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MATERNAL IODINE DEFICIENCY AND
PRENATAL BRAIN DEVELOPMENT

by

IAN DAVID HAY
B.Sc., M.B., Ch.B., M.R.C.P.
University Department of Medicine,
Royal Infirmary, Glasgow

VOLUME I

A thesis in two volumes submitted for the degree of
Doctor of Philosophy to the University of Glasgow
June 1978



“The Power in Iodine”

Warkany

.....That such a complicated disorder as endemic cretinism could be wiped out without explanation of the intricate mechanisms involved in its pathogenesis may be disappointing to some scientific purists but consoling to those who like shortcuts in the prevention of congenital disorders.

Josef Warkany (1971)

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DECLARATION

The work to be described in this thesis was performed in the University Departments of Medicine and Clinical Biochemistry at the Royal Infirmary, Glasgow between August 1974 and May 1978. In the first two years of study the author was employed by the Medical Research Council as a Training Fellow. Since August 1976 he has been employed by Glasgow University as Hall Fellow in Medicine. The detailed planning of the experimental work and its execution were performed entirely by the author. The only exception to this was the radioimmunoassay of thyroid hormones which was performed by Dr. W.A. Ratcliffe.

Preliminary results of the study were presented in 1976 to the European Thyroid Association and the Scottish Society for Experimental Medicine. Abstracts of these meetings have now been published and are as described below:

- (1) Hay, I.D., Ratcliffe, W.A. & Thomson, J.A. (1976)
Prenatal iodine deficiency and forebrain development.
Acta Endocrinologica, Suppl. 204: 56.
- (2) Hay, I.D., Ratcliffe, W.A. & Thomson, J.A. (1977)
The effect of prenatal iodine deficiency on forebrain
development in the rat. *Scottish Medical Journal*, 22:
106.

SUMMARY

Endemic cretinism is a congenital disorder of the nervous system whose postulated neuropathology involves principally the cerebral hemispheres and the eighth nerve system. Although the association of endemic cretinism with severe maternal iodine deficiency has long been recognised the pathogenesis of the neurological syndrome remains obscure. The experimental work described in this thesis represents an attempt to further define in an animal model the pathogenesis of the cerebral lesions assumed to cause many of the characteristic neurological defects.

The thesis is divided into three main sections. Part A is essentially a review of available data on the clinical and metabolic aspects of the syndrome of endemic cretinism. Chapter I deals with the early recognition of endemic cretinism, its association with endemic goitre and its relation to hypothyroidism and iodine deficiency. Chapter II is more concerned with thyroidal adaptation to endemic iodine deficiency and in particular the effects of varying degrees of maternal thyroid dysfunction on the outcome of pregnancy, the role of the placenta in thyroid hormone transfer and the effect of severe maternal iodine deficiency on foetal thyroid function. Lastly the chapter also reviews the currently held theories on the pathogenesis of endemic cretinism.

In Chapter III the neurological aspects of endemic cretinism are considered in greater detail. The clinical features and pathological findings in neurological cretinism are reviewed as are the reported experiments in mice and rats on the hearing defect induced in neonates by maternal administration of propylthiouracil.

Part B deals with the evolution of the animal model employed in this study. It was postulated in 1974 by the author that severe maternal iodine deficiency might act as a growth retarding stimulus on the developing foetal brain during the period of neuronal proliferation and that such a hypothesis might be tested by maintaining female rats on a Remington-type low iodine diet, mating the animals when significant

iodine deficiency was attained and then measuring in the neonatal forebrain (cerebrum) the DNA and protein content, considered accurate indices of cerebral neuronal number and size. In Chapter V the production of iodine deficiency in the rat by dietary means is described while Chapter VI is concerned with the development of techniques for the accurate measurement of cell number and size in the postnatal rat forebrain.

In Part C seven experiments performed with the rat model are fully described and the obtained results discussed. Chapter VII deals with the effects of prenatal iodine deficiency on forebrain development, Chapter VIII with the effects of combined prenatal and postnatal iodine deficiency and Chapter IX with the effects of pregnancy and iodine deficiency on maternal thyroid function.

Maternal iodine deficiency, severe enough to cause hypothyroxinaemia at mating had no apparent effect on prenatal neuronal proliferation as measured by DNA content of neonatal rat forebrain (Chapter VII). It was however associated with a significant reduction in forebrain protein content, implying a reduction in cerebral neuronal size. This qualitative defect was not seen in 22 day old pups born to mothers with a similar degree of iodine deficiency (Chapter VIII) and it was considered that this apparent reversibility was made possible by thyroidal adaptation to iodine deficiency during the period of maternal lactation.

Pregnancy in the rat was associated with significant falls in serum T_4 and T_3 concentration during the last two weeks of gestation, these changes occurring independent of the iodine status of the mother (Chapter IX). The principal difference found between iodine deficient (test) and iodine sufficient (control) pregnancies was the degree of hypothyroxinaemia seen throughout pregnancy in the test mothers. By contrast at no time during pregnancy or lactation were any differences in serum T_3 concentrations detected between test and control mothers.

The implications of these results are considered at length in Chapter X where further lines of investigation are described which could successfully employ this type of laboratory model.

ABBREVIATIONS

The abbreviations used in this thesis are in the main widely accepted. Less common abbreviations are listed below:

BEI	butanol-extractable iodine
BSA	bovine serum albumin
DH ₂ O	distilled water
DIT	diiodotyrosine
DNA	desoxyribonucleic acid
DPA	diphenylamine
fT ₃	free tri-iodothyronine
fT ₄	free thyroxine
GH	growth hormone
HClO ₄	perchloric acid
HPT	hypothalamic-pituitary-thyroid
ID	iodine-deficient
IT	iodine-treated
LID	low iodine diet
MF	maternal to foetal
MIT	monoiodotyrosine
MN	malnutrition
OD	optical density
P	probability
PAHO	Pan American Health Organisation
PBI	protein bound iodine
PTU	propylthiouracil
RAI	radioactive iodine
RAIU	radioactive iodine uptake
RIA	radioimmunoassay
RNA	ribonucleic acid
RT ₃	reverse T ₃

RTW	relative thyroid weight
T ₃	tri-iodothyronine
T ₄	thyroxine
TBG	thyroxine-binding globulin
TBP	thyroxine-binding proteins
TCA	trichloracetic acid
TG	thyroglobulin
TRH	thyrotrophin releasing hormone
TSH	thyroid stimulating hormone, thyrotrophin
TT ₃	total tri-iodothyronine
TT ₄	total thyroxine
WHO	World Health Organisation

Synonyms used in text

fetus	foetus
goiter	goitre
neuron	neurone

INTRODUCTION

Endemic cretinism has been defined as "a congenital disorder of the central nervous system which in its extreme form is associated with mental retardation, deaf-mutism, spastic diplegia and signs of bulbar damage" (161). Although first recognised in the sixteenth century (52) it was not until the 1920s, when deaf-mutism disappeared from Switzerland (284), that the intimate relationship of endemic cretinism to severe iodine deficiency was confirmed. In 1971 Pharoah et al. (219) reported that a single injection of iodised oil, if administered to the mother prior to conception, was effective in preventing the neurological syndrome. As a consequence they proposed that severe maternal iodine deficiency acting on the developing brain during the first trimester of pregnancy resulted in the clinical entity of "neurological cretinism" (154).

In 1974, when the work to be described in this thesis was commenced, it was considered that "no entirely satisfactory animal model" existed to subject the proposal of Pharoah et al. (219) to "rigorous proof" (258). The aim of the experiments to be described below was to investigate the effects in an animal model of maternal iodine deprivation on prenatal brain development. It was in other words an attempt to find a satisfactory animal model to test the foetal iodine deficiency theory of endemic cretinism.

The thesis based on this work has been divided into three main parts. Part A is principally a literature review which deals in three sections with the relation of endemic cretinism to endemic goitre and iodine deficiency, the adaptive mechanisms seen in mother and foetus during iodine deficiency and neurological aspects of endemic cretinism, both at clinical and experimental levels. Part B deals with the development of the animal model, particularly the production of iodine deficiency and the quantitation of brain development. Lastly, Part C describes in full the findings of seven experiments performed using the animal model.

CHAPTER I

ENDEMIC CRETINISM: HISTORICAL BACKGROUND

(i) Early recognition of endemic cretinism

Although endemic goitre was clearly described by Pliny in the first century it took almost another fourteen hundred years before Paracelsus first described what we now know as endemic cretinism (52). In notes taken at a lecture delivered in 1527 we read:

"...The strumous (goitrous) are seldom sensible because the brain also gets its liquor or nutriment from the minerals....and they are also deaf because the mineral humor occludes the ears..."

It is on the basis of this passage that Paracelsus is credited with first indicating the association between endemic goitre, mental retardation and deafness. Some three years before this lecture was published, however, Josiah Simler, a Protestant minister, had written about the prevalence of goitre in the Canton of Valais and suggested that in certain geographically defined areas "the waters when drunk injure the brain so much that they make (people) stupid and also....the throats swell" (51). Moreover he identified a group of fatuous people called "gouchen" and suggested that in the Valais midwives could recognise at birth the features of a "gouch" (fool), implying possibly an intra-uterine causation of the condition.

By the end of the sixteenth century it was widely recognised that cretinism was endemic and associated with goitre. It was also generally accepted that a peculiarity of the drinking water was responsible for both cretinism and goitre. As yet, however, no description of the distinctive clinical findings had been given. In 1602 the Swiss physician, Felix Platter, wrote that he had seen in the Valais and in Carinthia (Austria) infants who were dumb,

suffered from "innate folly", had misshapen heads, huge tongues and "often goitrous" throats (225). This statement is considered by some to be the first unequivocal description of endemic cretinism and is notable for its accuracy of observation.

Platter's description is all the more remarkable when we consider that as yet the thyroid gland had not been discovered. Indeed, until Wharton's book (285) appeared in 1656, a goitre was described as a "swollen throat", "hernia gutturis", "bronchocoele" or "struma" and throughout the seventeenth century cretinism was considered a traveller's curiosity and consequently omitted from the standard medical texts (51).

As for the actual word cretin this did not appear in print until 1754 when, in the Modern History section of Diderot's *Encyclopedie*, the entry for cretins read as follows:

"Cretins.... One gives this name to a species of men who are born in the Valais in rather great numbers.... They are deaf, dumb, imbecile.... and carry goitres hanging down to the waist.... The simplicity of the people of the Valais makes them regard the Cretins as the tutelary angels of the families and those who have none believe themselves to be on bad terms with heaven...." (71).

It is noteworthy that cretins are described here not as diseased but as "a species of men" and that again the Valais is mentioned as the region where cretinism was prevalent and prototypal. The angelic concept is also of interest since Fodere (120) was later to state in his treatise of 1800 that the word cretin derived from *chretien*, meaning good Christian, since cretins were "incapable of committing any sin".

Fodere and the brothers Wenzel did much to incorporate a reasonably clear and accurate knowledge of cretinism into the medical literature in the period 1789-1802 and it is to this period that one would readily date the medical "discovery" of cretinism.

In their memoir on cretinism the brothers Wenzel (283) wrote, on the subject of disease particularly indigenous to a country, the following:

"....Since the cause (of these illnesses)....can be removed only with difficulty or not at all one eventually permits the disease to go its way. Thus the physicians in these countries, accustomed to the sight of such misfortune, do not find the illness so striking and do not feel impelled to make a more exact determination of its causes. It therefore seems, for the most part, to be reserved to foreign physicians who travel with medical interest through such countries, to bring about a more exact investigation of such diseases....".

This statement, written in 1802, proved to be extremely relevant to the twentieth century investigation of endemic cretinism and particularly true for Captain McCarrison (207) who in 1908 started a new chapter in the study of endemic cretinism when he described endemic cretinism outside the Alpine region and made a distinction between a "nervous" and a "myxoedematous" type of endemic cretin.

(ii) Association with goitre and iodine

As has been stated above most early authors believed that endemic cretinism was somehow connected with endemic goitre and in their writings goitre was called the "father" of or the "first step" towards cretinism (120). Nowadays we recognise that the classic clinical attributes of endemic cretinism are only encountered when at least 20% of the population has significant thyroid enlargement (258). However, in the early nineteenth century such information was not available and theories of goitrogenesis abounded. By 1867 St. Lager (262) was able to enumerate forty three different goitre theories!

The substance iodine was first discovered in 1811 by the French chemist Courtois who found it in the waste ashes of seaweed. Soon thereafter it was recognised as an element and

in 1820 Coindet (47) suggested using the new element as a treatment for goitre. He himself had obtained some favourable results but he also had found some cases refractory and others who developed hyperthyroidism. Despite this the remedy was popularly used in ensuing years to treat goitre often with disastrous results.

This early use of iodine as a goitre treatment preceded the development of an iodine deficiency theory which probably had its origins in 1852 when Chatin (35) completed a systematic analysis of plants, animals, water, air, foodstuffs and soil and came to the conclusion that a causal connection existed between lack of environmental iodine and goitre formation.

Little belief existed in the merits of iodine, however, until 1895 when Baumann (17) recognised the element as an integral constituent of the thyroid gland. In 1908 Marine and Williams (201) explained the relation of iodine to the structure of the thyroid gland and concluded that iodine deficiency was the main cause of goitre. Further credence extended to the importance of iodine in 1915 when Kendall (174) isolated thyroxine as the active principle of the thyroid and showed it to contain 65% iodine by weight.

In 1926 Fellenberg (109) from Switzerland published a now classic study of the occurrence, circulation and metabolism of iodine. He showed by comparative examinations that in areas of high goitre prevalence the environmental iodine content was lower than in areas spared by goitre and demonstrated that differences in iodine availability were best shown by daily urinary iodide excretion.

In the United States McClendon (209) showed in 1939 that goitre rates were inversely correlated with the distribution of iodine. He made a comprehensive review of iodine occurrence in minerals, plants and animals and surveyed the prevalence of goitre and

cretinism on all continents. He repeatedly made the point that iodine was not only a drug but also an essential food constituent the lack of which, like the vitamins, could lead to deficiency diseases.

Finally in 1960 Kelly and Snedden (173), on behalf of the W.H.O. reviewed the goitre problem of 114 countries in the Americas, Europe, Africa, Asia and Oceania. Their review considered the evidence of more than 1300 papers and concluded that iodine deficiency, which at that time probably threatened more than 300 million people, constitutes the principal aetiological factor of endemic goitre.

(iii) Iodine prophylaxis of goitre

The history of iodine prophylaxis begins in 1831 when a French agricultural chemist named Boussingault (173) recommended to the government of Columbia, South America that iodised salt be sold by the government to prevent goitre. This date is of interest as only twenty years previously iodine had first been discovered and another twenty years were to elapse before any European authorities began to share Boussingault's thinking.

In 1855 Kostl (186) considered the problem of endemic cretinism in Austria a subject of public health care and recommended to the authorities either the addition of potassium iodide to rock salt or the introduction of iodide-containing sea-salt as a prophylactic measure. Supplementation of salt by iodine was again recommended in Vienna in 1898 (277) but it was not until 1923 that iodised salt was manufactured to any extent in Austria.

In the United States Marine and Kimball (199), impressed by the ease with which simple goitre could be prevented in lower animals, commenced in 1911 to carry out preventive treatment in man and in 1917 set up a prophylactic trial in the schoolchildren of Akron, Ohio. Prophylactic treatment consisted of the

administration of 2g sodium iodide (0.2g daily for 10 consecutive school days) repeated each spring and autumn. Results analysed after 30 months (200) showed that of 2190 pupils receiving iodine 5 developed goitre while of 2305 not receiving treatment 495 showed thyroid enlargement. They concluded that in man the disease is as easily prevented as in fish or in domestic animals.

Meanwhile in Switzerland Hunziker (164), a physician from a village near Zurich, had developed in 1914 a sound theory of the need for iodine as a foodstuff and by 1917 Bayard (18) had introduced iodised salt into three mountain communities of the Canton of Valais. At that time more than 70% of Valais schoolchildren were still affected by goitre. Beginning in 1924, after Bayard's pioneer experiments had impressed both the population and the authorities, iodised salt was used in increasing amounts and after 1933 the entire salt supply was iodised. By 1934 the incidence of goitre in schoolchildren had fallen to 30% and by 1954 the Canton of Valais (279) was "freed of goitre and cretinism". As Warkany has indicated although "one cannot give all the credit for the improvement of the situation to iodised salt, the complete eradication of goitre probably was due to this measure" (279).

Whenever iodised salt or other prophylactic agent has been introduced into a region of endemic goitre there has been a sharp reduction in the incidence of clinically significant goitre. To date, highly successful programmes have been carried out in Argentina, Czechoslovakia, France, Guatemala, New Zealand, Yugoslavia as well as Switzerland and U.S.A. (258). Iodised oil has also been used successfully as a prophylactic agent in Argentina, Ecuador, New Guinea, Peru and Zaire. Despite these successes, however, endemic goitre continues to be an important medical problem in many parts of the world and prevention of the disease is hardly known in some countries of the Middle East, in South East Asia

and throughout much of the endemic goitre region of the Himalayas.

(iv) Relation to hypothyroidism

By the early nineteenth century endemic cretinism was well described and in 1848 the Piedmontese Commission nominated by the King of Sardinia (234) defined the condition thus:

"....signs of abnormal body development and structure associated with a more or less marked degree of idiocy (in a person) born in an area where several persons suffer such defects of body and mind and where endemic goitre is widespread".

It is not surprising that this definition does not mention any thyroid dysfunction since at this time the concept of hypothyroidism did not exist. It was not until 1850 that Curling (56) described "two cases of absence of the thyroid body....connected with defective cerebral development" i.e. the first description of congenital hypothyroidism due to athyreosis.

Influenced by these cases Fagge (106) reported observations of infants and young children suffering from sluggish mentation combined with certain characteristic physical stigmata. Far from being the possessors of large goitres, these "cretins" had no palpable thyroid gland. Fagge, however, related the condition to defective thyroid function and to provide contradistinction to the endemic form he coined the term "sporadic cretinism". Since in both sporadic and endemic cretinism mental disability and certain physical deformities constituted the most conspicuous abnormalities Fagge in 1871 grouped both syndromes into the general category of "cretinism". By 1888 the London Myxoedema Commission (46) concluded that sporadic cretinism, endemic cretinism and post operative myxoedema were all caused by the same morbid process: thyroid failure.

Although thyroid failure was now considered essential for the appearance of "sporadic cretinism" it is interesting that as late as 1908 the thyroid's normal function was thought to be "to neutralise toxins produced in the ordinary cause of metabolism" and that cases

of "sporadic cretinism" were attributed to toxins produced by invading organisms (207).

It was armed with this background knowledge that Captain McCarrison surveyed the Gilgit area of Kashmir and in 1908 reported his findings on 203 endemic cretins (207). His report is of considerable interest for two reasons. First, he introduced the concept of thyroid atrophy in the non-goitrous cretin when he reported the appearances of a "struma fibrosa" in the thyroid of an autopsied child cretin. Secondly he identified two clinical pictures in Himalayan cretins: a "myxoedematous" type and a "nervous" type. With regard to the former, who comprised two thirds of all cases, McCarrison stated that "...few remarks are necessary. It corresponds to that form of affliction met with in Europe and it is described in any textbook of medicine". As regards the "nervous" cretins he stated that their disability "is more especially of the central nervous system in contradistinction to those of the myxoedematous type in whom the defect is more entirely physical". McCarrison noted that 87% of all cases had an associated degree of deaf-mutism and after a scholarly dissertation on the possible role of "toxins" in the pathogenesis he concluded that both syndromes were caused by a "congenital disability of the thyroid mechanism", the diversity of symptoms being "due to the extent to which the defect bears on the whole or part of that mechanism".

The thyroid histopathology in endemic cretinism was further studied in Switzerland by Wydler (292) who reported in 1926 a reduction in thyroid tissue identical to McCarrison's description. Follicles were seen to decrease in size, epithelial cells fused to form syncytial structures and the surrounding connective tissue showed gross fibrotic changes. There was, however, no evidence of any infiltration by lymphocytes.

In 1936 De Quervain and Wegelin (69) in their monograph "Der endemische kretinismus" critically assessed most of the reports on endemic cretinism in Switzerland and elsewhere in the world. They made a major distinction between cretins with and without goitre and also separated those without goitre into two groups, one showing "early atrophy (Fruhatrophie)" and the other showing so-called "late atrophy (Spatatrophie)". Only seven per cent of the cretins studied were classified as real dwarfs (under 1.4 metres) but De Quervain and Wegelin noted that stunted growth was almost exclusively a feature of the cases with early atrophy of the thyroid.

By 1936, as has been noted in the section on iodine prophylaxis, endemic cretinism in the Alpine region was already a "vanishing disease". Indeed Koenig (182) is on record as stating that since 1920 no endemic cretin has been born in Switzerland. Not surprisingly, therefore, since most of the reports on "cretinism" during the next twenty years emanated from physicians in the U.S. who had never seen the endemic syndrome (137), the hypothyroid aspect of the problem was probably over-exposed. In 1948 Means (202) equated cretinism with "athyreosis from intra-uterine life or early in infancy" and by 1956 Stanbury and Querido (260) had defined a cretin as "a patient who has permanent retardation in development of the skeleton or central nervous system resulting from thyroid deficiency which existed during foetal or early neonatal life".

1958, however, was to usher in a new chapter in the history of cretinism as once again the syndrome of endemic cretinism was "rediscovered". In that year American missionaries from the Unevangelised Field Mission were to chance upon a remarkable focus of endemic goitre in the remote Mulia region of West New Guinea where it was thought that the "entire population is apparently

defective in varying degrees, from barely detectable impairment of intelligence to extreme forms of cretinism and congenital deaf-mutism" (130). Simultaneously Belgian workers were starting their first investigation of hyperendemic goitre and "associated syndromes" in the Uele region of the then Belgian Congo while in Eastern New Guinea Australian investigators spearheaded by McCullagh, who had commenced work in 1956, were grappling with the problems posed by the Huon Peninsula goitre endemic and "associated congenital defect".

The Belgian investigators were first to report their findings on the cretins they found. In 1962 they stated that "the clinical aspect was that described in the classic papers on the old European goiter endemics (70). In a study of 21 cretins they found a uniformly dwarfed group who showed no evidence of "nervous cretinism" and with only one exception showed "unmistakable clinical and biological signs of hypothyroidism". They referred to the thyroid atrophy described from Alpine regions and suggested from their radioiodine studies that in the Uele endemic thyroid failure is due to reduction in the amount of functioning thyroid tissue (15).

Independent of the Belgian findings McCullagh had in 1961 submitted his thesis to Sydney University and in 1963 the Medical Journal of Australia published in four parts much of this thesis. McCullagh described the syndrome of goitre-associated congenital defect as "characterised by varying degrees of amentia (or hypomentia), partial or complete deaf-mutism, muscular incoordination and a general posture of flexion" (211). In contrast to the Belgians he noted "no cases of classical cretinism, no characteristically cretinoid face....and few protuberant abdomens". His cretins indeed more closely resembled those "Himalayan" cretins of McCarrison than those classically described from

Europe. McCullagh was inclined to view the differing clinical characteristics as evidence for a "spectrum of cretinism, its characteristics slowly changing as one moves from Europe through Asia to Oceania" (211).

The next two years were to provide further new information on endemic areas where patients with neurological defects outweighed those with clinical hypothyroidism. In 1963 Lobo et al. (191) presented the findings in 26 adult cretins from Goiaz, Brazil. A clinical picture of hypothyroidism was found in two cases while all patients shared a neurologic picture characterised by "marked defect of mental function (idiocy), deaf-mutism and evidence of upper motor neuron defect". Costa et al. in 1964 re-investigated adult cretins from Piedmont and, like the findings from Brazil, found that the predominant adult Alpine cretin was a mentally retarded deaf-mute with gross neurological deformities (49). He measured the radio-iodine uptake and PBI in seven infants born of cretinous mothers and showing stigmata of mental deficiency and somatic abnormalities. In six out of the seven he was able to demonstrate "normal thyroid function" (48).

In 1965 Choufoer et al. (43) reported on 80 cretins from the Mulia Valley and confirmed the neurological triad of mental deficiency, deaf-mutism and motor abnormalities which had previously been described in areas of endemic goitre by McCarrison (207), McCullagh (211) and Lobo (191). In this paper endemic cretinism was defined as follows:

"Endemic cretinism is the collective term for a number of developmental abnormalities, which geographically coincide with severe endemic goitre and are caused by lesions acquired before or shortly after birth. More precisely, it may be defined as the excess of these abnormalities, which is found in a goitrous population, as compared with a similar population without goitre, and, in due time, is abolished by adequate goitre prophylaxis".

This epidemiological definition was widely used in the subsequent ten years during a time of intense investigation when several groups (Dutch and Belgian, Australian, North and South American) met together on at least five occasions and jointly published three monographs with reports of their discussions (156, 254, 259). The main drawback of the definition was the difficulty in making a diagnosis in an individual patient. To this end Querido attempted in 1971 to find a descriptive definition. Eventually he concluded that "it is possible to recognise two major components in the syndrome of endemic cretinism - (i) damage of the central nervous system and (ii) hypothyroidism. Both occur in different degrees and therefore manifest a spectrum of symptoms". It is of interest here that the emphasis is on the neurological damage rather than the hypothyroidism and we note again that, like McCullagh (156) in 1963, Querido talks about a "spectrum" of cretinism. In 1972 at the Kroc Foundation Symposium Delange et al. (64) elegantly described the "extremely polymorphous" picture of endemic cretinism worldwide and attributed the spectrum of signs to varying degrees of impairment of the nervous system and thyroid function (Fig. 2).

