THE EFFECT OF HYPERTHYROIDISM ON RAT CARDIAC SARCOPLASMIC RETICULUM

by

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ABSTRACT

Hyperthyroidism is an endocrine disease which can affect the cardiovascular system. Cardiac function is typically augmented by the disease resulting in increased contractile force and a decrease in the relaxation time of the ventricular muscle. Since the cardiac sarcoplasmic reticulum (SR) has been shown to be intimately involved in both contraction and relaxation of the heart, it was investigated whether it was altered in the hyperthyroid rat heart. Hyperthyroidism was induced by subcutaneous injections of triiodothyronine (T_3) (dissolved in 0.01 N NaOH) at a dose of 500 μg/Kg/day for three days. The approach taken to investigate possible T_3 mediated alterations in cardiac sarcoplasmic reticulum was to study the progression of the disease from the euthyroid state up to a point which has previously demonstrated augmented cardiac function.

The effect of the treatment protocol was studied 12, 24, 48 and 72 hours after it was initiated. Ventricular weight was augmented at 48 and 72 hours ($p< 0.05$ and $p< 0.01$, respectively), and the SR yield was significantly increased 24 ($p< 0.05$), 48 ($p< 0.05$), and 72 ($p< 0.01$) hours after initiation of the treatment. The ratio of SR yield to ventricular weight was greater in the treated animals indicating that the SR yield was increased to a greater extent than the ventricular weight. The ATP-dependent oxalate-facilitated calcium transport activity of the SR preparation was determined at each of these times. There was no significant difference in the rate of calcium

uptake at 12 hours. At 24 hours, the T_3 treated rat SR calcium uptake activity was significantly ($p< 0.05$) higher at all free calcium concentrations assayed (range 0.1-5.3 μM). At 48 and 72 hours, the SR V_{Ca} was also significantly increased ($p< 0.01$ in each case). The K_{Ca} was not affected by the T_3 treatment at any of the time points studied. Phosphorylation of the SR at 24, 48 and 72 hours indicated that the increased calcium uptake activity was associated with a slight, but not significant, increase in the number of calcium pump sites at 24 hours, but significantly more calcium pump sites were labelled at 48 and 72 hours ($p< 0.01$ and $p< 0.05$, respectively). Therefore, the results of this study suggest that hyperthyroid rat cardiac SR may contribute to the cardiac manifestations of the disease.

Since long chain acylcarnitines (LCAC) are known to affect membrane transport proteins, and lipid metabolism and tissue carnitine content are affected by hyperthyroidism, it was investigated whether the carnitine derivatives localized in the SR were affected by the T_3 treatment. The total carnitine content (including free, acid soluble and long chain carnitine) was significantly decreased 24 ($p< 0.05$), 48 ($p< 0.05$) and 72 ($p <0.01$) hours after initiation of the treatment. Acid soluble carnitine levels were not affected. LCAC levels were slightly (but not significantly) decreased at 24 hours, and significantly decreased at 48 and 72 hours ($p< 0.05$ and $p< 0.01$, respectively). There was a strong negative correlation ($r= -.93$) between the increased V_{Ca} and the decreased LCAC content of

the SR. These results suggest a possible relationship between T_3 mediated alterations in lipid metabolism and the increased calcium transport activity of the SR. However other factors may also be involved which contribute to both the augmented calcium transport and the decreased LCAC content of the hyperthyroid cardiac SR.

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AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
ATPase	adenosine triphosphase
C	centigrade
^{14}C	carbon-14
^{45}Ca	calcium-45
cAMP	cyclic adenosine 5'-monophosphate
CoA	Coenzyme A
CPT I	carnitine palmitoyltransferase I
Da	dalton
DOC	deoxycholate
+dP/dT	rate of development of left ventricular pressure
-dP/dT	rate of decline of left ventricular pressure
et al	and others
FFA	free fatty acid
g	gram
K	kilo
K_{Ca}	assocation constant of the enzyme for calcium
L	liter
LCAC	long chain acylcarnitine
LVDP	left ventricular developed pressure
m	milli
μ	micro
M	molar

mg	milligram
min	minute
mL	millilitre
nmole	nanomole
^{32}P	phosphorus-32
pmole	picomoles
SDS	sodium dodecylsulphate
S.E.M.	standard error of the mean
SR	sarcoplasmic reticulum
T_3	triiodothyronine
T_4	thyroxine
TCA	trichloracetic acid
TRIS	tris (hydroxymethyl aminomethane)
w/v	weight per unit volume
V_{Ca}	maximal velocity of calcium transport

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DEDICATION

One father is more than a hundred schoolmasters.

George Herbert, Jacula Prudentum

That best academy, a mother's knee.

James Russell Lowell, The Cathedral

INTRODUCTION

Hyperthyroidism is an endocrine disease which can affect the cardiovascular system. The work of Parry in 1785 first described the association of thyroid enlargement with enlargement or palpations of the heart (Parry, 1825). These initial observations remain valid almost 200 years later as the cardiac manifestations of hyperthyroidism are amongst the more profound alterations and important clinical features of the disease (Williams and Braunwald, 1984). The heart is so typically affected that the absence of a bounding precordium is a point against the diagnosis of hyperthyroidism (DeGroot et al, 1984). Other signs and symptoms of this disease include: weight loss, weakness, dyspnea, increased thirst or appetite, irritability, profuse sweating, sensitivity to heat or increased tolerance to cold. Exopthalmopathy and goiter are also occasionally observed.

The evidence does not support a geographical variation to the incidence of hyperthyroidism (DeGroot et al, 1984). Epidemiological studies from England and Wales have shown the incidence of hyperthyroidism varies from 9.7 to 49.2 cases per 100,000 people (Barker and Phillips, 1984). In Iceland the incidence is 23.6 cases per 100,000 population (Haraldsson et al, 1985). External factors influence the incidence of the disease. The introduction of potassium iodate into commercial baking procedures caused an epidemic two months later in the exposed population (Connolly et al, 1970). The sex distribution

of the disease shows women to be from 4.2 (Harraldsson et al, 1985) to 12 (Tunbridge et al, 1977) times more frequently affected than males.

The causes of hyperthyroidism are most often Graves' disease, toxic multinodular goiter and toxic adenoma (Werner and Ingbar, 1978). In Toronto, Canada, the distribution of the various causes of hyperthyroidism are as follows: Graves' disease, 70%; toxic multinodular goiter and toxic adenoma, 8%; subacute thyroiditis, 15%. (Williams et al, 1983). In Wales, the percent distribution of Graves' disease is the same but toxic multinodular goiter and toxic adenoma account for 25% of all cases (Williams et al, 1983). Graves' disease presents as a diffuse enlargement of the thyroid gland, and toxic multinodular goiter as an unevenly enlarged gland. The cause of toxic multinodular goiter may be a single hyperfunctioning nodule, or a number of hyperfunctioning nodules (Werner and Ingbar, 1978). The exact etiology of Graves' disease is unknown, but a genetic predisposition, and abnormalities of the immune system have been implicated. Treatment of hyperthyroidism usually involves one of three forms of therapy: (1) destruction of the thyroid by irradiation, (2) blocking thyroid hormone synthesis with anti-thyroid drugs (e.g. propylthiouracil), or (3) partial surgical ablation of the gland (DeGroot et al, 1984).

The disease results from the excess production of the thyroid hormones, triiodothyronine (T_3) or thyroxine (T_4), or both. The thyroid gland is a butterfly-shaped organ located

anteriorly and laterally to the trachea and pharnyx, the isthmus lies across the trachea anteriorly just below the cricoid cartilage (DeGroot et al, 1984). The average weight of the euthyroid gland is 30 grams, increasing to between 30 and 120 grams in hyperthyroidism (Werner and Ingbar, 1971). The functional unit of the gland is the follicle, a spherical arrangement of cells responsible for the uptake, biosynthesis, storage and release of the thyroid hormones (DeGroot et al, 1984). Thyroid hormone secretion is under the influence of a negative feedback system, involving the hypothalamus, anterior pituitary and thyroid gland. The hypothalamus secretes TRH, thyrotropin-releasing hormone, which tonically stimulates the anterior pituitary. TRH receptor stimulation causes the release of TSH, thyroid-stimulating hormone. TSH binds to receptors on the surface of the follicular cells, stimulating the release of thyroid hormones. The primary inhibitor of TSH release is T_3 (Obregon et al, 1980), with circulating T_4 playing a role upon intrapituitary deiodination to T_3 (DeGroot et at, 1984). In Graves' disease TSH levels are usually low or normal, but circulating thyroid stimulators, i.e. long acting thyroid stimulator (LATS), mimic the effect of TSH at the gland and are not subject to negative feedback inhibition. Normal daily secretion of the thyroid hormones is 94-110 μg T_4 and 10-22 μg T_3 (DeGroot et al, 1984). Approximately 25% of T_4 is converted to T_3 via peripheral 5'-monodeiodination, accounting for 80% of circulating T_3. The mean steady state concentration range of T_4 is 60-100 μg/L, and for T_3 the range is 1-2.2 μg/L (DiStefano

and Fisher, 1979). Free concentrations of T_4 and T_3 in the euthyroid adult are 1-3.5 ng/dL and 0.25-0.65 ng/dL serum, respectively (DeGroot et al, 1984). The percentages of free T_4 and T_3 are 0.02-0.04% and 0.2-0.45%, respectively, with the higher percentage of free T_3 due to the relatively lower affinity of plasma proteins for the hormone. In the rat, only 3% of the total T_3 pool resides in plasma while the remainder resides in slowly equilibrating pools (e.g. muscle, skin and brain; 76%) and rapidly equilibrating pools (e.g. liver and kidney; 19%) (DiStefano et al, 1982).

Triiodothyronine is believed to be the physiologically active hormone (DeGroot et al, 1984). T_3 effects are dependent upon transport of free hormone from plasma to cytosol to nucleus. In rat heart, transport from plasma to cytosol is a non-energy dependent, non-stereospecific mechanism but transport to the nuclear compartment is energy dependent and stereospecific (Oppenheimer and Schwartz, 1985). The mechanism of action of T_3 involves an interaction with specific receptors within the nucleus (Oppenheimer, 1979). Cardiac receptors are high affinity (K_D 0.9×10^{-10} M to K_D 4.2×10^{-10} M) and low capacity (0.14 pmol/mg DNA to 0.5 pmol/mg DNA) (Ladenson, 1984). The receptor is a nonhistone nuclear protein of M_r 50,000 (Oppenheimer, 1985). That nuclear binding sites are the functional T_3 receptors is supported by data showing 1), the close correlation between the nuclear binding affinity and the thyromimetic effect of T_3 analogs and 2), increases in nuclear

processes such as the rate of formation of polyadenylated messenger RNA, RNA polymerase activity and the presence of mRNA encoding for T_3 inducible protein (α-2u globulin and pituitary growth hormone) (Oppenheimer, 1979). There is a positive correlation between nuclear binding of T_3 and stimulation of glucose uptake in cultured rat heart cells (Tsai and Chen, 1976). T_3 therefore exerts its hormonal effect through nuclear interactions in T_3 responsive tissues.

CARDIOVASCULAR EFFECTS OF HYPERTHYROIDISM

The cardiovascular consequences of experimentally induced hyperthyroidism are consonant with the expected hyperdynamic state. The maximum rate of tension development, the isometric developed tension and the rate of development of isometric tension are increased in isolated papillary muscle from hyperthyroid cat (Buccino et al, 1967; Taylor, 1970). Similar results have been found in hyperthyroid guinea pig papillary muscle (Goodkind et al, 1974). Hyperthyroid dog left ventricle develops less tension during isovolumetric contraction, and the time to peak tension is decreased (Taylor, 1969). As well left ventricular contractile element velocity is augmented in the hyperthyroid dog heart. The hyperthyroid isolated working rat heart has an elevated spontaneous heart rate, increases in both the rate of development (+dP/dT) and the rate of decline of left ventricular pressure (-dP/dT), and the left ventricular

developed pressure (Marriott and McNeill, 1983). This study also showed that the time to peak left ventricular pressure (LVDP), and ventricular relaxation time, are decreased. A more recent study (Brooks et al, 1985) confirmed the results of Marriott and McNeill (1983), and demonstrated increases in cardiac output and coronary flow in the hyperthyroid isolated working rat heart. The experimental evidence in hyperthyroidism, therefore, is equivocable with respect to changes in various indices of cardiac function in a number of different species.

In man, similar cardiac alterations are observed. Cardiac output, resting heart rate, rate of development of left ventricular pressure and velocity of contraction are all increased in the hyperthyroid state (Klein and Levey, 1984). Other cardiovascular manifestations of hyperthyroidism include palpitations and systolic hypertension (Braunwald, 1984). Resting left ventricular ejection fraction is also augmented in man (Shafer and Bianco, 1980; Fofar et al, 1982). The response of the hyperthyroid heart to exercise suggests the presence of a cardiomyopathy since left ventricular ejection fraction increases in response to exercise in controls, but does not increase, or declines, in hyperthyroid subjects. This is a reversible myopathy, as restoration to the euthyroid state returns the exercise response to normal; this return to normal lags behind the return of euthyroid hormone levels by several weeks (Fofar et al, 1984). These results are in

contradistinction to animal studies showing left ventricular function to be augmented (increased LVDP and +/- dP/dt) (Marriott and McNeill, 1983) with increasing left atrial filling pressures. However, because the left ventricular ejection fraction was not directly measured *in vitro* (rat heart), as it was *in vivo* (human heart), it cannot be denied that the left ventricular ejection fraction *in vitro* may have mimicked the *in vivo* response to the hyperthyroid condition. Further investigation measuring the left ventricular ejection fraction in rats will determine if the myopathy exhibits similar exercise responses in species other than man.

The cardiac manifestations of hyperthyroidism observed in both experimental animals and man could be due to either a direct effect of T_3 on myocardial nuclear receptors and the subsequent augmentation of protein synthesis and the physiological response or a physiological adaptation of the heart to the increased demand imposed upon it by T_3 induced changes in peripheral metabolism. There is evidence for both possibilities.

Isolated chick ventricular myocytes treated with T_3 respond with an increased rate of protein synthesis (10%-16%) and rate of cell growth (20%-40%) (Carter *et al*, 1985). Isolated ventricular myocytes also spontaneously contract at a faster velocity than control myocytes (Kim and Smith, 1985). These changes occur independently of an increased demand in work load and result from the T_3 treatment. Klein and Hong (1986), however, have shown that although the *in situ* heart responds to

T_4 treatment with increases in cardiac weight, protein, and total myosin content, the in vivo non-working heterotopically transplanted heart does not. The effect of hyperthyroidism on the heart may be a result of either possibility or a combination of the two. A number of parameters affecting cardiac contractility have been investigated in an attempt to define the biochemical mechanisms responsible for the augmented cardiac function in the disease state.

INVOLVEMENT OF THE AUTONOMIC NERVOUS SYSTEM

The cardiovascular manifestations of hyperthyroidism closely resemble conditions of catecholamine excess. The accelerated heart rate of hyperthyroid patients is attenuated by β-adrenergic receptor blockade (Grossman et al, 1971). These observations, and those demonstrating that, (1) circulating catecholamine levels are depressed in hyperthyroidism, (2) β-adrenergic receptors are not activated by thyroid hormones and (3) thyroid hormone levels are not affected by β-adrenergic antagonists (Williams and Lefkowitz, 1983), suggest the possibility that altered catecholamine responsiveness of the cardiac tissue may be responsible for the observed cardiac alterations of hyperthyroidism. Experimental studies show cardiac responsiveness to be either unaffected (Buccino et al,

1967; Levey et al, 1969; Carrioli and Crout, 1967; Goodkind, 1967; Aoki et al, 1967, 1972; Young and McNeill, 1974) or augmented (Coville and Telford, 1970; Hashimoto and Nakashima, 1978; MacLeod and McNeill, 1981; Fox et al, 1985) by the hyperthyroid state. Adenylate cyclase responsiveness is also controversial, as both an increase (Tsai and Chen, 1978; Tse et al, 1980) and no change (Sobel et al, 1969; McNeill et al, 1969) in the magnitude or the sensitivity of the response to catecholamines has been reported. The activity of cAMP-dependent protein kinase in response to both noradrenaline and isoproterenol is significantly decreased in hyperthyroid rat heart (Katz et al, 1977; Tse et al, 1980), suggesting that this step in the β-adrenergic response is not responsible for the possible supersensitivity of the heart to catecholamines.

Studies on the influence of hyperthyroidism on β-adrenergic receptor density in rat heart report that with very few exceptions, receptor binding increases (176 +/- 98%; mean +/- S.D.) in response to thyroid hormone (Bilezikian and Loeb, 1983). These changes occurr in the absence of any significant effect on membrane affinity for the binding ligand. In contrast to β-adrenergic receptor density, in rat heart the binding of α-receptor ligands decreases by 40%, and α-antagonist binding affinity decreases by a factor of two in rat heart.

It is apparent, therefore, that the hyperthyroid state alters adrenergic receptor pharmacology. The evidence showing an enhanced responsiveness of cardiac tissue to catecholamines and increased β-receptor density is consistent with an

adrenergic receptor mediated hypothesis of altered cardiac function. However the results concerning both the sensitivity of the hyperthyroid heart to catecholamines and the sensitivity of adenylate cyclase are not unequivicol. Until the technical, species and/or other experimental differences responsible for the discrepant results are resolved, the adrenergic receptor hypothesis does not present a sufficiently consistent case to explain the altered cardiac function of hyperthyroidism. This suggests that other mechanisms, perhaps more directly involved in the contractile process, may have a role to play.

EFFECTS OF HYPERTHYROIDISM ON CONTRACTILE PROTEINS

The augmented cardiac contractile force of hyperthyroidism may be related to changes in myosin ATPase activity of the heart. Several reports have documented an increase in ventricular myosin ATPase activity in response to thyroid hormone treatment in a number of different species including dog, rabbit and mini-pig (Conway et al, 1976; Takeo et al, 1984; Wiegand et al, 1985). The hyperthyroid rat, however does not demonstrate an enhanced myosin ATPase activity (Rovetto et al, 1972; Yazaki et al, 1975). Rat myosin ATPase activity is depressed in hypothyroidism but can be shifted to euthyroid levels by thyroid hormone treatment (Rovetto et al, 1972),

indicating that the rat myosin ATPase activity is also responsive to thyroid hormones under certain conditions. Kinetic analysis of the ATPase reaction of cardiac myosin subfragment-I of rabbit ventricle, has shown the major kinetic difference between euthyroid and hyperthyroid to be in the rate of ATP hydrolysis (Morkin et al, 1983). Other kinetic aspects of the reaction mechanism, such as the apparent ATP binding constants, and the rate constants for the binding process, were not affected by the hyperthyroid state.

More direct biochemical analysis has revealed that thyroid hormone controls the distribution of a family of ventricular myosin isoenzymes. Flink et al (1979) have shown that thyroxine stimulates the synthesis of a cardiac myosin isoenzyme which differs in the composition of its heavy chains, from that found in the euthyroid rabbit. Rat ventricular myosin has been separated into three isoenzymes (Hoh et al, 1977). The isoenzymes are distinct with respect to the composition of their respective heavy chains and ATPase activities. The isoenzymes, termed V_1, V_2 and V_3, have heavy chain subunits defined as $\alpha\alpha$, $\alpha\beta$ and $\beta\beta$, respectively, with V_1 having the highest, V_2 intermediate and V_3 the lowest ATPase activity. The three isoenzymes are also present in rabbit ventricle (Chizzonite et al, 1984). However, the relative amount of each isoenzyme under euthyroid status differs between rat and rabbit. Rabbit has a myosin distribution of 85% V_3 and 5-10% of V_2 and V_1, and the rat distribution is 80-85% V_1, 5-10% V_2 and 10-15% V_3 (Morkin et al, 1983). In the rabbit, the relative amount of V_1 increases

to 85% of the total following thyroxine administration (Martin et al, 1981). The three myosin isoenzymes are also present in both rat and rabbit atria, but in neither species is the distribution of the isoenzymes affected by thyroid hormone treatment (Chizzonite et al, 1984; Samuel et al, 1986). The isomyosin composition however does affect the velocity of cardiac muscle shortening (Schwartz et al, 1981; Pagani et al, 1984) and, therefore, in species such as the rabbit, where hyperthyroidism increases the proportion of V_1, the effect of an increase in myosin ATPase activity can contribute to the augmented force of contraction. However, because rat ventricle is predominantly V_1 in the euthyroid condition a possible increase in the proportion of the V_1 fraction in hyperthyroidism may not be sufficient to be responsible for the augmented contractile activity seen in the hyperthyroid state. Altered myosin ATPase activity cannot account for other functional changes occurring in hyperthyroidism such as the augmented rate of relaxation, hence other biochemical events such as cellular calcium handling during contraction and relaxation of the myocardium should be considered.

