PHYSIOLOGICAL EFFECTS OF β-LIPOTROPIC HORMONE

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Physiological Effects of β-Lipotropic Hormone

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SUMMARY

Sheep β-Lipotropic Hormone (β-LPH) caused an increase in free fatty acids (FFA) levels in rabbits. β-LPH also produced hypocalcemia. In thyroidectomized rabbits β-LPH administration caused lipolysis but did not affect the calcium. The lipolytic response of thyroidectomized rabbits was lower than the lipolytic response in normal rabbits.

During the lipolysis induced by β-LPH accumulation of calcium in fat tissue both in vitro and in vivo has been observed. Experiments with $^{45}Ca^{++}$ and ^{3}H-mannitol as well as the uptake of calcium by isolated fat cells are in favor of the view that calcium accumulates in fat cells rather than in the extracellular space.

β-LPH stimulated the adenyl cyclase in isolated rat and rabbit fat cells. Stimulation of lipolysis by β-LPH as well as the stimulation of adenyl cyclase by β-LPH was demonstrated as calcium dependent.

Differences in adenyl cyclase systems of rabbit and rat adipose tissue cells were described.

To Majka, Martina and Andrew

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by

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ABBREVIATIONS

The following abbreviations were used in this presentation:

AAS: Atomic Absorption spectrophotometry

ACTH: Adrenocorticotropin

ADH: Antidiuretic Hormone

AMP: Adenosine Monophosphate

c-AMP: Cyclic Adenosine Monophosphate

ATP: Adenosine Triphosphate

CMC: Carboxymethylcellulose

CPM: Counts per minute

DCB: 1-(2,4,-dichlorophenyl)-1-hydroxy-2-t-butylamino ethane hydrochloride

DEAE: Diethylaminoethyl

DPM: Disintegrations per minute

EDTA: Ethylenediaminetetraacetate

EGTA: (ethylenebis[oxyethylenenitrilo]-tetraacetic acid

FFA: Free Fatty Acids

FMS: Fat Mobilizing Substance (Chalmers)

GDP: Guanosine diphosphate

GMP: Guanosine monophosphate

c-GMP: Cyclic guanosine monophosphate

GK: Glycerokinase

GPD: α Glycerophosphate dehydrogenase

GTP: Guanosine triphosphate

c-IMP: Cyclic Inosine monophosphate

LATS: Long acting thyroid stimulator

LH; Luteinizing Hormone

LMF: Lipid Mobilizing fraction (Trygstad)

β-LPH: Beta-lipotropic hormone (unless otherwise stated the sheep β-LPH was used)

γ-LPH: Gamma-Lipotropic hormone

α- and β-MSH: α- and β-melanocyte stimulating hormone

NAD, NADH: Nicotinamide adenine dinucleotide and its reduced form.

NPS-ACTH: o-nitrophenyl sulfenyl ACTH

NPS-β-LPH: o-nitrophenyl sulfenyl β-LPH

POPOP: p-bis 2-(5-phenyloxazolyl)-benzene

PPO: 2,5-diphenyloxazole

PTH: Parathyroid hormone

TG: Triglycerides

1. INTRODUCTION

The study of the lipolytic properties of different pituitary hormones has previously been limited to known factors already isolated and more or less identified. However, numerous other fractions isolated from different species have been assayed for their lipolytic activity. These fractions were characterized only superficially and the apparent variability in results obtained from different laboratories has rendered comparison difficult. Recent progress in protein chemistry, particularly in the techniques of isolation and purification of peptides from complex and heterogenous biological materials as well as progress in the techniques of protein structure determination have made possible the precise characterization of some of these pituitary fractions.

Sheep β-lipotropic hormone was the first pituitary lipolytic factor to be chemically characterized (129). This was soon followed by the purification and characterization of a structurally related compound termed γ-lipotropic hormone (γ-LPH) (46). The most interesting features of the structure of these two peptides are the common sequences with α- and β-MSH and ACTH. The exact function of these pituitary peptides is not known. However, some data are now available that β-LPH is present in the circulation. For example β-LPH was successfully extracted from the sheep plasma (131) and its concentration was determined by radioimmunoassay as 10 ng/ml of sheep plasma (Chrétien & al - submitted for publication). Moreover similar peptides were isolated from porcine pituitaries (86) (73) (85) and also from human pituitaries (54).

The present study, utilizing β-LPH, was designed to contribute to the understanding of this question. Accordingly, experiments were

conducted in order to clarify the mode of action of β-LPH on fat tissue, as well as the mechanisms of hypocalcemia induced by β-LPH in rabbits and to determine the involvement of calcium in β-LPH action.

Certain aspects of lipolysis, including the importance of the pituitary gland in mobilization of fat tissue triglycerides and the possible function of calcium in lipolysis, will be discussed in the review of the literature.

2. REVIEW OF THE LITERATURE

2.1. Mechanism of mobilization of fat tissue triglycerides

2.1.1. Historical review

Until the past 40 years, fat tissue was considered as a static element, almost indeed as a type of connective tissue filled with fat droplets (224). The dynamic character of fat tissue and its unique properties in fat storage and mobilization processes were soon noted and related to particular physiological and pathological conditions (90) (222). A significant milestone in the understanding of metabolism and physiological function of fat tissue was the demonstration of release of free fatty acids both *in vivo* and *in vitro*, its stimulation by catecholamines and ACTH (59) (226) (225) (83), and the demonstration of lipogenic properties of glucose (198).

Following the introduction of the isolated fat cell technique of Rodbell (167) and the discovery of cyclic AMP (165) as the second messenger of hormonal action, a new period of research on adipose tissue had commenced. Adenyl cyclase activity has been localized in fat tissue (204) and its relation to the activation of hormone-sensitive lipase has been demonstrated (35). The system has been further analyzed and recently a preparation of purified lipase had been activated *in vitro* by the protein kinase isolated from muscle and the c-AMP (106).

Exhaustive reviews and bibliography are to be found elsewhere, for example (161) (108) on fat tissue or (165) on c-AMP.

In this presentation only the white adipose tissue will be discussed.

2.1.2. Ultrastructure of fat cells

Despite technical problems, caused by the presence of large amounts of intracellular lipids, the essential structures of fat cells are well described. Numerous mitochondria are usually observed in the cytoplasm of the fat cell. Smooth and rough endoplasmic reticulum (rough endoplasmic reticulum being generally scarce), and free ribosomes are also present. The nucleus is flattened and pushed to one side by a large lipid droplet (227) (147). Usually few ultrastructural changes are observed during and after lipolysis and/or lipogenesis (227) (147). Such morphological variations have been recently reported by Cushman (55). In attempting to classify the lipid droplets of the adipose cell cytoplasm he divided them into 3 distinct categories.

Type I: Fat droplets surrounded by fenestrated envelope. This fenestrated envelope has an appearance of flattened endoplasmic reticulum.

Type II: Small droplets characterized by the lack of any membraneous envelope and surrounded by an amorphous ground substance. This type appears to be adjacent to the large droplets or to the central droplet.

Type III: Represented by aggregates of very small lipid droplets of variable appearance associated with amorphous material of different electron opacity. Each aggregate is surrounded by fenestrated envelope which sometimes appears to be continuous with the envelope of the large droplet.

Cushman (55) made the significant observation that the type II fat droplets are more abundant in the epinephrine treated cells and that type III is more frequently observed in insulin treated cells. This

statement, however, should be regarded with caution as no objective or statistical criteria were reported.

Some fat droplets, called liposomes, were also isolated after cell disruption and their ultrastructure was shown to be similar to chylomicrons (4).

Murthy (145) observed that small membrane enclosed lipid droplets were broken off the central lipid vacuole of the adipose cell when lipolysis is accelerated.

An important feature of fat cell ultrastructure is the presence of a large number of different vesicles and smooth surfaced membraneous elements suggesting the operation of a complex, compartmentalized transport and metabolic system (55). The cellular membranes have been also observed to show numerous invaginations, vacuoles or rosettes (55). The isolated fat cells exhibit marked pinocytosis activity (56). It is not clear if this in vitro pinocytosis demonstrated on isolated fat cells is of equal importance in vivo.

2.1.3. Hormone sensitive lipase

Lipolysis can be observed in broken cell preparations when a suitable substrate preparation is used. This lipolysis could be stimulated by catecholamines or by ACTH. However, this stimulation occurs only when the hormones are administered prior to cell disruption (99). Several attempts have been made to isolate and characterize the hormone sensitive lipase from adipose tissue (104) (202) (50). The results of these attempts have confirmed that the enzyme is highly lipophilic, probably a lipoprotein (103) or a protein saturated with lipid substrate (216).

Variable and sometimes contradictory results were reported when the enzyme is purified by ultracentrifugation. The variable results could be attributed to the tendency of the hormone sensitive lipase to associate strongly with the neutral fat in homogenates. The yields of lipase in different fractions were therefore subject to substantial variability depending on the composition of the medium as well as the duration and force of centrifugation. Following the centrifugation of homogenate in Krebs-Ringer bicarbonate at 18,000 g for 30 minutes the lipase activity was found in the infranatant (184). When centrifuged for 30 minutes in 0.1 M phosphate buffer at 15,000 g it was recovered in the fat cake (201). In 0.25 M sucrose at 12,000 g it was found in the infranatant, using the same conditions at 105,000 g for 12 hours the activity was found in the pellet (162). In 0.15 M KCl at 15,000 g for 10-30 minutes it was localized in fat cake (216) and in Krebs-Ringer phosphate at 12,000 g for 10 minutes the activity has also been found in fat cake (52). Furthermore a high yield of triglyceride lipase activity was recovered in the clear 105,000 g supernatant when the tissue was homogenized in 0.25 M sucrose containing 10^{-3} M EDTA (104). More complications are evident from the fact that the hormone-sensitive lipase is not the only lipase to be found in fat tissue homogenates as lipoprotein lipase, monoglyceride lipase and possibly other enzymes are also present (99) (201) (216) (184) (116). The reported inhibitory effect on the lipase activity of infranatant (52) could further obscure the real distribution of the measured activities. Some indirect evidence has been also presented that the hormone-sensitive lipase is localized on the inner surface of the adipocyte plasma membrane (51).

2.1.4. Activation and inhibition of lipolysis

Surprisingly many substances of no apparent structural relation activate lipolysis when incubated with fat tissue preparations in vitro.

The best known and widely used lipolytic substances are the catecholamines. Their receptors on fat tissue were considered to be of mixed type, both α and β. These findings were, however, obscured by the nonspecific inhibition of lipolysis by both α and β-adrenergic blockers. Recently general agreement was reached and the receptors in fat tissue were declared to be of β character, more exactly of the β_2 nature as proposed (121) for the relaxation of trachea or uterus. As far as the white adipose tissue of the rat is concerned this question was well discussed by Himms-Hagen (94). The nonspecific inhibitory action of α blockers could probably be due to their direct effect on the lipase rather than to the inhibitory action at the receptor level (125).

Lipolytic compounds of pituitary origin will be discussed separately in part 2.3.

The other activators of lipolytic process are difficult to classify due to extreme variability of their origin and structure. For example lipolysis can be activated in vitro by such widely differing substances as cholera toxin (217), EDTA (141), serotonin (16) or LATS (89). This is only a random example of the variability of lipolysis stimulating compounds.

Even more variability is encountered with factors inhibiting the stimulation of lipolysis. At the present time the only physiologically important inhibitors of lipolysis reported are insulin (172), the prostaglandins (47) and, with appropriate caution, a yet unidentified c-AMP inhibitor released by stimulated fat tissue as described by Manganiello & al (135) and Ho & Sutherland (97).

Even insulin, which has been recognized as the antilipolytic substance, has been recently described to enhance the lipolytic effect of high doses of epinephrine (58).

2.1.5. Adenyl cyclase-3'5' adenosine monophosphate system in fat tissue

Following the important discovery of 3'5' adenosine monophosphate (c-AMP) significance in glycogenolysis (165) a deluge of potential applications to other fields has occurred during the past ten years. After the initial finding that c-AMP can mediate the glycogenolytic effect of epinephrine (204) other hormones have also been shown to act through c-AMP mediation in different organs e.g. insulin (64), ACTH, ADH, MSH, PTH, etc (165).

The intracellular level of c-AMP is now considered to be the result of the activity of two enzymes, one regulating its formation, the other regulating its degradation. The enzyme regulating the formation of c-AMP from ATP has been named adenyl cyclase. Because of its lability, and probable complex molecular arrangement, adenyl cyclase has not yet been purified (21). The properties of adenyl cyclase suggest that it is a lipoprotein (205) (150) and that it is localized in the membrane (205) (152). Moreover, some indirect evidence suggest that its activity is located on the inside part of the plasma membrane (150). Adenyl cyclase requires Mg++ (which could be replaced by Mn^{++}) and is stimulated by fluoride ion (205) (154) (153). Optimal pH for maximum enzyme activity is relatively broad and had been estimated to be approximately between pH 7.2 and 8.2 (205).

The most significant biological degradation of c-AMP is accomplished by phosphodiesterase which hydrolyses it to 5' AMP (43). Consequently changes in activity of phosphodiesterase will affect the c-AMP level and the duration of hormone action. Phosphodiesterase is present in almost all tissues examined (165). The enzyme is relatively stable and can be stored for months at -20°C (43). Its molecular weight is 200,000 or greater as determined by gel filtration on Sephadex G-200 (136). However results from different sources (43) indicate that the phosphodiesterase may be composed of subunits and that its molecular weight range could be somewhere between 130,000-750,000. Phosphodiesterase requires divalent ions for maximal activity (43) and the pH optimum is between 7.5-8.0 (43). Phosphodiesterase is inhibited by p-hydroxy mercuribenzoate, the inhibition being reversible by mercaptoethanol. This suggests the importance of its sulfhydryl groups (44). The other known inhibitors of phosphodiesterase are the methylxanthines (36) which are used in the majority of methods for measurements of adenyl cyclase or the c-AMP. Inhibitory effects are also reported for ATP, pyrophosphate (40), c-GMP, c-IMP (43), citrate and some other carboxylic acids (41), puromycin (8), triiodothyronine (134), diazoxide (139), and papaverine (149). Phosphodiesterase stimulation can be produced by imidazole (36), ammonium salts (146) and an activator or a protein cofactor (42).

It has been observed that the phosphodiesterase level is lower in some tissues of diabetic rats. The activity has been restored to normal levels by insulin. Effect of insulin was blocked by actinomycin D (194). Loten & Sneyd (133) observed in their preparation two K_m for an adipose tissue phosphodiesterase which could suggest either the presence of two enzymes or a single enzyme displaying negative cooperativity (63). Insulin raised V_{max} of the low K_m type of phosphodiesterase and lowered the K_m of

the high K_m type of activity without changing the V_{max} (133). In contrast to the findings of Senft & al (194) this type of insulin stimulation of phosphodiesterase has not been affected by cycloheximide (133). Observation with the two kinetics of phosphodiesterase activity (133) could probably explain the negative results of some earlier observations where considerably higher substrate concentrations were used (24) (92). Nevertheless because of some other conflicting results the exact role of insulin in the phosphodiesterase regulation remains unsettled.

In tissue homogenate the phosphodiesterase could be solubilized although a relatively high percentage of activity is usually retained in the particulate fraction (36).

In adipose tissue intracellular c-AMP concentration increased with the lipolytic response (35). However the rate limiting c-AMP concentration in fat cells was 180-300 picomoles per gm and the rate of lipolysis does not increase further at higher concentration (up to 1000 picomoles per gm) of c-AMP. This relationship between the intracellular levels of c-AMP and lipolysis is essentially the same with all the substances used to increase c-AMP levels (48). Another important pattern of c-AMP levels - lypolysis relationship is the timing. Maximal c-AMP levels were usually reached within 5 minutes, and significant changes noted as early as 15 seconds after epinephrine administration while the lipolytic response could be detected after 13 minutes (34).

Those methylxanthines known as phosphodiesterase inhibitors were reported to act synergistically with lipolytic hormones (218) (35).

Exogenous c-AMP usually did not stimulate lipolysis <u>in vitro</u> in intact fat pads, even slight inhibition has been noted (214). However, the derivative (N^6-2'-o-dibutyryl-cyclic AMP) is an effective lipolytic agent

in vitro in both intact fat pads or isolated fat cells (35). Exogenous c-AMP lipolytic activity has been, however, observed in isolated fat cells incubated in a modified incubation media (in buffer without Ca^{++} and Mg^{++}) (144).

Why the exogenous dibutyryl c-AMP is more active than c-AMP is not yet fully understood. Its resistance to degradation by phosphodiesterase has been described (136). Since the dibutyryl c-AMP can inhibit cellular phosphodiesterase, it was also proposed that by inhibiting phosphodiesterase, dibutyryl c-AMP might stimulate intracellular c-AMP (114). It was also assumed that the increased potency of the dibutyryl derivative of c-AMP is due to its more nonpolar nature which permits easier penetration of the cell membrane in the intact cells (199). Another explanation has been based on the fact that dibutyryl c-AMP showed no stimulatory effect on glucose oxidation whereas c-AMP stimulated glucose oxidation as well as lipogenesis so that the lipolytic effect is less evident (199).

Adenyl cyclase of fat cell ghosts of rat epididymal fat pads has been examined by the Rodbell group and their work has been published in a series of three extensive articles (20) (22) (170). Their findings could be summarized as follows: The enzyme has been characterized as bound to the membrane, unstable at 0^{o} and $37^{o}C$ and inactivated by trypsin. The enzyme requires divalent cations (Mn^{++}, Mg^{++} and Ca^{++}). Calcium inhibits its activity. The kinetic characteristics indicates two binding sites for Mg^{++}, one being at the catalytic site, the other probably allosteric. As a substrate for adenyl cyclase $Mg^{++}ATP$ has been proposed (the ATP alone proved to be inhibitory). No product inhibition by c-AMP has been noted. Activation by fluoride and by ACTH showed some differences. ACTH dose-response

curves were hyperbolae whereas fluoride curves were of sigmoid character. Thus it has been concluded that ACTH and fluoride ion activate the adenyl cyclase by different mechanisms (20).

Adenyl cyclase has been activated by such different lipolytic hormones as ACTH, epinephrine, glucagon, TSH and LH. When various combinations of these hormones were used their effects were not additive provided that maximally stimulating concentrations were used. From this finding Rodbell and his group (22) concluded that one enzyme only is activated. Propranolol inhibited specifically the action of epinephrine while a biologically inactive analogue of ACTH inhibited specifically and competitively the stimulatory action of ACTH. From these findings it was concluded that the hormones act at discrete hormone-specific receptors or discriminators (22).

The relation of glucagon and secretin has been furthermore compared in their activation of adenyl cyclase of adipose cell ghosts. Secretin was found inactive on liver plasma membrane preparations whereas both secretin and glucagon were active on the membranes of adipose tissue, secretin being more active than glucagon. EGTA, a calcium chelator, abolished the effect of ACTH and did not affect the actions of secretin, glucagon, TSH and LH. DCB(1-[2,4,-Dichlorophenyl] -1-hydroxy-2-t butylamino ethane hydrochloride), a β adrenergic blocking agent, inhibited the effects of epinephrine. DCB did not affect the action of secretin, glucagon and ACTH. Furthermore pretreatment of adipose tissue with trypsin decreased the lipolytic response to glucagon but did not affect the action of secretin and ACTH. Glucagon also failed to stimulate adenyl cyclase activity in ghosts prepared from trypsin treated fat cells, the effect of secretin and ACTH were reduced by 60% and 40% respectively. Trypsin did not change the

responsiveness of adenyl cyclase to fluoride ion and epinephrine. From these experiments it can be concluded that secretin, glucagon and other lipolytic hormones have distinct receptors or discriminators. It was also suggested that the receptors or discriminators of peptide hormones are proteins and are localized on the outer surface of the plasma membrane (170). Essentially similar findings concerning the nature of the receptors on fat cell membrane were reported by other groups (118) (13) (12). Some results concerning the role of calcium will be discussed in part 2.4.

A recent interesting finding indicates that, in isolated liver cellular membrane preparations, GTP and GDP stimulate the rate and degree of dissociation of labelled glucagon. They also decreased the uptake of glucagon by the membrane and the affinity of binding sites for glucagon (173). On the other hand GTP and GDP enhanced the initial rate of glucagon stimulated adenyl cyclase activity in liver cell membranes. In the same preparation guanyl nucleotides inhibited the response of adenyl cyclase to fluoride ion. It was concluded that guanyl nucleotides may play a specific and obligatory role in the activation of adenyl cyclase by glucagon. The nucleotides appear to bind at a distinct site from that of glucagon (171).

Protein kinase purified from rabbit muscle stimulated the lipolysis in fat tissue homogenated in the presence of c-AMP. The protein kinase from the muscle has been utilized because of technical difficulties in purifying the protein kinase from fat tissue (49). A more defined system has been arranged by other groups (106) (105). They utilized the protein kinase from muscle and partially purified lipase from fat tissue. The lipase has been activated in presence of c-AMP, ATP and protein kinase. Whether the protein kinase is phosphorylating the lipase directly as is the case of glycogen synthetase (23) or whether there are some other steps

as was described for glycogen phosphorylase (221) has not yet been clarified (105).

The experimental evidence for the interrelationship between lipolysis and c-AMP could be summarized as follows:

(a) Intracellular c-AMP increases with lipolysis.

(b) The intracellular c-AMP increase closely precedes the lipolysis.

(c) Lipolysis is stimulated by methylxanthines, which are known phosphodiesterase inhibitors. The activity of other lipolytic agents is potentiated in presence of methylxanthines.

(d) Exogenous dibutyryl c-AMP and (under some conditions) c-AMP stimulate lipolysis.

(e) All of the "fast acting" lipolytic hormones were reported to stimulate adenyl cyclase in fat tissue.

(f) An <u>in vitro</u> system has been prepared where c-AMP activated the partially purified lipase in the presence of protein kinase from muscle.

2.1.6. Species differences in the activation of hormone sensitive lipase

The fat tissues from different species present considerable variability in their response to various lipolytic hormones. Observations related to such species variability were tentatively summarized by Rudman (174) and by Shafrir & Wertheimer (197) as follows:

ACTH had adipokinetic activity in the rabbit, guinea pig, hamster, rat and mouse, but little or no activity in the dog, pig or man.

TSH has been found to be adipokinetic in guinea pig, rat, mouse, and dog whereas little or no activity was observed in the rabbit, hamster or pig.

α- and β-MSH are potent lipolytic agents in the rabbit and guinea pig but are inactive in the hamster, rat or pig.

Vasopressin is active in the rabbit and guinea pig whereas it is not in the hamster, rat, pig or dog.

Epinephrine and norepinephrine are very active in hamster, rat, mouse, dog, monkey and man, but are not lipolytic in the rabbit, guinea pig and pig.

Glucagon (176) is very active in rats, inactive in the hamster and slightly active in the rabbit.

Rudman (174) tried to classify the species specificity and proposed 3 distinct groups.

(a) Rabbit and guinea pig: responsive to nearly all the adipokinetic pituitary polypeptides and unresponsive to catecholamines.

(b) Hamster, rat and dog: responsive to certain of the pituitary polypeptides and also to the catecholamines.

(c) Pig: unresponsive to either pituitary peptides or to catecholamines.

Later human fat tissue was added as a new group (142) which is responsive to catecholamines but resistant to pituitary peptides.

As the mechanism of activation of hormone-sensitive lipase was studied in a more detail and as experimental techniques became more refined, conclusions derived from earlier investigations should be judged with extreme caution. In this regard, results reported by Rudman (174) (175) that rabbits are unresponsive to catecholamines were questioned (200) and it was subsequently demonstrated that there was indeed some change in the FFA/glycerol ratio after epinephrine administration to rabbits in vivo. Isolated fat cells of young rabbits have also been reported to respond to epinephrine essentially to the same degree as rat fat cells (65) (137).

Porcine adipose tissue, generally considered as a remarkably resistant to all lipolytic stimulation, has also been found to be responsive in vitro (32) when the lipolytic agents were incubated in presence of theophylline. Theophylline alone is inactive but it strikingly enhanced the lipolytic effect of ACTH, TSH, dibutyryl c-AMP, epinephrine and isoproterenol which were alone also inactive. The synergistic effect of theophylline was not seen with glucagon. Propanolol blocked the epinephrine and isoproterenol action on pig fat tissue (32).

α adrenergic receptors have been well identified in human adipose tissue, where phentolamine, an α blocker, enhanced the lipolytic response to epinephrine. These α adrenergic receptor sites were not demonstrated in rats (31).

It is obvious that the concept of responsive groups of animals proposed by Rudman needs some revision because under certain conditions some species could become responsive to lipolytic substances considered as inactive. One such example is the responsiveness of rat adipocytes to LH. Pretreatment of the rats with steroids (progesterone or dexamethasone being the most potent) induced the sensitivity to the LH (84). Lysine-

vasopressin is usually not lipolytic in rats but pretreatment of the rats with reserpine could sensitise the fat cells to vasopressin action (231).

From these results one can conclude that the non-responsiveness in some species (at least as far as mammals are concerned) does not necessarily imply the lack of an appropriate receptor but rather an influence of some other collateral regulatory or inhibitory systems not yet fully understood. The responsiveness to certain peptides could also be influenced by the presence of peptidase activity (181). Interestingly enough this activity was not detected in rabbit fat tissue where the pituitary peptides are usually the most active (181).

In assessing these results it should be noted that the anatomical source of fat tissue is important since the lipolytic rates could depend on localization (197). Also the age of the experimental animals is an important parameter since older animals are usually less active (197). The comparative physiology of white fat tissue should attract more interest as the rat adipose tissue model, which has been used extensively thus far, is not completely satisfactory. From the results on rat fat tissue few conclusions can be drawn concerning human adipose tissue.

It is still mandatory to define clearly what is unresponsiveness in certain species when all the known or at least partially known factors are considered. In this connection <u>in vitro</u> versus <u>in vivo</u> experiments, level of lipolytic compounds, the age of animals, the anatomical localization of fat, the species specificity of the pituitary lipolytic peptides (197) and even seasonal variations (124), all serve to influence rational experimental interpretation.

