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STUDIES ON THE IN VITRO LIPOLYTIC ACTIVITY OF PITUITARY GROWTH HORMONE

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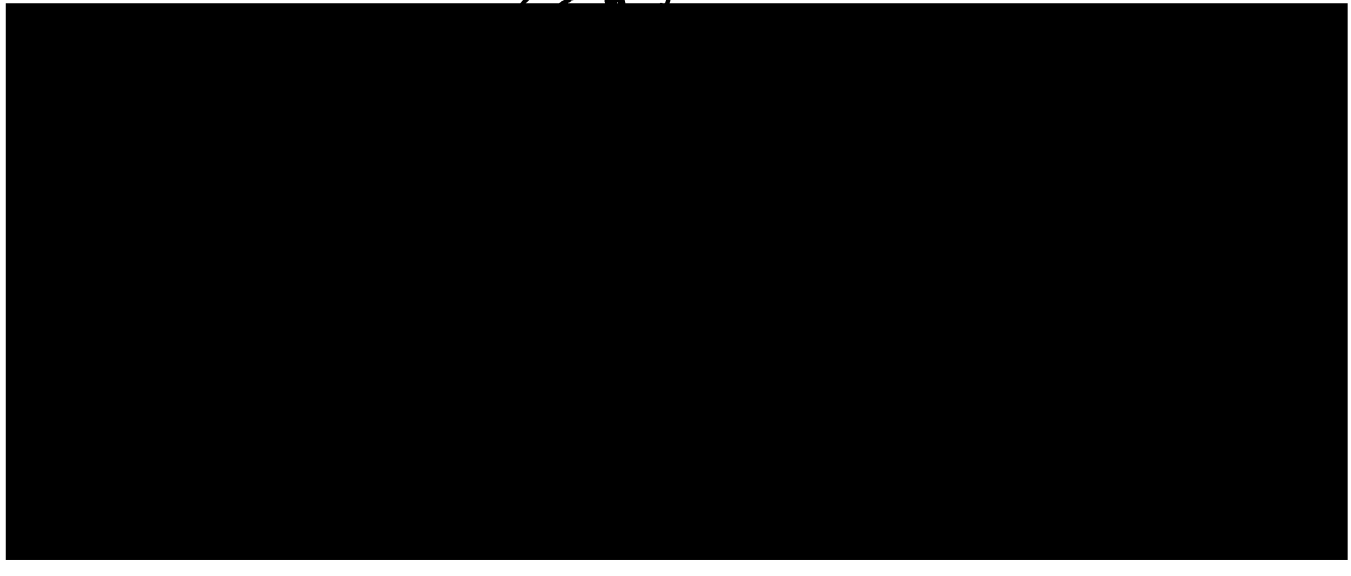

ABSTRACT

STUDIES ON THE IN VITRO LIPOLYTIC ACTIVITY

OF PITUITARY GROWTH HORMONE

by

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1. The growth promoting and rapid lipolytic activity associated with preparations of bovine growth hormone, can be separated by chromatography on QAE Sephadex A25. The contaminant responsible for the lipolytic effect was identified as thyrotropin by neutralization experiments with rabbit antiserum to thyrotropin. It was further shown that the lipolytic activity associated with bovine growth hormone can be removed by affinity chromatography on sepharose which was coupled to antibodies to the α subunit of ovine luteinizing hormone.

2. Incubation of human growth hormone with isolated rabbit fat cells but not rat fat cells produced a rapid stimulation of lipolysis. Evidence was presented to substantiate that the lipolytic actions of the hormone is an intrinsic property and not due to contamination with other lipolytically active pituitary hormones. Of the known pituitary hormones, human growth hormone and the melanotropins are the only hormones that activate lipolysis in the rabbit fat cells but not in the rat fat cells. However, human growth hormone exhibits no melanocyte-stimulating activity in the isolated frog skin assay.

The lipolytic effect of several preparations of human growth hormone have been studied and found to possess similar lipolytic activity. This lipolytic activity can be neutralized by rabbit antibodies to human growth hormone and chorionic somatomamnotropin. Treatment of human growth hormone (4.5×10^{-6} M) and adrenocorticotropin (0.03×10^{-6} M) with trypsin (4 µg/ml) for 5 minutes completely abolished the ability of human growth hormone to release glycerol but that of adrenocorticotropin was only decreased by 30%.

Human growth hormone increases the accumulation of cyclic AMP as well as glycerol release in the isolated rabbit fat cells. The lipolytic effect of human growth hormone is not inhibited by cycloheximide and requires Ca^{2+} ions. Human growth hormone also stimulates adenylate cyclase activity in intact rabbit fat cells by increasing the accumulation of labelled cyclic AMP from ^{3}H-adenine. However, it does not stimulate adenylate cyclase activity in rabbit fat cell ghosts. The reason for this discrepancy is not known.

Fat cells isolated from rabbits which have been fasted for 48 hours elicit a 3 to 6 fold increase in sensitivity to human growth hormone in the absence or presence of theophylline (32×10^{-6} M). The lipolytic specificity of known pituitary hormones was also studied in this system. Human growth hormone, adrenocorticotropin and melanotropins were found to be the only hormones which stimulated lipolysis in fat cells isolated from fasted rabbits.

3. Limited digestion of isolated fat cells from normal rabbits with trypsin (0.1 to 0.25 mg/ml) results in enhanced

sensitivity to subsequent lipolytic action of human growth hormone. Higher concentrations of trypsin (1 mg/ml) under similar conditions rendered the cells totally unresponsive to human growth hormone. Digestion of fat cells from fasted rabbits with trypsin (up to 4 mg/ml) was without effect on the subsequent lipolytic effect of human growth hormone.

Digestion of fat cells from both normal and fasted rabbits with phospholipase C causes lysing of the cells and destroys the lipolytic responsiveness of the cells to human growth hormone.

Mild digestion of isolated fat cells from both normal and fasted rabbits with neuraminidase (2.5 milli units per ml) results in enhanced lipolysis of these cells to human growth hormone. Digestion with higher concentrations of the enzyme (10 milli units per ml) abolishes this increase in sensitivity without destroying completely the lipolytic activity of human growth hormone.

Sequential digestion with neuraminidase and trypsin, and the studies on the digestion with individual enzyme alone on fed cells from fed rabbits and fasted rabbits indicate that the two enzymes probably act by a similar mechanism in increasing the sensitivity of the cell to human growth hormone. However, the effect of the two enzymes are not identical, since they are not interchangeable in sequential digestion, and also only neuraminidase, but not trypsin, is effective in increasing the sensitivity of fat cells from fasted rabbits.

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ABBREVIATIONS

NEFA - non-estrified free fatty acids

ACTH - adrenocorticotropin

TSH - thyroid stimulating hormone

BGH - bovine stimulating hormone

HGH - human growth hormone

MSH - melanotropins

FSH - folicle stimulating hormone

LH - luteinizing hormone

HCS - human chlorionic somatotropins

A/S - antibodies

LtH - prolactin

o - ovine

b - bovine

h - human

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CHAPTER I

INTRODUCTION

It was reported by Lee and Schaffer (1) in 1934 that growth promoting extracts of anterior pituitary glands administered to rats cause a shift in carcass composition accountable by a decrease in fat and an increase in protein. The pituitary factor responsible for these changes was later identified by Li, Simpson and Evans to be growth hormone (2).

Evidence has accumulated which suggests that growth hormone administration produces an elevation in plasma-free fatty acids (NEFA) (3-5), a mobilization of lipid to the liver (6,7) and an increase in production of ketone bodies (8). These effects of growth hormones on lipid metabolism coupled with the observation that fat depleted rats failed to grow in response to growth hormone administration led Greenbaum (9) to postulate that its primary action is on lipid metabolism and oxidation, thereby providing the caloric requirements for protein synthesis and growth. Since then, numerous investigations have been conducted to study the lipolytic effect of growth hormones on different experimental species both *in vivo* and *in vitro*. While the lipolytic effect of growth hormones in intact animals has been well documented (3,4,10,11), this effect of growth hormone in adipose tissue slices and isolated fat cell preparations is still under dispute primarily because of the high levels of growth hormone required in stimulation, lack of hormonal specificity of the adipose tissue and variation in species specificity.

The objective of this thesis is to determine whether the lipolytic effects of bovine and human growth hormone in fat cells isolated from the rat and rabbit is an intrinsic property or due to contamination with other pituitary hormones. If the lipolytic effect of these growth hormones was due to a contaminant, experiments were designed to determine the nature of this contaminant. On the other hand, if the lipolytic effect was an intrinsic property of the growth hormone, experiments were designed to substantiate this property. Below is a summary of the current studies of the lipolytic effects of growth hormones on fat metabolism.

A. In Vivo Effects

1. Slow-Acting Effects of Growth Hormone on NEFA Release

Raben and Hollenberg (10) originally showed that administration of physiological doses of growth hormone markedly increases the concentration of NEFA in the plasma of fasted dogs and humans. This was later confirmed by Goodman and Knobil (3) who injected a single dose of simian growth hormone (0.05 mg/ml) into fasting normal and hypophysectomized monkeys and observed a greater than 6-fold increase in plasma NEFA in 12 hours. Engel et al. (4) also observed that injection of growth hormone into fed and fasted hypophysectomized rats but not normal rats causes a significant increase in plasma NEFA concentration. Li et al. (11) injected a single dose of HGH (0.5 mg) into fasting rabbits and observed an over 3-fold increase in NEFA after a lag period of 45 minutes. Fain (15) administered growth hormone and dexamethasone into adrenalectomized rats and observed an increase in NEFA release over that

seen after treatment with either hormone alone. Glucocorticoid has been known to have a "permissive" effect on adipose tissue since adipose tissue from adrenalectomized animals is much less sensitive to lipolytic hormones than normal animals (16).

In general, the rise in plasma NEFA due to growth hormone only begins between 1 and 2 hours after injection, and the effect is maximal or near maximal at 3 to 4 hours (4,12-14). This in vivo effect of growth hormone on free fatty acid release is probably an intrinsic property of the hormone for the following reasons:

a. The effect can be observed with the administration of physiological doses of the hormone (3,10).

b. Knobil and Greep (12) reported that administration of ACTH (10 units), TSH (10 units), prolactin (10 units) and BGH (10-50 mg) did not produce this increase in fasting normal and hypophysectomized monkeys. The lack of response with BGH not only demonstrated species specificity of growth hormone, but also substantiated that this slow increase in NEFA is not due to contamination with ACTH or TSH, since many available preparations of BGH are contaminated with either hormone (17,18).

c. The kinetics of stimulation is different from other fast-acting lipolytic agents such as epinephrine, in that it requires a lag period of at least 1 to 2 hours (19,20).

d. The effect can be modified by metabolic adjustments such as fasting or exercise.

2. Effect of Fasting on NEFA Release.

Fasting seems to play an important role in the manifestation of the effect of growth hormone in vivo on lipid metabolism. Goodman and Knobil (2) reported with fasting normal and hypophysectomized monkeys, and Raben and Hollenberg (10) with fasting dogs and humans, that the increase in NEFA due to growth hormone was found to be decreased or completely abolished by the administration of food or glucose. Goodman and Knobil (3,21) also observed that fasting alone causes a marked increase in plasma NEFA within a few hours in both monkeys and rats. Scow and Chernick (22) suggested that this increase in NEFA during fasting is probably due to a decrease in re-esterification in response to a shortage of blood glucose. Goodman and Knobil (3,21) further observed that hypophysectomy, adrenalectomy, or thyroidectomy did not abolish this increase in plasma free fatty acids completely. Based on these findings, Knobil and Goodman concluded that the pituitary, adrenal or thyroid glands are not necessary to initiate FFA mobilization during fasting, but that optimal function of these glands is required for the normal metabolic response to food deprivation. The effect of growth hormone, then, is probably to accelerate this mobilization of fat initiated by fasting.

3. Acute Effects of Growth Hormone on NEFA Release.

As described above, the manifestations of the increase in free fatty acids with growth hormone administration required a lag period of at least 1 hour. There have been reports of an acute decline of plasma free fatty acids and glucose 20 to

30 minutes following injection of growth hormone. Zahnd et al. (23) observed an acute transient decline of NEFA and glucose following administration of HGH (8 mg) to fasting normal and humans. Beck et al. (24) have also observed this effect in fasting humans, Bergen et al. (25) in dogs, and Swislocki (26, 27) in fasting hypophysectomized rats. This transient hypoglycemia and hypolipemia occurs between 15 to 45 minutes after growth hormone administration and never persists for more than 1 hour. Objections have been raised that this effect of growth hormone could be due to the increase in secretion of insulin induced by growth hormone or that the growth hormone preparations may be contaminated with insulin-like peptides. However, Swislocki (26) suggested that this effect could not be due to the increase of insulin secretion because the transient decrease could still be detected in acute pancreatectomized dogs injected with growth hormone (28,29). Also, Beck et al. (24) could not detect any change in serum insulin level in fasting humans in conjunction with the transient decrease of NEFA and glucose after the administration of growth hormone. The other possibility could be that the growth hormone preparations used were contaminated with insulin-like peptides (30). Swislocki (27) used two preparations of BGH from different laboratories and found no difference in the transient decrease. However, the possibility of contamination with insulin-like peptides cannot be ruled out because the doses of growth hormone used in these studies were rather high (from 3 mg to 8 mg).

In summary, growth hormones have been reported to increase NEFA release into plasma of normal and hypophysectomized animals after a lag period of 1 to 2 hours. This effect of growth hormone can be modified by nutritional states. The basis for the acute hypolipemic and hypoglycemic effects of growth hormones detectable within 20 minutes after administration awaits further clarification.

B. In Vitro Effects of Growth Hormone:

White and Engel (31) and Gordon and Cherkes (32) in 1958 developed an *in vitro* technique to examine the effects of these hormones on NEFA release. Their method involves the incubation of adipose tissue fragments from rats in a medium of known composition and measurement of the NEFA released into the medium and accumulated in the tissue under the influence of lipolytic hormones. NEFA was determined by the method developed by Dole (33). Since then, a variety of hormones have been reported to exhibit lipolytic activities *in vitro*.

1 - Species Specificity

Numerous investigations and studies have been accumulated on the species specificity with respoect to animal variation (11,34) and hormone variations (11,12,35).

The original works of Rudman *et al.* (34) using the *in vitro* techniques developed by White and Engel (31) and Gordon and Cherkes (32) illustrated the specificity of lipolytic responses of a variety of hormones in different experimental species. Using adipose tissue slices from the rat, guinea

pig, hamster, rabbit, pig and dog, they found that lipolytic hormones possess different activities in different species. For example, ACTH was found to be very active only in rabbit and rat (minimum effective dose was 0.1 µg per ml) but not in the other species; α and β MSH extremely active in the rabbit only (minimum effective dose was 0.01 µg per ml and 0.1 µg per ml respectively) and b-TSH active in the rat BGH, prolactin, b-FSH and b-LH were reported to be inactive in the rat (up to 10 µg per ml) and rabbit (up to 100 µg per ml). It can be concluded from Rudman's observations that different pituitary hormones display tremendous species specificity in activating lipolysis in adipose tissue slices.

In 1964, Rodbell (36) succeeded in isolating preparations of hormonal-sensitive fat cells from rat adipose tissue by the digestion of the parent tissue with collagenase. These isolated fat cells were found to be at least 40 times more sensitive to the stimulation by lipolytic hormones than the parent tissue. Under the influence of these hormones, the fat cells released both free fatty acids and glycerol from triglyceride due to the activation of a hormonal sensitive lipase (37,38). Unlike free fatty acids which can be re-esterified by adipose tissue, glycerol is not utilized to a significant extent (39-42) owing to an apparent lack of glycerokinase in this tissue (43.44). Thus, glycerol release has been employed by most investigators as a measure of lipolysis.

With this more sensitive system, the lipolytic response of the pituitary hormones was re-examined. The results are summarized in Table I.

As indicated above, ACTH was found to be active in both rat and rabbit. αMSH, on the other hand, was about 20 times as active as ACTH in the rabbit, but was inactive even at a concentration 100 times that of ACTH in the rat. Among the glycoprotein hormones listed above, b-TSH, h-TSH and b-LH have higher activity in the rat than the others. None of these glycoprotein hormones showed any activity in the rabbit at concentrations up to 3×10^{-6} M. Prolactin was found to be inactive in either species (48).

Investigations with human fat cells were also carried out by Burn and Langley (128). Human adipose tissue seems to be even more selective, responding only to catecholamines and TSH, but not responding to ACTH, glucagon, or growth hormone plus dexamethasone.

From these studies, it can be concluded that pituitary hormones have tremendous variations in their lipolytic response in different experimental species.

In addition to the variation of response of adipose tissue from different species to different pituitary hormones, it was found that the lipolytic effect of adipose tissue from a single species also varied to the same hormone isolated from different species. The first observation of this variation was reported by Knobil and Greep (12) and Raben (35). Injection of porcine and bovine growth hormone into fasting monkeys has no effect on the release of NEFA, while simian and human growth hormone was effective. Subsequently, Li et al. (11),using adipose tissue slices from rabbit, observed that human growth hormone

is far more active than bovine growth hormone. Recently, we have observed that b-TSH is more effective (see Table above) than h-TSH on isolated fat cells from rats.

Therefore, it seems that species specificity plays an important role in the stimulation of lipolysis by lipolytic hormones. These observations were found to be extremely useful in documenting the lipolytic activity of HGH in the rabbit fat cells and the elucidation of the contaminant which is responsible for the observed lipolytic effect of many BGH preparations in the rat fat cells.

2 - Fast-Acting Lipolytic Hormones

The mechanism by which the above mentioned lipolytic hormones stimulate lipolysis has been well documented (49-51). The effect is rapid in onset and short in duration (51); glycerol or free fatty acid release can be detected within 15 minutes; maximum accumulation occurs at 2 hours (in the rat); requires Ca^{2+} ions; unaffected by inhibitors of RNA or protein synthesis. These fast-acting lipolytic hormones increase the cyclic AMP content of adipose tissue at the same time that they stimulate lipolysis (50,52,53). They also activate adenylate cyclase, the enzyme that catalyzes cyclic AMP formation, in subcellular preparations of fat cells (54). The action of these hormones on lipolysis is greatly enhanced by methyl xanthines (theophylline and caffeine) (53) presumably by decreasing the activity of cyclic nucleotide phosphodiesterase (50,52), the enzyme that degrades cyclic AMP (55). The fact that many different lipolytic hormones, including ACTH, TSH,

glucagon and MSH, can increase cyclic AMP in adipose tissue suggests that there may be several hormonal-receptors for activating adenylate cyclase activity (53,56,57).

In view of the observed *in vivo* effects of growth hormone on the release of NEFA, the *in vitro* effects of growth hormone on fat metabolism from several species has been investigated by many workers (11,17,18,31,58-65). Early studies by Buckle *et al.* (17), Jungas and Ball (18,61), Raben and Hollenberg (59), White and Engel (31), Winegrad *et al.* (60), and Knobil (58) incubating adipose tissue slices from normal or hypophysectomized rats with high doses of BGH demonstrated a fast-acting lipolytic effect of growth hormone. However, in view of the high dosage of BGH used (10^{-6}-10^{-5}M) and the lack of specificity of the rat adipose tissue (see above), these workers concluded that the effect was probably due to contamination with other pituitary hormones. The elegant sudies by Jungas and Ball (61) demonstrated the possibility of TSH contamination contributing to the observed BGH effect in increasing FFA release and oxygen consumption. Their suggestion was based on the following observations:

a. Less purified preparations of BGH exhibit greater lipolytic activity.

b. When crude pituitary fractions from different stages of purification were tested, the activity followed those fractions in which TSH was concentrated. Purified TSH as reported by Jungas and Ball possessed activity at a concentration as low as 3×10^{-9}M.

c. The possibility of contamination with other pituitary hormones was also examined. LH was found to possess low activity. ACTH, on the other hand, was highly active. However, when compared with standared ACTH, the degree of contamination of the BGH preparation by ACTH could not account for the observed activity in the BGH preparation.

Based on this experimental evidence, Jungas and Ball concluded that the in vitro lipolytic effect on BGH was probably due to TSH contamination.

Humid et al. (62) and Swislocki (63-65), using large doses of BGH, also reported an increase in lipolysis and oxygen consumption due to this hormone. However, they did not credit the effect to TSH contamination, but rather to a minor component of BGH. This theory derives from the observation that when BGH was subjected to disc electrophoresis at alkaline pH, several components could be detected (64). The heterogeneous behavior of BGH on electrophoresis was suggested to be due to the differences in the amide content of the native hormone (66).

Recently, Swislocki and Sonenberg (64) reported the isolation of an apparently homogeneous preparation of BGH which stimulated lipolysis when incubated with isolated fat pads derived from hypophysectomized rats. This preparation of BGH was obtained by chromatography on DEAE cellulose eluting with increasing concentration of NH_4HCO_3 buffer at pH 8.4. The criteria used for homogeneity was that it gave only one component on disc electrophoresis at pH 9.5 as opposed to the

usual three components observed for BGH under similar conditions. We have repeated the same chromatographic procedure and also found that this one component BGH has lipolytic activity in isolated fat cells from normal rats. However, the concentration used was still very high (5-20 µg/ml) and the possibility of contamination with other pituitary hormones could not be ruled out. The studies presented in the first chapter of this thesis were designed to examine whether BGH has any intrinsic lipolytic activity in the rat fat cells.

