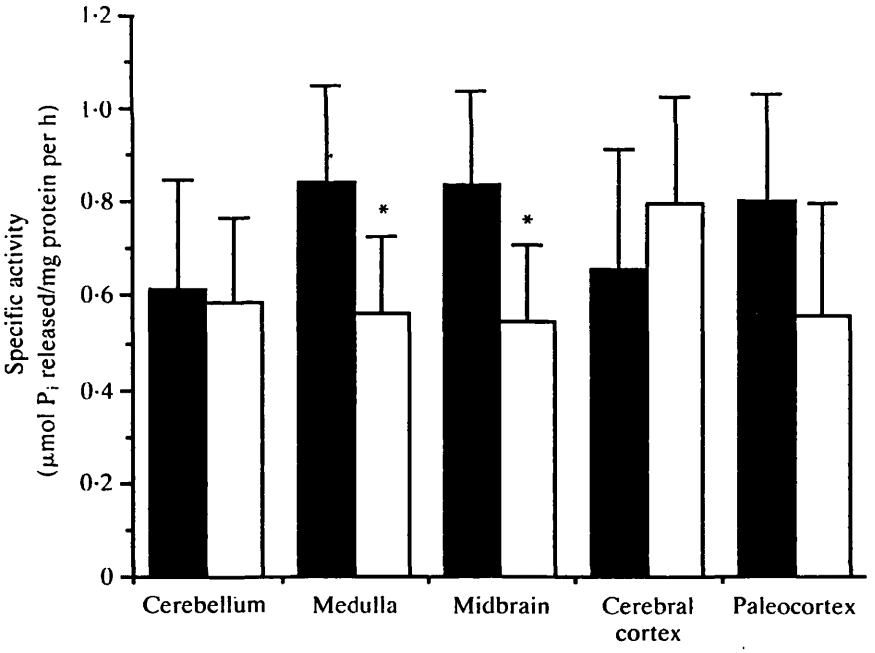

FIGURE 3. Activity of 5′-nucleotidase in brain regions from progeny of normal dams (solid bars) and partially thyroidectomized dams (open bars). Values are means ± S.D. (n = a minimum of five animals). *$P < 0{\cdot}05$ compared with progeny of normal dams (Student's t-test).

progeny after weaning was also similar. Preliminary work in our laboratory (A. K. Sinha & M. R. Pickard, unpublished results) would also tend to eliminate placental insufficiency as a major contributory factor. Comparison of placentae ($n=3$) from partially thyroidectomized and control dams at term revealed no obvious differences in a range of biochemical parameters (DNA, RNA, P_i and protein concentrations; N-acetyl-β-D-glucosaminidase, β-D-glucuronidase and acid phosphatase activities). The involvement of

