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THE EFFECT OF TRIIODOTHYRONINE ON MYOCARDIAL LIPOPROTEIN LIPASE ACTIVITY, PLASMA FREE FATTY ACIDS AND OXYGEN CONSUMPTION OF HYPOTHYROID RATS

by

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of the requirements for the degree of
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ABSTRACT

An acute injection of triiodothyronine (T_3:50, 125 and 250ug/100g body weight) was administered to hypothyroid rats and time courses of heart lipoprotein lipase (LPL) activity, plasma free fatty acid (FFA) level and total body oxygen consumption over 67 hr were recorded. In a preliminary experiment a time course of myocardial palmitylcarnitine acyl transferase (PCAT) activity after a single injection of 50ug T_3/100g was determined. The magnitude and duration of the increase in LPL activity was found to depend on the dose of T_3. Elevations in LPL and PCAT activity preceded changes in plasma FFA levels. In order to investigate the possibility of catecholamine participation in the stimulatory action of T_3 on LPL, FFA mobilization and oxygen consumption, the effects of reserpine pretreatment were studied. Reserpine (0.2 and 1.0mg/kg), administered 18 hr prior to T_3 injection did not alter the response of LPL to the hormone. Reserpine pretreatment had no effect on the calorigenic action of T_3. However, reserpine (0.2 mg/kg) significantly reduced the elevation in plasma FFA following T_3. Reserpine (1.0mg/kg) reduced the elevation in LPL activity 3-4 hr following an injection of epinephrine (0.5mg/kg) in normal rats but did not elevate enzymatic activity as it did

in hypothyroid rats. The results of these experiments indicate that thyroid hormone: i) exerts its calorigenic action independently of catecholamines, ii) mobilizes plasma FFA by interacting with catecholamines and iii) acts directly on heart LPL independently of the adrenergic system.

INTRODUCTION

Introductory remarks

In the hyperthyroid rat heart it is known that lipoprotein lipase (LPL) levels are elevated (Alousi and Mallov 1964). Furthermore, it has been shown that the increased LPL activity of hearts hypertrophied by hyperthyroidism is due to some metabolic change brought about by thyroid hormones. Hearts in which hypertrophy was induced by aortic constriction showed normal LPL levels (Mallov and Alousi 1967). In order to study metabolic changes which occur in the hyperthyroid animal, the sequence of the changes occurring in cardiac lipid metabolism as a result of thyroxine's [1] action was recorded. Thus, after a single injection of triiodothyronine (T_3), a time course of changes in lipoprotein lipase activity and plasma free fatty acid (FFA) levels, two important factors in cardiac lipid metabolism, was determined. Oxygen consumption was measured also, primarily as a method for ensuring that T_3 was producing a definite effect in the animal. Hypothyroid animals were used as a basal condition to provide a sensitive indicator of thyroxine's effects. Because catecholamines

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1. Throughout this paper "thyroxine" is used interchangeably with "thyroid hormones" unless otherwise specified.

have been reported to participate in many of thyroxine's actions, an examination of the possible catecholamine interaction with T_3 upon LPL, free fatty acid mobilization and calorigenesis was made. The following is a brief introductory discussion concerning the characteristics, location and measurement of activity of lipoprotein lipase. Also, some of the controlling factors of LPL activity and hormonal control of myocardial enzymes are discussed.

Characteristics of lipoprotein lipase

LPL hydrolyses the triglyceride portion of chylomicra and low density lipoproteins to glycerol and non-esterified or free fatty acids (Korn 1959, Robinson 1963). The presence of this enzyme, first known as clearing factor lipase, was demonstrated in 1943 when Hahn (1943) showed that the turbidity of plasma from dogs with alimentary lipemia was cleared after an injection of heparin. The turbidity, produced by fat particles or chylomicra, was later shown to be cleared *in vitro* also by adding post-heparin plasma from another animal (Anderson and Fawcett 1950). The post-heparin plasma was believed to contain clearing factor lipase which was released into the blood or activated there by some action of heparin. Tissue LPL was first demonstrated in extracts of acetone-powders of rat heart. The enzyme was shown to have many similar properties to

post-heparin serum or plasma LPL (Korn 1955). The recent purification of rat post-heparin plasma LPL (Fielding 1969) may prove helpful in further characterizing heart LPL. The molecular weight of plasma LPL was found to be 72,600. The optimum temperature was found to be 37°C and optimum pH, 8.5. Freezing the enzyme at 0°C for 24 hr reduced enzymatic activity only 10-15% while freezing at -20°C reduced activity only about 5%. These findings agree with those of Kessler and Senderoff (1962) and Mallov and Alousi (1969) who found that freezing heart tissue for several days had no significant effect on LPL activity.

Since heart LPL has not as yet been purified the assay methods used may be measuring the activities of other lipases. Such a consideration is particularly important when using crude homogenates as the enzyme source. There has been a previous report of two lipoprotein lipases in rat heart (Bjorntorp and Furman 1962a) and also of a monoglyceride-hydrolysing activity (Yamamoto and Drummond 1967). It was suggested by Yamamoto and Drummond that this monoglyceride hydrolase may act together with LPL to hydrolyse glycerides. Furthermore, there is recent evidence for separate monoglyceride hydrolase and triglyceride lipase (probably LPL) in post-heparin human plasma (Greten et el.1969). This finding supports the proposal that the so-called LPL of tissues is the source of the post-heparin plasma enzyme

activity (Brady and Higgins 1967). Although the use of inhibitors such as sodium chloride and protamine sulfate, believed to be specific for LPL, have indicated that the heart lipase whose activity is largely determined by such an assay system as was employed in the present studies is LPL (Mallov and Alousi 1969), there is strong evidence that other lipases are being measured as well . Thus, LPL must be considered a possible mixture of enzymes.

Site and sources of lipoprotein lipase

LPL is believed to reside on the endothelial surface of vessels, readily available to be released into the blood (Robinson and Harris 1959, Bjorntorp and Furman 1962). Such a location is consistent with the fact that heparin injection leads to a very rapid appearance of LPL in the plasma (Robinson and Harris 1959). It has also been shown by electron-microscopic studies that injected chylomicra, which serve as the substrate for LPL, were aligned along the endothelial capillary cell surface in adipose tissue (Robinson 1963). Such findings provide support for the proposal that the enzyme is found on the endothelial surface.

Although the greatest concentrations of LPL have been found in heart and adipose tissue (Korn 1959) other tissues have been shown to contain the enzyme: placentas of rats and humans (Mallov 1965), aorta, spleen and kidney of rats

(Korn 1959), guinea pig mam mary gland (Robinson 1963) and more recently and contrary to previous findings, in rat liver (Mayes and Felts 1968). Plasma LPL or clearing factor lipase which appears in the blood only after heparin or a heparin-like substance is injected is believed to be released from the tissues, primarily from adipose tissue (Brady and Higgins 1967).

Assay of lipoprotein lipase

The early methods of measuring clearing factor lipase activity in post-heparin plasma involved measuring colorimetrically the clearing of turbidity due to lipemia. This method, however, does not measure the rate of hydrolysis. Consequently, later methods are based upon the measurement of the rate of release of the products by the lipoprotein lipase hydrolysis reaction, glycerol and FFA. Glycerol production is not as good an indicator of LPL hydrolysis as is FFA production. It can be argued that when LPL activity is low, for example, if only one mole of fatty acid is hydrolysed from the triglyceride molecule, then glycerol is not formed and thus not measured (Korn and Quigley 1957). Also, in the early stages of the hydrolysis reaction there may be a lag behind the release of FFA if there is any accumulation of mono-or diglycerides (Robinson 1963).

Myocardial LPL activity has been measured using various tissue preparations. Crude homogenates (Mallov and Cerra 1966), microsomal and mitochondrial fractions (Alousi and Mallov 1964, Bjorntorp and Furman 1962), tissue slices (Hollenberg 1960) and acetone-dried tissue extracts (Korn 1959) have been used. The tissue preparation is incubated either with a chylomicron substrate using animal chyle as a source or with an artificial triglyceride emulsion such as Ediol. The artificial substrate must be activated by the addition of serum in order for optimum enzymatic activity whereas chylomicron-containing substrates do not require activation (Hollett 1964). Albumin is required in the incubation medium to act as an acceptor for free fatty acids (Korn 1959). The addition of heparin to the incubation mixture is used in some assays. Gartner and Vahouny (1966) found that the addition of heparin (30ug/ml) doubled LPL activity of heart homogenates. Greater LPL activity was found in heart homogenates by Bjorntorp and Furman (1962a) after pre-incubating the tissue with heparin (30ug/ml). Hollenberg (1960) added heparin (125ug/ml) to his incubation mixture when assaying heart homogenates and slices. The action of heparin on LPL is not known but it is thought that it may act as a component or a cofactor of the enzyme (Gartner and Vahouny 1966). There is also evidence that endogenous heparin may be responsible for

binding LPL at the capillary wall (Robinson 1963). A further description of the assay system used in the present study appears in the Methods section.

Myocardial lipid metabolism

It was shown as early as 1938 that the heart could utilize fatty acids as fuel (Bing 1965). Subsequent investigations have indicated that fatty acids are transported to the heart in the FFA-fraction of the plasma. Fatty acids are considered to be the most important fuel for the heart, providing more than 60% of the heart's energy requirements (Bing 1965). Using the isolated perfused rat heart it has also been estimated that about 60% of the carbon for oxidation is derived from lipids (Delcher et al. 1965). Bing has estimated that, in fasting man, there is an approximately equal extraction of free and of esterified fatty acids, with esterified contributing slightly more than 50%.

In addition to being able to utilize FFA in the plasma, the heart is believed to utilize the fatty acids of triglycerides (TG) associated with chylomicra and low density lipoproteins. It is possible that such triglyceride fatty acids (TGFA) may be a source of fatty acids for the heart under certain conditions (Delcher et al. 1965). TGFA may compete with other myocardial fuels such as FFA, lactate

and glucose (Opie 1969).

The possibility that chylomicron TG is hydrolysed in the circulating plasma has been examined and the findings indicate that this is probably not the case (Olivecrona 1962). In addition, there is no evidence for the intact passage of chylomicra across the vascular endothelium. It appears that TG associated with chylomicra are hydrolysed as they pass into the myocardial cells (Opie 1969). The proposed function of lipoprotein lipase is the facilitation of the passage of chylomicron TGFA into the cell (Robinson 1963) and the regulation of their supply to the cell (Robinson 1963, Enser et al. 1967).

Changes in activity of myocardial LPL occur under a variety of physiological and pathological conditions. Nutrional and endocrine factors are of major importance in the control of LPL activity. Myocardial LPL activity has been reported to be increased in the diabetic state (Kessler 1963) and in starvation (Hollenberg 1960), while decreased activity followed both high fat and high carbohydrate diets (Alousi and Mallov 1964). Exposure to cold (Grafnetter and Grafnetterova 1967) and exercise (Nikkila et al. 1963) have been shown to increase heart LPL activity. The administration of catecholamines increases LPL activity (Mallov and Cerra 1967), and hypercholesterolemized rabbits have been shown to have

elevated heart LPL levels (Huang and Kako 1970).

The mechanisms responsible for the observed changes in LPL activity occurring under various conditions have not, however, been characterized. For instance, the observed decreases in heart LPL activity following infarction and defibrillation are believed to be the result of an inactivation of the enzyme by disruption of electrostatic forces holding LPL to tissues (Kessler and Senderoff 1962). The rapid rise in myocardial LPL activity (15 min) after exercise was thought to be due to a stimulation of the enzyme already present rather than a change in its amount (Torsti 1965). In contrast to activation, Mallov and Cerra (1967) believe that the stimulation of heart LPL by epinephrine and alcohol is due to an increase in synthesis rate of the enzyme.

The enzyme, palmitylcarnitine acyl transferase (PCAT) converts long chain fatty-acyl CoA esters, which are impermeable to the mitochondrial membrane, to a carnitine derivative before they can be oxidized by the beta-oxidation process (Fritz 1967). The reaction catalysed by PCAT is as follows:

palmityl CoA + carnitine = palmitylcarnitine + CoA.

This was believed to be the rate-limiting reaction for fatty acid oxidation in the heart (Fritz 1967) although it has been recently questioned (Van Tol and Hulsmann 1969).

Under conditions of increased fatty acid supply the activity of this enzyme increases (Norum 1965). Some of the cases where plasma FFA are increased and heart PCAT activity is elevated are the same as when heart LPL activity is increased: in fasting or alloxan-diabetes, after fat-feeding (Norum 1965), and in the hyperthyroid state (Bressler and Wittels 1966). Under conditions of depressed fatty acid oxidation PCAT activity was found to be decreased (Fritz 1967). Fritz has hypothesized that hormones which affect myocardial fatty acid oxidation rates do so by influencing the activity of PCAT.

Action of thyroid hormones on lipid enzymes

It is known that LPL activity is elevated in the hyperthyroid rat heart (Alousi and Mallov 1964). Furthermore, it was demonstrated by Torsti (1965) that a single toxic dose of thyroxine (T_4) caused a significant increase in rat heart LPL activity after 24 hours. Alousi and Mallov proposed that the elevated myocardial LPL levels found in hyperthyroid rat heart are due to relatively slow changes in enzyme synthesis resulting probably from adaptations of the heart in accordance with its requirements for fatty acids.

Palmitylcarnitine acyl transferase activity was shown to be increased in the hyperthyroid heart (Bressler

and Wittels 1966). These workers proposed that the increase in activity was due to the increased supply of plasma FFA as a result of thyroxine's FFA-mobilizing action on adipose tissue rather than a direct effect of the hormone on PCAT.

The stimulation of adipose tissue lipolysis by thyroid hormones is believed to be due to an activation of the enzyme adipose tissue lipase. It appears that thyroxine directly stimulates adenyl cyclase synthesis in adipose tissue. As a result of this increase the level of cyclic 3',5'-AMP is elevated and the inactive form of adipose tissue lipase is converted to the active form at an increased rate. Consequently, the rate of lipolysis is elevated (Krishna et al. 1968). Further discussion of adipose tissue lipase and the adenyl cyclase system appears in later sections.

It was proposed that increases in hepatic cholesterol biosynthesis of rats as a result of thyroxine administration are due to elevated rates of synthesis of the enzymes, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Kritchevsky 1964). Indeed, there is mounting evidence that the primary action of thyroxine is on the synthesis of key, rate-limiting enzymes (Tata 1964). There is evidence that both the growth-promoting action and the calorigenic action of thyroid hormones are due to increases in protein (enzyme) synthesis (Tata 1964). Four inhibitors of protein synthesis

that act through different mechanisms were shown to inhibit the aforementioned actions of thyroxine. Enzymes such as succinoxidase, cytochrome oxidase and alpha-glycerophosphate dehydrogenase (alpha - GPDH) have been shown to have elevated activities in the livers of hyperthyroid rats and increases were due to increased protein synthesis (Price 1964). The increase in alpha-GPDH activity after thyroid hormones has been further shown to be preceded by the formation of messenger-RNA, a step indicating protein synthesis (Lee and Miller 1967). Tata and co-workers (1962) have shown that the stimulation of basal metabolic rate (BMR) after acute thyroid hormone administration was preceded (by several hours) by an enhanced turnover of nuclear RNA and a rise in the DNA-dependent RNA polymerase activity of rat liver nuclei. It was suggested by Tata (1964) that the genetic regulation of protein synthesis may be an important locus of action of thyroid hormones.

Thyroid-catecholamine interaction

The possibility that the sympathomimetic amines participate in the stimulatory action of thyroxine on heart LPL necessitates the examination of the complex field of thyroid-catecholamine interrelationships. Only an introductory examination of this field is attempted here.

i) Evidence for thyroid-catecholamine interaction

Two of the major lines of evidence for a thyroid-catecholamine interaction are the similarity of physiological effects and the potentiation of some responses to catecholamines by thyroid hormones (Waldstein 1966).

Thyroid hormones and the sympathomimetic amines have many common physiological effects. Epinephrine mimics some but not all of the actions of thyroxine. Some of these similar effects are increased heart rate, blood pressure, cardiac output, basal metabolic rate, accelerated lipolysis in adipose tissue and elevated plasma FFA. However, aspects of metabolism such as growth and maturation, which are profoundly influenced by thyroid hormones are unaffected by catecholamines (Waldstein 1966).

The main indication of an interaction between thyroxine and the sympathomimetic amines stems from the apparent potentiation of some of the actions of catecholamines by thyroid hormones. For instance, both thyroid hormones and catecholamines have been shown to elevate rat cardiac phosphorylase a activity (McNeill and Brody 1968). However, in rats treated with thyroid hormones, a potentiation of the norepinephrine-induced increase in phosphorylase a activity has been demonstrated (Hornbrook et al. 1965, McNeill and Brody 1968). Although Hess and Shanfeld (1965) could not show potentiation of epinephrine's effect

on phosphorylase a in thyroxine-treated rats, they felt that catecholamines were essential for thyroxine to exert its maximum stimulatory effect on the enzyme.

Brewster and his co-workers(1956) observed that the hemodynamic effects of norepinephrine and epinephrine were potentiated (in dogs) by thyroid hormones. However, more recent studies have proven to be contradictory. Van der Schoot and Moran (1965) could not show potentiation of the effects of catecholamines on the rate and force of contraction of isolated, spontaneously contracting atria from hyperthyroid rats and rabbits. Also, in a study on contractility, it was demonstrated that papillary muscles from hyperthyroid cats were not hypersensitive to the effects of added norepinephrine, indicating that this action of thyroid hormone was not dependent on catecholamines (Buccino et al. 1967).

ii) Modes of thyroid-catecholamine interaction

A brief presentation of three of the proposed modes of thyroid-catecholamine interaction will be given. One of the popular proposals which attempted to explain catecholamine potentiation by thyroid hormones concerns the alteration of receptor sites. It was held that thyroid hormones induced a hypersensitivity of the catecholamine receptor site with the result that the response to catecholamines was enhanced (Hornbrook et al.1965,

McNeill and Brody 1968). The potentiation of catecholamines by thyroxine on phosphorylase a was explained on this basis by Hornbrook et al. (1965). However, in the hemodynamic studies by van der Schoot and Moran (1965) in which potentiation was not found it was proposed that the inducement of hypersensitivity of receptor sites by thyroid hormones was unlikely as a mechanism for thyroid-catecholamine interaction.

In addition to the receptor site proposal, another possible mechanism of thyroid-catecholamine interaction which has received attention is the possibility that either thyroxine interferes with catecholamine catabolism or that catecholamines hinder thyroid hormone production or catabolism (Galton 1965). There does not appear to be much supporting evidence for the hypothesis that thyroxine hinders the destruction of catecholamines thus altering their effective concentration. The activities of monoamine oxidase (MAO) and catechol-o-methyl transferase, two of the prime enzymes concerned with catecholamine catabolism, have been reported to be decreased following thyroid hormone administration. However, such decreases are not considered of major consequence with respect to a thyroxine-catecholamine interaction (Hornbrook et al. 1965). Furthermore, consistent decreases in MAO activity have not been demonstrated after thyroid hormone treatment (D'Iorio and

Leduc 1960). A strong line of evidence against the theory that thyroxine hinders catecholamine catabolism is the finding that norepinephrine concentrations in hearts of hyper-and hypothyroid cats were not altered (Buccino et al. 1967).

Although it was shown that catecholamines increased the hepatic deiodination of thyroxine (T_4) in rats (Galton 1965), the significance of this finding with respect to a mechanism of interaction between the hormones is unknown (Waldstein 1966). Also, the effects of epinephrine on thyroid hormone secretion rate have been inconsistent (Hays and Solomon 1969). In his review on thyroid-catecholamine interrelations, Waldstein (1966) concluded that there was no substantial evidence of a direct effect of catecholamines on thyroid function or of thyroid hormones upon catecholamine production or metabolism.

One of the most promising theories of thyroid-catecholamine interaction is that involving the adenyl cyclase system. The so-called "second messenger", cyclic 3',5'-AMP, produced by the action of adenyl cyclase has been implicated in the mechanism of various hormone-enzyme interactions. A hormone such as thyroxine acts at the target tissue on the intracellular level of 3',5'-AMP. As a result of a change in the level of the second messenger the physiological response is brought about (Sutherland

et al. 1968). In adipose tissue, the hormones norepinephrine, epinephrine, ACTH, glucagon, LH and thyrotrophin are all believed to increase lipolysis by increasing levels of 3',5'-AMP (Kuo and DeRenzo 1969). Thyroid hormones have been shown to increase lipolysis in adipose tissue by interacting with the sympathomimetic amines at the level of 3',5'-AMP. Recent evidence indicates that thyroxine, by stimulating adenyl cyclase synthesis, renders adipose tissue lipase more sensitive to the action of endogenous catecholamines thereby increasing lipolytic activity (Krishna et al. 1968). As a result of thyroxine's action upon adipose tissue lipase plasma FFA levels are elevated.

In the heart, the positive inotropic, chronotropic and vasodepressor effects of sympathetic stimulation are thought to be mediated by increases in the intracellular level of 3',5'-AMP (Sutherland et al. 1968). Whether thyroxine interacts with catecholamines via adenyl cyclase in the heart, as it has been proposed for adipose tissue is not known. The recent finding of two separate adenyl cyclase systems in cat heart (Levey and Epstein 1969), one responsive to catecholamines and one to thyroid hormones, may lead to an explanation of the apparent interaction between these hormones. Further discussion of the possible role of adenyl cyclase in the action of thyroid hormones appears in a later section.