3 - Slow-Acting Lipolytic Hormones

In 1965, Fain and Kovacev (67) using physiological concentrations of growth hormone (0.01 to 0.1 µg/ml) in the presence of glucocorticoid (0.016 µg/ml) observed a slow-acting lipolytic effect of growth hormone in adipose tissue slices and isolated fat cells from fasted rats. This slow lipolytic action of growth hormone was judged not to be due to contamination with other pituitary hormones for the following reasons:

a. The amount of growth hormone required was low (in the range of 0.01 to 0.1 µg/ml) and therefore could not be attributed to TSH or ACTH contamination (67).

b. The kinetics of stimulation was very different from the fast-acting lipolytic hormone; lipolysis could only be detected after a lag period of 1 to 2 hours and reached maximum stimulation after a 4 hour incubation. This is somewhat similar to the <u>in vivo</u> situation.

c. This lipolytic action of growth hormone plus glucocorticoid is blocked by inhibitors of RNA and protein synthesis such as dactinomycin, puromycin, cycloheximide,

ultraviolet light and x-radiation (67-69). These inhibitors do not block the action of the fast-acting hormones.

d. Ca^{2+} and K^{+} were not required for the activation of lipolysis by growth hormone plus dexamethasone (70) but these ions are required for the action of the fast-acting pituitary hormones.

However, Fain did find some similarities between the action of growth hormone plus dexamethasone and the better established fast-acting lipolytic hormones. Theophylline potentiated the effect of growth hormone plus dexamethasone, suggesting that cyclic AMP might, in some way, be involved in the action of growth hormone (71). Incubation of isolated fat cells with growth hormone plus dexamethasone for 4 hours resulted in a 5 to 10 fold increase in the sensitivity of the lipolytic response to norepinephrine or theophylline without affecting the maximum response, and the lipolytic action of dibutyryl cyclic AMP was mildly depressed (71). The ability of growth hormone plus dexamethasone to increase the sensitivity of the fat cells to theophylline was prevented by the inclusion of cycloheximide in the incubation (72). Fain suggested that growth hormone plus dexamethasone probably affects the synthesis of some protein which can influence the formation of cyclic AMP rather than its action (70,72).

The findings of a delayed activation of lipolysis by growth hormone plus dexamethasone in the presence of theophylline have also been confirmed by Goodman using adipose tissue slices from hypophysectomized rats (73,74). However, Goodman did not

find this to be a cycloheximide sensitive process. The difference in Fain and Goodman's results could not be accounted for by the use of normal and hypophysectomized rats, since both Goodman and Fain found that the response of fat cells from hypophysectomized rats is essentially similar to normal rats except that the maximum response is greatly reduced (70,75). The exact explanation for the discrepancy is not known.

From the findings described by Fain and other workers and our own findings to be reported in Chapter III, it can be concluded that BGH probably does not have any "fast-acting" lipolytic effect in the rat. However, the possibility that some species of growth hormone can act as a fast-acting lipolytic agent in species other than the rat cannot be excluded.

Li et al. (11) in 1964 reported that HGH was extremely active in increasing the release of FFA in the rabbit, both in vivo and in vitro as compared to the rat. Rabbit adipose tissue also has been reported by Rudman et al. (34) and others to be more specific than rat adipose tissue since it is only responsive to small peptides such as ACTH and MSH, but not to glycoprotein hormones. When the lipolytic activity of HGH was examined in isolated fat cells from both rat and rabbit, we found that HGH was inactive in the rat even at 9×10^{-6} M and highly active in the rabbit (half maximum stimulation at about 2×10^{-7} M). Evidence that this fast-acting lipolytic effect of HGH in the rabbit is an intrinsic property and not due to contamination with other pituitary hormones is presented in Chapter IV.

4 - Effects of Enzyme Perturbations on Rat Fat Cell Membranes

In view of the fact that HGH is only active in the rabbit fat cells and not in the rat fat cells and also the high selectivity displayed by rabbit fat cells to lipolytic hormones, it was of interest to investigate the properties of the cellular receptors on the rabbit fat cell membrane for HGH. Such studies have been conducted extensively in the rat fat cell system by the perturbation of membrane receptors with specific enzymes (76-84). Macromolecular components of hormone receptors have been assumed to consist of proteins, lipoproteins and glycoproteins, but the exact distribution of these components within the cellular receptors is not known. However, information concerning those structural requirements which are essential for certain biological activities may be obtained by perturbation with enzymes specific for particular components. For example, membrane proteins are known to be digestable by protease (76-79); membrane phospholipids by phospholipases (80-82) and membrane glycoproteins by neuraminidases (83). Early studies by Kono (76,77), Fain (78), Kuo (84) and Cuatrecasas (79) indicated that pretreatment of isolated rat fat cells with trypsin completely abolished the antilipolytic effects of insulin and the lipolytic effect of glucagon without affecting that of ACTH, epinephrine and growth hormone plus dexamethasone. Catrecasas (82) also reported a perturbation of rat fat cells with phospholipase that led to the abolishment of subsequent lipolytic effects of low concentrations of ACTH, glucagon, theophylline, and also the

antilipolytic effect of insulin. Neuraminidase pretreatment on rat fat cells also led to a decrease in the subsequent lipolytic activities of epinephrine, ACTH, glucagon, theophylline and growth hormone plus dexamethasone (83). Chapter V describes the application of enzyme digestion methods to rabbit fat cells and examines the consequence of these perturbations on the lipolytic effect of HGH. The differences between the effect of these enzymes on rat and rabbit fat cells might indicate the reason why HGH is only active in the rabbit fat cells and not the rat fat cells.

CHAPTER II

MATERIALS AND GENERAL METHODS

A. Hormone Preparations

BGH was prepared in this laboratory by the procedure of Li (85). HGH was isolated according to the procedure of Li et al. (86,87). Highly purified ovine ACTH (88), synthetic gln^5-αMSH (89), ovine LH (90), HCS (91), ovine prolactin (92,93), performic acid oxidized HGH (94) and human TSH (95) were all prepared in this laboratory. Bovine TSH was a gift from Dr. J. G. Pierce.

B. Antisera

Antiserum against b-TSH was a gift from Dr. J.G. Pierce; antiserum against O-LH α a gift from Dr. J. Solis-Walkermann. Rabbit antisera to both HCS and HGH were obtained from Dr. A. Vinik, which were further purified by a 40% $(NH_4)_2SO_4$ precipitation twice followed by dialysis against 0.01M phosphate buffer pH 8.0 and finally chromatography on DEAE cellulose equilibrated with the same buffer.

C. Material

Collagenase (CLS grade), lima bean trypsin inhibitor, crystalline trypsin and clostridium perfringens neuraminidase (specific activity 1.13 μ/mg) were obtained from Washington. Clostridium perfringens neuraminidases (specific activity 2.4 μ/mg, 0.3 μ/mg) and cl. Welchii phospholipase C (specific activity 10 μ/mg) were obtained from Sigma, theophylline, creatine phosphate, creatine kinase, ATP (disodium salt),

cyclic AMP from Mann Research Laboratories; basic and neutral alumina (Merck, activity 1, for column chromatography) from Brinkman Instruments, Inc., Westburg, N.Y.; Sepharose 4B and QAE-Sephadex A25 from Pharmacia; ^{3}H cyclic AMP ([8-^{3}H] - cyclic AMP (20.8 Ci/mmole)) ATP-$\alpha^{32}P$ and α-^{3}H adenine from Schwarz Mann, International Chemical and Nuclear Corporation, respectively. Bovine serum albumin (Fraction V Armour) was purified as described earlier (48).

D. Preparation of Fat Cells from Rat and Rabbit

White fat cells were isolated from the perirenal fat pads of male New Zealand White rabbits (2 kg) or the epididymal fat pads of Sprague Dawley rats (160-180g) according to the procedure of Rodbell (36). Fat cells from the pooled adipose tissue of two rabbits or six rats were isolated by digestion with collagenase (2 mg/ml for rabbit fat tissue and 3 mg/ml for rat fat tissue) in Krebs Ringer bicarbonate buffer pH 7.4 containing 4% (w/v) bovine serum albumin, 0.1 mg/ml lima bean trypsin inhibitor. Fat cells were incubated at 37° in plastic tubes (17 x 100 mm) containing a total volume of 1 ml for one hour in the case of rabbit fat cells and two hours in the case of rat fat cells unless otherwise stated. Hormone was usually added in aliquots of 0.1 ml. Preparation of fat cells from fasted rabbits was essentially the same except the rabbits were fasted for 48 hours before sacrifice. The procedure for digesting fat cells from fasted rabbits with the different enzymes was also the same as described for cells from fed rabbits. Tables and figures shown are for fat cells from fed rabbits unless otherwise stated.

E. Glycerol Analysis

Incubation was terminated by immersing the incubation tubes in ice. After removal of the fat cells by aspiration, 0.25 ml aliquots of medium were assayed directly for glycerol release by the enzymatic procedure of Vaughan (96). The amount of cells present in each tube was determined by measuring the dry weight of the cells. This is obtained by passing 0.9 ml aliquots of cells through millipore filter (25 mm, 0.8 μ) and dried overnight in a desicator. The difference of the dried millipore filters with cells and without cells represented the dry weight of the cells. Glycerol was expressed as μ moles glycerol per gm dry weight of cells per hour (or 2 hours or otherwise stated).

F. Cyclic AMP Determination in Fat Cells and Medium

Cyclic AMP was determined by the method of Gilman (98) with a few modifications. The cyclic AMP binding protein and the inhibitor of the protein kinase was obtained from rabbit hind leg muscles by the procedure of Walsh et al. (99), followed by chromatography on DEAE cellulose according to Gilman (98). The binding protein has a Km of 1-2 nM in the presence of the inhibitor. For the measurement of cyclic AMP accumulated in the medium and the fat cells, incubation was terminated by transferring an aliquot of the medium alone or the entire contents of the incubation tubes (cell + medium) into 2 mls of ice cold 10 mM Tris-Hcl buffer pH 7.4 containing 5mM theophylline in glass tubes. The glass tubes were transferred immediately to a boiling water bath for 5 minutes. The samples were then

chilled and passed through a 3 cm column of basic alumina contained in a Pasteur pipet. The use of neutral and basic alumina for the separation of cyclic AMP from ATP, ADP, AMP and Pi has been described previously (100). Recovery of cyclic AMP was 60-70%. Aliquots of the eluate were lyophilized and then reconstituted with 0.1M sodium acetate buffer pH 4.0. Final steps for cyclic AMP determination were essentially the same as that of Gilman (98). Briefly, 50 µl sample aliquots were incubated in a total volume of 0.1 ml containing 50 mM sodium acetate buffer pH 4.0, 1.7 pmoles of ^{3}H cyclic AMP and sufficient kinase and inhibitor to bind 0.25 pmole cyclic AMP. Cyclic AMP was expressed as µ moles cyclic AMP per gm dry weight of cells per 20 min.

G. Measurement of Adenylate Cyclase Activity in Isolated Fat Cells

Perirenal fat pads from New Zealand White rabbits was digested as described above except that 15 µ Ci ^{3}H adenine (1 9.6 Ci per mmole) was present. At the end of the digestion period, fat cells were isolated and washed once and then incubated for another 30 min. in 10 ml of medium containing 15 µ c of ^{3}H adenine. At the end of the second incubation period, the cells were washed 4 times with 5 volumes of Krebs Ringer bicarbonate buffer containing 4% (w/v) bovine serum albumin and 0.1 mg per ml lima bean trypsin inhibitor, resuspended in the same buffer and distributed into plastic tubes for final incubation. At the end of the incubation, the entire content in each tube was transferred into glass tubes containing 1 ml of ice cold 10 mM cyclic AMP pH 7.4. The glass tubes were

then immediately immersed in a boiling water bath for 5 min. The samples were then chilled and 1.5 ml aliquots of the medium were passed through a 3 cm column of neutral alumina contained in a pasteur pipet. Recovery of cyclic AMP was 80-90%. 0.75 ml aliquots were added to equal volume of 10% TCA to remove precipitable proteins. After centrifugation, the supernatant was extracted 5 times with 3 volumes of ether to remove the TCA. Finally, 0.9 ml aliquots were lyophilized and then reconstituted with 20 λ of water. The reconstituted material was then spotted on tlc silica gel plates and developed in a solvent system of n-butanol: glacial acetic acid: water (4:1:1). After the plates were dried, the spots were detected by UV light, scraped onto tubes containing 1 ml of 20 mM Tris Hcl pH 7.4 for the final extraction of cyclic AMP. After centrifugation to remove the silica gel, the supernatant was counted with 15 ml Bray's scintillant in a Packhard liquid scintillation counter, Model 3320.

H. Measurement of Adenyl Cyclase Activity in Rabbit Fat Cell Ghosts

Fat cell ghosts were prepared from isolated rabbit fat cells according to the procedure of Birnbaumer and Rodbell (101). Reaction was initiated by the addition of 10 µl of adenyl cyclase preparation (40-60 µg protein) in 0.2 mM $KHCO_3$ to 5 µl hormone and 40 µl incubation mixture containing 3.2mM ATP-α^{32}P, 5 mM $Mgcl_2$, 10 mM theophylline, 0.1% (w/v) albumin, 30 mM Tris HCl buffer pH 7.4, 0.2 mg/ml of creatine kinase (40-50 units per mg), 10 mM creatine phosphate and maintained for 15 min. at 37°. Analysis of ^{32}P cyclic AMP formed from ATP-α^{32}P has

been described previously (110,102). Protein was determined by the method of Lowry et al. (103) using bovine serum albumin as standard. All incubations were performed in triplicate. Each experiment was repeated at least once, and in some cases as many as six times.

I. Neutralization of Lipolytic Activity with Antiserum

Neutralization experiments were performed by incubating the appropriate antiserum (sufficient to neutralize all the antigen as determined by precipitation curve) with the hormone preparations at 37° for 1 hour and in the cold for 16 hours to allow maximum cross reaction. Fat cells were added to antiserum-hormone mixture directly for final incubation.

J. Coupling of Antiserum to O-LH to Sepharose

Antiserum to O-LHα (1 ml) was coupled to 10 ml cyanogen bromide activated sepharose 4B according to the procedure of Cuatrecasas (104). The antibodies coupled sepharose were poured into small (0.9 x 10 cm) column and washed extensively with phosphate-saline buffer (0.01M phosphate, 0.15M saline, pH 7.8) and quanidine HCl (3M pH 3.1) alternately three times and finally with phosphate-saline buffer until no absorbance at 280 nm in the eluent was detectable. The bound material was eluted with 8M urea. The bound and unbound fractions were dialyzed immediately against several changes of distilled water and then concentrated on a Diaflo using a PM-10 membrane.

All other chromatographic procedures are described under each figure legend.

K. Digestion of Fat Cells with Enzymes

The specific procedures used for washing and digestion of cells with trypsin, phospholipase C and neuraminidase are described in the appropriate figure legends. Generally, in experiments designed to study the effect of trypsin, the cells after collagenase treatment were washed 3 times with 10 volumes of Krebs Ringer bicarbonate buffer containing 4% (w/v) albumin and free of lima bean trypsin inhibitor. 0.8 ml of cell suspension were dispensed into plastic tubes (17 x 100 mm) and incubated with trypsin (0.05 ml in Krebs Ringer bicarbonate-albumin buffer made up just before addition) with constant shaking at 37°. Tryptic digestion was terminated by the addition of lima bean trypsin inhibitor equal to twice the amount of trypsin by weight in 0.05 ml of Krebs Ringer bicarbonate-albumin buffer. For final incubation, 0.1 ml of water or hormone was added and incubated for 1 hour at 37° with constant shaking. In the control tubes, trypsin and lima bean trypsin inhibitor were added in the reverse order.

Digestion with phospholipase C or neuraminidase was performed at 37° in Krebs-Ringer bicarbonate buffer containing 4% (w/v) albumin and 0.1 mg per ml lima bean trypsin inhibitor. The digestions were terminated by washing the cells twice with 20 volumes of fresh buffer before dispensing for final incubation with hormone.

L. Miscellaneous Methods

Sialic acid released into the medium after neuraminidase or trypsin treatment was determined on 0.2 ml aliquots by the

thiobarbituric acid procedure of Wassens (105) using sialic acid as standard.

Melanocyte stimulating activity was measured using isolated frog skin according to the method of Shizume et al. (106) and Wright and Lerner (107).

ACTH activity was measured in hypophysectomized rats by the adrenal corticosterone concentration procedure of Vernikos-Danellis (108).

Free fatty acid released into the medium was determined on 0.25 ml aliquots of medium by the procedure of Duncombe (109).

The growth promoting activity of BGH preparations was investigated by the tibia assay (110,111).

CHAPTER III

PURIFICATION OF BOVINE GROWTH HORMONE: REMOVAL AND IDENTIFICATION OF LIPOLYTIC FACTOR

The evidence supporting the fact that the fast-lipolytic activity of BGH is probably due to contamination has been presented in Chapter I. Chapter III describes the identification and methods of separating this contaminant from BGH.

Results

A. Neutralization of BGH Stimulation on Lipolysis by Antiserum to O-LHα

Since BGH was found to be devoid of lipolysis in the rabbit fat cells even at 100 µg/ml, it can be deduced that the lipolytic activity observed in the rat fat cells could not be due to contaminant with small peptides such as ACTH or MSH (both are extremely active in rabbit fat cells) (Table 1). The lipolytic activity of BGH was compared with the activities of O-LH and b-TSH (Table 2). It is obvious that as small as 1% contamination with either glycoprotein hormone can account for the observed activities of BGH. That this is indeed the case is shown by the effect of the antiserum to O-LH-α. Antiserum to O-LHα was used because it has equal ability to cross-react immunologically with both LH and TSH from ovine and bovine glands (97). Therefore, if the increase in lipolysis with BGH was due to TSH or LH contamination, this increase should be abolished; conversely, if the stimulation was an intrinsic property of BGH, the lipolytic activity should

be retained. As shown in Table 2, in the absence of antiserum to O-LHα and in the presence of normal rabbit serum, BGH, b-TSH, O—LH and ACTH showed high lipolytic activities. In the presence of antiserum to O-LHα, the stimulation due to BGH, b-TSH and O-TSH was completely abolished, while that of ACTH was retained. This experiment suggests that the lipolytic activity of BGH is probably due to a contaminant of TSH or LH in nature, since it is removable by A/S O-LHα. ACTH was included as a control to demonstrate the specificity of the antiserum.

B. Preparation of BGH Devoid of Lipolytic Activity by Affinity Chromatography on Antiserum to LHα Coupled Sepharose

A preparation of BGH devoid of lipolytic activity was prepared by passing BGH (1 mg) through an antiserum to LHα ∿ sepharose column. Two fractions of protein were isolated; one which came through unadsorbed and a bound fraction. When the lipolytic activities of the fractions were examined as shown in Table 3, the unadsorbed material which was the major fraction (∿70%) did not stimulate lipolysis even at 100 μg/ml, whereas the bound material stimulated lipolysis ten-fold over basal at a concentration of 0.25 μg/ml. BGH (urea treated) was included as a control to show that urea treatment did not alter the activity of the bound fraction.

The growth promoting activity of the unadsorbed material was examined in the tibia assay and was found to be comparable to native BGH.

Experiments were also performed as described by Fain et al. (67) to examine whether unadsorbed BGH obtained from

antiserum to O-LHα column could stimulate lipolysis in the presence of dexamethasone. The results are shown in Table 4. BGH (0.1 µg/ml) produced a significant stimulation after 4 hours of incubation with isolated rat fat cells and was found to be higher than either hormone acting alone.

C. Isolation of BGH Devoid of Lipolytic Activity on a Preparative Scale

BGH, devoid of lipolytic activity, can be prepared by means of affinity chromatography using antiserum to O-LH α∿ sepharose as described above. However, there are limitations to this method. Availability of antiserum prevented batchwise production of pure hormone. Moreover, the sepharose coupled antibodies, in theory, could be used over and over again. In practice, the antibodies coupled sepharose column can only be used 3 or 4 times before the capacity of the column starts decreasing. Finally, the entire procedure is too lengthy and depended upon too many variables, such as the potency of the antiserum and the conditions for coupling. Various attempts were made to adopt chromatographic conditions developed by Pierce et al. (112,113) and Hennen et al. (114) for isolation of b-TSH to the purification of BGH.

1. Chromatography of BGH on DEAE Cellulose

Attempts were made to try and dissociate the lipolytic activity from BGH by chromatography on DEAE cellulose under different experimental conditions:

a. BGH (25 mg) was chromatographed on a DEAE cellulose column (0.9 x 30 cm) equilibrated with 1mM glycine buffer pH 9.5 according to the procedure of Pierce et al. (112). BGH was eluted with a stepwise gradient of glycine buffer

pH 9.5. The protein did not come out as a distinct peak; instead, it emerged as a very broad, indistinct peak.

b. BGH (50 mg) was chromatographed on a DEAE cellulose column (2 x 20 cm) equilibrated with 6.6 mM Na_2CO_3pH 8.5. BGH was eluted with a linear gradient of NaCl (0-0.5M). BGH came out as a distinct peak at 0.25 M NaCl. However, this material after dialysis against distilled water and lyophilization still possessed lipolytic activity comparable to the starting material.

c. BGH (20 mg) was chromatographed on a DEAE column (0.9 x 30 cm) equilibrated and eluted with 0.01 M tris HCl pH 8.4 in the presence of 8M urea. Two distinct peaks of protein emerged. After extensive dialysis against distilled water and lyophilization, both peaks were found to be equally potent in stimulating lipolysis and were also comparable to the starting material.