Since 1972 successive Australian and Dutch authors have placed increasing emphasis on the neurological features of endemic cretinism in defining the condition. Pharoah and Hornabrook (222) in 1974 described it as "a syndrome of organic neurological damage arising congenitally and occurring in association with endemic goitre" and made no mention of hypothyroidism in the definition. Hornabrook (161) went further in 1975 by excluding from his definition the cases of "cretinism" encountered in the Congo whom he considered to have an additional pathology resulting in "endemic childhood myxoedema rather than endemic cretinism".

This idea of endemic hypothyroidism occurring in non-cretinous subjects was also shared by Goslings et al. (138) who in 1977 reported a study of the occurrence of hypothyroidism in an area of endemic iodine deficiency in Central Java, Indonesia. They found that hypothyroidism could occur in subjects "lacking any signs of central nervous system damage classically related to endemic cretinism" and therefore they dropped hypothyroidism as a criterion for the diagnosis of endemic cretinism. This diagnosis was only made if at least two of the three following abnormalities were present: mental retardation, neuromotor abnormalities and bilateral hearing loss. Since "iodine prophylaxis" corrected or markedly improved thyroid function in all hypothyroid non-cretinous subjects studied Goslings et al. (138) stated that "iodine deficiency per se in postnatal life may lead to juvenile hypothyroidism, which can be corrected by iodine therapy". They cautioned that not all hypothyroid subjects living in an area of endemic iodine deficiency should be classified as cretins whom they defined in the light of their recently acquired knowledge as subjects "born in an area of endemic goitre, with irreversible damage to the central nervous system, resulting in mental retardation and/or perceptive deafness and/or neuromotor disorders, which may be accompanied by stunted growth and clinical hypothyroidism". This definition epitomises the state of current knowledge at the time of writing this thesis and the clinical features described in it are illustrated by the accompanying diagram (Fig. 3).

(v) Relation to iodine deficiency

As has been noted in previous sections of this review endemic cretinism and endemic deafness since the sixteenth century have been associated with endemic goitre (51, 52). In Switzerland the salt supply was iodised in the mid-1930s and by 1954 it had been

noted that the Canton of Valais, the traditional home of "cretins" (71), was not only rid of endemic goitre but also of endemic cretinism. Many authors took this type of observation as evidence that the pathogenesis of endemic cretinism depended critically on maternal iodine deficiency (279) but others felt that proof depended on observations on the frequency of endemic cretinism carefully studied in the same community before, during and after iodine prophylaxis (230).

Prior to 1968 only one report existed where the same investigators had studied a community before and after iodine prophylaxis. This was a Yugoslavian study by Kicic and coworkers (175) from the village of Gornja Josanica which had a goitre prevalence of 84%. In the period before 1930 the incidence of cretinism among the newborn was 13%. After the economic standard was raised it was about 7%. This level was maintained until 1954 when iodine prophylaxis was started. During the subsequent 14 years no cretins, "cretinoids", or deaf-mutes were born (233) and it was suggested that the iodine prophylaxis had also had a positive effect on the mean stature of children in the population (175).

The effect of iodine prophylaxis on the incidence of deaf-mutism, a marker for cretinism in all endemias except Zaire (230), was studied in Switzerland by Wespi (284). He collected his data through three Swiss organisations which for many years had provided care and education for deaf-mutes and deaf persons. These sources provided information about nearly all individuals who needed speech education in a period before, during and after iodine prophylaxis. From the studies done in detail in each canton there was, with one exception, always a correlation between both time of introduction and amount of iodised salt used and the disappearance of deaf-mutism (Fig. 4). Despite these two rather convincing correlations Trotter (269) criticised the study on the ground that the negative correlations

occurred at a time of very active social and economic change. Querido, however, felt in relation to this question that the burden of proof still lay with "anyone who states that endemic cretinism disappears spontaneously to prove that it did not happen through a small increase in (iodine) intake" (182).

To obtain an answer to this question one must again turn to New Guinea (155). Here in 1956-57 McCullagh studied a group of 10,252 Wain and Naba people in the Huon Peninsula area. He injected 7,881 of them half with saline and half with iodised oil in order to determine the possible value of the oil in the prevention of goitre. Three years later he returned to the area and examined 5,513 (70%) of the injected population (210). He was able to show a significantly lowered incidence of goitre in the oil injected group as compared with the group injected with saline, a finding which was subsequently confirmed by Hennessy in a follow-up patrol in 1962 (151).

Later work by Buttfield and Hetzel (29) demonstrated the severe iodine deficiency associated with the Huon Peninsula endemic and showed that a single injection of iodised oil was effective in the correction of this deficiency for as long as $4\frac{1}{2}$ years. Subsequently Pretell (227) demonstrated that within 48 hours of injection the serum thyroxine (T_4) level begins to rise and Thilly et al. (266) showed that this rise in T_4 was maintained in the longer term, still remaining within the normal range five years after a single injection.

In 1969 Buttfield and Hetzel, in a study of 254 endemic cretins in Eastern New Guinea, reported that since iodised oil prophylaxis had been introduced to the Huon region by McCullagh 13 endemic cretins had been born (28). Since in only one case was there a possibility that the mother of one of these cretins had received an injection of iodine before the birth of her child, the findings raised the possibility that iodine deficiency itself might be significant in the aetiology of endemic cretinism. Accordingly a controlled trial:

of the effect of iodine prophylaxis on the incidence of endemic cretinism was instituted in a limited area of the Western Highlands of Eastern New Guinea where a high incidence of cretinism had been recognised (218).

This controlled trial was to provide perhaps the most telling evidence to date incriminating iodine deficiency as a principal factor in the causation of endemic cretinism. The area chosen for the trial was the Jimi River Valley region where epidemiological evidence suggested that an acute iodine deficiency had arisen following European contact in the mid-1950s (222). The inhabitants had, for generations, prepared salt from certain mineral springs. The preparation involved much labour and the springs were in a remote and distant location. At the inception of contact with European patrols unpurified rock salt, poor in iodine, was employed in trade. It quickly supplanted the traditional salt, since shown to have a high iodine content. As a result within a few years a massive epidemic of endemic cretinism arose in the Valley (161).

In 1966, when the controlled trial was initiated, the prevalence of cretinism among newborns was in excess of 15%. For trial purposes each alternate family in twenty seven villages received intramuscular injections of iodised oil while control families received saline injections. Follow-up patrols were carried out regularly until the end of 1971 but, due to remoteness, only thirteen of the original twenty seven villages with a population of 8,000 were visited. Children born since 1966 were identified and initially examined, without knowledge of mother's treatment, for evidence of motor retardation. A diagnosis of endemic cretinism was made if motor retardation was present together with deafness and/or a squint.

The results of the trial were communicated in three papers (30, 219, 220) and Fig. 5 summarises the latest report. Six cretins out of a total 687 children were born to women who received

iodised oil; in five of these cases the mothers were noted to be pregnant at the time of injection. In the sixth the birthdate was uncertain and it was therefore possible that the mother was pregnant when the trial began. In the saline-treated group, by contrast, 31 endemic cretins were born out of a total of 688 children. In 5 of these 31 conception had occurred prior to the saline being given. Thus, in the Jimi Valley trial only one cretinous infant, as against 26 controls, was born to a mother treated with iodised oil. Pharoah et al. (219) concluded that intramuscular iodised oil, if given prior to conception, is effective in preventing endemic cretinism and therefore they suggested that iodine deficiency in the mother during the first trimester is probably the main factor in the causation of the neurological syndrome.

In 1972 Ramirez et al. (232) reported their findings from a similar trial performed in two isolated villages in the Ecuadorean Andes. They found no severely retarded children born to mothers treated with oil before the fifth month of pregnancy. By contrast, in the control village six instances of severe mental and motor retardation appeared over the same period.

In 1973 Thilly et al. (266) reported the findings from the Congo endemia. One case of cretinism had appeared among the offspring of more than 600 females injected with oil 5 years previously. Ironically, this was the child of one of the few women who had been absent from the village during the time of prophylaxis and presumably failed to receive the injection. In a control village where prophylaxis was not provided three new cases of myxoedematous "cretinism" were found. Similarly, in 1974 Ibbertson (166) reported that a six year follow-up study of iodine prophylaxis in Nepal had revealed the birth of only one cretin in a village where, prior to the institution of a prophylaxis programme, the annual incidence of cretinism was in excess of 13%.

Not surprisingly there is therefore now general agreement that correction of iodine deficiency results in the elimination of endemic cretinism. However, as Hornabrook (161) has pointed out, "considerable doubt remains as to the actual mechanism of the iodine action".

CHAPTER II

MATERNAL AND FOETAL IODINE DEFICIENCY IN MAN

(i) Thyroidal adaptation to lack of iodine

During the 1950s and early 1960s metabolic studies in areas of endemic iodine deficiency consisted of estimations of urinary iodide excretion, serum protein bound iodine¹²⁷ (PBI) and thyroidal radioactive iodine uptake (RAIU). Much of this work was described in detail by Stanbury et al. (257) in their monograph of 1954 entitled "Endemic Goiter and the Adaptation of Man to Iodine Deficiency". By 1961, when endemic cretinism was in the process of "rediscovery", it was clear that in areas of severe iodine deficiency the urinary iodide excretion was low, the RAIU elevated and in most instances the serum PBI significantly lowered (70). It had been shown that the administration of dessicated thyroid could reduce the elevated RAIU (257) and it was assumed that increased thyrotrophic drive by pituitary-derived thyroid stimulating hormone (TSH) was responsible for the increased thyroid activity demonstrable by the RAI studies. However, until 1966 it was not technically feasible to directly measure TSH under these circumstances.

Hetzel's team from the University of Adelaide (26) were first to demonstrate, using the McKenzie bioassay technique, that in an area of severe iodine deficiency (Huon, East New Guinea) the serum TSH is significantly elevated in goitrous, but not in non-goitrous, natives. This conclusion was confirmed in 1968 by Adams et al. (4) using the same bioassay in sera transported from Mulia, West New Guinea.

1968 also saw the first report, again from Hetzel's group (27), of moderately elevated immunoreactive TSH in the sera of goitrous subjects from New Guinea. They also showed that the administration of iodised oil resulted in a significant fall in TSH which was still

demonstrable 3 months after a single injection. Further reports of elevated immunoreactive TSH followed from Argentina and the Congo (65, 224), both these groups demonstrating a better correlation with geographical location rather than with the presence of goitre, a finding to be confirmed often by subsequent studies of larger groups of subjects (38, 223).

The apparent paradox of clinical euthyroidism in the presence of a low serum PBI or thyroxine and a moderately elevated TSH was in 1968 still considered "puzzling" (4). However, as early as 1961 De Visscher et al. (70) had shown in radiochromatographic studies of Congolese patients that tri-iodothyronine (T_3) in sera amounted to 6-15% of the total radioactivity. Since T_3 has the "highest activity per atom of iodine" they suggested that "its enhanced formation has the character of an adaptation reaction to iodine deficiency".

In 1968 Greer's group (265) reported a preferential T_3 secretion in the iodine deficient rat and in 1972 Delange et al. (63), using a competitive protein binding technique, showed that patients from Idjwi Island (Congo) with a normal PBI had elevated serum T_3 levels while those with a low PBI had T_3 levels which were markedly higher, as much as three times normal. They considered that these findings confirmed a preferential secretion of T_3 by the thyroid in endemic goitre.

In the subsequent three years, with the increasing use of specific radioimmunoassays for measuring circulating thyroid hormones, there have been no fewer than six papers reporting levels of T_3 , T_4 and TSH in subjects from the endemias of the Himalayas (166, 179), the Andes (246) and New Guinea (38, 216, 223). First, a Himalayan study (179) of 26 euthyroid patients showed high serum T_3 levels, bearing no relation to goitre size. Next a Brazilian group (246) demonstrated low normal T_3 levels in 12 clinically euthyroid patients. Patel et al. (216) in a study from New Guinea showed some evidence of relative T_3 hyper-

secretion in goitrous as compared to non-goitrous subjects but Pharoah et al. (223) in a larger series also from New Guinea found total T_3 maintained within the normal range in the majority (93%) of subjects whether goitrous or non-goitrous. The situation was further clarified or confused by Ibbertson (166) who reported in 1974 from a study of more than 200 patients from Nepal, where T_4 was low in 83% of subjects, that total T_3 levels were normal in 63% and only in a further 3% were elevated above normal.

A recent study of 322 subjects from two areas of endemic iodine deficiency in New Guinea probably provides the fullest account to date of thyroid/pituitary adaptation to iodine deficiency in man. In this report Chopra et al. (38) showed that an iodine-deficient population tends to have subnormal serum T_4 , supranormal serum T_3 , supranormal T_3/T_4 ratio and supranormal serum TSH. In those subjects where free hormone levels were directly measured the serum free T_4 (fT_4) was seen to be subnormal while free T_3 (fT_3) was significantly elevated. The serum T_4 in goitrous patients was significantly lower than that in non-goitrous patients while no differences in serum T_3 or TSH were noted in the presence or absence of goitre. Serum TSH correlated inversely with serum T_4 (Fig. 6) and no significant relationship existed between serum TSH and T_3 levels. Chopra et al. (38) indicated that in the region studied the combination of elevated serum T_3 and elevated serum TSH occurred in about 6% of the population and they concluded that under these circumstances the circulating T_4 may be more important than T_3 in the regulation of serum TSH levels.

It should be pointed out that in all of the studies, with one exception (246), described above the results refer to clinically euthyroid non-cretinous subjects living in areas of endemic iodine deficiency. Studies of thyroid function in endemic cretins are limited (153) but where in larger population studies cretins have particularly been investigated (4, 38, 246) the results have not

significantly differed from those seen commonly in other non-cretinous subjects from the same geographical region i.e. low or low normal serum T_4 , high normal or high serum T_3 and elevated serum TSH (38).

(ii) Thyroid dysfunction during pregnancy

Before considering how varying degrees of thyroid dysfunction can alter the maternal thyroid-pituitary axis and perhaps irreversibly affect the outcome of pregnancy it is only relevant that one first considers the changes in thyroid function seen during a normal euthyroid pregnancy. Recently Burrow et al. (24) have reviewed the changing concepts in this area which have evolved since the discovery 30 years ago (148) of an increase in the serum PBI during pregnancy.

In 1956 it was shown that the increase in PBI and serum thyroxine (T_4) was caused by an increase during pregnancy of thyroxine-binding globulin (TBG). Whether this increase in TBG affected the amount of free or metabolically active hormone was unclear until 1972 when Burrow (22) showed that although the total T_4 (TT_4) is increased throughout pregnancy the free T_4 (fT_4) concentration remains normal or slightly low. This finding has subsequently been confirmed by two independent groups of workers (8, 252).

In 1970 Malkasian and Mayberry (195) reported that baseline serum TSH values were slightly increased during the early months of pregnancy but returned to normal by term. Later studies using more specific TSH antisera have not found this (8, 24, 33).

In 1971 a progressive rise during pregnancy of total T_3 concentration, closely paralleling the increase in TT_4 and TBG, was reported (162). At the time of Burrow's review (24), however, no information on serum fT_3 concentration during pregnancy was available.

In 1975 Chan et al. (33) tackled the problem of free hormone levels by an indirect approach when they measured the urinary excretion of T_3 and T_4 , considered a close correlate of circulating free hormone concentrations. In 24 pregnant euthyroid patients during the third trimester the urinary excretion figures were found to lie within the accepted normal range for nonpregnant euthyroid females. In 1976 Avruskin et al. (8) measured serum T_3 by specific radioimmunoassay and also evaluated the free hormone concentrations by measuring the dialysable fraction. They were unable to confirm the progressive rise in T_3 reported by Hotelling and Sherwood (162) utilising the competitive binding assay and moreover, in contrast to the elevations of total hormone, found that both fT_3 and fT_4 actually fell during pregnancy, the fT_3 levels being below normal and the fT_4 levels at the lower limits. These interesting results, however, await confirmation.

In summary, then, normal pregnancy in man results in an increase in TBG which causes elevations in both TT_4 and TT_3 . Throughout pregnancy TSH does not change and it is likely that the free fractions of both T_4 and T_3 do not significantly deviate from normal. The effects of varying degrees of thyroid dysfunction during pregnancy on circulating thyroid hormone levels and outcome of pregnancy will now be considered under three headings viz. (a) maternal myxoedema (b) maternal iodine deficiency and (c) maternal "hypothyroxinaemia", (171).

(a) maternal myxoedema: pregnancy in myxoedema is rare (256). Chatfield (34) was able to collect only 26 reports of live births. However, Koenig (180), having himself observed four children born to hypothyroid mothers, searched the literature and found 74 women with 110 pregnancies. There were 49 normal children, 30 miscarriages or stillbirths and 28 abnormal children. Among these 28 were six having congenital hypothyroidism. These six children

came, however, from two families known to have an inborn error of thyroid metabolism. Of the other 22 children the abnormalities included neural tube defects and one case of oligophrenia. Among the 110 children there was no anomaly comparable to endemic cretinism and, with the exception of the six patients with inherited dysmorphogenesis, none of the children were hypothyroid.

Koenig concluded that endemic cretinism cannot be completely explained by maternal hypothyroidism during pregnancy.

(b) maternal iodine deficiency: little data is available on changes in thyroid function during pregnancy in iodine deficient areas.

However, some information has been culled from studies performed both in New Guinea and in Peru.

In 1965 Choufoer et al. (43) noted that the physiological rise in PBI normally seen in pregnancy frequently failed to appear in pregnant iodine-deficient women in Western New Guinea. By contrast in 1973 Patel et al. (216) reported in a study from Eastern New Guinea that in 9 pregnant women "as expected the mean serum T_3 and T_4 levels were elevated". Choufoer et al. (43) had suggested that in the pregnant women whom they studied the T_4 -binding proteins rose in the normal way during pregnancy. In 1974 Wellby et al. (282) showed that in Eastern New Guinea subjects TBG levels in pregnancy were significantly higher than nonpregnant levels and that the increase seen during pregnancy was of the same order as that seen in pregnant Australian subjects. Moreover they could show no difference during pregnancy in thyroxine binding proteins (TBP) between mothers who had received iodised oil and those who had not. The iodine-treated mothers did, however, have significantly higher total T_4 levels.

Similar findings had previously been reported by Pretell and Stanbury (229) who showed in a study from Peru that 41 out of 61

TT_4 determinations in iodine-deficient pregnant women were below the normal range and that all but one were less than the mean value. Although the mean value for all the iodine-deficient (I.D.) mothers was significantly higher than in the non-treated, nonpregnant population it was significantly lower than that of the iodine-treated (I.T.) mothers. Since at term the TBP were normal in the I.D. mothers it followed that in these subjects the maternal plasma fT_4 must have been low. Despite this, however, the cord blood of babies born to these subjects showed T_4 values similar to those from newborns of I.T. mothers and, indeed, fT_4 values in the babies were significantly higher than in the I.D. mothers. In only two instances did very low cord blood T_4 levels correspond to low plasma T_4 levels in the mother. In general a normal foetal thyroid hormone level existed despite iodine deficiency in the mother (228). Interestingly, the mean serum TT_3 level in iodine deficient mothers during pregnancy was no different from that seen in iodine treated mothers.

In another study from New Guinea Pharoah et al. (221) bled iodine deficient subjects during pregnancy and attempted thereafter to compare the outcome of pregnancy with measured indices of thyroid function. The offspring of mothers with "very low" total and free thyroxine and/or elevated TSH had a high mortality while the proportion of cretins born to mothers with "very low" TT_4 and fT_4 was greater than to those mothers with higher levels. Although numbers were too small for valid statistical analysis in the T_3 studies it appeared that pregnancy wastage was high when maternal T_3 was reduced. The foetal loss seen in iodine deficiency was late in pregnancy or postnatally and contrasted with the infertility or repeated abortions which were seen typically in untreated myxoedema or congenital hypothyroidism in women.

(c) maternal hypothyroxinaemia: this term was coined by Keating (171) to designate the observation of a low serum butanol-extractable iodine (BEI) during pregnancy. Hypothyroxinaemia had been found unexpectedly in at least 3% of women at a prenatal clinic in Providence, Rhode Island, i. e. in an area of iodine sufficiency. These women were enlisted between 1962 and 1967 in a prospective study of the effect of thyroid dysfunction during pregnancy on the psychological and neurological development of the progeny (196). The children were evaluated at 8 months, 4 and 7 years with standard psychological examinations and at 7 years with a complete neurological exam. The results of the project, published in 9 papers in the American Journal of Obstetrics and Gynecology, appeared over 15 years, the latest report being in 1976 (197).

For a variety of reasons, both medical and maternal, the hypothyroxinaemic mothers were sometimes inadequately treated with proloid (thyroglobulin). The progeny of these mothers were compared with those born to euthyroid controls and other adequately treated hypothyroxinaemic mothers. At 8 months and 4 years progeny of inadequately treated hypothyroxinaemic women had the lowest psychological scores. Fifteen progeny of adequately treated women and 21 born to inadequately treated hypothyroxinaemic women returned for the 7 year psychological examination. The progeny of inadequately treated mothers had the highest per cent (24) of IQs below 88 while progeny of adequately treated mothers had the highest per cent (47) of IQs of 110 and above. The full scale IQs of progeny born to inadequately treated mothers were lower and differed significantly from the IQs of progeny of adequately treated mothers. Some hypothyroxinaemic women who had delivered a retarded child were treated with adequate thyroid replacement therapy in a different pregnancy. In at least six sibling sets the outcome was better for the adequately-treated pregnancy.

Dr. Man concluded in her critical review of 1972 that "maternal hypothyroxinaemia, both in areas of adequate and inadequate iodine intake, may be a significant factor in retardation and neurological deficits of progeny".

(iii) Placental transfer during pregnancy

The passage of iodide through the placenta is a well established fact. In lower animal species the placenta traps iodide actively from the maternal to the foetal circulation and this transport can be blocked by thiocyanate (113). The ratio between foetal and maternal serum iodide concentration varies widely between species, being as much as 5:1 in guinea-pig or rabbit but being nearer unity in the dog, cat, horse, pig and ruminants (192). In man foetal values are about 2 to 3 times those observed in the mother following a ^{132}I iodide injection (50).

The situation is somewhat different as regards the placental transfer of T_4 , T_3 and TSH. Numerous investigations carried out in a number of species in the past decade indicate that placental transfer of thyroid hormones (T_4 and T_3) is limited (112). Geloso et al. (134) have reviewed the data for the rat. Recent studies, both from Fisher in California (104) and Thorburn in England (268) also show that in the sheep the placenta is essentially impermeable to T_4 , T_3 and TSH regardless of whether the foetus is euthyroid or hypothyroid.

The evidence that placental transfer of iodothyronines in man is minimal rests on three lines of argument: (i) the existence of significant transplacental hormonal gradients, (ii) the transfer of maternally ingested T_4 and T_3 to the foetus and (iii) the fate of radioiodine labelled iodothyronine administered to the pregnant mother (272).

As regards transplacental gradients there is no correlation at any time during gestation of serum TT_4 , fT_4 , TT_3 , fT_3 or TSH (116). Prior to 20 weeks there is a maternal to foetal (MF)

gradient of TT_4 , fT_4 and TSH (142) but throughout gestation the MF gradient of both TT_3 and fT_3 is marked (114). During the latter half of gestation there is a significant foetal-maternal gradient of TSH (116) and again near term the gradient of both TT_4 and fT_4 tends to favour the foetus (118).

Studies of T_4 and T_3 transfer from mother to foetus near term have suggested that such transfer does occur but is minimal in extent. Less than 1% of a large dose of T_4 given intravenously to women in labour was transferred to the foetus over an interval as long as 54 hours (117) and large doses of T_3 (300 μ g/day) given to women near term increased foetal serum T_3 levels only slightly and provided only minimal suppression of foetal T_4 concentrations (93).

Studies of MF transfer of tracer doses of radioiodine-labelled T_4 and T_3 have shown little or no placental T_4 transfer in early gestation (206) and significant but slow transfer near term (170). As for T_3 it is unlikely that transfer occurs because cord blood TT_3 and fT_3 are so much lower than maternal values (3, 204).

Finally, studies in small mammals have indicated that the placenta is also impermeable to TSH (178, 251). Direct data are not available from human studies but the significant differences between maternal and foetal serum TSH concentrations seen throughout pregnancy would lend support to this concept also applying to man.

(iv) Thyroid function in the foetus

Since, as has been described above, the mammalian placenta is essentially impermeable to the iodothyronines, T_4 and T_3 , as well as thyrotrophin, TSH, it follows that the foetal hypothalamic-pituitary-thyroid (HPT) system develops and functions autonomously of maternal control. It has been suggested that the general pattern of ontogenesis of the system is similar in the human foetus, the sheep

foetus and the foetal-infant rat. Fisher et al. (115) have recently examined the evidence for this in an extensive review.

The major events during the development of the HPT system have been classified into four stages. Stage I (Embryogenesis) is characterised by the development of the thyroid to the stage of mature follicles and the emergence in the pituitary of cell differentiation and TSH biosynthesis. The period of hypothalamic maturation (Stage II), which follows, includes an increase in the hypothalamic TRH concentration and a progressive maturation of the primary or hypothalamic plexus of the portal vascular system. Stage III characterises the development of neuroendocrine control which is demonstrable by increasing levels of TSH in both pituitary and serum and maturation of the negative feedback. Finally Stage IV represents maturation of thyroid hormone metabolism in peripheral tissues, characterised by increasing serum T_3 concentration, decreasing serum reverse T_3 (RT_3) level and equilibration of T_3 and RT_3 production rates.

