Influence of dietary linoleic acid (18:2, n6) on the effects of hyperthyroidism in Mus musculus

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DECLARATION

This thesis is submitted in accordance with the regulation of the University of Wollongong in fulfilment of the requirement for the degree of Master of Science. To the best of my knowledge, the work presented in this thesis has not been submitted at any other university or a similar institution except where specifically indicated.

Nalini Deshpande
June 1992
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INTRODUCTION

This investigation was stimulated by the earlier findings of Ershoff (1949). He reported that a diet containing 45% (w/w) full fat soybean meal counteracted the weight loss and other effects of hyperthyroidism on the gross appearance (for example scaly skin and hair loss) of the immature rats. However, he observed that this dietary treatment did not have any effect on the increased oxygen consumption caused by thyroid hormone treatment.

More recent studies have shown that unsaturated fatty acids inhibit thyroid hormone binding to nucleus (Van der Klis, 1989, Inoue et al., 1989, Wiersinga and Platvoet-Ter-Schiphorst, 1990). It has also been suggested that unsaturated free fatty acids in high concentrations may reduce the binding of thyroid hormones to plasma proteins resulting in an increase in their free level in the plasma (Lim et al., 1988). Benvenga et al., (1987) observed an increase in the plasma concentration of T4 when the ratio of free unsaturated fatty acid / thyroxine binding globulin was increased. These reports indicated a connection between polyunsaturated fatty acids and effects of thyroid hormones.

Hyperthyroidism is the most common endocrine disorder next to diabetes mellitus (Sterling, 1975). The treatment of this condition basically involves prolonged drug therapies, partial or total thyroidectomy or radioiodine treatment. The aim of this investigation was also to explore a possibility of a dietary treatment, if polyunsaturated fatty acids exert an effect on thyroid hormone action.

Soybean oil contains about 64 % linoleic acid (18:2, n-6) and about 6.5 % linolenic acid (18:3, n-3) (Hardwood and Geyer, 1964). Since neither of these fatty acids are synthesized by mammals, they are the essential fatty acids not only as precursors of prostaglandins, but also as essential constituents of phospholipids of metabolically active tissues. The present work, depending on Ershoff’s findings, was concentrated on the effects of dietary 18:2 (n-6) on hyperthyroidism.

The experiment was designed to compare the effects of dietary safflower oil (about 70 % linoleic acid) and coconut oil (saturated fat).

Basal metabolic rate (BMR) and growth were compared at the whole animal level. At cell level, activities of three thyroid hormone responsive enzymes located in different parts of the liver cell were measured. These enzymes are:

1. Cytosolic malic enzyme in the liver (ME)
3. Mitochondrial glycerolphosphate dehydrogenase (GPDH)
4. Fatty acid composition of the liver phospholipids was also measured. Activity of the liver malic enzyme is also influenced by type of dietary fat. The reports of dietary effects on the activities of ATPases are varied but it is not known whether mitochondrial GPDH is influenced by dietary fats.

Many reports have shown that hyperthyroidism causes depletion of 18:2 from liver mitochondrial membranes (Ruggiero et al., 1984a, 1984b, Hoch et al., 1988). Essential fatty acid deficiency has also been related to decreased level of 18:2 and 20:4 from various tissues. Increased activity of Na-K-ATPase in essential fatty acid deficiency has been related to lack of 18:2 and its derivatives. Activity of this enzyme has been shown to increase in hyperthyroid rats in vivo as well as in vitro (Ismail-Beigi and Edelman 1971 Ismail-Beigi et al., 1979).

Activity of mitochondrial GPDH is increased in hyperthyroidism (Horrum et al., 1985). Electron transport and mitochondrial respiration has been shown to require 18:2 (Yamaoka et al., 1990) but it is not known whether activity of GPDH is affected similarly by this fatty acid.

The first chapter of this thesis reviews the literature. Second chapter contains the materials and methods used in the experimental procedures, followed by chapter three, which includes the details of the results and discussion of BMR, growth, water intake and plasma hormone levels. Chapter four includes results of enzyme assays, protein contents and composition of fatty acids of the liver phospholipids and detailed discussions of these results. Chapter five contains a general discussion of the thesis.
CHAPTER ONE
Literature Review

1.1. **The Thyroid**

1.1.1. **Gross anatomy, histochemistry and hormone biosynthesis**

The mammalian thyroid gland is located on either side of the trachea, immediately below the larynx, and the two lobes are joined by a narrow isthmus (Ingbar, 1985). A normal thyroid in humans weighs about 25 to 30 g (Hardy, 1981). Thyroid weight of a euthyroid rat is about 9.2 mg / 100 g body weight (Gross and Pitt-rivers, 1953). Although there may be species specific variations, thyroid gland in most mammals exhibits similar structural and functional properties.

Thyroid follicles, parts of the lobular ingrowths of the connective tissue, are the functional units of the thyroid gland (Wollman, 1980). Each follicle contains a cavity or lumen, surrounded by a single layer of epithelial cells. The lumen is mainly filled with a viscous homogeneous 'colloid', an iodinated glycoprotein, thyroglobulin which contains the iodinated tyrosines (the inactive precursors) and the active thyroid hormones (Wollman, 1980). Externally, the follicles are connected to a very rich vascular network.

The plasma membrane of each epithelial cell surrounding the lumen is relatively smooth on the basal surface but the apical surface shows many projections or microvilli facing towards the colloid (Wollman, 1980). The epithelial cells and the colloid, together, participate in iodide metabolism, synthesis of thyroid hormones, thyroglobulin, and release of hormones into the circulation (De Nayer and Vassart 1980). Parafollicular or 'C' cells, in the thyroid, secrete calcitonin which controls calcium and phosphorous metabolism (Utiger, 1986).

Iodine obtained from water and food reaches circulation as inorganic iodide (Ingbar and Woeber, 1981). The thyroid gland extracts iodide from the blood by active transport against a concentration as well as an electrochemical gradient. (Wolff, 1964).

A membrane-bound hemoprotein, located near the apical surface of the follicular cell catalyzes further oxidation of iodide, its transfer to the tyrosine residues of thyroglobulin, forming mono (MIT) and diiodotyrosines (DIT) (Taurog et al., 1970) and also the final specific reaction of oxidative coupling of two DIT to form thyroxine (T4) and one MIT and one DIT to form triiodothyronine (T3), (Lamas et al., 1972).

Since the mechanism of iodide transport is not specific, ions of similar size, shape and charge (for example perchlorate and thiocyanate) can also enter the thyroid cell (Wolff, 1964). These are competitive inhibitors of iodide.
transport and are sometimes used as antithyroid drugs against gland hyperfunction and excessive production of thyroid hormones.

Thyroxine (T4) and triiodothyronine (T3), (Fig.1 and Fig.2) possess different structural and chemical properties. In a normal rat thyroid, the T4:T3 ratio is 4:1 but can decrease to 1:3 during iodine deficiency (Greer et al., 1968).

When injected subcutaneously, T3 is 4 to 5 times more effective than T4. However, oral administration of T3 is only twice as effective as T4 (Gross and Pitt-rivers, 1953). Biological half-life of T4 in normal humans is about 7 days, that of T3 one day (Tondury and Kistler, 1974 p 143).

In mammals, compared to the larger, slowly turning pool of T4, which has an intravascular distribution, T3 has a smaller body pool, higher turnover rate, and an intracellular distribution (Ingbar and Woeber, 1981).

Although T3 contains only 3/4 as much iodine, has a relatively shorter half-life and higher turnover rate than T4, it is more potent than T4. However, by producing T4 as its major hormone, the thyroid gland, in normal conditions, has successfully adapted to iodine economy by reserving a larger pool of a more stable hormone.

Conversion of T4 to T3 is considered to be essential for hormonal effects (Oppenheimer, 1972, Oppenheimer et al., 1972b) but the evidence cited below clearly demonstrates the importance of T4, independent of T3, in the finer regulation of thyroid activity, controlled by thyroid stimulating hormone (TSH):

Many cases of border-line hypothyroidism in endemic goiter regions, show normal to slightly elevated levels of T3 but low to subnormal levels of T4 and high levels of TSH, which shows that normal levels of T4 are essential for maintaining euthyroidism. (Chopra et al., 1973, Patel et al., 1973). In rat pituitary fragments T4 is reported to suppress TSH release without its detectable conversion to T3 (Chopra et al., 1978). Propylthiouracil, the antithyroid drug, inhibits hormone synthesis and T4 conversion to T3, and rT3 (type-1 deiodinase, Visser, 1988) without affecting T4-induced suppression of TSH release (Frumess and Larsen, 1975).

1.1.2. Regulation of secretion and deiodination

Thyroid hormones, stored extracellularly, are taken up by endocytosis of the colloid at the apical surface, are liberated by the lysosomal proteolytic enzyme action on thyroglobulin and secreted into circulation from the basal surface of the epithelial cells. (Wollman et al., 1964). The MIT and DIT released during the hydrolysis are specifically deiodinated further by deiodinases with in the thyroid, and the released iodide is reutilized for hormone synthesis (Dumas, 1973, cited by Tondury and Kistler, 1974).
Fig. 1 Thyroxine (T4)

\[
\text{HO} \quad \text{NH}_2
\]

Fig. 2 Triiodothyronine (T3)

\[
\text{HO} \quad \text{NH}_2
\]

Fig. 3. Pathways of thyroid hormone metabolism

Fig. 3 A Non-deiodinative metabolism

Fig. 3 B Deiodination of T4

Source: Fig. 1 and Fig. 2 Tepperman, J. (1968) p-90
Fig. 3 A and 3 B Visser, T. (1988) p-82, 83
As thyroid hormones are required by tissues according to the metabolic needs, regulation of hormone synthesis and release is closely controlled mainly by hypothalamus and anterior pituitary. Synthesis and release rates of T4 and T3 are modulated by Thyroid Stimulating Hormone (TSH) secreted by thyrotrophs, in the anterior pituitary. The negative-feedback from the plasma hormone levels to the anterior pituitary, primarily controls hormone secretion (Brown-Grant, 1957, 1967). Hypothalamic Thyroid Releasing Hormone (TRH) regulates TSH secretion (Nair et al., 1970) and synthesis (Kourides et al., 1984), supposed to be controlled at translational level (Magner, 1990). TSH binds to thyroid plasma membrane receptors and stimulates adenylate cyclase and generation of cAMP (Gilman and Rall, 1968). In human thyroid tissues, in vitro, TSH and 8-bromo-cAMP (a cAMP analogue) appear to increase thyroid activity by stimulating the gene expression of thyroid peroxidase and thyroglobulin (Nagayama et al., 1989).

The normal thyroidal secretion in humans is 115 nmol T4, 9 nmol T3 and 2 nmol rT3 (reverse T3) per day (Visser, 1988 p-81). In normal humans, about 75 % T4 is bound to TBG, 8 % to transthyretin (previously called prealbumin) and 16 % to albumin (Ekins,1986). Since albumin is present in a higher concentration in serum, despite its low affinity, a relatively large fraction of thyroid hormones is bound to albumin (Ekins, 1986).

Thyroid hormones are metabolised via many tissue specific processes (Fig. 3A). Sulfation, glucuronidation, deamination and decarboxylation and ether bond cleavage are the non-deiodinative pathways whereas monodeiodination (Fig. 3B) removes iodine atoms in a specific manner (Engler and Burger, 1984). Monodeiodination of the outer phenolic ring of T4 (5'-deiodination) produces T3 and the inner tyrosil ring is deiodinated (5-deiodination) to form reverse T3 (rT3) (Engler and Burger, 1984, Visser, 1988). In human beings about 80 % T3 and 95 % rT3 is produced from the enzymatic conversion of T4 (Visser, 1988). Monodeiodination systems also degrade 56 % of T3 and 28 % of rT3 per day (Engler and Burger, 1984).

In rat, the process of 5' monodeiodination of T4 was reported to be tissue specific and dependent on the thyroid status (van Doorn et al., 1984). In hyperthyroid rats local conversion of T4 has been shown to produce 72 % of total T3 in the liver (euthyroid value = 46 %) and 17 % in the kidney. The conversion in the pituitary (2.5 %) was similar in euthyroid, hypothyroid and hyperthyroid state (van Doorn et al., 1984).

Activities of the deiodinases are influenced by the thyroid status, fasting, and other hormones (Kaplan,1986). The process of outer ring deiodination (ORD) is an "up step" or activating reaction as it produces T3 which is more potent than T4.
Inner ring deiodination (IRD) forms rT3 which has no significant thyromimetic activity (Pitman et al., 1962) but it can inhibit T4 action when given in large doses (Pitman and Barker, 1959). Tseng and Latham (1984), have reported the antioxidant property of rT3 and related it to the abnormal increase in RBC size due to its deficiency in phospholipids during hypothyroidism. High rT3 levels are selectively formed by IRD of T4 in the placenta and developing fetus, where vitamin E level is found to be lower than in adults (Tseng and Latham, 1984).

Thyroid hormone uptake into cells is an active transport process, which was observed in the plasma membrane of human red cells (Holm, 1987, Holm and Kagdal, 1989), rat skeletal muscle (Pontecorvi and Robbins, 1986), rat skeletal myoblasts (Pontecorvi et al., 1987), rat hepatocytes (Krenning et al., 1981) and human cultured fibroblasts (Docter et al., 1987).

1.1.3. Transport of thyroid hormones

In human beings, both T3 and T4 circulate by noncovalent but strong binding with thyroxine binding globulin (TBG) but in the rat, transthyretin is the major binding protein (Navab et al., 1977). In some human males who are totally devoid of TBG, transthyretin, is the major thyroid hormone binding protein (Ekins, 1990).

Human plasma lipoproteins, transport about 3 and 6 % of T4 and T3 respectively. These have been suggested to facilitate the entry of lipophilic thyroid hormones by interacting specifically with cell surface receptors (Benvenga et al., 1988).

Opinions differ regarding the role of thyroid hormone binding proteins in hormone transport. It has been suggested that hormones bound only to specific proteins (for example albumin) are transported into tissues and in human beings and rats, thyroid hormones bound to TBG and transthyretin respectively are unavailable for tissue uptake (Padridge et al., 1981, 1985). Robbins and Rall (1957) and Mendel et al., (1988) believe that only the free, unbound form of hormone is active, and therefore the free hormone measurement would indicate the true thyroid status. However, Ekins (1990) has questioned above hypotheses on the basis that they do not fully take into account all other important parameters for example, capillary diameter and length, variations in the plasma level of specific binding proteins as observed during pregnancy, dissociation and rebinding of free hormones during capillary to tissue transit, and differences in rates of blood flow. The principle and methodology of using various hormone analogues in free hormone assays and serum dilutions could change the hormone and protein binding kinetics and give misleading values. The concept of measuring free hormones therefore, cannot deny the significance of measuring
total hormone concentration in the plasma (Ekins, 1990).

Unlike in liver, kidney and skeletal muscles, altered thyroid status does not affect the specific activities of certain thyroid responsive enzymes (Lee and Lardy, 1965) or the normal thyroid hormone levels in the brain of adult rats (van Doorn et al., 1984). In vitro studies of the rat choroid plexus (the blood / brain barrier) revealed the presence of high levels of transthyretin. Non-saturable accumulation of high levels of T4 in this tissue, determined by using fluorescence quenching technique confirmed the brain as a target organ (Dickson et al 1987). Compared to liver, choroid plexus contained 25% more mRNA for transthyretin. Dickson et al., suggested that choroid plexus may contribute towards maintaining appropriate T4 concentration in the cerebrospinal fluid by secreting high levels of transthyretin that has greater affinity for T4 than T3. The non-saturable uptake is thought to be due to the partitioning of hormones in the membrane lipids.

Iodothyronine homeostasis in rat brain was also observed by Dratman et al., (1983). Compared to liver, where a 11 fold increase in T4 was observed during the experimental transition from hypo to hyperthyroid state (thyroidectomized rats injected with 20 µg T4 / 100g / day for 3 weeks), brain T4 level indicated only a 3.3 fold increase. Intravenously administered labelled T4 was slowly accumulated and progressively concentrated in selected regions of brain (nerve terminals). Monodeiodination of T4 to T3 was accelerated in hypo and decreased in hyperthyroid state. Dratman et al., (1983) suggest that discrete nerve cell groups and neural systems selectively concentrate and retain the hormone in order to maintain the normal hormone levels in the brain.

1.2. Hyperthyroidism

Total T3 and T4 concentration in euthyroid humans is 3.2 and 135 nM respectively (Sterling 1975) Marsden and Mckerron (1975) reported a total T3 value of 3.0 and total T4 value of 124 nM. In euthyroid mice the total T3 and T4 level has been reported to be 1.1 and 61 nM respectively (Burgi et al., 1986). Tissue exposure to excessive quantities of thyroid hormones from the thyroid gland or other sources, results in many physiological and biochemical alterations. Hyperthyroidism is a specific condition caused by hyper secretion from the thyroid gland (Ingbar, 1985).

Hyperthyroidism is the most common endocrine disorder next to diabetes mellitus, (Sterling, 1975). Graves’ disease (uniform enlargement of the whole thyroid gland, a diffuse goiter due to an autoimmune disorder) is supposed to be caused by many thyroid stimulating immunoglobulins (TSIGS), interacting specifically as TSH agonists with the TSH receptor on the thyroid plasma membrane (Mehdi et al., 1973, Manly et al., 1974). It is suggested that the
combined agonistic activity of these TSIGS (Katakura et al., 1987, Mariotti et al., 1987) activates excessive production and secretion of thyroid hormones.

Selective pituitary resistance to thyroid hormones has also been shown to cause hyperthyroidism (Lamberg and Liewendahl, 1980, Beck-Peccoz et al., 1989, Hamon et al., 1989). It has been suggested that deficient transport of thyroid hormones into the thyrotrophs resulting in hypersecretion of TSH may cause this disorder (Hamon et al., 1989).

Bacterial and viral infections (Himsworth, 1985), presence of hyperfunctioning thyroid tissue in the ovary (Brown et al., 1973), hydatiform moles, carcinoma of the uterine tissue only during pregnancy (Ingbar, 1985), affect the functioning of thyroid gland and are known to cause hyperthyroidism. Iodine containing medication, Amiodarone, used for treating ischemic heart disease is also known to induce hyperthyroidism. It is a potent inhibitor of T4 and rT3 (reverse T3) deiodination (Jonckheer et al., 1978, Amico et al., 1984).

Ingestion of excessive thyroid hormones, deliberately or under experimental conditions induces iatrogenic or factitious thyrotoxicosis (Cohen et al., 1989). Secretion of TSH is suppressed because of the presence of exogenous hormone in excess. In an experimental situation where T3 is administered, T4 levels would diminish (Cohen et al., 1989).

Thyroid storm, an extremely severe condition of thyrotoxicosis where hypermetabolism and uncontrollable fever, delerium and coma may occur as a complication of pre-existing untreated, (or incompletely treated) thyrotoxicosis. Infection, or emergency surgery in hyperthyroidism can cause thyroid storm (Ingbar, 1985). Since T3 levels are not higher than in any other form of thyrotoxicosis, the mechanism for the onset of this crisis is not yet known (Ingbar, 1985).

T3-Toxicosis is the condition more common in elderly. It shows normal to low concentration of serum and free T4, while serum and free levels of T3 are elevated (Ingbar, 1985). Toxic adenomas (nodular proliferation of a single or a few follicular cells which secrete excessive hormones) seem to be associated with T3-toxicosis (Marsden and Mckerron, 1975).

In T4-Toxicosis serum as well as free T4 concentration is elevated. Inhibition of 5'-monodeiodination of T4 increases rT3 while T3 level may be normal to low (Ingbar, 1985). An inherited, and now a more commonly recognized disorder, Familial Dysalbuminemic Hyperthyroxinaemia (FDH), occurs due to the co-existence of raised levels of T4 affinity for transthyretin and an isoform of albumin (Docter et al., 1981, Barlow et al., 1982, Croxon et al., 1985).

Propylthiouracil, methimazole and carbimazole are the commonly used
antithyroid agents. These compounds inhibit the oxidation and coupling reaction in the formation of MIT and DIT in the thyroid. Propylthiouracil also inhibits T4 conversion to T3 in the peripheral tissues (Oppenheimer et al., 1972b).

Adrenergic antagonists (for example propranolol) which mainly block the effect of catecholamines can also inhibit some of the effects (for example increased heart rate, excessive sweating, and palpitation) observed in hyperthyroidism (Ingbar, 1985). Total thyroidectomy and radioiodine therapy inactivate the thyroid permanently therefore the treatment has to be followed by a daily calculated dose of hormone to maintain euthyroidism.

1.3. Effects of hyperthyroidism at cell level

Thyroid hormones are necessary to increase synthetic processes during early development and later, they are required to maintain metabolism. Severity of hyperthyroidism depends on the elevation in the serum concentration of thyroid hormones. Total T3 and T4 level in the serum of hyperthyroid humans can reach up to 19 nM and 350 nM respectively (Mareden and McKerron, 1975). Excessive secretion in hyperthyroidism leads to abnormal increases in basal metabolic rate (BMR) and cardiac output. Body weight loss and low levels of blood cholesterol are also observed. Activities of many lipogenic and oxidative enzymes are increased. Thyroid hormones have also been shown to alter the fatty acid composition of cellular membranes. These effects are discussed in further detail in the following sections of this chapter.

1.3.1. Basal Metabolic Rate (BMR)

Magnus-Levy's discovery in 1895, (Burgi and Lauhart, 1974) relating increased rate of oxygen consumption to excess thyroid hormones in the body, led to the belief that these hormones basically regulated the BMR. Boothby and Sandiford (1922) and Salter and MacKay (1944) used BMR as an index of thyroid status.

Administration of T3 and T4 separately in the drinking water was reported to elevate the metabolic rate approximately 169% and 26% above the basal value in the normal rats given about 12 μg T3 and 25 μg T4 respectively / 100g body weight per day for 13 days (Gemmil, 1956). In normal rats subcutaneous administration of about 16 μg T4 or 10 μg T3 / 100 g body weight every 4th and 3rd day for 3 and 2 weeks respectively, elevated the basal metabolic rate 45-75% above normal levels (Tata et al., 1963). Oppenheimer et al., (1991), observed 150% increase in oxygen consumption in normal rats made hyperthyroid by injecting 50 μg T3 / 100g body weight per day for 14 days. They also observed a lag of 24 h before the rise and a period of 4 days to reach the maximum oxygen consumption.

It has been shown that thyroid hormone effects on BMR become
apparent after a lag of time (Tata, 1964).

Tata in his review (1964) has summarized the following possible causes for this lag period:

1. metabolic transformation from inactive to active form of hormone.
2. differences in relative speeds of action due to different rates of absorption by tissues. This was observed in rat as a 24 hour earlier increase of mitochondrial Qo2 in liver than in kidney (Tata, 1964).
3. manifestation of a totally unknown, different initial action at the cell level that indirectly affects other final direct actions.

However, the biochemical and molecular mechanisms underlying the effect on elevating the metabolism are still a subject of much speculation.

1.3.2. Activity of sodium-potassium-ATPase (Na-K-ATPase)

Animal cells normally contain more potassium inside than outside. Ussing (1949) first suggested the active transport of potassium into the cells against an electrochemical gradient. Skou (1957) discovered Na-K-ATPase, the ATP dependent, ion transporting enzyme system now known as the 'sodium pump'. Its ubiquitous activity was reported in several tissues of cat, calf and rabbit (Bonting et al., 1961, 1963), in rabbit kidney (Jorgenson and Skou, 1969), and rat liver (Emmelot and Boss, 1965). Under normal physiological conditions the exchange of sodium and potassium has a ratio of 3:2 for each molecule of ATP hydrolysed by this enzyme (Glynn, 1962a). The active transport of Na+ is specifically inhibited by the cardiac glycoside ouabain but it does not inhibit the Mg++ or Ca++ stimulated ATPase (Glynn, 1962b, Skou, 1965, Stekhoven and Bonting, 1981). The enzyme complex contains the active site for ATP hydrolysis as well as the binding site for ouabain (Rouho and Kyte, 1974).

