THE EFFECT OF HYPOTHYROIDISM ON HEPATIC ADRENERGIC RECEPTORS IN THE RAT

by

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A B S T R A C T

Hepatic adrenoceptors were examined in different thyroid states. Activation of glycogen phosphorylase in hepatocytes from euthyroid and hyperthyroid rats was mediated by alpha adrenoceptors. In hypothyroid rats there was a significant beta receptor contribution to this response and enhanced beta receptor-mediated accumulation of cAMP. Responses to glucagon, a hormone which also activates phosphorylase via cAMP, were not similarly affected. The increase in beta receptor-mediated responses in the hypothyroid rat was accompanied by decreased alpha receptor activity as indicated by a marked supression of phenylephrine-stimulated phosphorylase activation and calcium efflux. Similar effects produced by vasopressin and the calcium ionophore, A23187, were changed only slightly or not at all. $Alpha_1$ receptors in liver plasma membranes were identified by [^{3}H]prazosin binding. Hypothyroidism did not influence the binding affinity of agonists or [^{3}H]prazosin, but reduced the density of $alpha_1$ receptors. This change was opposite to the increase in density of beta receptors in hypothyroid rat liver reported by others. All the changes observed in hypothyroidism were partially reversed by in vivo thyroid hormone replacement. It is concluded that hypothyroidism produces a selective enhancement of beta and suppression of alpha receptor responses in rat liver. These changes are most likely the result of corresponding alterations in the density of membrane receptor sites.

RÉSUME

Les récepteurs adrénergiques hépatiques furent examinés sous différents états thyroidiens. L'activation de la phosphorylase du glycogène dans les hépatocytes de rats euthyroidiens et hyperthyroidiens s'effectuait par-devers les récepteurs adrénergiques alpha. Il y avait, chez les rats hypothyroidiens, une participation significative du récepteur bêta a cette réponse. Ce dernier provoquait en outre une accumulation accrue de l'AMP cyclique. Les réponses au glucagon, une hormone qui active également la phosphorylase via l'AMP cyclique, n'étaient pas affectées de façon similaire. L'augmentation des réponses obtenues par l'intermédiaire des récepteurs bêta chez les rats hypothyroidiens était accompagnée d'une diminution de l'activité des récepteurs alpha, telle que démontrée par la suppression marquée de l'activation de la phosphorylase et de la sortie du calcium en réponse a la phényléphrine. Des effets comparables, produits par la vasopressine et l'ionophore calcique A23187, ne furent que légèrement ou nullement changés. Les récepteurs de type $alpha_1$ dans les membranes plasmatiques du foie furent identifiés par la liaison de prazosine tritiée. L'hypothyroidisme n'influencait pas l'affinité de la liaison des agonistes adrénergiques ou de la prazosine tritiee, mais reduisait toutefois la densite des recepteurs de type $alpha_1$. Ce changement était en opposition à l'augmentation de la densité des récepteurs bêta au niveau du foie du rat hypothyroidien, telle que notée par d'autres chercheurs. Tous les changements observés lors de l'hypothyroidisme étaient partiellement renversés, in vivo, par un apport exogène d'hormone thyroidienne. Il est de ce fait conclu que l'hypothyroidisme produit au niveau du foie une augmentation sélective des réponses reliées a l'activité des récepteurs bêta et une suppression de celles relevant des récepteurs alpha. Ces chahgements sont vraisemblablement le fruit d'une modification au niveau de la densité des sites récepteurs membranaires.

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ABBREVIATIONS USED

COMT	Catechol-O-methyl Transferase
MAO	Monoamine oxidase
cAMP, cyclic AMP	Adenosine-3',5'-monophosphoric acid, cyclic
cGMP	Guanosine-3',5'-monophosphoric acid, cyclic
ATP, ADP	Adenosine triphosphate, diphosphate
AMP, 5'-AMP	Adenosine-5'-monophosphate
Tx	Thyroidectomized
T_3	3,3',5'-Triiodothyronine
T_4	Thyroxine
IBMX	Isobutylmethylxanthine
DHA	Dihydroalprenolol
DHE	Dihydroergocryptine
IHBP	Iodohydroxybenzylpindolol
POB	Phenoxybenzamine
Gpp(NH)p	Guanyl-5'-ylimidodiphosphate
EDTA	Ethylenediaminetetracetate
EGTA	Ethylene glycol bis(-amino ethyl ether)-N,N,N',N'-tetra acetic acid
NAD, NADH	Nicotinamide adenine dinucleotide (oxidized, reduced)
TRIS	Tris(hydroxymethyl)-aminomethane
B_{max}	Maximum binding capacity
K_d	Dissociation constant
EC	Concentration of agonist causing half-maximal activation
pD_2	$-\log_{10}(EC_{50})$
n_H	Hill coefficient

SECTION ONE

INTRODUCTION

1.1 Historical Perspective and Preamble.

Many of the manifestations of thyrotoxicosis in man resemble hyperactivity of the sympathetic nervous system. That some relationship between the thyroid and adrenergic activity exists was first realized in the mid-nineteenth century (Harrison 1964). The earliest concept that alterations in the activity of the cervical sympathetic nerves resulted in enhanced glandular function gave way to the more widely held contemporary opinion that thyroid-induced changes in end-organ responses to sympathetic stimulation were important in the pathogenesis of thyroid disease (Harrison 1964). Indeed the effect of altered thyroid state on adrenergic responsiveness has been extensively studied especially in cardiac and adipose tissue (Kunos 1981). The recent introduction of the use of radiolabelled ligands in the study of adrenergic receptor properties has added an exciting dimension to these investigations (Hoffman and Lefkowitz 1980); it has been shown that, at least in some instances, thyroid-induced alterations in adrenergic responses can be attributed to changes at the level of the catecholamine receptor (Kunos 1981). However, in spite of the many excellent studies of this phenomenon, the underlying mechanism of these changes in adrenoceptor properties remains an enigma.

The concept of multiple types of adrenergic receptors was first proposed by Dale (1906) and later modified in the dual receptor theory of Alquist (1948). Subsequently, most adrenergic responses were classified as being mediated by either alpha or beta receptors. However, the glycogenolytic response of hepatic tissue to catecholamines could not easily be classified as belonging to the alpha or beta group of responses; the reasons for this are manifold (Hornbrook 1970). Recently it has become clear that both alpha and beta receptors can contribute to the glycogenolytic response of the liver. In the heart, where force and rate responses to catecholamines similarly involve both

alpha and beta receptors, hyperthyroidism was shown to increase beta and decrease alpha receptor activity. Therefore, it seemed logical to postulate that hepatic alpha and beta receptors would be modulated by thyroid hormones in a similar manner. This thesis describes the testing of this hypothesis and confirms an effect of thyroid state on hepatic adrenergic receptors, although the observed change is not as predicted.

The introduction which follows briefly reviews thyroid effects on the sympathetic nervous system (section 1.2), hepatic adrenergic receptors (section 1.3), the role of the sympathetic nervous system in the control of hepatic metabolism (section 1.4) and the metabolic effects of altered thyroid state with special emphasis on the liver and carbohydrate metabolism (section 1.5). No attempt has been made to comprehensively review here the huge literature that exists in any of these subject areas. Many controversial points arise, however, in the interest of clarity and brevity, only those that are felt to pertain directly to the present work are discussed at any length; most are taken up in Section IV: Discussion.

1.2 Sympathetic Nervous System-Thyroid Interactions.

1.2.1 General Effects

As might be expected for a subject that has been investigated for approximately 100 years, the vast amount of accumulated literature on sympathetic nervous system-thyroid interactions is often contradictory and confusing. Several excellent attempts have been made at organizing this information in recent reviews (Harrison 1964; Waldstein 1966; Leak 1970; Spaulding and Noth 1975; Landsberg 1977; Kunos 1981; Fain 1981).

Many clinical observations suggest that some manifestations of hyperthyroidism are due to over-activity of the sympathetic nervous system. Increased heart rate and contractility, sweating, muscle tremor and weight loss

are seen in patients with thyrotoxicosis. Similar effects can be produced by the administration of sympathomimetic agents to normal subjects and are also seen in patients with pheochromocytoma; indeed, the latter must be considered in the differential diagnosis of hyperthyroidism. Moreover, these manifestations may be ameliorated by the administration of adrenergic blocking agents. However, when sympathetic activity was determined directly by measuring noradrenaline turnover in hearts of hyperthyroid animals, it was shown to be normal or slightly decreased (Landsberg 1977). On the other hand, sympathetic activity is enhanced in hypothyroidism (Landsberg and Axelrod 1968).

Plasma and urinary noradrenaline levels are normal or low, and plasma dopamine-beta-hydroxylase activity is decreased in hyperthyroidism, whereas in hypothyroidism, plasma noradrenaline and dopamine-beta-hydroxylase are increased, and urinary noradrenaline levels are either increased or normal. Neither hypothyroidism nor hyperthyroidism appear to have a significant effect on urinary levels, plasma levels or the secretion of adrenaline. These findings (Landsberg 1977) suggest that sympathetic nervous activity is either decreased or unchanged in hyperthyroidism and increased in hypothyroidism. Neither state appears to produce a significant alteration in secretion of adrenaline by the adrenal medulla.

Therefore, the manifestations of thyrotoxicosis cannot be attributed to overactivity of the sympatho-adrenal axis and one must consider changes occurring at other sites.

1.2.2 Catecholamine Disposition: Effects of Thyroid State.

Alterations in tissue response to catecholamines could result either from changes in tissue sensitivity or from altered disposition of catecholamines causing local increases or decreases at receptor sites. Circulating catecholamines including adrenaline released from the adrenal medulla are destroyed mainly by catechol-O-methyl transferase (COMT) in the liver and

kidney (Mayer 1980). Since gross alterations in circulating adrenaline or adrenaline released from the adrenal medulla are not produced by changes in thyroid hormone levels (see above), it is unlikely that deranged metabolism could account for altered sympathetic activity in the intact animal.

Of perhaps greater significance, particularly with respect to thyroid-induced changes identified in isolated tissues, is the disposition of catecholamines in the vicinity of the adrenergic receptor. Normally, approximately 90% of noradrenaline released into the synapse is rapidly taken up again into the sympathetic nerve terminal by a specific transport system (Mayer 1980). Adrenaline, which finds its way into the synaptic space, is also a substrate for uptake via this mechanism. Some evidence exists to suggest that the activity of this transport system is decreased in hyperthyroidism and unchanged in hypothyroidism (Wurtman et al. 1963; Landsberg and Axelrod 1968). Wurtman et al. (1963) suggested that this could be the basis for apparently increased sympathetic activity in thyrotoxicosis. However, additional factors must be involved since tissue sensitivity to isoproterenol, an adrenergic agonist which is not a substrate for neuronal uptake, still showed thyroid-dependent changes (McNeill and Brody 1968; Kunos 1977). Moreover, inhibition of the extraneuronal uptake of isoproterenol in the heart by the addition of steroids did not modify the reduced sensitivity of the inotropic response in the hypophysectomized rat (Kunos et al. 1980). Thus at least in this example, alterations in extraneuronal uptake of the sympathomimetic could not account for alterations in adrenergic sensitivity.

Uptake into the noradrenergic nerve terminal is followed by storage of catecholamine in vesicles for subsequent re-release and, to a lesser extent, metabolism by monoamine oxidase (MAO), whereas extra-neuronal uptake results in metabolism by COMT (Mayer 1980). Effects of thyroid hormone levels on both MAO and COMT have been reported for many tissues and the findings are summarized in several recent publications (Youdim et al. 1976; Sourkes et al.

1977; Kunos 1981). These enzyme activities vary with species, age, sex and tissue examined. In general, the changes are small and while they may explain some aspects of altered tissue sensitivity to catecholamine, they are unlikely to be of major importance (Kunos 1981).

1.2.3 Thyroid Effects on End-organ Responsiveness to Catecholamines.

Present-day understanding of adrenergic receptors had its foundation in the dual receptor theory of Ahlquist (1948). He proposed that adrenergic receptors be divided into two groups based on the order of potency of agonists for producing the response: isoproterenol > adrenaline > noradrenaline for beta receptor-mediated responses and adrenaline $\geq$ noradrenaline > isoproterenol for alpha receptor-mediated responses. This theory was later refined to include two subtypes of beta receptors: $beta_1$ for which adrenaline = noradrenaline and $beta_2$ for which adrenaline > noradrenaline (Lands et al. 1967). Alpha receptors have also been sub-divided, originally on the basis of anatomical location as pre- and post-synaptic receptors (Langer 1974). The more recent terminology of $alpha_1$ and $alpha_2$ receptors reflects pharmacological rather than anatomical differences (Berthelson and Pettinger 1977). This modification in theory is justified since both receptor subtypes have been identified by pharmacological and ligand binding studies at post-synaptic sites (Fain and García-Sáinz 1980; Jard et al. 1981). The recent development of highly selective agonists and antagonists has proved useful in the differential study of these receptor types. It now appears clear that these receptors also differ in the mechanism by which they affect a tissue response. While both $beta_1$ and $beta_2$ receptors are coupled to adenylate cyclase and stimulation results in increased tissue cAMP levels (Ross and Gilman 1980; Limbird 1981), $alpha_1$ receptors mediate their effects through the mobilization of calcium and $alpha_2$ receptors inhibit adenylate cyclase activity (Fain and García-Sáinz 1980).

This concept of multiple subtypes of adrenergic receptors has been

emerging during recent years and has proven to be much more complex than one might have anticipated only a few years ago. Therefore, it is easy to appreciate the difficulties encountered in early attempts to understand the mechanism of thyroid effects on adrenergic responses. More recent studies of this problem attempt to analyze thyroid effects within this framework by examining specific receptor types (Kunos 1981; Fain 1981). Although controversies still exist, a clearer picture is emerging. Reasons for apparent discrepancies include sex; age; species differences, both with respect to catecholamine actions and sensitivity to thyroid hormones; the lack of complete dose-response studies and inadequate assessment of sensitivity changes, as well as differences in treatments used to produce hypothyroidism or hyperthyroidism in experimental animals (for a more complete discussion see Kunos 1981). Effects of thyroid hormones on adrenergic responsiveness of cardiac and adipose tissue have been examined extensively and will be briefly summarized here.

Cardiac Tissue.

Most studies have shown that hypothyroidism produced an increased alpha and decreased beta receptor contribution to adrenergic stimulation of the rate and force of contraction in isolated cardiac tissues (Nakashima et al. 1973; Kunos 1977; Kunos et al. 1980). The opposite was observed in hyperthyroidism (Kunos 1977; Hashimoto and Nakashima 1978). These results are supported by the finding of enhanced catecholamine-stimulated adenylate cyclase in cardiac preparations from hyperthyroid animals (Tse et al. 1980) and an attenuated activity in preparations from hypothyroid animals (Levey et al. 1969; Kunos et al. 1976; Brodde et al. 1980). Not all studies, however, are in agreement with these findings since some authors have found catecholamine-stimulated adenylate cyclase activity unaltered in hyperthyroid myocardium (McNeill et al. 1969; Young and McNeill 1974). The cardiac glycogenolytic effect of adrenergic agonists was also reported to be enhanced in hyperthyroidism, although these effects were not analyzed in detail with respect to receptor

type (Hornbrook et al. 1965; McNeill and Brody 1968; Hornbrook and Cabral 1972). In all of the above-noted studies thyroid state was altered in the intact animal. Tsai and Chen (1978) have demonstrated a T_3-mediated enhancement of beta receptor-stimulated cAMP accumulation in cultured fetal heart cells. This finding indicates that thyroid hormone could produce an increase in beta receptor activity in vitro.

Adipose Tissue.

Catecholamine-mediated activation of lipolysis in adipose tissue occurs via activation of the beta receptor/adenylate cyclase system (Himms-Hagen 1972; Young and Landsberg 1977). In addition, adipocytes also possess $alpha_2$ receptors that mediate inhibition of the adenylate cyclase and $alpha_1$ receptors that affect changes in glycogen metabolism analogous to those in liver (see section 1.3; García-Sáinz and Fain 1980). While the presence of all three types of adrenergic receptors have been shown in preparations of human and hamster adipose tissue, there is no evidence for $alpha_2$ receptors in rat fat cells (García-Sáinz and Fain 1980).

Debons and Schwartz (1961) showed that in adipose tissue from the rat the in vitro lipolytic response to catecholamine was enhanced in hyperthyroidism and suppressed in hypothyriodism. The decreased response in hypothyroidism was thought to be due to an attenuated cAMP accumulation during stimulation (Malbon et al. 1978b). Some investigators did show a decrease in adenylate cyclase response to catecholamine in hypothyroidism (Correze et al. 1974; Malbon et al. 1978b), while others found no change compared with euthyroid controls (Armstrong et al. 1974). Fain (1981) attributed these variable results to the use of different preparations and the inability to precisely control experimental conditions. Moreover, on the basis of unchanged beta receptor binding found in some studies (see section 1.2.4), he suggested that the impaired beta receptor responsiveness in hypothyroid adipocytes was due to a defective coupling of hormone receptor complexes to

adenylate cyclase. However, other effects cannot be discounted and probably contribute to the impaired response.

The presence of phosphodiesterase inhibitor potentiated adrenaline-induced lipolysis to a greater extent in adipocytes from hypothyroid than euthyroid rats (Goswami and Rosenberg 1978). Accordingly, others (Armstrong et al. 1974; Van Inwegen et al. 1975; Correze et al. 1976) reported increased phosphodiesterase activities in preparations from hypothyroid rat adipose tissue. These findings suggest that at least a portion of the impaired responsiveness of hypothyroid adipocytes may be due to increased metabolism of cAMP. However, Malbon et al. (1978b) were unable to find any change in the cAMP phosphodiesterase activity of fat cell ghosts. This may have been due to differences in the preparation used in that study compared with others described above.

Ohisalo and Stouffer (1979) observed that the impaired responsiveness of adipocytes from hypothyroid rats could be reversed by use of more dilute cell suspensions or the inclusion of adenosine deaminase in the incubation medium. This suggested that increased production or sensitivity to adenosine by hypothyroid adipocytes could contribute to the impaired responsiveness. On the other hand, Fain and Malbon (1979) found a decreased release and also impaired oxidative degradation of adenosine in hypothyroid adipocytes which tends to refute the importance of adenosine in this phenomenon.

Catecholamine-stimulated cAMP accumulation and lipolysis were enhanced in fat cells from T_3-treated rats, although this effect was not reflected in an enhanced adenylate cyclase response (Caldwell and Fain 1971; Malbon et al. 1978b). Thyroid state had an opposite effect on cAMP accumulation in hamster adipocytes. Isoproterenol-stimulated cAMP accumulation was enhanced in hypothyroidism and impaired in hyperthyroidism, the latter being attributed to a toxic effect of high doses of thyroid hormone (Fain 1981). Conversely, Giudicelli et al. (1980) also working with hamster adipocytes, inferred that the

decreased cAMP response in hyperthyroid animals was due to an alteration in alpha receptor activity. Alpha$_2$ receptor stimulation results in inhibition of adenylate cyclase (Fain and García-Sáinz 1980) and Rosenqvist et al. (1971) suggested that the impaired lipolytic response in human adipocytes could in part be due to a greater alpha$_2$ suppression of cAMP accumulation. However, it was later shown that neither hypothyroidism nor hyperthyroidism altered the alpha$_2$ receptor-medited inhibition of adenylate cyclase in hamster adipocytes (García-Sáinz et al. 1981). As mentioned before, thyroid effects on adrenergic receptors can show marked species variation and hence cannot readily be extrapolated. Therefore, the question of thyroid effects on alpha$_2$ receptors in humans and their role in altered sensitivity of the lipolytic response to catecholamines remains unanswered.

Alpha$_1$ receptor stimulation increases phosphatidylinositol turnover which is thought to be linked to an elevation of intracellular calcium (Fain and García-Sáinz 1980). Both alpha and beta receptor stimulation can produce activation of glycogen phosphorylase and inactivation of glycogen synthase in adipocytes (Lawrence and Larner 1977) just as in the liver (section 1.3). García-Sáinz and Fain (1980) have reported no change in alpha$_1$ receptor-mediated stimulation of phosphatidylinositol turnover or glycogen synthase inhibition in hypothyroid rat fat cells. However, Kunos (1981) has suggested that the lack of change in inactivation of glycogen synthase by the mixed agonist adrenaline in the face of markedly impaired inhibition by the beta agonist isoproterenol in the above study must suggest the presence of an increased alpha$_1$ receptor activity in the hypothyroid state. This point requires clarification. García-Sáinz et al. (1981) also reported a lack of effect of thyroid hormone on alpha$_1$ stimulation of phosphatidylinositol turnover in hamster adipocytes.

Hepatic Tissue.

Early studies demonstrated that the glycogenolytic effects of adrenaline

are potentiated in hyperthyroidism (Harrison 1964). In recent years it has been shown that both alpha and beta receptors can influence hepatic carbohydrate metabolism (see section 1.3). However, the glycogenolytic response in the intact animal is complex (Hornbrook 1970) and interpretation in terms of receptor effects is very difficult. At the outset of the present study there were only a few reports dealing with thyroid hormone effects on hepatic adrenergic responses. Experiments with isolated perfused rat liver were interpreted to suggest that beta receptor-mediated stimulation of gluconeogenesis in the normal rat was suppressed in hypothyroidism, while the contribution of alpha receptors was enhanced, (Hagino & Nakashima 1973, 1974). However, these findings do not agree with more recent studies which have shown that gluconeogenesis in the normal rat is mediated predominantly by alpha adrenergic receptors (for references see section 1.3). Nor are they in agreement with the findings to be presented here or those of Malbon and co-workers (Malbon et al. 1978a, 1980; Malbon 1980a). A more complete discussion of this is deferred to section 4.1.

Other Tissues.

Effects of thyroid hormones on catecholamine-mediated responses in a number of other tissues have been reported. These relatively few studies have been recently reviewed by Kunos (1981) and will not be described in detail here. However, of some relevance to the present studies, in as much as they deal with carbohydrate metabolism, are the effects of thyroid hormones on insulin secretion by the pancreas. Beta receptor stimulation enhances insulin secretion while alpha stimulation is inhibitory (Young and Landsberg 1977). Beta receptor activity dominates in hyperthyroidism and hence the mixed alpha/beta agonist adrenaline potentiates glucose-induced insulin secretion (Okajima and Ui 1978). In contrast, alpha receptors dominate in the hypothyroid pancreas, which results in an adrenaline-induced suppression of insulin release (Okajima and Ui 1978). Precisely how these changes relate to

the overall altered metabolic state of hypothyroidism or hyperthyroidism is unknown.

Summary.

From the foregoing it is clear that generalizations about the effects of thyroid hormones on adrenergic receptor-mediated responses must be made with caution. Most evidence suggests that hypothyroidism results in a decreased beta receptor activity in the heart and adipose tissue. Adipocytes from hamsters appear to represent a noteworthy exception (Fain 1981). Similar effects have been noted in pancreas (Okajima and Ui 1978). Alpha receptor activity is apparently enhanced in cardiac tissue from hypothyroid animals although this effect has not been analyzed in detail with respect to selective changes in alpha receptor subtypes. The question of how hypothyroidism affects alpha receptors in adipose tissue is unresolved. One group of investigators claim there is no effect on either $alpha_1$ or $alpha_2$ receptors (Fain 1981; García-Saínz et al. 1981; García-Sáinz and Fain 1982), although the suggestion has been made that such an effect has been overlooked (Kunos 1981).

Hyperthyroidism produces changes opposite to those found in hypothyroidism, although these effects are in general less clear. This is probably due to variations in species sensitivity as well as the use of different treatment regimens for the administration of thyroid hormone. The effects of hypothyroidism are in most cases reversible by thyroid hormone replacement (Kunos 1981; Fain 1981) and the effect of thyroid hormone level appears to follow a continuum. Thus, treatment of euthyroid animals with thyroid hormone produced opposite changes in cardiac adrenoceptor properties to those seen in hypothyroidism (Kunos 1977; Hashimoto and Nakashima 1978).

1.2.4 Thyroid Effects on Adrenergic Radioligand Binding.

Direct radioligand binding studies of adrenergic receptors have been of great value in precisely defining the role of receptors in thyroid-induced changes in tissue responsiveness. While providing a powerful tool to aid in the understanding of mechanisms which underlie these changes, studies using this approach appear to have introduced even more controversial and apparently contradictory findings into the literature. These investigations are subject to the same pitfalls and problems of variations in species, age, sex, etc. (see section 1.2.3) that have hampered the development of a clear understanding of thyroid effects on adrenergic responses. Even so, results of ligand binding studies have not in all cases supported the existence of changes in receptor properties that could explain the observed alterations in responsiveness even when the same tissue was examined for both (see below). There are two possible explanations for this: (1) thyroid hormones affect changes in responsiveness by altering components of the tissue response and have no effect on receptors per se or (2) the ligand binding study does not adequately assess receptor properties. Clearly the existence of a change in receptor properties which could explain the observed change in tissue response does not preclude the possibility of additional thyroid hormone effects on post-receptor events. Even in those situations where biochemical changes, other than at the receptor site itself, are not found, one cannot rule out possible additional effects on the coupling of receptor to the effector system. In the case of the beta receptor, for which the coupling to adenylate cyclase is relatively well understood (Ross and Gilman 1981; Limbird 1981), one can make assertions with greater confidence.

The first sucessful adrenergic binding studies were made in 1974 for beta receptors and in 1976 for alpha receptors (Hoffman and Lefkowitz 1980). Although this field has grown rapidly in a short period, the methodology is still being developed, as is an appreciation for the complexity of the receptor

systems being studied. Hence the over-zealous interpretation of earlier studies has undoubtedly contributed to incorrect and misleading conclusions. Moreover, the common practice of restating in print many times the results of a single study has led to the premature acceptance of data without adequate reinvestigation and confirmation. Hence, any dogmatic statements about adrenergic receptors based on binding studies must be viewed with skepticism.

Thyroid hormone effects on radioligand binding properties of adrenergic receptors has been reviewed and some of the common problems encountered discussed (Hoffman and Lefkowitz 1980; Kunos 1981; Fain 1981). The results of some of these studies in cardiac and adipose tissue will be summarized here.

Cardiac Tissue.

Most studies have shown an increase in beta receptor numbers in cardiac tissue from thyroid hormone-treated rats and a decrease in hypothyroid preparations (Hoffman and Lefkowitz 1980). In general there was no or little change in receptor affinity (Kunos 1981). This is the expected result on the basis of observed alterations in tissue responsiveness (section 1.2.3). Of particular interest are studies which have demonstrated increased binding sites for [^{3}H] DHA, a beta antagonist, when nanomolar amounts of thyroid hormone were added in vitro to either cultured rat myocardial cells (Tsai and Chen 1978), or ventricle slices of hypothyroid (Chang and Kunos 1981) or euthyroid rats (Kempson et al. 1978). The latter study showed an early (1.5-2 h) increase in binding sites that was not affected by protein synthesis inhibitors as well as a slower increase (15 h) which was. Although most investigations have demonstrated increased alpha adrenergic responsiveness of hypothyroid hearts (section 1.2.3), results of alpha receptor radioligand binding assays have been variable. Thus, studies using [^{3}H] DHE to evaluate alpha receptor changes in hypothyroid rats have shown either a decrease (Ciaraldi and Marinetti 1977, 1978) or no change (Williams and Lefkowitz 1979; McConnaughey et al. 1979) in alpha receptor binding site density. Reasons for these discrepant findings

are not clear, although it is possible that the presence of presynaptic alpha$_2$ receptor binding sites or differences in the physical-biochemical properties of preparations from normal and hypothyroid rats may have been factors. However, Noguchi and Whitsett (1983) using [^{3}H]prazosin, a selective alpha$_1$ antagonist, also found a decrease in alpha$_1$ receptor binding sites in hypothyroid rats hearts. On the other hand, Sharma and Banerjee (1978) had shown that T_3 treatment of thyroidectomized rats resulted in a decrease in [^{3}H] DHE binding site density. This finding was later confirmed by Chang and Kunos (1981) who found that the binding of [^{3}H]prazosin was decreased in hypothyroid rats after treatment with T_3 for 36 h, while [^{3}H] DHA binding was increased. Similarly, T_4 treatment of hypophysectomized rats for two days produced an increase in [^{3}H] DHA binding sites and a decrease in alpha$_1$ receptor binding sites identified as prazosin-suppressible [^{3}H] WB 4101 binding (Kunos et al. 1980).

Most studies on tissue responsiveness to sympathomimetics have shown that hyperthyroidism decreases the alpha receptor component in cardiac tissue (section 1.2.3). Those findings are supported by the results of receptor binding studies. Thus, Ciaraldi and Marinetti (1977, 1978), Williams and Lefkowitz (1979) and McConnaughey et al. (1979) found evidence of a decrease in alpha receptor binding in cardiac tissue from hyperthyroid rats.

Adipose Tissue.

[^{3}H] DHA has been used to assess thyroid-induced changes in beta receptors of adipocytes. Malbon et al. (1978b) and Goswami and Rosenberg (1978) reported no change in beta receptor binding sites with thyroid state. However, beta binding site numbers have been reported to be decreased in hypothyroid (Giudicelli 1978) and increased in hyperthyroid rats (Ciaraldi and Marinetti 1978). Non-linear Scatchard plots (Malbon et al. 1978b) in the absence of negative cooperativity (Malbon and Cabelli 1978) suggest that [^{3}H] DHA binding may be to heterogeneous sites and hence these studies must

be interpreted with caution. Moreover, Malbon (1980b) found that the well known effect of guanyl nucleotides on agonist affinity for the beta receptor (Ross and Gilman 1980; Limbird 1981) was lost in preparations from hypothyroid or hyperthyroid adipose tissue. This finding suggests that there may be a complex regulatory role for guanyl nucleotides in thyroid hormone effects on fat cells.

Alpha receptors have been little studied in adipose tissue. Giudicelli et al. (1980) have reported a decrease in binding site density for [^{3}H] DHE in adipose tissue from hyperthyroid hamsters and no change in hypothyroid hamster adipocytes. García-Sáinz et al. (1981) on the other hand, found no change in [^{3}H] DHE binding in adipocyte membranes from hyperthyroid hamsters. Neither of these studies differentiated alpha$_1$ and alpha$_2$ receptors, and a definitive statement about possible changes in alpha receptors in adipocytes must await studies with selective alpha$_1$ and alpha$_2$ ligands.

Findings of work on adrenergic receptor binding properties in hepatic tissue will be discussed later (section 1.3.4).

1.3 Hepatic Adrenergic Receptors.

1.3.1 Classification of hepatic adrenergic receptors.

The body stores glucose in the form of glycogen which can be rapidly mobilized, such as during strenuous muscular exercise, to supply fuel for glycolysis when local glucose or oxygen are in short supply. Hepatic glycogenolysis together with gluconeogenesis supply glucose for tissues such as brain and erythrocytes which utilize glucose as their basic metabolic fuel. Hence, the liver has the important role of maintaining the euglycemic state during short periods of fasting.

The control of liver glycogen metabolism has recently been reviewed (Hers 1976; Hems and Whitton 1980). In 1940, Soskin (1940) put forward the concept

that circulating glucose itself is the primary stimulus for glycogen breakdown or deposition in the liver. However, glucose storage and output is additionally modulated by hormonal and nervous influences, principally those of insulin and glucagon. These hormones act to control the activation or inactivation of glycogen phosphorylase, the rate-limiting enzyme for glycogen breakdown and glycogen synthase, the enzyme that catalyzes glycogen synthesis (Hems and Whitton 1980).

A variety of hormones in addition to glucagon can activate glycogen phosphorylase and hence rapidly stimulate hepatic glycogenolysis (table 1.1), however, the physiological importance of these is unknown. Adrenergic agonists are included in this list. Although the effects of catecholamines on hepatic carbohydrate metabolism have been studied for many years (Hornbrook 1970), it is only recently that the mechanism of adrenergic activation of hepatic phosphorylase has been clarified. This is particularly ironic in view of the important role the study of adrenaline effects on phosphorylase activation played in the discovery of cAMP by Sutherland and coworkers in the late 1950's (Robison et al. 1971). The historical development of our understanding of adrenergic receptors in the liver has been reviewed by Haylett (1979) and will be dealt with only briefly here.

Hepatic adrenergic receptors could not, until recently, be easily classified as alpha or beta (see section 1.2.3). At least part of this difficulty arose because of attempts to analyze the effect of catecholamines on the hyperglycemic response in the intact animal; catecholamine-induced hyperglycemia is a complex response involving direct effects on the liver, effects on insulin and glucagon secretion by the pancreas, glucose uptake and lactate production by muscle, lipolysis in adipose tissue, as well as a number of other minor effects (Hornbrook 1970; Young and Landsberg 1977; Himms-Hagen 1972). In addition, the response shows considerable variation with species and route of catecholamine administration (Ellis 1967). Some of the

difficulties with classification also arose because of the apparent refractoriness of the hyperglycemic response to conventionally accepted doses of adrenergic antagonists (Hornbrook 1970). However, studies from this laboratory (Kan et al. 1979) as well as others (Jenkinson et al. 1978) demonstrated the need for very high antagonist concentrations in order to block the response even in in vitro preparations. This is most likely due to the high capacity of the liver to take up antagonists thus reducing the effective concentration in the vicinity of the receptor (Jenkinson et al. 1978; Kan et al. 1979).

Although it was well accepted on the basis of the studies of Sutherland and co-workers (Robison et al. 1971) that adrenaline could activate phosphorylase via the beta receptor-adenylate cyclase pathway (see section 1.3.2), the role of alpha receptors in contributing to this response was questioned (Hornbrook 1970). Earlier evidence that, at least in the fed rat, adrenaline-induced hyperglycemia had the characteristics of an alpha receptor-mediated effect (Fleming and Kenny 1964) did not gain acceptance until the work of Sherline et al. (1972). These authors demonstrated that in the perfused rat liver catecholamines activate glycogenolysis by a beta receptor-mediated effect resulting in a rise in cAMP, but also by a cAMP-independent alpha receptor-mediated effect. However, they cautioned that phosphorylase activation by alpha receptors could be secondary to hypoxia resulting from alpha-mediated constriction of the liver vasculature (Sherline et al. 1972). At about the same time Haylett and Jenkinson (1972b) showed that catecholamine-stimulated glucose release from ginuea pig liver slices involved both beta and alpha receptors. Later, Exton and co-workers confirmed the existence of alpha receptor-mediated activation of glycogen phosphorylase and glucose output in suspensions of isolated rat hepatocytes, thus ruling out the possible effects of tissue hypoxia (Exton and Harper 1975; Hutson et al. 1976). In addition, studies showed evidence of alpha receptor-mediated activation of gluconeogenesis in isolated rat liver cells (Tolbert et al. 1973;

Kneer et al. 1974; Hutson et al. 1976) that was also independent of changes in cellular cAMP (Tolbert et al. 1973; Hutson et al. 1976).

It appears therefore, that both alpha and beta receptors are present in the liver. Adrenaline effects on carbohydrate metabolism in the rat liver are mediated predominantly via the alpha receptor with only a minimal contribution by the beta receptor (Exton and Harper 1975). However, it has been shown that the relative importance of these two receptor types varies with the species examined, which also accounts for some of the confusion in earlier attempts to classify the hepatic receptor. At present there is good evidence that (at least in vitro) both alpha and beta components contribute significantly in guinea pig (Haylett and Jenkinson 1972b; Osborn 1975) and rabbit liver (Haylett 1976). In the mouse, alpha receptors appear to play a relatively more important role (Seydoux et al. 1979). The dog and cat, long considered to mobilize hepatic glycogen stores via a beta receptor-mediated effect (Haylett 1979), have not been studied in detail since the importance of hepatic alpha receptors became accepted. However, Kuo et al. (1977) have demonstrated in vivo that the cat has both alpha and beta receptors that contribute to the glycogenolytic response.

In man, earlier studies comparing the effects of adrenergic agents on the hyperglycemic response in vivo indicated a major role for alpha receptors (see Exton 1979 for references). More recently, Rizza et al. (1980) analyzed this response in greater detail. These authors infused adrenaline into healthy volunteers after "clamping" glucose, insulin and glucagon by a continuous infusion of somatostatin. Under these conditions they could analyze the effects of selective alpha and beta blockade and concluded that hepatic glucose output was directly increased by a beta adrenergic mechanism. This effect could be augmented by alpha receptor-mediated inhibition of pancreatic insulin secretion and beta-stimulation of glucagon release. Alpha blockade was judged to be relatively ineffective in inhibiting adrenaline's direct hepatic effect. The

relative contribution of alpha and beta receptors to catecholamine effects in the liver also appears to be influenced by age (Hornbrook 1978; Blair et al. 1979a; Kawai and Arinze 1981), sex (Bitensky et al. 1970; Studer and Borle 1982), hormonal state (Wolfe et al. 1976; Chan et al. 1979a) and the presence of pathological changes in liver (section 4.5). These effects are discussed more fully in a later part of this thesis.