The maturational events described above, although occurring in the same sequence in man, sheep and the rat, do not occur at the same point in time relative to the period of gestation. Human and sheep foetuses are delivered towards the end of stage III and early in stage IV. By contrast the rat is delivered early in stage II of maturation i.e. at a time when "the system operationally resembles that of the adult animal with a transplanted pituitary gland" (115). The discussion which follows will mainly deal with foetal thyroid function in man but since the experimental part of this thesis deals with the rat this species will also be briefly considered.

In man stage I begins on day 16-17 of gestation when the embryonic thyroid is first visible in contact with the endothelium of the developing heart. As early as 29 days thyroglobulin is identifiable in the gland (135) and by 74 days iodine concentration and thyroid hormone synthesis are demonstrable (247). By the end

of the 7th week the thyroid has assumed its definitive shape and position while in the anterior pituitary lobe cell differentiation is proceeding, TSH being identifiable by bioassay and immunoassay by 10-12 weeks (125).

Stage II lasts from 10 to 30 weeks of gestation in man (115). The hypothalamic hormones, including TRH, appear at 8-10 weeks and the tufted capillaries of the primary plexus by 15-16 weeks. There follows a progressive maturation of the primary plexus between 15 and 30 weeks with increasing looping of the tufts and a progressive increase in the volume of median eminence capillaries. During this time also there is a progressive increase in the TRH concentration within the hypothalamus.

Stage III is characterised by two main developmental events: (i) an increase in pituitary and serum TSH and (ii) maturation of the negative feedback mechanism.

Pituitary TSH is detectable in the human foetus at 8-10 weeks but its concentration remains low until about 16 weeks when an increase is evident which continues to 28 weeks. In the foetal serum TSH is detectable as early as 10 weeks but concentrations remain relatively low until 20 weeks when, at mid-gestation, the TSH secretion rate increases. A marked increase in the level of foetal hypothalamic-pituitary function is noticeable between 16 and 22 weeks and this increase may possibly be triggered either by histological maturation of the hypothalamus or by maturation of the pituitary portal system (115).

No matter what is the mechanism for increased pituitary TSH secretion there is in response to the thyroid stimulation a progressive increase in foetal serum TT_4 and fT_4 during the second half of gestation. Most of this increase in T_4 occurs between 20 and 30 weeks, i. e. during stage II, but after 30 weeks the levels of both TT_4 and fT_4 continue to rise. Between 30 and 45 weeks, as TT_4

and fT_4 rise, mean serum TSH drops significantly suggesting that foetal TSH can be suppressed by increasing T_4 concentrations. That this is possible without change in foetal serum T_3 levels was proven by Fisher et al. (115) who injected T_4 into the amniotic fluid of women 24 hours before delivery and then measured the TSH response in cord and neonatal blood specimens.

The last stage of HPT maturation commences in man around 30 weeks of gestation but is not completed until one month post-partum when the ratios of T_3 and reverse T_3 (RT_3) to T_4 approximate adult values. During this period the predominant thyroid hormone produced by the foetus is T_4 . Serum TT_3 and fT_3 concentrations are unmeasurable during most of gestation but increase somewhat during the last 10 weeks. By contrast serum concentrations of RT_3 , derived from monodeiodination of the inner or α benzene ring of the T_4 molecule, rise at mid-gestation in parallel with T_4 and reach high concentrations in cord blood at term (see accompanying Fig. 7). This pattern of high serum RT_3 and low T_3 concentrations in the foetus probably represents decreased monodeiodination of T_4 to T_3 and relatively increased monodeiodination of T_4 to RT_3 in peripheral foetal tissues (41, 42).

In the rat, which has a gestation period of 22 days, stage I extends throughout the period of intra-uterine development (115). By 17 days the thyroid gland has descended to its adult location and between 17 and 19 days thyroglobulin (TG) is visible within and between cells (108). Radioiodine concentrating ability is observed by 17 days and between 17 and 20 days there is a progressive increase in radioiodine content and concentration (247). The foetal thyroid begins to secrete T_4 on day 18 (133) and by the 20th day TG is identifiable in extra-cellular follicles (108). Parallel development occurs in the anterior pituitary where by 17 days two types of secretory granules are seen which progressively increase

in number until the 6th postnatal day (115).

Stage II changes commence in the rat between 15 and 17 days of gestation. TRH is detectable in low concentration in the hypothalamus at birth and increases markedly during the first two postnatal weeks (91). During late foetal life the primary plexus of the pituitary portal system remains superficial and indeed capillary loops are not seen to penetrate the median eminence until late in the first postnatal week (115).

At birth the mean pituitary TSH concentration is low but thereafter it increases rapidly to attain a peak concentration by 10-12 postnatal days (91). The serum TSH at birth is measurable and like pituitary TSH continues to increase to peak levels at 7-8 days. Both pituitary and serum TSH concentrations fall progressively between 14-16 and 40 days (91).

Serum T_4 concentrations in the newborn rat are very low ($<1 \mu\text{g}/100 \text{ ml}$). After a transient decrease (176) they increase rapidly to about $6 \mu\text{g}/100 \text{ ml}$ at 15-20 days and fall subsequently to adult levels. The developmental events involving TRH, TSH and T_4 and occurring between 18 days of gestation and 10 postnatal days are illustrated by Fig. 8.

At the time of birth in the rat the serum T_3 concentration is not measurable (90, 145). However, during the first month it increases progressively to attain adult values, the rate of increase being much less than that for serum T_4 (91). Like the foetal human the foetal and newborn rat is relatively T_3 deficient and this is presumed due to a relatively low rate of production of T_3 by outer (β) ring monodeiodination in non-thyroidal tissue. To date there are no reports of RT_3 determination in the newborn rat.

Fisher et al. (115) have stated that during stage I development the foetus is "primarily hypothyroid" while during the early phase of stage II the HPT system "functions with features of both secondary

and tertiary hypothyroidism: the pituitary response to TRH remains relatively insensitive and hypothalamic control is minimal" (115). It is at this stage of development that the infant rat is born and thus one could compare the newborn rat in developmental terms to the human foetus at 12-16 weeks of gestation. To an extent therefore one could equate the intra-uterine phase of HPT maturation in the rat to the first trimester in man, an assumption which will be considered later in the planning of the present project.

(v) Foetal thyroid hypofunction

This may be classified into primary and secondary foetal thyroid hypofunction. Primary hypofunction implies inadequate foetal thyroid function in the face of maternal euthyroidism, both clinical and biochemical. Secondary foetal hypofunction, on the other hand, occurs as a consequence of maternal/placental dysfunction, maternal causes including inadvertent treatment with radioactive I^{131} during pregnancy and the overtreatment of maternal thyrotoxicosis with anti-thyroid drugs e.g. carbimazole and propylthiouracil (PTU). The possibility exists that severe maternal iodine deficiency may per se, in the absence of any other factors, cause secondary foetal hypothyroidism and this controversial issue will be considered in detail below.

It is now no longer disputed that thyroid agenesis or dysgenesis can result in primary foetal hypothyroidism. Screening programmes for congenital hypothyroidism have detected several dozen such infants at birth (92, 177) and all affected babies have had low serum T_4 ($<7 \mu g\%$) and high serum TSH concentration ($>100 \mu U/ml$). Fisher et al. (115) have shown that the serum T_4 concentrations in 17 of these infants' mothers have been normal.

Since 1957, when Russel et al. (240) described two cases, it has been recognised that foetal hypothyroidism may result from inadvertent treatment with I^{131} of maternal thyroid over-activity or carcinoma. In 1975 Battin et al. (16) reported a further case and reviewed seven cases from the literature. All 5 patients, who had had psychological assessment performed, were shown to be significantly mentally retarded as a consequence of hypothyroidism.

Until 1975 it had been standard practice, in an attempt to avert this tragic sequel of I^{131} treatment, to treat the mother with oral T_3 in the hope that some would cross the placenta thereby modifying the potentially damaging effects of foetal thyroid hypofunction. In 1975 two further reports of foetal hypothyroidism secondary to I^{131} exposure (167, 272) appeared and shed new light on this question. Van Herle et al. (272) treated their mother with 100 μg T_3 daily. Despite the mother at term having a T_3 level of 200 ng% the infant was born with $\langle 15$ ng% of T_3 , a serum T_4 of $\langle 2$ $\mu\text{g}\%$ and a TSH of 340 $\mu\text{U}/\text{ml}$. Ibbertson's patient (167) was treated with a combination of carbimazole and T_3 in doses sufficient to elevate the T_3 into the thyrotoxic range. Despite a maternal T_3 level prior to term of 550 ng% the babe was born with a T_3 of 31 ng%, a T_4 of 6 $\mu\text{g}\%$ and a TSH $\rangle 200$ $\mu\text{U}/\text{ml}$. Both groups of authors considered these cases as further evidence of the limited placental transfer of iodothyronines in man.

As regards the possible teratogenic effects of anti-thyroid drugs there have been, since their introduction in 1943 to thyrotoxicosis treatment, many reports alleging serious affects on human foetal thyroids when the drugs have been given during pregnancy (59, 152). On the other hand, the delivery of normal infants after the controlled use of these drugs in pregnant

hyperthyroid women has also been described (7). In 1968 Koenig reviewed the literature and found 187 successful pregnancies in women either on carbimazole or PTU (180). There were 15 "abnormal" children of whom 3 (1.6%) were considered hypothyroid or "cretinous". Koenig made the point that none of the offspring were called "deaf".

Few reports exist on the long-term sequelae of in utero exposure to anti-thyroid drugs. However, two reports from the U.S. on the use of PTU show no injurious effects on the subsequent growth and development of the children (23, 143) and recently McCarroll et al. (208), reviewing 25 children born to carbimazole-treated mothers, found growth, development and pituitary-thyroid function to be normal and could not identify any individual with an abnormal IQ using standard psychological tests.

The question of whether maternal iodine deficiency per se can cause foetal hypothyroidism is unsettled at present. The evidence for and against this possibility comes from studies performed within the past 5 years on newborns in the goitre endemias of Peru (228), Zaire (66) and Southern Germany (160).

Pretell et al. (228) studied paired cord and maternal sera and compared iodine deficient (I.D.) mothers with a group treated by iodised oil and another group living in relative iodine sufficiency in neighbouring Lima. They showed that both maternal and cord blood showed low serum T_4 levels in iodine deficient pregnancies. However, the mean level of T_4 in I.D. cord blood was no different from that seen in iodine-treated (I.T.) pregnancies whereas by contrast the mean fT_4 level in the I.D. cord blood was significantly lower than that in the I.T. group. As regards TSH levels the mean I.D. level was no different from the I.T. group, all except one in the I.D. group being within the normal range, i.e. $<12 \mu U/ml$.

Only 5 cord bloods were examined for serum T_3 and surprisingly the levels in the three I.D. bloods, although remaining within the normal range, were higher than those in the two I.T. specimens. However, only 22% of the I.D. neonates had a T_4 $<7 \mu\text{g}\%$ and TSH values did not differ from those seen in infants born to iodine-treated mothers. Thus none of these Peruvian iodine-deficient neonates would qualify for foetal hypothyroidism as diagnosed by the criteria of T_4 $<7 \mu\text{g}\%$ and TSH $>100 \mu\text{U}/\text{ml}$ (115).

Pretell et al. (228) emphasised that in the pregnant iodine-deficient woman there is relative unsaturation of TBG and thus a low fT_4 concentration. By contrast, on the foetal side of the placenta the fT_4 level is significantly higher. Assuming that the concentration gradient of fT_4 plays a major part in defining the direction of flux across the placenta they suggested that "the fetuses of iodine-deficient mothers are constantly in danger of having inadequate levels of thyroid hormone for normal development".

The question of foetal hypothyroidism was closely examined by Delange et al. (66) in a study of 96 neonates in the goitre endemic of Ubangi, Zaire. This area, like the Uele region (70) and Idjwi Island (65), has a high prevalence of the type of cretinism characterised by "severe thyroid failure, dwarfism and irreversible mental retardation" and here cretins "show extremely low intrathyroidal iodine reserves, indicating almost complete atrophy of the gland" (66).

Delange studied 54 infants born to iodine deficient mothers and 36 born to mothers treated with iodised oil on average during the 28th week of gestation. The infants born to untreated mothers showed lower cord blood levels of TT_4 , TT_3 and fT_3 . Moreover they also had a mean TSH of $26 \mu\text{U}/\text{ml}$ which was significantly

higher than in those born to treated mothers. In 38% of the untreated infants the cord TSH level exceeded $100 \mu\text{U}/\text{ml}$ indicating foetal thyroid hypofunction. Interestingly, in contrast to Pretell's findings, the iodine deficient infants in the Zaire study had undetectable fT_3 levels at birth and had a mean TT_3 level of $26 \text{ ng}\%$ which was significantly lower than in the iodine-treated group. Delange et al. (66) concluded that their data demonstrated the existence of perinatal hypothyroidism in severe endemic goitre and suggested that "this anomaly constitutes the causal mechanism of cretinism in Central Africa".

In Southern Germany, another area of endemic iodine deficiency, a more "subtle" type of perinatal thyroid hypofunction has recently been recognised. Homoki et al. (160) studied 45 neonates with congenital goitre attributed to iodine deficiency and compared these infants both biochemically and radiologically with 84 control non-goitrous neonates. Nineteen (42%) of the goitrous infants were clinically and biochemically euthyroid and had normal bone ages. The other twenty six (58%), although showing no clinical signs and symptoms of hypothyroidism, had biochemical and radiological evidence of foetal thyroid hypofunction. These 26 infants had low PBI and total T_4 -iodine, a retarded bone age and a serum TSH concentration above normal during the first 14 days of life. Homoki et al. (160) concluded that the 26 infants showed evidence of "subtle hypothyroidism" and they advised that for these infants substitution therapy with thyroid hormones is required "in order to avoid possible retardation of normal brain development".

(vi) Pathogenetic mechanisms in endemic cretinism

In the five years which followed the Pan American Health Organisation (PAHO) Meeting on Research in Endemic Goitre three

symposia, where groups of investigators spent a significant part of their time discussing the possible pathogenesis of endemic cretinism, were held. From these meetings monographs were prepared and much of the following discussion has been derived from these sources (89, 156, 254, 259).

In 1968 at the PAHO Meeting it fell to Dumont et al. (87) to provide a classification of endemic cretinism in the light of the new information being made available both from New Guinea (13) and the Congo (15, 70). They decided that "two very different syndromes" had been described and, using McCarrison's terminology "in a broader sense", they admitted that in 1968/69 "we do not know the pathogenesis of the defects in nervous cretinism or of thyroid atrophy in myxoedematous cretinism" (87).

They did, however, state that, like "sporadic cretins" the Uele dwarfs were mentally defective because of "hypothyroidism beginning in early infancy or in the fetus, (and) caused by thyroid hypoplasia" and on the second syndrome, as described from New Guinea, they said:

"....Nothing is known about the pathogenesis of deaf mutism, mental deficiency or neuromuscular disorders in nervous endemic cretinism. The relative independence of these defects may suggest different pathogenetic mechanisms operating at different periods of foetal life. Deaf mutism and the motor syndrome could be caused by a lack of maternal thyroid hormone supply during early pregnancy, while hypothyroidism due to this fact and to fetal deprivation of iodine could account for mental deficiency".

In 1969 Koenig (181) in the course of a review on endemic goitre and endemic cretinism defined 3 possible pathogenetic mechanisms:

- (i) iodine deficiency in the mother and also in the child compromising embryonic and foetal development

- (ii) iodine deficiency leading to deficient maternal T_4 production and an insufficient supply of maternal hormone for the child
- (iii) iodine deficiency leading to insufficient T_4 production in the foetus and resulting in foetal hypothyroidism

Because of the accumulating evidence (124) that maternal T_4 does not cross the placenta Koenig considered that foetal T_4 was "the decisive factor" and that most of the clinical picture of endemic cretinism could be explained on the basis of congenital hypothyroidism. However, he conceded that this did not explain the occurrence of deaf mutism which he likened to the hearing defect seen in Pendred's syndrome, an inherited association of perceptive deafness and defective thyroid iodine organification. As a possible explanation of its occurrence in association with thyroid dysfunction he proposed the hypothesis, originally put forward by Lenz (188), that the formation of the inner ear requires "an iodine-containing (unknown) molecule" and thus endemic cretinism could be considered "the phenocopy of the genetically determined defect (seen in Pendred's syndrome) and iodine deficiency the causative factor".

Koenig's first possible mechanism, viz. that iodine deficiency per se may "compromise" foetal development was a new one since until that time, as Querido was later to state, investigators had "followed the hypothesis that the only known physiological action mechanism of iodine was through thyroid hormones" (231).

In 1971 Pharoah et al. (219) reported that the administration of elemental iodine, if given prior to conception, effectively prevented the "neurological damage" of endemic cretinism. Challenging the views that maternal and/or foetal hypothyroidism were responsible for the syndrome they proposed that elemental iodine may be

"necessary for the embryological development of the nervous system quite apart from its role in the synthesis of the thyroid hormones". In the situation of severe iodine deficiency they envisaged that the avidity of the maternal and foetal thyroid glands for iodine would be such as to leave "insufficient elemental iodine available for neurological development" (217).

In the following year the same authors (220) added another two possible mechanisms to Koenig's original three. Firstly, they suggested that a mild degree of maternal hypothyroidism coupled with foetal hypothyroidism resulting from severe iodine deficiency might be sufficient to cause the syndrome and they also proposed that iodine deficiency might have to act in conjunction with some other factor, such as maternal protein-calorie malnutrition or other trace element deficiency, in order to produce foetal damage. However, they themselves still promoted the theory that elemental iodine may play an essential role in the early development of the normal brain.

During the next two years authors divided themselves into the majority who believed in iodothyronine deficiency as the principal mechanism and the minority who believed in deficiency of elemental iodine per se. Also some continued to think of two distinct syndromes while others, like Stanbury, looked at the different kinds of cretinism as being "different manifestations of the same fundamental disorder, perhaps modified by environmental, nutritional, genetic and other factors, but (sharing) the same aetiopathogenesis" (255).

In 1971 Stanbury wrote: "While a causal link between endemic cretinism and thyroid failure has been questioned by some, the evidence favouring a close relationship seems overwhelming.... the duration of hypothyroidism and the stage of embryonic or perinatal life when it appeared may determine the relative prominence of particular features of the cretinism" (255).

DeGroot in 1972 went further when discussing the evidence for iodine deficiency per se, he said:

"I don't think anyone has presented evidence so far which allows this to be more than pure speculation. Everything presented can fit with a concept of foetal hypothyroidism as a cause of cretinism. The difference in the clinical syndrome of sporadic and endemic cretinism doesn't mean much because none of us has seen a sporadic athyreotic cretin born of a hypothyroid mother which would be the test case. The degree of hypothyroidism in the sporadic cretin in utero is probably very different from that of the endemic cretin whose mother may be hypothyroid. . . . it seems to me that the burden of proof still rests on someone who suggests that there is a difference between severe foetal thyroid hormone deficiency and what we see in endemic cretinism" (61).

In 1972 Ibbertson et al. (168) tried to combine the majority and minority viewpoints when they proposed that "the neurological defect results from a severe intra-uterine deficiency of elemental iodine and the additional features of the myxoedematous cretin are dependent on the degree of postnatal iodothyronine deficiency". Moreover they divided the manifestations of endemic cretinism into prenatal - the nervous system abnormalities - and postnatal - the somatic abnormalities "which are predetermined only in so far as the thyroid development may be compromised before birth".

By the time of the PAHO Group Meeting in 1973 there was still no consensus regarding pathogenetic mechanisms. Indeed a select committee on further research problems said in relation to the pathogenesis of endemic cretinism: "...While much is known, our ignorance is still great. Does iodine deficiency per se affect neural growth? . . . Is neurocretinism a disease not caused by thyroid hormone deficiency?" (129).

In 1974 Stanbury et al. (258) summarised the situation as follows: "The cause or causes of endemic cretinism are less clear. The consensus is that endemic cretinism occurs when the fetus

receives insufficient iodine....While this view of the cause of the syndrome seems reasonable it has not yet been subjected to rigorous proof, nor is there an entirely satisfactory animal model in which to test its validity".

The experimental work to be described later in this thesis was commenced in an attempt to subject the foetal iodine deficiency theory to "rigorous proof", and with a particular aim to investigate, using an experimental rat model, the proposal of Pharoah et al. (219) that severe maternal iodine deficiency acting during the first trimester of pregnancy causes "damage" to the developing foetal nervous system resulting in "the clinical entity of neurological cretinism".

CHAPTER III

NEUROLOGICAL ASPECTS OF ENDEMIC CRETINISM

(i) Clinical findings

Since McCarrison identified "nervous cretinism" in the Gilgit area of Kashmir some seventy years ago (207) successive groups of investigators have consistently described a similar neurological syndrome occurring particularly in both West and East New Guinea but also in Brazil and Ecuador. This section of the literature review will summarise the neurological features seen in the cretins from these areas and will also attempt to locate the neuroanatomical sites of the lesions responsible for the findings. For a fuller clinical description of the syndrome the recent reviews by both Hornabrook (161) and Dodge et al. (83) are recommended.

McCarrison in 1908 was first to describe the neurological features and an extract from his original paper is given below (207).

"....There is, as a rule, complete deaf mutism. There is a knock-kneed spasticity of the lower limbs and the patient exhibits a complete or partial inability to stand upright. When supported on his feet he usually rests on his toes and the knees may be close together or actually crossed; There is an increased knee jerk and.... a peculiar stiffness of gait.... The spastic rigidity is always worse in the lower limbs.... In short, the condition is one of cretinous idiocy with associated cerebral diplegia".

This description of the three main features of neurological cretinism, viz. mental retardation (idiocy), deaf mutism and cerebral diplegia, was known to McCullagh when, in 1963, he wrote that the pattern of "goitre-associated congenital defect" seen in Eastern New Guinea was characterised by "varying degrees of amentia (or hypomentia), partial or complete deaf mutism, muscular incoordination and a general posture of flexion (211). He felt that the picture in

Huon bore many resemblances to that seen in "Himalayan Cretinism" but he contrasted the "rigid extension" seen by McCarrison with the characteristically flexed knees of the New Guinea neurological cretins.

In 1963 Lobo et al. (191) reported the clinical details of 26 cretins from Goiaz, Brazil. All 26 cretins had "marked defect of mental function (idiocy)" and the majority of patients showed evidence of "an upper motor neuron defect", implying pyramidal tract involvement. A similar picture was reported in 1965 by Choufoer et al. (43) who found in 80 cretins from Western New Guinea that the most frequent defects were mental deficiency, deaf mutism and motor abnormalities.

By 1969 the Australian workers in the Huon Peninsula had managed to identify 254 cretins and in this series, the largest to date, they found deaf mutism in 70%, brisk reflexes in 61%, an extensor plantar response in 48% and "mental abnormalities" in 47%. Since, because of language difficulties, only persons who were obviously mentally defective could be identified, Buttfield and Hetzel (28) felt that with more sensitive tests of mental function a greater percentage of the cretinous population would be found to be mentally subnormal.

In 1969 Dodge et al. (84), employed the formal psychological tests of Gesell and Leiter to critically assess the mental function of 28 cretins from two communities in the Ecuadorean Andes. All individuals in the series were markedly defective mentally while 27 out of the 28 had impaired hearing and speech. Some impairment of walking was present in 23 and the vast majority of subjects had pyramidal tract dysfunction involving predominantly the lower extremities. Because of the absence of sensory signs and bladder dysfunction Dodge et al. (84) suggested a "cerebral locus" for the spastic diplegia.

The findings of Dodge et al. (84) were extended by Fierro-Benitez et al. (110) to a final series of 94 subjects who were chosen for study because they had "an immediately obvious abnormality in walking, speech, hearing and mental capacity, separately or combined". Using formal tests they found that of 77 subjects aged 9 to 60 years 75 (97.4%) scored below the 7 year level i.e. they were severely mentally deficient. Of the total series of 94 subjects 26 were completely deaf, 72 had moderate to severe deafness and only 6 had normal hearing.

As regards "motor abnormalities" Fierro-Benitez et al. (110) found spasticity of the legs in 45%, sustained ankle clonus in 23%, exaggerated knee and ankle jerks in 62% and upper motor neuron signs in the arms in 25%. A general clumsiness of movement beyond that attributable to the pyramidal tract disease was also observed in the majority of subjects and in these patients there appeared to be "a spastic diplegia of variable severity which probably was of cerebral origin".

At the Symposium held at Goroka, Papua New Guinea in January 1971 Hetzel (156) summarised the neurological aspects in a manner which is still pertinent now, seven years later:

"Endemic cretinism is manifested clinically by degrees of mental retardation and hearing loss together with disorders of gait and stance, spasticity or hypotonia and a squint. The probable neuropathology involves damage to the cerebral hemispheres, the 8th nerve system and the brain stem".

(ii) Neuropathology

Further advances in our understanding of the pathogenesis of the neural damage in endemic cretinism have over the years been held back because of "a dearth of information regarding the neuropathology" (154). What few reports that exist are limited to descriptions of the post-mortem findings in the nervous system of elderly, institutionalised Alpine cretins, and, as Querido stated in

1971, to this day "the neuropathology of cretinism is still fully open for investigation" (156).

Despite a lack of confirmatory neuropathological reports it has been postulated on clinical grounds that "the two essential ingredients of the syndrome" (156) are damage to the cerebral hemispheres and the eighth cranial nerve system. These two areas of the nervous system will be considered in detail below.

