# HEART SARCOLEMMAL CHANGES IN HYPOTHYROIDISM

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by

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# IN HYPOTHYROIDISM

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## ABSTRACT

The influence of thyroid status on rat cardiac sarcolemmal composition, enzymatic activities and $Ca^{2+}$ binding was examined, $Na^+ - K^+$ ATPase activity of sarcolemma isolated by the hypotonic shock-LiBr method was depressed in propylthiouracil (P.T.U.) induced hypothyroidism but unaltered in triiodothyronine ($T_3$) induced hyperthyroidism. While Km and $V_{max}$ values of $Na^+ - K^+$ ATPase were decreased in hypothyroidism, $K_{aNa^+}$ and $K_{aK^+}$ values were unaltered. The sensitivity of $Na^+ - K^+$ ATPase to ouabain, calcium and pH was not affected in hypothyroidism. $K^+$ stimulated phosphatase activity, which is believed to represent the dephosphorylation step of $Na^+ - K^+$ ATPase activity, was inhibited to the same extent as $Na^+ - K^+$ ATPase whereas its sensitivity to ouabain was unchanged. The phospholipid composition of sarcolemma was not significantly altered in the hypothyroid state and gel electrophoretic pattern of sarcolemma from euthyroid and hypothyroid rats were qualitatively similar. $Na^+ - K^+$ ATPase activity of sarcolemma prepared by the KCl-sucrose density gradient method was also depressed in hypothyroidism. Thyroidectomy resulted in a similar depression of sarcolemmal $Na^+ - K^+$ ATPase activity. These changes in $Na^+ - K^+$ ATPase activity were not accompanied by changes in sarcolemmal $Mg^{2+}$ ATPase activity. The depression in $Na^+ - K^+$ ATPase activity in hypothyroid rats was not affected after 24 hours but was completely reversed after 48 hours of $T_3$ administration. Treatment with puromycin, an inhibitor of protein synthesis, prevented the $T_3$-induced reversal of changes in $Na^+ - K^+$ ATPase activity in hypothyroid animals. These results suggest an alteration of $Na^+ - K^+$ pump mechanism of the myocardial cell membrane in hypothyroidism.

In another series of experiments myocardial electrolyte contents were examined in hypothyroid rats, Intracellular sodium and potassium increased after thyroid hormone administration to hypothyroid rats; however the intracellular sodium and potassium did not change in hypothyroid and hyperthyroid rats. This observation suggests that a simple relationship between $Na^+ - K^+$ ATPase activity and intracellular monovalent cation concentrations did not exist under these experimental conditions. Intracellular calcium did not change significantly with thyroid status although there was a tendency for it to decrease in hypothyroidism and for this to be reversed after $T_3$ administration. Intracellular magnesium was significantly altered only when hypothyroid and hyperthyroid states were compared; however, it was found that $[Mg]_i$ / $[Ca]_i$ ratio was significantly increased in the hypothyroid state but not after 7 days of $T_3$ treatment. Increased $[Mg]_i$ / $[Ca]_i$ may play a role in the manifestation of depressed contractile state in hypothyroidism.

In hypothyroid rats, cardiac 5' nucleotidase activity was depressed in sarcolemma isolated by the hypotonic shock-LiBr method but not in sarcolemma isolated by the KCl sucrose density gradient method. As the former and latter methods have been shown to yield predominantly right-side out and inside out membrane vesicles respectively, it appears that 5'-nucleotidase located at the extracellular surface of sarcolemma was depressed in hypothyroidism. Passive calcium binding, sialic acid content and $Ca^{2+}$ dependent ATPase of rat cardiac sarcolemma were unaltered in hypothyroidism. However ATP dependent calcium binding was increased in hypothyroid animals. ATP dependent calcium binding and $Ca^{2+} - Mg^{2+}$ ATPase activity which are associated with calcium extrusion from the cell, were

activated by calmodulin to a greater degree in sarcolemma isolated from hypothyroid rats than in that from euthyroid rats. Trifluoperazine in concentrations, which completely blocked the effect of exogenous calmodulin, had no effect on these activities in the absence of exogenous calmodulin. Therefore, the differences in activation by calmodulin could not be ascribed to differences in the concentration of endogenous calmodulin in sarcolemmal preparations from euthyroid and hypothyroid rats. These results suggest an enhancement of $Ca^{2+}$ pump activity at the cell membrane and this may explain a slight reduction in myocardial calcium content and depressed contractile state of the hypothyroid heart.

Sarcolemmal adenylate cyclase activity was unaltered in the hypothyroid state but the washed particles preparation of hypothyroid rat hearts displayed higher basal adenylate cyclase activity in comparison to that in the euthyroid rat hearts. Fluoride stimulation was unaltered but guanylimidodiphosphate (GppNHp) stimulation was markedly depressed over a wide range of concentrations in the hypothyroid heart washed particles. Epinephrine stimulation in the presence of GppNHp was altered only at $10^{-5}$ to $10^{-4}$ M concentrations. Depressed responsiveness of cardiac adenylate cyclase to GppNHp and epinephrine was also found in washed particles of thyroidectomized rats. Depression of GppNHp response in hypothyroid animals was observed 48 hours after $T_3$ administration. It is proposed that altered guanine nucleotide binding to adenylate cyclase system may be an underlying mechanism of depressed positive inotropic action of catecholamines in the hypothyroid state.

In view of the important role of sarcolemma in the regulation of cardiac function and metabolism, it is suggested that changes in

sarcolemmal function described in this study may play a crucial role in altering cardiac function in hypothyroidism. These observations on sarcolemma extend our knowledge concerning the subcellular basis of hypothyroid-induced changes in cardiac function, which in the past emphasized the roles of sarcoplasmic reticulum, myofibrils and mitochondria.

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# I. INTRODUCTION AND STATEMENT OF THE PROBLEM

Circulating thyroid hormones have pronounced effects on the cardiovascular system in general and on the heart in particular (1,2). Hyperthyroidism is usually accompanied by increased cardiac activity whereas the opposite occurs in hypothyroidism. Although interaction of thyroid hormones with the heart has been studied at gross, tissue, cellular and subcellular levels, many areas of this interaction have not been fully characterized and other areas have been completely ignored. In this regard it is pointed out that thyroid hormone has been demonstrated to influence $Na^+$-$K^+$ ATPase activity in a variety of tissues (3,4,5,6,7). However, a full characterization of this enzyme, which is believed to serve as a $Na^+$-$K^+$ pump at the cell membrane, has not been undertaken in cardiac tissue and most studies have been performed on crude homogenates with a relatively low $Na^+$-$K^+$ ATPase specific activity. Under conditions such as hypothyroidism and hyperthyroidism, where dramatic alterations occur throughout the cardiac cell (8), the relative contribution of sarcolemmal proteins to total homogenate protein may be altered and this may distort the pattern of enzyme activities seen in the crude preparations. For these reasons and in view of the importance of $Na^+$-$K^+$ ATPase in the maintenance of transmembrane ionic gradients, it was thought worthwhile to characterize sarcolemmal $Na^+$-$K^+$ ATPase activity in hearts of animals with altered thyroid status. While attempts have been made to correlate thyroid associated changes in cardiac $Na^+$-$K^+$ ATPase activities with altered electrolyte balance, the results are conflicting (9,10). It was therefore decided to examine serum and cardiac electrolytes in euthyroid, hyperthyroid and hypothyroid animals as well as during reversal of hypothyroidism. Alterations in sarco-

lemmal $Na^+$-$K^+$ ATPase activity and subsequent changes in cellular contents of $Na^+$ and $K^+$ may serve as one of the important mechanisms for altered cardiac function due to thyroid hormones.

The interaction of catecholamines with thyroid hormones is another area which has attracted the attention of several investigators in the past. Since catecholamines are known to activate adenylate cyclase, which catalyzes the formation of cyclic AMP, various laboratories have examined the effect of catecholamines on adenylate cyclase activity of hearts from animals with different levels of thyroid hormones (11,12,13). However, the results on this aspect are inconclusive and conflicting. It was therefore the purpose of this study to gain detailed information in this regard. Since the activation of adenylate cyclase by epinephrine has been demonstrated to require guanine nucleotides, a special attention was paid to the presence of guanine nucleotides while studying the interaction of catecholamines and thyroid hormones. An investigation of adenylate cyclase, a well known membrane bound enzyme, in hearts of hypothyroid and hyperthyroid animals can be seen to complement studies on $Na^+$-$K^+$ ATPase in identifying sarcolemmal changes in myocardium due to a lack or excess of thyroid hormone. Since cyclic AMP has been implicated in opening sarcolemmal channels for the entry of calcium, any changes in adenylate cyclase activity can be conceived to affect the ability of the heart to generate contractle force.

The ability to influence sarcoplasmic calcium concentration is a mechanism by which the sarcolemma may exert control on cardiac contractile state. Calcium binds to the extracellular surface of sarcolemma and it has been suggested that this bound calcium or a portion thereof may be a source of calcium for influx during the cardiac action potential (14,15). The influence of thyroid state on the ability of sarcolemma to bind

calcium has not been examined. The sarcolemma has also been shown to possess properties associated with calcium extrusion. $Ca^{2+}$ stimulated, $Mg^{2+}$ dependent ATPase of sarcolemma has been associated with active $Ca^{2+}$ extrusion from the cells (16). The influence of thyroid state on this enzyme activity and its interaction with calmodulin, a known activator of sarcolemmal $Ca^{2+}$ - $Mg^{2+}$ ATPase and ATP dependent $Ca^{2+}$ binding have not been investigated in cardiac tissue. These properties will also therefore be examined in this study.

It should be pointed out that in addition to sarcolemma, other membrane systems such as mitochondria and sarcoplasmic reticulum are also believed to regulate myocardial contractility. The present study does not in any way undermine the importance of sarcoplasmic reticular and mitochondrial changes in the hearts of hypothyroid and hyperthyroid animals but instead is designed to evaluate and characterize sarcolemmal changes under these conditions. Furthermore, in view of the importance of sarcolemmal $Na^{+}$-$K^{+}$ ATPase, adenylate cyclase, $Ca^{2+}$-$Mg^{2+}$ ATPase and calcium binding activities in heart function, this investigation is limited to the examination of these systems following alteration in thyroid status. The sarcolemmal alterations in these situations were also assessed by monitoring the activities of some other membrane bound enzymes such as $Ca^{2+}$ dependent ATPase, $Mg^{2+}$ dependent ATPase and 5'-nucleotidase.

## II. REVIEW OF THE LITERATURE

### A. Thyroid Hormones: Source and Mode of Action

Thyroid hormones play an important role in growth and development of the young and act on peripheral tissue of the adult to regulate cellular metabolism. The thyroid gland is the principal source of naturally occurring iodinated compounds circulating in the blood; 3,5,3',5'- tetraiodothyronine (thyroxine or $T_4$) and 3,5,3'- triiodothyronine ($T_3$) are the major secretory products of the thyroid gland (18). Synthesis of thyroid hormones occurs at the apical surface of thyroid cells. Iodide, which enters the thyroid cell by an active process, is oxidized by a peroxidase enzyme in the apical microvilli. This oxidized form becomes organically bound to peptide linked thyrosine groups of intrafollicular thyroglobulin to form mono and diiodothyrosine (MIT and DIT respectively). It has been shown in vitro that thyroid peroxidase catalyses this reaction (19). Coupling of two DIT molecules or one MIT and one DIT results in the formation of $T_4$ and $T_3$ respectively.

Secretion of thyroid hormones is under the control of the anterior pituitary hormone, thyrotropin (TSH) (20), which in turn is secreted under the influence of hypothalamic TSH-releasing hormone (TRH) (21). Circulating thyroid hormones exert a negative feedback at the pituitary level (22). Under the influence of TSH, thyroglobulin is ingested and digested by the thyroid cells (3) and iodothyronines and iodothyrosines are released from peptide linkage. $T_3$ and $T_4$ are released from the cell; $T_4$ is present in concentrations 10-20 fold that of $T_3$ (23). Approximately four fifths of extrathyroidal $T_3$ is derived from peripheral monodeiodination of $T_4$ (24). In man, the mean total serum concentration

of $T_4$ in 8.5 µg/dl and of $T_3$ is 0.12 µg/dl. However, the free concentrations are 3.0 and 0.4 ng/dl respectively (25), the remainder being bound to specific serum proteins. Binding of hormones to serum proteins limits urinary loss and acts as a reservoir of readily available hormone which safeguards against sudden changes in free hormone levels. Catabolism of thyroid hormone occurs mainly by peripheral deiodination but approximately 20% is lost in feces (18).

A number of mechanisms have been proposed as the primary mode of action of thyroid hormones.

1. Direct effects on enzymatic activities

Inhibition of some zinc containing dehydrogenases including malic dehydrogenase (26,27) glutamic dehydrogenase (28), liver alcohol dehydrogenase (29) and 15' hydroxyprostaglandin dehydrogenase (30) by thyroid hormones has been demonstrated in vitro. However, carboxypeptidase which is also a zinc containing enzyme was not affected (26). Thus, the effect did not seem to be due to zinc chelation. The high level of hormone required for 50% inhibition ($10^{-6}$ - $10^{-5}$M) suggests that this may not be a physiologically significant mechanism. Incubation of erythrocytes with physiological concentrations of thyroid hormone was found to increase their 2,3- diphosphoglycerate content (31). However, conflicting results were obtained when direct effects of thyroid hormone on diphosphoglycerate mutase were studied (32,33).

Direct in vitro stimulation of cardiac adenylate cyclase by $T_3$ and $T_4$ has been reported by Levey and Epstein (34,35). The effect was not blocked by propranolol and was found to be additive with that of catecholamines. However, the high concentration of hormone required ($10^{-7}$ - $10^{-5}$M) for the effect and the activity of 3,3',5' triiodothyronine (reverse $T_3$) which has relatively low thyromimetic activity, argues

against the physiological relevance of this mechanism. This is supported by the lack of acute effects of thyroid hormone on cardiac contractility (36,37,38,39). Some investigators have failed to reproduce this finding (40). Will-Shahab et al (41) found no direct activation of adenylate cyclase by $T_3$ *in vitro* in the presence or absence of guanylimidodiphosphate (GppNHp). However, after preincubation for 15 minutes with GppNHp, $T_3$ in the concentration range of $10^{-8}$ - $10^{-6}$M increased adenylate cyclase activity. Activation of monkey spermatozoa adenylate cyclase by $T_3$ and $T_4$ has also been reported (42); however, high concentrations ($10^{-6}$ - $10^{-5}$M) were required. D-$T_4$ and other structurally related compounds were inactive.

2. Effects on Plasmalemma

Accumulation of certain amino acids and carbohydrates was increased within minutes of $T_3$ administration. For example, some investigators have reported an increase in the accumulation of deoxyglucose by $T_3$ in cultured chick embryo heart cells (43,44). The first phase of uptake was independent of protein synthesis whereas the second phase (after 6 hours) was dependent on protein synthesis (45). Increased accumulation was associated with increased $V_{max}$ without changes in $K_m$ values (46). Uptake of L-glucose, which enters by simple diffusion, was unaltered by $T_3$ (46). Thryoid hormone has also been demonstrated to increase the accumulation of the non-metabolizable amino acids, α-aminoisobutyrate and cycloleucine, by isolated rat thymocytes (47,48). *In vitro* $T_3$ stimulation of cycloleucine uptake in thymocytes was unaffected by the protein synthesis blocker, puromycin but was potentiated by epinephrine and norepinephrine; this potentiation was blocked by propranolol and practolol (49). Neither catecholamines nor β-blocker alone, in the absence of $T_3$, altered cycloleucine accumulation. The

effect of $T_3$ appeared to be an inhibition of cycloleucine efflux rather than a stimulation of its influx (49). Rat liver plasma membranes have also been demonstrated to possess saturable $T_3$ binding sites (50). However, it is unclear if these represent sites of action on glucose or amino acid transport or are receptors for the carrier mediated transport of the hormone into the cell (51,52).

3. Effects on Mitochondria

Mitochondria from thyrotoxic animals exhibit reduced phosphorylation (53). It has been demonstrated that thyroid hormones can uncouple oxidative phosphorylation *in vitro* (54). However, the concentration required for this effect is greater than that achieved *in vivo* even in hyperthyroid states. Physiological concentrations of $T_3$ *in vivo* increased $O_2$ consumption and phosphorylation without evidence of uncoupling (55). It has been demonstrated that a number of mitochondrial proteins, including α-glycerol phosphate dehydrogenase, are increased by thyroid hormones (56). Thyroid hormones increase amino acid incorporation into mitochondrial proteins (57,58). A specific thyroid hormone binding protein has been reported on the inner mitochondrial membrane and the relative binding affinities of $T_3$ and some of its analogues are directly proportional to their thyromimetic activities (59). However some workers have failed to confirm this finding (60,61).

4. Effects on Protein Synthesis

Inhibitors of protein and RNA synthesis block many of the effects of thyroid hormones. For example, the calorigenic response to $T_4$ was blocked by the administration of puromycin (62). Increases in the amount of specific enzymes and proteins such as carbamyl phosphate synthetase (63) and pituitary growth hormone (64) under the influence

of thyroid hormone have been demonstrated. These data support the theory that regulation of protein synthesis is a primary site of action of thyroid hormone. There is evidence that thyroid hormones act at both transcription and translation levels of protein synthesis. It has been shown that RNA synthesis was increased 24-48 hours after the administration of thyroid hormone in vivo (55). It has also been demonstrated in rat liver that RNA polymerase I activity, which is responsible for the formation of rRNA is increased after 10 hours while RNA polymerase II activity, which is responsible for the formation of mRNA was not increased until 24 hours after in vivo $T_3$ administration (65). Limas (66,67) found only small changes in rat myocardial RNA polymerase I and II activities 72 hours after $T_3$ injection, although RNA synthesis was increased to a much greater extent. However, he found the ratio of engaged to free RNA polymerase of hyperthyroid rats was almost twice that of euthyroid controls. It was suggested that in this model at least, increased RNA polymerase did not play a major role in determining RNA synthesis but it appeared to depend on increased number of transcription initiation sites. This may depend on changes in conformation of chromatin, which has been demonstrated to possess $T_3$ binding ability and has been suggested as a possible physiological receptor for the hormone (68,69). In fact, induction of specific mRNA species coded for individual proteins has been seen and this demonstrates the specificity of hormone action. mRNA coding for $\alpha_{2u}$-globulin was undetectable in the livers of hypothyroid rats but thyroid hormone administration results in its reappearance (70). Incubation of rat pituitary cells in culture with physiological concentrations of $T_3$ resulted in increased concentrations of mRNA specific for growth hormone

but not of the prolactin specific mRNA (18).

Ribosomal preparations from tadpoles treated with $T_4$ showed a higher rate of incorporation of amino-acyl tRNA than did ribosomes of untreated tadpoles (63). $T_3$ administration *in vivo* increased *in vitro* protein synthesis after 2 hours (70a). This effect was too rapid to be accounted for by the transcription related mechanisms. A mitochondrial requirement in this regard was reported, but has been called into question (71,71a).

5. Nuclear binding of $T_3$ and $T_4$

Specific binding of thyroid hormones by cell nuclei has been demonstrated. These binding sites exhibited a greater affinity for $T_3$ than for $T_4$ (68). They have been characterized as being high affinity-low capacity binding sites (68,69). Concentration of nuclear binding sites of different tissues correlated well with relative responsiveness of that tissue to thyroid hormone as judged by altered oxygen consumption (72). However, as the percentage of sites occupied in different tissues did not vary greatly in the euthyroid state (35-50%) (72), it has been suggested that the sites in different tissues have similar binding properties. Decreased affinity of lymphocyte nuclear binding sites has been reported in patients with tissue resistance to thyroid hormone activity (72a). Very good correlation existed between the affinity of binding sites for thyroid hormone analogues and their thyromimetic activity with the exception of triac, an acetic acid analogue of $T_3$ (73,73a). While nuclear binding sites have not been isolated, they have been identified as nonhistone proteins (74). These findings, together with the known effects of thyroid hormone on protein synthesis, suggest that these binding sites are true physiological receptors for

thyroid hormones and that thyroid hormones may act in a manner analogous to that of steroids.

B. Disorders of Thyroid Function

Disorders of thyroid function have been known for some time. Hyperthyroidism or thyrotoxicosis is a condition characterized by increased circulating levels of thyroid hormone. Hypothyroidism or myxedema is a condition resulting from decreased levels of circulating hormone or lack of responsiveness to the hormone. Weight loss in the presence of normal or increased appetite is characteristic of hyperthyroidism. Increased basal metabolic rate and skin temperature are classical symptoms. The patient suffering from hyperthyroidism is often restless and complains of fatigue. Reflexes are hyperkinetic. They are often irritable with a short attention span (75). The hypothyroid patient can be recognized by his/her lack of energy, intolerance to cold, dryness of skin, constipation, hoarseness of voice and reduced appetite (76,77). Paracelsus (1493-1542), a Swiss physiologist first noted the relationship between cretinism and endemic goitre (78). The Chinese scholar, Li Schi-chen recommended the use of ground thyroid gland for the treatment of goitre and cretinism in his text, "The Great Herbal" written between 1552 and 1578 (78). One of the earliest descriptions of myxedema appeared in 1657 written by the Bavarian, Wolfgang Hoefer. In 1820, Coindet recommended the use of iodine for the treatment of endemic goitre (79). Parry (1786), Graves (1835) and Basedow (1840) described the clinical features of hyperthyroidism (80). A specific type of hyperthyroidism bears the name of the latter two - "Graves" in English speaking countries and "Basedow" in continental European countries. In 1884, the Swiss physiologist Monitz Schiff demonstrated

experimental myxedema in dogs after the removal of the thyroid gland (80). This could be prevented by thyroid grafts, injection or ingestion of thyroid extract. Murray (1891) was the first physician to treat myxedema with thyroid extract (78). The isolation of thyroxine by Kendall (1914) made way for modern studies of thyroid function.

C. Thyroid Hormones and the Cardiovascular System

The level of circulating thyroid hormones have pronounced effects on the cardiovascular system in general and the heart in particular. Hyperthyroidism is accompanied by a general increase in cardiac performance as reflected by increased rate, stroke volume, cardiac output, cardiac index and pulse pressure (81,82). Systolic pressure is slightly increased and diastolic pressure decreased (75). Auscultation of large arteries may reveal intense and rough systolic bruit with clicks (75). Cardiac murmurs (usually systolic) are often present (1). ECG recordings reveal increased amplitude of P and T waves (75,83). Premature ventricular contractions and atrial fibrillation are common especially in the elderly (2). Hyperthyroid patients may have impaired functional cardiac reserve (84).

In hypothyroidism cardiac output is reduced as a result of decreased stroke volume and heart rate (76) and cardiac index is also reduced (82). Pericardial effusion is often present and the heart may be dilated without hypertrophy (77). ECG recordings reveal low voltage complexes and sometimes the ST segment may be depressed or the T wave inverted (76). Treatment of pericardial effusion has been reported to improve ECG recordings (76). These symptoms can be reversed with thyroid hormone therapy.

It has been suggested that changes in cardiac function during

alteration in thyroid status are secondary to increased demand for cardiac output due to increased oxygen utilization. However, it was found that increased metabolic rate induced by salicylate or dinitrophenol did not increase cardiac output to the same extent (85,86,87). It has been found that cardiac output increased to a greater extent than did metabolic rate in the hyperthyroid state, thereby causing a drop in arterio-venous oxygen difference (82,88,89). The opposite was true in hypothyroidism (88). It would appear, therefore, that these changes during altered thyroid status were not simply reflex adaptations to the increased metabolic demands of the body. The cardiovascular system itself was changed. In the examination of cardiovascular effects of altered thyroid status, however, it was not always possible to differentiate between direct effects and secondary effects of thyroid hormone (or the lack thereof). This is especially true of the cardiovascular system which can be influenced by numerous factors - neural, hormonal, metabolic - all of which are profoundly influenced by thyroid status. There is evidence of direct effects, as seen in cell culture preparations, but in general the effects described are induced by hormone administration or withdrawal. As all secondary effects can not be controlled under in vivo situations, their influence on the cardiovascular system must be considered.

1. Vascular Effects

In the hyperthyroid state, peripheral resistance was found to be decreased (90,91). However venomotor tone was unaltered (92). Coronary artery damage was not increased by thyrotoxicosis although increased cardiac work sometimes exacerbated angina pectoris (75). However, myocardial infarction in the absence of demonstrable disease of the

major arteries has been reported in thyrotoxic patients (93). Correlation between hypothyroidism and coronary disease has long been a matter of controversy. Severe hypothyroidism was accompanied by hypercholesterolemia in rats (94) and man (95). Although intermediate states of hypothyroidism did not result in significant changes (95,96), there are reports of hypercholesterolemia in patients who were euthyroid but who had thyroid antibodies (97,98).

Cholesterol synthesis from acetate was increased in liver slices after thyroid hormone treatment but synthesis from mevalonate in liver was unaltered. This suggests that thyroid hormone affected liver cholesterol synthesis by increasing conversion of acetate to mevalonate. This was supported by the finding that hepatic β-hydroxy-β methylglutaryl C.A reductase activity, which catalyzes the last step of mevalonate synthesis, was increased in hypophysectomized animals after thyroid hormone administration (100). Unlike the liver, renal conversion of mevalonate to cholesterol in rats was increased by $T_4$ administration in vivo while conversion was decreased in hypothyroidism (101). Elevated cholesterol levels, when they occur in hypothyroidism, were believed to be due to a greater decrease in degradation rate than in rate of synthesis (102,103).