2. Treatment of BGH with 1M Propionic Acid and Chromatography on Sephadex G100

Liao and Pierce in 1970 (113) developed a method for dissociating the subunits of b-TSH by means of propionic acid treatment. b-TSH at 1 mg/ml in 1M propionic acid was allowed to stand at room temperature for 16 hours. The mixture was then lyophilized and reconstituted in a small volume (1 to 2 ml) of 1M propionic acid. This material was chromatographed on a Sephadex G100 column equilibrated with the same buffer. Undissociated TSH came out first as a small peak followed by the α subunit then the β subunit. b-TSH having a molecular weight

of ∿ 32,000 under these conditions was dissociated into α and β subunits with molecular weight of approximately 16,000 each. Attempts were made to try and adopt this procedure for the removal of the contaminant from BGH. The rationale behind adopting this procedure was that if BGH was contaminated with b-TSH, under these conditions, b-TSH should be dissociated into subunits of smaller molecular weight and could be separated from BGH by chromatography on sephadex G100 column with 1M propionic acid. After treatment of BGH with 1M propionic acid and chromatography on Sephadex G100 column eluted with the same solvent, BGH emerged first as a polymer peak (Ve/Vo=1) and a dimer peak (Ve/Vo=1.65); no other peaks were detectable. The polymer peak was found to be inactive in stimulating lipolysis but the dimer peak was found to be active. Since the dimer peak corresponds to the biologically active BGH (115), this method was not pursued further.

Other chromatographic methods (114) which have been tried without success are as follows: Chromatography on CM-Sephadex C25 equilibrated with 0.01 M sodium phosphate buffer pH 6.2 and eluted with a continuous salt gradient (0.05 M NaCl) and chromatography on DEAE A25 equilibrated with 0.005 M sodium phosphate buffer pH 6.2 and eluted with a continuous salt gradient (0-0.5 M NaCl) and chromatography on DEAE Sephadex A25 equilibrated with 0.005 M glycine buffer pH 9.5 and eluted with a continuous salt gradient (0-0.5 M NaCl) were unsuccessful in dissociating the contaminant from BGH.

D. Preparation of BGH Devoid of Lipolytic Activity by Chromatography on QAE-Sephadex A25

A simple one-step chromatographic method was finally found to be successful in dissociating the contaminant from BGH. This is another method developed by Hennen (114) for the purification of b-TSH. BGH (50 mg) dissolved in 3 ml of 0.01 M tris HCl pH 8.4, was chromatographed on a column of QAE-Sephadex A25 (1.3 x 60 cm) equilibrated with the same buffer. Elution of the hormone was carried out with a linear gradient of NaCl (0 to 0.5 M). As illustrated in Fig. 1, BGH emerged as a sharp peak (Fraction 1) at a salt concentration of 0.22 M NaCl. A small second peak (Fraction 2) was found at a salt concentration of 0.32 M NaCl. The ratio of fraction 1 to fraction 2 varied for different batches of BGH (Table 5). This may be due to different degrees of contamination in the different batches of BGH. The material in fractions 1 and 2 were pooled separately, dialyzed extensively against distilled water and lyophilized. The lipolytic activity was determined. As indicated in Table 6, fraction 1 at 200 µg/ml has virtually no lipolytic activity, while fraction 2 at 5 µg/ml was able to stimulate lipolysis 25-fold over basal.

The identity of the lipolytic agent in fraction 2 was examined with the aid of A/S b-TSH which specifically neutralized b-TSH but not O-LH (112). The results given in Table 7 show that A/S b-TSH was able to neutralize the lipolytic activity of the starting material BGH, fraction 2 from chromatography on QAE-Sephadex A25, as well as b-TSH itself. ACTH was included as a control to show the specificity of the antiserum.

Fraction 1 was re-chromatographed on sephadex G100 eluted with 0.1 M Tris HCl pH 8.4 and found to have a Ve/Vo of 1.67 (Fig. 2). This agrees well with previous published data from this laboratory for dimeric BGH (116). The lipolytic activity was also tested, as illustrated in Table 6, and found to have no effect at 100 µg/ml.

The growth promoting activity of fraction 1 was also determined in the tibia assay and found to be comparable to the starting material (Table 8).

Discussion

The fact that the lipolytic activity of BGH can be removed by chromatography on QAE Sephadex A25, affinity chromatography with A/S O-LHα and neutralized by A/S b-TSH and A/S O-LHα, indicated that this rapid lipolytic effect was due to contamination with b-TSH. Our studies confirm that of Jungas and Ball (61) and others who first suggested the possibility of TSH contamination in the in vitro lipolytic effects of BGH. Our studies also suggest that the in vitro effects observed by Swislocki et al. (63-65), and Humid et al. (62) was not due to a minor component of BGH but to b-TSH contamination.

Recently, Sonenberg et al. (129) reported that limited digestions of BGH with trypsin yielded 2 fragments of molecular weight 16,000 and 5,000. Both of these fragments (at 250 µg/ml) were found to possess in vitro metabolic activities including lipolytic activity in fat pads isolated from hypophysectomized rats. Bornstein et al. (130) also prepared a

fragment of BGH by means of acid hydrolysis at pH 2.5 which was reported to stimulate lipolysis in adipose tissue from normal rats. In view of the fact that native BGH has no fast-acting lipolytic effect in the rat, it is very unlikely that these fragments obtained from the native hormone could possess this activity.

Fain et al. (67) reported a slow lipolytic effect of growth hormone. This effect could not be ascribed to b-TSH contamination, since low concentrations of growth hormone were used, and also the characteristics of this stimulation are very different from those reported for TSH (67). We have been able to confirm these findings using a preparation of BGH free of TSH contamination (Table 4). This b-TSH is probably associated rather firmly with BGH, since it is difficult to remove. Of all the chromatographic procedures developed by Pierce et al. (112,113) and Hennen et al. (114), chromatography on QAE Sephadex A25 was the only method successful in removing b-TSH from BGH.

CHAPTER IV

LIPOLYTIC ACTION OF HUMAN GROWTH HORMONE ON RABBIT FAT CELLS: AN INTRINSIC PROPERTY OF THE HORMONE

As described earlier, growth hormone has been known to stimulate lipolysis in the isolated fat cells by a slow-acting mechanism which is quite different from that of other fast-acting pituitary hormones such as ACTH, TSH and the gonadotropins (67,36,46). Li et al. (11) found that among isolated fat pads of rat, guinea pig and rabbit, the rabbit fat tissue was the most responsive to HGH. Preliminary investigations using the more sensitive fat cells from rabbit and rat indicated that HGH was active as a fast-acting lipolytic agent in the rabbit but not in the rat fat cells. However, the concentration of HGH required for stimulation of lipolysis was relatively high compared to the other lipolytic hormones of the pituitary. The present study was designed to examine whether this lipolytic action is an intrinsic property.

Results

A. Preparation of HGH-CMC-G100, HGH-HOAC-G100 and HGH-red-reox

HGH was isolated by previously published procedures (86, 87) and further purified by applying on a column of carboxylmethyl cellulose equilibrated with 0.01 M NH_4Ac (pH 6.8) and eluting with the same buffer. The hormone which came through

unadsorbed was dialyzed extensively against 0.1 M tris-HCl (pH 8.4), concentrated in a Diaflo cell using a PM-10 membrane (Amicon) and chromatographed on Sephadex G100 equilibrated with the same buffer as previously described (48). The main fraction which emerged as a monomer (Ve/Vo, 1.97) was dialyzed extensively and lyophilized. This preparation of HGH was designated HGH-CMC-G100 and shall be used throughout all the studies with HGH unless otherwise indicated. HGH-HOAc-G100 was prepared by treatment with 50% acetic acid for 6 hours at room temperature. After removal of the acetic acid by dialysis against water, then against 0.1 M Tris HCl pH 8.4, the hormone solution was concentrated on a Diaflo apparatus using a PM 10 membrane. Monomeric HGH (Ve/Vo 1.97) was isolated by chromatography on Sephadex G-100 in 0.1 M Tris HCl pH 8.4.

HGH-red-reox was obtained by reduction of the disulphide bonds in the HGH molecule followed by reoxidation, as described previously (117).

B. Comparison of Lipolytic Activities of HGH, ACTH, Prolactin in Rat and Rabbit Fat Cells

The effects of HGH on the stimulation of glycerol release in rat and rabbit fat cells are compared in Table 9. It is evident that HGH is inactive in rat fat cells at a concentration of 4.5×10^{-6}M but highly active in rabbit fat cells at this concentration. For comparison, the effects of ACTH and prolactin are included. ACTH is active in both species, but prolactin is inactive.

C. Comparison of Lipolytic Activity and Melanocyte Stimulating Activity of HGH Preparations

The lipolytic activity of most pituitary hormones in rat and rabbit fat cells have been compared (46,48). Farmer et al. (46) found that both b-TSH and O-LH were highly active in the rat at a concentration of 2.2×10^{-7} M but inactive even at a concentration of 3.33×10^{-6} M in the rabbit fat cells. We have shown above that O-prolactin was inactive while ACTH was extremely active in both species. HGH and αMSH were the only two known pituitary hormones that were both inactive in the rat fat cells but active in rabbit fat cells (Table 10).

To show that the lipolytic activity of HGH is not due to contamination with αMSH, the melanocyte stimulating activities of gln^{-5} αMSH and different preparations of HGH were compared. From Table 8 it can be seen that gln^{-5} αMSH at a concentration of 0.72 nM exhibits high melanocyte stimulating activity and stimulates lipolysis in rabbit fat cells 4-5 fold. All preparations of HGH at the concentrations tested (4.5×10^{-6} M) produced a 16 fold stimulation of lipolysis, yet showed no MSH activity.

D. Dose-Response Relationships of HGH

Various treatments of HGH designed to remove possible contamination with lipolytic peptides produced no change in the activity associated with HGH (Table 11). HGH (L3530F), HGH-CMC-G100, HGH-HOAc-G100 and HGH-red-ox, were 4 different preparations of HGH that were purified by different methods which have been described above. As illustrated in Table 11, all 4 preparations of HGH have similar, if not identical,

dose-response relationships. The concentration required for half-maximal stimulation is approximately 0.7×10^{-6} M. This suggests that the lipolytic activity of HGH is likely to be an intrinsic property of the hormone. The only treatment which abolished the lipolytic activity of HGH was oxidation with performic acid (Table 10). This treatment is known to destroy the growth-promoting activity of the hormone also (118).

Release of free fatty acids due to HGH in rabbit fat cells was measured by the procedure of Duncombe (109) and found to correlate well with glycerol release. The ratio of FFA to glycerol release was between 2-2.5:1.

All 4 HGH preparations were also found to be devoid of lipolytic activity in the rat fat cells (data not shown).

E. Neutralization of Lipolytic Activity of HGH with Purified Antibodies to HGH and HCS

The similarity (119) between primary structure of HGH and HCS has been established and the immunological-cross reactivity of HGH and HCS has been well documented (120). Antiserum raised against HGH cross-reacted with both HGH and HCS with almost identical affinity, and this was also true for antibody raised against HCS. From Table 12, it is seen that purified antibodies to HGH and HCS inhibited the lipolytic activity of HGH by 70% and 90%, respectively. The stimulation due to ACTH was not affected by either antibody. HCS, by itself, despite the close similarity to HGH (119) has no lipolytic activity in the rabbit fat cells (Table 10).

The specific neutralization of the lipolytic activity of HGH by antibodies raised against a structurally similar molecule

of non-pituitary origin (HCS) further implies that the lipolytic activity is an intrinsic property of the hormone and not due to contamination with pituitary peptides.

F. Effect of Trypsin on HGH

The effect of trypsin digestion on HGH has been described by Bewley et al. (121). As illustrated in Figure 3, the lipolytic activity of HGH was almost completely abolished by the treatment with 4 µg/ml trypsin for 5 minutes. At the same concentration of trypsin, the stimulation due to ACTH was only decreased by 30%. For complete abolishment of activity due to ACTH, it required 4 times as much trypsin as that for HGH. The results of this experiment further suggest that the lipolytic effect could not be due to contamination with ACTH-like molecules. The high sensitivity of HGH indicates that the integrity of the hormone is essential for the manifestation of the lipolytic activity.

G. Dose-Response Relationships of Glycerol Release and Cyclic AMP Accumulation in Response to HGH

In view of the role of cyclic AMP in the stimulation of lipolysis by fast-acting lipolytic agents (122), the effect of HGH on cyclic AMP accumulation and glycerol release was examined in the presence and absence of theophylline. From Figure 4a, it is apparent that theophylline potentiates the lipolysis of HGH in rabbit fat cells. HGH stimulates the accumulation of cyclic AMP significantly, and this response to HGH is also potentiated by theophylline (Figure 4b). The concentration of theophylline employed (3.2×10^{-5} M) had no

effect on the basal rates of cyclic AMP generation of glycerol release. These results suggest that HGH stimulates lipolysis in isolated fat cells by mechanisms similar to those found for the action of other fast-acting lipolytic agents like ACTH.

H. Effect of HGH on the Kinetics of Glycerol Release and Cyclic AMP Accumulation

The kinetics of glycerol release and cyclic AMP accumulation in both fat cells and medium are shown in Figure 5. HGH in the presence of theophylline (32×10^{-6} M) increased the intracellular accumulation after 5 minutes, reached maximum level at 20 minutes, but by 40 minutes, all the cyclic AMP was found only in the medium. The increase in glycerol release was only evident after 15 minutes and plateaued between 1 and 1 and 1/2 hours. These results show that the induction of cyclic AMP synthesis precedes glycerol release, indicating that the stimulation of lipolysis by HGH is probably mediated by cyclic AMP.

I. Effect of Cycloheximide and Ca^{2+} Ions on HGH Induced Lipolysis

Fain and Kovacev (67) reported previously that the lipolytic action of growth hormone plus dexamethasone was inhibited by inhibitors of both RNA and protein synthesis. As shown in Table 13, this lipolytic effect of HGH in the rabbit fat cells was not inhibited by cycloheximide (2 µg/ml). This dose of cycloheximide, however, inhibits ^{14}C leucine incorporation into rabbit fat cell proteins almost completely.

The dependence of Ca^{2+} was also investigated. HGH was incubated with rabbit fat cells in the presence of EGTA.

From Table 13, it can be seen that Ca^{2+} ions are necessary for the lipolytic action of HGH. Omitting Ca^{2+} ions from the incubation medium also abolished HGH activity. These results suggest that Ca^{2+} ions are necessary for the manifestation of the lipolytic activity of HGH in rabbit fat cells and are different from the results of Fain and Saperstein (70). In their hands, Ca^{2+} ions were not required for the lipolytic activity of HGH plus dexamethasone in rat fat cells.

J. Adenylate Cyclase Activity in Isolated Fat Cells and Rabbit Fat Cell Ghosts

In view of the fact that HGH stimulated total cyclic AMP accumulation in the fat cells, it was of interest to examine the effect of the hormone on adenylate cyclase activity in the isolated rabbit fat cells and rabbit fat cell ghosts. Adenylate cyclase activity in the intact cells can be measured by the conversion of ^{3}H-ATP to ^{3}H-cyclic AMP. ^{3}H-ATP was introduced into the cells by prelabelling the cells with ^{3}H-adenine, which was then converted into ^{3}H-ATP. From Table 14, it can be seen that both ACTH and HGH stimulate the conversion of ATP to cyclic AMP in intact cells prelabeled with ^{3}H-adenine to the same extent. The dose-response relationships are somewhat similar to the stimulation of lipolysis elicited by these two hormones. On the other hand, as indicated in Table 15, NaF and ACTH were potent stimulators of adenylate cyclase activity in fat cell ghosts, HGH was ineffective even at 4.5×10^{-6} M. At this particular concentration, both glycerol and cyclic AMP production in the intact rabbit fat cells are stimulated maximally (Figure 4a,b). These results

suggest that there is a discrepancy between the adenylate cyclase activity due to HGH in the isolated rabbit fat cells and fat cell ghosts. While in the former case, HGH was very active, it was totally ineffective in the latter case. This observation further confirms that the lipolytic response of HGH could not be due to contamination with small peptides such as ACTH or α-MSH since they are all extremely active in stimulating adenyl cyclase activity in the rabbit fat cell ghost system (102,103).

K. Effect of Fasting on the Lipolytic Activity of HGH

Fat cells were isolated from perirenal fat pads of rabbits which had been fasted for 48 hours. The lipolytic dose response relationships of HGH in white fat cells from normal and fasted rabbits are more responsibe to HGH than fat cells from fed rabbits. The concentration required for half-maximum stimulation decreased from approximately 2×10^{-6} M for fat cells from fed rabbits to 4.5×10^{-7} M for fat cells from fasted rabbits with HGH alone and from 9×10^{-7} M to 1.3×10^{-7} M with HGH in the presence of theophylline (32 μM). Cyclic AMP accumulation due to HGH and HGH plus theophylline (32 μM) were also compared in fat cells from fed and fasted rabbits and was found to be unchanged (data not shown).

L. Effect of Other Pituitary Hormones on Fat Cells Isolated from Fasted Rabbits

The lipolytic effects of HGH, b-TSH, b-LH, b-ACTH, βm-MSH, α-MSH, O-prolactin, HCS and BGH were compared in fat cells isolated from fasted rabbits as shown in Table 16. In this more sensitive system, b-TSH, b-LH, O-prolactin, HCS and

BGH were all found to be inactive at the concentrations indicated. Similar observations were also found in fat cells isolated from fed rabbits. HGH, b-ACTH, α-MSH, and βm-MSH were the only lipolytic active pituitary hormones in this system.

Discussion

These studies have clearly shown that HGH stimulates lipolysis in the isolated rabbit fat cells but not the rat fat cells, and this effect could not be attributed to contamination with other pituitary hormones. As indicated in Table 10, species specificity is very important in determining the fast lipolytic activity of a particular hormone. Contamination with the glycoproteins can be ruled out, since they display opposite specificity (46). Contamination with prolactin and ACTH cannot account for the lipolytic activity of HGH, since O-prolactin was found to be inactive in both rat and rabbit fat cells and ACTH was found to be extremely active in rat fat cells. Among the known pituitary hormones, MSH is the only one which displays the same specificity as that of HGH. However, 4 different preparations of HGH, namely HGH (L3530F), HGH-CMC-G100, HGH-HOAc-G100, and HGH-red-reox purified by different procedures exhibit no melanocyte stimulating activity, yet are highly potent in stimulating lipolysis in rabbit fat cells.

The specific neutralization of the lipolytic activity of HGH by antibodies raised against HCS further substantiates that this is an intrinsic property of the hormone and not due to contamination with pituitary peptides. HCS, a non-pituitary hormone, is known to be structurally similar to HGH.

Antisera raised against either hormone cross-reacted with both HGH and HCS with almost identical affinity. Therefore, it is not surprising that antibodies to HCS can neutralize the lipolytic activity of HGH.

The loss of activity following performic acid oxidation and limited digestion with trypsin indicate that the integrity of the HGH molecule is essential for the lipolytic action. The differences in susceptibility to tryptic digestion noted between HGH and ACTH suggest that the lipolytic activity of HGH is not associated with a small peptide fragment.

The observation that HGH stimulated adenylate cyclase activity in intact fat cells but not rabbit fat cell ghosts further lends indirect evidence that the lipolytic activity is an intrinsic property of HGH, since all the known small pituitary peptides are able to stimulate adenylate cyclase activity in both systems. The exact reason why HGH is only effective in the intact fat cells is not known. It is possible that the cellular receptor for HGH is damaged during lysing of the cells. Similar observations have also been made by Rodbell et al. (124) and Sutherland (50) for LH. While LH stimulates the intracellular accumulation of cyclic AMP in intact rat fat cells, it barely activates adenylate cyclase in rat fat cell ghosts. Another possibility is that the concentrations of HGH tested are inadequate. It is known that in the case of ACTH the concentration of hormone needed for half-maximal stimulation of rabbit fat cell ghost cyclase is at least 10 times that needed for half-maximal stimulation of lipolysis in intact fat cells. The highest concentration of HGH used in

the cyclase experiments was 4.5×10^{-6} M, which is less than 10 times the concentration of HGH needed for half-maximal stimulation of lipolysis.

The experiments in glycerol release, cyclic AMP accumulation, and effect of cycloheximide and Ca^{2+} ions gave some insight into the general characteristics of the lipolytic response in the rabbit fat cells. This effect is very similar to that of ACTH (in rabbit and rat fat cells (45) and the glycoprotein hormones (in rat fat cells) (46). It is fast acting; glycerol release can be detected within 15 minutes; it is not blocked by inhibitors of protein synthesis; it requires the presence of Ca^{2+} ions and seems to be mediated by cyclic AMP.

As discussed earlier, Fain and Kovacev have reported a slow lipolytic action of growth hormone in the isolated rat cells which requires a lag period of at least 1 hour, is potentiated by glucocorticoids and prevented by inhibitors of RNA and protein synthesis (67). This effect is very different from the fast lipolytic action of HGH and other pituitary hormones described above. Whereas HGH is inactive as a fast lipolytic agent in the rat, we have been able to confirm this slow action of HGH in the rat as reported by Fain and Kovacev (Table 17).