Although evidence exists indicating an interaction between thyroid hormones and catecholamines and various mechanisms have been proposed, a satisfactory explanation of such an interaction cannot be made at the present time.

Experimental protocol

The aim of this experiment was to observe the effects of thyroxine administration upon myocardial lipoprotein lipase activity in relation to the changes brought about in plasma free fatty acid levels and total body oxygen consumption. This study was undertaken to learn the mechanism of the action of thyroid hormone on LPL. A time course of changes in the activity of myocardial LPL, level of plasma FFA and total body oxygen consumption over 67 hours was determined after a single injection of three different doses of triiodothyronine to hypothyroid rats. In order to investigate the possibility of catecholamine mediation of the T_3-induced increases in LPL activity, plasma FFA levels and oxygen consumption, the effect of reserpine pretreatment was examined. The effect of reserpine on LPL was further studied in normal rats, together with its effect on the elevation of LPL activity following epinephrine injection.

Since the stimulation of heart LPL by thyroxine is similar to the elevation caused by catecholamines

(Alousi and Mallov 1964), the study of the second myocardial enzyme, palmitylcarnitine acyl transferase, was thought to be desirable in order to see a direct action of the hormone on the heart. In addition, the action of thyroid hormone on myocardial LPL cannot be stated with complete assurance since the enzyme has not been purified and the activity determined may include other enzymes. By showing changes in PCAT activity after thyroid hormone administration it could be stated with assurance that the hormone was acting on a specific enzyme concerned with fatty acid metabolism, since the characteristics of PCAT have been well-defined. In the case of LPL, such a statement cannot be made until the enzyme has been purified and more precisely characterized.

Many reports dealing with the effects of thyroid hormone examine its chronic effects. Since the period of chronic hormone administration is often longer than the latent period when a single dose is used it is impossible to determine which effects are manifestations of the primary action of the hormone and which are secondary or more remote actions (Tata 1964). By using a single dose some idea of the chronological order of events occuring may be learned. Furthermore, since the hypothyroid animal is more sensitive to the actions of exogenous thyroid hormone than the euthyroid animal, the physiological effects

of the hormone can be more clearly studied. A single dose of T_3 has a shorter latency and greater height of action than T_4 (Tata 1964) and consequently its administration to a hypothyroid animal provides a good indication of the physiological action of the hormone. Consequently, the effects of acute injection of T_3 to hypothyroid rats were examined.

METHODS

Animals

Male Wistar rats weighing 190-250g were used in the experiments (Robidoux Farms, Montreal). Rats were housed in individual wire cages and had tap water *ad libitum*. Control rats were fed on Master Lab Cubes in cube and in powdered form, *ad libitum*. It was found that growth rates differed slightly in the first week or so between rats on cubes and on powdered food. However, in the time required to produce hypothyroidism (minimum of 25 days) no significant difference in growth rates was observed between the rats on cubes and on powdered food. Consequently, cubes were used as the control diet. Surgically thyroidectomized rats were obtained from Robidoux Farms at approximately 120g. Various diets, to be described, were employed.

Triiodothyronine administration

Triiodothyronine (T_3)(sodium salt: Sigma), dissolved in saline, pH adjusted to 10.5 with sodium hydroxide, was injected intraperitoneally into hypothyroid rats at doses of 50, 125 and 250ug/100g body weight. Oxygen consumption was measured within 6 hr prior to T_3 injection. At 6, 12, 20, 44 and 67 hr after injection oxygen consumption was measured, plasma FFA and heart LPL activity were determined. Hypothyroid rats injected with alkaline saline served as controls.

Samples of heart homogenates from rats injected with 50ug T_3/100g were used for palmitylcarnitine acyl transferase activity determinations at 6, 12, 20, 44 and 67 hr after injection.

Reserpine and T_3 administration

Reserpine, dissolved in 10% ascorbic acid and injected intramuscularly, was administered to hypothyroid rats at doses of 0.2 and 1.0 mg/kg body weight. Eighteen hours later, after oxygen consumption was determined, T_3 was injected (ip) at a dose of 125ug/100g body weight. After 20 hr oxygen consumption, plasma FFA and LPL activity were measured. Values from reserpine-injected hypothyroid and saline-injected hypothyroid rats were also determined.

Reserpine and epinephrine administration

In a brief experiment, reserpine was injected intramuscularly into normal rats at doses of 0.2, 1.0 and 3.0 mg/kg. After 38 hr epinephrine suspended in peanut oil (Parke-Davis) was injected (im) at a dose of 0.5 mg/kg. Three to four hours later heart LPL activity was determined. Normal rats received peanut oil vehicle alone.

Inducement of hypothyroidism

a) Surgically thyroidectomized rats were put both on normal lab cubes (powdered and cubed) and on low iodine powdered diet (Remington Low-Iodine Test Diet, Nutritional Biochemicals).

b) Normal rats were administered the anti-thyroid drug, 6-propyl-2-thiouracil (PTU, Nutritional Biochemicals) mixed with powdered lab cubes in the proportion: 0.05 g PTU per 100 g diet. It was found that the drug did not mix uniformly with the powdered food in large quantities. Thus, PTU was measured per beaker of powdered lab cubes and mixed individually in each beaker so that the drug could be administered consistently and accurately.

c) Normal rats were injected with a single intraperitoneal dose of 800uc I^{131} per 100 g body weight (at approximately 150 g weight). These rats were put on a normal lab cube diet.

Rats were considered hypothyroid when oxygen consumption was at least 30% below control values. Initially, oxygen consumption determinations were taken every few days to determine the number of days required to produce the 30% decrease.

Oxygen consumption measurement

Oxygen consumption of the rat was measured in a metabolic cage described by Watts and Gourley (1953). The animals were anaesthetized by an intraperitoneal injection of Nembutal (sodium pentobarbital) at a dose at 40mg/kg. It was found by previous users of this particular apparatus that non-anaesthetized rats would not remain quiescent in the cage long enough for oxygen consumption measurements to be taken. Thus, the values for oxygen consumption obtained are somewhat lower than true basal metabolic rate values.

The procedure for measuring oxygen consumption involves flooding the cage with oxygen after the rat has been placed within and closing the cage with a large rubber stopper with a graduated pipette barrel inserted at one end. A soap bubble is placed at the open end of the pipette and the time is recorded for the bubble to move 5 ml along the pipette. A period of about 2 minutes is required for the animal to equilibrate in the oxygen-filled chamber. At least 4 separate readings are taken before the animal is removed. The oxygen consumption of up to 3 animals can be measured simultaneously. This method, although simple, provided reliable and consistent results. In over 400 oxygen consumption determinations only 8 animals died in the cage or shortly after having been removed.

Lipoprotein lipase assay

Rats were anaesthetized with an intraperitoneal injection of Nembutal (40mg/kg). After oxygen consumption determinations were taken and the animals were to be sacrificed, the abdomen was opened and 3 ml blood withdrawn into a heparinized syringe for FFA determination. While applying pressure to the cut abdominal aorta to reduce blood loss the thorax was quickly opened and the heart rapidly excised and placed into ice-cold Krebs Ringer Phosphate buffer (KRP), pH 8.5. The hearts were trimmed of atria and fat, blotted and weighed on a torsion balance. Approximately 5 ml of fresh KRP was used as the homogenizing medium for ventricles weighing between 350 and 500 mg. Some hearts were frozen in liquid nitrogen after weighing and used after periods of up to 48 hours. It was found that such freezing had no significant effect on LPL activity. Heart homogenates were also frozen (-10°C) overnight on occasion with no apparent change in enzyme activity. The heart ventricles were finely minced with scissors and homogenized using glass homogenizing tubes and Teflon pestles. All vessels were packed in ice at all times.

Protein content of the homogenates was determined by the method of Lowry et al.(1951) The volume of homogenate used in the incubation mixture was 0.5 ml which

corresponded to approximately 14mg protein per ml homogenate. The incubation medium, which was a modification of that of Alousi and Mallov (1964), consisted of the following:

2.7 ml of 5% bovine serum albumin (Sigma fraction V)
2.7 ml serum from dogs fasted overnight
0.6 ml of a coconut oil emulsion containing
5% Ediol (Calbiochem)
0.5 ml heparin (30 ug/ml medium)

It was found that when sera from different dogs were used the LPL activity values shifted considerably. Consequently, as many determinations as possible and especially from the same series of experiments were done using the same dog sera. Furthermore, LPL activity was expressed as per cent of paired control values so that shifting values due to changes in sera were taken into account. Since Ediol contains mono-and diglycerides as well as triglycerides (Greten et al. 1969) this assay system may determine the activities of other lipases in addition to LPL.

Heparin, which was added to a similar incubation mixture by Gartner and Vahouny (1966) was found to double LPL activity of homogenates. Heparin, at a concentration of 30 ug per ml incubation medium provided maximal enzyme activity, and thus was used in the present procedure. The pH of Ediol, albumin and KRP was adjusted to 8.5 with ammonium hydroxide. Incubations were carried out for 1 hour

at 37°C. Aliquots of 1 ml were taken at the beginning of and after one hour's time to be used for FFA determination. The amount of FFA liberated in one hour was determined by the method of Goss and Lein (1967), which will be described shortly.

In order to determine whether there was a direct relationship in the assay system between the amount of enzyme and amount of FFA released during incubation, various volumes of homogenate from the same source were incubated for one hour and the amount of FFA liberated by each volume determined. Also, samples from the same homogenate were incubated for 30, 60 and 90 minutes to ensure that there was a linear relation between time and FFA liberated for periods up to the 1-hour incubation time used in the assay procedure.

Plasma free fatty acid determination

Plasma FFA were determined by the method of Goss and Lein (1967). This modification of the more standard Dole method (Dole 1956) involves a one, rather than two-phase system for titration and employs phenolphthalein rather than thymol blue as an indicator. The procedure for the measurement of plasma FFA consists basically of two steps: extraction of the FFA with isopropanol-heptane-1N H_2SO_4 (20:5:1,v/v/v), and titration of the extracted

FFA with sodium ethoxide. The visual determination of the end-point was found to be much easier, and results more consistent using the one-phase system of Goss and Lein than in the 2-phase Dole procedure. In order to check the reliability of the method, known amounts of palmitic acid were extracted from heptane and from plasma and the percentage recovery of the added acid was determined.

It has been shown that this procedure for determining FFA does not measure short chain organic acids, since the addition of lactic acid had no effect on the titration of fatty acids (Goss and Lein 1967).

<u>Reliability of lipoprotein lipase assay method</u>

In an effort to ensure that the LPL assay method was able to show changes in LPL activity, other means which have been reported to increase LPL activity besides the one to be examined in these experiments were employed. One of the most reliable means of increasing heart LPL activity is to induce hyperthyroidism by the chronic administration of thyroid hormone. A brief experiment was carried out using three triiodothyronine-injected rats and two saline-injected controls. Oxygen consumption was measured at the beginning and after the end of 20 days of daily (ip) injections of 50 ug T_3 per 100 g body weight. Heart LPL was determined after the 20 days. The results

(Table I) show the oxygen consumption of two[1] T_3-injected rats after 20 days of injections as per cent of control rat oxygen consumption. Heart LPL is similarly expressed as per cent of control. The hearts of the T_3-injected, hyperthyroid rats were noticeably enlarged and the LPL activity was, as in the case of Alousi and Mallov (1964), increased.

TABLE I

Heart lipoprotein lipase activity and total body oxygen consumption of normal rats after 20 daily intraperitoneal injections of triiodothyronine (50ug/100g).

Animal	Oxygen Consumption (per cent of control)	Heart LPL activity (per cent of control)
1	240	212
2	203	230

It was reported by Mallov and Cerra (1967) that heart LPL activity in rats increased within 3 hours after ethanol injection. Such a rapid means of producing increased LPL activity seemed to be a valuable means of checking the

1. One T_3-injected rat died on the 18th day.

LPL assay method. A few experiments were carried out with normal rats using intraperitoneal injections of 25% and 50% ethanol; LPL activities were determined 5 hours later. Results are indicated in Table II. The results indicate that ethanol did cause heart LPL activity to increase, as expected and the assay system indicated this increase.

TABLE II

Heart lipoprotein lipase activity 5 hours after a single intraperitoneal injection of ethanol.

Dose of Ethanol	Heart LPL activity (per cent of control)
1 ml, 50%	163
	167
	240
2.5 ml, 25%	74
	100

The LPL-stimulating effect of ethanol was examined using rabbits also (Kikuchi and Kako 1970). Ethanol infusions (74 mg/min for 12 min and then 37 mg/min for 102 min) were given and heart homogenate LPL activity determined from 2 to 5 hours after the end of the infusion periods. Ethanol was

found to cause a decrease in rabbit heart LPL activity although not significant (Fig 1). These results were unexpected since ethanol appears to increase rat myocardial LPL activity in a consistent manner. However, it has been reported that an intramuscular injection of epinephrine which was effective in raising rat heart LPL activity in rats produced variable results in rabbits when given at the same dose (Mallov and Alousi 1969). It is known that an acute, toxic dose of ethanol raises the plasma catecholamine level (Perman 1960). That the activity of heart LPL is increased by catecholamines has been shown (Alousi and Mallov 1964). Thus, it is possible that the action of ethanol observed in rats, but not in rabbits, is due to a catecholamine - mediated increase in lipoprotein lipase activity.

A third method of increasing heart LPL activity is reported to be fasting (Hollenberg 1960). A few experiments were carried out to see if fasting did produce a reliable increase in LPL activity in the rat heart as measured by the assay system to be used in further experiments. It was found that only one of four rats fasted about 96 hours showed any significant increase in heart LPL activity (155% of fed control). The few number (3) of rats fasted for 48 hours showed no increased lipase activity. Alousi and Mallov (1964) found increased activity

FIGURE 1.

Ethanol was infused at 74 mg/min for 12 min, then 37 mg/min for 102 min. Heart homogenate LPL activity was determined 2 to 5 hr after the end of the infusion period. Indicated are the means ± S.E. together with the number of experiments.

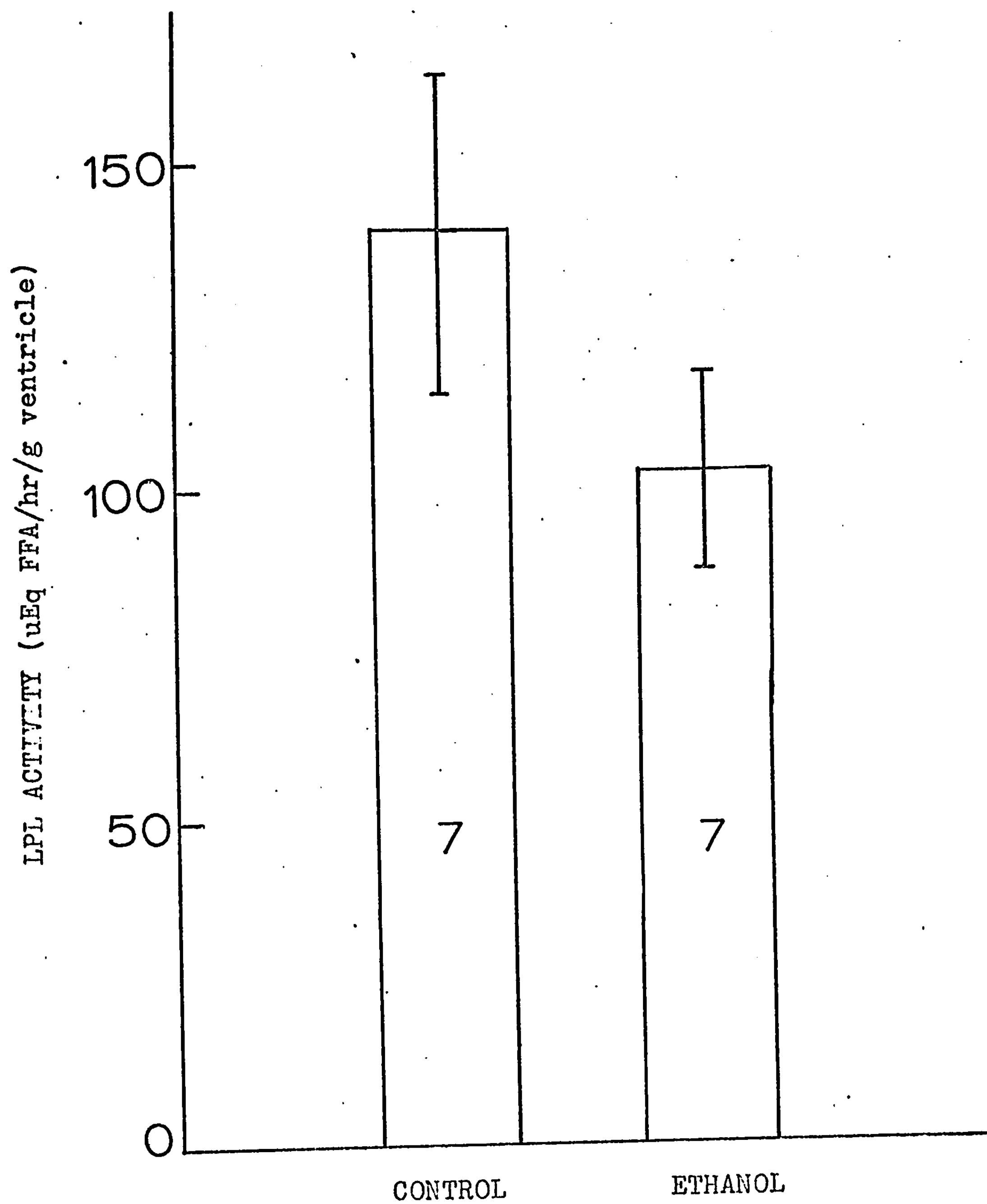

Fig. 1. Effect of ethanol on rabbit heart lipoprotein lipase (LPL) activity.

in heart homogenates only after 96 hours of fasting whereas Hollenberg (1960) found increases after 48, 72 and 96 hours. The phenomenon, however, does not seem clear cut since other experiments, besides those done in this lab, have not been able to verify the results of Hollenberg. Robinson and Jennings (1965) for example, found a decrease in heart LPL activity after 48 hours of fasting. An increase within 10 hr has also been shown (Borensztajn et al. 1970).

Thus, of those examined, the only seemingly reliable method to increase heart LPL activity is to produce a hyperthyroid animal. Since thyroid hormone treatment was to be used in the present investigation and since favorable results were obtained consistently, the assay system was considered reliable in showing increases in LPL activity. The expected increase found in LPL activity of rat heart after ethanol injection further supported the reliability of this assay procedure.

Palmitylcarnitine acyl transferase assay

Palmitylcarnitine acyl transferase (PCAT) activity was measured using samples from the same homogenate used for LPL determination. A modification of the ^{14}C-isotope-exchange method of Norum (1964) was used to measure PCAT activity. Hearts were homogenized in KRP buffer, pH 8.5, the same medium used for the LPL assay. The PCAT assay

involves incubating the tissue in a medium containing ^{14}C-carnitine and measuring the incorporation into ^{14}C-palmitylcarnitine.

The incubation medium contained the following:

DL-palmitylcarnitine, 0.5mM (prepared by the method of Brendel and Bressler 1967).

Co enzyme A (Sigma) 0.12mM

^{14}C-carnitine (Tracerlab) 0.25mM, 0.3uc/ml

glutathione (GSH, Sigma) 5mM

Tris 10mM pH7.5

Final volume of the incubation mixture was 1 ml. Incubations were carried out at 30°C for 15 min. Approximately 0.3mg tissue protein was incubated. This was obtained by diluting homogenates eight times with KRP and using a final volume of 0.2ml diluted homogenate containing about 0.3mg protein. The reaction was stopped with 0.1ml concentrated hydrochloric acid. Palmitylcarnitine was extracted with butanol as follows: 0.9ml water and 1.0ml butanol were added to the stopped reaction. The test tube was mixed on a Vortex mixer and then centrifuged at 2500 rpm for 10 min. A 0.4ml aliquot of the upper, clear butanol phase was carefully removed and added to 0.4ml butanol-saturated water. This mixture was shaken and allowed to settle into 2 phases. A 0.1ml aliquot of the upper phase was put into a liquid scintillation vial and dried using

mild heating in an atmosphere of nitrogen; 0.25ml of methanol was used to dissolve the sample and 10ml POPOP-PPO-toluene was added. Samples were counted in a Nuclear Chicago liquid scintillation counter using the channels ratio method.

Units of measurement

Oxygen consumption was measured in ml oxygen/hr/g body weight; plasma FFA in uEq FFA/ml plasma; LPL activity in uEq FFA/hr/g wet weight of ventricle; PCAT activity in DPM/mg protein. Oxygen consumption values were expressed as percentage change from time of injection of T_3 to sacrifice. Plasma FFA, LPL and PCAT activity were expressed as percentage of controls.

Values are given as averages with S.E. of mean unless otherwise indicated. Student's t test was used for testing for the significance of the difference between averages.

RESULTS

I METHODOLOGY

Inducement of hypothyroidism

Figure 2a shows the weight gain in 40 days of surgically thyroidectomized rats on a normal diet (Master Lab Cubes). Normal rats at the same initial weight (150-170g, n=5) gained 159.3±4.0 g (mean ± SE) whereas the operated animals' gain was 161.8 ± 14.6g (n=4).

Thyroidectomized rats on Remington Low-Iodine Test Diet gained less weight than normal rats (Fig. 2b) although not significantly. Initial weights of operated and control rats were 100-120g.