Triglyceride levels are believed to be influenced by thyroid status but the literature contains a number of contradictory findings. O'Hara et al (104) reported hypertriglyceridemia in myxedemic patients while Furman et al (105) found elevated triglyceride levels only in some myxedemic patients. Kutty et al (95) found a graded increase in mean serum triglycerides as thyroid failure progressed, although values did not achieve statistical significance. In cholesterol fed hypothyroid

rats, Redgrave and Snilson (106) found no increase in plasma triglycerides while cholesterol levels were raised 6 fold. Engelken and Eaton (107,108) found that rat plasma triglycerides were reduced in hypothyroidism and increased in hyperthyroidism; cholesterol levels were altered in the opposite direction. Hepatic triglyceride production was decreased in hypothyroid and increased in hyperthyroid animals. Peripheral triglyceride disposal was also reduced in hypothyroid rats but unaltered in hyperthyroid animals.

The question still arises as to whether or not the lipid changes seen are associated with the occurrence of vascular disease. In general the evidence supports increased atherosclerotic tendencies in hypothyroidism. A number of workers reported higher incidences of atherosclerosis in hypothyroid patients (109,110). One group reported increased propensity to develop atherosclerosis in women with subclinical hypothyroidism but not in males with a similar condition (111). Increased incidence of hypertension in both males and females with preclinical hypothyroidism was also reported while incidence of myocardial infarction increased in females but not in males with subclinical hypothyroidism (111). Others have observed relatively low incidence of infarction probably due to decreased cardiac work load (112). Tunbridge et al (113) reported a lack of correlation between hypothyroidism and coronary artery disease in males, although in female patients there was a correlation with "chest pain on effort" but not with ECG changes or past history of heart disease. They also failed to find a correlation between high thyrotropin hormone levels (a marker of hypothyroidism) and high cholesterol or triglyceride levels. High thyrotropin correlated with minor but not major ECG changes. Endo et al (114) have reported no increase in the

incidence of hypertension in female hypothyroid patients except among those in their 60's. Amide et al (82) reported increased mean arterial pressure in both hypothyroid and hyperthyroid patients but diastolic pressure was not significantly altered in either group.

2. Haematological Effects

In a study of 166 hypothyroid patients, approximately one third were found to be anaemic (115). 17 cases responded to iron administration without treatment of hypothyroidism. Pernicious anaemia was present in 13 cases. Horton et al (116) reported a 25% incidence of anaemia in hypothyroid patients but found true iron deficiency to be rare (5%). Pernicious anaemia was found in 8,5%. Two thirds of anaemic patients with normal iron, folate and Vit $B_{12}$ levels had large red blood cells which diminished in size with thyroid hormone treatment. Decreased affinity of haemoglobin for oxygen has been reported in hyperthyroid patients (117) as well as euthyroid man and rats treated with thyroid hormone (117,118). Increased levels of 2, 3 diphosphoglycerate was found in hyperthyroid patients (119) and in red blood cells incubated in vitro with $T_3$ (3). This may be the basis of the shift in oxygen affinity. Snyder and Reddy (120) reported increased 2,3 diphosphoglycerate synthesis in a haemoglobin free preparation of normal erythrocytes when incubated with $T_3$ or $T_4$. Studies on the effect of thyroid hormone on crude preparations of diphosphoglycerate mutase have produced conflicting results (32,33). Hypothyroidism per se has been shown to induce serum enzyme abnormalities in the absence of other known disorders. Creatine phosphokinase, lactate dehydrogenase and glutamine oxaloacetate transaminase levels were elevated (77) and thyroid hormone replacement was found to return creatine phosphokinase levels to normal (121).

3. Cardiac Ultrastructure

Quantitative methods were applied by Page and McCallister to examine ultrastructure changes in the myocardium of female hypothyroid rats given daily thyroid hormone injections for 24 days (8,122). Cell size increased 1.4 fold. While the total volume occupied by myofibrils increased, the volume relative to cell volume was unaltered. However both the total and relative volume of mitochondria increased. The area of cristae membrane per unit of mitochondrial volume also increased. The surface area of the cell to cell volume ratio was unaltered although the T tubular component of the surface area was markedly increased. Total volume occupied by sarcotubular system increased but this volume relative to cell volume was unaltered. In euthyroid female rats, the fractional volume of mitochondria was increased, the fractional volume of myofibrils decreased and mitochondria: myofibril ratio was increased after 9 days of $T_4$ administration (123). There was no change in capillary luminal area to myocyte area ratio (123). In rabbits, alteration in morphology of myofibrils was apparent after 6 days of thyroxine administration (124). In this study it was revealed that the ratio of thick to thin filaments was unchanged but the normal structural arrangement was altered. In the A band the normal hexagonal orientation often degenerated into a haphazard relationship between thick and thin filaments. T tubules were dilated and mitochondria enlarged without any decrease in matrix density. Severe chronic hypothyroidism in dogs was found to result in 2-3 fold thickening of cardiac capillary basement membranes (125). Histological and tracer methods both revealed an increase in cardiac extracellular space in hypothyroidism (9).

4. Hypertrophy and Protein Turnover

In the hypothyroid state, the heart weight: body weight ratio was decreased (122). Increased heart weight: body weight ratio in $T_4$ or $T_3$ treated animals has been well documented (122,126,127). In rats after 2 weeks of daily $T_3$ administration, both right and left ventricles were enlarged but the right ventricle was enlarged to a greater extent (1.4 fold) than the left (1.2 fold) (128,129). Hydroxyproline concentration decreased in this model of hypertrophy (128). As discussed earlier the regulation of protein synthesis is believed to be an important mechanism of thyroid hormone action. Oppenheimer et al (72) reported cardiac nuclear $T_3$ binding capacity to be 0.65 times that of liver but the binding association was the same. After thyroid hormone administration in rats, cardiac RNA and protein contents with respect to wet and dry weight were increased (126) RNA synthesis was increased in cardiac tissue after thyroid hormone treatment (66,67). Limas (66,67) reported significantly enhanced chromatin template activity after thyroid hormone administration. Increased synthesis was related to nonhistone proteins of the nuclear fraction in thyroid hormone treated rats, probably due to increased phosphorylation of these proteins, which is known to increase RNA synthesis. Nuclear protein kinase activity was found to be higher after thyroid hormone treatment. However RNA polymerase, which was reported to be dramatically increased in rat liver, was only modestly increased (15%) in cardiac tissue which would not account for the degree of increase in RNA synthesis.

Pyrimidine nucleotides are substrates for RNA synthesis and act as cofactors in other synthetic processes. Uridine kinase catalyzes the rate limiting step of the major uridine triphosphate (UTP) synthetic

pathway. It was found that UTP content of cardiac and skeletal muscle increased after thyroid hormone administration (130). Uridine kinase activity was also increased and this increase was blocked by a protein synthesis inhibitor, actinomycin D (130). Administration of $T_3$ to adult rats caused a rise in the rate of cardiac adenine nucleotide synthesis within 12 hours which preceded the rise in protein synthesis (131). This effect was attenuated by β blocking agents, which have been shown to reduce de novo synthesis of adenine nucleotides in the normal heart (131).

Cardiac protein turnover (synthesis and degradation) has been studied during the development and regression of thyroid hormone induced hypertrophy (132,133). After 3 days of thyroxine administration to rats, cardiac growth rate was at a maximum and protein synthesis increased by 22% above control whereas, protein degradation decreased by 12%. After 7 days, synthesis was still 8% above the control value but degradation rate had returned to control levels. At two weeks, when hypertrophy was stable, synthesis and degradation rates were not different from those in control animals. After termination of thyroid hormone administration, synthesis decreased significantly; protein degradation also decreased but to a much smaller extent. The finding that $T_3$ increased the rate of protein synthesis and decreased degradation in cultured fetal mouse hearts suggested that these effects are at least in part due to direct action of thyroid hormones and not secondary to neural or non-thyroid hormonal changes (133). Similar findings have been made in skeletal muscle (134). However skeletal, muscle was more prone to the catabolic effects of thyroid hormones so that at high concentrations catabolism exceeded anabolism (13.5), while similar concentrations resulted in continued cardiac growth (136).

Carter et al (136) proposed that degradation rate of specific proteins was altered to different extents. Pulse-labeling studies using $^{14}C$ carboxyl labeled aspartate and glutamate suggested that over an 18 day period, during which $T_3$ was administered daily, degradation in the "sarcoplasmic fraction" (supernatant after 1000 x g centrifugaion) was reduced by 22% while that of the "myofibrillar fraction" (pellet after 1000 x g centrifugation) was unchanged. Examination of the activity of cathepsin D, a lysosomal enzyme, which is a major proteinase in cardiac muscle, revealed a 15% decrease after 3 weeks of daily $T_3$ injections (137). One week after termination of $T_3$ administration, cathepsin D activity had risen to approximately 20% above control (40% above $T_3$ treated). Increase in cathepsin D after termination of $T_3$ administration exceeded the decrease in protein content. Therefore the increase was due to an absolute increase in enzyme activity and not a relative decrease in degradation rate of cathepsin D. During development of cardiac atrophy following hypophysectomy, cathepsin D activity of heart homogenate was elevated (138). One week after $T_4$ (L or D isomer) administration to rabbits, the number of cathepsin-D-positive lysosomes in myocytes was decreased, although in tissue homogenates the activity of cathepsin D but not cathepsin B was increased. The increased activity correlated with an increase in cathepsin D rich interstitial cells (139). Decrease in cardiac protease activity in rats after 10 days of $T_3$ administration was dose dependent and correlated well with the degree of hypertrophy (140). In skeletal muscle, the hyperthyroid state was associated with increased activity of the proteases cathepsin B and D as well as calcium activated neutral proteases (140,141).

Polyamines are involved in many steps of replication and transcription processes and are known to increase protein synthesis in cell free systems (142). Concentration of two polyamines, spermidine and spermine, were shown to rise in rat heart "post-ribosomal supernatant" preparation within 12 to 24 hours after $T_3$ treatment (143). Ornithine decarboxylase, which catalyzes the first and probably the rate limiting step of polyamine synthesis from ornithine, was found to be directly related to thyroid function (144). In neonatal rats, $T_3$ administration resulted in an initial (2 days) stimulation of both high and low affinity forms of the enzyme in the heart but further treatment resulted in a decline in activity which ultimately brought activity to a level below that of euthyroid rats (145). However treatment with 1,3- diaminopropanol prevented polyamine content increase without preventing hypertrophy (146). α-difluoromethyl ornithine, an inhibitor of ornithine decarboxylase attenuated isoproterenol-induced hypertrophy but did not prevent normal age-related growth or $T_3$ induced hypertrophy (147). Therefore although cardiac polyamine levels rise in hyperthyroidism, they do not appear to be primarily responsible for the thyroid hormone induced cardiac hypertrophy.

5. Myocardial Contractility

In hypothyroid humans systolic preejection period (S.P.E.P.) was prolonged and left ventricular ejection period (L.V.E.P.) was reduced (148, 149,150). Prolonged S.P.E.P. in the absence of altered L.V.E.P. has also been reported (82,149). However, when the effects of low stroke volume and slow heart rate were taken into consideration, hypothyroid patients had a longer L.V.E.P. than would be predicted (82,152). Even after such considerations S.P.E.P. was greater than predicted, which reflected lengthened

isovolumic contraction time. Thyroxine treatment reversed these changes and excessive doses of thyroxine further reduced S.P.E.P. (148) so that S.P.E.P. was found to be inversely related to plasma thyroxine levels. In the hyperthyroid state, S.P.E.P. was reduced below normal (82,153) and L.V.E.P. was less than predicted (82). Most workers agreed that S.P.E.P.: L.V.E.P. ratio was increased in hypothyroidism and decreased in hyperthyroidism (148,149, 153, 154). A linear correlation has been found between velocity of circumferential shortening as measured by echocardiography and serum $T_3$ and $T_4$ levels in a study of euthyroid, hyperthyroid and hypothyroid patients (152). In hypothyroid female rats, in vivo ventricular maximum rate of pressure development (dp/dt max) was reduced to 42% of dp/dt max of euthyroid rats (155). Exercise training did not significantly improve dp/dt max in these animals (155). It was also found that dp/dt max was depressed in hypophysectomized rats; thyroid hormones reversed this effect while growth hormone was ineffective (156,157). In sedated hypothyroid dogs with paced hearts, tension-velocity relationship of the left ventricle was displaced downwards and to the left with reduced maximum tension and velocity and prolonged time to peak tension (158). Opposite effects were found in the hyperthyroid state (158). Direct evidence of contractile changes has been obtained from isolated cardiac tissue of a number of species. Force-velocity relationship in guinea pig atria was altered by hyperthyroidism so that maximum velocity was increased but maximum force was unaltered (159). Buccino et al (160) demonstrated augmented velocity of shortening and rate of tension development in papillary muscles of hyperthyroid cats while the duration of the active state was reduced. The opposite occurred in

the hypothyroid state. These changes did not depend on temperature or frequency of stimulation but there were conflicting reports of norepinephrine dependence. Pannier et al (161) reported a loss of augmented maximum velocity after reserpine treatment while Buccino et al (162) observed no attenuation after reserpine treatment. This may relate to the difference in the treatment used since the former employed one large dose while the latter used several small doses. Creatine phosphate and ATP levels were unaltered except for a slight but significant increase in levels of ATP in the hyperthyroid state. Inotropic response to cardiac glycosides and norepinephrine varied inversely with the level of thyroid state but the same degree of maximal isometric tension was achieved under the influence of these drugs regardless of thyroid state (160). Increased maximum velocity of shortening in the hyperthyroid state was confirmed (163,164,165). Maximum load had also been reported to increase after thyroxine administration (163,164). Series elastic component, as measured by quick release method, was unaltered. Myocardial oxygen consumption per $g/mm^2$ developed tension was increased by 50% in hyperthyroidism (163). Other studies have revealed that papillary muscles from hyperthyroid cats utilized 40% more energy than those from euthyroid cats, while performing equal work at the same rate (166). Similarly, papillary muscles from hypothyroid animals utilized only 64% as much energy as those from euthyroid cats while performing 81% as much work (166). In hypophysectomized rats, peak tension developed and rate of tension development by papillary muscles were decreased (167). These changes were reversed by the administration of $T_4$ but not by growth hormone. In hyperthyroid rat papillary muscle (168), a small increase in rate of tension development was observed;

however, maximum tension did not change and time to peak tension was reduced. Further increase in thyroid hormone levels resulted in a decrease in time to peak tension to a greater extent than the increase in rate of tension development so that peak tension dropped. This seems to be a species related effect because in rat, time to peak tension was normally short and any further shortening dramatically curtailed tension development.

Time course of contractile changes after thyroxine administration was studied in guinea pigs (169). One day after $T_4$ administration developed tension and maximum rate of tension development were increased but time to peak tension was unaltered. Differences in developed tension were most obvious at lower frequencies. Time to peak tension was changed only after 8 and 21 days. At 21 days difference in developed tension was only seen at low frequencies but maximum rate of tension development was still seen at all frequencies. Increased ventricular myosin ATPase was seen after 8 and 21 days but not 1 or 3 days of treatment. In studies on isolated guinea pig working hearts, left ventricular dP/dt max was seen after 3 and 5 days of $T_4$ treatment but not earlier, although cardiac output was slightly increased after one day (170). In cat papillary muscle, increased rate of tension development and decreased time to peak tension were both first detected after 3 days of thyroxine administration but active tension development increased only after 7 days (36). However, in a study of contractile changes in rabbit papillary muscle (136), developed tension, rate of tension development and time to peak tension were found to be reduced after 6 days of thyroid hormone administration. 3 or 6 days of additional treatment further reduced these parameters. Myosin ATPase was found to be increased at

6,9 and 12 days; however, ultrastructural examination revealed loss of thick - thin filament arrangement in the A band. These observations are in contrast to the findings of Albert and Müliere (37) who found that a similar dose of $T_4$ resulted in increased velocity of shortening and rate of tension development, although peak tension was reduced to 72% of control after 14 days in rabbits. These workers also found tension development heat per unit tension was 75% greater than in the euthyroid state. These observations have been suggested to indicate faster cycling of crossbridges which could explain, in part, the inverse relationship between myosin ATPase activity and efficiency of tension development.

In left atrial preparations from hyperthyroid guinea pigs, the developed force varied to a lesser extent with variation in frequency, in comparison to preparations from euthyroid animals. At low frequencies the difference between force development in the two states was at a maximum (159) and this difference was diminished with increasing frequency of stimulation. Similar results were obtained in guinea pig papillary muscles (125).

Absence of acute effects of thyroid hormone on contractile force has been reported. Addition of $10^{-6}$ - $10^{-4}$ M $T_3$ or $T_4$ to isolated right ventricular muscle or left atria of cats or guinea pigs for up to 8 hours and to intact dog hearts for up to 45 minutes had no effects on contractile force development (36). Similarly, $T_3$ infused into the sinus node artery of isolated canine atria failed to have any inotropic or chronotropic effects or influence the action of norepinephrine (38). $T_3$ had no effect on the response of isolated sympathectomized cat hearts to norepinephrine (39).

6. Myocardial Metabolism

In addition to decreased efficiency of myocardium from hyperthyroid animals, cardiac basal oxygen consumption was observed to increase (163,166) and the opposite was true in hypothyroidism (166). The combined effects of increased basal demand, decreased efficiency and increased work load could place an overly great demand on myocardial energy supply in hyperthyroidism. Rachwitz et al (172) have reported a $T_4$ induced dose related decline in creatine phosphate levels and an increase in inorganic phosphate levels while ATP, ADP and AMP levels remained unchanged in the heart. Nishiki et al (173) reported unaltered ATP levels in perfused hearts of hyperthyroid and hypothyroid rats; however, ADP and inorganic phosphate (Pi) levels rose and creatine phosphate levels fell in hyperthyroidism. Cardiac creatine phosphate content rose and inorganic phosphate levels fell in the hypothyroid state (173). The changes in phosphorylation potential ([ATP]/[ADP][Pi]) were due mostly to changes in Pi. In a recent review Sestoft (174) suggested that increased energy supply and utilization in hyperthyroid tissue were accompanied by near normal cytosolic concentrations of ATP, ADP and Pi as seen in hyperthyroid dogs (175,176,177) and hypothyroid and hyperthyroid cats (160). In the latter case, however, ATP levels were slightly but significantly increased in hyperthyroid cats (160). Creatine phosphate levels of hyperthyroid dog ventricles were reported to be unaltered (176,177).

In vitro effects of thyroid hormones on energy supply have also been reported. Relatively high concentrations of thyroid hormones ($T_4$ or $T_3$) ($10^{-9}$ - $10^{-5}$ M) stimulated glucose oxidation without altering ATP, ADP and Pi levels in isolated myocytes (178). In vitro addition

of $5 \times 10^{-5}$ M $T_4$ had a protective effect on ATP levels of rat atria exposed to hypoxic challenge (179); this effect was only apparent at the plateau phase of the fall in ATP levels seen after 60 minutes. In established hyperthyroidism, however, the myocardium is more susceptable to hypoxic stress (180). In resting cat papillary muscle, exposure to iodoacetic acid and nitrogen resulted in a greater fall in high energy stores of hyperthyroid preparations than in euthyroid preparations (166); these stores in hypothyroid preparations fell to a lesser extent (166). These differences became more pronounced in beating cardiac muscle preparations (166). Hypoxia caused a relatively greater impairment of contractile force development and relaxation in hyperthyroid papillary muscle preparations as compared to that in euthyroid preparations (180). Although myocardium is faced with a dramatic increase in demand for energy in the hyperthyroid state, it appears that under normal conditions these demands are met with little or no change in high energy stores. Changes in the metabolic processes due to thyroid hormones have been reported which may account for this increased metabolic capacity. As noted earlier, administration of thyroid hormones was associated with increases in mitochondrial volume (8,122,123), mitochondria: myofibril ratio (123) and the area of cristae membrane per unit mitochondrial volume (8,122).

A number of glycolytic enzyme activities have also been shown to increase in cardiac tissue under the influence of thyroid hormones. Addition of thyroid hormone increased glucose utilization (181) and uptake of 2-deoxyglucose by cultured cardiac cells (43,44). In these studies increased uptake was apparent within minutes although Schwartz and Gorden (181a) reported a lag period of 12 hours before the effect was seen. After 24 hours, lactic acid accumulation was increased and

this effect reached a plateau after 72 hours and remained for at least 9 days of continued exposure to $T_3$. When $T_3$ was withdrawn after 3 days of exposure, the increased rate of lactic acid accumulation remained unaltered for at least 5 days (181). Incubation of rat cardiac myocytes with thyroid hormone increased oxidation with a significant decrease in fructose-6-phosphate and a slight increase in fructose-1-6-diphosphate (179). These observations led to the suggestion regarding stimulation of phosphofructokinase activity in hyperthyroidism. In vitro addition of $T_3$ or $T_4$ (approx. $10^{-6}$ g/ml) increased the rate of glucose decarboxylation in isolated guinea pig atria (182). Ali and Baquer (183) reported reduction in type II but not in type I hexokinase enzyme after thyroidectomy in adult rat heart. Depression of hexokinase following hypophysectomy could be reversed with either growth hormone or thyroid hormone replacement (184). Thyroid hormone deficiency resulted in depressed phosphofructokinase levels in neonate and adult rat cardiac and skeletal muscles (184,185,186). This effect was reversed with exogenous thyroxine replacement. However the depression of phosphofructokinase activity after hypophysectomy was not reversed by thyroid hormone alone (184). Others have failed to find phosphofructokinase depression in hypothyroidism (187). Myocardial lactic dehydrogenase was reported to be depressed in hypo - and hyperthyroidism (188) and in hyperthyroidism lactate/pyruvate ratio was increased in rat myocardium (189). Myocardial pyruvate kinase was depressed in hypophysectomized and hypothyroid rats (182). Administration of $T_3$ alone reversed this effect. However hexokinase, phosphofructokinase and pyruvate kinase have been reported to be independent of thyroid state (174).

While uncoupling of oxidative phosphorylation by thyroid hormones has been reported (53,54), the concentration required was thought to be higher than that achieved even under most pathological conditions. Physiological concentrations of $T_3$ (in vivo) increased $O_2$ consumption and phosphorylation without any evidence of uncoupling (55). In dogs, administration of $T_3$ in concentrations which uncoupled hepatic oxidative phosphorylation, did not uncouple cardiac mitochondria (176,177). In a study of euthyroid, hypothyroid and hyperthyroid rats, it was found that thyroid hormones caused an increase in cardiac mitochondrial mass, mitochondrial cytochome content per mg mitochondrial protein and mitochondrial respiratory rate without uncoupling oxidative phosphorylation (173). Thyroxine treatment of cats, guinea pigs and rabbits resulted in an increase in overall activity of the electron transport chain, mitochondrial mass and capacity of mitochondria for oxidative phosphorylation in the heart (190,191,192). $T_4$ administration raised succinate dehydrogenase activity in rat and guinea pig myocardium (193). Levels of cytochrome C and citrate synthetase fell in skeletal and cardiac muscle of neonate and adult thyroid deficient rats (185, 186). Thyroid hormone administration reversed these effects. Thyroid hormone related alterations in mitochondrial glycerol phosphate dehydrogenase, which participates in the glycerol phosphate shuttle system, have been reported. In hyperthyroid and hypothyroid rat hearts mitochondrial glycerol phosphate dehydrogenase activity (per mg mitochondrial protein) was altered (300% and 25% of euthyroid values, respectively) (194,195), although cardiac sarcoplasmic glycerophosphate dehydrogenase activity was independent of thyroid status. Kleitke et al (196) reported increased activity in rat heart mitochondria after thyroid hormone

treatment (in vivo) but not in dogs, guinea pigs, rabbits or mice. Paterson (187) failed to find any depression of glycerol phosphate dehydrogenase activity in whole heart homogenates of hypothyroid rats. In hyperthyroidism it has been found that cardiac lactate utilization was increased without changes in pyruvate utilization and it was proposed that this may be due to enhanced $NAD^+$ availability for lactate oxidation (194). After 5 days of $T_3$ administration to rats, glycerol phosphate dehydrogenase activity was increased 2 fold (197).

While the hyperthyroid myocardium was able to metabolize glucose at a rapid rate, free fatty acids were still the major substrate for the oxidative metabolism (174). In the hyperthyroid state, free fatty acid oxidation was greatly increased in the cat myocardium while glucose oxidation was decreased (198). However glycogen stores of liver, heart and skeletal muscle were reduced in hyperthyroidism (194,198,199). After the beginning of a fasting period, the rate of lipolysis in adipose tissue increased to a greater extent in hyperthyroid animals than in euthyroid controls because of the greater sensitivity of adipose tissue to catecholamines after thyroid hormone treatment (200,201) so that serum free fatty acid levels rose above those of euthyroid controls (202,203). In guinea pig heart, carnitine acyl transferase activity, which catalyzes the rate limiting step of free fatty acid transport across the mitochondrial membrane, was greatly increased by $T_4$ administration (198). In hyperthyroid rabbits it was found that free fatty acid uptake by the heart exceeded the amount utilized, resulting in increased lipid stores (204).