The effect of nutritional state on the in vivo lipolytic effect of HGH has already been described in detail. Fasting increases secretion of circulating growth hormone in human subjects (125,126). Goodman also observed that the administration of growth hormone for 2 days restored the responsiveness

of adipose tissue from hypophysectomized rats to growth hormone and dexamethasone in vitro (127). Our observation that fat cells from fasted rabbits were more sensitive to HGH suggests the possibility that this increase in sensitivity could probably be due to the adipose tissue responding to increasing level of circulating growth hormone. On the other hand, it is also possible that fasting decreases the availability of acetyl CoA which is necessary for the synthesis of prostaglandins. Prostaglandins are known to antagonize the stimulatory effects of lipolytic hormones (133). A decrease in the level of prostaglandins in the cell might bring about an increase in the sensitivity of fat cells from fasted rabbits to lipolytic hormones. The observation that fat cells from fasted rabbits have the same specificity as those from fed rabbits except for the increase in sensitivity should provide a better system for studying the structure-function relationships of HGH to fat cell receptors.

CHAPTER V

PERTURBATION OF RABBIT FAT CELL MEMBRANE WITH ENZYMES

The effect of digestion of rat fat cells with trypsin (76-79) phospholipase-C (80-82) and neuraminidase (83) on the subsequent lipolytic effects of a variety of hormones has been well documented and has been summarized in Chapter I.

The present studies describe the effect of these three enzymes on fat cells isolated from fed or fasted rabbits, and their subsequent lipolytic response to HGH. The purpose of this investigation was to study the nature of the HGH-receptors on the rabbit fat cells and at the same time compare the effects of these enzymes on the lipolytic responses of rat and rabbit fat cells to the hormone.

Results

A. Effect of Trypsin Treatment of Rabbit Fat Cells on the Lipolytic Effect of HGH

In the experiment shown in Figure 7, rabbit fat cells were exposed to various concentrations of trypsin for 10 minutes and lipolysis was determined after neutralization of the enzyme with lima bean trypsin inhibitor. Trypsin had no effect on the basal stimulation of the fat cells and only affected that due to theophylline (32×10^{-6} M) marginally. However, at a concentration of 100 µg/ml, it markedly sensitized the lipolytic response of the fat cells to HGH in the absence or presence of theophylline (32×10^{-6} M). Maximum sensitivity was obtained with trypsin treatment of 250 µg/ml

and was found to be about 3 fold over untreated cells. Pretreatment of cells with concentrations of the enzyme higher than 250 µg/ml led to a profound decrease in responsiveness of the cells to HGH. At about 1 mg/ml of trypsin, the treated cells were totally unresponsive towards HGH.

In the experiment shown in Figure 8, rabbit fat cells were pre-incubated for different time periods with trypsin (250 µg/ml). Cells treated for 5 minutes only showed a marked increase in sensitivity in lipolysis due to HGH. This increase in sensitivity peaked at 10 minutes and only decreased by 20% after 30 minutes. In fact, even after 1 hour of exposing the cells to trypsin, the treated cells were still more responsive to HGH than untreated cells (data not shown).

The experiments described above showed two distinct effects of trypsin on rabbit fat cells. At low concentrations it markedly sensitized the cells to HGH, while at high concentrations, it completely abolished all the lipolytic activity of HGH without affecting basal stimulation. The lipolytic response of ACTH was also studied under identical conditions and found to exhibit similar increase in sensitivity as that of HGH (unpublished observations).

B. Comparison of Dose-Response Relationships of HGH in Trypsin Treated and Untreated Rabbit Fat Cells

As illustrated in Figure 9, pretreatment of rabbit fat cells with trypsin (250 µg/ml) for 10 minutes markedly potentiated the lipolytic response of HGH in the absence and presence of theophylline (32×10^{-6} M). However, trypsin treatment

did not interfere with the maximum lipolytic activity of HGH. The concentration of HGH required for half-maximum stimulation was decreased from about 2×10^{-6} M to 4.5×10^{-7} M in the absence of theophylline and from about 9×10^{-7} M to 1.35×10^{-7} M in the presence of theophylline (32×10^{-6} M). The basal stimulation in the absence and presence of theophylline was very similar and was unaffected by trypsin treatment.

C. Cyclic AMP Accumulation in Response to HGH in Trypsin Treated and Untreated Rabbit Fat Cells

When cyclic AMP accumulation due to HGH was examined in trypsin treated and untreated rabbit fat cells, there was no change in the total cyclic AMP content (data not shown). However, there is a strong possibility that trypsin treatment might influence the rate of formation of cyclic AMP in the cells or the rate of disappearance of cyclic AMP from the cells, which could account for the increase in sensitivity in lipolysis. Therefore, the kinetics of cyclic AMP accumulation in the cells and in the medium and glycerol production of trypsin treated and untreated cells were studied. It was found that trypsin treatment did not change the rate of cyclic AMP formation in the cells or the rate of cyclic AMP accumulation in the medium. Maximum cyclic AMP production in treated and untreated cells occurred at 20 minutes and by 40 minutes, nearly all the cyclic AMP was found in the medium. The rate of glycerol production was not changed by trypsin treatment either (Table 18), except that treated cells released more glycerol than untreated cells in response to the same submaximal dose of HGH (Figure 9).

D. Lipolytic Response of HGH in Trypsin Treated and Untreated Fat Cells from Fasted Rabbits

It was previously reported in Chapter IV that fat cells isolated from fasted rabbits were more sensitive to HGH than fat cells isolated from fed rabbits. When fat cells from both fed and fasted rabbits were treated with trypsin (250 µg/ml) for 10 minutes and compared, there was no change in sensitivity in trypsin treated and untreated fat cells from fasted rabbit in response to HGH, whereas in fat cells from fed rabbits, the same "trypsin effect" was observed (Figure 10). The possibility exists that the conditions used for cells from fed rabbits may not be suitable for fat cells from fasted rabbits. Therefore the effect of different concentrations of trypsin treatment was examined again with various duration of time in fat cells isolated from fasted rabbits. The results in Table 19 indicate that pretreatment with trypsin up to 4 mg/ml for 10 minutes did not change the lipolytic effect of HGH. Moreover, pretreatment with trypsin (0.25 mg/ml) up to 1 hour has no effect on the lipolytic effect of HGH either (Table 20). However, since the pretreatment with trypsin was performed in Krebs Ringer bicarbonate buffer containing 4% (W/V) albumin, it is possible that the "effective" trypsin concentration was not high enough because of the high percentage of albumin in the medium. Then the trypsin pretreatment was performed in the presence of 1% (W/V) albumin. As shown in Table 21, fat cells from normal rabbits were rendered totally unresponsive to HGH when the trypsin digestion was carried out with 1% BSA, while

control digestion studies with 4% albumin gave the same increase in sensitivity. However, fat cells from fasted rabbits did not show any change in the response to HGH whether the digestion was carried out in 1% or 4% albumin.

E. Effect of Phospholipase C Digestion of Rabbit Fat Cells on the Subsequent Lipolytic Response of HGH

As described by various investigators (80-82), digestion of rat fat cells with phospholipase C readily destroys the capacity of these cells to increase lipolysis in response to ACTH, glucagon and theophylline. It may be seen that similar behavior was observed in the rabbit fat cells. Digestion of rabbit fat cells with 5 µg/ml of phospholipase C for 5 minutes completely abolished the ability of the cells to respond to HGH without affecting basal stimulation (Table 22). Fat cells isolated from fasted rabbits also behaved similarly when treated with phospholipase C (Table 22). The inability of the fat cells to respond to HGH may be related to lysis of the cells, since treatment with as little as 0.31 µg/ml of phospholipase C for 5 minutes showed detectable lysing and at least 80% of the cells were lysed when treated with 5 µg/ml of the enzyme.

F. Effect of Neuraminidase on HGH-Induced Lipolysis in Rabbit Fat Cells from Normal and Fasted Rabbits

When rabbit fat cells were exposed to neuraminidase (25 µg/ml) for various time periods, a progressive increase in sensitivity in response to HGH was observed (at 6.6×10^{-7} M) in the presence of theophylline (32×10^{-6} M), as shown in Figure 11. Maximum sensitivity occurred after pretreatment of

the cells with the enzyme for 45 minutes and only decreased slightly at 1 hour. Control cells were also preincubated for the same time periods in the absence of neuraminidase and showed no change in lipolytic response to HGH. Neuraminidase treatment did not alter the base-line stimulation of the fat cells. Similar increase in sensitivity was also observed with neuraminidase treatment on fat cells from fasted rabbits (data not shown).

Sialic acid released into the medium was followed after treatment with neuraminidase (25 µg/ml) for various time periods. Substantial quantities of sialic acid were released rapidly and a clear plateau was reached by 30 minutes (Figure 11). The release of sialic acid correlates well with the increase in sensitivity of the cells to HGH.

The effect of various concentrations of neuraminidase treatment was next examined (Table 23). Digestion of isolated rabbit fat cells with very low concentration of the enzyme (<5 µg/ml) up to 30 minutes showed no change in the lipolytic effect of HGH. Maximum sensitivity occurred when the cells were treated with 25 µg/ml of neuraminidase for 30 minutes to 1 hour and decreased when the concentration of the enzyme was increased. At 100 µg/ml, the lipolytic response of HGH fell below that of untreated cells but was not completely abolished.

G. Effect of Neuraminidase Treated Cells on the Subsequent Dose-Response Relationship to HGH in Isolated Fat Cells from Normal and Fasted Rabbits

As shown in Figure 12, pretreatment with neuraminidase (25 µg/ml) for 60 minutes increased the sensitivity of the

cells to HGH by about 3 fold without affecting the maximum level of stimulation. Half-maximum stimulation of HGH was decreased from 6.7×10^{-7} M for untreated cells to 2.0×10^{-7} M for treated cells. There was similar increase in sensitivity with neuraminidase treatment in fat cells isolated from fasted rabbits. There was no change in maximum stimulation, and the concentration of HGH required for half-maximal stimulation was decreased from 27×10^{-7} M for untreated cells to 0.50×10^{-7} M for treated cells.

H. Digestion of Cells with Different Preparations of Neuraminidase

Studies were performed on the effect of digesting rabbit fat cells with different preparations of neuraminidase in order to demonstrate the specificity of the enzyme. The exact quantity of neuraminidase added was determined by the specific activity of each preparation rather than by weight. In the experiment described in Figure 12, 2.5×10^{-3} U per millilitre of cell suspension (40-50 mg/ml) was added. As illustrated in Table 24, three different preparations of neuraminidase with different specific activity gave essentially similar increase in sensitivity. These results indicated that the effect of neuraminidase on rabbit fat cells is probably specific, since different preparations of the enzyme are equally effective in modifying the lipolytic activity of HGH. Catrecasas (83) also observed that the commercial, partially purified neuraminidase contained no detectable biologically active contaminants in his studies in rat fat cells.

I. Sequential Digestion with Neuraminidase and Trypsin

Earlier observations by Cuatrecasas (83) indicated that the effect of neuraminidase and trypsin on insulin-induced glucose transport in rat fat cells is probably identical since one enzyme can substitute for the other, and their effects are additive at the proper concentrations. We have found a difference in the effect of neuraminidase and trypsin on fat cells from fasted rabbits. Neuraminidase was able to modify the lipolytic effect of HGH but trypsin, on the other hand, is totally ineffective. However, in fat cells isolated from fed rabbits, both trypsin and neuraminidase acted in a similar manner; that is, they both increase the sensitivity of the cells in response to HGH. As shown in Figure 13, sequential digestion of fat cells from normal rabbits with trypsin followed by neuraminidase had the same effect as treatment with neuraminidase alone. However, when the enzyme treatments were reversed, that is, neuraminidase treatment first before trypsin, the increase in sensitivity was completely abolished. The response of the treated cells was found to be the same as that of untreated cells (Figure 13). The fact that sequentially treated cells and untreated cells elicit similar response to HGH may be a coincidence. It is possible that by increasing the concentration of trypsin, the lipolytic effect of HGH could be destroyed.

Isolated fat cells from fasted rabbits also respond in a similar fashion to sequential digestion with neuraminidase and trypsin. Digestion with trypsin followed by neuraminidase

gave similar increase in sensitivity as neuraminidase alone (Table 25). Digestion with trypsin in the reverse order abolished the increase in sensitivity. It is interesting to observe that trypsin alone has no effect on fat cells isolated from fasted rabbits. However, after neuraminidase digestion, the cells became susceptible to trypsin.

Discussion

The effect of trypsin digestion of rat fat cells on their subsequent lipolytic response to various hormones has been well documented (76-79). Trypsin treatment completely abolished the antilipolytic effect of insulin and the lipolytic effect of glucagon, while that of ACTH, epinephrine and growth hormone plus glucocorticoid were retained (78). It was previously reported in Chapter IV that HGH stimulated lipolysis in the rabbit fat cells but not the rat fat cells. The increase in sensitivity of HGH to trypsin pretreatment on rabbit fat cells further substantiates the differences between the receptor systems in the rat and rabbit fat cells. Unlike that in the rat fat cells, the lipolytic sensitivity of ACTH was also increased in the rabbit fat cells (unpublished observations). Whether tryptic digestion increases the affinity of the receptor for HGH or exposes new receptors can be ascertained only by binding studies with HGH. The inability of rabbit fat cells to respond to HGH after high concentration of trypsin treatment was probably due to severe damage or destruction of HGH receptors.

We have reported previously in Chapter IV that fat cells isolated from fasted rabbits were more sensitive to HGH than those from fed rabbits. The exact mechanism of this increase in sensitivity is not known. It has been reported from in vivo studies that fasting increases secretion of circulating growth hormone in human objects (125,126). Also, the injection of growth hormone to fasting animals elevates plasma free fatty acid level (59). It is possible that the adipose tissue from rabbits has already been sensitized before sacrifice in response to increasing circulating level of growth hormone. It is also possible that fasting decreases the availability of acetyl CoA which is necessary for the synthesis of prostaglandins. Prostaglandins are known to antagonize the stimulating effect of most lipolytic hormones. Therefore, the increase in sensitivity in fat cells from fasted rabbits may be in part due to the low level of prostaglandins present. However, these possibilities still do not offer an explanation of why these cells are resistant to trypsin digestion even at 4 mg/ml in 1% (W/V) albumin buffer.

Earlier studies have demonstrated that digestion of isolated rat fat cells with phospholipase C abolishes the lipolytic effects of low concentrations of ACTH, glucagon, theophylline and also the antilipolytic effect of insulin (81). The present data on digestion of rabbit fat cells with phospholipase C showed similar abolishment of lipolytic activity of HGH and ACTH. However, this loss of lipolytic response cannot be overcome by increasing the concentration of the hormones as was found by Rodbell for ACTH (81). Treatment of rabbit fat cells

even with low concentration of phospholipase C (0.31 µg/ml) causes visible lysing of the cells. When the cells were treated with 5 µg/ml of the enzyme, about 80 to 90% of the cells were found to be lysed. This can be detected by the formation of large droplets of fat floating on the surface of the cell suspension. It appears that the loss of ability of HGH or ACTH to stimulate lipolysis is related to extensive cell damages and lysing and therefore cannot be overcome by higher concentrations of hormones. The observation that phospholipase C is equally effective in fat cells isolated from normal and fasted rabbits suggests that the phospholipid structure in these two different cell preparations are probably very similar.

The effect of neuraminidase digestion on rat fat cells in response to a variety of lipolytic hormones has been reported by Cuatrecasas (83). In general, neuraminidase treatment decreases the lipolytic activities of epinephrine, ACTH, glucagon, theophylline and growth hormone plus dexamethasone. Cuatrecasas also reported a biphasic dependence on neuraminidase action as a function of enzyme concentration on base-line glucose transport (83). From our present data, neuraminidase digestion did not alter the base-line lipolytic activity of rabbit fat cells. However, similar biphasic dependence of neuraminidase concentration was observed on HGH-induced lipolysis in neuraminidase-treated rabbit fat cells. Treatment with neuraminidase (2.5 milli unit per ml) for about 1 hour increased the sensitivity of the cells to HGH by 3 to 6 fold. Digestion with higher enzyme concentrations led to a

progressive decrease in the responsiveness of the cells to HGH. As discussed previously for trypsin, this increase in sensitivity has no effect on the maximal response of the cells. Neuraminidase treatment may increase the affinity of the receptors for HGH or expose receptors previously unavailable. The identical behavior of fat cells from normal and fasted rabbits strongly suggests that the glycoprotein structures in the two cells are probably similar.

The lipolytic response of ACTH on neuraminidase-treated rabbit fat cells has also been studied and found to exhibit similar biphasic dependence on enzyme concentration as that reported for HGH (unpublished observation). These data provide further evidence for the differences between rat and rabbit fat cells.

Earlier studies by Cuatrecasas on the sequential digestion of rat fat cells with trypsin and neuraminidase indicated that these two enzymes may produce their effect by very similar, if not identical, mechanisms (83). Our present data suggest that this may not be entirely true for rabbit fat cells. Sequential digestion with trypsin first, then neuraminidase, showed comparable increase in sensitivity to that of neuraminidase alone. However, reversing the order of digestion showed a lipolytic response which was almost identical to that of nontreated cells; that is, no increase in sensitivity. This observation may well be a coincidence, since we have not explored the effect of various concentrations of the two enzymes on sequential digestion. It is possible

that higher concentrations of trypsin following neuraminidase treatment could abolish completely the lipolytic response of the cells to HGH.

As discussed earlier, we have found that fat cells from fasted rabbits were resistant to trypsin digestion. However, after neuraminidase treatment, these cells became susceptible to the effect of trypsin. As illustrated in Table 25, trypsin also abolished the increase in sensitivity due to neuraminidase on sequential digestion. The observation suggests that the cleavage of sialic acid from fat cell membranes by neuraminidase probably increases the susceptibility of the cells to trypsin digestion.

Previously, the species specificity of the lipolytic action of different pituitary hormones in different species was discussed. The present thesis concentrates on the differences between the rat and rabbit fat cells. Earlier studies by Ramachandran and Lee (45) with ACTH, NPS-ACTH, αMSH and NPS-αMSH have already indicated the differences in the rat and rabbit fat cell receptors for ACTH. Their results indicated that the tryptophan residue in the ACTH molecule is essential for the stimulation of the rat fat cell receptors, since modification of this residue by reaction with 0-nitrophenyl suphenyl chloride (NPS-ACTH) abolished the ability of the hormone to stimulate lipolysis completely. In contrast to this, NPS-ACTH was found to be more active than ACTH in stimulating lipolysis in rabbit fat cells (45) and rabbit fat cell ghost adenylate cyclase (102). These observations imply that the differences in the responsiveness of the rat and

rabbit fat cells must be due to differences in the structures of the hormonal receptors in the two species. In this present thesis, more evidence has been accumulated to substantiate the striking differences in the characteristics of the receptors on the rat and rabbit fat cell membranes (Chapters IV,V). The significance of these differences in the manifestation of lipolytic functions is not known. However, a deeper understanding of species receptors may explain why certain lipolytic hormones are active in some species and not others, and particularly, why HGH is only active in the rabbit fat cells, but not the rat fat cells.

CHAPTER VI

SUMMARY

1. The growth promoting and rapid lipolytic activity associated with BGH preparations can be separated by chromatography on QAE Sephadex A25. The contaminant responsible for the lipolytic effect was identified as b-TSH by neutralization experiments with A/S b-TSH. It was further shown that the lipolytic activity associated with BGH can be removed by affinity chromatography on A/S 0-LHα coupled to sepharose.

2. Incubation of HGH with isolated rabbit fat cells but not rat fat cells produced a rapid stimulation of lipolysis. Evidence was presented to substantiate that the lipolytic actions of HGH is an intrinsic property and not due to contamination with other lipolytically active pituitary hormones. Of the known pituitary hormones, HGH and the melanotropins are the only hormones that activate lipolysis in the rabbit fat cells but not in the rat fat cells. However, HGH exhibits no melanocyte activity in the isolated frog skin assay.

The lipolytic effect of several preparations of HGH have been studied and found to possess similar lipolytic activity. This lipolytic activity can be neutralized by antibodies raised against both HGH and HCS. Treatment of HGH (4.5×10^{-6} M) and ACTH (0.03×10^{-6} M) with trypsin (4 µg/ml) for 5 minutes completely abolished the ability of HGH to release glycerol but that of ACTH was only decreased by 30%.

HGH increases the accumulation of cyclic AMP as well as glycerol release in the isolated rabbit fat cells. The lipolytic

effect of HGH is not inhibited by cycloheximide and requires Ca^{2+} ions. HGH also stimulates adenylate cyclase activity in intact rabbit fat cells by increasing the accumulation of labelled cyclic AMP from ^{3}H-adenine. However, it does not stimulate adenylate cyclase activity in rabbit fat cell ghosts. The reason for this discrepancy is not known.

Fat cells isolated from rabbits which have been fasted for 48 hours elicit a 3 to 6 fold increase in sensitivity to HGH in the absence or presence of theophylline (32×10^{-6} M). The lipolytic specificity of known pituitary hormones was also studied in this system. HGH, ACTH, α- and β-MSH were found to be the only hormones which stimulated lipolysis in fat cells isolated from fasted rabbits.

3. Limited digestion of isolated fat cells from normal rabbits with trypsin (0.1 to 0.25 mg/ml) results in enhanced sensitivity to subsequent lipolytic action of HGH. Higher concentrations of trypsin (1 mg/ml) under similar conditions rendered the cells totally unresponsive to HGH. Digestion of fat cells from fasted rabbits with trypsin (up to 4 mg/ml) was without effect on the subsequent lipolytic effect of HGH.