Activity of the sodium pump can be measured by 1. counting the ouabain binding sites 2. release rate of inorganic phosphate (Pi) by the hydrolysis of ATP in the presence and absence of ouabain (Lo et al., 1976, Haber and Loeb, 1984, Lo and Lo, 1981, Esmann, 1988).

Apart from the concentration gradients of Na+, K+ and Mg++ some hormones also affect the activity of the sodium pump. Insulin (Clausen and Hansen, 1977) and catecholamines (Clausen and Flatman, 1977) act by increasing the activity of the existing molecules.

Thyroid hormones, injected into euthyroid rats, increased the in vitro oxygen consumption and specific activity of Na-K-ATPase in the liver by 70-90% (Ismail-Beigi and Edelman, 1971, Edelman and Ismail-Beigi, 1974). These authors postulated that thyroid hormone-mediated stimulation of the active transport of Na+ and elevation of oxygen consumption should result in an
increase in the cellular and therefore total body metabolic rate.

Increase in the number of ouabain binding sites associated with increased enzyme activity was observed in rat renal cortex, (Lo et al., 1976), skeletal muscle (Nørgaard et al., 1983), plasma membranes of liver, kidney and skeletal muscle but not of the brain (Lin and Akera, 1978). Increase in the incorporation of labelled methionine into both the sub-units of the ATPase molecule of the renal cortical tissue indicated the synthesis of extra units as well as an increase in the enzyme activity (Lo and Edelman, 1976).

However, an initial significant rise in the K⁺ efflux was observed in the isolated diaphragm of rat, within 6 hours after treatment with 50 μg T3 / 100g body weight for three alternate days and in the liver after a single injection of T3 (Haber and Loeb 1982, 1984). Sodium pumps were activated between 24 and 48 hours after T3 treatment. The efflux of K⁺ continued even after the inhibition of the sodium pump. Haber and Loeb suggest that increase in the number of sodium pump units is an adaptive response of the cells to the initial rise in K⁺ efflux, stimulated by thyroid hormones.

Ismail-Beigi (1988), and Gick et al., (1988) reported T3-induced changes at transcriptional as well as post-transcriptional level, causing increase in the number of sodium pumps / mg of membrane protein resulting in an augmented activity of Na-K-ATPase. Ismail-Beigi suggests that thyroid hormones, independently and perhaps coordinately increase the passive permeability of the cations and also the activity of the enzyme under nuclear control resulting in an augmentation of the overall active transport of Na⁺ and K⁺, eventually resulting in a higher respiratory rate.

However, maximum oxygen consumption (> 50 % of the total) under basal conditions appears to be used only by kidneys (Clausen et al., 1991). Brain was the next tissue but other tissues appear to use less than 10 % of the total oxygen consumed towards the activity of Na-K-ATPase which may increase up to 30 % in the liver, depending on the sodium concentration and cellular integrity (Clausen et al., 1991).

Activity of the sodium pump in different thyroid states also appears to be tissue specific. Leukocytes of experimental hyperthyroid human subjects showed an increase in ouabain sensitive ⁸⁶Rb (an isotope which mimics radioactive K) transport, independent of the increase in enzyme units (Turaihi et al., 1987). Prolonged exposure to thyroid hormones however, showed increased ouabain binding sites (Turaihi et al., 1987). In human erythrocytes, hyperthyroidism caused an increased degradation of the pump units resulting in a significant decrease in the activity of the sodium pump and other pathways of sodium efflux (Arumanayagam et al., 1990).
Conversely, hypothyroid human erythrocytes showed an increase in the activity of this enzyme (Dasmahapatra et al., 1985). These authors suggested that the increase in enzyme activity was due to the reduction in the degradation of existing units as an adaptive response since erythrocytes are devoid of nuclei and therefore unable to synthesize new proteins.

The sodium pump hypothesis has been questioned by Sestoft (1980). He suggests that the thermogenic effects of thyroid hormones are primarily exerted by the stimulation of a futile cycle of enhanced synthesis and oxidation of fatty acids at the expense of high energy phosphates.

A rise in the activity of Na-K-ATPase may not be a primary or even a universal effect of thyroid hormones but in certain thyroid responsive tissues such stimulation may result in an enhanced oxygen consumption. For example oxygen consumption in hypothyroid rat hepatocytes increased from 15 % of the total value to 31 % after T3 treatment (Clausen et al., 1991).

1.3.3. Lipogenesis and hepatic malic enzyme

Thyroid hormones exert a tissue specific effect on several lipogenic enzymes, liver being one of the main target organs. Experimental thyroidectomy significantly reduces the activities of the enzymes of the hexose monophosphate shunt, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in rat liver (Huggins and Yao, 1959). Activities of the above enzymes (Towle and Mariash, 1986), fatty acid synthetase (Volpe and Kishimoto, 1972, Roncari and Murthy, 1975) and acetyl-coA carboxylase (Roncari and Murthy 1975) are all increased in the presence of thyroid hormones.

Cytosolic malic enzyme (ME) in the liver is one of the lipogenic enzymes and has been used as a sensitive marker of thyroid status (Tepperman and Tepperman, 1964, Dozin et al., 1985b Clarke and Hembree, 1990). ME catalyzes NADP-dependent oxidative decarboxylation of malate to pyruvate and provides NADPH for reductive lipogenesis (Ochoa, 1955).

Dozin et al., (1985b) observed a 11-fold increase in the activity of ME per mg of total protein in rats, injected with 15 µg T3 / 100g body weight / day for 10 days.

Thakare et al., (1989) reported an increase in ME activity from normal 70 nmols to 284 nmols / mg of total protein / min. in adult rats injected with 50 µg T3 / 100g body weight on 2nd, 4th, and 8th day of treatment.

Hepatic malic enzyme on account of its extreme sensitivity to thyroid hormones has become a model system for studying and comparing thyroid states. Fat-free diets rich in carbohydrates also increase the activity of hepatic ME and the other lipogenic enzymes mentioned above.

Various reports show that the effects of the synergistic relationship
between thyroid hormones and carbohydrates, on the activity of ME and other lipogenic enzymes has provided a guideline to explore the molecular mechanisms that control thyroid hormone effects at the level of nucleus. For example see Mariash and Oppenheimer (1985) and Towle and Mariash (1986).

1.3.4. Lipolysis and fatty acid metabolism

The influence of thyroid hormones on catecholamine stimulation, the activity of adenylate cyclase, the accumulation of cAMP in cells and lipolysis are well documented (Fisher and Ball, 1967, Malbon et al., 1978, Bilezikian and Loeb, 1983). Many features of hyperthyroidism, e.g., increase in cardiac output, lipid and glycogen mobilization, and increased thermogenesis, resemble those of excess catecholamines (i.e., adrenal medullary activity) and in most cases can be inhibited by antiadrenergic agents such as propranolol (Ingbar, 1985). However, examination of the β-adrenergic receptors of membranes prepared from isolated fat cells from hyperthyroid rats did not indicate any increase in the number of receptors (Malbon et al., 1978). These authors suggested that in fat cells, thyroid hormones may regulate the transduction of information between hormone receptors and adenylate cyclase thus affecting the action of lipolytic hormones.

Since β-adrenergic blockers have no effect on fatty acid turnover rate and elevated RMR (resting metabolic rate) of hyperthyroid humans, Muller et al., (1990) suggested that perhaps the interaction of thyroid hormones and insulin may influence these parameters in hyperthyroidism.

Insulin basically controls lipid metabolism by its antilipolytic property and its effect on carbohydrate metabolism (Muller et al., 1990). At high plasma levels of T3, elevated fasting levels of both insulin and glucose, indicated thyroid-induced insulin antagonism resulting in impaired carbohydrate metabolism and decrease in glycogen synthesis (Muller et al., 1990). Fatty acid mobilization for energy is increased in hyperthyroidism (Sestoft, 1980). β-oxidation of long chain fatty acids from triglycerides (TG) is increased due to enhanced activity of carnitine acyltransferase, the rate-limiting enzyme which carries long chain fatty acids across the mitochondrial membrane (Fritz, 1963).

The oxidation and esterification of long chain fatty acids in the liver decides the turnover rate of fatty acids which is increased in hyperthyroidism and is associated with an increase in oxygen consumption (Heimberg et al., 1985). The total turnover rate of free fatty acids is elevated and accompanied by increased ketogenesis which is due to the elevated plasma levels of free fatty acids (Heimberg et al., 1985). Unmetabolized fat particles causing ketogenesis is a well-documented observation in hyperthyroid humans (Heimberg et al., 1985, Muller et al., 1990, Beylot et al., 1991).

Thyroid hormones reduce the serum cholesterol levels due to its increased
excretion in bile acids (Miettinen, 1968).

Thyroid hormones also activate microsomal enzyme systems for elongation and desaturation of long chain fatty acids (Landriscina et al., 1976). Newly synthesized fatty acids are mostly incorporated into phospholipids and less into TG (Heimberg et al., 1985), consequently synthesis of very low density lipoproteins (VLDL), that transport TG, is also reduced (Heimberg et al., 1985).

Activation of gluconeogenic enzymes, lipogenic enzymes, fatty acid oxidative enzymes and insulin antagonism has been shown to result in about 64% increase in energy expenditure by lipid oxidation to sustain a 65% rise in RMR in thyrotoxic humans (Muller et al., 1990).

1.3.5. Modulation of mitochondrial structure and function

Liver mitochondria of hyperthyroid rats show an increase in the number and size as well as inner membrane surface area and an increase in the number of functional respiratory-chain units (Muller and Seitz, 1984). Capacity of the adenine nucleotide (ATP / ADP) translocator is increased (Babior et al., 1973, Seitz et al., 1985). In thyroidectomized rats a decrease in the ability to rephosphorylate ADP was restored within 15 minutes after administration of thyroid hormones (Holness et al., 1984).

Electron micrography analysis of rat liver cells showed thyroid hormone effects on the morphofunctional properties of mitochondria (Goglia et al., 1989). Differential centrifugation separated three types: H (heavy, large and fully coupled), M (medium, loosely coupled), and L (small, fully uncoupled). Hypothyroid rats on PTU had an increase in the average volume of mitochondria and a major proportion of H and a minor proportion of L fractions. Treatment with T3 reduced the volume and slightly modified the number. Polarographic determinations performed on H, M and L fractions mentioned above, showed a significant effect of thyroid hormones on the formation of L fraction. Goglia et al., (1989) suggested that thyroid hormones could regulate the formation of L units from H units. These authors further suggested that subsequent maturation (synchronous activity of nuclear and mitochondrial DNA) of one L removes one H unit and T3 could regulate ATP synthesis by acting on the mass and functionality of H units and the activity of L units could contribute to thermogenesis.

Brand (1990b) has demonstrated that apart from the various electron transporting systems (including the mitochondrial glycerolphosphate dehydrogenase, discussed in the next section), thyroid status also affects the leak of protons back into the mitochondrial matrix. Proton leak consumes energy without ATP synthesis. It is decreased during state 3 as the electrons are transported when ATP synthesis occurs and increased during state 4 respiration.
(when ATP is not synthesized).

About 20-25% of the total oxygen consumption of the hepatocytes has been shown to drive the mitochondrial proton leak which only produces heat (Brand, 1990b). From the observations that state 4 respiration is increased in hyperthyroidism and comparison of proton leaks in different thyroid states, Brand calculated that compared to hypothyroid, the 7 fold higher proton leak observed in hyperthyroid mitochondria, was related to the high state 4 respiration. Brand (personal communication to Hulbert) has reported that the mechanism of the increased proton leak in hyperthyroid mitochondria was due to the change in the area of inner membrane per mg of protein and also a change in the intrinsic permeability of the phospholipid bilayer influenced by thyroid hormones.

1.3.6. Activity of mitochondrial glycerolphosphate dehydrogenase (GPDH)

It has been observed that FAD linked GPDH of the inner mitochondrial membrane (IMM) in the liver is a distinct marker of thyroid status. A significant increase in the respiratory rate of liver mitochondria of rats given 2% dietary desiccated thyroid for 10 days was observed by Lee et al., (1959) and Lee and Lardy (1965). A 20 fold increase in the GPDH activity was observed with increased oxidation of \( \alpha \)-glycerophosphate (\( \alpha \)-GP). The cytosolic isozyme was unaffected by thyroid status, indicating mitochondrial location of the GPDH activity, influenced by thyroid hormones.

A 5 to 7 fold increase in the GPDH activity, cytochrome aa3 (cytochrome oxidase) activity and a more reduced state of cytochrome c in rats injected with a daily dose of 15 \( \mu \)g T4 / 100g body weight for 10 days was observed by Horrum et al., (1985).

Mitochondrial GPDH catalyzes the delivery of the cytosolic reducing equivalents (produced during glycolysis), to the respiratory chain via the H shuttle (Estabrook and sacktor, 1958). Since the IMM is impermeable to NADH, the H shuttle acts as a transport mechanism, explained in the following steps of reactions:
(DHAP in cytosol) *DHAP (from glycolysis) + NADH \rightarrow \text{glycerol 3p + reducing equivalents + NAD inside mitochondria (IMM GPDH)} + FAD (Diffuses in to cytosol) \leftarrow \text{DHAP + FADH} + \frac{1}{2} \text{O}_2 \Rightarrow \text{FAD + H}_2\text{O}

* (Dihydroxyacetone phosphate)

The electron transport system present in the inner mitochondrial membrane contains the enzymes and their cofactors that control the respiratory rate and synthesis of ATP. Under normal conditions, if ATP stores are adequate, the coupling of electron transport and ATP synthesis would inhibit the oxidation of cytosolic NADH and mitochondrial FADH, the cofactors which initiate electron transport (Whittaker and Danks, 1978).

Under normal physiological conditions the activity of the IMM GPDH is a rate-limiting factor in the oxidation of \( \beta \)-GP (Lee and Lardy, 1965). Although increased oxidation of \( \beta \)-GP as such may not explain the phenomenon of thyroid-induced increased respiratory rate, Lee and Lardy suggested that the increased activity of IMM GPDH would facilitate the oxidation of cytosolic NADH via H shuttle and reduce the overall NADH : NAD ratio in the cytosol, causing carbohydrate degradation and prevention of glycogen resynthesis. Since addition of ethionine (inhibitor of protein synthesis) inhibited the increase in the activity of GPDH, these authors suggested synthesis of new enzyme units by thyroid hormone action.

Assays conducted by Wemette et al., (1981) with and without calcium in hyperthyroid rat mitochondria indicated that the mechanism of the activation of mitochondrial GPDH was by thyroid hormone-stimulated increase in catecholamine activity which caused the increase in cytosolic concentration of \( \text{Ca}^{++} \). These authors suggested that \( \text{Ca}^{++} \) inside the mitochondria binds directly to the enzyme molecule and activates it.

**1.3.7. Protein synthesis**

It is well known that thyroid hormones increase the synthesis of the enzymes for urea formation during amphibian metamorphosis (Cohen, 1970). Effects of thyroid hormones on some specific enzymes in mammalian systems have been discussed in the previous sections.
Effects of prolonged treatment with thyroid hormones on protein synthesis appear to be inconclusive. Liver homogenates of rats injected with 100 μg T4 for 6 to 16 days showed increased incorporation of D,L-leucine-1-C14 and D,L-valine-1-C14; but a higher dose decreased the rate of protein synthesis (Hoch, 1962, page-632). Injection of 20 μg T4 every fourth day for 35 days increased the metabolic rate in rats but total cellular RNA content was unchanged (Tata et al., 1963). Significant in vitro incorporation of [3H] leucine by isolated mitochondria of thyroidectomized rats, with in 10 hours, after treatment with 25 μg T3 / 100 g body weight, indicated thyroid hormone effect on mitochondrial protein synthesis (Bouhnik et al., 1979).

Towle in his review (1983), has stated that the increase in protein synthesis observed during the experimental transition from hypothyroid to euthyroid state does not continue into hyperthyroid state. This could be either due to the absence of further increase in RNA production or elevated degradation of the proteins in hyperthyroidism (Towle, 1983). However, Landriscina et al., (1976) reported that significant effects on synthesis of microsomal enzymes of fatty acid chain elongation and desaturation became apparent only after 22 days of treatment.

1.3.8. Metal ions and vitamin metabolism

A significant decrease in Zn concentration in the red blood cells (RBC) of hyperthyroid human beings was reported by Yoshida et al., (1990). Thyroid hormone-induced Zn deficiency inhibits the synthesis of RBC carbonic anhydrase β isozyme which catalyzes the hydration of CO2 to H2CO3 (Yoshida et al., 1990).

Requirements for water soluble thiamin, riboflavin, vitamin B12 and vitamin C and fat soluble vitamin D and E are also increased in hyperthyroidism (Ingbar, 1985). Smith et al., (1989) reported vitamin A deficiency in thyrotoxic human erythrocyte membranes in vitro. Retinol, the precursor of vitamin A is thought to be co-transported with thyroid hormones in the blood as a noncovalently transthryretin-bound protein complex. These authors suggested that as retinoic acid formed from vitamin A, bears structural similarity to thyroid hormones, it may compete for the same binding sites on the plasma membrane. Retinoids are also capable of altering membrane structure (Smith et al., 1989). Under normal conditions retinoic acid is perhaps capable of displacing thyroid hormones from the membrane but high levels of thyroid hormones may inhibit the transport of retinol competitively, thereby decreasing the synthesis of vitamin A and formation of retinoic acid (Smith et al., 1989).
1.4. Proposed mechanisms of action of thyroid hormones

From the preceding discussion it is evident that thyroid hormones act in a number of different ways on cellular metabolism but no single mechanism of action has been able to explain all the effects of these compounds. Theories based on specific effects have been proposed, which jointly appear to explain a pattern of interrelated mode of action on many metabolic processes.

1.4.1. RNA stimulation and protein synthesis

Nucleus as a possible site for initiation of thyroid hormone action was suggested by Tata et al., (1963). Nuclear binding sites with high affinity for T3 were reported in human, as well as rat liver and kidney (Schuster et al., 1979), nuclear envelope of rat liver cells (Lefebvre and Venkatraman 1984), preadipocytes of lean and obese mice (Anselment et al., 1984) and rat myocardial tissue (Ladenson et al., 1986). The nuclear receptor is reported to be a non-histone chromatin protein (Oppenheimer, 1979 Pascaul et al., 1982, Dozin et al., 1985a).

The recent review by Brent et al., (1991), cited often in this section, includes a comprehensive account of the recent progress made in this field of thyroid research.

Partial purification and photoaffinity studies indicated two forms of nuclear receptors of molecular weights 47000 and 57000. (Brent et al., 1991). The number of receptors per nucleus vary from 6000 to 8000 in rat pituitary and brown adipose tissues, 4000 in the liver and 2000 in the cerebral cortex (Brent et al., 1991).

Unlike steroid hormones, which have only one receptor, two different genes appear to code for very similar thyroid hormone receptors. The two nuclear receptors,T3Rα, isolated from chicken embryo (47,000 kd) by Sap et al., (1986) and T3Rβ, isolated from human placental cDNA by Weinberger et al., (1986) (about 57,000 kd) have been suggested to be analogous to the avian erythroblastosis virus v-erb-A. In vitro studies have shown that translated products of these cDNAs have high affinity for T3 and also bind to DNA in the presence of thyroid hormone response elements (T3RE) in DNA. T3Rα and T3Rβ appear to exhibit many variants, capable of altering hormone action by their interaction with T3REs (Brent et al., 1991).

Unlike steroid hormone receptors, T3 receptors are bound to chromatin even in the absence of a ligand. It is suggested that the receptors without hormones (aporeceptors) are capable of binding to T3REs and suppress the activity of other T3RE-containing promoters (Brent et al., 1991). It has also been suggested that the T3 receptor interacting with its response element in the absence
of hormone may be capable of influencing the expression of other genes not generally known to be responsive to T3 (Brent et al., 1991).

The receptors with an intact DNA-binding domain incapable of binding T3 can also act as dominant negative mutations (Brent et al., 1991). This possibility is being explored in identifying the genetic disposition of the condition known as generalized thyroid hormone resistance (GTHR), where thyroid hormone deficiency and associated symptoms are observed in spite of normal or increased levels of serum thyroid hormone levels (Brent et al., 1991).

In vivo studies on experimental animals have shown that hepatic malic enzyme activity is increased by thyroid hormones as well as high carbohydrate diet. Activity of this enzyme is believed to be controlled at pretranslational level (Winberry et al., 1983). Carbohydrate feeding or T3-mediated increase in spot 14 mRNA in rat liver has been linked with the nuclear T3 receptor and lipogenesis (Towle and Mariash, 1986, Oppenheimer et al., 1987).

However, mRNA S14 is supposed to be mainly associated with the activity of fatty acid synthase system, as both were suppressed by dietary polyunsaturated fats (Clarke et al., 1990). It was suggested by Usala et al., (1988), that thyroid hormone-stimulated malic enzyme synthesis can not be linked with mRNA S14 because thyroid hormones do not change the spot 14 gene hypersensitivity or increase its rate of transcription. These authors further suggested that thyroid hormone-induced malic enzyme synthesis is only partly controlled at transcriptional level. Their study on hepatocytes from hyperthyroid rats (50 μg T3 per 100 g body weight per day for 10 days) showed each liver cell with ME mRNAs whereas in hypothyroid animals some liver cells totally lacked mRNA for malic enzyme. These authors concluded that heterogeneity to respond to thyroid hormones could induce functional recruitment of hepatocytes and varying degrees of mRNA content could affect the enzyme activity in altered thyroid state.

Most of the investigations discussed above have been conducted by using the technology of genetic engineering. This has certainly provided information regarding the interactions of nucleus and thyroid hormones in vitro. Nevertheless, these findings have to be accepted with caution because under given physiological conditions they cannot be directly related to the reactions in vivo. As many of these findings are still inconclusive, the exact mechanism that occurs at nuclear level is not yet fully known.

1.4.2. Thyroid hormone receptors of mitochondria

The profound effect of thyroid hormones on respiration has stimulated a major part of thyroid research on mitochondrial function. In section 1.3.5. and 1.3.6. certain aspects of thyroid hormone effects on mitochondrial structure,
function and the futile cycle of respiration leading to lipolysis are already discussed.

Studies conducted on the direct effect on mitochondria showed specific binding sites for thyroid hormones on mitochondrial membranes.

A mitochondrial membrane protein that bound T3 with a comparatively very high $K_a$ of about $2 \times 10^{11} \text{ M}^{-1}$ was purified and sequenced by Sterling et al., (1983). They observed an immediate rise in oxygen consumption, prior to any protein synthesis in isolated rat liver mitochondria treated with a dose of T3 (5 μmol / L). Depending on their results of higher T3 binding affinity of mitochondria and the effect on mitochondrial oxygen consumption, these authors suggested that the mitochondrial membrane receptor is one of the major loci for hormone binding and subsequent action. Seitz et al., (1985) support the above hypothesis by stating that since the overall functional capacity of mitochondria is augmented by thyroid hormones, leading to a futile cycle of ATP synthesis, mitochondria are possibly a target of early thyroid hormone action.