Table 1.1

Agents that Stimulate Hepatic Glycogenolysis.*

Cyclic AMP-dependent Agents
beta adrenergic agonists
glucagon
parathyroid hormone
Cyclic AMP-independent Agents
alpha adrenergic agonists
vasopressin
oxytocin
angiotensin II
A23187

* for references see text and Hems (1977).

1.3.2 The mechanism of beta adrenergic activation of hepatic glycogen phosphorylase.

Our contemporary understanding of the mechanism of catecholamine activation of glycogen phosphorylase via the beta receptor is based on the classic studies of Sutherland and co-workers (Robison et al. 1971). Much of this information has been reviewed recently (Exton et al. 1981; Exton 1982) and will be briefly summarized here.

The presently accepted scheme as depicted in figure 1.1 is based on the contributions of a great many investigators working with various tissues and experimental systems. Interaction of catecholamine with the beta adrenergic receptor on the plasma membrane of the liver cell leads to the activation of adenylate cyclase. This interaction involves at least three separate proteinaceous membrane components: the receptor, a catalytic moiety and a guanine nucleotide-sensitive regulatory protein which is absolutely required for hormone-activation of the cyclase (Ross and Gilman 1980; Limbird 1981). The cyclase catalyzes the formation of cAMP, the so-called second messenger, from ATP. Cyclic AMP in turn activates protein kinase. Protein kinase exists in the cell as an inactive tetrameric structure composed of two catalytic and two regulatory subunits. Cyclic AMP binds to the regulatory sites and causes the complex to dissociate producing two active catalytic subunits. This reaction is reversible and activation is halted when the rate of cAMP extrusion from the cell or metabolism to 5'AMP by phosphodiesterase returns intracellular cAMP levels to their basal value.

Axelrod and coworkers (Hirata and Axelrod 1980) have recently proposed that coupling of the beta adrenergic receptor and adenylate cyclase involves local methylation of membrane phospholipids. They demonstrated that in several tissues stimulation of the beta receptor (as well as other cAMP-linked receptors) activated a membrane bound methyltransferase II which catalyzes methylation of membrane phospholipids. This effect is not cAMP-mediated and

results in a localized increase in membrane fluidity. They postulated that this increase in fluidity permits the coupling of receptor to adenylate cyclase and in so doing leads to cAMP generation. If indeed this hypothesis proves correct, it adds a vital link in the understanding of receptor mechanisms. Recent reports suggest, however, that this mechanism may not be generalized. In particular, it may not apply to the rat liver, where nearly complete inhibition of methyltransferase activity left hormone-induced stimulation of adenylate cyclase virtually intact (Colard and Breton 1981; Sanche et al. 1982).

Hormonal stimulation of adenylate cyclase results ultimately in the phosphorylation of a number of cellular components (Garrison et al. 1979). Two isoenzymes of cAMP-dependent protein kinase have been shown to exist in mammalian tissues and these appear to differ only in their regulatory subunits (Walsh 1978). Thus, the catalytic subunit possesses rather broad substrate specificity. Three substrates for the ATP-dependent phosphorylation by this protein kinase have thus far been identified (Exton 1979). As depicted in figure 1.1. phosphorylase kinase is phosphorylated to the active form by the catalytic subunit of protein kinase. This enzyme, in turn, activates glycogen phosphorylase b to the a or active form by a second ATP-requiring phosphorylation. Phosphorylase a catalyzes the breakdown of glycogen leading to increased glucose output. The second substrate for cAMP-dependent protein kinase is glycogen synthase. Phosphorylation of this enzyme results in inactivation and hence decreased glycogen synthesis. Most recently L-type pyruvate kinase has also been shown to be inactivated through phosphorylation by cAMP-dependent protein kinase. This enzyme represents an important control point in the Embden-Meyerhoff pathway. Its inactivation probably plays an important role in the stimulation of gluconeogenesis by cAMP-dependent hormones. A number of other hepatic enzyme activities are thought to be regulated by cAMP-dependent protein kinase phosphorylation, but these have not been as well studied (Exton et al. 1981). Also, there exist a number of

hepatic elements that undergo cAMP-dependent phosphorylation for which the function of this regulation is not yet known (Garrison et al. 1979).

In addition to beta receptor agonists, several other hormones are able to activate hepatic glycogenolysis via the adenylate cyclase-cAMP-dependent protein kinase pathway (Hems 1977; Hems and Whitton 1980). Those so far identified include glucagon and are listed in Table 1.1. Interaction of glucagon with its membrane-bound receptor activates adenylate cyclase in a manner exactly analogous to that described above for the beta receptor (Exton 1982). Hence post-receptor events that lead to specific protein phosphorylations are likely to be identical for these hormones. However, one cannot ignore the possible existence of additional effects of these hormones that might not involve the adenylate cyclase system. For example, high concentrations of glucagon have been shown to mobilize intracellular calcium, an effect which is undetectable at physiologic concentrations (Blackmore et al. 1978, 1979c). While most evidence suggests that this effect does not contribute significantly to the stimulation of glycogenolysis (Exton et al. 1981), one cannot rule out a role in regulating other metabolic processes in the hepatocyte. Côté and Epand (1979) have described the synthesis of a glucagon analogue which antagonizes glucagon stimulation of adenylate cyclase and yet is capable of activating glycogenolysis in isolated rat hepatocytes. Cardenas-Tanus et al. (1982) found that angiotensin II inhibited glucagon-stimulated cAMP accumulation in isolated hepatocytes, but did not alter the hormone's metabolic effects in the cell. The mechanisms underlying these findings are not yet established and until more is known about these effects it is reasonable to accept that cAMP is the major mediator of the effects of glucagon.

Insulin is able to counteract the effects of adrenaline and glucagon on hepatic glucose output (Soderling and Park 1974). Although the mechanism of this effect is not fully understood, insulin lowers glucagon-stimulated cAMP accumulation and activation of protein kinase, phosphorylase kinase as well as

glycogen phosphorylase by low concentrations of glucagon (for references see Blackmore et al. 1979a). Loten et al. (1978) have demonstrated that insulin will increase the "low K_m" cAMP phosphodiesterase of liver suggesting that this is the basis for the hormone's effects on cAMP-mediated processes. However, possible additional effects on adenylate cyclase itself in the intact cell or the regulation of phosphoprotein phosphatase activity may be important (Soderling and Park 1974). The effects on beta receptor-mediated glycogenolysis are likely to be same, but direct experimental evidence for this is lacking (Van de Werve et al. 1977). Inhibition of alpha receptor activation of phosphorylase by insulin will be discussed in section 1.3.3. There is also some evidence that stimulation of parasympathetic nerves to the liver can have effects similar to insulin infusion on cAMP accumulation (Shimazu 1968).

The properties and regulation of liver phosphoprotein phosphatases represent somewhat of a grey area in our understanding of hepatic metabolic regulation. This has been the subject of recent reviews (Lee et al. 1980; Li 1982; Krebs and Beavo 1979). Since the activity level of phosphoprotein enzymes is dependent on the net result of phosphorylation and dephosphorylation, phosphoprotein phosphatase activities may also play an important role in metabolic regulation by hormones. Numerous forms of phosphoprotein phosphatases varying in their physicochemical properties and substrate specificities have been described in many tissues including the rat liver (Li 1982). Early findings which suggested that each phosphoprotein possessed a corresponding specific phosphatase gave way to the concept of a single multifunctional phosphoprotein phosphatase with broad substrate specificity; the previously identified forms representing degradation products that retained some activity (Li 1982). However, most current evidence would support the existence of multiple forms of phosphoprotein phosphatase with distinct, but overlapping substrate specificities. For example, glycogen synthase phophatase and phosphorylase phosphatase have different properties and can be

separated by ion exchange chromatography (Lee et al. 1981). Phosphoprotein phosphatase activity can be regulated in a number of ways some of which may be important mechanisms in vivo (Krebs and Beavo 1979). Consideration of the regulatory phenomena described for glycogen phosphorylase phosphatase will serve to illustrate this point. The activity of phosphorylase a is inhibited by glucose which, by binding to phosphorylase, increases its affinity for deactivation by the phosphatase (Li 1982). Other metabolites such as glucose-6-phosphate have a similar so-called substrate-directed modulating influence (Krebs and Beavo 1979). Direct protein-protein interactions may also play an important role. Thus, phosphorylase kinase can interact with a phosphatase and inhibit the dephosphorylation of phosphorylase a (Krebs and Beavo 1979). Phosphorylase a can inhibit the phosphatase-mediated activation of glycogen synthase (Hers 1976). Hers and coworkers (Hers 1976) have proposed that these interactions play an important role in intracellular regulation. They point out that high levels of phosphorylase a may inhibit the concomitant activation of glycogen synthase. Their scheme, however, is not generally accepted (Krebs and Beavo 1979; Lee et al. 1981; Li 1982).

Specific proteinaceous phosphatase inhibitors have been isolated from muscle and liver as well as other tissues (Lee et al. 1981). These have been most extensively examined in muscle where two different protein inhibitors have been characterized. The finding that one of these inhibitors must be phosphorylated by a cAMP-dependent protein kinase to be able to inhibit a specific phasphatase activity, led Cohen (1978) to propose a central role for these inhibitors in the regulation of glycogen metabolism. In his scheme (Cohen 1978), the cAMP-dependent phosphorylation of inhibitor-1 would lead to inhibition of the phosphoprotein phosphatase responsible for the activation of glycogen synthase and inactivation of glycogen phosphorylase, assuming these are the same enzymes. While this scheme still lacks much supporting evidence, Foulkes and Cohen (1979) have demonstrated the in vivo phosphorylation of

muscle inhibitor-1 in response to adrenaline injection.

A similar scheme could be hypothesized to play a role in regulation of hepatic glycogen metabolism. Some sparce evidence for the possible hormonal regulation of phosphorylase phosphatase in liver does exist. Goris et al. (1978) have reported finding a phosphorylatable phosphoprotein phosphatase inhibitor in dog liver. Shimazu and Amakawa (1975) demonstrated that stimulation of sympathetic nerves to rabbit liver enhanced glycogenolysis and attributed this to inhibition of phosphorylase phosphatase they observed. Under those conditions, Shimazu and Amakawa (1975) found no increase in cAMP and suggested the presence of an unidentified neurotransmitter to account for the phosphatase inhibition. It is convenient to digress at this point from cAMP-mediated effects and deal briefly with phosphoprotein phosphatases in relation to hepatic alpha adrenergic actions which are discussed more fully in the next section. Hepatic alpha receptor-mediated activation of phosphorylase is independent of cAMP and proceeds via the release of intracellular calcium ions (section 1.3.3). Although phosphoprotein phosphatase activity is dependent on divalent cations (Li 1982), a role for calcium in regulating this activity has not been established (Khoo and Steinberg 1975). Furthermore, Proost et al. (1979) in experiments very similar to those of Shimazu and Amakawa (1975) found no evidence for a role of phosphatase in mediating the activation of phosphorylase by nerve stimulation. Blackmore and Exton (1981) found that alpha adrenergic stimulation of phosphorylase was impaired in hepatocytes from rats genetically deficient in phosphorylase b kinase activity. This finding suggests that it is modulation of the kinase activity that controls the level of activation of phosphorylase, but a possible additional role for the phosphatase cannot be ruled out. This area requires further study.

Before concluding this brief discussion of hormonal effects occurring via the cAMP system it is worth drawing attention to the fact that a number of cellular activities not directly related to carbohydrate metabolism are regulated

in a similar manner. Beavo and Krebs (1979) have compiled a list of 22 enzymes that have been reported to undergo phosporylation-dephosphorylation.

1.3.3 The mechanism of alpha adrenergic activation of hepatic glycogen phosphorylase.

Agents that are capable of stimulating hepatic glycogenolysis by a cAMP-independent mechanism are listed in table 1.1. While most studies have concentrated on alpha adrenergic effects in the liver, vasopressin and angiotensin II actions have also been examined in some detail. These latter two agents have effects similar to alpha adrenergic agonists and evidence for this will be summarized later in this section. Since the first clear demonstration by Exton and co-workers (Exton and Harper 1975) that alpha adrenergic agonists can activate glycogen phosphorylase without a concomitant increase in cellular cAMP levels, much effort has been expended in establishing a mechanism for this action. Considerable advances have been made and this work has been reviewed recently (Exton 1979, 1980, 1981; Exton et al. 1978, 1981). The development of current understanding of the cAMP-indepedent activation of glycogen phosphorylase will be summarized here.

Goldberg and co-workers (Goldberg et al. 1973) first suggested that alpha adrenergic effects were mediated by cGMP in a manner analogous to the involvement of cAMP in beta receptor-mediated events. Although, alpha stimulation does result in an increase in cGMP levels in the liver (Pointer et al. 1976), this effect can be produced by a number of other agents that do not activate glycogen phosphorylase (Pointer et al. 1976; Hems et al. 1978). Hence, this idea was discarded.

Many lines of evidence pointed to the involvement of calcium ions in the cAMP-independent activation of glycogen phosphorylase. Alpha adrenergic-activation of hepatic glycogenolysis is inhibited when extracellular calcium is removed (Keppens et al. 1977; Assimacopoulos-Jeannet et al. 1977;

Van de Werve et al. 1977; Chen et al. 1978). The divalent cation ionophore, A23187, mimicked alpha adrenergic effects on hepatic glycogenolysis in a calcium-dependent fashion (Keppens et al. 1977, Assimacopoulos-Jeannet et al. 1977). Blackmore et al. (1978) then demonstrated that the rapid chelation of extracellular calcium ion by the stochiometric addition of EGTA with the agonist did not significantly impair the alpha adrenergic activation of phosphorylase in isolated rat hepatocytes. The rapid addition of EGTA in this manner would presumably prevent the influx of extracellular calcium during stimulation without depleting intracellular calcium stores. In fact, Blackmore et al. (1978) were able to identify a net loss of cellular calcium occurring during stimulation that was even greater when EGTA was added as described. Soon afterward it was confirmed that alpha stimulation induced a loss of calcium from isolated hepatocytes (Chen et al. 1978; Burgess et al. 1979) or perfused liver (Blackmore et al. 1979b,c; Althaus-Salzman et al. 1980; Reinhart et al. 1982a). However, not all groups were able to identify a net loss of calcium after alpha stimulation (Foden and Laudle 1978; Murphy et al. 1980). The reason for this difference is not clear. Nonetheless, there was sufficient evidence to support the postulation of Exton and coworkers (Exton 1979) that the transient increase in cytosolic calcium was responsible for the activation of glycogen phosphorylase by phosphorylase kinase, an enzyme known to be sensitive to calcium (see below). This was supported by the findings of Murphy et al. (1980) who showed that alpha adrenergic stimulation of isolated hepatocytes produced a two- to three-fold increase in free cytosolic calcium that correlated well, with respect to time-course and dose-response, with phosphorylase activation.

While it is now well accepted that alpha receptor-mediated activation of phosphorylase occurs via the production of a transient increase in cytosolic calcium, there is still some disagreement about the source of this calcium. Studies using atomic absorbtion spectrometry (Blackmore et al. 1979b,c) or

chlortetracycline flourescence (Babcock et al. 1979) to measure mitochondria-associated calcium suggest that most of the calcium released during alpha receptor stimulation is mitochondrial in origin. On the other hand, studies using ^{45}Ca showed an increased mitochondrial uptake of ^{45}Ca after stimulation (Foden and Randle 1978; Althaus-Salzman et al. 1980; Poggioli et al. 1980). Unfortunately in these latter studies total mitochondrial calcium was not determined and it is unclear whether the increased ^{45}Ca reflects a true change in calcium content or merely increased exchange. One of these groups (Poggioli et al. 1980) in a subsequent study (Berthon et al. 1981) suggested that these discrepancies were most likely the result of differences between experimental protocols. In the later study, they demonstrated an initial, transient increase in mitochondrial calcium (measured by atomic absorbtion spectrometry) followed by a loss of mitochondrial calcium. They concluded that the transient increase in cytosolic calcium was the sum of sequential calcium release from endoplasmic reticulum and then mitochondria. However, these results were not confirmed in a detailed kinetic analysis of the effects of adrenaline on calcium distribution in isolated rat hepatocytes by Barritt et al. (1981). These authors demonstrated that adrenaline caused a loss of calcium from an intracellular compartment which includes mitochondria and also an early increase of calcium transport from the extracellular medium. Attempts to analyze a possible role for extracellular calcium in mediating the effects of alpha stimulation by the use of verapamil, a calcium channel blocker, met with difficulties owing to a direct antagonistic effect of this drug on the alpha receptor site (Blackmore et al. 1979d).

Blackmore et al. (1982) re-examined the role of extracellular calcium in the alpha receptor-mediated activation of phosphorylase in isolated hepatocytes. From the results of this study they concluded that net calcium influx does not occur during the initial response to alpha agonists since they could find no increase in cellular calcium measured by atomic absorbtion spectrometry as

early as 5s after stimulation. Also, rapid chelation of extracellular calcium, as described above, had no detectable effect on phosphorylase activation. They surmized that uptake of chemical calcium or ^{45}Ca must represent a recovery process. In support of these findings, Reinhart et al. (1982a) found that net efflux of calcium from perfused livers occurred as early as 7.1 $\pm$ 0.5 s after phenylephrine-stimulation. Based on measurements of chemical calcium after subcellular fractionation of perfused livers, these workers concluded that most calcium released by stimulation is mitochondrial and, to a lesser extent, from endoplasmic reticulum. A large portion of the calcium released is extruded from the cell by a calcium-sensitive ATPase located in the hepatic plasma membrane (Lotersztajn et al. 1981). Calcium is quickly reaccumulated which accounts for the influx of extracellular calcium noted by others. Based on existing evidence, this scheme provides the most reasonable mechanism for alpha receptor-mediated effects on hepatic calcium movements.

The locus of calcium's effect in the glycogenolytic cascade is most likely at the level of phosphorylase kinase (see figure 1.1). This enzyme is calcium sensitive, its activity being enhanced without undergoing phosphorylation (Khoo and Steinberg 1975; Shimazu and Amakawa 1975; Vandenheede et al. 1979). Blackmore and Exton (1981) examined the alpha adrenergic activation of phosphorylase in hepatocytes from rats genetically deficient in phosphorylase b kinase activity. They showed that stimulation of alpha receptors in hepatocytes isolated from these rats produced the same degree of calcium mobilization as in normal rats, but phosphorylase activation was impaired. Glucagon, vasopressin and the calcium ionophore, A23187, had similar effects. Calcium-dependent modulator protein (calmodulin) has been identified as the delta subunit of phosphorylase kinase from skeletal muscle (Cohen et al. 1978). A similar component has been identified in purified rat liver phosphorylase kinase (Chrisman et al. 1982) indicating the locus for the calcium-sensitive regulation of this enzyme. Taken together, these results suggest that

phosphorylase kinase is the most likely site of calcium's stimulation of the glycogenolytic cascade.

Alpha adrenergic stimulation has also been shown to inhibit glycogen synthase by a stable phosphorylation (Hutson et al. 1976; Murphy et al. 1980) and to stimulate gluconeogenesis (Pointer et al. 1976; Hutson et al. 1976; Chan and Exton 1978) probably in part as a result of phosphorylation and inactivation of pyruvate kinase (Chan and Exton 1978; Garrison and Borland 1979). Garrison and co-workers (Garrison et al. 1979) found that stimulation of hepatocytes in the presence of $^{32}PO_4$ with either noradrenaline plus a beta antagonist or with glucagon resulted in phosphorylation of the same 11 to 12 cytosolic proteins, three of which were identified as phosphorylase, glycogen synthase and pyruvate kinase (Garrison et al. 1979). Therefore, Exton (1980) has suggested that the liver possesses a calcium-sensitive protein kinase which has a substrate specificity similar to cAMP-dependent protein kinase. The possibility that phosphorylase kinase may be the multifunctional calcium-sensitive kinase has not been examined extensively. However, Payne and Soderling (1980) have isolated from rabbit liver a calmodulin-dependent glycogen synthase kinase that did not phosphorylate glycogen phosphorylase. This finding suggests the existence of at least two and possibly more calcium sensitive protein kinases in liver.

There are a number of alpha adrenergic effects in liver for which the role of calcium ions and protein phosphorylation is less clear. Haylett and Jenkinson (1972a,b) demonstrated that alpha stimulation caused a rapid efflux of potassium from guinea pig liver slices that was associated with a reversible 10 mV hyperpolarization; the resting membrane potential was approximately -30 to -40 mV. Activation of beta receptors had little effect on either of these responses although it was equally as effective as activation of alpha receptors in stimulating glucose output (Haylett and Jenkinson 1972a) or activating phosphorylase in the guinea pig (Osborn 1979). In a later study with

isolated guinea pig hepatocytes, Burgess et al. (1979) found that the calcium ionophore, A23187, was capable of producing a similar potassium efflux to that seen with alpha agonists, suggesting the presence of a calcium-sensitive potassium channel. Another group working with guinea pig liver slices (Weiss and Putney 1978) could not demonstrate a similar effect of A23187 on the potassium release response (measured as ^{86}Rb efflux), although these workers also identified a role for calcium in the response. The reason for this difference is not clear.

Not all species show a similar effect of alpha stimulation on ion movements in the liver (Jenkinson et al. 1978). Thus, rat liver cells do not respond to either alpha agonists or A23187 by potassium loss as seen in the guinea pig and it has been suggested that a calcium-sensitive potassium channel may be lacking in the rat (Burgess et al. 1979). Blackmore et al. (1979c) found that the alpha agonist phenylephrine as well as glucagon caused a rapid uptake of potassium in perfused rat liver. In addition, the hyperpolarizing effect of alpha stimulation observed in guinea pig (Haylett and Jenkinson 1972a,b) does not occur in all species; depolarization has been reported in the dog (Jenkinson et al. 1978). The reason for these interesting species differences is not known and is worthy of further study.

Alpha stimulating effects on amino acid transport (LeCam and Freychet 1978), oxygen consumption and lactate release (Jakob and Diem 1975; Blair et al. 1979b), ureagenesis (Corvera and García-Sáinz 1981), glycolysis and Na^+K^+transport (Becker and Jacob 1982), cGMP accumulation (Pointer et al. 1976) and the turnover of phosphatidyl inositol (Kirk et al. 1977; Billah and Mitchell 1978; Tolbert et al. 1980) have also been reported.

Although many of the effects of alpha receptor stimulation in liver appear clearly to be linked to calcium mobilization, the pathway whereby interaction of agonist with receptor leads to the release of intracellular calcium is unknown. Whiting and Barritt (1982) have listed some of the mechanisms that

have been proposed to account for this. Discussion of all of these is beyond the scope of this introduction. However, the possible role of phosphatidylinositol hydrolysis has received a great deal of attention and will be discussed briefly. Michell and co-workers put forward the hypothesis that receptor activation results in stimulation of phosphatidylinositol breakdown which is somehow coupled to calcium mobilization (Michell 1975). This hypothesis was based on observations that alpha receptor stimulation in liver (Kirk et al. 1977; Billah and Michell 1979; Tolbert et al. 1980) as well as a number of other tissues (for references see Exton 1980; Fain and García-Sáinz 1980; Michell and Kirk 1981) resulted in increased incorporation of $^{32}PO_4$ into phosphatidylinositol as a result of breakdown followed by resynthesis of this phospholipid (Michell and Kirk 1981). This response is thought to precede calcium mobilization, since it was still apparent afer removal of medium calcium and could not be reproduced by the calcium ionophore, A23187 (Billah and Michell 1978; Tolbert et al. 1980). Tolbert et al. (1980) have demonstrated that like the activation of hepatic phosphorylase (Hoffman et al. 1980b; El-Refai and Exton 1980; Aggerbeck et al. 1980a), alpha adrenergic stimulation of phosphotidylinositol breakdown is mediated by an $alpha_1$ receptor. While this hypothesis provides an attractive possible explanation for alpha effects on calcium homeostasis, it may have serious shortcomings. The most contrary evidence comes from a study by Exton's group. These authors (Prpić et al. 1982) carefully examined phosphatidylinositol breakdown induced by vasopressin, angiotensin II and adrenaline in isolated hepatocytes. Based on their study they concluded that the breakdown of phosphatidylinositol induced by these agents was too slow to play a causative role in mobilization of calcium; breakdown of the phospholipid was not seen before two min whereas a previous study (Blackmore et al. 1982) had shown that calcium movement and phosphorylase activation were at maximum by 10 s. Furthermore, the effect of these hormones on phosphatidylinositol hydrolysis was abolished in

calcium depleted hepatocytes indicating that the effect is calcium dependent (Prpić et al. 1982). Previous studies (see above) which found the opposite did not provide evidence of adequate calcium depletion (Prpić et al. 1982). If these findings are substantiated by further study, the phosphatidylinositol hypothesis (at least in the case of the liver) will have to be discarded and the search for the mechanism of calcium mobilization continued.

Insulin inhibits not only cAMP-mediated activation of glycogenolysis and gluconeogenesis (section 1.3.2), but also alpha adrenergic effects in both isolated hepatocytes and perfused liver (Massaque and Guinovart 1978; Blackmore et al. 1979a; Dehaye et al. 1981). Insulin inhibited the alpha receptor-mediated release of calcium suggesting that its effect is at or proximal to the production of the signal for intracellular calcium mobilization (Blackmore et al. 1979a; Dehaye et al. 1981). Interestingly, calcium release or phosphorylase activation by vasopressin, angiotensin II or A23187 was not inhibited in the presence of insulin. These observations would indicate that insulin's effects are not on intracellular calcium stores, but rather uniquely on the mechanism whereby alpha adrenergic agonists mobilize intracellular calcium, possibly on the alpha receptor itself.

Recent evidence from radioligand binding studies utilizing purified liver plasma membranes (see section 1.3.4) has suggested the existence of both $alpha_1$ and $alpha_2$ receptors in the rat liver (Hoffman et al. 1980a,b). The regulation of endogenous noradrenaline release by presynaptic alpha receptors has been demonstrated in the dog liver (Yamaguchi 1982); such receptors are usually of the $alpha_2$ type. However, $alpha_2$ receptors at post-synaptic sites have been described for a number of tissues and their stimulation has been shown to have an inhibitory effect on adenylate cyclase (Fain and García-Sáinz 1980). Hence, Jard et al. (1981) were able to demonstrate $alpha_2$ inhibition of adenylate cyclase in rat liver plasma membranes. Angiotensin II and vasopressin were also tested in this system, but only angiotensin II inhibited

adenylate cyclase activity.

There exists in the literature a number of earlier studies that also showed evidence of an inhibitory alpha effect on adenylate cyclase (Boyd and Martin 1976), cAMP accumulation in isolated hepatocytes (Chan et al. 1979a), as well as the perfused rat liver (Sherline et al. 1972). In none of these studies was the effect characterized with respect to alpha receptor subtype. More recently, Exton and coworkers (Assimacopoulos-Jeannet et al. 1982) demonstrated that $alpha_1$ receptor activation could inhibit glucagon-stimulated gluconeogenesis, phosphorylase activation and glucose output in isolated rat hepatocytes. These effects appeared to be due in part to inhibition of cAMP accumulation through $alpha_1$ receptor stimulation, although additional effects (possibly involving redistribution of intracellular calcium) are implied by the observed suppression of exogenous cAMP- or dibutyryl cAMP-mediated gluconeogenesis. Vasopressin and angiotensin II had inhibitory effects similar to catecholamine. This inhibitory $alpha_1$ effect is different from that described by Jard et al. (1981) for $alpha_2$ receptors in isolated membranes. One might expect that both phenomena may play a role in hepatic regulation. However, the rather precise conditions required to observe the $alpha_2$ effect (5 mM EDTA, 50-200 mM Na and 1-200 μM GTP) bring into question its physiological significance.

Under certain conditions alpha receptor stimulation has been shown to result in increases in hepatic cAMP accumulation. Chan and Exton (1977) have obtained evidence that alpha receptor stimulation resulted in cAMP accumulation in isolated hepatocytes that had been depleted of calcium by incubation in the presence of 1 mM EGTA. The increased levels of cAMP were associated with increased activation of glycogen phosphorylase. This response in calcium depleted hepatocytes was found to be somewhat less sensitive to agonists, but more sensitive to antagonists than the alpha mediated activation of phosphorylase in normal cells, but still appeared to be of the

alpha$_1$ subtype. It was suggested that the removal of calcium somehow alters the properties of the alpha receptor to allow coupling with adenylate cyclase. An alternate explanation would be that calcium depletion somehow unmasks the activity of a third type of alpha receptor which is positively coupled to adenylate cyclase. More recently, Jackowsky et al. (1982) demonstrated alpha$_2$ adrenergic stimulation of adenylate cyclase in calcium depleted liver plasma membranes. Vasopressin and angiotensin II did not have similar effects under identical conditions. Whether or not this could represent the same alpha receptor-mediated accumulation of cAMP in calcium depleted hepatocytes described above is not known. The descrepancy between these findings and those of Jard et al. (1981) is most likely due to differences in the preparations used and conditions of the assay; stimulation by alpha$_2$ receptors disappears in the presence of high GTP, magnesium or calcium ion concentrations (Jackowski et al. 1982), whereas inhibition by alpha$_2$ receptors required high GTP and sodium ion (Jard et al. 1981). Furthermore, Jackowski et al. (1982) claim that in spite of the presence of 5 mM EDTA, the conditions employed by Jard et al. (1981) do not represent significant calcium depletion. These considerations aside, since rather artificial conditions are required to demonstrate these actions, their physiological importance is dubious, but they might provide some insight into the biochemistry of the receptor-adenylate cyclase system. It is noteworthy that Blair et al. (1980b) have demonstrated alpha mediated cAMP accumulation in normal rat hepatocytes without calcium depletion and this finding was confirmed by Okajima and Ui (1982). This surprising result will require further investigation.

Hence, it appears that the liver possesses both alpha$_1$ and alpha$_2$ receptors which mediate different responses. Alpha$_1$ receptors cause the mobilization of calcium by a mechanism that is not yet established. The increased cytosolic calcium so produced can activate or deactivate a number of enzymes some of which are involved in hepatic carbohydrate metabolism.

Alpha$_2$ receptors inhibit or stimulate adenylate cyclase depending on the conditions of the assay. An effect of alpha$_2$ receptor stimulation on glucose output or phosphorylase activation has not as yet been described and the physiological role, if any, of hepatic alpha$_2$ receptors is still open to question.

Vasopressin and angiotensin II are thought to activate glycogen phosphorylase and hence stimulate glycogenolysis by a mechanism similar to that described for alpha receptors. The evidence for this is the following:

(1) The effect of the hormones on glycogenolysis is independent of cAMP accumulation (Kirk and Hems 1974; Keppens et al. 1977; Garrison et al. 1979).

(2) Their action in liver is critically dependent on extracellular calcium (Stubbs et al. 1976; Keppens et al. 1977; Garrison et al. 1979).

(3) Stimulation with angiotensin II or vasopressin causes marked efflux of calcium from either perfused liver (Blackmore et al. 1979c) or hepatocyte suspensions (Blackmore et al. 1978; Chen et al. 1979), and release appears to be from the same intracellular stores as are affected by alpha agonists (Chen et al. 1978; Blackmore et al. 1979b,c; Reinhart et al. 1982a).

(4) Most of this releasable calcium is of mitochondrial origin (Blackmore et al. 1979c; Murphy et al. 1980; Reinhart et al. 1982a).

(5) The pattern of phosphorylation of cytosolic protein in response to vasopressin or angiotensin II stimulation of isolated hepatocytes is the same as that observed with alpha receptor activation (Garrison et al. 1979; Garrison and Wagner 1982).

(6) Vasopressin or angiotensin II also stimulate phosphatidylinositol breakdown (Tolbert et al. 1980; Billah and Mitchell 1978; Kirk et al. 1977),

(7) potassium uptake by rat liver (Blackmore et al. 1979c), and

(8) gluconeogenesis in hepatocyte suspensions (Whitton et al. 1978).

A final point of evidence (9) comes from the studies of Bréant et al. (1981) who demonstrated that alpha adrenergic agonists and vasopressin

produced a dose-dependent heterologous desensitization of the phosphorylase response in isolated hepatocytes. However, others (Morgan et al. 1982) found that no desensitization of the alpha$_1$ glycogenolytic response was seen in perfused rat liver provided adequate calcium reaccumulation occurred before repeating the stimulus. These latter authors suggested that the heterologous desensitization found by Bréant et al. (1981) was due to depletion of an intracellular calcium pool(s) that is common for vasopressin, angiotensin II and alpha$_1$ agonists. Later, Keppens and DeWulf (1982) showed that desensitization occurred at low temperatures, while calcium fluxes could only be produced at higher temperatures. Hence they have proposed that this desensitization is a membrane phenomenon whereby an hypothetical hormone-effector complex is inactivated by a desensitizing stimulus. In any case, all of these findings support the existence of a common pathway for vasopressin, angiotensin II and alpha$_1$ agonists, although the issue of the mechanism of the heterologous desensitization remains unresolved.

Just as for alpha agonists (Dehaye et al. 1980), vasopressin (Cantau et al. 1980) and angiotensin II (Campanile et al. 1982) effects are mediated by interaction with a plasma membrane receptor. Hence, it seems reasonable to postulate that the common pathway for the hepatic action of these hormones may be established as early as the coupling of receptors to the effector system, just as it is for the beta adrenergic and glucagon receptor-adenylate cyclase systems (section 1.3.2). However, as discussed earlier in this section, insulin inhibits the hepatic actions of alpha$_1$ receptor agonists, but not angiotensin II or vasopressin suggesting some early divergence of the activating mechanism or selective action of insulin at the alpha$_1$ receptor site itself.

The calcium ionophore, A23187, has been much used in the study of the role of calcium in the actions of glycogenolytic hormones in the liver and a brief comment about its action seems in order before concluding this section.

A23187 produces a dose-dependent increase in phosphorylase a as a consequence of its ability to mobilize calcium (Blackmore et al. 1978). It has a biphasic effect on calcium content: at low concentrations the drug releases calcium from intracellular stores and produces marked calcium efflux from the liver cell (Blackmore et al. 1978). At higher concentration, influx of calcium from the extracellular medium is also produced (Blackmore et al. 1978). Hence, when extracellular calcium is low, only the efflux response is seen (Blackmore et al. 1978; Chen et al. 1978). The biphasic nature of this response may explain the inability of Friedman et al. (1979) to detect calcium efflux in response to A23187. These workers tested only relatively high doses of the drug and did not examine its effects in calcium-free medium.

Maximal stimulation with A23187 appears to deplete calcium from the hormone sensitive intracellular pool (Chen et al. 1978). After stimulation, there is a marked reduction in hormone-releasable mitochondrial calcium (Babcock et al. 1979), supporting the concept that this represents a significant portion of the hormone-sensitive intracellular calcium pool.

1.3.4 Identification of hepatic adrenergic receptors by radioligand binding.

Radiolabelled agonists and antagonists have been used to identify putative adrenergic receptor sites in various tissues including the liver (Exton 1980; Hoffman and Lefkowitz 1980). While most of these studies have met with considerable success, some controversy regarding the most suitable ligands to measure adrenergic receptors in the liver still exists (Hoffman et al. 1981). This is particularly true of the hepatic alpha receptor, which, owing to the great interest in the mechanism of alpha adrenergic effects, is being extensively studied. The task of measuring changes in the properties of this receptor system is made more complicated by the existence of $alpha_1$ and $alpha_2$ receptors in liver, as well as other receptor-like binding sites (see below). The results of these studies will be summarized here.

Early attempts to identify adrenergic receptors with ^{3}H-catecholamines have been reviewed by Cuatrecasas et al. (1974). These authors concluded that ^{3}H-catecholamine binding in intact tissues and microsomal preparations was predominantly to non-specific catechol binding sites and did not reflect receptor binding. U'Prichard and Snyder (U'Prichard 1981) found in several tissues that reliable receptor binding with ^{3}H-catecholamines could only be obtained when precautions were taken to minimize catechol-directed non-specific binding and auto-oxidation of the ligand. Hence, El-Refai et al. (1979) used ^{3}H-adrenaline and ^{3}H-noradrenaline in the presence of 10^{-5} M propranolol to study alpha receptor binding sites in rat liver plasma membranes. Catechol and ascorbate were included in the incubation mixture to reduce non-specific binding and oxidation of the catecholamines respectively. Using this approach, these authors were able to identify two classes of specific alpha receptor-like binding sites suppressible by 10^{-5} M phentolamine. One class (120 to 160 fmol/mg protein) had a high affinity for agonists and sensitivity to guanine nucleotides, and was thought to represent the physiological alpha adrenergic receptor. The second site showed a high affinity for antagonists, a markedly low affinity for agonists and was readily labelled by [^{3}H] DHE (1275 fmol/mg protein, Kd=7.2 nM). Both receptors were found to be of the $alpha_1$ subtype (El-Refai and Exton 1980a). Later these same authors demonstrated that trypsin treatment of isolated membranes resulted in a time-dependent increase in the high affinity agonist site and a concomitant decrease in low affinity sites (El-Refai and Exton 1980b). Geynet et al. (1980) also found that limited proteolysis of rat liver membranes increased the affinity and number of [^{3}H] noradrenaline binding sites. However, guanine nucleotide sensitivity was lost with treatment. It was suggested that trypsin treatment converted low affinity to high affinity sites and that trypsin may mimic an *in vivo* regulation of alpha receptors (El-Refai and Exton 1980b).