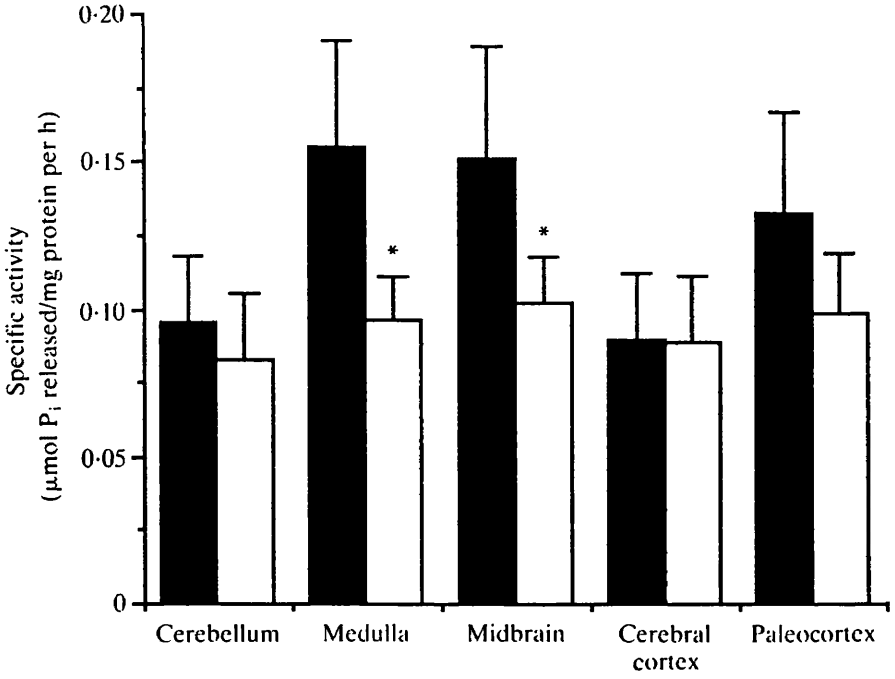

FIGURE 4. Activity of 2',3'-cyclic nucleotide 3'-phosphohydrolase in brain regions from progeny of normal dams (solid bars) and partially thyroidectomized dams (open bars). Values are means ± S.D. (n = a minimum of five animals). *$P<0·05$ compared with progeny of normal dams (Student's t test).

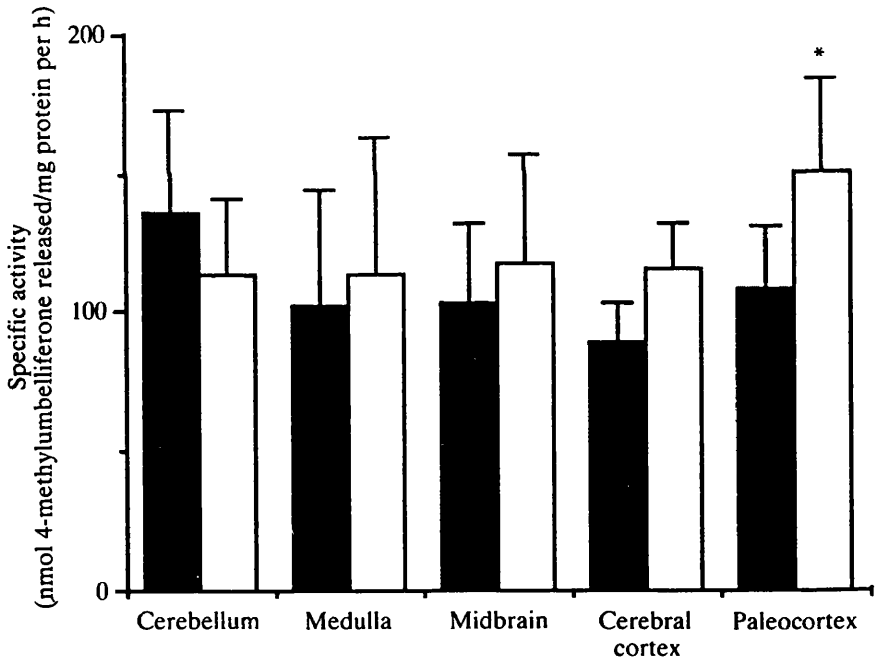

FIGURE 5. Activity of oleate esterase in brain regions from progeny of normal dams (solid bars) and partially thyroidectomized dams (open bars). Values are means ± S.D. (n = a minimum of four animals). *$P<0·05$ compared with progeny of normal dams (Student's t-test).

fetal iodine deficiency can be disregarded since iodine has a free transplacental passage and the diet used was iodine replete. Finally, an effect of maternal hypothyroxinaemia on thyroid function of the progeny also seems unlikely, since this was normal at least in adult animals.

What then may be the mechanism(s) for the observed effects? It is difficult to address this question