**1.4.3. Thyroid hormone receptors of plasma membrane**

Apart from the nucleus and mitochondria, thyroid hormone binding sites have also been located on cell plasma membrane. In rat, two types of thyroid hormone binding sites on liver plasma membrane have been identified as possible transport systems for hormone delivery into the cell (Pliam and Goldfine 1977, Gharbi-Chihi and Torresani 1981). Segal and Ingbar (1982, 1984), demonstrated the presence of two types; high affinity and high capacity binding sites on the plasma membrane of rat thymocytes. Synergistic binding of epinephrine and thyroid hormone was shown to increase the transport of the glucose analogues, 2-deoxy-D-glucose and 3-O-methylglucose. Increased activity of the intracellular effector, adenylate cyclase resulted in an increase of cAMP, independent of protein synthesis. Segal and Ingbar suggested that T3 stimulates the influx of Ca$^{++}$ into the cell which binds to calmodulin, a second messenger, to stimulate adenylate cyclase, resulting in excessive accumulation of cAMP and initiation of thyroid hormone effects.

This first messenger like activity of thyroid hormones and stimulation of membrane associated GPDH and Na-K-TPase suggest a strong possibility of hormone action on cell membrane composition, possibly membrane fatty acids.

In view of the earlier discussion on fatty acid oxidation and lipogenesis (section 1.3.3. and 1.3.4.) it is obvious that the predominant effect of thyroid hormones on lipid metabolism would also affect the fatty acid composition of phospholipids.

Hydrophobic bonding of thyroid hormones with phospholipids has been shown to result in the accumulation of a large proportion of thyroid hormones in
membranes (Hillier, 1970). Dickson et al., (1987) confirmed Hillier's findings by demonstrating with fluorescence quenching of the rat choroid plexus (discussed earlier in this chapter), a higher concentration of thyroid hormones in the middle of the bilayer where the hydrophobic fatty acids are located.

Hulbert (1978), has postulated that association of hydrophobic thyroid hormone molecules with membrane lipids changes the relative proportions of saturated and unsaturated fatty acids resulting in altered fluidity that could affect the functional properties of the cell membranes. Various studies discussed further in this section support the fact that membrane composition is altered by thyroid status. Hoch (1988) believes that these alterations are not the direct effects of hormones on membrane fatty acids but the result of thyroid hormone-induced altered activities of membrane associated desaturases.

1.4.4. Thyroid hormone effects on membrane composition

Effects of thyroid hormones on membrane fatty acid composition are well documented. Studies discussed below have been conducted on the fatty acid composition of mitochondria of liver, heart and brown adipose tissues (BAT). Hyperthyroidism appears to result in a loss of linoleic acid (18:2, n-6) and cholesterol and increase of arachidonic acid (20:4, n-6) content causing changes in membrane fluidity patterns (Ruggiero et al., 1984a, 1984b, Giuseppe and Ruggiero, 1988, Hoch., 1988).

Ruggiero et al., (1984a) reported a 14% increase in the phospholipid content of the liver mitochondrial membranes of hyperthyroid rats (30 μg T3 / day / 100g body wt. for 5 days). A significant 32.4% decrease in 18:2 but an increase in 20:4 and almost two fold increase in synthesis of 16:0 and 18:0 was observed. Increase in 20:4 was thought to be a compensatory response to excessive oxidation of 18:2. Since no significant changes in the microsomal phospholipids were observed, above authors suggested that polyunsaturated fatty acids synthesized by microsomes could be transferred to sites of oxidation. In another study Ruggiero et al., (1984b), reported a 30% decrease in esterified plasma cholesterol with its equal increase in the erythrocyte membranes of hyperthyroid rats (30 μg T3 / 100g / day for 5 days). Since cholesterol depletion via other channels (for example biliary excretion) occurs in hyperthyroidism, these authors suggested that the transfer of cholesterol from plasma to erythrocyte membranes in hyperthyroidism is an adaptive mechanism to maintain the stability of the erythrocyte membrane contour to prevent endocytosis. The same investigators examined the membrane composition of brown adipose tissues (BAT) of hyperthyroid rats (30 μg T3 / day / 100g body wt. for 7 days). Although 20:4 / 18:2 ratio remained high, BAT mitochondria contained 20% more cholesterol. Microsomes showed equal percentage reduction in cholesterol
and phospholipids but an overall increase of unsaturated fatty acids in the BAT of hyperthyroid rats has been related to resistance to cold in hyperthyroidism (Ruggiero et al., 1989).

Heart mitochondria of hyperthyroid rats given 30 μg T3 / day / 100 g body weight for 5 days showed an increase in 20:4 / 18:2 ratio (Giuseppe and Ruggiero, 1988). Pyruvate dependent oxygen uptake increased by 35-40% without a change in respiratory control or ADP / O ratio. Isolated pyruvate carrier activity was found to be dependent on cardiolipin. A thyroid hormone-mediated 50% increase in cardiolipin content with increased 20:4 and decreased 18:2 was suggested to be responsible for the changes in the kinetic parameters of this transporter. Absence of the increase in cyanocinnamate sensitive binding sites of the transporter indicated an absence of protein synthesis.

Beleznai et al., (1989) examined GPDH location and the fluidity of hyperthyroid rat liver mitochondrial and mitoplast membranes by steady-state fluorescence anisotropy measurements. High incorporation of saturated fatty acids in the cardiolipin and reduced cholesterol content of the hyperthyroid mitochondrial membranes was thought to cause highest order and a rigid membrane. Higher concentration of digitonin was required to release thyroid responsive GPDH from the mitochondrial membranes which is suggested to be due to the hormone-induced rigidification of the membrane surrounding the protein (Belznai et al., 1989).

Linoleic acid content of the liver mitochondrial membranes has been shown to increase in hypothyroidism (Hoch et al, 1981, Withers and Hulbert, 1987). Shaw and Hoch (1977) reported an increase in the energy of activation (Ea) of both state 3 and state 4 respiration between 21 and 38°C in the heart mitochondria from hypothyroid rats. Linoleic acid content increased by almost 9% in the hypothyroid membranes. Shaw and Hoch suggested that above changes in fatty acid composition of mitochondrial membranes would result in an increase in the Ea. Valdmarsson and Gustafson (1988) observed high 18:2 content in the plasma of hypothyroid humans before T4 therapy. The unsaturation index (UI) was 140. Thyroxine treatment reduced the 18:1 and 18:2 content of plasma lipids and elevated the 20:4 and 22:6 content which increased the UI to 153.

These findings indicate that thyroid hormones can affect membrane fatty acid composition which may in turn affect membrane associated enzyme functions.
1.5. Fatty acid effects on the activity of thyroid hormones

1.5.1. Fatty acid-mediated inhibition of thyroid hormone binding to nucleus

An increasing number of studies have shown that unsaturated fatty acids are capable of inhibiting the binding of thyroid hormones to nuclear binding proteins. A concentration of 33 μM oleic acid was shown to decrease T3 binding from 32% to less than 5% in 30 minutes (Van der Klis et al., 1989). It was also demonstrated that inhibition of nuclear binding of T3 by oleic acid was not due to any direct fatty acid hormone interaction. Oleic acid and T3 are thought to compete for the same binding site on the nuclear receptor. The inhibition was found to be reversible by bovine serum albumin (BSA) because oleic acid had greater affinity for BSA than the receptor.

Compared to a diet of mouse chow, mice fed high polyunsaturated diet (22% of calories as 18:2, n-6) showed a greater total and specific activity of the nuclear membrane ATPase (NTPase) (Venkatraman et al., 1986). The level of 18:2 was elevated whilst there was a decrease in 20:4 content of the phospholipids. Incorporation of high PUFAs in the membranes increased the activity of the NTPase at 37°C indicative of the increase in mRNA transport. However, binding of T3 to nuclear envelope which was comparatively higher at 22°C with both mouse chow and PUFA diet, decreased, as the temperature was increased to 30°C. These authors suggested decreased binding of T3 at increasing temperature. This is interesting because it suggests an activating effect of PUFA on NTPase at physiological temperature of 37°C but a negative effect on binding of T3 to nuclear envelope at a temperature higher than 22°C.

Wiersinga and Platvoet-Ter-Schiphorst (1990) and Inoue et al., (1989), have suggested that compared to saturated fatty acids, long chain fatty acids were more effective in inhibiting the binding of T3 to its nuclear receptor.

The above authors postulated that the inhibitory potency was high with free fatty acids and increased with the number of double bonds in these fatty acids. The kinks in the cis-isomers of unsaturated fatty acids were specifically effective in causing an allosteric inhibition of nuclear T3 binding.

By comparing the effects of saturated and polyunsaturated fatty acids, Wiersinga and Platvoet-Ter-Schiphorst (1990) suggested that the fully extended saturated fatty acid resembled the trans-isomer configuration which was not as effective as the cis form in inhibiting the binding of T3.

As thyroid hormones are very hydrophobic, their binding to the nucleus is suggested to be influenced by the interaction of fatty acids with the hydrophobic sites of the nuclear receptor; its conformational change resulting in a lower affinity for T3 (Wiersinga and Platvoet-Ter-Schiphorst 1990).
Experiments with isolated rat liver nuclei with added 16:1, 18:1, 18:2, 18:3 and 20:4 to the incubation medium indicated that 2.8 μM oleic acid (18:1) (which was the lowest concentration of all the above fatty acids used), inhibited 50% of the binding of T3 to its nuclear receptor. Inoue et al., (1989) therefore suggested oleic acid (18:1) to be structurally best fitted as an inhibitor. Concentration of 10 μM and 30 μM oleic acid inhibited the binding by 84% and 100% respectively. Free fatty acids are thought to be more potent inhibitors than esterified fatty acids (Wiersinga and Platvoet-Ter-Schiphorst, 1990, Inoue et al., 1989).

Although physiological concentration of oleic acid was not mentioned by the above authors, Henning and Watkins (1989) reported that free fatty acid concentrations of 180-1650 μmols L⁻¹ is commonly found in human plasma when the plasma albumin concentration is ~ 600 μmol L⁻¹. Wiersinga and Platvoet-Ter-Schiphorst (1990), and Inoue et al., (1989), did not report using any albumin in their assays. As reported by Van der Klis et al., (1989) and Mendel et al., (1986) if albumin modulates the effects of free fatty acids then inhibition of T3 binding by such low concentrations of free fatty acids in the absence of albumin, used by Wiersinga and Platvoet-Ter-Schiphorst and Inoue et al., cited above appears to be a non-physiological effect.

1.5.2. Effect of fatty acids on hormone binding to plasma proteins

The major function of plasma albumin is to transport free fatty acids through the blood (Ashbrook et al., 1975). Since albumin also transports thyroid hormones, it was proposed that physiological plasma concentrations of free fatty acids may affect concentration of free T4 in vivo (Hollander et al., 1967).

Since it is accepted that free rather than the protein-bound form of thyroid hormones are physiologically active, then the answer to the question whether free fatty acids influence the free thyroid hormone levels, requires to take into consideration, the levels of thyroid hormone binding proteins as well as free fatty acids in the plasma.

Mendel et al., (1986) used oleic acid (which is the most abundant of the circulating free fatty acids and all free fatty acids appear to exert the same effect on free T4 level) and suggested the following possible interaction of free fatty acids (FFA) with thyroid hormone binding proteins and the consequent effects on the free T4 concentrations in the serum:

1. Albumin rather than the FFA concentration is more important in modulating the effects of FFA.

2. Oleic acid had no effect on free T4 below the FFA to albumin molar ratio of about 5:1 (this concentration in vivo is considered extremely rare and not documented). [The normal free fatty acid to albumin ratio in humans is between 0.3 and 2.8. (Henning and Watkins, 1989)].
3. Studies on the concentrations of FFA and serum albumin in humans suffering from non-thyroidal illness revealed a mean FFA to albumin molar ratio of 1.5.

4. High concentrations of oleic acid resulted in higher free T4 level in the presence of transthyretin, only when TBG was absent in the serum, indicating that absence of TBG in the presence of high concentrations of FFA would increase free hormone level.

5. Physiological concentrations of long chain unsaturated free fatty acids indicated no effect on normal T4 binding unless the serum samples were diluted, during estimations by various assay methods.

It has also been reported that hypoalbuminemia in non-thyroidal illness or the presence of thyroid binding inhibitory activity (THBI) increase the FFA / TBG ratio resulting in elevated plasma T4 concentration (Benvenga et al., 1987).

Certain drugs eg., fenclofenac increased the free T4 fraction by 90 % (Lim et al., 1988). These authors suggested that very high levels of T4 in thyrotoxicosis or marked decrease in albumin concentrations would also result in elevation of FFA concentrations and raise the T4 level in the plasma.

1.5.3. Effects of fatty acids on thyroid hormone levels

In 1949, Ershoff had reported the beneficial effects of full-fat soybean meal (18–22 % fat) on counteracting the weight loss of immature hyperthyroid rats treated with 0.25 to 0.5 % desiccated thyroid in the diet for 28 days. He speculated that some component of the soybeans was somehow exerting this effect. The preceeding discussion on fatty acid effects on thyroid hormone activity pointed out that free fatty acids are capable of displacing thyroid hormones from their binding proteins but experiments conducted so far, indicated requirement of supraphysiological concentrations of free fatty acids to exert this inhibition; in which case high free hormone concentrations, not bound to plasma proteins have been estimated in the assays.

Addition of 5 mmol / L oleic acid to undiluted serum increased the FFA concentration 8-fold over that of albumin, resulting in increased free fraction of T3 and T4 by 88 and 122 % respectively (Lim et al., 1988). 1.5 mmol / L palmitic and stearic acid had no effect but same concentration of 18:2, 18:3 and 20:4 increased free T4 by 11, 23 and 26 % respectively. An increase of 32 % free T4 was observed in human serum after adding 3 meq / L oleic acid. Normal range is 1 meq / L (Mendel et al., 1986). In the absence of albumin, addition of 3 meq / L oleate increased the free T4 fraction by 120 %.

These investigations have been conducted in vitro. To my knowledge no report on total thyroid hormone levels influenced by dietary fatty acids in vivo, in experimental hyperthyroidism have been documented so far.
Thyroid hormones influence a number of physiological and biochemical parameters but exogenous factors, for example type of dietary fatty acids appear to influence thyroid hormone activity, causing changes in the metabolic functions influenced by these compounds.

1.6. Dietary fats

Incorporation of dietary fats into cellular membranes can alter their fatty acid composition, and may affect the activities of certain membrane associated proteins which act as enzymes. Dietary fats, membrane fatty acids, effects of altered fatty acid composition on membrane functions and possible interactions between fatty acids and thyroid hormone activities are discussed in the following sections.

Table 1 contains the list of major dietary fats which are also found in the cell membranes.

Oxidation of body fats provides considerably more energy compared to that from carbohydrates and proteins {\( \text{about } 39.3 \text{ kJ g}^{-1} \text{ and } 18 \text{ kJ g}^{-1} \) respectively (calculated from Schmidt-Nielsen 1983, p 179)}.

Fats are also required for transporting lipoproteins and fat soluble vitamins (eg. vitamin A, D and E).

Apart from being an important source of energy fats are also the major structural components of membrane lipids (phospholipids). The even numbered, hydrophobic long fatty acid chains are attached to the first and second hydroxyl groups of glycerol (Lehninger, 1982, p-310) of the lipid molecule. Major dietary saturated fatty acids, palmitic and stearic, are obtained from the diet or can be synthesized \textit{de novo}. They are used in triglyceride and phospholipid synthesis. They contain 16 and 18 carbon atoms respectively, with single bonds joining the carbon atoms. Unsaturated fatty acids possess double bonds between the carbon atoms and according to the distance between the terminal methyl group and double bond closest to it, are classified into these families: oleic (w9 or n-9), palmitoleic (n-7), linoleic (n-6) and linolenic (n-3) (Van Deenan, 1965). Other polyunsaturated species are derived by desaturation and elongation of these precursors (Brenner, 1971).

Animals cannot synthesize the precursors (linoleic 18:2 and linolenic 18:3 acid) of the longer chain n-6 or n-3 families. These fatty acids are the essential precursors in the synthesis of prostaglandins and some specific phospholipids (for example cardiolipin of mitochondrial membrane mainly contains 18:2 Daum, 1985).

Only dietary sources can provide 18:2. Inducing 18:2 deficiency in rats is found to result in retarded growth, scaly skin, fatty liver and an increase in the
1.7. **Membrane fatty acid composition**

Biological membranes contain proteins, lipids and cholesterol, bound in a noncovalent form. The main lipid constituents of membranes are phospholipids, glycolipids, sphingolipids and cholesterol (Lehninger, 1982, p 310-311).

The major phospholipids are phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine and phosphatidylinositol. As stated earlier, mitochondrial membranes mainly contain diphosphatidyl-glycerol or cardiolipin (Daum, 1985).

The two hydrophobic fatty acid chains esterified to the lipid molecules constitute the core of the bilayer which also contains intrinsic and integral proteins, many of which act as enzymes, receptors, transport systems and also ion channels (Tahin et al., 1981, Stubbs and Smith, 1984). Most membrane fatty acids in mammals contain 16 to 22 carbon atoms.

Of the two fatty acyl chains esterified to the lipid molecule, one is generally saturated. The double bonds between specific carbon atoms of the second unsaturated fatty acid create various movements of the acyl chains within the membrane bilayer thereby increasing its 'fluidity' (Housely and Stanley, 1982).

Desaturases (multienzyme complexes bound to microsomal membranes and present in animals and plants) insert double bonds between carbon atoms. Their activation is inversely related to the microsomal membrane fluidity, discussed further in this section. Animal membranes possess $\Delta^4$, $\Delta^5$, $\Delta^6$, and $\Delta^9$ desaturases but they cannot perform $\Delta^{12}$ or $\Delta^{15}$ desauration. Plants possess $\Delta^9$, $\Delta^{12}$ and $\Delta^{15}$ desaturases (Brenner, 1971). Therefore 18:2 (double bonds at carbon 9 and 12) and 18:3 (double bonds at carbon 9, 12 and 15), are essential fatty acids in mammalian diet.
### Table 1.
Major constituent fatty acids of the phospholipids

<table>
<thead>
<tr>
<th>Systematic name</th>
<th>Shorthand designation</th>
<th>Fatty acid family</th>
<th>Common name</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Hexadecanoic</td>
<td>16:0</td>
<td></td>
<td>Palmitic</td>
</tr>
<tr>
<td>n-Octadecanoic</td>
<td>18:0</td>
<td></td>
<td>Stearic</td>
</tr>
<tr>
<td>cis 9-Hexadecenoic</td>
<td>16:1</td>
<td></td>
<td>Palmitoleic</td>
</tr>
<tr>
<td>cis 9-Octadecenoic</td>
<td>18:1</td>
<td>n-9</td>
<td>Oleic</td>
</tr>
<tr>
<td>cis 11-Octadecenoic</td>
<td>18:1</td>
<td>n-7</td>
<td>Vaccenic</td>
</tr>
<tr>
<td>cis, cis 9,12 Octadecadienoic</td>
<td>18:2</td>
<td>n-6</td>
<td>Linoleic</td>
</tr>
<tr>
<td>All cis 9,12,15-Octadecatrienoic</td>
<td>18:3</td>
<td>n-3</td>
<td>ω-Linolenic</td>
</tr>
<tr>
<td>All cis 6,9,12-Octadecatrienoic</td>
<td>18:3</td>
<td>n-3</td>
<td>ω-Linolenic</td>
</tr>
<tr>
<td>All cis 5,8,11,14-Eicosatetraenoic</td>
<td>20:4</td>
<td>n-6</td>
<td>Arachidonic</td>
</tr>
<tr>
<td>All cis 4,7,10,13,16,19-</td>
<td>Docosahexaenoic</td>
<td>22:6</td>
<td>DHA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Source:
2. Sigma catalogue, 1991
Lipid systems, eg., biological membranes, exist between liquid-crystalline (fluid) and gel (rigid) phase. Preservation of fluid-gel state against reaching any extreme phase is important for the functional integrity of membranes (Hochachka and Somero, 1984).

Prokaryotes maintain their fluid-gel state by modifying the molecular arrangements and proportions of various fatty acids. Sinensky (1974), first discovered this capability in the membranes of *Eschrechia coli*, subjected to various temperature conditions in culture, and called it the 'homeoviscous adaptation'.

Even in eukaryotes, temperature (Cossins, 1981, Shinitzky, 1986), pressure (Cossins and Macdonald, 1984), and diet (Yamamato et al, 1965, Ehrstrom et al, 1981, Morson and Clandinin, 1986), may induce compensatory "homeoviscous" changes in the membranes. However, they cannot synthesize essential fatty acids and therefore, although the compensatory desaturation and elongation reactions maintain the overall saturation:unsaturation ratio, the types of fatty acids produced, are of different origin and contain different proportions, positions and number of double bonds. These alterations may produce changes in the orientation and activity of certain membrane associated enzymes.

1.8. **Effects of dietary fats on cellular functions**

1.8.1. **Mitochondrial respiration**

As pointed out in the previous section, it has been shown that dietary fatty acids can be incorporated into membranes. Mak et al., (1983) observed different effects of dietary 16 % w/w beef tallow (saturated fat, low in 18:2) and safflower oil (polyunsaturated fat high in 18:2) on the ATP /ADP carrier of rat liver mitochondria.

Liver mitochondrial fatty acids contained high levels of 18:2 and 20:4 and the degree of unsaturation in the lipids was 85 % higher in safflower oil diet group than the beef tallow group. At physiological temperature, the net ADP-dependent activity of the translocase was 13 % higher in high 18:2 diet group. Mak et al., (1983) concluded that the increase in unsaturated bonds in the phospholipids increased the maximum reaction rate (Vmax) and activity of the translocase due to the alterations in mitochondrial membrane composition.

Robinson, (1982) has related high affinity of cytochrome c oxidase (electron transporting intrinsic protein of the inner mitochondrial membrane) to the overall structure of cardiolipin. He also demonstrated that once the enzyme binds to the lipid, its activity of transporting electrons depends upon the hydrocarbon environment of the membrane. Electron transport was twice as high with unsaturated
than with saturated (16:0) fatty acids.

Heart mitochondria from rats fed an essential fatty acid deficient diet showed a significantly diminished activity of cytochrome c oxidase and respiratory function than those receiving 20 % corn oil (Yamaoka et al.,1990). The cis molecular species,18:2 (n-6) /18:2 (n-6) constituted 75.1 % of cardiolipin from corn oil fed as against 56.6 % in essential fatty acid deficient rats. Yamaoka et al., concluded that reduction in the specific molecular species, 18:2 / 18:2 from the cardiolipin due to essential fatty acid deficiency resulted in the significant decrease in the activity of the electron transporting enzyme thus causing a decrease in mitochondrial O_2 consumption.

**1.8.2. Dietary modulation of Na-K-ATPase and other ATPases**

Many studies have reported that essential fatty acid deficiency (EFAD) resulted in an increase in the activity of Na-K-ATPase. Comparisons of dietary fatty acids showed that polyunsaturated fatty acids added to the diet decreased the activity of this enzyme. Compared to a 10 % safflower oil diet (enzyme activity 10.5 μmols Pi / mg / h), a diet containing only saturated fat (e.g., 10 % butter) induced marginal EFAD but increased the ATPase activity (33.6 μmols Pi / mg / h) in salivary gland and kidney homogenates of rats treated for 45 weeks on the above diets. (Alam and Alam, 1983).

A diet deficient in essential fatty acids for three months resulted in an increased activity of Na-K-ATPase in the brain homogenates of mice (Sun and Sun 1974). Enzyme activity in controls, (fed 2 % corn oil in the diet) was significantly lower than in the EFAD mice. Phospholipids of the membranes of EFAD mice had an increase in 20:3 (n-9) and 22:3 (n-9) and decrease in 20:4 (n-6) 22:4 (n-6) and 22:6 (n-3), (an indicator of EFAD), but total polyunsaturates were increased in EFAD membranes than in controls on account of the increase in 20:3 (n-9) content. Sun and Sun suggested that the increase in membrane polyunsaturates in essential fatty acid deficiency would maintain the fluid-gel state but the increase in Na-K-ATPase activity may be the result of biological adaptation to altered brain function due to EFAD diet and therefore altered membrane fatty acid composition.