Similar [^{3}H] DHE binding results were obtained by another group

(1.7 pmol/mg protein, Kd=1.8 nM; Clarke et al. 1978); however, these authors implied that all of this binding was to the physiological receptor sites. Indeed, Aggerbeck et al. (1980b), have correlated [^{3}H] DHE binding to plasma membranes and activation of glycogen phosphorylase in isolated hepatocytes. While the relative potencies of both agonists and antagonists for inhibition of ligand binding and phosphorylase activation could be correlated (Aggerback et al. 1980b), agonists were approximately ten-fold more potent in activating phosphorylase than antagonizing [^{3}H] DHE binding. Antagonists were approximately ten-fold less potent in antagonizing phosphorylase activation than [^{3}H] DHE binding. Methodological problems complicate interpretation of these results. The low apparent inhibitory potency of antagonists was probably artifactual; these authors used preparations with high tissue to medium ratios at which the great capacity of liver cells to take up drugs results in a reduction of free drug concentration in the vicinity of the adrenergic receptor (Jenkinson et al. 1978; Kan et al. 1979). This leads to an overestimation of the K_d of the receptor based on phosphorylase activation. On the other hand, the difference between EC_{50}'s and binding K_d's of agonists was probably greater than estimated, as the EC_{50}'s were unusually high, suggesting that the hepatocytes used may have been of poor viability. This difference cannot be explained by the existence of spare receptors, since these are absent in rat liver cells (Kunos et al. 1983).

Hoffman et al. (1979) have quantified the relative number of $alpha_1$ and $alpha_2$ receptors in several tissues including the liver. Although [^{3}H] DHE binds both these receptor subtypes, analysis of displacement curves produced with prazosin, an $alpha_1$ receptor antagonist, yohimbine, an $alpha_2$ receptor antagonist and phentolamine, a mixed $alpha_1/alpha_2$ antagonist, could yield information about each subtype. Using this approach, these authors could not identify significant $alpha_2$ receptors in rat liver plasma membranes. In a subsequent later study (Hoffman et al. 1980a), it was found that guanine

nucleotides had a regulatory effect on agonist binding to $alpha_2$ but not $alpha_1$ receptors. A small, but significant effect of guanine nucleotide on [^{3}H] DHE agonist displacement curves (Hoffman et al. 1980a) prompted a re-examination of liver membranes for the presence of $alpha_2$ receptors. Approximately 20% of alpha receptor sites labelled by [^{3}H] DHE were $alpha_2$ (Hoffman et al. 1980a,b). Guanine nucleotides are apparently able to transform $alpha_2$ receptors from a state with high affinity for agonists to a low affinity state (Hoffman et al. 1980a,b). [^{3}H] Adrenaline, at low concentrations labels mainly the high-affinity $alpha_2$ site, and it was suggested that binding of ^{3}H-catecholamines described by El-Refai et al. (1979) was to these sites and not to $alpha_1$ receptors; however, at higher [^{3}H] adrenaline concentrations $alpha_1$ sites are also labelled (Hoffman et al. 1980b). The ability of trypsin to mediate the transformation of these sites (El-Refai and Exton 1980b) remains unexplained. Hoffman et al. (1981) compared the findings of [^{3}H] DHE binding in liver plasma membranes with [^{3}H] prazosin and [^{3}H] yohimbine as selective probes for $alpha_1$ and $alpha_2$ receptors respectively. These authors found this to be a usable approach to delineate alpha receptor subtypes.

Geynet et al. (1981) compared [^{3}H] noradrenaline, [^{3}H] prazosin and [^{3}H] DHE binding in hepatic plasma membranes. All three ligands displayed characteristics expected for binding to $alpha_1$ receptors. Antagonist competition studies suggested that [^{3}H] noradrenaline and [^{3}H] prazosin labelled different sites, while [^{3}H] DHE bound to both. Moreover, [^{3}H] prazosin sites could be converted to [^{3}H] noradrenaline binding sites by limited proteolysis with alpha-chymotrypsin. Based on these results these authors speculated that [^{3}H] noradrenaline binding sites represent the physiologically important $alpha_1$ receptor, while [^{3}H] prazosin binding is to a precursor form of the active receptor and/or an $alpha_1$ binding site not coupled to the response.

Thus, radiolabelled ligand binding studies directed towards identifying hepatic alpha receptors are complicated by the existence of several forms of

alpha receptor-like binding sites. The functional relevance of these forms and hence the most suitable ligand to measure the physiologically important alpha receptors remain debatable issues.

Another approach to the study of hepatic alpha adrenergic receptors involved the use of radiolabelled phenoxybenzamine ([^{3}H] POB), an irreversible alpha antagonist. Using this ligand it was feasible to label sites with the expected properties of alpha receptors in intact, viable hepatocytes (Kan et al. 1979) or purified liver plasma membranes (Guellaen and Hanoune 1979; Guellaen et al. 1979). However, the "specific" binding of [^{3}H] POB in these latter studies lacked stereoselectivity, which raises doubts about its relevance to the physiological alpha receptor. Guellaen et al. (1979) went on to solubilize [^{3}H] POB binding site and estimated the molecular weight under non-denaturing conditions at 96,000 daltons by gel filtration. In a subsequent study by the same group SDS-PAGE yielded three peaks labelled by [^{3}H] POB; one with a subunit molecular weight of 44,800 daltons, was identified by competition experiments as the alpha receptor binding protein (Guellaen et al. 1982). In a more recent study, Kunos et al. (1983) demonstrated that at nanomolar concentrations [^{3}H] POB bound to membrane sites with the characteristics of alpha$_1$ receptors including marked stereoselectivity for agonists. Micromolar concentrations of antagonists and millimolar concentrations of agonists protected a second set of sites from binding [^{3}H] POB similar to those identified by Guellaen et al. (1982). However, these latter sites lacked stereoselectivity and thus their physiological function is not known. Kunos et al. (1983) solubilized an hepatic membrane component labelled by [^{3}H] POB with properties of alpha$_1$ receptors including stereoselectivity and found a subunit molecular weight of approximately 80,000 daltons with a smaller 58,000 dalton protein thought to be a proteolytic fragment of the receptor site. Interestingly, a 58,000 dalton protein (SDS-PAGE) was also identified by Graham et al. (1982) in rat liver using affinity chromatography with an agarose-linked

analogue of prazosin. They also proposed that this protein was the ligand-binding subunit of the $alpha_1$ receptor. The above discrepancies in molecular weight may be due to variable success in inhibiting proteolytic degradation of a larger (approximately 80,000 dalton) membrane component that represents the native $alpha_1$ receptor.

The study of hepatic beta adrenergic receptors by the use of radiolabelled ligands is also not without difficulties. The beta antagonist, [^{3}H] DHA has been shown to bind specifically to sites on the liver plasma membrane with characteristics of $beta_2$ receptors (Munnich et al. 1981). The density of binding sites was low: 60 fmol/mg membrane protein as one would expect since the predominant adrenergic receptor in normal rat liver is of the alpha type. However, studies with a second potent beta antagonist, [^{125}I] IHBP gave values of 188 $\pm$ 27 fmol/mg protein (Wolfe et al. 1976) and 24 $\pm$ 3 fmol/mg protein (Malbon 1980) in plasma membranes purified from intact liver and isolated hepatocytes respectively. However, the unusually low affinity for [^{125}I] IHBP found in the study by Wolfe et al. (1976; K_d=2.6 + 1.5 nM) casts doubt on the validity of the binding results from this study.

Despite these reports of successful beta receptor binding studies in the liver, experiments in our laboratory were not successful in identifying high affinity, stereoselective sites in liver membranes from adult male rats using either [^{3}H] DHA or [^{125}I] IHBP. Similar negative results have been recently published (Dax et al. 1981). Therefore, further characterization of beta receptor binding sites will be necessary before the above results can be accepted without skepticism.

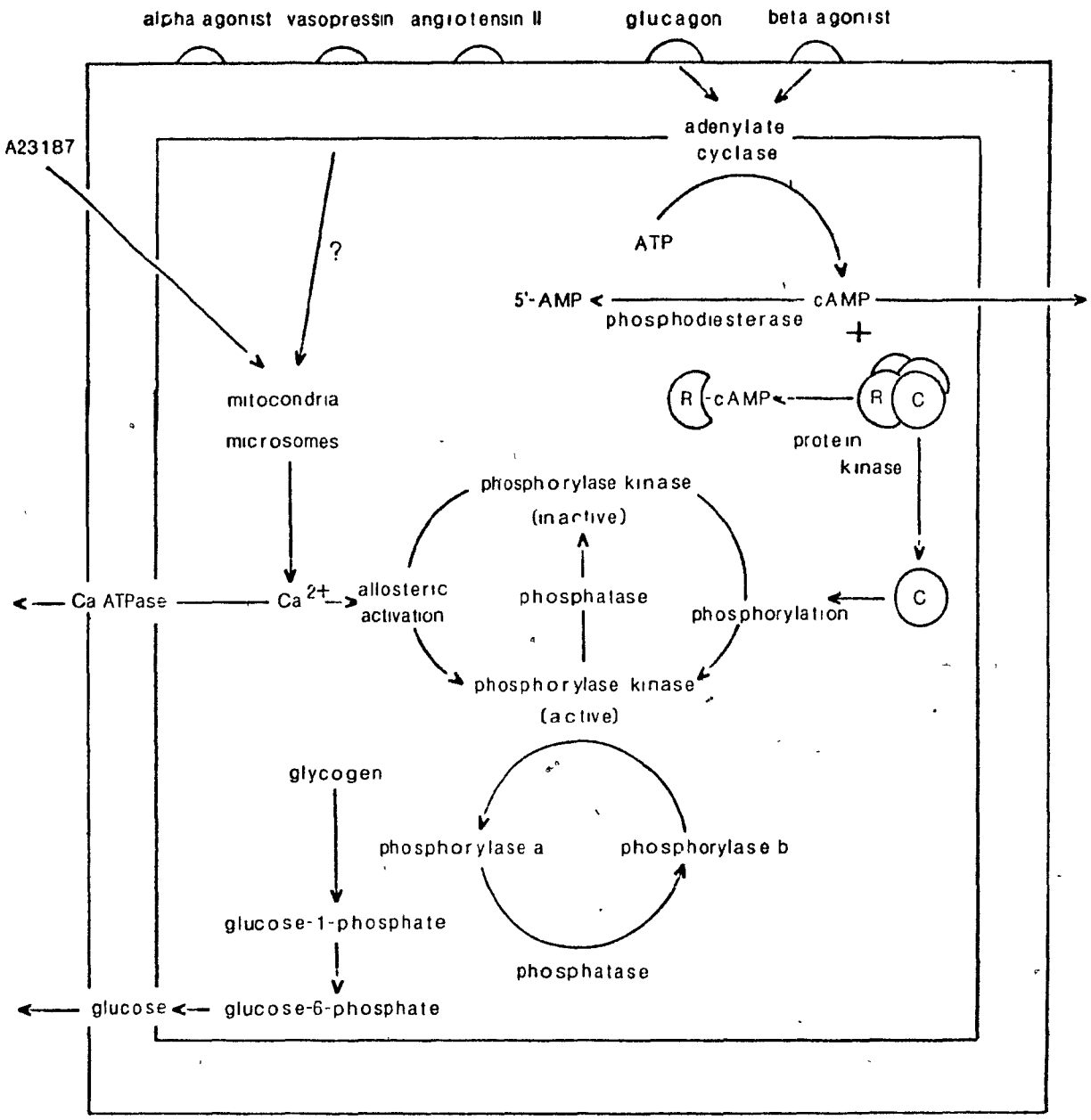


FIGURE 1.1 Calcium- and cAMP-dependent pathways for the activation of liver glycogen phosphorylase.

"C" and "R" are the catalytic and regulatory subunits of protein kinase respectively. For details concerning the calcium-dependent activation pathway refer to section 1.3.3. For details concerning the cAMP-dependent pathway see section 1.3.2.

1.4 Role of the sympathetic nervous system in the control of hepatic metabolism.

1.4.1 Anatomical Considerations

Claude Bernard (1849) discovered that "piqûre" of the floor of the fourth ventricle produced profound hyperglycemia and glycosuria, and concluded that this effect was mediated via nervous influences on the liver. In spite of this early demonstration, it has been only recently accepted that neuronal control of glucose output and hepatic metabolism in general may be of physiological importance. The anatomy and function of hepatic nerves has been extensively reviewed by Lautt (1980) and will not be detailed here.

Autonomic control pathways originate in the hypothalamus. Stimulation of the ventromedial hypothalamic nucleus produces hyperglycemia by neuronal activation of hepatic gluconeogenesis and glycogenolysis via the sympathetic system (Shimazu and Ogasawara 1975). Stimulation of the lateral hypothalamic nucleus produces slight hypoglycemia and can be linked to activation of hepatic glycogen synthesis via the vagus. These conclusions have also been supported by results of selective lesioning of hypothalamic sites which appear to have effects opposite to stimulation (Shimazu and Ogasawara 1975). Ventromedial hypothalamic lesioning produces a hyperglycemic-obese state and represents the modern counterpart of Claude Bernard's piqûre experiments (Hems and Whitton 1980).

1.4.2 The Effects of Splanchnic Nerve Stimulation.

Direct electrical stimulation of the splanchnic nerves results in hyperglycemia due to release of pancreatic glucagon, catecholamine release from the adrenal medulla and hepatic sympathetic nerve activity (Himms-Hagen 1972). Shimazu and Amakawa (1968a) demonstrated that splanchnic stimulation could produce a marked activation of liver glycogen phosphorylase in rabbits

that was not eliminated by adrenalectomy or pancreatectomy. These authors went on to demonstrate that this activation could not be blocked by the beta receptor antagonist, dichloroisoproterenol, whereas activation by injected catecholamine could be blocked (Shimazu and Amakawa 1968b). Later studies by the same investigators revealed that nervous activation of glycogen phosphorylase occurred independently of any change of cAMP, while levels of this cyclic nucleotide were elevated when phosphorylase was activated by injection of either glucagon or adrenaline (Shimazu and Amakawa 1975). No stable change in phosphorylase kinase activity could be demonstrated after nerve stimulation, but a two to three-fold increase was noted after glucagon or epinephrine injection. The activity of phosphorylase phosphatase decreased with nerve stimulation. These authors concluded, therefore, that splanchnic nerve stimulation elevated phosphorylase activity by inhibition of phosphorylase phosphatase rather than activation of phosphorylase kinase via a cAMP-mediated mechanism analogous to that occurring after adrenaline or glucagon injection. They further speculated that some unknown factor, not noradrenaline, was released from sympathetic nerve endings to affect this response. As already discussed (section 1.3.2), other workers (Proost et al. 1979) could not confirm a neurally mediated decrease in phosphorylase phosphatase. Moreover, it has been shown that in cat, mouse and rabbit, the activation of phosphorylase by nerve stimulation can be blocked by alpha antagonists, but is only slightly or not at all affected by beta antagonists (Lautt 1979; Proost et al. 1979; Seydoux et al. 1979).

In the light of more recent information about the role of alpha and beta adrenergic receptors in mediating hepatic glucose output (section 1.3.1), the above findings are best explained as follows. Neurally-mediated activation of hepatic phosphorylase occurs via release of noradrenaline and subsequent action mainly on alpha receptors. Thus no change in cAMP levels is detectable and phosphorylase kinase is allosterically activated by calcium, not by a stable

phosphorylation as occurs with cAMP-dependent protein kinase. Conversely, injected adrenaline activates glycogen phosphorylase via the beta adrenoceptor/adenylate cyclase system and the classical cAMP-dependant protein kinase cascade (Walsh 1978). A similar proposal has been made by Proost et al. (1979) and Osborn (1978) to explain the above-described findings.

There is evidence from several studies that in other tissues $alpha_1$ and $beta_1$ receptors are innervated while $alpha_2$ and $beta_2$ are not, as proposed for alpha receptors in vascular smooth muscle (Yamaguchi and Kopin 1980) and for beta receptors in cerebral cortex (Minneman et al. 1979). The glycogenolytic response in liver is mediated by $alpha_1$ receptors (section 1.3.3) or $beta_2$ receptors (see section 4.4).

1.4.3 The Effects of Stimulation of the Hepatic Parasympathetic Nerves.

The effects of hepatic parasympathetic stimulation have been less well studied. Shimazu and Amakawa (1975) demonstrated that simultaneous stimulation of the splanchnic nerves and vagi in the rabbit would block the activation of glycogen phosphorylase, but would have no effect on activation of this enzyme or elevated cAMP levels resulting from adrenaline or glucagon injection. Vagal stimulation by itself had no effect on these parameters, although it did produce a marked activation of glycogen synthase which could be counteracted by simultaneous splanchnic nerve stimulation (Shimazu 1971). As expected, incorporation of radioactive glucose into liver glycogen was enhanced by vagal stimulation independent of pancreatic release of insulin (Shimazu and Fujimoto 1971). Glycogen deposition is decreased in acutely vagotomized rats (Mondon and Burton 1971). In spite of the similarity of vagal stimulation and insulin action on glycogen synthase, Shimazu (1967) has shown that the neurally mediated effect is more rapid than the hormonal effect. Just as for the sympathetic nerves, an exact physiological role for the parasympathetic innervation of the liver in glucose control has not been

elucidated.

1.5 Effect of Thyroid State on Hepatic Carbohydrate Metabolism

1.5.1 Glycogen Levels and Phosphorylase Activity.

Hepatic glycogen levels tended to be normal in hypothyroid rats and markedly decreased in hyperthyroidism (Hoch 1974). These differences were maintained in the ex vivo perfused liver preparation (Laker and Mayes 1981) suggesting that hormonal or neural influences were not the only reason for this effect on glycogen levels. In spite of this difference, perfusate glucose stabilized at similar concentrations in hypothyroid, hyperthyroid and normal rats when pyruvate and lactate were present (Laker and Mayes 1981). In vivo hepatic glycogen levels appear to be dependent on nutritional state. Thus, Okajima and Ui (1979), by examining the kinetics of radiolabelled glucose in the intact rat fasted for 20 h, found that glycogen levels were decreased by 60% in hypothyroid and 90% in hyperthyroid rats compared with euthyroid controls. These authors concluded that enhanced hepatic gluconeogenesis and diversion of gluconeogenic products from liver glycogen to blood glucose helped to maintain circulating blood levels. On the other hand, a slower rate of gluconeogenesis in hypothyroid rats led to a slower release of glucose into circulation and suppressed glycogenesis.

Takahashi and Suzuki (1975) reported no effect of hypothyroidism or hyperthyroidism on total glycogen phosphorylase or synthase activities in rapidly excised and frozen rat livers. When total glycogen phosphorylase activity was assayed in isolated hepatocytes it was found to be decreased (30%) after T_3 treatment for two days and slightly increased in hypothyroidism although this latter change was statistically insignificant (Malbon and LoPresti 1981).

1.5.2 Hepatic Enzyme Activities.

Gluconeogenesis is enhanced in hyperthyroid rat liver and the activities of gluconeogenic enzymes are increased (Bottger et al. 1970). Conversely, the rate of hepatic gluconeogenesis is lower in the thyroidectomized rat (Menehan and Weiland 1969). The profile of gluconeogenic enzymes in the thyroidectomized rat resembles that of the diabetic rat (Baquer et al. 1976). This is not surprising since hypothyroidism results in a state of relative insulin resistance (Bray and Jakobs 1974). However, consideration of changes in enzyme levels alone will not adequately explain the alterations seen in either of these states. Thyroid hormone appears to be required for the full expression of glucagon or catecholamine effects, and therefore it is likely that altered response to hormonal stimuli may be of greater importance.

1.5.3 Effect on Redox State and Energy Charge.

The activities of a number of critical enzymes involved in carbohydrate metabolism are modulated by the redox state (NADH/NAD) or energy charge (ATP/ADP,AMP) of the cell. These factors are profoundly influenced by alterations in thyroid state.

Reducing equivalents produced during glycolysis are transferred from the cytoplasm to mitochondia by a cycle involving alpha-glycerophosphate dehydrogenase. The activity of this enzyme is low in thyroidectomized rats and increased several fold by thyroid hormone treatment (Bernal and DeGroot 1980). Thus, the hypothyroid liver is in a more reduced state (increased NADH/NAD) and hyperthyroid liver a less reduced state than normal. In addition to direct effects of NADH/NAD on enzyme activities there is some evidence that the redox state of the cell may have important consequences for the action of certain hormones. Clark and Jarret (1978) reported that under oxidized conditions the ability of glucagon to produce an increase in cAMP and stimulate glucose output is decreased and have attributed this to an effect of

nicotinamide nucleotides on cAMP phosphodiesterase activity.

While the total adenylate nucleotide pool is unchanged in hypothyroid animals, the "energy charge" (ATP/ADP+AMP) is elevated (Baquer et al. 1976) over a range for which the response of many regulatory enzymes is marked (Atkinson 1968). Hems and Whitton (1980) have summarized the effects of various metabolites on glycogen phosphorylase a and b. These authors point out that AMP has an activating effect on both forms of the enzyme, while ATP and ADP are inhibitory. Hence, the increased "energy charge" of the liver cell may contribute somewhat to the reduced glucose output seen in response to glucagon and catecholamines in the hypothyroid rat. Additional, as yet undocumented, effects may also be of importance.

1.5.4 Summary.

It appears that thyroid hormones have complex effects on carbohydrate metabolism in the intact animal. A number of studies have examined the various influences of these hormones on factors which may be important in producing these changes, but a complete explanation has not been forthcoming. This is in part due to the existence of multiple ways in which thyroid hormones might produce these effects. Of particular interest in the context of the present study is the ability of thyroid hormones to modulate the effects of other hormones.

1.6 Formulation of the Problem.

Thyroid hormones can influence the response of various tissues to catecholamines, but the mechanism of these interactions is not clear. Most studies of this phenomenon have examined effects of thyroid state on the properties of adrenoceptors in cardiac and adipose tissue. Both alpha and beta receptors are present in cardiac and adipose tissue, and it appears that in certain cases the altered sensitivity to catecholamines observed in

hyperthyroidism or hypothyroidism may be attributed to changes in the balance of these receptors. In the rat heart where both receptor types mediate the positive inotropic response to catecholamines, hypothyroidism increases the alpha and decreases the beta receptor component of this response. Excess thyroid hormone has the opposite effect.

The activation of liver glycogen phosphorylase by catecholamines is also mediated by both alpha and beta receptors. If the effects of thyroid state on cardiac adrenergic receptors can be generalized, one may hypothesize that hyperthyroidism would enhance the beta receptor contribution to the activation of hepatic glycogen phosphorylase, a predominantly alpha receptor-mediated response in the normal adult rat. Unexpectedly the opposite was found: the pattern of adrenoceptor activation of phosphorylase was unchanged in hyperthyroid rats, but hypothyroidism resulted in a shift from alpha to a beta type of response.

In order to further investigate the nature of the changes observed in the hypothyroid rat, the following questions were considered.

1. Is the effect of thyroidectomy the result of changes in adrenergic receptors or some post receptor event in the glycogenolysis cascade?

2. Is the effect selective for adrenergic receptors or are other glycogenolytic hormones similarly influenced?

3. Are these changes reversible by thyroid hormone replacement?

SECTION TWO

EXPERIMENTAL PROCEDURES

2.1 Animals

Male Sprague-Dawley rats (CD strain, Canadian Breeding Farms Ltd.) were housed in the McIntyre Animal Center under constant conditions (22°C, 12 h light-dark cycle), maintained on Purina Rat Chow and tap water ad libitum. Rats were rendered hypothyroid by surgical thyroidectomy under sodium pentobarbital anaesthesia (50 mg/kg). Success of the procedure was assessed by the absence of normal growth during the ensuing 10 to 14 weeks, after which time animals were used for this study. Hyperthyroidism was induced by daily intraperitoneal injections of T_4 (1 mg/kg) freshly dissolved in 0.01 N NaOH for seven days. T_3-treated hypothyroid rats received daily intraperitoneal injections of 0.25 mg/kg freshly prepared in 0.01N NaOH for 2 or 4 days, the last injection being given approximately 12 h before the experiment. Euthyroid control animals were maintained under identical conditions. In one series of experiments hypothyroid rats were treated with cortisol (50 mg/kg) every 12 h beginning 48 h before the experiment. All animals used in the present study weighed between 250 and 350 g.

2.2 Drugs and Chemicals.

Adrenaline (l-epinephrine bitartrate, Sigma), d-adrenaline (d-epinephrine bitartrate, Sterling-Winthrop), phenylephrine (l-phenylephrine HCl, Sigma), isoproterenol (d,l-isopropyl arterenol HCl, K&K) and noradrenaline (l-norepinephrine bitartrate, Sigma) were dissolved just prior to use in isotonic saline containing 1 mM HCl. dl-Propranolol HCl (Ayerst) and phentolamine (phentolamine methanesulfonate, Ciba) were dissolved in isotonic saline. Dihydrpergocryptine (Sandoz) and A23187 (Calbiochem) were dissolved in ethanol and kept at -20°C. Phenoxybenzamine (phenoxybenzamine HCl, SK&F) was kept at -20°C dissolved in acidified ethanol. Triiodothyronine (T_3, 3,3',5-triiodo-L-thyroinine, Sigma) and L-thyroxine (T_4, Sigma) were dissolved in 0.01 N NaOH for injection. Vasopressin (grade VII synthetic arginine

vasopressin, Sigma) and glucagon (Sigma) were dissolved in isotonic saline containing 1 g% BSA and in the case of glucagon 0.2 M glycine (pH 10).

Collagenase (type I), α-D-glucose-1-phosphate (grade V), BSA, cAMP and beef heart protein kinase were from Sigma and were used directly. Difco gelatin was obtained from Fisher. Glycogen (grade V-S) was also from Sigma and was used after purification as described by Thomas et al. (1968). Gpp(NH)p was from Boehringer-Mannheim.

$^{45}CaCl_2$, α-D-[U-^{14}C] glucose-1-phosphate, [^{3}H] Prazosin (17.1 Ci/mmol) and [^{3}H] cAMP were from New England Nuclear.

All other chemicals and reagents were from usual commercial sources.

2.3 Isolation of Hepatocytes.

Hepatocytes were isolated between 9:00 and 11:00 a.m. each day by the method of Berry and Friend (1962), with some modifications. Rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and heparinized (500 I.U./kg i.v.). The abdominal cavity was opened with a wide transverse incision. The vena portae was cannulated and perfused with calcium-free Krebs-Henseleit bicarbonate buffer (Krebs and Henseleit 1932; 115 mM NaCl, 3.7 mM KCl, 1.2 mM $MgSO_4$, 2.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and 20 mM glucose) for 10 min at a flow rate of 30 to 35 ml/min. During this period the liver was transferred to a perfusion-aeration apparatus. After this preperfusion, the medium was changed to 100 ml of Krebs-Henseleit buffer containing 2.5 mM calcium[1] and 0.05% crude collagenase and perfusion was continued for 10 to 20 min with recycling. The liver was then disrupted by gentle combing. Incubation of the collagenase-liver suspension was continued in a shaker bath for an additional period such that the liver cells were exposed to collagenase for a total period of 30 min. The perfusion and all subsequent incubations were done at 37°C, under an atmosphere of 5% CO_2 in O_2, and maintained at a pH between 7.0 and 7.4 by the addition of 150 mM NaOH as required. At

the end of the incubation, the cell suspension was filtered through nylon mesh and centrifuged for 2 min at 50 x g. The cell pellet was washed and centrifuged twice, and resuspended in Krebs-Henseleit bicarbonate buffer containing 2.5 mM calcium and 1.5% gelatin[2] pre-equilibrated with 5% CO_2 in O_2 at 37°C. Cells were either used immediately or kept briefly on ice. This method routinely yielded cells with 95% viability as assessed by exclusion of trypan blue[3]. Cell suspensions contained 30 to 40 mg wet weight of cells per ml unless otherwise specified. Wet weight was determined by centrifuging 1.0 ml aliquots of the cell suspension at 1000 rpms in a Sorval GLC-1 centrifuge for 2 min in tared 1.5 ml plastic tubes. The supernatant was removed, the tube walls carefully dried and the weight of the cell pellet determined.

[1]Calcium concentration was 4.2 mM in the collagenase solution in earlier experiments. The data for these experiments appears in all figures to 3.9 except 3.1. Occasionally a white precipitate would form in this solution making perfusion of the liver less efficient. Hence, for all subsequent experiments calcium content of the collagenase buffer was reduced to 2.5 mM. Viability and performance of hepatocytes was not significantly altered by this modification.

[2]It was found during the course of these experiments that the viability of isolated hepatocytes was enhanced and better preserved particularly through longer incubations if 1.5% gelatin was added to the Krebs-Henseleit incubation solutions. However, this necessitated a slight modification of the assay of glycogen phosphorylase a in hepatocytes incubated in this medium (see section 2.4). Figures showing results of experiments done without added gelatin are indicated as such in the respective figure caption.

[3]Viability was assessed by combining equal volumes of cell suspension and a solution of 0.4% trypan blue in isotonic saline. After one to two min at room temperature the total number of cells and the number taking up dye were counted in 3 or 4 low power (40 X) fields with a microscope for calculation of % viability.

2.4 Phosphorylase Assay

Freshly prepared hepatocytes were resuspended in previously equilibrated Krebs-Henseleit buffer containing 1.5 g% gelatin at 30 to 40 mg wet weight of cells per ml. Three ml aliquots of this cell suspension were gassed with 5% CO_2 in O_2 and incubated in a shaker bath at 37°C and 90 to 100 cycles

per min. After a 30 min preincubation, drugs were added in 10 µl volumes or as indicated in figure captions. Unless otherwise specified, incubations for glycogen phosphorylase assays were terminated 3 min after the addition of agonist by adding 1.0 ml of the cell suspension to 5.0 ml of ice-cold Krebs-Henseleit buffer not containing gelatin. After a brief period on ice, cell suspensions were rapidly centrifuged (30 s) in an IEC International Clinical Centrifuge at maximum speed, supernatant was discarded and the cell pellet was immediately homogenized in ice-cold phosphorylase assay buffer (50 mM morpholinosulfonic acid, 150 mM NaF, 2.5 mM EDTA, 2.0 mM dithiothreitol, final pH 6.5) with a Brinkman Polytron for twice 15 s seperated by 15 s of cooling. The homogenate was centrifuged at 3000 X g for 10 min and glycogen phosphorylase was assayed in duplicate aliquots of the supernatant by measuring the incorporation of [U-^{14}C] glucose into glycogen. Total incubation volume was 0.1 ml and the reaction was started by the addition of glycogen and [U-^{14}C] glucose-1-phosphate to give a final concentration of 1.0 g% and 15 mM respectively. The assay was rendered specific for phopsphorylase a by including 0.5 mM caffeine (Stalmans and Hers 1975). Incubation (30^{o}C, 20 min) was terminated by spotting 50 µl aliquots of the assay mixture on Whatman 41 filter paper discs which were further processed by sequential washing in 66% ethanol and a final wash in acetone (Thomas et al. 1968). The retained radioactivity was measured by liquid scintillation counting at an efficiency of 50 to 60%. Units of enzyme activity are nanomoles of [U-^{14}C] glucose incorporated into glycogen per min per mg protein.

Protein concentration in the 3000 X g supernatant was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

In experiments in which gelatin was omitted from the Krebs-Henseleit buffer (see footnote 2, section 2.3), 1.0 ml aliquots of cell suspension were homogenized directly in 1.0 ml of ice-cold assay buffer (100 mM morpholino-sulfonic acid, 300 mM NaF, 5.0 mM EDTA, 2.0 mM dithiothreitol final pH

6.5) with a Brinkman Polytron. The subsequent steps in the assay were done as described above.

Both maximally stimulated (10 μM adrenaline) and basal phosphorylase a activity was linear with respect to time for at least 20 min of incubation at 30°C and also directly proportional to protein concentration to levels greater than those in homogenates routinely prepared in the present experiments (figure 2.1).

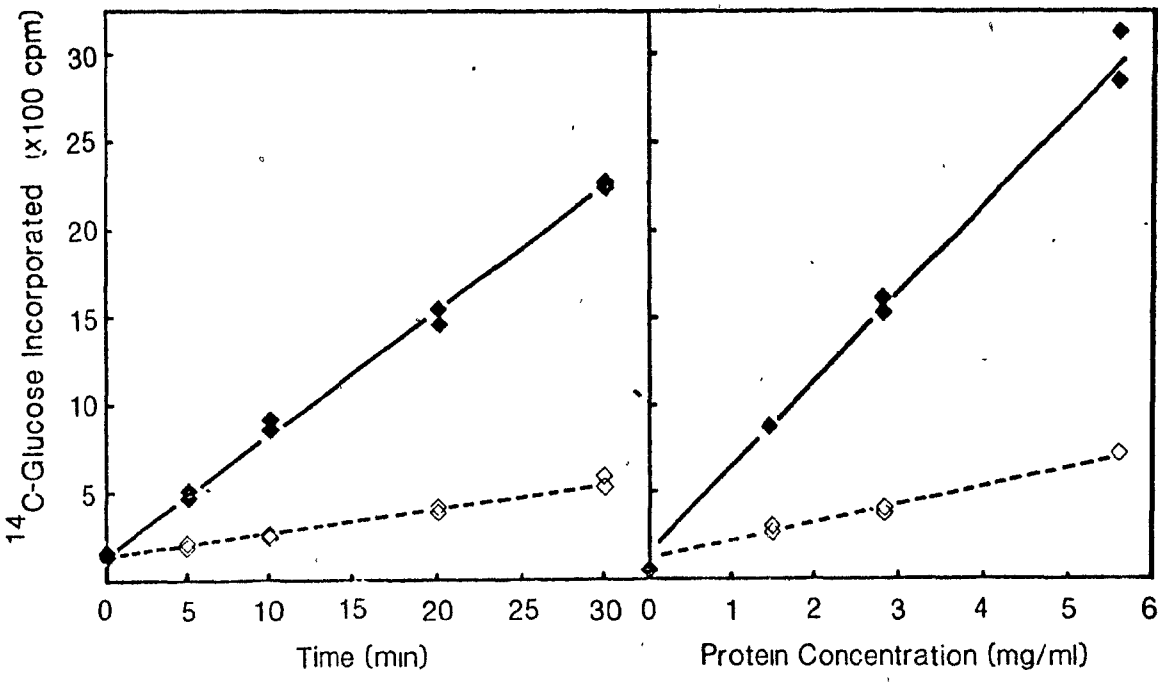


FIGURE 2.1 Time- and protein-dependent increase in [U-^{14}C] glucose incorporation into glycogen in the assay of hepatic glycogen phosphorylase a.

Cells were isolated from a normal rat and incubated as described in section 2.3. After 30 min of incubation 10 µl of saline alone (◇) or adrenaline (◆) was added to a 3.0 ml volume of cell suspension (80 mg/ml) to give a final concentration of 10^{-5}M adrenaline. After 3.0 min a 1.0 ml aliquot of this suspension was homogenized in an equal volume of assay buffer (section 2.4) and assayed for phosphorylase a activity as detailed in section 2.4. Panel A shows the amount of [U-^{14}C] glucose incorporated into glycogen when the reaction was stopped at the indicated times. Panel B shows the amount of [U-^{14}C] glucose incorporated as a function of homogenate protein concentration when the incubation was terminated after 20 min.

2.5 Cyclic AMP Determination

Cells were incubated as described for the determination of glycogen phosphorylase (section 2.4). At 1.5 min after the addition of agonist (or as indicated in figure legends) 1.0 ml aliquots of cell suspension were homogenized in 5.0% trichloracetic acid for 30 s with a Brinkman Polytron. Samples were centrifuged at 3000xg for 10 min. Precipitated gelatin was removed as a layer floating above the supernatant. One ml of supernatant was purified by passage over a 0.4 cm X 4.5 cm column of Dowex 50X8H^+. The column was eluted with deionized water and cAMP was recovered in the fourth to the sixth one ml fractions. Recovery was monitored with [^{3}H]cAMP for each batch of columns prepared and was usually 95 to 98%. Fractions containing cAMP were pooled and taken to dryness in a Brinkman Sample Concentrator. These were taken up in 0.2 to 5.0 ml of 50 mM TRIS buffer (pH 7.5) containing 4mM EDTA and cAMP was assayed in duplicate 50 µl aliquots by a radiobinding assay as described by Tovey et al. (1974).