In 1954 Warkany and Nelson (280) writing on "Prenatal effects of nutrition on the development of the nervous system" described endemic cretinism as a "nutritional experiment of nature" and summarised the neuropathology as follows:

"The cerebral changes vary a great deal. In the reports of the nineteenth century, gross anomalies of the brain were frequently described. Asymmetry of the hemispheres, underdevelopment of entire lobes, convolutional anomalies, marked wasting of the white with preservation of the gray matter, and internal hydrocephaly were repeatedly described in the older literature (245). In recent studies such gross changes have not been found, but microscopic developmental anomalies have been recognised. Lotmar (193) made a thorough study of the finer structure of the cerebral and cerebellar cortex of 14 cretins from Bern, Switzerland. He found irregularities in the development of the various cortical layers and variations of nerve cell distribution....Recent investigators agree with the older ones that the cerebral changes are prenatal in origin and date back to fetal life".

Despite the rediscovery of endemic cretinism in the early 1960s and the frequent symposia in the early 1970s no significant reports have since appeared on the cerebral lesion of endemic cretinism. In 1972 Crome and Stern (54) in a book entitled "Pathology of Mental Retardation" commented thus: "Various regressive neuronal changes have been described in cretinism, mainly by older authors, who also mentioned slight and possibly equivocal architectonic disturbances". They themselves were, however, unable to shed any further light on the central nervous changes.

A similar mystique unfortunately also surrounds the pathology of the otic lesions in endemic cretinism. Koenig and Neiger (183) in 1972 were unable to find any report published since 1927 and in their own review they relied heavily on the work of Nager (212) published in 1926.

In contrast to audiometric studies (139) which support a central derangement of hearing with perceptive deafness, Koenig and Neiger (183) found from the literature that the most typical and specific pathological findings occurred in the middle ear and included "a) hypertrophic bone changes of the promontorium, b) deformation of the ossicles with fixation of the stapes, c) distortions of the round and oval windows, and d) thickening of the mucous membrane of the tympanic cavity". They felt, however, that these middle ear changes were not the main cause of the deafness and that for technical reasons it was probable that the inner ear lesion had been overlooked.

(iii) Experimental models for hearing defect

To date there are no reported animal experiments showing that maternal iodine deficiency can cause damage to the developing "eighth nerve system". There are, however, reports both in mice (67, 68) and in rats (253) relating to the effect of the administration of propylthiouracil (PTU) to pregnant animals on the developing inner ears of their offspring. PTU is known to impair covalent binding of iodide to thyroglobulin (62) and, if administered during pregnancy, can cause an iodide organification defect not only in the mother but also in the foetus. Deol (67) has considered the experimental situation akin to that seen in a severe case of Pendred's syndrome (123). However, others (286) do not agree with this analogy and indeed Goslings et al. (139) have said that "there is no reason to suspect that this (Pendred's syndrome) or any other genetic metabolic disorder has anything to do with the hearing defect in endemic cretinism".

In Deol's experiments (67) mice were mated and given drinking water containing 0.1% PTU throughout pregnancy and until weaning. The offspring were allowed access to water containing PTU, were sacrificed from 14 to 120 postnatal days and thereafter their inner ears histologically examined. Control offspring were born to mothers who drank either water only or water containing both PTU and thyroxine (90 $\mu\text{g}/100\text{ ml}$). Animals from all three groups were subjected to hearing tests which depended on the pinna response to sharp, metallic clicks.

Deol found that the administration of PTU, if begun before day 15 of pregnancy, led to abnormalities of the cochlea and impaired hearing in the offspring. The cochlear duct in PTU-treated infants invariably displayed striking abnormalities of the organ of Corti, especially of the tectorial membrane which never came in contact with the hair cells. By contrast, the organ was normal in the offspring of untreated females and in those that had been given both T_4 and PTU at any time up to 9 days after birth. Deol concluded that the abnormalities were caused, not by PTU itself, but by the resultant abnormality in thyroid function. He felt that the loss of hearing was due to "congenital hypothyroidism" and that, at least in the mouse, thyroxine had a specific action on the epithelium of the inner sulcus, a deficiency of T_4 resulting in a failure of maturation of the tectorial membrane (68). The critical stage of cochlear development in the mouse appeared to last "from a little before birth to the age of about 10 days" and by analogy this was equivalent in man to a period extending from the fourth to the seventh month of pregnancy.

In 1976 Spindel et al. (253) extended Deol's findings to the rat. Using animals thyroidectomised by 0.5 mCi I^{131} and maintained in euthyroidism by T_3 supplementation of 1.2 $\mu\text{g}/\text{day}$ Spindel introduced 0.05% PTU in drinking water at day 1 of pregnancy.

PTU given during pregnancy and lactation produced a "severe foetal and neonatal hypothyroidism" and a loss of hearing in the offspring that was usually total. The "retardation" induced by in utero PTU treatment was reversible by T_4 treatment after birth implying that "the critical period in the rat is after birth and that any in utero changes resulting from foetal hypothyroidism in the rat may be reversible". Although goitrous but euthyroid infants were born to hypothyroid mothers maintained on low iodine diet (LID) the hearing in these animals was apparently not assessed.

Thus, in the mouse and in the rat there is accumulating evidence that thyroxine plays a role in the development of the sensory unit of the ear. That this may also apply to man is suggested by the association of familial deaf-mutism with a high PBI, due to target organ refractoriness to thyroid hormone, seen in the syndrome recently described by Refetoff et al. (235).

(iv) Experimentation on cerebral defect

Considering that the essential role of iodine deficiency in the pathogenesis of endemic cretinism has been recognised for at least 50 years it is rather surprising how few reports have been published on the possible teratogenic effects of severe maternal iodine deficiency. Indeed since 1939 only three papers (107, 274, 278), to the author's knowledge, have appeared which relate to effects of prenatal iodine deficiency other than those on thyroid function in the neonate (102, 145).

In 1945, in a survey of the manifestations of prenatal nutritional deficiency, Warkany (278) wrote, concerning iodine deficiency:

"In rats goiter can be produced experimentally on diets deficient in iodine. Remington has devised a goitrogenic diet which permits raising of females to maturity. When mated such rats produce a normal number of young which have hypertrophic and hyperplastic thyroids but show no signs of debility. Nelson and Warkany (in 1939) interbred such goitrous rats for five generations without observing any signs of injury to the young".

In 1960 Feldman (107) attempted "to produce cretinism in the newborn rat" by maintaining pregnant rats on a low iodine diet (LID) "throughout gestation or for longer". On his findings he commented:

"Iodine deficiency produced a nutritional disease which was transmitted from dam to newborn. In the neonate, this was manifested by a cretinoid appearance, thyroid enlargement and augmented activity as measured by I^{131} uptake. . . . The cretinoid appearance and the augmented thyroidal activity of iodine-deficient neonates were reversible. When newborn pups of iodine-deficient dams were transferred to dams on an adequate iodine intake, their body weight and metabolism returned to control levels within six weeks".

In neither of the above studies was brain examination apparently performed. Warkany (279) summarised the situation in 1971 as follows:

"It would be desirable to study the pathogenesis of endemic cretinism in animal experiments, but so far it has not been possible to obtain a model that simulates the disease picture of endemic cretinism. In rats prenatal iodine deficiency produces foetal thyroid hyperplasia but not cretinism".

The above statement epitomised the state of knowledge, as regards an animal model for endemic cretinism, at the time when this project was commenced. Since the completion of the present author's experimental studies, however, a report has appeared which may, for the first time, have identified an effect of prenatal iodine deficiency on the developing brain.

Van Middlesworth (274) has shown that rats exposed to severe iodine depletion in foetal and neonatal life exhibit a high incidence (89%) of audiogenic convulsions, in contrast to a 2% incidence in controls. This abnormal seizure susceptibility was found to persist even after the animal "matured on a normal diet". Van Middlesworth felt it "reasonable to suspect that severe iodine deficiency may have resulted in some degree of hypothyroidism of the offspring which

neither the newborn nor the mother's milk (263) was able to overcome" and also likely that there was a critical time of vulnerability to audiogenic seizures. He concluded by hoping that "testing for audiogenic seizure susceptibility may furnish a simple and reproducible method to study effects of severe iodine depletion on the early development of the rat".

PART B

THE EXPERIMENTAL MODEL

CHAPTER IV

PLANNING OF EXPERIMENTAL STUDIES

The general aim of this project was to investigate the pathogenesis of the cerebral lesion thought to be responsible for the most typical neurological features of endemic cretinism. Review of the literature on iodised oil prophylaxis suggested that the neurological damage must take place before 5 months of gestation (232) and probably commences during the first trimester (219). The damage to both the cerebral hemispheres and the inner ear could probably be timed to a period between 10 and 20 weeks of gestation.

Planning an experimental study to investigate this problem necessitated extensive reading in the area of developmental neurobiology and only after two months of searching was the author drawn to the works of Dobbing (72-81), Winick (287-291), Balazs (10-13) and Zamenhof (293-301). These authors were largely responsible for the promotion of the concepts of the "brain growth spurt" (72), "vulnerable periods" in development (72), "nutritional growth retardation" (78) and "distortion pathology" (74).

From his reading it seemed to the present author more than coincidental that at the time of probable neurological damage in endemic cretinism there was occurring in the human brain a miniature growth spurt in cell numbers probably representing the major portion of neuronal multiplication (79). This miniature growth spurt lasted from 10 to 18 weeks of gestation, i.e. at a stage not normally vulnerable to growth restriction, but, as Dobbing in 1974 pointed out, "probably and importantly

vulnerable to irradiation, chromosomal anomaly, viral infection and a host of other deleterious influences" (81). For the purposes of this study the author in October 1974 proposed that severe maternal iodine deficiency might be such a "deleterious influence" acting as a growth retarding stimulus on the developing foetal brain during the period of active neuronal proliferation.

To test such an hypothesis it became necessary to choose a suitable experimental animal. It was quickly obvious that the most studied animal as regards brain growth and development was the albino rat (157). It was fortuitous that this was also the animal most frequently employed for studies on adaptation to severe iodine deficiency (265). Both Balazs and Dobbing seemed in agreement that "with certain limitations" (13) it was "reasonable to expect help from the study even of rats in elucidating the human condition" (73).

One of the problems, however, in assessing a human problem with a rat model is that individual stages of development in the two species occur at different ages in relation to the time of birth. As Dobbing has said, it is essential in interpreting animal experiments to "play the concertina with the time scale" and compare "stages, not ages" (76). Fortunately, in relation to brain development, it is generally agreed that the 10 to 20 gestational week period in man is comparable to the last trimester of the rat pregnancy (77). Similarly, as has been outlined in Chapter II, the infant rat is born at the stage of hypothalamic maturation corresponding to that seen between 10 and 20 weeks of the human pregnancy (115).

For these reasons it was decided to use the laboratory rat in an attempt to test the hypothesis that maternal iodine deficiency results in "inhibition of neuronal multiplication in utero" (5). The basic plan devised in 1974 was to make female rats iodine deficient by means of a low iodine diet (LID), thereafter to mate them, allow the pregnancies to continue to full term and during the postnatal

period to sacrifice the pups and examine their forebrains. The next two chapters of the thesis will describe the production of iodine deficiency and the quantitation of perinatal brain development. Subsequent chapters will discuss the results obtained with the animal model.

CHAPTER V

PRODUCTION OF IODINE DEFICIENCY IN THE RAT

Historical Perspective

(i) Evolution of corn-based low iodine diets

In 1925 Steenbock and Black (261) discovered that the most effective dietary means of producing rickets in the rat was the use of a ration composed of yellow corn 76%, wheat gluten 20%, calcium carbonate 3% and sodium chloride 1%. In 1930 Krauss and Monroe (187) noted that rats fed this high calcium - low phosphorus diet were also goitrous and had a lowered content of iodine in their thyroids. Subsequently Remington and his colleagues from South Carolina (189, 190, 236, 237) took advantage of this discovery to describe a "dietary technique suitable for goitre studies employing the rat as an experimental animal".

In 1933 Levine et al. (189) were able to show, using Remington's recently developed methods (190), that the Steenbock ration was low in iodine, having an average content of 15 μg iodine per kilo. They calculated that their animals received on average 0.14 μg iodine daily and found that after 35 days on diet the thyroid glands were hyperplastic and had a low total iodine content. By feeding various groups of rats on low iodine diet (LID) to which different amounts of iodine were added they were able to establish that the smallest amount of iodine necessary to prevent any significant thyroid enlargement was approximately 1-2 μg per rat per day (236).

Although very effective in producing both rickets and hyperplastic thyroids the Steenbock ration did not, however, support normal growth. Remington (237) therefore resolved to improve the diet without loss of "goitre-producing power". To counteract

its rickets-producing property he reduced the proportion of CaCO_3 from 3 to 1% and to improve the vitamin and mineral supply he added small percentages of either dried pig liver or dried brewer's yeast. Equal gains in growth improvement were achieved with either 1% liver or 2% yeast and on this modified Remington diet rats were able to reach maturity and produce young "despite almost complete absence of iodine and colloid from the thyroid gland".

(ii) Introduction of radioisotopes of iodine

Although Hamilton and Soley (147) first introduced in 1939 iodine radioisotopes for use in experimental studies it was not until 1952 that Money, Rall and Rawson (203) undertook to determine "with modern techniques" the effect of LID on the growth and function of the thyroid gland. Using a diet containing $45 \mu\text{g}$ iodine per kilo they found that the 24 hour radioactive iodine uptake (RAIU) increased within 48 hours and had attained maximal values by 21 days. In the early stages of iodine deficiency the total thyroid iodine concentration did not fall markedly but by 34 days of treatment had reached a value about 50% of controls. Only when the level of thyroid iodine had dropped to this level did the thyroid weight increase markedly and it was not until 106 days that the serum protein bound iodine (PBI) fell to a level of about half of normal. Their conclusions were that an increased thyroidal collection of I^{131} appeared to be associated with a decreased level of thyroid iodine and a fall in the level of serum PBI appeared to be related to increased growth of the thyroid.

(iii) L- T_3 and its role in thyroid adaptation

That animals chronically fed LID were able, despite very low PBI levels, to grow normally and remain free of hypothyroidism was a difficult fact to accept in the light of knowledge available in 1952.

However, in that year Gross and Pitt-Rivers (146) reported the presence of a second thyroid hormone in human plasma and identified it as 3, 5, 3'-L-triiodothyronine (L-T₃). During the subsequent twenty years this hormone was to arouse increasing interest and its relevance to thyroid adaptation in iodine deficiency was well described in Studer and Greer's 1968 monograph (265).

In an extensive study of the temporal sequence of changes in thyroid function after initiation of a low iodine regime, Studer and Greer (264) found that significant thyroid hypertrophy was produced during the first week that is, at a time when the serum PBI was still normal. Temporally related, however, to the appearance of goitre was a rise in ¹³¹I uptake, MIT/DIT ratio and iodide clearance and a fall in thyroidal ¹²⁷I concentration. By contrast, a fall in the total content of thyroidal ¹²⁷I appeared later and was closely correlated with a decline in serum PBI concentration and a rise in the intra-thyroidal T₃/T₄ ratio.

Of the changes in thyroid hormone biosynthesis the most marked was the increased labelling to T₃. Studer and Greer argued that a biosynthetic "shift" in favour of T₃ over T₄ in iodine deficiency was teleologically sound since T₃ possesses approximately four times the potency of thyroxine while utilising only 75% as much iodine. Thus, in the face of limited iodine substrate, T₃ would be considerably more efficient than T₄ in maintaining a euthyroid state.

That such a preferential synthesis and secretion of T₃ over T₄ actually occurs in severe iodine deficiency was demonstrated by Greer and co-workers (144) in 1968. By measuring the thyroid effluent from prelabelled glands perfused in situ with non-radioactive blood they showed that the T₃/T₄ ratio of the thyroid effluent increased progressively with the length of time rats were

iodine deficient and they concluded that iodine deficiency results in at least as great an increase (and possibly greater) in the T_3/T_4 ratio of iodothyronines secreted as in those synthesised.

Although rats fed on LID for a period of more than one year remained healthy and gained weight normally, Greer could not know whether the animals were actually "euthyroid" in the face of severe iodine deficiency. It was left to Silva (249) to show in 1972 that iodine deficiency produced by feeding a diet containing 93 μg iodine per kilo for 3-4 months, altered neither the basal metabolic rate nor the metabolic response to cold in test animals.

(iv) Direct measurement of circulating thyroid hormones

Prior to the development of specific radioimmunoassay (RIA) methods, the estimation of plasma and tissue levels of T_3 and T_4 was dependent on isotope equilibrium methods. These techniques were notoriously tedious and prone to inaccuracy on account of their dependence on accurate estimates of iodine at very low levels in the diet.

In 1966 Henninger and Albright (149) using these methods quantified the absolute concentrations of T_3 and T_4 in thyroid and peripheral tissues. They showed that after 2 months on an LID containing 60 μg iodine per kilo the plasma T_4 level fell from 3.7 to 1.8 $\mu\text{g}/100\text{ ml}$ while the plasma T_3 level increased from 43 to 101 $\text{ng}/100\text{ ml}$.

In 1971 Chopra et al. (40) described the production of antibodies specifically binding T_3 and T_4 and in 1972 reported the first "rapid, specific, precise and reproducible" radioimmunoassays of both T_4 (37) and T_3 (39) in unextracted serum. These new methods were soon applied to the problem of iodine deficiency in the rat.

Volpert and Werner (276) were the first workers to study the effect of LID on serum T_3 concentrations measured by RIA. They

showed that feeding an LID for four to five weeks resulted in a decrease both in serum PBI and T_3 concentrations but an increase in T_3 :PBI ratio. By contrast, in the same year, Nejad et al. (213) reported no significant changes in T_3 values in rats fed LID for an unspecified period of time while T_4 levels decreased to less than 25% of control.

In 1973 Abrams and Larsen (2) measured T_3 and T_4 by immunoassay in the serum and thyroid hydrolysates of both control and iodine deficient rats. They were able to show that within 4 weeks of iodine restriction the mean serum T_4 concentration had fallen from $4.2 \mu\text{g}/100 \text{ ml}$ to undetectable levels ($<0.5 \mu\text{g}/100 \text{ ml}$). However, despite the profound decreases in serum T_4 , no changes were detectable in the levels of serum T_3 at either 1, 2 or 3 months after restriction of iodine intake.

Abrams and Larsen suggested, from comparison of the labelled T_4/T_3 ratio in thyroid and serum, that in the iodine-deficient rat the labelled thyroid hormones are secreted in the ratio in which they are present in the gland. Assuming that the T_4 to T_3 conversion remains constant despite iodine deficiency, they argued that, in contrast to iodine sufficiency where $2/3$ of the T_3 arises from T_4 conversion, in iodine deficiency about 90% of the T_3 arises directly from the thyroid. Thus, in the rat, they concluded that iodine deficiency results in a several fold increase in the absolute secretion of T_3 by the thyroid.

Studies Performed

(a) Introduction

From the literature cited above it seemed possible that within a short time after the introduction of an LID a rat could be rendered iodine deficient in a manner akin to that seen in the human in areas

of endemic iodine deficiency i.e. clinical euthyroidism in the face of a low serum T_4 , a normal serum T_3 , a high serum TSH and a goitre avid for iodine. Towards the end of 1974 the author commenced the studies described below in an attempt to achieve this hormonal profile.

Four well proven parameters of iodine deficiency were chosen for study. Measurements were made in vitro of the goitre size, as expressed by the relative thyroid weight (RTW), and the avidity of the thyroid for iodine as demonstrated by the radioiodine uptake (RAIU) at 4 hours. Blood was drawn in vivo for the direct measurement of total serum T_4 and T_3 which was performed by specific radioimmunoassay (RIA).

Groups of at least six animals were studied from the point of view of these parameters at regular intervals after the introduction of an LID. Both short-term (less than 4 weeks) and longer term (up to 6 months) studies were performed and these are described in full below.

(b) Materials and methods

Animals

Two hundred and ten rats were employed in these studies. In the main these were female rats of the Wistar strain. However, in the long-term experiment with test diet I male Sprague-Dawley rats were used. All animals weighed 150-200 g at the time of purchase and were reared on colony diet until the commencement of experiments when the diet was switched to one low in iodine. All diets were fed in the form of pellets and all animals received deionised distilled water for drinking.

Diets

The colony diet employed was the CNP Beta Rat and Mouse No. 1 Standard Maintenance diet (Cooper Nutrition Products Ltd.,

Stepfield, Witham, Essex). This diet is based on "Toprina" (BP single cell protein) and has a basic iodine content of $203 \mu\text{g}/\text{Kg}$ to which the makers add a supplementary $500 \mu\text{g}/\text{Kg}$ in the form of calcium iodate to give a final content of $700 \mu\text{g}/\text{Kg}$.

Two different low iodine test diets were employed. Both were prepared according to Remington and Remington (237) and contained 78% corn meal, 18% wheat gluten, 2% brewers yeast, 1% CaCO_3 and 1% NaCl. Diet I was that supplied by the Nutritional Biochemicals Division of ICN Pharmaceuticals Inc., Cleveland, Ohio while diet II was obtained from the Teklad Test Diets Division of the Mogul Corporation, Madison, Wisconsin. Both diets had been analysed by the Boston Medical Laboratory (2) and had been found to contain less than $165 \mu\text{g}/\text{Kg}$ of iodine.

In vitro parameters

All the animals employed in this study were injected intraperitoneally with $0.5 \mu\text{Ci}$ of carrier free ^{131}I 4 hours before sacrifice with ether anaesthesia. Groups of rats were killed at various intervals after starting the test diets. Thyroids were carefully dissected from the trachea and rapidly weighed. Thyroidal RAIU was measured by gamma counting and expressed as the percentage of the administered dose localising in the thyroid.

In vivo measurements

At each time interval groups of rats were exposed for 1-2 minutes to an infra-red heating lamp and then bled from the tail vein. Blood samples of about 1 ml were obtained and these after centrifugation regularly yielded serum samples of about $400 \mu\text{l}$ which were stored deep frozen until assay.

Serum T_4 and T_3 were measured by specific radioimmunoassays adapted from the method of Challand et al. (32). Hormone-free rat serum was added to the standard curve to equalise the protein concentrations. The intra- and inter-assay coefficients of variation

were 4.8% and 8.9% in the T_4 assay and 8.5% and 9.4% in the T_3 assay. The limits of detection of the assays were $0.7 \mu\text{g}/100 \text{ ml}$ for T_4 and $20 \text{ ng}/100 \text{ ml}$ for T_3 .

All samples from each individual experiment were analysed in the same assay to avoid inter-assay variation. Statistical analysis was made with Student's *t* test and values below the limit of detectability were for statistical purposes assigned that limit (e.g. $0.7 \mu\text{g}/100 \text{ ml}$, rather than 0). All results were expressed as the mean \pm one standard deviation.

(c) Results

Short-term deprivation with diet I

The effect of diet I for periods up to 4 weeks on RTW and RAIU is shown in Table 1. RTW increased significantly ($p < 0.001$) after 1 week, an increase which was maintained up to 4 weeks. The RAIU increased very rapidly to a figure of 23% at 1 week. There was no further change in uptake at 2 weeks but thereafter there was a further increase to a value of 43% at 3 weeks.

The effect of diet I in the short-term on serum T_4 and T_3 is shown in Table 2. Serum T_4 levels had not changed by 3 weeks but by 4 weeks had significantly ($p < 0.01$) dropped to a mean value of $3.1 \mu\text{g}/100 \text{ ml}$, only one third of the animals having at 4 weeks a T_4 level below the lower limit of normal, defined as $2.5 \mu\text{g}/100 \text{ ml}$. No change was observed in the levels of serum T_3 after 4 weeks on test diet I.

Longer term studies with diet I

Table 3 shows the results of RTW and RAIU estimations in rats maintained on diet I for periods up to 24 weeks. The RTW, shown in the short-term study to have increased by 1 week, continued to rise, being on average 28% increased at 12 weeks and 52% by 24 weeks. The RAIU which had attained a maximum value at 3 weeks appeared to rise again between 16 and 20 weeks.

However the difference in uptake between 4 and 16 weeks was not found to be significant.

Table 4 shows the results of RIA of serum T_4 and T_3 in the longer term diet I study. Serum T_4 , which was significantly decreased by 4 weeks ($p < 0.01$), continued to drop to a mean level of $1.9 \mu\text{g}/100 \text{ ml}$ at 20 weeks. By 12 weeks 50% of the T_4 values measured were less than lower limit of normal. However by 20 weeks all T_4 values were frankly low. By contrast throughout the period of study with diet I the serum T_3 concentration remained constant, varying between 92 and $113 \text{ ng}/100 \text{ ml}$.

Short-term deprivation with diet II

On the advice of Professor M.A. Greer an alternative Remington diet was also studied. The results of short-term deprivation with diet II are shown in Tables 5 and 6. RAIU increased significantly by 1 week ($p < 0.001$), and RTW by 2 weeks ($p < 0.05$). The serum T_4 was lowered to $3.2 \mu\text{g}/100 \text{ ml}$ by 4 weeks while the serum T_3 level remained unchanged.

Longer term studies with diet II

Groups of animals were studied at 2 week intervals until 8 weeks and thereafter every 4 weeks until 24 weeks. The results are shown in Tables 7 and 8. No further significant increase in RTW was noted after 4 weeks. However, between 2 and 10 weeks the serum T_4 fell progressively to a level of $2.1 \mu\text{g}/100 \text{ ml}$. Thereafter no further significant fall in T_4 was produced by deprivation for periods up to 24 weeks. Again, as with diet I, the serum T_3 concentration remained constant in a narrow range between 101 and $123 \text{ ng}/100 \text{ ml}$.