However, Bloj et al., (1973) from their observations of the erythrocyte membrane fluidity, irrespective of the dietary fatty acids used, suggested that it is not the type of fatty acid (essential or non-essential), but the double-bond / saturated fatty acid ratio which affects the allosteric interactions of the enzyme activity in an inverse manner i.e., a decrease in Hill coefficient with an increased membrane unsaturation. According to their observations, an increase in the number of double bonds and membrane unsaturation decreased the enzyme activity.
An increase in the intracellular sodium content and an increase in the rate of sodium transport across the cell membrane in thymocytes of rats fed diet high in coconut oil compared to rats fed diet high in corn oil content was reported by Murray and Patrick (1986).

Activites of the Ca\(^{2+}\) and Mg\(^{2+}\) ATPases appear to respond differently to a different dietary fatty acid. Swanson et al., (1989) reported a relative 100% increase in Ca\(^{2+}\) and Mg\(^{2+}\) ATPase activity in cardiac sarcoplasmic reticulum (SR) of mice fed 10% refined corn oil diet + 2% safflower oil for 2 weeks when compared to a 15% increase with refined menhaden oil + 2% safflower oil. These authors suggested that the high content of 22:6 (n-3) in menhaden oil perhaps changed the membrane composition, resulting in reduced calcium flux across cardiac SR in mice and also decreased the rapid changes in cellular calcium concentrations. Christon et al., (1988) observed a 30% decrease in the Ca\(^{2+}\) ATPase activity in liver microsomes of EFA-deficient rats (fed 5% hydrogenated coconut oil) compared to controls (5% corn oil).

Although dietary induction of essential fatty acid deficiency has been shown to increase the activity of Na-K-ATPase with simultaneous decrease of n-6 fatty acids in membranes, a definite conclusion about the inhibitory effect of linoleic acid on the activity of this enzyme cannot be drawn from these reports. In fact Hansen (1989) reported that in the pig kidney outer medulla, binding of vanadate (a high-affinity agonist of the cytosolic phosphorylation epitope of the sodium pump) was not affected by 18:2 and therefore this fatty acid cannot be considered as a ouabain-like inhibitor of the enzyme activity.

It appears that it is the number of double bonds and perhaps their positions in the fatty acids, rather than the type of fatty acids, may alter the membrane composition and affect the catalytic properties of certain membrane-bound enzymes.

1.8.3. Dietary fatty acids and lipogenesis

A high carbohydrate diet has been known to increase the activity of lipogenic enzymes (Tepperman and Tepperman, 1964, 1965, Towle and Mariash, 1986). Saturated fatty acids also exert similar effect. In one study saturated and unsaturated fatty acids (3%) were fed separately, as dietary supplements to rats for 7 days (Clarke et al., 1977). The activity of fatty acid synthesis and associated cytosolic fatty acid synthetase (FAS), malic enzyme (ME), and glucose-6-phosphate dehydrogenase (G6PD) were all increased by feeding saturated fatty acids (16:0 and 18:0). This was suggested to be due to the poor absorbability of these saturated fats. Fatty acid synthesis and activities of the above enzymes in rat liver were equally inhibited by 18:2 (n-6) and 18:3 (n-3). However, fatty acid synthesis in adipose
tissues was unaffected (Clarke et al., 1977).

When compared to 15% corn oil diet, a 11-day dietary treatment of 15% (w/w) coconut oil (mostly saturated fat) resulted in a 3-fold increase in the activity of hepatic fatty acid synthetase in mice (Schwartz and Abraham 1982). Polysaturated fatty acids (PUFA) with 18-carbon atoms and double bond position Δ⁹,₁² were the most effective inhibitors of the synthesis of this enzyme. In another report by these authors it was suggested that type of fatty acid did not affect the synthesis of ME mRNA but PUFA affected either the translation or caused a rapid degradation of the product of translation which resulted in decreased enzyme activity (Schwartz and Abrahams, 1983).

The inhibitory effect is supposed to be the direct effect of PUFA themselves and not their metabolic products (Szepesi et al., 1989). Fish oils and safflower oil effectively suppressed fatty acid synthase activity and gene expression of mRNA S-14, a member of lipogenic proteins coding for fatty acid synthase (Clarke et al., 1990).

Synthesis of lipogenic enzymes has been shown to be controlled at the level of nucleus by thyroid hormones and an unknown signal from the lipogenic carbohydrate diet (Towle and Mariash, 1986, Oppenheimer et al., 1987). Tepperman and Tepperman (1965) have suggested that augmentation in the activity of lipogenic enzymes by feeding high carbohydrate, fat free or saturated fat diets is basically due to the absence of unsaturated fatty acids in these diets. This triggers the enzymes of the desaturation and elongation systems which require NADPH. Activity of all lipogenic enzymes generates NADPH (Tepperman and Tepperman, 1965). Since corn oil and safflower oil supply the required unsaturated fatty acids, inhibition of the lipogenic enzymes by these fatty acids observed by others supports the above suggestion of Tepperman and Tepperman.

It has also been suggested that diets high in 18:2 content cause an increase in the activity of phospholipase A in mitochondria as well as other membranes. phospholipase activity was thought to result in breakdown of lipids and inhibition of weight gain (Mak et al., 1983).

1.8.4. Dietary fatty acids and activity of glycerolphosphate dehydrogenase (GPDH)

Integral proteins of mitochondrial membranes eg. cytochrome c oxidase are shown to require 18:2 in the cardiolipin for their activity (Yamaoka et al., 1990). However it is not known whether diet-induced alterations in mitochondrial membrane fatty acid composition affect the activity of GPDH.
CHAPTER TWO

Animals, experimental plan and methods

2.1. Animals

Male CBA mice, *Mus musculus* were bred in the animal house at the University of Wollongong. All the mice used in the experiment were 55 to 60 days old and weighed 20–26 g at the beginning of the treatment. Mice were housed in individual cages and were provided with wood shavings for flooring. A 12h:12h light: dark cycle and room temperature of 26°C was maintained. Food and water were available *ad libitum*. Shallow trays were filled with fresh food before 7 o’clock every evening. Drink bottles were cleaned and filled with fresh solutions every 4th day. Accurate measurement of food consumption was not possible under the experimental conditions but water consumption per day was recorded.

2.2. Chemicals

The source and grade of all chemicals and biochemicals are given in appendix 1.

2.3. Experimental plan

Littermates were randomly assigned to the experimental groups. Mice were placed in one euthyroid (no hormone) and two hyperthyroid groups. Hyperthyroid groups were treated with T3 and T4 separately. In each of these three groups mice were fed either a saturated fatty acid (SFA) or polyunsaturated fatty acid (PUFA) diet. All the animals were under treatment for 21–22 days. Figure 4 illustrates the general experimental plan.

Oxygen consumption was measured before starting and at the end of the treatment. Body weight was recorded prior to each measurement of oxygen consumption. Mice were killed at the end of the treatment, plasma was separated and livers were removed. Plasma hormone levels, total liver weight, total liver protein, and cytosolic and mitochondrial protein were estimated. Specific activities of the hepatic malic enzyme (ME) in the cytosol, sodium-potassium-ATPase (Na-K-ATPase) in the liver homogenate and glycerolphosphate dehydrogenase (GPDH) in the liver mitochondria were measured. Fatty acid analysis of the liver phospholipids was also conducted.
Fig. 4.

Experimental plan

(Mice treated for 21–22 days)

Euthyroid

No Hormone

SFA (n=7)

PUFA (n=7)

Hyper T3

Hormone Concentration 2.2 μM T3

SFA (n=7)

PUFA (n=7)

Hyper T4

Hormone Concentration 3.1 μM T4

SFA (n=7)

PUFA (n=7)

SFA (saturated fatty acid diet)

PUFA (polyunsaturated fatty acid diet)

(n = 7) indicates the number of mice.

Following parameters were investigated:

Body weight before and after the treatment.
Standard metabolic rate, measured before starting and at the end of treatment.
Final T3 and T4 levels in the plasma
Liver weight.
Total, cytosolic and mitochondrial protein concentration in the liver.
Specific activity of cytosolic liver malic enzyme.
Specific activity of Na-K-ATPase in the liver homogenate.
Specific activity of hepatic glycerol phosphate dehydrogenase of the inner mitochondrial membrane.
Fatty acid composition of hepatic phospholipids.
2.4. **Induction and maintenance of hyperthyroidism**

Hyperthyroidism was induced by administering T3 and T4 (see Appendix 1 for the details of chemicals) separately in the drinking water (Gemmil, 1956).

Concentrations of 2.3 μM T3 and 3.1 μM T4 were decided upon the basis of the results of preliminary experiments conducted on male CBA mice (results not shown). All the mice appeared to be normal healthy adults. An amount of 25 mg of T3 and T4, was dissolved separately in 8-10 ml of 0.02 M NaOH (Gemmil, 1956) and stock solutions were prepared by diluting these in 100 mls of distilled water and stored at 0–4°C. A fresh stock was prepared every three weeks. Normal tap water was used for diluting the stock for preparing fresh drinking solutions every 4th day. Final concentration of NaOH in all drinking solutions including the controls was about 0.02 mM. Animals did not appear to have any difficulty in consuming the water containing thyroid hormones or NaOH.

2.5. **Preparation of diets**

Two separate isocaloric diets with 18 % w/w total fat but different fatty acid composition were prepared using coconut oil and safflower oil which were the only sources of dietary fat.

SFA diet —— (coconut oil diet, high saturated fat, low linoleic acid)

PUFA diet ---(safflower oil diet, high polyunsaturated fat, high linoleic acid)

Diets were prepared according to the basal diet plan of the Association of Agricultural Chemists, Washington D.C. (1960). Details of diet composition can be seen in Table 2. Vitamins and salts (Appendix 1) were mixed according to the method in the diet plan. All the ingredients were mixed in a Kenwood blender. Fresh diets were prepared once a week and stored at -20°C in sealed containers from which small amounts were taken out and stored in the refrigerator in air-tight containers for feeding every day.

The calculated energy contents (joules / 100g) of each dietary component in the two diets were:

- Carbohydrates 843
- Proteins 396
- Fat 708

These were calculated from the energy value / g given by Schmidt-Nielsen (1983).
### Table 2.

**Composition of SFA and PUFA diets (g / 100 g)**

<table>
<thead>
<tr>
<th>Diet component</th>
<th>SFA diet</th>
<th>PUFA diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>22.0</td>
<td>22.0</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>48.0</td>
<td>48.0</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Salt mix</td>
<td>5.0</td>
<td>5.0</td>
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<tr>
<td>Cellulose</td>
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<tr>
<td>Water</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Safflower oil</td>
<td>1.5</td>
<td>18.0</td>
</tr>
<tr>
<td>Coconut oil</td>
<td>16.5</td>
<td>0.0</td>
</tr>
</tbody>
</table>

2.6. **Fatty acid composition of lipids in the diets**

Fatty acid composition was determined by the method of Hulbert and Else (1989) using Varian 3300 gas liquid chromatograph (Table 3). Fats from the diet samples were extracted in 2:1 chloroform: methanol containing 0.01% butylated hydroxy toluene (BHT). Linoleic acid content of safflower oil and coconut oil, analysed separately, was 72 and 0.8% respectively.

As coconut oil contained only 0.8% linoleic acid, in order that the SFA diet was not essential fatty acid deficient, 1.5% w/w safflower oil was added to the diet which increased the 18:2 content up to 6.7% of the total dietary fat. Analysis of the fatty acid composition of the dietary lipids showed the 18:2 content of SFA diet to be about 6% of the total fat and that of PUFA diet about 72% (Table 3). Mead (1980) suggests that in humans at least 5% of the total dietary fat should be linoleate, and in normal subjects about 40% of the total fat in the form of essential fatty acids should be considered as optimum.

Increased amount of polyunsaturated fat in the diet increases the degree of peroxidation and therefore the requirement of vitamin E. Briggs and Wahlqvist (1984) suggest an amount of 0.4 mg vitamin E for each gram of polyunsaturated fatty acid consumed. Vitamin E content of naturally occurring coconut oil and safflower oil is 0.5 and 39 mg/100 g respectively (Briggs and Wahlqvist, 1984). Hyperthyroidism is known to cause vitamin E deficiency (Ingbar, 1985) therefore the level of vitamin E in both the diets was adjusted to 40 mg/100 g fatty acid. Although naturally occurring safflower oil contains about 3.4% linolenic acid (18:3, n-3) (Hardwood and Geyer, 1964), analysis of the fatty acid composition of commercially purchased safflower oil used in the present experiment indicated no trace of 18:3.
<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>SFA diet</th>
<th>PUFA diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>12:0*</td>
<td>55.6</td>
<td>0.3</td>
</tr>
<tr>
<td>14:0</td>
<td>18.8</td>
<td>0.4</td>
</tr>
<tr>
<td>16:0</td>
<td>9.7</td>
<td>8.0</td>
</tr>
<tr>
<td>18:0</td>
<td>2.6</td>
<td>2.2</td>
</tr>
<tr>
<td>18:1</td>
<td>6.4</td>
<td>16.4</td>
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<tr>
<td>18:2</td>
<td>6.2</td>
<td>71.9</td>
</tr>
<tr>
<td>Total</td>
<td>99.3</td>
<td>99.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>SFA diet</th>
<th>PUFA diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monounsaturates</td>
<td>6.4</td>
<td>16.4</td>
</tr>
<tr>
<td>Polyunsaturates</td>
<td>6.2</td>
<td>71.9</td>
</tr>
<tr>
<td>Unsaturated fatty acids</td>
<td>12.6</td>
<td>88.3</td>
</tr>
<tr>
<td>Unsaturation Index §</td>
<td>19</td>
<td>160</td>
</tr>
</tbody>
</table>

* Number of carbon atoms: number of double bonds.

§ Average number of double bonds per 100 molecules.
2.7. **Measurement of standard metabolic rate**

In animals, resting oxygen consumption in a thermal neutral environment, measured in post-absorptive state (with minimum variations in the heat production) is considered as the basal metabolic rate (BMR) (Brody, 1945). Standard metabolic rate is the sum total of oxygen consumption of resting cells during the metabolic processes as well as the oxygen consumed by the animal to perform the mechanical functions required to continue the cellular metabolic processes (Brand, 1990a). In the present study SMR refers to the resting oxygen consumption of mice at 30°C ambient temperature after a 4 hour fast.

An open flow system was used to measure the oxygen consumption. Body weight was recorded before placing the animal in the metabolism chamber (about 350 cm\(^3\) capacity), maintained at a constant ambient temperature of 30°C. A constant flow of dry (moisture absorbed by drie-rite) (see Appendix 1. for chemicals) and CO\(_2\) free (absorbed by vivalyme) air was passed through the metabolism chamber. This constant flow was maintained with a calibrated flow regulator (Teledyne Hastings). Air leaving the chamber was also dried and CO\(_2\) was absorbed. The oxygen content of both “inlet” and “outlet” air was measured with a Taylor Servomex type OA 272 oxygen analyzer and the analyzer output was continuously recorded on a National chart recorder. The difference in O\(_2\) content between the “inlet” and “outlet” air was kept to less than 1 % by appropriate regulation of the flow rate. Readings were recorded when the mice were visually resting and the recorded trace of the oxygen content of “outlet” air was stable.

For each animal, standard metabolic rate was calculated as the average of 3 replicate readings of 5–7 minutes duration. Oxygen consumption was calculated as ml O\(_2\) h\(^{-1}\) using equation “A” from Hill (1972).

\[
O_2 \text{ consumption (ml O}_2\text{.h}^{-1}) = \text{Flow rate ml. h}^{-1} \times \Delta O_2
\]

For all euthyroid animals and all initial measurements, the flow rate was regulated in the range of 30–32 L min\(^{-1}\) whilst for hyperthyroid animals the flow was regulated at 60 L min\(^{-1}\).
2.8. Plasma hormone determination

Total T3 and total T4 were measured in the undiluted plasma by radioimmunoassay (RIA). T3 and T4 RIA kits were purchased from Spectria, Australia. The test tubes used in each hormone assay were geometrically optimized and coated with respective antibodies. Sealed contents of the kits stored at 0–4°C were used within a week of purchase. Heparin (sodium salt) was from Sigma.

Animals were killed on the 23rd day by cervical dislocation. About 1 to 1.5 ml of blood was collected from the heart in individual 3 ml eppendorf tubes containing heparin (12.5 iu for each ml of blood. Dacie and Lewis, 1975). The contents, after mixing gently, were spun in a Beckman microfuge for 3 to 4 minutes (3000 rpm). Separated plasma was collected in individual vials and stored in liquid nitrogen until tested.

The assay was performed according to the manufacturer's instructions. In the preliminary experiments, plasma T3 level of some hyperthyroid mice exceeded 12 nM (the highest concentration of the standard supplied in the kit). To avoid extrapolations, in the final experiment a 24 nM standard was made up by using an extra 12 nM lipophilized standard of T3 by dissolving it in 0.5 ml instead of recommended 1 ml of distilled water. To this, an equal volume of 24 nM T3, initially dissolved in a very small volume of dimethyl sulfoxide (DMSO) and diluted with distilled water, was added to give a final 24 nM T3 concentration in 1 ml. Kit standards were measured in triplicate whilst test samples were measured in duplicate as only small volumes of plasma were available. Residual radioactivity (i.e., the labelled hormone bound to antibody) was counted for at least 10,000 counts on a Packard Cobra 5 well gamma counter. The hormone concentrations of plasma samples were calculated by comparison of percent binding (after deduction of non-specific binding, NSB) to the curve obtained from the standard serum supplied with the kit. Representative standard curves for both T3 and T4 are shown in figures 5 and 6. Cricket graph programme on the Macintosh computer was used to calculate the final nM concentrations by mathematical transformations of the values obtained from the standard graphs.
Standard Graphs of T3 and T4 RIA

Fig. 5 Standard graph of T3

![Graph of T3](image)

Fig. 6 Standard graph of T4

![Graph of T4](image)
2.9. **Measurement of liver enzyme activities**

2.9.1. **Preparation of the homogenization buffer**

Since activities of three enzymes located in different parts of the liver cell were being assayed, it was important to prepare a homogenizing buffer that protected enzyme integrity and eliminated any interfering radicals. Sucrose (0.25 M) (Hsu and Lardy, 1969, Jorgensen, 1988, Johnson and Lardy, 1967), for osmotic support to the membranes (Schneider, 1972), was prepared with 2 mM imidazole, to remove traces of inorganic ions (Post and Sen, 1967) and EDTA, 1 mM was included to increase the specific activity of (Na\(^+\) + K\(^+\)) activated enzyme system (Skou, 1965). The buffer was neutralized to pH 7.0 (Esmann, 1988) with 2 M HCl and kept chilled (0–4°C) for 12 hours before use.

2.9.2. **Tissue preparation**

After collecting the blood for plasma separation, livers were excised immediately and placed in individual plastic tubes containing chilled buffer. Tubes were stored on ice. Care was taken to minimize the time and to keep the temperature close to 0°C throughout the procedure of tissue preparation. Livers were rinsed once in the buffer and weighed in pre-tared plastic dishes containing chilled buffer. Total wet weight of the livers was about 1.3–1.8 g. Approximately 0.2 g of each liver was stored in individual vials in liquid nitrogen for later fatty acid analysis of the phospholipids. One g of each liver was cut into small pieces and homogenized in 3 volumes of the buffer for 20 seconds on the highest speed of the Ultra Turax homogenizer. Tubes were immersed in ice water during homogenization. The total homogenate of 4 mls of each liver sample was then divided and diluted for individual assays as outlined in Fig. 7.
@ —— Ratio of the volume of the buffer to the initial weight of the liver in the homogenate volumes divided for individual assays.
2.10. **Specific activity of liver malic enzyme in the cytosol**

2.10.1. **Enzyme assay**

Cytosolic NADP dependent malic enzyme in the liver was measured by the method of Hsu and Lardy (1969), which is basically that of Ochoa (1955). In the assay, the rate of formation of NADPH during the conversion of malate to pyruvate, catalyzed by malic enzyme is proportional to the enzyme concentration up to a change of 0.2 optical density units. At a wavelength of 340 nm, the standard absorption coefficient value for 1 mM solution of pure NADPH in a cuvette of path length of 1 cm is $6.3 \times 10^3$ (from Bergmeyer and Graßl, 1983).

One ml of liver homogenate taken for assaying the ME activity (Fig.7) was centrifuged using TLA 100.3 rotor at 135, 240 x g for 1 hr. at 4°C in the Beckman TL-100 table top ultra centrifuge. Clear supernatants, about 300 µl were collected in individual vials and stored in liquid nitrogen. Enzyme activity was measured within 24 hrs. A volume of 30-50 µl of each supernatant was stored separately in eppendorf tubes at −20°C for later protein determinations. Concentrations of assay reagents were made to a final volume of 3 ml. (Table 4). Varian Techtron spectrophotometer was used to measure the enzyme activity as units per minute at 26°C and 340 nm. The reaction gave a linear time course when 50 µl of cytosolic supernatant was used from euthyroid and 10-25 (SFA diet) and 50 µl (PUFA diet) were used from hyperthyroid mice.

2.10.2. **Protein determination**

Bio-Rad reagent based on Bradford's method (1976) was used for protein determinations in a micro-assay (total volume 1 ml). Its compatibility with EDTA and also the simplicity of the microassay method using one dye reagent was useful for repetitive protein estimations. Bovine serum albumin (BSA), dissolved in the buffer was used as a standard protein which gave linear readings up to 20 µg / ml. Absorbance was read at 595 nm on LKB spectrophotometer. A separate standard graph was prepared for each assay. After appropriate dilutions, 3 to 5 µg cytosolic protein from test samples was assayed for determining protein concentrations.
Table 4. Liver malic enzyme assay, total volume = 3 ml

<table>
<thead>
<tr>
<th>Reagent solution</th>
<th>with malate (μl)</th>
<th>without malate (μl)</th>
<th>Conc.in assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triethanolamine buffer (0.4 M) pH = 7.4</td>
<td>500</td>
<td>500</td>
<td>66.66 mM</td>
</tr>
<tr>
<td>MnCl₂ (0.12 M)</td>
<td>100</td>
<td>100</td>
<td>4.00 mM</td>
</tr>
<tr>
<td>NADP (3.4 mM)</td>
<td>200</td>
<td>200</td>
<td>0.22 mM</td>
</tr>
<tr>
<td>L-Malate (30 mM) pH = 7.4</td>
<td>50</td>
<td>—</td>
<td>0.50 mM</td>
</tr>
<tr>
<td>H₂O</td>
<td>2100</td>
<td>2150</td>
<td>—</td>
</tr>
<tr>
<td>Homogenate *</td>
<td>50</td>
<td>50</td>
<td>—</td>
</tr>
</tbody>
</table>

¶ These solutions were always freshly prepared.
Stock solutions of L-Malate and MnCl₂ were stored in 3 ml eppendorf tubes at −20°C.

* Homogenate volumes were 10–50 μl for hyperthyroid and 50 μl for euthyroid mice. Volume of H₂O was adjusted accordingly.
2.11. **Specific activity of Na-K-ATPase in the liver homogenate**

Inorganic phosphate produced by the activity of the homogenate on ATP in the presence and absence of ouabain is an indirect and discontinuous assay method of measuring the activity of Na-K-ATPase in the given tissue preparation. Activity was measured by others, in the crude homogenate of rat kidney (Lo and Lo, 1981), membrane-rich fractions of various parts of kidney obtained by differential centrifugation and further dilution (Lo et al., 1976), isolated rat liver membranes (Emmelot and Bos, 1965), and repeated dilution-filtration of homogenized rat liver (Haber and Loeb, 1984).