2.1.7. Energy requirements for the lipolysis

Glucose addition to incubated adipose tissue usually inhibits FFA release (109). However, it was also observed that in addition to the decreased FFA there is an increased release of glycerol (10) which has been explained by increased reesterification due to the higher availability of α-glycerophosphate. Over all lipolysis was allegedly increased due to the reduction of intracellular FFA which could be inhibitory. The added pyruvate was found to be inferior to glucose in the formation of glyceride glycerol but under certain conditions pyruvate stimulated the lipolysis by essentially the same way as glucose (95). ATP cellular levels were usually decreased by lipolytic compounds and this decrease was restored by addition of pyruvate, glutamate or aspartate. Consequently it was proposed that the availability of ATP is essential for the activation of hormone sensitive lipase (95). Hollenberg & Patten (101) reduced intracellular ATP by oligomycin and 2-deoxy-D-glucose and they also found lower lipolytic rates after the stimulation of lipolysis by dibutyryl c-AMP. These results indicated that the activation of lipase by c-AMP required ATP and confirmed some previous experiments with fat tissue homogenates where ATP was also required for lipase activation by c-AMP (163). Interesting hypothesis concerning compartmentalization of ATP intracellular stores in fat tissue have been also proposed (101). Depressed ATP levels after application of lipolytic hormones are probably the consequence of intracellular FFA accumulation which depress ATP synthesis by uncoupling oxidative phosphorylation (5).

2.1.8. Intracellular concentration of free fatty acids. Possible metabolic consequences.

The FFA intracellular levels are dependent on essentially three rate limiting factors. These factors are the rate of lipolysis, availability of albumin as FFA acceptor outside the cell and the rate of reesterification (168). The basal cell FFA content of fed rats has been determined as 0.5 micro-mole per gram of cell lipid. Addition of lipolytic compounds rapidly increase this amount which reaches a plateau of about 2-2.5 micro-moles per gram of cell lipid (6). The maximum rate of lipolysis in isolated fat cell in vitro is approximately 600 µEq FFA per gram per hour (168) and the actual intracellular FFA concentration is relatively very low. However, the intracellular FFA level could be of importance and rate limiting for many vital functions of fat tissue. The FFA were reported to inhibit hexokinase, glucokinase, phosphofructokinase and pyruvate kinase. Acetyl-Co A, which is a degradation product of FFA also inhibits glucokinase and pyruvate kinase; this phenomenon has been called sequential feedback inhibition by Weber & al (223). In 200 g rats the adipose tissue critical FFA intracellular concentration is approximately 3 micro-moles per gram of cell lipid. When the intracellular FFA exceed this critical concentration the uncoupling effect of FFA probably occurs with subsequent lowering of intracellular levels of ATP (5). An interesting phenomenon was reported by Rodbell (169) who was able to demonstrate release of proteins as well as some nonparticulate enzymes by isolated fat cells secondary to the rise in intracellular concentration of FFA. It was suggested (169) that this release of cellular protein could represent a regulatory mechanism of the metabolic capacity of adipose tissue. The FFA intracellular regulatory

function was further proposed for α-aminoisobutyric acid uptake by fat cells in vitro (209) and for regulation of glucose metabolism within these cells (96).

2.2. Importance of pituitary gland in lipolysis

2.2.1. Physiological studies indicating the pituitary involvement in lipolysis

Marked physiological mobilization of fat occurs in starvation, in exercise, in adaptation to upright posture, in adaptation to extrauterine file, in psychological stress, and in exposure to cold (75).

Hypophysectomy seems to slow the depletion of lipid in fasted rats (128). The increase of FFA observed during fasting is significantly lowered by hypophysectomy (81). Also lipolysis induced by exercise is partially depressed by either hypophysectomy of by β-adrenergic blockade while both abolished it almost completely (74). The role of nonadrenergic regulation of lipolysis with possible pituitary involvement in fasting has been also suggested by in vitro studies with isolated fat cells of fasted rats (29). The adipose tissue removed from hypophysectomized rats exhibits lower rates of lipolysis as well as lower responsiveness to adrenaline (80) (82).

There are also some older observations about the action of crude pituitary extracts injected into animals. Administration of such extracts produced ketosis (7), body weight loss and hepatic lipid accumulation (15). Other such examples have been reviewed elsewhere (63) (70) (45).

2.2.2. Permissive effects of pituitary gland in lipolysis

The exact role of the pituitary gland in mobilization of fat stores is very difficult to assess since adrenals and thyroid gland which are themselves controlled by the pituitary, are involved in the lipolytic process. Nevertheless complex experiments were devised to analyze the role of individual hormones on fat tissue responsiveness to lipolytic agents.

As previously stated by Goodman (76) the effect of hypophysectomy on fat mobilization cannot be attributed to the loss of a single adipotropic hormone but rather appears to result from the loss of several factors acting either directly or indirectly. The impairement of in vitro lipolysis in fat tissue from hypophysectomized animals is caused by increased reesterification, decreased maximal glycerol production rate as well as a lower sensitivity to lipolytic hormones (76). However, these changes are partially corrected by glucocorticoids (196) (195) (76), triodothyronine (79) (76) (78), growth hormone (80) (195) (115) (76), and by their combinations (76). The effect of dexamethasone has been blocked by actinomycin D and was reproduced in vitro. Growth hormone potentiated the effect of dexamethasone (76). Pretreatment of hypophysectomized rats with triiodothyronine also potentiated the responsiveness of fat tissue in vitro. The effect of triiodothyronine was not inhibited by actinomycin D (76). In some experiments the effect of triiodothyronine on fat tissue responsiveness was reproduced directly in vitro (215). In other experiments only the in vivo pretreatment with triiodothyronine was effective (76). Growth hormone alone was also shown to decrease FFA reesterification (75). The interrelationships between permissive effects of glucocorticoids, triiodothyronine and growth hormone were further analyzed by Goodman (76) and he proposed that the mechanism by

which these hormones exert their actions might be different and that all three are probably required for optimal function of fat tissue. As the pituitary gland is involved in regulation of the secretory activity of both adrenal cortex and thyroid gland, the key role of pituitary gland in the optimal function of fat tissue could be postulated even if the direct lipolytic influence of pituitary hormones might be not physiologically important in all species.

2.3. Lipolytic compounds of pituitary origin

2.3.1. Pituitary Hormones

Surprisingly most pituitary hormones have been reported to increase lipolysis in preparations of fat tissue from different species.

ACTH has been shown to stimulate the release of FFA in rat adipose tissue in vitro (225). Following this initial observation of White & Engel (225) ACTH has been used as a lipolytic agent in the majority of in vitro experiments on rat adipose tissue. Later other pituitary hormones were found to possess lipolytic activity in vitro when incubated with fat tissue or with isolated fat cells. The lipolytic activity of MSH has been analyzed by Tanaka & al (207). The lipolytic activity of TSH was reported by Freinkel (68). Curiously enough LATS-IgC, which possess TSH activity but is structurally very different, is also lipolytic (89). Gospodarowicz (84) has recently shown that the α sub-unit of LH has some lipolytic activity in the mouse and under some conditions in the rat. Arginine-vasopressine is adipokinetic in rabbit adipose tissue (175). Growth hormone possesses lipolytic activity when combined with glucocorticoids. The

mechanism of the action of growth hormone is different from that of the other known lipolytic hormones and is not yet clear. However, it seems that it acts by increasing protein synthesis as indicated by its delayed action and by the blocking ability of actinomycin D or cycloheximide (66).

The physiological significance of lipolytic activity of these hormones remains to be elucidated but the species differences should be considered as discussed in part 2.1.6.

2.3.2. H fraction

Rudman & al (183) purified from pig pituitaries a lipolytic fraction which has been called fraction H. Later a more purified fraction H perparation has been called fraction L (182). This peptide is lipolytically active in rabbits both in vitro and in vivo, weakly active in guinea pigs but inactive in rat, mouse, hamster, dog and pig (180). Paper electrophoresis of fraction H revealed the presence of 3 components. Further purification on DEAE cellulose yielded 5 subfractions, one of which (fraction L) possessed all the adipokinetic activity of fraction H and was shown to be electrophoretically homogenous (182). The purification of fraction H from hog pituitaries as well as the measurement of its biological activities were later repeated by Schwandt & al (191) (190) (189) (192).

2.3.3. Peptide I and peptide II

After the initial observation of lipolytic activity of crude extracts of porcine anterior pituitaries (120) Astwood and Friesen tried to purify and to identify the active components (14) (9). They isolated two

peptides (peptide I and II). They published that peptide I had a molecular weight below 10,000 while peptide II had a molecular weight between 10,000 and 20,000. The biological properties of these two peptides were also extensively studied (72) (70). Peptide I and peptide II were lipolytically active in rabbits both in vitro and in vivo. After intravenous injection of 10-25 µg of peptide I to rabbit the FFA increase was seen within 5 minutes (70). As little as 0.02 µg/ml of peptide I caused a significant increase in the release of FFA in vitro when incubated with rabbit mesenteric fat (70). When large amounts of peptide I and peptide II (10-20 mg) were injected into dogs or human subjects only a slight FFA increase was sometimes observed (72) (70).

2.3.4. β-lipotropic hormone (β-LPH) and γ-lipotropic hormone (γ-LPH)

C.H. Li and his collaborators obtained from sheep pituitaries a lipotropic peptide which was chemically distinct from ACTH and other known adenohypophyseal hormones. They called it fraction L' (18). Later, following modifications in the extraction procedure they obtained another lipolytic peptide designated as β-LPH (129). In the course of the isolation of β-LPH certain side fractions were further examined which resulted in isolation and purification of a new peptide called γ-LPH (46). It should be noted that the complete amino acid sequence of these two peptides was also determined (129) (46). Consequently it is easier to compare them with other structurally known hormones and the structure-action relationship could be more easily followed than in the case of other lipolytic peptides only partially characterized. Bovine β-LPH was reported indistinguishable from ovine β-LPH on the basis of its amino acid composition, N-terminal

analysis, molecular weight and biological activity (240). β-LPH and γ-LPH were also found and purified from porcine pituitaries (86) (73) (85). Preliminary communication about the presence of a peptide similar to the porcine or sheep β-LPH in human pituitaries has been also reported (54).

2.3.5. Other lipolytic pituitary fractions reported

Trygstad observed the presence of lipolytic compounds in crude preparations of growth hormone and ACTH from human pituitary glands (210). These activities were much lower in pure preparations. Trygstad postulated the existence of a human pituitary lipid-mobilizing fraction (LMF). This LMF was further purified and was found active in vitro and in vivo on human fat (211). Electrophoretically pure LMF showed adipokinetic activity in rabbits and none of the hypocalcemic activity shown previously in less pure preparations. It was concluded that the hypocalcemic and adipokinetic activity are the actions of different molecules (212).

Rudman & al (177) reported the isolation of two lipolytic peptides from pig pituitary acetone extract. These peptides were labeled as a 7D6 and 7D7 respectively.

Other lipotropic principles have also been found by other groups. Ryshka & Khokhlov (185) purified a lipotropic substance from bovine, ovine and porcine pituitaries. Also reported are the "Fettstoffwechselhormon" of Schleyer & al (188) (187) (186) purified from porcine pituitaries, the "lipid mobilizer" found in the posterior lobe of the horse by Seifter & Baeder (193), the "lipid mobilizing factor" found in the human posterior pituitary by Lelek & al (127), the lipolytic substance purified from human pituitaries by Leites & Davtyan (126), and from porcine pituitaries by

Yudaev & Pankov (232). However, the exact comparisons of all these pituitary factors is very difficult as their chemical characteristics are not known and the possible contamination by other lipolytically potent hormones has not been always ruled out and the techniques of extraction and purification are also different.

In connection with the pituitary gland, one should also mention the "fat mobilizing substance" (FMS) of Chalmers (37) found, during fasting, in the urine of some animals as well as in humans. The finding of such FMS in the urine of fasted individuals seems to depend on the integrity of the adenohypophysis (37).

It should be also noted here that the pituitary gland is not the only tissue from which lipolytic substances have been extracted. Such compounds have also been found in calf mid-brain (102), in hypothalamus of different species (160), pineal (179) (178), in the thyroid and parotid glands (178).

Antilipolytic activity has also been reported (33) in pituitary fractions.

2.3.6. Structural relationship of pituitary lipolytic fractions

The most striking feature found in the structure of β-LPH and γ-LPH is fragment 41-58 (129) which is identical to the structure of β-MSH. Hypotheses that the β-LPH and the γ-LPH peptides might be the precursors of β-MSH has been supported by the presence of two basic amino acids immediately preceeding and immediately following the β-MSH sequence. This is similar in proinsulin (38) at the site of the C-peptide cleavage.

The structural relationships between pituitary fractions are summarized in table 2.3.6.1. The table was arranged with the data from references used in parts 2.3.2., 2.3.3., 2.3.4. and 2.3.5.

The pituitary lipolytic peptides could be divided into 2 groups. Peptide I, porc and sheep β- and γ-lipotropins, fraction L' and probably also the peptide of Ryshka & Khokhlov (with the exception of N-terminal indicated as methionin) are the peptides, structurally related to β- and α-MSH and ACTH, and containing the sequence of Met-Glu-His-Phe-Arg-Try-Gly. This sequence is essential for the lipolytic and melanotropic properties of these molecules (98). The other group comprises peptide II, peptide 7D6, fraction H and L, peptide 7D7. They are probably related to neurophysins (177) (71). Friesen & Astwood showed a marked decrease of peptide II in pituitary posterior lobe in dehydrated animals and they were able to form a dissociable ionic complex of peptide II with lysine-vasopressin in vitro (71).

A possible third group might be constituted with the lipolytic factors which cannot be classified in the previous two groups, since insufficient data are available to compare them (188) (193) (127) (126) (232).

The relationship of chemical structure to the in vitro lipolytic activity of the peptides related to α- or β-MSH and ACTH has been studied by Tanaka & al (207). They found the pentapeptide His-Phe-Arg-Try-Gly as a minimal requirement for lipolytic activity in rabbit fat tissue. The lipolytic activity of this pentapeptide gradually increased with the addition of the amino acids to give the sequence corresponding to the N-terminal portion of ACTH. ACTH 1-17 reached the full activity of the entire ACTH molecule. Tanaka & al (206) further followed the lipolytic activity of this pentapeptide corresponding to the positions 6-10 of N-terminal of ACTH

Table 2.3.6.1. STRUCTURAL RELATIONSHIP BETWEEN PITUITARY LIPOLYTIC FRACTIONS

PEPTIDE	MOL. WT.	N-TERMINAL	C-TERMINAL	A.A. COMPOSITION	IMMUNOCHEMICAL CHARACTERIZATION	BIOLOGICAL ACTIVITY (LIPOLYSIS)
SHEEP β-LPH & BOVINE β-LPH	9,958	Glu	Gln	LACK OF HALF-CYSTINE TRYPTOPHAN RESIDUE PRESENT	CROSS-REACT WITH PORCINE β-LPH BUT NOT WITH 7D6 AND TRYGSTAD'S LMF	ACTIVE IN RABBIT IN VITRO AND IN VIVO, ACTIVE IN RAT IN VITRO
PORCINE β-LPH	9,971	Glu	Gln	IDEM	CROSS-REACT WITH SHEEP β-LPH	ACTIVE IN RAT AND RABBIT
SHEEP γ-LPH	5,809	Glu	Asp	IDEM		ACTIVE IN RABBIT, NOT ACTIVE IN RAT IN VITRO
PORCINE γ-LPH	6,000-7,000	Glu	Asp	IDEM		ACTIVE IN RABBIT, NOT ACTIVE IN RAT
PEPTIDE I	LESS THAN 10,000		Asp	IDEM		ACTIVE IN RABBIT, NOT ACTIVE IN DOG AND HUMAN
PEPTIDE II	10,000-20,000			NO TRYPTOPHAN RICH IN HALF-CYSTINE	CROSS-REACT WITH FRACTION II	IDEM
FRACTION L' OF BIRK & LI	6,600	Glu	Lys	TRYPTOPHAN PRESENT LACK OF HALF-CYSTINE		ACTIVE IN VITRO IN RABBIT & RAT
FRACTION L (H) OF RUDMAN				RICH IN HALF-CYSTINE NO TRYPTOPHAN	CROSS-REACT WITH PEPTIDE II	ACTIVE IN RABBIT IN VITRO AND IN VIVO AND IN GUINEA PIG. NO EFFECT IN RAT, MOUSE, HAMSTER, CHICKEN, DOG AND PIG
LIPOTROPIN OF RYSHKA & KHOKHLOV	7,000	Met		TRYPTOPHAN PRESENT NO HALF-CYSTINE		ACTIVE IN RABBIT AND GUINEA PIG
7D6	8,900			NO TRYPTOPHAN RICH IN HALF-CYSTINE		ACTIVE IN RABBIT, GUINEA PIG AND CHICKEN, INACTIVE IN THE RAT, HAMSTER, CAT, AND OPOSSUM
7D7	5,500			IDEM		
LMF OF TRYGSTAD	2,100					ACTIVE IN HUMANS AND RABBIT

and its stereoisomers , They established that the minimal structure for lipolytic activity was a tetrapeptide His-Phe-Arg-Try and prepared the three stereoisomers which were inactive on rabbit tissue but active on rat tissue. However, in their experiments (206) rather high concentrations were used (1 mg of peptide per fat pad).

The minimum structure unit for lipolytic and melanocyte stimulating activities are essentially the same but for melanocyte stimulating activity the L-configuration of the basic amino acid residues (His-Arg) was necessary and the replacement of one of them or both by the corresponding D-amino acids brought about an anti-MSH activity (87).

2.4. Calcium and lipolysis

2.4.1. Calcium requirements for the activation of hormone sensitive lipase

Lopez & al (132) reported in 1959 that the lipolytic effect of ACTH was abolished when adipose tissue was depleted of ionized calcium. They did not observe such change of activity when epinephrine was used in the absence of calcium. More recently (143) this statement has been modified when it was found that the omission of calcium from Krebs-Ringer phosphate medium diminished slightly the lipolytic action of low concentrations of epinephrine (below 1 µg per ml) (143). This could explain the discrepancies between results which showed complete independence of epinephrine action to calcium when levels of 1-10 µg/ml of epinephrine were used. Mosinger & Vaughan (143) reported that stimulation of lipolysis by theophylline or dibutyryl c-AMP was little affected by the presence or absence of Ca^{++}, Mg^{++}, K^{+} or ouabain in the medium. This is compatible with the suggestion

that the calcium presence or absence could modify the lipolytic action of hormones by influencing it at some early step in hormone action on the cell membrane (143) (132). Different points of view have been also presented. In light of their experiments with human omental tissue in vitro, Efendic & al (62) are considering the possibility that calcium is necessary also for the intracellular activation of the lipase. In their preparation of fat tissue, repeated washing with a chelating agent (EGTA) resulted in a significant suppression of the lipolysis with any of the stimulating agent present, not only epinephrine, but also theophylline and dibutyryl c-AMP.

2.4.2. Calcium uptake by fat tissue during lipolysis

Akgun & Rudman (1), incubating rabbit adipose tissue with ACTH, observed a significant increase in calcium concentration in the tissue. Moreover, they postulated the presence of some serum factor as they observed calcium uptake only when serum was used as incubation medium. Similar findings were reported by Yanagi & al (229) (230) with rat adipose tissue whose calcium uptake increased with ACTH, and Alm & al (3) who observed the same phenomenon in fat tissue in vitro when incubated with ACTH, catecholamines, and theophylline. Alm & al (3) also proposed that calcium uptake could be regulated in fat tissue by intracellular concentration of FFA as the omission of albumin further enhanced the calcium concentration in the tissue.

The relationship between the calcium accumulation in fat tissue and the activation of the hormone sensitive lipase is not clear.

2.4.3. Calcium and 3'5' adenosine monophosphate in fat tissue

The general considerations on the possible relationship between the changes of calcium transport and c-AMP in different biological systems are extensively discussed in the articles of Rasmussen & Tenenhouse (159) and Rasmussen (158). The stimulation of adenyl cyclase by ACTH in fat cell ghosts seems to be fully dependent on the presence of calcium in the incubation medium. Epinephrine, TSH, LH, glucagon and secretin stimulate the adenyl cyclase even in the presence of EGTA, a calcium chelating agent (170). Similar findings were reported by other authors (118) (13) (12). There is,however,slight discrepancy concerning TSH as Kuo (79) reported that TSH stimulation of adenyl cyclase is also influenced by calcium presence or absence which is somehow contradictory to the findings of Rodbell group (170) or to previous observations of Freinkel (68) that the calcium presence is not necessary for the lipolytic action of TSH. However, this can depend on the concentrations of the hormone as was found by Mosinger & Vaughan (143) for epinephrine. Also different experimental conditions could account for this disagreement.

Although the basal activity of adenyl cyclase is inhibited by ionized calcium (20), this cation is necessary for its activation. This could be explained by the fact that the hormonal receptor site is localized on the outer surface of the cellular membrane while the enzyme is on the inner surface. Thus calcium can facilitate the binding of some peptides to their respective receptors, while it acts as an inhibitor on the inner surface of the membrane. On the other hand there are also some indications, as for example in brain preparations, that the calcium itself is able to activate the adenyl cyclase (26). It could indicate that the adenyl

cyclase complex is different from one tissue to the other.

The role of calcium in the regulation of the phosphodiesterase activity in fat tissue is not clear but there are some data indicating that the phosphodiesterase activity in brain can be divided by chromatographic procedures into two peaks: in one, the activity being Ca^{++} and Mg^{++} dependent, the other being Ca^{++} independent (112) (110) (111). It is not known if this applies to the adipose tissue.

A more obscure situation exists in the understanding of the calcium role in the lipase activation in cell free homogenates of fat tissue. There are some data showing that calcium can replace c-AMP for the activation of lipase in fat tissue homogenates (163). Even the hypothesis was suggested that the higher calcium influx into fat cells is the mechanism by which ACTH activates lipase since calcium increased the lipolysis in homogenates and ACTH stimulated calcium influx into the tissue (230). This point was also favorably discussed by Efendic & al (62). However, the opposite has been also reported and calcium was found inhibitory in fat homogenates on both basal and adrenaline stimulated lipase activity (cells pretreated with adrenaline before homogenization) (220).

3. EXPERIMENTAL SECTION

3.1. Material and Methods

3.1.1. Animals and in vivo experiments

Animals - Two hundred and fifty g male rats (COBS® CD strain, Charles River) were fed by Purina chow and tap water ad libitum.

Three kg male New Zealand white rabbits, fed by rabbit pellets (Maple Leaf Mills, Montreal) and tap water ad libitum, were utilized in experiments on rabbits. Unless otherwise stated they were fasted overnight before the experiments.

Male white pigs (18 kg, 14.5 kg, 15.5 kg) were used to follow the lipolytic action of porcine β-LPH. Pigs were fasted for two days before experiment.

Removal of fat tissue for in vitro experiments - Rat epididymal fat pads were removed immediately after decapitation. In each experiment the fat pads of 4-5 rats were pooled before cell isolation.

Rabbit perirenal and epididymal fat was removed under Nembutal anesthesia. Pooled tissue of three rabbits was utilized in each in vitro experiment.

Pig perirenal tissue was removed under Nembutal anesthesia.

Thyroidectomy - Rats were thyroidectomized under ether anesthesia and the rabbits were thyroidectomized under Nembutal anesthesia. Thyroidectomized animals were utilized 2 weeks after operation and the operation was verified at autopsy after the experiment. The controls were sham operated.

Blood samples - Blood was collected in rabbits from ear vein. The ears were shaved, cleaned by alcohol and lubricated by silicon grease (Dow Corning). The ear vein was opened by razor blade and the blood samples collected. Occasionally hyperemia was induced by heat using an infrared lamp. Bleeding was stopped by paper clips. In narcotized rabbits blood samples were withdrawn by syringe through a Silastic® (Dow Corning) cannula forming an arteriovenous anastomosis (carotid-jugular). In pigs the blood was collected from ear vein essentially by the same procedure as in the rabbits.

Blood samples were collected into heparin-containing chilled polyethylene tubes. Tubes were kept on ice and the samples were centrifuged as soon as possible. Plasma was kept frozen until the analysis.

In vivo samples of fat tissue - In one set of experiments the epididymal fat tissue was removed from conscious rabbits under epidural anesthesia with 2% Procain. In other experiments, perirenal fat was removed at different intervals from narcotized rabbits. Animals were narcotized by Nembutal (30 mg/kg i.v.) with chlorpromazine pretreatment (Largactil®, Poulenc, 25 mg i.m.). Assisted respiration was maintained throughout the narcosis.

Experiments on pigs - Pigs were fasted for two days before experiment and blood samples were collected from ear vein. The pigs were tranquilized by i.m. injection of chlorpromazine (1 mg/kg). Tranquilized pigs were kept in a wooden box without any restraint during all the manipulations.

3.1.2. Isolation of fat cells

Epididymal fat cells were isolated after collagenase (Worthington) digestion using the original Rodbell procedure (167) with minor modifications.

Incubation with collagenase was performed in Krebs-Ringer bicarbonate buffer without calcium. Isolated cells were washed and incubated in complete Krebs-Ringer bicarbonate buffer containing 0.5 mM sodium pyruvate (95) and 4% bovine serum albumin (fraction V, Pentex).

The cells were washed and uniformly distributed to polyethylene incubation vials by means of automatic Oxford sampler (Oxford Laboratories) with exchangeable polyethylene tips. Uniformity of the aliquots was verified by determination of the cell volumes with a micro-hematocrit centrifuge. Cell volumes were calibrated by their triglyceride content. The correlation between triglycerides and cell volume is shown in Fig. 3.1.2.1. Incubations were performed in a Dubnoff metabolic shaker at 38°C with a gas phase of 95% O_2 and 5% CO_2. Reactions were terminated by cooling the flasks on ice. Cell-free media were obtained by filtration through a Gelman Metricel GA-1 Cellular filter. EGTA (ethylenebis[oxyethylenenitrilo]-tetraacetic acid) (Baker) was used in some incubations. All incubations were performed in duplicate or quadruplicate and the experiments were repeated at least twice.

When the lipolytic potency of β-LPH was compared on isolated fat cell preparations the results are expressed as a ratio of glycerol released into the medium (E) to the glycerol concentration considered as a maximal response (E max). The maximal response was determined in each assay using 0.1 µg per ml of synthetic 1-24 ACTH (Cortrosyn, Organon).