7. Cardiac Myofibrils

Depression of cardiac myofibrillar ATPase activity in thyroidecto-

mized and hypophysectomized rats was reported by Lifschitz and Kagne (205). Thyroid hormone replacement reversed these changes. During the last ten years studies on the effect of thyroid states on myofibrillar function have focused on the role of myosin. Cardiac myosin $Ca^{2+}$ ATPase activity of hyperthyroid guinea pigs was found to be 30% greater than that of euthyroid controls (206). Myosin was also found to be structurally altered with increases in helical content as well as lysine and aspartic acid content but decreases in threonine and serine contents. Many reports have confirmed increased myosin ATPase activity (both $Ca^{2+}$ or $Mg^{2+}$ stimulated) after thyroid hormone treatment (37,124,169,207,208) and decreased activity in hypothyroid states (156,209); $K^{+}$ and $NH_3^{+}$ activated myosin ATPase activities did not change. The degree to which myosin ATPase activity was enhanced by thyroid hormone administration appears to depend upon the level of activity in the euthyroid state: the lower the activity in the euthyroid state, the greater the activation after thyroid hormone administration (210,211). For example, myosin ATPase activity of rabbit myocardium, which is relatively low, was markedly stimulated by thyroid hormone administration (210). Whereas rat heart myosin ATPase, which is relatively high, was altered very little in the hyperthyroid state (126). Thyroxine administration attenuated the reduction of cardiac myosin ATPase activity seen after aortic stenosis (212). Unlike myosin from control hearts, the myosin $Ca^{2+}$ ATPase activity of hyperthyroid animals was not stimulated by sulphydryl modification and displayed some of the characteristics of myosin of euthyroid controls after sulphydryl modification (210,212a,213). However, it has been shown that the SH-thiol group, which is modified, was accessible in the thyrotoxic state (212a).

One dimensional S.D.S. gels of cyanogen bromide peptides of S-carboxymethylated myosin from normal and hyperthyroid rabbits revealed differences in electrophoretic pattern and distribution of radiolabeled cysteine-containing peptides (214). Two dimensional electrophoresis of myosin heavy chain confirmed these changes (215). Peptide map of cardiac myosin heavy chain from euthyroid rabbits resembled the pattern of red skeletal muscle whereas the peptide map of cardiac myosin heavy chain from hyperthyroid rabbits did not resemble the pattern of red or white skeletal muscle myosin or normal cardiac muscle myosin (215). Staining of rabbit ventricular muscle fibers with anti-bovine atrial myosin antibodies (anti-bAm) was shown to be influenced by thyroid status. Ventricular fibres of euthyroid rabbits showed a variable response, while all fibers became strongly stained after $T_4$ treatment but were unstained or poorly stained after propylthiouracil treatment (216). These findings strongly support the suggestion of Thyrum et al (206) that a new myosin species appears in hyperthyroidism. Three rat ventricular myosin isoenzymes ($V_1$,$V_2$,$V_3$) have been differentiated by electrophoresis (217) with different heavy chain structure (218) but unaltered light chain content (217). $V_3$ predominates in hypothyroid rats (217,218) but after thyroxine treatment enzyme distribution shifts towards $V_1$ (218). $T_3$ has been shown to induce $V_1$ synthesis and suppress $V_3$ synthesis in ventricular strips within 6-12 hours (219); actinomycin pretreatment prevented these effects. The non-involvement of light chains was supported by the finding that substitution of light chain 1 from euthyroid animals for light chain 1 of hyperthyroid myosin subfragment 1 did not decrease myosin ATPase activity (220). Similarly light chain 1 from hypothyroid rabbit myosin did not increase ATPase activity

of cardiac myosin subfragment 1 of euthyroid rabbits.

Changes in myofibrillar activity did not appear to be modulated via altered phosphorylation. Covalent phosphate content of troponin I and myosin P light chain were unaltered in hyperthyroidism (221). Maximum activation of myosin ATPase by actin was enhanced 100% in hyperthyroid state and the affinity of actin for myosin was also increased 100% (222). The calcium dependence of myofibrillar ATPase of hyperthyroid rabbits was the same as for euthyroid animals (221). Similarly the calcium sensitivity of actomyosin as measured by superprecipitation was unaltered (223). However the time of onset of superprecipitation after the addition of ATP was shortened by 58% and the rate of the response increased 4 fold when actomyosin of hyperthyroid rabbit heart was compared with that of euthyroid preparation.

A study by Goodkind et al (169) on the time course of changes in guinea pigs revealed that peak tension and maximum rate of tension development increased after 1 and 3 days of $T_4$ treatment while cardiac myosin ATPase changes could not be detected until after 8 days of treatment. Myosin changes correlated best with decreased time to peak tension as far as could be judged by comparing time of onset. Earlier appearance of increased myosin ATPase activity (3 days) has been reported in rabbits (124). In a study of hypothyroid, euthyroid and hyperthyroid rats, changes in myosin ATPase activity correlated well with changes in rate of ventricular pressure development (224). The time course of rabbit cardiac myosin $Ca^{2+}$ ATPase activity enhancement after $T_3$ administration was found to parallel the enhancement of labeled lysine incorporation into myosin (225) suggesting the synthesis of new myosin with increased activity. In vitro incubation of thyroid hormone with myofibrils

did not alter ATPase activity (207).

8. Cardiac Sarcoplasmic Reticulum

The sarcoplasmic reticulum is believed to play an important role in the regulation of sarcoplasmic calcium levels. Calcium is released from this membrane network into the sarcoplasm during excitation and is re-sequestered during relaxation (16). After 24 days of $T_4$ administration to hypothyroid rats, the absolute area of sarcotubular membrane was increased but membrane area per unit cell volume and per unit myofibrillar volume remained unaltered (8,122). Nayler et al (175) found that microsomal preparations from hyperthyroid canine hearts accumulated calcium in the presence of oxalate and exchanged calcium more readily in comparison to preparations from euthyroid dogs. Basal ATPase was lower in hyperthyroidism but $Ca^{2+}$ stimulated ATPase was unaltered. Depression of calcium binding by cardiac microsomes of hypothyroid rat and stimulation of binding and uptake in hyperthyroid rats have also been reported (224). However large doses of desiccated thyroid gland, when fed to dogs, led to decreased calcium content and rate of calcium uptake by the cardiac microsomal fraction (208). In a study of sarcoplasmic reticular activities of hyperthyroid, euthyroid and hypothyroid rabbits, the rate of $Ca^{2+}$ uptake and $Ca^{2+}$ ATPase activity were increased in hyperthyroidism and reduced in hypothyroidism but the capacity to store calcium in the presence of oxalate was unaltered or reduced (226, 227). The Km of calcium transport was not significantly different in any thyroid state (227). Increased calcium uptake and $Ca^{2+}$ ATPase activity of cardiac sarcoplasmic reticulum of hyperthyroid rats has been confirmed by others (228,229,230). It has been suggested that increased calcium transport by sarcoplasmic reticulum may explain the increased rate of

relaxation seen in cardiac tissue of hyperthyroid animals and decreased rate in hypothyroid animals (224).

The steady state level of phosphoprotein intermediate found during ATP hydrolysis by sarcoplasmic reticulum was increased after $T_4$ administration and the calcium $K_a$ for formation of this intermediate was decreased (228); the affinity for ATP and the effects of pH were unaltered. These effects of $T_4$ administration were inhibited by treatment with protein synthesis inhibitors (228). Phospholipid content of cardiac sarcoplasmic reticulum was increased in the hyperthyroid state (244). The amount of rat cardiac sarcoplasmic reticular ATPase protein precipitated with antisera increased 5 days after $T_3$ administration as did the amount of labelled leucine incorporated into the protein (229). These results indicated that enhancement in sarcoplasmic reticulum $Ca^{2+}$ stimulated ATPase activity and calcium accumulation was due to induction of the enzyme synthesis. In hyperthyroid rats, cAMP dependent phosphorylation of cardiac sarcoplasmic reticulum by endogenous protein kinase was enhanced (230). Difference in phosphorylation was not apparent in the presence of excess exogenous protein kinase and this was interpreted to suggest that the level or activity of endogenous protein kinase was increased in the hyperthyroid state. The rate of dephosphorylation was unaltered (230). Phosphorylation of sarcoplasmic reticulum was associated with increased rate of calcium uptake and increased apparent calcium sensitivity (231).

9. Cardiac Sarcolemma

Of all subcellular fractions directly involved in regulation of cardiac contractile function, the roles of sarcolemma are probably most diverse. It provides both the barrier and communication system between

extracellular and intracellular environments and between adjacent cells. As a functional ionic barrier it maintains intracellular ions at their desired levels. This is achieved by a combination of selective permeability properties and ionic transport mechanisms. In the area of electrical activity, the "barrier" and "communication" functions can be seen to compliment and interact with each other. The property of selective and variable ion permeability endows sarcolemma with electrical properties which trigger intracellular events and act as a communication system between cells enabling them to act synchronously (16). Sarcolemma is also a site of action of neural, hormonal and pharmacological agents and may act in the transformation of such extracellular "messages" into a form recognizable intracellularly. Control exerted by sarcolemma on the contractile state generally depends on its ability to influence sarcoplasmic calcium concentration by a variety of methods. The most obvious of these is probably calcium influx across the sarcolemma during the cardiac action potential. This calcium, known as trigger calcium, plays a vital role in excitation contraction coupling (16). Sarcolemma binds calcium in the absence of ATP and it has been suggested that a component of this bound calcium may be a source of "trigger calcium" (14,15).

Unless the cell is to become overloaded with calcium, all the calcium which enters the cell during excitation must be extruded from the cell. As there is a considerable calcium concentration gradient across the sarcolemma favouring calcium entry, energy must be provided for calcium extrusion. Two processes have been suggested to be of primary importance in calcium efflux: active pumping of calcium associated with $Ca^{2+}$ - $Mg^{2+}$ ATPase (16) and $Na^{+}$ - $Ca^{2+}$ exchange (232). Energy derived

from the hydrolysis of ATP is utilized in the active pumping of calcium from the cell whereas the energy for calcium extrusion by $Na^+ - Ca^{2+}$ exchange is provided by the movement of sodium down its concentration gradient. The sodium gradient is maintained by the relative impermeability of the membrane to sodium at rest and by active pumping of $Na^+$ linked to $K^+$ in a process associated with $Na^+ - K^+$ ATPase (16). This process also helps to maintain the transmembrane $K^+$ gradient which is essential for the maintenance of normal resting membrane potential. Inhibition of $Na^+ - K^+$ ATPase activity by ouabain has been suggested to play a role in the inotropic effect of this agent (233). Inhibition of the $Na^+ - K^+$ pump is believed to increase intracellular $Na^+$ levels which in turn would reduce calcium extrusion by $Na^+ - Ca^{2+}$ exchange, thereby resulting in increased intracellular calcium levels. The β adrenergic receptor - adenylate cyclase system of sarcolemma is also believed to play a vital role in the regulation of cardiac contractility. Catecholamines, which bind to the β adrenergic receptor, stimulate adenylate cyclase activity and thereby increase intracellular cAMP levels (234). cAMP dependent protein kinases phosphorylate a variety of intracellular proteins which mediate a variety of intracellular events, which are believed to be responsible for the inotropic effects of catecholamines (234).

Interaction of thyroid hormone and cardiac sarcolemma has been studied at a number of levels. However, in many areas this interaction has not been fully characterized and other areas have been ignored. In a study of cardiac ultrastructural changes seen after 24 days of $T_4$ administration to hypothyroid rats, it was found that surface to

volume ratio of myocardial cells was unaltered by this treatment (8,122). The increased cell volume was compensated for by selective growth of the T-tubular system. Changes in sarcolemmal composition have not been examined.

Thyroid status has been reported to induce electrophysiological changes in cardiac tissues. Certain ECG changes were often seen in hyperthyroidism including increased P and T wave amplitude (75), premature ventricular contractions and atrial fibrillation especially in the elderly (2). Prolonged PR interval or second degree AV block, which disappear after treatment of thyroid disease, have been reported in hyperthyroid patients (235). In hypothyroidism low voltage ECG recording were often seen; S T segments may be depressed and T wave may be inverted (76). Treatment of pericardial effusion which may accompany hypothyroidism often resulted in improved ECG recordings. Incidence of conduction disturbance was approximately three times higher in hypothyroid than euthyroid patients (235). Tachycardia and bradycardia were features of hyperthyroidism and hypothyroidism, respectively. These have been shown to be independent of neural influences (236,237) i.e. the inherent heart rate itself has been shown to be influenced by thyroid status. The automatic rate of atria isolated from hyperthyroid rabbits was faster, and the effective refractory period shorter than in atria of euthyroid animals (238). Addition of $T_4$ to cultured chick embryo cardiac cells increased the inherent automatic rate after 12 hours but not 6 hours (239). After 2 days of thyroid hormone administration to euthyroid or hypothyroid rabbits, the rate of isolated atria was increased (240). Hypothyroid rabbit atria had a slower rate than those of euthyroid rabbits (240,241). Changes

due to thyroid state were accomplished by alterations in diastolic depolarization rate and action potential duration of the sinoatrial node. In hyperthyroidism diastolic depolarization rate was increased and action potential duration was shortened; the opposite occurred in hypothyroidism (240,241). No difference in maximal diastolic potential or threshold potential were seen in these studies, although increased and decreased maximum diastolic potential have been reported in hypothyroidism and hyperthyroidism,respectively (242). Lack of acute effects of thyroid hormone on automatic rhythm when infused into sinus node artery of isolated canine atria (38) or when added to chick embryo heart cells in culture (181) has been reported. However perfusion of isolated rabbit hearts with a high concentration of $T_4$ ($10^{-5}$M) lowered threshold and shortened the refactory period (238). High concentrations ($10^{-6}$-$10^{-5}$ M) of thyroxine have been reported to slow spontaneous pacemaker cells by reducing the slope of diastolic depolarization (243). Wollenberger (244) reported increased automatic rate of chick embryo cardiac cells 10 minutes after addition of high concentrations ($10^{-7}$ M) of thyroid hormone. This effect was blocked by veratramine and pronethalol, which also block the chronotropic effect of catecholamines.

Resting membrane potentials recorded from rabbit atrial muscle cells were not altered in hypothyroid or hyperthyroid states (241,245), although atrial resting membrane potential was reduced in hyperthyroid rats (246). Increased amplitude of atrial action potential was reported in hypothyroid rabbits but not in the hyperthyroid state (241). However, others report that atrial action potential amplitude was independent of thyroid state (245). In both the rat and rabbit atrial preparations, action potential duration was shortened in the hyperthyroid state (241,

245,246) and in rabbit, duration was increased in the hypothyroid state (242,245). These effects were independent of the effects of changes in heart rate on action potential parameters (241). Maximum rate of rise was not altered in hyperthyroid rat atria (246) but increased in both hypothyroid and hyperthyroid rabbit atria according to one report (241) and reduced in hypothyroidism at low frequencies and unaltered at higher frequencies according to a second report (245). It therefore appears that the only consistent finding among these studies is the dependence of action potential duration on thyroid status. Although prolonged P-R interval or second degree block was sometimes found in hyperthyroid patients, A V conduction time and refactory period were shortened in hyperthyroidism and lengthened in hypothyroidism in dogs (247). His bundle electrograms of hyperthyroid dogs revealed shortened A-H interval with normal H-V interval (248). Pacing hearts of euthyroid dogs to the same frequency did not reproduced these changes nor were these reversed by β-blockade (247,248).

Electrical properties of Purkinje fibers from hyperthyroid dogs were altered in a rate dependent fashion (249). At low stimulation frequency (1 Hertz) action potential duration was greater in hyperthyroid than in euthyroid states. Resting membrane potential, overshoot and plateau height were unaltered. As stimulation frequency rose, action potential was shortened to a greater extent in hyperthyroid than in euthyroid preparations so that at 3.1 Hertz no difference was seen. However, at 2 and 3 Hertz action potential overshoot was greater in hyperthyroid than in the euthyroid state. In hyperthyroid guinea pig papillary muscles stimulated at 1 Hertz, the rate of rise of the action potential was increased but this effect was attenuated in the

presence of propranolol. The action potential duration was reduced by approximately 20% in the hyperthyroid state (250). In hyperthyroid dog papillary muscle, action potential duration at 30% repolarization was shorter than in controls (251). Increased repolarization rate resulted in the appearance of a pronounced phase 1 and plateau phase which were not seen in normal ventricular tissue (251). At very low stimulation frequencies, action potential duration in hyperthyroid dogs was, however, longer than normal (251). Stimulation strength required to induce extrasystole in guinea pig hearts was not altered in the hyperthyroid state (252).

$Na^+ - K^+$ ATPase activity of a variety of tissues including heart, kidney, liver, skeletal muscle, jejunal epithelium and red blood cells has been shown to be responsive to thyroid hormones (3,4,5,6,7,253,254, 255). In general $Na^+ - K^+$ ATPase increased in hyperthyroidism and decreased in hypothyroidism. One study of the effects of thyroid hormone administration to hypothyroid rats revealed that the enzyme characteristics were not altered, although the enzyme activity was increased (256). However, a full characterization of thyroid dependent changes in this enzyme in cardiac tissue has not been undertaken and most work has been carried out by employing crude membrane fractions with relatively low $Na^+ - K^+$ ATPase specific activity. Under conditions such as hypothyroidism and hyperthyroidism where dramatic alterations occurred throughout the cell, the relative contribution of sarcolemmal protein to total homogenate protein may be altered and this may give a distorted idea of enzyme activities and the changes which may occur in these activities. In rat renal preparations, the phosphorylated intermediates of $Na^+ - K^+$ ATPase and ouabain binding increased to a similar degree after thyroid

hormone administration (257) which indicated that the number of $Na^+ - K^+$ ATPase units was increased. Lin and Akera (258) reported that ouabain affinity was unaltered in a number of tissues after thyroid hormone treatment but ouabain binding increased in kidney, liver and skeletal muscle, which are known to respond to thyroid hormones with increased $Na^+ - K^+$ ATPase activity, while in brain, which does not respond, ouabain binding was unaltered. Hegyvary (259) found that changes in $Na^+ - K^+$ ATPase activity of cardiac tissue paralleled changes in phosphorylated $Na^+ - K^+$ ATPase at steady state conditions. Banerjee and Sharma (260) failed to find increased ouabain binding in the hyperthyroid state of sympathectomized rats and concluded that induction of $Na^+ - K^+$ ATPase was not a molecular mechanism of thyroid hormone. Unfortunately, $Na^+ - K^+$ ATPase activity was not measured in this study and furthermore, it should be noted that all ouabain binding may not be to $Na^+ - K^+$ ATPase. In a recent study of rat cardiac membranes, two classes of ouabain binding sites were identified but only one of which had an apparent association constant similar to the $1D_{50}$ for $Na^+ - K^+$ ATPase (261). In rats it has been demonstrated that cardiac glycosides have a higher affinity for nervous tissue than for cardiac tissue (262) and in crude homogenates the contribution of nerve endings may be considerable (260).

Thyroid states have been shown to alter digitalis sensitivity of dog hearts in vivo (263). In humans it has been shown that in hyperthyroidism more digitalis is required to induce the same effects as compared to euthyroid patients; the opposite being true in hypothyroidism (264). In vivo studies, however, may be distorted by alterations in digitalis distribution. Hyperthyroid rats given digoxin intra-

venously had a lower serum level of digoxin than hypothyroid or euthyroid rats 2-3 hours after its administration. However in hyperthyroid rats the concentration of digoxin in cardiac tissue was higher than in euthyroid or hypothyroid rats. It did not appear as if these changes were dependent on altered $Na^+ - K^+$ ATPase activity, as serum and cardiac levels in euthyroid and hypothyroid rats did not differ from each other. Shimada and Yazaki (6) found that after thyroid hormone treatment, the concentration of ouabain required for half maximal inhibition of cardiac $Na^+ - K^+$ ATPase activity was increased four fold, although cardiac $Na^+ - K^+$ ATPase activity was not altered. In the same animals (rabbits) renal $Na^+ - K^+$ ATPase activity was increased but ouabain sensitivity was unaltered. In a study of contractile response of isolated rat ventricle strips, it was found that percentage increase in force was unaltered in strips from hyperthyroid animals but was decreased in strips from hypothyroid animals (266). However, Buccino et al reported depressed contractile response in hyperthyroid cat papillary preparations and enhanced response in hypothyroid preparations; such that all preparations achieved a similar maximum tension development regardless of thyroid state (160). Curfman et al (254) demonstrated that ouabain sensitive $Rb^+$ uptake, which is a measure of active monovalent cation transport, was increased 68% in left atria of guinea pigs after thyroid hormone treatment. $Na^+ - K^+$ ATPase activity of atrial homogenate of similarly treated animals was increased by 18% above control. Similar changes were observed in skeletal muscle (254,267).

The role of sodium transport in thyroid thermogenesis has been the subject of controversy for a number of years. Edelman and Ismail-Beigi were the main proponents of the view that the energy cost of

active $Na^+ - K^+$ transport accounts for a significant part of thyroid thermogenesis (268,269,270,271). They proposed that in the euthyroid state, active $Na^+$ transport uses a high proportion of the total energy supply (16 - 40%) and that increase in $O_2$ cost of $Na^+$ transport in kidney and liver due to altered thyroid status accounts for 30-90% of the total extra $O_2$ utilized. Others, however, have found that active $Na^+$ transport contributes only 5-10% of total heat production of skeletal muscle and the altered $Na^+$ transport dependent heat production seen after change in thyroid status, accounted for only 10% of thyroid induced heat production (267). Similarly, Falke and Sestoft (272) concluded that increased energy expenditure for $Na^+$ transport in liver due to thyroid hormone administration may be only a minor part of total thyroid calorigenesis. The contribution of $Na^+ - K^+$ transport to overall energy consumption has not been studied in cardiac tissue.

Total body potassium was found to be decreased in the hyperthyroid state but returned to normal after treatment of hyperthyroidism (273). Increased intracellular potassium levels have been reported after $T_3$ administration to euthyroid or thyroidectomized rats (274) or to hypothyroid rats (10). A concomitant decrease in intracellular sodium was reported in one case (274) but not in the other (10). Both authors attributed these changes to increased $Na^+ - K^+$ pump activity. However the lack of change or indeed increase in intracellular potassium after thyroidectomy (9,274) was ignored, although thyroidectomy is known to induce decreased $Na^+ - K^+$ ATPase activity (253). In both these cases thyroidectomy was used as the model of thyroid hormone deprivation and the accompanying loss of parathyroid glands may have

altered ionic distribution in which case the response to thyroid hormones may also be altered and the results may not reflect the true effects of thyroid hormone. Reports on the effects of thyroid hormone on serum or plasma electrolytes have also been variable. Plasma potassium and calcium levels were reduced after thyroidectomy in rats while sodium and chloride were unaltered (9). While confirming unaltered serum sodium and chloride levels, Ismail-Beigi and Edelman (274) found serum potassium levels to be independent of thyroid status. This may be due in part to leakage of $K^+$ from red blood cells during serum preparation which might mask true serum changes. Nishiki et al (173) reported serum calcium levels to be unaltered by thyroidectomy or $T_4$ treatment. However hypercalcaemia has been reported in thyrotoxic patients (275) which was reversed after treatment of the thyroid condition (276). Plasma magnesium has also been shown to be dependent or thyroid status. Increased plasma levels of magnesium have been found in hypothyroid patients and decreased levels in hyperthyroid patients (277,278,279). Treatment of thyroid disease reversed these changes (278,279). These changes may have been related to increased and decreased rate of urinary excretion of magnesium in hyperthyroidism and hypothyroidism respectively (279). However in thyroidectomized rats, plasma magnesium levels were found to be decreased (9). The discrepancy in results may again be related to lack of parathyroid hormone and calcitonin after surgical removal of thyroid gland, both of which have been reported to influence urinary calcium and magnesium excretion (280).

Interaction of thyroid hormones and the adrenergic system has been suggested as a mechanism by which changes due to altered thyroid states manifest themselves (1,240). However this remains a controversial

issue as is the role of adrenergic receptors and adenylate cyclase in such changes. Intravenous injection of epinephrine raised the heart rate of thyroidectomized cats and rats to a lesser extent than the heart rate of euthyroid animals (281,282). In humans, the cardiovascular system was found to be less responsive to catecholamines after thyroidectomy (283). However another study found increased apparent sensitivity to catecholamines in only one third of hypothyroid patients after thyroxine treatment (284). In acutely denervated dogs, the inotropic and chronotropic effects of catecholamines were not altered by thyroxine pretreatment (285). Similar findings were made by others in hypothyroid and hyperthyroid dogs (286,287). In spinal cats, chronotropic response to catecholamines was reduced after thyroxine treatment with or without reserpine treatment (288). Conflicting results have similarly been reported in isolated tissues. In isolated cat papillary muscle catecholamine responsiveness appeared to be inversely related to the level of circulating thyroid hormone. At maximal catecholamine concentrations, muscles from all three thyroid states reached a common ceiling (160,162). No potentiation of catecholamine inotropic and chronotropic effects on isolated rat or rabbit hearts were seen after thyroxine treatment. Isolated atrial or ventricular strips of hypothyroid rat hearts were not significantly less sensitive to the chronotropic or automaticity producing effects of norepinephrine (289). In general the chronotropic effects of epinephrine appeared to be independent of thyroid state in isolated rat atria (290,291,292). In all cases, both the resting and maximal rates were greatest in hyperthyroid and least in hypothyroid rats but percentage increase was unaltered or slightly reduced as thyroid hormone level increased. However, others have reported greater respon-

siveness of isolated rat hearts with respect to the chronotropic action of catecholamines after thyroid hormone treatment (293,294).