In the present study the activity of Na-K-ATPase was measured directly in the diluted homogenate of mouse liver.

2.11.1. **Enzyme assay**

Inorganic phosphate (Pi) measurement was performed in order of the following steps

a. Homogenate dilution.

b. Unmasking the latent activity with sodium deoxycholate (NaDoc).

c. Incubation with a known concentration of ATP in the presence of Na\(^+\), K\(^+\), and Mg\(^{++}\), with and without ouabain, for a specific time, so that the Pi liberated was within the linear range of standards.

d. Precipitation of proteins by centrifugation, after termination of the reaction by TCA.

e. Measurement of Pi as a reduced phospho-molybdate complex in the supernatant, by following the method of LeBel et al., (1978)

a. **Homogenate dilution**

One ml of the liver homogenate taken for Na-K-ATPase assay (Fig. 7), was diluted further in a ratio of 1g liver / 10 ml buffer (Lo et al., 1976, Lo and Lo, 1981). Diluted homogenates for measuring the activity of Na-K-ATPase were stored immediately for 24 hrs. at -20°C. A volume of 100 µl from diluted individual homogenates were stored separately at -20°C for later protein determinations.

b. **Unmasking the latent activity**

Tissue homogenization can transform plasma membranes into vesicles, some of which may be "inside out" resulting in the incomplete or lack of exposure of the substrate to the membrane associated enzymes, that have specific active and binding sites on a particular side of the bilayer, for example Na-K-ATPase (Jorgensen and Skou, 1971). Normally this enzyme requires an effect of K\(^+\) from outside and Na\(^+\), Mg\(^{++}\), and ATP act from inside the cell but the ouabain binding site is accessible only
from the outer surface of the plasma membrane (Skou, 1965, Stekhoven and Bonting, 1981). Although the ATP and ouabain binding sites are on the opposite side of the membrane, they are both present on the same larger sub-unit of the enzyme.

Detergents can activate Na-K-ATPase either by increasing membrane permeability to the substrates or by opening the vesicles, and they also cause a several-fold increase in the activity of Na-K-ATPase without affecting the activity of Mg++ ATPase (Jorgesen and Skou, 1971). These authors recommended sodium deoxycholate (NaDoc) as the ideal detergent because NaDoc at a comparatively higher concentration than the protein (0.25 mg protein / ml and 0.45 to 0.6 mg NaDoc / ml i.e., 0.045 – 0.060 % deoxycholate w / v ), did not change the critical micellar concentration of the buffer which is important for the maximum activation of the enzyme system. Maximum activation was observed at pH 7.1 and NaDoc concentration of 0.060 % (w / v). At pH 6.6 detergent concentration required was 0.045 % (w / v) indicating that the activation depended on the pH as well as the concentration of the detergent (Jorgensen and Skou, 1971). Esmann (1988) recommended a pH range between 6.9 and 7.2 at 37°C.

Protein concentration higher than 1.5 mg / ml reduced the activating effect of deoxycholate (Jorgensen and Skou, 1971). In the present study the activity was assayed after incubating the homogenate in 0.065 % NaDoc (w / v), in the buffer at pH 7.0 for 30 minutes at 20-25°C (room temperature) (Jorgensen, 1988). Total protein assayed was between 0.5 to 0.9 mg, comparable to the 0.25 to 1.0 mg used by Jorgensen (1988). Samples were vortexed gently for complete mixing of the detergent with the homogenate. On account of its high photoreductivity and insolubility in water, a fresh solution of ouabain was prepared for every assay by dissolving it in a small volume of DMSO. Final concentration of DMSO was 2.5 % (w / v), which was comparable with the less than 5 % concentration suggested by Esmann (1988) and it did not have any effect on the assay.

c. Assay with ATP and ouabain

Liver homogenates were preincubated in 0.065 % Nadoc at room temperature for 30 minutes. Preliminary experiments indicated that following addition of 3 mM ATP to the assay medium at 37°C (Esmann, 1988), reaction rate became non-linear after 5 minutes. Thus reaction period was set at 5 minutes.

Comparison of ouabain concentrations at 1, 5 and 10 mM showed that inhibition of ATPase activity was significantly greater with 5 mM than 1 mM ouabain but there was no significant difference in the inhibition between 5 and 10 mM ouabain. Thus 5 mM ouabain was used throughout the assays.
The release rate of Pi during 5 minutes of the reaction with 3 mM ATP in the presence and absence of 5 mM ouabain was measured. Na-K-ATPase activity is defined as the difference between these two measurements.

Concentration of ATP used by others appears to vary. Esmann (1988, shark rectal gland) Lo and Edelman (1976, rat renal cortex), Lo et al., (1976, parts of rat kidney) used 3 mM ATP whereas Haber and Loeb (1984) reported enzyme activity measured with 5 mM ATP in rat liver. In the present study Na-K-ATPase activity was measured in the presence of 3 mM ATP. The assay was carried out basically according to Esmann's method (1988) with slight modifications (Table 5).

All the solutions were brought to room temperature before use. Disposable eppendorf tubes were used throughout to avoid protein contaminations. Each test sample which included assay with and without ouabain, had four replicates. Since the activity was measured in the homogenate and not in the pure membrane preparations, 1 mM EGTA instead of 0.25 mM (Esmann, 1988) was included in the assay to ensure inactivation of counter cation Ca++. After adding 100 μl of 3 mM ATP, all eppendorf tubes were transferred immediately to a water bath maintained at 37°C. Reaction with ATP was continued for 5 minutes.

d. Protein precipitation

Reaction was terminated after 5 minutes by adding 100 μl of ice cold 50% trichloroacetic acid (TCA). The tubes were transferred to ice water and cooled for one minute. Proteins were precipitated by centrifugation in a Beckman microfuge at 3000 rpm for 3 minutes in the cold room at 4°C. The concentration of Pi in the supernatants was determined at room temperature without any delay to avoid increase in the background Pi concentration due to non-homogenate hydrolysis of ATP (Esmann, 1988).

e. Measurement of Pi concentration

Method of LeBel et al., (1978), was adapted to determine the Pi concentration in 100 μl of the supernatant, in a microassay. LeBel's method is shown to be unaffected by the presence of cations, Mg++, K+ and Na+, chelating agents EDTA and EGTA, 0.25 M sucrose and deoxycholate concentration up to 10% (w/v); TCA (10%) is shown to decrease the absorbance. Since 100 μl of 50% TCA was used to terminate the reaction in the present assay, an equal amount was included in the standards.

To 100 μl of the supernatant + 150 μl H2O, 750 μl of reagent A and 125 μl of reagent B were added. After vortexing, 125 μl of reagent C was added followed by
vortex mixing (Table 6). Absorbance was read immediately after 6 minutes on LKB spectrophotometer at 870 nm. The time of 6 minutes for the colour development was determined by recording the absorbance for 10 minutes by treating a known standard (54.5 nmols Pi) with 100 μl of 3 mM ATP and 100 μl of 50% TCA, and LeBel's reagents (Fig.8C).

Non-homogenate hydrolysis of ATP during the period of 6 minutes was determined by comparing the increase in absorbance in the assay reaction mixture with TCA without ATP and Pi (Fig.8 A) and with ATP+TCA minus Pi (Fig.8 B). The reading of absorbance B was deducted from all the test samples. Standard graphs of Pi for individual assays were determined from anhydrous potassium dihydrogen orthophosphate (KH₂PO₄) dissolved in distilled water. Readings of the standard graph were consistent and linear. Pi concentrations in the test samples were calculated from the difference between the absorbance, with and without ouabain (Lo and Edelman, 1976). The final concentration of total liver protein tested for enzyme activity was 0.5 to 0.9 mg/ml assay medium.

2.11.2. Protein determination

Bio-Rad reagent in a microassay was used to determine the protein concentrations. Homogenates were treated with NaDoc (0.065% w/v) for 30 minutes to solubilize the proteins from the membranes. After appropriate dilutions, about 12 to 20 μg protein was assayed. BSA standards were prepared in the buffer containing 0.065% NaDoc (w/v). Absorbance was read at 595 nm on LKB spectrophotometer.
<table>
<thead>
<tr>
<th>Reagent solution</th>
<th>Volume in the assay (µl)</th>
<th>Concentration in the assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>without ouabain</td>
<td>with ouabain</td>
</tr>
<tr>
<td>NaCl (1240 mM) §</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>KCl (200 mM)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>MgCl₂ (40 mM)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>ATP (30 mM) *</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>(pH 7.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histidine (150 mM)</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>(pH 7.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ouabain (200 mM)</td>
<td>—</td>
<td>25</td>
</tr>
<tr>
<td>EGTA (10 mM, pH 7.2)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>H₂O</td>
<td>200</td>
<td>175</td>
</tr>
<tr>
<td>Homogenate</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

§ ATP was a disodium salt therefore 1240 mM instead of 1300 mM NaCl was used (Esmann, 1988).

* Disodium salt.
Fig. 8

Time course of colour development and spontaneous hydrolysis of ATP

A - Assay medium + TCA
B - Assay medium + TCA + 3 mM ATP
C - Assay medium + TCA + 55.4 nmols Pi
<table>
<thead>
<tr>
<th>Reagent solution</th>
<th>Volume in the assay (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reagent A</strong></td>
<td></td>
</tr>
<tr>
<td>Copper acetate (PH 4.0) contained:</td>
<td>750</td>
</tr>
<tr>
<td>0.25 % copper sulfate pentahydrate in 2N acetic acid +</td>
<td></td>
</tr>
<tr>
<td>4.6 % sodium acetate trihydrate in 2N acetic acid</td>
<td></td>
</tr>
<tr>
<td><strong>Reagent B</strong></td>
<td>125</td>
</tr>
<tr>
<td>Ammonium molybdate (5 %)</td>
<td></td>
</tr>
<tr>
<td><strong>Reagent C</strong></td>
<td>125</td>
</tr>
<tr>
<td>Elon 2 % in 5 % sodium sulfite</td>
<td></td>
</tr>
<tr>
<td>H$_2$O</td>
<td>150</td>
</tr>
<tr>
<td>Supernatant</td>
<td>100</td>
</tr>
</tbody>
</table>

New reagents were made every 6 weeks and stored at 0 – 4°C (LeBel et al., 1978)
2.12. **Specific activity of mitochondrial glycerolphosphate dehydrogenase**

Mitochondrial glycerolphosphate dehydrogenase (GPDH) is specific for L-δ glycerolphosphate. Oxidation of this substrate is proportional to the enzyme concentration (Lee et al., 1959). This reaction in the mitochondria donates the reducing equivalents received from the cytosol via H shuttle, to the respiratory chain where reduction of molecular oxygen occurs. Blocking the oxygen by using a potent inhibitor such as cyanide and by employing an electron acceptor, INT (p-iodonitrotetrazolium violet), the enzyme activity can be measured spectrophotometrically by the colour intensity of reduced INT.

The method of Wernette et al., (1981) was adapted in this assay (Table 7).

2.12.1. Separation of mitochondria

Mitochondria were separated according to the method of Johnson and Lardy (1967). Two ml of homogenate (Fig.7) was diluted further to the ratio of 1g liver / 10 ml buffer and spun at 600 x g for 5 minutes using SS-34 rotor in a Sorvall 5B centrifuge at 0-4°C. Supernatants were saved (A). Pellets were dispersed in the buffer and diluted to the ratio of 1g liver / 2 ml buffer and centrifuged for 5 minutes at 600 x g. Supernatants (B) were saved and pellets discarded. A and B were mixed and centrifuged for 5 minutes at 15,000 x g. Supernatants were discarded and the mitochondrial pellets were suspended gently in 800 µl of sucrose buffer. Aliquots of the suspension (50-60 µl) were stored separately in eppendorf tubes at -20°C for later protein determinations. The remainder of the suspensions were collected in individual vials and stored in liquid nitrogen. Enzyme activity was measured within 48 hours.

2.12.2. Enzyme assay

The electron acceptor, INT is photoreductive and highly insoluble in water. The amount required to provide a concentration of 0.2 % in the final volume of 0.4 ml of the assay was freshly prepared by dissolving it in a small amount of DMSO and volume adjusted with distilled water. After adding the mitochondrial suspension to the assay medium (Table 7) and gentle mixing, the disposable eppendorf tubes were transferred to a 37°C water bath. In euthyroid mice, the reaction up to 35 minutes was linear with 25 µl of homogenate and 1.5 mM substrate. In hyperthyroid mice the enzyme activity was measured by adding 12 µl of mitochondrial suspension. Final volume of 0.4 ml was adjusted by adding required amount of distilled water. The reaction was terminated at 20 minutes by adding 0.6 ml of 3.2 M acetic acid. Since no turbidity was noticed, absorbance was read directly at 490 nm on LKB spectrophotometer, 2 minutes after stopping the reaction.
Activity was measured in triplicates and determined as the difference between respective test samples containing mitochondrial suspension with and without glycerophosphate. The extinction coefficient of $2.01 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ for the formazan product of INT used by Wernette et al., (1981) to calculate the activity in intact mitochondria was used in the assay.

### 2.12.3. Protein determination

Protein concentration was determined in a micro-assay using the Bio-Rad reagent. Since total mitochondrial membrane protein was assayed, the suspension was treated with 0.065 % NaDoc (w / v) for 20 minutes to solubilize the membrane bound proteins. Standard values were obtained with BSA (20 μg / ml), dissolved in the buffer with 0.065 % NaDoc (w / v).
<table>
<thead>
<tr>
<th>Reagent solution</th>
<th>Volume in the assay (μl)</th>
<th>Concentration in the assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>with glycerol p</td>
<td>without glycerol p</td>
</tr>
<tr>
<td>KH₂PO₄ 200 mM (PH 7.0)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>H₂O</td>
<td>117 *</td>
<td>197 *</td>
</tr>
<tr>
<td>EGTA 100 mM (PH 7.0)</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>KCN 20 mM</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>L-δ glycerophosphate 7.5 mM</td>
<td>80</td>
<td>-</td>
</tr>
<tr>
<td>INT 1.6 %</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Mitochondrial suspension</td>
<td>25 #</td>
<td>25 #</td>
</tr>
</tbody>
</table>

INT—p –iodonitrotetrazolium violet

* Euthyroid mice

# Euthyroid mice
2.13. **Measurement of fatty acid composition of hepatic phospholipids**

Fatty acid analysis of the liver phospholipids was conducted according to the method used by Hulbert and Else (1989).

Lipids were extracted from the liver samples using the method of Folch et al., (1957). These were dried under nitrogen and redissolved in chloroform. Silicic acid column chromatography was used to separate neutral and phospholipids (Sil-LC, 325 mesh). Phospholipids were eluted with three column volumes of chloroform : methanol (2 : 1) followed by six column volumes of methanol. Tissue phospholipid content was determined by measurement of the phosphorus content of a known aliquot of the phospholipid fraction by the method of Harris and Popat (1954). Phosphorus analysis confirmed that the fraction eluted with methanol contained only the phospholipids. Thin-layer chromatography confirmed the absence of neutral lipids in this fraction.

Methyl esters of the fatty acids from phospholipids were prepared with 20 % boron trifloride in methanol, extracted with petroleum ether (boiling point 40-60°C) and purified by hydrated florosil (florosil+7 % water) column chromatography. The pure methyl esters were eluted with 5 % diethyl ether in petroleum ether and separated on a Packard model 427 gas chromatograph with a capillary column coated with FFAP (S.G.E. column type 25QC2) and a flame ionization detector. Temperature of the column was maintained at 170°C for 20 minutes and raised to 195°C by increasing 1°C min⁻¹. Retention times of known standards were used to compare and identify the individual fatty acids in the test samples. A Shimadzu C-R3A chromatopac integrator was used to determine the relative composition (mol %).
2.14. Statistical analysis

Results were analysed with 2-way factorial analysis of variance from the Statview Statistical Programme Package. P values between 0.05 and 0.01 are considered significant and those less than 0.01 as highly significant.
CHAPTER THREE

Effects of dietary fats and hyperthyroidism on standard metabolic rate and body weight in mice

3.1. Results

3.1.1. Water intake

Effects of thyroid hormones and diets on water intake are presented in Table 8 and fig.9. Significant differences were observed between euthyroid and hyper T4 but not between hyper T3 and euthyroid mice. Difference in the type of dietary fat had no effect on water intake. Compared to euthyroid mice, the increased water consumption in hyperthyroid mice was probably related to their greater metabolic rate.

3.1.2. Plasma hormone levels

Hormone intakes are presented in table 9 and plasma levels of T3 and T4 can be seen in table 9 and Fig.10 A and 10 B respectively. Daily hormone intake of hyper T3 mice on SFA and PUFA diet was about 100 nmols / 100g body weight and hyper T4 mice on both diets consumed about 150 nmols of T4 / 100g body weight respectively. Total T3 level in hyper T3 and hyper T4 mice were significantly higher than the euthyroid levels. Mice receiving T3 showed a diminished level of T4, indicating the presence of the normal negative-feedback mechanism at the anterior pituitary, i.e., inhibition of TSH and therefore T4 secretion from the thyroid gland. Administration of T4 significantly elevated T4 as well as T3 levels in the plasma, indicating the activity of the 5’ deiodination system. In spite of a higher concentration of the T4 dose (relative to T3), the plasma T3 levels were similar in both hyperthyroid groups.

3.1.3. Standard metabolic rate

Results of the SMR are presented in table 10 and Fig.11. There was no significant difference in the SMR of various groups before the beginning of treatment. The SMR of 1.8 –1.9 ml O2 g⁻¹ h⁻¹ in euthyroid mice measured at 30°C (body weight 24–25g) in the present study is slightly higher than 1.6 ml O2 g⁻¹ h⁻¹ (body weight 35.7g) measured at 32°C (Federation of American Societies, Experimental Biology, 1974 p1615). This could be due to the slightly lower ambient temperature and also the lower body weight of the mice used in the present experiment. Compared with controls, all animals receiving thyroid hormones showed a significant increase in their SMR after 21–22 days of treatment. There was no significant difference in the SMR of euthyroid mice at the end of treatment. Hyper T3 and T4 animals on SFA diet had an SMR increase of 44 %. With PUFA diet, T3
and T4-induced SMR increase was 42 and 37% respectively.

From these results it can be seen that thyroid hormones elevated the metabolism in mice significantly. However, there was no effect of diet on oxygen consumption.

3.1.4. Body weight

Results of body weight gain are presented in Table 10 and Fig.12. Both T3 and T4 had highly significant effect on loss of body weight. Hyper T3 mice had a greater body weight loss than hyper T4 on the same diet. Significant differences were observed between groups and within groups when dietary effects were analysed. Comparison of euthyroid and hyperthyroid groups showed an overall loss of body weight in hyperthyroid animals fed PUFA diet. After 21–22 days of dietary treatment, the weight gain in euthyroid animals fed SFA and PUFA diet was 12 and 7.4% respectively. Hyper T3 animals on SFA diet showed 1.2% loss in body weight when compared to 18.4% loss on PUFA diet. Hyper T4 mice on SFA diet had an average weight gain of 3.2% when compared to the average weight loss of 13.7% on PUFA diet.

It is interesting to note that hyper T4 mice on SFA diet and higher plasma T3 level (plasma T3 conc. 16.1nM) gained weight when compared to the significant weight loss with PUFA diet and lower plasma T3 level (plasma T3 conc. 13.1nM). Similarly hyper T3 mice, in spite of a higher T3 level (17.7 nM) lost less weight on SFA diet when compared to a greater body weight loss with PUFA diet and lower T3 level (15.0 nM). It can be seen that euthyroid and hyper T4 mice receiving SFA diet gained weight whereas with PUFA diet euthyroid mice gained less weight and hyperthyroid mice had a greater body weight loss.

3.1.5. Liver weight (total weight and as % of final body weight)

There was no significant difference in the total liver weight of euthyroid and hyperthyroid groups (Table 11 and Fig.13). However, because of the greater loss of total body weight in the hyper T3 group, the liver was a significantly greater percentage of the final body weight.
Table 8

Water consumption in euthyroid and hyperthyroid mice on SFA and PUFA diet

<table>
<thead>
<tr>
<th>Thyroid status</th>
<th>Diet</th>
<th>Water intake (ml/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euthyroid</td>
<td>SFA</td>
<td>9.1 ± 1.6</td>
</tr>
<tr>
<td>Euthyroid</td>
<td>PUFA</td>
<td>7.8 ± 0.9</td>
</tr>
<tr>
<td>Hyper T3</td>
<td>SFA</td>
<td>11.3 ± 1.3</td>
</tr>
<tr>
<td>Hyper T3</td>
<td>PUFA</td>
<td>10.5 ± 1.1</td>
</tr>
<tr>
<td>Hyper T4</td>
<td>SFA</td>
<td>11.9 ± 1.8 *</td>
</tr>
<tr>
<td>Hyper T4</td>
<td>PUFA</td>
<td>12.0 ± 1.3 *</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 7) for all groups

Statistics * Significant effect of hormones on water intake.

Fig. 9

Water consumption

![Water consumption graph](image)
Table 9
hormone intake and plasma concentrations of thyroid hormones in euthyroid and hyperthyroid mice on SFA and PUFA diet

<table>
<thead>
<tr>
<th>Thyroid status</th>
<th>Diet</th>
<th>Hormone intake (nmols / day)</th>
<th>Plasma Concentration T3 (nM)</th>
<th>Plasma Concentration T4 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euthyroid</td>
<td>SFA</td>
<td>0.00</td>
<td>2.6 ±0.2</td>
<td>241.3 ± 4.8</td>
</tr>
<tr>
<td>Euthyroid</td>
<td>PUFA</td>
<td>0.00</td>
<td>3.2 ±0.2</td>
<td>42.2 ± 3.2</td>
</tr>
<tr>
<td>Hyper T3</td>
<td>SFA</td>
<td>24.9 ±2.1</td>
<td>17.7 ±1.0 *</td>
<td>10.4 ± 0.5 *</td>
</tr>
<tr>
<td>Hyper T3</td>
<td>PUFA</td>
<td>23.5 ±2.4</td>
<td>15.0 ±1.3 *</td>
<td>10.4 ± 0.4 *</td>
</tr>
<tr>
<td>Hyper T4</td>
<td>SFA</td>
<td>36.8 ±5.0</td>
<td>16.1 ±1.2 *</td>
<td>255 ±17.0 *</td>
</tr>
<tr>
<td>Hyper T4</td>
<td>PUFA</td>
<td>37.4 ±3.7</td>
<td>13.1 ±1.9 *</td>
<td>250 ±18.6 *</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 7 for all groups).

Statistics: * Highly significant effect of hormone intake on plasma hormone level.

Fig. 10 A  
Total T3 in the plasma

Fig. 10 B  
Total T4 in the plasma
Table 10
Body weight gain and SMR change in euthyroid and hyperthyroid mice on SFA and PUFA diet

<table>
<thead>
<tr>
<th>Thyroid state</th>
<th>Diet</th>
<th>Initial SMR</th>
<th>SMR increase</th>
<th>Initial body Wt (g)</th>
<th>Body Wt gain (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euthyroid</td>
<td>SFA</td>
<td>1.80 ±0.09</td>
<td>0.06 ±0.07</td>
<td>25.7 ±1.3</td>
<td>3.1 ±0.9 #</td>
</tr>
<tr>
<td>Euthyroid</td>
<td>PUFA</td>
<td>1.90 ±0.11</td>
<td>-0.12 ±0.15</td>
<td>24.4 ±1.3</td>
<td>1.8 ±0.8</td>
</tr>
<tr>
<td>Hyper T3</td>
<td>SFA</td>
<td>1.80 ±0.08</td>
<td>1.30 ±0.09 *</td>
<td>24.4 ±1.0</td>
<td>-0.3 ±0.7</td>
</tr>
<tr>
<td>Hyper T3</td>
<td>PUFA</td>
<td>1.90 ±0.06</td>
<td>1.20 ±0.19 *</td>
<td>24.5 ±1.0</td>
<td>4.5 ±0.9**</td>
</tr>
<tr>
<td>Hyper T4</td>
<td>SFA</td>
<td>1.90 ±0.10</td>
<td>1.20 ±0.09 *</td>
<td>25.0 ±0.7</td>
<td>0.8 ±0.9 #</td>
</tr>
<tr>
<td>Hyper T4</td>
<td>PUFA</td>
<td>1.80 ±0.05</td>
<td>1.10 ±0.02 *</td>
<td>24.8 ±0.7</td>
<td>-3.4 ±0.9**</td>
</tr>
</tbody>
</table>

≠ (ml O₂ g⁻¹ h⁻¹)

Values are mean ± SEM (n = 7 for all groups)

Statistics
* Highly significant effect of hormones on SMR increase.
# Significant effect of SFA diet on weight gain.
** Highly significant effect of hormone and significant effect of diet on body weight loss.

