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# HORMONE-ACTIVATED LIPOLYSIS AND CYCLIC 3'5' ADENOSINE MONOPHOSPHATE


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Thesis submitted for the degree of Doctor of Medicine

December 1967

(ii)

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# Introduction

Growth hormone has been reported to produce a wide variety of seemingly unrelated metabolic effects on adipose tissue. When the hormone is injected into the intact animal there is prompt mobilization of adipose tissue stores with a concomitant increase in the level of circulating plasma free fatty acids. In contrast to these *in vivo* findings, *in vitro* studies using physiological concentrations of growth hormone have frequently failed to demonstrate a lipolytic effect.

The present study was undertaken to assess the characteristics of the action of growth hormone on various metabolic parameters in adipose tissue, and to examine the relationship of hormone-activated lipolysis to changes in glucose and fatty acid metabolism. Due to the similarities between growth hormone and human placental lactogen ( a placental peptide circulating in maternal plasma during pregnancy ), the metabolic effects of this latter peptide were also examined.

*In vitro* observations that adipose tissue lipose was activated by cyclic 3'5' adenosine monophosphate led to an assessment of the role of this cyclic nucleotide as a mediator for the hormonal control of lipolysis *in vivo*. The intracellular concentration of cyclic 3'5' adenosine monophosphate was raised by the injection of theophylline, a methyl xanthine which inhibits the degradation of cyclic 3'5' adenosine monophosphate by 3'5' cyclic nucleotide phosphodiesterase.

The injection of theophylline into rats produced a prompt increase in the plasma free fatty acid concentration, but was also noted to cause an immediate outpouring of insulin from the pancreas. The evidence suggested that cyclic 3'5' adenosine monophosphate was involved, not only in the activation of lipolysis, but also as a mediator of insulin release. To determine the definite role of this cyclic nucleotide it was necessary to develop a new sensitive assay to permit measurement of cyclic 3'5' adenosine monophosphate in microgram quantities of tissue.

Subsequently the role of cyclic 3'5' adenosine monophosphate, as a mediator of the cellular response to hormone action, was examined in three tissues - rat adipose tissue, pancreatic islets, and toad bladders. The specific responses of these tissues to certain hormones were correlated with the concentration of cyclic 3'5' adenosine monophosphate and the mechanisms of these responses were evaluated.

## Review of Published Data

Growth Hormone. Since the early experiments of Welt and Wilhelmi in 1950 (1), numerous metabolic actions have been reported for growth hormone. Most confusing is the variety of metabolic effects on adipose tissue. Using large doses of hormone, Jungas and Ball in 1960 (2) and Leboeuf and Cahill in 1961 (3) have demonstrated that growth hormone simulated the action of insulin on the rat epididymal fat pad *in vitro*, as reflected by accelerated rate of glucose uptake, oxidation and incorporation into fatty acids. More recently, in adipose tissue from hypophysectomized rats maintained on a high carbohydrate, fat-free diet, Goodman (4,5) demonstrated similar insulin-like changes with physiological concentrations of growth hormone (as little as 10 mμg/ml). In the studies by all three groups the duration of incubation of the tissues was limited to 1-3 hours, and no fat mobilizing activity was demonstrated.

In support of these *in vitro* studies are the *in vivo* reports of Knobil et al in 1961 (6) and Huggins and Ottaway in 1961 (7) in which growth hormone was shown to produce transient insulin-like effects immediately after injection. Within 30 minutes of injection into man or animals, there was a fall in the plasma glucose and free fatty acid concentration followed by a gradual rise to pre-injection levels over the next 30-45 minutes.

In contrast to these insulin-like actions of growth hormone is the decrease in lipogenesis from glucose, pyruvate and acetate reported by Winegrad et al (8,9), subsequently confirmed by Orth et al (10). These <u>in vitro</u> studies required 30-1000 µg of growth hormone / per millilitre of incubation medium. Consistent with decreased lipogenesis are the well demonstrated adipokinetic and insulin antagonistic effects observed in the intact animal several hours after injection of growth hormone. This lipid mobilizing action and the consequent elevation of incubating plasma free fatty acids have been demonstrated in fasting dogs (11), humans (12), rats (13-15), monkeys (16) and more recently in guinea pigs (17). The response in different species is variable. In most cases there is a delay of 1-3 hours after either intramuscular or intravenous administration of the hormone before the plasma free fatty acid concentration rises. However, in the fasting monkey there is a prompt early rise in the circulating plasma free fatty acid level after an intramuscular injection of 0.05 mg of growth hormone/Kg (16). It is perhaps possible that this prompt response is due to epinephrine release as a result of excitation of the animal.

It has been difficult to demonstrate direct lipolytic activity of growth hormone added to adipose tissue <u>in vitro</u> unless extremely large concentrations were used (12,17-19),

but Knobil in 1959 (20), and White and Engel in 1959 (21) showed in normal rats, that fat pads removed 3-24 hours after the injection of growth hormone into the intact animal exhibited an accelerated rate of lipolysis when incubated in vitro. Furthermore, in 1963 and 1965, using hypophysectomized rats maintained on a high carbohydrate, fat-free diet, Goodman (22,23) demonstrated decreased glucose uptake, decreased oxidation of glucose-U-$^{14}$C and impaired lipogenesis in fat pads removed 3-48 hours after injection of relatively small amounts of growth hormone. Similar inhibition of glucose uptake, utilization and incorporation into fatty acids has been reported by MacLeod (24) in fat pads removed from rats bearing a transplantable somatotrophin-secreting tumour. These findings are completely opposed to the insulin-like action produced by adding growth hormone to adipose tissue incubated in vitro.

Recently however, using the Rodbell preparation of isolated rat fat cells (25), Fain etal (26) have demonstrated that growth hormone, in concentrations approaching physiological levels, did accelerate lipolysis in vitro after an initial lag period.

The diversity of metabolic actions ascribed to growth hormone by these investigations indicated the necessity for a study of the temporal characteristics of growth hormone action on adipose tissue, together with an examination of the relationship of hormone-activated lipolysis to changes in glucose and fatty acid metabolism.

Human Placental Lactogen. A peptide produced by the normal placenta has been reported recently to be distinct from chorionic gonadotrophin. This material has been referred to by various investigators as human placental lactogen (27), chorionic growth protein (28), growth factor placental origin human * and purified placental protein (29). The peptide is present in human plasma only during pregnancy (30), and may be responsible for the lipolytic activity of pregnancy plasma (31). It increases in maternal plasma after about six weeks' gestation, reaches maximal levels at term and rapidly disappears after parturition (30). Human placental lactogen ( HPL ) shares with growth hormone certain physico-chemical properties such as size (29), amino acid composition (32) and migration characteristics on electrophoresis (33), as well as some immunological properties (27,30). When assayed against antisera to growth hormone, HPL gives a reaction of partial identity with growth hormone (27) and the two peptides cross-react in a radio-immunoassay system (27,30).

* FOOTNOTE: Growth factor placental origin human ( GFPOH ) was the term used by the Lederle Company for early placental protein fractions supplied to approved investigators.

The biological properties of HPL are also similar to those of growth hormone, although the potency of HPL is only approximately one thousandth the potency of growth hormone. Friesen demonstrated that HPL injected into hypophysectomized immature rats or adult female mice produced an increase in body weight (34). In vitro studies have shown that HPL increased the width of the tibial epiphyseal plate (28), stimulated the uptake of radioactive sulphate by rib cartilage (28,29) and increased protein synthesis (29). Furthermore in vivo, the injection of an ineffective amount of HPL potentiated the weight gain observed with a minimally effective dose of growth hormone (29). Similar HPL potentiation of the response to growth hormone in the tibial epiphyseal plate test had been noted by Josimovich and Atwood in 1964 (35).

Crude placental fractions were first noted by Ehrhardt in 1936 (36) to possess prolactin-like activity and this was confirmed later by Higashi (37), and Kurosaki (38). Josimovich and Brande (39) showed that HPL exhibited some prolactin-like activity and that this peptide was probably responsible for the activity seen with crude placental fractions. The lactogenic activity of HPL has since been confirmed by other workers (27,28,34).

Recently Grumbach et al (40) reported that the injection of HPL increased the level of circulating plasma free

fatty acids in children with hypopituitarism, although Beck et al (41) did not show any alteration in plasma free fatty acid concentration after administration of HPL either by intravenous infusion or intramuscular injection to normal male subjects. In animals an extensive series of experiments by Riggi et al (42) showed that either intramuscular injection (1-50 mg/Kg) or intravenous injection (10 mg/Kg) of HPL increased the plasma free fatty acid concentration in fasted rabbits six hours after administration. Similar findings were observed by these investigators using fasted monkeys (42). In all cases the pattern of elevation of plasma free fatty acid concentration was similar to that produced by growth hormone (43), although HPL was less potent. Similar experiments were attempted by Friesen (34) using rats, but no alteration in plasma free fatty acid concentration was noted.

Human placental lactogen circulates in the maternal plasma during pregnancy, appears at 6-8 weeks gestation and reaches maximal levels at term (30). At term the concentration reaches 2-5 μg/ml (41). Assuming a half-life of approximately 19 minutes, as has been observed (41) the placenta produces 2-3 g of HPL daily, yet no physiological role has yet been demonstrated for this peptide. It is confined entirely to the maternal circulation and cannot be detected in foetal plasma (30). The similarities between HPL and

growth hormone, together with its increasing concentration in the plasma during pregnancy, have led to considerable interest in the possible role of HPL as a mediator of certain physiologic and biochemical changes associated with pregnancy. During pregnancy there is a progressive rise in the plasma free fatty acid level (44-46), and a decreased insulin sensitivity (41,44) which remain unexplained. The present study was extended to survey further the properties of HPL and specifically to determine whether it exerts a lipid mobilizing effect on adipose tissue.

## Outline of the Problem

In view of the conflicting data on the adipokinetic effects of growth hormone, the metabolic actions of this peptide hormone and the similar peptide, human placental lactogen, were examined on adipose tissue. A suitable preparation was required to exclude the variations which occur in studies using whole tissue such as the rat epididymal fat pad. For this reason the isolated rat fat cell preparation of Rodbell (25) was used, and this permitted accurate, reproducible estimations of the dynamic changes in adipose tissue metabolism during lipolysis.

Following observations that:

1. The primary effect of both growth hormone and placental lactogen was activation of lipolysis in isolated fat cells.

11. All other delayed actions on adipose tissue were secondary to primary activation of lipolysis and in view of recent reports that cyclic 3'5' adenosine monophosphate (cyclic 3'5' AMP) activates lipolysis <u>in vitro</u> (47), it was decided to investigate the role of this cyclic nucleotide in the physiologic control of lipolysis <u>in vivo</u>.

The <u>in vivo</u> infusion of theophylline, an antagonist of 3'5' cyclic nucleotide phosphodiesterase, caused a moderate rise in the circulating plasma free fatty acid concentration which fell to normal or subnormal levels 1-3 minutes after injection.

This fall in the plasma free fatty acid concentration coincided with a marked outpouring of pancreatic insulin. These findings suggested that the elevation of the intracellular level of cyclic 3'5' AMP produced by theophylline was implicated in both the activation of lipolysis and pancreatic insulin release in vivo.

To pursue these observations further it was necessary to develop a technique for the assay of cyclic 3'5' AMP suitable for the measurment of the cyclic nucleotide in extremely small amounts of tissue; for example a suspension of $3\text{-}5 \times 10^6$ fat cells or 100-200 pancreatic islets containing approximately 0.1 to 1.0 $\mu\mu$moles of cyclic 3'5' AMP. A satisfactory procedure was developed which increased the sensitivity at least one hundred fold over that obtained with earlier assay techniques.