Comparison of diets I and II

There were no significant differences in the effects on RAIU and RTW up until 12 weeks (Figs. 9 and 10). However, between 8

and 16 weeks diet II produced significantly lower values of serum T_4 as compared to diet I (Table 9). By 20 weeks there was no significant difference in the levels of serum T_4 and neither diet seemed able to lower the mean level below $2 \mu\text{g}/100 \text{ ml}$ even after a period of 24 weeks. Fig. 11 shows the sequential changes in serum T_4 on both diets and highlights the difference in effects demonstrable until 20 weeks.

The fall in serum T_4 in the first 20 weeks with diet I and in the first 10 weeks on diet II may be represented as straight lines ($y = mx + c$) and Fig. 12 demonstrates the relative superiority of diet II in producing hypothyroxinaemia. By contrast, neither diet was able to cause a fall in the serum T_3 level and this is illustrated by Fig. 13.

(d) Conclusions

From the studies described above it seemed clear that both test diets were capable of increasing thyroidal radioiodine uptake, causing goitre formation and eventually lowering the serum thyroxine (T_4) value. Of the four parameters initially chosen for study the serum T_4 was clearly the most relevant and on the basis of this value the degree of iodine depletion achieved was graded into mild, moderate and severe categories (Fig. 14). Using diet II these varying degrees of iodine deficiency were consistently attained at 4, 10 and 24 weeks after commencing diet and because of this facility and also its relative superiority over diet I, the test diet II, as recommended by Professor M.A. Greer, was employed in all subsequent studies.

It was disappointing at first to discover that after 4 weeks of test diet II, although the serum T_4 had fallen, it had not attained undetectable levels as had been reported by Abrams and Larsen (2) employing apparently the same General Biochemicals diet. It was therefore of some consolation to the author to find out from

Drs. Greer and Taurog at the 1975 International Thyroid Conference that they too had noted a drop in the efficiency of their low iodine test diets. It was suspected that the iodine content of the diet had altered between 1973 and 1975 but it was not until the publication in 1976 of the paper by Riesco et al. (238) that it was universally appreciated that the reason for an increase in dietary iodine content was the fact that the source of the corn which made up 78% of the diet, had changed in 1974 from Ohio to Wisconsin, resulting in a corn-based diet with a higher iodine content.

Employing an analytical procedure sufficiently sensitive to measure 15 μg of iodine per kilo of diet Riesco et al. (238) were able to show that diet I contained $104 \pm 17 \mu\text{gI/Kg}$ while diet II purchased in 1975 contained only $67 \pm 6 \mu\text{gI/Kg}$. Although this latter value is acceptably low it falls short of the content of $16 \pm 5 \mu\text{g/Kg}$ which characterised the Ohio-derived corn extensively used between 1965 and 1974 by the groups of workers in Portland and Dallas led by Greer (265) and Taurog (238) and also successfully employed by Abrams and Larsen (2) in their 1973 study. Since, however, by 1975 the Ohio corn-based diet was no longer obtainable it was necessary for the author to use the Teklad (Wisconsin) test diet (containing about $70 \mu\text{gI/Kg}$) for all his experiments. Although the content of iodine was only relatively low compared with Remington's original modification of the Steenbock ration (189), it was adequate to cause the desired alterations in thyroid physiology, namely iodine deficiency without hypothyroidism, and because it produced these changes gradually it allowed the author to study the effects of milder degrees of iodine deficiency before going on to evaluate the effects of severe iodine depletion.

Discussion

At the time that the above studies were performed there was little data in the literature on the effect of iodine deficiency on plasma

thyroid hormones (2, 276) and none reporting an effect on plasma TSH levels. Since 1975 there have been a number of important reports which have extended knowledge not only on the relation between daily iodide intake and tissue levels of thyroid hormone but also on the short and longer term changes in circulating thyroid hormones, especially T_3 , during adaptation to elemental iodine deficiency.

Henninger and Albright (150) compared the established isotopic equilibrium method with the newer radioimmunoassay techniques in the evaluation of the effect of increasing iodine intake on tissue and serum concentrations of thyroid hormones. Their data indicated that an iodine intake of $1 \mu\text{g}/\text{day}$ results in extremely low hormone concentrations in tissues whereas maximal tissue concentrations are generally reached at an iodide intake no greater than $10 \mu\text{g}$ per day. In general there was good agreement between the two methods of hormone estimation and the values obtained by RIA for serum T_4 and T_3 in animals maintained on an iodine intake of $10 \mu\text{g}$ per day were $6.2 \pm 0.8 \mu\text{g}/100 \text{ ml}$ and $92 \pm 21 \text{ ng}/100 \text{ ml}$ respectively, compare favourably with those described above.

Using RIA techniques Fukuda et al. (127) reported temporal changes in plasma T_4 , T_3 and TSH in rats fed LID for periods up to 3 months. The T_4 level remained between 4 and $6 \mu\text{g}/100 \text{ ml}$ until the tenth day of LID when it rapidly decreased to a value of $<0.4 \mu\text{g}/100 \text{ ml}$ at 1 month. TSH, initially $50 \mu\text{U}/\text{ml}$, increased linearly to $165 \mu\text{U}/\text{ml}$ on day 16. Thereafter there was a much more rapid rate of rise to reach a value of $640 \mu\text{U}/\text{ml}$ at 38 days. Throughout the experiment there was a highly significant negative correlation of plasma T_4 with plasma TSH. By contrast plasma T_3 did not change significantly in any experiment, remaining at 60-90 ng/100 ml throughout. It was suggested that, "at least in iodine deficiency, plasma T_4 may be a more important regulator of TSH

secretion through negative feedback effects than is T_3 ". Moreover, since radioiodine uptake increased after one day on LID i. e. at a time when plasma TSH level was still normal, it was concluded that the activity of the thyroid iodide pump is to a degree independent of TSH drive and to a large extent dependent on the intrathyroidal concentration of organically iodinated material.

Later in 1975 Fukuda et al. (128) reported their findings in rats fed LID for 1 year. In contrast to their early studies they found that at 1 year the T_3 level of 54.9 ± 5.3 ng/100 ml was significantly lowered and they presumed that in these severely depleted rats the absolute quantity of iodide substrate had fallen below the threshold necessary to maintain a normal plasma T_3 concentration.

Similar results were reported in 1977 by Riesco et al. (239) who also attempted to evaluate the effect of severe iodine deficiency on thyroid status by measuring body temperature and survival rates when animals fed on LID for 3-4 months were exposed to a cold environment. In contrast to control iodine sufficient animals those rats fed LID alone showed significant decreases in body temperature after 5 days of cold exposure and by 15 days the majority had died. Riesco et al. (239) concluded that thyroid function in severely iodine deficient rats is inadequate to meet the challenge of acute cold stress and in this sense therefore these animals could be considered to display signs of hypothyroidism.

CHAPTER VI

QUANTITATION OF PERINATAL BRAIN DEVELOPMENT

Neurobiological Background

(i) Cellular growth during brain development

Serious interest in the experimental study of brain development began seventy years ago with the work of Donaldson from the Wistar Institute (85). The approach of Donaldson and his colleagues was primarily anatomical and, although they examined various animal species, the albino rat was always the species of comparison. In 1973 Himwich (157) reviewing early studies on the developing brain dated 1913 as the inception of the chemical approach to neurobiology and 1949 as "the end of the early chemistry of the developing brain". He singled out the 1931 paper of Donaldson and Hatai (86) as "a paradigm for the anatomical study of developing brain".

It took Donaldson and co-workers more than 20 years to collect the material for "the definitive paper on the maturation of the brain and brain parts" (157). They studied the brains of 250 male and 213 female albino rats from the day of birth to 529 days of age. At day 1 the average brain weight of male rats was 228 mg. Thereafter a gradual increase in weight occurred until by 529 days the brain weighed 2.04 g.

From Donaldson and Hatai's data it was clear that there were "three postnatal periods characterised by predominant rates of growth" (157). In the first 8 days of life (the neonatal period) there was an increase in brain weight of 90 mg/day. From 8 to 45 days (the pre-pubescent period) growth was slower with an average gain of 18 mg/day and during the final period until 529 days the growth was slowest at about 1 mg/day. No sex differences were noted until 50 days when the male brain became heavier than the female.

Donaldson and Hatai added a further dimension to their study by supplying data on growth within the various regions of the rat brain. They divided the brain into 4 areas (i) forebrain (essentially cerebral hemispheres), (ii) stem, including the brain axis from the level of the first spinal nerve to a plane passing in front of the quadrigemina (iii) cerebellum and (iv) olfactory bulbs.

Throughout the rat's life the forebrain represented at least 60% of the entire brain weight. Within the first 8 days the forebrain weight increased from 64 to 71% of the whole brain weight. The absolute weight is shown in relation to that of the total brain in Fig. 15. It is obvious from this figure that the early rapid increase in brain size is chiefly due to growth of the forebrain and that, as Himwich has indicated (157) despite "the lower position of the rat in the evolutionary ladder the size of rat forebrain is remarkably large".

Donaldson and Hatai (86) were responsible for providing data on the postnatal growth of the brain as an organ and the forebrain as part of that organ. However, before studies of cellular growth within these brain regions could become possible further advances in basic cellular biochemistry were necessary.

In 1948 Boivin et al. (19) showed in cattle that the amount of desoxyribonucleic acid (DNA) in the cell nucleus was constant for any one species. In 1953 Thomson et al. (267) from Davidson's laboratory in Glasgow confirmed this finding in the rat, showing that the DNA content was constant at about 6 $\mu\mu\text{g}$ per diploid nucleus. It was obvious soon after Boivin's discovery that the number of diploid nuclei in an organ could be obtained by first measuring the total DNA content in $\mu\mu\text{g}$ and then dividing this value by the constant for that species (58). Thus total DNA content could be taken as a measure of cell number, thereby avoiding the laborious technique of cell counting.

Enesco and Leblond (103) in 1962 were the first workers to study cellular growth in the rat using the new techniques. Their investigation consisted of measurements of total weight and DNA content in a series of tissues and organs which did not include brain. Total DNA content was used as a measure of cell number while weight per nucleus (total weight divided by number of nuclei), which varied with cell size and/or proportion of intracellular material, was used as an index of weight gain "not due to addition of new cells".

Taking weight gain as an index of growth Enesco and Leblond concluded that "three periods of growth may be considered in the young male rat:

- (i) Until about 17 days of age growth of organs and tissues is due to rapid cell proliferation (hyperplasia) with little or no change in cell size.
- (ii) Between about 17 days and 34-48 days of age cell proliferation continues but at a slower rate. Meanwhile cell size increases in most organs (concomitant hypertrophy).
- (iii) Finally, after 34-48 days of age, cell proliferation slows down or even stops, while cell enlargement proceeds in most tissues but is slight or absent in organs".

In 1965 Winick and Noble (289) further defined cellular growth in the rat from 10 days after conception through to maturity. Like Enesco and Leblond (103) they employed total organ DNA as an index of cell number but also used both weight and protein per nucleus as an indication of cell size. Their findings included for the first time cellular data on the whole brain (Fig. 16).

Like the findings of previous workers (86, 103) Winick and Noble identified three distinct phases of growth about which they said:

"The first (phase) consists of rapid cell division with cell size remaining constant; the second consists of an increase in cell number and cell size as both DNA

and protein content rise....and the third consists of an increase in cell size only, as DNA synthesis stops and protein continues to accumulate. Growth finally ceases when protein synthesis and degradation come into equilibrium" (289).

Winick and Noble noted that DNA synthesis and hence cell division had stopped in brain by 20 postnatal days (289). A year later Fish and Winick showed that various regions of rat brain had different rates of cell division and that the entire pattern of cellular growth was regionally specific. The differences were particularly marked when cerebrum and cerebellum were directly compared (111).

In the cerebellum there was a marked increase in cell number between 6 and 17 days while in the cerebrum cell number increased more slowly but for a longer period viz. to 21 days. Regional analysis also revealed differences in the protein:DNA ratio (cell size). In the cerebrum the ratio increased steadily, especially after 10 days while, by contrast, in the cerebellum the protein:DNA ratio actually decreased during growth.

Thus far, in the years which had followed Donaldson's 1908 paper (85) almost all neurobiological research had been on laboratory animals, particularly the albino rat. In 1968 Winick (287) extended the cellular approach to the examination of human brains. He determined the nucleic acid and protein content of 31 human brains, the earliest being from a foetus of 13 weeks and the latest from a 13 month old infant. Total brain weight and protein content increased linearly from 13 weeks of gestation until 13 months of age. By contrast, increase in DNA content began to level off at about the time of birth and reached a maximum at 5 months of age. Winick commented that "the sequence of cellular changes during the growth of human brain is qualitatively similar to that seen in the rat brain" and from his DNA data concluded that "very little" cell division occurs in the human brain after 5 months of age.

(ii) "Artificial changes in the architectonic"

The title of this section derives from a book published in 1940 by Stephen Zamenhof from Columbia University and entitled "On present possibilities of increasing the higher functions of the cortex through artificial changes in its architectonic" (293). In this treatise Zamenhof demonstrated that "the number of neurons seems to be, next to the complexity of nerve fibers, one of the most important factors in determining the level of psychical functions" (295). In contradistinction to the complexity of nerve fibres which he considered could not be influenced artificially Zamenhof stated that "the number of neurons can probably be artificially increased". In 1941 he showed that in tadpoles it was possible to stimulate the proliferation of prospective neurones by the intraperitoneal injection of a growth hormone preparation and "concluded that, by means of the growth hormone, it is possible to increase artificially and intentionally the number of brain cells at least in amphibians" (294).

In 1942 Zamenhof extended his approach to the albino rat (295). Pregnant rats were injected subcutaneously daily from day 7 to day 18-20 of gestation and pups delivered by caesarian section at term. The offspring at birth showed increases of 18.7% in body weight, 36% in cerebral hemisphere weight, 21% in cortical thickness and 70.4% in cortex volume. Cell density increased 9.3% over the control value and the increase in cell numbers per volume of cortex was 86.5%. This pioneering work indicated that experimental alteration of neurone number and density was feasible but it was unclear from Zamenhof's description whether neurones and glia were clearly identified.

Clendinnen and Eayrs in 1960 applied purer forms of growth hormone (GH) in the same manner as Zamenhof and achieved results clearly in contrast to this earlier work. Rather than hyperplasia or

numerical increase in cells these authors noted a hypertrophy of neurones (45). This was reflected in "an enlargement of the perikarya and an expansion of protoplasmic processes resulting in an increase in the statistical probability of interaction between neurones". To assess the possibility of alterations in behavioural capacity they submitted the animals to tests of both innate responses and adaptive behaviour. Although the maturation of innate and reflex behavioural responses was little affected the performance of cortically mediated behaviour, as assessed by a closed-field test, was significantly enhanced.

In 1962 Enesco and Leblond published their paper on total DNA as an accurate index of cell number (103) and by the following year Zamenhof had set himself to finding the best available technique for the determination of DNA in brain tissue (296). In 1966 he combined the techniques of DNA determination and quantitative histology in a reassessment of the effect of prenatally administered GH on the proliferation of cortical neurones.

Subcutaneous or intravenous injections daily of purified bovine GH into pregnant rats from day 7 to day 20 resulted in offspring with unchanged body weight but with significant increases in brain weight and DNA content. Quantitative histology revealed that cortical cell density per unit volume was 63% higher in the offspring of treated mothers and the neurone-glia index in 20 day old rats was 71% higher than in controls. Zamenhof concluded that "the final number of cortical neurons can be significantly increased by stimulation with pituitary growth hormone administered before the neurons cease to divide" (297). Some nine years later Sara and Lazarus employing a selective isotope labelling technique confirmed these findings and showed that, at maturity, learning performance on a series of conditional discrimination tasks was enhanced in the offspring of the mothers treated during pregnancy (242).

1966 also saw another significant contribution from Winick's group at Cornell. To investigate the cellular events underlying growth restriction due to postnatal malnutrition (MN) Winick and Noble (290) exposed rats to 21 days of caloric restriction either at birth or at weaning. MN from birth to weaning resulted in a proportional decrease in weight, protein, and DNA content of whole brain, indicating a reduction in cell number without alteration in cell size. By contrast MN from weaning to 42 days resulted in reductions in weight, protein and RNA but did not affect DNA content of brain. Refeeding resulted in recovery of normal brain weight in the animals deprived from weaning but not in those deprived from birth. From these findings it was concluded that:

"....malnutrition during hyperplastic growth will retard the rate of cell division and result in an organ that is reduced in size and contains a reduced number of cells. Moreover this change is not reversible after the period when cell division normally stops. By contrast, malnutrition during the period of hypertrophic growth prevents the increase in cell size which normally occurs and again results in a smaller organ. This change, however, is reversible at any time. The cells simply fill up again with protein and the individual cells and entire organ regain their normal size.... The old observation that the earlier the growth failure the less likely the recovery can now therefore be explained in cellular terms" (291).

In 1968 Zamenhof et al. (298) studied the effect of earlier growth retardation by maintaining female rats on a protein restricted diet for one month prior to mating and throughout pregnancy. Newborns were killed within 6 hours of delivery and forebrains (cerebral hemispheres) stored for later determination of DNA and protein content. Because cerebral neurones essentially do not replicate after birth the DNA content assayed at birth is indicative of final cerebral neurone number. On the other hand, the protein content of the neonatal brain reflects the size of perikaryon (44) and thus can be correlated with the potential for subsequent neuronal differentiation e.g. development of an extensive

neuronal dendritic tree (301).

Dietary protein restriction resulted in a 30% lowering of body weight in newborn offspring which was accompanied by a 23% reduction in brain weight. Determination of DNA revealed a 10% reduction in content, implying a reduction in total brain cell number and probable "permanent brain-neuron deficiency". An accompanying reduction in protein content, twice as large as that in DNA, was felt to indicate that not only was the number of cells altered but also the cells were "qualitatively different". Zamenhof et al. (298) were unclear whether the qualitative changes were irreversible or whether they represented a "delay in maturation". However, when at 3 months of age the test offspring were evaluated they manifested "abnormalities of gait and response to environmental stimuli" suggesting that manipulation of prenatal nutrition had resulted in a permanent alteration in behaviour.

By 1968, therefore, some 28 years after his original treatise, Zamenhof had shown that by prenatal manipulation one could not only increase but also decrease "artificially and intentionally" the number of brain cells, these alterations in cell number apparently resulting in permanent behavioural differences. The work of Winick and Zamenhof led to a world-wide explosion in experimental research into the influence of maternal nutritional restriction on growth and development of the nervous system. Extensive reviews of the relevant literature are now available and four recently published surveys are particularly recommended (36, 53, 82, 226). For the purposes of this thesis, however, an adequate insight into this area of development can be obtained from a survey of the work performed between 1968 and 1971 by Zamenhof and his group at the UCLA Brain Research Institute.

In 1969 Van Marthens and Zamenhof (273) studied the effect on neonatal rat cerebral DNA of increased feeding (overnutrition) before

birth. This was performed by unilateral ligation of the uterine horn so that effectively the litter size was halved. The neonatal rats showed highly significant increases in body weight, cerebral weight and content of cerebral DNA and protein. This work was later extended to rabbits (301) where it was found that, as in rats, the increases of body weight were up to 50% and the increase in DNA up to 21%. An accompanying increase in cerebral protein was on average twice higher than the increase in DNA. This was considered to imply a higher protein content per cerebral cell and a larger cerebral cell size which might conceivably enhance "post-natal cell development (dendritic tree)" (301).

In 1971 Zamenhof and colleagues (299) investigated the effect on cerebral DNA of protein restriction of short duration during various periods of pregnancy. Regardless of when complete protein deprivation occurred the neonate showed significant decrease in body and cerebral weight, and cerebral content of DNA and protein. In general, the decreases tended to be larger if the period was longer and if it was imposed later during pregnancy. The most pronounced effects were obtained by protein deprivation from day 10 to day 20 of gestation.

Employing knowledge gained from the above experiment Zamenhof et al. (300) maintained pregnant rats from day 10 to day 20 on a diet with one third of the normal caloric value but with normal vitamin and protein content. The neonates showed changes similar to those due to protein deprivation viz. 11% decrease in DNA and a 17% decrease in protein (i. e. a quantitative and qualitative change). By contrast, when mothers on a low calorie diet received daily intravenous injections of GH no decreases in either DNA or protein appeared. Moreover, compared with calorie restricted animals the GH-treated offspring showed a 9%

increase in DNA and a 16% increase in protein. Interestingly, treatment of normal animals with GH did not produce a significant increase in neonatal cerebral DNA (cell number) in contrast to the results previously reported (297). However, GH treatment did produce in the offspring of control mothers an 8% increase in cerebral weight and also a 10% increase in protein content implying a qualitative rather than quantitative effect on the neonatal cerebral neurones.

(iii) Vulnerability of brain growth spurts

Ten years ago Davison and Dobbing (60) reviewed the problem of "critical periods" (119) during brain development and stated that the concept was only valid provided it did not distract attention from "the overlapping and sequential nature of the pattern of events". They indicated that amongst the different mammalian species the broad developmental sequence of events did not vary fundamentally and that the only important species difference, apart from the degree of complexity of the final product, was the timing of the developmental processes in relation to birth. On the assumption that birth was not a significant milestone in brain development they attempted to describe a general pattern of development applicable to all mammalian species.

Four stages of development were recognised. Stage I was from conception until the attainment of an adult complement of larger neurones. Stage II encompassed the period of the "brain growth spurt" and lasted until the end of the period of rapid myelination. The transition from stage II to stage III was "very gradual" but in the latter stage the rate of growth gradually slackened to be replaced by a growth process designed simply to keep pace with increase in body size. Lastly stage IV represented the stage of senile regression.

It was stage II which Davison and Dobbing felt corresponded to Flexner's critical period of development (119) and they described it thus:

"....The second stage....embraces the period during which there is a rapid increase in the size of the brain, which undergoes a "growth spurt" at this time, just as the whole body does somewhat later....The major events in stage II from the morphological viewpoint are the growth of axons and dendrites and the establishment of neuronal connections, the almost explosive multiplication of oligodendroglial cells and the subsequent deposition by these cells of myelin sheaths....Thus stage II necessarily embraces all the multiplicity of biochemical changes associated transiently with the processes of neuronal maturation as well as with myelination, and also the important, permanent changes from foetal and neonatal metabolism characteristics into the mature, adult state of brain metabolism" (60).

The period of the brain growth spurt was further considered in the same volume by John Dobbing (72) who felt that the whole period represented a transient phase of heightened sensitivity. Rather than use "the more academic terms "critical" or "sensitive" " (80) he chose the term "vulnerable" to imply both lasting distortion and lasting deficit consequent on the growth retarding stimulus of under-nutrition.

From Davison and Dobbing's developmental sequence (60) it was clear that glial mitosis occurred later than neuronal mitosis and that the brain growth spurt began at about the time neuroblast multiplication ended and the adult number of neurones had already been achieved. This obviously had clear implications for the interpretation of animal experiments on growth restriction during the vulnerable period of the growth spurt. As Dobbing and Smart in 1973 stated:

"most experimental proof of the vulnerability of "brain cell" mitosis has been confined to the later phase of glial multiplication, which happens to occupy the first part of the brain growth-spurt. Therefore most of the lasting "brain cell" deficit in these experiments must be glial, and it seems unlikely that a numerical glial deficit would be functionally important. It is even possible that a numerical neuronal cell deficit might not be very significant for brain function, compared with a deficit

(for example) in subsequent dendritic branching and in the establishment of synaptic connections" (80).

A second consequence of the timing difference between neuronal and glial mitosis was that theoretically any growth curve showing the increase in "brain cell" numbers (DNA counts) should exhibit two consecutive phases of cell division. Prior to 1973 such a phenomenon had not been demonstrated in any of the small laboratory species studied although it was in these species that the phenomenon was originally described by histologists (6, 21). In 1973 two consecutive peaks of brain cell proliferation were demonstrated in both rats (55) and man (79).

Crosskerry and colleagues (55) showed, by carefully drawing the cellular accumulation in the rat brain from foetal to postnatal life, that two peaks existed, the cut off point of the first peak being around birth. In 1973 Dobbing and Sands (79) reported their findings from the estimation of total DNA in 139 human brains ranging in age from 10 weeks of gestation to 7 postnatal years. Fig. 17 shows the data on total DNA in forebrain between 10 gestational weeks and 4 postnatal months. It is clear that cell multiplication occurs in two quite distinct phases with a sharp cut off point at 18 weeks of gestation. To Dobbing's eye it seemed "almost certain that the period from 10 to 18 weeks is the major period of human neuroblast multiplication, differentiation to non-dividing neurons occurring towards the end of this time. Glial division then takes over, and occupies the remainder of the multiplicative phase until well into the second postnatal year" (74). It therefore appeared that, in contrast to Winick's previous data (287), the human brain growth spurt continued well into the second postnatal year and that since at least 5/6 of the human brain growth spurt was postnatal "in this respect humans resemble rats much more closely than previously thought" (79).

In 1968 it was Dobbing's impression that the period of vulnerability consisted of the brain growth spurt of developmental

stage II (72). However, by 1974, consequent on the publication of the human brain data, he reconsidered his stance by identifying two possible periods of brain vulnerability: "the first, and probably much less common, related to the 12 to 18-gestational-week period of neuronal multiplication; and the second, the much more recognised later period of the main brain growth spurt during which the processes at risk probably include dendritic arborisation and the establishment of synaptic connectivity as well as glial multiplication and myelination" (75).

(iv) Experimental hypothyroidism and brain development

An awareness of a relationship between the thyroid gland and brain development has been evident since at least the middle of the nineteenth century (106) particularly in relation to the mental retardation associated with congenital hypothyroidism. However, beyond this observation, attempts to understand further the role of thyroid hormones in brain development were seldom undertaken in the first half of this century and the present day interest probably represents a part of the overall upsurge in investigation of the nervous system which started soon after World War II (121).