Fig. 11

<table>
<thead>
<tr>
<th>SMR increase</th>
<th>SFA</th>
<th>PUFA</th>
</tr>
</thead>
</table>

Fig. 12

<table>
<thead>
<tr>
<th>Body weight change</th>
<th>No Hormone</th>
<th>Hyper T3</th>
<th>Hyper T4</th>
</tr>
</thead>
</table>

![Graph showing SMR increase and body weight change](image)
Table 11
Liver weight (as % of final body wt.) in euthyroid and hyperthyroid mice on SFA and PUFA diet

<table>
<thead>
<tr>
<th>Thyroid Status</th>
<th>Diet</th>
<th>Final body wt. (g)</th>
<th>Total liver wt. (g)</th>
<th>Liver wt. as % of final body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euthyroid</td>
<td>SFA</td>
<td>28.8 ± 1.7</td>
<td>1.71 ± 0.09</td>
<td>5.9 ± 0.2</td>
</tr>
<tr>
<td>Euthyroid</td>
<td>PUFA</td>
<td>26.2 ± 1.4</td>
<td>1.72 ± 0.10</td>
<td>6.5 ± 0.6</td>
</tr>
<tr>
<td>Hyper T3</td>
<td>SFA</td>
<td>24.1 ± 1.3</td>
<td>1.82 ± 0.14</td>
<td>7.5 ± 0.3</td>
</tr>
<tr>
<td>Hyper T3</td>
<td>PUFA</td>
<td>20.0 ± 1.1 **</td>
<td>1.43 ± 0.13</td>
<td>7.0 ± 0.4</td>
</tr>
<tr>
<td>Hyper T4</td>
<td>SFA</td>
<td>25.8 ± 1.3</td>
<td>.80 ± 0.10</td>
<td>6.9 ± 0.5</td>
</tr>
<tr>
<td>Hyper T4</td>
<td>PUFA</td>
<td>21.4 ± 1.0 **</td>
<td>1.34 ± 0.13</td>
<td>6.2 ± 0.4</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 7 for all groups)

Statistics
* Significant effect of T3
** Highly significant effect of hormone and PUFA diet on body weight loss

Fig. 13

Liver weight as % of body weight

![Graph showing liver weight as % of body weight for No Hormone, Hyper T3, and Hyper T4 groups.](image_url)
3.2. Discussion

Oral administration is probably a more physiological way of inducing and maintaining hyperthyroidism than daily injections of hormones. Although oral administration is not as effective as subcutaneous injections (Gross and Pitt-rivers, 1953), administering thyroid hormones in drinking water minimizes the sudden fluctuations in the body hormone levels that can occur following injections of thyroid hormones (Connors and Hedge, 1980).

Induction of hyperthyroidism was confirmed by the presence of significantly high plasma levels of both T3 and T4. It was observed that although the mice on T4, consumed about 1.9 times higher amount of the hormone than the mice on T3 in their drinking water, the difference in plasma level of T3 between various hyperthyroid groups was not as great.

It has been suggested that free unsaturated fatty acids inhibit the binding of thyroid hormones to plasma proteins leading to low values of serum T3 and T4 (Benvenga et al., 1987). It has also been suggested that the competitive inhibitory potency (on a molar basis) is related to the number of double bonds in the fatty acid. Arachidonic acid (20:4) appears to be more effective than linoleic (18:2) or oleic (18:1) acid (Benvenga et al., 1987). However Hollander et al., (1967) and Chopra et al., (1984) suggest that oleic acid, (18:1), the most abundant plasma free fatty acid, also exerts similar effects to other unsaturated fatty acids. Mendel et al., (1986), suggest that because of their normal plasma concentrations, free fatty acids in vivo, commonly do not influence the circulating free thyroid hormone concentration.

While stearic and palmitic acid were found to be ineffective, linoleic acid, (1.5 mmol / L in vitro) added to undiluted serum of euthyroid humans, increased the mean free fraction of T4 by 11 percent (Lim et al., 1988). Plasma concentration of free fatty acids in humans ranges from 180 to 1650 μmol / L (Henning and Watkins, 1989). Although the fatty acid concentration used by Lim et al., (1988) was at its high extreme of normal concentration, they suggested that significant increase in serum fatty acids can occur in certain conditions (for example non-thyroidal illness, increased levels of T4 concentration and marked decrease in plasma albumin). These conditions could influence the binding of T3 and T4 to plasma proteins in vivo (Lim et al., 1988). It is also known that elevation of plasma free fatty acids is one of the major effects of hyperthyroidism (Heimberg et al., 1985, Sestoft., 1980).

Assuming that type of dietary fatty acids and hyperthyroidism affected plasma fatty acids, the results of this study suggest that the influence of fatty acids on total thyroid hormone levels are not significant. Since very small volumes of plasma were
available, only total hormone levels could be measured. It is not known whether the free hormone levels were increased due to the effect of unsaturated free fatty acids.

In euthyroid rats, treated with oral administration of 25 μg T4 and 12 μg T3 / 100 g body weight, for 13 days, the BMR increase reported by Gemmil (1956), was 26 % with T4 and 169 % with T3. As pointed out by him, BMR values of T3 treated rats in his study were very high. Tata et al., (1963) observed a 45-75 % increase in oxygen consumption above basal value in the rats treated with subcutaneous administration of about 16 μg T4 for 3 weeks and 10-11 μg T3 / 100 g body weight for 2 weeks. He injected T4 and T3 at regular intervals of 4 and 3 days respectively. The "field metabolic rate" (average rate of energy utilization of an animal during its daily activities in its natural surroundings) of euthyroid rats, injected with 50 μg T3 / 100 g / day for 14 days showed an increase of 150 % (Oppenheimer et al., 1991).

Amounts consumed by the mice in the present study were about 67 μg T3 and 131 μg T4 per 100 g body weight for 21 days. Rise in the metabolism of hyperthyroid mice was about 44 % with T3 and T4 with SFA and 42 and 37 % with T3 and T4 respectively with PUFA diet.

These results compared with those of Tata et al., and Oppenheimer et al., cited above, showed that compared to their lower dose of subcutaneous injection in rats, higher doses of orally administered thyroid hormones were relatively less effective in elevating the oxygen consumption in mice in the present study.

Gross and Pitt-rivers (1953) have reported that parenteral administration of thyroid hormones was more effective than giving them orally. They compared the decrease in thyroid weight as a measure of thyroid hormone potency and demonstrated that when given orally, T3 and T4 were only 86 and 39 % as effective as their parenteral administration respectively. Therefore, it is possible that the method of oral administration I used was perhaps less effective in raising the metabolic rate. On the other hand it is also possible that stimulation of oxygen consumption by thyroid hormones in mice is relatively less prominent than in rats.

In their latest study Oppenheimer et al., (1991) did not report the hormone levels which would have helped to compare the effects of oral administration of thyroid hormones in mice and subcutaneous administration in rats.

Loss of body weight is one of the major effects of hyperthyroidism. Gemmil reported 16–17 % body weight loss in rats fed normal rat food and treated with orally administered thyroid hormones.

Increased dietary unsaturated fatty acids have also been reported to cause loss of body weight in hyperthyroidism. When linoleic acid was fed as 28.8 % of total
fats, compared to euthyroid rats hyperthyroid rats injected with 1 μg T3 / g body weight / day for 3 days had a weight loss of 8.4 g (Hoch, 1982).

Irrespective of the type of dietary fat (6 % w / w corn, coconut or menhaden oil), compared to controls, a 5 % lower final body weight was observed in BHE rats receiving 10 μg T4 / 100 g body weight / day for 7 days (Pan and Berdanier, 1990). In another study, compared to the diet of 16 % beef tallow, rats subjected to a dietary treatment of 16 % safflower oil for 4-5 weeks gained 20 % less weight (Mak et al., 1983).

Ershoff (1949) treated immature rats for 28 days with massive doses of desiccated thyroid and 45 % full fat soybean meal in the diet (soybeans contain about 64 % linoleic acid). He did not observe any beneficial effect of the diet on the increased rate of oxygen consumption but reported that compared to the diet without any soybean meal, 45 % full fat soybean meal completely counteracted the weight loss and other visual symptoms (Loss of hair, scaly skin) in hyperthyroid rats.

Except Ershoff's findings where soybean meal and immature rather than adult rats were used, others, cited above, have reported that hyperthyroid adult animals, irrespective of the dietary fatty acids (corn oil, safflower oil coconut oil and menhaden oil) and duration of treatment, lost body weight.

In the present study, mice treated with T3 and T4 for 21-22 days and feeding on a diet high in PUFA, lost 18.0 % and 14.0 % body weight respectively. However, with T4 treatment SFA diet showed an average 3.0 % increase in body weight. Similarly euthyroid mice on SFA diet gained 5.0 % more weight than on PUFA diet. This showed that PUFA diet containing higher amount of 18:2 had an overall negative effect on body weight gain.

Effect of PUFA diets on the inhibition of lipogenic enzymes is discussed in section 1.8.3. in chapter one. The results of enzyme activities, discussed in the next chapter, showed that malic enzyme activity was significantly decreased in mice on PUFA diet. Activities of other lipogenic enzymes are also decreased by PUFA diet (Schwartz and Abraham, 1982, 1983, Clarke et al., 1977, 1990). Therefore it appears that lipogenesis on the whole was impaired by PUFA diet which aggravated the body weight loss in hyperthyroid mice.

Sestoft in his review (1980), has stated that the capacity of simultaneous triglyceride synthesis and lipolysis is increased in many tissues of hyperthyroid animals and this effect is prominent in adipose tissues of hyperthyroid rats. The futile cycling of triglycerides in thyroid-stimulated liver results in increased turnover of free fatty acids and a net decrease in triglyceride synthesis (Sestoft 1980). This could also be the cause of the body weight loss observed in hyperthyroid mice in the
Significantly higher levels of fatty acids in the livers of T4 treated BHE rats on coconut oil than on corn oil diet were reported by Pan and Berdanier (1990). Thus it may be that the higher total liver weights of hyperthyroid mice on SFA than on PUFA diet, observed in the present study were due to the increase of fatty acids in the liver.

Although body weight was decreased in PUFA treated hyperthyroid mice, there was no decrease in their liver weights. It may be that the total body weight loss in these mice was largely due to a decrease in adipose tissue, although this was not measured.

Mice treated with T3 and SFA diet had higher total as well as % liver weights than the rest of the groups. This could be due to T3-induced increased activity of lipogenic malic enzyme with SFA diet (Discussed in chapter 4), greater body weight loss with T3 than T4 and perhaps due to increased level of fatty acids in the hyperthyroid livers with coconut oil diet as reported by Pan and Berdanier (1990).

In conclusion: orally administered thyroid hormones induced hyperthyroidism in mice. This was confirmed by significant increase in plasma hormone levels, elevation of oxygen consumption, and body weight loss. Increased dietary PUFA did not counteract these effects of hyperthyroidism. Comparison of the effects of dietary fatty acids suggests that although PUFA diet aggravated body weight loss, and did not indicate any beneficial effects, increased intake of saturated fatty acids (SFA diet) in hyperthyroidism resulted in increased liver weight in spite of a body weight loss.
CHAPTER FOUR

Effects of dietary fats and hyperthyroidism on liver enzyme activities and liver phospholipid fatty acid composition in mice

4.1. Results

4.1.1. Specific Activity of cytosolic malic enzyme (ME)

There were highly significant effects of both thyroid hormones and dietary fats on liver malic enzyme activity in mice. (Table 12 and Fig. 14). There was also a highly significant interaction between the type of dietary fat and effect of hormones (for all \( P < 0.001 \)). From the results it can be seen that the activity of malic enzyme, irrespective of the thyroid state increased with the SFA and decreased significantly with the PUFA diet.

Both SFA and PUFA diets contained 18 % w/w fat. The linoleic acid content of SFA and PUFA diet was about 6 and 72 % of total fat respectively. Thus the SFA diet was not deficient in its 18:2 content. After 21–22 days of treatment, ME activity in euthyroid mice on SFA and PUFA diet was 9.8 and 6.8 nmols mg\(^{-1}\) cytosolic protein min\(^{-1}\) respectively. Compared to euthyroid mice, thyroid hormone treatment on SFA diet resulted in a 2.5 and 2.4 fold increase in the activity of ME with T3 and T4 respectively which showed that linoleic acid (as 6 % of total fat in the diet) did not counteract the effects of thyroid hormones on the activity of malic enzyme. In hyperthyroid mice although the enzyme activity was significantly lower with the PUFA than SFA diet, it was significantly higher than the euthyroid levels.

Compared to euthyroid mice on the same diet, the enzyme activity with T3+SFA and T4+SFA increased by 148 and 139 % respectively in hyperthyroid mice. With T3+PUFA the increase was only 50 % higher than euthyroid whilst T4+PUFA treatment resulted in a 75 % increase. These results showed that although thyroid hormones stimulated the activity of malic enzyme in all hyperthyroid groups, the high level of 18:2 in the PUFA diet caused a significant decrease in the stimulation.
Table 12

Specific activity of hepatic malic enzyme in euthyroid and hyperthyroid mice on SFA and PUFA diet

<table>
<thead>
<tr>
<th>Thyroid Status</th>
<th>Diet</th>
<th>nmols mg(^{-1}) cytosolic protein min(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euthyroid</td>
<td>SFA</td>
<td>9.8 ± 0.4</td>
</tr>
<tr>
<td>Euthyroid</td>
<td>PUFA</td>
<td>6.8 ± 0.3</td>
</tr>
<tr>
<td>Hyper T3</td>
<td>SFA</td>
<td>24.3 ± 0.9 $</td>
</tr>
<tr>
<td>Hyper T3</td>
<td>PUFA</td>
<td>10.2 ± 0.3 **</td>
</tr>
<tr>
<td>Hyper T4</td>
<td>SFA</td>
<td>23.4 ± 0.7 $</td>
</tr>
<tr>
<td>Hyper T4</td>
<td>PUFA</td>
<td>11.9 ± 0.7 **</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (\(n = 7\) for all groups)

Statistics

$\quad$ Highly significant interaction of thyroid hormones and SFA diet on ME activity.

**$\quad$ Highly significant interaction of thyroid hormones and PUFA diet on ME activity.

Fig. 14

Specific activity of malic enzyme

![Graph showing specific activity of malic enzyme](image-url)
4.1.2. Specific Activity of sodium potassium ATPase (Na-K-ATPase)

Compared to euthyroid controls, the Na-K-ATPase activity in hyper T3 and hyper T4 mice was increased significantly (Table 13 and Fig.15). The activity measured by the release of inorganic phosphate (Pi), was 17-19 nmols in euthyroid and 30-37 nmols mg\(^{-1}\)min\(^{-1}\) in hyperthyroid mice. The stimulation of Na-K-ATPase was basically the same whether hyperthyroidism was caused by T3 or T4. The degree of stimulation was 75-80\% with T3 and 76-84\% with T4 (P<0.02). The two dietary treatments did not exert any significant effects on this enzyme activity in euthyroid or hyperthyroid mice.

One of the difficulties of measuring Na-K-ATPase activity in liver is the very high background ATPase activity. Inhibition with ouabain generally, only decreases the total ATPase by about 7-11\%. I have called the ATPase activity without Na-K-ATPase as "other ATPases" activity and the results of the other ATPase activity are shown in table 14 and Fig.16. There was no effect of T4 but a significant effect of T3 on the other ATPases was observed. Exactly what other liver ATPases were being stimulated by T3 is not known (but it may be Ca\(^{++}\) or Mg\(^{++}\) ATPase).
### Table 13

Specific activity of Na-K-ATPase in the liver homogenates of euthyroid and hyperthyroid mice on SFA and PUFA diet

<table>
<thead>
<tr>
<th>Thyroid status</th>
<th>Diet</th>
<th>nmols mg(^{-1}) total liver protein min(^{-1})</th>
<th>% of total ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euthyroid</td>
<td>SFA</td>
<td>17.2 ± 1.5</td>
<td>6.6 ± 2.4</td>
</tr>
<tr>
<td>Euthyroid</td>
<td>PUFA</td>
<td>19.9 ± 4.2</td>
<td>7.1 ± 1.5</td>
</tr>
<tr>
<td>Hyper T3</td>
<td>SFA</td>
<td>30.9 ± 7.1*</td>
<td>9.8 ± 2.1</td>
</tr>
<tr>
<td>Hyper T3</td>
<td>PUFA</td>
<td>34.9 ± 4.5*</td>
<td>10.3 ± 4.3</td>
</tr>
<tr>
<td>Hyper T4</td>
<td>SFA</td>
<td>31.7 ± 3.0*</td>
<td>11.0 ± 0.6</td>
</tr>
<tr>
<td>Hyper T4</td>
<td>PUFA</td>
<td>35.1 ± 5.8*</td>
<td>11.0 ± 1.4</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 7 for all groups).

Statistics: *Significant effect of thyroid hormones on enzyme activity.

![Specific activity of Na-K-ATPase](image)
Table 14

Specific activity of the other ATPases (excluding Na-K-ATPase) in the liver homogenates of euthyroid and hyperthyroid mice on SFA and PUFA diet

<table>
<thead>
<tr>
<th>Thyroid status</th>
<th>Diet</th>
<th>nmols mg⁻¹ total liver protein min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euthyroid</td>
<td>SFA</td>
<td>265.0 ± 22.9</td>
</tr>
<tr>
<td>Euthyroid</td>
<td>PUFA</td>
<td>281.0 ± 17.8</td>
</tr>
<tr>
<td>Hyper T3</td>
<td>SFA</td>
<td>317.0 ± 23.5 *</td>
</tr>
<tr>
<td>Hyper T3</td>
<td>PUFA</td>
<td>341.0 ± 13.7 *</td>
</tr>
<tr>
<td>Hyper T4</td>
<td>SFA</td>
<td>287.0 ± 19.3</td>
</tr>
<tr>
<td>Hyper T4</td>
<td>PUFA</td>
<td>310.0 ± 19.5</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 7 for all groups)

Statistics: * Significant effect of T3

Fig. 16

Specific activity of other ATPases

SFA

PUFA

No hormone  Hyper T3  Hyper T4
4.1.3. Specific Activity of mitochondrial glycerolphosphate dehydrogenase

Compared to euthyroid groups all the hyperthyroid mice had a significant increase in the activity of mitochondrial glycerolphosphate dehydrogenase (GPDH) (Table 15 and fig.17). Both T3 and T4 had a significant effect on the enzyme activity (P< 0.002). When compared to euthyroid on SFA, hyper T3 mice on the same diet showed 345 % higher activity and T3 + PUFA diet, compared to euthyroid, showed a 303 % increase. Even in euthyroid mice the enzyme activity with PUFA diet was slightly higher than with SFA diet. Enzyme activity in hyper T4 mice on SFA diet increased by 345 % whilst with T4 and PUFA diet, the significant increase was 399 % higher than euthyroid controls. The activity with T4 and PUFA diet was 96 % higher than with T3 and PUFA diet. From the results it is seen that with T3, the dietary treatment did not alter the activity of GPDH. However, T4 treatment and PUFA diet resulted in a comparatively 54 % higher increase than T4 and SFA diet. This significant increase with T4+PUFA treatment appears to be influenced by T4 (P< 0.02), dietary fat (P< 0.02), and a significant interaction between dietary fat and T4 (P < 0.05).
Table 15
Specific activity of GPDH in euthyroid and hyperthyroid mice on SFA and PUFA diet

<table>
<thead>
<tr>
<th>Thyroid status</th>
<th>Diet</th>
<th>nmols mg(^{-1}) mito.prot. min(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euthyroid</td>
<td>SFA</td>
<td>6.5 ± 0.5</td>
</tr>
<tr>
<td>Euthyroid</td>
<td>PUFA</td>
<td>7.4 ± 0.3</td>
</tr>
<tr>
<td>Hyper T3</td>
<td>SFA</td>
<td>29.0 ± 1.9 *</td>
</tr>
<tr>
<td>Hyper T3</td>
<td>PUFA</td>
<td>29.9 ± 1.3 *</td>
</tr>
<tr>
<td>Hyper T4</td>
<td>SFA</td>
<td>29.2 ± 1.6 *</td>
</tr>
<tr>
<td>Hyper T4</td>
<td>PUFA</td>
<td>37.1 ± 2.3 **</td>
</tr>
</tbody>
</table>

All values are mean ± (n = 7 for all groups).

Statistics
* Highly significant effect of hormone on enzyme activity.
** Highly significant effect of hormone + PUFA diet and interaction of diet + hormone on enzyme activity.

Fig. 17
Specific activity of mitochondrial glycerolphosphate dehydrogenase
4.1.4. Fatty acid composition of liver phospholipids

Fatty acid composition of the liver phospholipids is presented in table 16. Figure 18 shows the differences in the levels of fatty acids which were significant (i.e., the 18:0, 18:2 (n-6), 20:4 (n-6) and 22:6 (n-3) contents).

Compared to euthyroid mice the unsaturation index (UI) was lower in hyperthyroid mice in the respective dietary treatments (see table 16). The high UI observed in mice on the PUFA compared to the SFA diet was significant due to dietary effects (P< 0.02); changes in the UI due to thyroid hormone effects were not significant. There was no significant interaction between the effects of diets and thyroid hormones.

Although monounsaturates were generally lower in PUFA than SFA treated mice, compared to euthyroid and hyper T4 mice on SFA diet, hyper T3 mice on the same diet showed a significantly higher level of monounsaturates (P< 0.02). With PUFA diet the monounsaturates were lower in hyperthyroid mice than euthyroid mice; the significantly lower level was observed in hyper T3 group. The interaction between diet and hormone in this respect was significant (P< 0.02).

A significant effect of diet but no effect of hormone or any interaction was observed on polyunsaturate contents. These were primarily affected by dietary fatty acids i.e., lower levels were observed with SFA and higher with PUFA diet, as expected. Thyroid hormones did not appear to exert any significant effect on total polyunsaturates. The overall percent unsaturation was comparable in all groups.

From the above observations it can be seen that membrane composition on the whole was maintained, irrespective of the dietary treatment or thyroid state. However, some individual fatty acid contents (discussed below) were significantly different due to an effect of the diet, hormone or both.

Although no difference in the 18:2 content with SFA diet was observed between hyperthyroid and euthyroid mice (see fig.18), compared to euthyroid+PUFA, hyperthyroid+PUFA mice had a significantly higher level of 18:2 (n-6). Both T3 and T4 (P< 0.05) and the PUFA diet (P< 0.002) had a significant effect and there was also an interaction between diet and hormone effects (P< 0.05).