Using the new procedure, the concentration of cyclic 3'5' AMP was measured in three tissues - isolated rat fat cells, isolated pancreatic islets, and toad bladders. The cyclic 3'5' AMP concentration was correlated with the specific metabolic response patterns of the tissues to theophylline, epinephrine and adrenergic blocking agents. Evidence is presented that the metabolic response patterns depended on the cellular concentrations of cyclic 3'5' AMP. Furthermore the effects of some hormones were mediated via an alteration in the synthesis of cyclic 3'5' AMP by a direct effect on adenyl cyclase.

For clarity in the presentation of this data, the experimental procedures and results are presented in three parts;

1. Hormone-activated lipolysis

A. The lipolytic action of human growth hormone and placental lactogen.

B. A mechanism for the hormonal control of lipolysis <u>in vivo.</u>

11. Development of a new assay for cyclic 3'5' adenosine monophosphate in tissues.

111. A mechanism correlating the control of cyclic 3'5' adenosine monophosphate synthesis with the specific pattern of metabolic response in tissues.

A discussion and summary follow the third part.

## Publications

The following papers have been published, or submitted for publication, on the material presented in this thesis:

1. TURTLE, J.R., BECK, P. and DAUGHADAY, W.H.
   Purification of Human Placental Lactogen
   Endocrinology 79 : 187, 1966
2. TURTLE, J.R. and KIPNIS, D.M.
   The Lipolytic Action of Human Placental Lactogen on Isolated Fat Cells
   Biochem et biophys Acta - 1967. In press.
3. TURTLE, J.R. and KIPNIS, D.M.
   Effect of Growth Hormone and Fatty Acids on the Metabolism of the Isolated Rat Fat Cell
   Biochemistry - submitted for publication.
4. TURTLE, J.R., LITTLETON, G.K. and KIPNIS, D.M.
   Stimulation of Insulin Secretion by Theophylline
   Nature 213 : 727, 1967
5. WILBER, J.F., TURTLE, J.R. and CRANE, N.A.
   Inhibition of Insulin Secretion by Phaeochromocytoma
   Lancet 7466 (11) : 733, 1966
6. TURTLE, J.R. and KIPNIS, D.M.
   An Adrenergic Receptor Mechanism for the Control of Cyclic 3'5' Adenosine Monophosphate Synthesis in Tissue
   Biochem Biophys. Research Comm. 28 : 797, 1967

7. TURTLE, J.R. and KIPNIS, D.M.
A New Assay for Cyclic 3'5' Adenosine Monophosphate in Tissue.
Biochemistry - submitted for publication.

8. TURTLE, J.R., LITTLETON, G.K. and KIPNIS, D.M.
Lipolytic Action of Human Placental Lactogen on Isolated Fat Cells (Abstract)
Clin. Research 15 : 289, 1966

9. TURTLE, J.R. and KIPNIS, D.M.
Stimulation of Insulin Secretion by Theophylline (Abstract)
Med. J. Aust. 2 : 516, 1967

10. TURTLE, J.R. and KIPNIS, D.M.
An Adrenergic Receptor Mechanism for the Control of Cyclic 3'5' Adenosine Monophosphate Synthesis (Abstract)
Proc. Aust. Soc. Med. Research 2 : 131 , 1967

## Acknowledgements

The material presented in this thesis was undertaken by the writer in the laboratories of Dr. William H. Daughaday (Professor of Medicine), and Dr. David M. Kipnis (Professor of Medicine) in the Metabolism Division, Department of Internal Medicine, Washington University, St. Louis, Missouri, U.S.A. The work was performed during the tenure of a post-doctoral fellowship from the National Institutes of Arthritis and Metabolic Diseases, and as the R.T. Hall Fellow in Diabetes from The Royal Australasian College of Physicians.

The writer is particularly grateful to Drs. Kipnis and Daughaday for provision of the facilities to undertake this project and for their continued encouragement, advice and support.

Mr. George K. Littleton and Mrs. Ingeborg Pommer provided expert technical assistance.

# PART I

## HORMONE-ACTIVATED LIPOLYSIS

### A. The Lipolytic Action of Human Growth Hormone and Human Placental Lactogen

The isolated rat fat cell preparation was used for the studies of lipolysis *in vitro* in order to reduce tissue variations in studies using the whole epididymal fat pad.

#### Experimental Procedure

**Materials:**

Human growth hormone ( HS 530A ) was obtained from Dr. Alfred Wilhelmi and Growth Factor Placental Origin Human ( GFPOH ) was supplied by Dr. Virgil A. Place of the Lederle Laboratories. Purified HPL, at least four times the potency of previous preparations, was prepared as described below. Collagenase ( CLS 099-108 ) was purchased from Worthington Biochemical Corporation and glucose, acetate and pyruvate isotopes from New England Nuclear Corporation. Insulin $I^{131}$ was purchased from Abbott Laboratories and nicotinic acid from Eastman Organic Chemicals.

**Methods:**

*The Isolated Fat Cell Procedure.* Epididymal fat pads were obtained from Charles River rats weighing 180-220 grams which had been maintained on Purina rat chow *ad libidum*. Animals were in the fed state at the time the fat pads were removed unless otherwise specified.

Isolated fat cells were prepared according to the procedure of Rodbell (25), except that all solutions in which fat pads or isolated cells were suspended contained 2% bovine serum albumin. Fraction V bovine serum albumin, freed by fatty acids by the glacial acetic acid-heptane method of Garland et al (48), or the charcoal method (49), was used in all studies. After treatment the fatty acid content was less than 1 uEq per gram of albumin. Epididymal adipose tissue from five to ten rats was cut into 50-100 mg fragments, distributed into four siliconized 25 ml conical flasks, and incubated for one hour at 37°C with collagenase dissolved in 1.5 ml albumin-bicarbonate buffer pH 7.4. Glucose was present during treatment with collagenase at the concentration to be used in the subsequent incubations. Following treatment with collagenase, the cells were washed twice in buffer and suspended in a volume of warm albumin-bicarbonate buffer to give a final cell count of $2.5 - 3.5 \times 10^6$ cells/ml. Incubations were carried out in 20 ml siliconized glass counting vials (Packard Instrument Company Inc.) capped with rubber stoppers. One millilitre aliquots of the cell suspension were used in all incubations; the various hormones and isotopically labelled substrates were added in a volume of 20 ul or less. The vials were gassed for 5 minutes with 95% $O_2$-5% $CO_2$ and incubated in a Dubnoff metabolic shaker at 37°C for varying periods.

Addition of isotopes during incubation was accomplished by injection through the rubber stopper with a microsyringe.

Lipolysis was measured by determining the rate of both glycerol and free fatty acid (FFA) release. Free fatty acids were extracted by the procedure of Dole (50), and measured colourimetrically on the dried heptone extract by the method of Duncombe (51). Glycerol was assayed, after deproteinization by boiling, using the enzymatic method of Wieland (52).

Carbon dioxide was trapped at the end of incubation by the addition of 0.5 ml Hyamine 10X (Packard Instrument Company Inc.) to small polyethylene cups suspended from the rubber stoppers, and by the addition of 0.2 ml 10N $H_2SO_4$ to the medium. The flasks were gently agitated for an additional hour. The polyethylene cups were then transferred to 10 ml of scintillation fluid (0.5% PPO, 0.03% POPOP) in toluene and counted in a liquid scintillation counter. The rate of incorporation of $^{14}C$ labelled precursor into fatty acids was used as a measure of fatty acid synthesis. Fatty acids were isolated by hydrolysis of the cells and medium in 5 ml 5 N KOH and 95% ethanol (5:1 by volume) for 4 hours, followed by neutralization with concentrated HCL, then extraction with two washings of n-hexane. The extracts were combined, dried under nitrogen and redissolved in 1 ml chloroform for analysis of FFA concentration and $^{14}C$ incorporation.

Deoxyribonucleic acid was determined by a modification of the fluorimetric method of Kissane and Robins (53). One millilitre of the fat cell suspension was added to 5 ml of 5.6% trichloracetic acid and homogenised. The precipitate was removed by centrifugation, then the lipids were extracted with 5 ml cold IM alcoholic potassium acetate, followed by two washings with 5 ml absolute ethanol. The precipitate was dried in an air stream then subjected to the DABA reaction as described by Kissane and Robins. An average DNA content of $7.9 \pm 0.2 \times 10^{-12}$ g DNA/cell was obtained. This is slightly lower than the DNA content reported for other rat tissues by Slater (54), but is in good agreement with rat liver nuclei DNA content found by Sakurai and Kipnis (unpublished observations) using the colourimetric procedure of Burton (55). Cell counts were performed on 1:20 dilutions of the cell suspension using a siliconized Neubauer haemocytometer chamber. Twenty 0.1 µl volumes were counted for each determination. Results are generally expressed in terms of metabolic activity per $10^6$ cells. This expression can be converted to metabolic activity per mg DNA by multiplication by a factor of 127.

Preparation of HPL:

The preparations of human placental lactogen (HPL) have all shown evidence of heterogeneity when examined on starch gel electrophoresis (29,34,56). It was considered desirable to

attempt further purification to obtain a suitable preparation for the studies on lipolysis. A modification of the technique described by Cohen, Grumbach and Kaplan (56) was used, which resulted in HPL with increased potency and reduced contaminants.

A crude placental extract was precipitated with ammonium sulphate according to the procedure of Cohen et al (56). The 20-40% ammonium sulphate precipitate was fractionated on a DEAE cellulose column by stepwise elution with sodium chloride in 0.1 M TRIS buffer, pH 9.0. Two preliminary elutions were performed with 0.05 M Nacl/0.1 M TRIS and 0.1 M Nacl, then the active fraction was eluted with 0.33 M Nacl/0.1 M TRIS. This fraction was dialyzed against distilled water for 48 hours and lyophilized. The dried material contained 46 % protein. Dialysis for 72 - 96 hours yielded material with a higher protein content.

250 - 300 mg of the dried material (46% protein) was dissolved in 2 ml 0.01 M TRIS buffer pH 9.0. 4N hydrochloric acid was added dropwise to adjust to pH 5, and the suspension was stirred for one hour at 5°C. The pH was adjusted at 15 minutes and remained stable thereafter.

The precipitate was removed by centrifugation, the supernatant was adjusted to pH 9.0 with 4N sodium hydroxide, and the material was dialyzed overnight at 5°C against 0.01 M TRIS buffer pH 9.0, and further fractionated by elution through a 40 x 1 cm column of sephadex G - 200 previously equilibrated

with 0.01 M TRIS pH 9.0. Elution was performed at 5°C. The retarded fraction was dialyzed against distilled water and lyophilized.

HPL for iodination was prepared by the procedure of Cohen, Grumbach and Kaplan (56), and iodinated with $I^{131}$ by the method of Hunter and Greenwood (57). Separation of the HPL-$I^{131}$ from free $I^{131}$ and other materials was achieved by starch gel electrophoresis at 5°C. In some assays material prepared by the present technique was used for iodination. Potency was determined by a double antibody precipitation radioimmunoassay technique (30), with either guinea pig anti-HGH or guinea pig anti-GFPOH as the primary antibody, and rabbit anti-guinea pig gamma globulin as the precipitating antibody. Activity is expressed in terms of the reference standard described by Cohen et al (56). Protein was determined by the procedure of Lowry (58).