2.6 Calcium Efflux Measured by Calcium Electrode

Drug effects on calcium efflux from intact hepatocyte suspensions were determined with the use of a calcium-sensitive electrode as described by Chen et al. (1978) with some modifications. Cells were washed once and resuspended in a nominally calcium-free Krebs-Henseleit buffer. Fifteen ml aliquots were incubated in 50 ml plastic Ehrlenmeyer flasks at 37°C under an atmosphere of 5% CO_2 in O_2. To determine drug effects, a calcium-sensitive electrode (Orion Model 93-20) and a reference electrode (Orion Model 90-01) were fixed into each Ehrlenmeyer. Electrodes were connected to a pH meter (Orion Model 701) and a Varian A-25 chart recorder. A 1000 µF capacitance was included between the pH meter output and the chart recorder to reduce the noise in the recordings. This had no effect on the drug responses measured. Cells were incubated until a stable baseline calcium level was obtained: usually 30

to 60 min. Baseline free calcium concentrations were checked by comparing the pH meter output to a standard curve for calcium prepared in 150 mM KCl (approximately isotonic) at 37°C. These values ranged between 0.068 and 0.096 mM for hepatocytes prepared from normal rats, and 0.053 to 0.108 mM for hepatocytes from thyroidectomized rats. Cummulative concentration-response curves were produced by the addition of increasing amounts of drugs as detailed in figure captions for each series of experiments. At the conclusion of each experiment, changes in external calcium concentrations were determined by the addition of 3.3 mM $CaCl_2$ calibration pulses. One ml aliquots of cells were then removed for determination of wet weight and viability (section 2.3). Only results from preparations with >85% viability were included. Results were expressed as nanomoles of calcium efflux per mg wet weight of cells. Preliminary experiments to test this method were done and representative tracings are shown in figure 3.13.

2.7 ^{45}Ca Efflux Studies

Freshly prepared hepatocytes were resuspended in Krebs-Henseleit buffer containing 1.5 g% gelatin and 2.5 mM ^{45}Ca (0.5 Ci/ml), and incubated in a shaker bath at 37°C and 80 cycles per min. Throughout this ^{45}Ca loading, the suspension was continuously gassed with 5% CO_2 in O_2. After 60 min, cells were rapidly washed twice (50xg for 1 min) and resuspended in Krebs-Henseleit solution containing 2.5 mM non-radioactive calcium. Sixty min of preloading hepatocytes with ^{45}Ca has been shown to label most of the exchangeable calcium in rat hepatocytes (Chen et al. 1978). Duplicate 1.0 ml aliquots were rapidly centrifuged (1500xg for 20 s) in an IEC International Clinical Centrifuge and ^{45}Ca was determined in 0.7 ml aliquots of supernatant by liquid scintillation spectrometry at approximately 65% efficiency (determined by the addition of a known amount of ^{45}Ca). The mean of this value was called the zero-time efflux. The remaining suspension was incubated in 1.0 ml aliquots

with varying concentrations of agonists added in 10 µl volumes. After 3 min of incubation samples were centrifuged and 0.7 ml aliquots of supernatant were assayed for ^{45}Ca activity as described above. Data are expressed as % ^{45}Ca efflux, where 100% is assigned to the increase in ^{45}Ca activity from zero-time to 3 min in the absence of added drug. With care, this method was reproducible and duplicate determinations agreed to within 5%.

This procedure for the determination of ^{45}Ca efflux is analogous to the "calcium efflux-exchange" procedure reported by Blackmore et al. (1978), except that ^{45}Ca released from cells is measured in the present experiments rather than ^{45}Ca cell content in the latter. The loss of ^{45}Ca from cells is measured after 3 min of stimulation with agonists to correspond to times at which phosphorylase a activity was determined.

2.8 Thyroid Hormone Assays.

Serum thyroxine (T_4) was measured in blood samples collected at the time of hepatocyte isolation by radioimmunoassay with the use of a kit commercially available from Diagnostics Biochem Canada Inc., London, Ontario.

2.9 Preparation of Liver Plasma Membranes

Plasma membranes were prepared from freshly excised livers by the method of Neville (1968) as modified by Wolfe et al. (1976). Membranes were used for the binding assay on the day of preparation.

2.10 [^{3}H] Prazosin Binding Assay.

Binding of (^{3}H)prazosin was assayed in triplicate at 31°C. Incubation was for 30 min in 50 mM potassium phosphate buffer at pH 7.5 containing 0.080 to 0.160 mg of protein, 20 to 1000 pM [^{3}H]prazosin the indicated adrenergic agonist or antagonist and 4 mM $MgSO_4$ in a final volume of 1.0 ml. The use of protein concentrations less than 0.2 mg/ml was necessary to avoid

significant (<10%) reduction of the free concentration of [^{3}H]prazosin in the incubation medium. Specific binding was defined as the difference between binding in the absence and presence of 2.0 μM phentolamine. Incubation was terminated by rapid vacuum filtration onto a single Whatman GF/C glass fiber filter followed by three 5.0 ml washes with the same buffer at 22°C. The filters were air-dried, incubated with 0.3 ml of NCS tissue solubilizer (Amersham) overnight and the radioactivity determined by liquid scintillation spectrometry in a toluene based cocktail at an efficiency of 50 to 55%. Efficiency for each sample was verified by the channels ratio technique.

2.11 Statistics and Expression of Results.

All data shown is either the result of a single representative experiment or the mean ± standard error of the mean (s.e.m.) from three or more separate cell preparations as detailed in the captions to figures and tables.

Statistical comparisons were made by either a two-sample rank test: the Mann-Whitney U-test or the Kruskal-Wallis test where more than two groups are compared (Sokal and Rohlf 1969). A probability value of less than 0.05 was taken to imply a statistically significant difference between groups.

SECTION THREE

RESULTS

3.1 Activation of glycogen phosphorylase by adrenergic agonists in hepatocytes from euthyroid, hypothyroid and hyperthyroid rats.

Figure 3.1 shows the time-course of activation of glycogen phosphorylase by 10^{-6}M adrenaline in hepatocytes from euthyroid, hypothyroid and hyperthyroid rats. Thirty minutes of preincubation resulted in stable basal phosphorylase a activity. Addition of agonist was followed by a rapid (less than one min) increase in activity which remained at plateau for at least five minutes. Similar results were obtained in all three groups of animals.

Complete concentration-response curves for adrenaline, isoproterenol and phenylephrine for each group of rats are shown in figure 3.2. For all experiments shown in this figure, phosphorylase a activity was determined in aliquots of cell suspension taken immediately before (zero agonist concentration) and 3.0 min after the addition of agonists. The type of adrenoceptor involved in the activation of phosphorylase was deduced from the relative potencies of these three agonists. Basal phosphorylase a activity was not changed by either thyroidectomy or thyroxine treatment. The order of potency of agonists in hepatocytes from normal rats was adrenaline > phenylephrine > isoproterenol (figure 3.2 and table 3.1) which agrees with earlier reports that the response is mediated by alpha adrenoceptors (Sherline et al. 1972; Hutson et al. 1976). Thyroxine treatment decreased somewhat the maximum phosphorylase a response to adrenaline and phenylephrine (figure 3.2), which resulted in a slight decrease in the EC_{50} to all three agonists (table 3.1), but their relative potencies remained unaltered. However, in hepatocytes from hypothyroid rats the order of potency was markedly changed to isoproterenol > adrenaline > phenylephrine (table 3.1). This order of potency is characteristic of beta adrenoceptors. Figure 3.2 shows that in normal rats isoproterenol was not only the least potent agonist, but its efficacy was also significantly less than that of the other two agonists. In hypothyroid rats, the efficacy of isoproterenol was greater while the efficacies of adrenaline and phenylephrine were lower than

in euthyroid controls.

The change in response pattern developed slowly. Changes in agonist potencies were minimal in two rats tested at 2 wk following thyroidectomy although serum thyroxine levels were as low as at three months (table 3.1).

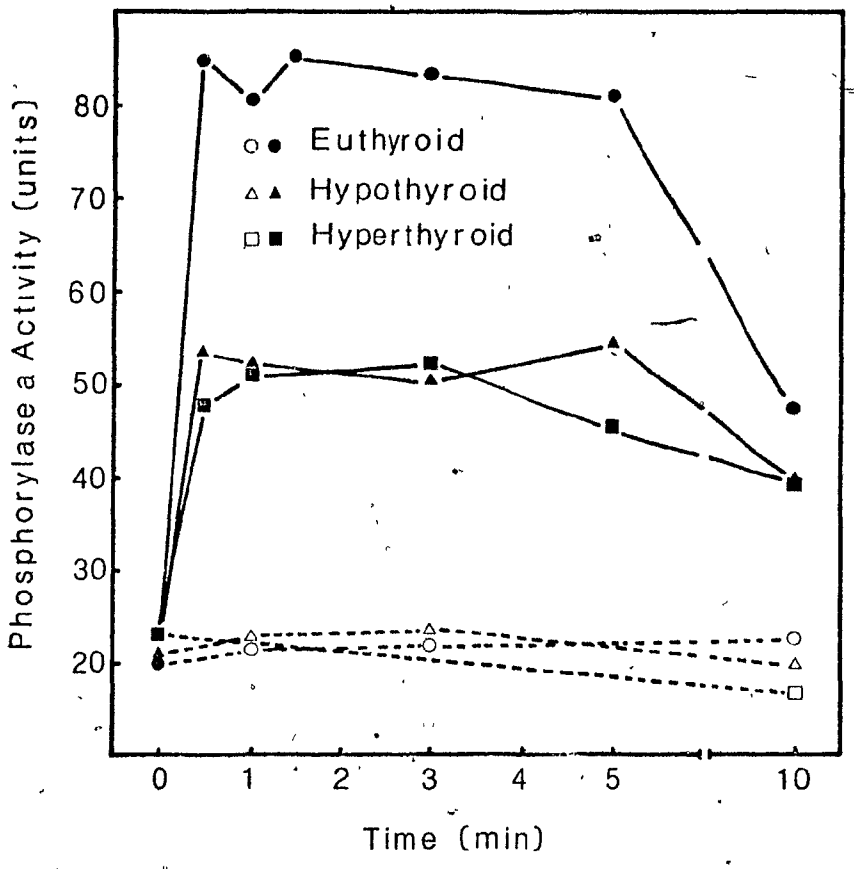


FIGURE 3.1 Time-course of activation of glycogen phosphorylase a by adrenaline in hepatocytes from euthyroid, hypothyroid and hyperthyroid rats.

Cells were isolated as described in section 2.3. After 30 min of incubation in a medium without added gelatin (time 0), 10 µl of saline or adrenaline plus saline was added to a 10 ml volume of cell suspension to give a final concentration of 10^{-6} M adrenaline. At the indicated times 1.0 ml aliquots of this suspension were homogenized in an equal volume of assay buffer (section 2.4) and assayed for phosphorylase a activity as detailed in section 2.4. Units of enzyme activity are nanomoles of [U-^{14}C] glucose incorporated into glycogen per min per mg protein. Each set of data points are the results from a single rat from each group.

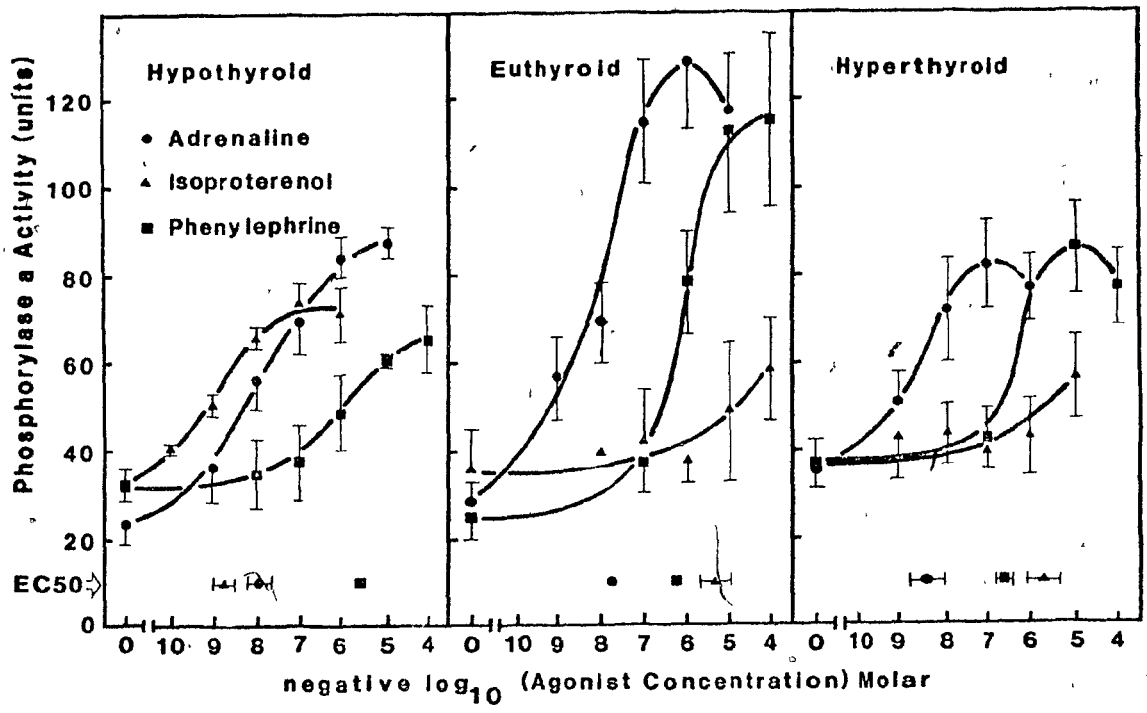


FIGURE 3.2 Activation of glycogen phosphorylase a by adrenaline, isoproterenol and phenylephrine in hepatocytes from euthyroid, hypothyroid and hyperthyroid rats.

Cells were isolated as described in section 2.3. After 30 min of incubation in a medium without added gelatin, adrenaline, isoproterenol or phenylephrine (10 µl) were added to 3.0 ml of cell suspension to give the final concentrations of agonist indicated. Phosphorylase a activity was determined (section 2.4) in 1.0 ml aliquots of cell suspension taken immediately before (zero agonist concentration) and 3.0 min after the addition of agonist. Units of enzyme activity are as noted in section 2.4). Each point represents the mean ± s.e.m. for 3 to 13 separate cell preparations. The EC_{50} is also shown. pD_2 values for these experiments are given in table 3.1.

Table 3.1

The influence of thyroid state on the potency of adrenergic agonists in activating hepatic glycogen phosphorylase.

	Normal	Hyperthyroid	Hypothyroid	
			10-14 wk	2 wk
Serum T4 Level (ng/ml)	50.3 ± 9.9	98.3 ± 2.0*	8.2 ± 2.4*	9.9 ± 2.1
Agonist Potencies				
Isoproterenol	5.35 ± 0.38 (5)	5.75 ± 0.34 (5)	8.77 ± 0.25* (4)	6.02 ± 0.36 (2)
Adrenaline	7.73 ± 0.11 (13)	8.41 ± 0.39 (8)	7.96 ± 0.27 (5)	7.45 ± 0.12 (2)
Phenylephrine	6.21 ± 0.10 (8)	6.58 ± 0.20 (5)	5.54 ± 0.15* (3)	5.77 ± 0.12 (2)

Data are from the same experiments shown in figure 3.2. Values shown are the mean ± s.e.m. of the pD_2 values for the number of experiments shown in parentheses. The pD_2 is the negative logarithm of the agonist concentration producing half-maximal activation of hepatic glycogen phosphorylase. For details concerning the determination of phosphorylase activity, refer to the caption for figure 3.2 and section 2.4. Serum T_4 levels were determined as described in section 2.8. * indicates a significant difference from corresponding values in normal rats.

3.2 Effects of adrenergic antagonists on adrenaline activation of glycogen phosphorylase in hepatocytes from euthyroid, hypothyroid and hyperthyroid rats.

The apparent change in the type of adrenoceptor mediating phosphorylase activation was further examined by comparing the effects of adrenergic antagonists on the phosphorylase response to the mixed alpha/beta receptor agonist adrenaline in hepatocytes from each group of animals.

The altered potency of agonists in hypothyroid rats was associated with a corresponding change in the effectiveness of alpha and beta receptor antagonists. Figure 3.3 illustrates that the increase in phosphorylase activity produced by adrenaline in normal hepatocytes was substantially inhibited by the alpha antagonists phenoxybenzamine (POB) and dihydroergocryptine (DHE), but was not affected by the beta antagonist propranolol. In cells from hypothyroid animals the alpha receptor antagonists did not significantly inhibit phosphorylase activation by adrenaline, whereas propranolol was an effective inhibitor at low adrenaline concentrations. Beta receptors are generally more sensitive to stimulation by adrenaline (Kunos 1977) and the absence of block by propranolol at higher adrenaline concentrations may be due to remaining alpha receptor activity.

In hepatocytes from hyperthyroid rats both DHE and POB were effective antagonists of adrenaline-induced phosphorylase activation, while the beta receptor antagonist, propranolol, was again ineffective. This pattern of drug response is the same as that seen in hepatocytes from normal rats and confirms results obtained with adrenergic agonists: the response is mediated predominantly by alpha receptors in preparations from the hyperthyroid rat liver.

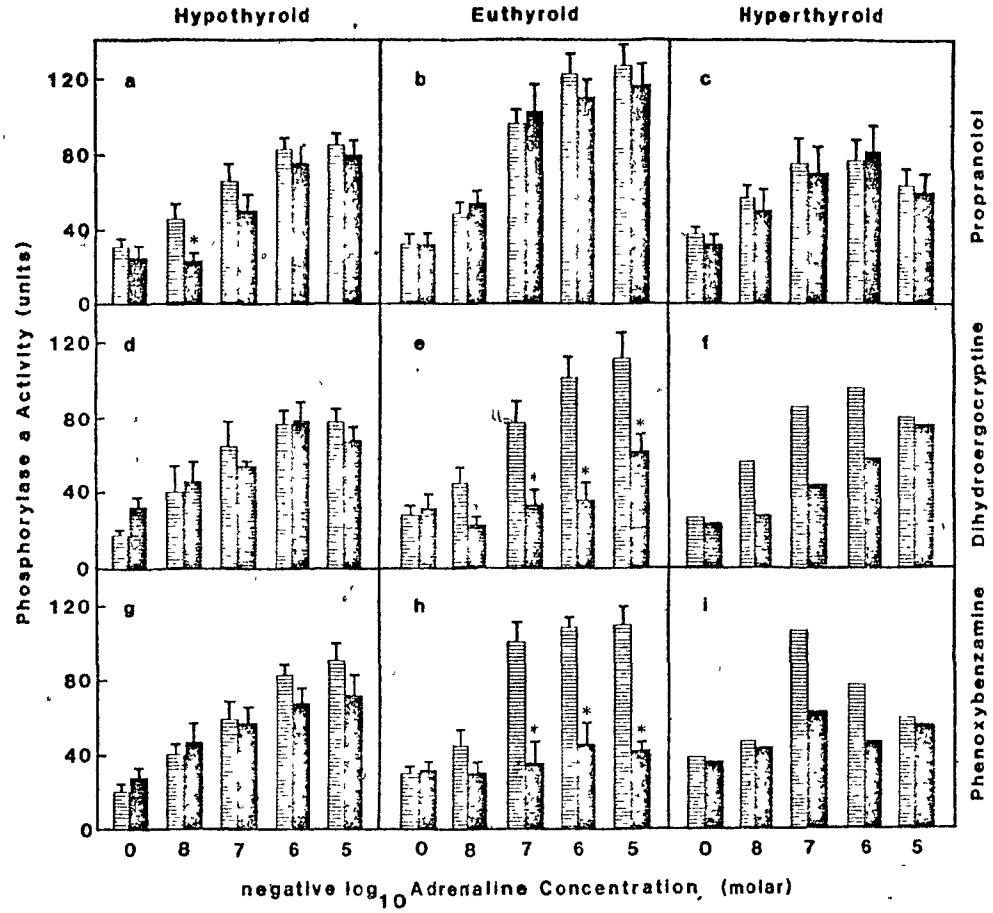


FIGURE 3.3 Effects of adrenergic antagonists propranolol, phenoxybenzamine and dihydroergocryptine on activation of glycogen phosphorylase a by adrenaline in hepatocytes from euthyroid, hypothyroid and hyperthyroid rats.

Experimental details are as described in the caption to figure 3.2 except that antagonists were added at the start of the 30 min incubation period to give a final concentration of 10^{-5}M in each case. Antagonists were added, 1 µl per ml of cell suspension in vehicle as specified for each in section 2.2. Units of enzyme activity are as noted in section 2.4. Each point represents the mean ± s.e.m. for 3 to 7 separate cell preparations except for panels f and i where the results of a single representative experiment are shown. Solid bars: antagonist present. Striped bars: no antagonist present.

3.3 The effect of cortisol treatment on glycogen phosphorylase activation by adrenaline, isoproternol and phenylephrine in hepatocytes from thyroidectomized rats.

Changes in hepatic adrenergic receptors similar to those observed after thyroidectomy have been described following adrenalectomy (Chan et al. 1979a). In order to test the possibility that the effect of thyroidectomy reported here was secondary to thyroid-induced changes in the adrenal-pituitary axis, the effect of cortisol treatment on the phosphorylase response in thyroidectomized rats was examined. Results are shown in figure 3.4. Treatment of thyroidectomized rats with cortisol (50 mg/kg) every 12 h beginning 48 h before the experiment did not significantly change the response to the three agonists when compared with untreated thyroidectomized rats. A similar treatment regimen has been reported to reverse the effects of adrenalectomy on hepatic beta adrenergic receptors (Wolfe et al. 1976).

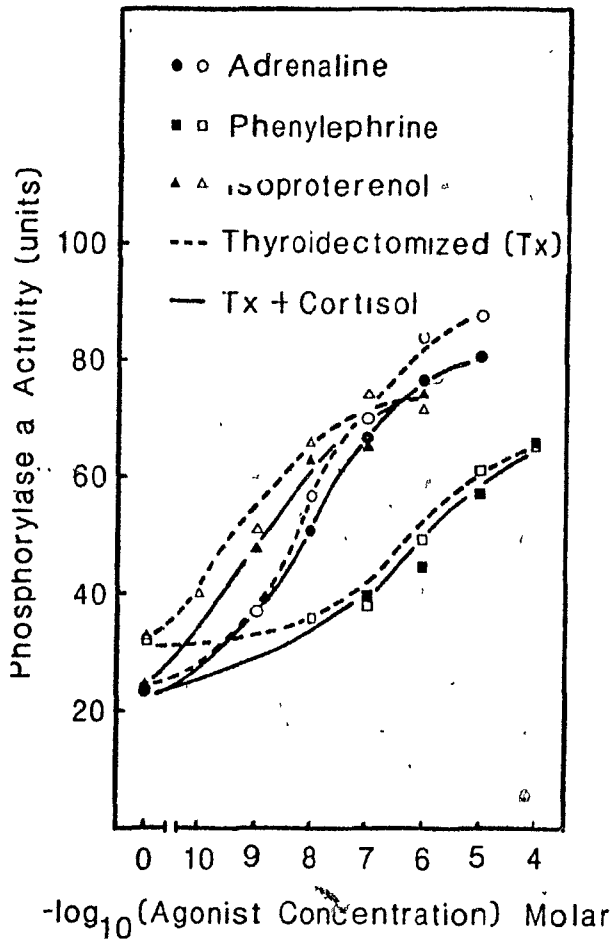


FIGURE 3.4 The effect of cortisol treatment on the activation of glycogen phosphorylase a by adrenaline, isoproterenol and phenylephrine in hepatocytes from hypothyroid rats.

Cells were isolated from hypothyroid rats that had received intraperitoneal injections of cortisone acetate (50 mg/kg) every 12 h beginning 48 h before the experiment. Isolation, incubation of hepatocytes and assay of glycogen phosphorylase a activity were done as described in the caption to figure 3.2. Open symbols and filled symbols represent the means of 3 to 8 separate cell preparations from hypothyroid and hypothyroid, cortisol-treated rats respectively. Data for hypothyroid rats is the same as that appearing in figure 3.2. Standard error bars have been omitted for clarity.

3.4 Accumulation of cAMP in response to stimulation by adrenergic agonists in hepatocytes from normal and thyroidectomized rats.

In order to further investigate the nature of the emergent beta receptor contribution to phosphorylase activation in thyroidectomized rats, cAMP accumulation in response to adrenaline, isoproterenol and phenylephrine was measured and compared with the response in hepatocytes from normal rats. Preliminary time-course experiments are shown in figure 3.5. Isoproterenol (10^{-6}M) produced a rapid (less than one min) increase in cAMP that remained at plateau for at least 5 min in hepatocytes from thyroidectomized rats. Similar results were obtained with normal hepatocytes except that magnitude of the response was very much less. In subsequent experiments hepatocyte suspensions were sampled for the determination of cAMP at 1.5 min after the addition of agonists.

Complete concentration-response curves were produced for adrenaline, isoproterenol and phenylephrine in hepatocytes from normal and thyroidectomized rats (figure 3.6). Basal cAMP levels were not different for both groups: 1.85 $\pm$ 0.76 pmol/mg protein for normal (n=3) and 2.25 $\pm$ 0.93 pmol/mg protein for thyroidectomized rats (n=3). All three agonists produced concentration-dependent increases in cAMP levels, however, in both groups of animals the magnitude of the response to adrenaline and isoproterenol was very much greater in hepatocytes from thyroidectomized rats; the response to isoproterenol stimulation was approximately 20-fold greater than in normal rats. The order of effectiveness in producing this response was isoproterenol > adrenaline > noradrenaline, which is consistent with a $beta_2$ adrenergic receptor response in the thyroidectomized rat (figures 3.6 and 3.7). The difference in the maximum response to isoproterenol in the two groups could not be accounted for by a difference in metabolism of the drug since exposure was brief, and as shown in figure 3.8, the level of cAMP accumulation in response to isoproterenol was directly proportional to the density of hepatocyte

suspensions from both a thyroidectomized and normal rat.

In order to test the possibility that differential metabolism of cAMP by phosphodiesterase in the two groups was the basis for this difference, the response to isoproterenol was examined in the presence of isobutylmethylxanthine (IBMX), a phosphodiesterase inhibitor. Results of these experiments are summarized in table 3.2 and demonstrate that although cAMP accumulation in response to isoproterenol was enhanced in the presence of this inhibitor, the difference between the response in normal and thyroidectomized rat hepatocytes remained. A difference in phophodiesterase activity, therefore, cannot account for the above observations.

Comparison of the cAMP response to isoproterenol and adrenaline in the thyroidectomized rats showed that at maximal concentrations of these drugs, isoproterenol consistently produced greater cAMP accumulation than adrenaline. However, both of these drugs are are considered to be "full" beta receptor agonists. Adrenaline is also a "full" alpha receptor agonist, while isoproterenol has only a very slight effect on alpha receptors. This raised the possibility that the difference in their effectiveness could be due to an inhibitory influence of alpha receptor stimulation on cAMP accumulation as noted in the liver by others (section 1.3.3). To test this possibility the effect of phenoxybenzamine, an alpha receptor antagonist, on adrenaline-stimulated cAMP accumulation was examined. As the results summarized in table 3.3 show, adrenaline-stimulated cAMP accumulation in hepatocytes was significantly enhanced after blockade of alpha receptors with phenoxybenzamine in the thyroidectomized rat. However, phenoxybenzamine blockade did not potentiate cAMP accumulation in response to adrenaline in the normal rat. This suggested that inhibitory alpha receptor activity was responsible for the difference in maximal cAMP accumulation induced by isoproterenol and adrenaline. Furthermore, such inhibitory alpha receptors appear to be active only in the hypothyroid and not in the normal rat.

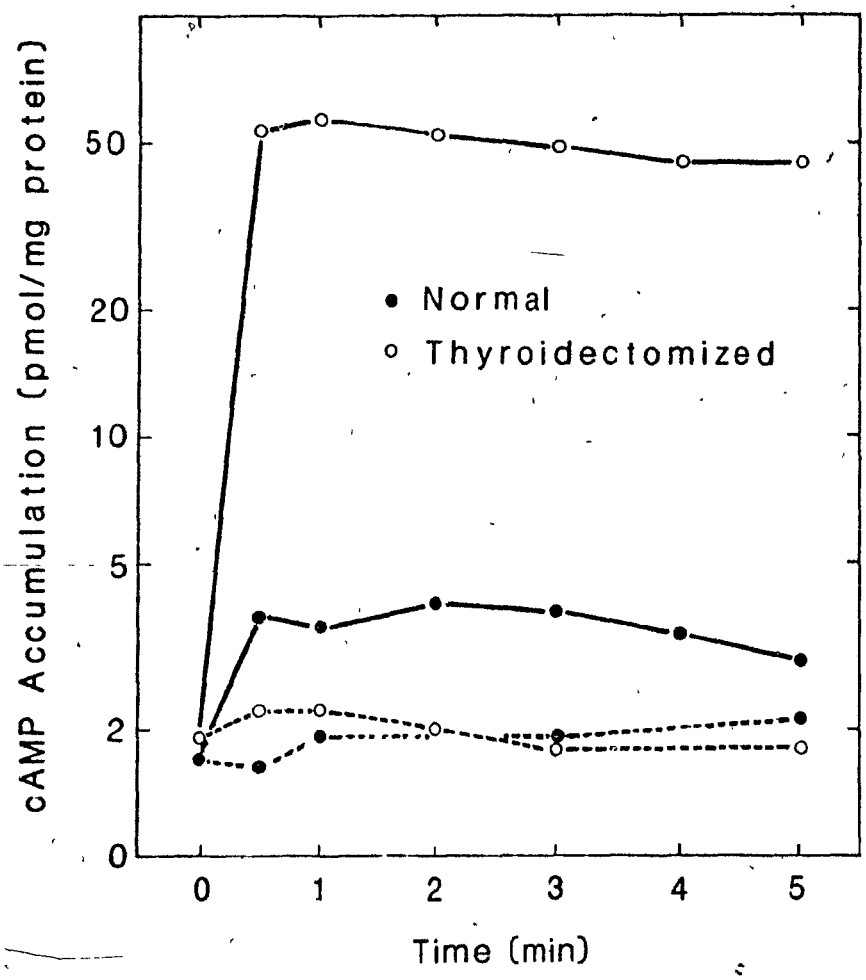


FIGURE 3.5 Time-course of cAMP accumulation in response to isoproterenol in hepatocytes from normal and thyroidectomized rats.

Cells were isolated as described in section 2.3. After 30 min of incubation in a medium without added gelatin (time 0), 10 µl of saline (broken line) or isoproterenol (solid line) was added to a 10 ml volume of cell suspension (approximately 40 mg/ml) to give a final concentration of 10^{-6}M isoproterenol. At the indicated times, 1.0 ml aliquots of this suspension were homogenized in an equal volume of 10% trichloracetic acid and assayed for cAMP as detailed in section 2.5. Results of a single representative experiment are shown with cells from a normal and thyroidectomized rat.

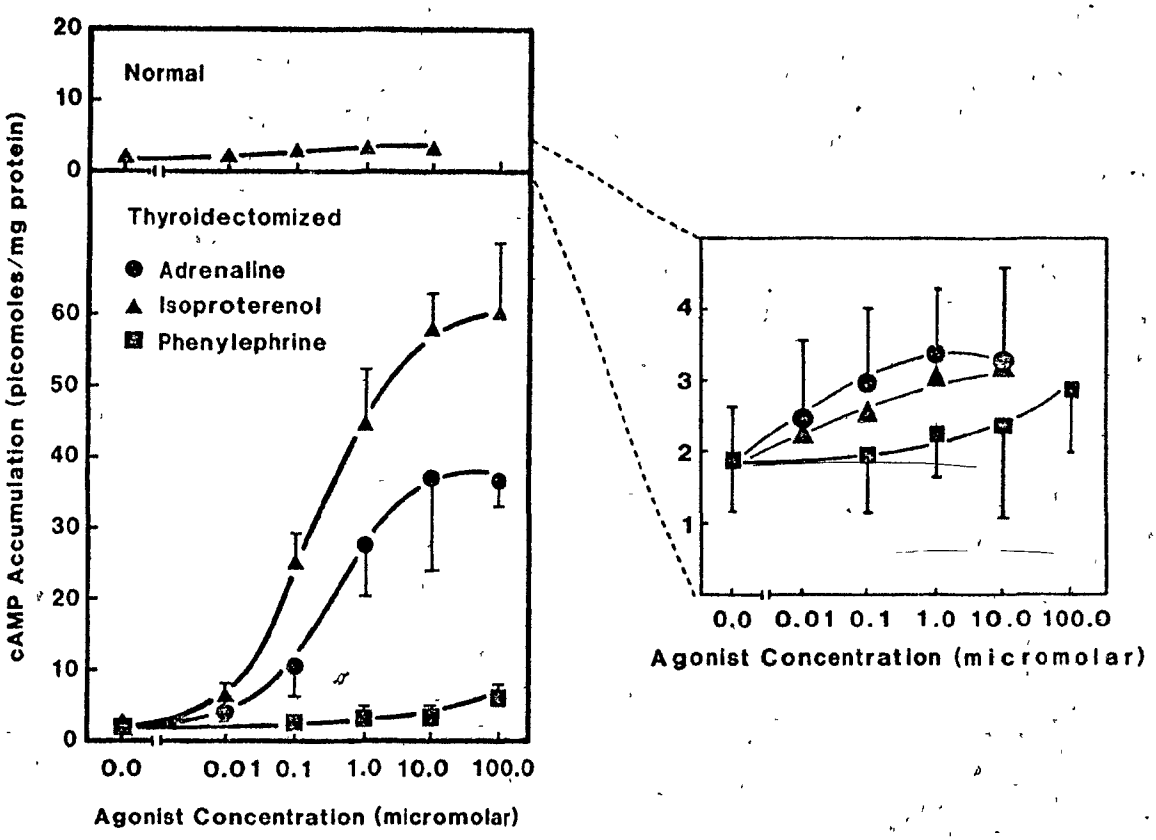


FIGURE 3.6 Accumulation of cAMP in response to stimulation by adrenaline, isoproterenol and phenylephrine in hepatocytes from normal and thyroidectomized rats.

Cells were isolated as described in section 2.3 and resuspended in medium without gelatin. After 30 min of preincubation, drug was added in a 10 µl volume to 3 ml of cell suspension to give the indicated final concentrations. After 1.5 min, 1.0 ml aliquots of this suspension were removed and assayed for cAMP as detailed in section 2.5. Results are the mean ± s.e.m. for three separate cell preparations from thyroidectomized and normal rats (upper and right panel).

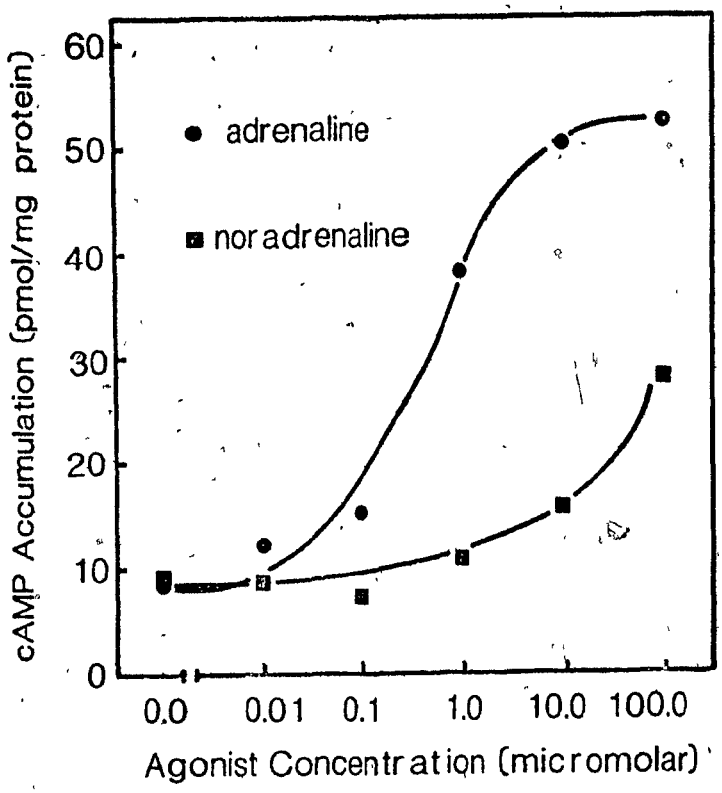


FIGURE 3.7 Accumulation of cAMP in response to stimulation by adrenaline and noradrenaline in hepatocytes from a thyroidectomized rat.

Cells were isolated as described in section 2.3. After 30 min of preincubation, adrenaline or noradrenaline was added in a 10 µl volume of saline to 3.0 ml volume of cell suspension to give the indicated final agonist concentrations. After 1.5 min, 1.0 ml aliquots of this suspension were removed and assayed for cAMP as detailed in section 2.5. Results shown are of a single cell preparation from a thyroidectomized rat.