The Percentage of 20:4 was significantly increased in euthyroid mice on the PUFA than on the SFA diet. Hyper T3 and T4 mice on PUFA diet showed a significantly lower level of this fatty acid compared to euthyroid mice on the same diet. However, the 20:4 content of the hyperthyroid mice on the SFA diet was comparable with that of euthyroid mice on the same diet. The interaction between the diet and thyroid hormones was significant with T4 (P> 0.02) but not with T3.
The percentage of 22:6 (n-3) in hyperthyroid mice on T4 and PUFA diet was significantly higher than euthyroid and hyper T3 group on the same diet. However, with SFA diet, hyper T4 mice showed a lower level of this fatty acid than their euthyroid counterparts and there was a significant interaction between the diet and the hormone (P< 0.05). In hyper T3 mice, the 22:6 content was lower on SFA and higher on PUFA diet than the euthyroid mice and only the dietary effect was significant.

The effect of both thyroid hormones and PUFA diet on the higher 18:0 content in hyperthyroid than euthyroid mice was significant with T3 (P< 0.02). However, with T4, only a significant effect of hormone (P< 0.02) was evident.

Compared to the PUFA diet, SFA diet resulted in a higher level of 16:0 in euthyroid and hyperthyroid mice. This was influenced significantly only by the diet (P < 0.05).

4.1.5. Liver Protein contents

Results of protein contents are presented in table 17. No significant differences between euthyroid and hyperthyroid mice due to hormone and / or diet treatments were observed. Protein contents were measured in the homogenate (total protein), mitochondrial pellet and cytosolic supernatant.
Table 16
Fatty acid composition of the liver phospholipids of euthyroid and hyperthyroid mice on SFA and PUFA diet

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Eu. SFA</th>
<th>Eu PUFA</th>
<th>T3 SFA</th>
<th>T3 PUFA</th>
<th>T4 SFA</th>
<th>T4 PUFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>0.8 ±0.4</td>
<td>0.4 ±0.4</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>16:0</td>
<td>22.9 ±2.8</td>
<td>18.0 ±2.1</td>
<td>20.5 ±0.5</td>
<td>14.9 ±1.6</td>
<td>20.9 ±1.4</td>
<td>14.6 ±0.5</td>
</tr>
<tr>
<td>16:1*</td>
<td>1.9 ±0.05</td>
<td>0.6 ±0.6</td>
<td>2.0 ±0.4</td>
<td>–</td>
<td>1.9 ±0.2</td>
<td>–</td>
</tr>
<tr>
<td>18:0</td>
<td>15.7 ±0.1</td>
<td>17.0 ±0.6</td>
<td>19.0 ±0.8</td>
<td>22.8 ±1.1</td>
<td>19.1 ±1.6</td>
<td>20.9 ±1.2</td>
</tr>
<tr>
<td>18:1(n-9)</td>
<td>11.3 ±0.7</td>
<td>8.3 ±1.7</td>
<td>17.3 ±1.1</td>
<td>4.7 ±0.4</td>
<td>14.6 ±1.0</td>
<td>5.8 ±0.5</td>
</tr>
<tr>
<td>18:1(n-7)</td>
<td>3.5 ±1.1</td>
<td>1.6 ±0.7</td>
<td>2.4 ±0.4</td>
<td>–</td>
<td>2.3 ±0.4</td>
<td>–</td>
</tr>
<tr>
<td>18:2(n-6)</td>
<td>14.1 ±0.3</td>
<td>20.3 ±3.3</td>
<td>14.1 ±0.6</td>
<td>29.3 ±2.0</td>
<td>14.3 ±1.1</td>
<td>32.2 ±2.8</td>
</tr>
<tr>
<td>20: 2(n-6)</td>
<td>0.3 ±0.3</td>
<td>0.4 ±0.4</td>
<td>–</td>
<td>0.4 ±0.4</td>
<td>–</td>
<td>0.4 ±0.4</td>
</tr>
<tr>
<td>20: 3(n-9)</td>
<td>2.1 ±0.3</td>
<td>1.9 ±0.1</td>
<td>1.4 ±0.1</td>
<td>0.9 ±0.0</td>
<td>1.7 ±0.1</td>
<td>0.4 ±0.4</td>
</tr>
<tr>
<td>20:4(n-6)</td>
<td>19.9±0.7</td>
<td>23.9 ±1.5</td>
<td>21.1 ±0.3</td>
<td>20.6 ±1.1</td>
<td>20.4 ±0.0</td>
<td>18.5 ±1.1</td>
</tr>
<tr>
<td>22:5(n-6)</td>
<td>3.3 ±0.5</td>
<td>2.8±0.3</td>
<td>1.4 ±0.1</td>
<td>1.2 ±0.1</td>
<td>1.5 ±0.1</td>
<td>–</td>
</tr>
<tr>
<td>22:6(n-3)</td>
<td>4.1 ±0.2</td>
<td>4.5 ±0.3</td>
<td>2.9 ±0.2</td>
<td>5.4 ±1.1</td>
<td>3.2 ±0.2</td>
<td>6.8 ±1.1</td>
</tr>
</tbody>
</table>

| monunsat. | 16.7 ±1.7| 10.6 ±2.2| 21.7 ±0.8| 4.7 ±0.4| 18.8 ±1.4| 5.8±0.5|
| polyunsat. | 44.0 ±1.6| 54.1 ±4.9| 41.0 ±1.2| 57.5 ±0.8| 41.2 ±1.4| 58±0.5|
| % unsat.   | 60.7 ±3.3| 64.7 ±2.8| 62.7 ±1.6| 62.2 ±0.7| 60.0 ±0.5| 64.1 ±0.7|
| UI         | 173 ±7| 196 ±10| 164 ±4| 187 ±8| 161 ±2| 187 ±6|

Values are mean ± SEM (number of liver samples tested = 3 for all groups). Unsaturation index is the average number of double bonds per 100 molecules of fatty acids. * number of carbon atoms : number of double bonds
Fig. 18
Percentage of 18:0, 18:2, 20:4, and 22:6 in the liver phospholipids of euthyroid and hyperthyroid mice on SFA and PUFA diet
Liver protein contents in euthyroid and hyperthyroid mice on SFA and PUFA diet

<table>
<thead>
<tr>
<th>Thyroid status</th>
<th>Diet</th>
<th>Total protein (mg. g⁻¹)</th>
<th>Cytosolic protein (mg. g⁻¹)</th>
<th>Mitochondrial protein (mg. g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euthyroid</td>
<td>SFA</td>
<td>120.2 ± 12.4</td>
<td>54.1 ± 6.6</td>
<td>16.6 ± 1.2</td>
</tr>
<tr>
<td>Euthyroid</td>
<td>PUFA</td>
<td>117.0 ± 12.0</td>
<td>60.3 ± 4.3</td>
<td>15.8 ± 1.5</td>
</tr>
<tr>
<td>Hyper T3</td>
<td>SFA</td>
<td>113.0 ± 11.0</td>
<td>47.1 ± 4.1</td>
<td>14.5 ± 1.2</td>
</tr>
<tr>
<td>Hyper T3</td>
<td>PUFA</td>
<td>111.0 ± 8.1</td>
<td>49.2 ± 4.9</td>
<td>15.4 ± 1.4</td>
</tr>
<tr>
<td>Hyper T4</td>
<td>SFA</td>
<td>123.0 ± 11.3</td>
<td>62.3 ± 5.9</td>
<td>16.2 ± 0.9</td>
</tr>
<tr>
<td>Hyper T4</td>
<td>PUFA</td>
<td>113.0 ± 10.3</td>
<td>51.0 ± 4.2</td>
<td>12.1 ± 1.0</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 7 for all groups).
No significant differences were observed in the protein contents of the groups.
Protein contents were measured in the homogenate, cytosolic supernatant and the mitochondrial pellet.
4.2. Discussion

Activity of lipogenic malic enzyme and also the other lipogenic enzymes (for example fatty acid synthetase and enzymes of the hexose monophosphate shunt) are elevated by a saturated fat diet as well as by thyroid hormones (Volpe and Kishimoto, 1972, Roncari and Murthy, 1975, Clarke and Hembree, 1990, Schwartz and Abraham, 1982, 1983). In the present study, compared to euthyroid mice, the activity of malic enzyme in hyperthyroid mice on SFA diet increased significantly in spite of the presence of about 1.5% (w/w) safflower oil in the diet.

Polyunsaturated fatty acids particularly with double bonds in Δ⁹,¹² position, have been shown to decrease the activity of many fatty acid synthesizing enzymes including ME (Clarke et al., 1977). This effect of polyunsaturated linoleic acid on decreasing ME was observed in both, euthyroid and hyperthyroid mice fed PUFA diet containing 18% safflower oil (w/w) in the diet.

The activity of ME reported by Schwartz and Abraham (1983) was 30-45 nmols mg⁻¹ soluble liver protein min⁻¹ (measured at 30°C) in euthyroid mice on a diet containing 15% (w/w) corn oil for 12 days. Mice fed a diet containing the same amount of saturated hydrogenated cottonseed oil without any unsaturated fat had a 3 fold increase in the activity. Compared with the present study, the duration and intensity of treatment were considerably less in the investigation cited above. The total fat in the PUFA diet used by Schwartz and Abraham contained about 55% linoleic acid compared to 72% used in the present investigation. The SFA diet used by them did not contain any unsaturated fatty acid whereas the SFA diet I prepared, contained about 6% linoleic acid. Their duration of treatment was 12 days compared to 21-22 days in the present treatment. They also measured the enzyme activity at a higher temperature of 30°C compared to 26°C used in the present assay. All of these reasons probably contributed to the lower enzyme activities recorded in the present study compared to the values of Schwartz and Abraham cited above.

With 18% safflower oil (w/w) in the PUFA diet, although the ME activity decreased significantly in euthyroid and hyperthyroid mice, it was not totally inhibited. In hyperthyroid mice the activity was significantly higher than euthyroid mice on the same diet which suggests that even with 18% safflower oil, thyroid hormones (about 67 μg T3 and 131 μg T4/100g body weight) increased the activity of malic enzyme.

Comparison of the 18:2 content of the two diets in the present study showed that there was a significant interaction between thyroid state and dietary fats on the activity of ME.

The present data suggests that the stimulation of ME (and perhaps other
lipogenic enzymes) by thyroid hormones is decreased by PUFA, and probably depends on the concentration of polyunsaturated fat in the diet.

Clarke and Hembree (1990) have reported that compared to beef tallow, 10% safflower oil in the diet could suppress ME activity in rats at a lower dose concentration of 2.4 and 8 μg T3 / 100 g body weight, injected daily for 7 days, but 15 μg T3 / 100 g given for 7 days overcame the inhibitory effect of 10% safflower oil diet. This suggests that the ME activity may also depend on the thyroid hormone concentration. Schwartz and Abraham (1982) have reported that in rats, both mono and polyunsaturated fatty acids decreased the activity of ME but in mice only polyunsaturated fats caused this effect, suggesting a species specific difference in the activity response of this enzyme to different dietary fatty acids.

Thyroid hormone action on lipogenic enzymes has been studied in detail. Activity of ME, particularly in the liver, has been used as a marker of thyroid status. However, various studies (for example those cited above), clearly show that hepatic malic enzyme activity is not controlled by thyroid hormones alone. Carbohydrate rich and fat free diets also increase the activity of this enzyme (Schwartz and Abraham 1982, Towle and Mariash 1986, Mariash and Oppenheimer, 1985, Oppenheimer et al., 1987 Clarke and Hembree, 1990). Tepperman and Teppertman (1965) suggest that increased activity of lipogenic enzymes (generating NADPH) is mainly associated with NADPH utilizing reactions, for example desaturation and chain elongation. These authors suggested that above reactions occur during essential fatty acid deficiency (for example when only saturated fat is included as a dietary fat component), resulting in increased requirement for NADPH and thus the increased activities of lipogenic enzymes.

It has been strongly suggested that thyroid hormone-stimulated activity of ME is controlled at the level of nucleus (Towle and Mariash, 1986, Mariash and Oppenheimer, 1985). These authors suggest that its induction by carbohydrate-rich diet appears to show similarity to the effects of thyroid hormones.

Although malic enzyme has been accepted as a marker of thyroid status, since its activity appears to be influenced by factors other than thyroid hormones and since it appears to exhibit a species specific difference, its use as an indicator of thyroid state in experimental situations requires to take these other factors (for example type of dietary fat, amount of unsaturated fat in the diet, species, and duration of dietary treatment) into consideration.

Contrary to the effects on cytosolic malic enzyme discussed above, polyunsaturated fatty acids in the diet did not cause any decrease in the elevated activity of hepatic Na-K-ATPase of the hyperthyroid mice.
An increase in the activity of Na-K-ATPase in the kidney and the salivary glands of rats fed an essential fatty acid deficient diet for over 45 weeks was observed by Alam and Alam (1983). Fatty acid composition of the tissue homogenates showed a significant decrease in double bond index and decrease in 18:2 (n-6) and 20:4 (n-6) was partially compensated by an increase in 20:3 (n-9) content. These authors suggested altered membrane fluidity and allosteric modifications of the enzyme as the cause of the increased activity of Na-K-ATPase. Sun and Sun (1974) reported that the prolonged absence of essential fatty acids in the diet presumably resulted in an increase of 20:3 (n-9) and 22:3 (n-9) to maintain membrane fluidity and the increased activity of the enzyme was an adaptive response to altered membrane structure. Although these authors suggested that it was probably the deficiency of essential fatty acids in the diet that caused an increase in the activity of Na-K-ATPase, Bloj et al., (1973) suggested that a decrease in fluidity and a relative increase in the Hill coefficient (n value) of Na-K-ATPase in erythrocytes of euthyroid rats, was due to the lack of double bonds in general and not because of the lack of any specific fatty acid in the membranes.

Similarly De Mendoza et al., (1977) observed a significant increase in the Hill coefficient of Na-K-ATPase, when erythrocytes of rats fed 5% corn oil for 15 weeks were subjected to physiological levels of T3 (1 x 10^-9 M). These results support the observation of Bloj et al., cited above that linoleic acid, although a specific essential polyunsaturated fatty acid, was unable to maintain membrane fluidity in rat erythrocytes treated with thyroid hormones in vitro.

However, Stubbs and Smith (1984) suggested that membrane fluidity and fatty acid unsaturation, induced in the membranes by dietary means cannot be combined to define physical states of membranes. The effects of various levels of unsaturation on the physical parameters (related to fluidity) such as acyl chain motion which includes describing the rate as well as orientational range of motion within the bilayer and phase transition, do not indicate a direct relation of fluidity and the unsaturation index (Stubbs and Smith, 1984).

Schroeder (1982) in his in vitro study of physical parameters of rat liver plasma membrane showed that physiological concentration of T3 affected the fluorescent polarization of membrane probes, indicative of a decreased membrane fluidity.

In the present study, the unsaturation index (UI) of liver phospholipids and the activity of Na-K-ATPase activity showed that compared to the UI of euthyroid mice on PUFA diet (196), hyper T3 and T4 mice on the same diet showed a lower UI index (187 with both hormones) and an increased activity of Na-K-ATPase. It was
also observed that hyperthyroid mice on T3 and PUFA had a higher UI (187) than mice on T3 and SFA diet (164) but the activity of Na-K-ATPase in both hyperthyroid groups was significantly higher than euthyroid mice. Similarly euthyroid mice on SFA diet had a lower unsaturated index (173) than on PUFA diet but did not show any increase in the enzyme activity.

As demonstrated by Haber and Loeb (1982, 1984) thyroid hormones increased the passive permeability of isolated rat diaphragm and liver of rats. A significant efflux of K+ with in six hours of inducing hyperthyroidism was observed. The sodium pump was activated between 24 and 48 hours which was suggested to be a secondary response as an adaptation to a primary increase in the permeability, in order to maintain the condition of high K+ and low Na+ inside the cell. According to the recent observations of Haber et al., (1988), the mechanism of thyroid hormone effects on the pathways of these Na+ and K+ fluxes are still unknown.

The results of the present study have shown that thyroid hormones caused a significant increase in the Na-K-ATPase activity but the dietary treatment did not have any significant interaction with the effects of thyroid hormones.

The fatty acid composition of liver phospholipids showed that compared to SFA diet, the effect of PUFA diet resulted in a higher content of not only 18:2 but also 18:0 in euthyroid mice. This dietary effect was accentuated by thyroid hormones, to a comparatively higher degree by T3. These results suggest that alterations in the membrane fatty acid composition are influenced by both, thyroid hormones and dietary fat. Thyroid hormone effect on membrane fatty acid composition has been suggested by Hulbert (1978). The comparatively increased levels of 18:0 and 18:2 in the liver phospholipids of hyperthyroid mice observed in this study demonstrate a distinct effect of thyroid hormones on membrane fatty acid contents. However, the unsaturation index which is sometimes used as an indication of fluidity, does not appear to relate to the activity of Na-K-ATPase. These observations indicate that perhaps the double bond index may not be related with the activity of Na-K-ATPase in hyperthyroidism. The nuclear hypothesis of thyroid hormone effects has been applied to the activity of Na-K-ATPase. Chapter one contains a more detailed discussion on the nuclear control of thyroid hormone effects. Ismail-Beigi (1988) and Gick et al. (1988) have demonstrated that the increase in the activity of this enzyme was due to T3-induced changes at the transcriptional as well as post-transcriptional level. Gick et al., (1988) suggested that thyroid hormones stimulated the transcription of the $\delta$ and the $\beta$ gene of the respective sub-units of the enzyme.
Since the increase in the activity of the total ATPase due to Na-K- activation was about 9-11% in all hyperthyroid groups (about 3-4% higher than euthyroid), it appears that the increase in the oxygen consumption which was in the range of 37-44% due to thyroid hormone effects was not mainly due to the elevation of the Na-K-ATPase activity in the liver. Clausen et al., (1991), have also suggested that in intact liver or liver cells, under basal conditions, <5% of the total oxygen is used for active transport of Na+ which may increase up to 30%, depending on the sodium content and integrity of the cell. The studies reviewed by Clausen et al., (1991) also showed variations in results which were due to difference in tissues, species and also the assay conditions.

Results of the activity of mitochondrial glycerolphosphate dehydrogenase (GPDH) showed that thyroid homones caused a significant increase in the activity of this enzyme irrespective of the dietary treatment. This effect of thyroid hormones on elevating the activity of mitochondrial GPDH has been reported by others (Lee et al., 1959, Lee and Lardy, 1965, Wernette et al., 1981, Nakamura et al., 1979, Horrum et al., 1985). Effects of dietary 18:2 and other unsaturated fatty acids on mitochondrial oxygen consumption and electron transport chain (discussed below) have also been reported.

The GPDH of the H shuttle and the cytochromes of the respiratory chain are all located on the inner mitochondrial membrane. Many studies, discussed in the following account have demonstrated that activities of these electron transporting enzymes are influenced by thyroid hormones and also by the nature of fatty acids in the mitochondrial lipids.

The ‘H’ shuttle (Estabrook and Sactor, 1958), connects the mitochondrial GPDH to the respiratory chain. Its activation elevates the transfer of electrons to the cytochromes and thus the transfer of reducing equivalents from cytosol to the ‘H’ shuttle or the ‘glycerol phosphate cycle’ increases. This augmented transfer of reducing equivalents decreases cytosolic NADH:NAD ratio. Glycolysis is increased in order to maintain this ratio which results in decreased glycogen resynthesis (Lee and Lardy, 1965).

An increased rate of respiration and increased activity of GPDH in T4 treated liver mitochondria of rats injected with 15 μg T4 / 100g body weight daily for 10 days was observed by Horrum et al., (1985). The specific activity of GPDH reported by them was 28.4 in hyperthyroid and 4.9 nmols mg⁻¹ min⁻¹ in euthyroid rats (it is assumed that the activity is expressed as mg⁻¹ of mitochondrial protein), which is comparable with the results of the present study. Although the plasma T4 concentration in their study was 200 nM which was lower than the 250-255 nM
observed in the present study (see table 7), their values of GPDH activity are comparable with the results of the present study.

Horrum et al., (1985) also observed an increase in the concentration of cytochrome oxidase which directly accepts molecular oxygen and cytochrome c was in a more ‘reduced’ state due to enhanced electron flow from substrate oxidation. Thus the effect of thyroid hormones either directly on the respiratory chain or via ‘H’ shuttle appears to enhance the activity of mitochondrial GPDH. Cytochrome c concentration and its activity is also increased in heart and kidney mitochondria of hyperthyroid rats Hoch (1962).

Activity of the cytochrome c oxidase system which spans the inner mitochondrial membrane has been shown to depend on the membrane lipid composition. In beef heart mitochondria, cytochrome c oxidase had greater affinity for cardiolipin than other phospho- lipids (Robinson, 1982). A two fold increase in the electron transport activity was observed when the boundary phospholipids of the inner mitochondrial membrane contained unsaturated rather than saturated fatty acids (Robinson,1982). Cardiolipin (a specific mitochondrial lipid) is almost entirely located in the inner mitochondrial membrane (Marinetti et al., 1958) and 18:2 is a major constituent of mammalian cardiolipin (Daum, 1985). Yamaoka et al., (1990) reported that oxygen consumption of the rat heart mitochondria decreased when the dietary corn oil (containing 18:2 n-6) was replaced by sardine oil (containing 20:5 n-3 and 22:6 n-3). When cardiolipin showed significantly high level of n-3 than n-6 fatty acids (caused by dietary means) the cytochrome c oxidase activity was significantly lower in these rats.

Above findings suggest that both thyroid hormones and 18:2 augment oxygen consumption and the activity of electron transporting systems in mitochondria.

The results of Ruggiero et al., (1984a,1984b and 1989) and Giuseppe and Ruggiero (1988) (these were discussed earlier in chapter one) showed that although mitochondrial cardiolipin content increased, its 18:2 content decreased significantly in hyperthyroid rats. Significant increases in 16:0, 18:0 and 20:4 in the total phospholipids and an increase in the 20:4 / 18:2 ratio but compared to euthyroid a decrease in unsaturation index was observed in hyperthyroid rats (Ruggiero et al., 1984a).

In the present study, fatty acid composition of the total liver phospholipids showed that on PUFA diet, compared to euthyroid mice, 18:2 (n-6) content was significantly increased in hyperthyroid mice. Hyper T4 mice on PUFA diet showed the most significant increase among the groups. It was observed that dietary 18:2 did not influence the activity of GPDH in euthyroid mice. In hyper T4 mice, compared
to SFA, PUFA diet appeared to play a permissive role where a highly significant increase in the activity of GPDH was influenced by thyroid hormones and an interaction between T4 and PUFA diet.

Evidence from the present study suggests that although a depletion of 18:2 may occur in hyperthyroid liver mitochondria as reported by Ruggiero et al., (1984a); dietary 18:2 as an essential fatty acid present in significant amounts in liver phospholipids did not counteract the effect of thyroid hormones on mitochondrial GPDH activity but perhaps caused an augmentation of the T4 effects. With reference to the literature cited above and the results of the present study it is suggested that increasing the 18:2 content in the diet did not counteract the increased rate of respiration in hyperthyroid mice.

The report of Yamaoka et al., (1990) that cytochrome c oxidase in the heart required 18:2 for its activity which was decreased by replacing it with dietary n-3 fatty acids suggests that n-3 fatty acids perhaps play a distinct role, different to 18:2 (n-6) fatty acids in mitochondrial membrane function.

Studies conducted on thyroid state and membrane fatty acid composition have shown that in rat liver, hypothyroidism increases (Shaw and Hoch 1977, Withers and Hulbert 1987) and thyroid hormone administration decreases the linoleic acid content of mitochondrial membranes (Shaw and Hoch 1977, Ruggiero et al., 1984a). In the present study although the 18:2 content of PUFA diet was significantly higher, mice on SFA were receiving a diet that was not deficient in linoleic acid. It was observed that 18:2 content of the liver phospholipids of euthyroid and hyperthyroid groups on SFA diet was comparable, but compared to euthyroid mice, the higher levels of 18:2 in hyperthyroid mice on PUFA diet showed a significant interaction between thyroid hormones and dietary fat.