Rat Infusion Technique. Male Charles River rats weighing 200-400 grams and fasted for 24-48 hours were adrenalectomized under ether anaesthesia three hours before use to exclude acute variations in catecholamine secretion during the experimental procedure. Under anaesthesia with intraperitoneal sodium pentobarbitone 3 mg/100 g body weight, a polyethylene catheter was inserted through the jugular vein into the right atrium for injection of test materials and subsequent blood sampling.

Portal venous blood samples were obtained by inserting a 25 gauge needle attached to a short length of polyethylene tubing into the portal vein without interrupting the portal circulation. 0.4 ml blood samples were drawn into heparinized tubes three minutes before and again immediately before injection. Theophylline ethylenediamine dihydrate (Sigma Chemical Co.) 15 mg in physiological saline was injected through the jugular catheter in a five second period. Subsequently, plasma samples were obtained at frequent intervals for insulin assay by the double antibody precipitation radioimmunoassay of Morgan and Lazarow (59). Glucose was determined by the method of Somogyi (60), and FFA concentration was estimated by the Dole extraction procedure (50) and a modification of the Duncombe (51) colourimetric assay to permit estimation in micro quantities of plasma. 0.1 ml plasma was extracted using 5 ml of the Dole extraction mixture, and the heptane fraction was dried in a stream of nitrogen. The dried extract was dissolved in 1 ml of chloroform, shaken with 0.5 ml of the copper reagent (Duncombe), and the colour reaction developed using 0.5 ml of the chloroform phase. The mixture was read at 440 u wavelength in 0.5 ml cuvettes.

Insulin $I^{125}$ disappearance, using porcine insulin iodinated with $I^{125}$ by the method of Hunter and Greenwood (57), was estimated by injection into the iliac vein and removing samples from the right atrium. Test substances were infused into the tail vein using a controlled infusion pump.

Results:

Growth Hormone;

Effect of Growth Hormone on Lipolysis.

Fat cells obtained from fed rats and incubated in a buffer containing 3 mM glucose and 4 x $10^{-8}$M dexamethasone, maintained a linear rate of glycerol release at 7.0 m μmoles/$10^6$ cells/hour throughout the course of a five hour control incubation (Figure 1). Growth hormone-activated lipolysis appeared after a lag period of 60-90 minutes, then glycerol (44 m μmoles/$10^6$ cells/hour) and FFA (21 m μmoles/$10^6$ cells/hour) release continued at an accelerated rate for 150-180 minutes. The rate of glycerol and FFA release was increased 6-7 fold. During the last hour of incubation the rate of glycerol and FFA release fell to control levels. This decrease was not prevented by additional growth hormone (0.5 μg/ml) added during the third to fourth hour of incubation. The presence of actinomycin D (1.5 x $10^{-6}$M) or Puromycin ( 1 x $10^{-4}$M) completely abolished the lipolytic action of growth hormone (Table 1).

FIGURE 1

TIME SEQUENCE FOR APPEARANCE OF HGH-ACTIVATED LIPOLYSIS IN ISOLATED FAT CELLS

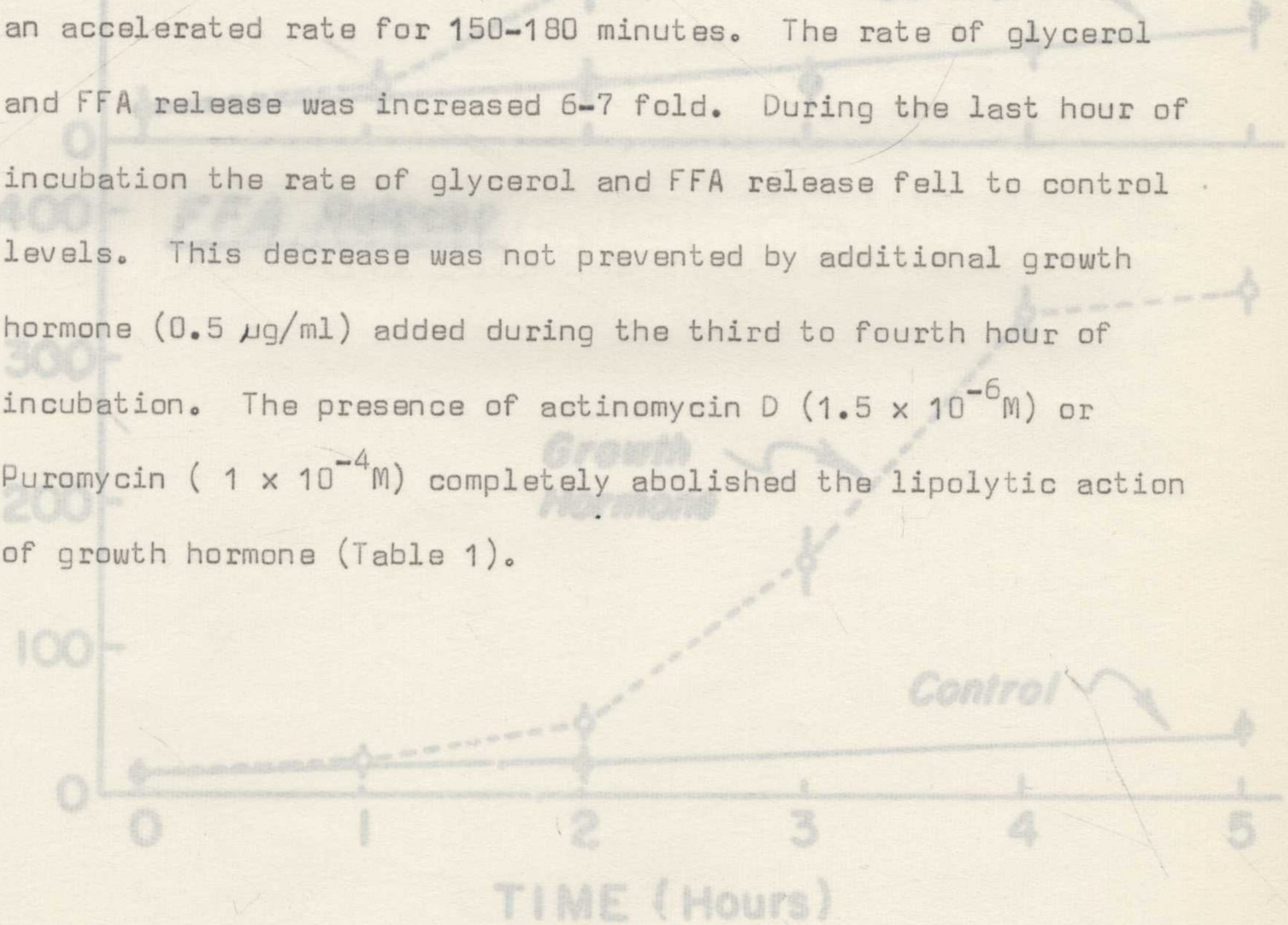


FIGURE 1

TIME SEQUENCE FOR APPEARANCE OF HGH-ACTIVATED LIPOLYSIS IN ISOLATED FAT CELLS

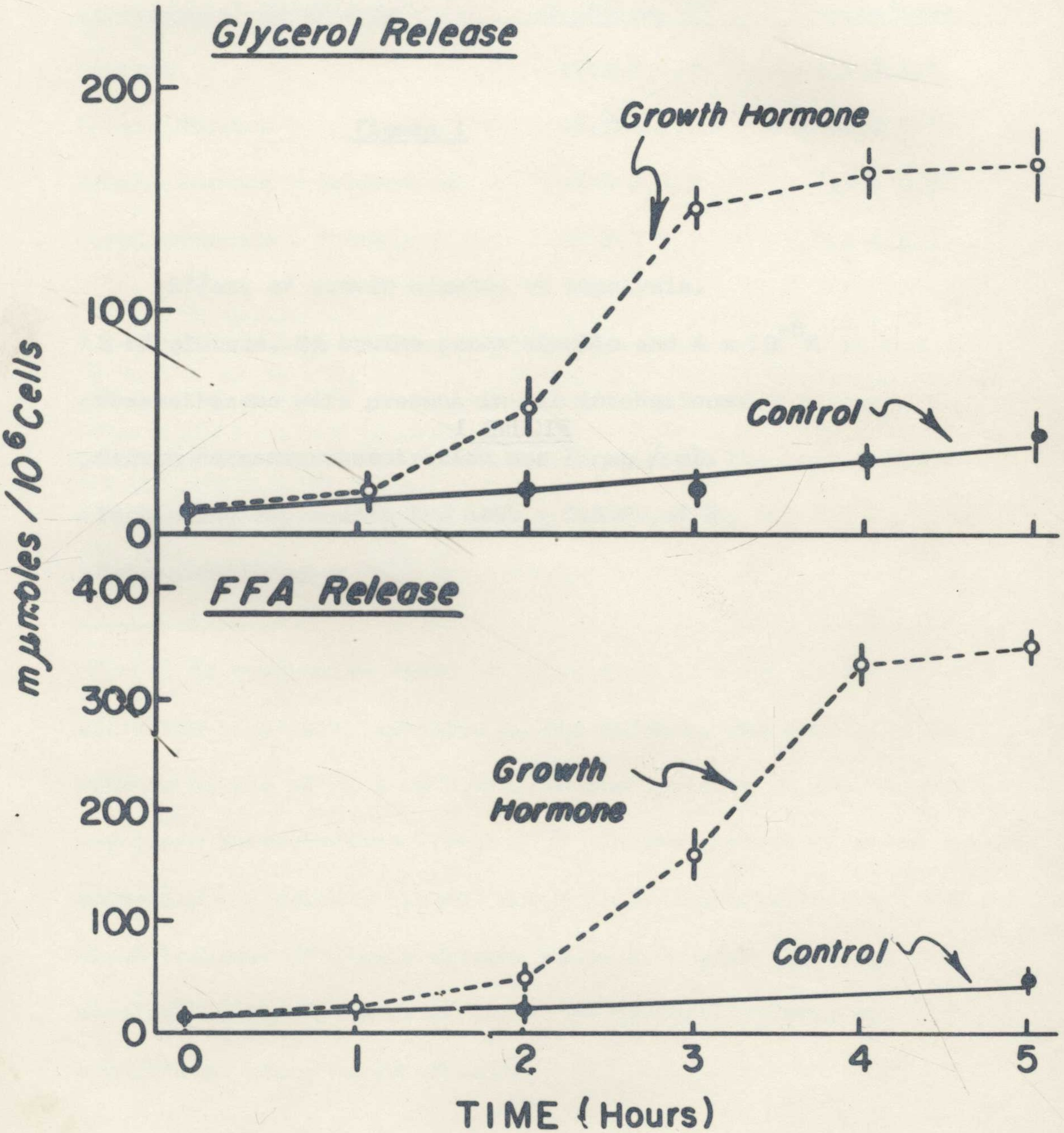


FIGURE 1

Table 1

Effect of Actinomycin D and Puromycin on Growth-Hormone Stimulated Lipolysis*

| Experimental Conditions | Glycerol Release m µmoles/$10^6$ cells/hour | FFA Release |
|---|---|---|
| Control | 11.2 ± 1.7 | 4.1 ± 0.5 |
| Growth Hormone | 45.2 ± 2.7 | 24.1 ± 2.1 |
| Growth Hormone + Actinomycin D | 10.3 ± 0.9 | 3.8 ± 0.3 |
| Growth Hormone + Puromycin | 10.9 ± 1.1 | 4.2 ± 0.3 |

Figure I

Effect of growth hormone on lipolysis.