Digestion of fat cells from both normal and fasted rabbits with phospholipase C causes lysing of the cells and destroys the lipolytic responsiveness of the cells to HGH.

Mild digestion of isolated fat cells from both normal and fasted rabbits with neuraminidase (2.5 milli units per ml) results in enhanced lipolysis of these cells to HGH.

Digestion with higher concentrations of the enzyme (10 milli units per ml) abolishes this increase in sensitivity without destroying completely the lipolytic response of HGH.

Sequential digestion with neuraminidase and trypsin, and the studies on the digestion with individual enzyme alone on fed cells from fed rabbits and fasted rabbits indicate that the two enzymes probably act by a similar mechanism in increasing the sensitivity of the cell to HGH. However, the effect of the two enzymes are not identical, since they are not interchangeable in sequential digestion, and also only neuraminidase, but not trypsin, is effective in increasing the sensitivity of fat cells from fasted rabbits.

TABLE 1

Hormone Preparation	Half Maximum Stimulation (M)	
	Rat	Rabbit
ACTH	1.5×10^{-9} (123)	1.6×10^{-8} (123)
αMSH	Inactive at 10^{-7} (123)	$.71 \times 10^{-9}$ (123)
βMSH	Inactive at 10^{-7} (123)	7.1×10^{-9} (123)
b-TSH	1×10^{-8} (46)	Inactive at 3×10^{-6} (47)
h-TSH	3×10^{-8} (47)	" " " (47)
b-LH	2.8×10^{-8} (46)	Inactive at 3×10^{-6} (46)
h-LH	1.4×10^{-7} (46)	" " " "
O-LH	2.2×10^{-7} (46)	" " " "
O-FSH	2.6×10^{-6} (46)	" " " "
O-LtH	Inactive at 90×10^{-6} (48)	Inactive at 9.0×10^{-6} (48)
BGH*	Inactive at 9×10^{-6} (132)	Inactive at 9×10^{-6} (132)

*Highly purified BGH as described in Chapter IV has no lipolytic effect in both the rat and rabbit fat cells.

TABLE 2

Neutralization of Lipolytic Activity with A/S 0-LH α

Hormone	Dose	Glycerol Production		
	µg/ml	no A/S*	Normal Serum	A/S 0-LH α
None		1.5 ± 0.3	3.5 ± 0.08	4.1 ± 0.08
BGH	100	29.9 ± 0.3	29.0 ± 0.3	4.8 ± 0.3
b-TSH	1	31.1 ± 0.4	33.0 ± 0.2	4.3 ± 0.1
0-LH	1	27.8 ± 0.2	23.0 ± 0.1	4.2 ± 0.02
ACTH	0.05	45.5 ± 0.5	44.1 ± 0.08	39.8 ± 0.1

Neutralization and incubation procedures are described under Materials and Methods. Each value is the mean of triplicate incubation of 2 experiments. Glycerol production is expressed as µ moles glycerol per g cells per 2 hours ± S.E.

*In the absence of both normal serum and A/S 0-LHα.

TABLE 3

Lipolytic Activity of Bound and Unadsorbed Fractions from A/S-0-LHα-Sepharose

Hormone	Dose µg/ml	Glycerol Production
None		0.9 ± 0.09
BGH (urea treated)	20	25.1 ± 0.01
BGH-unadsorbed	25	1.2 ± 0.06
BGH-unadsorbed	100	1.3 + 0.07
BGH-bound	0.25	9.2 ± 0.03
BGH-bound	1.25	27.1 ± 0.06

Preparation of bound and unadsorbed BGH from A/S 0-LHα coupled sepharose and incubation procedure are described under Materials and Methods. Each value is the mean of triplicate incubation of 2 experiments. Glycerol is expressed as µ moles glycerol per g cells per 2 hours ± S.E.

TABLE 4

Lipolytic Effect of BGH plus Dexamethasone in Rat Fat Cells

Hormone	Dose	Glycerol Production	
	µg/ml	Without Dexamethasone	With Dexamethasone
None		3.1 ± 0.09	5.1 ± 0.05
BGH-unadsorbed	0.1	4.7 ± 0.03 ($p<0.001$)	7.2 ± 0.1 ($p<0.001$)
BGH-unadsorbed	1	5.0 ± 0.08 ($p<0.001$)	8.0 ± 0.04 ($p<0.001$)

BGH in the absence or presence of dexamethasone (0.016 µg/ml) were added at the start of the incubation. Values are the mean of triplicate of 2 experiments. Glycerol production is expressed as µ moles glycerol/g dry weight cells/4 hours ± S.E.

TABLE 5

Recovery of Highly Purified BGH from QAE-Seph A25 Chromatography

Preparation*	Yield (mg)	
	Fraction 1	Fraction 2
BGH (L3665B)	16	15
BGH (L3512A)	18	12
BGH (3517E)	19	7

*Amount of hormone employed was about 40 mg.

TABLE 6

Lipolytic Activities of BGH Fractions from Chromatography QAE-Sephadex A25 and Sephadex G100

Hormone	Dose µg/ml	Glycerol Production
None		0.7 ± 0.07
BGH (L3512A) Starting Material	25	9.0 ± 0.1
BGH (L3512A)	100	20.5 ± 0.1
BGH-QAE Fraction 1	25	0.8 ± 0.02
BGH-QAE Fraction 1	100	0.9 ± 0.04
BGH-QAE Fraction 1	200	0.8 ± 0.01
BGH-QAE Fraction 2	1	9.3 ± 0.09
BGH-QAE Fraction 2	5	20.8 ± 0.06
BGH-QAE G100	25	0.8 ± 0.03
BGH-QAE G100	100	0.9 ± 0.04

BGH-QAE Fraction 1 and BGH-QAE Fraction 2 were obtained from chromatography on QAE-Sephadex A25 as described in Fig. 1. BGH-G100 was prepared as described in Fig. 2. Glycerol production is expressed as µ moles glycerol per g cells per 2 hours ± S.E.

TABLE 7

Neutralization of Lipolytic Activities with A/S b-TSH

Hormone	Dose	Glycerol Production	
	µg/ml	Without A/S bTSH	With A/S TSH
None		0.8 ± 0.07	2.2 ± 0.05
BGH (L3512A)	100	23.5 ± 0.08	2.7 ± 0.1
BGH-QAE Fraction 1	100	1.3 ± 0.06	2.3 ± 0.05
BGH-QAE Fraction 2	5	25.6 ± 0.2	2.8 ± 0.2
b-TSH	1	19.3 ± 0.06	2.2 ± 0.07
ACTH	0.5	31.5 ± 0.03	30.5 ± 0.2

Conditions of neutralization and incubation procedures were the same as that described under Table 2. Glycerol production is expressed as µ moles glycerol per g cells per 2 hours ± S.E.

TABLE 8

Bioassay by the Tibia Test of Purified BGH from QAE-Sephadex A25 Chromatography

Preparation	Total Dose µg	Tibia Width* µ
Control		178 ± 3 (5)
BGH	30	239 ± 1 (4)
BGH-QAE-G100	10	227 ± 3 (5)
BGH-QAE-G100	30	245 ± 5 (5)

*Average ± S.E. (number of animals)

TABLE 9

Stimulation of Lipolysis in Rat and Rabbit Fat Cells by HGH, ACTH and Prolactin

Hormone	Concentration ($\times 10^{-6}$M)	Glycerol Production	
		Rat*	Rabbit*
None		0.9 ± 0.04	1.0 ± 0.2
HGH	1.13	0.9 ± 0.1	6.5 ± 0.3
HGH	4.50	1.3 ± 0.1	24.1 ± 0.6
ACTH	0.03	24.3 ± 0.2	22.9 ± 0.5
Prolactin	4.50	0.44± 0.2	2.2 ± 0.1

[a]μmoles glycerol/g cells/2 hours

[b]μmoles glycerol/g cells/hour

[c]mean ± S.E.

TABLE 10

Melanocyte Stimulating Activities and Lipolytic Activities of ACTH, gln-5α MSH HGH-(L 353 0F), HGH-CMC-G100 and HGH-red-reox in Frog Skin Assay and Rat and Rabbit Fat Cell Lipolysis

Hormone	Concentration ($X10^{-9}$ M)	Percent Decrease in Reflectance	Glycerol Production	
			Rat	Rabbit
None	--	6 ± 1 (5)	1	1
ACTH	2.1	28 ± 2 (5)	14.5 ± 1	13.7 ± 0.3
gln^5 - MSH	0.72	31 ± 3 (5)	1.2 ± 0.2	7.5 ± 0.7
HGH-L3530F	4500	8 ± 1 (5)	1.3 ± 0.1	16.3 ± 1
HGH-CMC-G-100	4500	7 ± 1 (5)	0.97± 0.1	15.9 ± 2
HGH-red-reox	4500	3 ± 1 (5)	1.1 ± 0.05	16.3 ± 0.9
HGH-performic 0X	4500	---	0.98± 0.2	0.97± 0.9
HCS	4500	---	1.2 ± 0.1	0.99± 0.7

*Values are the mean ± S.E.; number of skins in parenthesis. Method for measuring melanocyte stimulating activities was described under "Experimental Procedures."

Glycerol production is expressed as number of times over basal value as 1. White fat cells (40-50 mg/tube) was incubated for 2 hours for rat fat cells and 1 hour for rabbit fat cells in 1 ml Krebs Ringer bicarbonate buffer containing 4% albumin, 0.1 mg/ml lima bean trypsin inhibitor.

TABLE 11

Dose Response Relationships of Different Preparations of HGH in Isolated Rabbit Fat Cells

Concentration ($X10^{-6}$ M)	Glycerol Production			
	HGH-L3530F	HGH-CMC-G100	HGH-HOAC-G100	HGH-red-reox
0.56	3.6 ± 0.7	4.4 ± 0.3	4.6 ± 0.4	4.8 ± 0.5
1.13	7.0 ± 0.8	7.6 ± 0.7	7.7 ± 0.7	7.5 ± 0.7
2.25	9.2 ± 0.7	9.5 ± 0.7	9.5 ± 0.3	9.3 ± 0.4
4.5	11.8 ± 0.2	11.7 ± 0.6	11.4 ± 0.4	11.8 ± 0.7

White fat cells (40-60 mg/ml) was incubated for 1 hour in 1 ml of Krebs Ringer bicarbonate buffer containing 4% albumin. HGH-L3530F, HGH-CMC-G100, HGH-HOAC-G100, and HGH-red-reox were 4 different preparations of HGH which were purified by 4 different procedures described. The basal value was 2.4 ± 0.1 µmole glycerol/g of cells/hour. Each value is the average ± S.E. of 3 experiments.

TABLE 12

Neutralization of Lipolytic Activity of HGH by Purified Antibodies to HGH and HCS

Hormone	Concentration ($X10^{-6}$ M)	Glycerol Production		
		Hormone Alone	+A/S HGH	+ A/S HCS
HGH	2.3	6.3 ± 0.2	1.7 ± 0.04	0.72 ± 0.02
		6.7 ± 0.4	1.69± 0.05	0.95 ± 0.03
ACTH	0.03	10.4 ± 0.6	11.87± 0.6	9.42 ± 0.5
		10.8 ± 0.7	11.88± 0.7	9.65 ± 0.6

The specific antibodies to HGH or HCS were incubated with the different hormones for 1 hour at 37° and stored in the cold for 16 hours to allow maximum cross reaction. Sufficient antibodies were added to neutralize all the antigen (determined from the precipitation curve). The antigen-antibody mixtures were added directly to the isolated rabbit fat cells for final incubation without centrifugation. Glycerol production is expressed as µmole glycerol/g dry wt cells/hour ± S.E. Subtracting the basal stimulation without hormone and the slight stimulation due to the purified antibodies to HGH and HCS. Basal stimulation with no antibodies was 0.980 ± 0.2, 0.882 ± 0.15. Basal stimulation due to A/S HGH 1.249 ± 0.1, 1.4 ± 0.1. Basal stimulation due to A/S HCS 3.29 ± 0.2, 3.01 ± 0.3.

TABLE 13

Effect of Cycloheximide, EGTA and Ca^{2+} Ions on Lipolytic Action of HGH in Isolated Rabbit Fat Cells

Hormone	Concentration ($X10^{-6}$ M)	Glycerol Production			
		No Addition	+ Cycloheximide	+EGTA	$-Ca^{2+}$
Basal	--	0.4 ± 0.1	1.2 ± 0.2	1.3 ± 0.1	1.5 ± 0.06
HGH-L3530F	4.5	22.4 ± 0.2	23.5 ± 0.1	1.5 ± 0.1	1.7 ± 0.05
HGH-CMC-G100	4.5	22.2 ± 0.2	24.1 ± 0.3	1.9 ± 0.05	1.6 ± 0.04
ACTH	0.03	20.1 ± 0.05	22.5 ± 0.4	1.8 ± 0.1	1.6 ± 0.02

Cycloheximide (2 µg/ml) and EGTA (1 mM) were present at the start of the experiment. Ca^{2+} was omitted from the incubation medium. Glycerol production is expressed as µmoles glycerol/g dry wt cells/hour ± S.E.

TABLE 14

Adenyl Cyclase Activity in Isolated Rabbit Fat Cells

Hormone	Concentration ($X10^{-6}$ M)	^{3}H Cyclic AMP/g Dry Wt Cells/20 Min $(cpm)_{x10^{3}}$
Basal		9.6 ± 0.7
ACTH	0.008	23.2 ± 3.3
ACTH	0.03	104.9 ± 4.4
HGH-L3530F	0.9	56.7 ± 4.3
HGH-L3530F	4.5	120.2 ± 6.7

Isolated rabbit fat cells (40-50 mg) previously labeled with ^{3}H adenine were incubated for 20 minutes at 37°. 3.2×10^{-6} M theophylline was present in all tubes.

TABLE 15

Adenyl Cyclase Activity in Rabbit Fat Cell Ghosts

Hormone	Concentration ($X10^{-6}$ M)	Cyclic AMP Production
None		0.79 ± 0.11
NaF	9×10^{3}	5.86 ± 0.61
ACTH	0.008	1.54 ± 0.32
ACTH	0.03	2.58 ± 0.2
HGH-L3530F	0.56	0.71 ± 0.10
HGH-L3530F	1.13	0.60 ± 0.12
HGH-L3530F	2.25	0.67 ± 0.08
HGH-L3530F	4.5	0.66 ± 0.15

Fat cell ghosts (40-60 µg of protein) was incubated for 15 minutes at 37°. Cyclic AMP production is expressed as nanomoles/mg/protein/15 minutes. Data are the mean ± S. E. of 6 experiments.

TABLE 16

Lipolytic Effect of Various Hormones in Fat Cells from Fasted Rabbits

Hormone	Concentration ($X10^{-6}$ M)	Glycerol Production
None	--	1.15 ± 0.06
HGH-L3517E	0.02	1.3 ± 0.05
HGH-L3517E	0.07	5.4 ± 0.10
HGH-L3517E	0.28	11.8 ± 0.03
HGH-L3517E	1.1	19.0 ± 0.09
HCS	0.9	1.3 ± 0.09
HCS	4.5	1.5 ± 0.08
h-TSH	1.05	1.4 ± 0.1
h-TSH	3.5	1.20 ± 0.1
h-ICSH	3.5	1.4 ± 0.08
h-ACTH	0.001	1.7 ± 0.04
h-ACTH	0.004	12.4 ± 0.09
αMSH	0.0017	4.0 ± 0.2
αMSH	0.0058	12.5 ± 0.03
m-βMSH	0.0015	2.3 ± 0.1
m-βMSH	0.01	11.5 ± 0.1
O-Prolactin	4.5	1.9 ± 0.05
BGH	4.3	1.2 ± 0.04

Fat cells were isolated from rabbits which had been fasted for 48 hours.

Glycerol production was expressed as moles glycerol/g dry wt cells/hour ± S.E.

TABLE 17

Lipolytic Effect of HGH Plus Dexamethasone in Rat Fat Cells

Hormone	Dose	Glycerol Production	
	µg/ml	Without Dexamethasone	With Dexamethasone
None		2.2 ± 0.07	3.2 ± 0.09
HGH	0.01	2.5 ± 0.09	4.9 ± 0.02
HGH	0.1	3.3 ± 0.1	7.2 ± 0.08
HGH	1	3.5 ± 0.07	7.9 ± 0.07

HGH in the absence or presence of dexamethasone (0.016µg/ml) were added at the start of the incubation. Values are the mean of triplicate of 3 experiments.

Glycerol production is expressed as µmoles glycerol/g cells) 4 hours ± S.E.

TABLE 18

Kinetics of the Subsequent Response to HGH of Trypsin Treated and Untreated Rabbit Fat Cells

Time	Glycerol Production		Cyclic AMP Accumulation			
min	Untreated	Treated	Untreated (t)*	Treated (t)*	Untreated (m)*	Treated (m)*
0	1.69 ± 0.2	2.9 ± 0.2	0.46 ± 0.03	0.44 ± 0.08	0.52 ± 0.6	0.45 ± 0.04
5	--	--	2.39 ± 0.2	1.88 ± 0.15	0.55 ± 0.0	0.66 ± 0.09
10	--	--	3.16 ± 0.02	2.97 ± 0.02	0.88 ± 0.03	1.03 ± 0.05
20	--	--	3.76 ± 0.14	3.46 ± 0.20	1.32 ± 0.01	1.43 ± 0.1
30	7.0 ± 0.09	8.6 ± 0.1	--	--	--	--
40	9.7 ± 0.02	10.9 ± 0.08	1.84 ± 0.03	1.88 ± 0.12	2.1 ± 0.04	2.1 ± 0.03
60	12.1 ± 0.1	13.3 ± 0.2	1.60 ± 0.05	1.53 ± 0.03	2.4 ± 0.2	2.3 ± 0.3
90	12.9 ± 0.1	16.2 ± 0.2	--	--	--	--

(t)* total amount of cyclic AMP accumulated (i.e., cell + medium)

(m)* amount of cyclic AMP accumulated in the medium only. Glycerol production is espressed as μmoles glycerol/g cells ± S.E. Cyclic AMP accumulation is expressed as nanomoles cyclic

AMP/g cells ± S.E. HGH (50 µg/ml) in the presence of theophylline was added to all tubes at zero time. Treated cells were digested with 250 µg/ml trypsin for 10 minutes and neutralized with a 500 µg/ml lima bean trypsin inhibitor before final incubation with hormone. Untreated cells were preincubated for the same amount of time before the addition of hormone.

TABLE 19

Effect of Various Concentrations of Trypsin on Fat Cells from Fasted Rabbits

Trypsin Concentration (mg/ml)	Glycerol Production*
0	12.7 ± 0.01
0.5	12.8 ± 0.06
1	12.3 ± 0.18
2	12.4 ± 0.2
4	12.3 ± 0.1

Rabbit fat cells (40-50 mg per tube) were incubated for 10 min with trypsin at the concentrations shown. Tryptic digestion was terminated by the addition of lima bean trypsin inhibitor (4 mg/ml). HGH was present at 9×10^{-7} M. Glycerol production is expressed as μmoles/g dry wt of cells/hour. The basal rate of glycerol production in the absence of HGH was 0.5 ± 0.04. Each value is the mean ± S. E. of 2 experiments.

TABLE 20

Effect of Duration of Trypsin Treatment on Fat Cells Isolated from Fasted Rabbits

Time (Min)	Glycerol Production
0	17.4 ± 0.2
5	17.4 ± 0.1
10	17.8 ± 0.1
20	18.4 ± 0.06
30	18.2 ± 0.05
60	18.1 ± 0.07

Fat cells from fasted rabbits were pretreated with trypsin (0.25 mg/ml) for various time periods. Tryptic digestion was terminated by the addition of lima bean trypsin inhibitor (0.1 mg/ml). HGH (9×10^{-7} M) was added to the cell suspension for final incubation. Basal stimulation without HGH was 0.75 ± 0.1 µmoles glycerol/g cells/hour.

TABLE 21

Comparison of Trypsin Effect on Fat Cells Isolated from Normal and Fasted Rabbits with 2 Different Concentrations of Bovine Serum Albumin

Trypsin Concentration (mg/ml)	Glycerol Production			
	Fed		Fasted	
	4% BSA	1% BSA	4% BSA	1% BSA
Basal	2.5 ± 0.05	2.4 ± 0.04	2.6 ± 0.7	2.7 ± 0.05
0	3.8 ± 0.09	3.5 ± 0.04	5.9 ± 0.02	5.7 ± 0.02
0.25	10.9 ± 0.09	2.9 ± 0.09	5.8 ± 0.3	5.8 ± 0.06
0.5	6.3 ± 0.2	2.5 ± 0.02	6.6 ± 0.03	5.9 ± 0.09
0.75	3.8 ± 0.05	2.4 ± 0.09	6.8 ± 0.06	6.0 ± 0.04
1.0	2.5 ± 0.02	2.3 ± 0.1	6.5 ± 0.04	5.5 ± 0.06

Rabbit fat cells (40-50 mg per tube) from normal and fasted rabbits were pretreated with various concentrations of trypsin in either 4% (W/V) or 1% BSA for 10 min. The action of trypsin was terminated by the addition of lima bean trypsin inhibitor (2 mg/ml). HGH (9×10^{-7} M) was present in all tubes except basal. Final incubation of fat cells after digestion in 1% (W/V) BSA was performed with Krebs Ringer bicarbonate buffer containing 4% BSA and 2 mg/ml lima bean trypsin inhibitor.