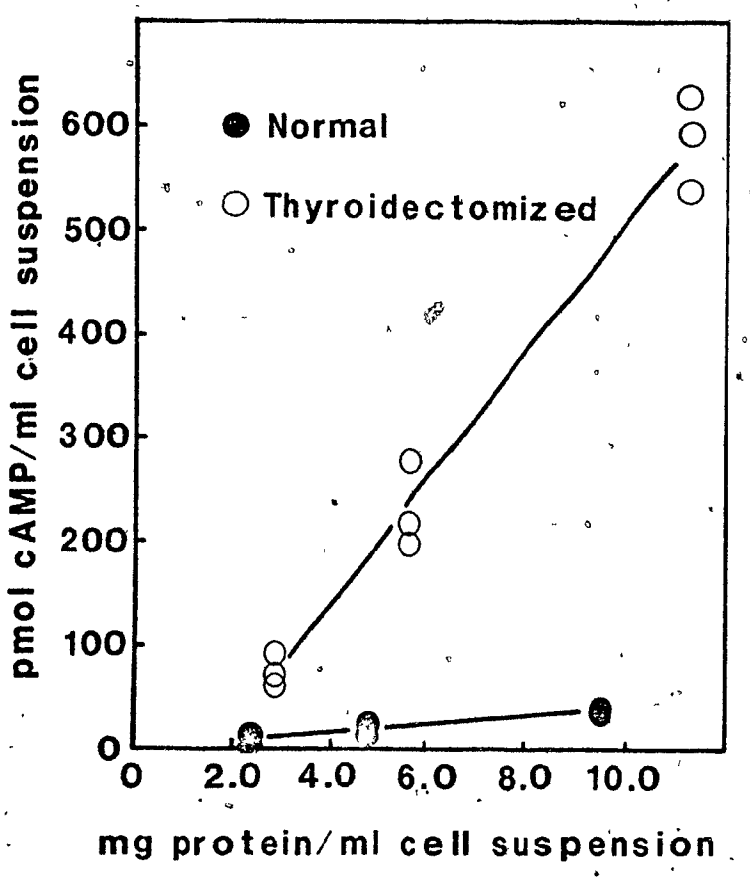


FIGURE 3.8 Isoproterenol-stimulated cAMP accumulation as a function of tissue amount in hepatocytes from a normal and a thyroidectomized rat.

Cells were resuspended at approximately 80 mg/ml. These were then diluted twice by 50% to give three concentrations of cell material. Three ml aliquots of each of these concentrations were incubated and cAMP accumulation in response to 10^{-6}M isoproterenol was determined as described in the caption to figure 3.6. Each data point reflects the value obtained from a single aliquot of cell suspension for cells isolated from a normal and a thyroidectomized rat.

Table 3.2

Effect of phosphodiesterase inhibitor (IBMX) on isoproterenol- and glucagon-stimulated cAMP accumulation in hepatocytes from normal and thyroidectomized rats.

	cAMP Accumulation (pmol/mg protein)	
	Normal	Thyroidectomized
Control	1.9 ± 0.4	1.6 ± 0.3
+ IBMX	2.7 ± 0.4	2.7 ± 0.5
10^{-8}M Isoproterenol	2.3 ± 0.6	3.8 ± 0.6
+ IBMX	3.1 ± 0.6	8.1 ± 0.6 *
10^{-5}M Isoproterenol	2.5 ± 0.4	10.0 ± 1.1 *
+IBMX	3.8 ± 0.6	42.8 ± 5.6 *
10^{-9}M Glucagon	3.9 ± 0.7	4.2 ± 1.3
+ IBMX	11.2 ± 2.9	15.2 ± 4.5
10^{-7}M Glucagon	11.3 ± 1.8	24.5 ± 5.4 *
+ IBMX	40.6 ± 8.8	75.2 ± 12.4 *

Cyclic AMP accumulation in response to stimulation with glucagon or isoproterenol at the indicated concentrations was determined as detailed in the caption to figure 3.10 and 3.12 except that 10^{-4}M isobutylmethylxanthine (IBMX) or vehicle alone (2 µl ethanol/ml of cell suspension) was added 10 min before the addition of agonist. Values shown are the mean ± s.e.m. for four to six cell preparations. * indicates values significantly different from that obtained with normal hepatocytes.

Table 3.3

Effect of phenoxybenzamine on adrenaline-stimulated cAMP accumulation in hepatocytes from a normal and a thyroidectomized rat.

	cAMP Accumulation (pmol./mg protein)		
	Control		Phenoxybenzamine
Thyroidectomized			
Saline	2.03 ± 0.24		1.87 ± 0.28
10^{-6}M Adrenaline	3.75		6.50
10^{-5}M Adrenaline	5.25 ± 0.40	*	7.10 ± 0.60
Normal			
Saline	2.40		2.25
10^{-6}M Adrenaline	3.47		2.96
10^{-5}M Adrenaline	3.60		3.50

Cyclic AMP accumulation in response to stimulation with adrenaline at the indicated concentrations was determined as detailed in the caption to figure 3.6 except that 10^{-5}M phenoxybenzamine or vehicle alone (1 µl acidified ethanol/ml of cell suspension) was added 10 min before the addition of adrenaline (10 µl./ml). Values shown are the mean ± s.e.m for quadruplicate incubations of a single cell preparation from a thyroidectomized rat or the results of duplicate determinations where values without standard errors are shown. Similar results were obtained in two more cell preparations from thyroidectomized and one from normal rats. * indicates a significant difference between adjacent values.

3.5 Glucagon-induced activation of glycogen phosphorylase and cAMP accumulation in hepatocytes from thyroidectomized and normal rats.

In order to examine the selectivity of changes in beta adrenoceptor activity brought about after thyroidectomy, glucagon activation of phosphorylase was compared in normal and thyroidectomized rats. Figure 3.9 illustrates that glucagon produced an identical phosphorylase activation in hepatocytes from these two groups over the entire range of concentrations tested. Thus the action of a second glycogenolytic hormone, known to activate phosphorylase via a similar cAMP cascade (section 1.3.2) is not affected by thyroidectomy in the same way as beta receptor agonists are.

However, when cAMP accumulation in response to glucagon was examined, it was found to be significantly enhanced in hepatocytes from thyroidectomized rats, but only at relatively high glucagon concentrations (figure 3.10). At 10 nM glucagon, a concentration that was maximally effective in stimulating phosphorylase activity (figure 3.9), no difference in cAMP accumulation was observed (figure 3.10). This indicates that over the range of glucagon concentrations that can be considered physiologically important, there is no difference in cAMP accumulation when thyroidectomized rats are compared with normal animals. The addition of phosphodiesterase inhibitor (IBMX, table 3.2) enhanced glucagon-stimulated cAMP accumulation in both groups. The difference noted at higher glucagon concentrations in the absence of this inhibitor was even greater, suggesting that differential metabolism of cAMP in these two groups could not account for the observed difference.

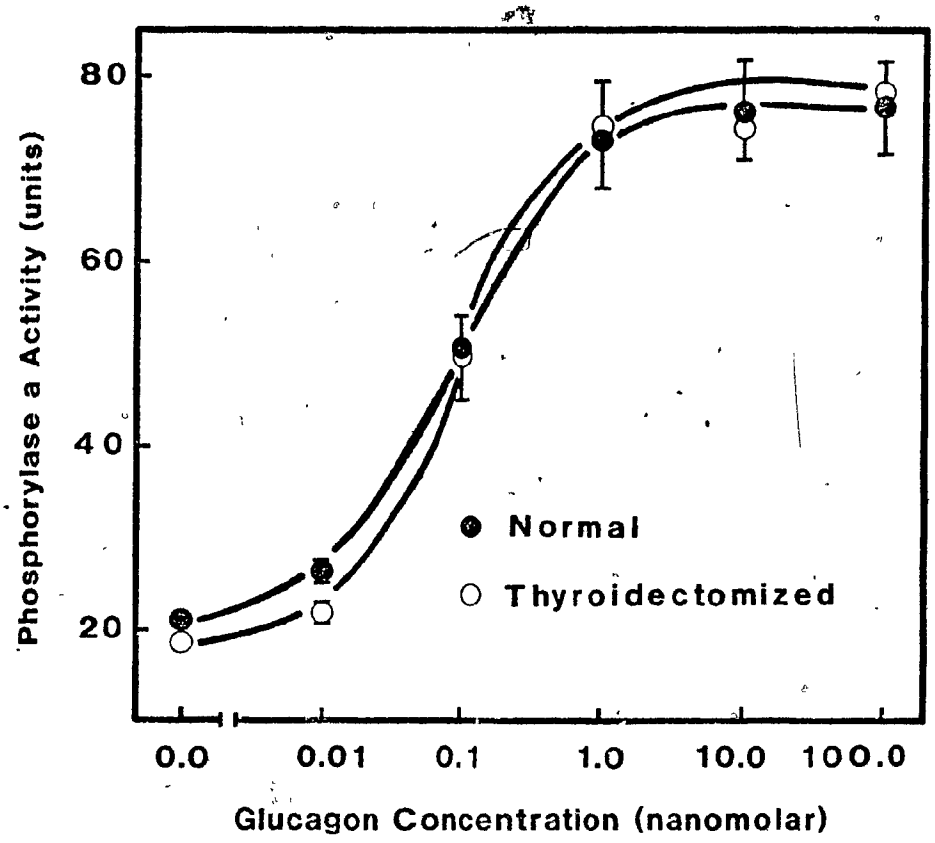


FIGURE 3.9 Activation of glycogen phosphorylase by glucagon in hepatocytes from normal and thyroidecomized rats.

Cells were isolated as described in section 2.3. After 30 min of incubation in a medium with added gelatin, glucagon (10 µl/ml) was added to give the indicated final concentrations. After 3 min 1 ml of cell suspension were removed, diluted into 5 ml of ice cold Krebs-Henseleit buffer, rapidly centrifuged and prepared for assay of phosphorylase a as detailed in section 2.4. Values shown are the mean ± s.e.m. of four separate cell preparations from normal rats and five in the case of thyroidecomized rats.

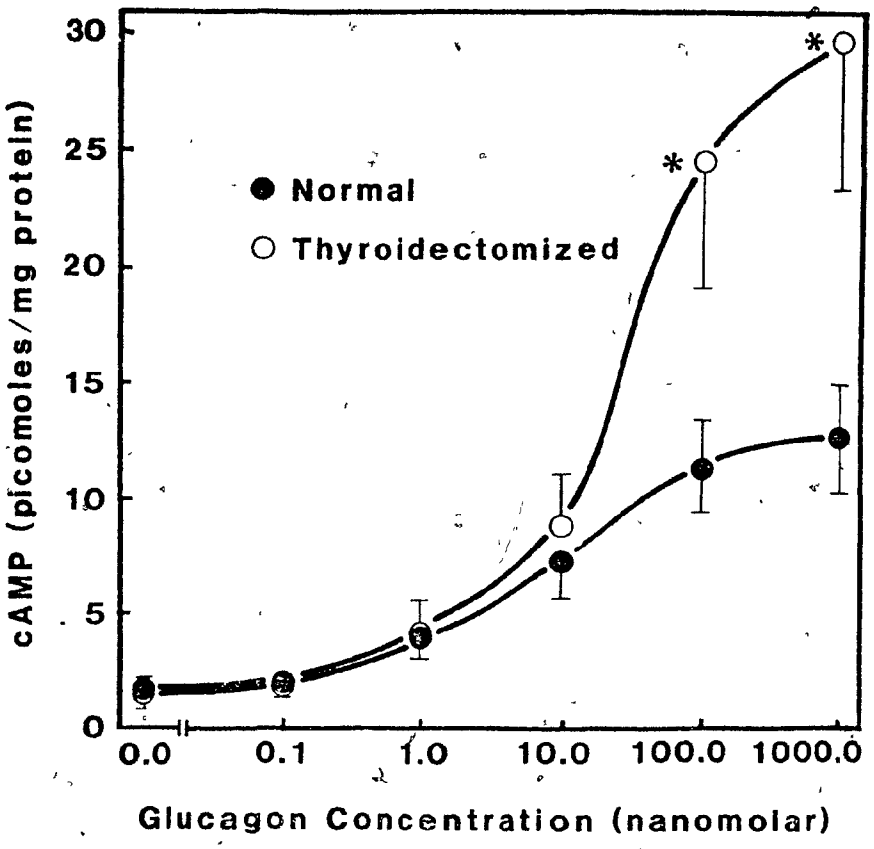


FIGURE 3.10 Accumulation of cAMP in response to stimulation by glucagon in hepatocytes from normal and thyroidectomized rats.

Cells were isolated as described in section 2.3 and resuspended in medium containing gelatin. After 30 min of preincubation glucagon was added to give the indicated final concentrations. After 1.5 min, 1.0 ml aliquots of this suspension were removed and assayed for cAMP as detailed in section 2.5. Results shown are the mean $\pm$ s.e.m. for six and eight separate cell preparations from thyroidectomized and normal rats respectively. *indicates values significantly different from normal.

3.6 Effect of triiodothyronine replacement on isoproterenol-induced cAMP accumulation and glycogen phosphorylase activation in hepatocytes from thyroidectomized rats.

Figure 3.11 summarizes the effect of T_3 replacement on isoproterenol-induced cAMP accumulation in thyroidectomized rats. Animals received 0.25 mg T_3 per kg body weight by daily intraperitoneal injections for either two or four days. This dose of T_3 is known to completely saturate hepatic nuclear thyroid hormone binding sites (Oppenheimer et al. 1978). As shown in figure 3.11, isoproterenol-induced cAMP accumulation was greatly enhanced in hepatocytes from thyroidectomized compared with normal rats. Thyroid hormone replacement for two days reduced the response somewhat, while after four days the effect of isoproterenol was not different from that of normal rat hepatocytes.

The effect of four days of treatment with T_3 on isoproterenol activation of glycogen phosphorylase is shown in figure 3.12. In agreement with earlier results (section 3.1), isoproterenol was significantly more effective in stimulating phosphorylase in hepatocytes from thyroidectomized rats when compared with normal. The response of hepatocytes from T_3-treated, thyroidectomized rats was returned partially, but not completely to normal levels, in contrast to the effect of four-day treatment on cAMP responses (figure 3.11). This is most likely due to the apparently greater sensitivity of phosphorylase activation than cAMP accumulation to stimulation by glycogenolytic hormones (section 4.2).

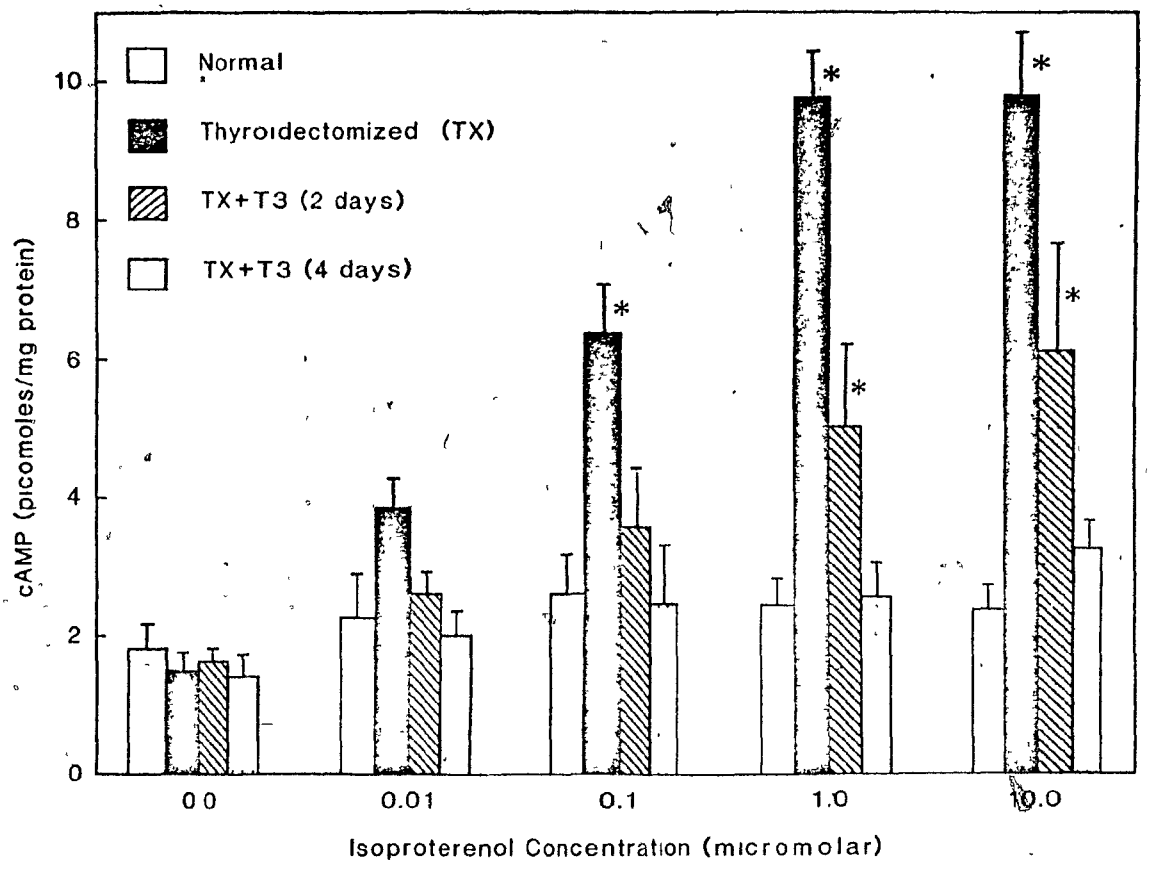


FIGURE 3.11 Effect of triiodothyronine replacement on isoproterenol-stimulated accumulation of cAMP in hepatocytes from thyroidectomized rats. Comparison with response in normal and untreated thyroidectomized rats.

Thyroidectomized rats received 0.25 mg T_3 per kg by intraperitoneal injection for two or four days as indicated. Approximately 12 h after the last injection hepatocytes were isolated from these as well as normal and untreated, thyroidectomized rats as described in section 2.3. Cyclic AMP accumulation in response to the indicated isoproterenol concentrations was determined as detailed in the caption to figure 3.6. Results shown are the mean $\pm$ s.e.m. for four cell preparations in the case of four day-treated rats and five for the others. * indicates a significant difference from normal at a given concentration.

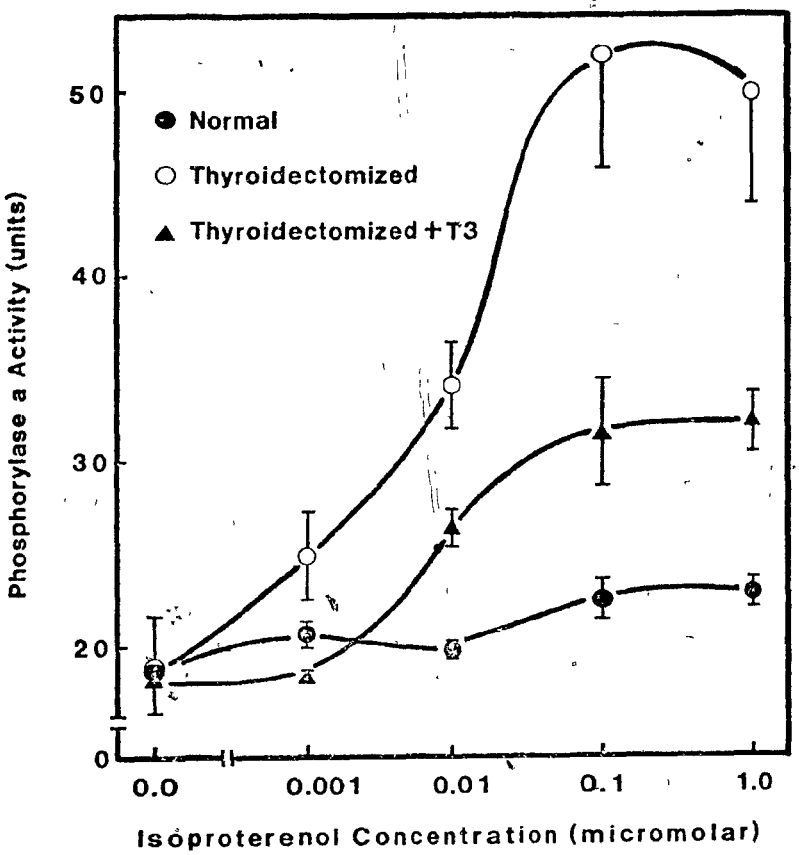


FIGURE 3.12 Effect of triiodothyronine replacement on activation of phosphorylase by isoproterenol in hepatocytes from thyroidectomized rats. Comparison with response in normal and untreated, thyroidectomized rats.

Cells were isolated from normal, thyroidectomized, and thyroidectomized rats treated for four days with T_3 as described in the caption to figure 3.11. Cells were preincubated for 30 min after which time isoproterenol was added (10 µl/ml) to give the indicated final concentrations. After 3 min phosphorylase was determined as described in the caption to figure 3.9. Values shown are the mean ± s.e.m. for four cell preparations in each case.

3.7 Effect of phenylephrine on calcium efflux in hepatocytes from normal, thyrodectomized and thyrodectomized, triiodothyronine-treated rats.

As described above (section 3.1), thyroidectomy produced a marked decrease in the efficacy of both adrenaline and phenylephrine in activating glycogen phosphorylase. A likely explanation for the decreased efficacy of these two agonists is a decrease in alpha receptor activity. Stimulation of alpha receptors has been shown to lead to the release of calcium from isolated hepatocytes (section 1.3.3). Thus, to further investigate this possible decrease in alpha receptor activity, phenylephrine-stimulated calcium efflux from intact hepatocytes was examined by the use of a calcium-sensitive electrode as described by Chen et al. (1978).

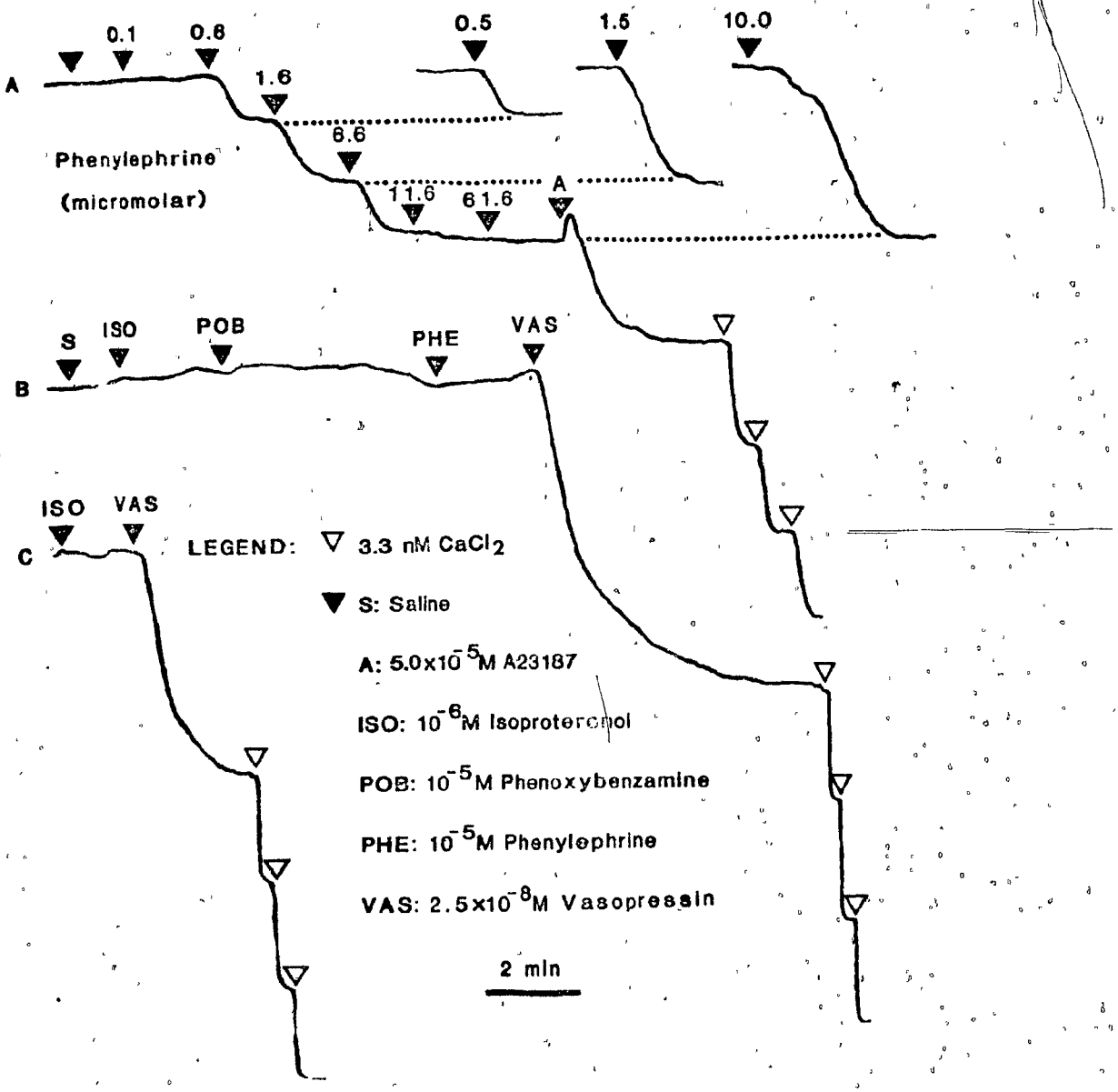
In agreement with those authors, preliminary experiments (figure 3.13) showed that phenylephrine produced a concentration-dependent increase in calcium release from intact hepatocytes which could be blocked by the alpha antagonist phenoxybenzamine. The calcium inophore, A23187, induced the release of additional calcium, above that released by maximal phenylephrine concentrations (figure 3.13 A). Isoproterenol, at a concentration that is maximally effective in stimulating beta receptors, had no effect on calcium efflux in either normal or hypothyroid rats, whereas a second glycogenolytic hormone, vasopressin, produced significant calcium release even after blockade by phenoxybenzamine (figure 3.13 B and C). Figure 3.13 also shows that phenylephrine could be measured in a cumulative manner without the emergence of significant tachiphylaxis: responses to single doses were not significantly different from those to cumulative doses.

Hence, complete concentration-response curves were produced for phenylephrine in hepatocyte suspensions prepared from thyroidectomized rats and compared with triiodothyronine-treated, thyroidectomized rats and normal rats. These results are summarized in figure 3.14. As expected on the basis of results obtained for phenylephrine stimulation of glycogen phosphorylase,

thyroidectomy resulted in a marked suppression of the phenylephrine concentration-response curve for calcium efflux when compared to that for normal rats. Triiodothyronine treatment (0.25 mg/kg for four days) returned the concentration-response curve of the thyroidectomized rats towards normal. The mean EC_{50} values for these three groups are shown in table 3.4. The decrease in efficacy of phenylephrine was accompanied by an approximately six-fold decrease in potency of this agonist after thyroidectomy. T_3-treatment also normalized this shift in potency.

FIGURE 3.13 Effects of various glycogenolytic agents on calcium efflux from hepatocytes isolated from normal and thyroidectomized rats as measured by a calcium-sensitive electrode.

Hepatocytes were isolated (section 2.3) and prepared for determination of calcium efflux using a calcium-sensitive electrode as described in section 2.6. Tracing A shows the calcium efflux produced by incremental doses of phenylephrine in a suspension of cells from a normal rat. Adjacent are tracings showing the efflux produced by approximately equivalent single phenylephrine doses in separate suspensions of cells from the same cell preparation. A23187 caused the efflux of more calcium than a maximum dose of phenylephrine. Tracing B shows that isoproterenol, at a maximally effective glycogenolytic dose, produced no calcium efflux in a cell suspension from a second normal rat. Phenoxybenzamine completely inhibited phenylephrine-induced efflux, but had no effect on the vasopressin response. Tracing C is produced with cells from a thyroidectomized rat and shows that isoproterenol was again without effect on calcium efflux, while vasopressin produced a rapid calcium loss.


A
0.1
0.6
1.6
6.6
11.6
61.6
A
0.5
1.5
10.0
Phenylephrine
(micromolar)
B
S
ISO
POB
PHE
VAS
C
ISO
VAS
LEGEND:
3.3 nM CaCl2
S: Saline
A: 5.0x10-5M A23187
ISO: 10-6M Isoproterenol
POB: 10-5M Phenoxybenzamine
PHE: 10-5M Phenylephrine
VAS: 2.5x10-8M Vasopressin
2 min

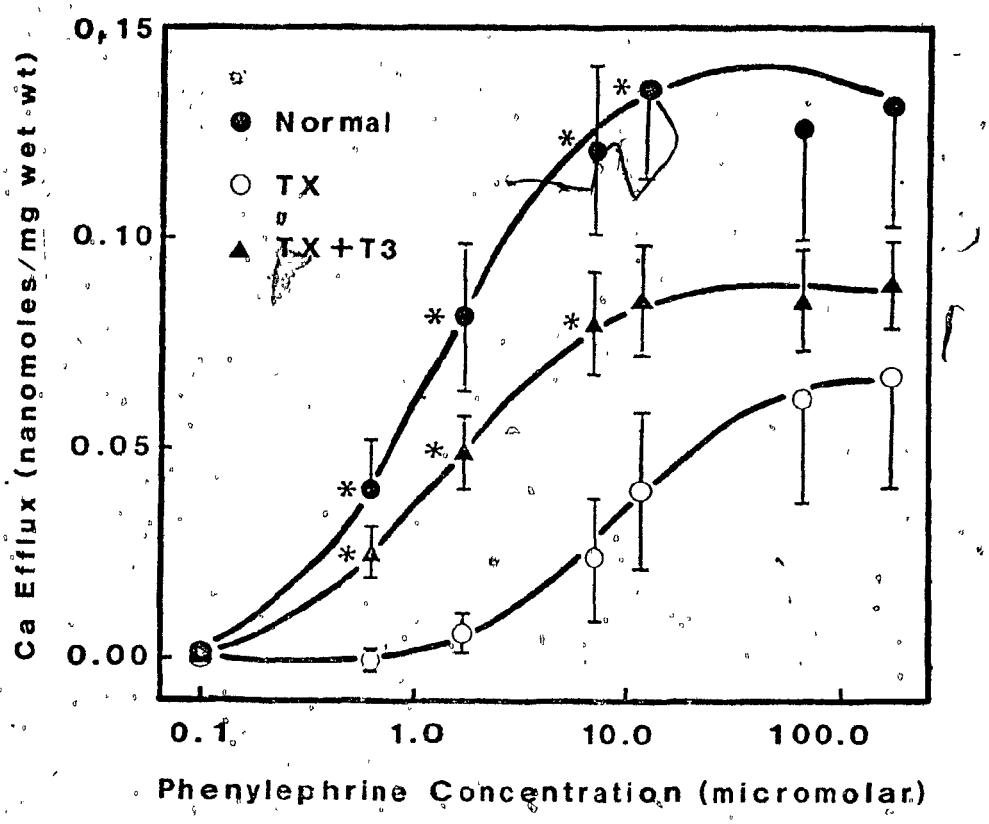


FIGURE 3.14 Phenylephrine-stimulated calcium efflux in hepatocytes from normal, thyroidectomized (Tx) and thyroidectomized, T_3-treated rats (Tx + T_3).

Hepatocytes were isolated and prepared for determination of calcium efflux by calcium-sensitive electrode as described in the caption to figure 3.13. After a stable baseline was obtained, phenylephrine was added to give the cummulative concentrations shown. At the conclusion of each concentration-response curve, calibration pulses of calcium were added and the amount of calcium efflux calculated. Values shown are the mean $\pm$ s.e.m. of five cell preparations from T_3-treated rats and six from each of the other groups. * indicates values significantly different from those obtained with cells from normal rats. Mean EC_{50} values with standard errors are given in table 3.4.

Table 3.4

Potency of phenylephrine and vasopressin in stimulation of glycogen phosphorylase and calcium efflux in hepatocytes from normal, thyroidectomized (TX). and thyroidectomized, triiodothyronine-treated ($Tx + T_3$) rats.

	Normal	TX	TX + T3
Phenylephrine (μM)			
Phosphorylase	0.8 ± 0.3 (4)	6.0 ± 1.6* (4)	1.3 ± 0.7 (4)
Calcium Efflux (Ca-free)	1.1 ± 0.4 (6)	6.2 ± 1.7* (6)	1.3 ± 0.2 (5)
^{45}Ca-Efflux (2.5 mM Ca)	1.6 ± 0.7 (4)	n.d.	1.6 ± 0.3 (4)
Vasopressin (nM)			
Phosphorylase	0.5 ± 0.2 (4)	1.2 ± 0.2 (4)	n.d.
Calcium Efflux (Ca-free)	1.9 ± 0.7 (5)	5.1 ± 1.4* (6)	n.d.
^{45}Ca-Efflux (2.5 mM Ca)	0.5 ± 0.1 (4)	3.0 ± 0.4* (4)	n.d.

Data are from the same experiments reported in figures 3.14, 3.15, 3.19 and 3.20. Each value is the mean ± s.e.m. of the EC_{50} value for the number of experiments shown in parentheses. Phosphorylase activities were determined with 2.5 mM extracellular calcium as was ^{45}Ca efflux. Calcium efflux (measured by calcium-sensitive electrode) was determined in nominally calcium-free medium. * indicates values significantly different from that obtained in normal rats. For details about the measurement of these responses in hepatocyte suspensions refer to the legends to the above-indicated figures.

3.8 The effect of vasopressin on calcium efflux from hepatocytes of normal and thyroidectomized rats.

Vasopressin has been shown to activate hepatic glycogen phosphorylase by a cyclic AMP-independent mechanism (Kirk and Hems 1974). Similar to alpha adrenergic agonists, vasopressin stimulates calcium release from isolated hepatocytes (Blackmore et al. 1978; Chen et al. 1978) and the accompanying transient increase in cytosolic calcium is believed to mediate the activation of glycogen phosphorylase (section 1.3.3). Since the mitochondrial pool from which calcium is released and the post-receptor mechanisms for phosphorylase activation are thought to be the same for alpha adrenergic agonists and vasopressin (section 1.3.3), vasopressin was used to test whether the influence of hypothyroidism on the responses to phenylephrine is selective for alpha adrenergic stimulation. The results of the effect of vasopressin-induced calcium efflux from hepatocytes of normal and thyroidectomized rats are shown in figure 3.5. Although the response to vasopressin is somewhat suppressed in the thyroidectomized rat this difference approaches, but does not attain statistical significance. Comparison of this change with that for phenylephrine (figure 3.14) reveals a different pattern of change with a more significant suppression for the alpha receptor agonist. The EC_{50} for the vasopressin response is shifted 2.7-fold to the right (table 3.4) compared with the six-fold shift observed for phenylephrine after thyroidectomy (section 3.7).

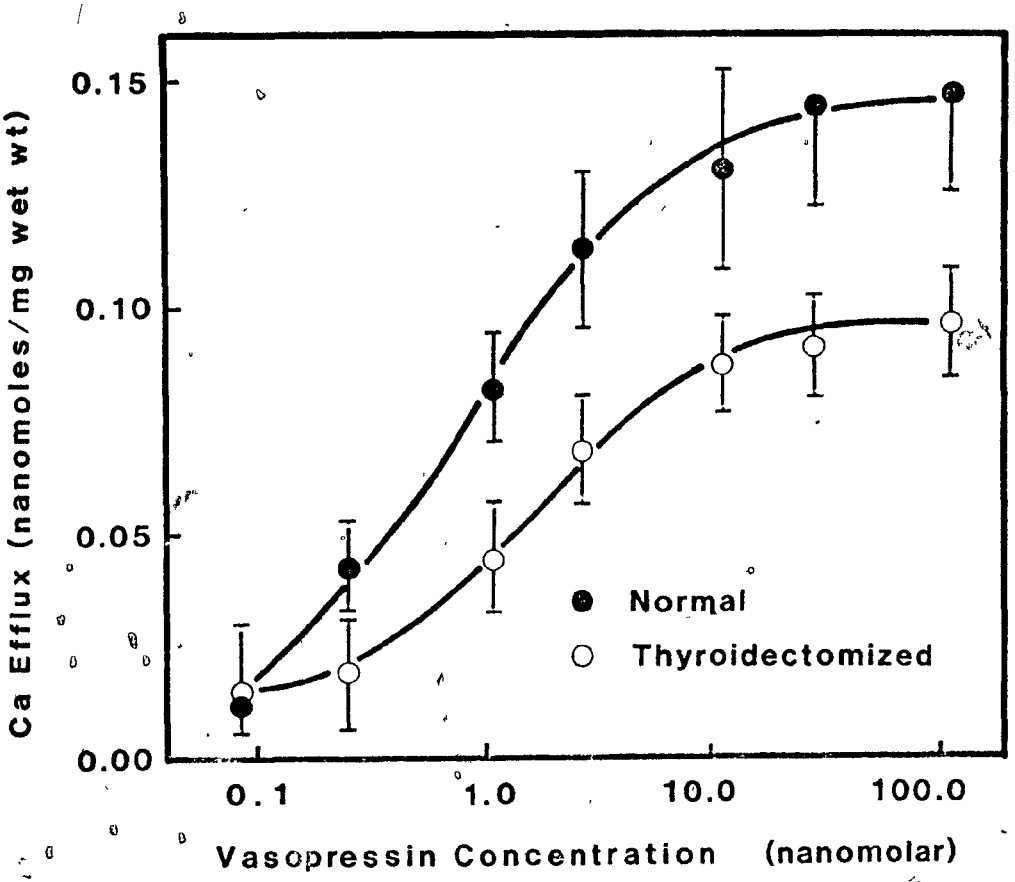


FIGURE 3.15 Vasopressin-stimulated calcium efflux in hepatocytes from normal and thyroidectomized rats.