EFFECT OF HYPERTHYROIDISM ON CALCIUM METABOLISM

Cardiac function is dependent upon the temporal flux of calcium ions through membrane systems governing the concentration of ionic calcium within the myoplasm. The three major membrane systems of the cardiac cell are the sarcolemma, sarcoplasmic reticulum and mitochondria. Mitochondrial calcium handling does not contribute to the normal regulation of cardiac contraction because (1) the inhibition of mitochondrial calcium uptake in intact hearts does not alter the time course of relaxation and (2) the kinetics of calcium uptake by the mitochondria is too slow for the organelle to be important in normal calcium cycling (Winegrad, 1982). In certain pathological conditions, such as myocardial ischemia, mitochondrial calcium metabolism can affect contractility (Bourdillon et al, 1981). The sarcolemmal membrane contributes to the regulation of free calcium through a number of mechanisms including the Na/Ca exchanger and the Ca-ATPase (Winegrad, 1982) and the glycocolyx coating external to the sarcolemmal membrane (Langer, 1978). Thyroid hormone mediated alterations to any of these sarcolemmal processes has not been reported. Of pivotal importance is the cardiac sarcoplasmic reticulum (SR), a membrane limited reticular structure of continuous vesicles and tubules forming a network through the myocyte surrounding the myofibrils. The SR is responsible for the accumulation of calcium to promote relaxation (Tada et al, 1978) and calcium release from the SR is responsible for inducing contraction

(Fabiato, 1983).

The enzyme responsible for the energy-dependent vectoral transport of calcium from the myoplasm into the lumen of the sarcoplasmic reticulum is the calcium-activated, magnesium-dependent adenosine triphosphatase (Ca + Mg ATPase). The enzyme accounts for up to 40% of cardiac SR protein and has a molecular weight of approximately 100,000 daltons (Tada et al, 1978). Other protein components of the SR membrane are phospholamban, a 22,000 dalton molecular weight protein involved in the regulation of calcium transport and ATPase activity (Ambudkar and Shamoo, 1984), calsequestrin, a 57,000 dalton calcium binding protein (Campbell et al, 1982), a 53,000 dalton band, accounting for 10-15% of SR protein (Chamberlain et al, 1983), and high-affinity calcium-binding protein (Coll and Murphy, 1984). Depending on the preparative technique used, between 20 and 40 protein bands have been detected in isolated SR membranes (Jones and Besch, 1979; Chamberlain et al, 1983).

The activity of the calcium pump enzyme in the SR is regulated by ions and regulatory proteins within the cell. Calcium ions autoregulate calcium transport by the SR such that increases in cytoplasmic free calcium stimulate ATP hydrolysis and calcium transport (Hasselbach, 1964). The rate of calcium uptake by the SR follows typical Michealis-Menton kinetics, with a half-maximal stimulation by 1 μM calcium (K_{Ca} 1 μM) (Tada et al, 1974). Calcium transport by cardiac SR is stimulated by monovalent cations; potassium augments both calcium ATPase and

calcium transport five-fold (Jones et al, 1977). Potassium ion enhances the rate of dephosphorylation of the enzyme, thereby increasing the rate of enzyme turnover, expressed as increases in ATPase and calcium uptake activity.

The augmentation of cardiac contractility and relaxation by β-adrenergic receptor stimulation is well documented (Tsien, 1977). β-adrenergic receptor stimulation increases adenylate cyclase activity, resulting in elevated cAMP levels (Tsien, 1977). Cyclic-AMP promotes the release of the catalytic subunit of cyclic AMP dependent protein kinase from the regulatory subunit. The catalytic subunit catalyses the phosphorylation of phospholamban (Tada et al, 1975). The close association of phospholamban with the Ca-ATPase enzyme, and the structural perturbation induced by phosphorylation, results in augmentation of calcium uptake, with phospholamban serving as either an activator (Tada et al, 1978) or derepressor (Hicks et al, 1979) of the enzyme.

SR calcium transport and calcium ATPase activity are also regulated by calmodulin, a 17,000 dalton acidic protein (Katz and Remtulla, 1978; Lopaschuk et al, 1980; Davis et al, 1983). Calmodulin requires the presence of calcium and possibly a specific calmodulin dependent protein kinase to augment SR calcium transport. The mechanism by which calmodulin stimulates calcium transport is similar to that of cAMP-dependent protein kinase: the receptor protein for the kinase mediated phosphorylation is phospholamban (LePeuch et al, 1979), and the increased pump activity is due to the interaction of the

covalently modified phospholamban with the enzyme. Cyclic AMP-dependent protein kinase and calcium-calmodulin dependent protein kinase phosphorylate phospholamban at distinct sites and their effects are additive. It has also been suggested that calmodulin acts directly on the Ca-ATPase enzyme itself and not through the kinase phospholamban system (Katz, 1980).

Cardiac SR is also regulated by a calcium-activated, phospholipid-dependent protein kinase (protein kinase C). This enzyme catalyses the phosphorylation of SR resulting in augmented calcium transport (Limas, 1980) and calcium ATPase (Movsesian et al, 1984) activity. This effect may also be mediated by phospholamban phosphorylation (Movsesian et al, 1984).

The cardiac sarcoplasmic reticulum is, therefore, intimately involved in contraction and relaxation processes that take place in the heart. SR calcium transport is a dynamic process affected by calcium, potassium and the three regulatory proteins described above. Previous studies have shown augmented cardiac SR calcium uptake and calcium ATPase from hyperthyroid rat and rabbit heart (Suko, 1974; Limas, 1978a; Guarnieri et al, 1980). The results describing changes in hyperthyroid cardiac SR are not unequivocal however, as a decreased calcium uptake activity has been shown in both rat (Takacs et al, 1985) and canine (Conway et al, 1976) heart. The investigations of Limas (1978a) probed for possible mechanisms of the augmented SR function, and demonstrated an increase in the steady-state levels of the

calcium transport phosphoprotein intermediate formed during the translocation reaction. This response, in addition to calcium transport, was prevented by inhibitors of protein synthesis. The enhanced cAMP-dependent protein kinase phosphophorylation of phospholamban observed in hyperthyroid rat heart SR may indicate increased levels, or activity of, endogenous cAMP-dependent protein kinase (Limas, 1978b). These results are at variance with a subsequent report (Guarnieri et al, 1980) where, although basal calcium uptake was augmented in hyperthyroid rat heart SR, the response to exogenous cAMP-dependent protein kinase was not significantly different from controls. This latter study had results in accord with those of Katz et al (1977), as no difference in contractile response to dibutryl cAMP could be discerned between control and hyperthyroid rat heart. Katz et al (1977) also showed that the activation of cAMP-dependent protein kinase in response to dibutyrl cAMP was not altered by the hyperthyroid state. These studies on the effect of thyroid hormone on the SR calcium pump indicate that more information is required before all mechanisms can be defined which explain the enhanced SR function in hyperthyroidism.

METABOLIC ASPECTS OF HYPERTHYROIDISM

Hyperthyroidism induces changes in cardiac protein, carbohydrate and lipid metabolism (Muller and Seitz, 1984a, 1984b, 1984c) and results in increased cardiac protein content. This is due in part to a stimulation of protein synthesis (Carter et al, 1985) and no concomitant, or a reducing effect on, protein catabolism (Carter et al, 1980). The effects of hyperthyroidism on protein metabolism are organ-specific, as skeletal muscle mass and sarcoplasmic reticular protein levels are decreased in response to the hormone. With respect to carbohydrate metabolism, cardiac glycogen catabolism is sensitive to the tissue levels of phosphorylase a, the active form of the enzyme responsible for the formation of glucose -1-phosphate from glycogen. The catalytic activity of phosphorylase is dependent upon it being in the active phosphorylase a, or phosphorylated, state. Phosphorylation of the enzyme is mediated by phosphorylase kinase, which in turn is regulated by calcium and cAMP-dependent protein kinase (Taegtmeyer, 1985). Adrenergic agents which increase tissue cAMP levels, as well as the influx of calcium during depolarization, therefore, influence the catabolism of glycogen. In the hyperthyroid rat heart, it has consistently been demonstrated that basal phosphorylase a levels are increased and that the elevation in phosphorylase a in response to norepinephrine is augmented (McNeill and Brody, 1968; McNeill et al, 1969; Young and McNeill, 1974). Calcium ion mediated

activation of phosphorylase a is not affected by hyperthyroidism (Hartley and McNeill, 1976). The metabolic consequence of elevated phosphorylase a activity would be decreased tissue glycogen stores which has been reported in hyperthyroid guinea pig heart by Bressler and Wittels (1966). In spite of lowered glycogen, which may have raised the oxidizable pool of glucose, glucose metabolism is decreased. Lactate metabolism is also depressed in hyperthyroid rat heart owing to the inhibition of pyruvate dehydrogenase resulting from an elevation in free fatty acid metabolism (Fintel and Burns, 1982).Long chain fatty acid metabolism is elevated in hyperthyroid guinea pig heart as well (Bressler and Wittels, 1966).

Hyperthyroidism may affect cardiac lipid metabolism because of the increased serum free fatty acid concentration occurring in the disease (Muller and Seitz, 1984). This would result in greater substrate delivery to the heart, as the heart derives all of its free fatty acids from the circulation either via passive diffusion or active transport across the sarcolemma (Bieber and Fiol, 1985). Hyperthyroidism also influences lipid metabolism by its effect on carnitine palmitoyltransferase I. Carnitine palmitoyltransferase I is an inner mitochondrial membrane bound enzyme responsible for the transacylation of acyl-CoA thioesters to acylcarnitine esters (Schulz, 1985). Acylcarnitine formation is required for transfer of the acyl groups into the mitochondrial matrix by carnitine:acylcarnitine translocase for subsequent β-oxidation. Carnitine

palmitoyltransferase I has been suggested to be the rate limiting enzyme for free fatty acid metabolism (McGarry et al, 1978). In hyperthyroid rat liver (Stakkestad and Bremer, 1982) and guinea pig heart (Bressler and Wittels, 1966) the activity of the enzyme is enhanced. Malonyl-CoA, an endogenous inhibitor of the enzyme (McGarry et al, 1978), retains its inhibitory effect in hyperthyroidism but the magnitude of inhibition is reduced (Stakkestad and Bremer, 1982).

Long chain acylcarnitines, metabolic intermediates of fatty acid metabolism, have been shown to be potent endogenous inhibitors of a number of membrane bound enzymes. Palmitoyl carnitine is the most abundant of the acylcarnitines and most frequently studied. DL-palmitoylcarnitine has been shown to inhibit calcium-independent phosphodiesterase (Katoh et al, 1982), calcium-dependent phospholipid-sensitive protein kinase (Wise and Kuo, 1983) and Na,K-ATPase (Wood et al, 1977) from cardiac tissue. Sarcoplasmic reticular calcium uptake and calcium ATPase activities are also inhibited by palmitoylcarnitine (Pitts et al, 1978). Accumulation of long chain acylcarnitines in the ischemic heart has been documented (Idell-Wenger et al, 1978) and inhibition of sarcoplasmic reticular function by these intermediates proposed as a mechanism for the loss of contractility observed following ischemia (Pitts et al, 1978). Accummulation of long chain acylcarnitines in SR fractions of diabetic rat heart and the concomitant depression both in SR calcium transport and cardiac function has been proposed as a contributing factor to the

depressed cardiac function observed in diabetes (Lopaschuk et al, 1983). Long chain acylcarnitine levels are, therefore, dynamic. In vitro studies have demonstrated that long chain acylcarnitines have the capacity to inhibit calcium fluxes across the sarcoplasmic reticulum. In hyperthyroid rat heart tissue total carnitine levels are depressed (Suzuki et al, 1983) and free and short-chain acylcarnitine levels are depressed in mouse cardiac tissue (Cederblad and Engstrom, 1978); long chain acylcarnitine levels were not reported in either paper. Levels of long chain acylcarnitines are detected in SR membrane fractions from control heart indicating that the metabolic intermediates are not a consequence of disease alone. The possibility exists that the presence of endogenous long chain acylcarnitines in SR membranes could play a role in calcium transport in vitro, and possibly in cardiac function in vivo.

As mentioned earlier, cardiac contractility is increased and relaxation time decreased in hyperthyroid rat heart. The close association of cardiac SR in these processes indicates that it may be involved in these changes. Since studies have shown both an increase (Limas,1978a) and a decrease (Takacs et al, 1985) in hyperthyroid rat cardiac SR calcium transport, it was decided to determine the effect of hyperthyroidism on rat cardiac SR using an experimental model which has previously demonstrated the above cardiac alterations. To more closely monitor the possible effect of hyperthyroidism on the SR it was decided to study the SR during the progression of the disease. It has been suggested

that lipid metabolic changes could affect cardiac function through their effects on membranes (Katz and Messineo, 1981). Since there is an alteration in cardiac free fatty acid metabolism and a decrease in the levels of the essential cofactor carnitine in the hyperthyroid heart, it was decided to determine if the levels of the carnitine derivatives associated with the SR membrane are affected by the hyperthyroid state, and if alterations can be detected, the relationship to possible changes in calcium transport activity determined.

MATERIALS AND METHODS

I.Materials

A. Animals

Male Wistar rats in the weight range of 250-300g were utilized in this study. The rats were obtained from the U.B.C. Animal Care facilities.

B. Chemicals

Chemicals, proteins and materials used were purchased from the following sources:

1. Sigma Chemical Co.
   - Acetyl Coenzyme A
   - Bovine Serum Albumin
   - L-Carnitine
   - Carnitine Acetyl Transferase
   - Copper Sulfate
   - Deoxycholic Acid
   - Disodium ATP
   - Dowex 1X8-400
   - EGTA
   - Folin-Ciocalteu Phenol Reagent
   - Histidine
   - Hydroxylamine
   - Magnesium Chloride

Sodium Azide

Sodium Bicarbonate

Sodium Potassium Tartrate

Sucrose

Trichloroacetic Acid

L-3,5,3'-Triiodothyronine

Tris-ATP

Tris-Base

Tris-HCl

Tris-Oxalate

2. BDH

Calcium Chloride

Charcoal (Norit A (alkaline))

Potassium Chloride

Sodium Acetate

Sodium Hydroxide

3. Amersham

Amerlex T-3 RIA kit

^{45}Ca

^{32}P-ATP

4. Fisher

Potassium Hydroxide

Scintiverse II

5. Chemonics Scientific
   Sodium Chloride

6. Amachem
   Potassium Phosphate Monobasic

7. Pierce Chemical Co.
   Sodium Tetrathionate

8. J.T.Baker Chemical Co.
   Hydrochloric Acid

9. New England Nuclear
   Aquasol

10. ICN
    1-^{14}C-Acetyl Coenzyme A

11. Allied Chemical
    Perchloric Acid

12. Millipore Corporation
    HA 0.45 μ filters

13. Whatman Ltd.
    GF/A 2.4 cm filters

II. Methods

1. Hormone Treatment Protocol

Animals were randomnly assigned to either control (vehicle treated) or test (L-3,5,3'-triiodothyronine (T_3) treated) groups. To induce the hyperthyroid state, the male Wistar rats were treated with T_3 at a dose of 500 µg/Kg/day. The T_3 was dissolved in 0.01 N NaOH (1.0 mg/mL) and injected subcutaneously. Following treatment (between 0800 and 0900 hours) the animals were housed in group cages (3-4 animals/cage) where food and water were available ad libitum. Animals were sacrificed for study 12, 24, 48 and 72 hours after hormone treatment was initiated.Animals in the 12 and 24 hour groups received a single dose of T_3 and were studied 12 and 24 hours after this single dose, respectively. Animals in the 48 hour group received two doses and were sacrificed 24 hours after the second dose. Those in the 72 hour group received three doses and were sacrificed 24 hours after the last dose.

2. Preparation of Cardiac Sarcoplasmic Reticulum

Cardiac sarcoplasmic reticulum (SR) was prepared as previously described (McConnaughy et al, 1979) with slight modifications (Dr.G.D.Lopaschuk, personal communication). Following decapitation of the rat, the heart was quickly removed and rinsed of blood in ice cold 10 mM $NaHCO_3$, pH 7.4. The heart was trimmed of pericardial fat, connective tissue, large vessels and atria, blotted dry on absorbent tissue paper and weighed.

The ventricular tissue was first minced with scissors, followed by two 15 second homogenizations with a Kinematica^{R} tissue homogenizer (5 seconds rest between homogenizations) at speed 4. The homogenate was diluted to a volume of 25 mL with the homogenization buffer, and centrifuged at 500 xg for 5 minutes (all centrifugation at 4^{o}c). The resulting pellet was discarded and the supernatant centrifuged at 7000 xg for 15 minutes. The pellet was discarded and the supernatant centrifuged at 31,000 xg for 30 minutes. Following this step the supernatant was discarded and the pellet resuspended in 12 mL of media of 0.6 M KCl and 30 mM histidine-Cl, pH 7.0, and centrifuged at 31,000 xg for 30 minutes. The final pellet was resuspended in 0.75 mL of a media consisting of 0.25 M sucrose, 0.3 M KCl and 0.1 M Tris-Cl, pH 7.2. Aliquots of the SR were either quick frozen in liquid nitrogen and stored at -80^{o}c or, assayed immediately following isolation.

3. Measurement of Calcium Uptake Activity

SR calcium uptake activity was determined as follows: 5-10 μg of SR protein was incubated at 30^{o}c for 5 minutes in a reaction media containing (in mM): histidine-Cl, pH 6.8 40; KCl 110; $MgCl_2$ 5; NaN_3 5; tris-oxalate 2.5; tris-ATP 5. The reaction was started with the addition of EGTA buffered calcium (0.1 - 5.3 μM free, final concentration, 126 nmoles total added, containing $^{45}CaCl_2$; 100,000-200,000 dpm/tube to monitor calcium transport). The reaction proceeded for 5 minutes and was terminated with the filtration of an aliquot of the reaction

mixture through a Millipore^{R} HA 0.45 μ filter. The filter was washed once with 15 mL 40 mM Tris-Cl, pH 7.2, dryed and counted in 5 mL liquid scintillation cocktail.

The rate of calcium uptake activity was determined according to the following equation:

Calcium Uptake (nmoles/mg SR protein/minute)=

(S.C.-B.C.)/(T.C.-B.C.) x (D.F./R.T.) x (Total Ca^{2+}/mg SR)

Where,

S.C. = Sample Counts = dpm obtained in sample

T.C. = Total Counts = total dpm added to reaction

B.C. = Background Counts = dpm present in 5 mL scintillation cocktail alone

D.F. = dilution factor to correct for the volume of the reaction counted = 1.22

R.T. = reaction time = 5 minutes

Total Ca^{2+} = total calcium present in the reaction media

mg SR = mg of SR protein present in the reaction

4. Measurement of SR Carnitines

The determination of the levels of the total, acid soluble and long chain acyl carnitines were made following isolation of these fractions from an aliquot of SR.