TABLE 22

Effect of Digestion of Fat Cells Isolated from Normal and Fasted Rabbits with Various Concentrations of Phospholipase C

Phospholipase C Concentration (mg/ml)	Glycerol Production	
	Fed	Fasted
Basal	0.33 ± 0.07	1.123 ± 0.04
0	27.7 ± 0.84	26.85 ± 0.74
0.08	27.04± 1.12	26.11 ± 0.68
0.31	25.54± 1.30	25.19 ± 0.13
1.25	19.47± 1.74	17.97 ± 1.5
5	2.77 ± 0.99	2.19 ± 0.45

Isolated fat cells (40-50 mg per tube) from normal and fasted rabbits were treated with various concentrations of phospholipase C from Cl. Welchii (sp. act. 10 U/mg) for 5 min. The cells were washed twice with 20 volumes of fresh Krebs Ringer bicarbonate buffer containing 4% (W/V) albumin before the final incubation with hormone. HGH (4.5×10^{-6} M) was present in fat cells from normal rabbits; HGH (9×10^{-7} M) was present in fat cells from fasted rabbits; theophylline (32×10^{-6} M) was present in all incubation tubes.

TABLE 23

Lipolysis of Rabbit Fat Cells Pretreated with Neuriminidase

Neuraminidase Concentration (µg/ml)	Glycerol Production Time	
	30 Min	60 Min
	In the Absence of HGH	
0	0.98 ± 0.06	0.92 ± 0.05
10	0.72 ± 0.1	0.81 ± 0.09
25	0.84 ± 0.02	0.92 ± 0.02
50	0.91 ± 0.04	0.94 ± 0.01
100	0.73 ± 0.05	0.99 ± 0.07
	In the Presence of HGH	
0	18.10 ± 0.1	18.24 ± 0.13
10	20.42 ± 0.14	21.35 ± 0.11
25	25.72 ± 0.09	27.42 ± 0.07
50	20.51 ± 0.02	12.92 ± 0.39
100	13.93 ± 0.08	7.64 ± 0.06

Rabbit fat cells (40-50 mg per tube) were digested with various concentrations of neuraminidase for 30 min or 60 min. The digestion was stopped by washing the cells twice with 20 volumes of Krebs Ringer bicarbonate buffer containing 4% albumin and 0.1 mg per ml lima bean trypsin inhibitor before final incubation in the absence or presence of HGH (9×10^{-7} M). Theophylline (32×10^{-6} M) was present in all incubation tubes.

Lipolysis was expressed as µmoles glycerol/g dry wt cells/ hour ± S.E.

TABLE 24

Effect of Different Preparations of C. Perfringens Neuraminidase on the Subsequent Lipolytic Response of HGH in Isolated Fat Cells

HGH Concentration	Glycerol Production			
(10^{-6} M)	No Neuraminidase Treatment	Neuraminidase (sp. act. 0.03 U/mg)	Neuraminidase (1.13 U/mg)	Neuraminidase (2.5 U/mg)
0	0.38 ± 0.06	0.53 ± 0.03	0.45 ± 0.1	0.60 ± 0.02
0.07	0.86 ± 0.01	12.26 ± 0.07	11.52 ± 0.12	11.88 ± 0.14
0.22	5.21 ± 0.06	18.79 ± 0.04	20.24 ± 0.09	19.91 ± 0.02
0.66	15.89 ± 0.04	22.41 ± 0.06	24.18 ± 0.12	24.15 ± 0.12
1.98	22.07 ± 0.03	24.25 ± 0.04	25.69 ± 0.05	25.83 ± 0.12
5.94	26.23 ± 0.24	25.86 ± 0.09	25.86 ± 0.09	26.12 ± 0.22

Three different preparations of C. perfringens neuraminidase with different specific activity (0.03 U/mg; 1.13 U/mg; 2.4 U/mg) were incubated with fat cells for 60 minutes at 37°. The amount of neuraminidase added from each preparation was 0.75 x 10^{-3}U/ml. After

preincubation with neuraminidase, the cells were washed twice with 20 volumes of fresh Krebs Ringer bicarbonate buffer containing 4% (W/V) albumin, and 0.1 mg/ml lima bean trypsin inhibitor before the final incubation with HGH.

TABLE 25

Lipolysis of Fat Cells from Fasted Rabbits Digested Sequentially with Trypsin and Neuraminidase

Neuraminidase Concentration	Trypsin Concentration	
	0 µg/ml	200 µg/ml
In the absence of HGH		
0 µg/ml	0.56 ± 0.08	0.59 ± 0.07
25 µg/ml (1)	0.61 ± 0.09	0.49 ± 0.04
25 µg/ml (2)	----	0.52 ± 0.05
In the presence of HGH		
0 µg/ml	14.31 ± 0.03	14.99 ± 0.34
25 µg/ml (1)	23.71 ± 0.03	22.3 ± 0.21
25 µg/ml (2)	----	15.16 ± 0.31

Fat cells (40-50 mg per tube) were digested with trypsin (200 µg/ml) for 10 min or neuraminidase (25 µg/ml) for 1 hour. (1) indicates cells treated with trypsin (200 µg/ml) for 10 min first before neuraminidase (2 µg/ml) digestion for 1 hour. (2) indicates the

reversed order of treatment. Tryptic digestion was stopped by adding lima bean trypsin inhibitor (0.4 mg/ml), and neuraminidase actin was stopped by washing cells twice with Krebs Ringer bicarbonate buffer containing 4% (W/V) albumin. HGH was present at 2.3×10^{-7} M and theophylline at 32×10^{-6} M. Lipolysis was expressed as µmoles glycerol/g cells/hour $\pm$ S. E.

Figure 1. Chromatography of BGH (L3512A) on QAE-Sephadex A25.

50 mg of BGH was chromatographed on QAE-Sephadex A25 (1.3 x 60 cm) column equilibrated with 0.01 M Tris HCl pH 8.4. Elution was carried out with a linear gradient of NaCl (0-0.5 M). Fractions collected was 3 ml per tube, and flow rate was 50 ml per hour. Fraction 1 and Fraction 2 were pooled as indicated, dialyzed extensively against distilled water and lyophilized.

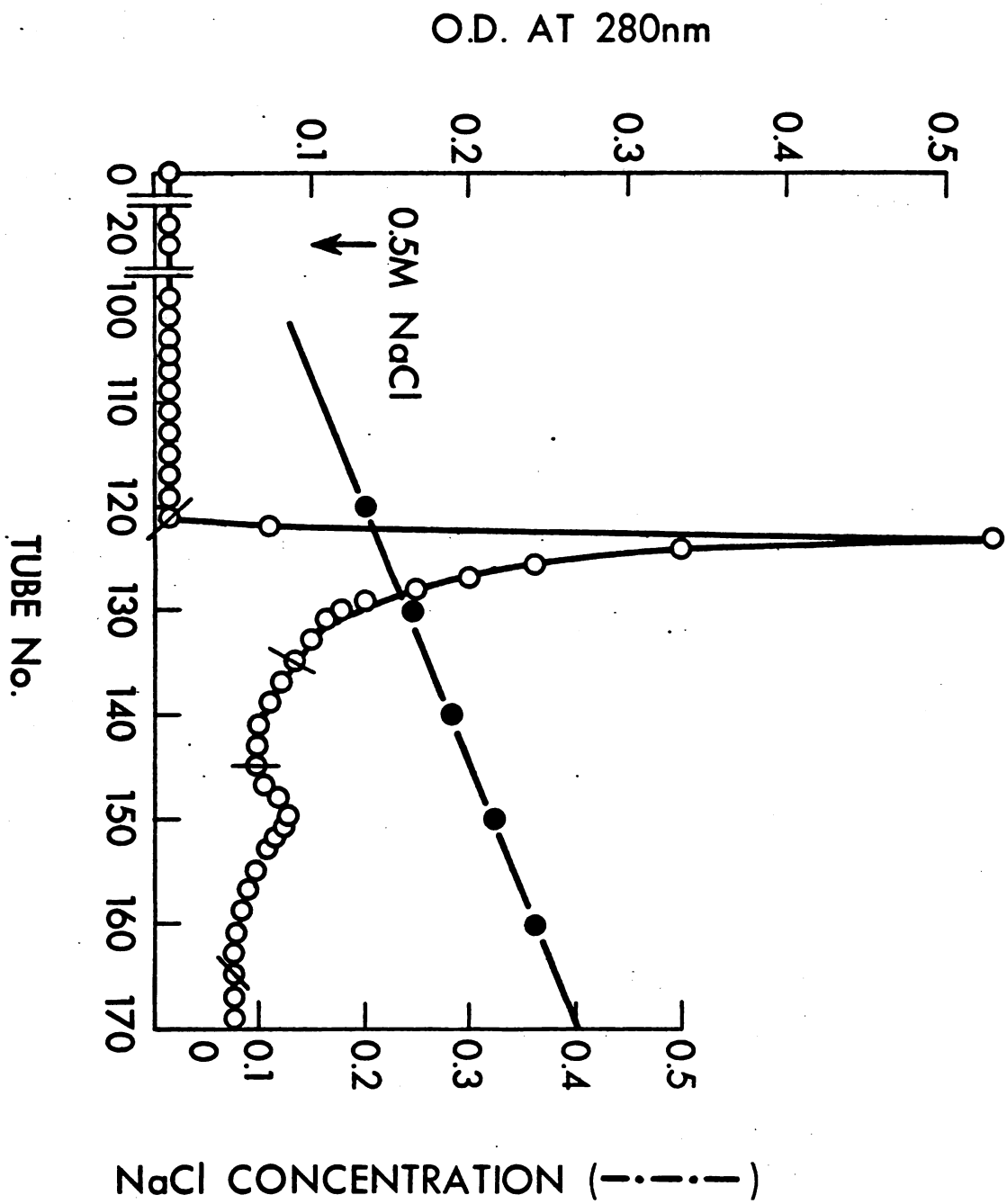

O.D. AT 280nm
0.1
0.2
0.3
0.4
0.5
0.5M NaCl
0
20
100
110
120
130
140
150
160
170
TUBE No.
0
0.1
0.2
0.3
0.4
0.5
NaCl CONCENTRATION (—·—·—)

Figure 1

Figure 2. Chromatography of BGH-Fraction 1 on Sephadex G100

BGH-Fraction 1 (12 mg) was chromatographed on Sephadex G100 column (Vo = 90 ml) equilibrated with 0.1 M Tris HCl pH 8.4. Elution was carried out with the same buffer. Volume collected in each tube was 3.1 ml and flow rate was 40 ml per hour. Ve/Vo was calculated to be 1.67.

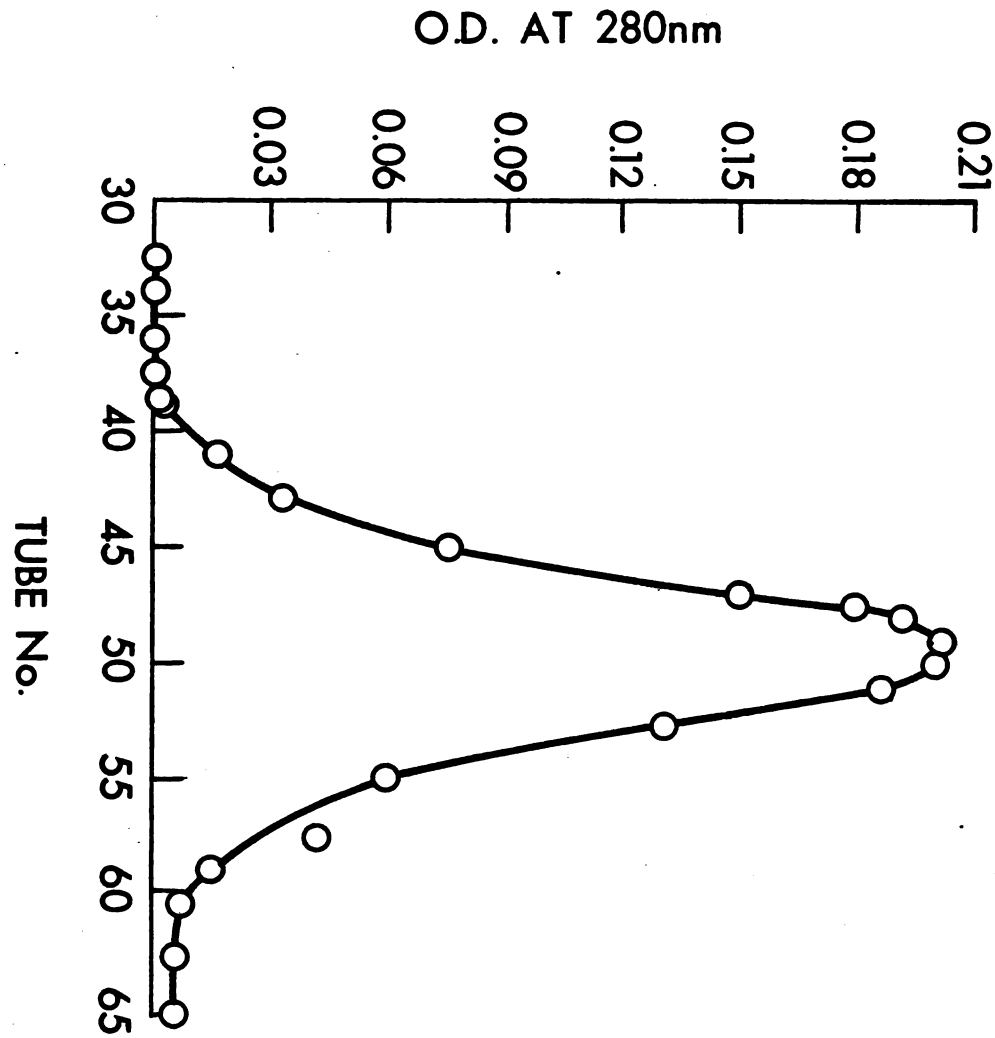

O.D. AT 280nm
0.03
0.06
0.09
0.12
0.15
0.18
0.21
30
35
40
45
50
55
60
65
TUBE No.

Figure 2

Figure 3. Effect of Trypsin on HGH and ACTH in Stimulating Rabbit Fat Cells Lipolysis

O——O HGH (4.5×10^{-6} M) and O——O ACTH (0.03×10^{-6} M) were treated with various concentrations of trypsin for 5 minutes. The reaction was stopped by the addition of 0.1 mg/ml of lima bean trypsin inhibitor. Isolated rabbit fat cells (40-50 mg/ml) was then added for final incubation. Details of incubation and glycerol analysis were described under "Experimental Procedure." The basal stimulation without any hormone was 1.7 ± 0.2. Each value is the mean $\pm$ S.E. of 3 experiments.

Figure 3

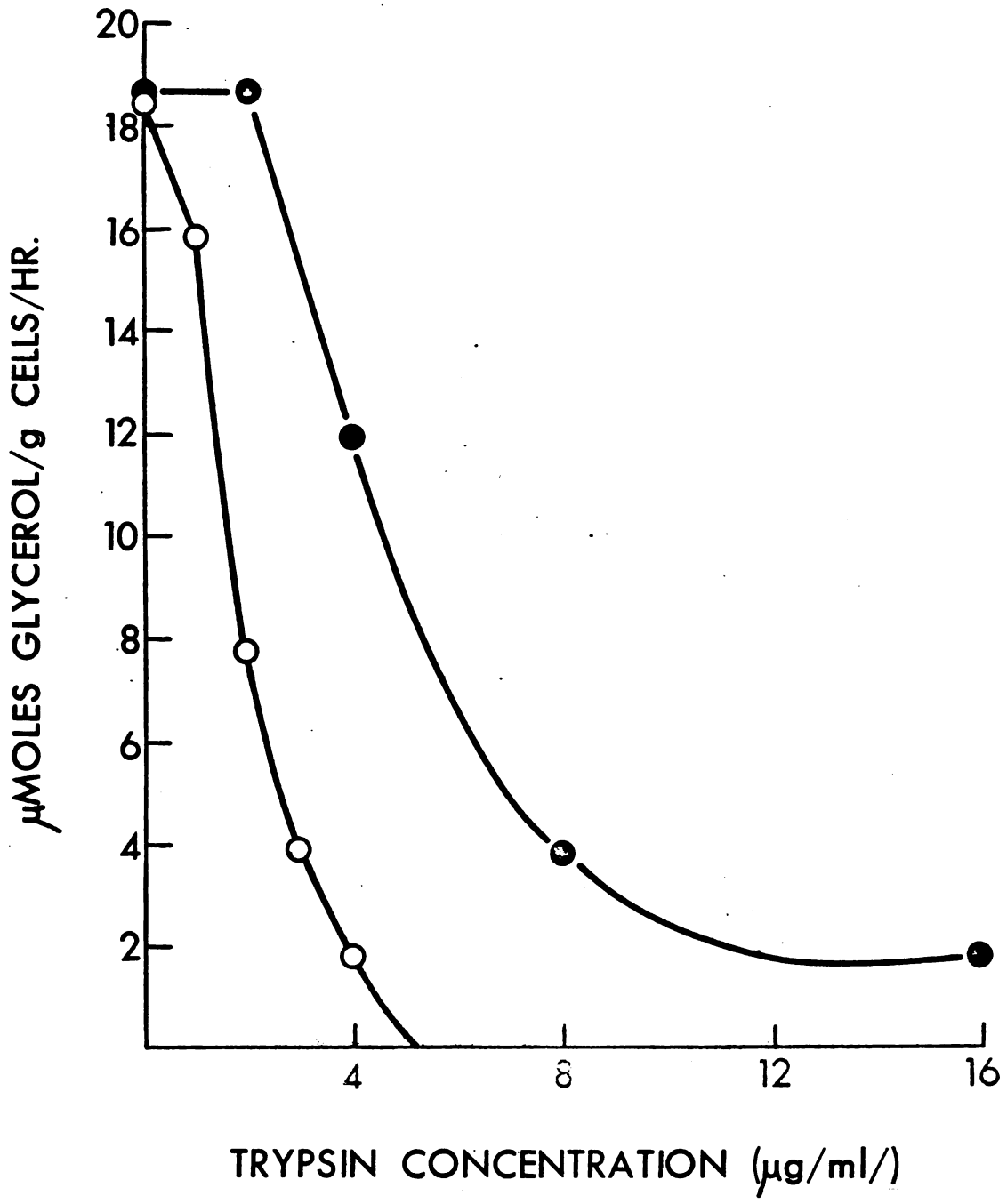

20
18
16
14
12
10
8
6
4
2
μMOLES GLYCEROL/g CELLS/HR.
4
8
12
16
TRYPSIN CONCENTRATION (μg/ml/)

Figure 4A,B. Effect of Theophylline on the Stimulation of Lipolysis (A) and Cyclic AMP Accumulation (B) in Isolated Rabbit Fat Cells by HGH

Theophylline was present at a concentration of 3.2×10^{-5} M. The basal rate of cyclic AMP accumulation in the absence and presence of theophylline was 0.4 and 1.6 pmoles/g cells 20 min respectively. The basal rates of glycerol release were 1.6 and 1.8 µmoles/g cells/hour in the presence and absence of theophylline respectively; ---, absence of theophylline; ——, presence of theophylline.

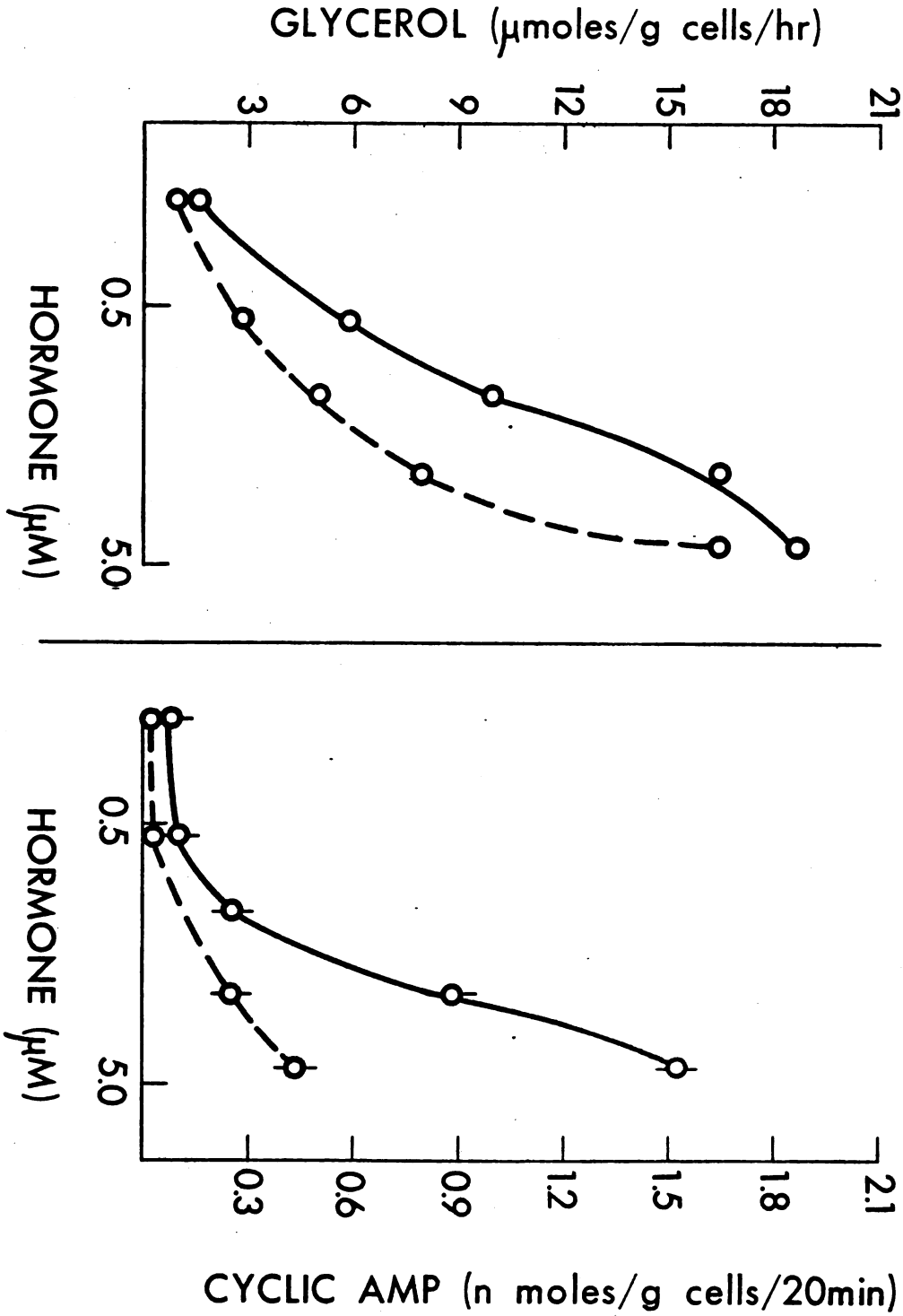


Figure 4A

Figure 4B

Figure 5. Glycerol Release and Cyclic AMP Accumulation as a Function of Time

Isolated rabbit fat cells (40-50 mg per tube) were incubated for various times with 2.3×10^{-6} M HGH and 3.2×10^{-6} M theophylline. Incubation procedure and cyclic AMP analysis have been described under "Experimental Procedure."