The modern experimental approach to the problem probably dates from 1948 when Russell Barrnett (14) submitted to Yale University School of Medicine a thesis entitled "Some aspects of the physiology of the experimental cretin-like animal". In this thesis he demonstrated that animals born to mothers fed 0.25% of the diet as thiouracil during pregnancy developed an ataxic and rigid gait about 35 days after birth. Animals fed 0.5% thiouracil developed similar signs earlier and those fed 1% thiouracil failed to walk and died from maternal cannibalism. Using the Nissl method of staining Barrnett showed that "nerve cells of the cretinoid animals" were at one day of age fewer in numbers, less well differentiated and more closely packed than in the controls.

In the 30 years, which have passed since the publication of Barrnett's thesis, a massive literature has emerged on the effects of thyroid deficiency on nervous system development. The vast majority of studies have dealt with the postnatal development of the brain, particularly the cerebellum, in the rat while relatively few studies have been devoted to the intra-uterine phase of possible thyroid hormone dependency.

In the rat three main methods of inducing experimental hypothyroidism have been employed, either (i) administration of anti-thyroid drugs, e.g. PTU, to mother (215), neonate (57) or mother and neonate (9), (ii) surgical thyroidectomy of neonate (241) or (iii) neonatal radiothyroidectomy (12, 132) by the method of Goldberg and Chaikoff (136). Intra-uterine thyroidectomy for technical reasons has not gained popularity in the rat. However this technique has recently been successfully employed both in sheep (105) and in the rhesus monkey (158).

In the early 1950s Eayrs and his colleagues from Birmingham, England used both techniques (i) and (iii) in their extensive studies (94-101). In their hands quantitative assessment of the size and development of neurones in the cerebral cortex revealed profound changes. Not only were the perikarya of cortical neurones smaller and more densely packed but the development of cell processes was also impaired. Their branching and peripheral extension were considerably reduced and the form of decay in the density of the dendritic field with distance from the perikaryon departed from that in normal rats. The developmental abnormalities were seen to be a distortion, rather than a retardation, of growth (98) resulting in a marked diminution of the probability of interaction between the axonal and dendritic components of the neuropil (96), this reduced "connectivity" causing a considerable degree of behavioural impairment (97).

In 1967 Geel and Timiras, taking advantage of Enesco and Leblond's 1962 formulae (103), determined the RNA and DNA concentrations in the cerebral cortex of 22 day old rats given 100 μCi I^{131} on day 1 (132). They showed that the DNA concentration was significantly increased while RNA, expressed as a ratio of DNA to give an average cellular concentration, was significantly lowered. The weight per nucleus, an index of cell size, was also reduced implying that the cortical cells were smaller and more closely packed. The decrease in RNA concentration coupled with the finding of a depressed cerebral protein turnover prompted Geel and Timiras (132) to suggest that impaired protein synthesis might underly the cerebral hypoplasia observed by Eayrs (94) and that "the altered structure of conducting elements which arises from neonatal hypothyroidism has a biochemical basis as a result of the participation of thyroid hormones in protein biosynthesis" (132).

In the following year, 1968, Balazs et al. (12) confirmed and extended these findings by estimating DNA, RNA and protein not only in cerebral cortex but also in whole cerebrum. They showed that at 35 days the weight of cerebral hemispheres in the hypothyroid rat was reduced by 20%. Although the DNA concentration was significantly increased at both 24 and 35 days, at no time did the DNA content in whole cerebrum differ from normal. By contrast, there were striking differences in the RNA and protein values at 35 days, whether expressed as ratios of DNA or as total cerebral content. At 35 days the RNA and protein content were reduced in cerebrum by 15% and 22% respectively. Balazs et al. (12) inferred that thyroid deficiency affected the size of cells in cerebrum rather than their total number. Since a reduction in protein synthesis was demonstrable despite apparently normal incorporation of labelled pyrimidine nucleotides into RNA (11) it was suggested that "in the

maturing brain thyroid hormones are mainly involved at the level of translation of the genetic message in protein" (12).

Thus, in the first twenty years of study of the role of thyroid hormones in brain development the state of knowledge had progressed from a histologic observation of closely packed cortical cells (14) to an implication that thyroid hormones were intimately involved in cellular protein synthesis (12). The five years which followed Balazs's paper were notable for the frequency of publications in this area. No attempt will be made to summarise these as adequate reviews have recently been published (13, 20, 121). Instead, the author will identify four areas of investigation which in the past three years have emerged as growth areas for study.

The first of these is the work reported by Holt et al. (159) on the effect of prenatal hypothyroidism on brain composition in a primate, the rhesus monkey. These workers injected I^{131} intravenously at 71-88 days of gestation into pregnant animals and delivered the foetuses at day 150 by caesarian section. Both mother and infant showed hypothyroxinaemia at delivery and DNA determination revealed that total number of cells and cellular density were normal in both cerebrum and cerebellum. By contrast, total RNA and total protein, were significantly decreased, strongly suggesting that RNA and/or protein synthesis had been affected. Moreover the non-morphologic measures of mean cell size (protein:DNA ratio) and cell volume (nonchloride space) supported these findings.

The second area of interest has been the work of Davenport et al. (57) on the timing of the critical period for the induction of maze learning deficit by thyroid deficiency. These workers performed direct comparison of the effects of prenatal-only, postnatal-only, and combined pre and postnatal thyroid deficiency produced by feeding rats varying doses of thiouracil-treated mash diet. In comparing combined prenatal and postnatal treatment with postnatal only they found that the greater deficit was to be found

in the combined group. In a second experiment they systematically manipulated the duration of thiouracil exposure in an attempt to determine the lower age boundary of the critical period for the induction of learning deficit. Their major finding was that the lower age boundary for the critical period was the 18th day of gestation i.e. at the time of onset of foetal thyroid function (133).

The third area has been the further investigation of the role of thyroid hormone in protein synthesis in the developing brain (270). In its role as "a prime mover in cerebral differentiation" (140) it is now believed that thyroid hormone acts to control the rate of several critical enzyme steps in the flow of encoded genetic information from storage in DNA to expression in structural and enzymatic proteins. Latest estimates suggest that thyroid hormones may function in at least four basic ways during transcription and translation viz. (i) by affecting RNA polymerase II which assembles messenger RNA; (ii) by affecting transfer RNA (tRNA) sulfurtransferase which confers codon specificity on tRNA; (iii) by affecting release of nascent polypeptide chains from ribosomes and (iv) by affecting the activity of thymidine kinase and other enzymes which assemble nucleotides (122).

Lastly there have, in the past year, been attempts to define by new histological and biochemical techniques the basis of reduced neuronal connectivity in experimental hypothyroidism. Sanchez-Toscano et al. (241) have studied the effect of postnatal thyroidectomy on the number and the pattern of distribution of spines along the apical shaft of pyramidal neurones in the cerebral cortex. They have found both a reduction in the number of spines/segment and also evidence of abnormal development in the distribution of the spines along the shaft. From a more biochemical viewpoint, Francon et al. (122) have shown that hypothyroidism produces a decrease in the concentration within the developing rat brain of an initiator of

microtubule assembly. They considered that thyroxine probably represents "a regulatory signal for neurotubule assembly" and, since the process of microtubule assembly is fundamental to axonal growth and the formation of nerve connections, that the results of their work "might contribute to the understanding of the effects of thyroid hormone in brain development and in the aetiology of cretinism" (122).

Studies Performed

(a) Introduction

Having chosen the albino rat as appropriate for experimentation and having achieved iodine depletion by dietary means the next step for the author was to acquire techniques for evaluating the development of the brain in animals exposed prenatally to iodine deficiency. Three planning decisions had to be made early on:

- (i) When to sacrifice the offspring?
- (ii) Which part of brain to sample?
- (iii) How to quantitate "development"?

The first decision made was to sacrifice the animals either as neonates or as weanlings. As indicated in the earlier part of this chapter the time of birth in the rat marks the end of major neuronal multiplication while by day 20 postnatally DNA replication in brain has ceased. A day 22 brain is therefore considered both chemically and histologically to be "mature".

With regard to the anatomical dissection of brain, since this project was designed to investigate a possible cerebral lesion, it was necessary to sample the cerebral hemispheres. The area of brain called "forebrain" by Donaldson (86) and Dobbing (79) and whole cerebrum (163) or cerebral hemispheres (301) by others was therefore chosen for study.

Lastly, it was clear from the review of the neurobiological literature that the most relevant approach to the present problem was

a cellular biochemical one such as employed by Winick (290), Dobbing (78) and particularly Zamenhof (298). Because the types of experiments planned by the author to test his hypothesis were similar to those pioneered by Zamenhof and colleagues it was decided to analyse the cerebral tissue in a biochemical manner exactly similar to these workers.

(b) Materials and methods

Animals and diets

Female virgin Wistar rats weighing 150-200 g were employed in these studies. The animals were maintained on the colony diet described in Chapter V and were given deionised distilled water ad libitum to drink. After four weeks on diet all animals had vaginal smears and those animals found to be in the pro-oestrous or oestrous phase were mated. The pregnancies were allowed to continue to term and the offspring were sacrificed by decapitation either as neonates (within 6 hours of birth) or on postnatal day 22 as weanling pups (weaned at day 21). In one particular litter three pups were also sacrificed at days 5 and 10 postnatally.

Dissection of forebrain

As Howard has pointed out, "the whole cerebrum can be isolated from the brain stem by a transection at a precisely defined position near the colliculi, an anatomical level at which the area of the cut surface is small in relation to the area of the cerebrum, and hence operational variability in the weight of the tissue isolated will be a small fraction of the total weight" (163). The precisely defined points of transection were those originally described by Donaldson and Hatai (86) and thereafter employed by Zamenhof (295). The method of dissection employed by the author is illustrated by Fig. 18.

After decapitation the cerebral hemispheres without cerebellum, olfactory bulbs and brain stem (301) were rapidly removed and

immediately weighed in a plastic storage tube. The forebrain preparation was then snap-frozen in dry-ice and stored at -20°C pending analysis.

Forebrain DNA and protein estimation

To allow comparability with reported studies the author planned to perform the biochemical assessment of development as per Zamenhof's group. To extract the brain tissue these workers employ the Margolis adaptation (198) of Zamenhof's modified Schneider procedure (296), and subsequently they determine total DNA by a modified Burton procedure (25, 296) and total protein by a modified Lowry technique (31, 194). An outline of the scheme employed by Zamenhof and subsequently by the author is given in Fig. 19. The three main steps in this scheme viz. brain extraction, DNA assay and protein determination, will be considered individually below while detailed instructions for the day-to-day methods employed by the author are given in the appendices which follow the main text.

- (i) Extraction of forebrain: cold acid extraction of the brain homogenate with 6% trichloroacetic acid (TCA) was followed by two hot acid extractions at 70°C using 1M perchloric acid (HClO_4). A third hot extraction accounted for less than 1% of the total DNA and was therefore not employed. In the technique described in Appendix II the main limitation was the maximum capacity of the centrifuge head which was restricted to six tubes. As a result it was feasible only to extract six brains in any one morning or afternoon and for that reason the methods in Appendix II refer particularly to "six brains".
- (ii) DNA Assay: the first step in setting up the assay was to adjust the concentration of the extracts to a final one of 0.5M HClO_4 . All samples were then set up in triplicate and incubated for 16-18 hours with the Burton's reagent to which had been added acetaldehyde on the day of assay.

The method was dependent on the reaction of purine-bound deoxyribose with diphenylamine to produce a blue colour which could be quantitated by spectrophotometry (205). With this technique the optical density (O.D.) peak for DNA was at 600 nm. However, because of impurities and dirt (tubes, glass) a slight shift was often seen. To eliminate these problems readings were taken at two separate wavelengths and for calculation purposes the difference in O.D. reading between 610 and 650 nm was employed (296).

(iii) Protein determination: in this method the final colour developed in two stages viz. (1) reaction of the peptide bonds in the protein with copper in the alkaline solution and (2) reduction of the Folin reagent by tyrosine and tryptophan residues in the protein (31). The calibration curve was found to be linear with protein concentrations between 0 and 70 μg per tube. Adjustments in the dilution with 1N NaOH (step 8 of method in Appendix II) were made in order that with brains more concentrated in protein than those of neonates the standard curve would lie within this range.

(c) Results

Familiarity with the techniques described above and in the Appendices were first obtained with neonatal material. Subsequently the method was adapted as described in the protein determination section to allow analysis of weanling material. In the analysis of brains older than 10 postnatal days the forebrains were divided into two hemispheres prior to storage. Subsequent analysis was made of each hemisphere separately and the two results combined to provide a value for the whole forebrain.

Total DNA content

As outlined in Appendix II the process from brain extraction to calculation of DNA (μg), although requiring only 5 hours of work, took more than a day because of the requirement for a 16-18 hr incubation. However, since the DNA content of acid extracts stored at 4°C was

found to be stable up to 48 hrs it became routine practice to accumulate up to 18 (3 x 6) brains for incubation in one batch. A sample of standard DNA solution was always hydrolysed in the last extraction prior to setting up the Burton assay and used for calibration purposes.

A typical standard curve is shown in Fig. 20. On this occasion five standards were employed but S_1 - S_3 were the standards used routinely. Using these methods the total DNA content of neonatal forebrain was, in early experiments, found to be $582 \pm 24 \mu\text{g}$ ($n = 20$) which corresponded very well to the values of 544 ± 20 and $518 \pm 33 \mu\text{g}$ reported by Zamenhof's group in 1968 (298) and 1973 (301). For 22 day old forebrain material a figure of $844 \pm 78 \mu\text{g}$ was obtained. This value is not significantly different from that obtained by Zamenhof et al. (301) for 30 day cerebrum ($913 \pm 52 \mu\text{g}$) although somewhat less than the value reported by Sobotka et al. (250) for 22 day old telencephalon ($1240 \pm 50 \mu\text{g}$).

Total protein content

In contrast to the stability of the acid extracts it was found that the brain pellets hydrolysed by 1N NaOH required analysis within 24 hours. Where two extractions were performed on one day twelve brains (2 x 6) were analysed on the afternoon of the subsequent day. As indicated in the methods section, where more mature brains were analysed, dilutions of the alkaline extract were performed in order to allow work at protein concentrations within the linear portion of the calibration curve. A typical curve is shown in Fig. 21.

Using this method the neonatal forebrain protein content varied between 7 and 8 mg. These values were somewhat less than those of 8.8-9.7 mg reported by Zamenhof et al. (301), this difference also being seen in the 22 day old material where values between 47 and 55 mg contrasted with reported values (301) at 30 days of $80 \pm 7 \text{ mg}$.

Postnatal growth and development

Employing the techniques described analysis was made of brains from a litter of 12 pups where 3 animals were sacrificed at days 0, 5, 10 and 22. The brains were weighed immediately prior to storage at 4°C, subsequently analysed for content of total DNA and protein and a ratio of total protein to total DNA (mg:mg) derived from the individual assay values. The results obtained from this experiment are illustrated by Fig. 22 which shows the mean values for wet weight, total DNA, total protein and protein:DNA ratio.

The most striking change in the first three postnatal weeks is the sigmoid-shaped change in forebrain weight. This curve is closely paralleled by the total protein content which contrasts with the unimpressive change in DNA content. Taking total DNA as an index of cell number and total protein or protein:DNA ratio as indices of cell size it is clear that the suckling period in the rat is notable for an increase in cell size in the forebrain without a concomitant increase in cell number i.e. this period is marked by a predominant cellular hypertrophy.

The change between birth and day 22 is further illustrated by Table 10 where the mean values for wet weight and chemical composition are compared. It is of interest to see how closely the increasing protein content is related to the increase in forebrain weight and also how the increase in cell size (approximately 300-600%) contrasts with the meagre increase (36%) in cell number during the same period.

Discussion

By employing methods for forebrain DNA and protein estimation adapted from those of Zamenhof et al. (301) it was possible for the author to use the assayed values of DNA and protein as parameters of perinatal rat brain development. In the next two chapters the effects of prenatal and postnatal iodine deficiency, produced as

described in Chapter V, on both the neonatal (Ch. VII) and weanling (Ch. VIII) brain will be considered, the emphasis being placed on possible alterations in the number and size of "brain cells" involved.

PART C

THE MODEL IN PRACTICE

CHAPTER VII

EFFECTS OF PRENATAL IODINE DEFICIENCY ON FOREBRAIN DEVELOPMENT

Introduction

This chapter will describe the results of four experiments designed to investigate the effect of varying degrees of prenatal iodine deficiency on neonatal forebrain development. The four experiments shared a common experimental design, the only significant variable being the duration of exposure to iodine deprivation. The experimental design is shown schematically in Fig. 23 where the descriptions of mild, moderate and severe refer to the categories of iodine deficiency defined in Chapter V (see Fig. 14).

Experiment 1: Effects of LID from mating until parturition

(a) Experimental design

In this pilot study the offspring of two rats fed low iodine diet (LID) from mating were compared with the offspring of two mothers on colony diet. The aim of the experiment was to establish ground-rules for the 4, 10 and 26 weeks studies and also to assess the unlikely possibility that the degree of iodine deficiency produced by LID introduced at mating was capable of being teratogenic to the developing nervous system.

Six mature female virgin Wistar rats were employed in the experiment. All animals were maintained until mating on a colony diet (700 $\mu\text{gI/kg}$). At the time of mating three animals were chosen to be the test group and their diet was switched to LID II which contained about 70 $\mu\text{gI/kg}$. All six animals were mated in the oestrous phase and the presence of a copulation plug was considered suggestive evidence of successful mating. The pregnancies were allowed to continue to term and the pups allowed to deliver naturally.

At birth the offspring were examined for gross deformities and weighed. Sacrifice by decapitation was performed within 6 hours of birth and the forebrain dissected from the cranium as fully described in Chapter VI. After weighing in storage tubes the forebrains were snap-frozen in dry ice and stored deep frozen to await analysis of DNA and protein content. When chemical analysis was performed by the methods described in Appendix II an equal number of control and test brains were included in the procedure to minimise inter-assay variation.

On the day of parturition the mothers were bled from the tail vein to provide samples of sera for estimation by RIA of serum thyroid hormones. On day 1 post-partum the mothers were injected intra-peritoneally with 0.5 μ Ci of I^{131} and 4 hours thereafter were sacrificed with ether anaesthesia. After sacrifice the thyroids were carefully removed and rapidly weighed. Thyroidal RAIU was measured by gamma counting and expressed as the percentage of the administered dose localising in the thyroid.

(b) Results

Litter size and body weights

Of the six animals mated four became pregnant. Two control and two test litters, consisting of 19 (12 + 7) and 14 (10 + 4) pups respectively, were produced. Although the test pups tended to be heavier the difference was not significant (Table 11). However, when male pups were analysed separately (Table 12) it was found that these test pups were significantly heavier ($p < 0.01$).

Forebrain weight and chemical composition

Taken as a group of 14 the absolute weight of forebrain in test animals was significantly greater than controls ($p < 0.005$). This difference was due to heavier test male pups (Table 12) and was not seen in the females. When expressed in relation to the body weight the differences between test and control animals disappeared (Tables 11 and 12).

Analysis of DNA and protein content was performed in all 33 forebrains. Table 13 shows that no significant difference existed between the two groups as regards total DNA, total protein and protein: DNA ratio.

Maternal thyroid function

The LID introduced at mating resulted in goitrogenesis and a significant rise in 4 hr RAIU (Table 14). However, the degree of iodine depletion was insufficient to cause a lowering of either the mean serum T_4 or T_3 (Table 15).

(c) Discussion

This pilot experiment showed that LID introduced at mating had no apparent effect on maternal fertility and did not cause any gross anatomical/teratogenic deformities in the offspring. Moreover the degree of iodine depletion attained, although adequate to cause a significant goitre and to increase maternal avidity for radioiodine, was insufficient to affect any alteration in either the weight or chemical composition of the neonatal forebrain (cerebrum).

Experiment 2: Effects of LID introduced 4 weeks prior to mating.

(a) Introduction

This experiment was designed to study the effect of feeding LID for a period of one month prior to mating on the outcome of pregnancy with particular reference to litter size, individual pup body weight and forebrain size and composition. Additionally the experiment was designed to correlate the above effects with maternal thyroid function as assessed by goitre size, radioiodine uptake and circulating thyroid hormones. The effects of pregnancy on serum T_4 and T_3 in both test and control mothers will be considered in greater detail in Chapter IX. The results section below will, however, consider the results of thyroid function tests performed both after one month of LID and at the end of experiment.

(b) Experimental design

Thirty virgin female Wistar rats weighing 150-200 g at commencement of experiment were employed. The animals were divided into two groups: 15 receiving ad libitum low iodine diet and deionised distilled water (test group) and the other 15 (control group) receiving the same diet and deionised water supplemented with $0.1 \mu\text{g}$ of elemental iodine, as potassium iodide, per ml (estimated intake 20-30 ml water daily). Each animal was weighed at commencement and thereafter weekly to the end of experiment.

After 4 weeks on the LID all animals had vaginal smears and those animals found to be in the pro-oestrous or oestrous phase were mated, each test mating being matched with a control. Those 10 animals found to be in least favourable phase of oestrous, 5 from each group, were bled for serum thyroid hormones, injected with radioiodine and 4 hours thereafter sacrificed. Thyroidectomy was rapidly performed, thyroids weighed and RAIU measured by gamma counting. Serum T_4 and T_3 were subsequently determined by specific radio-immunoassay.

Fifteen days after mating all 20 animals were bled from the tail vein for circulating thyroid hormone levels. The pregnancies were allowed to proceed to term and those animals not delivering spontaneously by day 23 were sacrificed and their pups delivered by uterotomy. All animals who gave birth naturally were bled for thyroid hormones within six hours of parturition. On day 1 postnatally all mothers were injected with radioiodine and 4 hours later sacrificed, this procedure also being carried out on those animals who were not found to be pregnant.

Pups were removed from their mothers within six hours of birth and individually weighed. Immediately after weighing they were sacrificed by decapitation and the cerebral hemispheres without olfactory bulbs, cerebellum or brain stem were rapidly removed and weighed. The forebrains were stored deep frozen to await

subsequent chemical analysis. When analysis was performed an attempt was made always to include an equal number of test and control brains in any one assay batch.

Results are expressed below as mean values \pm one standard deviation (S.D.). Statistical analysis was performed using standard Student's t test. Because of the spontaneous variability between litters known to occur in experiments involving manipulations of pregnant rats (163) litter means were calculated and used as the units for computation of the standard deviations as recommended by Abbey and Howard (1).

(c) Results

Growth rates on diet

Both test and control animals were weighed weekly throughout the experiment. The mean body weights during the first four weeks on LID are shown in Fig. 24. The growth rate was identical in both groups of animals.

Litter size and perinatal mortality

Of the 20 animals mated five control and six test animals proved to be pregnant. One litter from each group had to be delivered by uterotomy on day 23 but statistical analysis of newborn data was restricted to these litters naturally born on day 22.

The effect of LID, with or without iodine supplementation, on litter size and perinatal mortality is shown in Table 16. Of the 87 liveborn pups 57 were born to test animals and 30 to control. There were only 5 stillborn pups, all being born to the one control mother. Of those two litters which were postmature both were small in number, the test litter having one and the control four pups.

Body and forebrain weights

The weights of body and forebrain were determined for each individual pup and the relative forebrain weight was calculated. The average value for each litter was then derived from the individual values and statistical comparison made between the litter means

(Table 17). No significant differences in body weight, forebrain weight or relative forebrain weight were found between the control and test litters.

Forebrain chemical composition

The results of chemical analysis of both control and test brains are shown in Table 18. No significant difference was found between the two groups as regards total DNA, total protein and protein:DNA ratio.

Changes in thyroid function

Both in vitro and in vivo measurements of thyroid function were made at 4 and at 7 weeks after commencing diet. At 4 weeks, i. e. at a time just prior to mating, the test animals showed significant increases in thyroid weight and were markedly avid for radioiodine (Table 19). The iodine depletion was sufficient to lower the serum T_4 by 23% but the mean T_4 level still remained within the normal range. The serum T_3 was not significantly changed at 4 weeks.

At the end of the experiment, i. e. at 7 weeks after commencing diet, the changes in goitre size and RAIU shown by test animals were more marked. The serum T_4 level had further fallen to a mean level of $2.5 \mu\text{g}/100 \text{ ml}$, i. e. the lower limit of the normal range. Although T_3 was lower than at 4 weeks in both groups no significant difference existed between control and test animals (Table 20).

Both at 4 and at 7 weeks the 4 hour RAIU in control animals, although significantly less than in test animals, was elevated when compared to the basal figure of $4.88 \pm 2.16\%$ reported in Chapter V from animals maintained on colony diet with a high iodine diet.

Changes of pregnancy

The changes seen in both T_4 and T_3 levels during pregnancy are shown in Fig. 25. The mean T_4 level in test animals fell by 42% to a figure of $1.8 \pm 0.9 \mu\text{g}/100 \text{ ml}$ which is below the normal lower limit. A similar fall of 26% was seen in the control pregnancies

although the mean T_4 in the post-partum period was not outwith the normal range. No difference was noted in control T_3 but in test pregnancies, although the post-partum T_3 was not abnormally low, there had been a fall in mean levels during pregnancy of 42%.

(d) Discussion

Four main conclusions were drawn from Experiment 2.

(1) The mild iodine deficiency produced by 4 weeks feeding with LID did not adversely affect the fertility of test animals who produced litters of normal size with no increased incidence of stillbirths or postmaturity.

(2) Mild iodine deficiency produced by feeding LID for 4 weeks prior to mating and throughout gestation had no effect on the intra-uterine development of forebrain as measured by brain weight or content of DNA and protein.