A. Isolation of Total, Acid Soluble and Long Chain Acyl Carnitines

An aliquot of SR (100-200 μg protein) was centrifuged at 40,000 xg for 45 minutes, the supernatant discarded, and the pellet resuspended in 0.6 mL ice cold 6% perchloric acid. From this suspension an aliquot of 0.1 mL was isolated for determination of total carnitine. This aliquot was neutralized with 0.075 mL 2 M Tris base and placed on ice. The remaining suspension was centrifuged at 12,000 xg for 10 minutes. A 0.20 mL aliquot of this supernatant was isolated for the determination of acid soluble carnitine, neutralized with 0.15 mL 2 M Tris base and placed on ice. The pellet, containing the long chain acylcarnitine fraction, was washed with ice cold 6% perchloric acid, and resuspended in 0.1 mL distilled water. To both the total carnitine and the long chain acylcarnitine fractions, 0.1 mL 1 M Tris base and 0.05 mL 0.4 N KOH were added and the samples hydrolysed at 70°C for one hour. Following the hydrolysis the samples were neutalized with 0.2 mL 0.575 N HCl, and assayed as free carnitine.

B. Determination of Carnitine Content

The method used to determine the amount of carnitine present in the sample was that of McGarry and Foster, (1976). A volume of 0.15 mL of the carnitine sample (total, acid soluble or long chain) was added to 1.05 mL of reaction media containing 120 μM Tris-Cl pH 7.3, 2 μM sodium tetrathionate and 25 nM acetyl-CoA (0.025 μCi 1-^{14}C-acetyl-CoA). The reaction was initiated by the

addition of 0.01 mL carnitine acetyl transferase suspension (1 unit), and proceeded for 30 minutes at room temperature. The reaction was terminated by the addition of 0.3 mL of a suspension of Dowex^{R} 1X8-400 anion exchange resin (0.22 mL water in 0.3 mL suspension),vortexed and the reaction vessel placed in an ice bath. The tubes were vortexed twice more at 10 minute intervals, each time being replaced into the ice bath. Following the vortexing the tubes were centrifuged at 1500 xg for 10 minutes. A 0.5 mL aliquot of the supernatant was added to 5 mL liquid scintillation fluid and counted. The amount of ^{14}C-acetyl-L-carnitine formed is stoichiometrically related to the amount of carnitine present in the sample.

5. Determination of SR Phosphoprotein Levels

The SR (0.15 - 0.35 mg/mL) was preincubated at 30^{o}c for 5 minutes, then transferred to a 10^{o}c water bath for a further 5 minute preincubation. The reaction was initiated by the addition of the SR membranes to the reaction media containing (final concentration) histidine-Cl, pH 6.8, 40 mM; $MgCl_2$, 0.01 mM; Tris-EGTA, pH 7.4, 0.1 mM and Tris-ATP 2 μM (containing ^{32}P-ATP at a specific activity of 2500 dpm/pmole total ATP). The phosphorylation reaction proceeded for 15 seconds and was terminated by the addition of 0.4 mL of an ice cold stop solution (5% w/v TCA, 5 mM Na_2ATP and 2 mM KH_2PO_4), vortexing and placing the reaction vessel in an ice bath.

The reaction mixture was filtered through a Whatman^{R} GF/A

filter, the filter washed with 30 mL ice cold 5% w/v TCA and dryed and counted in 5 mL liquid scintillation fluid.

The amount of phosphoprotein formed is calculated according to the following equation:

Phosphoprotein (pmol/mg protein) = (S.C.-B.G.)/(S.A.)(R.V.)(P.T.)

Where,

S.C. = Sample Counts = dpm obtained in sample

B.G. = Background = dpm from scintillation cocktail alone

S.A. = Specific Activity = (Media Counts - Background)/Total ATP

R.V. = Reaction Volume = 0.2 mL

P.C. = Protein Concentration (mg SR protein/mL)

6. Hydroxylamine Treatment

The SR was phosphorylated under the reaction conditions described above and the reaction stopped by the addition of 0.4 mL of ice cold 15% w/v TCA. The SR membranes were centrifuged at 1500 xg for 10 minutes, and the supernatant discarded. The pellet was resuspended in 0.5 mL of either 0.6 M hydroxylamine/ 6.8 M sodium acetate, pH 5.2, or 0.6 M sodium chloride/ 6.8 M sodium acetate, pH 5.2, and incubated at room temperature for 10 minutes, followed by the addition of 2.0 mL of ice cold 5% w/v TCA. The SR was pelleted by centrifugation at 1500 xg for 10 minutes, the supernatant discarded and the pellet resuspended in 0.5 mL 5% w/v TCA. The suspension was filtered on a Whatman^{R} GF/A filter which was dryed and counted in 5 mL of liquid scintillation cocktail.

7. SDS-Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed in 1.5 mm thick gels according to a modification of the method of Laemmli and Favre (1973): The sample buffer was identical to the 0.0625 M Tris-Cl, pH 6.8 buffer used by these workers. The stacking gel was 5% (w/v) acrylamide, 0.1% (w/v) SDS and 0.315 M Tris-HCl, pH 6.8; the separating gel was a 5-20% (w/v) acrylamide and a 0.13-1.0% (w/v) bis-acrylamide gradient in 0.375 M Tris-HCl, pH 8.8 and 0.1% (w/v) SDS. Following electrophoresis at 15 ma/slab for 16 hours, gels stained with $AgNO_3$ (0.1%), according to Morrisey (1981).

8. Protein Assay

SR protein was quantified using a DOC/TCA precipitation modification of the Lowry (Lowry et al, 1951) protein assay, as described by Peterson, (1977). A 10 µL aliquot of the final SR suspension was added to 1.490 mL of distilled water. To this was added 12.5 µL of 2% DOC, the test tube vortexed and allowed to stand 10 minutes at room temperature. Following the incubation, 0.5 mL of ice cold 24% TCA was added, the tube vortexed, then centrifued at 1500 xg for one hour. The supernatant was carefully aspirated and discarded. To the pellat was added 1.0 mL of copper reagent (1 mL 2% $NaHCO_3$ in 0.1 N NaOH, 0.01 mL 2% NaK tartrate and 0.01 mL 1% $CuSO_4$ per mL of reagent), the tubes vortexed and incubated at room temperature for 10 minutes. This was followed by the addition of 0.1 mL of a 50/50 Folin-Ciocalteu phenol reagent/water mixture. After a minimum of one

hour incubation at room temperature, the absorbance was read at 660 nm.

9. Determination of the Free Calcium Concentrations

Free calcium concentrations were calculated using a FORTRAN program, TCATIONS.BC (modified slightly from Goldstein, (1979)). Association constants were taken from Martell and Smith (1979-1982); except in the case of monoprotonated ligands which were calculated as described by Blinks et al (1982). Prior to application of the program, constants were corrected for temperature according to Tinoco et al (1978), using enthalphy values tabulated in Martell and Smith (1978-1982). The constants were also adjusted for ionic strength as described by Martell and Smith (1978-1982) and Blinks et al, (1982). These latter three corrections were done using a BASIC program, APPK.

10. Determination of Serum Free Triiodothyronine Concentration

The concentration of free T_3 in rat serum samples collected at the time of sacrifice were determined with an Amersham Amerlex^R T-3 RIA kit.

11. Statistical Analysis

When two groups were compared, statistical analysis was performed using the unpaired Student's t-test. For multiple comparison, one-way analysis of variance followed by Newman-Keuls' test was used. Probabilities of $p< 0.05$ and $p< 0.01$ were used to define the level of significance.

RESULTS

1. Characterization of the SR Preparation Employed in the Study

A comparison was made of the SR calcium uptake activity from two different SR preparative techniques; Sumida et al (1978) vs. McConnaughey et al (1979). The results shown in figure 1 demonstrate that at all free calcium concentrations tested, the SR prepared according to the modified McConnaughey et al (1979) method transported calcium with a greater velocity and with a V_{Ca} approximately five-fold greater than the Sumida et al (1978) preparation. The enhanced calcium transport activity of the preparation (McConnaughey et al, 1979) was considered important when attempting to discern possible differences in SR calcium transport activity between the control and T_3 treated animals. The McConnaughey et al (1979) preparation was therefore used throughout the study.

To ascertain the possible contribution of mitochondrial membrane contamination in the SR preparation, calcium uptake activity in the presence and absence of 5 mM NaN_3 was determined. Figure 2 shows that the calcium uptake activity determined under the experimental conditions employed is independent of mitochondrial calcium transport processes.

Since the objective of the study was to determine if differences could be detected between control and T_3 treated rat heart SR with respect to a number of parameters, it was decided that all parameters would be assayed from each individual SR sample prepared. Using this technique interanimal variation in

Figure 1.

Comparison of the Sumida et al (1978) and the McConnaughey et al SR preparative techniques with respect to calcium transport activity. Results shown are a typical experiment. Sumida (●-●), McConnaughey (o-o).

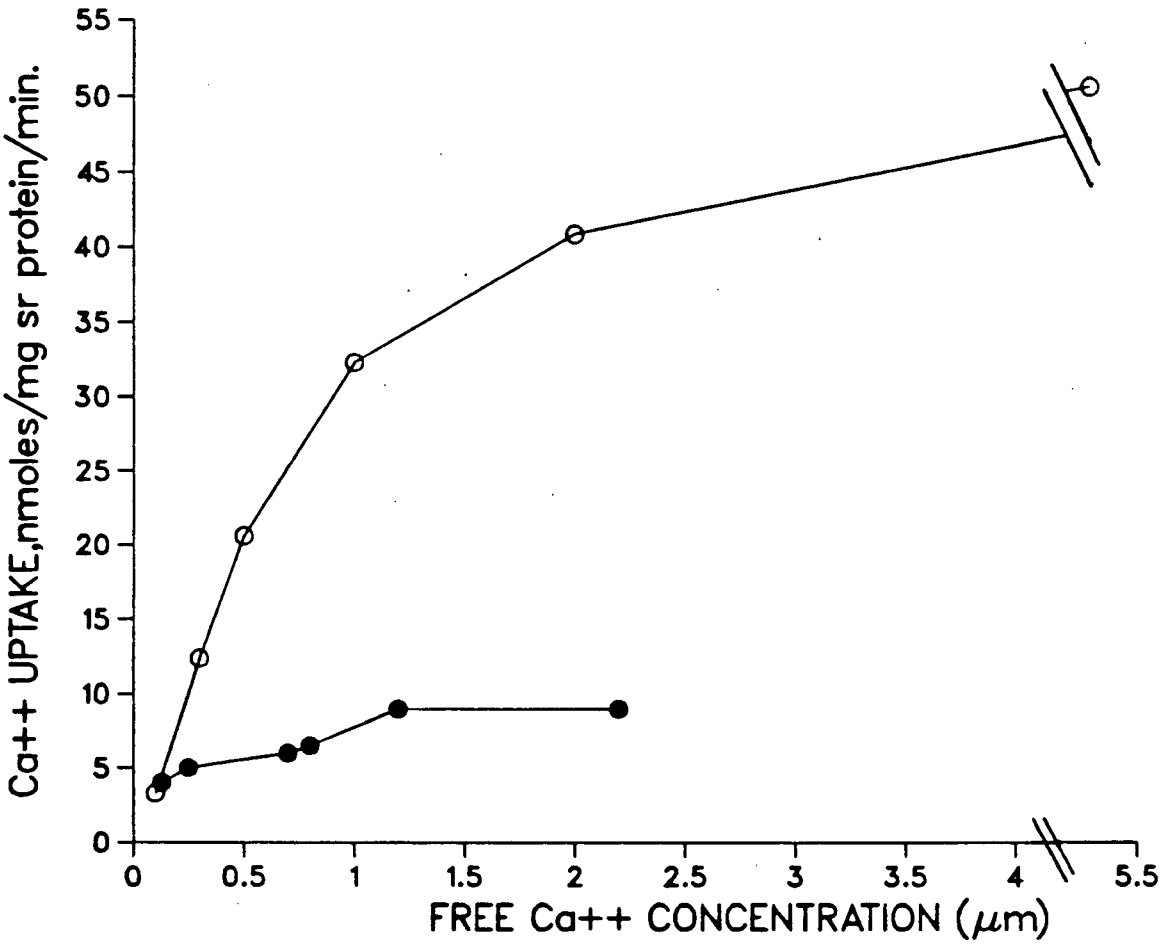
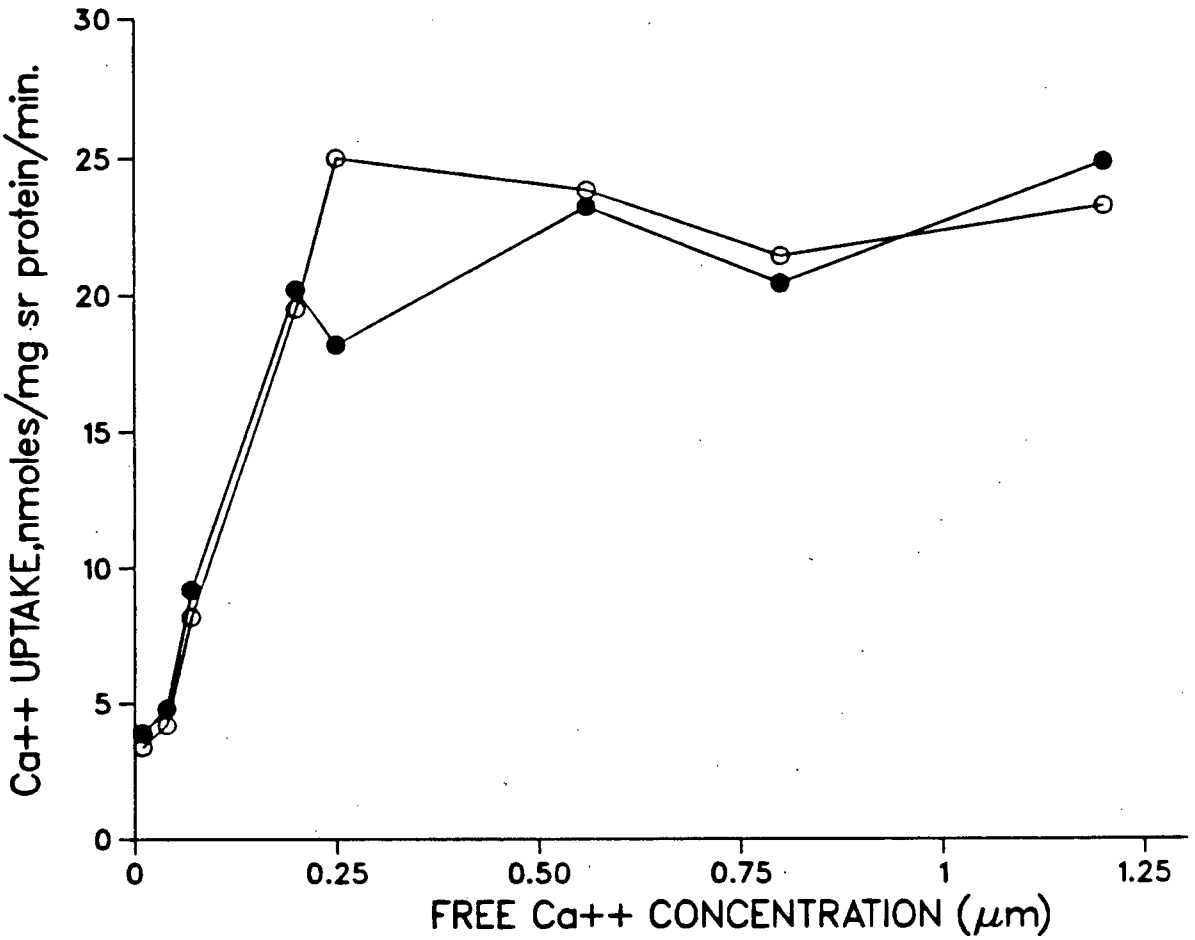

Ca++ UPTAKE,nmoles/mg sr protein/min.
55
50
45
40
35
30
25
20
15
10
5
0
0
0.5
1
1.5
2
2.5
3
3.5
4
5.5
FREE Ca++ CONCENTRATION (μm)

Figure 2.

Calcium activation curve of calcium uptake in the absence (o-o) and presence (●-●) of 5 mM sodium azide. Calcium uptake activity determined as described in Methods. Result shown is a typical experiment.

CALCIUM UPTAKE +/− 5 mM SODIUM AZIDE

vs FREE [Ca++]



the cardiac response to hyperthyroidism was more readily apparent, and differences lost or gained were not masked by a pooling of the cardiac tissues. Therefore, for each group described below, the n value represents the results obtained from separate determinations of the various parameters from the SR yield of single hearts. Preliminary results (data not shown) indicated that the SR yield using the modified McConnaughey et al,(1979) SR preparation would be sufficient for these purposes.

2. Studies on the Effect of T_3 Treatment on Serum Free T_3 Body Weight, Ventricular Weight and SR Protein Yeild

Table 1 shows the serum free T_3 concentrations of the control and treated groups. The data indicate that the animals were in fact clinically hyperthyroid, as they had significantly elevated serum concentrations of T_3. The effect of T_3 treatment on body weight is shown in Table 2, where the vehicle treated controls are compared to the T_3 treated rats. Except for the 12 hour time point, where a slight drop in the mean body weight was observed, the control rats gained weight over the period of study (2%-4%). The mean body weight of the T_3 treated rats decreased over the study period (2%-6%). At no point was the mean body weight observed at sacrifice, significantly different from the initial body weight.

The wet ventricular weight of the control and T_3 treated groups is shown in Table 3. There was no significant difference between the wet ventricular weight of the control and the T_3 treated group 12 hours after a single dose of T_3. At 24 hours,

Table 1.

The Effect Of T_3 Treatment On Serum Free T_3 Concentration

TIME POINT (HR)	SERUM FREE T_3 CONCENTRATION (pmol/l)	
Control	4.9+/- 0.56	(9)
12	23.5+/- 7.5	(3)
24	16.0+/- 1.9	(3)
48	29.8+/- 3.2	(4)
72	121.7+/- 9.6	(7)

Results expressed as the mean +/- S.E.M., Bracketed values (n)= the number of animals.

Table 2.

The Effect of T_3 Treatment on Mean Body Weight

TIME POINT (HR)	GROUP: CONTROL BODY WEIGHT (g)		GROUP: T_3 TREATED BODY WEIGHT (g)	
	INITIAL	AT SACRIFICE	INITIAL	AT SACRIFICE
12	289+/-14	287+/-8 (-1)	288+/-8	275+/-4 (-5)
24	287+/-14	293+/-11 (+2)	286+/-12	280+/-8 (-2)
48	256+/-5	266+/-6 (+4)	253+/-4	246+/-4 (-3)
72	310+/-7	317+/-6 (+2)	308+/-12	289+/-9 (-6)

Control refers to those animals receiving vehicle (0.01 N NaOH) alone. Results are mean +/- S.E.M.,n=6. The bracketed values represent the percentage increase (+) or decrease (-) in mean body weight at sacrifice relative to initial weight.

Table 3.

The Effect of T_3 Treatment on Ventricular Weight (mg)

TIME POINT (HR)	GROUP CONTROL	T_3 TREATED	
12	903+/-23	861+/-16	(N.S.)
24	808+/-84	860+/-30	(N.S.)
48	841+/-16	917+/-20	*
72	906+/-21	1046+/-29	**

The ventricular weight was determined as described in Methods. The results shown are the mean +/- S.E.M., n=6. N.S., non-significant; *, significantly different from control $p< 0.05$; **, significantly different from control $p< 0.01$.

there was a slight but not significant increase (9%) in the wet ventricular weight of the T_3 treated animals. At the 48 hour ($P< 0.05$) and 72 hour ($P< 0.01$) time points the wet ventricular weight was significantly greater in the T_3 treated groups relative to the respective controls.

The heart weight to body weight ratio is an indication of the relative size of the heart. Administration of thyroid hormones has been associated with cardiac hypertrophy and increases in the heart weight to body weight ratio (Marriott and McNeill, 1983; Morkin et al, 1983). Table 4 shows that the ventricular weight to body weight ratio (mg:g) increased with T_3 administration after 24 hours of treatment. The difference increased with the duration of the T_3 treatment. However because the control animals gained weight and the T_3 treated animals lost weight, the ratios in Table 4 may not be a reliable index of hypertrophy . To correct for this, a comparison was made based on initial body weight where there was no difference between the treated and control groups. The ratio of the T_3 treated animals remained greater than the respective controls. The magnitude of the difference was, however, decreased.