* 3 mM glucose, 2% bovine serum albumin and $4 \times 10^{-8}$ M dexamethasone were present in all incubations. Growth hormone concentration was 1 µg / ml. Each value represents the mean ± S.E.M. of 4 determinations.

Table 1

Effect of Actinomycin D and Puromycin on Growth-Hormone Stimulated Lipolysis*

| Experimental Conditions | Glycerol Release m μmoles/$10^6$ cells/hour | FFA Release cells/hour |
|---|---|---|
| Control | 11.2 ± 1.7 | 4.1 ± 0.5 |
| Growth Hormone | 45.2 ± 2.7 | 24.1 ± 2.1 |
| Growth Hormone + Actinomycin D | 10.3 ± 0.9 | 3.8 ± 0.3 |
| Growth Hormone + Puromycin | 10.9 ± 1.1 | 4.2 ± 0.3 |

* Incubations were carried out for five hours at 37°C in the presence of $4 \times 10^{-8}$ M dexamethasone. The initial glucose concentration was 3mM. Each value represents the mean ± SEM of 4 determinations. Growth hormone concentration was 0.5 μg/ml, actinomycin D $1.5 \times 10^{-6}$ M, puromycin $1 \times 10^{-4}$ M.

As originally reported by Fain et al (26), growth hormone activated-lipolysis, measured as FFA release, was demonstrated with as little as 10 m μg HGH/ml in the presence of the glucocorticoid dexamethasone (Table 11). In the absence of dexamethasone, increased FFA release did not occur consistently with the concentrations of growth hormone below 0.5 μg/ml, although accelerated lipolysis as reflected by glycerol release was observed at lower concentrations.

Table II

Effect of Growth Hormone on Lipolysis in Isolated Fat Cells*

| Growth Hormone in µg/ml | FFA Release in µmoles/$10^6$ cells | Glycerol Release in µmoles/$10^6$ cells |
|---|---|---|
| 1000 | 317 ± 2.9 | 167 ± 9.4 |
| 100 | 103 ± 5 | 73 ± 5.0 |
| 10 | 54 ± 8 | 45 ± 3.7 |
| 1 | 22 ± 8 | --- |
| 0 | 20 ± 7 | 38 ± 2.3 |

*Incubations were carried out for five hours at 37°C. 2% bovine serum albumin, 3 mM glucose and $4 \times 10^{-8}$M dexamethasone were present in all incubations. Each value represents the mean ± S.E.M. of 6 - 20 determinations.

The potentiating effect of dexamethasone on the lipolytic activity of growth hormone, in terms of FFA release, appears to be primarily a result of inhibition of FFA re-esterification rather than a direct effect on lipolysis. At a concentration of $4 \times 10^{-8}$M, dexamethasone produced a slight increase in the basal rate of lipolysis as reflected by glycerol release. However, the fat cell still re-esterified the FFA's release as a consequence of lipolysis. In the presence of a FFA load, added exogenously or produced endogenously by lipolysis, the esterification mechanism was challenged and the inhibitory effect of the glucocorticoid on the re-esterification mechanism became evident (Table III).

Table III

Effect of Dexamethasone on FFA Esterification in Isolated Fat Cells*

| Glucose | Palmitate | Dexamethasone | Glycerol Release | Fatty Acid Esterified |
|---|---|---|---|---|
| mM | μM | | m μmoles/$10^6$ cells/4hours | |
| 3 | - | - | 29 ± 1.4 | 70 ± 8 |
| 3 | - | + | 42 ± 2.1 | 98 ± 16 |
| 3 | 645 | - | 8 ± 0.4 | 160 ± 16 |
| 3 | 1260 | + | 0 | 5 |

*Incubations were carried out for 4 hours at 37°C in the presence of 2% bovine serum albumin. The concentration of dexamethasone was $4 \times 10^{-8}$ M of four determinations.

Fatty acids esterified were calculated from the difference between the expected release (assuming FFA : glycerol ration of 3 : 1 for triglyceride hydrolysis) and the actual FFA release into the medium.

---

The impaired ability to esterify FFA produced by dexamethasone may be secondary to a decreased production of ∝ -glycerophosphate, since the same concentration of the steroid markedly depressed glucose uptake from 39 ± 5 m μmoles / $10^6$ cells/ hour to 29 ± 3 m μmoles/$10^6$ cells / hour when cells were incubated in 3 mM glucose.

Effect of Glucose Concentration on Growth Hormone-Activated Lipolysis

The endogenous rate of lipolysis, as measured by glycerol release, increased approximately 50 per cent from control levels of 19.6 ± 0.9 m μmoles / $10^6$ cells/hour when the glucose concentration was raised to 150-300 mg % (Figure 2 ). At all glucose concentrations used, growth hormone still stimulated lipolysis; however, the accumulation of FFA in the incubation medium and the rate of FFA re-esterification were markedly affected by the glucose concentration (Table IV).

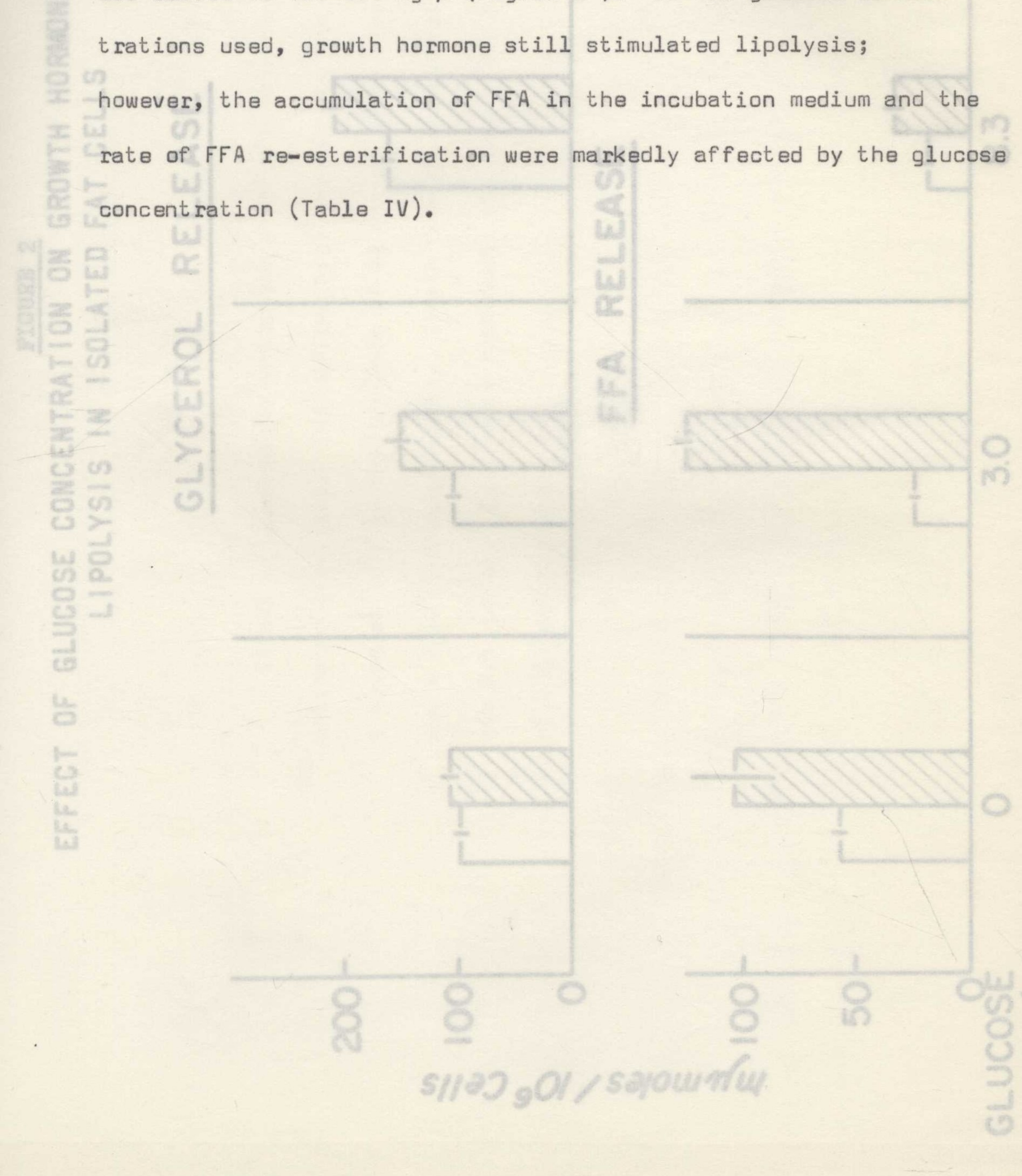


FIGURE 2

EFFECT OF GLUCOSE CONCENTRATION ON GROWTH HORMONE ACTIVATED LIPOLYSIS IN ISOLATED FAT CELLS

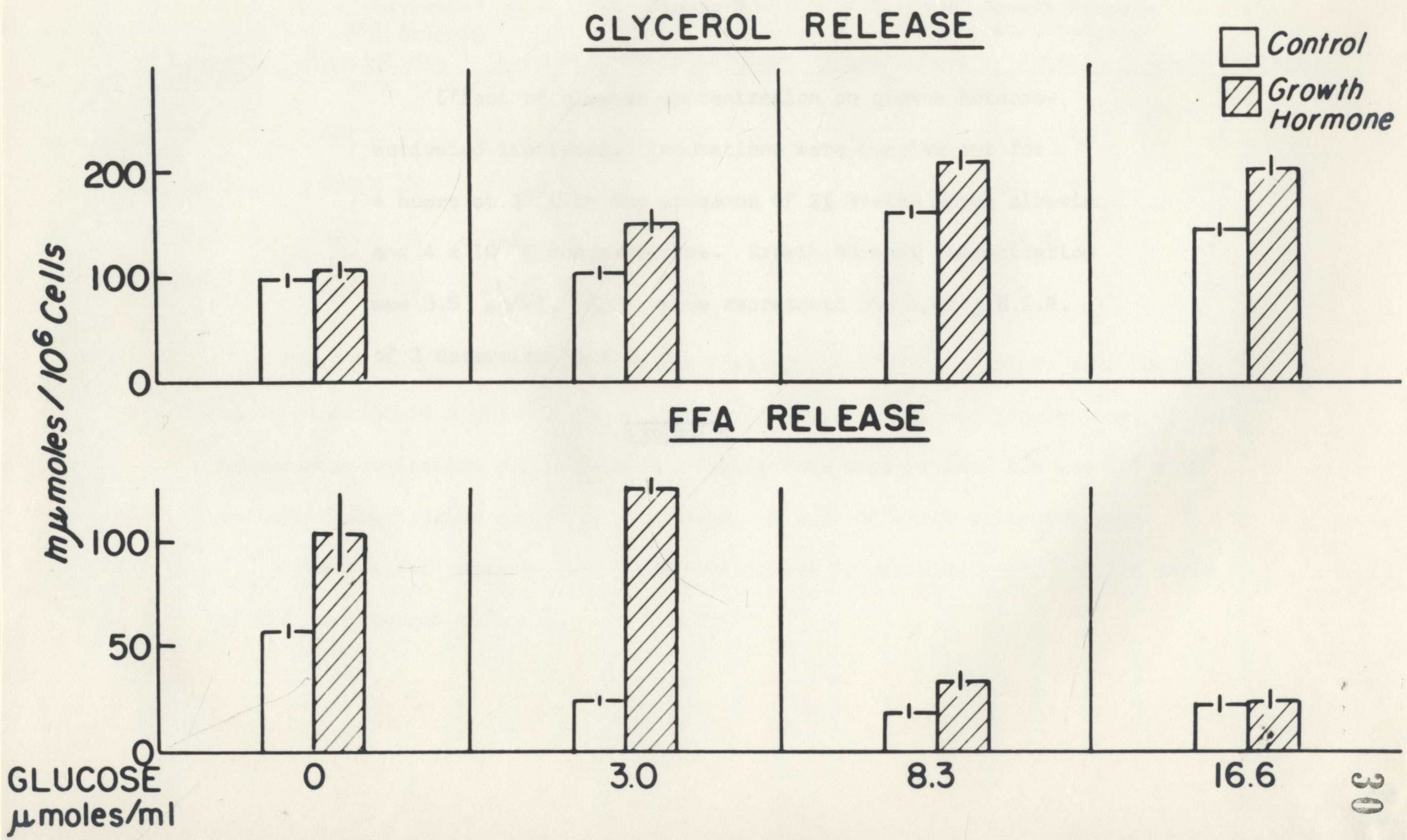


FIGURE 2

Figure 2

Effect of glucose concentration on growth hormone-activated lipolysis. Incubations were carried out for 4 hours at 37°C in the presence of 2% bovine serum albumin and 4 x $10^{-8}$M dexamethasone. Growth hormone concentration was 0.5 µg/ml. Each value represents the mean ± S.E.M. of 3 determinations.