3.1.3. Incubation of fat tissue with ^{45}Ca and ^{3}H-mannitol

Pieces of epididymal rabbit fat tissue weighing about 50 mg were preincubated for 30 minutes and then incubated in Krebs-Ringer bicarbonate buffer containing 2% albumin, 0.3 µCi per ml of $^{45}Ca^{++}$ ($^{45}CaCl_2$ New England

Nuclear, 14,6 mCi per mg) and 0,6 µCi per ml of D-mannitol-1-^{3}H (New England Nuclear, 3140 mCi per mM). Incubations were performed in polyethylene vials in a Dubnoff metabolic shaker at 38°C. The volume of incubation medium was 1 ml. After incubation, the medium was withdrawn with a Pasteur pipette and the tissue was quickly washed with 5 ml of ice-cold non-radioactive medium. The tissue was then transferred to weighed Parafilm squares. The tissue was blotted with filter paper and then quickly frozen. It was weighed in the frozen state and transferred to the 20 ml vials, where it was dissolved at 65°C in 0.5 ml of NCS® solubilizer, a quaternary ammonium base soluble in toluene (Amersham Searle). Twenty ml of scintillation liquid - 6 g PPO (Amersham Searle)/ 1 liter of toluene and, for correction of possible chemiluminiscence, 2 drops of a solution of ascorbic acid (15%) were added to each counting vial as recommended by Bransome & Grower (27). Samples were counted in a Packard liquid scintillation spectrophotometer. Counting efficiency and quenching were determined by means of external standard ratio. For calibration, we used ^{45}Ca secondary standard (kindly supplied by Dr. Milligan from Queen's University, Kingston) and tritiated toluene standard (New England Nuclear).

The distribution spaces of Ca^{++} and mannitol were determined by dividing the total radioactivity for each isotope in the tissue in d.p.m. by the corresponding specific activities in the medium expressed as per µl.

Similar method was used by Milligan & Kraicer (138) for the measurement of calcium uptake by rat pituitary glands in vitro.

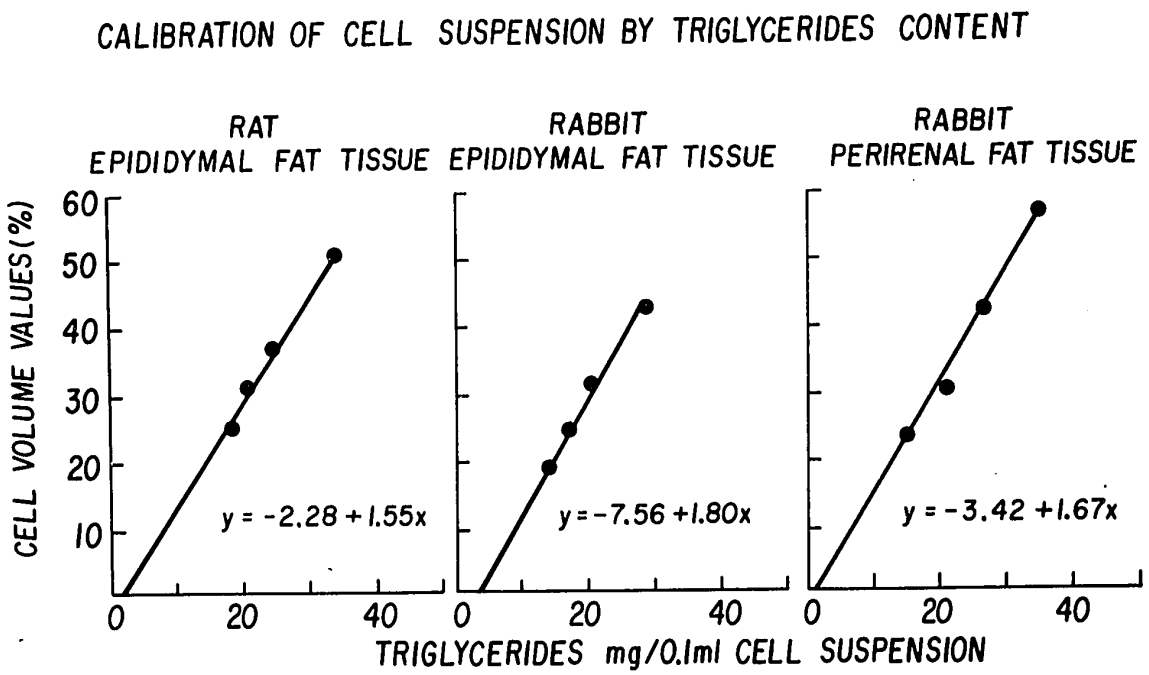

Fig. 3.1.2.1. Isolated cells from 6 g of fat were suspended in 7 ml of Krebs-Ringer bicarbonate buffer. Aliquots were collected and each time the suspension was diluted by additional 2 ml of buffer. Hematocrit values and triglycerides were determined at each dilution. Each point represents 5 measurements. Two hundred and fifty g male rats and 3 kg New Zealand white rabbits were used.

3.1.4. Determination of adenyl cyclase activity in isolated fat cells

I used a modified method based on those previously described (119) (208). Adipose tissue (5 g) cut in 50 mg fragments was incubated in about 4 ml of Krebs-Ringer bicarbonate buffer containing 0.5 mM sodium pyruvate (Baker) and 10 µCi of Adenine-8-^{14}C (New England Nuclear - specific activity of 50.7mCi/mM) at 38°C with 95% O_2 and 5% CO_2 as gas phase. After one hour of incubation, 10 ml of Krebs-Ringer bicarbonate buffer containing 4% of bovine serum albumin (Fraction V, Pentex) and 30 mg of Collagenase (Worthington) were added. Following an additional 60 minutes of incubation, the isolated cells were treated as proposed in the original Rodbell procedure (167). Uniformity of the cell suspension was measured in micro-hematocrit capillaries. Cell volume values were calibrated by their triglyceride content as is shown in Fig. 3.1.2.1. Incubation with hormones were performed in 1 ml volumes of Krebs-Ringer bicarbonate buffer containing pyruvate, 2% Albumin and 10 mM theophylline. The reaction was terminated by the transfer of incubated medium and the cells into the test tubes containing 1 ml of recovery mixture and by immersion of the tubes into a boiling water bath for 4 minutes. The recovery mixture contained "carrier" ATP (Sigma), c-AMP (Sigma) and 0.5 µCi/ml of tritiated c-AMP (New England Nuclear, specific activity 24.1 Ci/mM). Precipitate was then extracted with 2 ml of hexane and after centrifugation 3 fractions were obtained: a hexane phase, a water phase and a precipitate. Fifty µl of the water phase was then chromatographed on silica gel impregnated glass fibre paper (Gelman ITLC-type SG) in the system n-butanol: isopropanol: NH_4OH (7:2:1) (208). Spots corresponding to c-AMP were visualized in UV light, transferred into counting vials and counted in a Packard 3 channel liquid scintillation counter in

15 ml of toluene containing 5 g PPO and 50 mg POPOP per liter. C-AMP was neatly separated from AMP, ATP and adenine as shown in the table 3.1.4.1.

Because of the absence of significant quenching in the system as judged by external standard ratio the results are expressed as CPM. Recovery of tritiated c-AMP varied between 90-100%.

In some incubations, EGTA (ethylenebis-[oxyethylenenitrilo]-tetraacetic acid) (Baker) was added at different concentrations. Every incubation was done in duplicate and every experiment was repeated at least twice to assure good reproducibility.

The only disadvantage of this method is that the phosphodiesterase is not completely inhibited by theophylline even at the concentration of 10 mM as it was utilized in the present experiments. However, no lipolytic hormone was yet reported to affect directly phosphodiesterase activity. Consequently we may consider the accumulation of ^{14}C-c-AMP in fat cells after β-LPH stimulation as adenyl cyclase dependent.

3.1.5. Preparation of the o-nitrophenyl sulfenyl derivative of β-LPH (NPS-β-LPH)

NPS-β-LPH has been prepared by essentially the same procedure published by Ramachandran for the preparation of o-nitrophenyl sulfenyl ACTH (156). This procedure is known to modify the indole ring of the tryptophan residue. Modification of tryptophan is quantitative (239).

Twenty-five mg of sheep β-LPH (purified in our laboratory from frozen sheep pituitaries according to the method of Li & al (129) was dissolved in 0.2 ml of water and 1.8 ml of glacial acetic acid and 25 mg of o-nitrophenyl sulfenyl chloride (Eastman Organic Chemicals) was added.

Table 3.1.4.1.

Rf VALUES ON GELMAN ITLC-TYPE SG

N-BUTANOL: ISOPROPANOL: NH_4OH (7:2:1)

ATP	0
AMP	0
c-AMP	0.53
Adenine	0.93

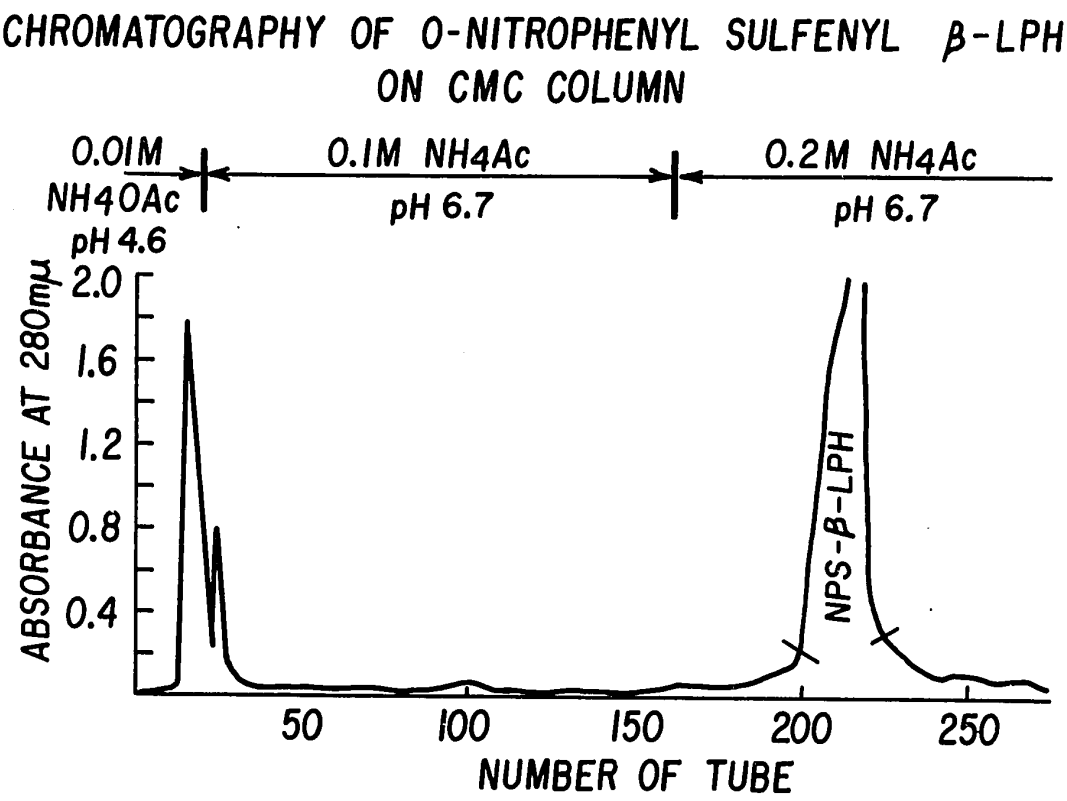

Fig. 3.1.5.1. CMC column size was 20 x 1 cm, mixing chamber volume was 125 ml. Ammonium acetate gradient was changed as indicated. One ml fractions were collected. NPS-β-LPH from the tubes (200-225) was lyophilized and used for biological assays.

After 3 hours of incubation at room temperature 10 ml of ethyl acetate was added. Yellow precipitate was dissolved in 0.1 N acetic acid, and lyophilized. The lyophilized material was purified on a column of carboxy-methyl cellulose using a gradient elution formerly used for the purification of β-LPH (129). NPS-β-LPH emerged from the column as a single peak as is shown in the Fig. 3.1.5.1.

3.1.6. Analytical methods

Glycerol determination - I modified the enzymatic procedure of Chernick (39) and Laurell & Tibbling (123) for semiautomatic determination using the standard Auto Analyzer (Technicon Corporation) equipment (sampler, pump II, fluorometer II).

Principle of the assay:

Glycerol is converted to α-glycerophosphate by glycerokinase and ATP. Under the influence of glycerophosphate dehydrogenase and NAD, dihydroxyacetone phosphate and NADH are then formed. Dihydroxyacetone phosphate is trapped by hydrazine. NADH formed is proportional to the glycerol content of the sample.

Reagents:

1. Glycine 0.2 M
2. $MgSO_4$ 0.15 M
3. Hydrazine-$MgSO_4$ Buffer (10.5 g of Hydrazine HCl dissolved in 70 ml of water. Adjust pH to 9.4 with KOH. Add 1 ml of $MgSO_4$ 0.15 M and make volume to 100 ml).
4. EDTA 1 mM in 0.1 N NaOH
5. NAD (DPN) 20 mg in 1 ml of water)
6. ATP (30 mg in 1 ml of 5% $NaHCO_3$)

7. Glycerokinase (GK) (Sigma) 2 mg/ml - 1 mg will convert approximately 90 μ moles of glycerol per minute.
8. α-Glycerophosphate dehydrogenase (GPD) 10 mg/ml (1 mg will reduce approximately 160 μM of α-glycerophosphate per minute).
9. Standard stock solution - 0.1 M Glycerol.

Solutions 5, 6, and 9 are kept frozen in small volumes. Solutions 1, 2, 3, 4, 7, and 8 are kept at about $0^{o}C$.

Samples:

Incubation medium from in vitro experiments.

0.05 ml of medium and 0.15 ml of water is added to make volume to 0.2 ml.

Samples of blood plasma or serum.

To 0.1 ml of plasma or serum, 0.7 ml of water is added and the solution is heated in boiling water bath for 3 minutes. 0.2 ml of the solution is used for measurement.

Standards:

One ml of the glycerol stock solution (0.1 M) is first diluted to 100 ml with water. Then 0.5 ml, 1 ml, 2 ml, 4 ml, 8 ml are taken and their volume is adjusted to 10 ml with water. 0.2 ml is used for measurement. These dilutions represent 10,20, 40, 80, 160 nanomols per 0.2 ml. Diluted standards can be used for 1 week. Water is used as a blank. The reagent mixture (Table 3.1.6.1.) is prepared immediately before use and cannot be stored.

Procedure:

To a 0.2 ml sample or standard, 0.1 ml of reagent mixture is added and incubated for 1 hour at room temperature in capped Autotechnicon tubes. After incubation 1 ml of EDTA is added and samples are measured by Auto Analyzer fluorometer as shown in Fig. 3.1.6.1.

The method is specific for glycerol as was shown by Chernick (39). None of the other triose intermediates appeared to interfere with enzymatic method of this kind.

Free Fatty Acids - Numerous methods for the measurement of FFA in biological materials were described. They are, however, essentially based on two principles. One is the determination by titration (60) or by an acid-base indicator system (140), the other is the copper soap method as described by Duncombe (61). The disadvantage of the first method is its sensitivity to carbon dioxide dissolved in the serum. The copper soap method, on the other hand, might be influenced by lecithine. I choosed the semiautomatic copper method as modified by Dalton & Kowalski (57) with extraction procedure of Itaya & Ui (107). This extraction procedure was proposed specifically for copper soap method to overcome the lecithine interference. However, in extensive series of samples the extraction method was found too laborious with some difficulties due to the occasional formation of emulsions. When the work presented in this thesis was almost completed, the automated method was developed based on the extraction procedure of Kashket (113) but with detection system of Dalton & Kowalski (57). The flow diagram of the complete Auto Analyzer (Technicon Corporation) system is shown in Fig. 3.1.6.2.

Determination of calcium - Calcium in fat tissue was measured by Perkin-Elmer 303 atomic adsorption spectrophotometer (233) after the tissue had been ashed in platinum crucibles at 600°C. When I measured FFA and Ca^{++} in the same piece of adipose tissue, the extraction procedure of Bligh & Dyer (25) was used. The fat tissue fragments were thoroughly homogenized in a mixture of methanol, chloroform and 0.2 M EGTA by teflon pistle homogenizer. After the separation of two phases Ca^{++} was determined in the aqueous phase and free fatty acids (FFA) in the organic phase.

Table 3.1.6.1.

PREPARATION OF REAGENT MIXTURE

	20 samples	30 samples
Glycine (ml)	0.2	0.3
Hydrazine (ml)	1.4	2.1
ATP (ml)	0.2	0.3
DPN (ml)	0.2	0.3
GK* (µl)	2	3
GPD* (µl)	10	15

* When the enzymes are stored for a long time or when the activity is lower, their volume should be readjusted.

MEASUREMENT OF GLYCEROL BY SEMIAUTOMATIC ADAPTATION OF ENZYMATIC METHOD - FLOW DIAGRAM

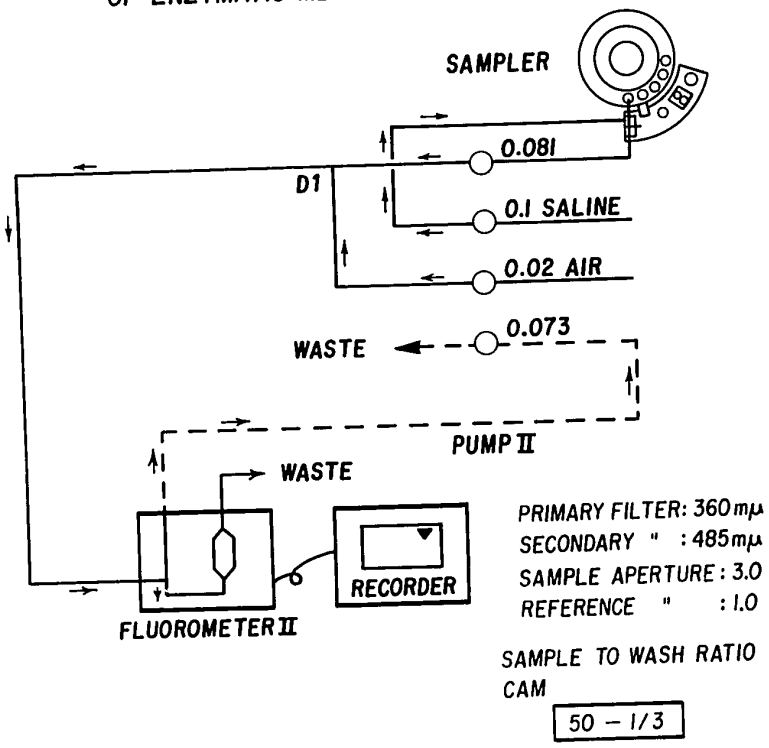

Fig. 3.1.6.1.

FLOW DIAGRAM OF THE AUTOMATED FFA DETERMINATION

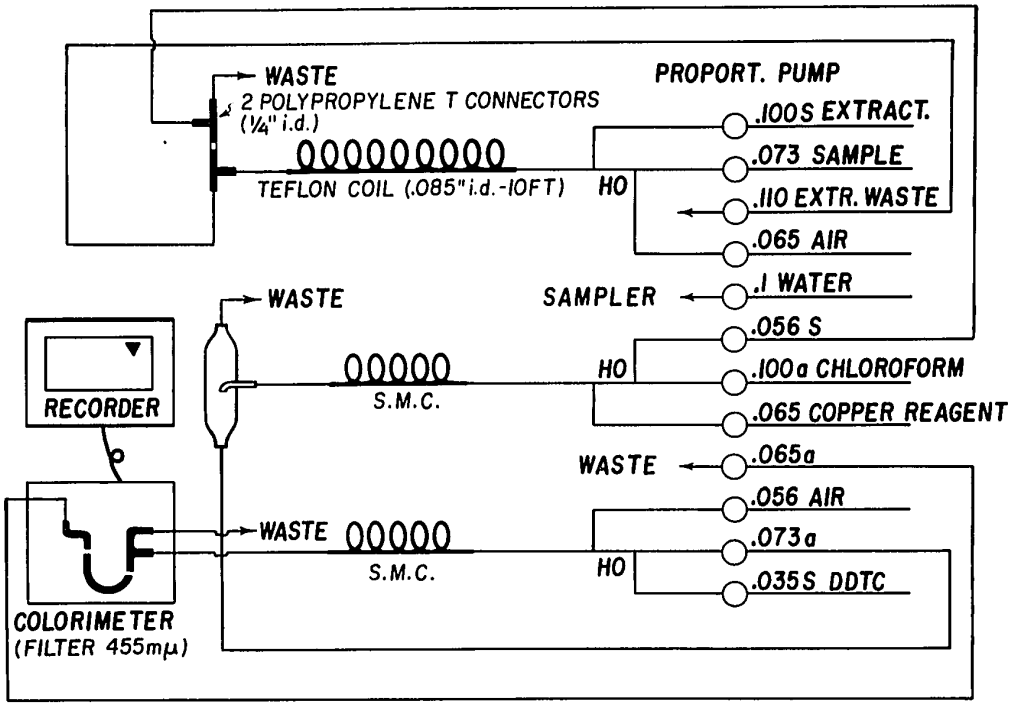

Fig. 3.1.6.2. Extractant (isopropanol-500, heptane-500, H_2SO_4 1N-40) copper reagent (2 M triethanolamine-450, 10% copper nitrate-500, 2N acetic acid-50) DDTC (sodium diethyldithiocarbamate - 1 g in 500 ml isobutanol). Palmitic acid is used as a standard. Cam 30 1:5 is used on sampler. The method was developed by combining the automatic extraction procedure of Kashket (113) with detection system of Dalton & Kowalski (57).

s - solvaflex tubing

a - acidflex tubing

Recovery of Ca^{++} in EGTA containing water phase has been determined using $^{45}Ca^{++}$. Negligible radioactivity has been found in the lower organic phase. The recovery of added $^{45}Ca^{++}$ in aqueous phase of fat tissue extracts is shown in table 3.1.6.2. Calcium in plasma and in the incubation medium was measured by the automated method of Hill (93) (Auto Analyzer method N 31 P).

Some data indicating the reproducibility of the methods for determination of FFA, glycerol and calcium are summarized in table 3.1.6.3.

Other measurements in plasma - In rabbit plasma the magnesium glucose, cholesterol and triglycerides were measured. Triglycerides were measured by the method of Laurell (122). Magnesium was measured by the atomic absorption spectrophotometer (Perkin-Elmer) essentially according to the procedure proposed by Hansen & Freier (88). Glucose and cholesterol were measured by Auto Analyzer methods (Technicon Corporation) (cholesterol method N-24 a) (glucose method N-2 b).

3.1.7. Hormones tested

Sheep, porcine and human β-LPH were purified from fresh frozen pituitary glands according to the procedures of Li & al (129) and Gilardeau & Chrétien (73). Synthetic 1-24 ACTH (Cortrosyn®, Organon) was used. Synthetic α-MSH and β_h-MSH were generous gifts from Dr. Y. Yajima (University of Kyoto).

Criteria of purity of β-LPH purified in our laboratory are as follows: Disc electrophoresis at pH 4.6 and pH 8.3, determination of N-terminal amino acid, and sedimentation analysis.

Mr, P, Doddridge technically participated in AAS determination of calcium and magnesium and in determination of FFA. Mrs. F. Rousseau technically collaborated in isolation of fat cells, in determination of adenyl cyclase, determination of FFA, glycerol and calcium. Triglycerides, glucose and cholesterol were determined in the laboratory of Dr. J. Davignon by Mrs. N. Patard, Mrs. D. Giroux and Mr. M. Tremblay.

Table 3.1.6.2.

RECOVERY OF ADDED $^{45}Ca^{++}$

IN AQUEOUS PHASE OF FAT TISSUE EXTRACTS

	$^{45}Ca^{++}$ C.P.M.	
Organic Phase	31.1 ± 4.6	20.8 ± 1.25
Aqueous Phase	8478 ± 662	20.3 ± 0.57
	^{45}Ca added	Non radio-active control

Fifty mg fragments of rabbit perirenal fat tissue were incubated in 1 ml of Krebs-Ringer bicarbonate buffer containing 0.2 µCi of $^{45}Ca^{++}$ for 30 minutes. After homogenization of the fragments with a teflon pistle homogenizer (25), the radioactivity in aqueous and organic phases was determined. The aqueous phase consisted of 0.2 M EGTA. The numbers are the means ± SE of four incubations (table 3.1.6.2.).

Table 3.1.6.3.

REPRODUCIBILITY OF METHODS FOR DETERMINATION OF CALCIUM, GLYCEROL AND FFA

	Coefficient of variation % $\frac{s}{m} \times 100$
Calcium (Atomic Absorption)	2.5%
Calcium (Auto Analyzer N31P)	3.5%
FFA	2.2%
Glycerol	4.7%

Determination of Calcium in LAB-trol (DADE)	Calcium (Atomic Absorption) mg/100 ml	10.7 ± 0.136
	Calcium (Auto Analyzer N31P) mg/100 ml	10.09 ± 0.012
	Calcium Value for LAB-trol mg/100 ml	10.0 ± 0.42

Coefficient of variation was calculated from the difference between 10 pairs of identical samples. Lab-trol mean values were determined from 20 measurements. The means of 20 samples ± SE are indicated for the measurements; Lab-trol value is ± 95% confidence limits.

3.2. Results and Discussion

3.2.1. Lipolytic and hypocalcemic activity of β-LPH in vivo

Following its intravenous injection to rabbits sheep β-LPH produced a marked lipolysis as shown in Fig. 3.2.1.1. In this figure, the elevation of FFA reached its maximum approximately between 40-80 minutes. Measurement of calcium in the same samples revealed that β-LPH also caused a marked hypocalcemia (Fig. 3.2.1.2.). Maximal decrease in calcium occured approximately between 120-180 minutes following the injection. This effect was not observed in rabbits thyroidectomized 14 days previously (Fig. 3.2.1.3.).

The lipolytic response in thyroidectomized animals and in normal rabbits is almost identical if one compares the net change in FFA (Δ FFA). This is shown in Fig. 3.2.1.4. However, when the absolute values of FFA in plasma are compared, the thyroidectomized rabbits showed lower FFA values as is shown in Fig. 3.2.1.5.