A further indication that the T_3 treatment affected the rat heart is given in Table 5, where the amount of SR protein isolated is shown. There was no significant increase in the amount of SR protein isolated 12 hours after a single dose of T_3. However, 24 hours after a single dose, the amount of SR protein isolated from the T_3 treated hearts was significantly greater ($p< 0.05$) than that of the control group. At 48 and 72

Table 4.

The Effect of T_3 Treatment on Cardiac Ventricle Weight to Body Weight Ratio (mg:g)

TIME POINT (HR)	GROUP CONTROL	T_3 TREATED	DIFFERENCE
12	3.15	3.13	0.1
24	2.76	3.07	0.3
48	3.16	3.73	0.5
72	2.86	3.62	0.7

Table 5.

The Effect of T_3 Treatment on the Amount of SR Protein Isolated

TIME POINT (HR)	GROUP CONTROL	T_3 TREATED		n
12	1046+/-76	1005+/-59	(N.S.)	6
24	792+/-38	902+/-36	*	6
48	960+/-60	1146+/-75	*	13
72	1108+/-42	1494+/-107	**	6

The results shown are in μg and are the mean +/- S.E.M., n= sample size. N.S., non-significant difference between control and treated; *, significantly different from control $p< 0.05$; **, significantly different from control $p< 0.01$.

hours there was a significant ($p< 0.05$ and $p< 0.01$, respectively) elevation in the amount of SR protein isolated from the T_3 treated group relative to the controls.

To address the question of whether the changes in the amount of SR protein isolated are due to the overall increased cardiac mass associated with the T_3 treated animals or to a T_3 mediated alteration in the SR protein, the ratio of SR protein isolated to ventricular weight (μg:mg) was calculated (Table 6). The ratio of SR protein to ventricular weight increased in the T_3 treated groups with increasing duration of the treatment, indicating that the SR was altered by the treatment.

Table 6.

The Effect of T_3 Treatment on SR Protein to Ventricular Weight Ratio (µg:mg)

TIME POINT(HR)	GROUP CONTROL	T_3 TREATED	DIFFERENCE
12	1.13	1.17	0.04
24	0.98	1.05	0.07
48	1.14	1.25	0.11
72	1.22	1.43	0.21

3. Studies on the Effect of T_3 Treatment on SR Calcium Transport

The rate of calcium transport was determined in SR assayed immediately after isolation over a free calcium concentration range of 0.1 μM - 5.3 μM. At twelve hours following a single injection of T_3, there was no significant difference in the rate of calcium transport at any of the free calcium concentrations assayed (Figure 3). At 24 hours following the initial dose of T_3, the rate of calcium transport was significantly ($p< 0.05$) greater at all free calcium concentrations assayed (Figure 4). At 48 hours, (Figure 5) there was a further increase in calcium transport in the T_3 treated group ($p<0.01$ at all free calcium concentrations, except 0.5 μM, where the significance remained at $p< 0.05$). At 72 hours, the calcium transport rate was further augmented and was significantly ($p< 0.01$) greater in the T_3 treated SR at all free calcium concentrations assayed (Figure 6). It was determined that the differences observed in the rates of calcium transport between control and T_3 treated groups were not due to differences in non-ATP dependent calcium uptake (data not shown).

Figure 7 shows that when the maximal calcium transport rate (at 5.3 μM free calcium) is plotted as a function of time, the augmentation of calcium transport by T_3 treatment correlates positively ($r=0.997$) with the progression of the disease.

Figure 3.

The effect of T_3 treatment on the rate of calcium transport 12 hours after the first dose. Calcium transport activity of control (o-o) and T_3 treated (●-●) SR was determined as described in Methods. The results are the mean +/- S.E.M. with an n=6 in each group.

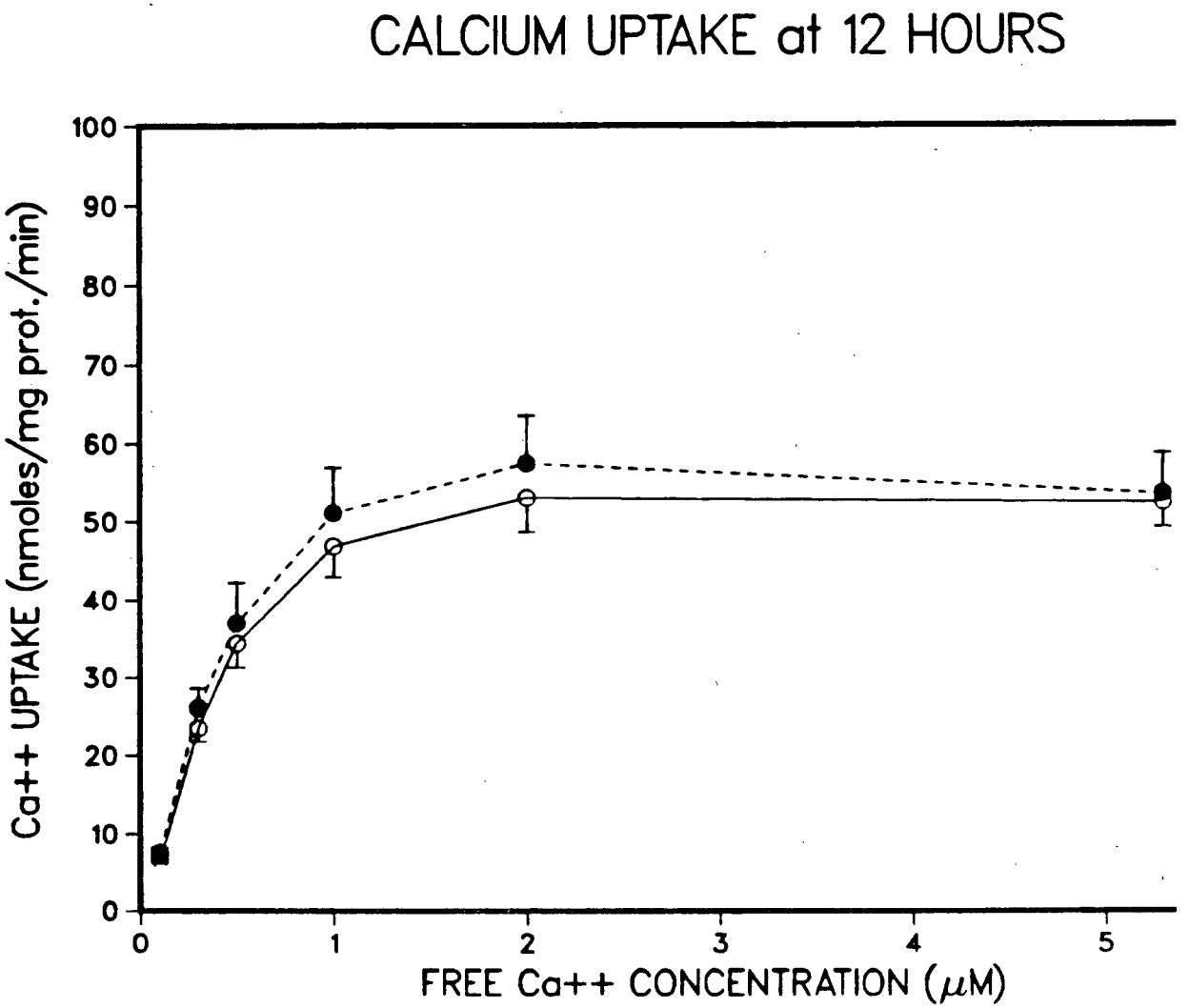

CALCIUM UPTAKE at 12 HOURS
Ca++ UPTAKE (nmoles/mg prot./min)
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
FREE Ca++ CONCENTRATION (μM)

Figure 4.

The effect of T_3 treatment on the rate of calcium transport 24 hours after initiation of the treatment. Calcium transport activity of control (o-o) and T_3 treated (●-●) SR was determined as described in Methods. Results are the mean +/- S.E.M. with an n=6 in each group. *, significantly different from control $p< 0.05$).

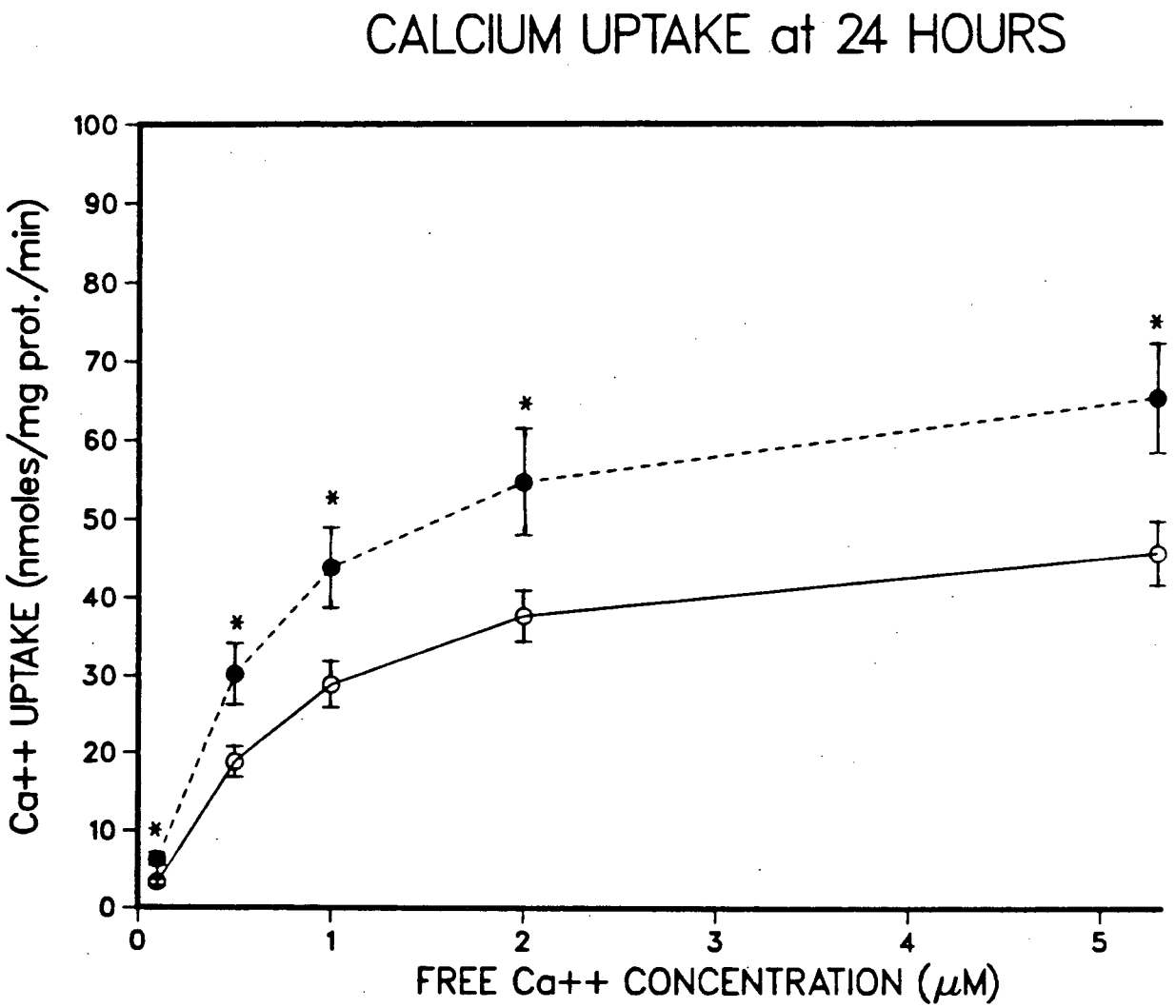

CALCIUM UPTAKE at 24 HOURS
Ca++ UPTAKE (nmoles/mg prot./min)
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
FREE Ca++ CONCENTRATION (μM)

Figure 5.

The effect of T_3 treatment on the rate of calcium transport 48 hours after initation of treatment. Calcium transport activity of control (o-o) and T_3 treated (●-●) SR was determined as described in methods. Results are the mean +/- S.E.M., with an n=6 in each group. *, significantly different from control $p< 0.05$; **, significantly different from control $p< 0.01$.

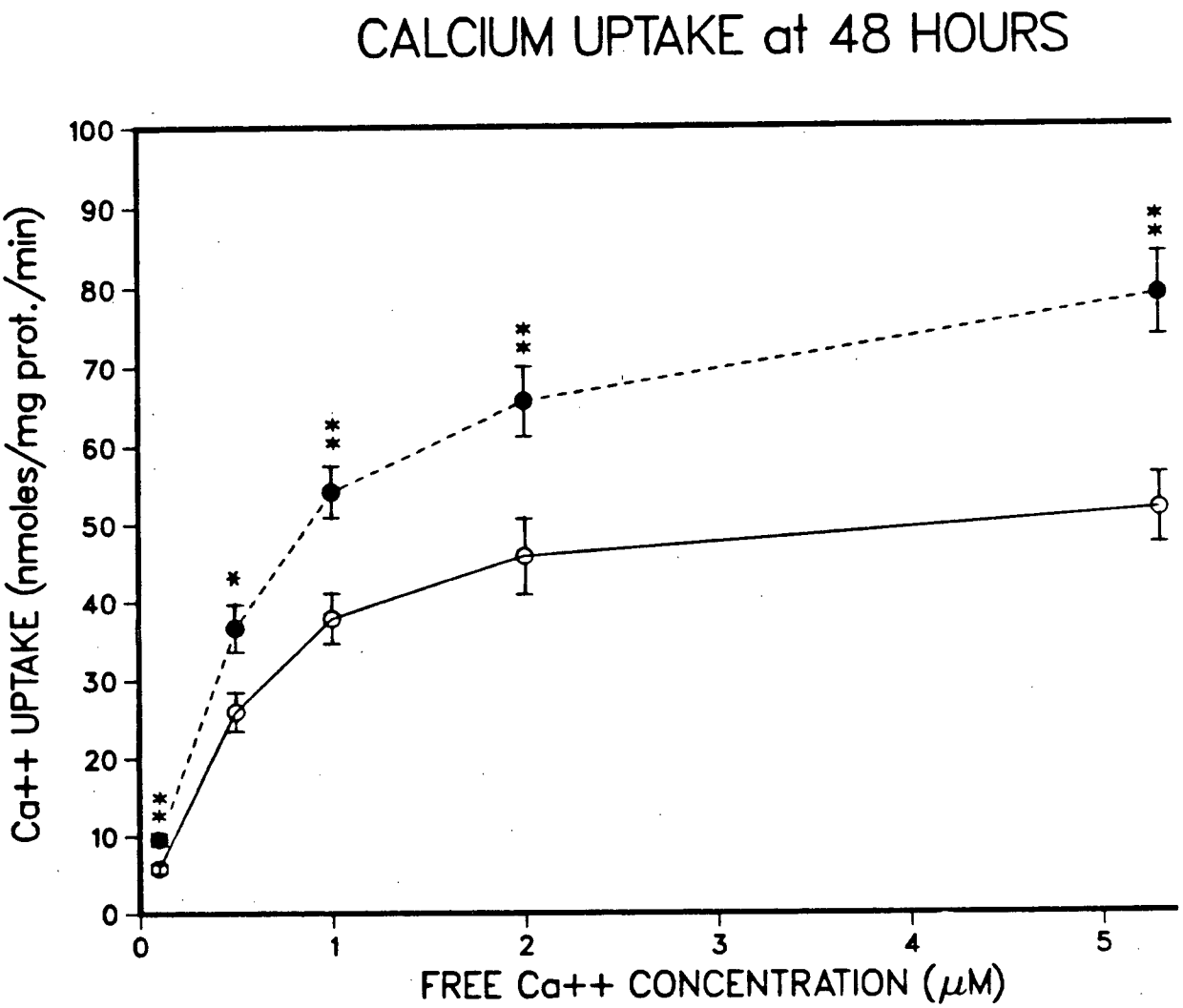

CALCIUM UPTAKE at 48 HOURS
Ca++ UPTAKE (nmoles/mg prot./min)
FREE Ca++ CONCENTRATION (μM)
0
10
20
30
40
50
60
70
80
90
100
1
2
3
4
5
*
**

Figure 6.

The effect of T_3 treatment on the rate of calcium transport 72 hours after initiation of the treatment. Calcium transport activity of control (o-o) and T_3 treated (●-●) SR was determined as described in Methods. Results are the mean +/- S.E.M., with an n=6 in each group. **, significantly different from control $p< 0.01$.