Table IV

Effect of Glucose on Fatty Acid Re-esterification by Isolated Fat Cells in the Presence and Absence of Dexamethasone*

| Glucose in M | Control FFA Release Theoretical | Actual | Re-esterified | Growth Hormone FFA Release Theoretical | Actual | Re-esterified |
|---|---|---|---|---|---|---|
| | in $\mu$ moles/ml | | % | in $\mu$ moles/ml | | % |
| 0 | 882 ± 36 | 187 ± 20 | 79 | 132 ± 7 | 134 ± 20 | 0 |
| 3 | 993 ± 22 | 71 ± 3 | 93 | 378 ± 38 | 305 ± 12 | 19.3 |
| 8.3 | 1465 ± 44 | 54 ± 3 | 97 | 228 ± 6 | 44 ± 2 | 80.7 |
| 16.6 | 1293 ± 25 | 66 ± 3 | 95 | 303 ± 24 | 4 | 99 |

*Incubations were carried out for five hours at 37°C. Two per cent bovine serum albumin and $4 \times 10^{-8}$M dexamethasone were present in all incubations. Growth hormone concentration was 0.5 µg/ml. Cell counts were between 2.8 and $3.1 \times 10^{6}$ cells/ml. Each value represents the mean S.E.M. of three determinations.

Theoretical release of FFA was calculated by assuming a 3 : 1 molar ratio of FFA to glycerol released.

Under control conditions, greater than 90 per cent of the fatty acids released during five hours incubation (calculated by assuming a 3:1 molar ratio of fatty acids to glycerol released) were re-esterified when the extracellular glucose level was 3 mM or greater. Fatty acid esterification appeared to be operating at a near maximal capacity in the absence of glucose or at low glucose concentrations ( 3 mM ), since at these levels, growth hormone-accelerated lipolysis resulted in an accumulation of free fatty acids which approximated the expected concentration calculated on the basis of glycerol release. At higher levels of glucose (> 8.3 mM), re-esterification was sufficiently accelerated to prevent the accumulation of FFA.

Immediate Effects of Growth Hormone on Glucose and Acetate Metabolism.

During the first two hours of incubation, growth hormone increased glucose uptake 155 per cent, but did not significantly affect the rate of lipolysis (Figure 3). During the same period, glucose-1-$^{14}$C oxidation increased 22 per cent, but the oxidation of glucose-6-$^{14}$C and acetate-1-$^{14}$C remained unchanged (Figure 4 ). Lipogenesis from all three precursors was accelerated during this immediate phase, the increase ranging from 84 per cent for glucose-1-$^{14}$C to 30 per cent for glucose-1-$^{14}$C and acetate-1-$^{14}$C. These effects were observed regularly with 0.5 µg GH/ml, but were not consistently seen with concentrations below 0.1 µg GH/ml.

FIGURE 3

EFFECT OF GROWTH HORMONE ON GLUCOSE UPTAKE, FFA AND GLYCEROL RELEASE IN ISOLATED FAT CELLS

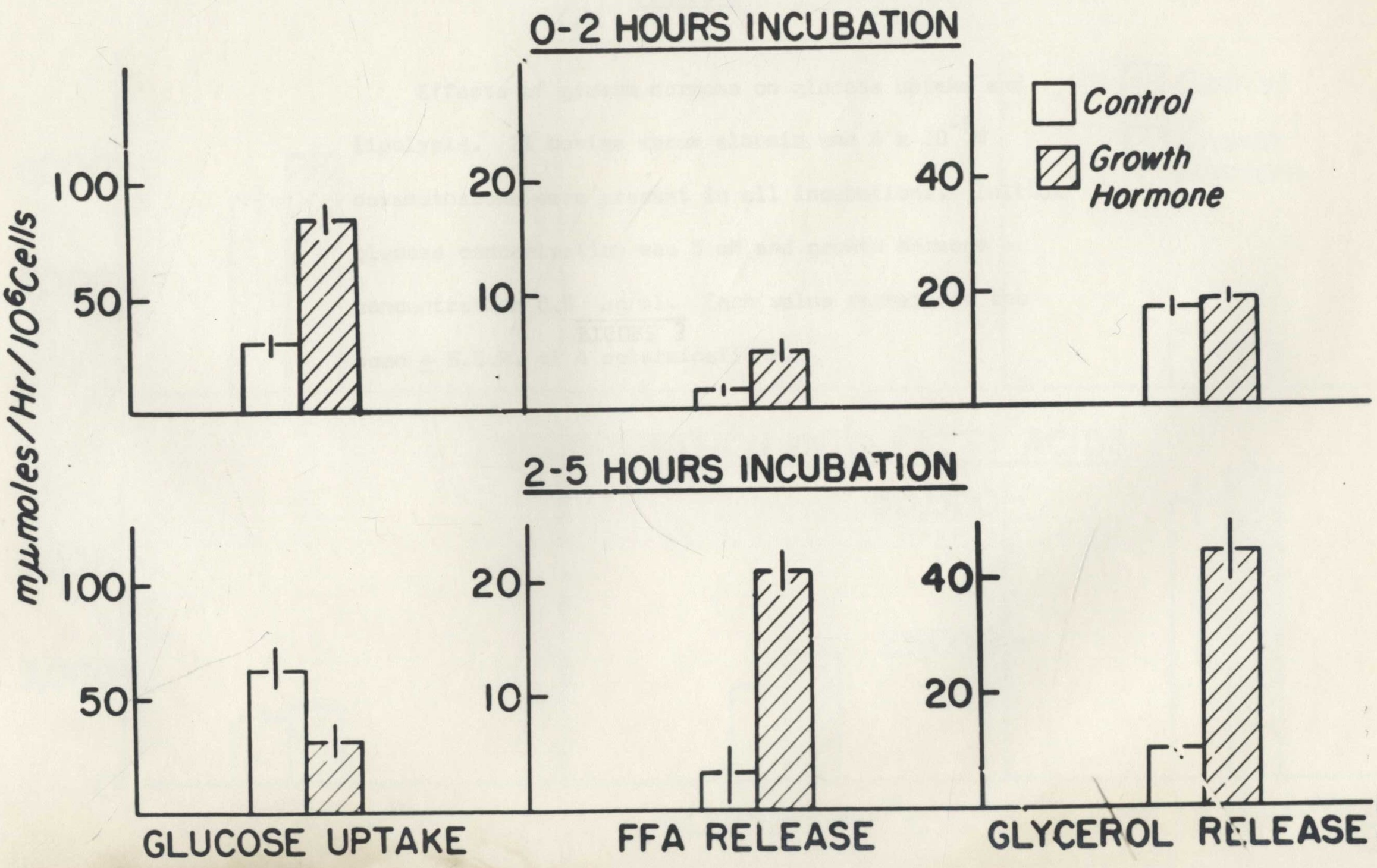


FIGURE 3

FIGURE 4

EFFECT OF GROWTH HORMONE ON GLUCOSE AND ACETATE OXIDATION AND INCORPORATION INTO FATTY ACIDS DURING THE FIRST TWO HOURS OF INCUBATION.

Figure 3

Effects of growth hormone on glucose uptake and lipolysis. 2% bovine serum albumin and 4 x $10^{-8}$ M dexamethasone were present in all incubations. Initial glucose concentration was 3 mM and growth hormone concentration 0.5 µg/ml. Each value represents the mean + S.E.M. of 4 determinations.

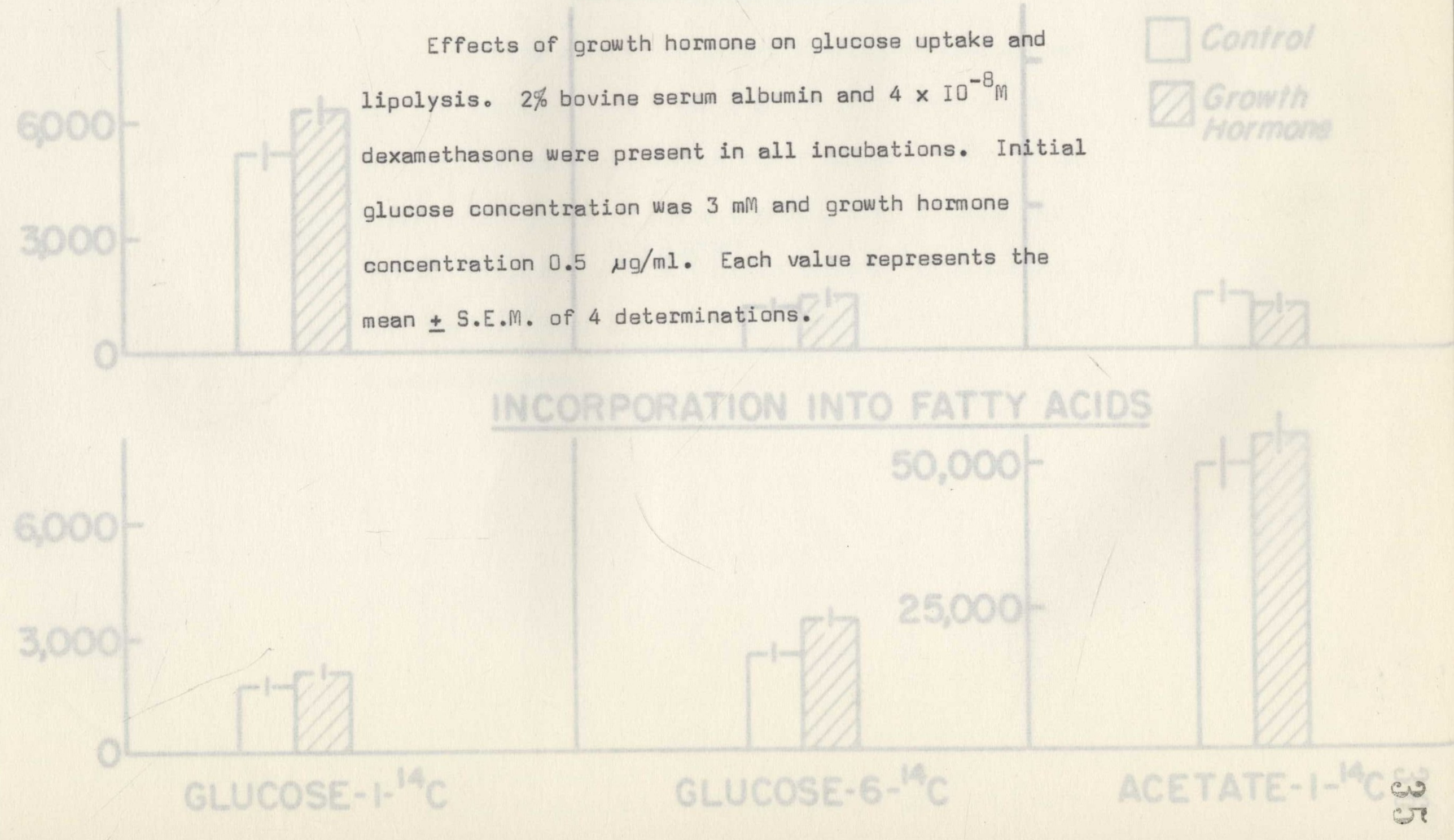


FIGURE 4

EFFECT OF GROWTH HORMONE ON GLUCOSE AND ACETATE OXIDATION AND INCORPORATION INTO FATTY ACIDS DURING THE FIRST TWO HOURS OF INCUBATION.