(3) Iodine supplementation at a level of $0.1 \mu\text{gI/ml}$ drinking water was adequate to maintain normal circulating thyroid hormone levels and a normal thyroid weight but appeared inadequate to maintain a normal thyroid radioiodine uptake. As a result control animals in all subsequent experiments received $1 \mu\text{gI/ml}$ water, i.e. a ten-fold increase in iodine supplementation.

(4) During pregnancy a fall in serum T_4 level occurred. This was especially marked in test pregnancies but could also be seen in controls. With the degree of iodine depletion produced by feeding LID for 4 weeks prior to mating and throughout gestation the test mother, who at mating showed a normal but lowered T_4 level, developed during the course of pregnancy a significant degree of hypothyroxinaemia.

Experiment 3: Effects of LID introduced 10 weeks prior to mating.

(a) Introduction

This experiment had two main aims: (i) to study the effect of moderate iodine deficiency on forebrain development in the neonate

and (ii) to further examine the fall in thyroid hormone levels seen during pregnancy. It was estimated from the preliminary work of Chapter V that by 8 weeks on LID the mean T_4 level would be at the lower limit of normal and thus approximately half of the test animals would by that time be hypothyroxinaemic. To achieve the first aim it was planned to mate only those animals whose T_4 levels had fallen by 8 weeks to less than $2.5 \mu\text{g}/100 \text{ ml}$. As regards the second aim, animals both pregnant and nonpregnant were bled at times equivalent to days 8 and 15 of pregnancy. The detailed results of this second aspect will be further considered in Chapter IX.

(b) Experimental design

Eighty virgin female Wistar rats weighing 150-200 g were used in Experiments 3 and 5 which commenced simultaneously. The animals were divided into a test group of 40 animals receiving LID and deionised distilled water and a control group who received the same diet and water containing $1 \mu\text{gI}/\text{ml}$. All animals were weighed weekly throughout the experiment and were bled from the tail vein for thyroid hormone RIA at 8 weeks. On the basis of the serum T_4 level at 8 weeks test and control animals were selected for mating. For Experiment 3 ten test animals were chosen from those who showed hypothyroxinaemia ($<2.5 \mu\text{g}/100 \text{ ml}$) while ten control animals were picked at random from those with normal T_4 levels after 8 weeks on diet supplemented by iodised drinking water.

All twenty selected animals were mated after 10 weeks on diet. Mating was performed in the oestrous phase, one female being exposed to a single male for the duration of one night. After mating the animals were divided into one group of ten, who were to be bled for serum thyroid hormones 8 days after mating, and another ten who would be bled at 15 days. Those animals who were mated but did not conceive were bled 22 days after mating and sacrificed on the following day after injection with $0.5 \mu\text{Ci}$ of radioiodine. In all other respects

the later details of this study closely resembled those described above for Experiment 2.

(c) Results

Growth rates on diet

The results of mean body weights at commencement and after 5 and 10 weeks of diet are shown in Table 21. No difference existed between the two groups, implying an identical growth rate independent of the degree of iodine deficiency.

Changes in thyroid function

The results of the determination of serum T_4 in all 80 animals at 8 weeks are shown in Fig. 26. The broken line denotes the lower limit of normal and is seen to divide almost equally the test animals into those with normal levels and those with frank hypothyroxinaemia. Also shown in the figure are the mean values of the 20 animals chosen for mating in the present study.

Table 22 shows the results of serum T_3 and T_4 and T_3/T_4 ratio in animals fed LID for 8 weeks. The test animals had on average lower T_4 and higher T_3 levels and as a result higher T_3/T_4 ratios. The difference between the groups was more striking in the animals selected for mating where test T_4 was reduced by 47% while T_3 was significantly higher, resulting in a T_3/T_4 ratio more than twice normal (Table 23).

Mated but infertile animals had both in vivo and in vitro parameters of thyroid function assessed after 13 weeks on diet (Table 24). Thyroid weight was significantly increased in test animals while RAIU estimates confirmed the adequacy of the $1 \mu\text{gI/ml}$ supplementation. Serum T_4 in test animals was significantly lowered to a mean level of $1.9 \mu\text{g}/100 \text{ ml}$. Biochemical euthyroidism, however, was maintained by a high normal level of serum T_3 .

Litter size and postmaturity incidence

Of the ten litters born as a result of the twenty matings six

arrived on day 22, two on day 21 and two were delivered by uterotomy on day 23 (Table 25). The average litter size was similar in both groups, being 5 in controls and 6 in test litters. No difference was found in the incidence of postmaturity between the two groups.

Body and forebrain weights

These parameters were considered both for all non-postmature neonates (born days 21 and 22) and also for those naturally born on day 22. The test animals in both groups were shown to have smaller body and absolute forebrain weights. However, statistically, the only significant difference was in the forebrain weight for all neonates (Table 26) where the mean value for test litters was 16% less than controls. The fact that this difference was no longer significant when only the day 22 litters were considered (Table 27) was due to the exclusion in the latter group of the premature test litter which had a mean forebrain weight of 124.76 mg, i. e. 20% less than the mean for day 22 test litters.

Cell number and cell size

Test neonates showed no evidence of reduction in forebrain cell number as measured by total DNA. However, in both statistical groups analysed (Tables 28 and 29) the test animals showed a significant ($p < 0.05$) reduction in forebrain protein content. In the face of a constant DNA value the protein:DNA ratio, an index of cell size, was reduced by 11% (Table 30) while in the same test pups the forebrain weight:DNA ratio, another index of cell size (289) was reduced at a significance level of $p < 0.001$.

Changes in pregnancy

The mean levels of both serum T_4 and serum T_3 in successfully mated animals after 8 weeks of diet and within 6 hours of parturition are illustrated in Fig. 27. As in Experiment 2 there was a significant drop in T_4 shown by both control and test animals. In the test group there was a 60% fall to an almost undetectable mean level

of $0.8 \mu\text{g}/100 \text{ ml}$. This was paralleled in controls by a 41% fall in mean T_4 which by day 22 had reached the lower limit of the normal range viz. $2.5 \mu\text{g}/100 \text{ ml}$. Similar significant falls in serum T_3 were seen in both groups during the period of pregnancy. The percent fall in test animals was 45% as compared to 34% in controls. By day 22 neither group showed a mean T_3 below the normal lower limit and thus the animals could still be considered in biochemical "euthyroidism".

(d) Discussion

The main conclusion to be drawn from this experiment was that moderate maternal iodine deficiency, although having no effect on forebrain cell number as measured by total DNA, did apparently significantly diminish the forebrain content of total protein and as a consequence lowered indices of mean cell size such as protein:DNA and weight:DNA ratios. Since in the rat prenatal cerebral growth was almost entirely neuronal it was implied that, without any effect on neuronal multiplication, a moderate degree of prenatal iodine deficiency might in the rat result in a reduction in size of cerebral neurones.

Experiment 4: Effects of LID introduced 6 months prior to mating.

(a) Experimental design

Sixty virgin female Wistar rats weighing 150-200 g at commencement were employed in this experiment. The test group of 30 animals received LID and deionised distilled water while the control animals fed on an identical diet but received iodine supplement as $1 \mu\text{gI}/\text{ml}$ drinking water. All animals were bled for thyroid hormone estimation after 24 weeks on diet. The ten test animals with the lowest T_4 levels were chosen for mating as were ten control animals picked at random from those with normal T_4 levels after 24 weeks.

All twenty selected animals were mated after 6 months on diet. Mating was performed in the oestrous phase, one female being exposed

to a single male for the duration of one night. To avoid increasing maternal morbidity during pregnancy no attempt was made to take maternal blood samples at days 8 and 15. Instead the mothers were bled within 6 hours of parturition and sacrificed after injection with radioiodine on the following day. The dissection, storage and analysis of forebrain were exactly as described for Experiment 2.

(b) Results

Selection for mating

The serum T_4 in test animals fell by 49% after 24 weeks on diet to a mean level of $2.1 \mu\text{g}/100 \text{ ml}$ (Table 31). By contrast serum T_3 in test animals remained normal allowing the maintenance of biochemical euthyroidism. In those animals selected for mating the mean serum T_4 in test animals was 32% of that in controls. No significant difference, however, was found between the groups as regards serum T_3 concentrations (Table 32).

Litter size and perinatal mortality

Of the twenty animals mated only 3 control and 3 test animals produced litters. Test litters were on average no smaller in number than controls and both groups showed a high incidence of stillborn pups (Table 33). Nineteen of the 29 test pups had apparently died in utero shortly below parturition and this high incidence contrasted with a 5% incidence of intra-uterine death in control litters.

Forebrain size and chemical composition

No significant differences were found between the test and control groups as regards either body or forebrain weight (Table 34). However, although not having an effect on cell number as measured by total DNA, the severe prenatal iodine deficiency did decrease the content of protein by 13% resulting in a decrease of 18% in protein:DNA ratio (Table 35). Thus, as in Experiment 3, it appeared that a severe degree of maternal iodine deficiency was associated in the forebrain with a reduction in cell size without a concomitant effect on cell number.

Thyroid function and pregnancy

Test mothers sacrificed in the post-partum period showed significant increases in RTW and RAIU and frankly low mean values of both serum T_4 and T_3 (Table 36). During the period of pregnancy the T_4 dropped in controls by 43% and in test animals by 33% (Fig. 28). At the same time the mean T_3 level dropped in the control and test animals by 52 and 70% respectively.

(c) Discussion

The degree of iodine depletion achieved in this experiment was adequate to lower the serum T_4 by 68% to a mean level of $1.5 \mu\text{g}/100 \text{ ml}$ but this significant hypothyroxinaemia was not associated at the time of mating with any lowering of mean serum T_3 level. Pregnancy provided an added strain on the already severely depleted iodine stores and apparently resulted in a significant lowering of T_3 to a mean level in test animals during the puerperium of $33 \text{ ng}/100 \text{ ml}$ which is outwith normal limits. Thus, in the severely iodine deficient animal during the later stages of pregnancy it appeared that T_3 compensation would eventually fail if the degree of iodine depletion prior to mating was sufficiently extreme.

With regard to forebrain development it appeared that, as seen in the study of moderate iodine deficiency (Experiment 3), feeding with LID for a period of time long enough to cause hypothyroxinaemia prior to mating did not result in any decrease in forebrain cell number but did significantly decrease the total protein content implying a significant reduction in the size of cerebral neurones and, as a result, a limited capacity for development of an extensive dendritic tree, so essential for the establishment of inter-neuronal connections.

Conclusions from Experiments 1-4

The four experiments described above were designed primarily to examine the possibility that severe maternal iodine deficiency might cause in the developing rat forebrain an inhibition of neuronal

multiplication. A secondary aim of the experiments was to investigate the effect of iodine deficiency on the changes in thyroid function seen during pregnancy in the rat.

The experiments provided no evidence that maternal iodine deficiency resulted in infertility. However, as the degree of iodine deficiency worsened so did the incidence of perinatal mortality, particularly the number of intra-uterine deaths in the severely iodine deficient litters.

Analysis of forebrain DNA content effectively ruled out any significant effect of maternal iodine deficiency on neuronal multiplication as measured by cell numbers (total DNA) in neonates. An unexpected finding, however, was that the degree of prenatal iodine deficiency sufficient to cause hypothyroxinaemia at the time of mating resulted in a significant reduction in forebrain protein content suggesting that severe maternal iodine deficiency could cause a reduction in the average size of cerebral neurones.

Another unexpected finding was that pregnancy apparently caused a fall in serum T_4 and T_3 not only in test animals but also in controls. In the most severe degree of iodine deficiency studied, that produced by feeding LID for six months prior to mating, the serum T_3 was also lowered by the time of parturition and therefore at that time the test animals could be considered frankly hypothyroid.

CHAPTER VIII

EFFECTS OF COMBINED PRENATAL AND POSTNATAL IODINE DEFICIENCY ON FOREBRAIN DEVELOPMENT

Introduction

In the previous chapter it was shown (Experiments 3 and 4) that a moderate to severe degree of maternal iodine deficiency caused a reduction of forebrain protein content in the newborn. The experimental work to be described in this chapter was aimed at discovering whether this qualitative change in forebrain composition was permanent or whether it was reversible in the postnatal suckling period. Accordingly study was made of weanlings whose mothers had been fed LID for 10 weeks prior to pregnancy until the time of weaning (postnatal day 21).

Experiment 5: Effects of LID introduced 10 weeks prior to mating, continued through pregnancy and until postnatal day 22

(a) Experimental design

The design of this experiment from mating until parturition was identical to that described for Experiment 3. On the day of delivery litters were identified where the number of pups exceeded or equalled 8. On day 1 those litters in excess of 8 were culled to that number. The litters of 8 were nursed by their natural mothers and were separated only at the time of weekly weighings. Throughout the first 21 days of the pups' lives test and control mothers continued on LID and deionised or iodised drinking water respectively.

On day 21 the pups were removed from their mothers and individually caged. The mothers were bled for serum thyroid hormone estimation and then sacrificed 4 hours after injection of radioiodine. On day 22 the pups were allocated either to be injected with radioiodine or bled for serum thyroid hormone estimation.

Those pups who were injected with ^{131}I were sacrificed using ether anaesthesia to allow the careful removal of thyroids while animals selected for thyroid hormone estimation were sacrificed by decapitation. Forebrains were dissected as previously described and stored deep frozen prior to chemical analysis.

(b) Results

Selection of pups

From the twenty animals mated six suitable litters (8 pups/litter) were produced. During the first postnatal week 4 test and 5 control pups were eaten by their mothers and thus by weaning only 39 pups (20 test and 19 control) were available for study.

Postnatal growth of pups

At birth the mean body weight of test pups was 6% lower than controls (Table 37). However, in the first postnatal week the test animals grew 30% more than controls (Fig. 29) and by day 21 were no longer significantly different in weight from the controls.

Body and forebrain weights

Although body and absolute forebrain weights were no different in test and control pups at day 22 the mean relative forebrain weight was 11% less in test animals than in controls (Table 38). Male pups had significantly heavier weights of body and forebrain than females (Table 39). Test males also showed a significant reduction in relative forebrain weight (Table 40).

Forebrain chemical composition

No significant differences in total DNA, total protein or protein:DNA ratio were found between test and control weanlings whether both sexes (Table 41) or male pups only (Table 42) were considered. No sex difference was noted in test pups as regards either DNA or protein content (Table 43).

Maternal thyroid function

When sacrificed at weaning the test mothers showed increases in thyroid weight and RAIU when compared to control (Table 44).

Changes occurred during the experiment in both serum T_4 and T_3 and these are illustrated by Fig. 30.

Control and test mothers showed a similar pattern of change in T_4 concentrations. In controls the T_4 dropped during pregnancy from 4.2 to 1.9 $\mu\text{g}/100\text{ ml}$ while in test mothers the drop was from 2.3 to 0.8 $\mu\text{g}/100\text{ ml}$. However, after delivery there was an apparent recovery period and serum T_4 rose in both groups to achieve levels at weaning comparable to those seen prior to mating (Table 45).

During pregnancy the serum T_3 dropped in control and test mothers by 54 and 42% respectively. During the period of lactation a trend upwards was seen in both groups. However, in contrast to the restoration of T_4 levels, the upward trend in T_3 was not significant and the T_3 levels at weaning remained lower than those seen prior to mating (Table 46). Moreover, when compared to unsuccessfully mated animals fed LID for 16 weeks under identical conditions the serum T_3 in both control (Table 47) and test (Table 48) nursing mothers was significantly lowered ($p < 0.005$) at the time of weaning.

Weanling thyroid function

The results of thyroid function assessment in 22 day old weanlings are shown in Table 49. Test pups showed significant increases in goitre size, RAIU and serum T_3 concentration. Surprisingly no difference existed between the two groups as regards the serum T_4 concentration the mean of which was 5 $\mu\text{g}/100\text{ ml}$ in both.

Comparing the results of serum T_4 and T_3 and 4 hour RAIU in control mothers and their pups no differences were found (Table 50). However, when test animals were compared it was clear that the test pups achieved a normal serum T_4 level despite the continuing presence of significant maternal hypothyroxinaemia. Moreover at the time of weaning the mean serum T_3 in test pups was significantly higher than that seen in their mothers (Table 51).

(c) Discussion

Although the test pups were lighter at birth they soon caught up with controls due to a marked body growth spurt in the first postnatal week. By day 22 there was no difference in body weight between the two groups although the relative forebrain weight in test pups was significantly less.

The most striking finding of this experiment, however, was that no difference existed between test and control brains as regards chemical composition. In particular the findings in Experiments 3 and 4 of a reduction in protein content were not confirmed in the day 22 forebrain. It therefore appeared that during the period of lactation it was possible for the forebrain cells to "fill up" (291) with protein suggesting that the neonatal abnormality was reversible.

The period of lactation was also marked by a restorative phase in serum T_4 since, in both control and test mothers, although the T_4 dropped during pregnancy, it progressively increased between birth and weaning to achieve levels at day 21 comparable to those found prior to mating. This tended to imply that during pregnancy there was an added strain on maternal iodine stores resulting primarily in a drop in serum T_4 but also, to a lesser extent, in a decrease in serum T_3 levels. Interestingly, despite the recovery of T_4 levels the mean serum T_3 in both groups of mothers remained low during the period of lactation.

Another finding of interest was that, despite the continuing significant difference in T_4 level between test and control mothers (2.1 as against 4.3 $\mu\text{g}/100\text{ ml}$), no difference existed between the pups of these mothers as regards serum T_4 which was 5 $\mu\text{g}/100\text{ ml}$ in both groups. This contrasted with the serum T_3 and 4 hour RAIU which were significantly increased in the test weanlings. Thus it seemed that, independent of maternal iodine status, the test pups

were able, possibly due to the activity of the iodide trap, to maintain normal levels of circulating thyroid hormones.

The overall conclusion from Experiment 5 therefore was that the strain of pregnancy, which effectively increased the degree of iodine deficiency prior to term, was removed as a result of delivery and in the ensuing three postnatal weeks, during the period of maternal lactation, not only were the pups able to compensate for reduced iodine intake but also seemed able to overcome the forebrain protein deficit seen in the neonate.

CHAPTER IX

EFFECTS OF PREGNANCY AND IODINE DEFICIENCY ON MATERNAL THYROID FUNCTION

Introduction

From the results described in Chapter VII it was clear that during pregnancy in the rat the circulating levels of total T_4 and total T_3 dropped. The changes in both control and test mothers are summarised in Fig. 31. In the case of the iodine sufficient (control) mothers the T_4 level dropped towards the lower limit of the normal range. However, in the situation of significant maternal iodine deficiency (Experiments 2-4) the drop in T_4 resulted in significant hypothyroxinaemia during pregnancy. By contrast no differences in T_3 levels existed between test and control mothers except when the degree of iodine deficiency prior to mating was particularly severe.

The purpose of the experiments to be described in this chapter was to further define in rats with varying degrees of iodine deficiency the changes in circulating thyroid hormone levels occurring during pregnancy.

Experiment 6: Effects of mild maternal iodine deficiency

(a) Experimental design

Twenty female Wistar rats, who had been fed LID for 4 weeks prior to mating, were studied in this experiment. Ten test animals drank deionised distilled water while 10 controls received deionised distilled water containing $0.1 \mu\text{g}$ of elemental iodine per ml. All 20 animals were mated at 4 weeks and both fertile and infertile animals were bled for serum thyroid hormones at day 15 of gestation and within 6 hours of parturition. Serum T_4 and T_3 were subsequently measured by specific radioimmunoassays adapted from the method of Challand et al. (32). All the sera from the one experiment were analysed in a single batch to avoid inter-assay variation.

(b) ResultsChanges due to pregnancy

The serum T_4 dropped in both control and test mothers during the first two weeks of pregnancy (Fig. 32). However, only in the test mothers was the mean T_4 significantly lower ($p < 0.02$) than non-pregnant controls (Table 52). During the last week of pregnancy no significant change in T_4 occurred although there appeared to be a downward trend in levels in test mothers and an upward trend in controls.

No difference in serum T_3 levels were found between pregnant and non-pregnant animals in both control and test groups at day 15 of gestation (Fig. 33). However, at day 22 test mothers had a significantly lower mean T_3 value ($p < 0.025$) than non-pregnant controls (Table 53).

Differences due to iodine deficiency

The changes occurring during pregnancy in both iodine sufficient (control) and mildly iodine deficient (test) mothers are shown in Fig. 34. At the time of mating the test T_4 level was significantly lower ($p < 0.05$) and, as pregnancy proceeded, this differential between test and control animals persisted (Table 52). By contrast, at no time before or during pregnancy did a difference in serum T_3 levels exist between the two groups of mothers (Table 53).

Differences due to litter size

The changes occurring in both serum T_4 and serum T_3 levels during the last week of pregnancy are shown for the six test mothers in Fig. 35. During this period 4/6 (67%) of the animals showed a drop in T_4 while all six showed a fall in T_3 levels. At day 22 all but one of the animals showed hypothyroxinaemia but only 2/6 (33%) had T_3 levels lower than normal. Those two mothers with low levels of T_3 at term also showed the lowest T_4 levels at day 15 and almost undetectable levels at term. These animals were probably the most severely iodine deficient of the group and as a result of the lack of iodine substrate even the intra-thyroidal switch from T_4 to T_3 production was inadequate compensation.

The only animal which had normal serum T_4 levels in the last week of pregnancy was also unusual from a second standpoint: in contrast to the other mothers whose litters numbered from 9 to 13 pups this animal was carrying a single pup which required delivery by uterotomy on day 23. Of the two test mothers who had at day 15 a serum T_4 of $1.8 \mu\text{g}/100 \text{ ml}$ the animal carrying 13 pups had a T_3 of $70 \text{ ng}/100 \text{ ml}$ while the other with 9 pups had a T_3 of $80 \text{ ng}/100 \text{ ml}$. At the time of delivery the mother with the larger litter had a T_4 of $0.8 \mu\text{g}/100 \text{ ml}$ and an undetectable serum T_3 . By contrast the mother with the smaller litter had a higher T_4 ($1.3 \mu\text{g}/100 \text{ ml}$) and a normal T_3 of $67 \text{ ng}/100 \text{ ml}$.

(c) Discussion

From Experiment 6 it appeared that the fall in serum T_4 , previously observed during pregnancy in Experiments 2-4, took place during the first two weeks of gestation and that between days 15 and 22 no further changes in T_4 level occurred. By contrast, the serum T_3 remained unchanged during the first two weeks but after day 15 dropped in all test pregnancies to a level at day 22, possibly dependent on two factors: firstly, the degree of maternal hypothyroxinaemia at day 15 and secondly, the size of litter being carried. The degree of maternal iodine deficiency did not affect the changes in maternal T_3 levels occurring during pregnancy. However, pregnancy in iodine deficient animals was characterised by significant maternal hypothyroxinaemia for at least one third of the duration of gestation.

Experiment 7: Effects of moderate maternal iodine deficiency

(a) Experimental design

Eighty virgin female Wistar rats weighing 150-200 g at commencement were maintained on a Remington LID containing approximately $70 \mu\text{gI}/\text{kg}$ (238). Half of this group (test animals) received deionised distilled water and the other half (controls) distilled water containing $1.3 \mu\text{g}$ iodide/ml (estimated intake 20-30 ml water daily). After 8

weeks on LID all animals were bled from the tail vein and the concentrations of total T_4 and T_3 in serum were measured by specific radioimmunoassays (32).

On the basis of the RIA results twenty test animals with a serum T_4 concentration of $<2.5 \mu\text{g}/100 \text{ ml}$ and an equal number of control animals were selected for mating at 10 weeks. All animals were mated with a single male for only one night and the day of gestation was estimated from the day of mating.

On the eighth day after mating 11 of the test and 7 of the control animals were bled from the tail vein and on day 15 a further 9 animals from each group were also bled. On the day of delivery all successfully mated animals were bled within 6 hours of parturition. Infertile animals were also bled for estimation of serum thyroid hormones on the twenty second day after mating.

At birth the size of each litter was noted and 3 litters from each group were reduced in size to 8. Their mothers continued to feed on LID with or without iodine supplementation and on day 21 post-partum the 6 nursing mothers were bled from the tail.

The four groups of sera from test and controls, both pregnant and non-pregnant were stored deep frozen and subsequently analysed in one assay to avoid inter-assay variation. Statistical analysis of the data was made with Student's *t* test.

(b) Results

Changes due to pregnancy

The changes in maternal serum T_4 and T_3 occurring during pregnancy are compared with corresponding levels in non-pregnant animals in Figs. 36 and 37. The effects of pregnancy on serum T_4 , T_3 and T_3/T_4 ratio are analysed for control and test animals in Tables 54 and 55.

In control animals serum levels of both T_4 and T_3 were reduced in the pregnant animals at day 15 of gestation (Table 54). At the time

of parturition the T_4 was not significantly different from non-pregnant controls but T_3 was significantly reduced ($p < 0.001$) resulting in a significant lowering of the serum T_3/T_4 ratio ($p < 0.02$).

In test pregnancies the maternal serum T_3 level was lower than non-pregnant controls at days 8, 15 and 22. No difference in mean serum T_4 level was demonstrable at day 8 but at both days 15 and 22 the pregnant animals showed significantly lowered T_4 levels (Table 55). Since the reduction in T_4 was greater than that seen in T_3 the T_3/T_4 ratio was significantly reduced at both days 15 and 22.

Differences due to iodine deficiency

The changes in serum T_3 , T_4 and T_3/T_4 ratio occurring during the duration of Experiment 7 are shown for both control and test mothers in Fig. 38. It is clear that there is a striking qualitative resemblance between the two groups of animals. What difference exists is a quantitative one related to the effect of iodine deprivation on serum thyroxine levels.