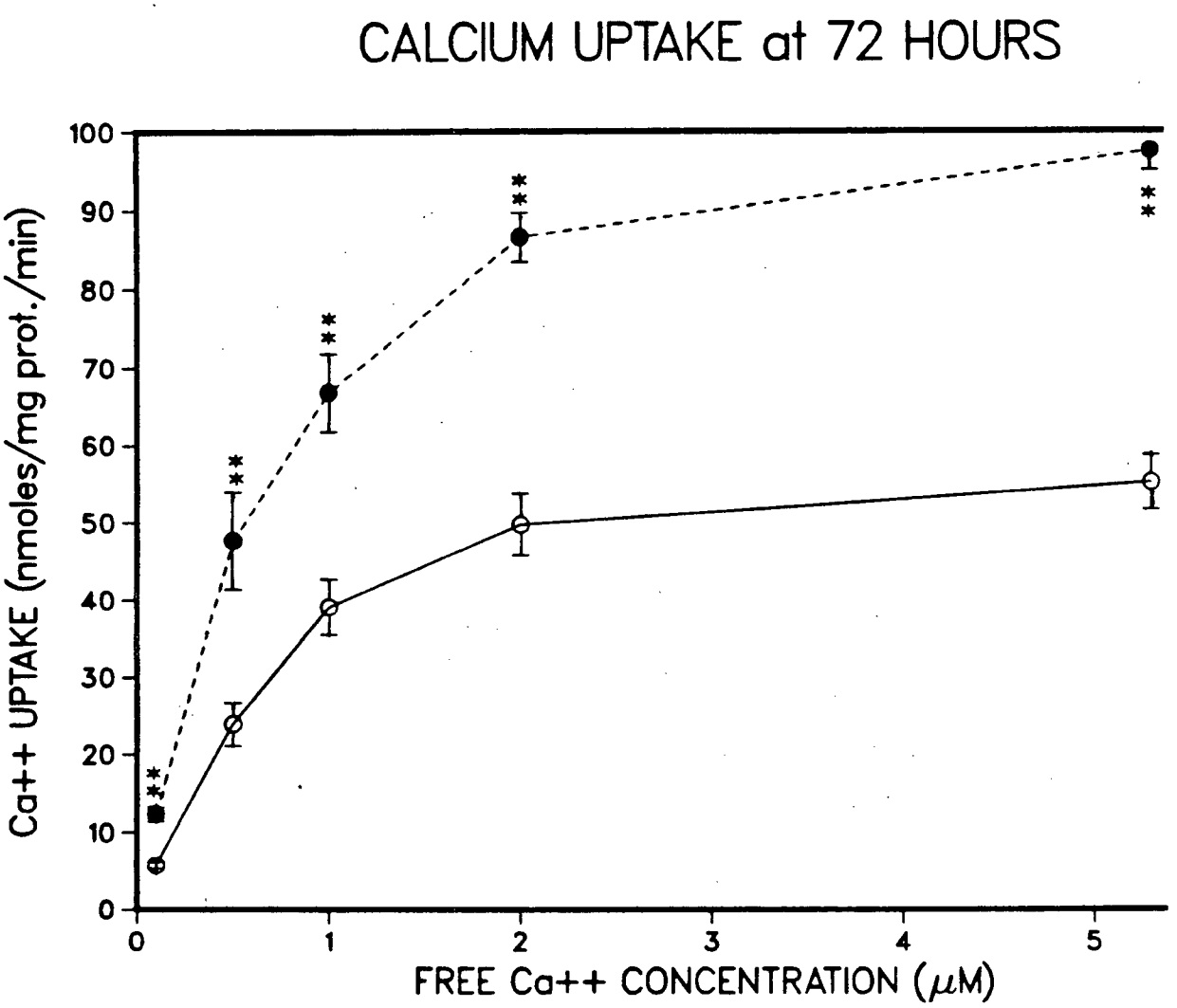
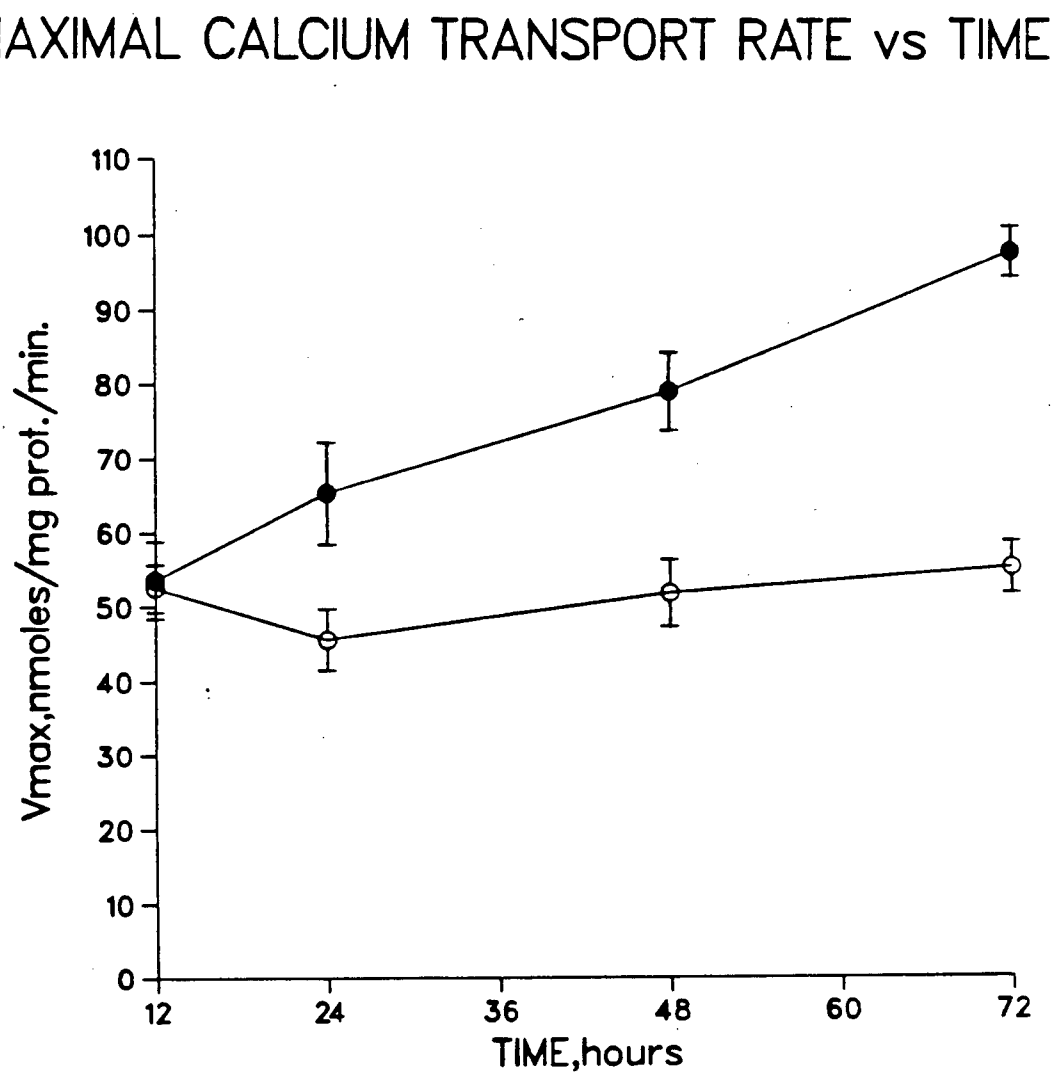

CALCIUM UPTAKE at 72 HOURS
Ca++ UPTAKE (nmoles/mg prot./min)
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
FREE Ca++ CONCENTRATION (μM)

Figure 7.

Graph showing the correlation of disease progression and augmented calcium transport activity. Control (o-o) and T_3 treated (●-●) values were obtained from the 5.3 μM free calcium point on figures 3-6. Results are expressed as mean +/- S.E.M..



The K_{Ca} was calculated from the slope of Eadie-Hoffstee plots of the calcium activation curves at 12, 24, 48 and 72 hours (Figures 8-11, respectively. The K_{Ca} of the SR calcium transport process was not altered by the T_3 treatment at any of the time points studied, and remained within the range of previously reported values (Tada et al, 1978). The V_{Ca} was augmented at 24, 48 and 72 hours (y intercept).

Table 7 shows that for both control and T_3 treated groups there is no significant difference in calcium uptake activity either in the presence or absence of 5.0 mM sodium azide.

Figure 8.

Eadie-Hofstee plots of the 12 hour calcium uptake data represented in Figure 3, of control (o-o) and T_3 treated (●-●) groups. The K_{Ca} determined from these plots for both the control and T_3 treated groups was 0.6 μM.

EADIE–HOFSTEE PLOT OF 12 HOUR

CALCIUM UPTAKE CURVE

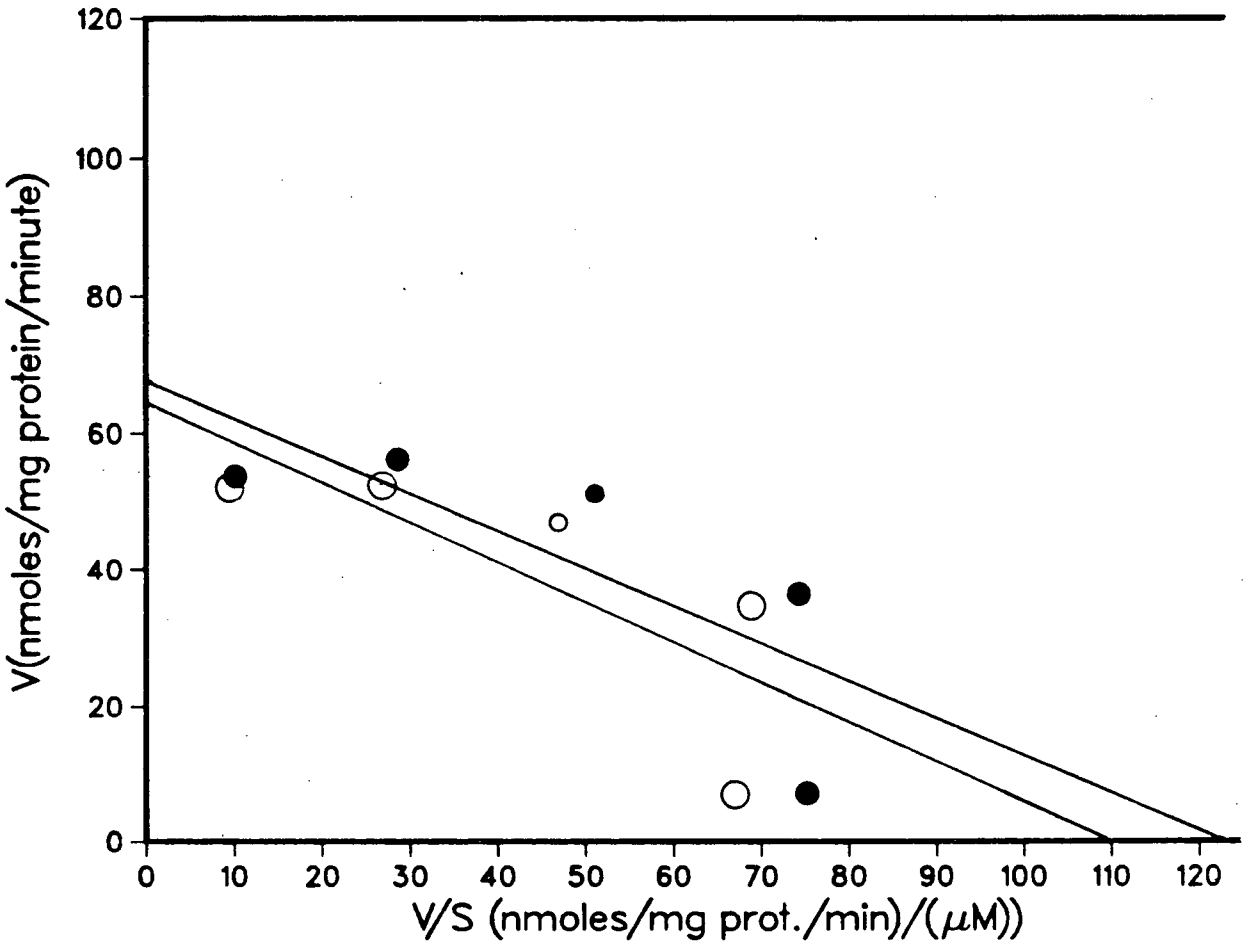


Figure 9.

Eadie-Hofstee plots of the 24 hour calcium uptake data represented in Figure 4 of control (o-o) and T_3 treated (●-●) groups. The K_{Ca} determined from these plots for control and T_3 treated groups were 1.2 μM and 1.0 μM, respectively.

EADIE–HOFSTEE PLOT OF 24 HOUR

CALCIUM UPTAKE CURVE

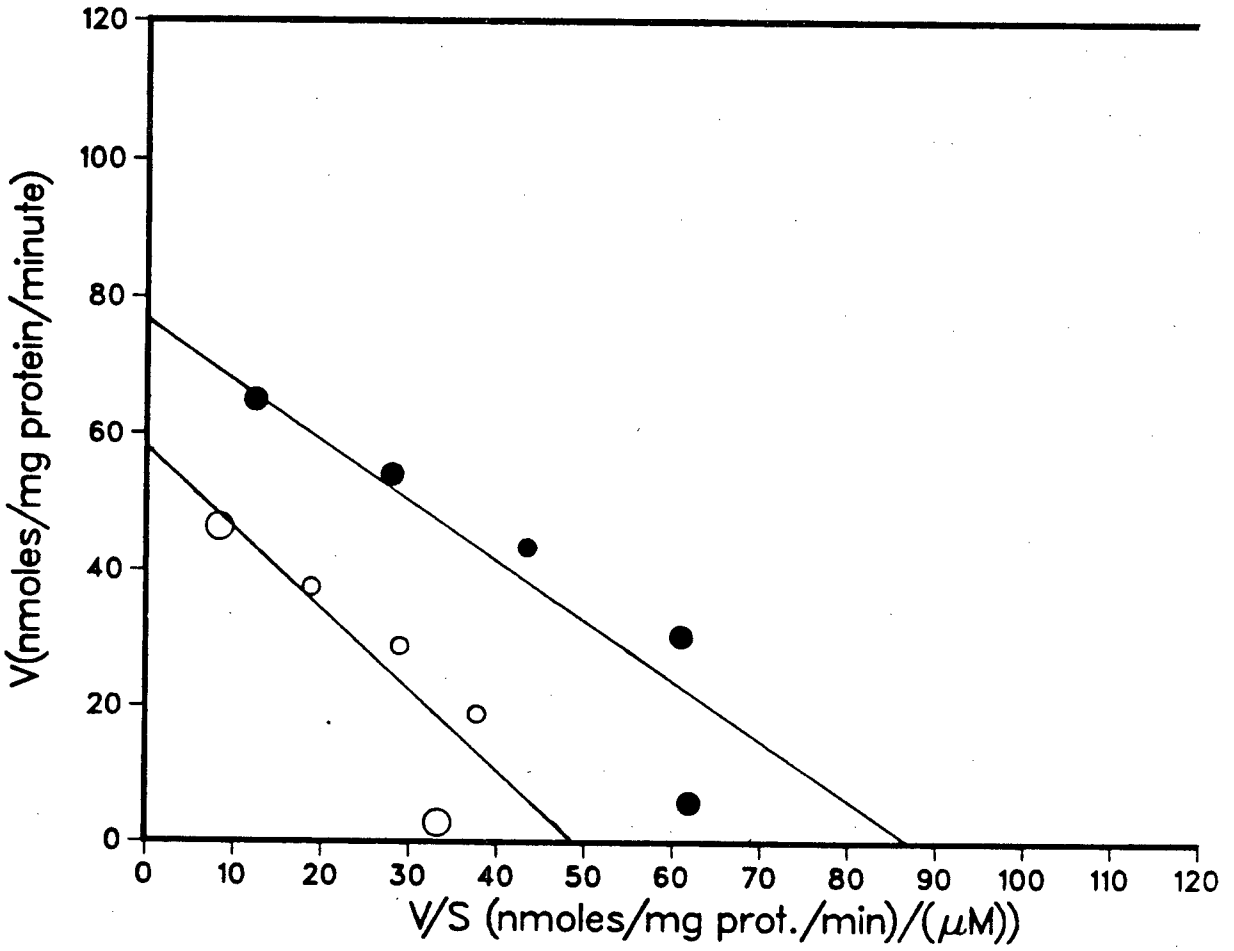


Figure 10.

Eadie-Hofstee plots of the 48 hour calcium uptake data represented in Figure 5, of control (o-o) and T_3 treated (●-●) groups. The K_{Ca} determined from these plots for the control and T_3 treated groups were 0.9 μM and 0.8 μM, respectively.

EADIE HOFSTEE PLOT OF 48 HOUR

CALCIUM UPTAKE CURVE

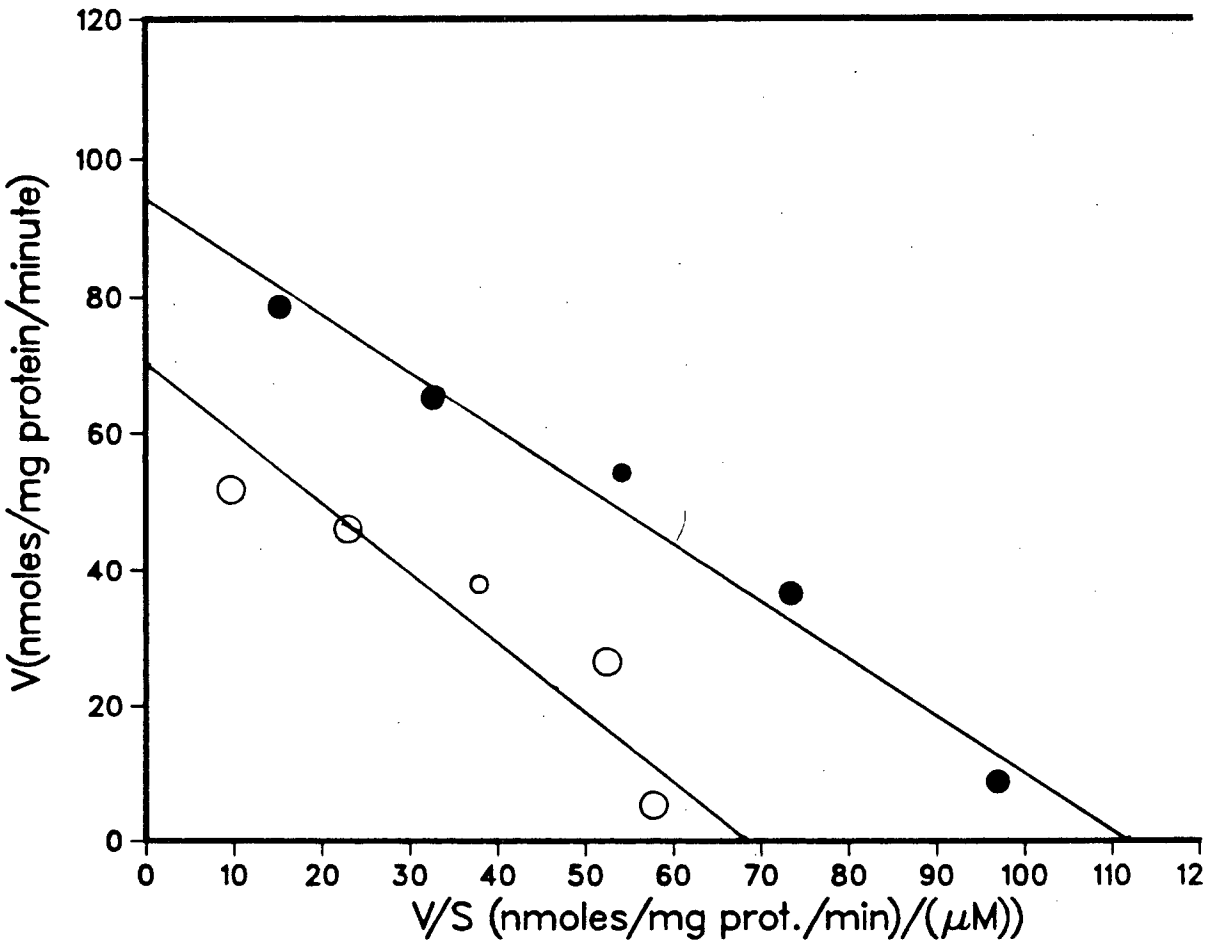


Figure 11.

Eadie-Hofstee plots of the 72 hour calcium uptake data represented in Figure 6, of control (o-o) and T_3 treated (●-●) groups. The K_{Ca} determined from these plots for the control and T_3 treated groups were 0.9 μM and 0.8 μM, respectively.

EADIE–HOFSTEE PLOT OF 72 HOUR

CALCIUM UPTAKE CURVE

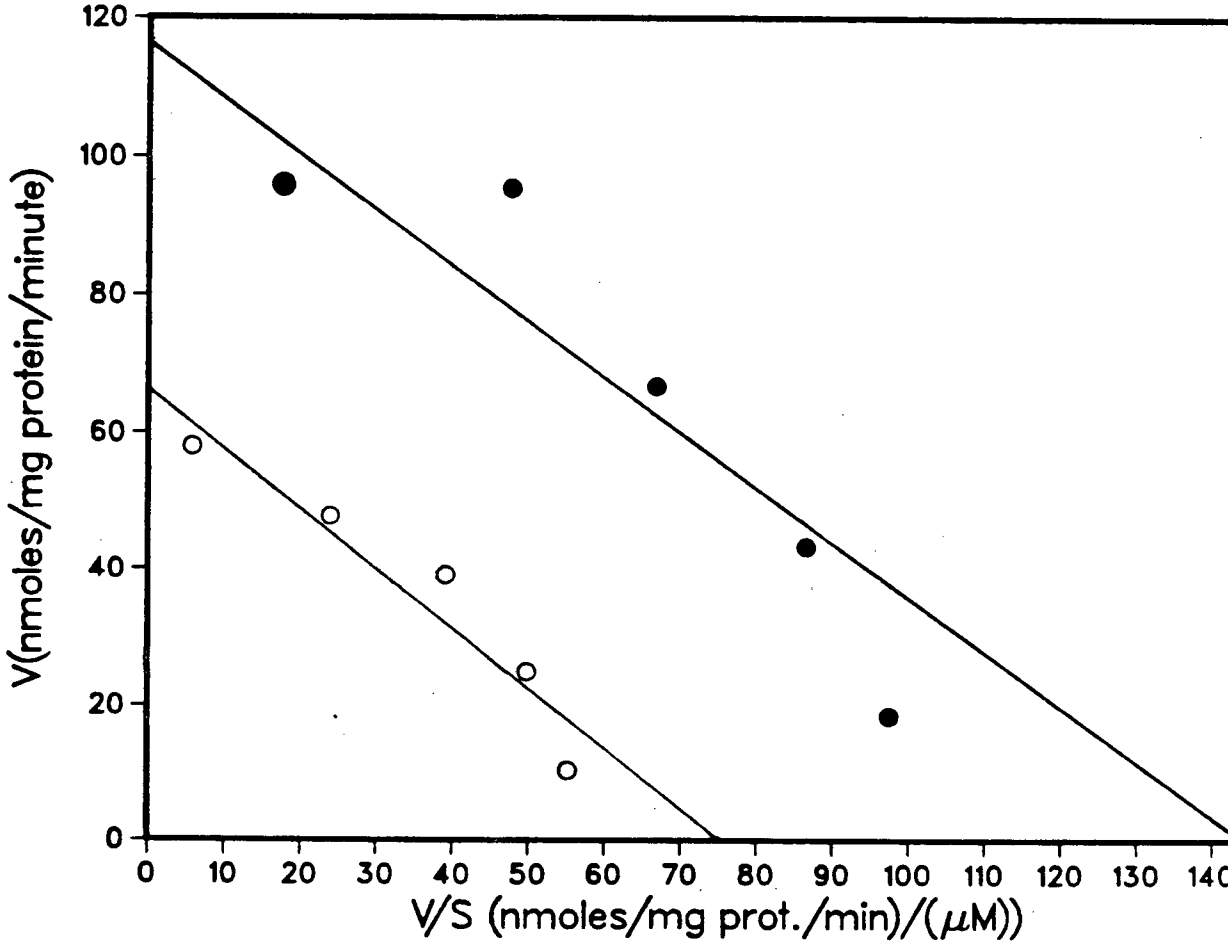


Table 7.