Simultaneous measurements of serum glucose, cholesterol, magnesium (table 3.2.1.1.) and triglycerides (Fig. 3.2.1.6.) revealed that β-LPH had no effect on these parameters except for the triglycerides which markedly increased 7 hours after the injection.

The effect of β_h-MSH and α-MSH is demonstrated in Fig. 3.2.1.7. When compared with the β-LPH response, α and β-MSH seem to provoke only very short response which return to control values at 120 minutes after intravenous administration. The lipolytic effect of an identical amount of β-LPH (0.1 mg) lasted for more than 200 minutes (Fig. 3.2.1.1.)

Prolonged lipolysis has been demonstrated in rabbits after the subcutaneous injection of 0.5 mg of β-LPH dissolved in 16% gelatine. The

lipolysis then lasted for at least 12 hours.

Porcine β-LPH was also administered to the pigs as indicated in Fig. 3.2.1.8. Relatively high dose of porcine β-LPH showed some lipolytic activity in vivo. However, it did not show any lipolytic effect in vitro on the isolated fat cells of pig's perirenal fat (as it will be demonstrated in table 3.2.2.3.).

DISCUSSION

Lohmar & Li (131) published some data on biological properties of sheep β-LPH but they were concerned mainly with its melanophore-stimulating and lipolytic activities in vitro. In this part of my work, I followed its lipolytic activity in vivo in rabbits. In one experiment the lipolytic activity of newly isolated porcine β-LPH (73) has been examined in pigs and it was active. However, a relatively high amount of porcine β-LPH was injected in order to obtain a response (5.4 mg to 18 kg pig) and the trial to reproduce the lipolysis in vitro on isolated fat cells of the pig failed (as will be shown in part 3.2.2.).

Of particular interest is the marked hypocalcemic activity of β-LPH. Hypocalcemic activity in rabbits was also reported for corticotropin, epinephrine, vasopressin and anterior lobe pituitary extracts (148), for crude preparations of corticotropin (151) and also for purified corticotropin, peptide I and peptide II, α-MSH and β-MSH (69). Friesen discussed the hypocalcemic effects of the substances he utilized (69) and his points could be summarized as follows:

(a) All pituitary factors which were shown to be hypocalcemic in rabbits are also lipolytic.

(b) Lipolytic and hypocalcemic effects are related since fasting potentiates both effects.

(c) The species specificity is the same for hypocalcemic and lipolytic effects.

(d) In heavier rabbits with more substantial fat depots, both lipid mobilization and hypocalcemic effects are increased.

(e) Hypocalcemia occured after peptide I and II administration despite the injection of large amounts of parathyroid extracts.

My results could add to Friesen's conclusion that the lipolytic response is faster than hypocalcemia since the maximal FFA level is reached in about 40-80 minutes after β-LPH administration while the maximal hypocalcemia occured between 120-180 minutes. It could then be stated that the hypocalcemia closely follows the lipolysis.

The absence of hypocalcemia in thyroidectomized rabbits could be explained by a overall lower FFA levels. The relative increase (Δ) of FFA was about the same but the absolute FFA concentration is probably critical. The problem of the critical cellular FFA level in calcium accumulation in fat tissue will be discussed in part 3.2.4.

Up to now two main hypotheses have been raised concerning the relation of hypocalcemia and lipolysis. Friesen (69) mentioned the possibility of the formation of insoluble soaps between FFA and calcium. Akgun & al (2) on the other hand, explained the calcium change by the competition of calcium with FFA for the binding sites on serum albumin. According to this point of view the free calcium in the blood is the same whereas the

total calcium is lower due to the occupation of calcium binding sites on albumin by FFA.

There are, however, data which are in opposition to the concept of the interdependence of lipolysis and hypocalcemia. Trygstad (212) claimed that his LMF conserved its lipolytic activity while its hypocalcemic activity has been lost in the late steps of its purification.

The absence of change in plasma cholesterol, following β-LPH injection, could be explained by the relatively short time of the experiments. The last blood samples were collected 7 hours after β-LPH injection. Friesen (70) found that cholesterol increased 12 hours after administration of pituitary extract. Seven hours interval was, on the other hand, sufficient to detect the plasma triglyceride increase. We detected no changes in glucose levels which have sometimes been found following injections of some lipolytic compounds and we did not detect any significant changes in magnesium levels as were reported by Akgun & al (2).

The less persistent lipolysis of α- and β-MSH when compared to β-LPH in rabbits in vivo could be probably explained by faster elimination or inactivation of these peptides. It could also be due to the transformation of β-LPH into β-MSH.

LIPOLYTIC ACTIVITY OF β-LPH IN RABBITS

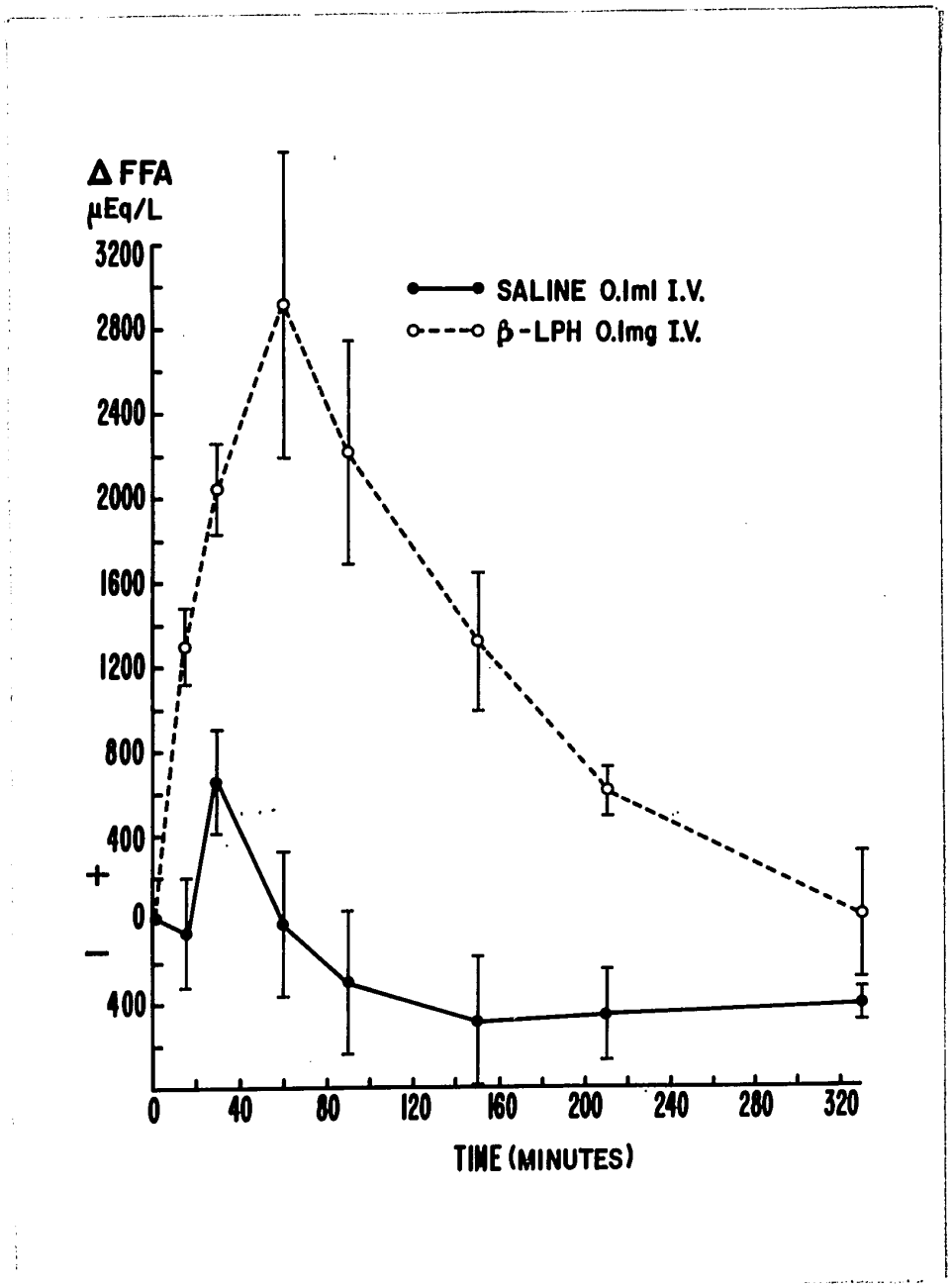

Fig. 3.2.1.1. Effects of intravenous injections of 0.1 mg of β-LPH on serum FFA level in five different rabbits. Each animal served as its own control, being tested one week previously with saline injections. Plotted is the net change of FFA in plasma (Δ FFA). Each point represents the mean ± SE.

CHANGES IN BLOOD CALCIUM AFTER β-LPH ADMINISTRATION

TO RABBITS

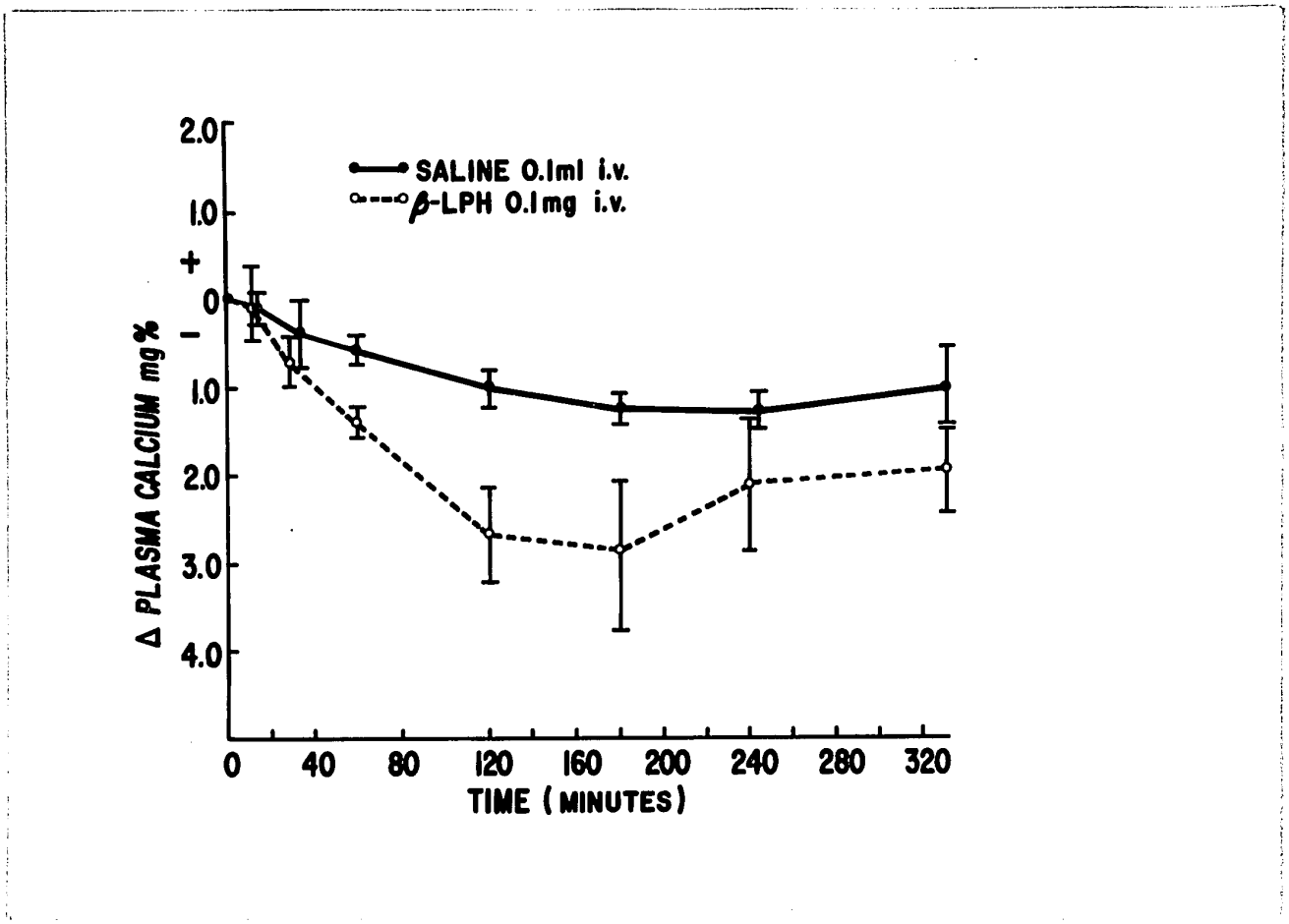

Fig. 3.2.1.2. Effects of 0.1 mg of sheep β-LPH injected i.v. on plasma calcium in five different rabbits. Each animal was tested after a control injection of saline 1 week previously. Ordinate: changes of plasma calcium. Abscissa: time following injections.

CHANGES IN BLOOD CALCIUM AFTER β-LPH ADMINISTRATION

TO NORMAL AND THYROIDECTOMIZED RABBITS

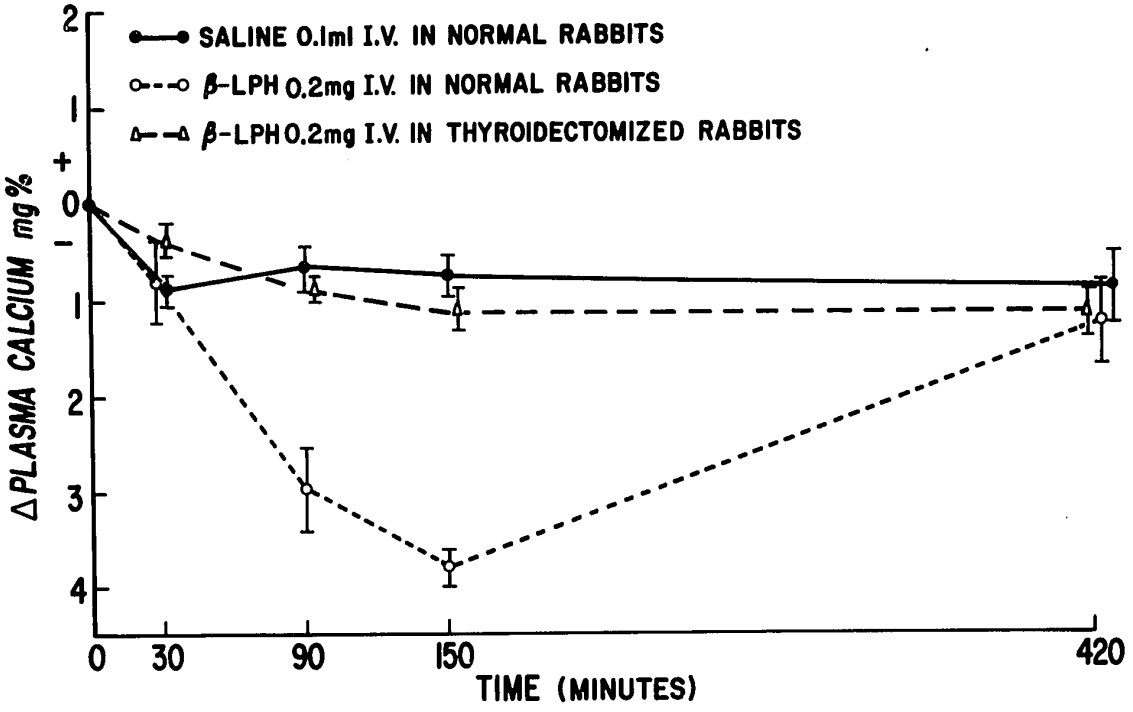

Fig. 3.2.1.3. Effects of 0.2 mg of sheep β-LPH injected i.v. on plasma calcium in four normal rabbits and in four rabbits 2 weeks after thyroidectomy. Controls were injected by saline. Ordinate: changes in plasma calcium. Abscissa: time following injection.

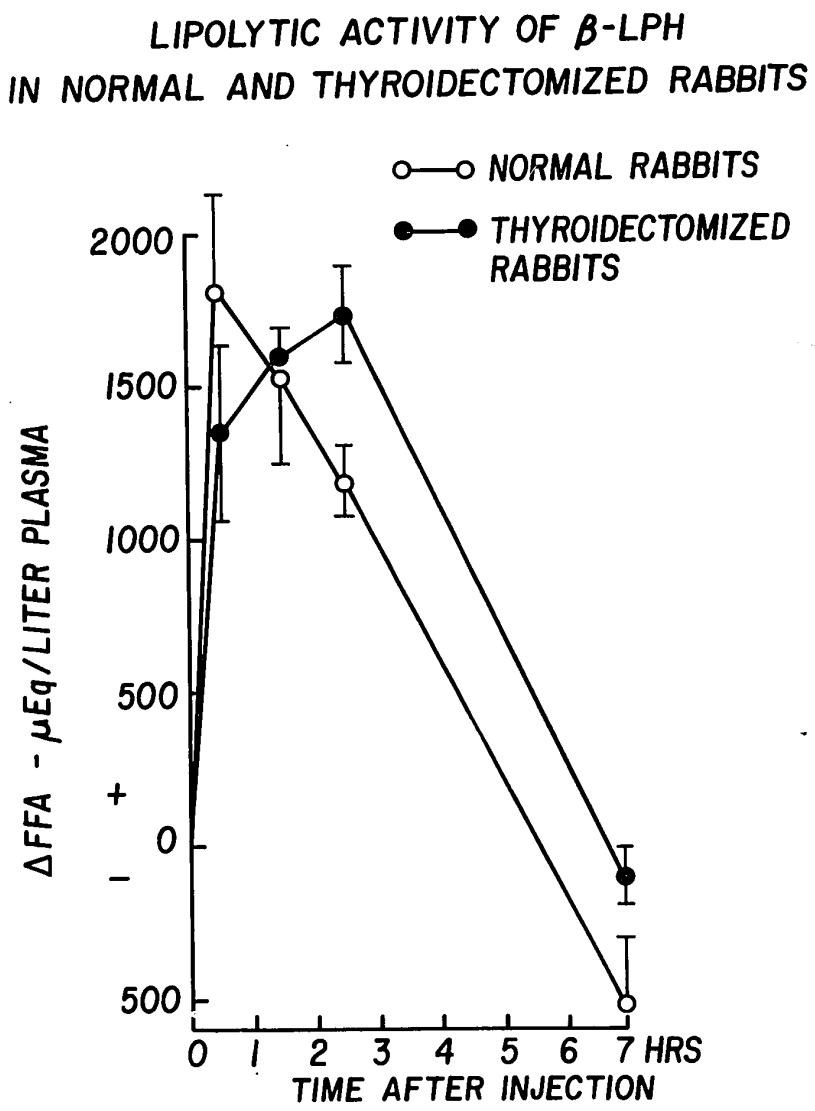

Fig. 3.2.1.4. Lipolytic response in 3 kg rabbits following intravenous injection of 0.2 mg of β-LPH. Thyroidectomized rabbits were injected 2 weeks after operation. Plotted is the net change in FFA (Δ FFA) at the time indicated. The values at time 0 were substracted from absolute values to obtain Δ FFA. The same data are used as in Fig. 3.2.1.5. Each point represents the mean of 4 samples from four different rabbits ± SE.

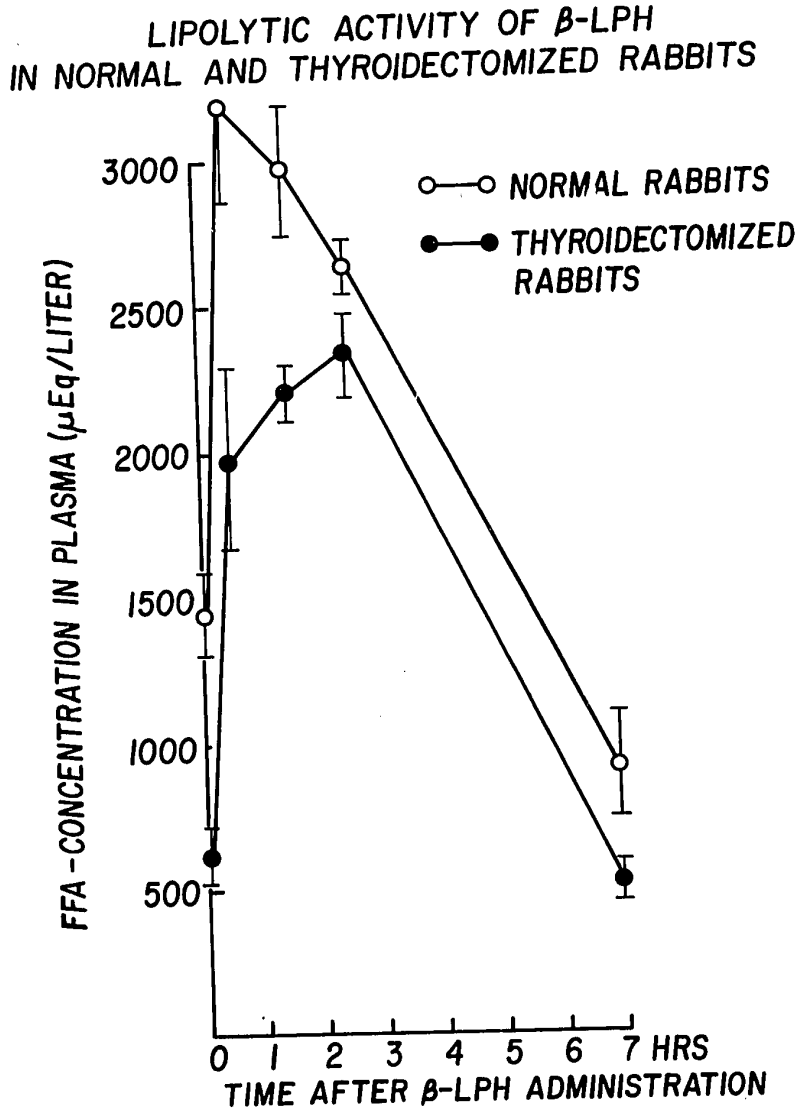

Fig. 3.2.1.5. Three kg rabbits received intravenous injections of 0.2 mg of sheep β-LPH intravenously. FFA concentration in plasma was measured at indicated intervals. Plotted are the absolute values in μEq per 1 liter of plasma. Each point represents the mean of 4 samples from four different rabbits ± SE. Thyroidectomized rabbits were injected 2 weeks after operation.

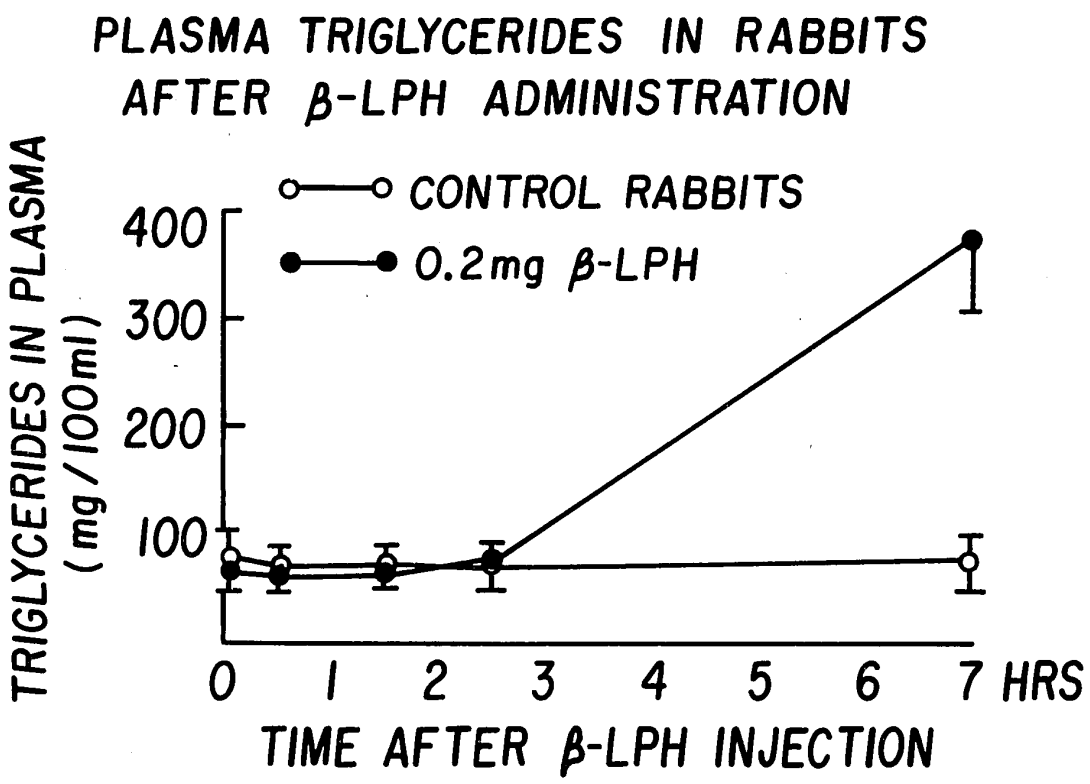

Fig. 3.2.1.6. Three kg rabbits were injected by 0.2 mg β-LPH intravenously. Controls were injected by saline. Blood was collected from ear vein at indicated intervals. All points are the means of 4 observations in four different rabbits ± SE.