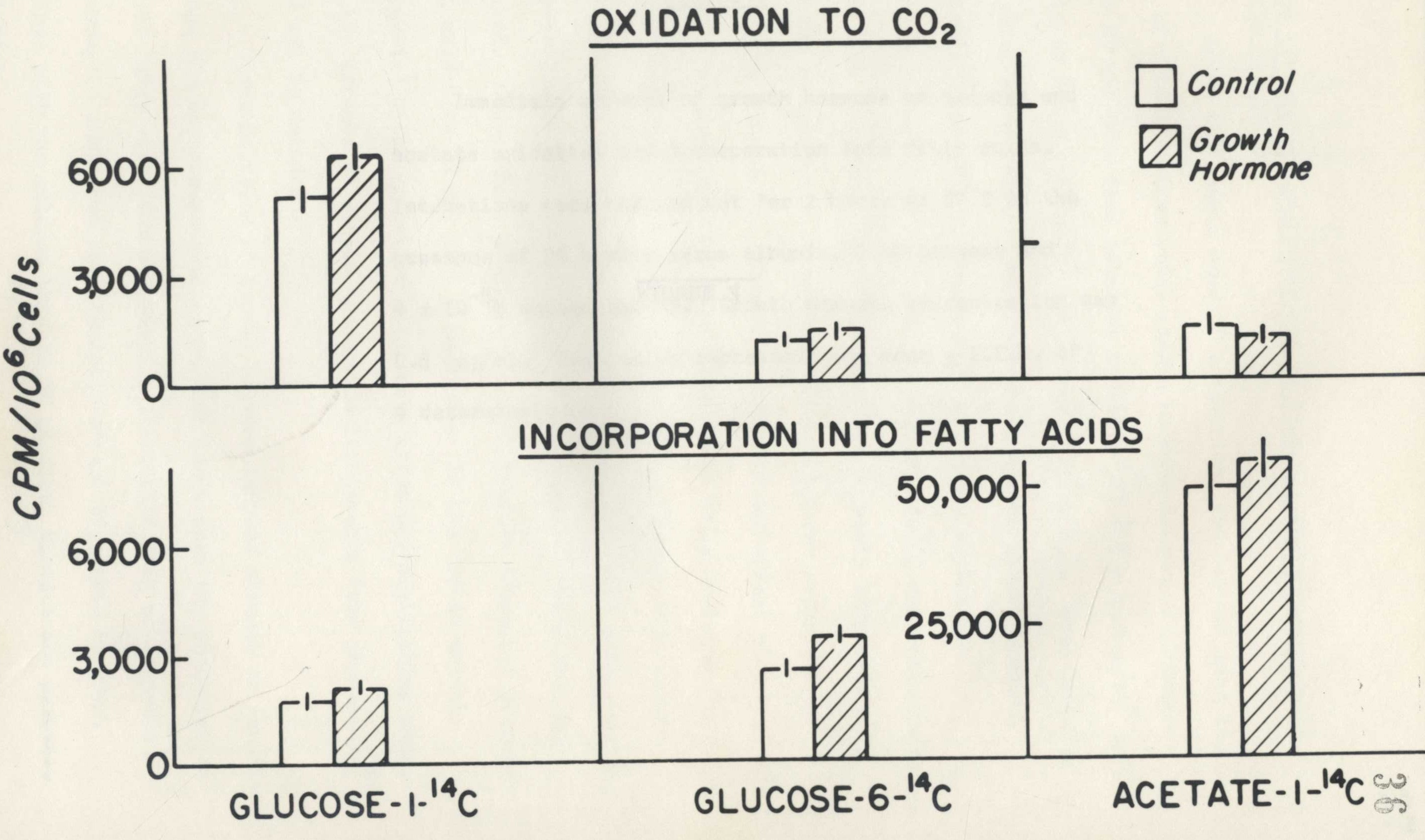


FIGURE 4

Figure 4

Immediate effects of growth hormone on glucose and acetate oxidation and incorporation into fatty acids. Incubations were carried out for 2 hours at 37°C in the presence of 2% bovine serum albumin, 3 mM glucose and $4 \times 10^{-8}$ M dexamethasone. Growth hormone concentration was 0.5 µg/ml. Each value represents the mean ± S.E.M. of 4 determinations.

Delayed Effects of Growth Hormone on Glucose, Acetate and Pyruvate Metabolism

After the second hour of incubation, concomitant with the onset of activated lipolysis, growth hormone decreased glucose uptake 50 per cent from baseline levels of 33 ± 3 m μmoles/$10^6$ cells/hour to 16 ± 4 m μmoles/$10^6$ cells/hour (Figure 3 ). When labelled were added after three hours preincubation with growth hormone, glucose-1-$^{14}$C oxidation increased 90 per cent, adetate-1-$^{14}$C oxidation increased 44 per cent and pyruvate-2-$^{14}$C oxidation increased 85 per cent (Figure 5 ). Lipogenesis was markedly impaired, as reflected by decreased incorporation of all three precursors into fatty acids (Figure 5). Growth hormone did not affect glucose-1-$^{14}$C oxidation of incorporation into fatty acids using isolated fat cells prepared from fed rats. If fat cells were prepared from donor animals fasted for 15 hours, the hexose monophosphate shunt activity increased, thus the control level of glucose-1-$^{14}$C oxidation was elevated. Under these conditions growth hormone decreased glucose-1-$^{14}$C oxidation 28 per cent (Figure 5). In the fasted animal glucose-1-$^{14}$C incorporation into fatty acids was negligible; therefore the effects of growth hormone on lipogenesis from glucose-1-$^{14}$C could not be detected.

These patterns of growth hormone induced changes in labelled glucose and acetate metabolism did not occur when the accumulation of free fatty acids was prevented. Incubation with actinomycin D or puromycin abolished the lipolytic effect of growth hormone, together with the delayed changes on glucose, acetate and pyruvate metabolism.

FIGURE 5

EFFECT OF GROWTH HORMONE ON GLUCOSE, ACETATE AND PYRUVATE METABOLISM FOLLOWING THE ONSET OF HORMONE ACTIVATED LIPOLYSIS.

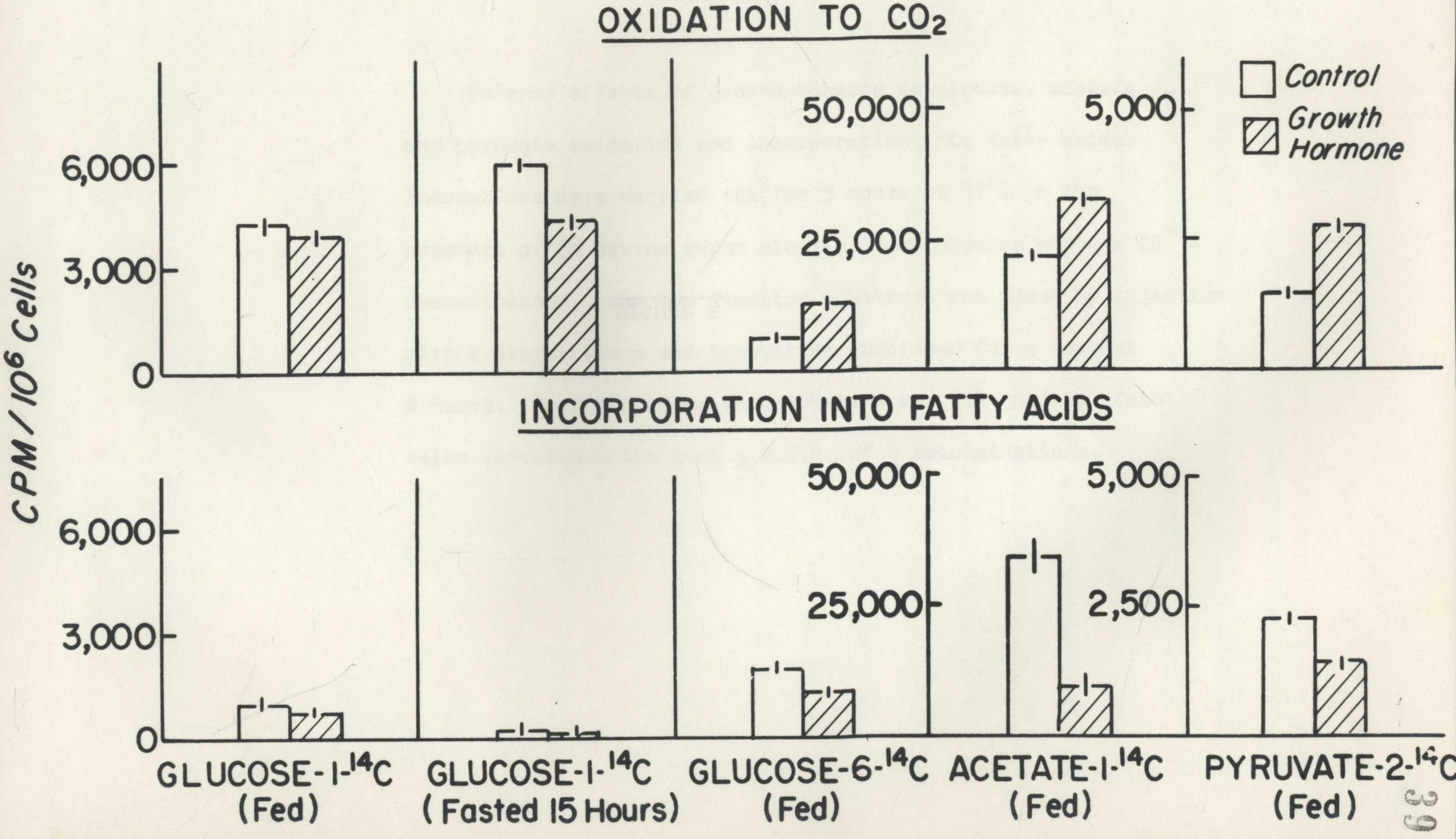


FIGURE 5

Figure 5

Delayed effects of growth hormone on glucose, acetate and pyruvate oxidation and incorporation into fatty acids. Incubations were carried out for 3 hours at 37°C in the presence of 2% bovine serum albumin, 3 mM glucose and 4 x $10^{-8}$M dexamethasone, then the labelled substrate was added by injection with a microsyringe and incubation continued for a further 2 hours. Growth hormone concentration was 0.5 µg/ml. Each value represents the mean ± S.E.M. of 3 determinations.