The Effect of Sodium Azide (5 mM) on SR Calcium Transport At 2 μM Free Calcium

TIME POINT (HR)	GROUP	CALCIUM TRANSPORT (nmoles/mg protein/minute) (+) NaN_3	(-) NaN_3
12	Control	41.2+/-2.5	37.9+/-3.8
	T_3 Treated	39.7+/-2.3	38.1+/-4.3
24	Control	40.2+/-6.5	43.7+/-4.7
	T_3 Treated	60.1+/-11.4	60.4+/-11.3
48	Control	43.0+/-3.6	40.3+/-4.5
	T_3 Treated	57.9+/-2.7	54.3+/-4.4
72	Control	40.7+/-3.5	39.3+/-4.3
	T_3 Treated	71.5+/-2.8	74.2+/-3.4

Results shown are the mean +/- S.E.M..

4. Studies on the Effect of T_3 Treatment on SR Carnitine Level

Previous reports have indicated that palmitoylcarnitine inhibits cardiac SR calcium uptake (Pitts et al, 1978) and that diabetic rat heart SR demonstrates depressed calcium uptake activity concomitant with increased long chain acylcarnitine levels (Lopaschuk et al, 1983). It was therefore investigated whether the SR levels of carnitine esters could play a role in the calcium transport alterations associated with the hyperthyroid state. The SR levels of total carnitine (which includes free, short chain acyl and long chain acyl carnitines), acid soluble (short chain) and long chain acylcarnitines were determined from a fraction of the SR isolated for calcium transport studies.

There was no significant difference in total carnitine levels 12 hours after the initial dose (Figure 12). The T_3 treatment though, resulted in a significant depression in the level of total carnitine (Figure 12) present in the SR at 24 hours ($p< 0.05$), 48 hours ($p< 0.05$) and 72 hours ($p< 0.01$) following initiation of the treatment. The level of acid soluble carnitine (Figure 13) was not affected by the T_3 treatment, as no significant difference between the control and T_3 treated groups could be discerned at all time points studied. The levels of long chain acylcarnitines (Figure 14) were not altered at 12 hours and only slightly depressed (not significant) at 24 hours. At 48 and 72 hours, there was a significant depression in the levels of long chain acylcarnitines ($p< 0.05$ and $p< 0.01$, respectively). The absolute value of the total, acid soluble

Figure 12.

The effect of T_3 treatment on the level of total carnitine measured in the SR. Total carnitine levels were determined as described in Methods. Results shown are the mean +/- S.E.M.. The sample sizes, for both control and T_3 treated groups, at the various time points are as follows: 12 hours, 5; 24 hours, 5; 48 hours,4; 72 hours, 6.

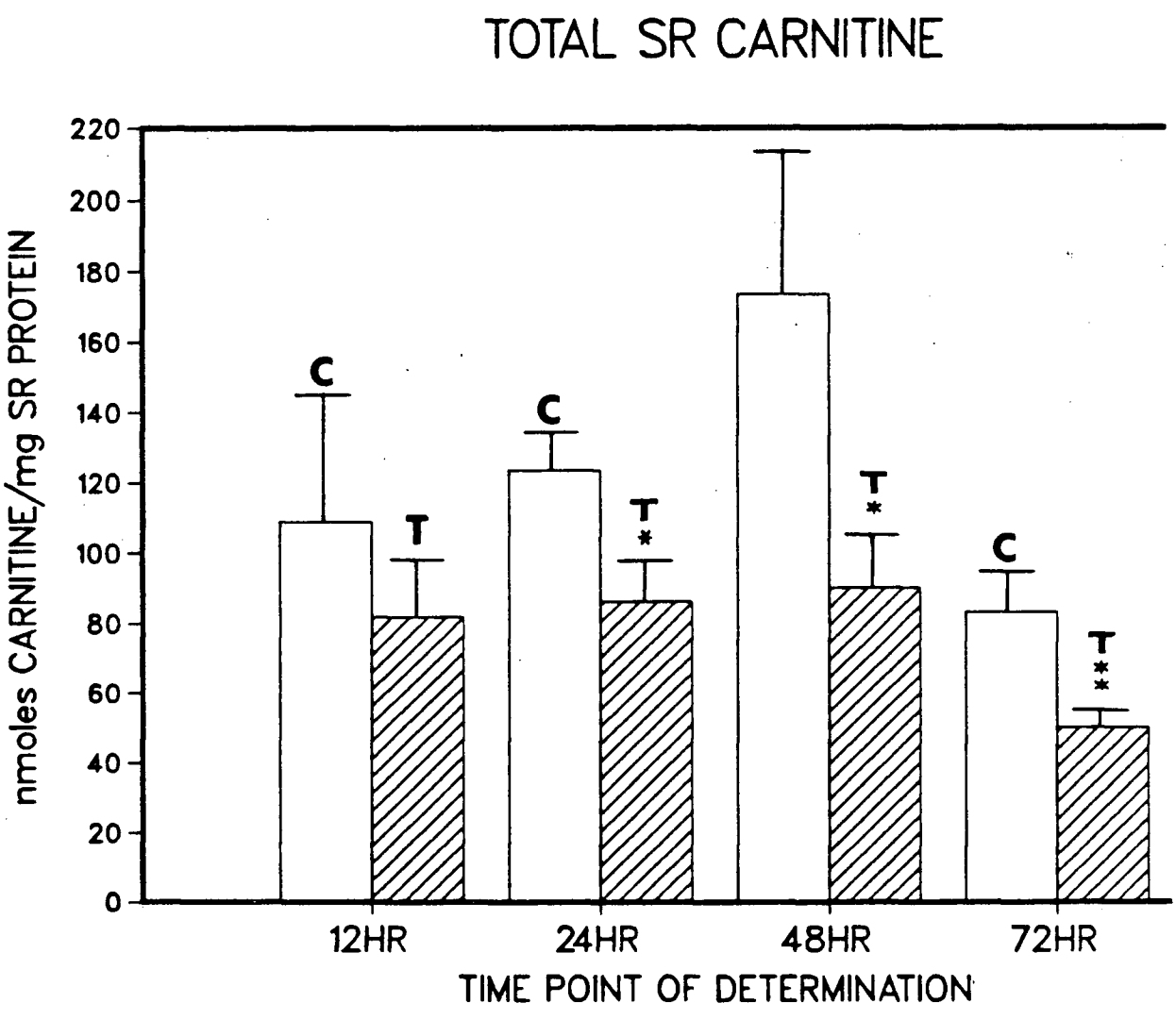

TOTAL SR CARNITINE
nmoles CARNITINE/mg SR PROTEIN
220
200
180
160
140
120
100
80
60
40
20
0
C
T
C
T*
T*
C
T**
12HR
24HR
48HR
72HR
TIME POINT OF DETERMINATION

Figure 13.

The effect of T_3 treatment on the level of acid soluble carnitine measured in the SR. Acid soluble carnitine levels were determined as described in Methods. Results shown are the mean +/- S.E.M.. The sample sizes for control (C) and T_3 treated (T) groups at the various time points are as follows: 12 hours, (C)=(T)=5; 24 hours, (C)=5, (T)=6; 48 hours, (C)=(T)=4; 72 hours, (C)=(T)=6.

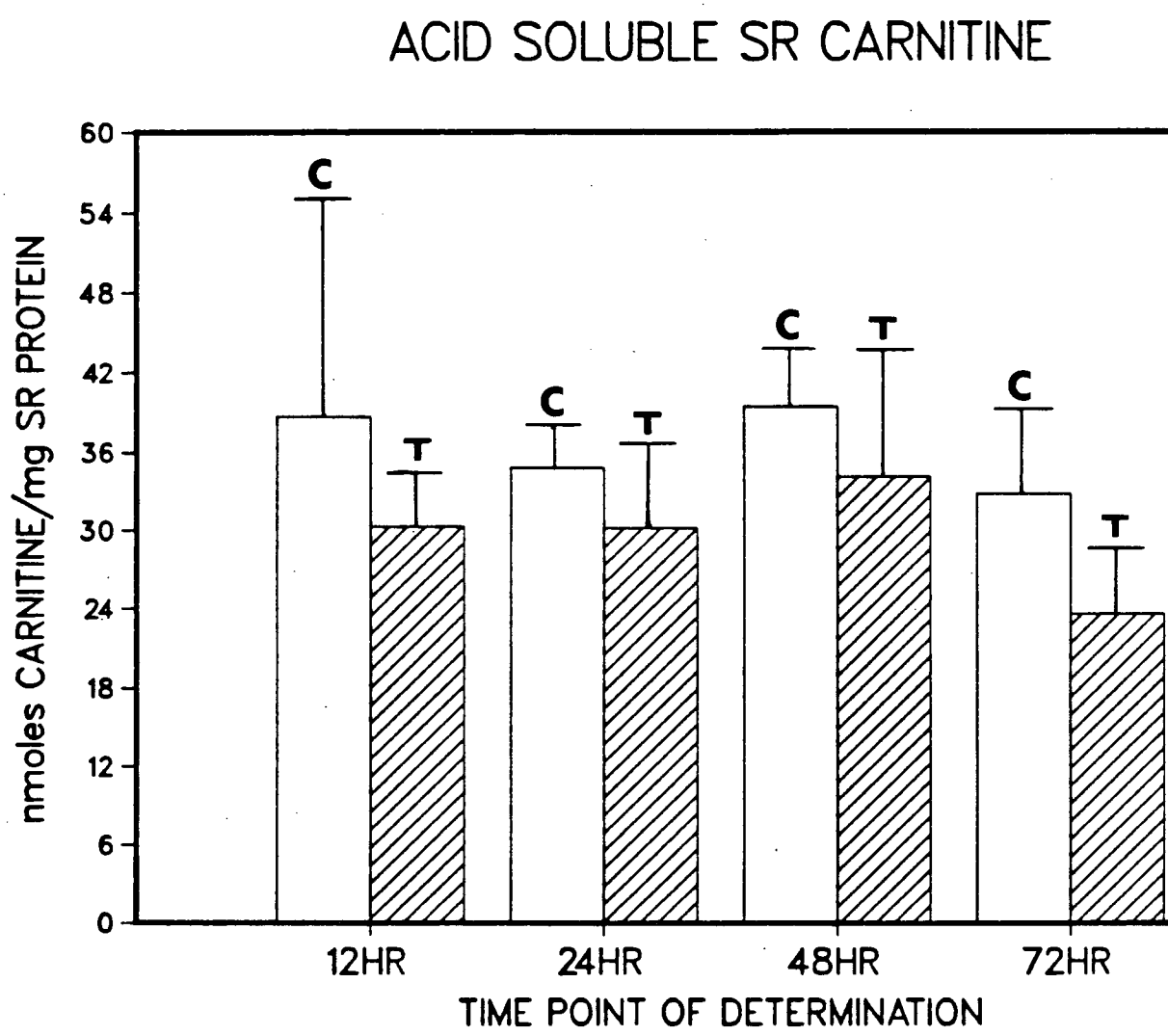
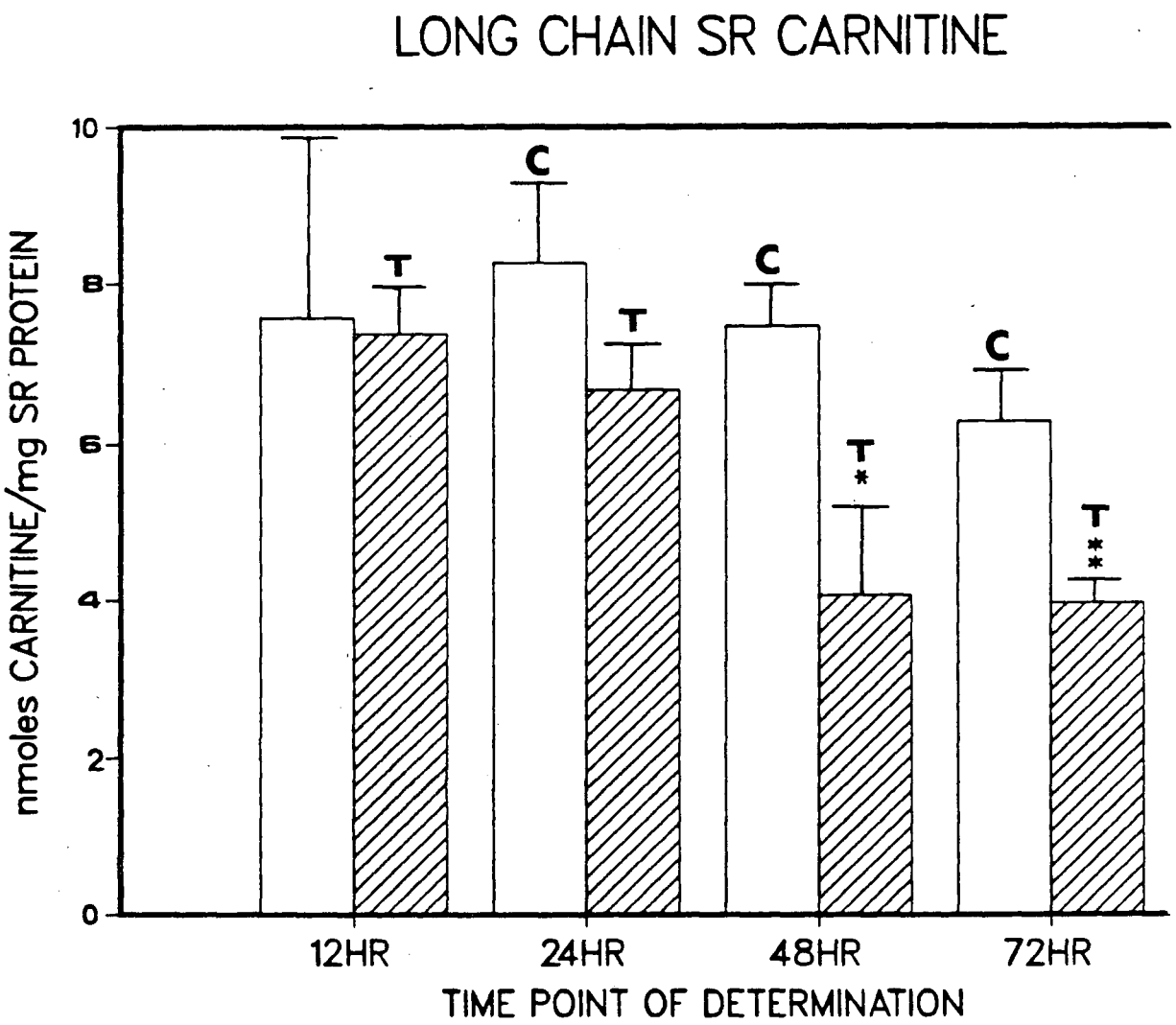

ACID SOLUBLE SR CARNITINE
nmoles CARNITINE/mg SR PROTEIN
60
54
48
42
36
30
24
18
12
6
0
C
T
C
T
C
T
C
T
12HR
24HR
48HR
72HR
TIME POINT OF DETERMINATION

Figure 14.

The effect of T_3 treatment on the level of long chain acylcarnitines determined in the SR. Long chain acylcarnitine levels were determined as described in Methods. Results shown are the mean +/- S.E.M.. The sample sizes for the control (C) and T_3 treated (T) groups are as follows: 12 hours, (C)=(T)=5; 24 hours, (C)=5, (T)=6; 48 hours, (C)=(T)=4; 72 hour, (C)=(T)=6.


LONG CHAIN SR CARNITINE
nmoles CARNITINE/mg SR PROTEIN
10
8
6
4
2
0
C
T
C
T
C
T*
C
T**
12HR
24HR
48HR
72HR
TIME POINT OF DETERMINATION

and long chain acyl carnitines varied quite significantly, as shown by the scale of the y-axis of Figures 12, 13 and 14. The reason for this variation is not known at this time.

There is a strong negative correlation (r= -0.93) between the rate of calcium transport at maximal free calcium (5.3 μM) and the level of long chain acylcarnitines present in the SR (Figure 15). Thus, it appears that the augmented rate of SR calcium transport observed in the T_3 treated animals may in some way be related to the depression in the SR level of long chain acylcarnitines.

5. The Effect of T_3 Treatment on The Level of Calcium ATPase Phosphoprotein Intermediate.

Figure 16 shows SR phosphoprotein formation as a function of free calcium concentration (range 0.1 μM-10 mM) in SR from control heart tissue. The reaction time was 15 seconds to maximize steady state calcium ATPase phosphoprotein formation and to minimize kinase-mediated phosphorylation of SR protein. Under these conditions, the phosphoprotein formed was primarily the phosphoprotein intermediate associated with the calcium pump as hydroxylamine treatment resulted in a marked reduction in the calcium dependent acyl phosphate phosphoprotein levels (68%) yet had little effect on calcium independent phosphoprotein formation (15%).

Calcium-dependent phosphoprotein formation increased with increasing free calcium. Subsequent phosphorylation experiments

Figure 15.

Correlation of maximal calcium transport activity (determined at 5.3 μM free calcium as described in Methods) with the SR level of long chain acylcarnitines. Results are expressed as the mean +/- S.E.M., control (o-o) and T_3 treated (●-●) groups.