The effect of a 0.05% PTU diet on growth of normal rats is shown in Figure 2c. Animals on a normal diet gained an average of 146.2 ± 16.2g in 30 days (n=5) whereas rats on the PTU diet gained 32.7 ± 3.7g (n=13). Although rats on the PTU diet ate less than control rats they ceased to gain weight after about 30 days.

Although normal control rats on the powdered form of Master Lab Cubes gained weight more rapidly during the first week or so in the cages, the growth rate over a longer period of time was not significantly different from that of rats on cubes (Fig. 3). Thus, since cubes provided

a more convenient method of feeding, they were employed as the control diet for rats on the 0.05% PTU diet.

Oxygen consumption

The measurement of oxygen consumption was used to determine the degree of hypothyroidism (Fig. 4). Surgically thyroidectomized rats on a normal diet showed a slight decrease in oxygen consumption over a period of 50 days. The oxygen consumption of surgically thyroidectomized rats on the low-iodine diet was recorded after 32 days and was found to be only slightly below that of thyroidectomized rats on a normal diet. Since the required criterion for a hypothyroid animal was at least a 30% decrease in oxygen consumption the method of surgical thyroidectomy with normal and with low-iodine diet was abandoned.

Rats on the powdered lab cube diet containing 0.05% PTU showed a rapid decrease in oxygen consumption. Consistent decreases of at least 30% below the lower limit of normal levels were obtained after 25 days on the PTU diet. The lower limit of the normal range (1.05 ± 0.15, n=12) was found to be 0.90 ml oxygen per hour per gram body weight.

A single intraperitoneal injection of 800 uc I^{131} per 100g body weight resulted in an oxygen consumption of 0.58 ± 0.03 ml oxygen/hr/g (n=8) after 22 days on the normal

FIGURE 2

Growth of rats on different procedures for inducement of hypothyroidism. Control rats were normal in each weight group and had normal diet. Mean ± S.E. and number of animals are indicated.

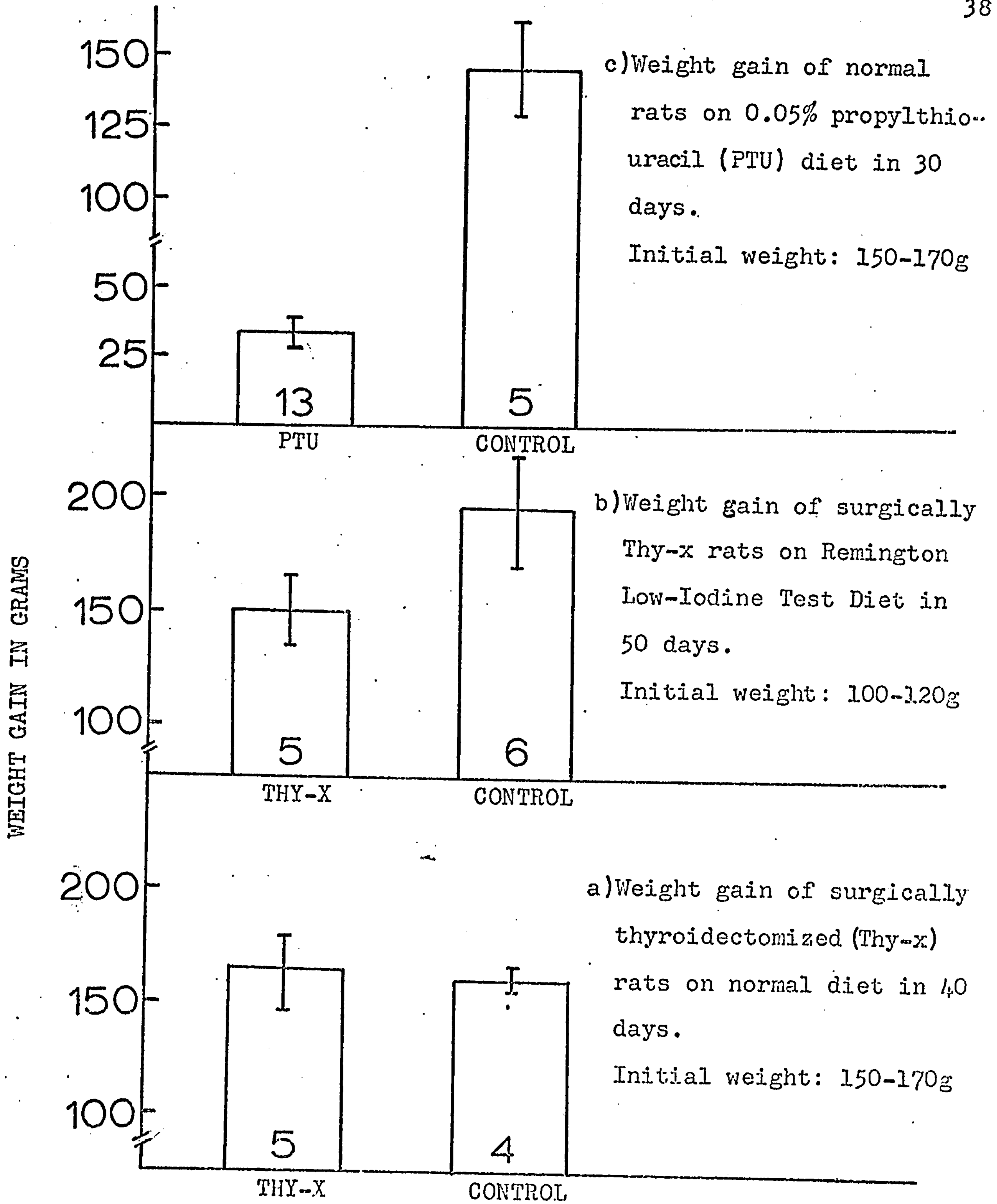

Figure 2

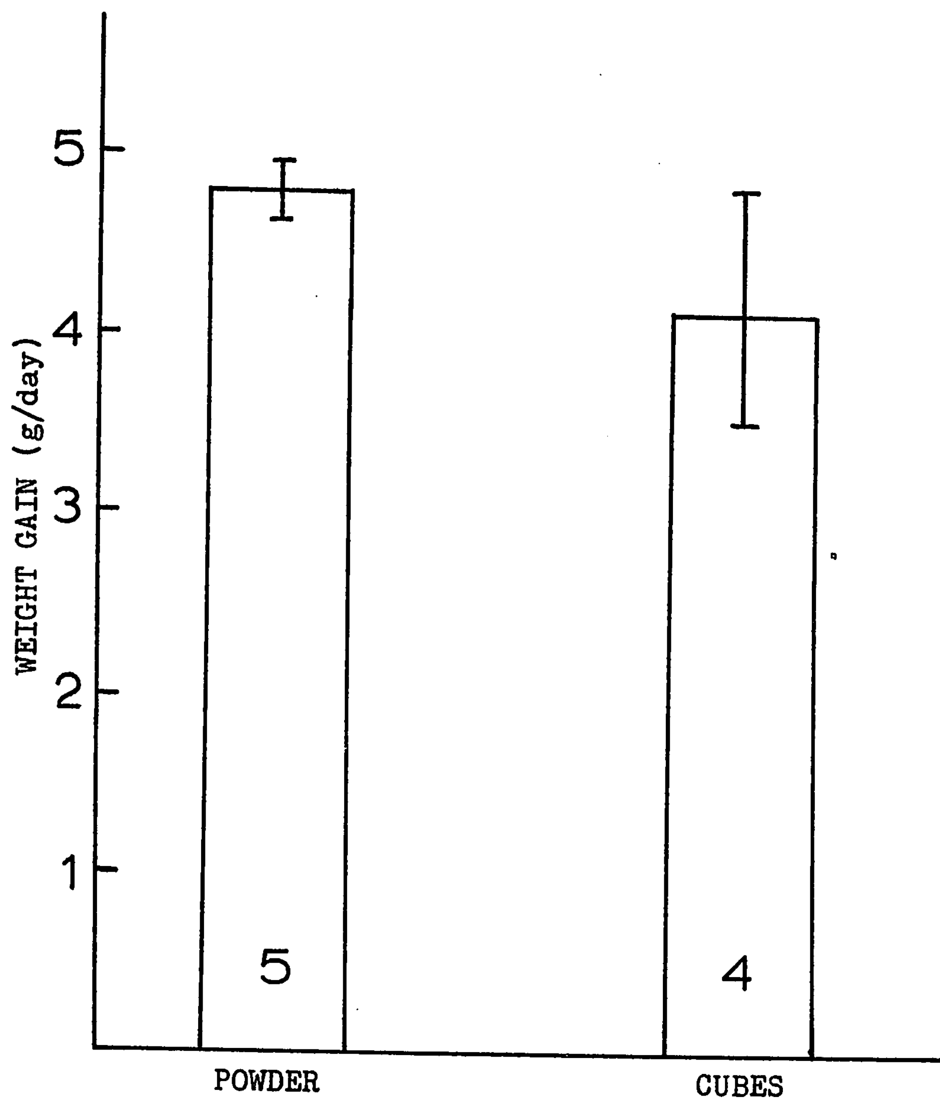

Fig. 3. Growth rate of normal rats on powdered and cubed lab feed over 40 days. Initial weight: 120-150g. Mean ± S.E. and number of animals are shown.

FIGURE 4

○ Surgically thyroidectomized rats on normal diet (n=6-8).

▲ Surgically thyroidectomized rats on Remington Low-Iodine Diet (n=5).

● Normal rats on 0.05% propylthiouracil diet (n=5-9, except 25 day: n=26, 40 day: n=19).

△ Normal rats injected with a single intraperitoneal dose of 800uc I^{131}/100g body weight (n=8).

Means ± S.E. are shown.

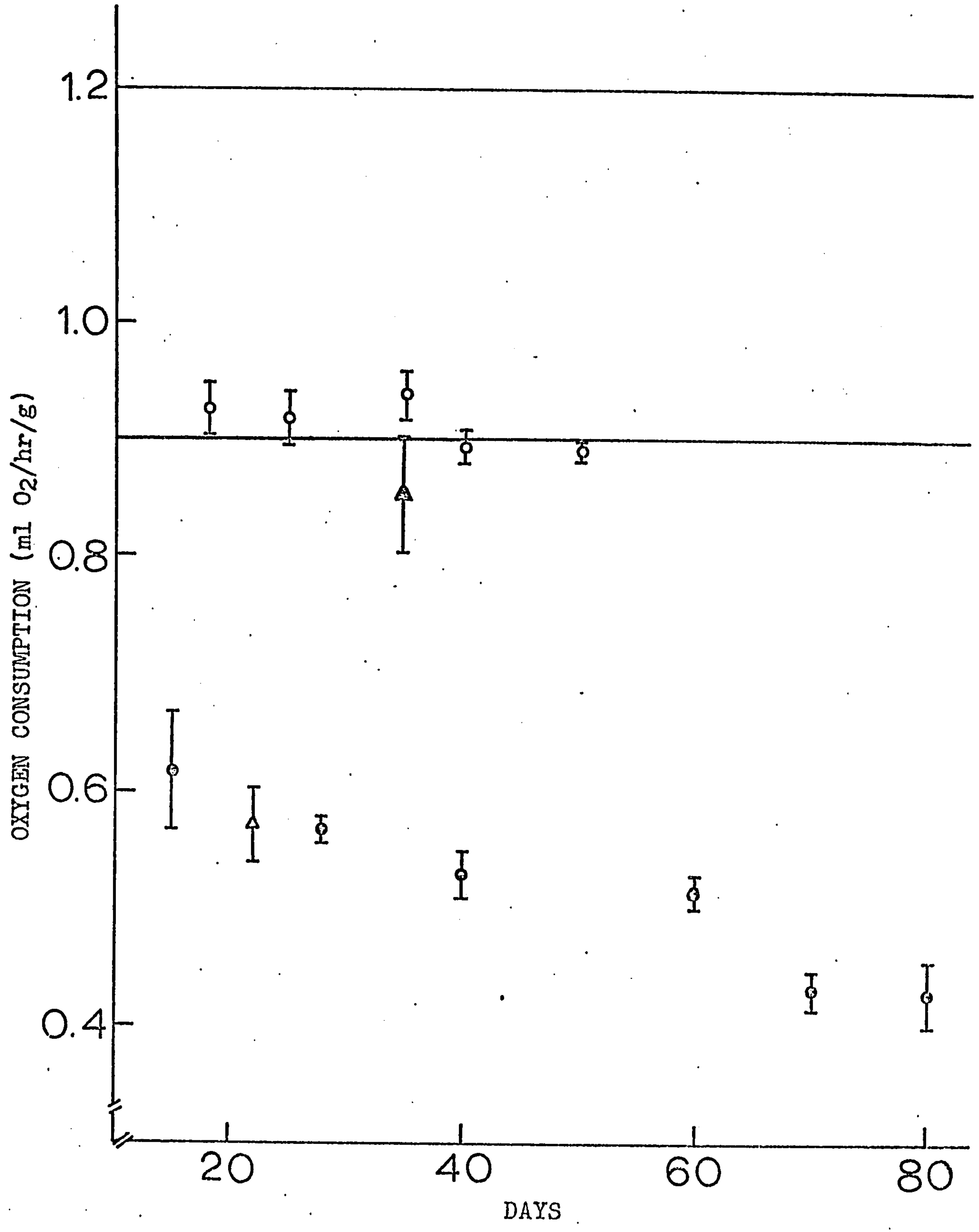

Fig. 4. Oxygen consumption of rats under various treatments for inducement of hypothyroidism.

lab cube diet.

Lipoprotein lipase assay

Various volumes of heart homogenate were incubated in the medium for 1 hour (37°C) as described in the Methods section. A typical relationship between lipase activity and amount of lipase present is shown in Figure 5. A linear relationship was found for volumes up to 1.0 ml; 0.5 ml was the volume employed in the assay.

The relationship between LPL activity and incubation time is shown in Figure 6. Volumes of 0.5 ml homogenate were incubated for 30, 60 and 90 min at 37°C and the amount of FFA released was determined. A linear relation was found up to 60 min of incubation. This was the period used for the LPL assay.

Free fatty acid determination

In order to determine the reproducibility of the method of Goss and Lein (1967), a known amount of a palmitic acid standard was added to heptane and to rat plasma and the percentage recovery of the acid was calculated. Table III shows that our results compare favourably with those obtained by Goss and Lein.

TABLE III

Recovery of added palmitic acid standard from heptane and rat plasma

	Percentage recovery of palmitic acid standard	Percentage recovery by Goss and Lein (1967)
From heptane	90.7 ± 0.5 (n=7)	98.6 ± 1.6 (n=12)
From rat plasma	104.2 ± 2.3 (n=6)	103.0 ± 2.7 (n=14)

Palmitylcarnitine acyl transferase assay

A linear relationship was found between heart homogenate PCAT activity and incubation time up to 15 min (Fig 7); this incubation time was used in the assay. High radioactivity was found in samples without tissue and in samples with tissue but not incubated. Primarily because of this reason further studies on PCAT were discontinued.

FIGURE 5

Each point represents the average of duplicate determinations of heart homogenate incubated 1 hr at 37°C. Protein content of homogenate was 14 mg/ml.

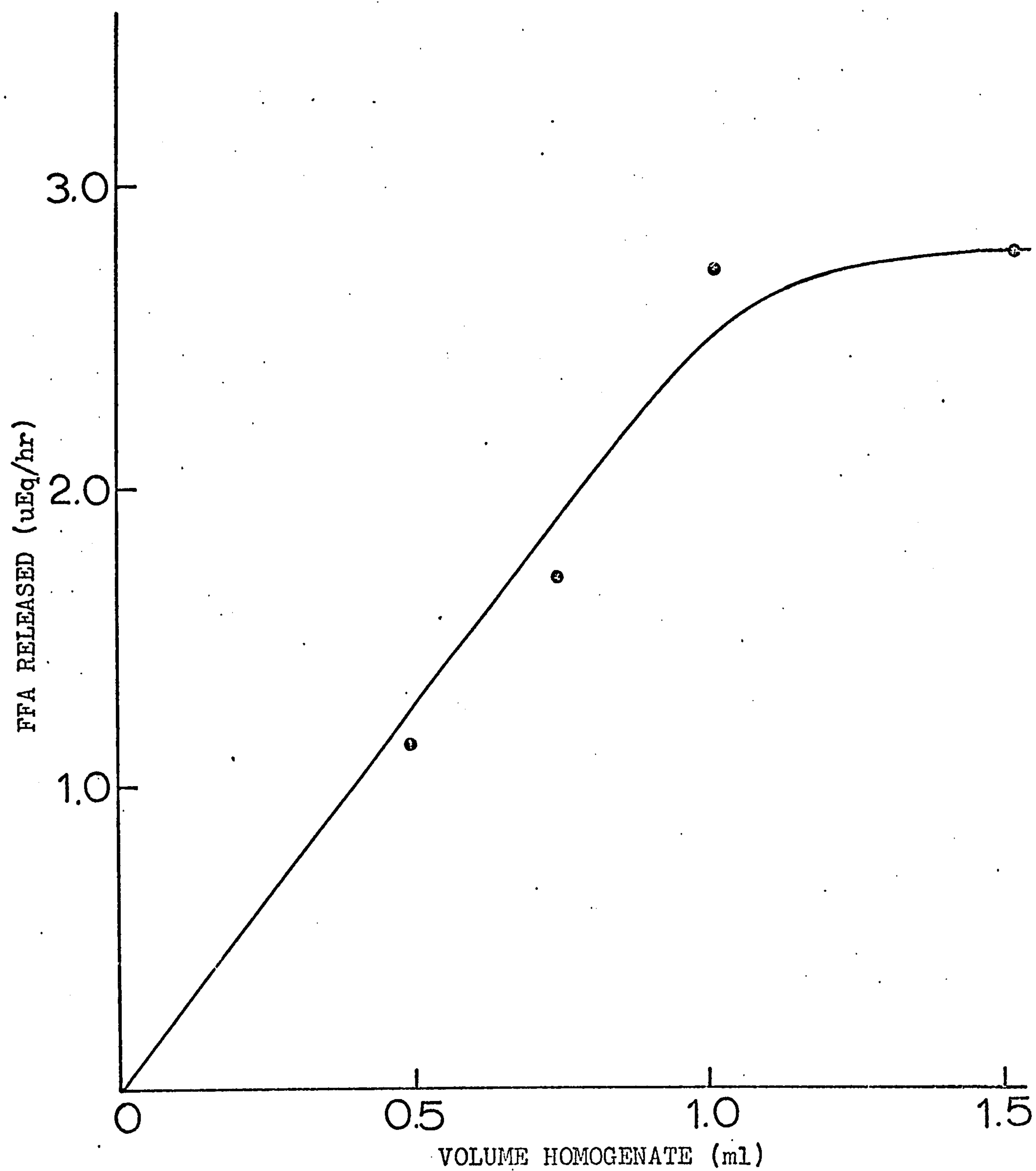

Fig. 5. Typical relationship between volume of heart homogenate and activity of lipoprotein lipase.

FIGURE 6

Aliquots of 0.5 ml homogenate were incubated at 37°C. Each point represents the average of duplicate determinations.

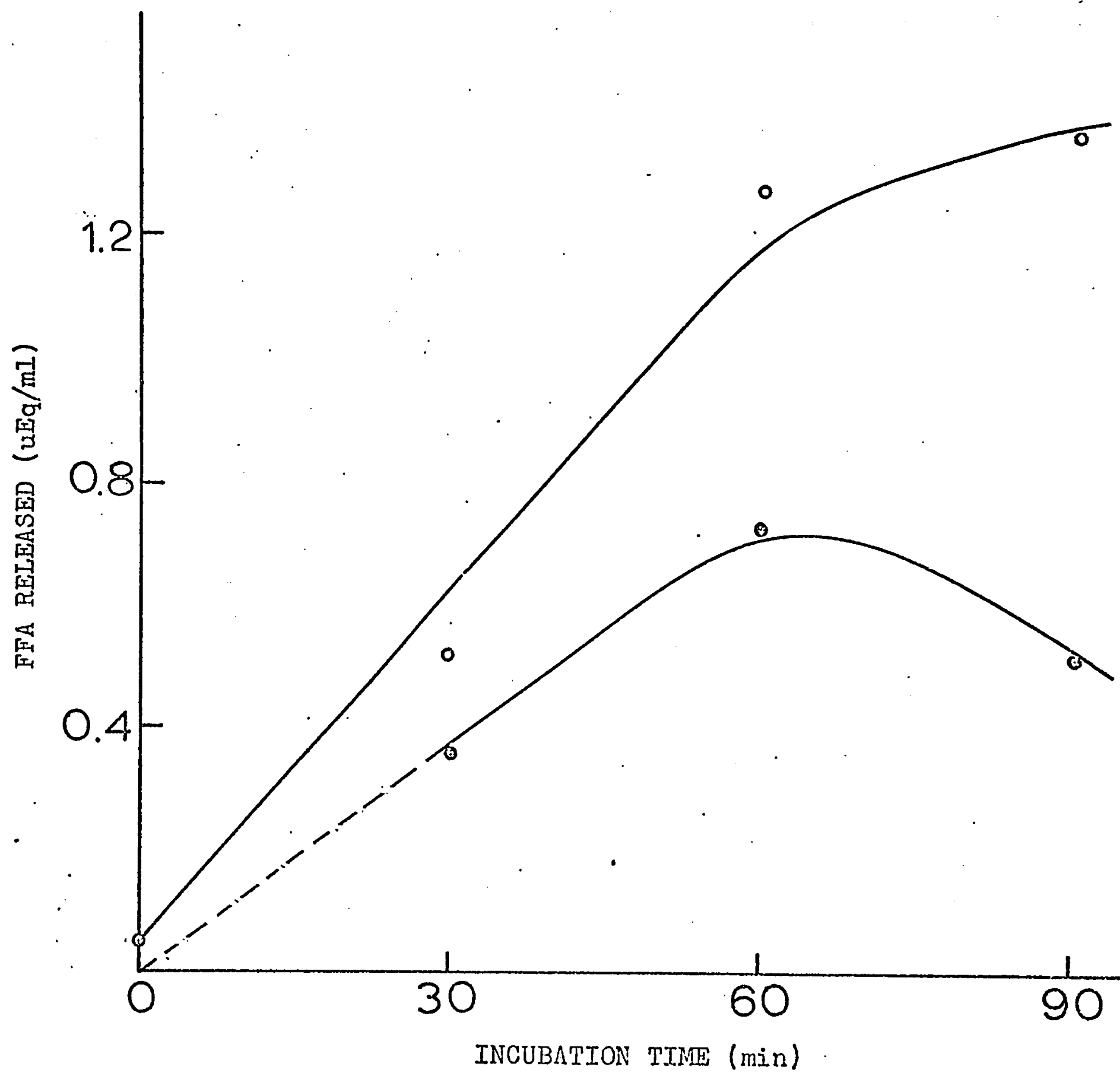

Fig. 6. Relationship between lipoprotein lipase activity of heart homogenates and incubation period.