The maximal contractile response of perfused interventricular septa of hyperthyroid rats to isoproterenol was less than that of preparations from euthyroid hearts. However, the maximum rate of force development increased to a greater extent in response to near threshold levels of isoproterenol in hyperthyroid preparations (295). There was no difference in the response of euthyroid and hyperthyroid rat septa to exogenously added dibutryl cAMP which suggests that the enhanced response was at or proximal to cAMP generation (295). In ventricular strips from hyperthyroid rats, the maximal isoproterenol induced increase in force was less than in euthyroid rats; however the concentration required to stimulate half maximally was reduced in the hyperthyroid state (296). The potency of isoproterenol with respect to both its inotropic and chronotropic effects on isolated rat atria from hypothyroid rats was less but maximum responses were similar in euthyroid and hypothyroid states (297,298). In atria from hypothyroid rats, the dose response curves for the positive inotropic effect of isoprenaline and phenylephrine measured in the presence of an α-blocker, yohimbine, were shifted to the right. However in both cases the maximal increase in tension in both absolute and relative terms was greater in the hypothyroid state (12). Hypothyroidism increased the potency and efficiacy of the mixed agonist phenylephrine with respect to its inotropic effects on isolated rat atria (297). Hypothyroidism also increased the efficacy of methoxamine, an α - agonist, with respect to positive inotropic and chronotropic responses (299). In one study positive chronotropic effect could be demonstrated

only in the hypothyroid state (298). Nakashima et al (299) found no alteration in methoxamine potency when examining its inotropic effect on rat atria isolated from euthyroid and hypothyroid rats but Simpson and McNeill (298) found a significant increase in the hypothyroid state.

Basal activity of rat cardiac cAMP dependent protein kinase was unaltered in hypothyroidism but isoproterenol induced increase in activity was not seen. Administration of $T_3$ for 48 hours restored isoproterenol sensitivity (300). Rat cardiac cAMP dependent protein kinase (isozyme I) activity (basal and cAMP stimulated) was increased within 2 hours of thyroid hormone administration but isozyme II was not increased until 2-3 days after hormone administration (301). Direct stimulation of cardiac adenylate cyclase by thyroid hormones has been demonstrated and stimulation by catecholamines was found to be additive (34,35); thyroid induced increase in adenylate cyclase was found to be uninfluenced by reserpine treatment before membrane isolation (302). Others have failed to reproduce these results (40) or found that the membranes must first be incubated with GppNHp (41) for demonstrating the effect. It is unlikely that the direct action on adenylate cyclase is an important mechanism for thyroid hormone effect as the concentrations required were extremely high and thyroid hormone analogues with low thyromimetic activity were found to be almost equipotent stimulators of adenylate cyclase (34). Wollenberger (244) reported increased inherent rate in chick embryo cells 10 minutes after the addition of high concentrations of thyroid hormones. Isoproterenol stimulation of maximal rate of left ventricular pressure development in euthyroid and hypothyroid dogs was potentiated by the infusion of relatively small amounts of thyroid hormone (240). However, in general

the addition of even high concentrations of thyroid hormones to isolated cardiac tissue preparations resulted in neither inotropic nor chronotropic effects (36,38,39) and did not potentiate the effects of exogenous catecholamines (38,39).

Myocardial stores of catecholamines were not altered in dogs or rabbits by $T_4$ administration (175,303,304). However, myocardial uptake of epinephrine, norepinephrine and dopa were increased 24 hours after $T_4$ administration (303) and the rate of thyroxine incorporation into catecholamines was diminished in rats treated with $T_4$ for 10 days (304). Cardiac turnover rate of catecholamines was found to be higher in hypothyroid rats and lower in hyperthyroid rats (305). Cardiac monoamine oxidase (MAO) activity was increased in hyperthyroid rats (306, 307,308,309) and decreased in hypothyroid rats (306,308,309). The relative change in activity produced was greater with serotonin or tyramine than benzylamine as a substrate and it has been suggested that changes in MAO type A were largely responsible for overall changes in specific activity of MAO (306,307,309).

More recently, interest has been focused on the effect of altered thyroid hormone levels on adenylate cyclase activity, its activation by catecholamines and adrenergic receptor density. Altered sensitivity to catecholamines has been demonstrated in a number of non cardiac tissues. Catecholamine responsiveness of rat fat cell and turkey erythrocyte adenylate cyclase was reduced in hypothyroidism (309a,310) but potentiated in liver cells of hypothyroid animals (311). Addition of $T_3$ to the incubation medium of cultured heart cells of newborn rats for a 24 hour period following 24 hours of thyroid hormone deprivation resulted in enhanced epinephrine stimulation of intracellular cAMP accumulation

(312). This effect was dose dependent in the range of $5 \times 10^{-11}$ to $5 \times 10^{-9}$ M $T_3$; basal cAMP levels were unaltered. However McNeill et al (11) found no difference in either basal or epinephrine stimulated adenylate cyclase activities in washed particulate preparations of hyperthyroid and euthyroid rat hearts. cAMP contents in the hyperthyroid rat heart at rest were not different from that of euthyroid rat heart, although catecholamine induced increase in phosphorylase a activity was enhanced in both ventricles and atria of hyperthyroid rats (11,313). In hypothyroid rat atria, neither basal nor maximal isoproterenol stimulated levels of cAMP were different from those of control rats (314). Brodde et al (12) also found that basal and maximal isoproterenol or phenylephrine stimulated levels of cAMP were similar in atria of euthyroid and hypothyroid rats. However submaximal doses of isoproterenol or phenylephrine increased cAMP levels in atria of hypothyroid rats to a lesser extent than in atria of euthyroid rats. Adenylate cyclase activity of homogenates prepared from these atria revealed decreased basal, isoproterenol and fluoride stimulated activity in the hypothyroid state. However, if isoproterenol or fluoride stimulation was examined in terms of percentage increase above basal, they were not significantly different in hypothyroid or euthyroid states. In contrast an examination of adenylate cyclase activity of ventricular homogenates from euthyroid, hyperthyroid and hypothyroid rats revealed a series of very complex changes (13). While basal, maximal epinephrine and maximal fluoride stimulated activity was unaltered in the hyperthyroid state, the dose response curve to epinephrine was shifted to the left. In the hypothyroid state, the dose response curve was shifted to the right. However, the basal activity was markedly increased in hypothyroidism above control ( + 200%) and maximal epinephrine and

fluoride stimulation expressed as percentage stimulation were unaltered. Increased basal activity in hypothyroidism, which was reversed after $T_4$ administration, has also been reported by others (40). Tse et al (296) reported unaltered basal and fluoride stimulated adenylate cyclase of hyperthyroid rat ventricular homogenate; however, adenylate cyclase of homogenates from hyperthyroid rats was stimulated to a greater extent by isoproterenol and the concentration required for half maximal stimulation was less than that required in the euthyroid state. Sharma et al (313a) found GppNHp stimulation of adenylate cyclase activity of rat ventricular homogenate was reduced in PTU induced hypothyroid state but only one concentration of GppNHp was used. On the other hand, Robberecht et al (314a) failed to find any difference in GppNHp concentration over a wide range of concentrations when euthyroid, thyroidectomized and PTU treated rat ventricular homogenates were examined; the maximum glucagon stimulation was somewhat reduced and secretion stimulation was dramatically reduced in hypothyroidism (314a).

Studies on alteration in adrenergic receptor binding capacity and affinity of cardiac tissue due to altered thyroid status have produced many conflicting results. Wollenberger and Will-Shahab (13) reported decreased affinity of noradrenaline binding to rat cardiac membranes in hypothyroidism and increased affinity in hyperthyroidism; receptor number was unaltered in hypo - and hyperthyroidism. Ciaraldi and Marinetti (315) reported that β receptor number increased markedly (200%) but the affinity was unaltered in cardiac membranes of hyperthyroid rats. In hypothyroidism receptor number decreased slightly (30%) and affinity was increased slightly. α-adrenergic receptor number decreased to 50% of control in hyperthyroidism and to less than 20% of control

in hypothyroidism; α-adrenergic receptor affinity increased in hypothyroidism and decreased in hyperthyroidism. In two studies by Williams et al (316,317), it was found that β receptor number increased in hyperthyroidism but affinity for isoproterenol and dihydroalprenolol was unaltered and that α receptor affinity was decreased in hyperthyroidism but neither number nor affinity of binding to α receptors was altered in hypothyroidism. While McConnaughay et al (318) confirmed the increased β receptor number and decreased α receptor number in the hyperthyroid state in rats, they found that α and β receptor numbers were both decreased in hypothyroid rats. Tse et al (296) confirmed increased β receptor number without change in affinity in hyperthyroid rats. It has also been reported that β receptor number was reduced without change in affinity in hypothyroidism (319); however, in hyperthyroidism β receptor number was increased but agonist displacement curves were shifted to the left (319). In female rats, thyroid hormone treatment for 3 days resulted in increased cardiac β receptor number independent of the age (3-24 months) of the animal (320). However thyroid hormone administration for 3 days was found to be ineffective in altering basal, epinephrine stimulated and fluoride stimulated adenylate cyclase of neonatal or adult rats (321). Incubation of rat ventricular slices with $T_4$ for 2 hours increased dihydroalprenalol binding in membrane fractions prepared from these slices. This effect was uninfluenced by protein synthesis blockers; however, cyclohexamide inhibited a "long term" increase in receptor binding seen 15 hours after $T_4$ addition. Exogenous amino acids must be added during the incubation in order to see these effects (322). Incubation of cultured heart cells from newborn rats with $T_3$ for 24 hours after 24 hours of thyroid hormone

deprivation resulted in increased dihydroalprenalol binding (312). This was dose dependent in the range of $5 \times 10^{-11} - 5 \times 10^{-9}$ M $T_3$ and paralleled the enhanced epinephrine induced increase in intracellular cAMP. In general studies on adrenergic receptors do not support the hypothesis of thyroid hormone induced $\alpha - \beta$ receptor interconversion as a basis for altered catecholamine sensitivity. This hypothesis was based on altered chronotropic and inotropic effects of $\alpha$ and $\beta$ adrenergic agonists (323).

From the foregoing discussion it is evident that cardiac contractile activity, ultrastructure and metabolism are under the influence of thyroid status. A great deal of work concerning changes in contractile proteins, sarcoplasmic reticulum and mitochondria in hypothyroid and hyperthyroid animals has been accumulated but relatively little information regarding changes in heart sarcolemma is available in the literature. Although some data on changes in some cardiac enzymes such as $Na^+ - K^+$ ATPase and adenylate cyclase as well as adrenergic receptors are available for hypo- and hyperthyroid animals the results are conflicting. Electrophysiological studies on heart muscle preparations from hypothyroid and hyperthyroid animals also suggest the involvement of sarcolemmal changes in these conditions. This study was therefore undertaken to gain further insight into changes in heart sarcolemma in hypothyroid and hyperthyroid states.

# III. METHODS

## A. Animals

Adult male Sprague-Dawley rats were randomly distributed between euthyroid and hypothyroid groups. The hypothyroid groups received a 0.05% solution of propylthiouracil (P.T.U.) in their drinking water for 6 - 8 weeks while the euthyroid group received only water for the same period of time (324). In order to induce a hyperthyroid condition or reverse hypothyroidism, animals were injected intraperitoneally with saline containing 25 µg thyroxine ($T_4$) per 100 gm body weight or 50 µg triiodothronine ($T_3$) per 100 gm body weight per day. It is pointed out that hypothyroid animals receiving thyroid hormone were maintained on 0.05% P.T.U. during hormone administration. Control animals were injected with the vehicle only. Surgically thyroidectomized animals were purchased from Canadian Breeding Farm and Laboratory, Quebec, Canada and maintained on 1% $CaCl_2$ drinking water. During studies of reversal hypothyroid state, some animals were also injected intraperitoneally with 5 mg puromycin/ 100 gm body weight/ day in order to inhibit protein synthesis (343).

## B. Isolation of Sarcolemmal Fractions

### 1. Hypotonic Shock-LiBr Extraction Method

Sarcolemma isolated by the hypotonic shock-LiBr method was employed for most experiments unless otherwise stated. The isolation method was essentially that described by McNamara et al (325) with a KCl extraction step added (326). Rats were decapitated and their hearts rapidly removed and placed in ice cold 10 mM Tris HCl, 1 mM ethylenediaminetetracetate (E.D.T.A.), pH 7.4. All isolation steps were performed at temperatures between 0°C and 4°C. Atria and connective tissue were removed. The

ventricles were coarsely minced with a scissors and then homogenized in a Waring blender at speed 5 for 2 periods of 30 seconds. The homogenate was filtered through 4 layers of gauze and centrifuged for 10 minutes at 1,000 x g. The supernatant was discarded. There then ensued a series of washing of the pellet in different media in the following order: 10 mM Tris HCl- 1 mM E.D.T.A. (pH 7.4), 10 mM Tris HCl (pH 8.0), 10 mM Tris HCl (pH 7.4), 0.4 M LiBr- 0.4 mM E.D.T.A.- 18 mM Tris HCl (pH 7.4), 10 mM Tris HCl (pH 7.4), 0.6 M KCl- 10 mM Tris HCl (pH 7.4) and 10 mM Tris HCl- 1 mM E.D.T.A. (pH 7.4). At each step the pellet was suspended in 10 volumes (V/W) of the appropriate medium and the suspension stirred for 15 minutes (except for the washing with LiBr where 30 minutes was employed). At the end of each washing step, the suspension was centrifuged at 1,000 x g for 10 min. After the final step the pellet was suspended in 1 mM Tris HCl (pH 7.0) and assays performed on this sarcolemmal suspension. Earlier studies from our laboratory (327) have shown that this preparation is minimally (2 - 3%) contaminated with other subcellular organelles.

In some cases, enzyme activities were assayed at different stages of sarcolemmal isolation. In such studies the term "homogenate" refers to the crude homogenate obtained after filtration before the first centrifugation. "Washed particles" preparation refers to the pellet obtained after washing in 10 mM Tris HCl (pH 7.4) before its suspension in LiBr containing medium.

2. KCl Extraction - Sucrose Density Gradient Method

Sarcolemma was isolated by the KCl extraction - discontinuous sucrose density gradient method as adapted by Nayler et al (328) from the method of St. Louis and Sulakhe (329). This preparation will

be referred to as "sucrose-gradient" sarcolemma. Rats were decapitated and their hearts rapidly removed and placed in ice cold buffer containing 10 mM Tris HCl and 2 mM dithiothreitol (D.T.T.), pH 7.5 (T-D buffer). The ventricles were minced and then homogenized in 8 volumes (V/W) T-D buffer using a Polytron homogenizer at setting 9 for 4 seconds. The homogenate was gently stirred on ice for 10 minutes after KCl had been added to a final concentration of 1.25 M. The homogenate was then centrifuged at 9,000 x g for 10 minutes and the resultant pellet suspended in 10 volumes of T-D buffer containing 1.25 M KCl. After 10 min the suspension was centrifuged for 10 min at 5,000 x g. The pellet was suspended in 8 volumes T-D buffer and then centrifuged for 10 minutes at 4,000 x g. The pellet was suspended in 8 volumes T-D buffer containing 10% sucrose and centrifuged at 4,000 x g for 10 minutes. The pellet was suspended in approximately 3 volumes 10% sucrose in T-D buffer and then layered in a discontinuous sucrose gradient of 7 ml each of 45%, 50%, 52.5%, 55% and 8 ml of 60% sucrose in T-D buffer (pH 8.2). The tubes were then centrifuged by using swing rotor at 40,000 x g for 90 minutes. The fraction banding between 52.5% and 55% sucrose layers was removed, diluted 3 fold in T-D buffer and centrifuged at 11,000 x g for 20 minutes. The pellet was suspended in T-D buffer containing 10% sucrose and used for enzyme assays. Protein concentrations of membrane suspension were obtained using the method described by Lowry et al (330).

C. Enzymatic Activity Measurements

1. Adenosine Triphosphatases (ATPases)

Assays for adenosine triphosphatases (ATPases) of hypotonic shock - LiBr sarcolemmal preparations were performed in a total volume of

1 ml at 37°C pH 7.4. The assay medium for $Ca^{2+}$ dependent ATPase contained 50 mM Tris HCl, 4 mM $CaCl_2$ and 4 mM Tris ATP. The assay medium for $Mg^{2+}$ ATPase contained 50 mM Tris HCl, 4 mM $MgCl_2$, 1 mM EDTA and 4 mM Tris ATP. In both cases a nonspecific ATPase activity (i.e. one assayed in absence of both $Mg^{2+}$ and $Ca^{2+}$) was measured and the value subtracted before calculating the final $Mg^{2+}$ or $Ca^{2+}$ dependent ATPase activities. Total ATPase activity was assayed in a medium containing 100 mM NaCl and 10 mM KCl and all other components as described for $Mg^{2+}$ ATPase. $Na^+ - K^+$ ATPase was calculated by subtracting $Mg^{2+}$ ATPase activity from total ATPase activity. Ouabain sensitive $Na^+ - K^+$ ATPase activity was taken as the $Na^+ - K^+$ ATPase activity inhibited in the presence of 2 mM ouabain.

Total ATPase activity of the sucrose gradient sarcolemmal preparation was assayed at 37°C in a medium containing 2 mM $MgCl_2$, 0.25 mM ethyleneglycol-bis- (β amino ethyl ether) N, N'-tetraacetic acid (EGTA), 0.641 mM $CaCl_2$, 7.5 mM histidine and 2 mM ATP, pH 7.0. This gives a free calcium concentration of 100 μM (331). Basal ATPase activity in this preparation was assayed in the same medium except $CaCl_2$ was omitted. $Ca^{2+}$ stimulated $Mg^{2+}$ dependent ATPase [$(Ca^{2+} - Mg^{2+})$ATPase] was calculated by subtracting basal ATPase activity from total ATPase activity.

After 3 minutes preincubation in the presence of the membrane protein, the reaction was initiated by the addition of ATP. The reaction was terminated after 6[$(Ca^{2+} - Mg^{2+})$ ATPase] or 10 (others) min. by the addition of 1 ml ice cold 12% trichloroacetic acid (T.C.A.). The tubes were then centrifuged at 1,000 x g for 10 minutes and the inorganic phosphate concentration of the supernatant measured by the method of

Taussky and Shorr (332). All these reactions were linear with respect to the time of incubation and membrane protein concentration employed in this study.

2. Potassium Stimulated Phosphatase

Potassium stimulated phosphatase activity was measured by the method described by Lamers et al (333) in a volume of 1 ml at 37°C It was calculated by measuring the activity stimulated by 15 mM KCl in the presence of 100 mM Tris HCl, 4 mM $MgCl_2$, 1 mM EDTA and 3 mM p-nitrophenyl phosphate. The reaction was terminated after 20 min by the addition of 2 ml ice cold, 24% T.C.A. The tubes were centrifuged at 1,000 x g for 10 min and 0.75 ml of supernatant added to 1.5 ml of 0.5 M Tris (base). The concentration of p-nitrophenolate ion was estimated by measuring the extinction at 405 nm Ouabain sensitivity was estimated by measuring the activity in the presence of 2 mM ouabain.

3. 5' Nucleotidase

The method of Avruch and Wallach (334) was employed to measure 5' nucleotidase activity. To 200 µl of assay medium, 50 µl of membrane suspension was added (after 3 min preincubation) to give a final concentration of 50 mM Tris HCl, 2 mM $MgCl_2$, 1.5 mM p-nitrophenyl phosphate and 200 µM $^{14}$C-AMP at pH 8.4. After 10 min incubation the reaction was terminated by the addition of 50 µl 0.25 M $ZnSO_4$ and 50 µl 0.25 M $Ba(OH)_2$. 200 µl $H_2O$ was added and the tubes centrifuged at 1,000 x g for 10 minutes. 200 µl of the supernatant was added to 10 ml scintillation cocktail and the radioactivity measured in a Beckman scintillation counter.

4. Adenylate Cyclase

Adenylate cyclase activity was measured using in essence the method

of Drummond and Duncan (335). The assay medium contained 50 mM Tris maleate, 8 mM caffeine, 5 mM KCl, 15 mM $MgCl_2$, 22 mM creatine phosphate, 75 units/ml creatine kinase, 2 mM cAMP and 0.4 mM $^{14}C$-ATP at pH 7.4 in a total volume of 150 µl. The activity was also assayed in the presence of L epinephrine, guanylimidodiphosphate (GppNHp) or sodium fluoride. The reaction was carried out at 37°C and was terminated by placing the assay tubes in boiling water for 3 min after the addition of 25 µl 15 mM cAMP. The tubes were centrifuged at 1,000 x g for 10 min, 100 µl of supernatant was spotted on chromatographic paper and descending chromatography performed using a 30:70 (V/V) mixture of 1 M ammonium acetate and 95% ethyl alcohol. After 14-18 hr the papers were dried and the cAMP spots identified under ultraviolet light. These were then removed and the $^{14}C$ content measured using the Beckman liquid scintillation counting system.

5. Calcium Binding

Calcium binding properties of the sarcolemma were measured by the Millipore filtration method (336). ATP independent binding was measured by incubating sarcolemma for 5 min at 37°C will 50 mM Tris HCl pH 7.4 and either 0.1 mM or 1.25 mM $^{45}Ca\ Cl_2$. For ATP dependent calcium binding, sarcolemma was incubated for 5 min at 37°C in a medium containing 50 mM Tris HCl (pH 7.4), 5 mM $MgCl_2$, 100 mM KCl, 100 µM $^{45}Ca\ Cl_2$ and 2.5 mM Tris ATP. Reactions were terminated by Millipore filtration and the $^{45}Ca$ concentration of the filtrate measured using liquid scintillation counting.

D. Phospholipid Analysis

Membrane lipids were extracted by the method described by Folch et al (337). 2.5 mg of membrane preparation was centrifuged at 1,000 x g for 10 minutes. The pellet was homogenized in 5 ml chloroform:

methanol (2:1) using a glass/glass potter. This suspension was then transferred to a graded conical glass tube which was then sealed and left overnight at room temperature. Non-lipid contamination was removed by adding 1 ml 0.1N HCl. The mixture was stirred and then centrifuged at low speed. The upper phase was discarded and the lower phase washed three times with synthetic upper phase. To the lower phase, two drops of concentrated ammonia were added which was then evaporated almost to dryness in a stream of nitrogen. The residue was quickly dissolved in 70 µl chloroform:methanol:water (75:25:2) and then spotted on a silica gel plate (Anasil H; 250 µM) which had been activated at 110 °C for 1 hr. Two dimensional thin layer chromatography was then performed according to the method of Pumphrey (338) by using chloroform:methanol: 7N ammonium hydroxide (12:7:1). The plates were then dried and subjected to chromatography with chloroform:methanol:glacial acetic acid:water (80:40:7.4:1.2) at right angles to the first run. The plates were air dried, sprayed with 5% $H_2SO_4$ and heated at 180°C for 15 minutes. The spots were visualized under ultraviolet light, removed and digested in 0.7 ml 70% perchloric acid at 160°C for 2 hr. The inorganic phosphate liberated was measured by the method of Bartlett (339). After the tubes had cooled, 4 ml water, 0.2 ml 5% ammonium molybdate and 0.2 ml of (0.1 M 1 amino-2-naphthol-4-sulfonic acid, 0.79 M sodium bisulphate, 0.04 M sodium sulphate) mixture were added. The samples were placed in boiling water for 25 min and then centrifuged at 1,000 x g for 10 minutes. The absorbance of the supernatant was measured at 830 nm.

E. Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate (S.D.S.) - polyacrylamide gel electrophoresis

was performed on isolated membranes according to the procedure of Weber and Osborn (340). Membrane proteins were precipitated by centrifugation and then dissolved in 0.1 M sodium phosphate buffer which contained 1% S.D.S., 1% mercaptoethanol, 0.015% bromphenol blue and 35% urea. Gel buffer contained 0.78% $NaH_2PO_4$-$H_2O$, 3.8% $NaHPO_4$ -7 $H_2O$ and 0.2% S.D.S. Acrylamide solution contained 22.2% acrylamide and 0.6% methylenebisacrylamide. Ammonium persulphate solution was made by dissolving 100 mg in 15 ml $H_2O$. 15 ml of gel buffer was mixed with 13.5 ml of acrylamide solution. 1.5 ml of ammonium persulfate and 0.045 ml of N,N,N',N'- tetramethylethylenediamine were added. The solution was pipetted into electrophoresis tubes and a few drops of water layered on top. The tubes were allowed to stand for 2 hr in order polymerize. The tubes were placed in the electrophoresis apparatus and the water removed. Protein samples were applied and electrophoresis performed at 50 milliamps for 6 tubes in a Buchler Polyanalyst with the buffer reservoir containing twice diluted gel buffer. Gels were fixed overnight in 40% (V/V) methanol, 7% glacial acetic acid and then stained for 2 hr in 0.25 (W/V) coomassie brilliant blue in 45.5% (V/V) methanol and 9.2% (V/V) glacial acetic acid. Gels were destained in 5% (V/V) methanol, 7.5% (V/V) glacial acetic acid. Gels were scanned for protein at 560 nm in a Pye Unicam absorbance spectrophotometer.