Table 56 summarises the data on serum T_4 throughout the experiment. Between days 8 and 15 the T_4 level dropped by 56% in iodine deficient mothers and by 47% in controls. During the last week there was no further significant change in T_4 . However, between delivery and weaning there was a restoration of T_4 in both groups to pre-mating levels. At all five points in time the differences in T_4 between test and control mothers were statistically significant.

Table 57 shows the effect of moderately severe iodine deficiency on changes in serum T_3 during pregnancy and lactation. No change in T_3 levels occurred in the second week at a time when T_4 levels were falling by about 50%. However, in the last week of pregnancy T_3 fell in both control and test mothers by 25%. In contrast to the recovery of T_4 levels during the suckling period the T_3 levels were not significantly different at weaning from those levels found at delivery. At no point during the experiment were any differences detected in mean T_3 levels between test and control mothers.

The serum T_3/T_4 ratios to an extent reflected the fall in T_4 during the second week and the fall in T_3 during the third week (Fig. 38). As a result, between days 8 and 15, as T_4 fell, the ratio rose while between day 15 and weaning the ratio dropped as T_4 stabilised and T_3 remained low. At all times during the experiment the differences in T_3/T_4 ratio between the two groups of pregnant animals were statistically significant (Table 58).

(c) Discussion

Few reports exist on the changes in thyroid function during pregnancy in the rat. Iino and Greer's 1961 paper (169), which dealt particularly with maternal RAIU and thyroid size, was probably the first in the field. These authors found no differences in RAIU between pregnant and non-pregnant until day 20 when there was a sudden marked drop of 50%. This decreased RAIU persisted throughout lactation. Foetal RAIU was negligible until day 20 when it reached 25% of that of mother. No consistent differences in thyroid weight were found throughout the experimental period and no correlation was found between any of the parameters and the size or weight of the litter. Iino and Greer concluded that "it seems reasonable that with the sudden competition for iodine by the mammary glands, particularly if borderline iodine deficiency exists, increased thyroid activity will be required to preserve normal levels of thyroid hormone" (169).

In 1968 Galton (131) examined the urinary excretion of I^{131} and T_4 metabolism in fertile rats between 14 and 28 days after mating. The mean 24 hour urinary excretion of I^{131} was significantly increased in the pregnant rats during the last 2-3 days of pregnancy, values returning to normal immediately after parturition. This increased urinary excretion of iodide derived from T_4 was associated with a marked fall in the concentration of maternal serum T_4 . At day 13 Galton estimated the maternal serum T_4 to be $2.1 \mu\text{g}/100 \text{ ml}$ and at day 20 $2.7 \mu\text{g}/100 \text{ ml}$. These figures compare favourably with those of 1.9 and $2.5 \mu\text{g}/100 \text{ ml}$ found in Experiment 7 at days 15 and 22.

Galton showed that the clearance of T_4 from plasma was significantly enhanced by day 13 and increased at least three fold in the 20 day pregnant rat. She concluded that pregnancy in the rat was associated with an increase in the fractional and probably the absolute rate of turnover of T_4 in the tissues. The increase was due in part to alterations in the T_4 -binding activity in serum but might have also resulted from "hormonal deiodination in fetal tissues". Further studies in 1974 by Gray and Galton (141) revealed that the placenta was readily permeable to T_4 when the hormone was present in maternal serum in concentrations close to physiological. It was therefore felt a "plausible possibility" that the fall in maternal T_4 was due to deiodination of maternal T_4 in foetal tissues.

Galton's serum T_4 data had been derived from PBI determinations divided by 0.65. Hershman's group from UCLA (185) were the first to apply specific radioimmunoassays of T_4 , T_3 and TSH to the quantification of changes in the pituitary-thyroid axis during pregnancy in the rat. Pregnant rats at day 21 of gestation were studied and shown to have increased pituitary TSH secretion, accompanied by decreases in serum T_4 and T_3 of 45 and 42% respectively. Kojima et al. (185) felt that the reductions of serum T_3 and T_4 levels were too large to be explained by the "slight physiological dilution of plasma proteins during pregnancy" and considered their findings "suggestive of hypothyroidism, increased peripheral disposal of thyroid hormone or both possibilities" (185).

The results derived from Experiment 7 confirm and extend the earlier observations of Galton (131) and Kojima et al. (185). Like these workers' findings significant reductions in both T_4 and T_3 have been found in the last trimester of the rat pregnancy. To date no comparable study has been reported on the changes in thyroid function seen in the iodine deficient rat pregnancy.

Since Experiment 7 was completed two further relevant reports have been published in abstract form. Zaninovich and Matty (302) studied T_3 metabolism in conditions similar to those used for T_4 by Galton (131). They demonstrated in pregnant rats a reduced T_3 half-time, a lowered fractional T_3 turnover rate and a decrease in both metabolic and urinary T_3 clearance. They concluded that the decreased T_3 utilisation in the pregnant rat appeared to be reciprocal to the increased T_4 deiodination and was probably related to "the foetal handling of both hormones" (302).

In 1976 Fukuda and Greer (126) reported to the 5th International Endocrine Congress a study of the sequential changes in plasma TSH, T_4 and T_3 during pregnancy and lactation. They showed that during pregnancy the T_4 level dropped by 59% from $6.3 \mu\text{g}/100 \text{ ml}$ to $2.6 \mu\text{g}/100 \text{ ml}$ at term, while T_3 only "slightly decreased". Despite this dramatic drop in T_3 , TSH remained at normal levels until day 16 when it significantly increased. During late pregnancy the fT_4 fraction doubled but fT_4 concentration was lowered. After delivery the high TSH and low thyroid hormone levels, "particularly T_4 " persisted until weaning but returned to normal thereafter. These workers concluded that during pregnancy the decrease in thyroid hormones could be explained in part by a decrease in thyroid hormone binding capacity. By contrast thyroid hormone decrease during lactation was presumed to result from a loss of thyroid hormones into the milk.

Conclusions from Experiments 6-7

Sequential study of the changes occurring in serum levels of T_4 and T_3 during pregnancy and lactation in the rat revealed several significant changes which occurred independent of maternal iodine status. During the second week of pregnancy there was a significant fall of about 50% in maternal serum total T_4 concentrations (Table 59). During the last seven days of pregnancy the mean level of maternal serum T_3 fell by 25% (Table 60). During the three week period of lactation there was a restoration of maternal serum T_4 levels to those

found prior to mating (Table 61) while during the same time no change in serum T_3 concentration occurred.

The changes in T_4 levels were more pronounced in the iodine deficient mothers who were hypothyroxinaemic throughout pregnancy in Experiment 7. By contrast, at no time during pregnancy or lactation were any differences detected between iodine deficient and iodine sufficient animals as regards serum total T_3 concentrations.

CHAPTER X

GENERAL CONCLUSIONS

The experiments described in Chapters V to IX were designed to investigate using an experimental rat model the proposal of Pharoah et al. (219) that severe maternal iodine deficiency acting during the first trimester of human pregnancy causes damage to the developing foetal nervous system resulting in the clinical entity of neurological cretinism. Translated into the framework of neurobiological experimentation on the albino rat the experiments were designed to test the hypothesis that maternal iodine deficiency acts as a growth retarding stimulus on the developing foetal brain resulting in the intra-uterine inhibition of neuronal multiplication within the forebrain.

Analysis of forebrain DNA content in the experiments described in Chapter VII effectively ruled out a significant effect of maternal iodine deficiency on neuronal multiplication as measured by the acquisition of cell numbers in the neonatal forebrain. An unexpected finding, however, was that the degree of prenatal iodine deficiency, sufficient to cause hypothyroxinaemia at the time of mating, resulted in a significant reduction in forebrain protein content implying that severe maternal iodine deficiency caused a reduction in the average size of cerebral neurones and, as a result, a limited capacity for development of an extensive dendritic tree.

This qualitative rather than quantitative defect was in effect the reverse of the changes seen by Zamenhof et al. (300) in the forebrains of offspring born to mothers treated during pregnancy with daily injections of growth hormone. Indeed the reduction in total protein content was rather reminiscent of the biochemical defect thought due to thyroid hormone lack and seen in the cerebral hemispheres of both the neonatally radiothyroidectomised rat (12, 132) and the 150 day-gestation rhesus monkey foetus treated during intra-uterine life with maternally administered radioiodine (159).

The fact that the change seen in neonatal forebrain was qualitative rather than quantitative prompted the thought that the effect of iodine deficiency might represent a "distortion" rather than a deficit (74) and thus would be potentially reversible (291). The experiment described in Chapter VIII proved that the reduction in protein content demonstrated in Experiments 3 and 4 was not permanent and that in respect of "total" protein the effect of prenatal iodine deficiency was reversible during the nursing period of the first three postnatal weeks.

Experiment 5 also showed that, despite the continuation of significant maternal iodine deficiency, the pups being nursed by test mothers were capable of normal growth and through adaptive mechanisms, including an increased activity of the iodide pump, were able to acquire iodine substrate in quantities sufficient to allow normal serum T_4 concentrations at the age of 22 days.

Lastly the changes in maternal thyroid function during pregnancy and lactation were further characterised in both iodine-sufficient and iodine-deficient animals in the experiments described in Chapter IX. Here it was shown that, independent of maternal iodine status, pregnancy resulted in a fall in serum T_4 during the second gestational week and a fall in T_3 during the last seven days of intra-uterine life. During the period of lactation the maternal serum T_4 was restored to levels equivalent to those immediately prior to pregnancy, a recovery which was not paralleled by changes in maternal serum T_3 levels. Moderate and severe degrees of maternal iodine deficiency resulted in hypothyroxinaemia throughout pregnancy but, by contrast, at no time during pregnancy or lactation were any differences in serum T_3 concentrations detected between iodine deficient and iodine sufficient animals.

The changes demonstrated in these experiments suggest to the author a critical sequence of events occurring in the last week of the rat pregnancy, probably during the last 4-5 days of gestation (57). During this latter period the adult complement of cerebral neurones

is nearing completion and a number of neurones are beginning to hypertrophy by increase in cytoplasmic protein content. At this time too there are gross changes in both T_4 and T_3 metabolism in the mother (131, 185, 302) and from day 18 competition exists between maternal and foetal thyroids for the limited available iodine substrate (133). The period is also characterised by an increasing leak of iodine from maternal urine (131) and from day 20 further competition for iodine by the iodide trapping mechanism of the lactating breasts (169). During the last few days of pregnancy, however, a state of iodine deficiency does not appear to affect the concentration of maternal serum T_3 and, moreover, at this time the foetus apparently does not make T_3 in significant amounts and is considered relatively T_3 deficient (90, 145).

If prenatal iodine deficiency during this critical period results in a reduced forebrain cell size it seems unlikely that this effect would be caused by elemental iodine deficiency per se but much more likely to be secondary to effects on maternal and particularly foetal thyroid function and probably mediated by hypothyroxinaemia in view of the absence of demonstrable differences in T_3 concentrations between test and control pregnancies. Furthermore, if one were to extrapolate from the animal model to the 10-20 gestational week period in man then the results of these experiments would suggest an effect of prenatal iodine deficiency on cerebral protein synthesis probably mediated by a lack of thyroid hormone derived from both maternal and foetal thyroid glands, the timing of the insult being largely dependent on the emergence or non-emergence of autonomous foetal thyroid hormone manufacture.

In some respects it is unfortunate that in the rat the time of birth comes so soon after the commencement of foetal thyroid function and as a result the length of the intra-uterine period following this event, when compared to the human, is so short lived. From this aspect the laboratory rat does not provide an ideal model for determining the pathogenesis of the cerebral lesion in endemic cretinism. However,

on the other hand, it is fortuitous that in the rat birth marks the end of cerebral neuronal multiplication and the beginning of major glial multiplication. Thus to an extent one is satisfied that any defect discovered in the rat forebrain at birth and attributable to an intra-uterine influence is likely to prove of greater functional significance than one demonstrable later and affecting glial cells only (57).

Overall the preliminary results found in these experiments have been encouraging and probably merit further work using this animal model. It would clearly be of interest to see whether any reduction in cerebral protein content would be mirrored by a reduction in cerebral neuronal size as seen by light and electron microscopy using the techniques of the quantitative histologists. It would also be relevant to study the protein synthetic capacity of the day 20 foetal forebrain and a regional study of protein synthesis within defined areas of the forebrain might also provide additional information about areas of the cerebrum particularly sensitive to the effects of prenatal iodine deficiency.

No attempt was made in the experiments described in this thesis to determine whether the pups of severely iodine deficient mothers displayed any hearing or learning defect. In any future experiments using this model, however, it would be useful to preserve tissue for histological examination of the organ of Corti (68) and also to determine the hearing capabilities of the animals using the technique of Deol (67, 253). During the nursing period susceptibility to audiogenic seizures could perhaps be assessed as described by Van Middlesworth (274) and, lastly, the opportunity should be taken to determine whether mature pups conceived in an environment of severe maternal iodine deficiency show any lasting functional deficit as elicited by standard behavioural testing (5, 57, 242).

APPENDICES

APPENDIX I

PREPARATION OF SOLUTIONS FOR DNA ASSAYS

(a) Molar Perchloric Acid

1. To 700 ml of distilled water (DH_2O) in a 1 l volumetric flask add drop-wise 96.6 ml of Analar grade 60% perchloric acid.
2. Make up with DH_2O to 1 litre.

(b) Stock Standard DNA

1. Obtain highly polymerised calf thymus DNA (e.g. Type 1, Sigma London Chemical Company Ltd.).
2. Weigh out about 47-48 mg of DNA in a small (e.g. 15 g) beaker.
3. Dessicate for one week with phosphorus pentoxide.
4. After constant dry weight is reached transfer dessicated DNA to 20+ml universal bottle.
5. To bottle add 20 ml DH_2O followed by 2 ml N NaOH.
6. Put bottle on shaker overnight.
7. Next morning use funnel to pour solution into 100 ml volumetric flask.
8. Wash out universal bottle with at least 3 washes of 0.1N NaOH and make up DNA solution to 100 ml with washes.
9. The DNA concentration should be around 400 $\mu\text{g}/\text{ml}$.
10. Store solution in flask in fridge for up to 6 months.

(c) Burton's Diphenylamine Reagent

1. Recrystallise diphenylamine (DPA) by dissolving 100 g in 777 ml of 70% ethanol.
2. Heat to 70°C in shaking waterbath.
3. Vacuum filter hot solution and transfer filtrate to a conical flask.
4. Allow to crystallise in the dark over at least 24 hours at room temperature.
5. Filter recrystallised liquor with Buchner funnel.

6. Place filter paper on petri dish and put crystals on top of filter paper.
7. Weigh dish and crystals.
8. Dessicate with silica gel in the dark to constant weight.
9. To make 500 ml DPA solution, dissolve 7.5 g of DPA in about 200 ml Aristar grade Acetic Acid and pour this through funnel into a 500 ml volumetric flask.
10. Wash out beaker with Acetic Acid, discard washings.
11. Use same beaker to pour Acetic Acid into volumetric flask to make up to 500 ml.
12. Add to this 500 ml via pipette 7.5 ml of Analar grade Sulphuric Acid.
13. Mix contents of flask thoroughly.
14. Divide contents into five 100 ml reagent bottles.
15. Pipette 5.075 ml Acetaldehyde into about 200 ml DH_2O in volumetric flask, make up to 250 ml.
16. Store 100 ml bottles of DPA in Acetic Acid and 250 ml flask of 16 mg/ml Acetaldehyde solution in the dark under refrigeration (4°C) for up to six months.

APPENDIX II

RAT FOREBRAIN DNA AND PROTEIN ESTIMATION

<u>Day 1</u>	Morning:	Brain extraction (4 hrs)
	Late afternoon:	Setting up Burton assay ($\frac{1}{2}$ hr)
<u>Day 2</u>	Morning:	Reading Burton assay (1 hr)
	Early afternoon:	Lowry protein assay (2 hrs)

(a) Brain Extraction

Requirements

<u>Hardware:</u>	1 water bath at 70°C.
	1 Tri-R homogeniser (motor + six pestles).
	1 Whirlimixer.
	1 ice bath, 1 stopwatch.
	1 Oxford 1 ml sampler + tips.
<u>Solutions:</u>	1 MSE Mistral 2L freezing ultracentrifuge.
	6% Trichloroacetic acid (TCA).
	1M Perchloric acid (HClO ₄)
	1N NaOH
<u>Glassware:</u>	7 x 7 ml MSE polypropylene tubes.
	6 x 10 ml S31 glass Tri-R tubes.
	6 x 10 ml graduated glass test tubes (for supernatant).
	5 x 100 ml glass beakers (for solutions and tubes).
	1 x 1000 ml glass beaker (for homogenisers).

Procedure

1. Switch on MSE Mistral 2L centrifuge at 5°C.
2. Switch on both water baths, 30°C and 70°C.
3. Transfer Burton's reagent from fridge to 37°C oven.
4. Take out 6 frozen brains from deep freeze.
5. Record details of brains as per tube label.
6. Transfer each brain to a 10 ml S31 tube on ice.

7. Add 4 ml (i. e. 4 x 1 ml) 6% TCA to each tube.
8. Place appropriate pestle in each tube.
9. Homogenise first sample with 15 up and down strokes at speed 4 on Tri-R homogeniser.
10. Pour off 4 ml into 7 ml polypropylene tube on ice.
11. Add 3 ml (via sampler) 6% TCA to 10 ml S31 tube.
12. Homogenise the 3 ml by 10 up and down strokes to remove debris from pestle.
13. Pour 3 ml into centrifuge tube to make a total volume of 7 ml.
14. Repeat steps (9) to (13) for other five samples.
15. Transfer all 6 x 7 ml tubes to MSE centrifuge at 5°C.
16. Spin the samples at 10,500 rpm (18,000 g) for 40 minutes.
17. After 40 mins. remove tubes from centrifuge, place on ice.
18. Pour off supernatant into beaker and discard.
19. To each pellet add 2 ml 1M HClO₄ via Oxford 1 ml sampler and cover each tube with parafilm.
20. "Buzz" each tube with "whirlimixer" for 60 secs.
21. Place tubes on 7 tube steel rack for water bath.
22. Incubate tubes on rack in vibrating 70°C water bath for 15 mins.
23. After 15 mins. take off hot tubes and transfer to 5°C centrifuge.
24. Centrifuge 6 hot tubes for 40 minutes at 10,500 rpm.
25. After 40 mins. take off tubes and place on ice.
26. Pour off supernatant into 6 graduated 10 ml tubes.
27. Store 6 tubes containing supernatant in fridge.
28. Add 2 ml 1M HClO₄ to 2 ml standard DNA in 7 ml tube.
29. Repeat steps (19) to (23) for 6 samples.
30. Also include standard in step (22), but store hydrolysed standard DNA 4 ml sample in fridge after 15 mins. in water bath.
31. Switch off 70°C water bath.
32. Repeat steps (24) to (27).

33. Add 2 ml 1N NaOH to precipitate in each tube.
34. Cover tube with parafilm and buzz for 1 minute.
35. Take off parafilm and store 6 tubes in fridge.

(b) Setting up Burton Assay

Requirements

Hardware: 30°C water bath.

Eppendorf 100 µl sampler + tips.

Oxford samplers and yellow, green, white tips.

Oxford 2 ml dispenser.

Solutions: 0.5M HClO₄.

0.375M HClO₄.

Standard DNA solution (hydrolysed).

Glassware: 28 x 10 ml graduated glass test tubes.

Procedure

1. Empty Oxford dispenser of water and allow to dry on rack.
2. Mark standard tubes in triplicate S₁-S₃ and test tubes in triplicate T₁-T₆.
3. Leave one tube unmarked for blank.
4. Add 1 ml of 0.5M HClO₄ to blank tube.
5. To each standard tube add (500 + 200 µl) of 0.5M HClO₄.
6. To S₂ and S₃ add 100 µl of 0.5M HClO₄.
7. To S₁ and S₂ add 50 µl of 0.5M HClO₄.
8. To S₁ and S₃ add 25 µl of 0.5M HClO₄.
9. To tubes T₁-T₆ add 500 µl of 0.375M HClO₄.
10. To tubes T₁-T₆ add 300 µl of 0.375M HClO₄.
11. To S₁, S₂ and S₃ add 100 µl of hydrolysed standard.
12. Add (1 x 25 µl), (2 x 25 µl) and (3 x 25 µl) of hydrolysed standard to S₁, S₂ and S₃ respectively.
13. To T₁ add 200 µl of test sample 1.
14. Add 200 µl of samples 2-6 to T₂-T₆.
15. Put 28 stoppered tubes in refrigerator.

16. At approx. 16.15 hrs remove Burton's reagent from oven.
17. Pour reagent into 100 ml measuring cylinder in fume cupboard.
18. To Burton's reagent add 100 μ l of 16 mg/ml acetaldehyde.
19. Pour modified reagent through glass funnel into Oxford dispenser.
20. Add 2 ml of reagent via dispenser to each of 28 glass tubes.
21. Discard Burton's reagent down sink and fill empty dispenser with tap water.
22. Stopper tubes and "buzz" with whirlimixer.
23. Transfer tubes to 30°C water bath and incubate from 16.30 to 09.00.

(c) Reading Burton Assay

Requirements

Unicam SP 500 Spectrophotometer.

Cuvette holder + 4 glass cuvettes.

Procedure

1. After at least 16 hrs incubation remove tubes from water-bath.
2. Switch on spectrophotometer.
3. Pour blank sample into a cuvette and place this cuvette into position 1 of the holder.
4. Pour triplicates of S_1 into positions 2-4.
5. Alter slit width to read O.D. of zero at 610 nm with blank sample.
6. Read and record values for $S_1 \times 3$.
7. Adjust wavelength to 650 nm and again zero blank.
8. Read S_1 samples at 650 nm.
9. Take out cuvettes 2-4 and return contents to graduated tubes.
10. Repeat (4) to (9) for other 8 \times 3 samples.
11. Scan results and repeat readings for inconsistent values.
12. Discard samples.
13. Subtract O.D. 650 from O.D. 610 for each sample.
14. Average triplicate results for each specimen.
15. Draw standard curve with O.D. as vertical axis (0-250) and μ g/tube of DNA as horizontal axis (0-50 μ g). Standards

S_1 , S_2 and S_3 for author's Sigma DNA standard solution were 26.3, 31.6 and 36.8 μg respectively.

16. Calculate slope from standard curve and total DNA by formula below. The dilution factor for neonatal brain was 20.

$$\text{Total DNA } (\mu\text{g}) = \frac{\text{O.D.}}{\text{Slope}} \times \text{dilution factor}$$

(d) Lowry Protein Assay

Requirements

Hardware: 1 x 1 ml glass pipette.
1 x 5 ml glass pipette.
Unicam SP 500 Spectrophotometer.
Cuvette holder + 4 glass cuvettes.
Oxford samplers + tips.

Solution: 2% sodium carbonate.
2% NaK Tartrate.
1% Copper sulphate.
30% Bovine Serum Albumin (BDH).
Folin Reagent (BDH).

Glassware: 1 x 250 ml volumetric flask.
1 x 100 ml volumetric flask.
9 x 5 ml glass thick walled tubes.
29 x 10 ml graduated glass tubes.
4 x 100 ml glass beakers.

Procedure

1. To 100 ml 2% Sodium Carbonate in volumetric flask add via sampler 1 ml 2% NaK tartrate, 1 ml 1% Copper Sulphate and mix thoroughly.
2. To 5 ml of distilled water in 10 ml glass tube add via glass pipette 5 ml Folin reagent. Mix thoroughly.
3. To approximately 240 ml distilled water (DH_2O) in 250 ml volumetric flask add 1 ml of 30% bovine serum albumin (BSA) to give 1200 $\mu\text{g}/\text{ml}$ solution. Make up with DH_2O to 250 ml and mix thoroughly.

4. Mark 9 x 5 ml glass tubes as S_1-S_3 and T_1-T_6 .
5. To tubes S_1-S_3 add 1 ml 2N NaOH.
6. By means of 200 μ l pipette add 400, 600 and 800 μ l of BSA solutions to S_1-S_3 .
7. By means of 200 μ l pipette add 600, 400 and 200 μ l of DH_2O to S_1-S_3 and mix.
8. To each of T_1-T_6 add 1.8 ml N NaOH (variable amount depending on age of brain involved).
9. Remove brain extracts from fridge, add 200 μ l of extract to tubes T_1-T_6 and mix.
10. Mark in triplicate standard 10 ml tubes as S_1-S_3 and test as T_1-T_6 .
11. To a 28th tube (blank) add 100 μ l of N NaOH.
12. To each of S_1-S_3 10 ml tubes (empty) add 100 μ l from appropriate S_1-S_3 5 ml tubes.
13. Similarly, to each of T_1-T_6 10 ml tubes add 100 μ l from T_1-T_6 5 ml tubes.
14. Pour alkaline copper solution (prepared in step 1) into auto-dispenser and add 2 ml to each of standard and test tubes as well as blank i. e. 28 tubes.
15. "Buzz" each tube and wait 10 minutes.
16. To each tube add 200 μ l of Folin reagent and "buzz".
17. Wait 40 mins. during which switch on spectrophotometer.
18. After 40 mins. place blank into cuvette 1 and read all samples in triplicate at 540 nm.
19. Draw standard curve with 0-250 as vertical axis (O.D.) and 0-50 as horizontal axis (μ g/tube of Protein), using $S_1 = 24$, $S_2 = 36$ and $S_3 = 48$ μ g for BSA standards.
20. Calculate test protein values from:

$$\text{Total protein (mg)} = \frac{\text{O.D.}}{\text{Slope}} \times \text{dilution factor}$$

For neonatal brain dilution factor was 200.

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