However, incubation of the cells in the presence of high glucose concentration (18 μmoles/ml) to stimulate FFA re-esterification and prevent the accumulation of FFA in the medium, completely reversed the delayed pattern of growth hormone effects on glucose, acetate, and pyruvate (Figure 6), although activated lipolysis still occurred as reflected by glycerol release (Figure 3).

Further evidence of the role of FFA accumulation in the delayed pattern of growth hormone effects was obtained when no glucocorticoid was omitted from the medium. In the absence of dexamethasone, and at a glucose concentration of 5.0 mM, growth hormone did not decrease glucose uptake or glucose-1-$^{14}$C oxidation (Table V).

FIGURE 6

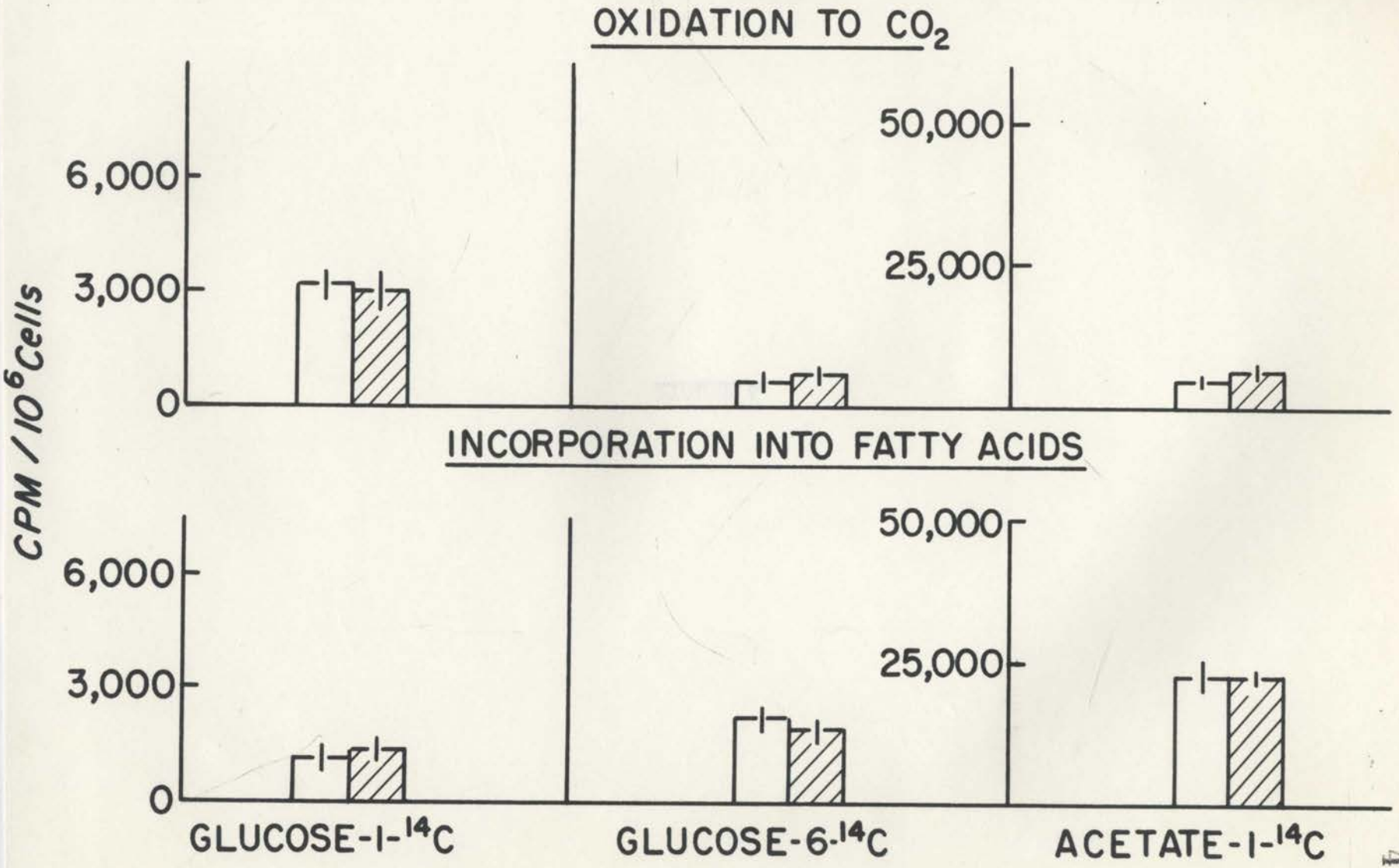

OXIDATION TO $CO_2$
6,000
3,000
0
50,000
25,000
INCORPORATION INTO FATTY ACIDS
CPM / $10^6$ Cells
GLUCOSE-1-$^{14}$C
GLUCOSE-6-$^{14}$C
ACETATE-1-$^{14}$C

FIGURE 6

Figure 6

Delayed effect of growth hormone on glucose and acetate oxidation and incorporation into fatty acids in the presence of high glucose concentration. Incubations were carried out as described in Figure 5, only the initial glucose concentration was I8 mM. Growth hormone concentration was 0.5 µg/ml.

Control incubations are represented by clear bars and growth hormone incubations by hatched bars. Each value represents the mean ± S.E.M. of 4 determinations.

Table V

Delayed Effects of Growth Hormone and Dexamethasone on Lipolysis and Glucose Metabolism*

| Experimental Conditions | | | | | | |
|---|---|---|---|---|---|---|
| | Dexamethasone | Growth Hormone | FFA Release | Glycerol Release | Glucose Uptake | Glucose-1-$^{14}$C Oxidation |
| | | | m μmoles/$10^6$ cells/hour | | m μmoles/$10^6$/hr | cpm/$10^6$ cells/hour |
| EXP I | - | - | 4.7 ± 0.4 | 7.2 ± 0.4 | 45.7 ± 1.8 | 1079 ± 10 |
| | - | + | 5.5 ± 0.5 | 39.2 ± 2.5 | 57.7 ± 1.9 | 1261 ± 27 |
| EXP II | + | - | 5.0 ± 0.4 | 10.5 ± 0.6 | 39.5 ± 2.8 | 1523 ± 64 |
| | + | - | 80.2 ± 3 | 41 ± 0.3 | 24.5 ± 2.0 | 1118 ± 51 |

*Cells were incubated in the presence of 2% bovine serum albumin and 5.6 mM glucose for four hours. The results for glucose uptake and glucose-1-$^{14}$C oxidation represent the change during the third and fourth hours of incubation. Cells were prepared from fed animals except for the experiment on glucose-1-$^{14}$C oxidation, in which cells were prepared from animals fasted for fifteen hours. The specific activity of the glucose-1-$^{14}$C was greater in experiment II than in experiment I. Dexamethasone concentration was $4 \times 10^{-8}$M and growth hormone concentration 1 μg/ml.

Each value represents the mean ± S.E.M. of five determinations.

Under these conditions lipolysis was still accelerated as indicated by glycerol release, FFA did not accumulate due to rapid re-esterification, and growth hormone actually increased glucose uptake 27 per cent ( $p < 0,05$).

Effect of Nicotinic Acid on the Delayed Action of Growth Hormone

Nicotinic acid 1 µg/ml inhibited completely the lipolytic effect of growth hormone measured by either FFA or glycerol release (Figure 7). Nicotinic acid increased both glucose-1-$^{14}$C and acetate-1-$^{14}$C oxidation by 30-33 per cent, but did not significantly affect glucose-6-$^{14}$C oxidation or the incorporation of any of these precursors into fatty acids. Inhibition of the lipolytic action of growth hormone with nicotinic acid blocked the delayed effects of the hormone on glucose-1-$^{14}$C and glucose-6-$^{14}$C metabolism. Furthermore, in the presence of nicotinic acid, growth hormone stimulation of acetate-1-$^{14}$C oxidation was reduced markedly, and no significant inhibition of acetate incorporation into fatty acids was noted.

Effect of Added Palmitate on the Endogenous Rate of Lipolysis and Glucose Uptake

Lipolysis, measured by glycerol release, was markedly inhibited by the addition to the incubation medium of palmitate in the form of the fatty acid-albumin complex, prepared by the method of Bowman (61) (Figure 8 ). In these experiments the albumin concentration was maintained constant at 2 per cent so that the FFA/albumin molar ratio ($\bar{V}$) varied from 0 to 3.7.

FIGURE 7

THE EFFECT OF NICOTINIC ACID ON LIPOLYSIS AND GLUCOSE AND ACETATE MEBABOLISM

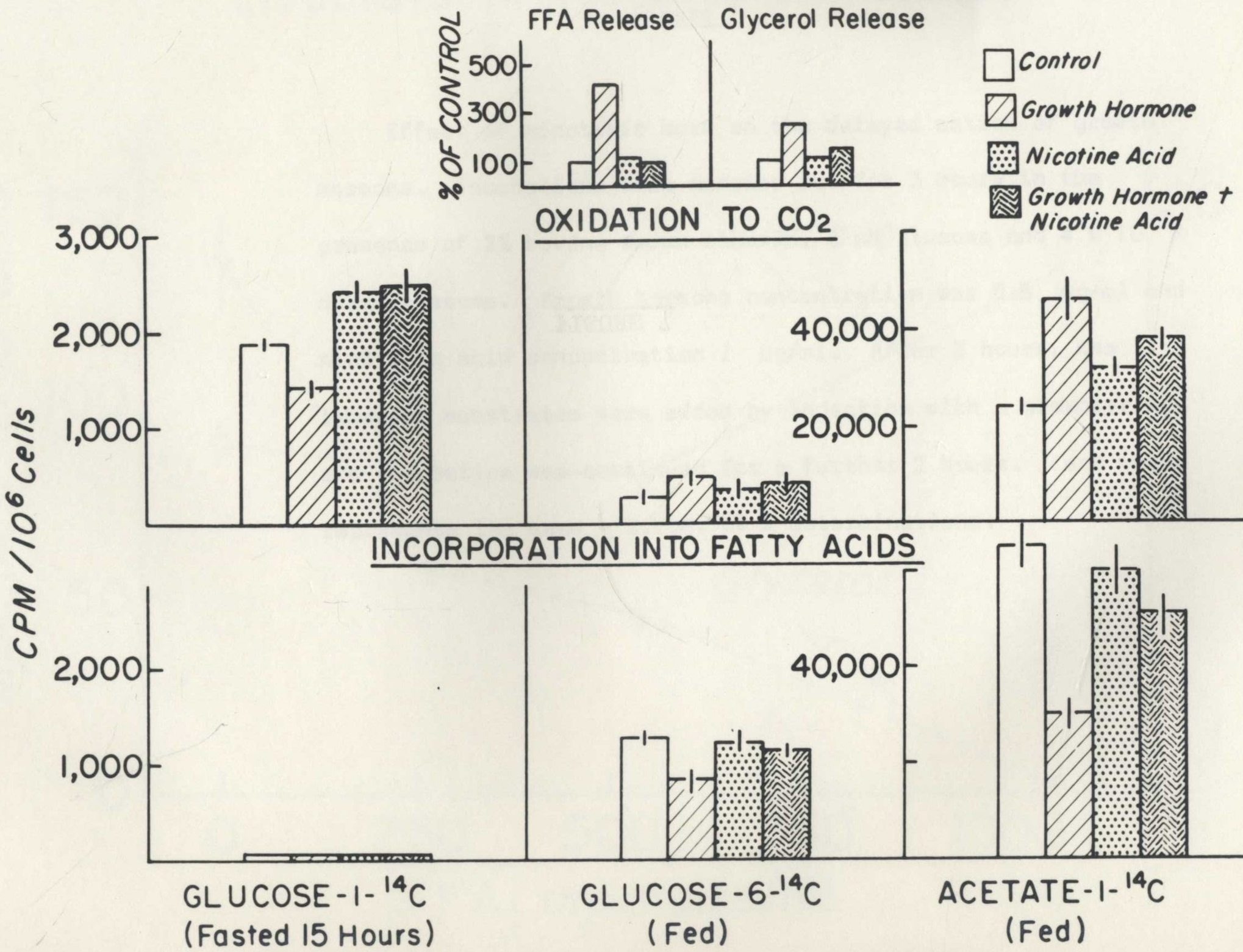


FIGURE 7

FIGURE 8

# EFFECT OF ADDED PALMITATE ON GLUCOSE UPTAKE AND LIPOLYSIS IN ISOLATED FAT CELLS

Figure 7

Effect of nicotinic acid on the delayed action of growth hormone. Incubations were carried out for 3 hours in the presence of 2% bovine serum albumin, 3 mM glucose and 4 x $10^{-8}$ M dexamethasone. Growth hormone concentration was 0.5 µg/ml and nicotinic acid concentration I µg/ml. After 3 hours, the labelled substrates were added by injection with a microsyringe and incubation was continued for a further 2 hours. Each value represents the mean ± S.E.M. of 3 determinations.