F. Sialic Acid Content

Isolated membranes were assayed for sialic acid using the thiobarbituric acid assay of Warren (341). A 2 ml sample of membrane suspension (1 mg protein/ml) was mixed with 1 ml 0.2 N $H_2SO_4$ and heated for 1 hr at 80°C. The sample was centrifuged at 1,000 x g for 10 min and 0.2 ml

of supernatant added to a tube containing 0.1 ml 0.2 M sodium meta-periodate in 53% (V/V) phosphoric acid. After standing for 20 min, 1 ml of a solution containing 10% sodium arsenite, 0.5 M sodium sulphate and 0.1 N $H_2$ $SO_4$ was added to the tube and mixed well. 3 ml of a 0.6% thiobarbituric acid, 0.5 M sodium sulphate mixture was added and the tubes boiled for 15 min. After cooling, 4 ml cyclohexanone was added and mixed well. The tubes were centrifuged at 1,000 x g for 5 min. The top phase was removed and the absorbance at 549 nm measured in an absorbance spectrophotometer.

G. Myocardial Electrolyte Content

Myocardial levels of sodium, potassium, calcium and magnesium were measured using the methods described by Polimeni (342). $^{35}SO_4$ was employed as an extracellular marker in order to estimate the extracellular space (E.C.S.).

1. Collection of Tissue and Plasma Samples

Under ether anaesthesia, bilateral nephrectomy was performed on the animals and 100 µCi of $^{35}SO_4$ in 200 µl 0.9% saline was injected into the femoral artery. Animals were allowed to equilibrate for periods of from 30 min to 4 hr and then placed under anaesthesia again. The thorax was opened and a blood sample taken in a heparinized syringe from the inferior vena cava. The blood was centrifuged at 1,000 x g for 20 min. The plasma was removed and stored for later analyses. The heart was rapidly excised, the ventricles were opened and blotted dry of surface blood. The tissue was divided into two samples of approximately equal size, one of which was placed in a preweighed polyethylene vial and the other in a preweighed crucible. Both were immediately reweighed and the former was tightly sealed. It should be noted that all polyethylene ware and glass ware employed in this study were washed

in acid and repeatedly rinsed in double distilled, deionized water before use.

2. Estimation of Extracellular Space (E.C.S.)

After weighing, the tissue sample, which was placed in the crucible, was dried overnight in a vacuum oven at 95°C, 20 inches Hg. The tissue was then reweighed in order to obtain its dry weight. The tissue was transferred to and sealed in a polyethylene vial. From these weights the water content per gram dry weight or wet weight was calculated.

5.0 ml 0.1 N $HNO_3$ was added to the tissue sample which had not been dried. This was then placed in an orbital shaker at 2,000 r.p.m. for 48 hr at room temperature. 500 µl of this extract was mixed with 100 µl 10% T.C.A., allowed to stand for 30 min and then centrifuged for 30 min at 2,000 r.p.m. at room temperature. Duplicate 250 µl aliquots of supernatant were placed in 10 ml scintillation cocktail. 100 µl of plasma was mixed with 1.0 ml 10% T.C.A. and 0.9 ml 0.1 N H $NO_3$, allowed to stand for 30 min and centrifuged for 30 min at 2,000 r.p.m. at room temperature. The samples were placed in a scintillation counter (alternating each muscle sample with its appropriate plasma sample) and read using the appropriate settings for $^{35}S$.

E.C.S. was then calculated and expressed as gram of extracellular water per gram muscle water.

$$E.C.S. = \frac{(H_2O)o}{(H_2O)m}$$

$(H_2O)o$ = extracellular water content of sample

$(H_2O)m$ = total water of muscle sample

In full,

$$E.S.C. = \underline{\phi_o \; d_o \; V_p^o \; e^{\lambda_o (tm-tp)}} \; \frac{(R_m - R_b)\; [H_2O]p \; \{Ve + (H_2O)m\}}{(R_p - R_m)\; (H_2O)m}$$

Where $\phi_o$ = correction factor for uptake of $^{35}S$ by blood cells in the myocardial sample = 0.975

$d_o$ = dilution factor of muscle extract and plasma for scintillation counting.

$t(n-p)$ = time between counting of muscle and plasma surface

$V_p$ = volume of plasma samples.

$R_m$ = radioactivity (c.p.m.) in muscle vial

$R_b$ = radioactivity (c.p.m.) in appropriate blank

$[H_2O]p$ = gm water/ ml plasma (0.946 in rat)

Ve = volume of 0.1 N H $NO_3$ used for muscle extraction

$(H_2O)m$ = water content of muscle sample used for extraction

$R_p$ = radioactivity (c.p.m.) in plasma vial

$R_b$ = radioactivity (c.p.m.) in appropriate blank.

3. Analysis of Myocardial Sodium and Potassium Content

After the extraction with 0.1 N $HNO_3$ (as described in G2), 0.1 ml of extract was diluted 50 fold with 15 m Eq Li/1. Plasma samples were diluted 200 fold in the same medium. Muscle and plasma samples were then assayed for sodium and potassium using a Jarrell Ash 1L Model 433 Flame Photometer after calibration with suitable standards (140 mM Na/5mM K diluted 200 fold for plasma samples and 100 mM Na/ 100 mM K diluted 200 fold for muscle samples. A calibration curve was generated using serial dilutions of the latter). Calibration was checked periodically throughout the assay. Plasma concentrations ($[K]_p$, $[Na]_p$) could be

read directly as the flame photometer assumes a 200 fold dilution.

Potassium content and concentration were calculated as follows:

$[K]_p^1 = \frac{[K]_p}{[H_2O)_p}$ when $[K]_p^1$ = plasma potassium concentration in units of plasma water.

Potassium content (m moles) of the muscle sample extracted is $(K)_m = \theta_{Na,K} [Ve + (H_2O)m] r_m^K$, where $\theta_{Na,K}$ = slope of calibration line, $r_m^K$ = instrument reading for muscle potassium.

Muscle content per gm wet weight = $\frac{(K)m}{W_w}$

Muscle content per gm dry weight = $\frac{(K)m}{W_D}$

Where $W_w$ and $W_D$ are the wet and dry weight respectively of the muscle sample extracted. An intracellular concentration $[K]_i$ (m mol K/kg cell water) was also calculated.

$$[K]_i = \frac{(K)m - (H_2O)o\ [K_p]^1}{(H_2O)i}$$

when $(H_2O)o$ and $(H_2O)i$ are the extracellular and intracellular water content of the muscle sample extracted, respectively.

In a similar fashion, muscle content and concentrations of sodium were calculated.

4. Analysis of Myocardial Magnesium Content

50 µl samples of plasma and 0.1 N H $NO_3$ muscle extract (as described in section G2) were diluted 100 fold in 0.1% La $Cl_3$. Assays for magnesium were then performed on a Jarrell Ash 850 atomic absorbance spectrophotometer using the magnesium lamp and the following settings; wave length - 2852 Å; slit size - 5 Å; averaging period - 3 seconds; hollow cathode lamp - 5 m amps.

Before the assay began, instrument settings (nebulizer flow rate, flame and lamp positions) were optimized using 0.5 µg/ml magnesium chloride in 0.1% $LaCl_3$ standard. A calibration curve was then established using a concentration range from 0 to 20.6 n moles magnesium/ml and the samples were then read. Calibration was checked at regular intervals during the assay. Magnesium content and concentration were then calculated. $[Mg]p = D_p \, a_p \, / \, \frac{a_s}{[Mg]_s}$

$[Mg]p$ = plasma magnesium concentration (mM)

$D_p$ = dilution factor

$a_p$ = plasma absorbance reading

$a_s$ = standard absorbance reading

$[Mg]_s$ = standard magnesium concentration

Magnesium content of the muscle sample $(Mg)m = D_m \, [V_e + H_2O)m] \, a_m / \frac{a_s}{[Mg]_s}$

Magnesium content per gm wet weight = $\frac{(Mg)m}{W_w}$

Magnesium content per gm dry weight = $\frac{(Mg)m}{W_D}$

Intracellular magnesium concentration $[Mg]_i$ was also calculated.

$$[Mg]_i = \frac{(Mg)m - (H_2O)_o \, [Mg]_p^1}{(H_2O)_i}$$

5. Analysis of Calcium Content

5.0 ml of a solution containing 0.1% $LaCl_3$, 0.1 M NaCl and 0.03 M T.C.A. was added to the muscle sample which had been dried (as described in Sec. G.2). The vials were then sealed and placed on an orbital shaker at 2,000 r.p.m. for 60 hr at room temperature. 2 ml of extract were transfered to a centrifuge tube and spun at 2,000 r.p.m. for 30 min. 4 fold, 10 fold and 20 fold dilutions of the supernatant were prepared using extraction medium as dilutent. 200 µl of plasma was diluted

50 fold in extraction buffer. Assays for calcium were performed on a Jarrell Ash 850 atomic absorbance spectrophotometer using a calcium lamp and the following settings: wave length - 4227 Å; slit size - 2 Å; averaging period - 3 seconds; hollow callode lamp '10 milliamps. Before beginning the assay, instrument settings (nebulizer flow rate, flame and lamp positions) were optimized using 10 µg/ml calcium standard in 0.1% $LaCl_3$. The instrument was then calibrated using standards ranging from 0 to 248.8 n moles calcium/ml prepared in the extraction medium described earlier. Samples were then read. Calibration was checked at regular intervals. Calcium content and concentration were then calculated:

$$[Ca]_p = D_p \, a_p \, / \, \frac{a_s}{[Ca]_s}$$

$$[Ca]_p^1 = [Ca]_p \, / \, [H_2O]_p$$

Calcium concentration of the muscle sample extracted is $(Ca)_m = \frac{D_m \, a_m \, V_e}{a_s \, / \, [Ca]_s}$

Calcium content per gram wet weight = $\frac{(Ca)m}{W_w}$

Calcium content per gram dry weight = $\frac{(Ca)m}{W_D}$

Intracellular calcium concentration [Ca]i could be calculated, although this represents only intracellular mean concentration and not sarcoplasmic calcium concentration

$$[Ca]_i = \frac{(Ca)m - (H_2O)_o \, [Ca]_p^1}{(H_2O)i}$$

H. Plasma Triiodothyronine($T_3$) and Thyroxine ($T_4$) Levels

Plasma $T_3$ and $T_4$ levels were assayed by radioimmunoassay in the Endocrine and Metabolism unit of the Health Sciences Center, Winnipeg

and by Dr. Eales of the Department of Zoology, University of Manitoba.

L. Statistical Analysis

Statistical analyses of the data was carried out using analysis of variance (ANOVA), and examined for significant difference with Duncan's New Multiple Range Post Hoc Test. Significant difference between two groups was detected using Students t-test. A p level of 0.05 was employed to determine significant differences.

# IV. RESULTS

## A. Ventricular Weight and Hypothyroid Status:

Successful induction of hypothyroidism with the use of propylthiouracil (P.T.U.) is indicated by reduced plasma levels of $T_4$ (Table 1). It is also supported by the characteristic reduction of body growth and decreased ventricular weight and ventricular weight: body weight ratio (Table 1). A ventricular weight: body weight ratio of 0.198 ± 0.007 (ventricular weight as a percentage of body weight) was found in thyroidectomized animals which is very similar to the value for P.T.U. induced hypothyroidism. Treatment of hypothyroid animals with $T_3$ for 2 days increased ventricular: body weight ratio (%) to 0.25 ± 0.01 which is very close to the euthyroid value. After 7 days of $T_3$ treatment of hypothyroid and euthyroid animals ventricular weight: heart weight ratios (%) of 0.350 ± 0.013 and 0.355 ± 0.012 were observed respectively. No difference in sarcolemmal yields from euthyroid, hypothyroid and hyperthyroid rat hearts was noted in this study.

## B. Sarcolemmal $Na^+$-$K^+$ ATPase

Rat heart $Na^+$-$K^+$ ATPase activity was reduced in the hypothyroid state, $Mg^{2+}$ ATPase activity was not altered (Table 1). This indicates that the sarcolemmal changes in $Na^+$-$K^+$ ATPase may be specific and do not reflect generalized depression of sarcolemmal parameters. However, sarcolemmal $Na^+$-$K^+$ ATPase was not increased above euthyroid levels in hyperthyroidism (17.42 ± 1.77 μmoles Pi/mg prot/hour). This disagrees with the finding in guinea pigs (254) but is in agreement with the finding in rabbits (3). Because of this it was decided to concentrate on $Na^+$-$K^+$ ATPase activity in the hypothyroid state. $Na^+$-$K^+$ ATPase activity of sarcolemma from euthyroid and hypothyroid rat hearts was studied by varying the ATP concentration in the incubation medium.

Table 1. Plasma thyroxine ($T_4$) levels, ventricular weight: body weight ratios, ventricular sarcolemmal yields and ATPase activities.

| | Euthyroid | Hypothyroid |
|---|---|---|
| $T_4$ (μg/dl) | 4.10 ± 0.38 | 0.13 ± 0.04* |
| Body wt (gm) | 388 ± 7.3 | 277 ± 7.8* |
| Ventricular Wt (gm) | 0.93 ± 0.05 | 0.56 ± 0.03* |
| $\frac{\text{Ventricular Wt}}{\text{Body Wt}}$ % | 0.24 ± 0.01 | 0.20 ± 0.01* |
| Sarcolemmal yield (mg prot/gm ventricular wt) | 3.60 ± 0.17 | 3.67 ± 0.16 |
| Sarcolemmal $Mg^{2+}$ ATPase (μ moles Pi/mg prot/hr) | 48.4 ± 1.90 | 44.6 ± 1.45 |
| Sarcolemmal $Na^{+}$-$K^{+}$ ATPase (μ moles Pi/mg prot/hr) | 17.4 ± 0.57 | 14.5 ± 0.47* |

Values are means ± S.E. of 4 - 8 experiments.

* $p < 0.05$

Lineweaver-Burk analysis of the data revealed alteration in both $K_m$ and $V_{max}$ values (Fig. 1). $V_{max}$ was reduced from 27 to 16 µmoles/ mg prot/ hour and $K_m$ reduced from 1.34 to 0.64 mM ATP in the hypothyroid state. $K_a$ values for $Na^+$ and $K^+$ were obtained by varying $Na^+$ and $K^+$ concentrations respectively in the incubation medium and subjecting the results to Lineweaver-Burk analysis. Although $V_{max}$ value under these conditions was also decreased in hypothyroidism, no alteration in activation by $Na^+$ or $K^+$ was seen, as revealed by unaltered $K_{aNa^+}$ and $K_{aK^+}$ (Table 2). The $Na^+$-$K^+$ ATPase activity was also measured in media buffered at different pH values ranging from 6.6 to 7.8. In both hypothyroid and euthyroid states maximum activation occurred at pH 7.4 (Fig. 2). A decrease in pH values below 7.4 resulted in a steep drop in the activity. Increasing pH by 0.4 units above 7.4 also decreased activity but to a lesser extent. When the activity was calculated as a percentage of maximum (Fig. 2), the plots were virtually superimposable for euthyroid and hypothyroid hearts.

The inhibitory effect of ouabain on $Na^+$-$K^+$ ATPase was unaltered by the induction of hypothyroidism (Fig. 3). No significant difference existed between the enzyme activity (expressed as percent of maximum) of sarcolemma isolated from the hearts of hypothyroid and euthyroid rats. This is not as would be expected from the findings of Shimada and Yazaki (6) who have reported a four fold increase in the concentration of ouabain required for half maximal inhibition of rabbit cardiac $Na^+$-$K^+$ ATPase after the administration of thyroxine. The $Na^+$-$K^+$ ATPase activity was also measured in the presence of different concentrations of calcium, which is a known inhibitor of $Na^+$-$K^+$ ATPase and it is believed that inhibition by calcium is dependent upon sodium and potassium components of $Na^+$-$K^+$ ATPase (343). Calcium was found to depress

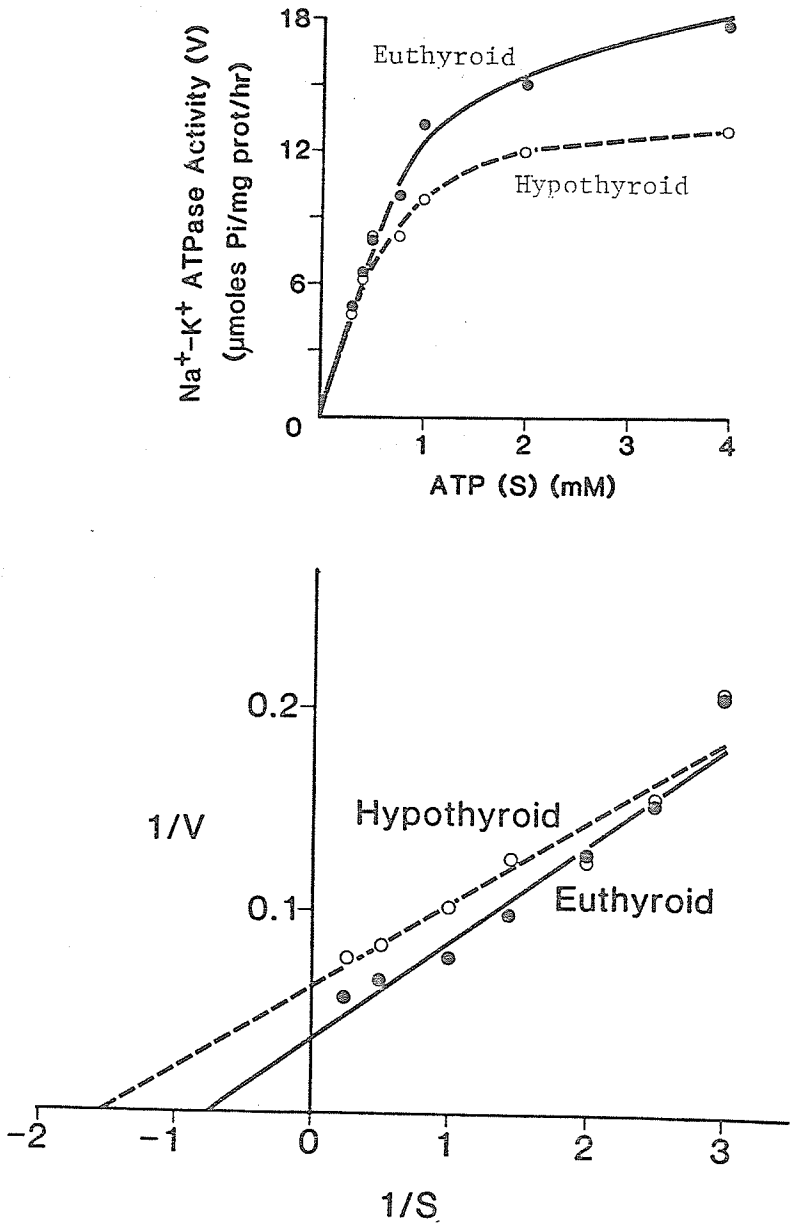


FIGURE 1. Heart sarcolemmal $Na^+ - K^+$ ATPase activities from euthyroid and hypothyroid rats at different concentrations of ATP. Values are means of 5 to 6 experiments.

Table 2. Kinetics of $Na^+ - K^+$ ATPase activity of euthyroid and hypothyroid rat heart sarcolemma studied by altering the concentration of $Na^+$ or $K^+$ in the incubation medium.

| | Euthyroid | Hypothyroid |
|---|---|---|
| $K_{a\ Na^+}$ (mM) | 12.9 ± 4.1 | 12.6 ± 2.8 |
| $K_{a\ K^+}$ (mM) | 1.26 ± 0.27 | 1.56 ± 0.17 |
| $V_{max}$ (μ moles Pi/mg prot/hr) | 25.3 ± 2.5 | 15.8 ± 1.65 * |

Values are means ± S.E. of 3-4 experiments.

* $p < 0.05$

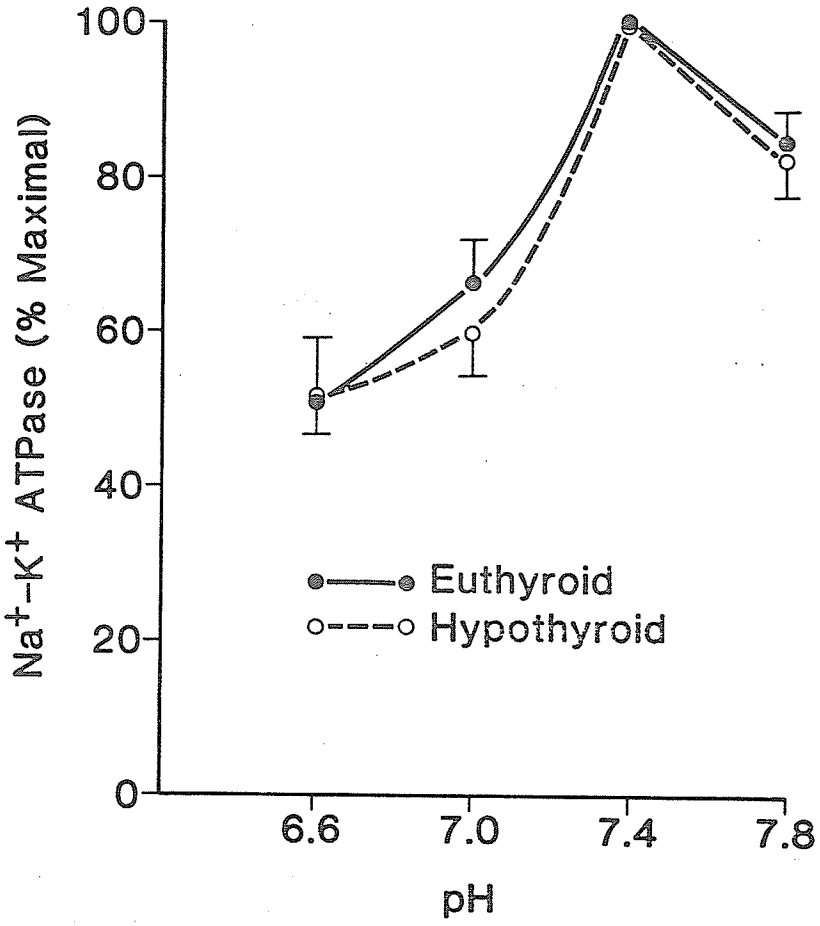


FIGURE 2. Effect of varying pH on sarcolemmal $Na^+$ - $K^+$ ATPase from euthyroid and hypothyroid rat hearts. Values are means ± S.E. of 6 experiments. No statistically significant difference ($p < 0.05$) exists between the two groups at any pH examined.

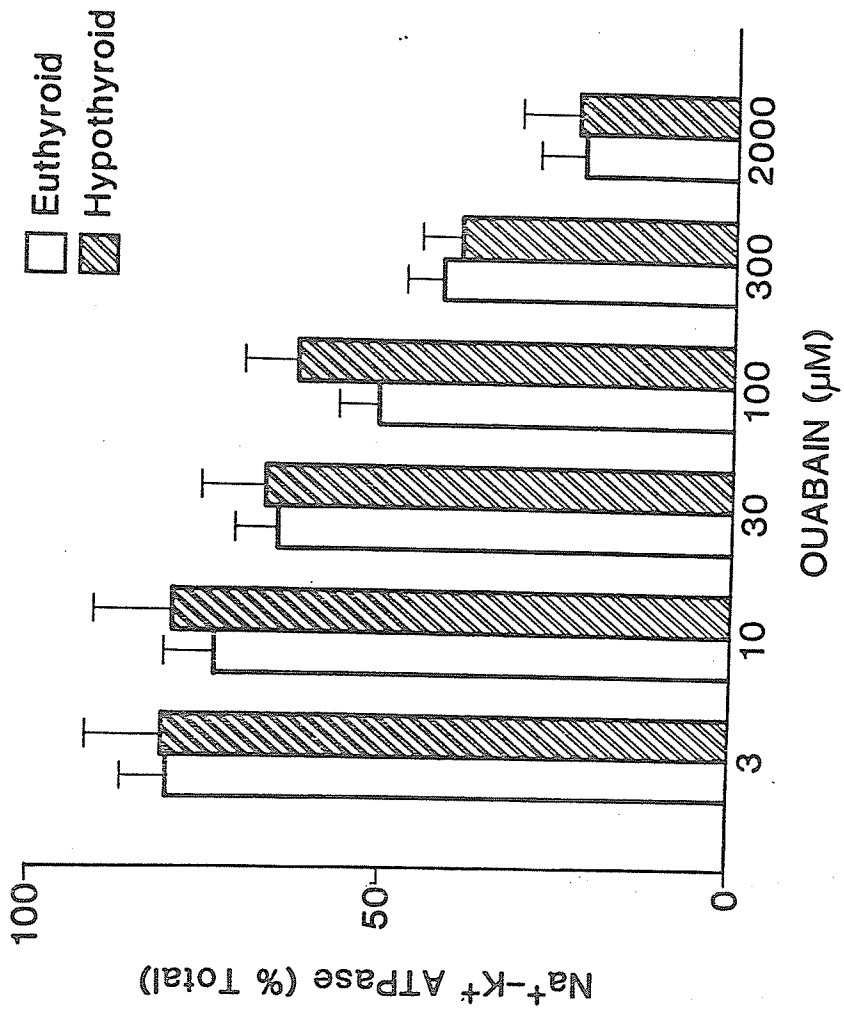


FIGURE 3. Sarcolemmal $Na^+ - K^+$ ATPase activity from euthyroid and hypothyroid rat hearts in the presence of varying concentrations of ouabain. Values are expressed as percentage of $Na^+ - K^+$ ATPase activity in the absence of ouabain and are means ± S.E. of 4 - 6 experiments. No statistically significant difference ($p < 0.05$) exists between the two groups at any concentration of ouabain.

sarcolemmal $Na^+$-$K^+$ ATPase activities in both euthyroid and hypothyroid states (Fig. 4). While inhibition in the hypothyroid state tended to be slightly greater than in the euthyroid state, the difference was not statistically significant at any concentration of calcium employed in this study.