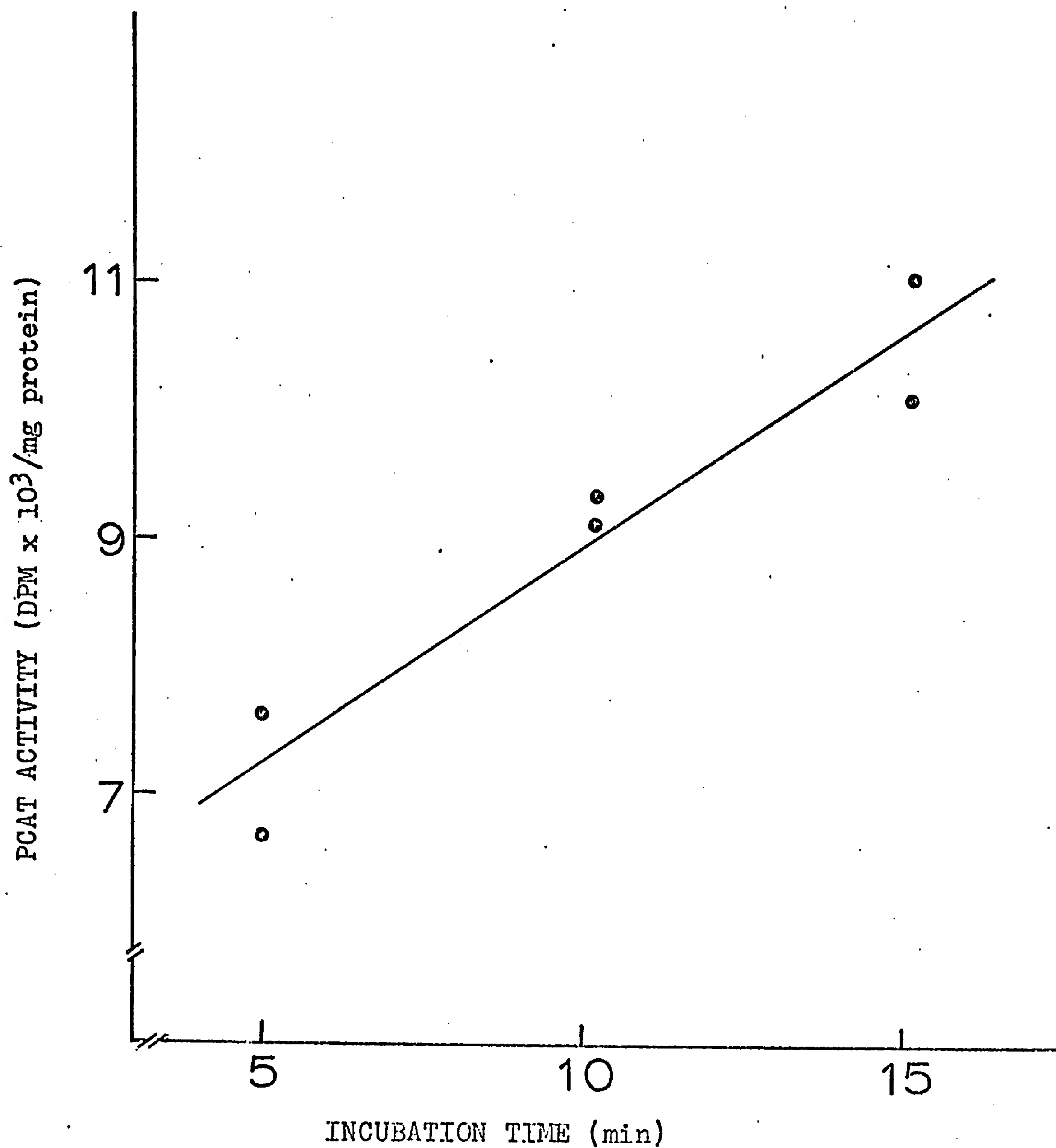

Fig. 7. Relationship of palmitylcarnitine acyl transferase (PCAT) activity in crude rat heart homogenate to incubation period. Each point represents PCAT activity of a single determination from homogenate.

II. EXPERIMENTAL RESULTS

Time courses - LPL, plasma FFA, oxygen consumption

After a single dose of 50ug T_3/100g (Fig. 8) LPL activity was found to be significantly elevated ($P<0.05$) only at 44 hr. At this time plasma FFA levels were only slightly above control values. The increase and subsequent decrease in LPL activity by 67 hr was accompanied by similar changes in oxygen consumption (Fig. 8A).

LPL activity increased more rapidly with the 125ug/100g dose of T_3 (Fig. 9) than with the 50ug dose. Activity was increased significantly ($P<0.05$) at 20 hr. Similarly, plasma FFA levels were above control values ($P<0.05$) by 20 hr. Plasma FFA levels returned to control values by 67 hr whereas LPL activity was still elevated at this time. Oxygen consumption (Fig. 9A) was increased by 20 hr ($P<0.01$) and remained elevated.

After the 250ug/100g dose of T_3 (Fig. 10) the most rapid increase in LPL activity occurred. LPL activity was increased at 12 hr, although not significantly, while plasma FFA were not elevated until about 20 hr. As in the case of the 125ug dose, plasma FFA levels returned to control values by 67 hr while LPL activity was still elevated. An increase in oxygen consumption of 66.1 ± 7.8 per cent ($n=7$) was found at 12 hr after the 250ug dose of T_3 (Fig. 10A).

A decrease and a more gradual increase from 20 hr followed the rapid elevation at 12 hours.

The combined results of the three doses of T_3 on LPL activity and plasma FFA levels over 67 hr is shown in Figure 11. It can be clearly seen that at all 3 doses, LPL activity was stimulated before any elevation in plasma FFA. Also, the magnitude of the response of LPL and plasma FFA to T_3 was found to be dependent upon the dose of the hormone. Such a dose-dependent relationship was not found with oxygen consumption (Fig. 11A).

The relationship between LPL activity and the dose of T_3 and that between plasma FFA level and dose of T_3, at 20 hr, (Fig. 12) appears to be linear. The response of LPL to approximately 500 ug T_4 per 100g body weight was taken from the work of Torsti (1965). Although tissue slices from normal rat heart were used as the enzyme source and LPL activity was determined at 24 hr after T_4 injection, it is interesting to note that the response of LPL appears to fit the linear dose-response relationship. However, Torsti's value for plasma FFA response to thyroxine at 24 hours does not coincide with our results.

Effects of reserpine pretreatment

a) Oxygen consumption and LPL- Reserpine (1.0mg/kg body weight), administered 18 hr prior to T_3 (125ug/100g), was found to have no effect on the calorigenic action of the

hormone at 20 hours (Fig. 13). Furthermore, reserpine itself had no effect on oxygen consumption (38 hours after administration). LPL activity increased significantly ($P<0.05$) following reserpine administration. However, the stimulatory effect of T_3 on LPL activity was not altered by reserpine pretreatment.

The effect of reserpine pretreatment, 0.2mg/kg, on LPL activity and oxygen consumption 20 hr after T_3 is shown in Figure 14. Oxygen consumption was not affected by reserpine nor was the calorigenic action of T_3. These findings confirm the results with 1.0mg/kg reserpine. Reserpine treatment lowered LPL activity slightly ($P<0.10$). The percentage increase in LPL activity from the lowered basal levels of reserpinized rats was approximately the same as that in untreated hypothyroid rats following T_3 injection (54.9 and 49.2 respectively).

b)Plasma FFA- Reserpinized (0.2mg/kg) rats showed a decreased response to the lipolytic action of T_3 (Fig. 15b). The level of plasma FFA of the group pretreated with reserpine was significantly below ($P<0.05$) that of the untreated group following T_3 administration. In contrast, 1.0mg/kg of reserpine failed to reduce the elevation in plasma FFA following T_3 (Fig. 15a). Reserpine treatment alone (0.2 and 1.0mg/kg) had no significant effect on plasma FFA levels.

FIGURE 8

Time course of heart lipoprotein lipase (LPL) activity and plasma free fatty acid (FFA) level after a single intraperitoneal injection of triiodothyronine (T_3,50 ug/100g body weight). The means ± S.E. are shown together with the number of experiments. S.E. of controls (saline-injected hypothyroid rats) are indicated by arrows. NS - not significantly above control.

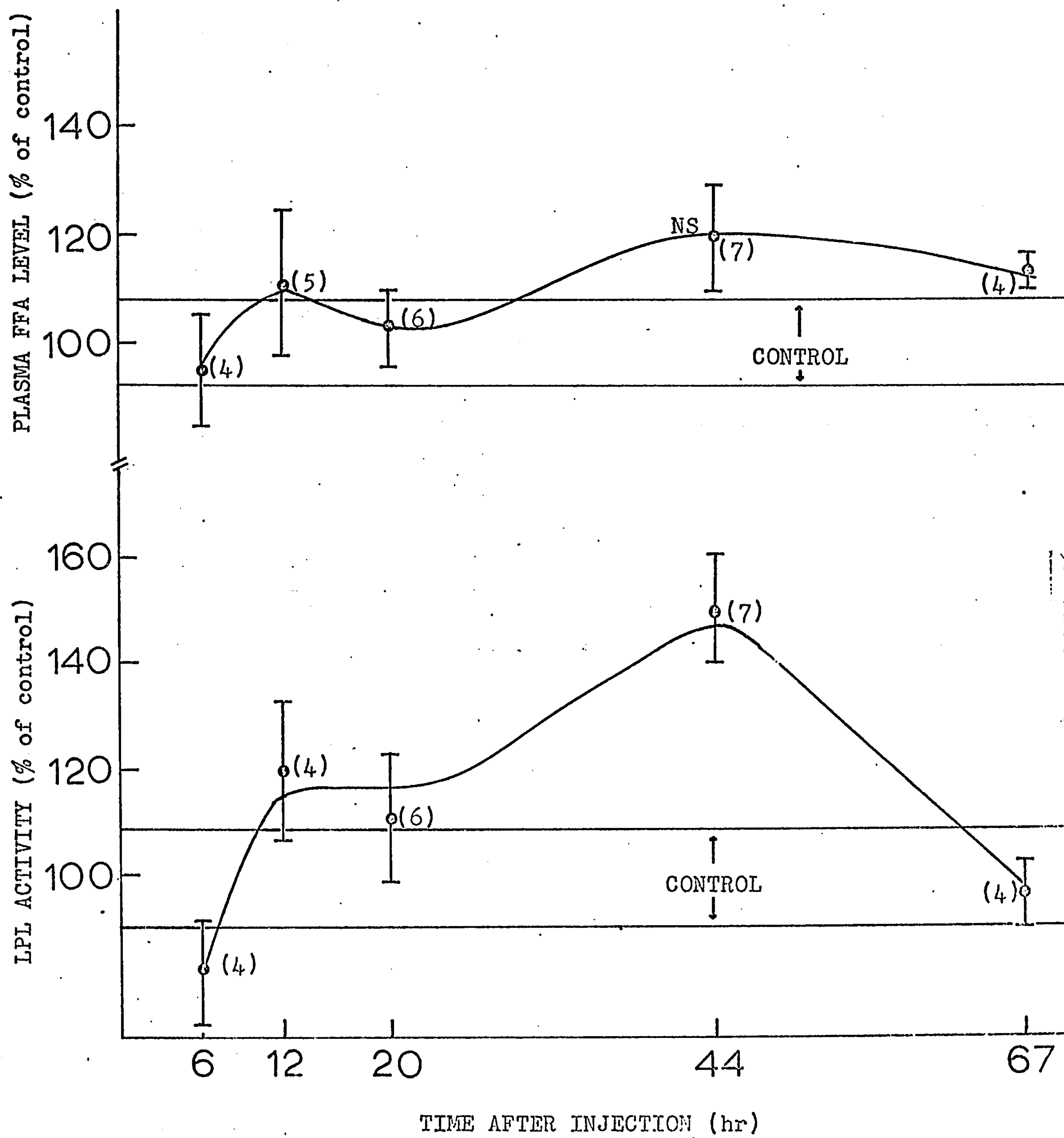

Figure 8

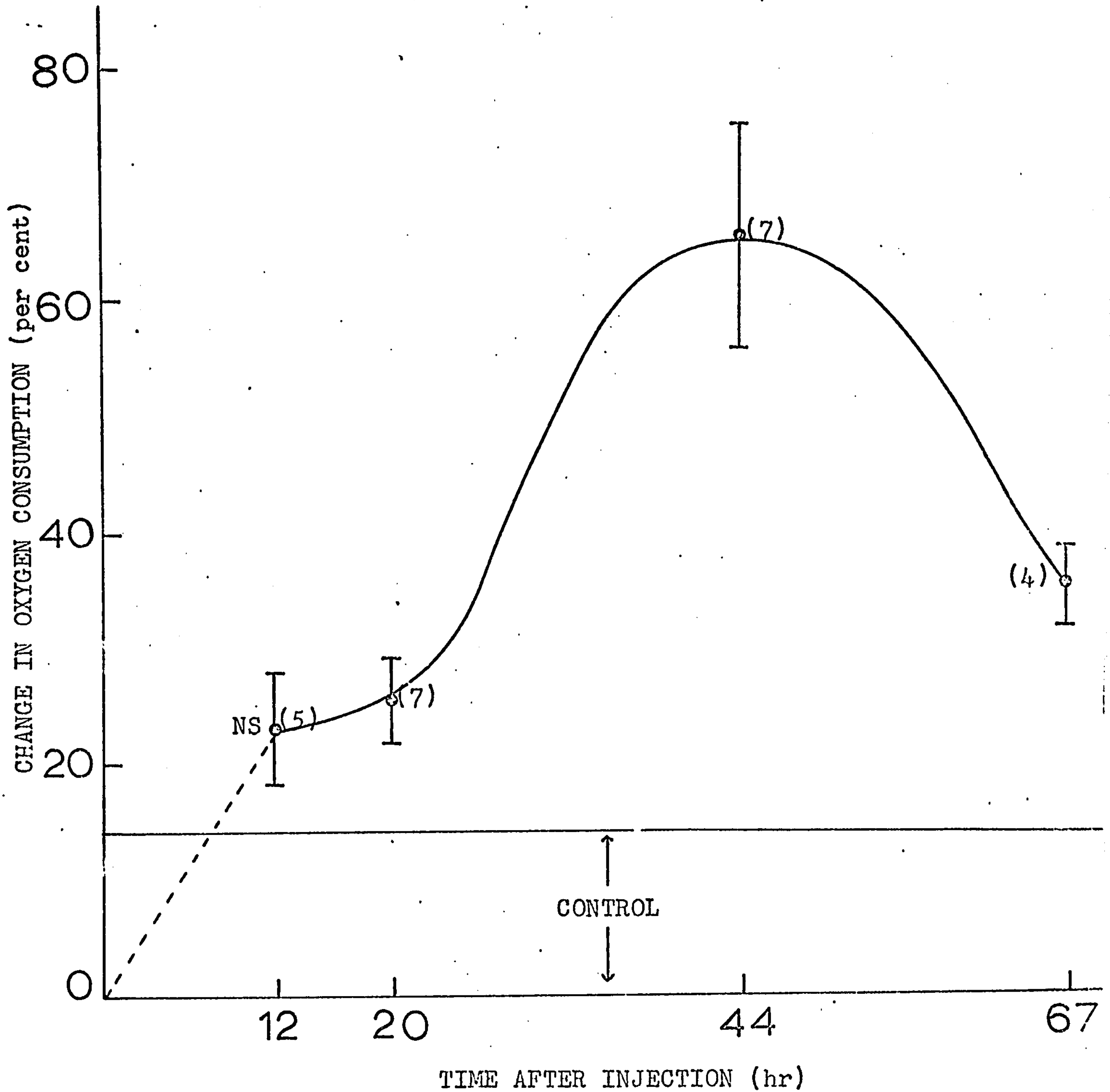

Fig. 8A. Time course of oxygen consumption change after a single injection of T_3 (50ug/100g).

Area between arrows indicates positive mean change in control oxygen consumption. Change was ± 14.2% (S.E.±2.2%) NS - Not significantly above control.

FIGURE 9

Time course of heart LPL activity and plasma FFA level after T_3 (125ug/100g). Indicated are means ± S.E., number of experiments and S.E. of controls.

NS - Not significantly above control.