●——● Glycerol accumulation in the medium

O---O Total cyclic AMP (cells and medium)

O——O Cyclic AMP accumulation in the medium alone.

Figure 5

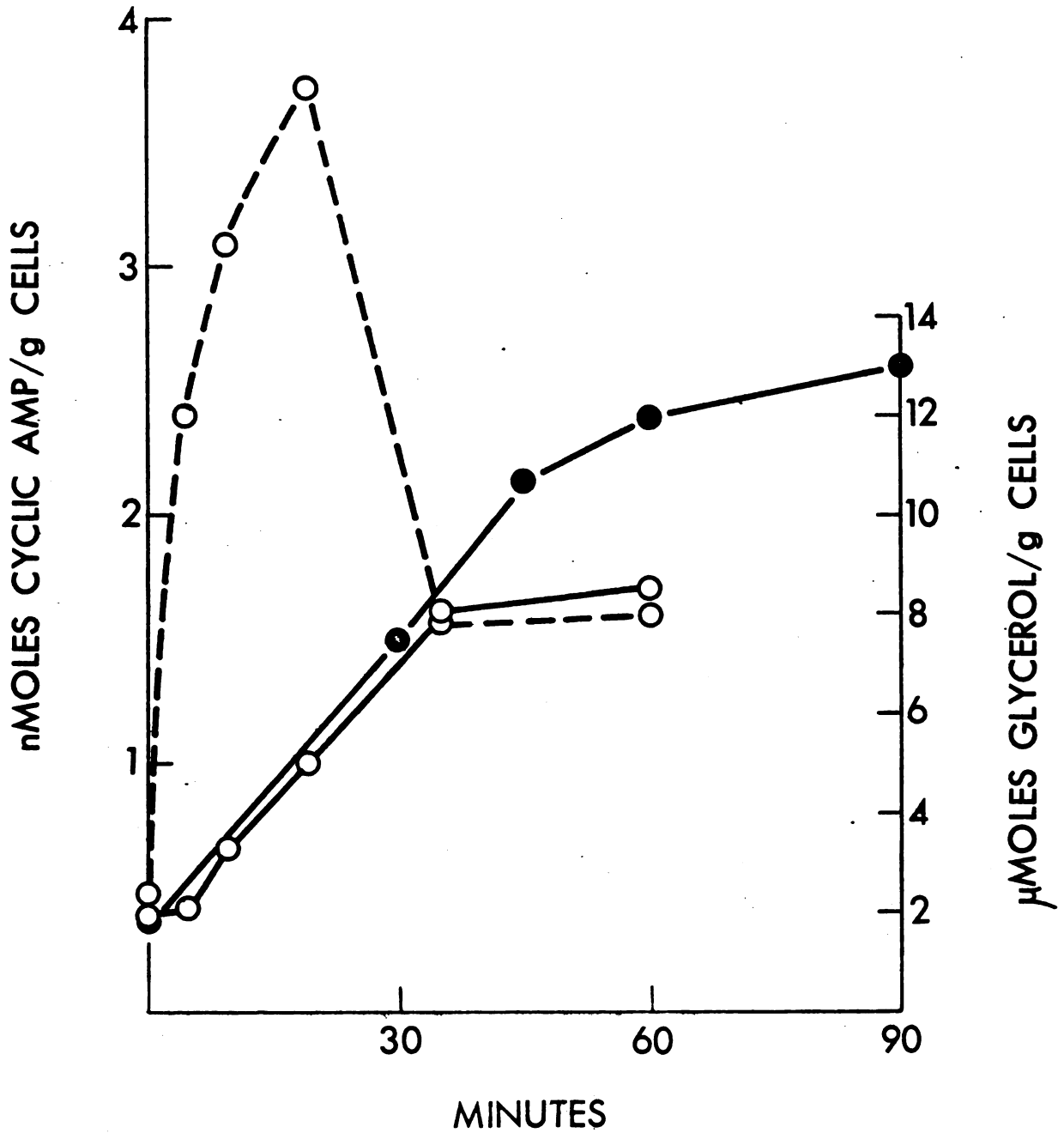

4
3
2
1
14
12
10
8
6
4
2
30
60
90
MINUTES
nMOLES CYCLIC AMP/g CELLS
μMOLES GLYCEROL/g CELLS

Figure 6. Comparison of Dose-Response Relationships in Isolated Fat Cells from Fed and Fasted Rabbits

Fat cells were either isolated from fed rabbits or rabbits which had been fasted for 48 hours. Incubation procedure and glycerol analysis have been described under "Experimental Procedure."

●---● Dose-response curve of fat cells from fed rabbits.

O——O Dose-response curve of fat cells from fasted rabbits.

Δ---Δ Dose-response curve of fat cells from fed rabbits in the presence of theophylline (32×10^{-6} M).

□——□ Dose-response curve of fat cells from fasted rabbits in the presence of theophylline (32×10^{-6} M).

Figure 6

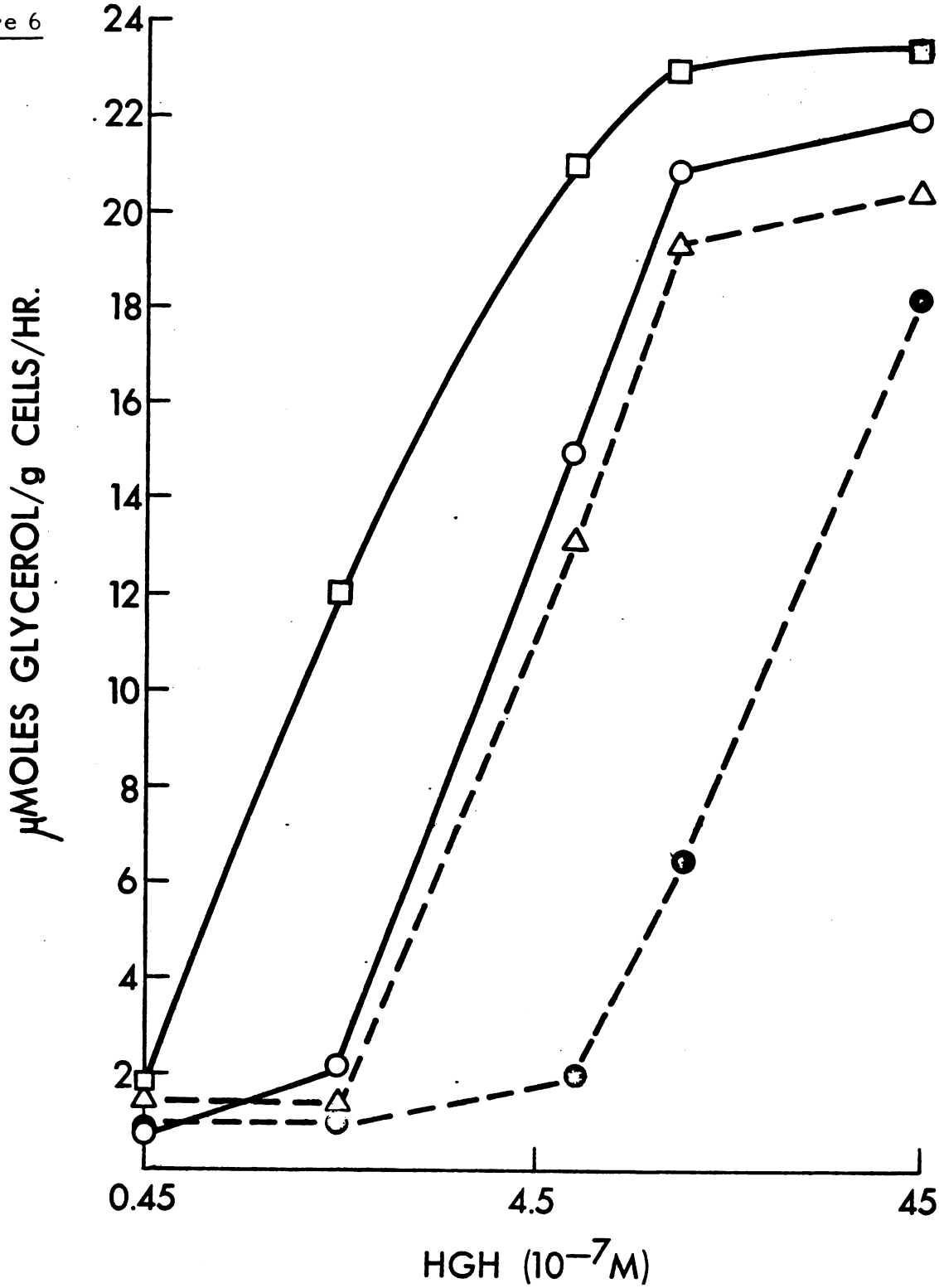

24
22
20
18
16
14
12
10
8
6
4
2
μMOLES GLYCEROL/g CELLS/HR.
0.45
4.5
45
HGH (10⁻⁷M)

Figure 7. Effect of Various Trypsin Concentrations on Rabbit Fat Cells

Fat cells (40-50 mg/ml) were incubated with various concentrations of trypsin for 10 minutes at 37° with constant shaking. Tryptic digestion was terminated by the addition of lima bean trypsin inhibitor (2 mg/ml). HGH (2.3×10^{-7} M) was added in the absence (O---O) or presence (▫--▫) of theophylline (32×10^{-6} M) and allowed to incubate for 1 hour. Details of incubation and glycerol analysis were described under "Experimental Procedure."

●——● Basal stimulation of treated fat cells

▫——▫ Basal stimulation of treated fat cells with theophylline (32×10^{-6} M) only.

Figure 7

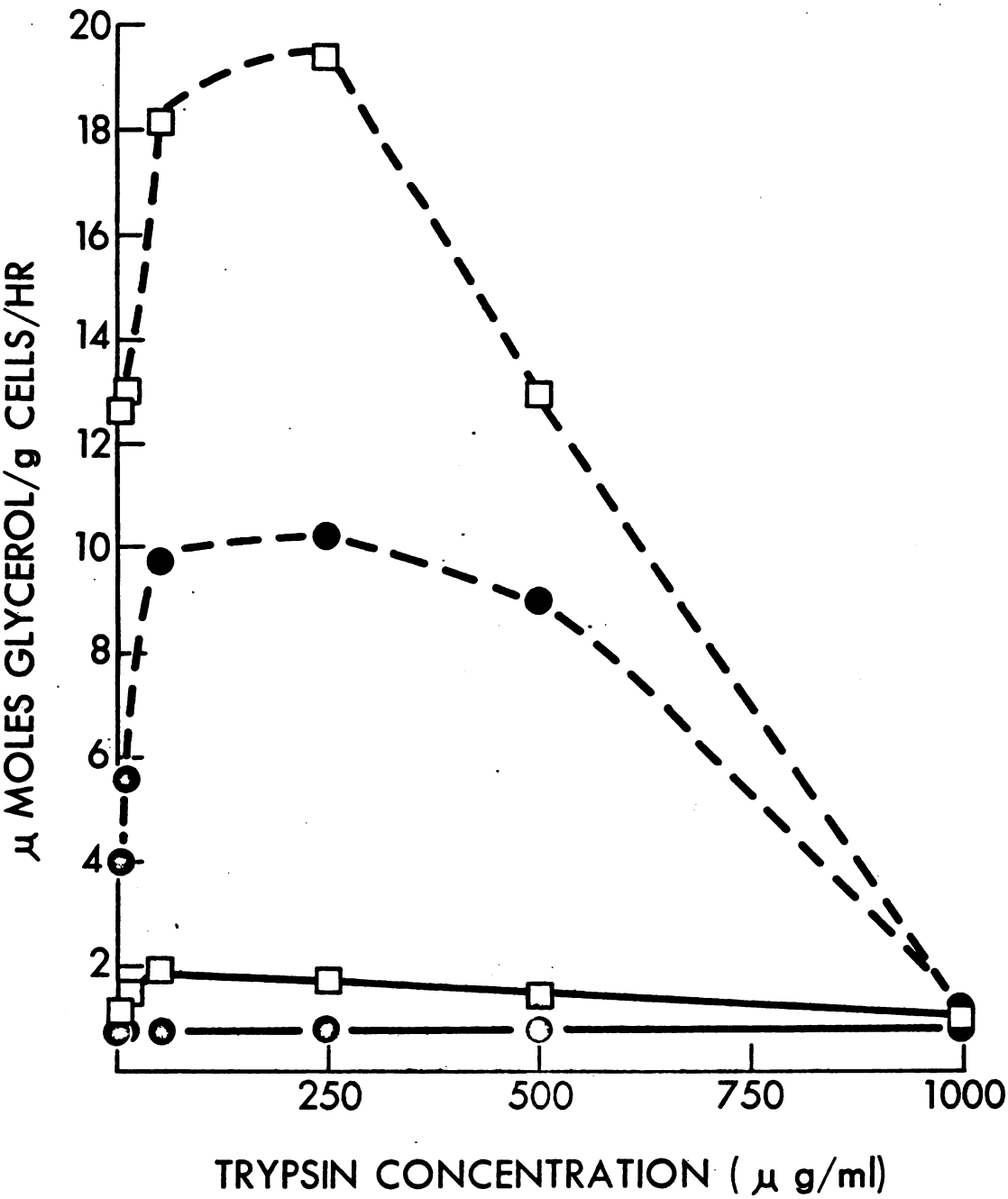

20
18
16
14
12
10
8
6
4
2
μ MOLES GLYCEROL/g CELLS/HR
250
500
750
1000
TRYPSIN CONCENTRATION (μ g/ml)

Figure 8. Effect of Trypsin Pretreatment for Various Time Periods on Rabbit Fat Cells

Rabbit fat cells (40-50 mg per tube) were treated for various time periods with trypsin (250 µg/ml) at 37° with constant shaking. Digestion was stopped by the addition of lima bean trypsin inhibitor (0.5 mg/ml). The cells were incubated for 1 hour in the absence (●——●) or presence (O——O) of HGH (9 x 10^{-7} M) at 37°. Incubation procedure and glycerol analysis were essentially that described under "Experimental Procedure."

Figure 8

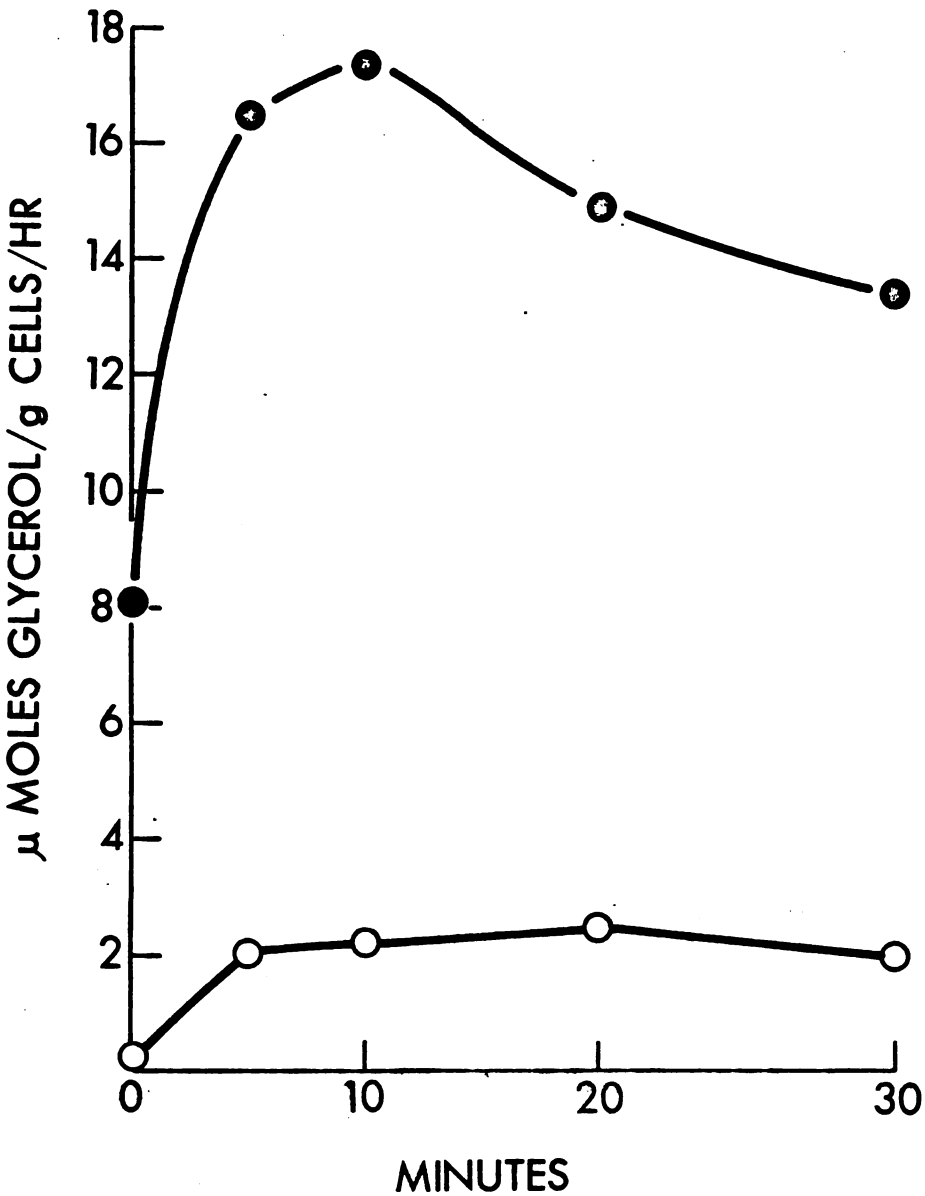

18
16
14
12
10
8
6
4
2
0
10
20
30
μ MOLES GLYCEROL/g CELLS/HR
MINUTES

Figure 9. Effect of Trypsin Pretreatment on Dose Response Relationship of HGH in the Presence or Absence of Theophylline

Rabbit fat cells (40-50 mg per tube) were pretreated with trpsin (250 µg/ml) for 10 minutes at 37° with constant shaking. After termination of trypsin activity with lima bean trypsin inhibitor (0.5 mg/ml), the cells were incubated with various doses of HGH in the absence (O——O) or the presence (O---O) of theophylline (32 x 10^{-6} M). Untreated cells were also incubated with HGH in the absence (●——●) or presence (●---●) of theophylline (32 x 10^{-6} M). Incubation procedure and glycerol analysis have been described under "Experimental Procedure."

Basal stimulation in the absence of hormone was about 0.9 µmoles glycerol/g cells/hour.