$K^+$ stimulated phosphatase activity was measured in sarcolemmal preparations from euthyroid and hypothyroid rats. This activity has been suggested by some investigators to be related to the dephosphorylation step of $Na^+$-$K^+$ ATPase activity (344, 345) whereas others have concluded that these two activities are different entities (346, 347). As shown in Fig. 5, $K^+$ stimulated phosphatase was significantly decreased in hypothyroidism to a very similar degree as $Na^+$-$K^+$ ATPase. Ouabain sensitive potassium stimulated phosphatase was also significantly depressed in hypothyroid rat hearts (Fig. 5) but the percentage of inhibition by ouabain was similar in both cases (32.5 ± 0.5% and 29.5 ± 1.25% for euthyroid and hypothyroid conditions, respectively). Ouabain sensitive $Na^+$-$K^+$ ATPase was also measured at different stages of sarcolemmal isolation (Table 3). While differences did not achieve statistical significance except in the sarcolemmal preparation, a trend can definitely be seen with values of ouabain sensitive activity of hypothyroid preparations being consistently less than those of euthyroid preparations.

Thyroid state has been shown to be intimately involved in lipid metabolism (95,104,105,106,107,108) and such changes may account for the observed alteration in $Na^+$-$K^+$ ATPase activity. Limas (228) found increased phospholipid content of sarcoplasmic reticulum of hyperthyroid dogs. It was therefore decided to examine the phospholipid content of sarcolemma in the hypothyroid and euthyroid states. The

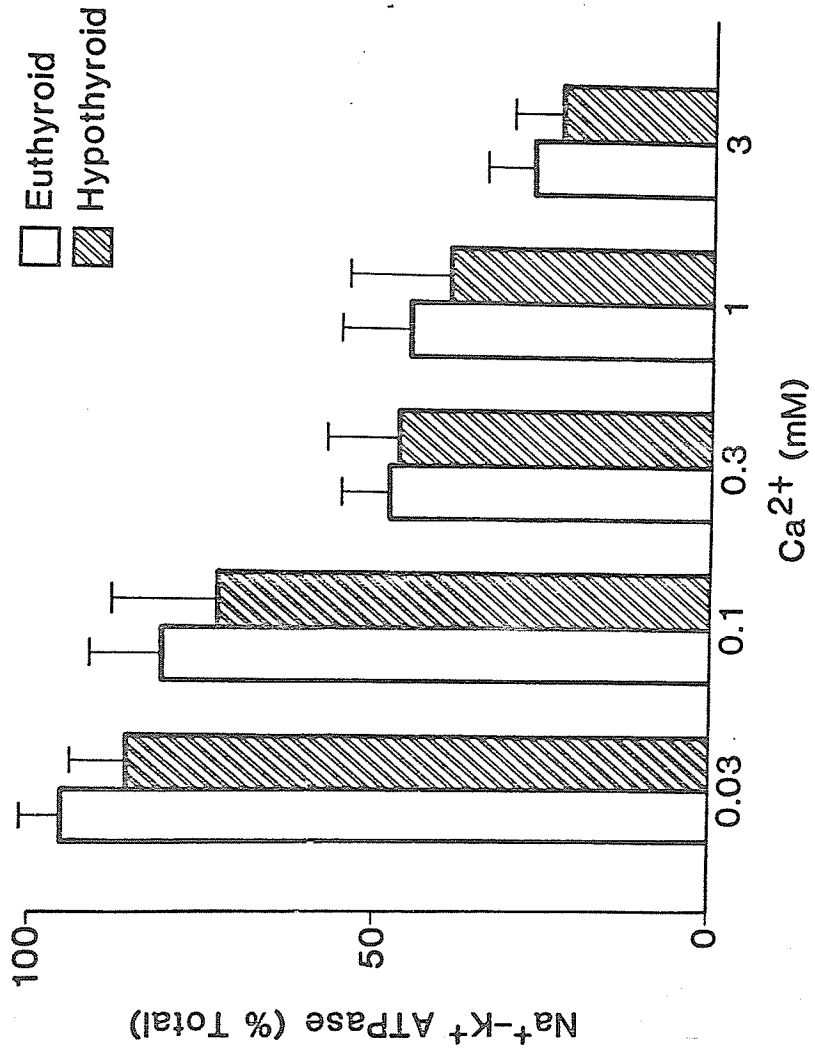


FIGURE 4. Effect of different concentrations of calcium on sarcolemmal $Na^+$ - $K^+$ ATPase activity from euthyroid and hypothyroid rat hearts. Values are expressed as percentage of $Na^+$ - $K^+$ ATPase activity in the absence of ouabain and are means ± S.E. of 4 - 6 experiments. No statistically significant difference ($p < 0.05$) exists between the two groups of concentrations of calcium.

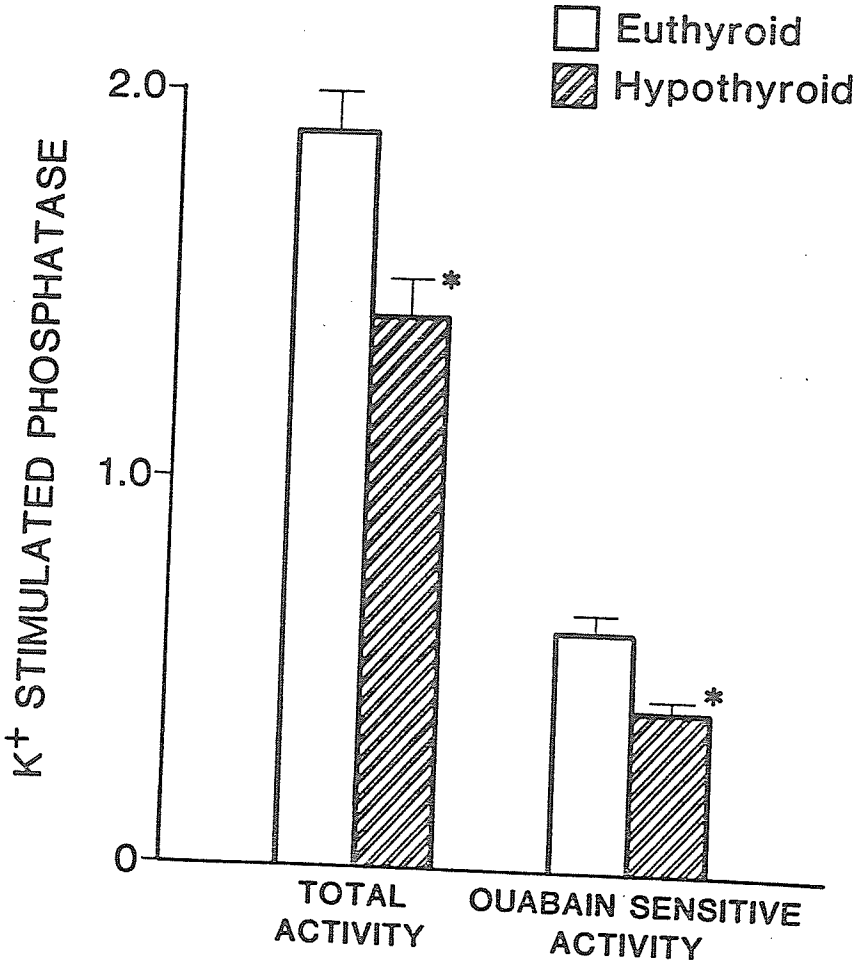


FIGURE 5. Total and ouabain sensitive sarcolemmal potassium stimulated phosphatase activities from euthyroid and hypothyroid rat hearts. Activities are expressed in μmol phenolate/mg prot/hr and values are means ± S.E. of 10 experiments, * $p < 0.05$.

Table 3. Ouabain sensitive $Na^+ - K^+$ ATPase activities at different stages of sarcolemmal isolation from euthyroid and hypothyroid rat hearts.

| | Euthyroid | Hypothyroid |
|---|---|---|
| Homogenate | 2.37 ± 0.49 | 1.62 ± 0.19 |
| Washed Particle | 3.37 ± 0.51 | 2.95 ± 0.33 |
| After LiBr Treatment | 9.06 ± 0.91 | 7.11 ± 0.67 |
| Sarcolemma | 13.93 ± 0.99 | 10.47 ± 0.80* |

Values are given as means ± S.E. Activities are given in units of μ mole Pi/mg prot/ hr.

* $p < 0.05$

major phospholipid components of sarcolemma isolated from euthyroid and hypothyroid rat ventricles are given in Table 4. No change in phospholipid composition was detected. The SDS gel electrophoretic profiles of sarcolemma from euthyroid and hypothryoid rats are shown in Fig. 6. The profiles are qualitatively similar although certain subtle changes can be seen. These findings suggest that changes seen in sarcolemmal $Na^+$-$K^+$ ATPase activities are not due to major contamination by other subcellular fractions. In order to further establish that sarcolemmal $Na^+$-$K^+$ ATPase activity is in fact depressed in hypothyroidism, $Na^+$-$K^+$ ATPase was measured in another sarcolemmal preparation obtained by the sucrose gradient method. As shown in Table 5, a depression in $Na^+$-$K^+$ ATPase was observed in the hypothyroid state while $Mg^{2+}$ ATPase was unaltered.

In order to verify the effect of hypothyroidism on $Na^+$-$K^+$ ATPase and rule out other non-thyroid mediated effects of P.T.U., a second model of hypothyroidism, thyroidectomy, was studied. $Na^+$-$K^+$ ATPase of sarcolemma isolated from thyroidectomized rat heart was significantly reduced compared to euthyroid controls (Table 6) and the depression was very similar to that found in P.T.U. treated rats. As in P.T.U. treated animals, $Mg^{2+}$ ATPase activity and percentage inhibition of $Na^+$-$K^+$ ATPase by ouabain were unaltered in the sarcolemma from thyroidectomized rat hearts (Table 6).

Sarcolemmal $Na^+$-$K^+$ ATPase activity was examined after $T_3$ administration to P.T.U. treated animals. 24 hours after $T_3$ administration no change in activity was detected but after 2 days of $T_3$ treatment activity was significantly increased. The effect of a protein synthesis inhibitor, puromycin, was examined on this $T_3$ induced increase in the enzyme activity. Sarcolemmal $Na^+$-$K^+$ ATPase activity was measured after

Table 4. Phospholipid composition of sarcolemma isolated from euthyroid and hypothyroid rat hearts.

| | Euthyroid | Hypothyroid |
|---|---|---|
| Total Phospholipid | 224.7 ± 24.2 | 208.9 ± 18.5 |
| Sphingomyelin | 18.46 ± 2.78 | 20.84 ± 2.36 |
| Phosphatidyl serine | 17.69 ± 3.62 | 21.87 ± 2.61 |
| Phosphatidyl inositol | 3.40 ± 0.05 | 3.96 ± 0.93 |
| Phosphatidyl choline | 64.87 ± 4.75 | 61.79 ± 3.27 |
| Phosphatidyl ethanolamine | 67.56 ± 6.24 | 62.66 ± 2.28 |
| Diphosphatidyl glycerol | 1.98 ± 1.4 | 1.70 ± 0.86 |
| Phosphatidyl glycerol | 14.61 ± 2.81 | 12.93 ± 0.65 |

Values are means ± S.E. in units of n moles/mg protein

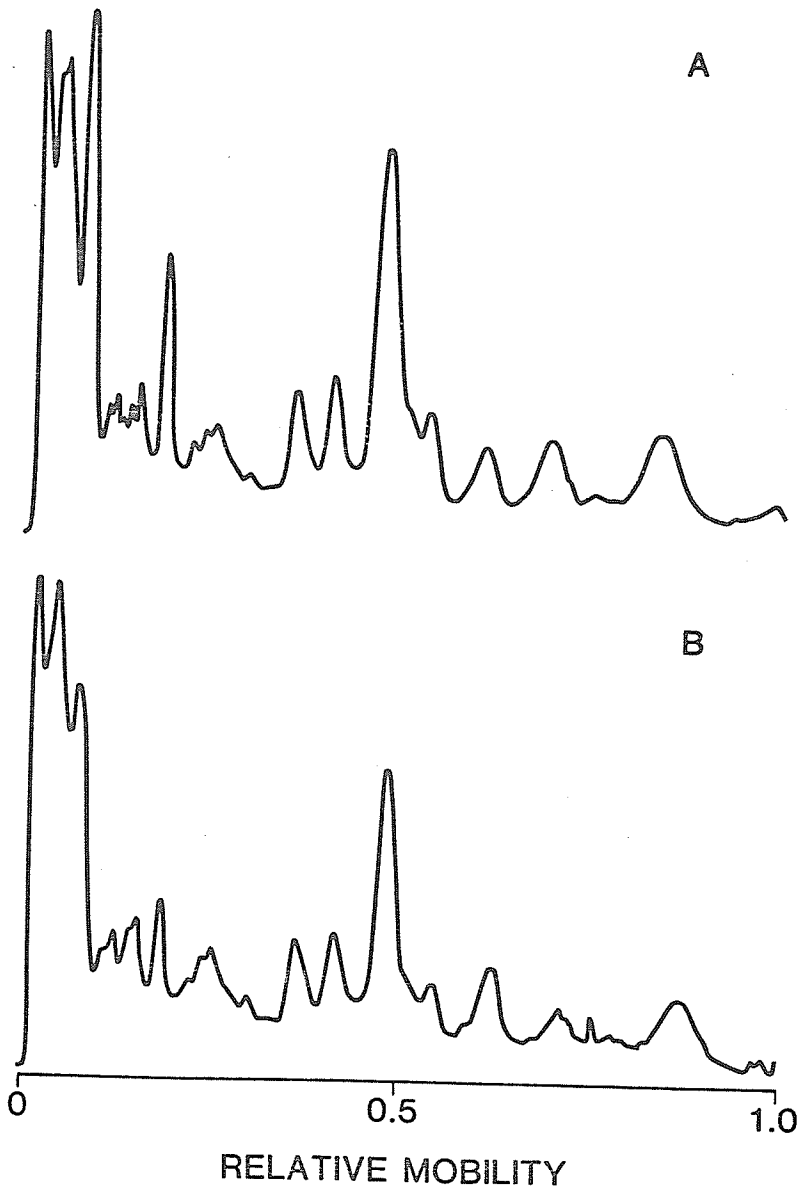


FIGURE 6. Densitometric scans of sarcolemmal protein bands separated by S.D.S. gel electrophoresis. A, Sarcolemma from euthyroid rat heart. B. Sarcolemma from hypothyroid rat heart.

Table 5. $Mg^{2+}$ ATPase and $Na^{+} - K^{+}$ ATPase activities of euthyroid and hypothyroid rat heart sarcolemma isolated by sucrose density gradient method.

| | Euthyroid | Hypothyroid |
|---|---|---|
| $Mg^{2+}$ ATPase | 36.5 ± 0.67 | 36.8 ± 2.13 |
| $Na^{+} - K^{+}$ ATPase | 9.9 ± 0.81 | 5.4 ± 0.61* |

Values are means ± S.E. of 5 - 8 experiments. Activities are in μ moles Pi/mg protein/ hr

* $p < 0.05$

Table 6. Effect of thyroidectomy on plasma thyroxine ($T_4$) levels and $Mg^{2+}$ ATPase and $Na^+ - K^+$ ATPase activities in rat heart sarcolemma

| | Euthyroid | Hypothyroid |
|---|---|---|
| $T_4$ levels (µg/dl) | 4.64 ± 0.34 | 1.18 ± 0.18 * |
| $Mg^{2+}$ ATPase (µ moles Pi/mg prot/hr) | 39.6 ± 2.68 | 33.9 ± 2.81 |
| $Na^+ - K^+$ ATPase (µ moles Pi/mg prot/hr) | 17.96 ± 1.27 | 13.28 ± 1.21 * |
| Ouabain sensitive $Na^+ - K^+$ ATPase ( % of control) | 70 ± 5.3 | 68 ± 5.3 |

Values are means ± S.E. of 5 - 11 experiments

* $p < 0.05$

4 different treatment regimens; Group a- P.T.U. + 2 x daily saline injection; Group b - P.T.U. + 2 x daily puromycin injection, Group c - P.T.U. + 2 x daily $T_3$ injection, and Group d - P.T.U. + 2 x daily $T_3$ + puromycin injections. Activity was measured 48 hours after first injection of $T_3$ and the results are shown in Fig 7. Puromycin alone resulted in an insignificant depression of $Na^+$-$K^+$ ATPase activity. $T_3$, when administered alone, resulted in a significant increase in activity but when in conjunction with puromycin, no stimulation was seen. It was found that activity in group c was significantly ($p < 0.05$) different from that of group a, b and d but no significant difference existed between groups a, b and d. Addition of $T_3$ in vitro during incubation of membranes from P.T.U. treated animals did not significantly change $Na^+$-$K^+$ ATPase activity.

C. Myocardial Cation Contents

In order to better understand how alterations in $Na^+$-$K^+$ ATPase activity are related to functional alterations in terms of the ability of membrane to maintain electrolyte gradients across the sarcolemma, electrolyte contents of the myocardium were studied. Five groups of animals were examined: a) Euthyroid, b) Hyperthyroid (7 days $T_3$ treatment), c) Hypothyroid (P.T.U. treated), d) Hypothyroid (P.T.U.) + 2 days $T_3$ treatment, and e) Hypothyroid (P.T.U.) + 7 days $T_3$ treatment. All animals were initially weight matched and maintained for the same period before being sacrificed. For the last 7 days, all animals received daily intraperitioneal injections ($T_3$ or saline as appropriate)- the last injection was administered 24 hours before removing the heart.

From the data in Table 7, it can be seen that the extracellular space (E.C.S.) was increased by hypothyroidism as reported after thyroparathyroidectomy (9). However, there was no reversal of this effect

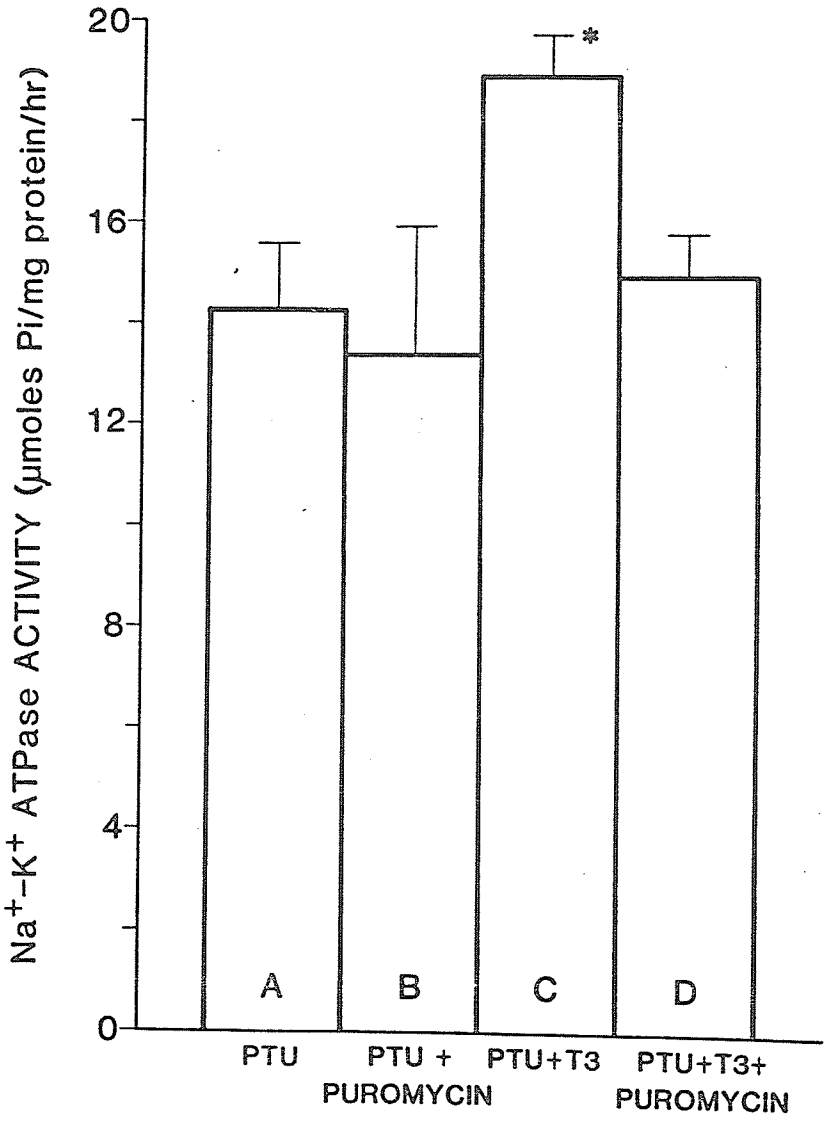


FIGURE 7. Sarcolemmal $Na^+ - K^+$ ATPase activity from 4 groups of rat hearts: A. - Hypothyroid (P.T.U.), B.- Hypothyroid treated with puromycin for 48 hours (P.T.U. + Puromycin), C.- Hypothyroid treated with $T_3$ for 48 hours (P.T.U. + $T_3$) and D. - Hypothyroid treated with $T_3$ and puromycin for 48 hours (P.T.U. + $T_3$ + Puromycin). Activities are expressed in μmoles Pi/mg protein/ hours and values are means ± S.E. of 7 - 10 experiments. * $p < 0.05$ when compared to group A.

Table 7. Effect of altered levels of circulating thyroid hormone on rat ventricular extracellular space and tissue water content.

| | (a) Euthyroid | (b) Hyperthyroid | (c) Hypothyroid | (d) Hypothyroid + 2 days $T_3$ | (e) Hypothyroid + 7 days $T_3$ |
|---|---|---|---|---|---|
| E.C.S. | 0.153 ± 0.003 | 0.160 ± 0.012 | 0.194 ± 0.005* | 0.204 ± 0.008* | 0.212 ± 0.003* |
| $(H_2O)_m/W_D$ | 3.09 ± 0.02 | 3.12 ± 0.01 | 3.33 ± 0.05 * | 3.45 ± 0.05 * | 3.23 ± 0.04 * |
| $(H_2O)_o/W_D$ | 0.627 ± 0.014 | 0.657 ± 0.049 | 0.836 ± 0.022* | 0.901 ± 0.041* | 0.897 ± 0.015* |
| $(H_2O)_i/W_D$ | 2.47 ± 0.02 | 2.46 ± 0.05 | 2.49 ± 0.05 | 2.54 ± 0.03 | 2.34 ± 0.04 * |

Values are means ± S.E. of 7 - 8 experiments. E.C.S. is expressed as gm extracellular water per gm wet weight. $(H_2O)_m/W_D$ is gm muscle water per gm dry weight. $(H_2O)_o/W_D$ is gm extracellular water per gm dry weight. $(H_2O)_i/W_D$ is gm intracellular water per gm dry weight.

*Statistically significant ($p < 0.05$) when compared to euthyroid values.

within the 7 days period of $T_3$ treatment studied. E.C.S. was unaltered by hyperthyroidism. Muscle water contents with respect to dry weight $[(H_2O)/W_o]$ are also given in Table 7. In general the tendency was for muscle water to increase in hypothyroidism. On treatment with thyroid hormone, muscle water increased further after 2 days (group d) but at 7 days (group e) it had fallen below that of hypothyroid animals. There was no difference between water content of hyperthyroid and euthyroid rat ventricles. An examination of extracellular and intracellular water with respect to dry weight revealed that changes in water content can be accounted for by changes in extracellular water as would be expected by the change in E.C.S. noted earlier. Intracellular water content was significantly changed in hypothyroid animals after 7 days of treatment (group e).

During estimation of E.C.S., time of equilibration, between $SO_4^{2-}$ injection and excision of the heart, was varied from 30 minutes to four hours. If the myocardial membrane became permeable to the sulphate marker, calculated E.C.S. would increase with increased equilibration time as intracellular levels of sulphate increased. However, it was found that no such relationship existed in any group. It therefore appears that all groups are equally impermeable to sulphates. In one group (c, hypothyroid) an anomalous relationship between equilibration time and E.C.S. existed i.e. a decrease in calculated E.C.S. with increased time. No explanation for this phenomenon was found. As sodium is a predominantly extracellular ion, improper estimate of E.C.S. would tend to have a dramatic effect on calculated intracellular sodium. A study of intracellular sodium at different equilibration times revealed no relationship. It therefore appears that the increase in calculated E.C.S. with time is in fact real i.e. that E.C.S. is being

correctly estimated at each stage.