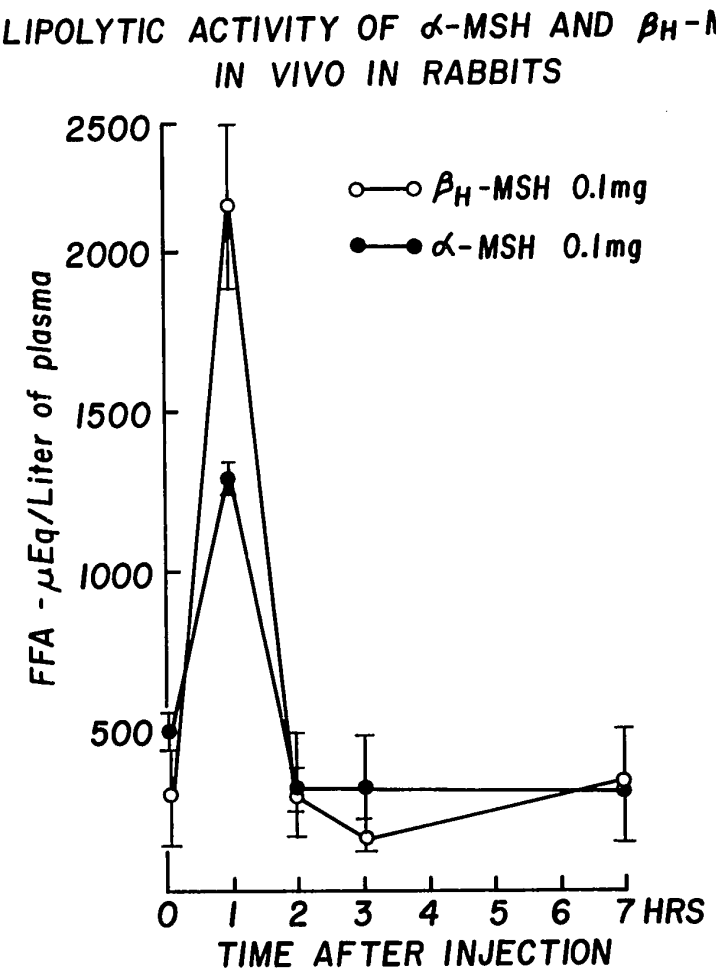

Fig. 3.2.1.7. Three kg rabbits were injected intravenously with 0.1 mg of β_h-MSH and 0.1 mg α-MSH. The points are the means of two injected animals. The bars indicate the respective individual values.

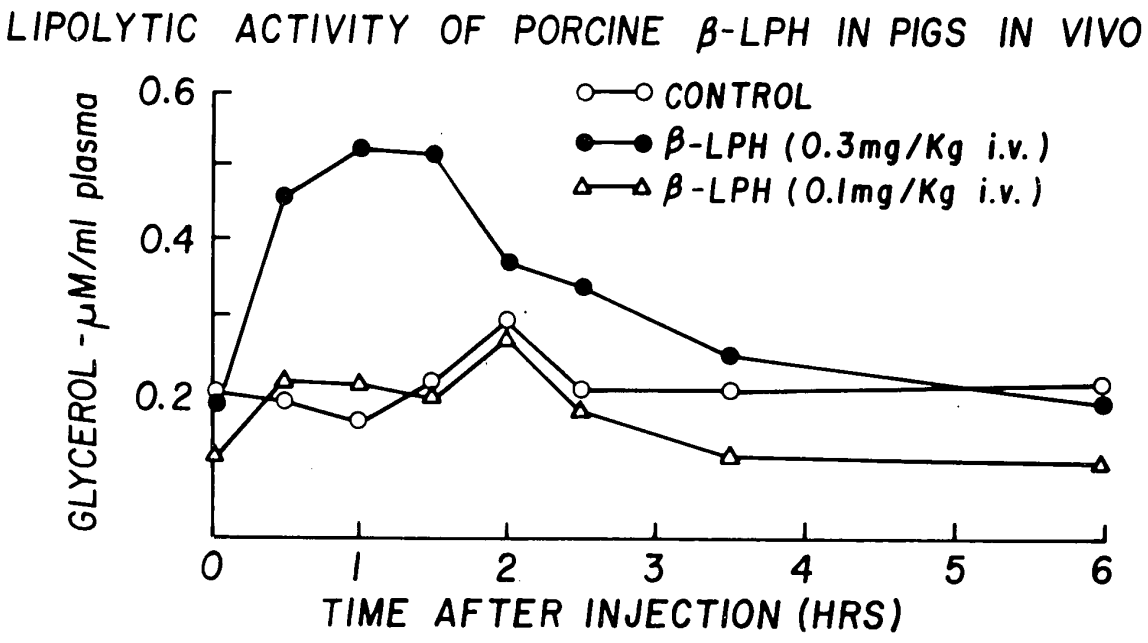

Fig. 3.2.1.8. Three pigs (white, male, 18, 14.5 and 15.5 kg) were fasted for 48 hours and injected by saline (control) and by 0.3 mg or by 0.1 mg per kilogram of body weight of porcine β-LPH intravenously. Animals were tranquilized by intramuscular injection of chlorpromazine (Largactil) 1 mg/kg, 30 minutes before β-LPH injection. Blood samples were collected from ear vein.

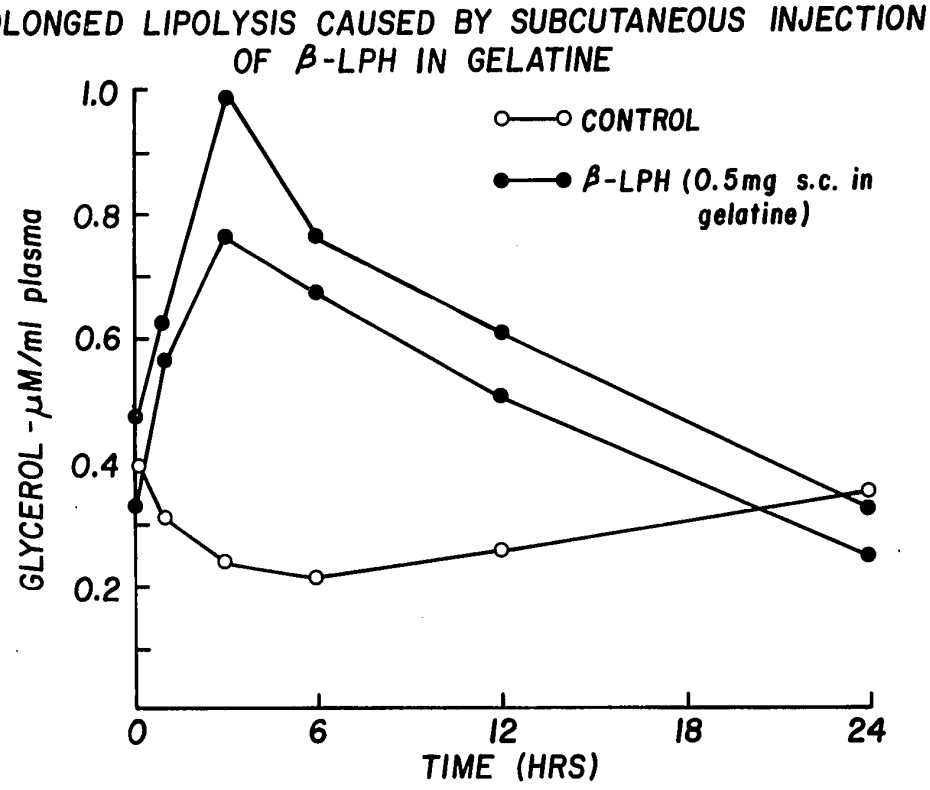

Fig. 3.2.1.9. Two rabbits (3 kg, male) were injected subcutaneously by 0.5 mg of sheep β-LPH in gelatine diluent (0.5 ml of 16% gelatine and 0.5% phenol). One control rabbit was injected by gelatine diluent only. Blood samples were collected at indicated intervals from the ear vein.

Table 3.2.1.1.

GLUCOSE, MAGNESIUM AND CHOLESTEROL IN THE BLOOD OF THE RABBITS INJECTED BY β-LPH

		Time (minutes) after β-LPH injection				
		0'	30'	90'	150'	420'
Glucose	β-LPH	122 ± 2.1	130 ± 4.3	127 ± 5.6	133 ± 8.6	136 ± 2.7
mg/100 ml	Control	123 ± 1.7	129 ± 6.8	132 ± 5.9	134 ± 4.3	136 ± 6.7
Magnesium	β-LPH	2.43 ± 0.187	2.20 ± 0.096	2.52 ± 0.178	2.57 ± 0.173	2.65 ± 0.142
mg/100 ml	Control	2.34 ± 0.126	2.21 ± 0.088	2.27 ± 0.106	2.43 ± 0.125	2.61 ± 0.121
Cholesterol	β-LPH	59.4 ± 8.66	59.8 ± 8.64	54.2 ± 8.64	50 ± 7.37	49.8 ± 8.05
mg/100 ml	Control	47 ± 4.76	44.6 ± 4.92	45.4 ± 5.36	44.4 ± 4.98	44.8 ± 4.96

Glucose, Magnesium and Cholesterol were determined in plasma of 3 kg male rabbits injected intravenously by 0.2 mg of β-LPH. Controls were injected by saline. Values are the means of 5 measurements ± SE.

3.2.2. Lipolytic activity of β-LPH *in vitro*

The lipolytic activities of β-LPH and ACTH *in vitro* are compared in Fig. 3.2.2.1. ACTH is apparently more active in both rabbit and rat isolated fat cells but in rabbit cells β-LPH specific activity approached ACTH. Fig. 3.2.2.2. shows the lipolytic response of isolated rat fat cells at different time of incubation.

Human isolated fat cells were incubated with different concentrations of β-LPH. As demonstrated in Fig. 3.2.2.3. and Fig. 3.2.2.4., no lipolytic response was obtained. The highest concentration used was 4 μM for both sheep and human β-LPH. Human fat cells responded, however, to 10 μg/ml of epinephrine. The results obtained with isolated fat cells of thyroidectomized rats and rabbits are presented in table 3.2.2.1. and in table 3.2.2.2. respectively. Comparing these two tables it seems that the responsiveness of rabbit fat tissue is less affected by thyroidectomy than the fat tissue of rats. Isolated perirenal fat cells from porc did not respond to any of the hormones utilized (table 3.2.2.3.).

DISCUSSION

The lipolytic activity of sheep β-LPH was assayed on fat tissue fragments of rat and rabbit by Lohmar & Li (131). They estimated the so-called minimal effective dose for β-LPH and ACTH in both tissues. However, the comparison between the stimulation of lipolysis in rat and rabbit adipose tissue presents some difficulties. In rat isolated fat cells, the maximal response is usually obtained after 1 hour of incubation with lipolytic substance. The increase of FFA or glycerol sometimes observed in the incubation medium after additional 1 hour of incubation does not represent an increase since the controls are usually also more elevated

(Fig. 3.2.2.2.). Rabbit isolated fat cells, on the other hand, respond more slowly and the maximal response is usually obtained after 2 hours of incubation. The examples of the different lipolytic rates in rabbit and rat fat cell preparations could be also observed in Fig. 3.2.4.7. and Fig. 3.2.5.3.

In part 3.2.1., I reported the lipolytic response after the injection of β-LPH to thyroidectomized rabbits. It has already been published that thyroidectomy in rats almost completely reduced the lipolytic response of fat tissue (117). In table 3.2.2.1. and table 3.2.2.2. are shown the results of incubation of fat tissue from thyroidectomized rats and rabbits with different concentrations of β-LPH and ACTH. It is obvious that thyroidectomy affected to a lower extent the response of rabbit compared to rat fat tissue. Nevertheless the absolute lipolytic response was reduced in thyroidectomized rabbits both in vitro (table 3.2.2.2.) and in vivo (Fig. 3.2.1.5.).

The lack of any response in the incubations of isolated porcine perirenal fat cells is presented in table 3.2.2.3. This is consistent with previously reported resistance of porcine fat tissue to every lipolytic compound assayed (174) (197) but contradictory to the reported sensitivity (minimal effective dose 5×10^{-3} µg) of porcine fat tissue to the porcine β-LPH by Cseh & Graf (53). I found no significant response at the concentrations up to the 40 µg per ml porcine β-LPH (table 3.2.2.3.) prepared in our laboratory. The results of incubations with isolated human fat cells (Fig. 3.2.2.3.) likewise did not confirm the lipolytic activity of human β-LPH on human fat tissue reported by Cseh & al (54). It is, however, impossible to compare these results since the authors (54) did not publish any details of their lipolytic assay on human adipose tissue.

A slight lipolytic response was obtained after the injection of a relatively high dose of porcine β-LPH to pigs (Fig. 3.2.1.8.) and no response has been observed in vitro on isolated porcine fat cells. The physiological role of β-LPH in mobilization of triglycerides in the pig seems therefore uncertain.

Lohmar & Li (131) assayed β-LPH for adrenal stimulating activity in vitro. β-LPH consistently evoked a response corresponding to 1 IU/mg. They are, however, not sure if this response is due to the intrinsic adrenal stimulation activity of β-LPH, due to contamination of β-LPH by ACTH, or due to some nonspecific type of response which is sometimes encountered in similar in vitro assay system (131).

The relatively slight lipolytic activity (Fig. 3.2.2.1.) of sheep β-LPH in rat fat cells raise the question whether this activity is due to an ACTH contaminant. It should be, however, noted that this lipolytic activity of β-LPH in rats is consistent and does not change if β-LPH is repurified (131). β-LPH was further found to have no detectable growth-promoting, gonadotropic or thyrotropic activity and minimal lactogenic activity which was lost after additional purification by countercurrent distribution. It possess, on the other hand, about 50% of the MSH activity of ACTH (131).

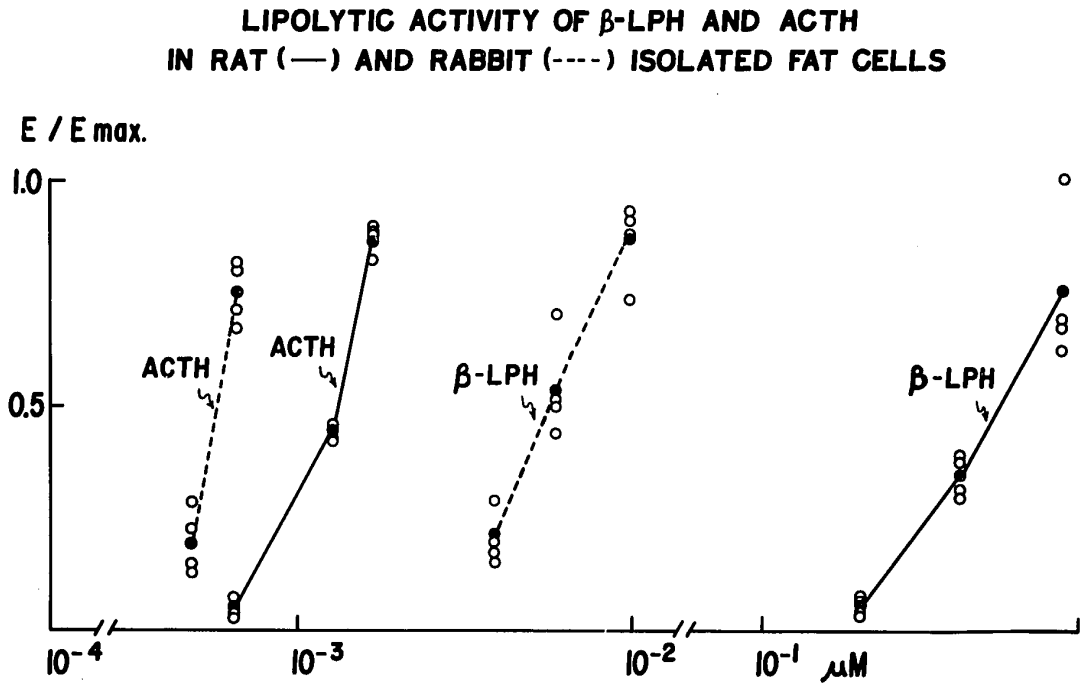

Fig. 3.2.2.1. Lipolytic activity was determined as a glycerol release from isolated fat cells into the incubation medium. E max. is the maximal response of given cell preparation as determined by the lipolytic effect of 0.1 μg/ml ACTH. E is the lipolytic effect of given concentration. Concentration in μM is on logarithmic scale.

Rabbit cells were incubated for 2 hours, rat cells for 1 hour. Open circles are the individual responses, filled circles are the means at each dose level.

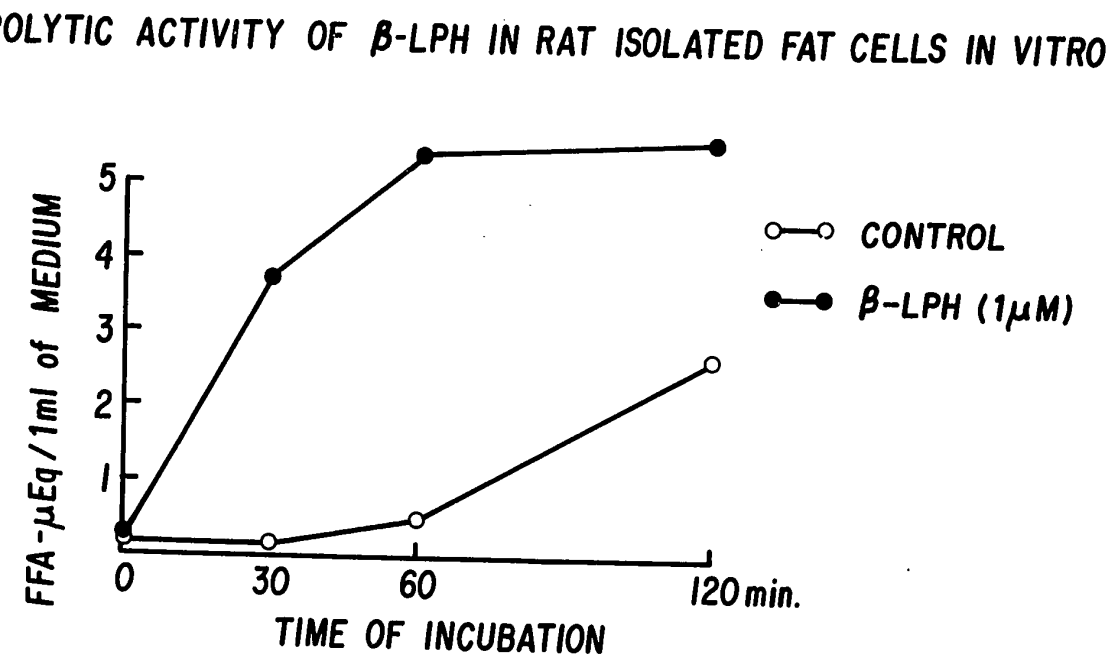

Fig. 3.2.2.2. Cells from 100 mg of epididymal fat tissue were incubated in 1 ml of medium (Krebs-Ringer bicarbonate with 4% albumin) containing 1 μM β-LPH. At the end of incubation period the medium was filtered through cellular filter and FFA were determined in the medium.

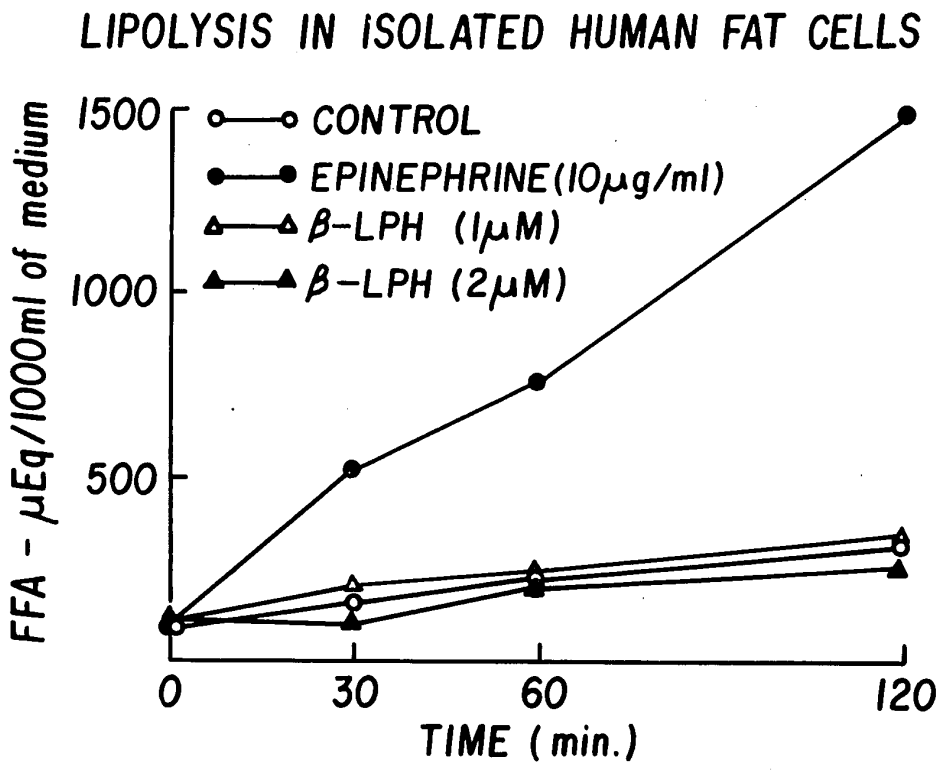

Fig. 3.2.2.3. Isolated fat cells were prepared from subcutaneous fat tissue removed from 16 years old male obese patient (104.2 kg, 1.72 m, adiposo genital syndrome). Fat tissue was removed under local anesthesia (1% xylocaine without epinephrine).

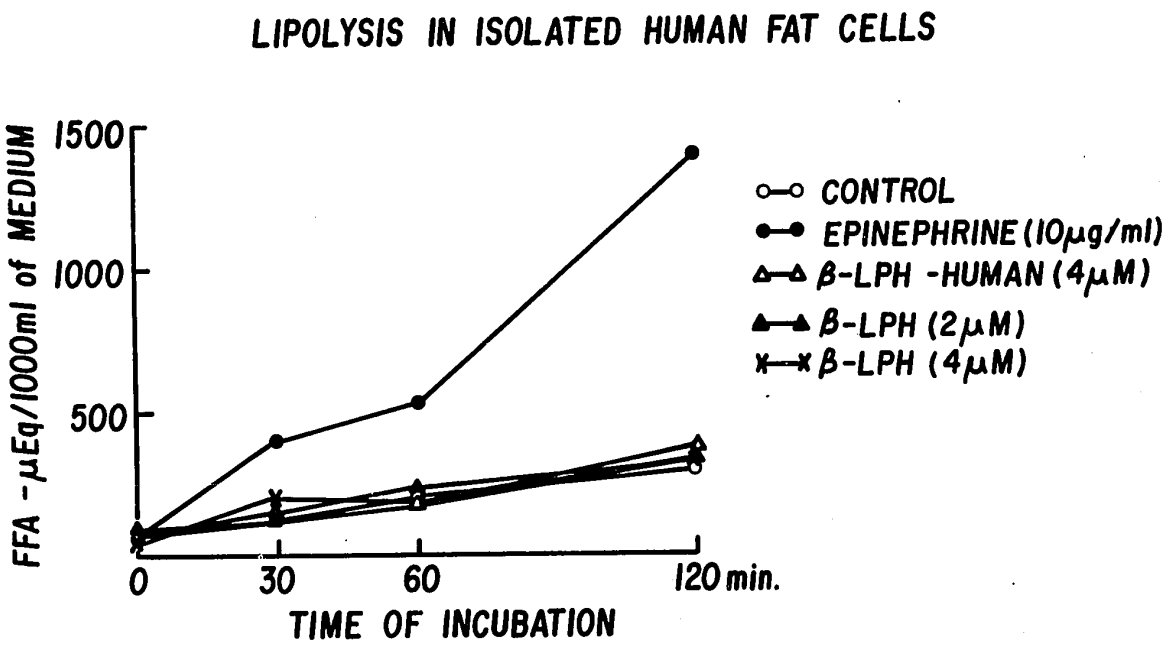

Fig. 3.2.2.4. Isolated fat cells were prepared from subcutaneous fat tissue removed from 16 years old male obese patient (130.5 kg, 1.81 m). Fat was removed 4 minutes after the induction of narcosis. Volume of individual incubations was 4 ml. Concentration of cells corresponds to 100 mg of fat tissue per 1 ml of medium.

Table 3.2.2.1.

LIPOLYTIC ACTIVITY OF β-LPH AND ACTH IN ISOLATED FAT CELLS FROM NORMAL AND THYROIDECTOMIZED RATS

Nanomoles of glycerol per 1 ml of incubation medium

	Fat Cells from		% of inhibition
	Normal Rats	Thyroidectomized Rats	by thyroidectomy
β-LPH (4 µg/ml)	179 ± 2.0	52 ± 2.5	100%
β-LPH (20 µg/ml)	1320 ± 84.8	69 ± 3.5	99%
ACTH (0.004 µg/ml)	1005 ± 40.3	66 ± 2.0	99%
ACTH (0.1 µg/ml)	1642 ± 119	196 ± 5.4	91%
Control	56 ± 6.5	52 ± 2.5	

Thyroidectomized rats were used 2 weeks after operation. Incubation volume of fat cells was 1 ml with the cell concentration represented by 30 mg of triglycerides per incubation. Each value is a mean of four incubations ± SE. Incubation time was 90 minutes.

Table 3,2,2,2.

LIPOLYTIC ACTIVITY OF β-LPH AND ACTH IN ISOLATED FAT CELLS FROM NORMAL AND THYROIDECTOMIZED RABBITS

	Nanomoles of glycerol per 1 ml of incubation medium		
	Fat Cells from		% of inhibition
	normal rabbits	thyroidectomized rabbits	by thyroidectomy
β-LPH (4 µg/ml)	1430 ± 142	837 ± 88.9	43%
β-LPH (20 µg/ml)	1437 ± 219	1005 ± 173	31%
ACTH (0.004 µg/ml)	1421 ± 204	576 ± 43.4	61%
ACTH (0.1 µg/ml)	1532 ± 94.2	1032 ± 109	34%
Control	50 ± 9.1	44 ± 5.7	

Thyroidectomized rabbits were used 2 weeks after operation. Incubation volume of fat cells was 1 ml with the cell concentration represented by 30 mg of triglycerides per incubation. Each value is a mean of four incubations ± SE. Incubation time was 90 minutes.

Table 3.2.2.3.

LIPOLYSIS IN ISOLATED PERIRENAL FAT CELLS OF PIG

			Nanomoles of glycerol per 1 ml of incubation medium		
Control			124	±	5.77
Norepinephrine	-	10 µg/ml	143	±	2.36
β-LPH (sheep)	-	10 µg/ml	115	±	3.31
β-LPH (sheep)	-	20 µg/ml	106	±	7.88
β-LPH (porcine)	-	2 µg/ml	107	±	11.08
β-LPH (porcine)	-	4 µg/ml	110	±	5.73
β-LPH (porcine)	-	8 µg/ml	122	±	4.3
β-LPH (porcine)	-	10 µg/ml	119	±	1.0
β-LPH (porcine)	-	20 µg/ml	128	±	4.24
β-LPH (porcine)	-	40 µg/ml	132	±	10.03
ACTH	-	1 µg/ml	102	±	3.82
ACTH	-	10 µg/ml	123	±	2.36
Theophylline	-	10 mM	105	±	6.6

Perirenal fat tissue was removed under Nembutal anesthesia (20 mg/kg) in 23 kg pig 2 days fasted. Incubation time was 120 minutes.