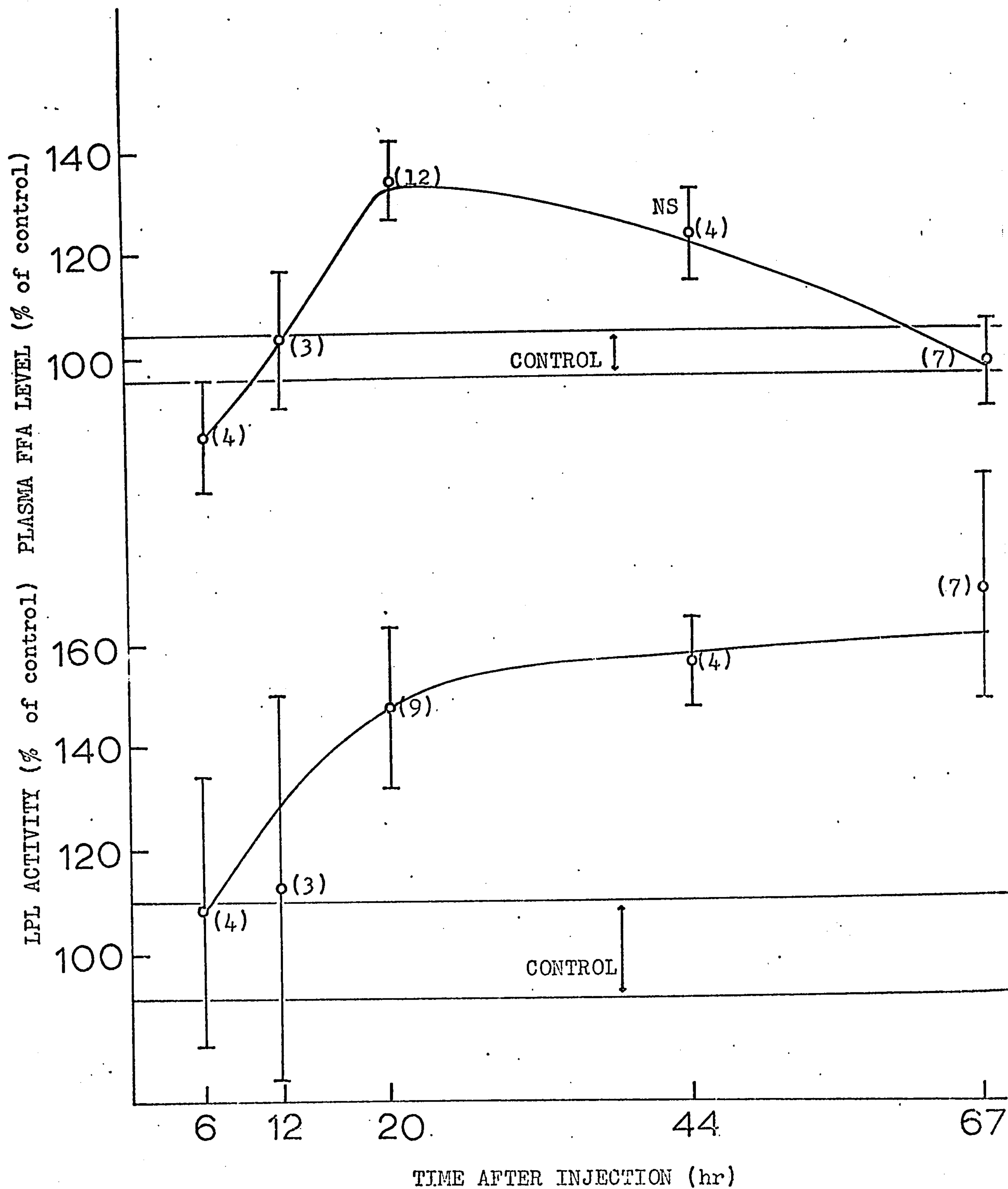

Figure 9

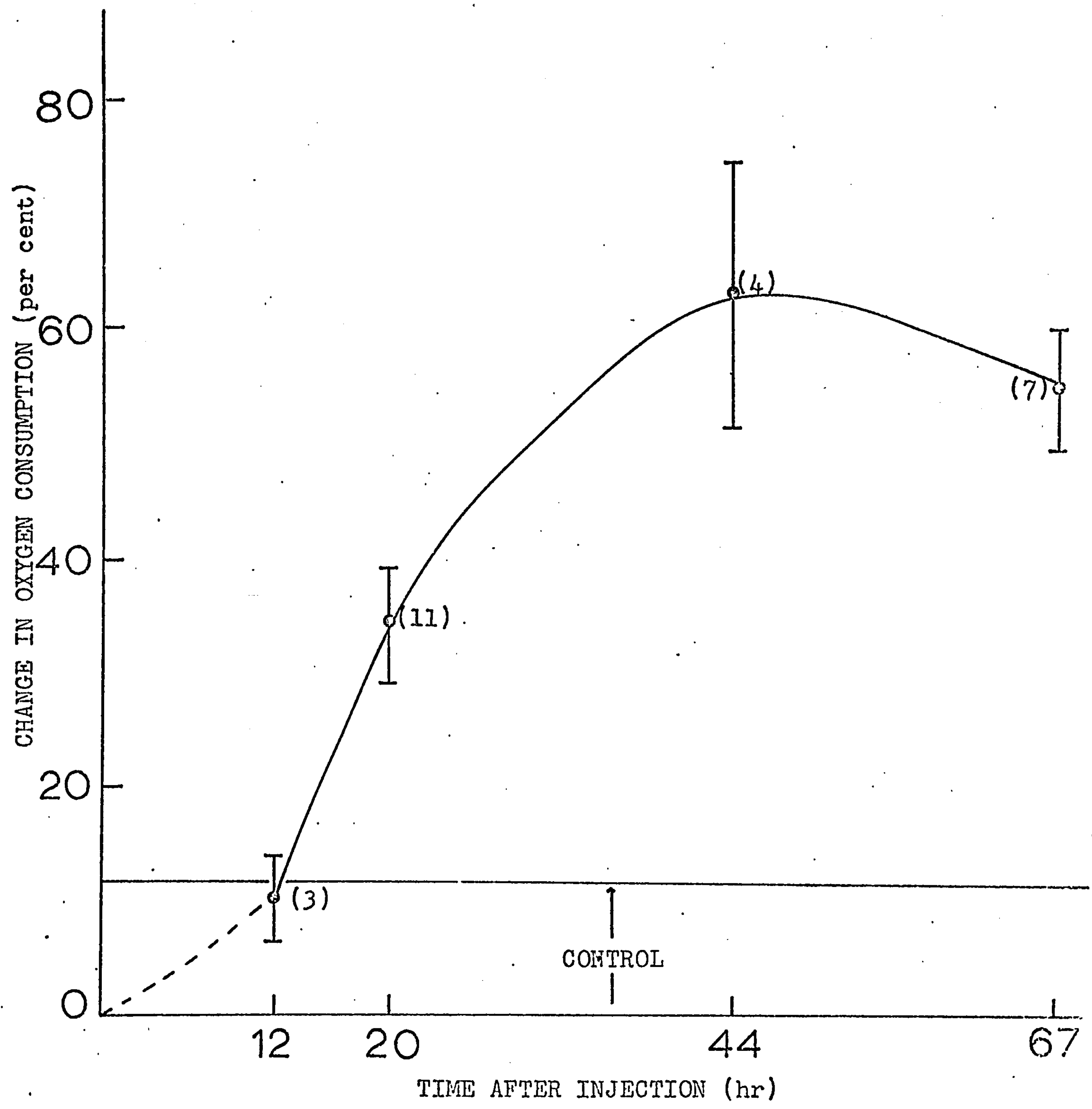

Fig. 9A. Time course of oxygen consumption change after T_3 (125ug/100g). Mean change in control oxygen consumption is shown.

FIGURE 10

Time course of heart LPL activity and plasma FFA level after T_3 (250ug/100g). Indicated are means $\pm$ S.E., number of experiments and S.E. of controls.

NS - Not significantly above control.

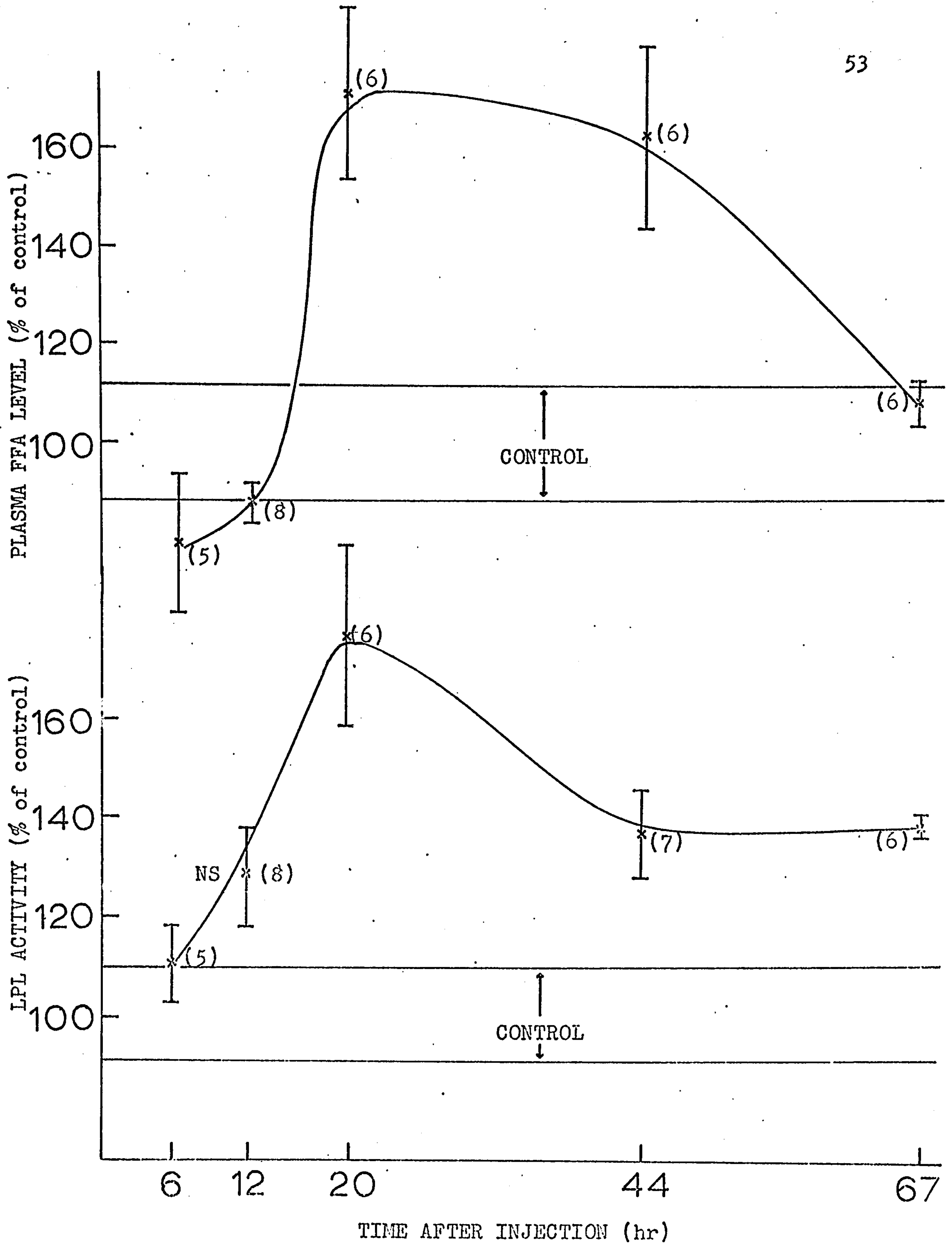

Figure 10

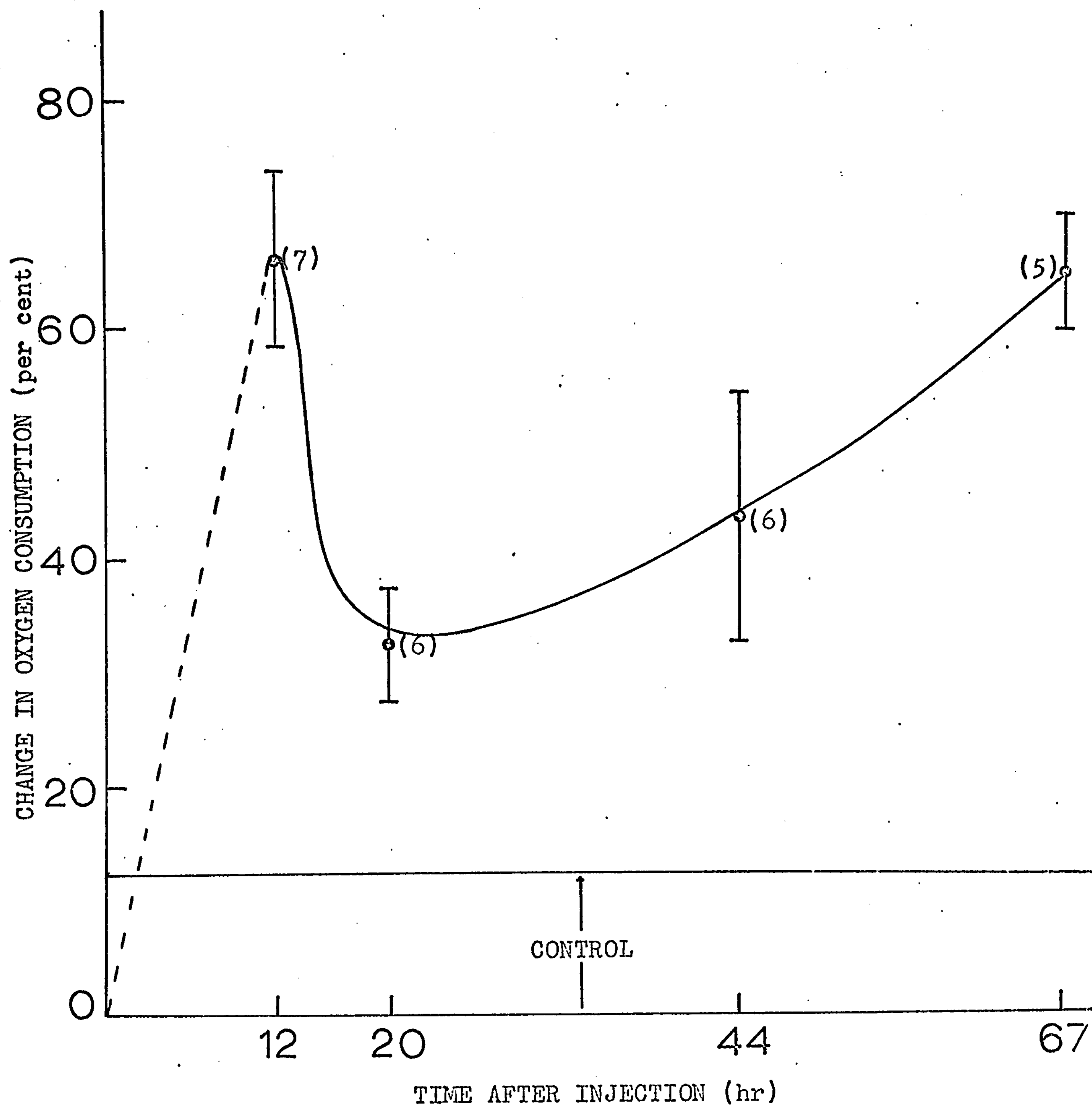

Fig. 10A. Time course of oxygen consumption change after T_3 (250ug/100g). Mean change in control oxygen consumption is shown.

FIGURE 11

Time courses of heart LPL activity and plasma FFA level after three doses of T_3. 50ug/100g ● 125ug/100g o 250ug/100g × Means and S.E. are shown together with S.E. of controls.

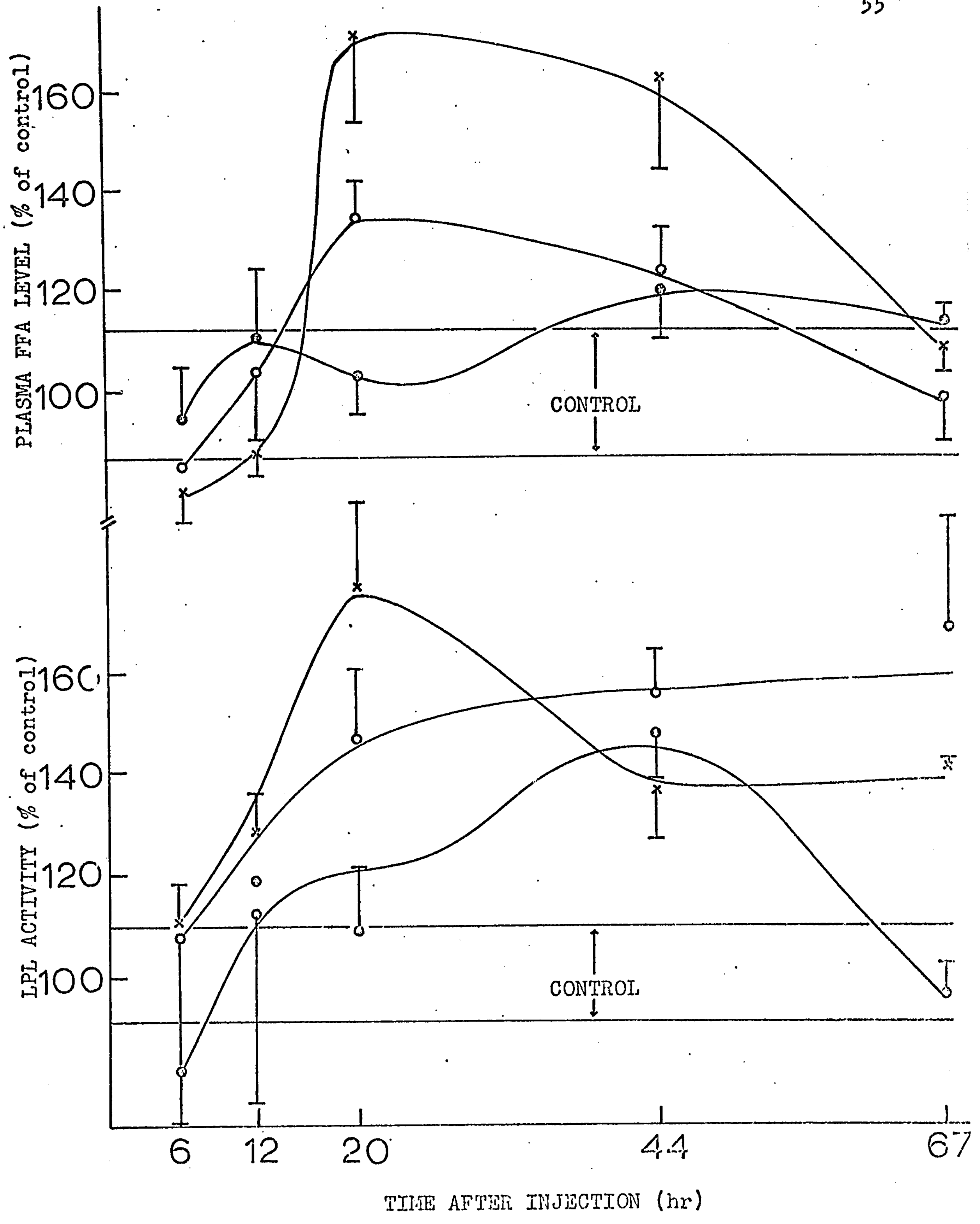

Figure 11

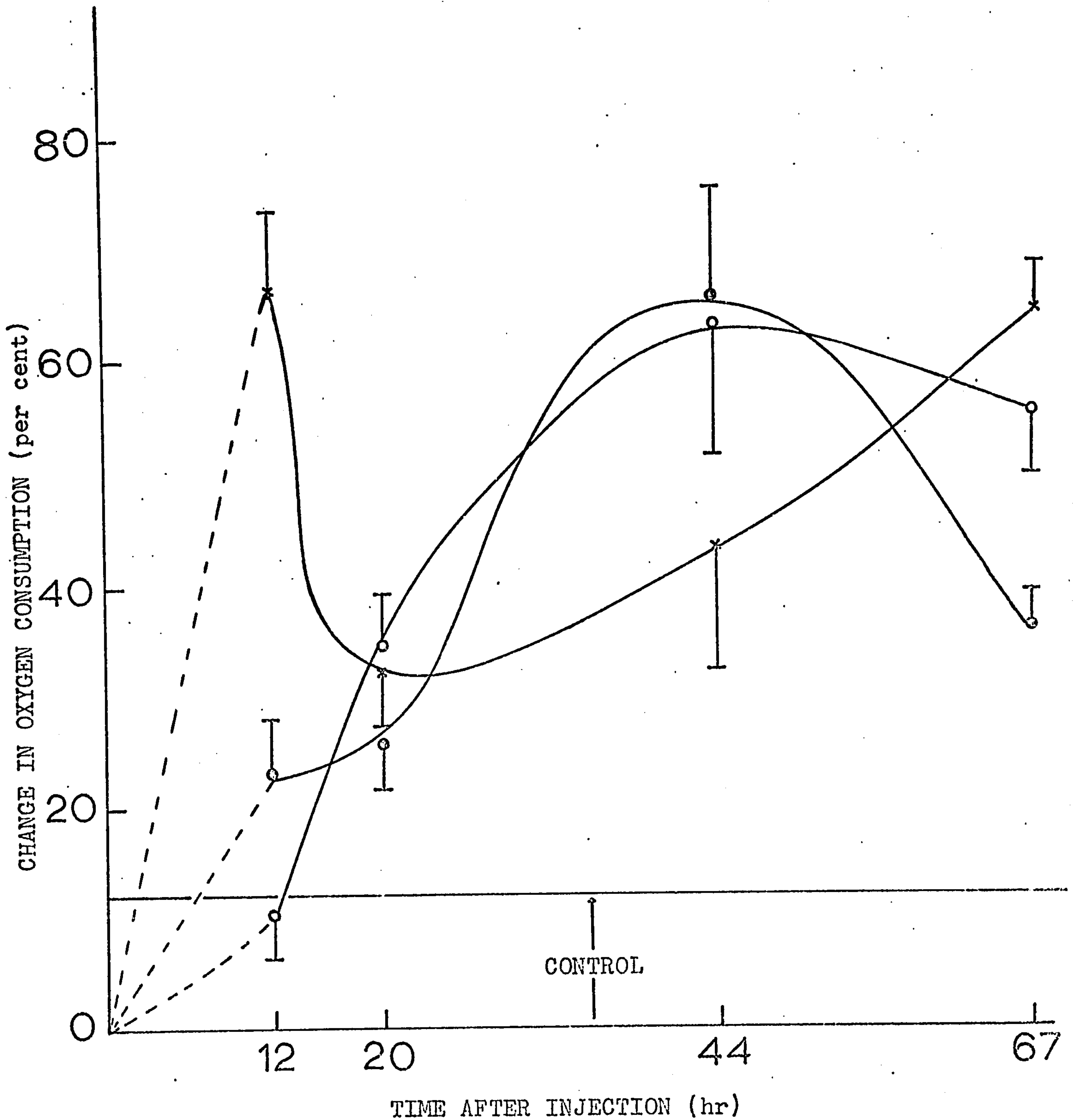

Fig. 11A. Time courses of oxygen consumption change after three doses of T_3. Doses: 50ug/100g o 125ug/100g o 250ug/100g x Means and S.E. are shown together with mean change in control oxygen consumption.

FIGURE 12

Means of LPL activity(●) and plasma FFA level(o) are shown. Number of experiments are indicated.

* Results of Torsti (1965) 24 hr after a single injection of T_4 (500ug/100g) to normal rats.