Plasma electrolyte concentrations are shown in Table 8. Plasma potassium concentration appeared to be directly related to thyroid status. Hyperthyroidism resulted in hyperkalemia while hypokalemia occurred in hypothyroidism. The latter was reversed by $T_3$ treatment. Decreased plasma potassium has been reported in thyroidectomy (9). but others have found plasma potassium to be independent of thyroid status (274). Plasma sodium was independent of thyroid status, as reported elsewhere (9, 274). Hypocalcemia accompanied hypothyroidism and this was readily reversed by $T_3$ treatment. After only 2 days of $T_3$ treatment, calcium levels had risen above those of euthyroid rats. While hyperthyroidism was accompanied by a tendency for calcium to increase, statistically significant difference was not achieved. Nishiki et al (173) found that serum calcium was unaltered by thyroidectomy although using a similar model, Polimeni (9) found decreased serum calcium. Bajusz et al (348) found serum calcium changes in thyroidectomy to be dependent on lack of parathyroid hormone rather than thyroid hormone. However hypercalcemia has been reported in thyrotoxic patients (275). Plasma magnesium levels were found to be related to thyroid state. Levels rose in hyperthyroid state and fell in hypothyroidism. The latter effect was reversed by $T_3$ administration so that after 2 days levels were close to those of euthyroid controls and after 7 days close to those of hyperthyroid rats. Other than E.C.S. of group c, magnesium plasma level was the only parameter studied which was shown to be related to equilibration time. It was found that plasma magnesium levels rose significantly with increased equilibration time. However if values were extrapolated to time zero, the same relative differences between the groups were seen. This time dependent

Table 8. Effect of thyroid status on plasma cation concentration in the rat.

| | (a) Euthyroid | (b) Hyperthyroid | (c) Hypothyroid | (d) Hypothyroid + 2 days $T_3$ | (e) Hypothyroid + 7 days $T_3$ |
|---|---|---|---|---|---|
| $[K]_p$ | 2.59 ± 0.12 | 3.54 ± 0.23* | 2.22 ± 0.10* | 2.49 ± 0.13 | 2.70 ± 0.16 |
| $[Na]_p$ | 143 ± 0.6 | 143 ± 1.8 | 141 ± 0.9 | 143 ± 2.2 | 146 ± 1.6 |
| $[Ca]_p$ | 2.97 ± 0.05 | 3.24 ± 0,16 | 2.60 ± 0.06* | 3.36 ± 0.10* | 3.53 ± 0.12* |
| $[Mg]_p$ | 1.28 ± 0.09 | 1.64 ± 0,10* | 0.84 ± 0.04* | 1.19 ± 0.09 | 1.59 ± 0.07* |

Cation concentration given in mM units.

* Statistically significant ($p < 0.05$) difference when compared to euthyroid values.
Values are means ± S.E. of 7-8 experiments.

alteration may be related to the stress of surgery. It has been shown in humans that stress or pain may be associated with changes in serum magnesium levels (349). Decreased plasma magnesium has been reported in thyroidectomized rats (9) but these do not agree with the clinical findings of decreased serum magnesium in hyperthyroidism and increased levels in hypothyroidism (277,278,279).

Intracellular monovalent cation concentrations and their ratio are listed in Table 9. It must be remembered that these do not represent sarcoplasmic concentrations of free cations since the calculations do not take compartmentalization of cations into consideration. Intracellular potassium was not significantly altered in hypothyroidism or hyperthyroidism. However, treatment of hypothyroid animals with $T_3$ resulted in a significant increase in potassium levels. Ismail- Beigi and Edelman (274) found intracellular potassium levels rose after thyroid hormone administration to both euthyroid and thyroidectomized rats. Significant difference in intracellular sodium levels was only revealed when hyperthyroid and hypothyroid states were compared. Treatment of hypothyroid animals led to further decrease in intracellular sodium. The $[K]_i/[Na]_i$ was unaltered in either the hypothyroid and hyperthyroid state in contrast to the findings in thyroidectomized animals (9,274). The only significant change was seen after 7 days of thyroid hormone administration to hypothyroid rats when $[K]_i/[Na]_i$ increased significantly.

No significant change in calcium concentration was revealed (Table 10), although there did appear to be a tendency for the level to fall in hypothyroidism and to rise after thyroid hormone administration. Significant difference in intracellular magnesium could be seen only when hyperthyroid and hypothyroid conditions were compared. The increase in hypothyroidism was partially reversed by $T_3$ treatments. These results

Table 9. Effect of thyroid status on intracellular monovalent cations

| | (a) Euthyroid | (b) Hyperthyroid | (c) Hypothyroid | (d) Hypothyroid + 2 days $T_3$ | (e) Hypothyroid + 7 days $T_3$ |
|---|---|---|---|---|---|
| $[K]_i$ (mM) | 163 ± 3.3 | 165 ± 3.9 | 168 ± 3.2 | 174 ± 2.8* | 183 ± 2.8* |
| $[Na]_i$ (mM) | 21.1 ± 1.7 | 26.3 ± 3.1 | 19.1 ± 1.0 | 20.6 ± 1.9 | 16.7 ± 1.1* |
| $[K]_i/[Na]_i$ | 7.73 ± 0.17 | 6.83 ± 0.88 | 9.00 ± 0.62 | 9.02 ± 0.62 | 11.32 ± 0.80* |

* Statistically significant ($p < 0.05$) difference when compared to euthyroid value

Values are means ± S.E. of 7-8 experiments.

Table 10. Effect of thyroid status on intracellular divalent cations

| | (a) Euthyroid | (b) Hyperthyroid | (c) Hypothyroid | (d) Hypothyroid + 2 days $T_3$ | (e) Hypothyroid + 7 days $T_3$ |
|---|---|---|---|---|---|
| $[Ca]_i$ (mM) | 0.969 ± 0.098 | 1.043 ± 0.147 | 0.743 ± 0.052 | 0.712 ± 0.083 | 0.972 ± 0.161 |
| $[Mg]_i$ (mM) | 14.1 ± 1.27 | 13.2 ± 0.94 | 17.8 ± 0.91 | 16.2 ± 1.77 | 16.4 ± 1.52 |
| $[Mg]_i / [Ca]_i$ | 14.9 ± 1.94 | 15.3 ± 3.2 | 25.7 ± 2.78* | 24.5 ± 3.04* | 18.95 ± 1.66 |

* Statistically significant ($p < 0.05$) difference when compared to euthyroid values.

Values are means ± S.E. of 7-8 experiments.

do not agree with those from thyroidectomized rats (9) where magnesium content was decreased. $[Mg]_i$ depletion in hyperthyroidism and the reverse in hypothyroidism may reflect the failure of $Mg^{2+}$ controlling mechanisms to keep pace with the rapidly expanding or constricting cell volume. $[Mg]_i/[Ca]_i$ ratio was increased in hypothyroidism. After 7 days, but not after 2 days, of $T_3$ administration this ratio was decreased so that it was no longer significantly different from control.

D. Sarcolemmal 5' Nucleotidase, Calcium Binding and Calcium ATPase

5' Nucleotidase activity was found to be significantly ($p < 0.05$) less in the hypothyroid state than in the euthyroid state in sarcolemma prepared by the hypotonic shock-LiBr method (Table 11). This decrease in enzyme activity was not associated with significant change in $K_m$ (34 ± 2.2 μM adenosine in hypothyroidism and 42 ± 4 μM adenosine in euthyroid preparations) (Fig. 8). On the other hand, no difference in 5'-nucleotidase activity was seen when sarcolemma was prepared by the KCl-sucrose gradient method from euthyroid and hypothyroid rat hearts.

ATP independent calcium binding ability of sarcolemma prepared from euthyroid and hypothyroid animals by hypotonic shock-LiBr method was examined at 0.1 mM and 1.25 mM calcium concentrations. As shown in Table 12 no difference in calcium binding ability was seen at either calcium concentrations. Similarly the content of sialic acid, which is known to bind calcium, of these membranes was unaltered in hypothyroid rats (Table 12). ATP independent calcium binding of sarcolemma prepared by the KCl-sucrose gradient method in the presence of 0.1 mM $CaCl_2$ was also unaltered (3.33 ± 0.44 and 3.78 ± 0.50 n moles calcium/mg protein/5 minutes for euthyroid and hypothyroid states, respectively). Sarcolemmal $Ca^{2+}$ dependent ATPase activity was also examined in euthyroid and hypothyroid states. Hypotonic shock LiBr method was employed for sarcolemmal

Table 11. Sarcolemmal 5' Nucleotidase activities of euthyroid and hypothyroid rat hearts.

| | Sucrose Density Gradient | Hypotonic Shock–LiBr |
|---|---|---|
| Euthyroid | 56.8 ± 2.07 | 130 ± 8.5 |
| Hypothyroid | 52.62 ± 3.64 | 97 ± 7.1* |

Values are means ± S.E. of 6 - 10 experiments. 5' Nucleotidase activity is given in units of n moles adenosine/ mg protein/ min

* $p < 0.05$

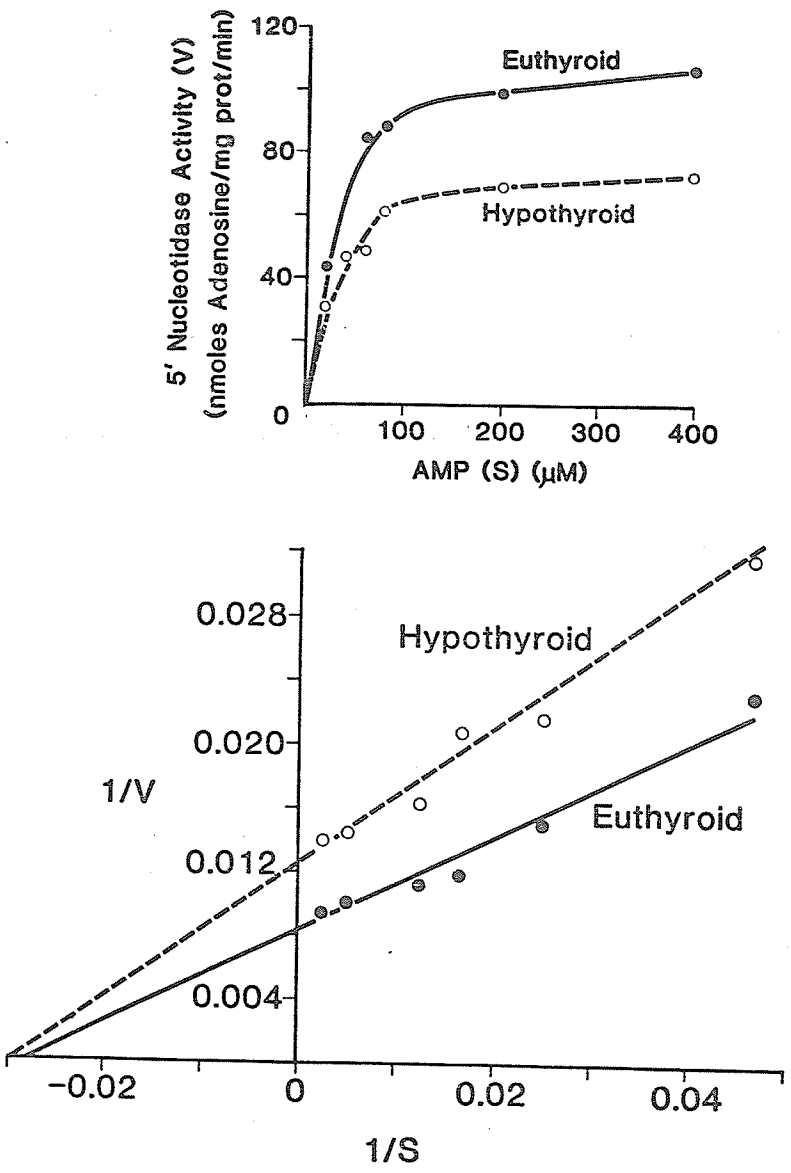


FIGURE 8. Sarcolemmal 5' Nucleotidase activities from euthyroid and hypothyroid rat hearts at different concentrations of AMP. Values are typical of 3 experiments.

Table 12. $Ca^{2+}$ dependent ATPase activity, ATP independent calcium binding capacity and sialic acid content of sarcolemma isolated by hypotonic shock-LiBr treatment from euthyroid and hypothyroid rat hearts.

| | Euthyroid | Hypothyroid |
|---|---|---|
| $Ca^{++}$ ATPase (μ moles Pi/mg prot/hr) | 51.2 ± 1.70 | 47.1 ± 1.34 |
| $K_a$ $Ca^{2+}$ (mM) | 1.12 ± 0.17 | 1.01 ± 0.18 |
| Calcium Binding at 0.1 mM $Ca^{2+}$ (n moles $Ca^{2+}$/mg prot/ 5 min) | 16.3 ± 3.18 | 16.58 ± 1.43 |
| Calcium Binding at 1.25 mM $Ca^{2+}$ (n moles $Ca^{2+}$/mg prot/5 min) | 72.8 ± 7.99 | 69.4 ± 8.20 |
| Sialic Acid Content (n moles sialic acid/ mg prot) | 32.3 ± 2.03 | 37.0 ± 3.06 |

Values are means ± S.E. of 4 - 6 experiments. No statistically significant difference exists between euthyroid and hypothyroid values.

isolation. Activities were not significantly different in the two states (Table 12). Examination of activation by calcium revealed no difference in $K_a$ values for $Ca^{2+}$ dependent ATPase in euthyroid and hypothyroid rat hearts.

ATP dependent calcium binding of the KCl-sucrose gradient sarcolemma prepared from hypothyroid animals was greater than that prepared from euthyroid animals (Table 13). In the presence of 5 µg/ml calmodulin, a known activator of the calcium pump, the difference in activity became even more apparent with the hypothyroid preparation being stimulated by 44.4 % while the euthyroid was stimulated by only 24.4%. 1 µM trifluoperazine (T.F.P.), an inhibitor of calmodulin action, completely blocked calmodulin stimulation while having an insignificant effect on basal ATP dependent calcium binding (Table 13). Higher concentrations of T.F.P. were found to have a general depressant effect. ($Ca^{2+}$ - $Mg^{2+}$) ATPase activity was also measured in sarcolemma (KCl-sucrose gradient isolation) of hypothyroid and euthyroid animals. Basal ($Ca^{2+}$ - $Mg^{2+}$) ATPase activity was unaltered in the hypothyroid state (Table 14). However in comparison to control, calmodulin stimulation of the enzyme was significantly greater in hypothyroid preparations. As in the case of ATP dependent calcium binding, calmodulin stimulation of the ($Ca^{2+}$ - $Mg^{2+}$) ATPase was completely blocked by 3.3 µM T.F.P. At this concentration the ($Ca^{2+}$ - $Mg^{2+}$) ATPase activity in the absence of calmodulin was not significantly altered.

E. Sarcolemmal Adenylate Cyclase

Adenylate cyclase activity was examined in two sarcolemmal preparations obtained by the hypotonic shock-LiBr and KCl- sucrose gradient methods from euthyroid and hypothyroid rat hearts. Stimulation by fluoride, epinephrine and guanylimidodiphosphate (GppNHp) were also

Table 13. ATP dependent calcium binding of sarcolemma isolated by sucrose density gradient from euthyroid and hypothyroid rat hearts.

| | ATP Dependent $Ca^{2+}$ Binding (n moles $Ca^{2+}$/ mg protein/ 5 min) | | | |
|---|---|---|---|---|
| | Control | + 5 µg/ml Calmodulin | + 1 µM T.F.P. | + 1 µM T.F.P. 5 µg/ml Calmodulin |
| Euthyroid | 12.93 ± 0.58 | 15.91 ± 0.71 (24.4 ± 3.7%) | 11.4 ± 0.75 | 12.1 ± 0.74 |
| Hypothyroid | 15.64 ± 0.87* | 22.63 ± 1.74* (44.4 ± 5.7%)* | 14.35 ± 0.44* | 15.41 ± 1.06* |

Values are means ± S.E. of 4 - 6 experiments.

Binding in the presence of calmodulin expressed as percentage stimulation are given in brackets below actual values.

* $p < 0.05$ (hypothyroid compared to euthyroid)

Table 14. $Ca^{2+}$ stimulated - $Mg^{2+}$ dependent ATPase of sarcolemma isolated by sucrose density gradient method from euthyroid and hypothyroid rat hearts.

| | $Ca^{2+}$ Stimulated ATPase | | |
|---|---|---|---|
| | Activity (μ moles Pi/mg/prot/hr) | | % stimulation by Calmodulin |
| | Control | 5 μg/ml Calmodulin | |
| Euthyroid | 2.88 ± 0.18 | 3.52 ± 0.16 | 23 ± 2.2 |
| Hypothyroid | 2.94 ± 0.10 | 4.43 ± 0.23* | 51 ± 5.1* |

Values are means ± S.E. of 4 experiments.

* $p < 0.05$ (hypothyroid compared to euthyroid)

examined in both preparations and the results are shown in Table 15. In both preparations there was no difference in basal adenylate cyclase activities between euthryoid and hypothyroid states although hypotonic shock-LiBr preparation resulted in basal activities approximately twice that of KCl- sucrose gradient sarcolemma in both conditions. Hypotonic shock-LiBr preparations was characterized by poor fluoride, epinephrine and GppNHp responses; these was no difference between euthyroid and hypothyroid groups in any of these parameters. While fluoride, epinephrine and GppNHp responses were more pronounced in KCl-sucrose gradient preparations, no significant difference was seen between euthyroid and hypothyroid activities.

In view of the poor sensitivity of sarcolemmal adenylate cyclase to different activators, perhaps due to the loss of certain factors required for the expression of enzyme activation, it was decided to examine the enzyme activity in washed cell particles from euthyroid and hypothyroid rat hearts. Basal activity was found to be higher ($p < 0.05$) in hypothyroid washed particles than that from euthyroid animals (Table 16). While stimulation by 8 mM fluoride was similar in both cases, 30 μM GppNHp resulted in significantly greater stimulation in euthyroid than in hypothyroid states (Table 16). Stimulation by 100 μM epinephrine alone was rather small but this was greatly potentiated when assayed in the presence of GppNHp. Epinephrine stimulation was slightly greater in the euthyroid state, than in the hypothyroid state at this concentration.

A more detailed study of fluoride, GppNHp and epinephrine responses was also performed by using different concentrations of each activator. Dose response relationship for fluoride shown in Fig. 9 indicates no significant difference between euthyroid and hypothyroid states at any

Table 15. Sarcolemmal adenylate cyclase activity of euthyroid and hypothyroid rat hearts.

| | Sarcolemma Preparation | | | |
|---|---|---|---|---|
| | Hypotonic Shock-LiBr | | Sucrose Density Gradient | |
| | Euthyroid | Hypothyroid | Euthyroid | Hypothyroid |
| Basal | 501 ± 28 | 462 ± 47 | 218 ± 4.4 | 249 ± 27.1 |
| 8 mM Na F % Basal | 115 ± 18.7 | 124 ± 11.9 | 188 ± 26.6 | 196 ± 10.5 |
| 100 µM Epinephrine % Basal | 117 ± 3.3 | 112 ± 10.8 | 109 ± 10.2 | 96 ± 3.9 |
| 30 µM GppNHp % Basal | 126 ± 1.3 | 131 ± 8.3 | 169 ± 9.2 | 160 ± 8.2 |
| 100 µM Epinephrine + 30 µM GppNHp } % Basal | 146 ± 12.4 | 149 ± 9.0 | 233 ± 15.4 | 200 ± 8.3 |

Basal adenylate cyclase values are in units of p moles cAMP/ mg prot/min.

Values are means ± S.E. of 5 - 6 experiments.

No statistically significant difference exists between the hypothyroid and euthyroid groups.

Table 16. Adenylate cyclase activity of washed particulate preparations from euthyroid and hypothyroid rat hearts.

| | Euthyroid | Hypothyroid |
|---|---|---|
| Basal Activity | 91 ± 3.3 | 124 ± 8.9 * |
| + 8 mM Na F % Basal | 367 ± 22.8 | 349 ± 34.3 |
| + 100 µM Epinephrine % Basal | 112 ± 6.3 | 116 ± 12.1 |
| + 30 µM GppNHp % Basal | 273 ± 22 | 210 ± 6.2* |
| + 100 µM Epinephrine, 30 µM GppNHp } % Basal | 390 ± 20 | 307 ± 18 * |

Basal adenylate cyclase activity in p moles cAMP/ mg protein/ minute.

Each value is a mean ± S.E. of 6 - 7 experiments.

* $p < 0.05$

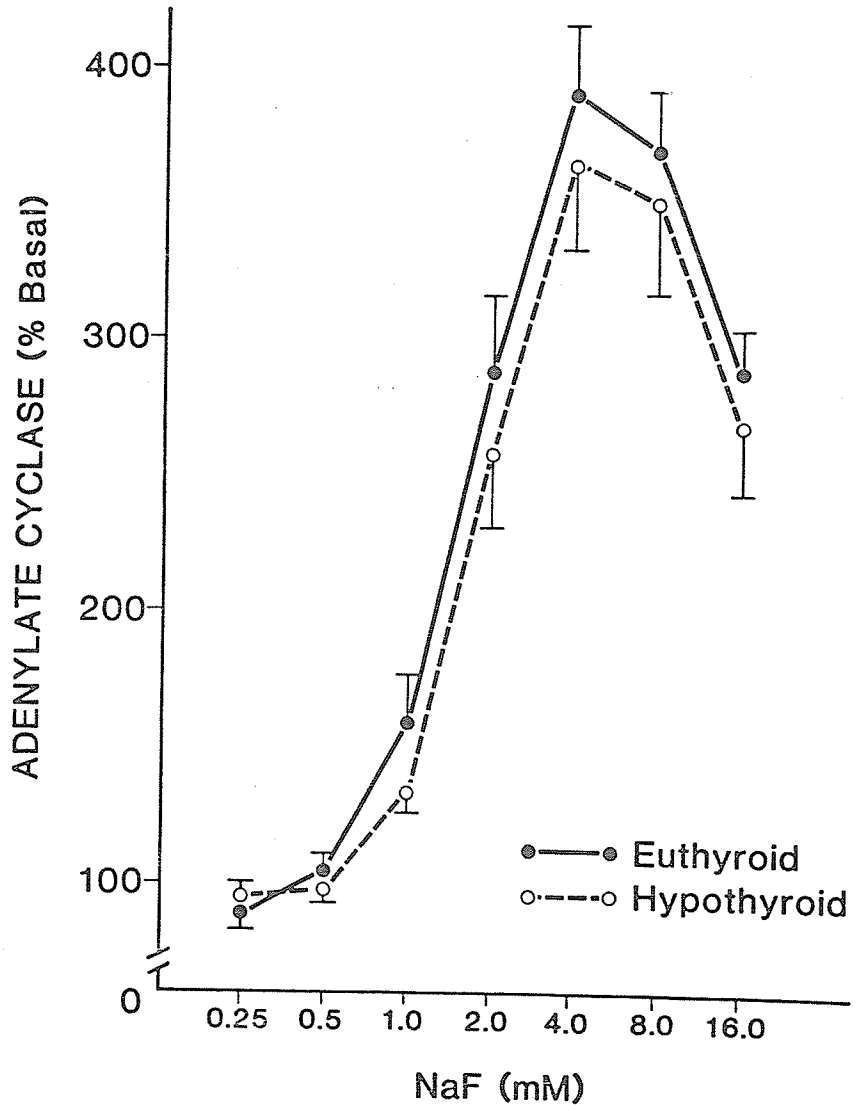


FIGURE 9. Fluoride stimulation of adenylate cyclase activity of euthyroid and hypothyroid rat heart washed particles. Values are expressed as percentage of basal activity and are means S.E. of 4 - 5 experiments. No statistically significant difference ($p < 0.05$) exists between the two groups at any concentration of fluoride.

fluoride concentration. Under both conditions fluoride stimulation increased dramatically as the concentration rose above 0.5 mM and reached a maximum at 4 - 8 mM. Further increase in fluoride concentration resulted in decreased stimulation. On the other hand, responses to GppNHp were dramatically different in euthyroid and hypothyroid preparations (Fig. 10). At virtually all concentrations studied, GppNHp stimulation was greater ($p < 0.05$) in the euthyroid state. 30 μM GppNHp resulted in maximum stimulation in both cases. There also appears to be a tendency for the concentration which stimulated half maximally to shift to the right in hypothyroidism (Fig. 10); $ED_{50}$ shifted from approximately 0.68 μM to 1.25 μM GppNHp. Responses of adenylate cyclase in preparations from euthyroid and hypothyroid animals to epinephrine are shown in Fig. 11. Stimulation of $10^{-5}$, $3 \times 10^{-5}$ and $10^{-4}$ M epinephrine was significantly greater in euthyroid animals, while at $10^{-3}$M concentration no significant difference was seen.

To confirm that the major differences recorded earlier were due to the hypothyroid state and not caused by its mode of induction (P.T.U.), adenylate cyclase activity was examined in another model of hypothyroidism induced by thyroidectomy. As can be seen in Table 17, the changes seen after thyroidectomy follow closely those of P.T.U. induced hypothyroidism. In washed particles there was a definite tendency for basal activity to be higher in thyroidectomized than euthyroid preparations. GppNHp stimulation was dramatically reduced after thyroidectomy to an even greater extent than in P.T.U. induced hypothyroidism. Stimulation by 100 μM epinephrine in the presence of GppNHp achieved values very close to those for hypothyroidism (P.T.U.). The characteristics of the adenylate cyclase in sarcolemmal preparations obtained from thyroidectomized rats were similar to those of the P.T.U. treated rat hearts.