Experimental details are as described in the caption to figure 3.14. Each data point is the mean ± s.e.m. of five and six experiments on separate cell preparations from normal and thyroidectomized rats respectively. The differences in vasopressin-stimulated calcium efflux in the two groups of rats approach statistical significance at some points. Mean EC_{50} values with standard errors are given in table 3.4.

3.9 Effect of calcium ionophore A23187, on calcium efflux and glycogen phosphorylase activation in hepatocytes from normal and thyroidectomized rats.

Like vasopressin, the divalent cation ionophore, A23187, has been shown to activate glycogen phosphorylase by a cAMP-independent mechanism similar to alpha agonists (Blackmore et al. 1978). Accordingly, the effect of the ionophore on glycogen phosphorylase activation was compared in thyroidectomized and normal rats. Figure 3.16 shows that the ionophore produced a dose-dependent increase in phosphorylase a activity in hepatocytes from normal rats that was not altered after thyroidectomy. The slight increase in phosphorylase activity at lower A23187 concentrations in the thyroidectomized group was most likely due to an increased sensitivity of this group to the vehicle, ethanol. This is supported by the fact that phosphorylase activity is significantly greater in thyroidectomized rats when alcohol alone was added (Zero A23187 concentration).

The effect of A23187 on calcium efflux was also examined in hepatocytes from normal and thyroidectomized rats. These results are summarized in figure 3.17 and show that the concentration-dependent, A23187-induced calcium efflux is identical in the two groups of animals.

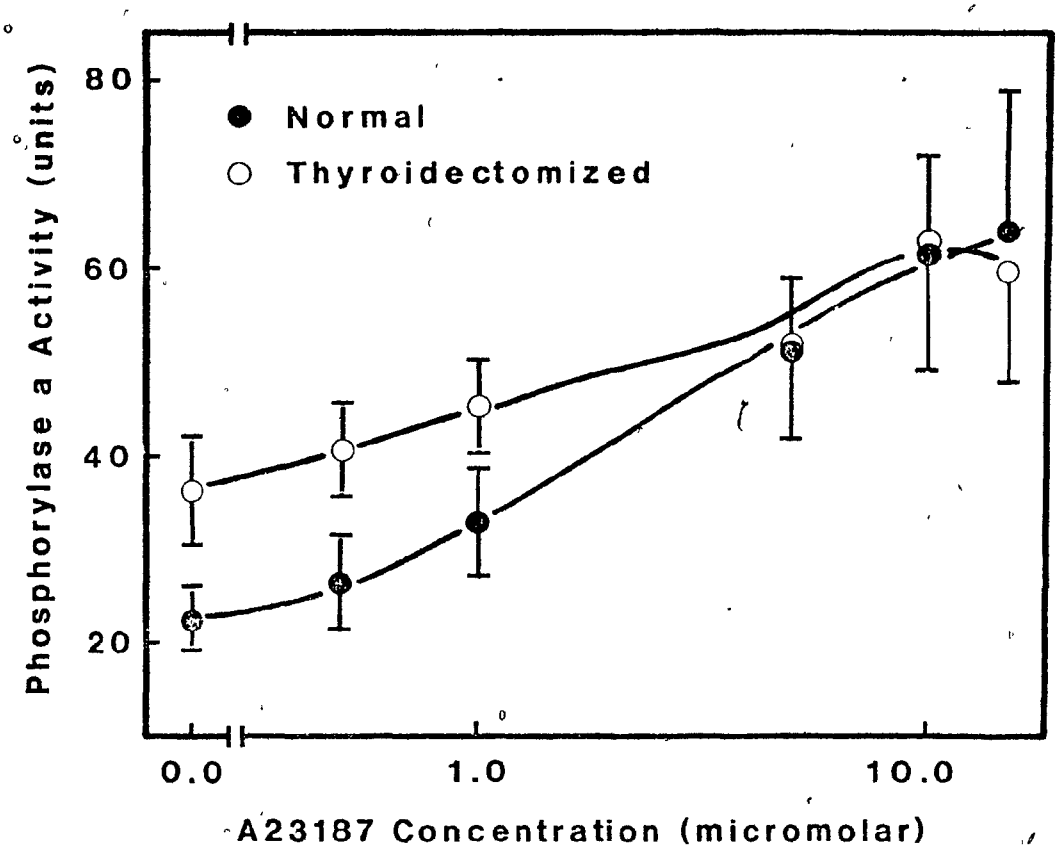


FIGURE 3.16 Activation of glycogen phosphorylase by calcium inophore, A23187, in hepatocytes from normal and thyroidecomized rats.

Cells were isolated as described in section 2.3. After 30 min of incubation in a medium with added gelatin, A23187 or vehicle alone (zero concentration, 2 µl ethanol/ml) was added to give the indicated final concentrations. After 3 min 1 ml of cell suspension were removed, diluted into 5 ml of ice cold Krebs-Henseleit buffer, rapidly centrifuged and prepared for assay of phosphorylase a as detailed in section 2.4. Values shown are the mean ± s.e.m. of four separate cell preparations in each group.

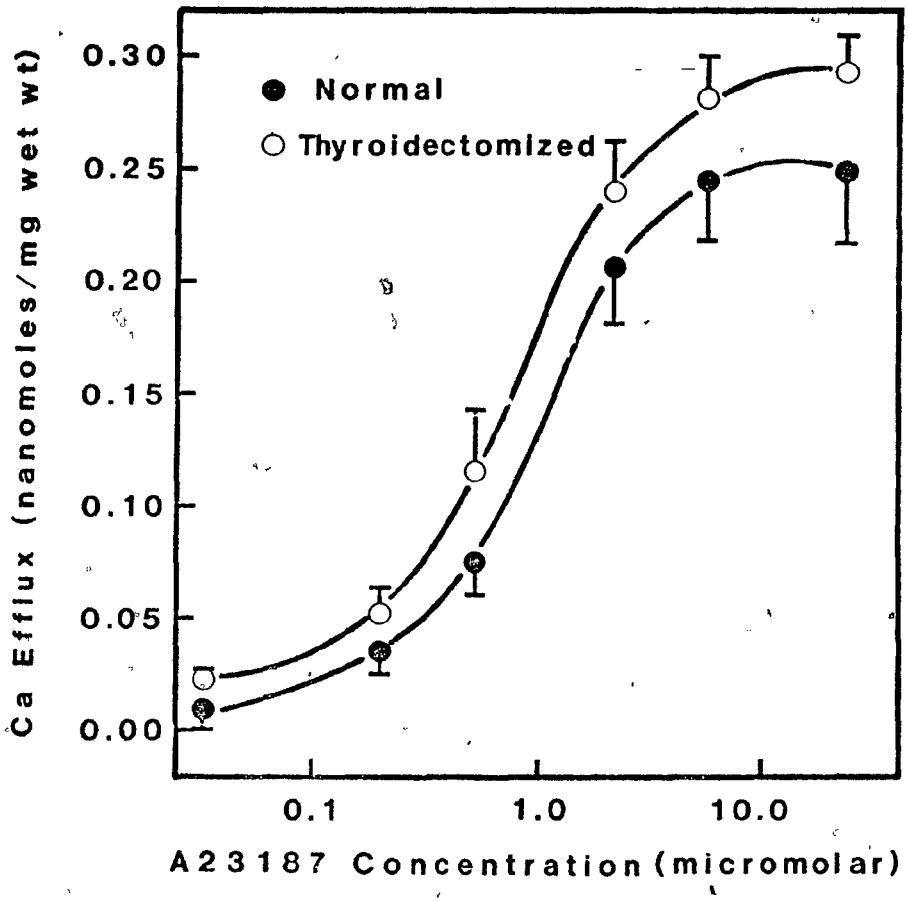


FIGURE 3.17 Stimulation of calcium efflux in hepatocytes from normal and thyroidectomized rats by calcium ionophore, A23187.

Experimental details are as described in the caption to figure 3.14. A23187 or vehicle (2-50 µl ethanol/15 ml of cell suspension) were added to give the indicated cumulative concentration of drug. Each data point is the mean ± s.e.m. of six experiments on separate cell preparations from normal and thyroidectomized rats.

3.10 Phenylephrine-induced activation of glycogen phosphorylase and ^{45}Ca efflux in hepatocytes from normal, thyroidectomized and thyroidectomized, triiodothyronine-treated rats.

The calcium efflux experiments described above (sections 3.7, 3.8 and 3.9) were made with hepatocyte suspensions in nominally calcium-free medium. Figure 3.18 shows that phosphorylase activation by either phenylephrine or vasopressin is significantly suppressed under these conditions. Similar results have been obtained by others (Stubbs et al. 1976; Keppens et al. 1977; Van de Werve et al. 1977). Thus in order to rule out the possibility that differences in phenylephrine-stimulated calcium efflux observed when normal and thyroidectomized rats were compared (section 3.7) were due to low calcium conditions, ^{45}Ca efflux was examined at physiological calcium concentrations.

Figure 3.19 A shows that, in agreement with the results described in section 3.7, the effect of phenylephrine in activating phosphorylase was suppressed in the hypothyroid liver: the maximal response was reduced by 37% and the EC_{50} increased 8-fold (table 3.4). Euthyroid control and thyroidectomized, T_3-treated values were similar (table 3.4) indicating that the effect of hypothyroidism was reversed by T_3 replacement (figure 3.19).

In support of the findings with phosphorylase and calcium electrode efflux experiments (section 3.7), figure 3.19 B shows that phenylephrine-stimulated ^{45}Ca release was markedly suppressed in cells from thyroidectomized rats compared with controls: the maximal response was reduced by 82% so that reliable estimates of EC_{50} could not be obtained in these preparations. Nevertheless, the increase in ^{45}Ca release over baseline was only significant at 10^{-4}M phenylephrine, which is 100 times higher than the effective threshold concentration in controls. T_3 treatment of thyroidectomized rats again partially corrected this suppression (figure 3.19). Thus the effect of thyroidectomy on phenylephrine-stimulated calcium efflux described in section 3.7 was confirmed at the same ambient calcium concentrations at which

phosphorylase responses were studied.

3.11 Vasopressin-induced activation of glycogen phosphorylase and ^{45}Ca efflux in hepatocytes from normal and thyroidectomized rats.

The data in figure 3.20A illustrate that, in contrast to phenylephrine (figure 3.19A), the maximal response to vasopressin was not influenced by hypothyroidism, but there was a small, 2.4-fold increase in EC_{50} (table 3.4) that approached statistical significance. The maximal ^{45}Ca release by vasopressin was similarly unaffected, whereas the EC_{50} increased 6-fold (table 3.4). Thus, hypothyroidism caused a moderate reduction in the potency of vasopressin, but the effects on phenylephrine were much more substantially reduced.

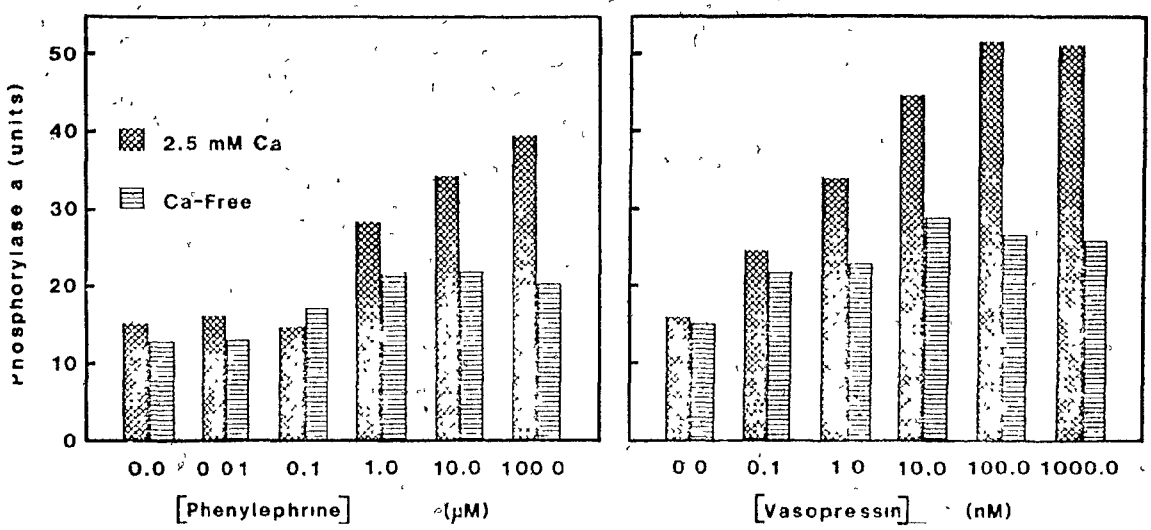


FIGURE 3.18 Effect of extracellular calcium concentration on vasopressin and phenylephrine activation of glycogen phosphorylase in hepatocytes from a normal rat.

Cells were isolated as described in section 2.3. Half of the yield from a single isolation was resuspended in nominally calcium free medium as for calcium electrode studies (section 2.6), while the other half was resuspended in medium containing normal calcium (both with gelatin). After 30 min of incubation vasopressin or phenylephrine was added to give the indicated final concentrations. After 3 min 1 ml of cell suspension was removed, diluted into 5 ml of ice cold Krebs-Henseleit buffer, rapidly centrifuged and prepared for assay of phosphorylase a as detailed in section 2.4. Values shown are the mean of duplicate determinations with cells from a single rat. There was no difference in viability of cells determined at the end of incubation. The experiment was repeated once with the same result.

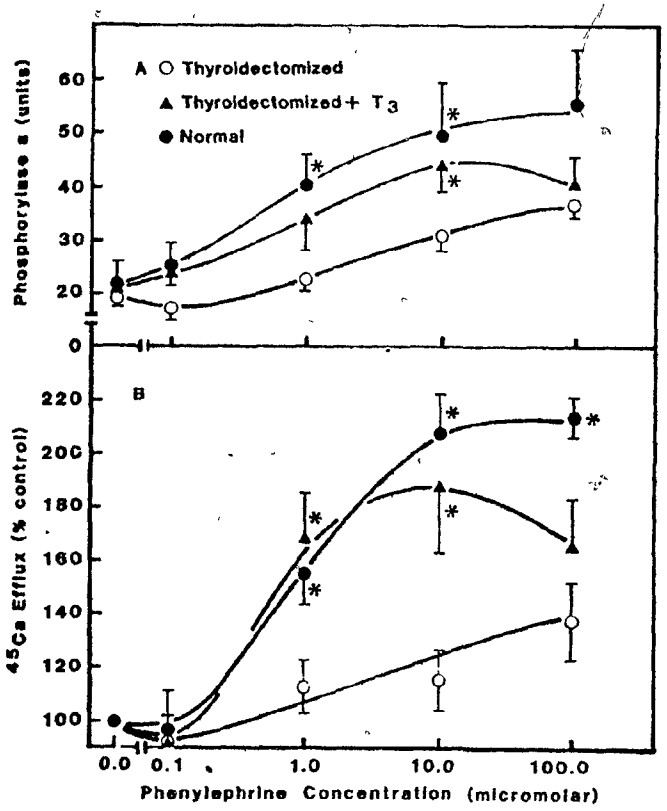


FIGURE 3.19 Phenylephrine stimulation of phosphorylase (A) and ^{45}Ca efflux (B) in hepatocytes from normal, thyroidectomized and thyroidectomized, T_3-treated rats.

Hepatocytes were isolated and incubated as described in section 2.3. One ml aliquots were taken for the determination of phosphorylase a 3 min after the addition of phenylephrine or saline. Determination of ^{45}Ca efflux was made at zero time (see section 2.5) and 3 min after addition of phenylephrine or saline. 100% represents the difference in radioactivity in the medium from zero time to 3 min in the presence of saline alone. Each data point is the mean ± s.e.m. of four experiments on seperate cell preparations. * indicates values significantly different from those obtained with thyroidectomized rats. Mean EC_{50} values with standard errors are given in table 3.4.

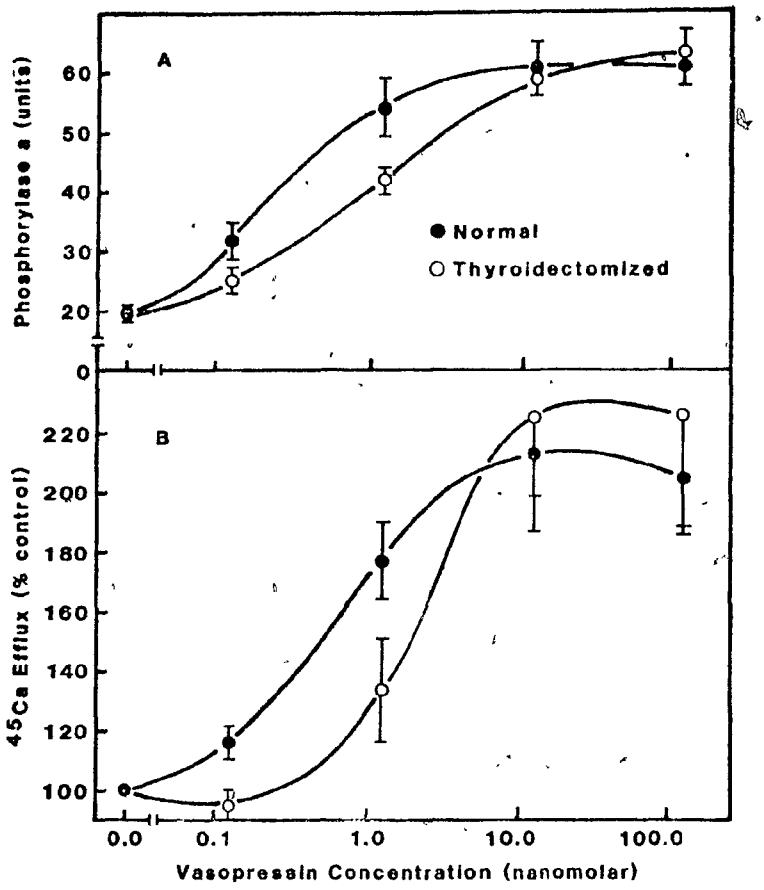


FIGURE 3.20 Vasopressin stimulation of phosphorylase (A) and ^{45}Ca efflux (B) in hepatocytes from normal and thyroidectomized rats.

Experimental details are as described in the caption to figure 3.19. Each data point is the mean $\pm$ s.e.m. of four experiments on separate cell preparations. At no concentration of vasopressin are values in thyroidectomized rats significantly different from controls. Mean EC_{50} values with standard errors are given in table 3.4.

3.12 Results of [^{3}H]prazosin binding to plasma membranes isolated from normal, thyroidectomized and thyroidectomized, triiodothyronine-treated rat livers.

Activation of phosphorylase and stimulation of calcium release in the normal rat liver are known to occur via stimulation of alpha$_1$ adrenoceptors (section 1.3.3). In view of the effects of hypothyroidism on these responses, the specific binding of [^{3}H]prazosin, a selective alpha$_1$ receptor antagonist, to liver plasma membranes from normal, thyroidectomized and thyroidectomized, T_3-treated rats was examined. Previous studies have shown that [^{3}H]prazosin is a useful probe for alpha$_1$ receptors in various tissues (Hornung et al. 1979; Barnes et al. 1979; Karliner et al. 1979) including the liver (Hoffman et al. 1981; Geynet et al. 1981). Figure 3.21 illustrates that the binding of [^{3}H]prazosin is saturable in membranes from all three groups. Specific binding was 80 to 85% at saturating [^{3}H]prazosin concentrations.

Binding of [^{3}H]prazosin to liver plasma membranes from normal rats was rapid and reversible. Figure 3.22 illustrates the kinetics of specific [^{3}H]prazosin binding. From the time-course of association, pseudo-first order association plots were produced and the apparent association rate constant. K_{ob} determined by linear regression analysis (Williams and Lefkowitz 1978). Dissociation initiated by the addition of excess phentolamine (10^{-5}M) was first-order (figure 3.22) and the dissociation rate constant, k_2, could be determined by linear regression analysis. From these two values the kinetically derived dissociation constant, K_d, of [^{3}H]prazosin could be calculated as $K_d=k_2/k_1$, where $k_1=(K_{ob}-k_2)/200$, 200 being the concentration (pM) of [^{3}H]prazosin in the assay (Williams and Lefkowitz 1978). The mean $\pm$ s.e.m. of k_1 and k_2 thus determined in three seperate experiments are 6.6 $\pm$ 0.4 x 10^{10} $M^{-1}min^{-1}$ and 0.031 $\pm$ 0.004 min^{-1} respectively. The resulting kinetically derived K_d value of 41.9 $\pm$ 8.2 pM was close to the equilibrium K_d obtained from saturation experiments (table 3.5). Both these values agree well with the

apparent dissociation constant for prazosin determined by Schild plot for antagonism of the phosphorylase response to adrenaline in intact hepatocytes (89 ± 11 pM[1]).

In order to determine the maximum binding capacity (b_{max}) and the affinity of [^{3}H]prazosin in purified liver plasma membranes from each group of animals, steady state binding data were analyzed by the method of Scatchard (1949). Typical Scatchard-plots are illustrated in figure 3.23 and the cumulated results from several experiments in each group are shown in table 3.5. These indicate that receptor density was reduced by more than 40% in thyroidectomized preparations, but reversed to near normal levels after T_3-treatment. There was no change in the K_d of [^{3}H]prazosin. The Hill-plots in figure 3.23 indicate that binding of [^{3}H]prazosin was to a single population of non-cooperative sites (n_H: 0.92 to 1.03).

In order to determine if thyroidectomy had any effect on the affinity of adrenergic agonists for the alpha$_1$ receptor site, competition experiments were made with increasing concentrations of agonists and [^{3}H]prazosin (0.2 nM, figure 3.24 and table 3.5). The K_d of agonists was determined by the method of Cheng and Prusoff (1973):

$$K_d = \frac{IC_{50}}{1 + \frac{0.2}{K^*}}$$

where IC_{50} is the concentration of agonist producing 50% suppression of [^{3}H]prazosin and K* is the equilibrium dissociation constant of [^{3}H]prazosin.

[1]Experiments done by W.H. Kan using low hepatocyte concentrations (1 mg/ml) to avoid significant uptake of prazosin by liver cells (section 1.3.1).

The data in table 3.5 show that the affinities of agonists were unaffected by thyroid state, except for a small decrease in the K_d for phenylephrine in thyroidectomized, T_3-treated rats. Gpp(NH)p, which is known to influence the affinity of adenylate cyclase coupled receptors (Limbird 1981), did not alter the K_d for adrenaline in any of the groups at either 30 or 100 μM (table 3.5).

Figure 3.24 illustrates the stereoselectivity of [^{3}H]prazosin binding sites in preparations from normal rats, as well as the absence of effect of 100 μM Gpp(NH)p on l-adrenaline suppression of [^{3}H]prazosin binding in any of the three groups.

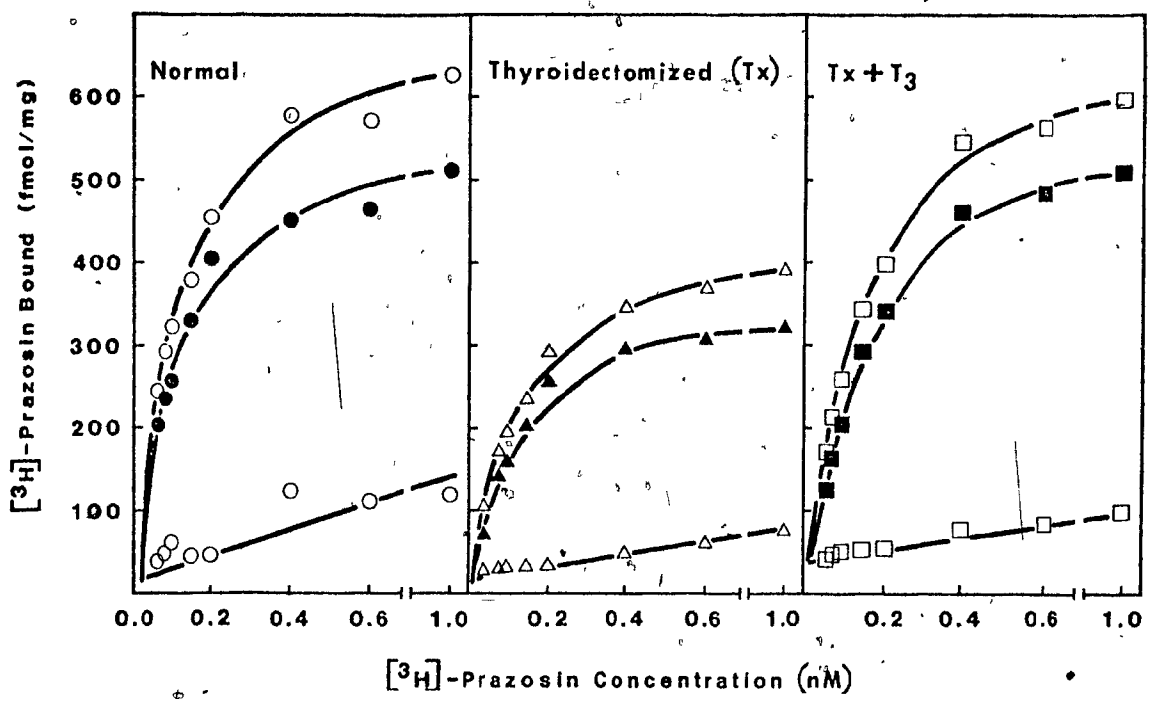


FIGURE 3.21. Saturation curves for [^{3}H]prazosin binding in liver plasma membranes isolated from normal, thyroidectomized and thyroidectomized, T_3-treated rats.

Plasma membranes were prepared from freshly excised livers as described in section 2.9. Binding of [^{3}H]prazosin in the absence (total binding, upper curves) and presence (nonspecific binding, lower lines) of 2 μM phentolamine was determined as described in section 2.10. Specific binding (middle curves) was calculated by taking the difference between total and nonspecific binding. One representative experiment from each group of rats is shown. Means of several similar experiments are given in table 3.5.

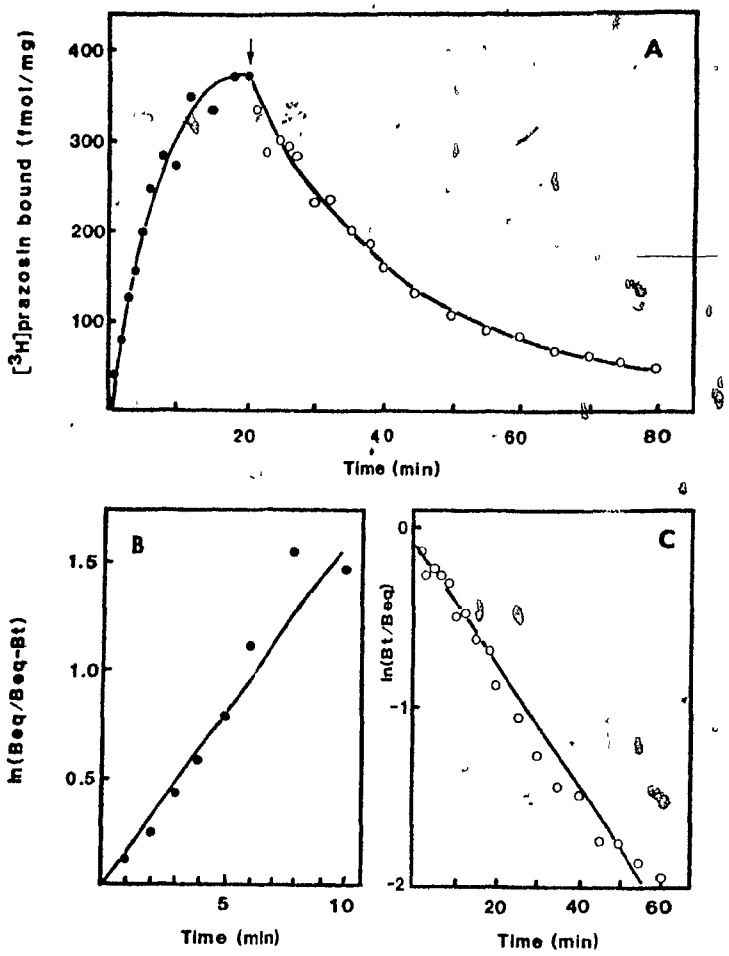


FIGURE 3.22 Kinetics of specific binding of [^{3}H]prazosin to purified plasma membranes from a normal rat liver.

Membranes were incubated with 200 pM [^{3}H]prazosin in the absence or presence of 2 μM phentolamine. A: time-course for specific binding of [^{3}H]prazosin during association (●) and dissociation (○) after the addition of 10 μM phentolamine (↓). B: a pseudo-first order association plot whose slope (r=0.98) represents the apparent association rate constant, K_{ob} = 0.153 min^{-1}, C: a first-order plot of dissociation, where the slope (r=0.98) is the first order dissociation rate constant, k_2 = 0.033 min^{-1} The kinetically derived K_d (section 3.12) was 55 pM and the equilibrium K_d derived from a Scatchard plot was 52 pM in the same membrane preparation. The mean ± s.e.m. of these values determined in three separate experiments are given in section 3.12.

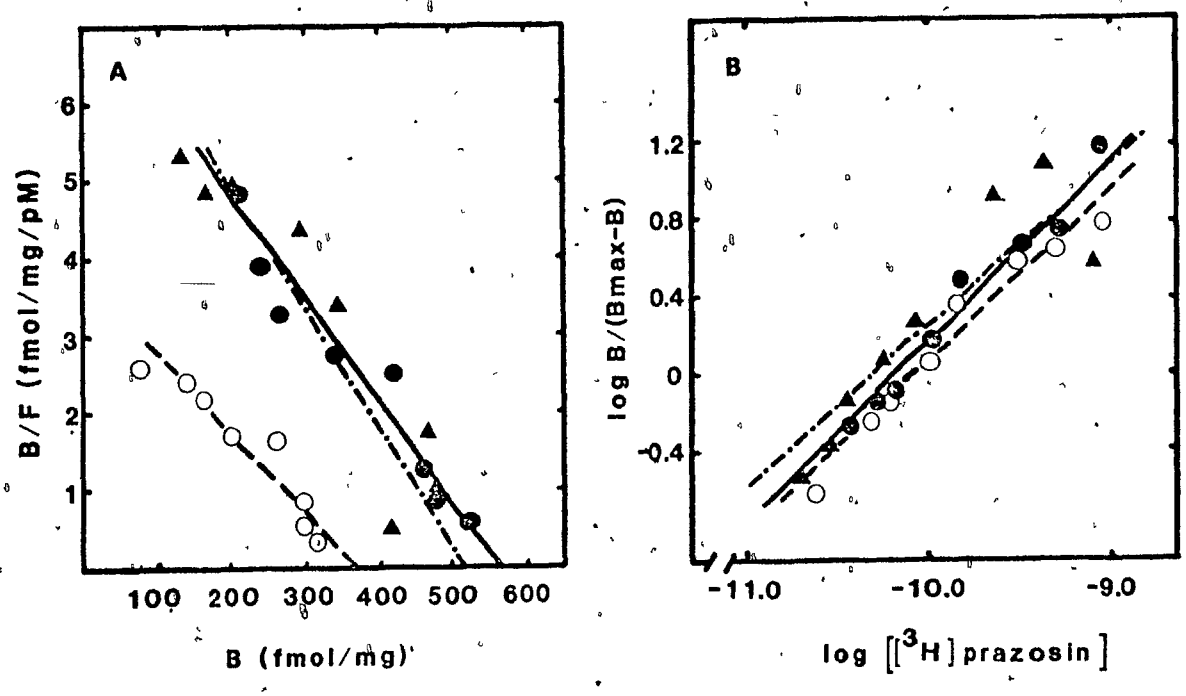


FIGURE 3.23 Effects of thyroid state on putative alpha$_1$ adrenoceptors labelled by [^{3}H] prazosin in purified liver plasma membranes.

Liver plasma membranes were prepared from freshly excised, whole livers of normal (●——●), thyroidectomized (○- - -○) and thyroidectomized, T_3-treated rats (▲-·-Δ) as described in section 2.9. Equilibrium binding of [^{3}H]prazosin was assayed as detailed in section 2.10. A: Scatchard plots from the same three experiments shown in figure 3.21. The K_d values were 77.4 (pM) (●), 98.4 pM (○) and 64.6 pM (▲). B: Hill plots from the same experiments, with n_H values of 1.03 (●), 0.92 (○) and 0.93 (▲). Data shown are from the same experiments in figure 3.21.

Table 3.5

The effect of thyroid state on hepatic alpha$_1$ receptors labelled by [^{3}H]prazosin.

	Normal		Tx		Tx + T$_3$
b_{max} (fmol/mg protein)	567 ± 25 (5)	*	326 ± 51 (8)	*	498 ± 23 (5)
K_d [^{3}H]prazosin (pM)	69.8 ± 6.1 (5)		53.7 ± 11.9 (8)		61.6 ± 10.2 (5)
K_d l-adrenaline (μM)	0.68 ± 0.17 (9)		0.67 ± 0.15 (7)		0.89 ± 0.11 (6)
+ 30 M Gpp(NH)p	1.06 ± 0.45 (3)		0.73 ± 0.36 (3)		0.98 ± 0.28 (3)
+ 100 M Gpp(NH)P	0.73 ± 0.23 (3)		0.76 ± 0.39 (3)		0.94 ± 0.16 (3)
K_d l-phenylephrine (μM)	2.58 ± 0.15 (3)		2.56 ± 0.40 (3)	*	4.05 ± 0.10 (3)

Liver plasma membranes were incubated with [^{3}H]prazosin in the absence or presence of competing ligands as described in section 2.10. The K_d and b_{max} of [^{3}H]prazosin was determined by Scatchard analysis of specific binding as described in the caption to figure 3.23. In competition experiments with agonists, of [^{3}H]prazosin was 0.2 nM and the K_d of agonists was determined as described in section 3.12. Means ± s.e.m are shown with the number of experiments in parentheses. * indicates a significant difference between adjacent values.

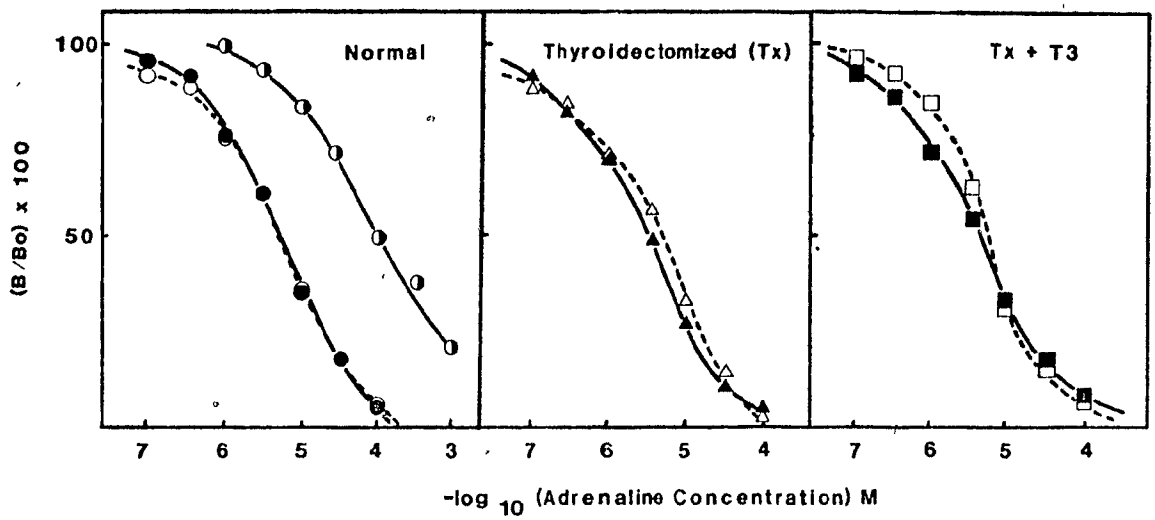


FIGURE 3.24 Adrenaline suppression of [^{3}H]prazosin binding to putative alpha$_1$ receptors in rat liver.