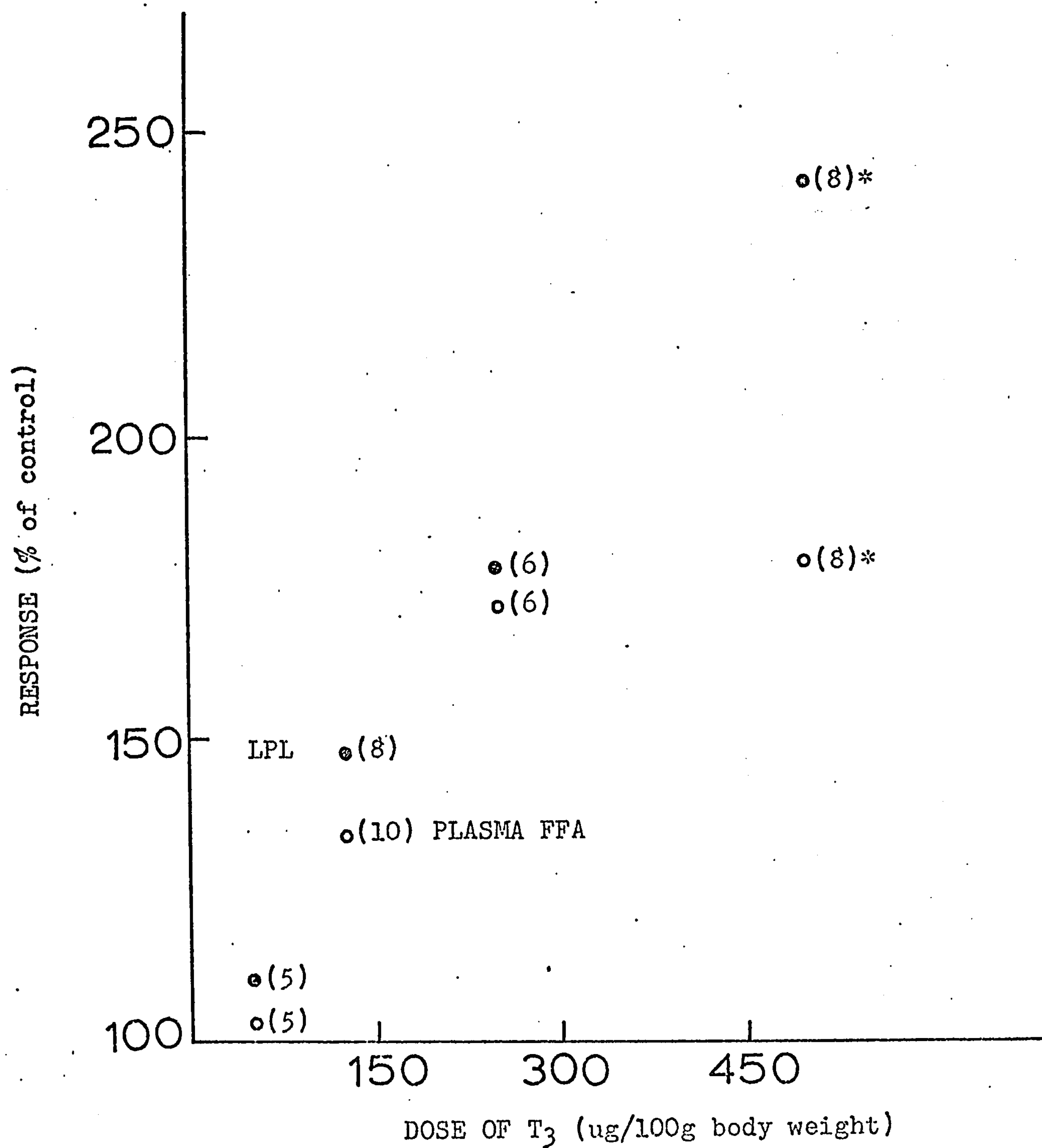

Fig. 12. Relationship between dose of T_3 and response of heart LPL and plasma FFA level 20 hr after a single intraperitoneal injection to hypothyroid rats.

FIGURE 13

Reserpine was administered (intramuscularly) 18 hr prior to T_3 injection. Mean ± S.E. of each group is shown together with the number of experiments. S.E. of control values, in the case of LPL, and positive mean change in oxygen consumption of controls are shown.

* Significantly above control ($P < 0.05$)

** ($P < 0.01$)

*** ($P < 0.001$)

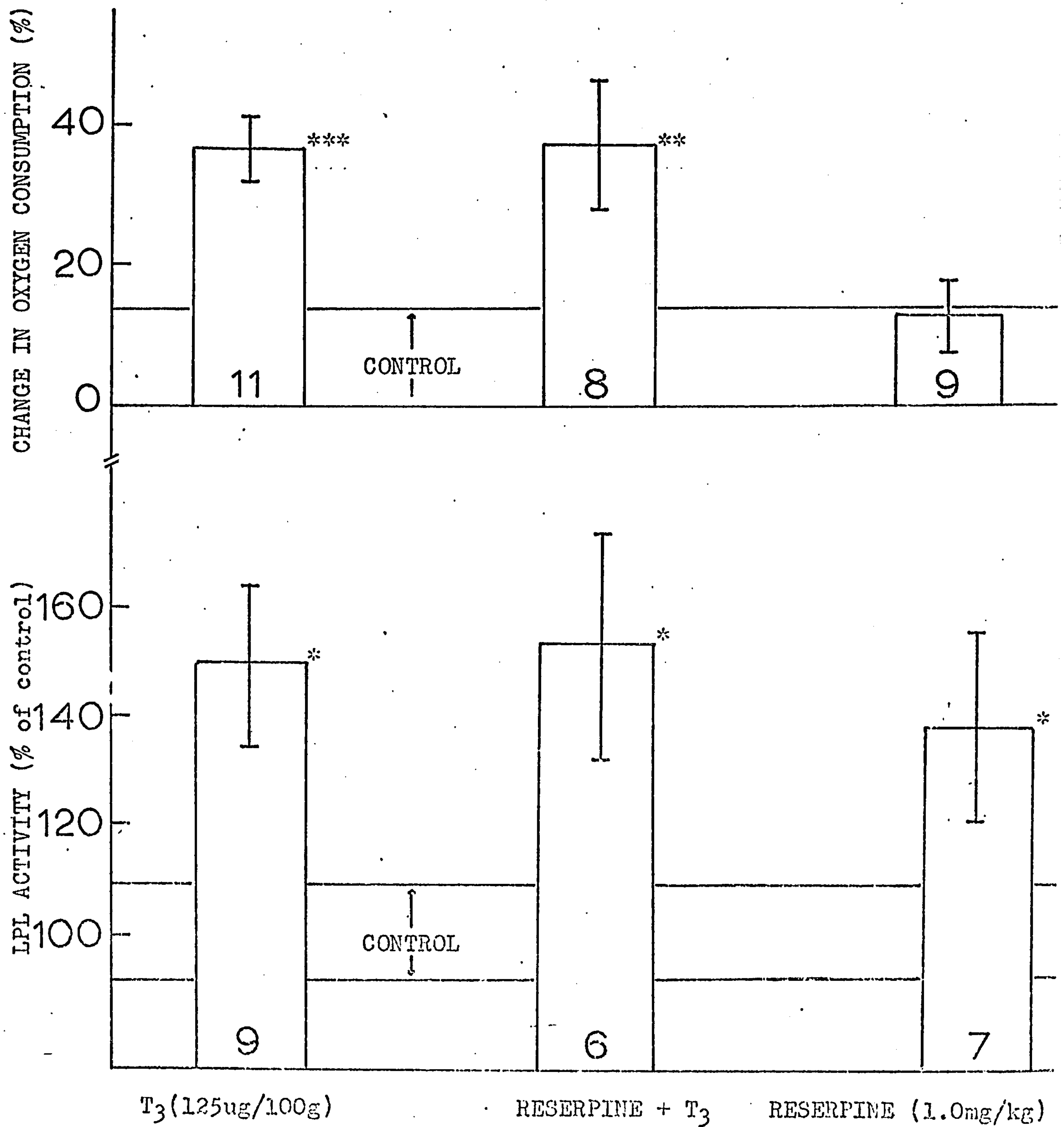

Fig. 13. Effect of reserpine pretreatment (1.0mg/kg) on heart LPL activity and bodily oxygen consumption 20 hr after a single intraperitoneal injection of T_3.

FIGURE 14

Effect of reserpine pretreatment (0.2mg/kg) on heart LPL activity and bodily oxygen consumption 20 hr after T_3. S.E. of controls (LPL activity) and positive mean change in oxygen consumption of controls are shown.

* Significantly above control ($P < 0.05$)

** ($P < 0.001$)

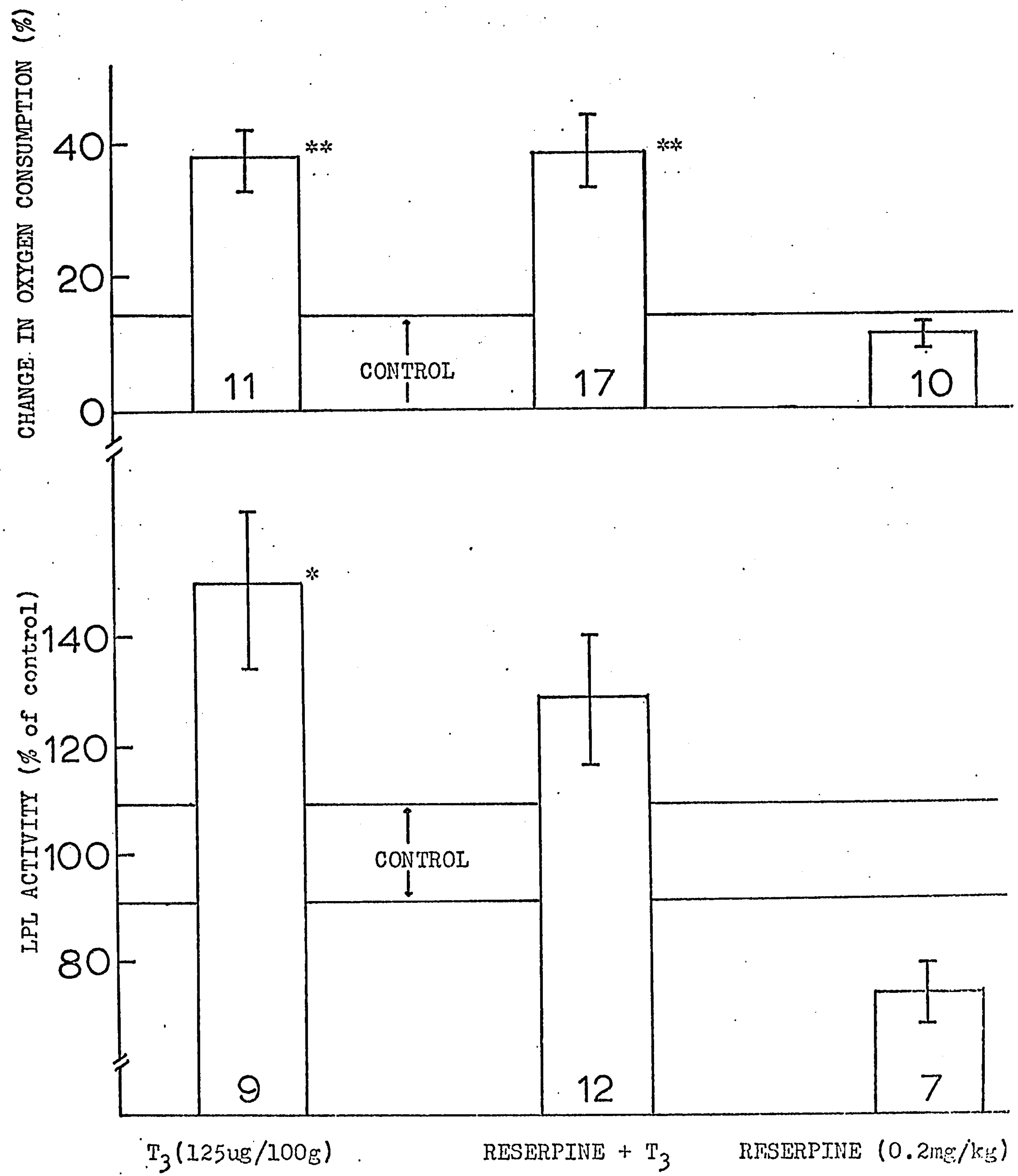

Figure 14

FIGURE 15

Effect of reserpine pretreatment on plasma FFA level 20 hr after T_3. Reserpine was administered 18 hr prior to T_3 injection.

* Significantly above control ($P < 0.05$)

** ($P < 0.01$)

*** Significantly lower than T_3-treated group ($P < 0.05$)

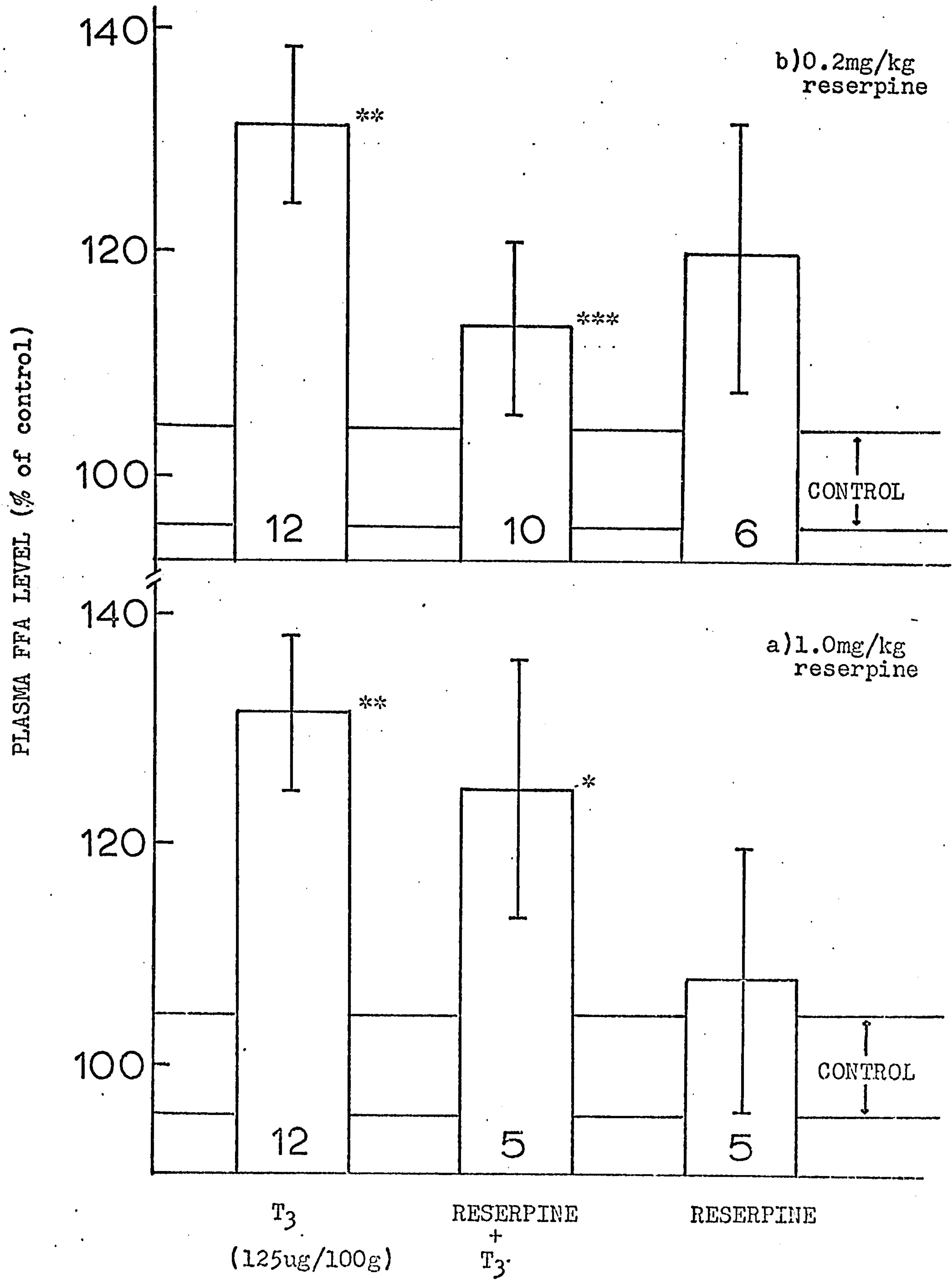

Figure 15

Effect of reserpine and epinephrine on LPL activity in normal rats

A single intramuscular injection of 0.5mg/kg epinephrine raised LPL activity within 3 to 4 hours (Fig. 16). The response of LPL to epinephrine, however, was reduced in rats pretreated with reserpine (1.0mg/kg). Doses of reserpine of 0.2 and 1.0mg/kg reduced LPL activity whereas 3.0mg/kg did not.

Time course - PCAT

The time course of activity change in palmityl-carnitine acyl transferase over 67 hours is shown in Fig. 17. The activity of PCAT appeared to be increased by 20 hours after 50ug T_3/100g and then returned to control levels by 67 hours. The apparent increase in PCAT activity was found to precede any change in plasma FFA levels after 50ug T_3/100 grams body weight.

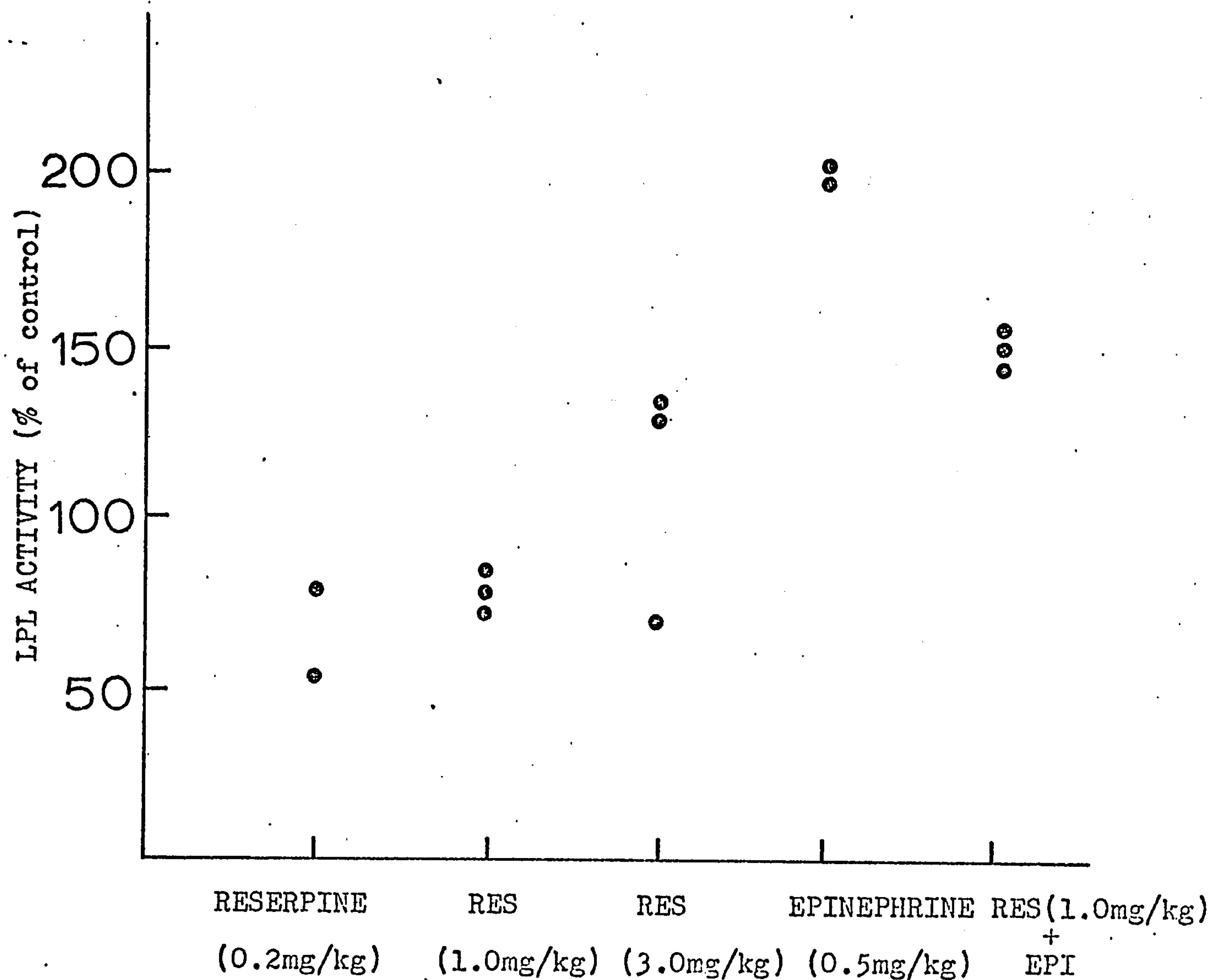

Fig. 16. Effect of reserpine and epinephrine on heart LPL activity of normal rats. Epinephrine, suspended in peanut oil, (0.5mg/kg) was injected (im) into rats 38 hr after a single im injection of reserpine. LPL activity was determined 3 to 4 hr later. Each point represents the LPL activity of a single heart expressed as a percentage of peanut oil-injected controls.

FIGURE 17

Time course of myocardial palmitylcarnitine acyl transferase (PCAT) activity and plasma FFA level after a single injection of T_3 to hypothyroid rats. Each point depicting PCAT activity represents the determination from a single heart following injection of 50ug T_3/100g. The time course in plasma FFA level is taken from Fig. 8. Shown are the number of experiments, means $\pm$ S.E. and S.E. of controls.