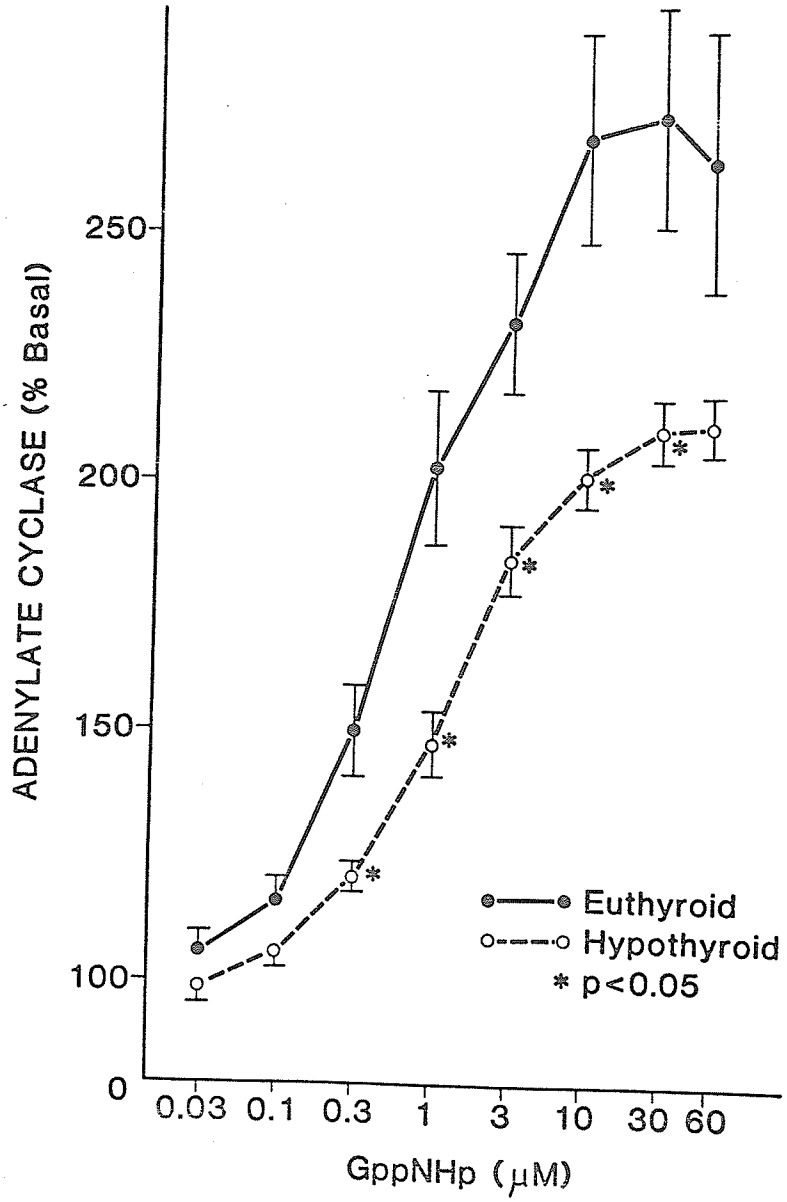


FIGURE 10. GppNHp stimulation of adenylate cyclase activity of euthyroid and hypothyroid rat heart washed particles. Values are expressed as percentage of basal activity and are means ± S.E. of 4 - 5 experiments. * statistically significant difference ($p < 0.05$) when compared to euthyroid value.

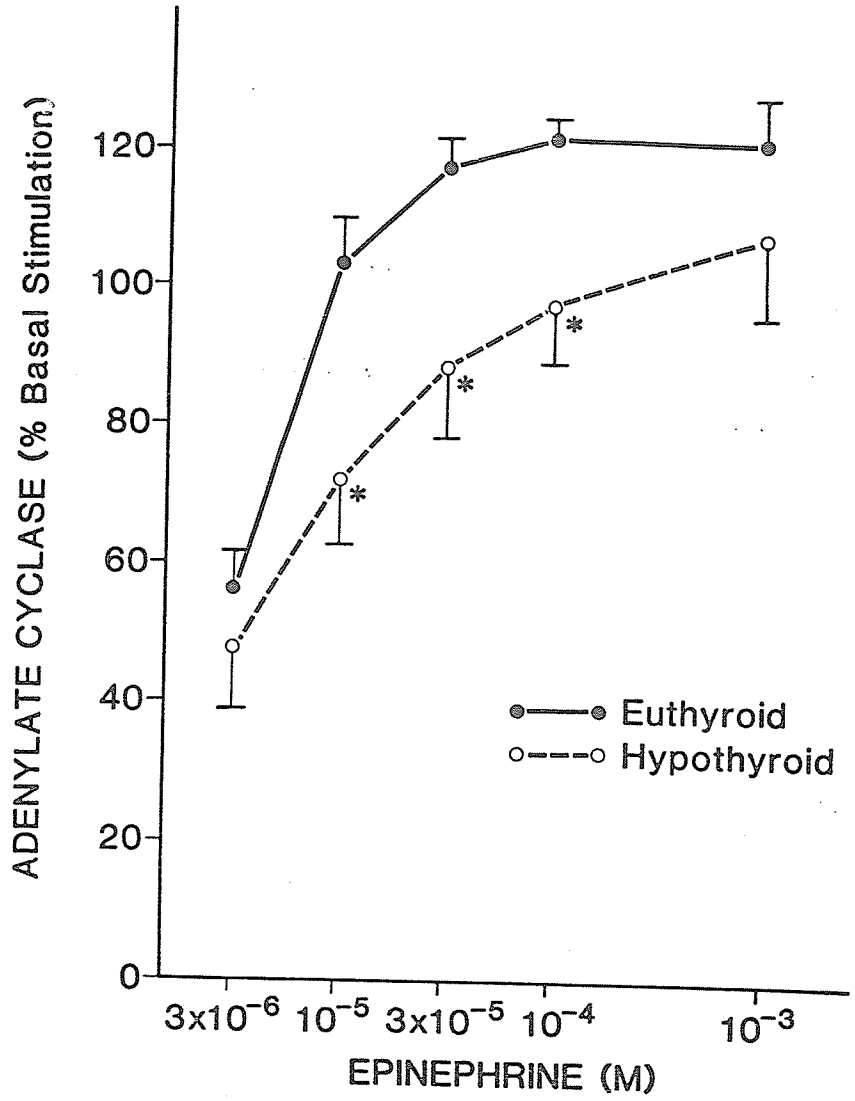


FIGURE 11. Stimulation of adenylate cyclase activity of euthyroid and hypothyroid rat heart washed particles by epinephrine in the presence of 30 μM GppNHp. Stimulation is expressed as a percentage of basal above that seen in the presence of 30 μM GppNHp alone. Values are means ± S.E. of 5 - 6 experiments. * statistically significant difference ($p < 0.05$) when compared to euthyroid value.

Table 17. Adenylate cyclase activity of washed particles obtained from euthyroid and thyroidectomized rat hearts.

| | Euthyroid | Thyroidectomized |
|---|---|---|
| Basal Activity | 105 ± 2.2 | 155 ± 25.0 |
| + 30 μM GppNHp % Basal | 276 ± 14.6 | 188 ± 12.5 * |
| 100 μM Epinephrine + 30 μM GppNHp } % Basal | 390 ± 16 | 286 ± 10.8 * |

Values are means ± S.E. of 3 experiments.

Basal adenylate cyclase activity in p moles cAMP/ mg prot/min.

* $p < 0.05$

Washed particles were also prepared after 1 and 2 days of $T_3$ administration to hypothyroid animals. After 24 hours neither GppNHp nor epinephrine stimulations were significantly altered (Table 18). However after 48 hours, GppNHp stimulation had increased to above the euthyroid levels. Stimulation by 100 μM epinephrine in the presence GppNHp was slightly increased (104 ± 3.58 vs 119 ± 3.26% basal for saline and $T_3$ injected respectively). Basal adenylate cyclase activity was not significantly altered 24 or 48 hours after $T_3$ administration. In vitro addition of $T_3$ to incubation medium did not significantly alter adenylate cyclase activity, in contrast to the findings of Levey and Epstein (34,35) but in agreement with others (40,41).

Table 18. Epinephrine and GppNHp stimulation of adenylate cyclase activity of washed particles of hypothyroid rat ventricles 24 hours and 48 hours after $T_3$ administration.

| | | 24 hours | | 48 hours | |
|---|---|---|---|---|---|
| | | $- T_3$ | $+ T_3$ | $- T_3$ | $+ T_3$ |
| 30 μM GppNHp | % Basal | 205 ± 4.4 | 221 ± 9.2 | 232 ± 23 | 316 ± 4.6* |
| 100 μM Epinephrine + 30 μM GppNHp | % Basal | 292 ± 5.0 | 327 ± 20.5 | 324 ± 23 | 414 ± 9.3* |

Values are means ± S.E. of 3 - 5 experiments.

* $p < 0.05$

## V. DISCUSSION

In this study it was observed that $Na^+ - K^+$ ATPase activity in sarcolemma prepared from hearts of hypothyroid rats was less than that from euthyroid animals. Since this change was seen in preparations from animals treated with P.T.U. and thyroidectomy, it is obvious that alterations in $Na^+ - K^+$ ATPase were a reflection of the thyroid status of the animal. Furthermore, depression in the enzyme activity was seen in sarcolemmal preparations obtained by hypotonic shock LiBr treatment and sucrose-density gradient methods, which have been shown to yield predominantly right-side out and inside out vesicular preparations, respectively, (342a) and thus cannot be regarded as due to differences in the orientations of the membrane preparations. The yields and cross contamination of the sarcolemmal preparations with other subcellular organelles (3 to 5%) were found to be similar in control and experimental hearts. In this regard, it is noteworthy that there was neither any qualitative difference in the electrophoretic pattern nor was there any significant difference in the phospholipid composition of the membrane preparations from control and hypothyroid hearts. Since sarcolemmal $Mg^{2+}$ ATPase activities in the euthyroid and hypothyroid rat hearts were not different from each other, it appears that the observed decrease in $Na^+ - K^+$ ATPase activity in the hypothyroid state may be of some specific nature.

It has been demonstrated that sarcolemmal $Na^+ - K^+$ ATPase activity was depressed in hypothyroidism but did not increase in hyperthyroidism. While rat cardiac sarcolemmal $Na^+ - K^+$ ATPase may be under the influence of thyroid status, the magnitude of changes was less than that predicted from the examination of crude microsomal preparations (7) and

maximal thyroid hormone induced activity was achieved in the euthyroid state. In the hypothyroid state Km was reduced but other parameters such as $K_a$ for $Na^+$ or $K_a$ for $K^+$ as well as pH optimum were unaltered. The finding that ouabain sensitivity was unchanged is in contrast to the decreased sensitivity of thyroxine treated rabbits (6). The reduced inotropic effect of ouabain on ventricular strips of hypothyroid rats (266) can not be accounted for by altered sensitivity of ventricular $Na^+ - K^+$ ATPase to ouabain. In this regard, it is pointed out that two classes of ouabain binding sites have recently been demonstrated in rat cardiac membranes (261) but only one of these has an apparent dissociation constant similar to the $1D_{50}$ for $Na^+ - K^+$ ATPase. However, the dissociation constant of the second site is similar to the concentration required to induce half maximal inotropic effect. Altered ouabain sensitivity of the inotropic process may be associated with altered characteristics of this second binding site or at some stage of the coupling process between binding of ouabain and its inotropic effect.

It has been proposed that $K^+$ stimulated phosphatase represents the dephosphorylation step of $Na^+ - K^+$ ATPase cycle (344,345) while others (346,347) have concluded that these activities are different entities. The finding that both $K^+$ stimulated phosphatase and $Na^+ - K^+$ ATPase activities are depressed to the same degree in cardiac sarcolemma of hypothyroid rats without alteration in maximum ouabain inhibition of either activity provides support to the hypothesis that $K^+$ stimulated phosphatase activity is linked to the enzyme $Na^+ - K^+$ ATPase. Furthermore, the reversal of hypothyroid induced depression of $Na^+ - K^+$ ATPase after 48 hours but not 24 hours of thyroid hormone treatment is consistent

with the induction of synthesis of new $Na^+ - K^+$ ATPase moieties. Puromycin, which did not significantly alter the sarcolemmal $Na^+ - K^+$ ATPase of hypothyroid rats, completely blocked the reversal of hypothyroid induced depression by $T_3$ administration. It therefore seems that $T_3$ induced reversal depends on the synthesis of new $Na^+ - K^+$ ATPase moieties or a protein activator of $Na^+ - K^+$ ATPase. The synthesis of new $Na^+ - K^+$ ATPase molecules has been demonstrated in kidney (350, 351). The lack of effect of $T_3$ added *in vitro* to sarcolemmal preparations and the lag period of more than 24 hours before activation is detected after *in vivo* $T_3$ administration, indicate that direct activation of enzyme moieties, which are present but inactive, is not a mechanism by which $T_3$ acts. At any rate, the difference in activities of sarcolemma from hypothyroid and euthyroid rats can not be considered to be due to the presence of different levels of endogenous $T_3$ in the isolated membranes from euthyroid and hypothyroid animals.

Sarcolemmal $Na^+ - K^+$ ATPase is probably an essential part of the $Na^+$ pump in the myocardial cell (Bonting 352). It is therefore possible that alterations in $Na^+ - K^+$ ATPase activity may be reflected in alterations of ionic distribution in the cell. Alternatively, changes in the enzyme activity may be such that they exactly balance the requirements of the cell and no intracellular electrolyte changes are seen. This is analogous to metabolic changes which enable the cell to increase energy supply to match increased demand so that cytosolic high energy compounds remain at or near normal levels. Thus it is not absolutely necessary that changes in cardiac $Na^+ - K^+$ ATPase activity must be accompanied by changes in the myocardial electrolyte contents.

Plasma electrolyte changes are believed to occur due to complex changes in absorption and excretion by the body. Expansion or reduction of cell volume or extracellular volume and alteration in intracellular levels may also influence extracellular levels. In general the myocardium can be conceived to have little or no role in regulating plasma electrolyte levels; however, extracellular electrolytes may have profound effects on myocardial function. Whether changes in heart function in hypothyroid and hyperthyroid states are due to alterations in extracellular or intracellular electrolytes cannot be stated with certainty on the basis of the data at hand as well as in the literature.

As in other studies (10,274), the administration of thyroid hormone to hypothyroid rats for 48 hours or more caused an increase in intracellular potassium. This has been interpreted by other authors (10,274) as reflecting enhancement of the $Na^+$ pump associated with $Na^+ - K^+$ ATPase activity. However, in this study, intracellular potassium concentration did not differ among the hypothyroid, euthyroid and hyperthyroid states in spite of the fact that $Na^+ - K^+$ ATPase activity of hypothyroid rats was depressed. These observations indicate that a simple relationship does not exist between intracellular potassium and $Na^+ - K^+$ ATPase activity measured <u>in vitro</u> or indeed the concomitant alterations in active monovalent ion uptake measured in isolated tissue (254). Similarly intracellular sodium did not vary as expected in euthyroid, hyperthyroid and hypothyroid states. Intracellular sodium levels of hypothyroid rats were not different from those of euthyroid rats but were less than those of hyperthyroid rats. Intracellular sodium only changed as predicted in hypothyroid rats after $T_3$ administration.

Decreased intracellular sodium accompanied by increased potassium is expected when $Na^+ - K^+$ ATPase activity increases. But this type of relationship was only found after $T_3$ administration to hypothyroid rats. It may reflect only the acute phase of $Na^+ - K^+$ ATPase change. The hypothyroid state was developed and maintained over a five week period and after this time intracellular levels were at or near normal. It may be that over a period, demands on transport mechanism and transport ability are modified so as to better match each other. Tachycardia, which is a characteristic of hyperthyroidism and increases the frequency of action potentials of the myocardium and the influx of $Na^+$ and efflux of $K^+$, will put greater demand on the $Na^+ - K^+$ pump; the opposite will occur in hypothyroidism. In ventricular tissue of the frog it has been demonstrated that increasing $[K^+]_o$ increases the $K^+$ efflux from the cell (353). In this study, extracellular potassium was found to be related to thyroid state. Therefore if a similar mechanism exists in the rat, the increased extracellular potassium of hyperthyroidism will increase $K^+$ efflux; the opposite will occur in hypothyroidism.

Intracellular calcium content did not vary with thyroid state, although there appeared to be a tendency for it to decrease in hypothyroidism and for this to be reversed after thyroid hormone administration. However, the values of intracellular calcium represent total calcium content of the cell and not cytosolic concentrations which may be influenced by a number of processes. The magnitude of $Ca^{2+}$ influx has been shown to be influenced by extracellular calcium levels (354). Since plasma calcium concentration has been shown in this study to be influenced by thyroid status, this may tend to increase intracellular

calcium contents. We have also demonstrated that in the presence of calmodulin, $Ca^{2+}$ - $Mg^{2+}$ ATPase and ATP dependent calcium binding of cardiac sarcolemma were enhanced in the hypothyroid state. Therefore extrusion of calcium from the cell will be increased in hypothyroidism. Intracellular magnesium levels were only altered when hypothyroid and hyperthyroid states were compared. Mechanisms which control intracellular magnesium levels are poorly understood. $Mg^{2+}$ ATPase which has been suggested to be associated with a pumping mechanism (16) was not altered by thyroid status. Increased or decreased $[Mg]_i$ may reflect the inability of the cell to extrude or acquire magnesium at the same rate as the expansion or constriction of the cell volume.

$[Mg]_i$ / $[Ca]_i$ ratio was found to be significantly increased in hypothyroidism. After 2 days but not 7 days of thyroid hormone treatment of hypothyroid animals, $[Mg]_i$ / $[Ca]_i$ ratio was still above normal. Although it did not decrease in the hyperthyroid state, myocardial $[Mg]_i$ / $[Ca]_i$ appeared to correlate well with thyroid status. $Mg^{2+}$ has been demonstrated to influence calcium metabolism in muscle. In skinned skeletal fibers increasing free $Mg^{2+}$ above 0.3 mM decreased submaximal tension development (355). Caffeine induced release of calcium from isolated sarcoplasmic reticulum was inhibited by high magnesium levels (356) and it has been suggested that $Mg^{2+}$ can affect the rate of $Ca^{2+}$ uptake by the sarcoplasmic reticulum (357). These results were obtained in skeletal muscle but it is conceivable that $Mg^{2+}$ can have similar effects in cardiac muscle. Therefore the increased $[Mg]_i$ / $[Ca]_i$ ratio of hypothyroid myocardium may play a role in the induction of depressed contractility in this state. However,

in thyroidectomy no such change was seen (9) which would appear to rule out an obligatory role of altered $[Mg]_i$ / $[Ca]_i$ in the depression of myocardial contractility.

5' nucleotidase activity of rat cardiac sarcolemma isolated by isotonic shock-LiBr treatment was depressed in the hypothyroid state induced by P.T.U. On the other hand, in sucrose gradient sarcolemma, 5' nucleotidase activity was not depressed. It may be that alterations in the sarcolemmal 5' nucleotidase may only become evident in the predominantly right-side out preparations. Since two populations of 5' nucleotidase, one on either side of the membrane, have been demonstrated (358), it would therefore seem that only the extracellular population of 5' nucleotidase is altered in hypothyroidism. These results agree with the finding in thyroidectomized sheep (253) and indicate a fair degree of selectivity in sarcolemmal alterations in hypothyroid state.

Passive calcium binding properties and $Ca^{2+}$ dependent ATPase of rat cardiac sarcolemma, both of which have been implicated in the control of calcium influx (359,360), were unaltered by the hypothyroid state. However, alterations were found in $Ca^{2+}$ - $Mg^{2+}$ ATPase and ATP dependent calcium binding which have been associated with extrusion of calcium from the cell (16). Basal $Ca^{2+}$ - $Mg^{2+}$ ATPase was unaltered but ATP dependent calcium binding was increased in hypothyroid heart sarcolemma. However, the presence of calmodulin, a known activator of these activities (17,361), stimulated both activities by approximately 24% in the euthyroid state but 50% in the hypothyroid state. Since trifluoperazin inhibited activation by calmodulin without altering basal activity, the presence of varying amounts of endogenous levels of

calmodulin in the isolated membranes from control and experimental hearts cannot be considered to account for the difference in activation by exogenous calmodulin. As calmodulin has been shown to be present in cardiac sarcolemma (361), it probably plays an integral role in the regulation of calcium extrusion process. Therefore the calcium extrusion will be greater from cardiac cells of hypothyroid than euthyroid rats , thereby lowering intracellular calcium to a greater extent. This may play a role in development of depressed contractile state of hypothyroid myocardium,

Basal adenylate cyclase activity was higher in washed particles of hypothyroid state than euthyroid state. This may be due to a difference in relative contribution of sarcolemma protein to the preparation. This is supported by the finding that sarcolemmal basal activity is unaltered and that reversal of other enzyme related changes 48 hours after thyroid hormone administration does not significantly alter basal activity. While stimulation of fluoride was unaltered, stimulation by GppNHp, a GTP analogue, was depressed in washed particles of hypothyroid rats over a wide range of concentrations. Stimulation by epinephrine alone was very poor indicating that levels of endogenous guanine nucleotides were very low in preparations from euthyroid and hypothyroid rats. While these results agree with the finding of Sharma et al (313) of depressed stimulation by 100 μM GppNHp in hypothyroid rats, they are in marked contrast to Robberecht et al (314a) who reported unaltered GppNHp stimulation but enhanced fluoride stimulation in hypothyroid rat heart homogenate. It is difficult to account for these contradictory results. However, Robberecht et al employed

homogenates prepared from frozen tissue which may have altered the enzyme activity as suggested by their low values for the basal activity. In our study all assays were performed within 1 1/2 - 2 hours of preparation of washed particles. The ability to restore GppNHp sensitivity without altering basal or fluoride stimulated activities within 48 hours of $T_3$ administration confirms that the results reported are truly thyroid status related. Maximal GppNHp stimulation was also depressed in thyroidectomized rat hearts.

Hormone activation of adenylate cyclase is a guanine nucleotide sensitive process which is mediated by a regulatory protein (G/F) distinct from the catalytic protein (362,363,364,365). While it has been suggested that fluoride acts through a separate regulatory protein (366), it has been recently demonstrated that fluoride stimulation also requires the presence of the G/F component (367). The G/F component purified to homogeneity was capable of restoring guanine nucleotide and fluoride stimulated adenylate cyclase activity of lymphoma cells deficient in this protein (368). In the hypothyroid state it would appear that G/F component - catalytic component coupling capacity is not altered as fluoride stimulation is not affected. It would therefore seem that binding of guanine nucleotide is somehow affected as the coupling process is altered but only in the presence of guanine nucleotide and not fluoride. Maximal epinephrine stimulation was not affected which suggests that the number of functional $\beta$ receptors was not reduced. However at certain submaximal concentrations ($10^{-5}$ - $10^{-4}$ M), epinephrine stimulation was depressed although the $ED_{50}$ was unaltered. This shift in dose response curve to epinephrine in the presence of GppNHp may also be related to differences in guanine

nucleotide binding. The unresponsiveness of the sarcolemmal adenylate cyclase to GppNHp or epinephrine may be due to a loss of factors required for this action during the isolation and purification of membranes. As noted earlier epinephrine activation of adenylate cyclase is guanine nucleotide dependent and is mediated through G/F component. It has also been demonstrated that guanine nucleotides influence the binding of agonists to β adrenergic receptors (369,370). We feel that alterations in guanine nucleotide binding proteins with respect to the binding of nucleotides or coupling with the catalytic protein in the presence of guanine nucleotides is an underlying mechanism of depressed catecholamine responsiveness in the hypothyroid state.

From the foregoing discussion it is evident that $Na^+ - K^+$ ATPase and 5' nucleotidase activities were depressed whereas $Ca^{2+}$ stimulated $Mg^{2+}$ ATPase activity and ATP-dependent $Ca^{2+}$ binding were increased in hypothyroid rat hearts. These observations provide evidence regarding alterations in sarcolemmal functions in hypothyroidism. This view is further substantiated by the fact that the responses of adenylate cyclase, another sarcolemmal bound enzyme system, to GppNHp and epinephrine were depressed in hypothyroid rat heart washed particles. The observed changes in sarcolemma from hypothyroid animals appear to be of specific nature since the activities of other sarcolemmal bound enzymes such as $Ca^{2+}$ dependent ATPase and $Mg^{2+}$ ATPase as well as as ATP-independent $Ca^{2+}$ binding were not altered under the experimental conditions employed in this study. In view of the important roles of sarcolemmal $Na^+ - K^+$ ATPase, 5'-nucleotidase, $Ca^{2+}$ stimulated ATPase and adenylate cyclase in regulating heart function

and metabolism, it is suggested that changes in sarcolemmal functions may play a crucial role in altering cation movements across the cell membrane in hypothyroid state. These changes thus can partly account for depressed cardiac function as well as altered responsiveness of myocardium to some hormones and drugs in hypothyroidism.

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