3.2.3. Lipolytic activity of o-nitrophenyl sulfenyl β-LPH (NPS-β-LPH)

NPS-β-LPH showed lipolytic activity in rabbits in vivo. This lipolytic activity seems to be lower than that of unmodified β-LPH (Fig. 3.2.3.1.). There is no effect of NPS-β-LPH on isolated rat epididymal fat cells (Fig. 3.2.3.2.) but NPS-β-LPH inhibited the response to unmodified β-LPH.

DISCUSSION

Results with NPS-β-LPH are essentially comparable to those of Ramachandran & Lee (156) with NPS-ACTH. The modified ACTH lost its activity on rat fat cells and was found inhibitory when co-incubated with nonmodified ACTH. As is shown in Fig. 3.2.3.2., NPS-β-LPH had no activity on rat fat cells and has inhibitory effect towards β-LPH.

Ramachandran & Lee (155) further followed the activity of NPS-ACTH in rat and rabbit fat cell ghosts. They found that NPS-ACTH did not stimulate the adenylate cyclase activity in rat adipose tissue ghosts, but was fully active on the same preparation from rabbit fat tissue. Ramachandran & Lee (155) postulated that the modification of the tryptophan residue in ACTH does not affect the ability of ACTH to bind to its receptor. On the other hand the full activity of NPS-ACTH found in rabbit fat cell ghosts could be explained by the difference in the structure of the receptors in the rat and rabbit fat tissue (157). Species differences in the responsiveness of rat and rabbit fat tissue to pituitary hormones has been already mentioned (part 2.1.6.). ACTH was found active in both rat and rabbit. α- and β-MSH on the other hand are active only in rabbit. Ramachandran & Lee (157) pointed out that there is relatively good correlation between melanocyte stimulating activity in amphibians and lipolytic activity in

rabbits. The same is true about NPS derivative of ACTH. NPS-ACTH was found equally active as ACTH in stimulating amphibian melanophores (155).

NPS-β-LPH behaved essentially the same way as NPS-ACTH as far as the lipolytic activity is concerned. The only difference found was the relative lower activity of NPS-β-LPH in rabbits in vivo when compared with β-LPH. However, it could be concluded that the receptors for β-LPH in fat tissue are related, if not identical, to those of ACTH and α- and β-MSH.

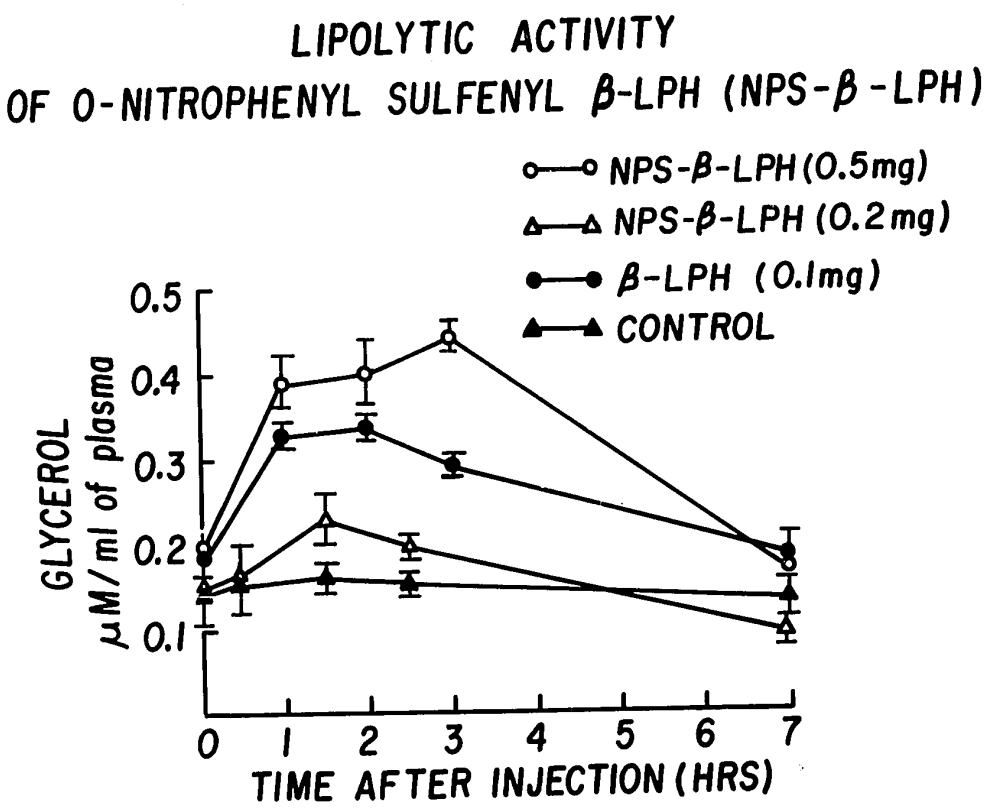

Fig. 3.2.3.1. 1.5 kg fed rabbits were injected intravenously by 0.5 mg NPS-β-LPH, 0.2 mg NPS-β-LPH and 0.1 mg β-LPH respectively. Controls were injected by saline. Each point is the mean of two samples from different animals. The bars indicate the individual values.

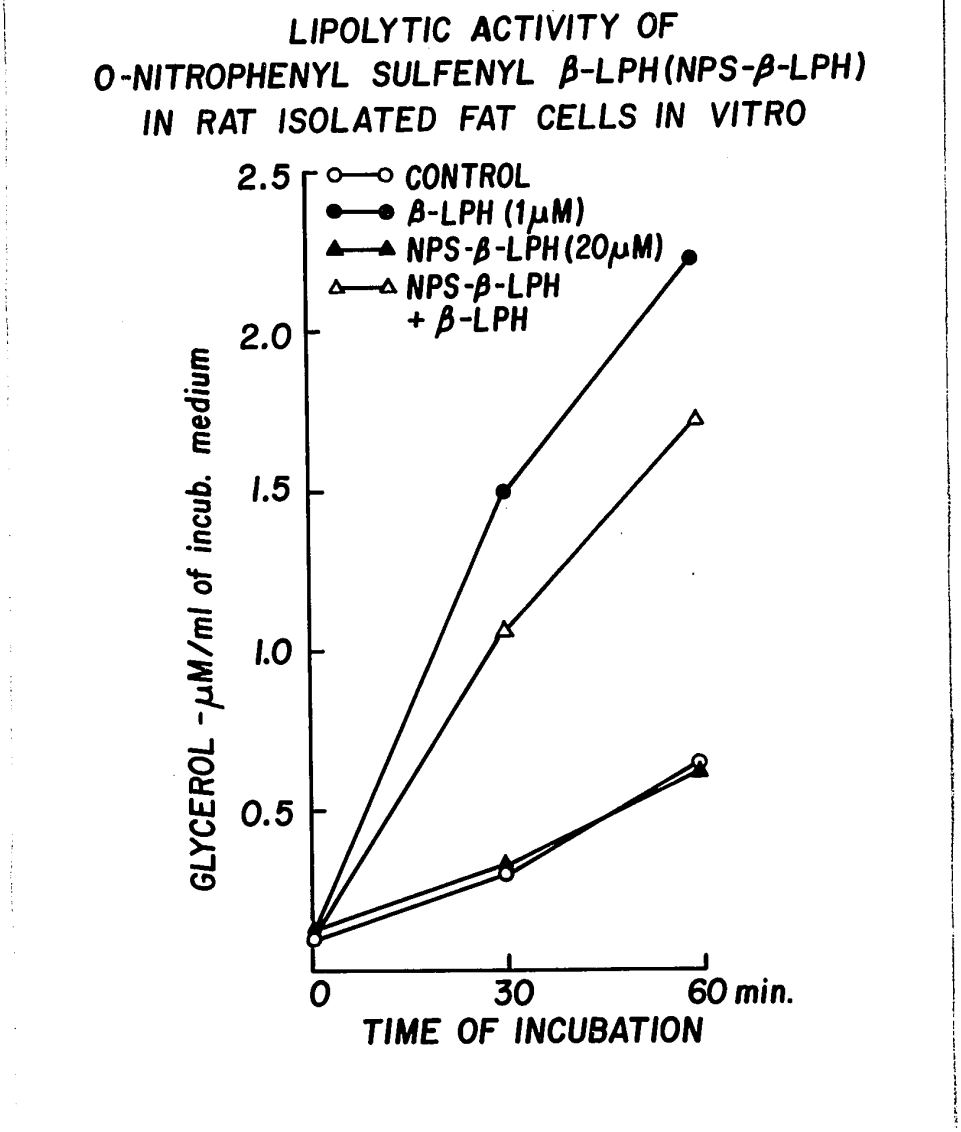

Fig. 3.2.3.2. Isolated rat epididymal fat cells were incubated with β-LPH and NPS-β-LPH. When both peptides were incubated the NPS-β-LPH was added 10 minutes before β-LPH. Concentration of fat cells in 1 ml of Krebs-Ringer bicarbonate buffer corresponds to the 50 mg of triglycerides.

3.2.4. Calcium uptake by adipose tissue in vitro and in vivo

In vivo - Following the intravenous injection of sheep β-LPH the calcium concentration increased in rabbit epididymal fat pads as shown in Fig. 3.2.4.1. and 3.2.4.2. In both figures the calcium increase became evident about 180 minutes after the injection. Fig. 3.2.4.2. also show the concomittant decrease of plasma calcium and the increase of FFA in plasma and in the adipose tissue.

In vitro, fat tissue - I subsequently tried to reproduce the calcium increase in fat tissue by incubating it with β-LPH in presence of $^{45}Ca^{++}$ and ^{3}H-mannitol. ^{3}H-mannitol has been used in the incubations in order to measure the extracellular space since mannitol does not enter the cell (217). Results are shown in Fig. 3.2.4.3. It is evident from this figure that β-LPH does not change the mannitol space during lipolysis. Since mannitol does not enter the cells, the difference between the ^{45}Ca space and ^{3}H-mannitol space represents the intracellular distribution of $^{45}Ca^{++}$.. However, the possibility that Ca^{++} is being complexed on the cellular membrane cannot be excluded by this approach. The term "intracellular" should be considered here in a rather broad sense e.g. including cell membrane compartment and presumably also the cell coat.

In vitro, isolated cells - The calcium uptake by isolated fat cells has been also measured by the decrease of calcium in incubation medium. Results are shown in Fig. 3.2.4.4., 3.2.4.5., 3.2.4.6. and 3.2.4.7.

DISCUSSION

Although our experiments with sheep β-LPH indicate that Ca^{++} uptake by the fat cell is quantitatively correlated with lipolysis, nothing proves that Ca^{++} influx causes lipolysis.

Our results are in agreement with recent work of Alm & al (3) who found a significantly higher Ca^{++} uptake by fat tissue. They concluded that the intracellular increase of FFA is the main cause of Ca^{++} accumulation.

Since Ca^{++} concentration decreases in the medium after incubation with isolated fat cells it might be also concluded that calcium moves into the cells. However, the exact location and the nature of the binding are not known. Moreover, we do not know whether the Ca^{++} accumulation in the fat cell is due to increased influx of Ca^{++}, decreased efflux or both. The shift of Ca^{++} into the fat tissue of rabbits <u>in vivo</u> seems to correspond to hypocalcemia, but other tissues could be involved.

It is difficult to assess to what extent the hypocalcemia in rabbits is connected with accumulation of calcium in fat tissue. Some aspects of hypocalcemia in rabbits, following the injection of pituitary extracts, were discussed in part 3.2.1. The indirect evidence that the hypocalcemia is caused by calcium uptake during lipolysis could be summarized as follows:

(a) Hypocalcemia and lipolysis are related (see part 3.2.1.). So are the lipolysis and the increased calcium uptake by fat tissue or isolated fat cells (Fig. 3.2.4.1., Fig. 3.2.4.7.).

(b) Attempts to prove that the hypocalcemia in rabbits is caused by a sudden shift of calcium into bone or by an increase in urinary excretion of calcium were not successful (69).

(c) The timing of hypocalcemia and the calcium increase in fat tissue are almost identical (Fig. 3.2.4.1., Fig. 3.2.4.2., and part 3.2.1.)

(d) The hypocalcemia seems to be more intensive in heavier rabbits which have proportionally more fat tissue (69).

(e) If it is assumed that adipose tissue constitutes about 25% of body weight, an approximate calculation suggests that the redistribution of calcium from extracellular fluid into adipose tissue may account for the hypocalcemia (1).

Akgun & Rudman (1) postulated, from their results, that some serum factor, different from albumin, was essential for a calcium shift into the adipose tissue. They found that the increased calcium concentration in fat tissue, stimulated by lipolytic hormones in vitro, was occurring only in presence of serum (rabbit, rat and hamster). No calcium shift was observed when the tissue has been incubated in Krebs-Ringer phosphate buffer with 4% albumin. This requires some comments since the FFA concentration in their incubations is increased by the addition of serum. For example with rabbit serum the initial FFA concentration has been estimated as 1073 ± 314 µEq/1 and after 2 hours 30 minutes of incubation with lipolytic hormone 4869 ± 157 µEq/1. When hamster serum was used, the FFA values before and after the experiment were 2413 ± 30 µEq/1 and 5719 ± 313 µEq/1 respectively. On the other hand, in Krebs-Ringer phosphate buffer with albumin, an initial FFA concentration was 406 ± 39 µEq/1 and after 2 hours 30 minutes of incubation with lipolytic hormone 3863 ± 156 µEq/1. Alm & al (3) reported the increase of calcium uptake by fat tissue when albumin was absent from the

medium. They (3) also hypothetized that the increased intracellular concentration of FFA is crucial for the stimulation of calcium uptake by fat tissue, Akgun & Rudman (1) results can be therefore also explained by higher intracellular FFA levels since FFA concentration in incubations with serum was higher than corresponding values with Krebs-Ringer bicarbonate buffer and the available binding sites on albumin were consequently more satured.

As was discussed in part 2.1.8. the critical FFA concentration in fat cells was established as 3 micro-moles per gram of cell lipid in rats (5). When the critical concentration is exceeded, metabolic consequences usually take place, one of which is probably the increased calcium uptake.

This could also offer an explanation for some other conflicting observations reported as for example the preparation of purified LMF with full lipolytic activity but completely free of hypocalcemic activity (212) as well as the lack of hypocalcemia in thyroidectomized rabbits (Fig. 3.2.1.3.) In the examples where lipolytic response without hypocalcemia or without calcium uptake by fat tissue was observed, the critical FFA intracellular concentration was probably not reached. Intracellular levels of FFA could not be evaluated by means of the release of FFA or glycerol into the medium. It is known that the intracellular FFA level depends not only on the rate of lipolysis but also on the availability of FFA acceptor outside the cell and the rate of re-esterification (168). The rate of re-esterification is probably important in thyroidectomized rabbits since triiodothyronine was reported to lower re-esterification in fat tissue (77). The FFA level in the medium could be almost the same but the intracellular FFA level and consequently the calcium uptake by fat tissue will differ. In Fig. 3.2.4.7., the FFA release at 0.5 μM and 1.0 μM β-LPH is almost the same but the calcium uptake by fat cells differ substantially.

The mechanism for the uptake of calcium by adipose tissue during lipolysis is not known but probably one of the following explanations could be suggested:

(a) The uncoupling effect of increased FFA concentration (5) with subsequent lowering of ATP intracellular levels can impair the activity of Ca^{++} dependent ATPase; allegedly involved in calcium, energy dependent, efflux from the cells.

(b) A high concentration of intracellular FFA can result in the formation of insoluble soaps between calcium and FFA in the cells and subsequent intracellular retention of calcium.

(c) It was reported that increased FFA intracellular level caused the release of proteins from isolated fat cells (169). Such release could be the consequence of a change in the membrane permeability which could also increase the calcium influx.

CALCIUM ACCUMULATION BY RABBIT EPIDIDYMAL ADIPOSE TISSUE

IN VIVO

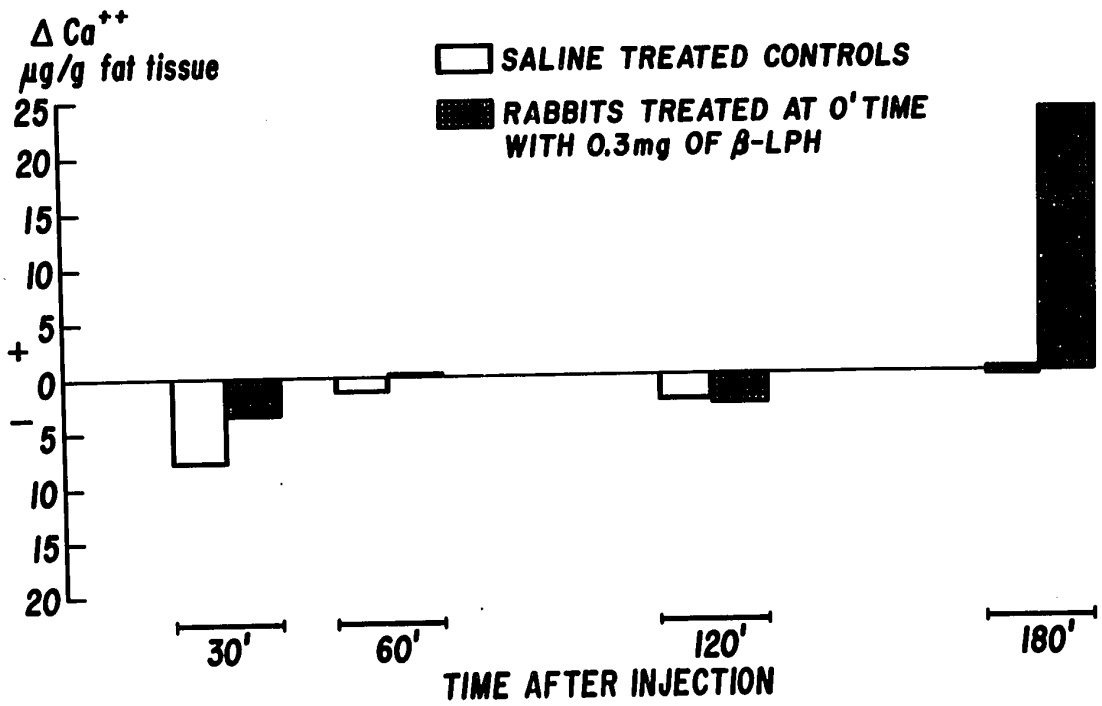

Fig. 3.2.4.1. At time zero 0.3 mg of sheep β-LPH was injected intravenously into 3 kg male rabbits. Controls were injected with the equivalent volume of saline. Epididymal fat was removed under epidural anesthesia. Plotted is the difference between the Ca^{++} concentration in the epididymal fat removed at the time indicated and the Ca^{++} concentration in the contralateral pad removed before injection.

β-LPH INDUCED LIPOLYSIS, HYPOCALCEMIA AND Ca^{++} UPTAKE BY RABBIT ADIPOSE TISSUE IN VIVO

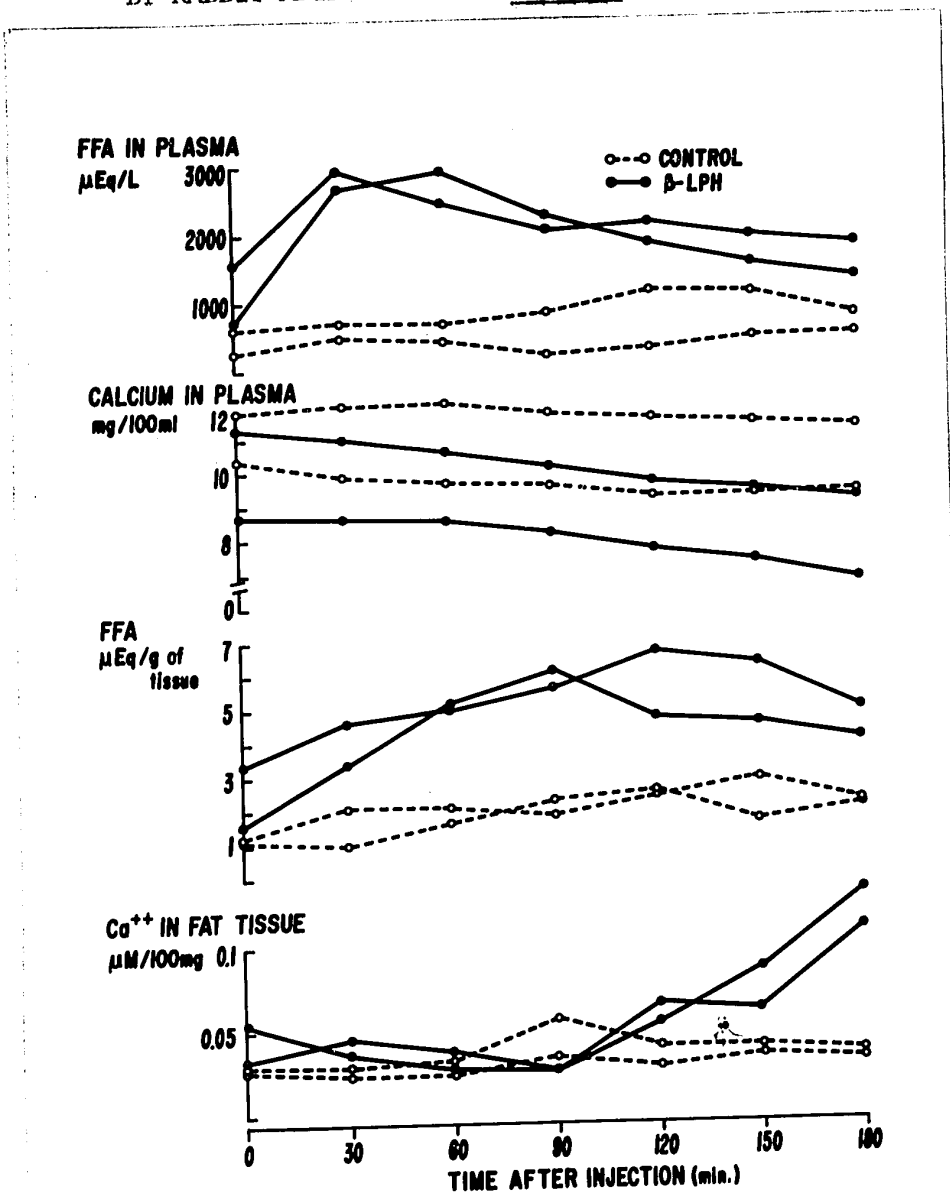

Fig. 3.2.4.2. Male rabbits weighing 3 kg were anesthetized with Nembutal and Chlorpromazine and injected intravenously with 0.2 mg of β-LPH. Controls were injected with saline. At the indicated intervals, blood samples were taken and 100-mg pieces of perirenal fat tissue were removed. Ca^{++} and FFA were determined in the blood and in the fat tissue. Each individual line represents the data obtained with one animal.

UPTAKE OF $^{45}Ca^{++}$ AND ^{3}H-MANNITOL BY RABBIT FAT TISSUE IN THE PRESENCE OR ABSENCE OF β-LPH

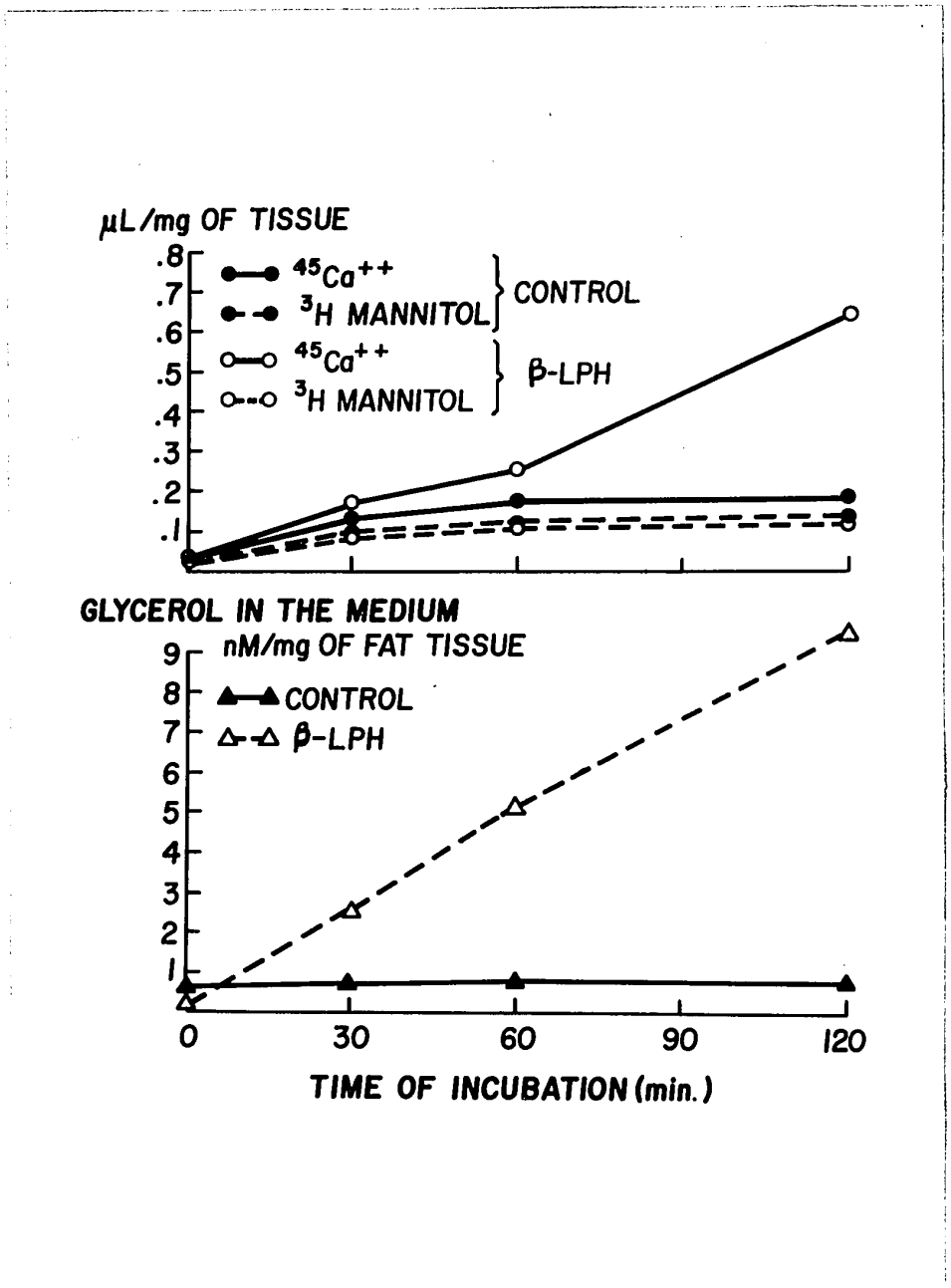

Fig. 3.2.4.3. Slices (about 50 mg) of epididymal rabbit fat tissue were incubated in 1 ml of Krebs-Ringer bicarbonate with 2% albumin containing 0.1 µCi per 1 ml of ^{45}Ca and 0.4 µCi of ^{3}H-mannitol. Distribution spaces in the tissue are expressed in µl (dpm in the tissue divided by dpm of isotope per 1 µl of incubation medium). Lipolysis was measured by means of glycerol release into the medium. One µM β-LPH was used.