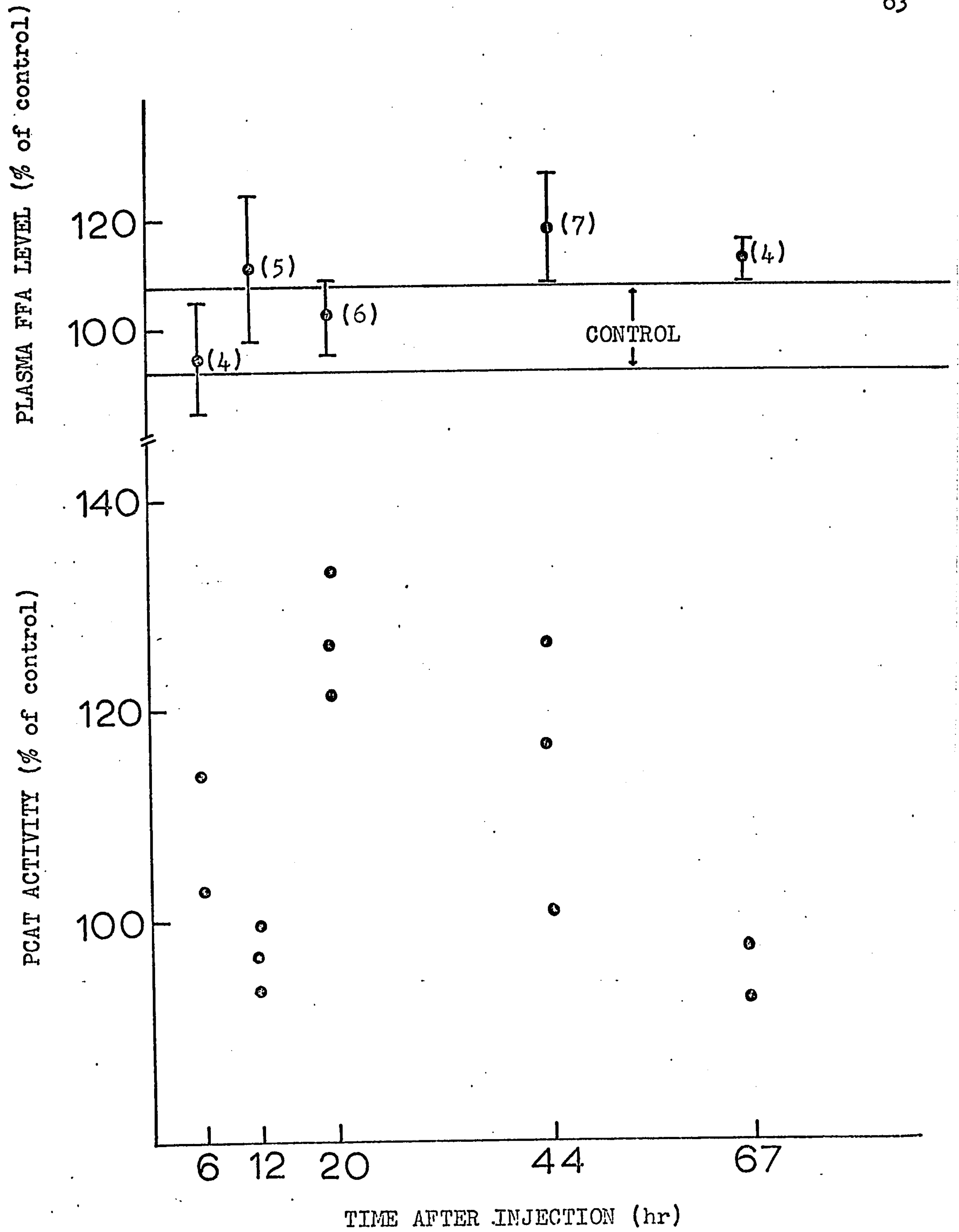

Figure 17

DISCUSSION

I. METHODOLOGY

In order to study a particular physiological response to administered thyroid hormone a sensitive indicator is needed. Such a condition is provided by the acute administration of the hormone to an animal in the hypothyroid state. Tata (1964) reported that the latency of a particular response can best be recorded after a single small dose of thyroxine to a thyroidectomized (hypothyroid) subject. In order to examine the chronological sequence of the actions of triiodothyronine upon heart lipoprotein lipase, plasma free fatty acids and total body oxygen consumption, such a condition was used. By examining time courses in the changes of LPL activity, plasma FFA and oxygen consumption after T_3 administration, some idea of their interrelationships and of their individual responses over a period of several days can be gained.

a) Inducement of hypothyroidism

A number of methods of producing a hypothyroid animal were compared in this study. In looking for a satisfactory method the criteria which were sought were the rapid and the consistent production of hypothyroidism. The degree of hypothyroidism was based upon total body

oxygen consumption.

Since surgical thyroidectomy with normal and with low-iodine diets (Fig. 4) did not produce the desired degree of hypothyroidism within a 50-day period these procedures were abandoned. Although reports of thyroidectomized rats being regarded as hypothyroid after 4-6 weeks on normal diets have appeared (Lee and Miller 1967, Grad 1952), hypothyroidism was based upon a slower weight gain than controls. Eartly and Leblond (1954) found that thyroidectomized rats on a low-iodine diet stopped growing after two weeks. Oxygen consumption at this time was reported to be decreased by about 14%. We found that the oxygen consumption of thyroidectomized rats on low-iodine diet had decreased by only 19% after 32 days (Fig.4). Furthermore, our results indicated that a 14% change in oxygen consumption was not significantly different from euthyroid levels. Beznak (1960) also, found that basal metabolic rates of normal rats fluctuated by 15% (S.E. of mean was ± 15%). In all of these cases rats of approximately the same weight (150-220g) were used. Although Nuttall (1968) considered thyroidectomized rats hypothyroid by a decrease in weight gain, he waited 17 weeks after thyroidectomy. Thus, it is apparent that the growth rate of the rat may not always be a valid indicator of the true thyroid state. Since BMR is so closely associated with thyroid function (Tata 1964) it was regarded as a more accurate indicator of the thyroid

state.

Chemical thyroidectomy, by the use of I^{131} injection is another common means of producing hypothyroidism. This treatment has been employed with normal rats (Krause et al. 1967), and with surgically thyroidectomized rats (Tata et al. 1963). Also, I^{131} treatment of thyroidectomized rats put on low-iodine diets has been used to produce a hypothyroid state (Hoch 1965). Krause and his co-workers (1967) reported a 29% decrease in BMR of normal rats (on normal diet) injected with 800 uc I^{131} per 100g body weight within 4 weeks. In the present studies, rats injected at this dose showed a 30% decrease in oxygen consumption within a minimum time of 22 days. Most of the animals were, however, used after at least 4 weeks.

The antithyroid drug, 6-propyl-2-thiouracil (PTU) is also used to produce a hypothyroid state. This drug inhibits the binding of iodide to thyroglobin and also interferes with the de-iodination of thyroxine in the tissues (Ruch and Patton 1965). PTU, mixed in the diet at a concentration of 0.05% has been employed by Yasumura et al. (1967) and by Mallov and Alousi (1967). Mallov and Alousi found that the oxygen consumption of normal rats on this diet decreased by 26% after 7 to 8 weeks. In the present work normal rats on a 0.05% PTU diet showed a 30% decrease in oxygen consumption after approximately 25 days.

The majority of the animals used in this study were rendered hypothyroid by PTU-feeding; a smaller number were used after inducement of hypothyroidism by I^{131} injection. No differences in either LPL activity or plasma FFA were found between rats from these two groups when at the same level of hypothyroidism.

b) Lipoprotein lipase assay

The assay method employed in the present experiments may determine the activity of at least one other lipase, a monoglyceride-hydrolysing lipase (pp. 3-4). Ediol, the artificial triglyceride substrate, has been shown to contain significant amounts of mono-and diglycerides (Greten et al 1969). Crude heart homogenates were used as the source of LPL and consequently it must be realized that a mixture of lipases may contribute to the "lipoprotein lipase activity".

In order to determine whether the assay system was a valid indicator of lipolytic activity, various amounts of heart homogenate were incubated for 1 hour and the amount of FFA liberated was measured. A linear relationship was found between the volume of homogenate, up to 1.0 ml, and FFA released (Fig. 5). The incubation volume used in the experiments, 0.5 ml, was within this linear range. A linear relationship between incubation time and

amount of FFA liberated was found for times up to 1 hour (Fig. 6).

c) <u>Reserpine</u>

Reserpine was administered in order to deplete the tissues of their catecholamine content. The action of T_3 on LPL, plasma FFA and oxygen consumption could subsequently be examined in the absence of a fully functioning sympathetic nervous system. However, since reserpine has been shown to have a multitude of complex effects on various biological systems, it is desireable to supplement the observation showing an absence of sympathetic function with supporting experiments using other means.

d) <u>Palmitylcarnitine acyl transferase assay</u>

The assay for palmitylcarnitine acyl transferase activity involves the determination of the incorporation of ^{14}C-carnitine into ^{14}C-palmitylcarnitine in the equilibrium reaction:

palmityl CoA + carnitine = palmitylcarnitine + CoA

It was found that greater PCAT activity was obtained using hearts homogenized in KRP, pH 8.5, than in a 10% sucrose buffer containing 10mM Tris, pH 7.4, similar to that used by Norum (1964) for calf liver. He found the pH optimum to be 7-8.2. Reproducibility of the assay

system using tissue homogenized in either buffer, however, was found to be unsatisfactory and therefore the results presented in this study must be considered only as preliminary.

II. LIPOPROTEIN LIPASE

a) Thyroxine and heart lipoprotein lipase

In the present study it was found that the time course of LPL activity change after T_3 varied with the dose of the hormone. The latency and the magnitude of the response of LPL appeared to be dependent upon the dose of T_3. Pretreatment of hypothyroid animals with reserpine (1mg/kg) had no effect on the response of the enzyme to T_3. Pretreatment of normal animals with this dose of reserpine reduced the elevation in LPL activity following epinephrine injection. Although this dose of reserpine increased LPL levels in hypothyroid rat hearts, it did not elevate the levels in the hearts of euthyroid (normal) animals. These findings indicate that the action of thyroid hormone on myocardial lipoprotein lipase is a direct one, independent of the sympathetic nervous system.

It has been shown that chronic (Alousi and Mallov 1964) as well as acute (Torsti 1965) thyroid hormone treatment resulted in elevations of heart LPL activity. Alousi and Mallov suggested that the change in LPL activity in hyperthyroidism was a relatively slow process requiring several days or weeks. This process was thought to be the result of an adaptation of the heart in accordance with its fatty acid energy requirements. Torsti showed a great

increase in heart LPL activity (243% of controls) 24 hr after T_4 but the high dose employed (500 ug/100g) most likely caused the physiological latent period to be shortened. This characteristic of large doses of thyroxine is discussed elsewhere (page 91). We found that after a single injection of T_3 to hypothyroid rats, the latency and the magnitude of the elevation in LPL activity was dependent upon the dose of the hormone. (Figs. 11,12). The "most physiological" dose, 50 ug/100g, did not raise LPL levels until 20-44 hr after injection and the maximum increase observed (at 44 hr) was 149% of control. Our findings indicate that the physiological action of thyroid hormones on LPL takes several days and is a direct action on the enzyme.

A direct mode of action by thyroxine on the enzyme was not favoured by Alousi and Mallov (1964) since in vitro experiments failed to show any stimulation of LPL activity. However, T_3 was used at concentrations of 10^{-6} to 10^{-4}M; estimates of tissue levels of thyroid hormones are 10^{-7} to 10^{-8}M (Tata 1959) and 10^{-11}M (Tata 1964). Challoner (1969) showed in vitro stimulation of oxygen consumption in rat fat pads using T_3 at concentrations of 5×10^{-6} to 10^{-8}M. Levey and Epstein (1969) demonstrated in vitro stimulation of myocardial adenyl cyclase employing T_3 at a concentration of 5×10^{-7}M. Indeed, Tata (1964) has pointed

out that the weaknesses of *in vitro* experiments with thyroid hormones are i) it has not been possible to allow a latent period of a long duration in *in vitro* experiments and, ii) very high concentrations of the hormones are used. In some cases the *in vitro* effects of low concentrations are opposite to those at high concentrations. For instance, increased microsomal protein synthesis induced by T_4 *in vitro* is inhibited at a concentration above 6x10-4M (Sokoloff and Kaufman 1961). Also, the effects of a direct interaction between thyroxine and a system often bear no relation to the *in vivo* action. An example is the usual failure of thyroxine to raise mitochondrial respiration *in vitro*, when *in vivo*, respiration is elevated after thyroid hormone treatment (Tata 1964). Thus, although Alousi and Mallov (1964) concluded that thyroid hormones probably do not act directly on myocardial LPL *in vivo*, such an extrapolation of data indicating the *in vitro* failure of T_3 to stimulate the enzyme is not warranted.

The existence of a long latent period suggests that the hormone may act through the protein-synthesizing machinery of the cell and that synthesis of new protein is a relatively slow process. Thus, a direct action of thyroxine may be the *de novo* synthesis of LPL or alternatively, the activation of the enzyme. Further discussion of these possible mechanisms appears in a later section.

b) Thyroxine and post-heparin plasma lipoprotein lipase

Although post-heparin plasma (or serum) LPL activity was not determined in the present study, a brief examination of the reported effects of thyroid hormones and altered thyroid states upon this lipase may be useful since it has been proposed that post-heparin plasma LPL may be a reflection of the tissue enzyme (Mallov and Alousi 1967). In rats, thyroxine treatment for one week was reported to result in an inhibition of post-heparin plasma LPL (Cairns and Constantinides 1955). However, the crude method of measurement of turbidity was used in this study to assay LPL. Thyrotoxic rabbits have been shown to have decreased plasma LPL levels (Kirkeby 1968). Surgical thyroidectomy in rats was reported to have no effect on plasma LPL (Cairns and Constantinides 1955). However, the magnitude of the hypothyroid state of these animals may not have been very great since thyroidectomy, as has been previously mentioned does not produce any noticeable degree of hypothyroidism for at least several weeks. Kirkeby (1968) reported that decreased plasma LPL levels have been found in human hypothyroidism.

Since post-heparin plasma LPL activity appears to be decreased both in hypo-and hyperthyroidism it is unlikely that it is the same enzyme as myocardial LPL;

heart LPL is decreased in hypothyroidism (Mallov and Alousi 1967) and elevated in hyperthyroidism (Alousi and Mallov 1964). It must be borne in mind, however, that in none of these reports was a purified enzyme preparation used and it is possible that the lipolytic activity determined included not only LPL but other enzymes as well. Furthermore, it was postulated that the major source of plasma LPL is not the heart but adipose tissue (Brady and Higgins 1967).

c) Possible mechanisms of thyroxine stimulation of myocardial lipoprotein lipase

Some of the possible mechanisms of thyroid hormone stimulation of myocardial lipoprotein lipase examined are the following: 1)alteration in plasma FFA, 2)elevation of oxygen consumption and 3) mediation by catecholamines. Also, the possibility that thyroxine acts directly on the enzyme, either by the stimulation of LPL synthesis or by activation via the adenyl cyclase system is examined.

1. Alteration in plasma FFA level

Plasma FFA have been shown to alter the activities of various enzymes. Glucose-6-phosphate dehydrogenase and acetyl-CoA carboxylase have been reported to be inhibited by FFA (Ho and Jeanrenaud 1967). Also, adipose tissue lipoprotein lipase activity is believed to be regulated by

FFA (Nikkila and Pykalisto 1968). Thus, the possibility that the altered levels of plasma FFA following T_3 administration affected heart LPL was examined.

The findings of the present work indicate the increase in myocardial LPL activity following T_3 administration is independent of the plasma FFA level. At all 3 doses of T_3 employed, LPL activity was found to increase before any noticeable change in plasma FFA (Fig.11). Further evidence that increased levels of plasma FFA are not responsible for elevated LPL activity is provided by the following findings:

i) The rates of fatty acid oxidation in heart homogenates of thyroxine-treated rats were increased before BMR was increased (Hoch 1962). Although LPL is not directly involved in the oxidation of FFA, it is believed to regulate the supply of fatty acids derived from chylomicron-triglycerides to the myocardial tissues (Robinson 1963).

ii) In hypothyroid rats, after a low dose of T_3, BMR was elevated before any change in plasma FFA (Myant and Witney 1967). Thus, the chronological order of these actions of thyroxine appears to be an elevation of myocardial fatty acid oxidation and LPL activity, increase in metabolic rate, and an increase in plasma FFA concentration. Another strong line of evidence against plasma FFA being responsible for changes in myocardial LPL is the demonstration that

nicotinic acid activated heart LPL and decreased plasma FFA at the same time (Grafnetter and Grafnetterova 1967).

It was suggested by Bressler and Wittels (1966) that the stimulatory action of thyroxine on myocardial fatty acid oxidation was due to the increased levels of FFA in plasma resulting from a stimulation of adipose tissue lipolysis. However,based on the evidence presented, it appears that fatty acid oxidation is stimulated by thyroxine independently of the mobilization of fatty acids. The enhancement of LPL activity by the hormone allows the myocardium to utilize fatty acids from blood lipids before an increased supply of plasma FFA is made available by the lipolytic action of thyroxine on adipose tissue. However, the reason for increased heart LPL activity under conditions of elevated plasma FFA is not clear.

2. Elevation of oxygen consumption

It appears that increased myocardial LPL activity is not due to a general increase in metabolic rate as a result of thyroxine treatment. Although the present studies did not demonstrate a clear cut increase in LPL activity preceeding elevations in oxygen consumption, there is evidence that thyroid hormones act on the heart in general before increasing BMR. As mentioned previously

thyroxine treatment was shown to increase fatty acid oxidation rates in rat heart homogenates before elevating BMR (Hoch 1962). Evidence that increased metabolic rate is not responsible for elevated myocardial LPL activity is provided by the demonstration that, in the fasting state, when myocardial LPL levels have been generally shown to be elevated, BMR is depressed (Donati _et al_. 1963). Our findings have confirmed that the oxygen consumption of rats fasted 96 hr are lower than controls although consistent increases in myocardial LPL activity were not found.

Barker (1956) demonstrated in thyroidectomized rats injected with T_3 that oxygen consumption of heart slices responded at 12 hr after injection whereas the oxygen consumption of skeletal muscle, liver and kidney didnot increase until after 24 hr. It has also been shown that heart rate was stimulated before BMR in myxedematous rabbits treated with thyroxine (Guz _et al_. 1961). Since it is apparent that the heart is more sensitive to thyroid hormones than is overall metabolic activity in other tissues (Tata 1964) our belief that myocardial LPL is not stimulated by the increase in metabolic rate following thyroxine is justified.

3. Mediation by catecholamines

The question of whether or not catecholamines participate in thyroxine's stimulatory action on heart LPL

is a complex one. Such a possibility was raised by Alousi and Mallov (1964) because of the similar effects of adrenalin and thyroxine on LPL. In contrast, the increases in lipoprotein lipase activity associated with fasting and after exercise are believed to occur independently of catecholamines since prior treatment with reserpine had no effect on these increases (Nikkila et al. 1965). Also, the elevation of heart LPL activity during cold exposure is thought to be independent of the action of the sympathomimetic amines (Grafnetter and Grafnetterova 1967).

One of the difficulties in assessing the effect of the catecholamine-depleting drug, reserpine, upon thyroxine's stimulatory action on LPL is that reserpine itself may effect the enzyme. Reserpine treatment (2mg/kg) for 4 days was reported to result in a considerable decrease in normal rat heart LPL activity, and led to the conclusion that the amount of lipase was reduced (Drury 1961). Nikkila et al. (1963), using the same dose for 2 days showed no decrease in LPL activity. In fact, the reserpine-treated group had higher LPL activity than controls. However, such treatment with reserpine was found to result in a loss of appetite over the 2 days. Consequently, this group was compared to a group of (two-day) fasted rats whose LPL levels were the same as the reserpine-treated group. Mallov and Cerra (1967) found that reserpine (1 and 2 mg/kg)

produced an increase in heart LPL activity of normal rats after about 20 hours; no explanation of this increase was made. In the present study, a 1 mg/kg dose of reserpine administered to normal rats appeared to decrease LPL activity after 38 hours (Fig. 16). In hypothyroid rats this dose elevated enzymatic activity (Fig.13) whereas 0.2 mg/kg reserpine decreased levels slightly (Fig. 14).

Doses of reserpine in the range of 1-5 mg/kg have been reported to deplete the rat heart of 75-90% of its catecholamines within a few hours (Mallov and Cerra 1967). Furthermore, it was shown that approximately 2 weeks were required for norepinephrine-replentishment in the rat heart after a single 5 mg/kg dose of reserpine (Paasonen and Krayer 1958). Neuvonen and co-workers (1969) showed that a 0.2 mg/kg dose of reserpine produced a 20% depletion in norepinephrine content of normal rat heart 16 hr after administration; a 1 mg/kg dose resulted in a 60% depletion in the same time. Since the catecholamine-depleting action of reserpine wears off relatively slowly, it is likely in the present experiments that, at 38 hr after the 1 mg/kg dose, the heart is considerably depleted. The heart is less depleted of its catecholamines 38 hr after the 0.2mg/kg dose although levels are probably still significantly below non-reserpinized levels.

Large doses of reserpine (such as 1-5 mg/kg) may have severe side effects on an animal. The functioning of the central nervous system and pituitary are affected (Westermann 1963);symptoms of hypotensive shock, diarrhea and somnolence are not uncommon (Goodman et al. 1955). Indeed, in the present studies, most of the rats treated with 1 mg/kg reserpine showed the latter two symptoms. The 1 mg/kg dose of reserpine produced severe side effects which may have obscured the effect of catecholamine-depletion on LPL.

The finding that reserpine pretreatment did not prevent the stimulatory action of T_3 on lipoprotein lipase supports the hypothesis that thyroid hormone acts on this enzyme independently of catecholamines. The response of LPL to T_3 was the same in both reserpine-pretreated (1 mg/kg) and non-treated hypothyroid rats (Fig. 13). The 0.2 mg/kg dose of reserpine slightly decreased basal levels of LPL activity (Fig. 14) but the percentage increase from these lowered levels following T_3 was approximately the same as the increase in activity of LPL in non-reserpinized animals.