Binding assays were done as described in section 2.10. Assay tubes contained [^{3}H]prazosin (0.2 nM) in the absence (Bo) or presence (B) of various concentrations of adrenaline. Data points represent the means of three separate experiments. In each preparation, l-adrenaline suppression of binding was tested in the absence (filled symbols, solid lines) and presence (open symbols, dashed lines) of 100 μM Gpp(NH)p. d-Adrenaline suppression in the absence of Gpp(NH)p was tested in controls (◑). The mean K_d values in the absence and presence of Gpp(NH)p (calculated as described in section 3.12), respectively, were 0.88 ± 0.14 and 0.73 ± 0.23 μM in controls, 0.60 ± 0.12 and 0.73 ± 0.39 μM in Tx, and 1.02 ± 0.25 and 0.94 ± 0.16 μM in Tx +T_3. The n_H values of corresponding Hill plots (95% confidence limits) were 0.77-0.87 and 0.68-0.82 (controls); 0.70-0.84 and 0.75-0.89 (Tx); 0.65-0.75 and 0.82-0.98 (Tx + T_3).

SECTION FOUR

DISCUSSION

4.1 General Considerations: The effect of altered thyroid state on hepatic adrenergic receptors.

The observations presented here indicate a change in the adrenoceptor-mediated activation of hepatic glycogen phosphorylase from an alpha adrenergic response in hepatocytes from normal rats to a predominantly beta adrenergic effect in hepatocytes from hypothyroid rats. This was shown by an increase in both the potency and efficacy of the selective beta receptor agonist, isoproterenol, and by the ability of the beta receptor antagonist propranolol to reduce activation of glycogen phosphorylase by adrenaline in hepatocytes from hypothyroid, but not normal rats. Alpha receptor antagonists, which were effective in euthyroid rats, were without effect in hepatocyte preparations from thyroidectomized rats. As well, there was a decrease in the efficacy of adrenaline and phenylephrine, a relatively selective alpha receptor agonist, in the activation of phosphorylase in cells prepared from hypothyroid rats.

Changes in thyroid state are known to influence the activity of enzymes involved in catecholamine metabolism and altered disposition of agonists could conceivably be involved in the observed changes in their effectiveness. Results of studies in which this question has been addressed are variable and often contradictory (see section 1.2.2). The effect of thyroid state on the activities of these enzymes specifically in rat liver has been extensively studied in numerous preparations, but no one to date has examined this problem using collagenase dispersed hepatocytes. Zimon et al. (1976) examined the metabolism of ^{14}C-labelled noradrenaline in isolated perfused livers from hypothyroid, euthyroid and hyperthyroid rats. They inferred from their results that both COMT and MAO activities are slightly decreased in hypothyroid rat livers, while in hyperthyroidism increased metabolism is observed. Sourkes et al. (1977) examined the metabolism of a series of ^{14}C-labelled monoamines in whole liver homogenates and reported reduced MAO activity in

hyperthyroidism with no change after thyroidectomy. Note that the observations of neither of these studies may be directly applicable to experiments with isolated parenchymal cells; it is not clear what the relative importance of other tissue elements in catecholamine metabolism may be. Even so, it is unlikely that altered metabolism of drugs used in this study could account for the apparent differences in adrenoceptor activity between groups of rats for the following reasons.

(1) The amount of tissue relative to extracellular medium was lower in the present experiments than in those described above making it unlikely that concentrations of agonists were significantly depleted, particularly since phosphorylase activity was determined after only 3 min of stimulation.

(2) Adrenergic agonists and antagonists are metabolized by very different pathways (Weiner 1980). That enzyme activities would be altered in such a manner as to produce compatible changes in agonist and antagonist actions would be an unlikely coincidence.

(3) When beta receptor activity was evaluated by comparing cAMP accumulation in normal and thyroidectomized rats (section 3.4), the magnitude of the response was found to vary directly with the amount of cell material present.

(4) The major change in the hepatic adrenoceptor response pattern was observed in the hypothyroid state where either no change in enzyme activity or a similar small decrease in both MAO and COMT was noted (see above). These observations cannot explain the opposite changes in the effectiveness of phenylephrine, which is a substrate for MAO, and isoproterenol, which is preferentially metabolized by COMT (Weiner 1980).

The increase in beta receptor activity in hepatocytes from hypothyroid rats has been confirmed by others (Malbon et al. 1978a; Malbon et al. 1980; Malbon 1980a). Malbon (1980a) has extended these studies by examining both isoproterenol-stimulated adenylate cyclase activity and specific binding of

[^{125}]iodohydroxybenzylpindolol ([^{125}I]IHBP), a selective beta receptor antagonist, to plasma membranes purified from hepatocytes isolated from euthyroid, hypothyroid and hypothyroid, T_3-treated rats. As expected on the basis of the enhanced beta receptor-mediated stimulation of phosphorylase and cAMP accumulation in isolated hepatocytes, beta receptor activation of plasma membrane adenylate cyclase was increased in hypothyroid rats (Malbon 1980a). The specific binding of [^{125}I]IHBP was also increased in preparations from hypothyroid rats and Malbon (1980a) suggested that this increase in the number of putative beta receptor sites was the major factor responsible for enhanced beta adrenergic activity.

Malbon and Fain (1978) also described findings of a marked reduction in the ability of adrenaline and phenylephrine to activate glycogen phosphorylase in hypothyroid rat hepatocytes suggestive of a reduced alpha adrenergic activity. However, in subsequent reports (Malbon et al. 1978a, 1980), this group did not confirm the decreased alpha receptor contribution in hypothyroid hepatocytes described here and in their earlier work (Malbon and Fain 1978). Some possible reasons for this discrepancy will be discussed later (section 4.3).

Hepatic gluconeogenesis is regulated by adrenergic receptors in a manner analogous to the glycogenolytic response since both beta and alpha receptors are involved, the latter being more important in the rat (Tolbert et al. 1973; Kneer et al. 1974; Hutson et al. 1976). The effects of adrenergic agonists and antagonists on gluconeogenesis from lactate have been examined in perfused hypothyroid rat livers (Hagino and Nakashima 1973, 1974). The findings of those studies suggested that gluconeogenesis is mediated by both alpha and beta receptors in normal rats and that the contribution of beta receptors to this response becomes less important in hypothyroidism. Those results appear to contradict the present findings in isolated hepatocytes as well as studies that have clearly shown catecholamine-mediated gluconeogenesis in the normal rat to be predominantly an alpha receptor response (see above). The findings of

Hagino and Nakashima (1973, 1974) are difficult to evaluate; agonists were used at only single doses and neither alpha nor beta antagonists were effective in normal rat livers even at very high concentrations (0.4 mM). Moreover, in a later study, Hagino and Shigei (1976) found that cAMP accumulation in response to in vivo injection of catecholamine was enhanced in livers of hypothyroid rats. This latter finding contradicts their conclusions and is more compatible with the interpretation of the present discussion: an increased beta receptor activity in hypothyroidism. However, it is important to bear in mind that conclusions regarding adrenergic receptors made on the basis of the glycogen phosphorylase activation response alone may not be extrapolated to other adrenergic responses in the liver without caution (see section 1.3.3 for a list of these other responses). This is particularly true of the gluconeogenic response, which probably involves more than one enzymatic step under adrenergic control. This point is discussed in more detail in section 4.5.

The effect of hypothyroidism on hepatic adrenoceptors developed slowly and was not apparent at two weeks after surgical thyroidectomy even though serum thyroxine levels were as low as those seen at 10 to 14 wk. Malbon and coworkers identified similar effects on hepatic beta adrenoceptors after 18 to 25 days of iodine-deficiency and propylthiouracil administration (Malbon et al. 1978a; Malbon 1980a); however, effects on alpha adrenergic receptors were not apparent after this shorter period of thyroid deficiency (Malbon et al. 1978a). Although there may be other reasons why these latter investigators could not identify changes in the alpha receptor response (secton 4.3), these findings suggest that beta receptor function may be more sensitive to serum thyroid hormone levels than alpha. On the other hand, the effects of thyroid deficiency were almost completely reversed by only four days of T_3 treatment in the present study and two days in that of Malbon (1980a). Taken together, these results suggest that both alpha and beta receptor functions have a relatively high sensitivity to regulation by thyroid hormones; detectable changes

in these receptors are likely to occur at low hormone levels.

The effect of thyroid hormone excess was also examined in the present study. Hyperthyroidism, induced by the administration of thyroxine, produced a similar decrease in the efficacy of adrenaline and phenylephrine to that seen in hypothyroid rat hepatocytes. Basal phosphorylase activity was unaltered and the slight stimulatory effect of a high concentration of isoproterenol (probably an alpha receptor-mediated effect) was not changed. These findings could suggest that hyperthyroidism in the rat results in a decrease in alpha adrenoceptor activity, without a concomitant change in the beta receptor contribution to the phosphorylase response in the hyperthyroid rat.

Similar findings were reported by Malbon and LoPresti (1981) except that those authors found a 70% reduction in basal phosphorylase activity in hepatocytes from hyperthyroid rats. In agreement with the present findings, adrenaline stimulation of phosphorylase was suppressed in hyperthyroid rats. Malbon and LoPresti (1981) extended their study by demonstrating an increased phosphorylase phosphatase activity and decreased number of [^{3}H] DHE binding sites in plasma membranes from hyperthyroid rats. They suggested that these effects could account for the attenuated phosphorylase response. In the preparations studied by Malbon and LoPresti (1981), there was a significant beta receptor contribution to phosphorylase activation even in the euthyroid rat and this was also suppressed in hyperthyroidism. That this change was not detected in the present study may be be related to the fact that the beta receptor component of adrenergic activation of phosphorylase is more important in female as compared to male rats (Studer and Borle 1982); Malbon and LoPresti (1981) used female rats, whereas only males were used in the present investigations. In a second study, Malbon and Greenberg (1982) found that the reduced beta receptor activity in hyperthyroid hepatocytes could be localized to the hormone-sensitive adenylate cyclase. The properties of the beta receptor-adenylate cyclase complex were altered at several loci by thyroid

hormone excess, including a 35% reduction in the number of [^{125}I] IHBP binding sites. The effect of hyperthyroidism on hepatic adrenergic receptors was not further investigated in the present study.

4.2 The effect of hypothyroidism on hepatic beta adrenergic receptors. Comparison with glucagon.

The emergent beta adrenergic contribution to glycogen phosphorylase activation in hypothyroid rat hepatocytes was found to be associated with an increased accumulation of cAMP (section 3.4). Thus, the difference between normal and thyroidectomized rats is apparent in production of the second messenger, an early step in the glycogenolytic cascade. However, decreased metabolism of cAMP in hepatocytes from hypothyroid rats could also contribute to this enhanced response. As described in section 1.2.3, beta receptor-mediated stimulation of lipolysis and cAMP accumulation in adipose tissue is suppressed in hypothyroidism and at least part of this suppression is thought by some workers to be due to an increased activity of the particulate high affinity form of phosphodiesterase in fat cells from hypothyroid rats (Armstrong et al. 1974). Accordingly, theophylline, a phosphodiesterase inhibitor, enhanced adrenaline-stimulated lipolysis and cAMP accumulation to a greater extent in adipocytes from hypothyroid than normal rats (Goswami and Rosenberg 1978; Van Inwegen et al. 1975). In the present experiments on isolated hepatocytes, the difference in isoproterenol-stimulated cAMP accumulation between thyroidectomized and normal rats became even more pronounced in the presence of a phosphodiesterase inhibitor, isobutyl-methylxanthine (section 3.4). Thus, unlike the situation in adipocyte preparations, the differences described could not be attributed to changes in phosphodiesterase activity. Furthermore, Gumma et al. (1977) have directly examined the effect of thyroidectomy on cyclic nucleotide phosphodiesterases

in rat liver. They found that the activities of both the high and low affinity phoshodiesterases were increased after thyroidectomy. Hence, on this basis one would have expected a decreased cAMP accumulation in response to hormonal stimulation. However, the present findings show a clearly increased response after thyroidectomy. Gumma et al. (1977) point out that these changes in hepatic cAMP phosphodiesterase activity parallel those found in adipose tissue from hypothyroid rats and also suggest that the observed changes in phosphodiesterase activity in adipose tissue account (at least in part) for the decreased cAMP and lipolytic response of these animals. The results presented here show that this is not a general property of adenylate cyclase/cAMP-dependant systems. Furthermore, one must keep in mind the fact that findings in unstimulated tissues may not reflect the true situation during hormonal activation: there is some evidence that cAMP phosphodiesterase activity may itself be subject to hormonal influences (Francis and Kono 1982). In addition, because of the possible compartmentalization of the cAMP rise and cAMP metabolism that has been postulated to exist in some tissues (Brunton et al. 1981), homogenates of whole tissue may not reflect accurately physiologic mechanisms at work in the cell.

The enhanced isoproterenol-stimulated cAMP accumulation in hepatocytes from hypothyroid rats was reduced towards normal levels by in vivo administration of T_3 (section 3.6). Although four days of T_3 treatment resulted in a cAMP response that was indistinguishable from that observed in normal rat hepatocytes, isoproterenol-stimulated phosphorylase activity, while less than that seen in hypothyroid hepatocytes, was still enhanced above normal (section 3.6). A similar dissociation of the cAMP response and phosphorylase activation has been noted under other circumstances (Birnbaum and Fain 1977) and can be explained by the fact that very small, often inapparent, changes in cAMP are capable of producing marked increases in phosphorylase activity (see below).

Very similar results to those presented here have been simultaneously

reported by Malbon and co-workers[1]. Beta receptor-mediated activation of glycogen phosphorylase and stimulation of cAMP accumulation was enhanced in hepatocytes from hypothyroid rats (Malbon et al. 1978a). This difference was not abolished in the presence of IBMX (Malbon et al. 1978a). A later study showed that beta adrenergic activation of adenylate cyclase was enhanced in homogenates and purified plasma membranes prepared from hypothyroid rat hepatocytes when compared to euthyroid controls (Malbon 1980a). Basal and fluoride-stimulated cyclase activities were unchanged. Specific binding of [^{125}I] IHBP, a potent beta adrenergic antagonist, was increased in those preparations. Both the increased [^{125}I] IHBP binding and adenylate cyclase activity were reversed by two days of in vivo T_3-pretreatment of hypothyroid rats.

Conversely, Jones et al. (1972) had earlier reported no change in basal and adrenaline-stimulated adenylate cyclase activity in hypothyroid rat liver homogenates, but an increased fluoride-stimulated activity in these preparations. Reasons for this discrepancy are not entirely clear. Differences cannot be accounted for by the type of preparation used in each study; Malbon (1980a) also examined fluoride-stimulated adenylate cyclase in whole liver homogenates and still found the same activity in hypothyroid and euthyroid rats. One possible reason for the similarity of agonist-induced adenylate cyclase activation in these two groups observed by Jones et al. (1972) could be due to the use of adrenaline, a mixed alpha/beta receptor agonist, rather than isoproterenol, a pure beta receptor agonist. The presence of inhibitory alpha activity (Jard et al. 1981; Assimacopoulos-Jeannet et al. 1982) could have masked an increase in beta adrenergic activation of adenylate cyclase.

[1]The report by Malbon et al. (1978a) appeared on Dec. 25, 1978 and our own study (Preiksaitis and Kunos 1979) on Jan. 1, 1979.

Although in the present study both adrenaline and isoproterenol stimulation resulted in greatly enhanced cAMP accumulation in hypothyroid compared to normal hepatocytes, isoproterenol consistently produced greater maximal increases than adrenaline in hypothyroid hepatocytes (section 3.6). A similar difference in response to adrenaline and isoproterenol could not be observed in the much smaller cAMP response of the euthyroid rat. The alpha receptor antagonist, phenoxybenzamine, enhanced the adrenaline-induced cAMP response in the hypothyroid, but not in the normal rat. These observations may suggest that not only the beta adrenergic stimulatory influence on adenylate cyclase is greater in hypothyroidism, but an alpha receptor-mediated inhibitory effect may be enhanced as well.

Remarkably similar results were obtained in hepatocytes from adrenalectomized rats (Chan et al. 1979a): enhanced adrenaline-stimulated cAMP accumulation was even greater after phenoxybenzamine blockade, which had no effect on this response in hepatocytes from normal control rats. Evidence for the existence of alpha receptor-mediated inhibition of adenylate cyclase has also been reported in liver preparations from carcinogen-treated rats (Boyd and Martin 1976), as well as a number of other tissues (for references see Fain and García-Sáinz 1980). Jard et al. (1981) have provided evidence that stimulation of angiotensin II or alpha$_2$ receptors inhibits normal rat liver adenylate cyclase in plasma membrane preparations. However, the conditions required to demonstrate this alpha$_2$-mediated inhibition of adenylate cyclase were rather precise: membranes were washed in 5 mM EDTA and activity had to be assayed in the presence of GTP and monovalent cations, which raises some doubt about its role under more physiologic conditions. Exton and coworkers (Assimacopoulos-Jeannet et al. 1982) characterized a similar effect in intact hepatocytes, but concluded that it was mediated predominantly by alpha$_1$ receptors. This point has been discussed in more detail in an earlier part of this thesis (section 1.3.3).

Glucagon and beta adrenergic agonists share a common mechanism of action (Exton 1982). However, in contrast to the effect of beta receptor stimulation, no difference in glucagon activation of glycogen phsophorylase could be found in hypothyroid and normal rat hepatocytes (section 3.5). On the other hand, cAMP accumulation was enhanced in hypothyroid hepatocytes, but only at glucagon concentrations above that required for maximal phosphorylase activation (10 nM; section 3.5). Just as for isoproterenol, IBMX enhanced glucagon-stimulated cAMP accumulation in both normal and hypothyroid rats, but the difference at higher glucagon concentrations was maintained (section 3.5).

Both isoproterenol (section 3.4) and glucagon (section 3.5) were more potent in stimulating phosphorylase activation than cAMP accumulation. A similar disparity in cAMP-mediated effects and cAMP accumulation in other systems has been noted. This finding in hepatocytes led Fain et al. (1977) to propose the existence of a cAMP-independent mechanism for phosphorylase activation by low glucagon concentrations. Cherrington et al. (1977) were able to identify small but reproducible increases in intracellular cAMP at low glucagon concentrations that resulted in the most sensitive changes in glycogenolysis and phosphorylase. It would appear therefore, that these apparent dissociations of cAMP response and phosphorylase activation are the result of the great amplification between the primary receptor signal (production of cAMP) and the final response, resulting as a consequence of an efficient enzyme cascade (figure 1.1). Morevover, careful measurement of intracellular cAMP levels is required to detect changes at threshold drug concentrations. In the present study, as in many others, cAMP was determined in the incubation medium plus cells homogenized together. Exton et al. (1972) have found that cAMP released into the medium from perfused liver correlated better with glucose output than did tissue levels of cAMP. Intracellular cAMP alone was not assayed in the present experiments and hence it is not known

whether the increased accumulation at higher glucagon concentrations in cells from hypothyroid rats would also be observed in the physiologically important intracellular compartment. The role of the 'extra' cAMP released from cells or produced by agonist at levels greater than those required to maximally activate phosphorylase is unknown and hence, it is difficult to attribute physiological significance to this change.

Several other groups have examined the effect of thyroid state on glucagon actions in the liver. The results are quite variable and often contradictory. In agreement with results of the present experiments, Malbon et al. (1978a) found no change in glucagon-mediated phosphorylase activation in hepatocytes from hypothyroid compared with normal rats. They also found no difference in cAMP accumulation, but glucagon concentrations above 10 nM were not tested by Malbon et al. (1978a); the difference between hypothyroid and normal hepatocytes in the present study was apparent only at higher glucagon levels. In a subsequent report, plasma membranes purified from hypothyroid rat hepatocytes showed an increased specific binding of [^{125}I]glucagon when compared with euthyroid controls (Malbon 1980a). However, only a single concentration (2.0 nM) of [^{125}I]glucagon was examined and hence these results must be interpreted with caution; single point binding determinations cannot be used to differentiate changes in affinity and binding site density, and are prone to errors introduced by cooperativity or the existence of multiple binding sites (Sperling et al. 1980). Glucagon-stimulated adenylate cyclase activity was examined in the same preparations (Malbon 1980a). While 1 μM glucagon produced greater cyclase activity in liver homogenates and membranes from hypothyroid rats, this difference was not statistically significant.

These latter findings agreed with those of earlier investigations that showed no change in basal, fluoride- or glucagon-stimulated adenylate cyclase in homogenates of hypothyroid rat liver compared with euthyroid controls

(Harkcom et al. 1978). Treatment of hypothyroid rats with thyroxine resulted in higher basal and glucagon-stimulated adenylate cyclase activity than that of either hypothyroid or euthyroid rat preparations (Harkcom et al. 1978).

Sperling et al. (1980) demonstrated a substantial reduction of glucagon receptor number and adenylate cyclase response in plasma membranes from hypothyroid rats without any significant difference in these parameters between hyperthyroid and euthyroid rats. Scatchard analysis of specific [^{125}I]glucagon binding data revealed evidence for high and low affinity glucagon receptors, which may account for some of the discrepancies in the above-described findings (Sperling et al. 1980).

An additional reason for these variable findings may be the fact that some of the above preparations were whole tissue homogenates, while others were from collagenase-dispersed liver cells as in the present experiments. Hanoune and co-workers have demonstrated the presence of a proteolytic contaminant in commercial preparations of crude collagenase which produced at least a two-fold increase in adenylate cyclase activity (Hanoune et al. 1977; Lacombe et al. 1977). This factor, which was shown not to be collagenase itself, enhanced basal as well as catecholamine-, glucagon-, fluoride- or GTP-stimulated activity. Activation was time-dependent and irreversible. It may be noted that some variation in maximal cAMP reponses to isoproterenol were observed in the present experiments (sections 3.4 and 3.6). The most likely explanation for these differences is variation in the amount of contaminating proteases in collagenase batches used in preparing hepatocyte suspensions. Although care was taken to prepare all cells in a given series of experiments with a single batch of collagenase, one cannot rule out the possibility that cyclase activity in hypothyroid preparations has an increased sensitivity to activation by the protease contaminant. This possibility was not investigated in the present study.

Glucocorticoid deficiency has been shown to have similar effects on

hepatic adrenoceptors as thyroidectomy in the present study (section 4.4). In contrast to the effect of hypothyroidism, glucagon-stimulated phosphorylase activity is impaired in adrenalectomized rats, while cAMP accumulation remains normal (Chan et al. 1979b). Further investigation revealed that phosphorylase b kinase activation by glucagon was suppressed after adrenalectomy and this offers the best explanation for the impaired phosphorylase response (Chan et al. 1979b). Thus, while adrenalectomy and thyroidectomy have similar effects on hepatic adrenergic receptors, their effects on glucagon are quite different.

In summary, the present findings indicate that beta receptor activity is unequivocally increased in preparations from hypothyroid rats when compared with normal. This increase was apparent at the level of production of the second messenger, cAMP, and could not be attributed to differential metabolism by phosphodiesterase in the two groups of rats. A concomitant increase in putative beta adrenergic receptor binding sites has been identified by others (Malbon 1980a) and is the most likely reason for the increased activity in hypothyroidism, although additional changes in the coupling of beta receptor to adenylate cyclase cannot be ruled out. These changes were reversible by in vivo administration of T_3.

The effects of hypothyroidism on the hepatic actions of glucagon are less clear. Nonetheless, it appears that glucagon-stimulated phosphorylase activation and cAMP accumulation are not influenced in the same way as beta receptor-mediated responses. The fact that glucagon activation of glycogen phosphorylase was the same in normal and hypothyroid rat hepatocytes indicates that changes in the sensitivity of the glycogenolysis cascade to cAMP did not contribute significantly to the altered beta adrenergic response.

4.3 The effect of hypothyroidism on hepatic alpha adrenergic receptors. Comparison with vasopressin and A23187.

The observations presented in this thesis indicate that hypothyroidism in rats results in a decrease in the alpha adrenergic stimulation of glycogen phosphorylase b to a conversion and calcium release in isolated hepatocytes. These changes were accompanied by a decrease in the density of plasma membrane binding sites for [^{3}H]prazosin, a selective alpha$_1$ receptor antagonist, which may be the basis of the altered physiological responsiveness observed in the intact cells. Furthermore, the change in both binding site density and alpha adrenergic responses could be returned towards normal levels by in vivo administration of T_3 to thyroidectomized rats.

Work from several laboratories has indicated that alpha adrenergic stimulation of rat liver results in the release of intracellular calcium from mitochondrial stores and its subsequent extrusion into the extracellular space (for references see section 1.3.3). The transient increase in cytosolic calcium levels so produced is believed to activate glycogen phosphorylase kinase, which in turn phosphorylates glycogen phosphorylase to the active a form of the enzyme. Vasopressin has been shown to activate glycogen phosphorylase by a cAMP-independent mechanism (Kirk and Hems 1974) and to cause changes in calcium distribution similar to those produced by alpha receptor agonists (Blackmore et al. 1978; Chen et al. 1978). The divalent cation ionophore, A23187, can also cause release of calcium from the same intracellular pool (Chen et al. 1978; Blackmore et al. 1978; Babcock et al. 1979) which results in the activation of glycogen phosphorylase (Blackmore et al. 1978). Accordingly, the effect of thyroidectomy on phosphorylase activation and calcium release in response to A23187 and vasopressin were examined

In contrast to the effects of phenylephrine, neither vasopressin nor A23187 activation of glycogen phosphorylase and stimulation of calcium release were

greatly influenced by thyroidectomy (sections 3.8 and 3.9), although the response to vasopressin was moderately reduced. Since the source of released intracellular calcium and the subsequent post-receptor events are believed to be the same for phenylephrine, vasopressin and A23187 (section 1.3.3), these findings suggest that the locus of the impaired alpha adrenergic response in the hypothyroid hepatocyte is at a site proximal to the release of intracellular calcium.

The fact that A23187 produced identical calcium release responses in hepatocytes from normal and hypothyroid rats (section 3.9) indicates that releasable calcium stores were not affected by thyroidectomy. Rosenqvist (1978) has examined the effect of thyroidectomy on the amount and distribution of exchangeable calcium in isolated rat liver cells. He identified two distinct intracellular calcium pools: a large, slow turnover pool and a smaller, more rapidly exchangeable pool. Thyroidectomy did not significantly alter the pool with rapid turnover, but markedly reduced the slow turnover pool. Another group of workers (Barritt et al. 1981) found a similar intracellular calcium distribution. They suggested that each kinetically defined compartment is likely to be heterogeneous with respect to anatomical composition, but that the larger compartment represents calcium sequestered in organelles and the smaller, exchangeable calcium in the cytoplasm. Stimulation with alpha agonists resulted in an increase of about 100% of exchangeable calcium in the small compartment and a decrease of 20% in the large (Barritt et al. 1981). The time-course and dose-response relationship of cytosolic free calcium changes during alpha adrenergic stimulation closely follow the increase in phosphorylase a (Murphy et al. 1980). Thus it would appear that it is changes in the smaller kinetically defined calcium pool that are important in the activation of glycogen phosphorylase. Rosenqvist (1978) found no change in this pool after thyroidectomy. The 20% decrease in the larger pool during stimulation (Barritt et al. 1980) most probably represents release of calcium from mitochondrial

stores (Babcock et al. 1979; Blackmore et al. 1979b,c; Murphy et al. 1980). This larger compartment was decreased after thyroidectomy (Rosenqvist 1978), but unfortunately, changes in calcium pools during hormonal stimulation have not been compared in thyroidectomized and normal rats. Whatever this effect may be, the present findings that maximal stimulation of calcium efflux and phosphorylase activation by A23187 and vasopressin were not or only slightly affected by hypothyroidism indicates that a change in the hormone sensitive calcium pool cannot fully account for the marked reduction in the effect of phenylephrine.

Experiments in which calcium efflux was measured with a calcium-sensitive electrode were made in nominally calcium-free medium (sections 3.7, 3.8 and 3.9). Total cellular calcium can be directly related to the concentration of calcium in the medium (Borle 1975). Hence the possibility arises that the decreased phosphorylase response to vasopressin and phenylephrine observed under these conditions (section 3.10) could result from a depletion of calcium from the hormone sensitive calcium pool, which may be greater in hypothyroid than in normal rat hepatocytes. A similar decreased responsiveness under low calcium conditions has been reported by others (Keppens et al. 1977; Stubbs et al. 1976; Van de Werve et al. 1977). However, the net calcium loss in response to agonist stimulation in the present experiments (approximately 0.15 to 0.30 nmol of calcium per mg cells) compares well with that determined by measuring total calcium in isolated hepatocytes at normal medium calcium concentrations (approximately 0.20 to 0.30 nmol per mg cells; Blackmore et al. 1978) or the estimated total alpha adrenergic agonist-mobilized pool in isolated perfused liver (approximately 0.14 nmol per mg of liver; Reinhart et al. 1982a).

Kneer et al. (1979) have found that like phosphorylase activation, stimulation of gluconeogenesis from lactate by noradrenaline was suppressed at low extracellular calcium concentrations, whereas it was enhanced when the

substrate was fructose. These authors proposed that calcium influenced the rate of transfer of reducing equivalents between cytosol and mitochondria. Thus the increased cytosolic NADH/NAD ratio under low calcium conditions would favour gluconeogenesis from oxidized substrates (fructose) but not reduced substrates (lactate). It is possible, therefore, that suppression of phosphorylase response under low calcium may be the result of secondary alterations in the metabolic state of the hepatocyte and not an effect on calcium homeostasis per se.

In order to rule out the possibility that the observed differences in calcium release were related to the low calcium conditions under which these experiments were made, ^{45}Ca efflux from prelabelled hepatocytes was examined with normal calcium levels in the medium. Results of these experiments confirm those obtained with the calcium-sensitive electrode (sections 3.7 and 3.8): phenylephrine-stimulated ^{45}Ca release was suppressed after thyroidectomy and returned towards normal by T_3 replacement.

While both the use of the calcium sensitive electrode and ^{45}Ca efflux are felt to reflect alpha adrenoceptor activity, methodological differences preclude a direct comparison of these results. Nonetheless, one observes that by using either approach, phenylephrine-induced calcium efflux is suppressed in the thyroidectomized rat. However, the results obtained with vasopressin are somewhat less straightforward. The concentration-response curve for ^{45}Ca is somewhat supressed at lower vasopressin concentrations and is significantly shifted to the right in the thyroidectomized rat (figure 3.10, table 3.4). On the other hand, in the calcium electrode studies the effect of vasopressin curve in thyroidectomized rats was somewhat suppressed over the entire concentration range, but this difference is not statistically significant (section 2.8). Hence, one cannot conclude that vasopressin-mediated calcium release is unaffected by thyroidectomy. Nevertheless, it is clear that the reduction of the alpha-mediated response is substantially greater.

Using [^{3}H]prazosin a decrease in putative alpha$_1$ receptor binding sites in liver plasma membranes from hypothyroid rats could be demonstrated (section 3.11). Just as for the above-described effects on alpha adrenergic stimulation of phosphorylase and calcium mobilization, the decrease in [^{3}H]prazosin binding was reversible by T_3 replacement. The observed decrease in the density of putative alpha$_1$ receptor binding sites points to a decreased alpha$_1$ receptor number as the likely basis for the altered response to alpha adrenergic stimulation in hypothyroid hepatocytes. No change in the affinity of binding sites for [^{3}H]prazosin or antagonist could be detected.

In the absence of significant "spare" alpha receptors in rat liver (Kunos et al. 1983), a decrease in receptor number is expected to result in a decreased maximal response to agonists as observed (section 3.7). However, one cannot rule out additional effects of thyroidectomy on the coupling of receptor activation and the release of intracellular calcium, since the nature of this process is not fully understood. Indeed, the smaller decrease in the effectiveness of vasopressin in hypothyroid rats could suggest such a possibility. Alternatively, an independent effect of hypothyroidism on vasopressin receptors could also account for this. The latter effect would be analogous to the effect of hypothyroidism on adenylate cyclase-coupled receptors in rat liver, which involve a marked increase in beta adrenergic and a much smaller, but significant increase in glucagon receptor number (section 4.2; Malbon 1980a). Direct binding of [^{3}H]vasopressin to isolated hepatocytes and liver membranes has been examined by Cantau et al. (1980). A comparison of [^{3}H]vasopressin binding in euthyroid and hypothyroid rats would clarify the effect on vasopressin-mediated responses. It is of interest to note that adenylate cyclase response to vasopressin stimulation in renal tissue particulate fractions was reversibly decreased by thyroid hormone deficiency (Harkom et al. 1978), although this response is mediated by a different subtype of vasopressin receptor (Cantau et al. 1980).

Recent studies have implicated alpha$_1$ adrenergic effects on phosphotidylinositol in the activation of glycogenolysis, possibly as an intermediate in the release of intracellular calcium (section 1.3.3). García-Sáinz and Fain (1980) have examined the effect of hypothyroidism on the alpha adrenergic stimulation of phosphotidylinositol labelling in rat fat cells and find no difference in this parameter when compared with euthyroid controls. These observations cannot be extended to the present results since effects of altered thyroid state are highly tissue dependent. The effect of hypothyroidism on phosphotidylinositol turnover in rat liver remains to be determined.

Changes similar to those occurring after thyroidectomy have been demonstrated in the adrenalectomized rat (see section 4.4). Chan et al. (1979a) found that adrenalectomy produced a reciprocal change in alpha and beta adrenergic activation of hepatic glycogen phosphorylase, including a decreased alpha receptor-mediated mobilization of intracellular calcium. However, binding of [^{3}H] noradrenaline to liver plasma membranes showed no significant change after adrenalectomy in the initial study (Chan et al. 1979a). This was interpreted as evidence for no change in alpha receptor binding capacity or affinity. Similar results were obtained by Guellean et al. (1978) using [^{3}H] DHE as a binding probe for alpha receptors. However, after re-examining the binding of ^{3}H-catecholamines the former authors (Chan et al. 1979a) have unpublished results showing a decrease in alpha receptor binding sites after adrenalectomy (Exton et al. 1981).

As described in section 1.3.3 and 1.3.4, it has recently been shown that rat liver has at least two populations of alpha receptors (Hoffman et al. 1980a,b; Hoffman et al. 1981): alpha$_2$ receptors that can, under certain conditions, inhibit adenylate cyclase (Jard et al. 1981) and also alpha$_1$ receptors responsible for the activation of glycogen phosphorylase (Hoffman et al. 1980b; Aggerbeck et al. 1980a). ^{3}H-Catecholamines were shown to

label alpha$_1$ (El-Refai 1980a) as well as alpha$_2$ receptors in the rat liver (Hoffman et al. 1980b), and [^{3}H] DHE also labels both alpha receptor subtypes (Hoffman et al. 1980a,b; 1981). As discussed above, an inhibitory alpha component (possibly mediated by the alpha$_2$ receptor) may be enhanced in the hypothyroid liver and this could have influenced the above results. In the present experiments this difficulty was avoided by the use of an alpha$_1$ receptor-selective ligand: [^{3}H] prazosin.

A question of obvious importance is the identity of [^{3}H] prazosin binding sites with functional alpha$_1$ receptors, and the evidence for this is necessarily circumstantial. While the binding K_d values of antagonists determined by their competition for [^{3}H] prazosin were identical with their K_B values for antagonising adrenaline activation of phosphorylase in isolated hepatocytes[1], the EC_{50} of adrenaline was 20 times and that of phenylephrine 3 times lower than their respective K_d values (section 3.I2). This discrepancy, which is similar to that noted with other radiolabelled antagonists (El-Refai et al. 1979), cannot be attributed to spare receptors, since experiments with the irreversible alpha antagonist phenoxybenzamine indicated a lack of significant receptor reserve in the rat liver (Kunos et al. 1983). Hanoune and coworkers (Geynet et al. 1981) recently compared the binding characteristics of [^{3}H] prazosin, [^{3}H] DHE and [^{3}H] noradrenaline in liver plasma membranes. On the basis of their findings they proposed that [^{3}H] noradrenaline labelled the physiologically active form of the alpha receptor and that [^{3}H] prazosin labelled either a precursor of the active receptor or an alpha$_1$-like binding site not coupled to the effector system. The present findings do not rule out the possibility that only a small fraction of the [^{3}H] prazosin binding sites with high affinity for agonists is functionally relevant, the rest being precursors for such sites or sites with undefined function (Geynet et al. 1981; El-Refai and Exton 1980b).

[1]Results of experiments done by W.H. Kan at low hepatocyte concentrations.