Figure 9

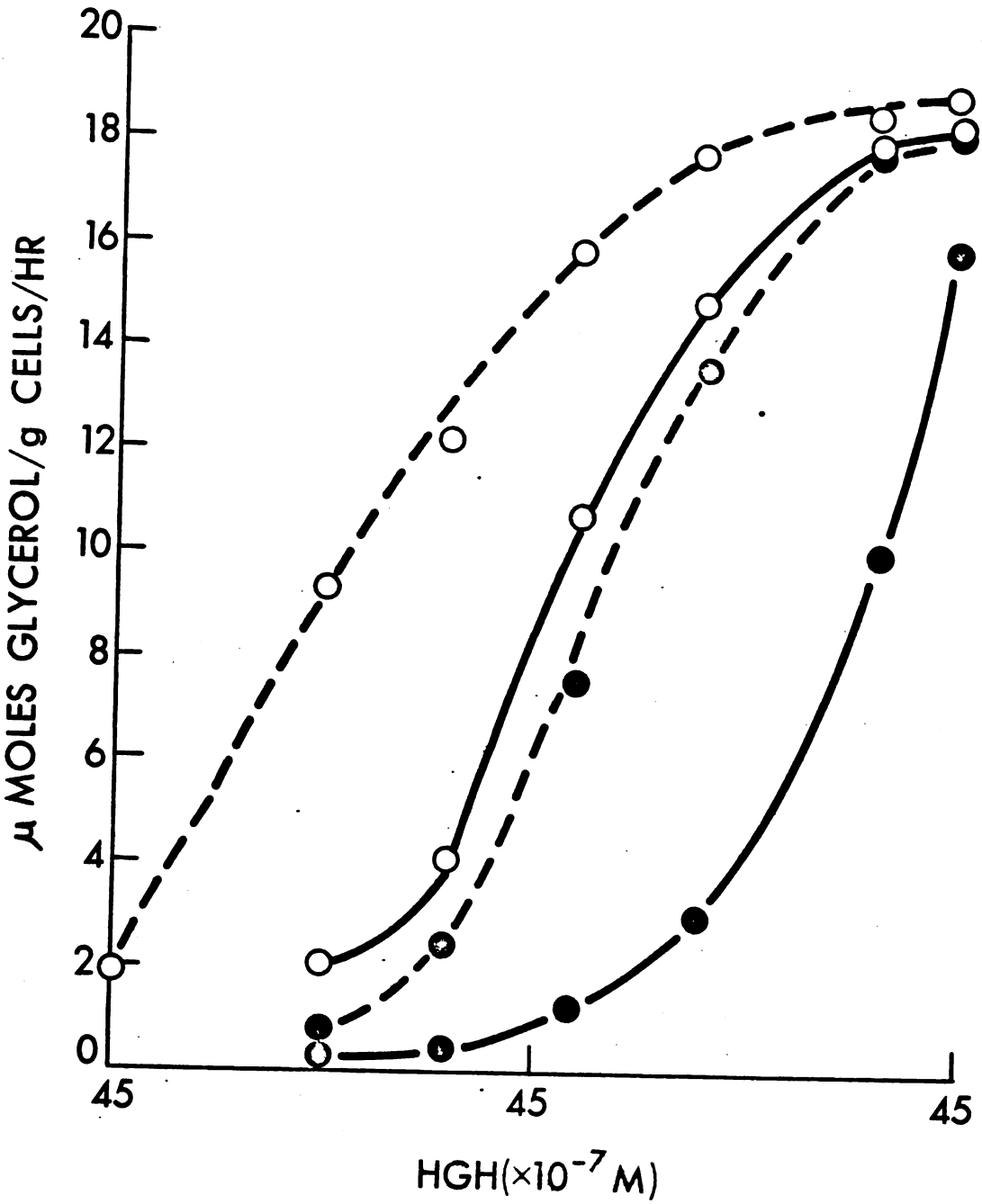

20
18
16
14
12
10
8
6
4
2
0
μ MOLES GLYCEROL/g CELLS/HR
45
45
45
HGH(×10⁻⁷ M)

Figure 10. Comparison of Trypsin Pretreated in Fat Cells from Normal and Fasted Rabbits

White fat cells from normal or fasted rabbits were treated with trypsin (250 µg/ml) for 10 minutes at 37°. Tryptic digestion was terminated by the addition of lima bean trypsin inhibitor (0.5 mg/ml). HGH at various concentrations were added to untreated fat cells from normal rabbits (●——●) and fasted rabbits (O---O) and trypsin-treated fat cells from normal (Δ——Δ) and fasted rabbits (□--□). Glycerol release was determined as described under "Experimental Procedure."

Figure 10

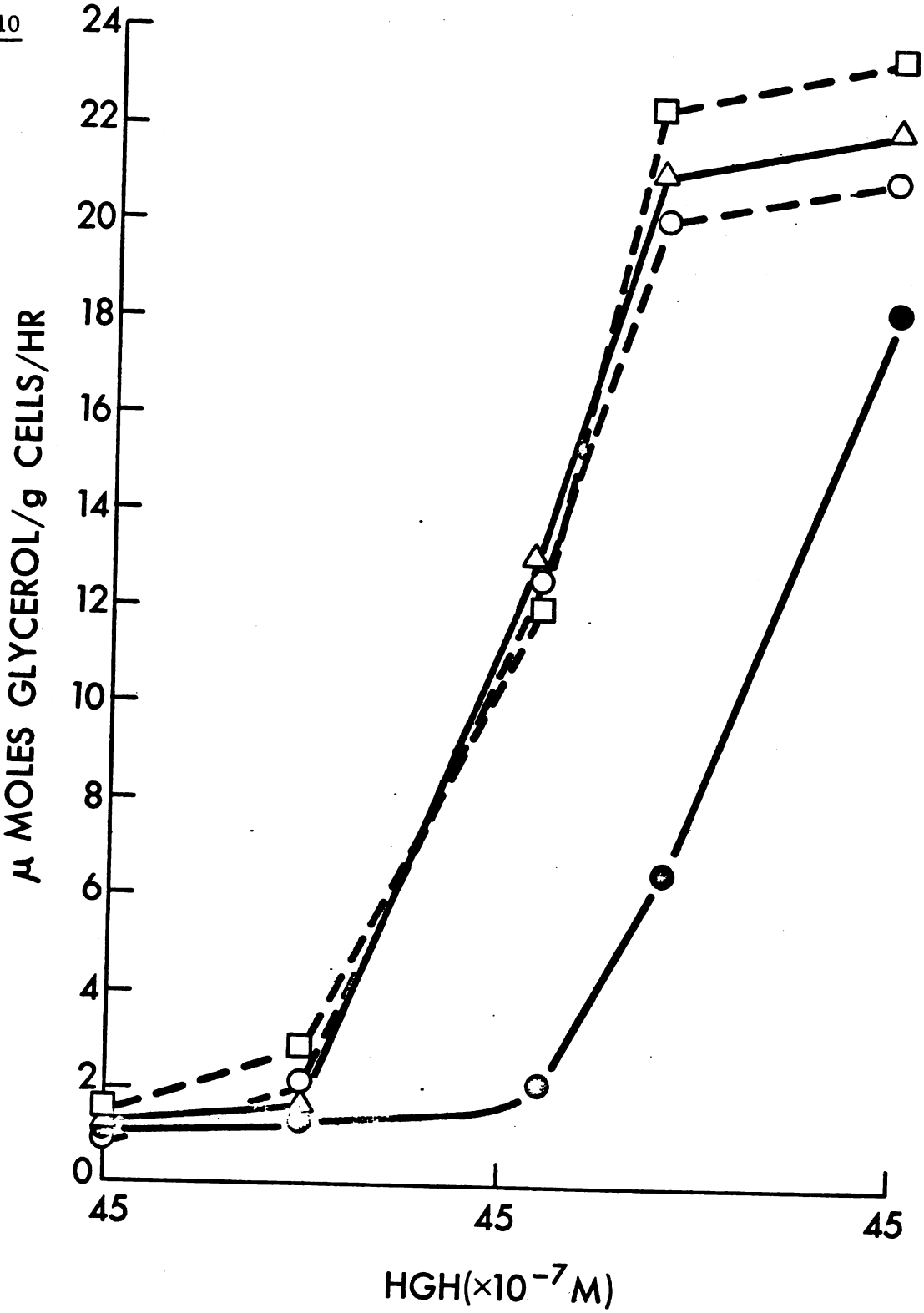

24
22
20
18
16
14
12
10
8
6
4
2
0
μ MOLES GLYCEROL/g CELLS/HR
45
45
45
HGH(×10⁻⁷M)

Figure 11. Time Course of Neuraminidase Treatment in the Subsequent Lipolytic Response to HGH in Isolated Fat Cells from Normal Rabbits

Isolated rabbit fat cells (40-50 mg/tube) were incubated with neuraminidase (25 μg/ml) for various times. The cells were washed twice with 20 volumes of fresh Krebs Ringer bicarbonate buffer before final incubation with HGH. Theophylline (32×10^{-6} M) was present in all tubes.

□---□ No neuraminidase treatment in response to HGH (6.6×10^{-7} M).

O---O Neuraminidase treated cells in response to HGH (6.6×10^{-7} M).

●——● Sialic acid release.

Δ—— Δ Basal stimulation of neuraminidase treated cells.

Figure 11

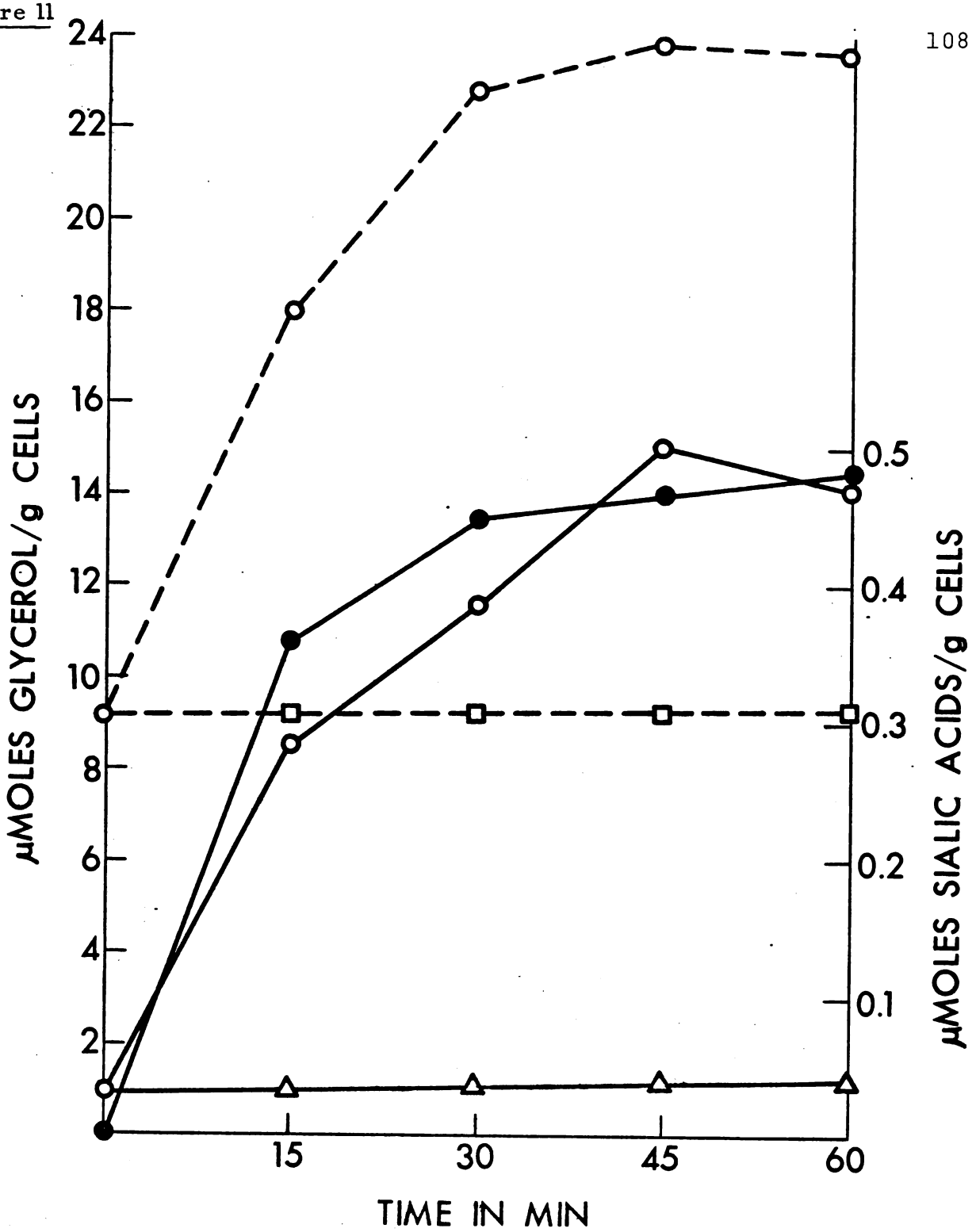

24
22
20
18
16
14
12
10
8
6
4
2
μMOLES GLYCEROL/g CELLS
0.5
0.4
0.3
0.2
0.1
μMOLES SIALIC ACIDS/g CELLS
15
30
45
60
TIME IN MIN

Figure 12. Effect of Neuraminidase Treated Cells on the Subsequent Dose Response Relationship to HGH in Isolated Fat Cells From Normal Rabbits

Rabbit fat cells were treated with C. perfringens neuraminidase (25 µg/ml; specific activity of 0.03 unit/mg) for 60 minutes. The cells were washed twice with 20 volumes of fresh Krebs Ringer bicarbonate buffer containing 4% albumin and 0.1 mg/ml lima bean trypsin inhibitor before final incubation with various doses of HGH. Theophylline (32×10^{-6} M) was present in all tubes

O——O No neuraminidase treatment

O---O Neuraminidase treated cells.

Figure 12

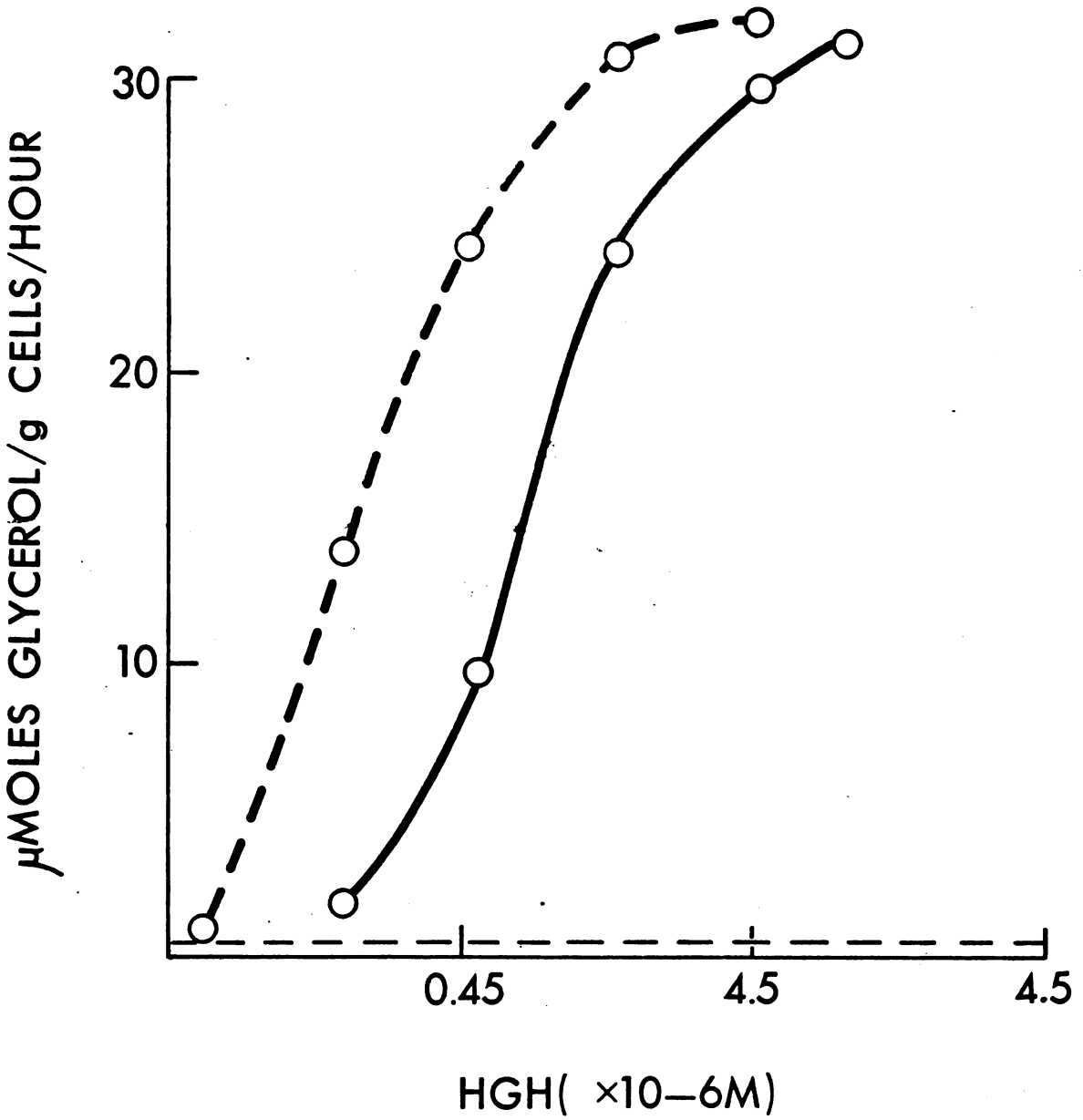
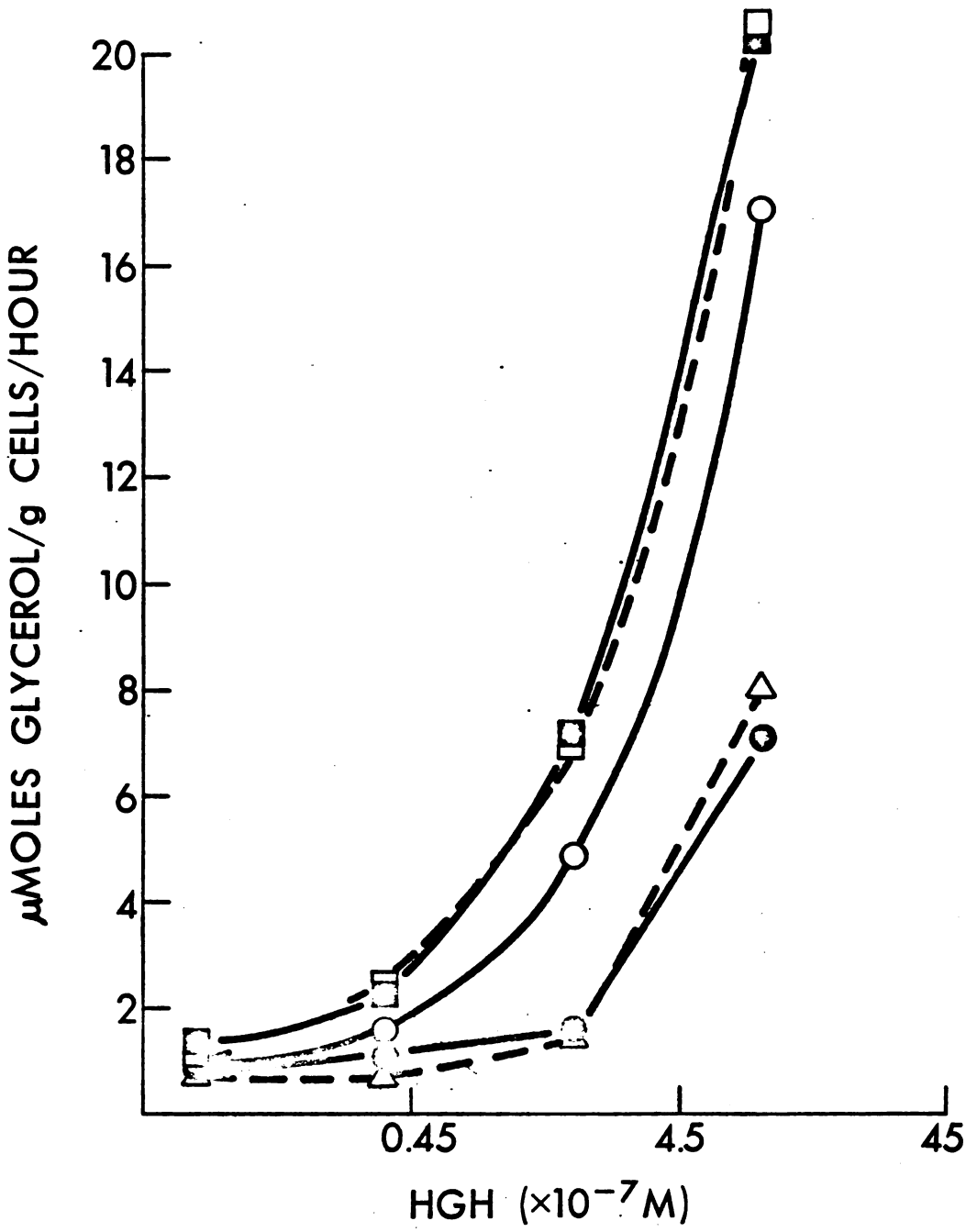


Figure 13. Effect of Digestion of Isolated Fat Cells with Neuraminidase and Trypsin on the Subsequent Lipolytic Response to HGH

Fat cells (40-50 mg/ml) were incubated without either enzyme (●——●); with trypsin (200 µg/ml) for 10 minutes (O——O); with neuraminidase (25 µg/ml, specific activity 0.03 units per mg) for 60 minutes (▣——▣); with trypsin (200 µg/ml) for 10 minutes, then neuraminidase (25 µg/ml) for 60 minutes (■--■) and neuraminidase (25 µg/ml) for 60 minutes, then trypsin (25 µg/ml)for 10 minutes Δ--- Δ in Krebs Ringer bicarbonate buffer containing 4% albumin at 37°. Trypsin action was stopped by the addition of lima bean trypsin inhibitor (0.2 mg/ml) and neuraminidase action by washing the cells twice with 20 volumes of fresh buffer before final incubation with HGH. Details of incubation and glycerol analysis were described under "Experimental Procedure."

Figure 13


20
18
16
14
12
10
8
6
4
2
μMOLES GLYCEROL/g CELLS/HOUR
0.45
4.5
45
HGH (×10⁻⁷M)

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