DECREASE OF CALCIUM IN INCUBATION MEDIUM

DURING LIPOLYSIS IN INCUBATED RAT FAT CELLS IN VITRO

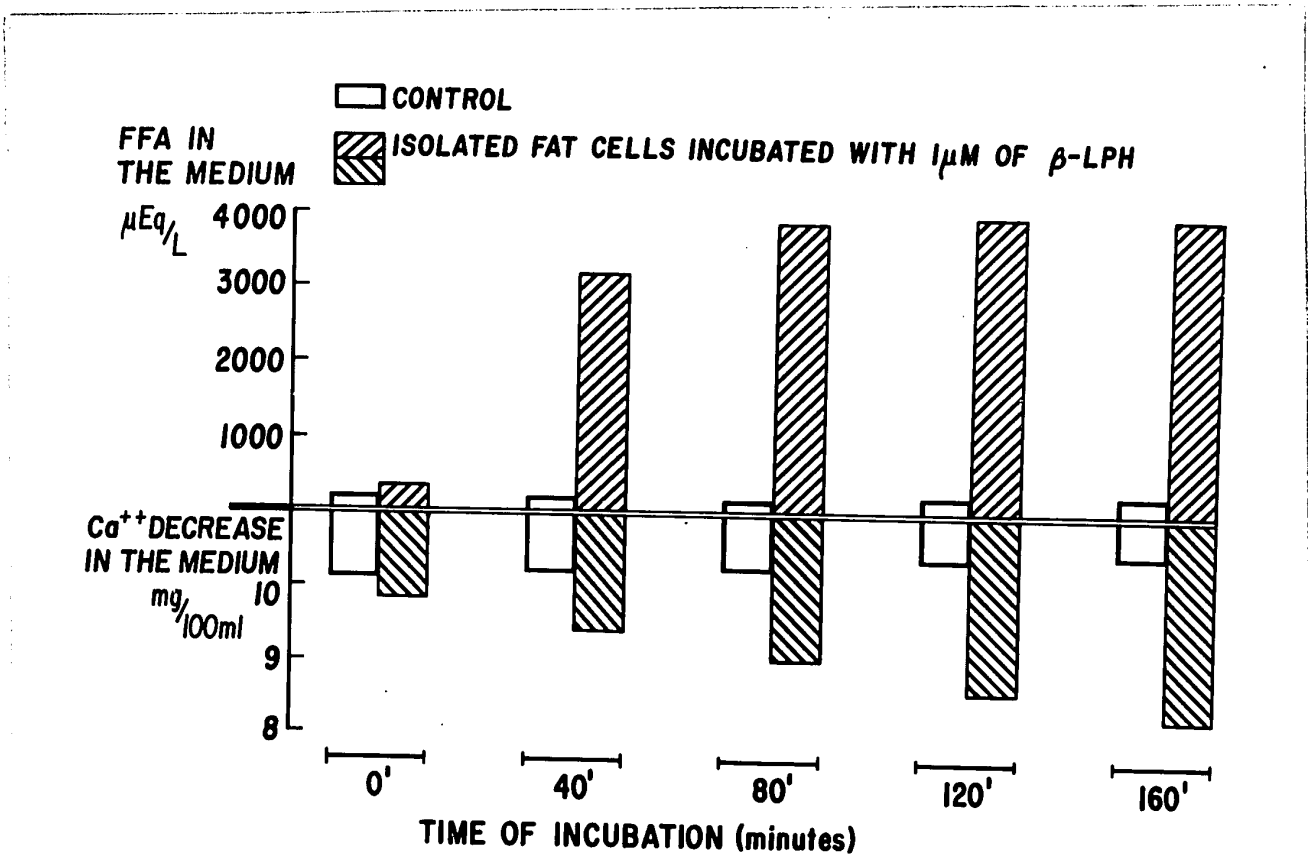

Fig. 3.2.4.4. Isolated rat epididymal fat cells were incubated with 1 μM β-LPH in total volume of 2 ml. Cell concentration corresponds to the 100 mg of tissue per 2 ml of medium. At the end of incubation period the medium was filtered through cellular filters and FFA and calcium determined.

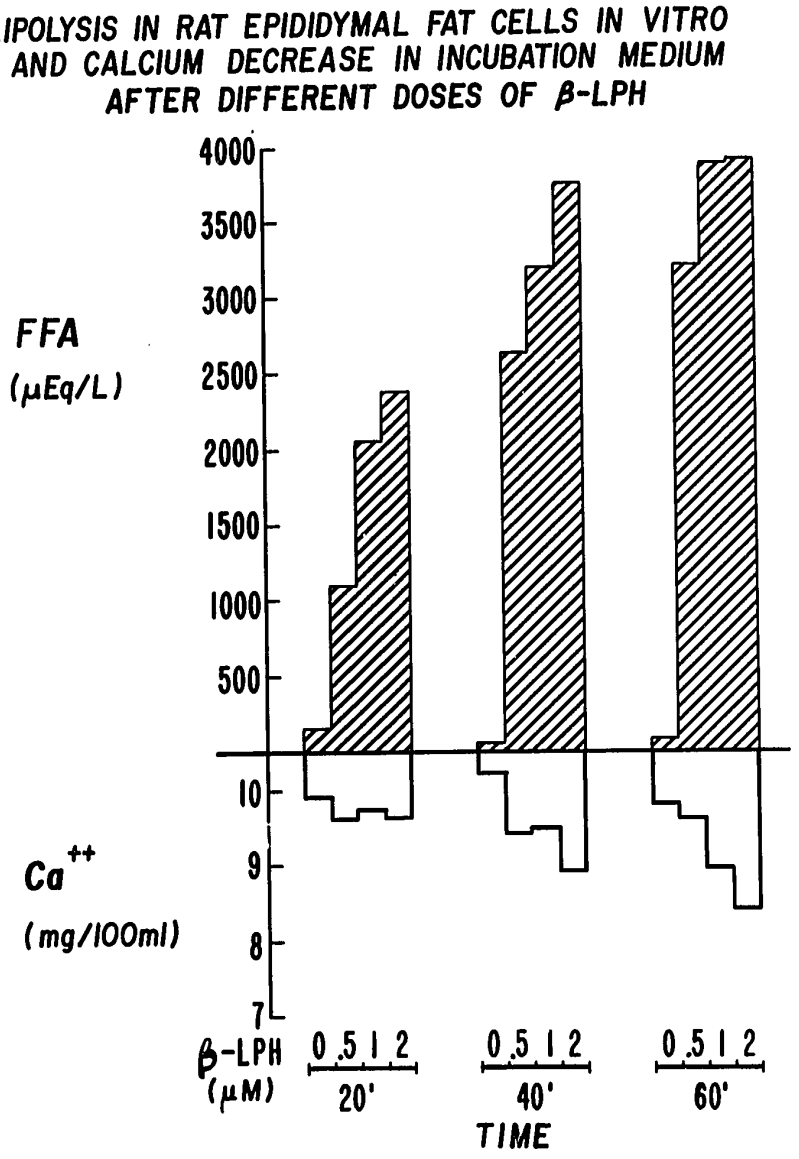

Fig. 3.2.4.5. Isolated rat epididymal fat cells were incubated in total volume of 2 ml. Cell concentration corresponds to the 100 mg of tissue per 1 ml of medium. At the end of the incubation period medium was filtered through cellular filters and FFA and calcium determined.

DOSE-RESPONSE OF LIPOLYSIS AND Ca^{++} UPTAKE

BY RABBIT PERIRENAL FAT CELLS

STIMULATED BY β-LPH

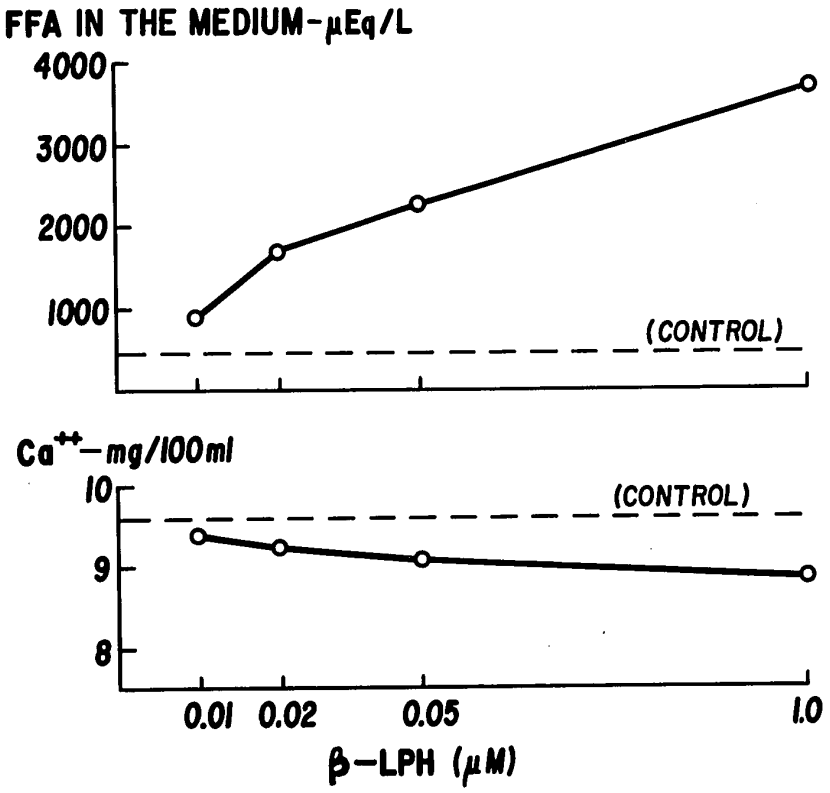

Fig. 3.2.4.6. Cells from 100 mg of perirenal fat tissue were incubated in 1 ml of medium (Krebs-Ringer bicarbonate with 4% albumin) containing β-LPH. Concentrations of FFA and Ca^{++} in the medium were determined after 90 minutes of incubation followed by filtration to remove fat cells.

TIME COURSE OF LIPOLYSIS AND Ca^{++} UPTAKE

BY RAT FAT CELLS IN VITRO

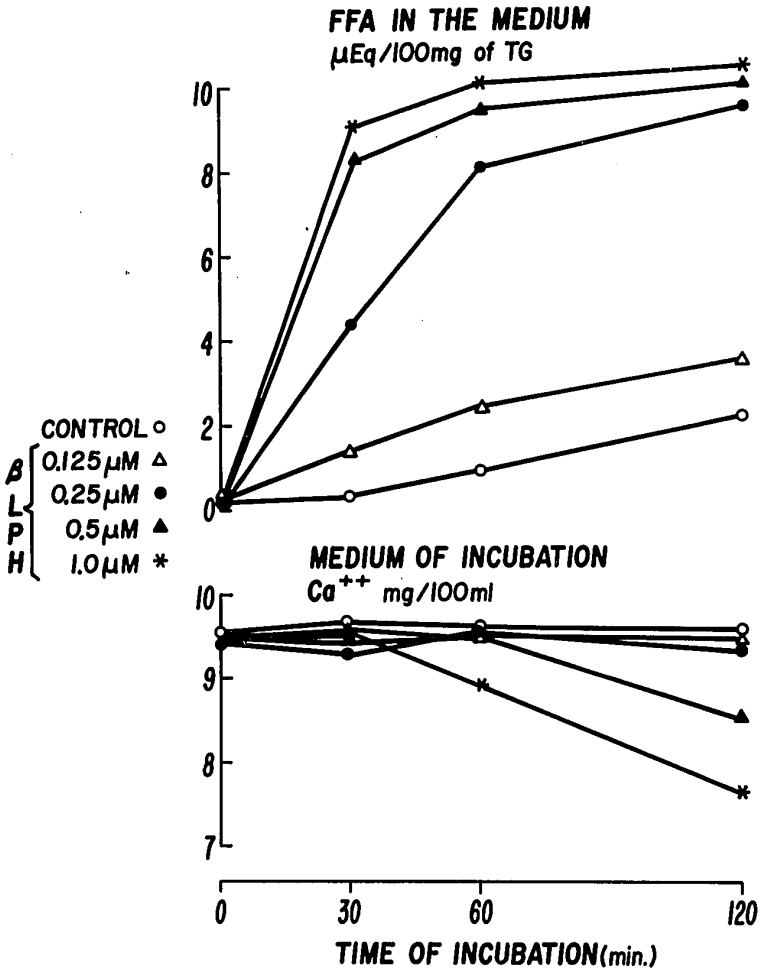

Fig. 3.2.4.7. Isolated rat epididymal fat cells were incubated in total volume of 2 ml. Final cell concentration corresponds to the 100 mg of tissue per 1 ml of medium. At the end of the incubation period medium was filtered through Gelman Metricel GA-1 cellular filters and FFA and Ca^{++}determined. FFA are related to the concentration of triglycerides in fat cells.

3.2.5. Calcium requirements for lipolytic effect of β-LPH

Rat isolated fat cells - The absence of Ca^{++} in the medium inhibited the lipolytic effect of β-LPH in isolated rat fat cells (Fig. 3.2.5.1.). The same phenomenon was observed when EGTA was added to calcium containing incubation mixture (Fig. 3.2.5.2.).

Rabbit isolated fat cells - In isolated rabbit fat cells the dependence of β-LPH induced lipolysis on the presence of calcium, has also been verified (Fig. 3.2.5.3.). The presence of 2 mM EGTA slightly inhibited the lipolytic response while 4 mM EGTA inhibited it completely. When 4 mM calcium was added to incubations inhibited by 4 mM EGTA, the lipolysis was fully restored. When 4 mM EGTA was added to the incubation mixture in which the lipolysis had already proceeded for 20 minutes, the glycerol level in the medium did not increase further (Fig. 3.2.5.3.).

DISCUSSION

Bally & Tilbury (11) concluded that Ca^{++} is required in the incubation medium for the lipolytic effect of ACTH but not for epinephrine, TSH and glucagon. This difference between epinephrine and ACTH was observed for the first time by Lopez & al (132) who concluded that Ca^{++} is necessary for the attachment of ACTH to the fat cell, probably at the receptor site on the cell membrane. Yanagi & al (229) (230) demonstrated the inactivity of ACTH in a homogenate of fat tissue, and they obtained a lipolytic response when Ca^{++} was added. This led them to conclude that ACTH acts by increasing Ca^{++} influx and that the ACTH-induced lipolysis is the result of the increased intracellular Ca^{++} concentration.

According to Mosinger & Vaughan (143) the lipolysis induced by epinephrine is inhibited when Ca^{++} is absent from the medium. Inhibition of lipolysis was, however, only partial and did not occur at higher concentration of epinephrine. The presence or absence of calcium does not affect the lipolytic effect of theophylline or dibutyryl c-AMP (143). It was also reported (62) that, in fat tissue, the lipolytic response to any activation, including that by theophylline and dibutyryl c-AMP, is suppressed by repeated washing with EGTA. It is, however, possible that the repeated washing with EGTA solution could induce changes in the membrane structure and function.

The calcium dependence of lipolysis in vitro could be summarized as follows:

(a) The absence of calcium in the medium blocked completely the lipolytic response to ACTH and β-LPH.

(b) The absence of calcium in the medium slightly inhibited the lipolytic activity of epinephrine, which is apparent only at low concentration of epinephrine.

(c) Repeated washing of fat tissue preparation with EGTA resulted in a significant suppression of the lipolysis with other agents not affected by the previous conditions (theophylline, dibutyryl c-AMP).

At least for the conditions summarized in (a) and (b), it could be suggested that the calcium is involved in the lipolytic action of hormones at some early steps of their effect on the cell membrane (143) (132).

DEPENDENCE OF β-LPH INDUCED LIPOLYSIS IN RAT FAT CELLS ON Ca^{++}

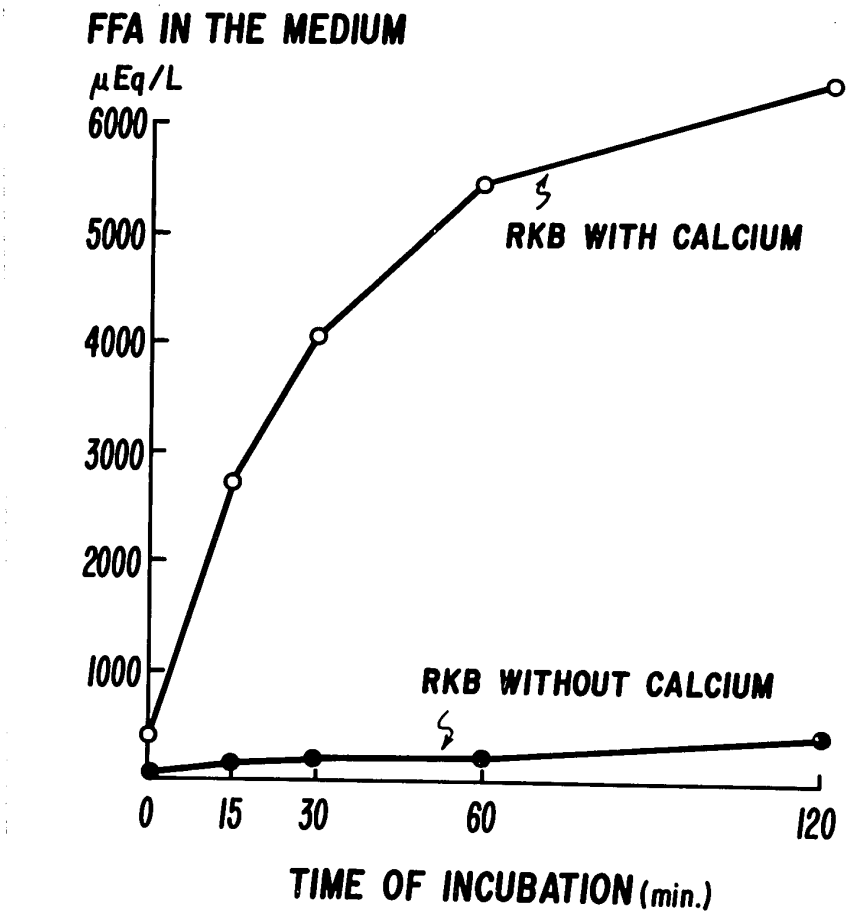

Fig. 3.2.5.1. Isolated rat epididymal fat cells were incubated with 1 μM β-LPH. Cells were washed 4 times in calcium free Krebs-Ringer bicarbonate medium. FFA were determined in the medium. RKB means Krebs-Ringer bicarbonate buffer with 4% albumin.

LIPOLYTIC ACTIVITY OF β-LPH IN ISOLATED RAT FAT CELLS.

EFFECT OF EGTA

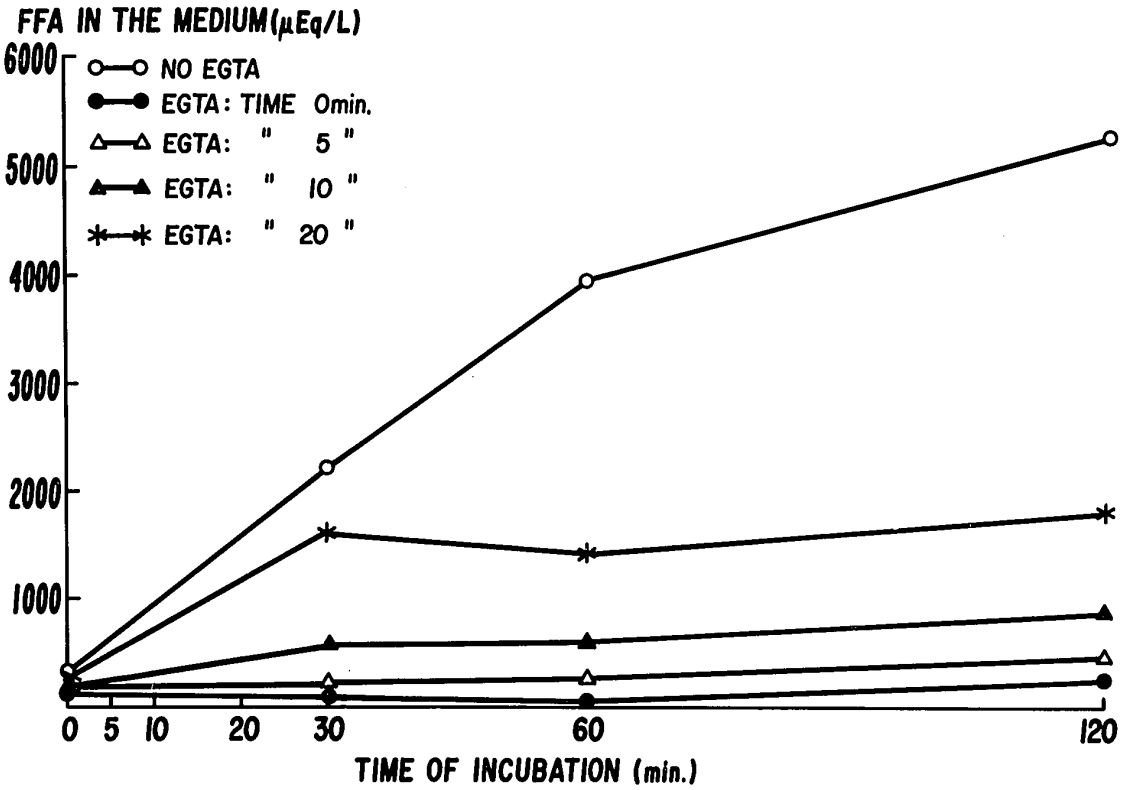

Fig. 3.2.5.2. Isolated rat epididymal fat cells were incubated with 1 μM β-LPH in total volume of 4 ml. Concentration of cells was equivalent to 100 mg of tissue per 1 ml of medium. EGTA was added to bring the final concentration to 2.5 mM. EGTA was added at intervals 0, 5, 10 and 20 minutes of incubation.

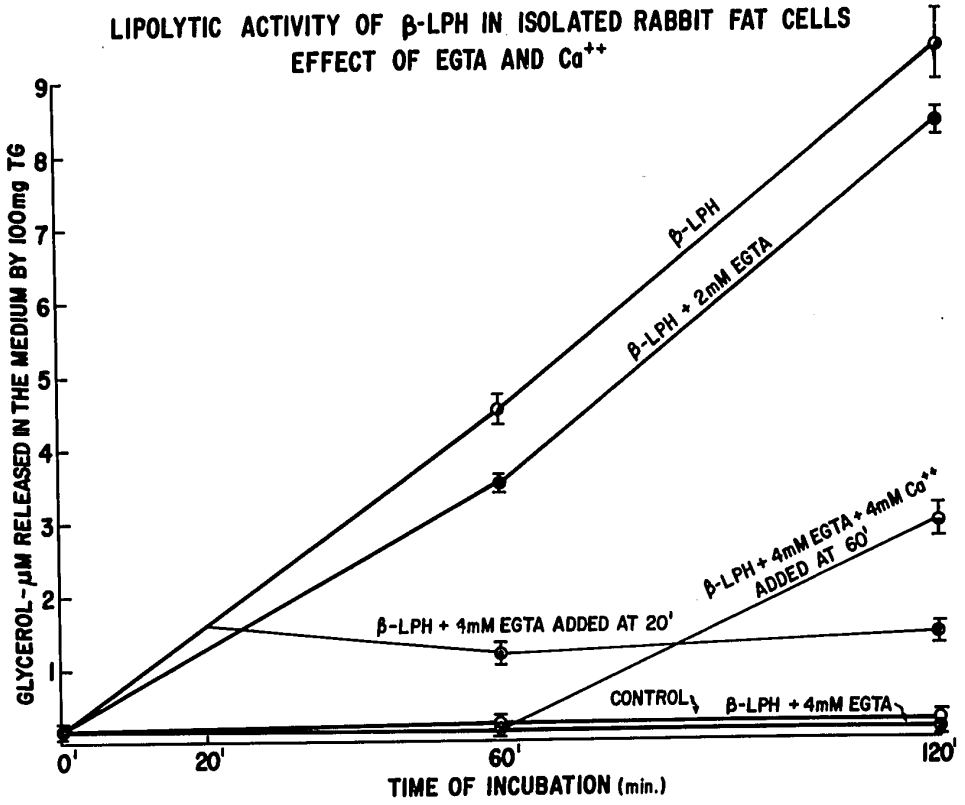

Fig. 3.2.5.3. Fat cells isolated from rabbit epididymal fat tissue were incubated in 1 ml of Krebs-Ringer bicarbonate buffer with 4% albumin. Lipolysis was determined as glycerol release into the medium per 100 mg of triglyceride content of incubated cells. Each point represents a mean of 4 incubations ± SE. In all incubations except control 0.02 μM β-LPH was used.