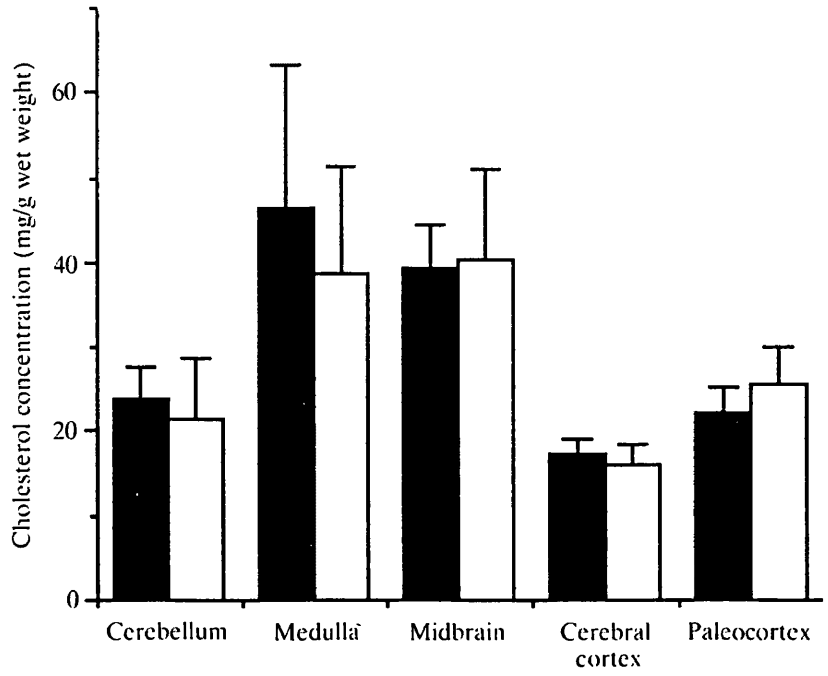

FIGURE 6. Cholesterol content of brain regions from progeny of normal dams (solid bars) and partially thyroidectomized dams (open bars). Values are means ± S.D. (n = a minimum of three animals).

on the basis of experiments conducted solely in adult progeny but nonetheless this age represents the end of brain development and certain points are worthy of further comment.

Although there is a wide body of evidence documenting the influences of thyroid hormone on postnatal brain development in the rat (Ford & Cramer, 1977; Dussault & Ruel, 1987; Timiras, 1988), including the ontogenesis of the myelin metabolic systems, very little is known regarding their control of prenatal development, especially before the development of independent fetal thyroid function (17·5 gestational days in the rat). In general, the thyroid hormones initiate their effects by combination with specific nuclear receptors, resulting in changes in the levels of selective mRNA species and the proteins that they encode (Oppenheimer, 1979; Oppenheimer, Schwartz, Mariash *et al.* 1986). Since fetal brain nuclear T_3 receptors can be detected as early as day 14 of gestation in the rat (Perez-Castillo, Bernal, Ferreiro & Pans, 1985), then a similar mechanism of action is possible. However, the damage we observed was not only confined to those regions (medulla and midbrain) which develop largely prenatally in the rat, but the paleocortex (a phylogenetically recent brain region) was also affected. Furthermore, it should be noted that the majority of glial cell division and differentiation occurs postnatally in the rat. Given the complex interaction of a variety of factors (cell migration, cell–cell interaction, autocrine and endocrine influences) in determining the overall outcome of brain development, it is not surprising that the maintenance of an adequate fetal thyroid hormone environment *in utero* is of critical importance for the expression of functions in later life.

The effects we observed in this study are irreversible, since the animals were at adulthood, and they persist despite the presence of a normal thyroid state. This points to the possible existence of a 'window' or 'windows' for the thyroid hormones in early brain development. Insufficiency of thyroid hormone at this critical stage, due to maternal hypothyroxinaemia, cannot be corrected by subsequent thyroid hormone sufficiency. It is emphasized that this postulate remains speculative at present since little is known regarding the thyroid state of the progeny in late fetal/early postnatal life.

Several features of the animal model used in our experiments resemble the human conditions that prevail in iodine-deficient endemias, where an increased incidence of a variety of neurological disorders has been reported (Pharoah *et al.* 1980; Hetzel, 1983; Connolly, 1986; Stanbury, 1986). Although the women in these areas are apparently normal and clinically euthyroid, they are severely hypothyroxinaemic, similar to the experimental rat dams used in our study. The regional compromise observed in our animal model is also a primary feature in neurological cretinism in man. For example, the auditory dysfunction in man points to damage of the cochlear division

of the auditory nerve, auditory cortex and/or auditory projection areas. Mental retardation, cognitive deficits and behavioural disorders suggest functional lesions of the neocortex, whereas gait disorders perhaps reflect impaired cerebellar function. Finally, it should be noted that electron microcscopy of brains from severe neurological cretins in China has revealed changes in the appearance of the myelin sheath (Ma, Lu, Tan & Chen, 1986). It is possible therefore, that the biochemical deficiencies observed in our experiments may partly explain the disorders found in cretinism.

ACKNOWLEDGEMENTS

The authors are indebted to the Thyroid Hormone Physiology research group for constructive criticism and continuous encouragement during the time of this work. The work was partly supported by a grant from NETRHA, which is gratefully acknowledged.

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