MAXIMAL CALCIUM TRANSPORT RATE

vs SR LCAC CONCENTRATION

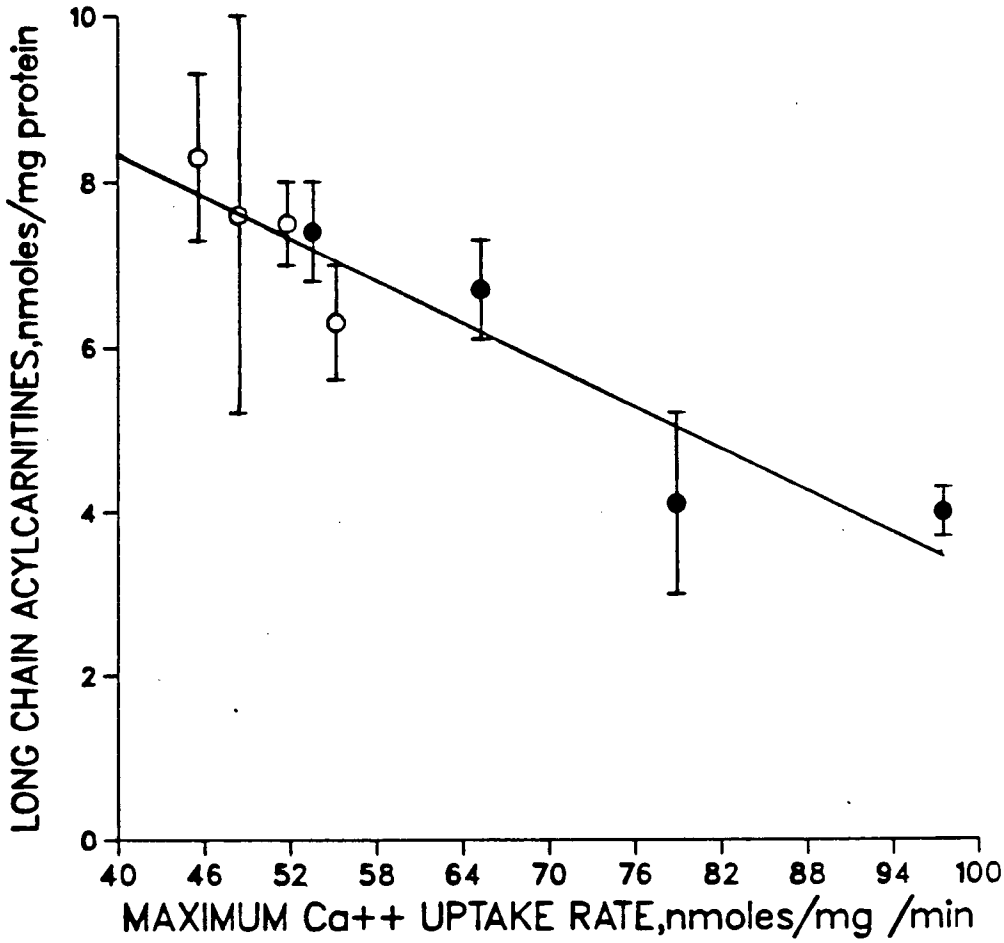
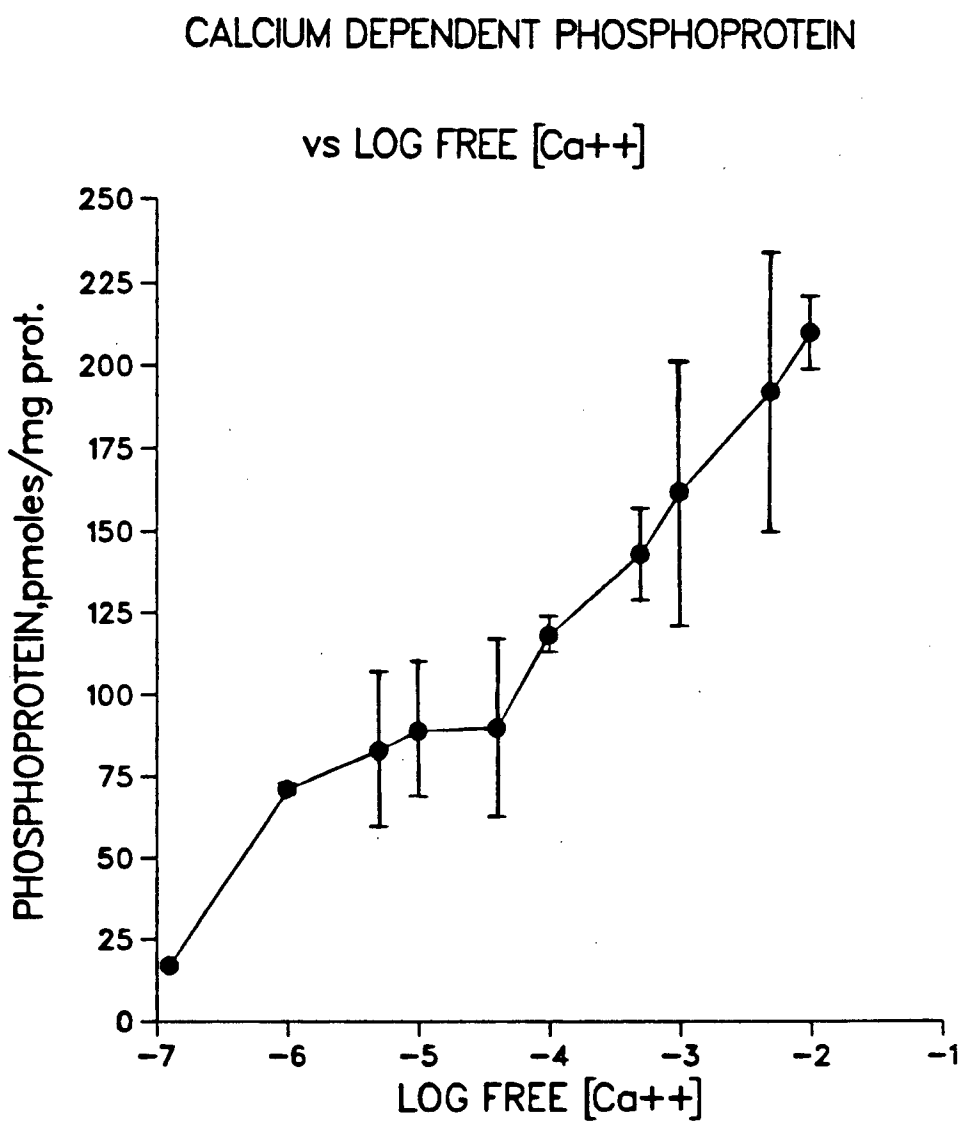


Figure 16.

SR phosphoprotein formation as a function of free calcium concentration. Phosphorylation performed according to protocol described in Methods. Results shown are the mean +/- S.E.M. of two separate experiments performed in duplicate.


CALCIUM DEPENDENT PHOSPHOPROTEIN
vs LOG FREE [Ca++]
PHOSPHOPROTEIN,pmoles/mg prot.
250
225
200
175
150
125
100
75
50
25
0
−7
−6
−5
−4
−3
−2
−1
LOG FREE [Ca++]

were carried out at 10 mM free calcium to label a maximal number of sites under these conditions.

Figure 17 shows the calcium dependent phosphoprotein formation in control and T_3 treated SR membranes at 10 mM free calcium. There was no significant difference between the phosphoprotein levels determined in any of the three control groups. Twenty-four hours following the first dose there was a slight but not significant increase in the phosphoprotein levels detected in the T_3 treated group. By 48 hours, phosphoprotein levels in the T_3 treated group were significantly ($p< 0.01$) greater than control levels and at 72 hours the T_3 treated phosphoprotein levels remained significantly ($p< 0.05$) greater than the control level. The hydroxylamine sensitivity and the calcium-dependence of the phoshoprotein formed indicated that it was primarily the calcium pump protein that was labeled. The number of active pumping sites was, therefore, increased after 24 hours and significantly increased after 48 and 72 hours of the T_3 treatment regimen.

Figure 18 shows the results of the SDS polyacrylamide gel electrophoretic separation of the SR proteins visualized by silver staining. Track A shows the Biorad^R molecular weight marker proteins; myosin (200 kDa), B-galactosidase (116.3 kDa), phosphorylase B (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). Tracks B, C, D, E, and F represent control, 12, 24, 48 and 72 hour SR respectively. The gel indicates that there is no qualitative

Figure 17.

Phosphoprotein levels in control and T_3 treated SR fractions determined at 10 mM free calcium. Phosphoprotein determinations were performed as described in methods, and the results shown are the mean +/- S.E.M., N.S.= non-significant, *= significantly different from control, $p< 0.05$, **= significantly different from control, $p<0.01$. n values: 24 hour control and T_3 treated= 10, 48 hour control and T_3 treated= 9, 72 hour control and T_3 treated= 10.

CALCIUM–DEPENDENT PHOSPHOPROTEIN vs TIME

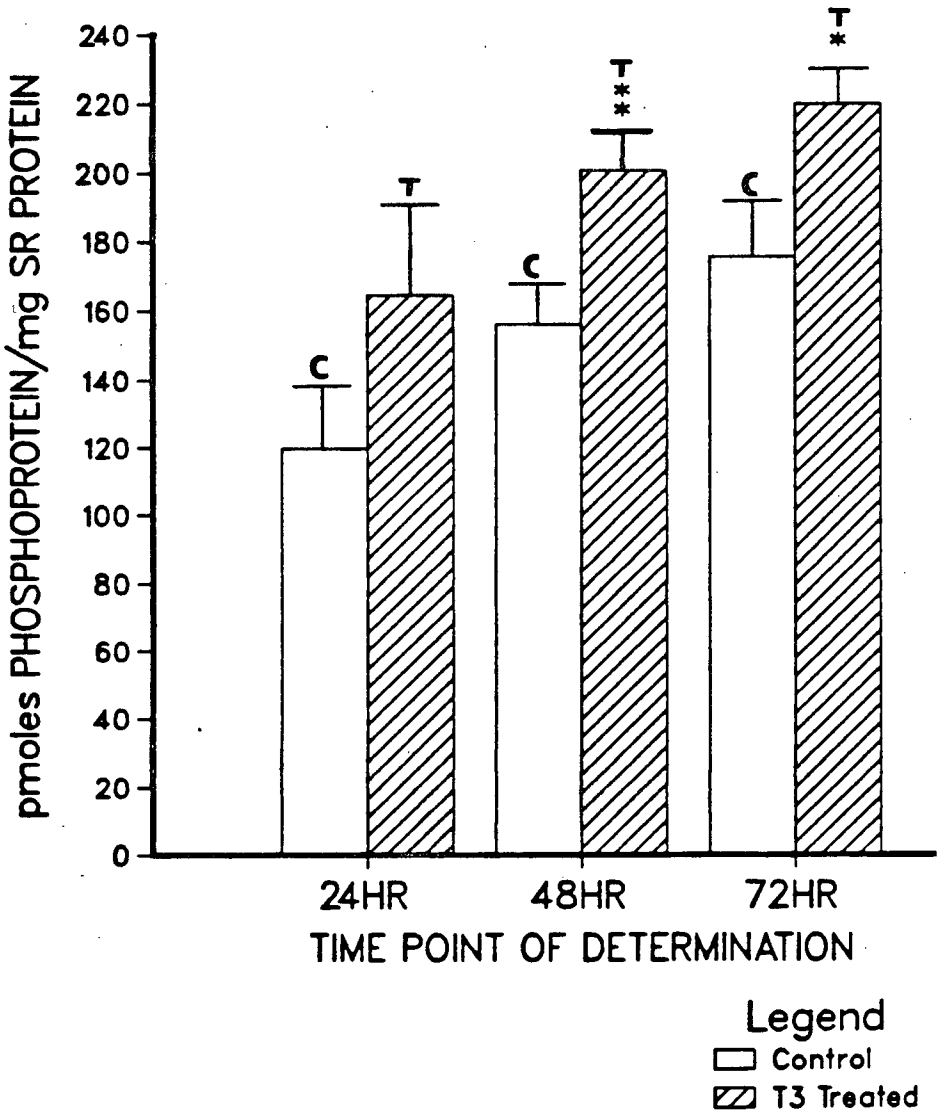
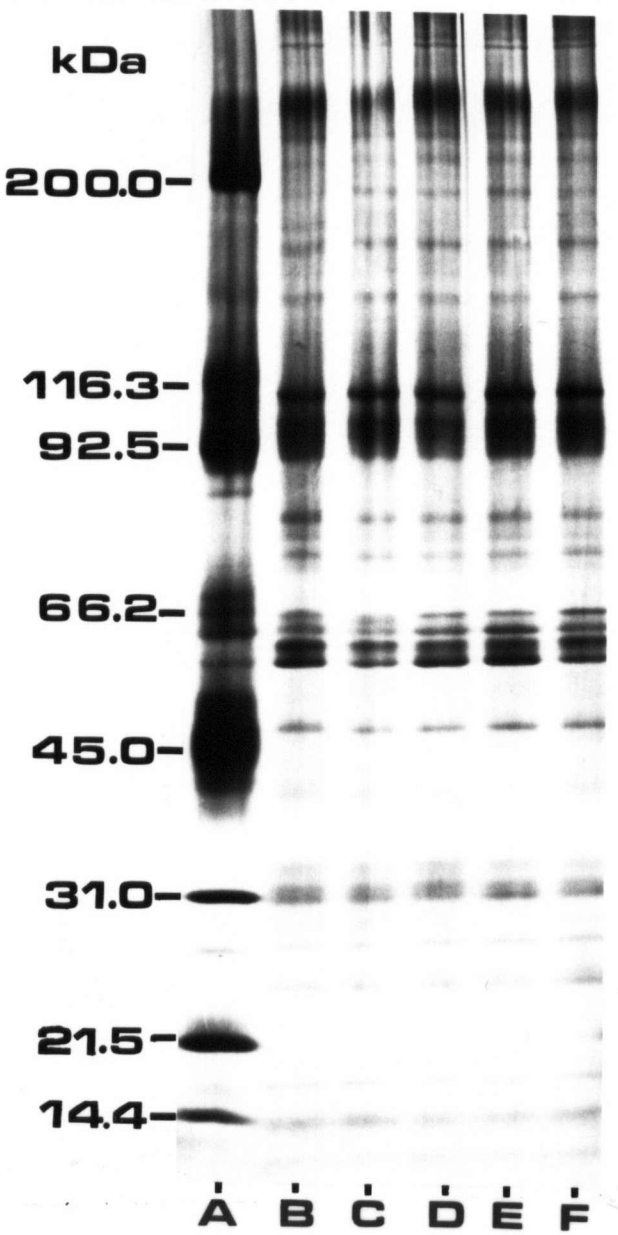


Figure 18.

SDS gel electrophoresis and silver stain of control, 12, 24, 48 and 72 hour SR membranes. Gel electrophoresis and staining were done as described in methods. Track A represents the protein standards: Myosin, 200 kDa; B-galactosidase, 116.3 kDa; phosphorylase B, 92.5 kDa; bovine serum albumine, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa. Tracks B, C, D, E and F represent control, 12, 24, 48 and 72 hour SR samples, respectively.

Figure 18



differences in the proteins isolated from either control or any of the T_3 treated groups. Protein bands indicative of SR can be detected at approximately 100 kDa (calcium ATPase), 57 kDa (calsequestrin) and 22kDa (phospholamban).

DISCUSSION

The Effect of T_3 Treatment on Serum Free T_3, Body Weight, Ventricular Weight and Sarcoplasmic Reticulum Yield

The aim of this project was to investigate biochemical alterations in the heart occurring during the progression of the hyperthyroid state. Therefore, an appropriate experimental model was required in which hyperthyroidism could be reproducibly induced. The animal chosen was the male Wistar rat and hyperthyroidism was induced by the daily subcutaneous injection of 500 μg/Kg T_3. This particular treatment protocol had been utilized previously to study cardiac alterations due to hyperthyroidism (McNeill and Brody, 1968; McNeill et al, 1969; Marriott and McNeill, 1983) and shown to produce cardiac alterations closely resembling aspects of the human condition. The T_3 treatment protocol used in this study did in fact result in elevated serum free T_3 values (Table 1) at the 12, 24, 48 and 72 hour time points studied. The serum half-life of T_3 is 24 hours (DeGroot et al, 1984), which may account for the observed decrease in serum free T_3 at 24 hours compared to the 12 hour concentration.

As in the human disease, the rats in this study responded to the T_3 treatment with a loss of body weight (Table 2); the maximum loss was after the full treatment, at 72 hours, where the treated rats lost an average of 19 g. This is approximately the same weight loss experienced by the same sex and strain of

rat subjected to the identical treatment in a previous study (Marriott and McNeill, 1983). The T_3 treated rats also demonstrated increased "irritability", in that they were more sensitive to external stimulii than controls. This parameter was not measured, but only observed, and since it is subjective is intended only as an indication of hyperthyroidism in the rat paralleling a sign of the human disease.

The wet ventricular weight was increased by the T_3 treatment 48 and 72 hours after initiation of the treatment (Table 3). Others have also reported significant increases in heart weight following the treatment protocol used in this study (Marriott and McNeill, 1983) and with other thyroid hormone treatment protocols (Korecky and Beznak, 1971; Morkin et al, 1983; Fox et al, 1985). Although the finding of cardiac hypertrophy in experimental hyperthyroidism is common, this anatomical change may represent a difference between the animal model and the human disease. Ventricular hypertrophy is generally absent in patients dying from thyrotoxicosis (Morkin et al, 1983), and the transverse diameter of the heart is usually normal in living patients (DeGroot et al, 1984). However, cardiac hypertrophy can occur in humans with long standing hyperthyroidism (Klein and Levey, 1984). The rapidly induced hypertrophy seen experimentally is likely to be a result of the pharmacological doses of thyroid hormones typically administered and the short onset of thyrotoxicosis associated with these doses, relative to the longer onset of the human disease. The ventricular weight to

body weight ratios represented in Table 4 are a further indication that there was cardiac hypertrophy in the T_3 treated group, and that the degree of hypertrophy increased with increasing duration of the disease. Therefore, the various indices of hyperthyroidism expressed by the rats indicate that they were hyperthyroid, and that the T_3 treatment protocol was appropriate for the purposes of the study.

There was a differential effect of the T_3 treatment on the ventricular weight and the yield of SR protein. There was no significant increase in the ventricular weight 24 hours after the first dose, yet the yield of SR was significantly greater at this time in the T_3 treated group. At the later time points of 48 and 72 hours, the yield of SR was also significantly greater than in the controls. Thyroid hormone treatment has been shown to increase the absolute area of sarcotubular membrane in rat ventricle (McCallister and Page, 1973). This study also showed that the membrane area per unit cell volume and per unit myofibrillar volume remained constant, indicating that myofibrillar volume and sarcotubular membrane both respond to thyroid hormone. The results of this project indicate that the ratio of SR protein yield to ventricular weight increases with increasing duration of T_3 treatment (Table 6). This result may imply that the T_3 treatment is increasing SR protein to a greater extent than overall cardiac mass. The SDS- gel electrophoretic separation of SR from control and hyperthyroid samples (Figure 18) though, shows that there are no qualitative differences in distribution of the SR proteins isolated from any

of the time points studied. In contrast to the results shown in Table 6, Limas (1978a) did not observe any substantial difference in the yield of SR protein between control and hyperthyroid groups. The reason for this difference is not known.

The Effect of Hyperthyroidism on SR Calcium Transport and Phosphoprotein Levels

The finding in this study that cardiac SR calcium transport is increased following thyroid hormone treatment is in agreement wiht previously published reports (Suko, 1974; Limas, 1978a,b; Guarnieri et al, 1980). Enhanced SR calcium transport activity is consistent with the thesis that this organelle may in part be responsible for the augmented cardiac function typically observed in both experimental and human hyperthyroidism. Currently, there are two possible mechanisms by which functional and biochemical changes in the heart could be induced in the hyperthyroid state: The first is that cardiac function is altered due to an intreraction between cardiac nuclear T_3 receptors and the subsequently augmented protein synthesis. The second is that cardiac manifestations of hyperthyroidism are the result of the increased work load imposed upon the heart by increased peripheral metabolism. With respect to the first possibility, the well defined control over isomyosin distribution by thyroid hormones (Morkin et al, 1983), and the

augmented contractile properties of each of these isomyosins (Schwartz et al, 1981; Pagani et al, 1983), suggest that this may be the case in species other than rat. In rat, myosin V_1 accounts for about 85% of total myosin in the euthyroid state, therefore, the augmented contractility cannot result from the shift in isomyosins as seen, for example, in rabbit. In this study, the latency period for T_3 mediated augmentation of SR calcium transport was between 12 and 24 hours after administration (significant increase at 24 hours, Figure 4). This period of time is in agreement with T_3 mediated increases in rat cardiac function (Brooks et al, 1985), and the induction of the synthesis of T_3 responsive enzymes (Oppenheimer, 1983). Brooks et al (1985) stated the T_3 mediated increases in cardiac contractility occurred prior to changes in whole body function. In this study the increased calcium transport activity was seen to occur prior to increases in ventricular weight. Therefore, the results of this study suggest a direct effect of T_3 on the heart, and specifically on SR calcium transport.

SR calcium transport was increasingly augmented with the duration of the hyperthyroid treatment (Figure 7). The differences in the observed rates of calcium transport between the control and T_3 treated groups were not due to alterations in mitochondrial membrane contamination, as there was no significant difference in the rate of calcium transport in either the absence or the presence of 5mM sodium azide (Table 7). The maximum increase in calcium uptake activity occurred at

72 hours, which is the same time that cardiac contractility has been shown to be augmented following the same T_3 regimen (Marriott and McNeill, 1983). Therefore, augmented SR calcium transport probably contributes to both the increased contractile activity and the decreased relaxation time observed in the hyperthyroid rat heart.