3.2.6. Stimulation of adenylate cyclase by β-LPH

The maximal and half maximal doses for β-LPH and ACTH were determined from the Fig. 3.2.2.1. for rabbit and rat fat tissues. These doses were used for adenylate cyclase stimulating activity. Results are shown in Fig. 3.2.6.1. for rat tissue and in Fig. 3.2.6.2. for rabbit tissue. I also verified in rat adipose cells the effect of EGTA (at different concentrations, 0.5 to 4 mM) and found out (Fig. 3.2.6.3.) that the β-LPH stimulation of adenylate cyclase activity seems to be calcium dependent.

Since 4 mM EGTA inhibited completely the stimulation of adenylate cyclase by β-LPH (Fig. 3.2.6.3.), I verified the effects of 4 mM EGTA added at different times of incubation. Results revealed (Fig. 3.2.6.4.) that the stimulation of adenylate cyclase activity is inhibited as soon as EGTA is added to the medium.

DISCUSSION

β-LPH, like ACTH, stimulated the adenylate cyclase system in adipose tissue. However, the lipolytic activity of these two hormones shows some species difference when incubated with rabbit and rat adipose tissue. The maximal stimulation of rat adenylate cyclase was reached after 10 minutes of incubation and fell rapidly thereafter. In rabbits a continuous increase up to the 40 minutes was observed as shown in Fig. 3.2.6.2. We previously observed that in rabbit isolated cells the maximal lipolytic response is usually obtained after 2 hours of incubation. In rat cells maximal response is obtained in 1 hour. It is possible that this difference just reflects different timing of the adenylate cyclase system activation.

When equipotent lipolytic doses of ACTH and β-LPH were used for adenylate cyclase stimulation, ACTH was found to be more active in rat cells while β-LPH was found to be more active in rabbit cells preparation.

This difference is difficult to interpret by present knowledge. There is a possibility of some collateral regulatory mechanism of lipase activation not yet described and different in species utilized. However, this will require separate and more detailed study.

While this study was in progress, Braun & al (28) published, in abstract form, that β-LPH stimulated the adenylate cyclase in rabbit adipose cell ghosts but did not stimulate it in an identical preparation of cells from rat adipose tissue. However, there were no details about doses or procedures utilized and we cannot compare their results with my own experiments which are already published (236).

It was shown in part 3.2.5. that calcium is necessary for the lipolytic activity of β-LPH. The present results revealed that calcium is essential for the stimulation of the adenylate cyclase system. The calcium dependence of adenylate cyclase activation by ACTH was previously reported (170) (118) (13) (12). In this respect the receptor site for β-LPH behave as the receptor site for ACTH.

The present results are also of interest from the point of view of recent findings about the formation and release of a hormone antagonist by rat adipocytes (135) (97). The characteristic of c-AMP response in rat fat cells correspond to those previously reported. This sharp rise of c-AMP level at the very beginning of adenylate cyclase stimulation has been reported in many preparations from rat adipose tissue (97). This finding led to the demonstration of an inhibitor which is formed by fat cells

stimulated by lipolytic hormone. This inhibitor was partially purified and some evidence has been presented that the inhibition is not due to prostaglandin nor to a higher level of FFA.

From Fig. 3.2.6.2. could be observed, however, that the intracellular c-AMP increase in rabbit fat cells is different from those reported for rat fat cells. From the completely different character of c-AMP level after stimulation of rabbit fat cells, it can be concluded that in this tissue the inhibitor is probably absent. On the other hand, when the possible biological importance of this still somehow hypothetic inhibitor is considered, it should be mentioned that the c-AMP intracellular level, which is correlated with lipolysis, is relatively very low and could be roughly estimated as about 15% of usually measured c-AMP intracellular increase (48).

Results comparable to this presentation were reported by Magnaniello & al (135). They used, for example, propranolol which they added to isolated cell preparation at several times after epinephrine-stimulated lipolysis had begun. After propranolol addition the rate of glycerol production fell essentially to zero within 1-2 minutes. In the same period of time they observed that c-AMP usually fell to basal level. These results are compatible with the observations found in this study on the effect of EGTA addition on lipolysis (Fig. 3.2.5.2. and Fig. 3.2.5.3.) or on the accumulation of labelled c-AMP in fat cells after β-LPH stimulation (Fig. 3.2.6.4.). This similarity of propranolol-blocked epinephrine lipolytic action and EGTA blocked β-LPH lipolytic action also favours the suggestion that the calcium is involved in the lipolytic action of some hormones at early steps of their effect on the cell membrane.

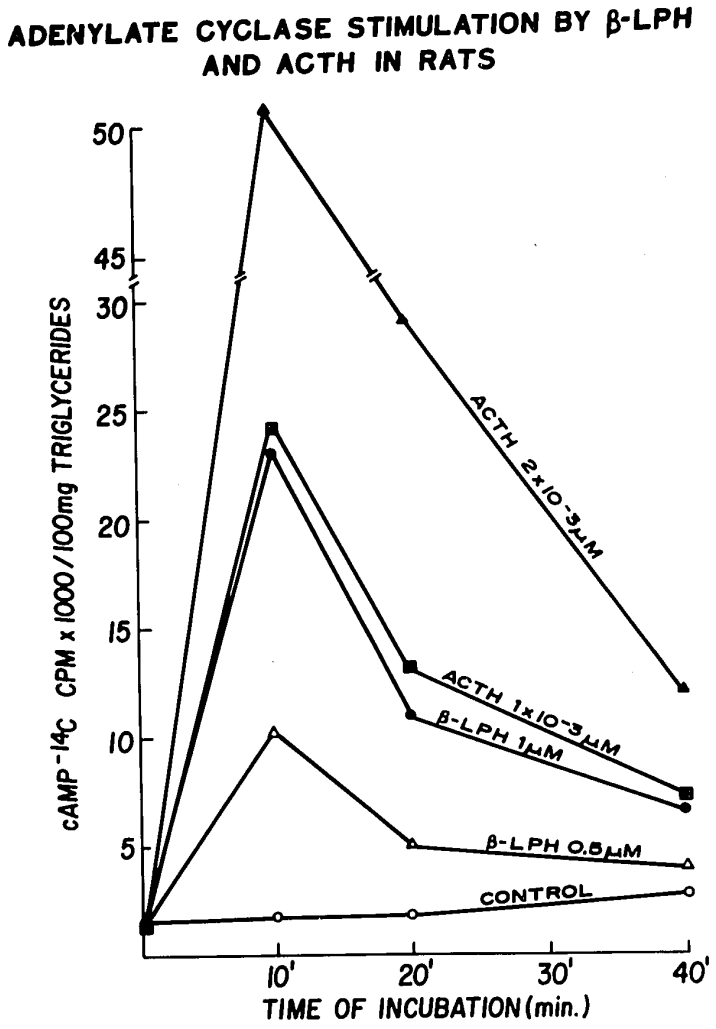

Fig. 3.2.6.1. Adenylate cyclase activity is determined as of ^{14}C-c-AMP in isolated fat cells labelled previously with ^{14}C-adenine. Cpm determined in the incubation were adjusted to 100 mg of triglycerides of adipose cells. The ACTH and β-LPH doses were chosen from log. dose-response relationship as shown in Fig. 3.2.2.1. The doses giving maximal and half-maximal lipolytic responses were used.

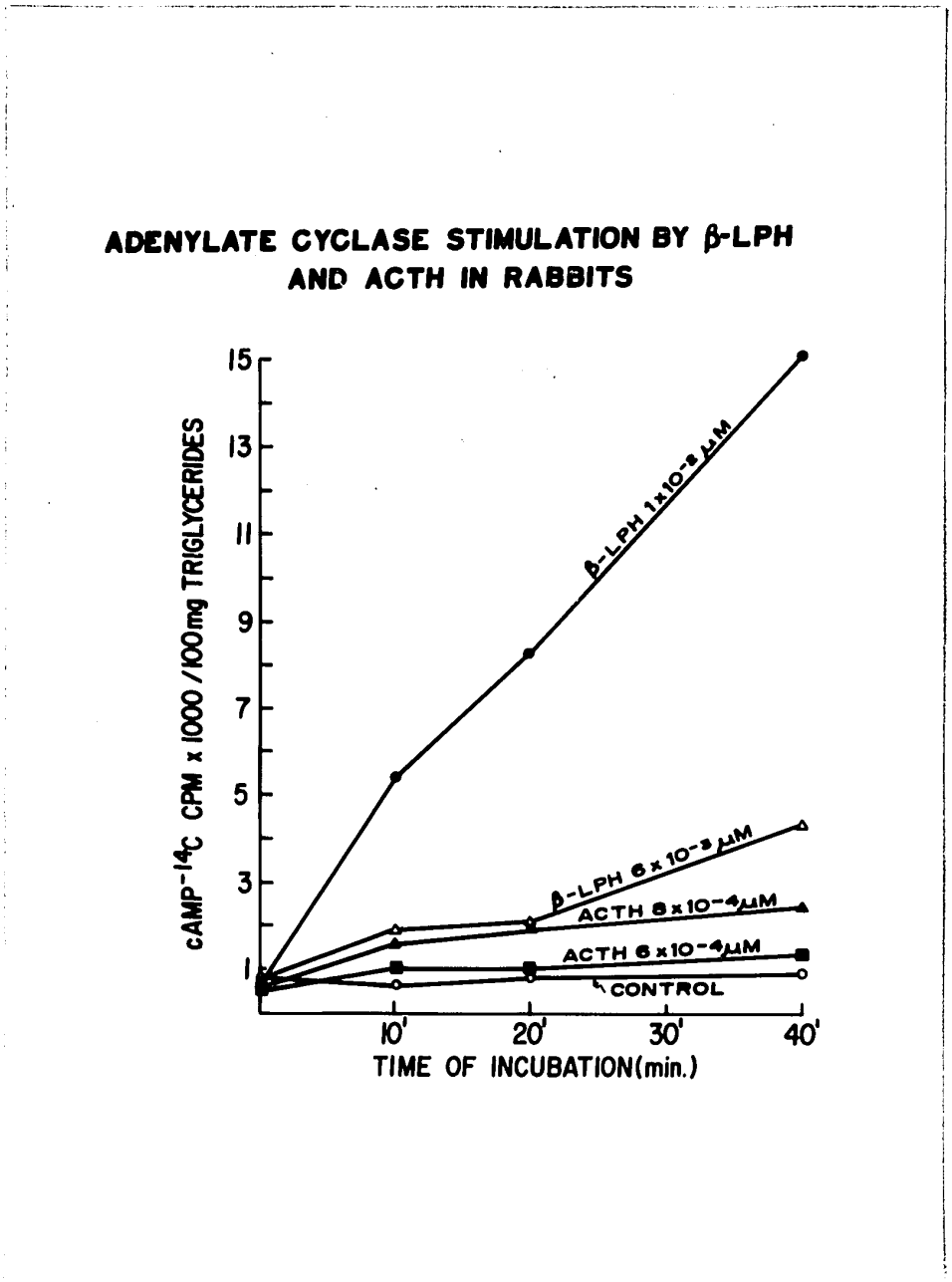

Fig. 3.2.6.2. Experimental conditions are the same as in Fig. 3.2.6.1. The ACTH and β-LPH doses were determined on rabbit isolated cells by the same way as in Fig. 3.2.2.1.

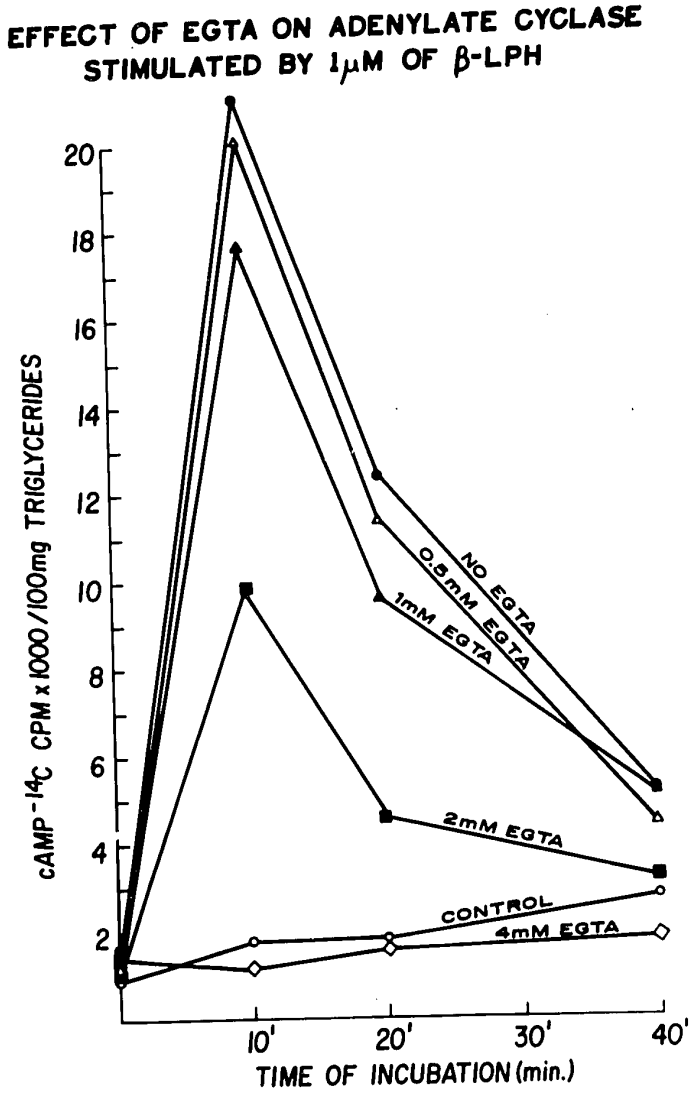

Fig. 3.2.6.3. Experimental conditions are the same as in Fig. 3.2.6.1. Rat isolated fat cells were used in incubations containing different concentrations of EGTA.

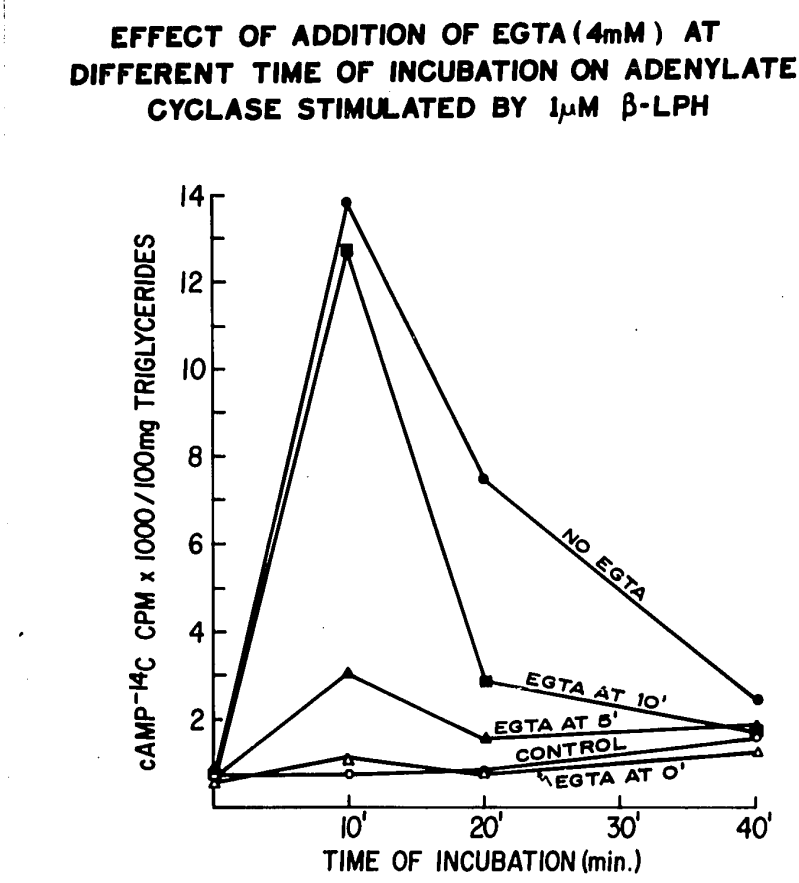

Fig. 3.2.6.4. Experimental conditions are the same as in Fig. 3.2.6.1. EGTA (4 mM) was added to the incubated rat fat cells at 0 minute, 5 minutes and 10 minutes.

4. GENERAL DISCUSSION

Some lipolytic compounds were reported to cause hypocalcemia in rabbits. β-LPH, when injected to rabbits, is also hypocalcemic and the hypocalcemia is closely related to the lipolysis. This relationship has been already discussed by Friesen (69). His conclusions were summarized in section 3.2.1. and my data are in agreement with the view that there may be a relationship between lipolysis and hypocalcemia. Moreover, in section 3.2.4., the accumulation of calcium in fat tissue after β-LPH administration was reported. In section 3.2.4., the evidence was summarized that the hypocalcemia in rabbits, after the administration of β-LPH, is partly caused by the shift of calcium into adipose tissue. This was already proposed for ACTH by Akgun & Rudman (1) but they were not using isolated fat cells or tritiated mannitol to assess the space of calcium accumulation.

The hypothesis was proposed in section 3.2.4. that the increased uptake of calcium by fat tissue is probably the consequence of increased concentration of FFA in fat cells. The same hypothesis was also proposed by Alm & al (3) who observed calcium accumulation in fat tissue when albumin, a FFA acceptor, was absent from the incubation medium and the lipolysis stimulated.

Observation on thyroidectomized rabbits in section 3.2.1. revealed that β-LPH did not cause hypocalcemia in these rabbits. The lipolysis in thyroidectomized rabbits was stimulated by β-LPH but the FFA levels were lower than in controls. Lower rate of lipolysis in fat cells of thyroidectomized rabbits was described in section 3.2.2. Thyroidectomy was also previously reported to increase re-esterification of FFA in fat tissue (77).

Lower rate of lipolysis and increased re-esterification might keep the intracellular FFA at low level and consequently neither calcium accumulation in fat tissue nor hypocalcemia occurs. Proposed hypothesis could also explain some of the results of Akgun & Rudman (1). They postulated the existence of a serum factor, different from albumin, and essential for a calcium shift into the adipose tissue. Their results might be also the consequence of higher intracellular FFA levels. In their experiments FFA concentration was higher in incubations with serum than corresponding values in incubations with Krebs-Ringer bicarbonate buffer. Consequently the available binding sites on albumin were more saturated and this probably contributed to higher intracellular FFA concentrations. The existence of the hypothetic "serum factor" of Akgun & Rudman (1) was not confirmed by the experiments presented in this thesis (section 3.2.4.) as well as by the results of others (3) (229) (230).

A constant finding in the experiments with pituitary lipolytic principles, including β-LPH, has been the responsiveness of rabbit adipose tissue and the nonresponsiveness in other species. Contrary to these previous findings were the experiments of Cseh & Graf (53) who reported that the porcine β-LPH is lipolytic on pig fat tissue and they also claimed (54) that human β-LPH is active in human fat tissue.

The results of Cseh & Graf (53) (54) were not confirmed in this thesis since the human β-LPH prepared in our laboratory was not active in the preparation of human isolated fat cells. Porcine β-LPH preparation isolated in our laboratory showed some <u>in vivo</u> lipolytic effects in pigs but was not active <u>in vitro</u> on isolated porcine perirenal fat cells.

When α- and β-MSH are injected into rabbits the lipolytic response is less prolonged than when an equipotent dose of β-LPH is used. This could be explained either by faster inactivation or elimination of MSH or by transformation of β-LPH into β-MSH.

The β-LPH molecule modified by o-nitrophenyl sulfenyl chloride (NPS-β-LPH) had a lipolytic activity comparable to that of NPS-ACTH as reported by Ramachandran & Lee (156). The modification of both ACTH and β-LPH impaired the lipolytic activity on rat fat cells without affecting the activity on rabbit adipose cells. These findings suggest that the receptors for β-LPH in fat tissues are related, if not identical, to those of ACTH and they also suggest that rat and rabbit receptors are different.

The requirement of calcium for the lipolytic activity of β-LPH in vitro was demonstrated in section 3.2.5. on both rat and rabbit isolated fat cells. The addition of EGTA, a calcium chelator, to the incubations of fat cells in which the lipolysis had already proceeded, quickly stopped the release of glycerol into the medium.

The results presented in this thesis are in agreement with the view of some authors (143) (132) that the calcium is involved in the lipolytic action of hormones at some early steps of their effect on the cell membrane.

As was demonstrated in section 3.2.6., β-LPH stimulated the adenyl cyclase in both rat and rabbit isolated fat cells. The calcium dependence of adenyl cyclase activation confirmed further the conclusions of section 3.2.5. that calcium is involved at some early steps in the activation of the lipolytic process. The apparent difference in the response of the two species used is interesting in view of recent publication of Ho & Sutherland (97) who demonstrated that an adenyl cyclase inhibitor is formed by rat fat cells stimulated by lipolytic hormone. The results presented in section 3.2.6. favor the hypothesis that the rabbit adipose cells are probably lacking such an inhibitor.

5. CONCLUSIONS

Sheep β-lipotropic hormone (β-LPH) is a polypeptide consisting of 90 amino-acid residues with a sequence (41-58) corresponding to β-MSH (129). Similar peptides were isolated from porcine (86) (73) (85) and human pituitaries (54). Sheep β-LPH has been measured in sheep plasma by Chrétien & al (submitted for publication). Its concentration is about 10 ng/ml.

In this thesis the lipolytic activity of highly pure β-LPH was studied in vivo and in vitro and the following conclusions could be presented:

(a) Sheep β-LPH, when injected to rabbits, caused an increase in FFA blood levels and caused hypocalcemia. Plasma triglycerides increased in injected rabbits at 7 hours after injection. No change in cholesterol, glucose and magnesium were found up to 7 hours after β-LPH injection.

(b) In thyroidectomized rabbits β-LPH administration caused lipolysis but the hypocalcemia was absent. The lipolytic response in thyroidectomized rabbits was generally lower than the lipolytic response in normal rabbits especially when the absolute FFA levels were compared. However, this difference was not significant when the net change of FFA increase (ΔFFA) was compared in the two groups of animals. Incubation of isolated fat cells from thyroidectomized rabbits showed lower lipolytic response to β-LPH and ACTH. The same was observed in the fat cells from thyroidectomized rats. However, rabbit fat cells were less affected by thyroidectomy than the rat fat cells.

(c) Porcine β-LPH was found to be lipolytic in the pig (fasted for 2 days), only when a relatively high dose (0.3 mg per kg of body weight) was injected. On the other hand, the same porcine β-LPH failed to act in isolated porcine perirenal fat cells in vitro.

(d) α-MSH and β-MSH when injected into rabbits provoke only a very short lipolytic response when compared to the lipolytic effect of an identical amount of β-LPH.

(e) In vitro lipolytic activity of β-LPH was compared to ACTH in isolated fat cells of rats and rabbits. ACTH is generally more active in both species but in isolated rabbit fat cells the activity of β-LPH approached that of ACTH.

Sheep and human β-LPH was found to be inactive on isolated human fat cells.

(f) Modification of the indole ring of the tryptophan residue of β-LPH by o-nitrophenyl sulfenyl chloride (NPS-β-LPH) did not affect the lipolytic activity in rabbits but abolished it in rat isolated fat cells. This NPS-β-LPH also inhibited the lipolytic activity of unmodified β-LPH in rat fat cells in vitro.

(g) Following β-LPH administration to rabbits the calcium concentration in fat tissue increased. This calcium increase coincided with hypocalcemia. This phenomenon was further studied by incubating rabbit fat tissue in presence of ^{45}Ca and ^{3}H-mannitol. β-LPH increased the ^{45}Ca concentration in fat tissue without changing the ^{3}H-mannitol uptake. This favored the assumption that the calcium pool in fat cells increased. The calcium decrease in the medium during the β-LPH activated lipolysis in isolated fat cells also supports such hypothesis.

These results as well as those of Friesen (69) and Akgun & Rudman (1) favor the idea that the hypocalcemia in rabbits is probably the consequence of calcium accumulation in fat tissue during lipolysis. It was also discussed that the increased uptake of calcium by fat tissue during lipolysis could be caused by the excessive intracellular FFA level.

(h) β-LPH did not induce lipolysis in vitro in the absence of calcium in the medium or in the presence of EGTA, a calcium chelator. Addition of EGTA to the medium almost immediately stopped the lipolysis. When excess calcium was added to EGTA-containing incubations β-LPH lipolysis was restored.

(i) β-LPH stimulated the adenyl cyclase in isolated rat and rabbit fat cells. Stimulation of adenyl cyclase activity in isolated fat cells is also calcium dependent, since the EGTA addition blocked the β-LPH stimulation immediately. Differences in adenyl cyclase systems of rabbit and rat adipose cells were also demonstrated.

6. CLAIMS TO ORIGINALITY

These can be summarized as follows:

(a) One original contribution of this thesis to the field of lipolytic peptides of pituitary origin is the finding that β-LPH stimulates the adenyl cyclase of fat cells, that the stimulation of adenyl cyclase is calcium dependent and finally that rabbit and rat adenyl cyclase system behave differently. When the work on this subject proceeded, Braun & al (28) published in abstract form similar results on rabbit isolated cells, but one part of their results was contradictory to our findings. They declared that sheep β-LPH is neither lipolytic nor adenyl cyclase stimulating in rat fat cells. This is in contradiction not only with our findings but also with all previous publications about lipolytic activity of β-LPH in the rat (131). During the determination of adenyl cyclase stimulation by sheep β-LPH and ACTH, important species differences in the kinetics of the adenyl cyclase system of rat and rabbit were noted. These species differences were not previously described. From the different character of adenyl cyclase activity in rabbit fat cells it can be concluded that the c-AMP inhibitor, described recently in rat fat cells by Ho & Sutherland (97), may be absent in rabbits.

(b) Another original contribution of my work is related to the β-LPH and ACTH receptors. NPS-β-LPH lipolytic activity was assayed and the changes were found to be similar to those found with NPS-ACTH. This is compatible with the hypothesis that the lipolytic receptors of ACTH and β-LPH are closely related or identical.

(c) Calcium accumulation in fat tissue during lipolysis was reported previously but, evidences were presented here that calcium accumulates in fat cells rather than in the extracellular space of fat tissue.

Indirect evidence was presented and discussed that the hypocalcemia in rabbits after β-LPH administration is caused by calcium accumulation in fat tissue. Other indirect evidence and discussion contributed to the hypothesis that the calcium accumulation in fat cells could be a consequence of an increase of intracellular FFA.

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