However, neither the agonist displacement curves (figure 3.24) nor the kinetics of [^{3}H]prazosin binding (figure 3.22) gave any indication of binding site heterogeneity that could suggest such a possibility. An alternative explanation of the above anomaly is offered by the hypothesis put forward by Franklin (1980). He proposed that the nonproductive interaction of antagonists with receptors is fully translated into tightness of binding, which results in K_d values similar to physiological K_B values. However, the productive binding energy of strong agonists is largely used to cause conformational changes necessary to initiate the biological response. This may explain the apparent paradox of low binding affinity of biologically highly potent agonists as seen in the present as well as in many previous studies (for references see section 1.3.4). Finally, the binding characteristics of [^{3}H]prazosin are compatible with seperate interacting agonist and antagonist alpha$_1$ receptor sites, the latter being labelled by [^{3}H]prazosin. The low binding potency of agonists could reflect their low affinity for the antagonist site. The possibility of distinct agonist and antagonist forms of the alpha receptor was first proposed by U'Prichard et al. (1977), although their findings were later attributed to differential binding to alpha$_1$ and alpha$_2$ receptors (U'Prichard et al. 1978). The present findings are more consistent with the original proposal, since [^{3}H]prazosin is highly selective for the alpha$_1$ receptor.

Comparison of [^{3}H]prazosin binding in membranes prepared from normal, thyroidectomized and thyroidectomized, T_3-treated rats showed that the decreased density of binding sites in thyroidectomized rats was unaccompanied by any change in affinity for this ligand (section 3.11). Similarly, the affinity of adrenaline for these sites was unchanged even in the presence of guanine nucleotide analogue, Gpp(NH)p (section 3.11); agonist displacement curves were shown to be shifted to lower affinity in the presence of Gpp(NH)p when binding was to alpha$_2$ receptor sites (Hoffman et al. 1980a). A similar effect on alpha$_1$ receptor sites in liver has not been demonstrated (Hoffman et al.

1980a,b) which supports the contention that the present experiments examined only binding to alpha$_1$ receptor sites.

Recoveries of specific marker enzymes for plasma membranes were not examined in the present study. Sperling et al. (1980) showed that basal and fluoride-stimulated adenylate cyclase activity were the same in euthyroid, hypothyroid and hyperthyroid rat hepatic plasma membranes isolated from whole livers by the same method used in the present experiments. Malbon (1980a) also found no difference in recovery of basal and fluoride-stimulated adenylate cyclase or in 5'-nucleotidase activity in membranes prepared from hypothyroid and normal hepatocytes. However, the latter results are not directly applicable to the present experiments since in Malbon's study, membranes were prepared from isolated hepatocytes rather than whole liver.

The present experiments clearly show a change in phenylephrine-induced activation of glycogen phosphorylase (section 3.10). However, as stated in section 4.1, others were unable to identify similar effects of thyroidectomy on alpha receptor activity (Malbon et al. 1978a, 1980). A lack of thyroid regulation of hepatic alpha receptors was suggested (García-Sáinz and Fain 1980) on the basis of the absence of changes in the effect of phenylephrine (Malbon et al. 1978a; 1980). While classically considered an alpha receptor agonist, phenylephrine can stimulate both beta and alpha receptors in various tissues (Chahl and O'Donnell 1969; Wagner and Schumann 1979). Its EC_{50} for relaxation of guinea pig trachea, an effect mediated by beta$_2$ receptors is 3.1 μM (Chahl and O'Donnell 1969), which is close to its EC_{50} of 0.8 $\pm$ 0.3 μM for alpha receptor-mediated activation of phosphorylase in normal rat liver (table 3.4). Since hepatic beta receptors are also of the beta$_2$ subtype (section 3.3), it is very likely that a significant beta receptor activation by phenylephrine contributes to its effect on phosphorylase in the hypothyroid rat liver. It may be noted that in the present study the difference in the effects of phenylephrine in cells from euthyroid and hypothyroid rats is

greater for calcium release, a pure alpha adrenergic response, than for phosphorylase activation, a mixed alpha/beta effect. The observation of Malbon et al. (1978a) that propranolol reduced the phenylephrine-induced activation of phosphorylase in hypothyroid, but not in normal hepatocytes (Table I in Malbon et al. 1978a), also indicates potentiation of the beta receptor component in the effect of phenylephrine in the hypothyroid liver. In this group of rats, phenylephrine was more effective in hypothyroid than euthyroid controls and propranolol abolished this difference; the similar residual activity of phenylephrine in the presence of propranolol indicated that alpha receptor activity was unchanged. However, in another group of rats used in the same study, phenylephrine tested in the absence of propranolol was not more, but even less effective in hypothyroid than in normal cells (Figure 1 in Malbon et al. 1978a). Thus in these latter animals, the increased beta receptor activity must have been offset by a similar decrease in alpha receptor activity, which is consistent with the conclusions of the present study.

Some additional difference between the present study and that of Malbon et al. (1978a; Malbon 1980a) could account for the absence of an identifiable effect of hypothyroidism on alpha activation of phosphorylase in their studies. (1) In the present experiments rats were surgically thyroidectomized and used after 10 to 14 weeks while Malbon et al. (1978a; Malbon 1980a) induced hypothyroidism by feeding an iodine-deficient diet and propylthiouracil for 18 to 25 days before the experiment. A comparison of thyroid effects produced in these two different ways on hepatic metabolism has not been made.

(2) The present study used exclusively male rats, whereas the latter used only females. Bitensky et al. (1970) have found that adrenaline-stimulated hepatic adenylate cyclase activity was greater in preparations from female rats when compared to males. More recently, Studer and Borle (1982) demonstrated that in hepatocytes isolated from female rats beta receptor-mediated stimulation of cAMP accumulation and its contribution to glycogen phosphorylase activation

was enhanced when compared to males. Conversely, the alpha receptor-mediated calcium efflux was greater in males than females and accounted for virtually all the activation of phosphorylase by adrenaline in the former. Since the female rats in their euthyroid state appear to possess hepatic adrenoceptor properties similar to the hypothyroid male, it is possible that a further reduction in alpha receptors in the hypothyroid female rat may be difficult to demonstrate clearly.

(3) Animals used in the present experiments were older (250-350 g) than those used by Malbon et al. (1978a; 100-150 g). Blair et al. (1979a) have examined the adrenergic control of glucose output in hepatocytes from juvenile (100-150 g) and adult (greater than 300 g) rats. They found aging to be accompanied by a loss of functional beta receptors with adrenaline acting primarily through the alpha adrenergic receptor system. Hence, it is possible that the alpha response was more pronounced in the older animals used in the present study than it may have been in younger animals.

(4) Malbon et al. (1978a) express their reults for phosphorylase activation as % change over basal. In spite of the fact that basal enzyme activities were not different when hypothyroid and euthyroid rats were compared, small changes in basal activity in a given experiment could profoundly influence the calculated stimulated enzyme activity when expressed in that way. Actual enzyme activities are reported in the present experiments.

These consideration notwithstanding, one must point out that changes in the beta adrenergic activation of phosphorylase after thyroidectomy are more profound than for the alpha receptor system. The mechanism underlying these changes and how either the emergence of beta receptors or the suppression of alpha responses in the hypothyroid rat might influence overall metabolic regulation in vivo remain matters of speculation. Each of these questions will now be briefly considered.

4.4 Similar changes in hepatic adrenoceptors produced by non-thyroidal influences.

Thyroid hormone effects on adrenergic receptors in a number of other tissues have been described (section 1.2.3). Most of these studies have involved either adipose or cardiac tissue and have shown that beta adrenergic responsiveness is decreased and alpha responsiveness increased in hypothyroidism. (for a more detailed discussion and references, see section 1.2.3). Thus, the effect of hypothyroidism on hepatic adrenergic receptors is opposite to that seen in these tissues indicating that the effects of thyroid hormones on these receptors are highly tissue specific and cannot be generalized. Indeed, in some preparations no effect of altered thyroid state on adrenergic receptors could be found (for references see section 1.2.3).

As already discussed in section 4.3, similar changes in hepatic adrenergic receptors have been described in adrenalectomized rats. Wolfe et al. (1976) found an increase in isoproterenol-stimulated adenylate cyclase activity and specific binding of [^{125}I]IHBP, a potent beta antagonist. However, this latter finding is of questionable reliability because of the unusually low affinity for this antagonist observed in that study (see section 1.3.4). These studies were extended by Chan et al. (1979a) who reported a greater beta receptor-mediated cAMP accumulation and phosphorylase b to a conversion in hepatocytes from adrenalectomized rats. Alpha adrenoceptor-mediated phosphorylase activation and calcium mobilization decreased after adrenalectomy. The fact that these changes are analogous to those reported here after thyroidectomy suggests the possibility that they may have occurred as a consequence of a thyroid hormone effect on the pituitary-adrenal axis. Indeed, it has been shown that in the rat, deficiency of thyroid hormone is associated with subnormal concentrations of corticosteroids in plasma (Bray and Jacobs 1974). Also, the effects of adrenalectomy were more rapid than thyroidectomy occuring after only 3 to 8

days. However, administration of cortisol to thyroidectomized rats using a treatment regimen that was shown to reverse adrenalectomy-induced changes in hepatic adrenoceptors (Wolfe et al. 1976) had no effect on the pattern of phosphorylase response to adrenergic agonists (section 3.3). These findings argue against thyroid effects being secondary to changes in the adrenal-pituitary axis.

But what of the converse of this hypothesis: could the effects of adrenalectomy on hepatic adrenoceptors be secondary to an effect on thyroid hormone action? Thyroid hormone receptor levels and consequently tissue sensitivity to T_3 and T_4 can be influenced by a variety of factors (Samuels et al. 1982). It may be then, that the liver becomes "functionally hypothyroid" in the presence of normal circulating thyroid hormone levels, and hence shows changes similar to those seen following thyroidectomy. Unfortunately, there is no simple approach to testing this hypothesis, since treatment of adrenalectomized rats with excess thyroxine may not reverse the effect in the face of reduced tissue responsiveness. It would be of interest to know how thyroid hormone receptors are influenced by adrenalectomy. To the best knowledge of the author, neither of these experiments has been done.

Hepatic adrenergic receptors can also be modulated by non-hormonal influences. Hornbrook (1978) has shown that alpha-mediated activation of glycogenolysis in hepatocytes from normal rats takes on the characteristics of a mixed alpha/beta response when examined 24 h after partial hepatectomy. In support of these findings, Brønstad and Christoffersen (1980) later demonstrated that adrenaline-stimulated cAMP accumulation and adenylate cyclase activity was enhanced after partial hepatectomy. Again, there exists a relationship with thyroid hormone effects on the liver. Partial hepatectomy in the rat reduces the number of nuclear thyroid hormone receptors (Dillman et al. 1977). Plasma glucagon levels are markedly elevated after partial hepatectomy (Morley et al. 975) and glucagon administration has been reported

to lower hepatic nuclear T_3 binding capacity (Dillman et al. 1978). Thus, partial hepatectomy might also produce a situation in which the liver becomes "functionally hypothyroid" and hence, manifests changes in adrenoceptor properties analogous to those observed following thyroidectomy. Starvation also produces an elevation in circulating glucagon levels (Grey et al. 1970) as well as a decrease in T_3 nuclear binding sites (Burman et al. 1977). It would, therefore, be of interest to see whether either starvation or exogenous glucagon administration would produce changes in hepatic adrenergic receptors similar to those produced by thyroidectomy. Burns et al. (1979) have observed that after only one day of fasting, the adrenergic receptor activity of human adipocytes is altered in a way similar to that seen in hypothyroid subjects.

Treatment of adult rats with the hepatic carcinogen 2-acetylaminofluorene resulted in a marked increase in hepatic adenylate cyclase response to adrenaline (Christoffersen et al. 1972). Hornbrook (1979) later found that feeding of the same carcinogen to rats shifted the adrenergic activation of hepatic phosphorylase in isolated hepatocytes from an alpha to a mixed alpha/beta response. Increase in beta adrenoceptor activity in isolated hepatocytes has also been reported following feeding of a second hepatic carcinogen, 3'-methyl-4-dimethylaminobenzene (Christoffersen and Berg 1975). Boyd and Martin (1976), studying the action of this second carcinogen, found an increased catecholamine- and decreased glucagon-responsive adenylate cyclase activity in preneoplastic liver. These changes were seen in non-turmor as well as tumor tissue (Boyd et al. 1978) which suggest that they may be the manifestation of some reactive change in non-tumor tissue analogous to that seen following partial hepatectomy.

Of some relevance to these studies of hepatic tumors are reports comparing adrenoceptors in normal rat liver and the Zajdela ascites hepatoma cell line (Lacombe et al. 1976). The adrenoceptor mediating adenylate cyclase activation in the normal rat liver is typically of the $beta_2$ subtype (Lacombe

et al. 1976; Vanquelin et al. 1976). However, in the Zajdela hepatoma, adenylate cyclase stimulation occurs predominantly via activation of a $beta_1$ adrenergic receptor; moreover, the response is greatly enhanced when compared with normal rat liver (Lacombe et al. 1976; Hanoune et al. 1977). The present results indicate that enhanced beta receptor activity observed after thyroidectomy is mediated by receptors of the $beta_2$ subtype since adrenaline is more potent than noradrenaline in stimulating cAMP accumulation (section 3.4). Leray et al. (1973) reported that enhanced adrenaline-stimulated adenylate cyclase in preparations from adrenalectomized rat liver was also mediated by $beta_2$ adrenoceptors. And, data shown in the study of preneoplastic liver tissue also suggests that the receptor remains the $beta_2$ subtype; noradrenaline was less potent than adrenaline (Boyd and Martin 1976). Thus, unlike the Zajdela hepatoma, where enhanced beta receptor activity is associated with a $beta_1$ adrenoceptor type, preneoplastic liver tissue, preparations from adrenalectomized rat liver and hepatocytes from thyroidectomized rats show the characteristics of $beta_2$ adrenergic receptors as in the normal rat liver. The beta receptor subtype in liver after partial hepatectomy or in young rats (see below) has not been analyzed in detail.

Extrahepatic cholestasis produced by ligation of the common bile duct in the rat has been shown to produce a 2.6-fold increase in isoproterenol-stimulated adenylate cyclase activity in isolated liver plasma membranes after two days (Schmelck et al. 1979). This increase in activity was associated with a four-fold enhancement of beta receptor binding site density as measured by [^{3}H] DHA specific binding (Schmelck et al. 1979). Although the characteristics of alpha receptor responses have not yet been reported in cholestatic livers, Guellaen et al. (1982) have found a two- to three-fold decrease in the number of [^{3}H] DHE and [^{3}H] prazosin binding sites. Apparently only single concentrations of [^{3}H] prazosin (1 nM) were examined in the latter study and detailed reports of binding data are lacking.

Another parameter that is known to influence hepatic adrenergic receptor properties is age. Hornbrook (1978) reported that catecholamine-mediated activation of glycogen phosphorylase occurred predominantly via beta adrenoceptors in weanling rats. In a later report, Blair et al. (1979a) examined adrenergic control of glucose output and cAMP levels in juvenile rats. They found that in hepatocytes from young rats (27 to 35 days old, weighing 100 to 150 g), adrenaline stimulated glucose output from endogenous glycogen stores and cAMP accumulation via predominantly beta adrenergic receptor activation. Maturation (60 to 120 days, 300 g and greater) was accompanied by a loss of functional beta receptors with adrenaline acting primarily through alpha receptors. Butlen et al. (1980) used [^{3}H] DHE as an alpha receptor probe to study the ontogenic development of alpha receptors in rat liver. They found that in 19-day-old fetuses the density of [^{3}H] DHE binding sites was about 1.5 times that of the adult, but decreased to one quarter in late fetal life and the first two weeks after birth. Unfortunately, beta receptors have not as yet been studied in a similar way. On the other hand, Kalish et al. (1977) found that adreraline-sensitive adenylate cyclase activity was doubled in liver membranes prepared from 24 month old rats when compared to that found at 3 or 12 months. Those findings could imply the existence of an enhanced hepatic beta receptor activity in senescent rats, although the receptor mediating adrenaline's effect was not characterized in that study. Moreover, basal, fluoride- and glucagon-stimulated activities were also enhanced, but not to the same degree as adrenaline.

Three points about these observations that relate to the present study are noteworthy.

(1) Properties of hepatic adrenoceptors are similar in normal young rats and adult rats following thyroidectomy.

(2) This suggests that thyroid hormones may exert a primary effect on hepatic adrenoceptors by influencing the developmental state of the liver (for further

discussion of this point refer to section 4.5)

(3) Normal euthyroid controls used throughout the present experiments were weight-matched, and hence hypothyroid rats were on average 10 to 14 weeks older than controls. However, the fact that in adult rats (300 g and greater) the predominant hepatic adrenoceptor becomes and remains an alpha receptor (Blair et al. 1979a) argues against the possibility that the observed difference between these groups was due to age. Kalish et al. (1977) found no difference in adrenaline-stimulated adenylate cyclase in liver plasma membranes from three month and 12 month old male rats.

A final factor that is known to influence the balance of hepatic adrenergic receptors is sex and has already been briefly discussed in section 4.3. Recall that Studer and Borle (1982) have shown that hepatic adrenoceptors in female rats resemble those described for hypothyroid males in the present study; activation of glycogen phosphorylase is by catecholamine is shared by alpha and beta receptors, alpha receptor-mediated calcium mobilization is suppressed and beta receptor-mediated cAMP accumulation is enhanced when compared to males. How these effects might be related to thyroid influences is not clear, although, sex differences in the metabolism and actions of thyroid hormones have been described (Harris et al. 1979), and one might postulate that female rats behave such that their livers are "functionally hypothyroid" as compared to males. This interesting sex-related difference and what it might mean physiologically invites further study.

Changes in the properties of hepatic adrenergic receptors have also been described in the ex vivo state. Okajima and Ui (1982) have examined the adrenergic regulation of glycogen phosphorylase and synthase in rat hepatocytes in primary culture. They found that beta receptor contributions become predominant over alpha after only 8 h in culture. This shift in adrenoceptor properties could be prevented by the addition of cycloheximide to the culture medium suggesting a role for protein synthesis in the observed change. It

would be of interest to examine the effect of addition of thyroid hormone or glucocorticoid in a similar experiment.

The findings presented here as well as those of Malbon (1980a) suggest that the alterations in hepatic adrenoceptor properties in hypothyroidism are the result of changes in the density of adrenergic receptors in the plasma membrane. A similar conclusion can be drawn about changes occurring after adrenalectomy (Guellaen et al. 1978, Exton et al. 1981) or hepatic cholestasis (Schmelck et al. 1979; Guellaen et al. 1982). Unfortunately the effects of the other factors described above on alpha and beta receptor densities has not been examined directly. Thus one cannot conclude that these changes are completely analogous to the hypothyroid state. Nonetheless, it appears that hepatic adrenoceptors are influenced by a number of physiological and pathological changes of which hypothyroidism is a single example. Moreover, several of these can be hypothetically related to altered thyroid hormone function and hence, may share a common mechanism. Although evidence supporting this notion is at present only circumstantial, some aspects are worthy of further experimental consideration.

4.5 Physiological implications of thyroid-induced changes in hepatic adrenoceptor properties.

The physiological role of the sympathetic nervous system in the control of hepatic carbohydrate metabolism remains enigmatic. Therefore in the context of this broader question, any discussion of how changes in hepatic adrenoceptor properties of the kind described here influence in vivo metabolic regulation would be pure conjecture. Nonetheless, some interesting aspects of this problem are worth considering in a speculative way.

Why two different pathways whereby catecholamines stimulate hepatic glucose output should coexist is problematic. Exton and Harper (1975) have

speculated that the duality of this system may have survival value, pointing out that the liver plays a vital role in the "fight or flight" situation. However, the developmental, sex and species dependent variation in the balance of alpha and beta receptors remains unexplained; duality of the system if it has survival value should be of benefit under any circumstances. The shift in this balance observed in various pathological states such as carcinogenesis, hepatectomy, adrenal insufficiency, cholestasis and hypothyroidism (section 4.4) can be viewed in two ways. Either it is an undesirable change and contributes to the pathologic state or it represents an adaptive response that allows the animal to optimize its metabolic machinery in this pathological state. If alpha and beta receptors represent mere duplications that contribute survival value, one would expect these changes in the balance of receptors to gain little advantage in acute pathological states. One must, therefore, presume that alpha and beta receptor activation must have different effects on the overall metabolism of the cell.

Hormones affecting hepatic metabolism can be considered in two broad groups (see section 1.3): those which, like beta adrenoceptor agonists, produce effects that are mediated via the adenylate cyclase/cAMP-dependent protein kinase system and others, such as alpha receptor agonists, whose effects are calcium-mediated. While most studies have stressed the similarities of the effects on cellular metabolism of these two pathways some differences have been noted. The identification of metabolic effects that are not shared by these two groups suggests possible points at which alpha and beta receptor stimulation would have different metabolic consequences. Unfortunately, there are few studies in which the metabolic effects of hepatic beta and alpha receptor activation have been compared within the same experimental preparation. However, cAMP-dependent hormonal effects (usually glucagon stimulation) and calcium-dependent hormonal effects (alpha adrenergic, vasopressin and angiotensin II) in hepatocyte preparations have been compared.

By analogy, these observations provide support for the hypothesis that alpha and beta receptor stimulation can alter cellular metabolism in very different ways, and hence probably provide the animal with a metabolic "flexibility" that under some circumstances may be essential for survival rather than a mere duplication of receptor systems.

Garrison et al. (1979) have compared the pattern of phosphorylation of rat liver proteins after stimulation with glucagon and noradrenaline. These authors were able to resolve 11 to 12 cytoplasmic proteins that were phosphorylated in response to hormonal stimulation. Protein peaks whose phosphorylation was increased by noradrenaline in the presence of 20 μM propranolol were the same as those increased by glucagon both in position and magnitude. Three of these peaks were identified as phosphorylase a, glycogen synthase and pyruvate kinase. Vasopressin, angiotensin II and A23187 gave similar results. However, one-dimensional SDS gels used in that study lack sufficient resolution to determine if the proteins phosphorylated by calcium-linked hormones were distinct from those affected by glucagon stmulation. Thus, when two-dimensinal gels were analyzed in a subsequent series of experiments (Garrison and Wagner 1982), it was observed that calcium-dependent hormones and glucagon were able to affect the phosphorylation of distinct substrates. These authors found six substrates uniquely phosphorylated by glucagon, one by the calcium-linked hormones and seven by both.

Garrison and Wagner (1982) draw particular attention to some of the enzymes of the gluconeogenic pathway. In contrast to glucagon, the calcium-dependent hormones did not stimulate the phosphorylation of phosphofructokinase, fructose-6-phosphate 2 kinase or fructose-1,6-bisphosphatase and had only a slight effect on pyruvate kinase. Others (Chan and Exton 1978; Garrison and Borland 1979; Hems et al. 1978b) have observed that while gluconeogenesis can be stimulated by both cAMP-dependent and

calcium-dependent pathways, hormones functioning via the latter stimulate gluconeogenesis to a lesser extent at a glycogenolytic equivilant dose.

In this context the results of one study where indirectly the effects of alpha and beta stimulation were compared is of interest. Blair et al. (1979b) have shown that adrenaline markedly inhibits pyruvate kinase and lactate production in juvenile rats, but is only slightly effective in adults and yet, adrenaline-stimulation of glucose output in these two groups is comparable (Blair et al. 1979a). These authors suggest that this difference is due to to the fact that catecholamine effects in younger animals are mediated predominantly by beta adrenoceptors, whereas alpha receptors are relatively more important in older rats (Blair et al. 1979b).

Others have shown that alpha-mediated inhibition of pyruvate kinase is less effective than that produced by glucagon (Chan and Exton 1978; Garrison and Borland 1979). It has been suggested that this may be the reason for the generally observed less effective stimulation of gluconeogenesis by catecholamines compared with glucagon (for references see Garrison and Borland 1979). However, Rognstad and Katz (1977) have reported that in cells from fasted rats, maximal concentrations of adrenaline stimulated glucneogenesis as effectively as glucagon, but had no effect on flux through pyruvate kinase, while glucagon inhibited flux through this step by approximately 60%. This observation led Chan and Exton (1978) to suggest that alpha adrenergic stimulation of gluconeogenesis could occur at another control point in the pathway, possibly fructose-1,6-bisphosphatase. It is interesting to note that recently Van Schaftingen et al. (1980) have shown that fructose-2,6-bisphosphate is an important regulator at this point in the gluconeogenic pathway. This metabolite inhibits fructose-1,6-bisphosphatase and activates phosphofructokinase (Hers and Van Schaftingen 1982). Glucagon stimulation of isolated hepatocytes causes a marked decreases in levels of this metabolite and is thought to be in part responsible for the enhanced

gluconeogenesis under these conditions (Van Schaftingen and Hers 1982). However, A23187, phenylephrine and vasopressin cause an increase in fructose-2,6-bisphosphate content of the hepatocyte and yet the latter two agents also stimulate gluconeogenesis (Whitton et al. 1978). The interesting possibility that this regulator exerts effects on other metabolic pathways and control points requires further investigation.

Still other mechanisms not involving enzyme phosphorylation directly may be at work in the cell. For example, Merryfield et al. (1982) have shown that phosphénolpyruvate carboxykinase, another key enzyme in the gluconeogenic pathway, is activated by the release of Fe^{++} from rat liver mitochondria mediated by free calcium. These authors speculate that alpha receptor agonists as well as other calcium-dependent hormones may exert a regulatory influence on this enzyme via this mechanism. Whether cAMP-dependent hormones may have a similar effect is unknown.

Another indication of profound differences in the effect of these two groups of hormones on hepatocyte metabolism comes from the study of Reinhart et al. (1982b). These authors demonstrated that phenylephrine, vasopressin and angiotensin II administered to perfused rat liver produced a rapid parallel increase in glucose output and oxygen uptake. On the other hand, glucagon's effect on glucose output was slower and occurred in the absence of any large change in respiration. These authors concluded that mitochondria play a vital role in mediating the glycogenolytic effect of the calcium-dependent hormones, but not glucagon.

Although these two groups of hormones appear to have different effects on cellular metabolism, evidence exists to suggest that these pathways do not function independently: hormones of one group may modify or override the effects of the second. Exton and coworkers (Assimacopoulos-Jeannet et al. 1982) demonstrated that phenylephrine and adrenaline could suppress glucagon-mediated elevation of cAMP and gluconeogenesis from lactate in

isolated hepatocytes. In contrast to the studies of Jard et al. (1981) who demonstrated that adenylate cyclase inhibition in isolated plasma membranes occurred via $alpha_2$ receptors, Exton's group (Assimacopoulos-Jeannet et al. 1982) showed that the inhibitory effect on cAMP accumulation in hepatocytes suggested an $alpha_1$ receptor mechanism was more important. The contradictory nature of these findings may be a consequence of the different preparations used. Whether this inhibitory effect is mediated by $alpha_2$ or $alpha_1$ receptors, the ability to also inhibit stimulation of gluconeogenesis by exogenous cAMP suggests that other loci in addition to adenylate cyclase may be involved (Assimacopoulos-Jeannet et al. 1982). The elucidation of the true nature of this interaction and its possible role in vivo require further study.

Assuming that alpha and beta receptor stimulation do affect the cell's metabolic machinery in different ways, how and under what circumstances does differential stimulation of these receptors occur? As can be seen from table 3.1, the sensitivity to glycogen phosphorylase stimulation by adrenaline is not markedly changed in the hypothyroid rat; half-maximal phosphorylase activation occurs at 7.73 (pD_2) and 7.96 (pD_2) for normal and hypothyroid rats respectively. Since at least some residual alpha receptor activity remains, one would not expect the threshold for phosphorylase activation to be greatly altered in the hypothyroid rat liver. However, hypothyroid rats possess adrenoceptors of the $beta_2$ subtype, (section 4.4) which are very much less sensitive to noradrenaline released from hepatic sympathetic nerves (section 1.2.3) than are alpha receptors. One may then speculate that in the hypothyroid rat the major pathway for adrenergic activation of liver phosphorylase is through stimulation of $beta_2$ receptors by adrenaline released from the adrenal medulla, whereas in normal rats noradrenaline released from sympathetic nerve endings and acting on $alpha_1$ receptors would be more important. As described in section 1.4 there is some evidence that such an arrangement might exist in the rabbit and the guinea pig which normally

possesses both alpha and beta hepatic adrenoceptors.

In conclusion, one must concede that in the face of only sparse information about the role of sympathetic nervous system in control of hepatic metabolism there is little that can be said about the physiological role of thyroid-induced changes in adrenoceptor properties. However, as described above, logical reasons do exist for expecting that such changes may have important physiological consequences.

4.6 Possible mechanisms of thyroid induced alterations in hepatic adrenoceptor properties.

The present study documents a clear effect of thyroid state on hepatic adrenoceptor properties, but unfortunately provides little insight into the possible mechanism of such changes. However, isolated hepatocytes provide an ideal model system which might be used to reproduce and study these effects under controlled in vitro conditions. Thus far there is some evidence that thyroid hormones can produce changes in adrenergic receptor properties in isolated cardiac tissue (see section 1.2.3), but attempts to affect similar changes in isolated adipocytes has met with little success (see discussion in Fain 1981).

One must consider that thyroid hormones could influence adrenergic receptors other than by a direct effect on tissue for which the change is identified. For example, thyroid-induced alterations in the metabolic state that result in changes in adrenergic receptors could represent part of an overall adaptive response of the animal to its new physiologic state. There is at present little that can be said to refute or support this possibility other than to reiterate the facts that thyroid hormones have profound effects on almost every metabolic system studied and that adrenergic receptors exhibit significant "plasticity" in their ability to change in order to accommodate new stimuli

(Hoffman and Lefkowitz 1980). Furthermore, the fact that similar changes are observed in a number of situations not directly related to thyroid hormone action argues for a generalized nature of the phenomenon (section 4.4).

Thyroid hormones can modulate the actions of a number of other hormones and may influence adrenergic receptors secondarily via one of these. This possibility has been discussed with respect to corticosteroids (section 4.4). Results presented here suggest that thyroid hormone effects on hepatic adrenoceptors do not occur because of corticosteroid deficiency in hypothyroidism. However, the possibility that part of the effect of adrenalectomy on hepatic adrenergic receptors may be due to an effect on thyroid hormone actions cannot be ruled out (see section 4.4).

One possible clue to the mechanism relates to the apparent effect of thyroid hormones on the developmental state of the tissue. Adequate thyroid function is necessary for normal growth and differentiation of many tissues (Greenberg et al. 1974) as are glucocorticoids. Hepatic beta receptors are relatively more important in young rats and there is an apparent shift in the predominant hepatic adrenoceptor from alpha to beta after partial hepatectomy, exposure to hepatocarcinogens or in hepatocytes maintained in primary culture (see section 4.4). The similarity between these situations and the hypothyroid rat model suggests that the presence of thyroid hormone may be required to maintain the state of differentiation of the tissue. When thyroid hormone is removed the hepatocyte may revert to a less well differentiated form in which the beta receptor contribution is more significant than in the normal adult animal.

An effect of thyroid hormone directly on the tissue could influence adrenergic reponsiveness by an action on the receptor site or some post-reeptor event. The loss of beta receptor activity in fat cells from hypothyroid rats provides an example of the latter (see section 1.2.3); the refractoriness of adipose tissue to adrenergic stimuli was due in part to an increase in cAMP

phosphodiesterase (Armstrong et al. 1974) and possibly also a loss of coupling between the beta receptor and adenylate cyclase (Fain 1981). In the present experiments the increased beta and decreased alpha receptor responsiveness are accompanied by an increase in beta and decrease in alpha receptor binding site densities. Although this is the most likely explanation for the altered tissue responsiveness the possible existence of additional thyroid effects on receptor coupling or other post-receptor events cannot be ruled out.

How then might thyroid state modulate adrenoceptor numbers in the liver? Thyroid hormone receptors have been described in a number of sub-cellular structures including the plasma membrane and the nucleus (Sterling 1979; Bernal and DeGroot 1980). There is evidence that thyroid hormone stimulates protein synthesis at both the level of translation and transcription (Bernal and DeGroot 1980). Hence, it is possible that thyroid hormones may directly influence the production of receptor protein or some other gene product that has a regulatory effect on the adrenergic receptor. If these effects could be reproduced in vitro one could test the role of protein synthesis directly by the use of specific inhibitors. Recall that the emergence of beta receptor activity in hepatocytes maintained in primary culture could be prevented by the addition of cycloheximide, a protein synthesis inhibitor (Okajima and Ui 1982).

Kempson et al. (1978) reported changes in beta receptor binding in cardiac tissue incubated in vitro with thyroid hormone that appeared not to be dependent on protein synthesis (section 1.2.4). Thus one could speculate that a specific effect of thyroid hormone at the level of the cell membrane may be important. The incorporation or activation of cryptic receptors from an inactive membrane or cytoplasmic pool may be regulated by thyroid hormone through either a direct effect on such receptors or as a consequence of a more generalized change such as alteration of membrane fluidity (Mendoza et al. 1977).

Of particular interest with respect to possible direct effects of thyroid

hormones on adrenergic receptors is the proposal that alpha and beta receptors represent two forms of a single protein which can interconvert in response to certain stimuli such as temperature or thyroid state (Kunos 1977, 1978). However, in light of recent demonstrations of the existence of at least four types of adrenergic receptors, some with very different mechanism for response activation (section 1.2.3) this interconversion hypothesis has become somewhat strained. Wood et al. (1979) have solubilized from hepatic plasma membranes seperate macromolecules that have the property of binding [^{3}H] WB 4101 and [^{3}H] DHA respectively, and concluded that these receptors, therefore, do not reside on the same macromolecule. However, recovery of alpha receptor-like binding sites was low (1-5%) and may not be truly representative of the hepatic receptor. Furthermore, as discussed in section 1.3.4, the most appropriate ligands for identifying alpha and beta receptors in liver are unsettled issues. Kunos (1980, 1981) has broadened the definition of "interconversion" to imply a close functional coupling between receptors. Hence, a decrease in one receptor type would result in a concomitant increase in the second type to which it is coupled. Such coupled regulation may be mediated indirectly by a thyroid hormone-dependent cellular signal other than the adrenergic receptor itself. Indeed, observations in many experimental systems have shown that in many cases when either alpha or beta receptor activities change they do so in a reciprocal manner. The explanation for this is not known.

In conclusion, a variety of possible mechanism whereby thyroid hormones could modulate adrenergic receptor activity exist. Some of these have been described above. The elucidation of the actual mechanism(s) involved in this regulation will require a greater understanding of both thyroid hormone action and adrenergic receptor properties. Such studies would be facilitated by a reliable in vitro model system in which thyroid regulation of adrenergic receptor properties can be demonstrated. The isolated rat hepatocyte might provide such a model.

SECTION FIVE

SUMMARY AND CONCLUSIONS

(Contributions of Original Knowledge)

1. The effect of thyroid hormones on hepatic adrenergic receptors was examined in hepatocyte suspensions prepared from euthyroid, hyperthyroid, hypothyroid and hypothyroid, triiodothyronine-treated rats.

2. Experiments with adrenergic agonists and selective antagonists showed that activation of liver glycogen phosphorylase in cells prepared from euthyroid and hyperthyroid rats was mediated by alpha adrenergic receptors.

3. The induction of hypothyroidism revealed the emergence of a significant beta receptor contribution to this response, as well as an enhanced accumulation of cAMP in response to beta receptor stimulation. The presence of a phosphodiesterase inhibitor did not eliminate this difference.

4. The response to glucagon, which also activates glycogen phosphorylase via a cAMP-dependent mechanism, was not similarly affected; phosphorylase activation by glucagon was the same in hepatocytes from hypothyroid and euthyroid rats. Cyclic AMP accumulation was enhanced in hypothyroid hepatocytes at high glucagon concentrations and this difference was maintained in the presence of a phosphodiesterase inhibitor.

5. This emergence of beta receptor activity in hypothyroidism was accompanied by a decreased alpha receptor activity, as demonstrated by a suppression of phenylephrine-stimulated phosphorylase activation and calcium efflux from isolated hepatocytes.

6. Responses to vasopressin and the divalent cation ionophore, A23187, which like alpha agonists produce activation of liver glycogen phosphorylase by a release of intracellular calcium, were not similarly affected; the effectiveness of vasopressin in stimulating calcium efflux was slightly reduced and that of A23187 unaffected in hypothyroid hepatocytes.

7. Analysis of specific binding of [^{3}H]prazosin, a selective alpha$_1$ antagonist, to liver plasma membranes revealed a decrease in the density of putative alpha receptor sites in preparations from hypothyroid rats. Hypothyroidism did not influence the binding affinity of adrenergic agonists or [^{3}H]prazosin to alpha$_1$

receptor sites. This change was opposite to the increase in density of beta receptors in hypothyroid rat liver reported by others.

8. All of these changes: the increased beta receptor activity, the decreased alpha receptor activity and the decreased density of $alpha_1$ receptor binding sites could be returned towards normal by prior administration of triiodothyronine to hypothyroid rats.

9. It is concluded that hypothyroidism produces a selective and reversible enhancement of beta receptor activity and supression of alpha receptor activity in rat hepatocytes. These changes are most likely the result of alterations in the density of receptor sites in the plasma membrane.

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