The percentage of 20:4 (n-6) was high only in euthyroid mice on the PUFA diet. Since the ratio of 20:3 (n-9) / 20:4 (n-6) was lower than 0.4 in other groups, these mice were not considered to be deficient in n-6 essential fatty acids (Holman, 1960). However, a significant interaction between diet and thyroid hormones was apparent in the T4 group. de G’omez et al., (1977) have demonstrated that hepatic Δ6 desaturase activity which converts 18:2 to 20:4 is decreased in hyperthyroid rats. Ruggiero et al., (1984a) reported an increase in 20:4 / 18:2 ratio in hyperthyroid rats injected with 30 μg T3 / 100g / day for 7 days indicating an increase in the activity of Δ6 desaturase. Hoch (1988) suggested that a decrease in the activity of this enzyme occurs at thyrotoxic level of thyroid hormones.

The dose (4000 μg / 100g body weight injected daily for 12 days) administered by de G’omez et al., was obviously supraphysiological. The hormone...
intake of mice in the present study (67 \( \mu \)g T3 and 131 \( \mu \)g T4 / 100g body weight / day) also appears to have caused a decrease in the activity of \( \Delta^6 \) desaturase; significantly in hyper T4 mice fed PUFA diet. With SFA diet, the difference in 20:4 content was not significant between euthyroid and hyperthyroid mice.

The level of 22:5 (n-6) decreased in all hyperthyroid mice. Compared to euthyroid mice, a significant rise in 22:6 (n-3) was observed in hyperthyroid mice on PUFA diet. On the contrary, on SFA diet, compared to euthyroid, hyperthyroid mice showed significantly lower level of 22:6 (n-3). This fatty acid was suggested to be essential in synaptosomal plasma membranes to maintain its liquid crystalline state (Sun and Sun 1974). Essentiality of 22:6 (n-3) in the membranes of photoreceptor cells was demonstrated by Anderson et al., (1992) and Brown et al., (1992). Docosahexaenoic acid, 22:6 (n-3) or DHA is essential in proper visual function and 47 \% of the total rod outer segment lipids in mammals contain DHA (Brown et al., 1992).

No 18:3 (n-3) was found either in the fatty acid composition of the SFA and PUFA diets or the liver phospholipids examined at the end of the present treatments. Since these animals were fed commercial laboratory diet for at least 4 weeks before they were put on the experimental diets, sufficient n-3 fatty acids were probably stored in their membranes during that time (Leat, 1992). It is possible that the stored 18:3 was converted to DHA. Requirement of DHA could be increased in hyperthyroidism resulting in its higher synthesis in the liver from stored precursors for transport to specific areas such as photoreceptor cells and other neural membranes. However, the reason for the comparatively lower level of 22:6 in hyperthyroid mice on SFA diet is not clear. It appears that the SFA diet promoted lipogenesis and synthesis of higher level of monounsaturates but some other mechanism, perhaps increased oxidation, reduced the proportion of 22:6 in the mice on SFA diet.

Compared to the SFA diet, euthyroid mice on PUFA diet showed higher levels of 18:2 (as expected), but it was observed that these mice also had a higher content of 18:0 in the liver phospholipids. Although 18:2 and 18:0 contents were significantly higher in all hyperthyroid groups, hyper T3 mice on PUFA diet showed significantly higher levels of these fatty acids which were influenced by T3 and diet. With T4 however, statistically, only hormone effect appeared to cause the increase in 18:0 content. This specific effect of thyroid hormones on increased level of 18:0 in total liver phospholipids in rat was also reported by de Gomez et al., (1977). These authors reported that there was also a decrease in 18:2 content (but these animals were not on high 18:2 diet) and in spite of the increase in 18:0 content they observed
that microsomal elongation system was not stimulated by T4.

Influence of thyroid hormones on membrane fatty acids (Hulbert 1978) and increase in membrane permeability to Na+ and K+ under thyroid hormone effect (Haber and Loeb 1984) have been discussed in the preceding section. Henning and Watkins (1989) have reported that compared to 18:3 (n-3), exposure of euthyroid porcine endothelial cells to excess 18:2 (8-fold higher than controls) increased the membrane permeability to 16:0, 18:0 and 18:2, resulting in their higher incorporation in the membranes. This report, similar to the observation of Yamaoka et al., (1990), cited earlier, suggests a different effect of n-3 fatty acids to 18:2 (n-6) on membrane properties.

From the results of the present study however, it is not possible to say whether the comparatively higher level of 18:2 and 18:0 observed in the liver phospholipids of hyperthyroid mice on PUFA diet was an effect of thyroid hormones on membrane permeability to fatty acids or a more direct effect on membrane fatty acid composition or an effect of both thyroid hormones and dietary 18:2. Further work in this area would certainly be more informative.
CHAPTER FIVE

General Discussion

Orally administered thyroid hormones, T3 and T4 induced hyperthyroidism in *Mus musculus*. The two hormones were administered separately in the drinking water. The degree of hyperthyroidism induced by this method was considered to be within physiological levels because the plasma T3 and T4 levels of 13-17 nM and 250-255 nM respectively, recorded in the present study have also been reported in the serum of hyperthyroid humans (Marsden and McKerron 1975). From the plasma levels of T3 and degree of stimulation, it appears that T3 was the active hormone in all hyperthyroid groups; although T4 effects in certain areas were different to T3. These will be pointed out in the appropriate sections of the following account.

When a comparison of the potency of oral administration used in the present study and parenteral administration used by Tata et al., (1963) and Oppenheimer et al., (1991) was made (this was discussed in chapter three), it was observed that smaller doses of hormones injected by these authors (10 μg T3 and 16 μg T4 / 100g body weight and 50 μg T3 / 100 g body weight respectively) were more effective in causing a greater increase in oxygen consumption. Gross and Pitt-rivers (1953) have reported that orally administered T3 was about twice as effective as T4. These authors also suggested that T3 and T4 when administered orally, exert about 86 and 39 % of the potency of their respective subcutaneous doses. Hormone intake in the present study was about 67 μg T3 and 131 μg T4 / 100g body weight / day. Although T4 intake was almost twice that of T3, (because of a higher T4 concentration in the drinking water), plasma T3 levels in all hyperthyroid groups were comparable and the intensity of thyroid hormone effects was almost similar. Although orally administered thyroid hormones exhibited a lesser degree of potency in increasing the oxygen consumption, it can be seen that this method has induced hyperthyroidism within physiological limits. Here it is also stated that oral administration of thyroid hormones in drinking water probably maintained hyperthyroidism by eliminating the sudden fluctuations in the hormone levels that may occur following an injection, given at regular intervals (Connors and Hedge, 1980).

As expected, standard metabolic rate, body weight, and growth in general were influenced by thyroid hormones. In this respect T3 was more potent than T4 in causing body weight loss (table 10 and Fig.12) on both diets.

Also as expected, activities of malic enzyme, Na-K-ATPase and GPDH in liver cells were increased as a result of thyroid hormone administration. However,
dietary treatment produced specific results.

As this study was undertaken as an extension of Ershoff’s findings (1949), dietary approach was continued but instead of soybean meal used by Ershoff {which contains about 64 % linoleic acid (n-6) and 6.5 % linolenic acid (n-3)}, safflower oil which mainly contains linoleic acid (n-6) was used in the diets. An increase in the activity of Na-K-ATPase in essential fatty acid deficiency has been generally related to the deficiency of linoleic acid and its derivatives. Its depletion from mitochondrial membranes in hyperthyroid rats and increase in hypothyroid mitochondrial membranes suggested that perhaps there was a connection between this fatty acid and various effects of thyroid hormones.

The study also provided the possibility of examining a nutritional approach towards counteracting the effects of hyperthyroidism. However, the results and discussions in the previous chapters have confirmed that linoleic acid can not be considered as a dietary treatment for counteracting the effects of hyperthyroidism.

One of the major outcomes of this investigation is that it has examined some of the effects of hyperthyroidism on the major gross physiological parameters as well as the effects at cell level, caused separately by T3 and T4 under the influence of saturated and polyunsaturated fatty acids in the diet. This has allowed to examine the parameters that had similar effects of T3 and T4 but were influenced differently by dietary fatty acids. For example, the activity of the liver malic enzyme which increased due to thyroid hormone effects, was counteracted by the PUFA diet but SFA diet + thyroid hormones caused a significant increase in the activity of this enzyme. On the other hand measurement of body weight change showed that PUFA diet treatment resulted in a significant decrease whereas SFA diet had a counteractive effect on body weight loss.

The possible causes of body weight loss in hyperthyroid mice on PUFA diet were 1. the increased energy waste as a result of an increased metabolic rate 2. increased lipolysis caused by hyperthyroidism 3. significant decrease in lipogenesis due to the PUFA effect, confirmed by the marked decrease in the activity of malic enzyme (and perhaps other lipogenic enzymes as well). Even in euthyroid mice the decreased activity of malic enzyme was associated with a reduced weight gain (compare table 10 and 12 and Fig 12 and 14). The results showed that the most significant interaction between thyroid hormones and dietary fatty acids was on ME activity and presumably lipogenesis, where dietary treatment with 18:2 resulted either in reduced weight gain (in euthyroid) or increased weight loss (in hyperthyroid). Although the hyperthyroid mice on SFA diet compared to PUFA diet showed a weight gain, compared to euthyroid mice, their livers also showed a higher
weight gain (expressed as a % of body weight) which could be due to increased level of fatty acids in the liver. Pan and Berdanier (1990) have reported significant accumulation of fatty acids in the livers of hyperthyroid rats on a hydrogenated coconut oil diet.

Compared to euthyroid mice, oxygen consumption in hyperthyroid mice was increased by up to 44 percent. However thyroid hormone-induced increase of total ATPase due to Na-K-ATPase activity was not more than 11 % (about 3-4 % higher than euthyroid level). These results agree with the observations of Clausen et al., (1991) that thyroid hormone effect on increased activity of Na-K-ATPase in the liver does not contribute significantly to the increased oxygen consumption.

Effect of dietary treatment on the activity of Na-K-ATPase and membrane fatty acids showed that the increased UI in PUFA treated hyperthyroid mice had no significant effect on the activity of this enzyme. The overall results showed that the UI of the phospholipids was not related to the activity of Na-K-ATPase in the mouse liver. This result does not agree with that of Alam and Alam (1983) and Bloj et al., (1963) who suggested an increase in the activity of Na-K-ATPase in essential fatty acid deficiency due to decrease in double bonds in membrane fatty acids. It is possible that the mechanism of activation of this enzyme in essential fatty acid deficiency is different to that in hyperthyroidism.

Results of the activity of mitochondrial GPDH showed that its activity was stimulated by thyroid hormones and it was also evident that compared to T3, T4 and PUFA diet interaction caused a significantly greater increase in the activity of this enzyme. Here it can be seen that T4 effects were different to that of T3. From these results it is evident that dietary 18:2 did not counteract the increased transport of electrons to the respiratory chain, mediated by increased GPDH activity. This is in agreement with the observations of Yamaoka et al., (1990) who reported a significant decrease in the activity of cytochrome c oxidase and oxygen consumption in the heart mitochondria of rats fed a diet containing sardine oil, rich in n-3 fatty acids (20:5 and 22:6) compared to the diet containing corn oil containing 18:2 (n-6).

The above discussion is mainly related to the increased rate of respiration due to enhanced electron transport, increased activity of GPDH and the cytochromes of the respiratory chain in hyperthyroidism. A different theory and its demonstration by Brand (1990a, 1990b) suggests that the proton leak across the mitochondrial membrane back in to the mitochondrial matrix has an important influence on oxygen consumption. In resting hepatocytes (i.e., during state 4 respiration) the proton leak has been shown to represent 20-30 % of the oxygen consumed and this process produces only heat. Brand has demonstrated that proton leak and therefore state 4
respiration is increased by 7 fold in hyperthyroidism.

Brand has (personal communication to Hulbert) also reported that in rats, various levels of thyroid hormones changed the proton flux per mg of protein in isolated mitochondria.

The surface area of the inner mitochondrial membrane was significantly increased, and the membrane proton permeability of liposomes prepared from phospholipids of inner mitochondrial membranes of hyperthyroid mitochondria was 3 times greater than hypothyroid mitochondria. Brand suggests that these changes may be due to the differences in fatty acid composition of the bilayer in different thyroid states.

Results of the present study showed that fatty acid composition of the liver phospholipids were influenced by thyroid hormones and dietary fatty acids and there was a significant interaction between the two. However, an increased 18:2 content of liver phospholipids was not associated with a decrease in the metabolic rate or the activity of Na-K-ATPase in hyperthyroid mice, suggesting that these parameters may not be affected by changes in membrane fatty acid composition. On the contrary, the activity of GPDH, within the hyperthyroid groups, showed a significant increase with PUFA diet and only with T4 which suggested that this effect of significant increase in 18:2 and increased activity of GPDH was related specifically to the interaction of T4 and PUFA.

When body weight loss is examined, possible effects of thyroid hormones on lipolysis due to increased oxidation are evident. Decreased lipogenesis with PUFA diet discussed earlier has probably also contributed towards this effect. Heimberg et al., (1985) have reported that in hyperthyroidism more fatty acids are incorporated into phospholipids than in the synthesis of triglycerides. Since the turnover rate of fatty acids in phospholipids is rapid (Berdanier, 1988), their higher incorporation in membranes in hyperthyroidism would result in an overall decrease in triglyceride synthesis. Thus the effect of PUFA diet on decreased lipogenesis has magnified the lipolytic effects of thyroid hormones on weight loss.

The decrease in the activity of malic enzyme could have occurred by two ways:

1. As suggested by Tepperman and Tepperman (1965), presence of excessive polyunsaturated fatty acids in PUFA diet could have reduced the requirement for NADPH, resulting in decreased activity of lipogenic enzymes which generate NADPH.

2. The nuclear receptor (s) of thyroid hormones have been under constant investigation. Inhibitory effect of unsaturated fatty acids on binding of thyroid
hormones to nuclear binding proteins has been demonstrated by Van der Klis et al., (1989), Wiersinga and Platvoet-Ter-Schiphorst (1990), and Inoue et al., (1989). From these recent reports it can be assumed that if the activity of malic enzyme is initiated at nuclear level (Winberry et al., 1983, Dozin et al., 1985a, 1985b, Schwartz and Abraham 1983), then the dietary safflower oil used in this study may have been a potential inhibitor of thyroid hormone binding and activation of malic enzyme via nuclear pathway.

Although an increase in the activities of various lipogenic and oxidative enzymes and the desaturases and elongases are observed in hyperthyroidism, Landriscina et al., (1976) have reported that no difference in the protein content between thyroid hormone treated and euthyroid rats was apparent until after 22 days of treatment. Results of the protein contents obtained in the present study did not show any significant differences between euthyroid and hyperthyroid groups. Towle's review (1983) on the effects of thyroid hormones on cellular RNA metabolism also suggests that from the reports of various authors, and the period of treatment in their studies, effects of thyroid hormones on overall protein synthesis are inconclusive.

From the above overall discussion of the present results it is quite clear that the specific effects of thyroid hormones and the diets on the various parameters examined, were influenced by the different interactions of thyroid hormones with the type of fatty acids in the diets. These are compared below in table 18.

Table 18
Comparison of the interactions of thyroid hormones and dietary fatty acids

<table>
<thead>
<tr>
<th>No interaction between hyperthyroidism &amp; diet</th>
<th>Significant interaction between hyperthyroidism &amp; diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolic rate</td>
<td>Body weight loss</td>
</tr>
<tr>
<td>Na-K-ATPase activity</td>
<td>Malic enzyme activity</td>
</tr>
<tr>
<td>GPDH activity (only with T3)</td>
<td>GPDH activity (only with T4)</td>
</tr>
<tr>
<td>Protein content</td>
<td>Fatty acid composition of liver phospholipids</td>
</tr>
</tbody>
</table>

In conclusion, thyroid hormones influenced the membrane fatty acid composition. However, the dietary treatment of 18:2 which also appears to have influenced the membrane fatty acid composition did not counteract the effects of
thyroid hormones on membrane associated enzymes although the PUFA diet significantly decreased the thyroid hormone-induced activity of malic enzyme.

Since no dietary 18:3 was used in the treatment, the results of this study relate solely to the effects of 18:2 (n-6) essential fatty acid. Although safflower oil has been shown to contain about 3.5 % 18:3 (Hardwood and Geyer, 1964), it was absent in the safflower oil used in the present study. With reference to the results of Yamaoka et al., (1990) where a decrease in respiration due to the presence of dietary n-3 fatty acids in the sardine oil and the results of Henning and Watkins (1989) where compared to 18:2, a significant decrease in endothelial cell permeability to saturated fatty acids, triglycerides and albumin occurred in the presence of 18:3, it appears that 18:3 (n-3) as an essential fatty acid has a different function to that of 18:2 (n-6).

The 18:3 content of soybean oil is about 6.5 % (Hardwood and Geyer, 1964). Therefore it is possible that the antithyroid effects of full fat soybean meal observed by Ershoff (1949) were perhaps related to the presence of not only 18:2 but also 18:3 in the dietary fat. A further study of the above parameters using 18:3 will certainly provide more information regarding the effects of n-6 and n-3 fatty acids on hyperthyroidism and membrane fatty acid functions.
Effects of both T3 and T4 were significant on the thyroid hormone responsive parameters measured in *Mus musculus*. Comparison of the present results of oxygen consumption with those of others showed that orally administered thyroid hormones were less potent than their subcutaneous administration. However, from the plasma levels of the hormones it was evident that this method induced hyperthyroidism within physiological levels. Activities of malic enzyme, Na-K-ATPase and mitochondrial glycerolphosphate dehydrogenase (GPDH) in the liver were significantly elevated in hyperthyroid mice. Dietary treatment did not influence the activity of Na-K-ATPase. However, compared to SFA diet, PUFA diet and T4 elevated the activity of GPDH to a significantly higher level than T3 and PUFA diet. Activity of malic enzyme was influenced differently by dietary fatty acids. The significant interaction of thyroid hormones and dietary fatty acids showed that with SFA diet the comparative activity of malic enzyme was 148 and 139 % higher with T3 and T4 respectively. With PUFA diet the significant decrease in the activity of this enzyme (and perhaps also other lipogenic enzymes) and the lipolytic effects of thyroid hormones caused a greater body weight loss in hyperthyroid mice on this diet.

Unsaturation index was not related to the activity of Na-K-ATPase. Higher level of unsaturated fatty acids in hyper T3 and hyper T4 mice on PUFA diet were probably related to an increase in the activity of GPDH which was significantly higher in T4 +PUFA treated group.

Fatty acid analysis showed that thyroid hormones as well as 18:2 diet caused an increase in the level of 18:2 and 18:0 in the liver phospholipids. Significant effects of diet and thyroid hormones were observed on 18:0, 18:2, 20:4 and 22:6 contents. These results showed that thyroid hormones influenced the fatty acid composition of the membranes. However, the mechanism of this effect is not yet known.

Linoleic acid (18:2 n-6) is an essential fatty acid and hyperthyroidism appears to decrease the level of this fatty acid from the mitochondrial membranes. However, excess amount of dietary 18:2 did not counteract increased BMR, weight loss or activities of membrane associated Na-K-ATPase and GPDH. Its inhibitory effect on malic enzyme activity magnified the overall effects of hyperthyroidism on lipolysis. It is suggested here that the antithyroid effects of soybean meal on hyperthyroid rats reported by Ershoff (1949) were perhaps related to the influence of 18:3 (n-3) or some other components of the soybeans.
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**Appendix 1.**

<table>
<thead>
<tr>
<th>Chemical / Ingredient</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>Ajax</td>
</tr>
<tr>
<td>Adenosinetriphosphate (disodium salt)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Ammonium Molybdate</td>
<td>Sigma</td>
</tr>
<tr>
<td>Biorad reagent</td>
<td>Bio-rad, USA</td>
</tr>
<tr>
<td>Biotin</td>
<td>Roche</td>
</tr>
<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Calcium Carbonate</td>
<td>BDH, Australia</td>
</tr>
<tr>
<td>Ca-Pantothenate</td>
<td>Roche</td>
</tr>
<tr>
<td>Casein</td>
<td>Cottees, Australia</td>
</tr>
<tr>
<td>Cellulose</td>
<td>Sigma</td>
</tr>
<tr>
<td>Choline Chloride</td>
<td>Sigma</td>
</tr>
<tr>
<td>Cobalt Chloride</td>
<td>Sigma</td>
</tr>
<tr>
<td>Coconut oil</td>
<td>ETA, Australia</td>
</tr>
<tr>
<td>Copper Sulfate</td>
<td>BDH</td>
</tr>
<tr>
<td>Dextrose</td>
<td>Sigma</td>
</tr>
<tr>
<td>Dimethylsulfoxide (DMSO)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Drierite</td>
<td>BDH</td>
</tr>
<tr>
<td>EDTA</td>
<td>Sigma</td>
</tr>
<tr>
<td>(Ethylendiamine tetra-acetic acid)</td>
<td>Sigma</td>
</tr>
<tr>
<td>EGTA</td>
<td>Sigma</td>
</tr>
<tr>
<td>(Ethyleneglycol-bis- (β aminoethyl ether)</td>
<td>Sigma</td>
</tr>
<tr>
<td>N,N,N’N’-Tetraacetic acid</td>
<td>Sigma</td>
</tr>
<tr>
<td>Elon (P-methyl-aminophenol sulfate)</td>
<td>Kodak</td>
</tr>
<tr>
<td>Ferrous Sulfate</td>
<td>Ajax, Australia</td>
</tr>
<tr>
<td>Folic acid</td>
<td>Roche</td>
</tr>
<tr>
<td>Heparin</td>
<td>Sigma</td>
</tr>
<tr>
<td>Histidine</td>
<td>Sigma</td>
</tr>
<tr>
<td>Imidazole</td>
<td>Sigma</td>
</tr>
<tr>
<td>Inositol</td>
<td>Sigma</td>
</tr>
<tr>
<td>INT (2-p-iodophenyl-3p-nitrophenyl-5phenylmonotetrazolium chloride)</td>
<td>Sigma</td>
</tr>
<tr>
<td>L-Malate</td>
<td>Sigma</td>
</tr>
<tr>
<td>L-δ glycerophosphate</td>
<td>Sigma</td>
</tr>
<tr>
<td>Magnesium Sulfate</td>
<td>BDH</td>
</tr>
</tbody>
</table>
L-3 glycerophosphate Sigma
Magnesium Sulfate BDH
Manganese Sulfate Ajax
Manganese Chloride BDH
Menadione (K) Sigma
Nicotinic acid Roche
NADP (Nicotin Adenine Dinucleotide Phosphate) BDH
Ouabain Sigma
P-Amino Benzoic acid Merck
Potassium Cyanide Ajax
Potassium Dihydrogen Orthophosphate Sigma
Potassium Oxypxide Sigma
Potassium Iodide BDH
Pyridoxin HCl Roche
Riboflavin Roche
RIA kits Spectria, Australia
Safflower oil Becel
Sodium Acetate Sigma
Sodium Deoxycholate Sigma
Sodium Chloride BDH
Sodium Sulfite Sigma
Sucrose (analytical grade) Mallinckrodt,
Australia
Sucrose (for diet preparation) Local supermarket
Thiamin HCl Roche
Triethanolamine buffer Sigma
Triiodothyronine (T3) Sigma
Thyroxine (T4) Sigma
Vitamin A Roche
Vitamin B12 Roche
Vitamin D3 Roche
Vitamin E Roche
Vivalyme CIG Medishield,
Australia
Zinc Sulfate Sigma