To further characterize the effect of T_3 treatment on the SR, the levels of the calcium-ATPase phosphoprotein intermediate were determined. A high calcium concentration which competitively inhibits the magnesium-dependent dephosphorylation of the ATPase (Tada et al, 1978) was used in order that all the calcium ATPase sites present in the SR could be labelled with substrate. In this way the number of sites in the SR of control and T_3 treated rats could be compared. Under these conditions there was maximal inhibition of dephosphorylation; 68% of the phosphoprotein formed was hydroxylamine-sensitive, indicating that the calcium pump was accounting for the majority of the phosphoprotein detected. At 48 and 72 hours, the phosphoprotein levels detected in hyperthyroid SR were significantly greater than control levels ($p< 0.01$ and $p<0.05$, respectively). This indicates that there are more phosphorylated calcium pumps in the hyperthyroid SR. This may result from, (1) an increased number of active pumping sites (2) an increase in the total number of pumping sites or, (3) an increased turnover of the pump. Calcium ATPase activity was not determined, therefore the distinciton between these possibilities cannot be made. However, because of the increased yield of SR protein at 24

hours, it is probable that there is an increased number of calcium ATPase sites. Other, indirect evidence supporting this possibility comes from work showing that thyroid hormone induced increases in calcium transport are prevented by the administration of inhibitors of RNA and protein synthesis (Limas, 1978a). Thyroid hormone has also been shown to influence the activity and expression of the Na/K-ATPase transport enzyme (Curfman et al, 1977; Guernsey and Edelman, 1983).

Calcium transport in cardiac SR is regulated by a number of different mechanisms. Conceivably, different degrees of regulator activity could at least partly account for the differences in calcium uptake activity observed between control and hyperthyroid cardiac SR. Cyclic AMP-dependent protein kinase, calcium-calmodulin dependent protein kinase and calcium-sensitive phospholipid-dependent protein kinase all augment calcium transport through phospholamban phosphorylation (LePeuch et al, 1979; Movsesian et al, 1984). Since only 68% of the phosphoprotein detected was hydroxylamine sensitive, differential phospholamban phosphorylation could have contributed to the remaining 32% since the phosphate bond associated with phospholamban is hydroxylamine insensitive. As mentioned earlier the effect of hyperthyroidism on the responsiveness of SR calcium uptake activity to cAMP-dependent protein kinase is controversial. Limas (1978b) has shown that hyperthyroid rat cardiac SR calcium transport responds to cAMP-

dependent protein kinase and the magnitude of the augmentation is approximately two-fold greater than the control response. The cAMP-dependent protein kinase-mediated incorporation of phosphate into a 22 KD SR protein (phospholamban) paralleled the calcium uptake response. An increased level or activity of endogeneous cAMP-dependent protein kinase was suggested as the explanation for these results. The work of Guarnieri et al (1980), showed that the contractility of hyperthyroid rat interventricular strips in response to dibutyrl cAMP did not differ from that of controls, and that hyperthyroid tissue could not respond to the same extent as control in the presence of isoproterenol. They also showed that the SR calcium uptake response of hyperthyroid rats to exogeneous cAMP and protein kinase was significantly less than controls. Basal calcium uptake activity in the hyperthyroid rats was greater than control by two-fold. The lack of response to cAMP-dependent protein kinase was suggested to be due to this high basal activity. Calmodulin may exert some effect over the observed calcium transport activity, as the amount of this protein in heart has been shown to be increased in hyperthyroidism (Segal et al, 1985). Studies in our laboratory, though, indicate that calmodulin does not stimulate SR calcium transport in rats (R.Mahey, personal communication).

The Effect of Hyperthyroidism on Total, Short Chain and Long Chain Carnitine in Cardiac SR

The inhibitory effect of long chain acylcarnitines (LCAC), particularly palmitoylcarnitine, on membrane bound enzymes was discussed in the introduction. To reiterate briefly, isolated cardiac SR incubated in the presence of exogenous palmitoylcarnitine, exhibited depressed calcium uptake and ATPase activity (Pitts et al, 1978). Cardiac sarcolemmal Na/K-ATPase activity was also found to be depressed in the presence of exogenous palmitoylcarnitine (Adams et al, 1979). Since the amount of LCAC in the cell is increased both following an ischemic episode (Idell-Wenger et al, 1978) and in the diabetic myocardium (Feuvray et al, 1979), it has been suggested that these lipid metabolic intermediates play a role in the cardiomyopathies associated with these conditions. Lopaschuk et al, (1983) has also shown increased SR LCAC levels concomittant with depressed calcium transport in SR isolated from diabetic rat heart.

The results presented in this thesis indicate a possible relationship between reduced SR LCAC and an increase in SR calcium transport activity in hyperthyroid rat heart. The metabolic alterations associated with hyperthyroidism and possible mechanisms of augmentation of SR calcium transport under conditions of reduced sarcoplasmic reticular LCAC, warrant further discussion. To support this hypothesis there is

evidence that metabolic alterations associated with hyperthyroidism favour a reduction in cardiac LCAC.

As previously mentioned, prior to mitochondrial oxidation, the activated fatty acyl-CoA derivatives of FFA's are transacylated by carnitine acyltransferase I (CAT I). An essential cofactor in the transacylation reaction is carnitine (4-trimethylamino-3-hydroxybutyrate) (Bremer, 1983). Carnitine metabolism is influenced by thyroid hormones in a way which, in conjunction with other metabolic alterations, may support the decrease of SR LCAC content: Thyroid hormone administration to rats and mice results in decreased carnitine synthesis (Suzuki et al, 1983), with hyperthyroid rat cardiac tissue having reduced total carnitine content. These results are in agreement with those of Cederblad and Engstrom (1978) which show decreased short chain and free carnitine content in heart tissue from hyperthyroid mice. The results concerning cardiac carnitine levels are not consistent: Bressler and Wittels (1966) have shown free and long chain acylcarnitine to be increased in hyperthyroid guinea pig heart. The differences may represent a species variation in cardiac carnitine metabolism. Carnitine has been shown to be metabolized to β-methylcholine by an intramitochondrial enzyme, carnitine decarboxylase, the activity of which is controlled by the rate of free fatty acid oxidation (Khairallah and Wolf, 1967). In man hyperthyroidism has been associated with increases in urinary carnitine excretion (Maebashi et al, 1977). Therefore it has been documented that carnitine levels in the hyperthyroid heart are

either decreased (Suzuki et al, 1983; Cederblad and Engstrom, 1978) or increased (Bressler and Wittles, 1966), that under a metabolic state occurring in hyperthyroidism carnitine metabolism is increased (Khairallah and Wolf, 1967), and that in man hyperthyroidism results in increased excretion of carnitine. However, because Khairallah and Wolf (1967) did not determine mitochondrial degradation of carnitine in hyperthyroid animals, the extrapolation of their observations to the hyperthyroid state may not be accurate; there may be changes associated with hyperthyroidism which reduce the catabolism of carnitine under conditions of increased free fatty acid metabolism. More recent results suggest that the actual metabolite determined was trimethylaminoacetone, and that the conclusion regarding carnitine degradation may not be accurate (Bremer, 1983). Therefore, data concerning the cardiac content of carnitine in hyperthyroidism are controversial. In none of the three reports describing the cardiac carnitine levels were attempts made to ascribe the levels detected to either cytosolic or subcellular compartments. The distribution of the various carnitine derivatives may be influenced by the hyperthyroid state. Further research is required to answer the question of whether or not cellular distribution and the relative amounts of the various carnitine derivatives are affected by hyperthyroidism, and if so, what the significance of such changes to either overall cardiac metabolism or cardiac function may be.

In addition to alterations in carnitine metabolism, the hyperthyroid heart also exhibits an increase in free fatty acid metabolism (Bressler and Wittels, 1966; Fintel and Burns, 1982). This increase may in part be accounted for by the increased serum free fatty acid concentrations associated with hyperthyroidism (Muller and Seitz, 1984c) and the thyroid hormone stimulated activity of carnitine palmitoyltransferase I (Bressler and Wittels, 1966; Stakkestad and Bremer, 1983). Since the heart derives its FFA supply from the circulation, increased circulating FFA results in increased substrate delivery to the heart. Although the heart is an omnivorous organ capable of adapting to a number of different substrates for energy production, the preferred substrate is generally that most available in the arterial circulation (Berne and Levey, 1981). Under euthyroid conditions, approximately 60% of the metabolic energy is supplied by various free fatty acids (Opie, 1968). Increased supply of free fatty acids, in addition to the lack of other substrates, results in an increase in FFA utilization. The rate limiting step of FFA oxidation is the transacylation reaction leading to the synthesis of LCAC from long chain acyl-CoA. By increasing CPT I activity, thyroid hormones are accelerating the flux of activated FFA into the mitochondria. Thyroxine treatment also increases mitochondrial content in rat heart (McCallister and Page, 1973), so that there is an increased capacity for β-oxidation of acyl-CoA. The decrease in available carnitine and increase in substrate delivery and free fatty acid metabolism are conditions which may

provide a rationale for the decreased LCAC detected in the SR of hyperthyroid rat heart. Since carnitine synthesis may be decreased, and/or metabolism and excretion increased, cardiac levels of this cofactor are decreased. In conjunction with this, CPT I activity and free fatty acid metabolism are increased and lactate metabolism concomittantly is decreased. The following hypothesis is therefore suggested: since (1) the rate of synthesis of LCAC is augmented and, (2) the metabolic state of the heart is such that the demand for intramitochondrial transport of LCAC is high, and (3) carnitine levels are decreased, the probability that LCAC will diffuse away from the inner mitochondrial membrane locale, where the carnitine pool is the most metabolically active, is decreased, hence, LCAC deposition into other subcellular organelles' is decreased.

This hypothesis, and the metabolic alterations observed in hyperthyroidism upon which it is based, appear to be unique to the hyperthyroid state. In other disease states, carnitine deficiency is associated with cardiomyopathies. Diptheritic myocarditis is assoicated with triglyceride accummulaiton, a depressed rate of FFA oxidation and decreased carnitine content (Opie, 1968). Familial cardiac carnitine deficiency has been associated with a cardiomyopathy (Tripp et al, 1981). Treatment of the patients surviving this cardiomyopathy with L-carnitine resulted in restoration of cardiac function, although, cardiac levels of total, free and esterfied carnitine were not changed

from pretreatment values. There are other examples of heart failure and successful treatment with oral carnitine (Rebouche, 1986). The influence of hyperthyroidism on carnitine metabolism in one respect follows the above observations. Of the total body carnitine, at least 90% resides in skeletal muscle (Rebouche, 1986). There may be a relationship between the augmented excretion of carnitine in hyperthyroid patients (Maebashi et al, 1977) and the skeletal muscle weakness observed in hyperthyroidism (DeGroot et al, 1984). It is paradoxical that cardiac carnitine is decreased in hyperthyroidism (Cederblad and Engstrom, 1978; Suzuki et al, 1983) and that cardiac function is augmented in the disease. However, the cardiac dysfunction ameliorated by carnitine treatment was not associated with an increase in cellular carnitine, and the beneficial effect has been attributed to replacement of intracellular LCAC with free carnitine (Rebouche and Engel, 1983). Therefore a consideration that may be more important than the total carnitine content of the heart, is the relative amount of the various carnitine derivatives (free, short and long chain).

A less subjective hypothesis concerning the observed decrease in SR LCAC from hyperthyroid heart relates to metabolic processes ongoing during the isolation of the SR. Oxidation of long chain acyl derivatives may be taking place between the time of isolation of the ventricular tissue and the time when the SR is separated from mitochondrial components of the cell. The addition of cyanide, to prevent the oxidation of acyl

carnitines, results in an approximate three-fold increase in the level of long chain acylcarnitine determined in a mitochondrial fraction of rat heart (Idell-Wenger et al, 1978). Addition of cyanide did not affect the level of total carnitine in the mitochondrial compartment (the increase in long chain carnitine was reflected by a decrease in free carnitine), nor did it affect the levels of total carnitine in the post-mitochondrial supernatant. Presumably, this latter fraction would contain the SR membranes, however the distribution of the various carnitine derivatives was not determined. It is possible that the procedures employed in this study would allow mitochondrial oxidation of long chain acylcarnitine after removal of the heart from the rat. Since cyanide was not included in the homogenization media, long chain acylcarnitine may have been metabolized, and since there is evidence of enhanced CPT I activity in the hyperthyroid heart, the rate of metabolism of long chain acylcarnitine in the hyperthyroid homogenate may have been greater than in control. The net effect, therefore, being decreased LCAC detected in the SR. Although this possibility provides an alternate explanation for the decreased levels of LCAC detected in hyperthyroid SR, it does not neccessarily obviate the possible relationship between increased SR calcium transport and decreased SR LCAC.

There are a number of considerations to be made in extrapolating in vitro data to the in vivo situation, not the least of which is the effect of the isolation techniques on the

organelle, enzyme or other biochemical parameter under study. When the abscissae of figures 12, 13 and 14 are compared, it is apparent that there is a large variation in the values of the various carnitine derivatives detected. The isolation technique utilized to separate the acid soluble and long chain fractions from total carnitine may account for the variation. As described in the methods, the isolation of LCAC involves washing the acid insoluble precipitate prior to hydrolysis to remove residual free and acetyl carnitine. Other workers have documented a loss of LCAC due to this washing step (Idell-Wenger et al, 1978). Of the total LCAC carnitine present in the cytosol, the majority is primarily bound to membrane structures since its hydrophobic character makes it thermodynamically more favourable to reside in the hydrophobic domains they afford. Figure 12 shows a significant depression in total carnitine 24,48 and 72 hours after initiaion of the T_3 treatment, and figure 14 shows a depression in LCAC at 24 (non-significant), 48 and 72 hours. The concentration of LCAC determined in the control cardiac SR (6.3-8.3 nmoles/mg SR protein) were approximately 6-fold higher than those reported by Lopaschuk et al (1983), yet are lower than the estimated concentration suggested by Pitts et al (1978) of 30 nmoles LCAC/mg SR protein. Acid soluble carnitine remains unaffected by the duration of T_3 treatment (Figure 13). The obvious trend is that both LCAC and total carnitine levels decline with increasing duration of T_3 treatment. It may be possible that the primary determinant of total carnitine in the SR is the LCAC

fraction, and that the discrepency between total and LCAC arises because of the isolation procedures employed. Further studies which avoid the washing of the acid insoluble precipitate may provide evidence supporting this hypothesis.

Another consideration to be made in interpreting these data is that the levels of the carnitine derivatives are reported as nmoles/mg SR protein. Since the study also showed increased SR yeild from hyperthyroid rat heart, it may be prudent to consider that the results obtained based on a protein standard are not a true reflection of the amount of carnitine in the SR. Although the results reported are accurate, when basing the value of one parameter on the amount of another, it is more ideal if the latter does not change between control and diseased groups. Under circumstances where an appropriate base parameter can not readily be acquired, basing the parameter of interest on a number of different bases may further indicate the actual situation. If the results reported in this study are skewed because of an unreliable standard, then the situation concerning the acid insoluble carnitine fraction would change such that these levels could be increased rather than not changed by the hyperthyroid state. Hypothetically, if the long chain acylcarnitine in the SR interacted in some specific manner with the calcium pump to inhibit its activity, then a relative increase SR protein in the absence of a concommitant increase in long chain acylcarnitine could account for the observed increase in calcium uptake reported in this study. There is no evidence

in this study to support such a hypothesis.

The inhibitory effect of palmitoylcarnitine on SR calcium transport and ATPase acitvity is due to its detergent effects on the SR membrane (Adams et al, 1979). The acyl ester inserts into the lipid matrix of the SR exerting its effects through physical disruption of the lipid bilayer. Adams et al (1979) have proposed that at low concentrations (below the critical micelle concentration of approximately 15 μM) the acylcarnitine perturbs the SR membrane in such a way as to increase the "leakiness" of the membrane to calcium. Figure 15 shows a correlation (r=-0.93) between the rate of calcium transport and the SR level of LCAC. The figure suggests a relationship between augmented calcium transport and reduced endogenous LCAC in the SR membrane. Under euthyroid conditions, the amound of LCAC isolated with the SR may result in a vesicle population with a permeability to calcium. As the hyperthyroid state progresses, the metabolic alterations induced result in a decreased amount of LCAC isolated in the SR, reducing the permeability of the vesicle to calcium and, therefore, increasing the apparent rate of calcium transport.

Extrapolating these in vitro results to the in vivo situation, a lowered calcium permeability of the SR would allow more efficent sequestration of calcium during relaxation, and therefore more calcium available for release during depolarization. However, because the contribution of LCAC to the total SR lipid must be small, as judged by the lipid composition of the SR, this may not be true. Phospholipids

account for approximately 80% of SR lipid, and neutral lipids the remainder, of which 95% is cholesterol (Tada et al, 1978). This leaves about 1% of the total lipid, of which a component will be LCAC. Therefore when viewed in the perspective of the total lipid content of the SR, and since passive permeability to calcium is extremely low under normal conditions, it does not seem probable that the decreased LCAC content could alter the permeability of native SR membrane to calcium.

CONCLUSIONS

1. The experimental model of hyperthyroidism used in this study resulted in alterations in the cardiac tissue. The effect of the T_3 treatment protocol on the ventricular weight was apparent after 48 hours. Ventricular weight remained significantly above the control 72 hours after the treatment was started. The degree of hypertrophy, as reflected by the cardiac ventricular to body weight ratio, increased with the progression of the disease. The T_3 treatment had a different effect on the SR compared to the ventricle as a whole. Significantly greater yields of SR protein were obtained 24, 48 and 72 hours after initiation of the treatment. The early alteration in the SR, occuring between 12 and 24 hours after the first T_3 injection, has a time course similar to other thyroid hormone inducible effects and may represent a direct effect on SR protein synthesis. SDS-polyacrylamide gel-electrophoresis indicated no obvious difference between control and treated SR membrane proteins at any of the time points assayed. Therefore, if T_3 does have an effect on SR protein it is without a change in the protein profile of the organelle.

2. The SR calcium uptake activity was determined during the progression of the disease. It was found that the V_{Ca} was significantly increased 24, 48 and 72 hours after the initiation of the hormone treatment. The increase in V_{Ca} was progressive, correlating positively with the time of the disease. The T_3

treatment was without an effect on the K_{Ca} of the calcium pump at any of the time points studied. Phosphorylation experiments indicated a slight increase in the number of calcium pump sites 24 hours after the first dose, and after 48 and 72 hours the number of calcium pump sites in the treated groups was significantly greater than the respective controls. These results, in conjunction with those showing an increased yield of SR protein in the T_3 treated groups, suggest that hyperthyroidism in the rat is associated with an augmentation in the amount of SR in the heart, and in the calcium transport activity of the SR. These alterations in the SR are consistent with the hypothesis that altered SR function in hyperthyroidism can explain the augmented contractile behaviour of the hyperthyroid rat heart.

3. The levels of total carnitine and long chain acylcarnitine detected in the SR were decreased following T_3 treatment. Total carnitine was significantly decreased 24 hours after the treatment was initiated, and remained so at 48 and 72 hours as well. Long chain acylcarnitine levels were not significantly lower than control until 48 and 72 hours. The T_3 treatment did not affect the levels of acid soluble carnitine detected in the SR at any of the time points studied. There was a strong negative correlation between the maximal calcium uptake activity determined in the SR at each of the time points studied and the level of long chain acylcarnitine detected in the SR. These

results suggest the possible relationship between the level of long chain acylcarnitine in the SR and the calcium pump activity. These results are different from previously published reports concerned with the effect of long chain acylcarnitines on SR function, where the norm is that long chain acylcarnitines are increased in cardiomyopathies and contribute to the depressed cardiac function. It is recognized that these results should be interpreted with caution because of possible technical reasons and the contribution of long chain acylcarnitine to the total membrane lipid.

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