If thyroxine stimulated LPL via the release of endogenous catecholamines one might expect a higher concentration of catecholamines in the heart after thyroid hormone treatment. Decreased catecholamine levels have been reported in rat heart after thyroid hormone treatment

(Goodkind 1966), and Hornbrook and Brody (1963) showed no increases in hyperthyroid rat heart. Furthermore, Buccino et al. (1967) recently showed no significant changes in norepinephrine content of ventricles from either hypo- or hyperthyroid cats. The failure to demonstrate elevated catecholamine levels in heart tissue as a result of thyroid hormone administration supports the proposal that thyroxine stimulates LPL independently of catecholamines.

Strong evidence exists indicating a direct stimulatory action of catecholamines on myocardial LPL. It has been demonstrated that epinephrine not only rapidly stimulates rat (Mallov and Cerra 1967) and rabbit heart LPL *in vivo*, but also in isolated perfused preparations (Mallov and Alousi 1969). In agreement with the work of Mallov and Cerra (1967), we found elevated LPL activity 3 to 4 hours after epinephrine injection (0.5 mg/kg) to normal rats (Fig. 16). Although large doses of reserpine have been reported to release catecholamines from their storage sites (Himms Hagen 1967) it is unlikely that the 1 mg/kg dose increased LPL activity by this mechanism since, as it has been mentioned, myocardial catecholamines are probably in a depleted state 38 hours after administration of the drug.

Another possibility for the elevated LPL activity produced by the 1 mg/kg dose of reserpine is that higher

doses of the drug may cause a hypersensitivity of the enzyme to catecholamines. However, our findings that in normal rats both 0.2 mg/kg and 1 mg/kg reserpine depressed LPL activity (3 mg/kg produced variable results) and that the response to epinephrine in reserpinized (1 mg/kg) rats was reduced (Fig. 16), do not support this proposal.

A third possibility for the elevation of heart LPL activity produced by 1 mg/kg reserpine is that this dose, administered to a hypothyroid animal, may cause a decrease in food intake. The elevation in LPL activity may consequently be due to the animal being in a fasted state. Doses of reserpine greater than 0.5 mg/kg have been reported to decrease the food intake of rats (Premachandra 1965). Although reports of the duration of fasting required to elevate heart LPL levels differ (page 33), an increase due to such a mechanism after reserpine (1 mg/kg) is a likely possibility.

A Direct action of thyroxine -

i) Protein synthesis - The findings that the heart rate and rate of oxygen utilization (q O_2) of myocardial tissue were stimulated before BMR and q O_2 of other tissues (Tata 1964) as well as the demonstration of fatty acid oxidation in heart homogenates being stimulated before BMR (Hoch 1962) support the hypothesis that thyroxine acts

directly on the heart. The work of Buccino et al. (1967) indicates that thyroid hormones act on myocardial contractility independently of catecholamines. Further support for this hypothesis is provided by the demonstration that the addition of T_3 to isolated myocardial cells of a 24-hr chick embryo culture produced an immediate increase in the pulsation rate (Wollenberger 1965).

That the primary action of thyroid hormones on the heart is a stimulation of protein synthesis is supported by the work of Beznak (1967). She demonstrated that, whereas the hearts of untreated, hypophysectomized rats underwent atophy, T_4 treatment prevented atrophy. Furthermore, cardiac hypertrophy induced by unilateral nephrectomy in young hypophysectomized rats did not occur unless the animals were treated with thyroxine. Such findings suggest that protein synthesis indeed may be a primary action of thyroid hormones on the heart.

Strong evidence that thyroxine acts on protein synthesis is provided by the work of Widnell and Tata (1963) in which hypothyroid rats were injected with 20ug T_3/100g and time courses of hepatic nuclear RNA polymerase, mitochondial amino acid incorporation and cytochrome oxidase activities were determined. Comparing our time course of LPL activity after 50ug T_3/100g it was found that the increase in nuclear RNA polymerase activity preceeded, and mitochon-

drial amino acid incorporation and cytochrome oxidase activity closely coincided with the elevation in LPL activity. These investigators showed the former activity to be increased by 20 hours whereas the latter two activities and LPL activity did not increase until between 20 and 40 hours after T_3. Moreover, it was found that thyroxine increased mitochondrial alpha-glycerophosphate dehydrogenase activity of those tissues in which oxidative rate is elevated by thyroid hormones. Thyroidectomy resulted in decreased alpha-GPDH activity in liver, kidney and in heart tissue (Lee and Lardy 1965). The indication of these findings is that the fundamental action of thyroxine is upon protein synthesis and thus the hormone may act upon heart LPL by stimulating its rate of synthesis.

ii) Activation via adenyl cyclase - Levey and Epstein (1969) suggested that the cardiac manifestations of the hyperthyroid state may, in part, be caused by the direct activation of myocardial adenyl cyclase by thyroid hormone. Such an hypothesis may explain the mechanism of thyroxine's action on heart lipoprotein lipase. Their findings point to the existence of at least two adenyl cyclase systems in the heart, one responsive to catecholamines and one to thyroid hormone. This further adds support to the theory that thyroid hormones affect the

heart independently of the adrenergic system.

It has been demonstrated that beta-adrenergic blocking agents prevent the activation of myocardial adenyl cyclase by catecholamines (Sutherland et al. 1968). Since T_3 and T_4 stimulated cat heart adenyl cyclase in the presence of a concentration of the beta-adrenergic blocking agent, propranolol, sufficient to block completely the effects of norepinephrine on this enzyme, Levey and Epstein (1969) proposed that thyroid hormones act on a separate adenyl cyclase. Their demonstration that maximal stimulatory doses of T_4 and norepinephrine produced an additive effect on 3',5'-AMP production further supports the proposal of two adenyl cyclases.

It is possible that thyroid hormones activate heart LPL via the stimulation of adenyl cyclase synthesis. The stimulation of adenyl cyclase would result in an increase in the level of 3',5'-AMP; LPL might be activated by the elevated level of this nucleotide. A similar mechanism has been proposed for the action of thyroid hormones on adipose tissue lipase (Krishna et al. 1968); this mechanism will be discussed shortly.

An increase in cyclic AMP was demonstrated in rat heart homogenates in which lipolytic activity was stimulated by epinephrine and norepinephrine (Kruger et al. 1967). A catecholamine-sensitive lipase which was stimulated by 3',5'-AMP

has also been shown in perfused rat heart by Christian and co-workers (1968). However, it is not known whether this lipase is exclusively LPL or other lipases. It must be borne in mind also that the lipolytic activity referred to as LPL may be a mixture of enzymes.

d) Summary of section II

The time course of LPL activity varied with the dose of T_3, with activity increasing more rapidly and to a greater extent with a higher dose. This finding together with that of increased LPL activity preceeding changes in plasma FFA and oxygen consumption indicates that thyroid hormone acts directly on the enzyme. Depletion of tissue catecholamines by prior treatment with reserpine did not alter the stimulatory effect of T_3 on LPL, indicating that thyroxine acts on this enzyme independently of catecholamines. There is considerable evidence that the primary action of thyroxine is upon protein synthesis and thus the hormone may stimulate the synthesis rate of LPL. Another possible mechanism of action, activation of LPL via the adenyl cyclase system was also examined. The adenyl cyclase found in cat heart, sensitive to thyroxine, may prove to be of importance as a mechanism of action of thyroid hormone on LPL. However, at present such a proposal is speculative.

III. THYROXINE AND PLASMA FREE FATTY ACIDS

The level of FFA in the plasma is governed by the balance between utilization by tissues and output from adipose tissue. FFA are utilized by tissues for oxidation and for esterification to glycerides and phospholipids (Shapiro 1967). Thyroid hormone administration increases fatty acid oxidation in the heart and results in decreased glucose oxidation. Also, it is held that the rate of fatty acid oxidation is regulated by the availability of FFA (Bressler and Wittels 1966). Thus, the plasma level of FFA is primarily controlled by the rate of lipolysis, rather than by the rate of utilization. Nutritional and endocrine factors are responsible for mobilizing FFA from adipose tissue by acting upon the hormone-sensitive adipose tissue lipase (Shapiro 1967). Epinephrine, norepinephrine, glucagon, ACTH and thyroxine have been shown to stimulate lipolysis in adipose tissue (Shapiro 1967, Vaughan 1967).

If FFA utilization was increased, either by the direct action of thyroxine or through the elevation in BMR, then the plasma FFA level would be expected to be low. The fact that plasma FFA are increased following thyroid hormone administration means that lipolysis increases concurrently since the plasma FFA level reflects the balance between input and output of FFA. However, because the

turnover rate of FFA in the plasma is great (Hollander et al. 1967), fluctuations in concentration are not uncommon. This, in part, discredits the plasma FFA level as an accurate indicator of bodily FFA metabolism.

The changes in plasma FFA concentration after T_3 followed a similar pattern to that of LPL activity. The highest concentrations of plasma FFA parallelled the peak activities of the enzyme (Fig. 11). However, whereas LPL activity at 67 hr had only returned to control levels at the 50ug/100g dose, plasma FFA levels at all 3 doses were back to control values. The rapid rise and fall of plasma FFA is compatible with a mechanism of activation of adipose tissue lipase by the interaction of thyroxine and catecholamines.

Thyroid hormones have been shown to interact with the sympathomimetic amines at the level of adenyl cyclase. Recent evidence suggests that thyroxine increases the amount of adenyl cyclase in adipose tissue. The increased amount of this enzyme allows endogenous catecholamines to stimulate the formation of cyclic 3',5'-AMP at an increased rate, the level of 3',5'-AMP being the limiting factor in activating adipose tissue lipase (Brodie et al. 1966). Thus, thyroxine acts to activate adipose tissue lipase rather than to increase its synthesis by stimulating the synthesis of adenyl cyclase (Krishna et al. 1968).

As has been mentioned, an adenyl cyclase system may be responsible for the actions of thyroxine on heart LPL. However, an interaction with catecholamines upon LPL has not been shown. Also, since LPL appears more sensitive to thyroxine than adipose tissue lipase, as evidenced by the elevation in LPL activity but not in plasma FFA following 50ug T_3 per 100g (Fig. 8), the mechanisms of action of the hormone on these enzymes are not likely the same. The proposed thyroxine-sensitive adenyl cyclase system found in cat heart (Levey and Epstein 1969), if present in rat myocardial tissue, may be more sensitive to thyroid hormones than the adenyl cyclase system in adipose tissue. Of course, further studies are necessary in order to propose such an adenyl cyclase system as the mechanism responsible for thyroid hormone stimulation of heart LPL.

The results of our experiments with reserpine indicate that catecholamines do participate in the FFA-mobilizing action of T_3. Reserpine pretreatment (0.2mg/kg) prevented the rise in plasma FFA as a result of T_3 injection (Fig. 15b). An increase in plasma FFA after T_3 was still found in the group pretreated with 1mg/kg reserpine. Although neither dose of reserpine alone elevated plasma FFA significantly, Neuvonen et al. (1969) found a 32% increase in plasma FFA 16 hr after an injection of 1mg/kg reserpine to normal rats. It is possible that a FFA-mobilizing action

of 1mg/kg reserpine, via the liberation of catecholamines in adipose tissue, caused plasma FFA levels to be elevated in the above experiment even though no significant increases were shown in the group treated only with reserpine.

Another alternative for the elevated plasma FFA in the reserpinized (1mg/kg) group treated with T_3 is that, as was mentioned in regards to LPL, reserpine may have caused the animal to be in a fasted condition. Plasma FFA would have increased as a result of fasting. The fact that the group administered 1mg/kg reserpine alone did not show elevated plasma FFA levels may be due to an insufficient number of animals.

IV. CALORIGENIC ACTION OF THYROXINE

a) Action of acute triiodothyronine administration

Upon examination of the changes in oxygen consumption over 67 hours after T_3 injection (Fig. 11A) two features are apparent. The first is the rapid increase in oxygen consumption found at 12 hours after the 250ug/100g dose, followed by a decrease and then more gradual increase. Secondly, a dose-dependent relationship was not found.

The rapid increase in oxygen consumption found 12 hr after the 250ug/100g dose is not an uncommon effect of a large dose of thyroid hormone. Tata (1964) reported that large doses of thyroxine can result in elevations in oxygen consumption with short or with no latent periods. The cellular sites reached by such large doses of the hormone may be quite different from those affected by more physiological doses. These "extra-physiological" sites may respond immediately and mask the physiological response.

In the present work, the most physiological dose of T_3 (50ug/100g) produced an increase in oxygen consumption between 20 and 30 hours after injection (Fig.8A). Tata and co-workers (1963) similarly found a latency of 20-30 hr using a 20ug/100g dose of T_3. In both cases hypothyroid rats were used in which oxygen consumption was at least 30% below normal values. Tata's group found an

increase of eleven per cent 25 hr after injection. Our 50ug dose produced an increase of 25% after 20 hr. The difference in sensitivity of metabolic rate to T_3 depends not only on the dose but also on such factors as the strain of rats, the degree of hypothyroidism and the depth of anaesthesia if measured when anaesthetized. The more hypothyroid the animal, the more likely its BMR will increase to a greater extent with a given dose of T_3. It was previously reported that in hypothyroid rats sudden and unpredictable changes in oxygen consumption occurred during sleep and basal conditions (Bramante 1960). During the course of our work the oxygen consumption of rats, measured under anaesthesia, gradually decreased while the animals became more hypothyroid (Fig. 4) and seldom showed any "sudden or unpredictable" changes.

It is unlikely that the rapid rise in oxygen consumption at 12 hr after the high dose is due to the release of endogenous catecholamines, since we have found that the sympathomimetic amines are not involved in the calorigenic action of T_3 (pp. 94-97). Furthermore, heart LPL activity and adipose tissue lipolysis (as indicated by plasma FFA levels), which are both stimulated by the action of catecholamines, were not increased at 12 hours after T_3 injection (Fig. 10).

The oxygen consumption of the rat has been shown to follow a dose-dependent increase up to doses of T_4 of about 8ug/100g (Eartly and Leblond 1954), and 50ug/100g (Grad 1952, Swanson 1956). Perhaps at the relatively large doses used in the present experiments such a relationship does not exist. A physiological dose of T_4 has been estimated at between 2 and 4ug per 100g body weight (Johansson and Jonsson 1968, Eartly and Leblond 1954, Fregly 1959). Another factor to be considered in the explanation of the apparent lack of a dose-dependent relation is that in the works cited, T_4 was used and in our experiments T_3 was administered. Although Hirvonen and Lang (1962), using very high doses (1000ug/100g) found no difference in the duration of increased oxygen consumption when comparing the effects of T_4 and T_3, it is generally held that the duration of thyroxine's (T_4) effect on oxygen consumption is longer than that of T_3 (Tata 1964). Triiodothyronine is a more potent stimulator of BMR however, (Tata 1964, Evans et al. 1964), and it acts more rapidly than thyroxine (Hoch 1962).

b) Mechanism of thyroid hormone calorigenesis

As has been mentioned in the introduction (p. 11) it is believed that thyroxine's calorigenic effects are due to a stimulation of protein synthesis (Tata 1964). In a study in which Widnell and Tata (1963) injected hypothyroid

rats with 20ug T_3/100g it was found that the chronological sequence of events, in liver, which preceeded elevations in BMR was as follows: an increase in nuclear RNA polymerase, increased mitochondrial amino acid incorporation, and increase in cytochrome oxidase activity. Further evidence that calorigenesis is dependent upon protein synthesis is provided by the demonstrations that i) puromycin reduced the elevated basal metabolic rates of thyrotoxic rats to normal levels (Weiss and Sokoloff 1963) and that ii) actinomycin-D blocked the T_3-induced increase in BMR of thyroidectomized rats (Tata 1963). Although demonstrations of protein synthesis being a prerequisite for thyroid hormone calorigenesis have appeared, the precise mechanisms whereby thyroxine stimulates protein synthesis and calorigenesis are unknown.

Since both catecholamines and thyroxine appear to have similar stimulatory effects on myocardial LPL and since it is known that these hormones have similar calorigenic effects on the whole animal, a closer examination of the relationship between thyroid hormones and catecholamines with respect to calorigenesis is warranted. The results of our experiments using reserpine to attempt to deplete body stores of catecholamines indicate that the sympathomimetic amines are not involved in the calorigenic action of T_3 since i) reserpine treatment had no effect on the oxygen

consumption of hypothyroid rats, and, ii) the calorigenic effect of T_3 (at 20 hr after injection) clearly was not altered by either dose of reserpine (Figs. 13, 14).

Bray (1964), using chronic administration of T_3 (6.6ug/100g) and reserpine (0.3mg/kg) similary showed that reserpine had no effect on BMR of thyroidectomized, hypothyroid rats and had no effect on the increase in BMR produced by T_3. Others have indicated that sympathetic blocking agents and reserpine can prevent the calorigenic effect of thyroxine (Ramey et al. 1955, Kuschke and Gruner 1954). Kuschke and Gruner administered reserpine (0.5 and 0.3mg/kg) and T_4 (150 and 200ug/100g) together over a period of 6 days to normal rats and reported that reserpine lowered the increased BMR due to T_4 to normal levels. However, these investigators did not examine the effect of reserpine alone on the BMR of the rats. It has been demonstrated that reserpine, administered at the same dose schedule as used by Kuschke and Gruner for 3 days instead of 4 caused a significant decrease in the oxygen consumption of euthyroid guinea pigs (De Felice et al. 1957). Furthermore, reserpine treatment (0.5mg/kg daily for 3 days) has been reported to lower the oxygen consumption of euthyroid rats (Taylor and Fregly 1962). Thus, the decrease in BMR shown by Kuschke and Gruner may have been partially due to a lowering of the euthyroid level and not due to a blocking action of reserpine on the calorigenic action of

administered thyroxine. De Felice and co-workers (1957) have shown that reserpine lowered the oxygen consumption of hyperthyroid guinea pigs although they reported patients on prolonged reserpine treatment have shown no change in metabolic rate. Furthermore, most sympathetic blocking agents do not alter the increased metabolic rate of human hyperthyroidism (Himms Hagen 1967).

Since reserpine was used to help clarify the actions of thyroid hormones it is necessary to examine briefly the relationship between this drug and thyroid function. As with many of reserpine's effects conflicting results exist. It has been shown that reserpine does not interfere with the peripheral utilization of thyroxine but may block the response of the thyroid to TSH (Taylor and Fregly 1962). In man, there are reports that reserpine does not affect thyroid function (Goodman and co-workers 1955, De Felice et al. 1957). Reserpine has been shown both to decrease (Moon and Turner 1959), and not to alter (Premachandra 1965) thyroid secretion rate. Similarly, thyrotrophin secretion has been reported to be depressed (Moon and Turner 1959) and unaltered (Yamazaki et al. 1961, Premachandra 1965) as a result of reserpine treatment. The dose of reserpine employed appears to be the critical factor with respect to a change in thyrotrophin or thyroxine secretion (Premachandra 1965). Thus, the question of whether reserpine affects thyroid

function is unresolved.

Based upon available evidence and supported by our findings it appears that thyroid hormones exert their calorigenic action independently of catecholamines.

V. THYROXINE AND PALMITYLCARNITINE ACYL TRANSFERASE

The resulting time course of PCAT activity suggests that T_3 acts directly on this enzyme. The finding of elevated PCAT activity before an increase in plasma FFA (Fig. 17) suggests that the hormone acts on the enzyme before raising plasma FFA levels. Bressler and Wittels (1966) proposed that the increase in myocardial fatty acid oxidation as a result of thyroid hormones was due to the increased supply of plasma FFA, resulting from thyroxine's lipolytic action on adipose tissue. They showed that PCAT activity was elevated in the hearts of hyperthyroid guinea pigs. Since PCAT is believed to be the rate-limiting enzyme for fatty acid oxidation in heart muscle (Fritz 1967) then the above results suggest that the level of FFA is the factor controlling PCAT activity in response to thyroid hormone treatment. It is held by Fritz that hormones which affect the rate of fatty acid oxidation in the heart do so by influencing the activity of PCAT. The stimulation of myocardial fatty acid oxidation by thyroxine before elevating plasma FFA (p. 75) together with the finding that thyroxine elevates qO_2 of myocardial tissue before that of other tissues supports the hypothesis that thyroid hormones act directly on myocardial enzymes; the findings of the present work, in support of Fritz's theory, indicate that

thyroid hormones act directly on myocardial palmitylcarnitine acyl transferase.

CONCLUDING REMARKS

The findings of the present study indicate that thyroid hormones act directly on myocardial lipoprotein lipase. Such a direct stimulation allows the myocardium to utilize fatty acids associated with plasma lipids in the face of increased energy demands by the heart before plasma FFA levels are elevated. The demonstration of *in vitro* stimulation of LPL by thyroxine would confirm that the hormone acts directly on the enzyme. In addition, in order to show more conclusively that thyroid hormones stimulate LPL independently of the sympathetic nervous system, the demonstration of an unaltered response of the enzyme to administered thyroxine in the presence of beta-adrenergic blocking agents would be extremely valuable. Studies using such agents could also help confirm that thyroxine's FFA-mobilizing action is dependent upon an interaction with catecholamines.

Further investigations of the type mentioned for LPL are necessary in order to show that thyroid hormone acts directly on myocardial palmitylcarnitine acyl transferase. The use of inhibitors of protein synthesis would be useful in determining whether thyroxine stimulates these myocardial enzymes via the acceleration of enzyme synthesis rate. The other mechanism of action of thyroid hormones on LPL discussed

in this study, activation via the adenyl cyclase system, would have strong support if it could be shown that cyclic 3',5'-AMP stimulated LPL activity. Finally, further studies on the mode of action of thyroid hormones on heart lipoprotein lipase, to be conclusive, require the use of a purified form of the enzyme which up until this time has not been prepared.

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