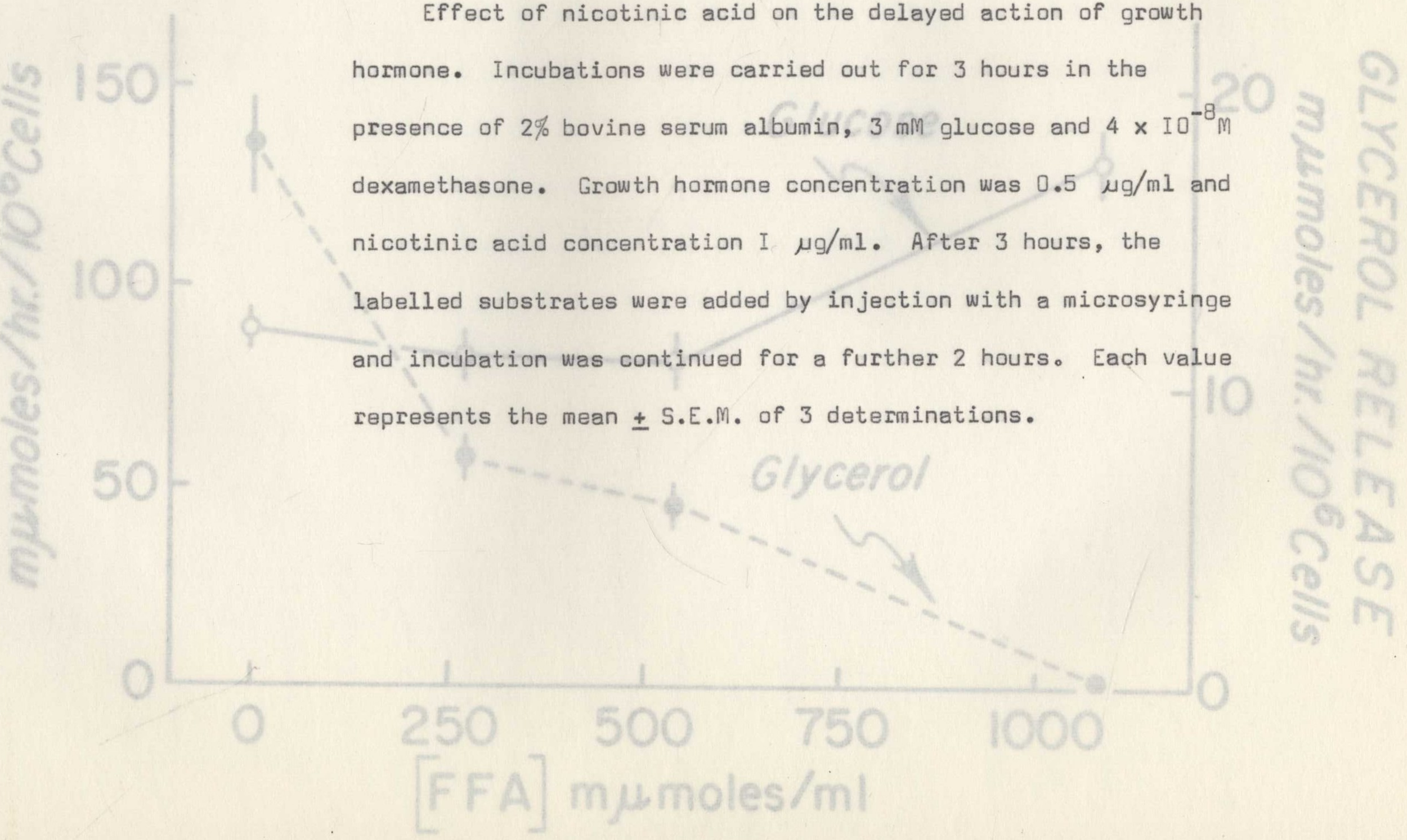


FIGURE 8

# EFFECT OF ADDED PALMITATE ON GLUCOSE UPTAKE AND LIPOLYSIS IN ISOLATED FAT CELLS

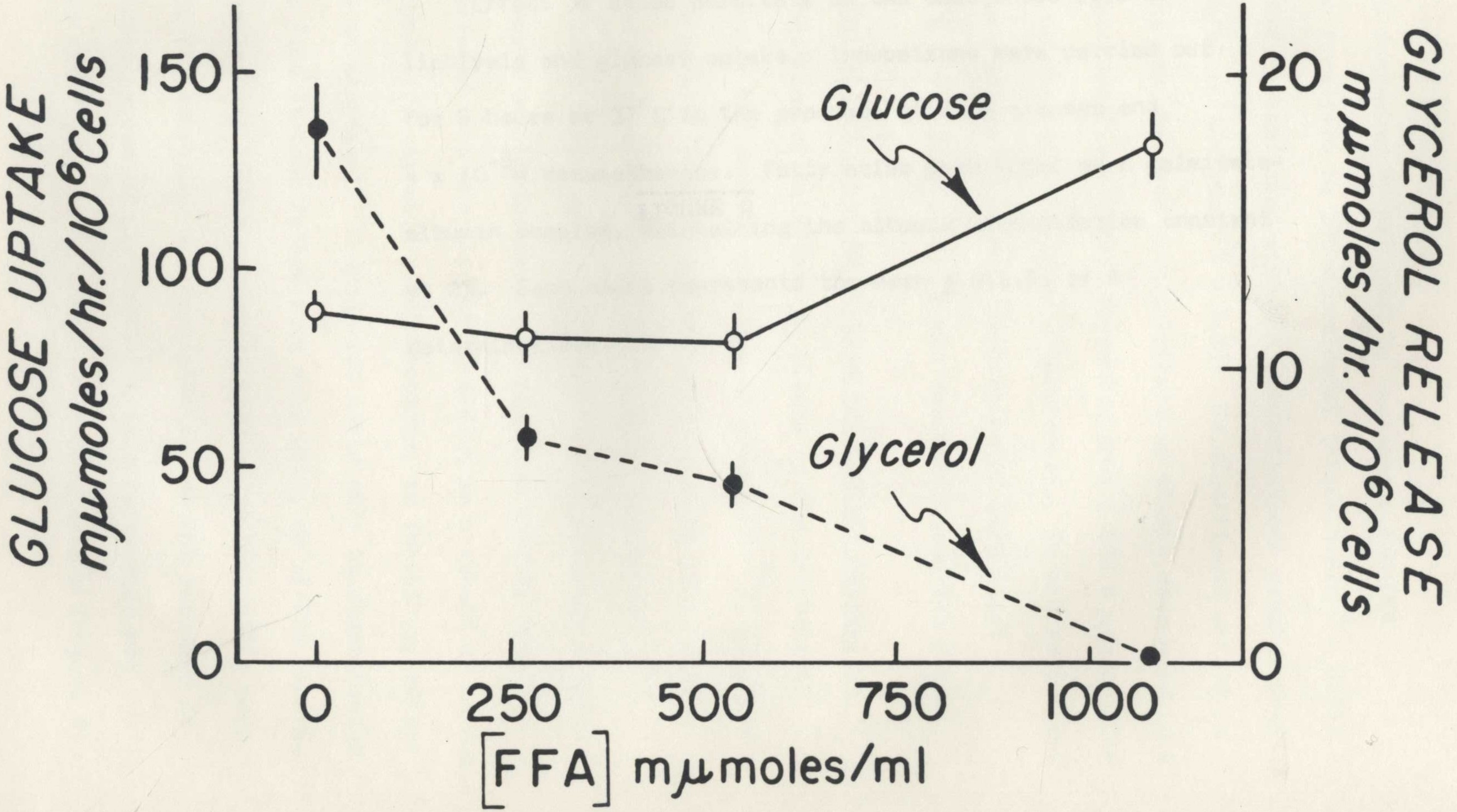


FIGURE 8

Figure 8

Effect of added palmitate on the endogenous rate of lipolysis and glucose uptake. Incubations were carried out for 5 hours at 37°C in the presence of 3 mM glucose and $4 \times 10^{-8}$ M dexamethasone. Fatty acids were added as a palmitate-albumin complex, maintaining the albumin concentration constant at 2%. Each value represents the mean ± S.E.M. of 4 determinations.

Lipolysis was decreased 58 per cent by increasing the FFA concentration from zero to 250 m μmoles/ml (FFA/albumin $\bar{V}$ - 0.75) and was completely inhibited with FFA concentrations greater than 1100 m μmoles/ml (FFA/albumin $\bar{V}$ - 3.3). No significant effect on glucose uptake was observed with palmitate or oleate concentrations up to 500 m μmoles/ml. At higher concentrations ( 500 m μmoles FFA/ml), glucose uptake was stimulated consistently. In one experiment in which oleate was added at a concentration of 2000 μEq/1 (FFA/albumin $\bar{V}$ - 6.0 ), glucose uptake was stimulated 2-3 fold.

Effect of Palmitate on Labelled Glucose and Acetate Metabolism

The addition of palmitate or oleate to the incubation medium duplicated the delayed patterns of glucose-1-$^{14}$C, glucose-6-$^{14}$C and acetate-1-$^{14}$C oxidation and incorporation into fatty acids produced by growth hormone (Figure 9 ). The amount of palmitate (250-500 m μmoles/ml) required to reproduce the effects of growth hormone was approximately 50 per cent greater than the FFA Level released into the incubation medium as a result of hormone-activated lipolysis. As with growth hormone, an inhibitory effect of palmitate on glucose-1-$^{14}$C oxidation could only be demonstrated if fat cells from 15 hour fasted rats were used, or if FFA concentrations greater than 1000 m μmoles/ml were added when cells were prepared from fed animals. The concentration of FFA required to stimulate incorporation of glucose-1-$^{14}$C into fatty acid was comparable to that required to increase glucose uptake (Figure 8).

FIGURE 9

THE EFFECT OF ADDED PALMITATE ON GLUCOSE AND ACETATE OXIDATION AND INCORPORATION INTO FATTY ACIDS

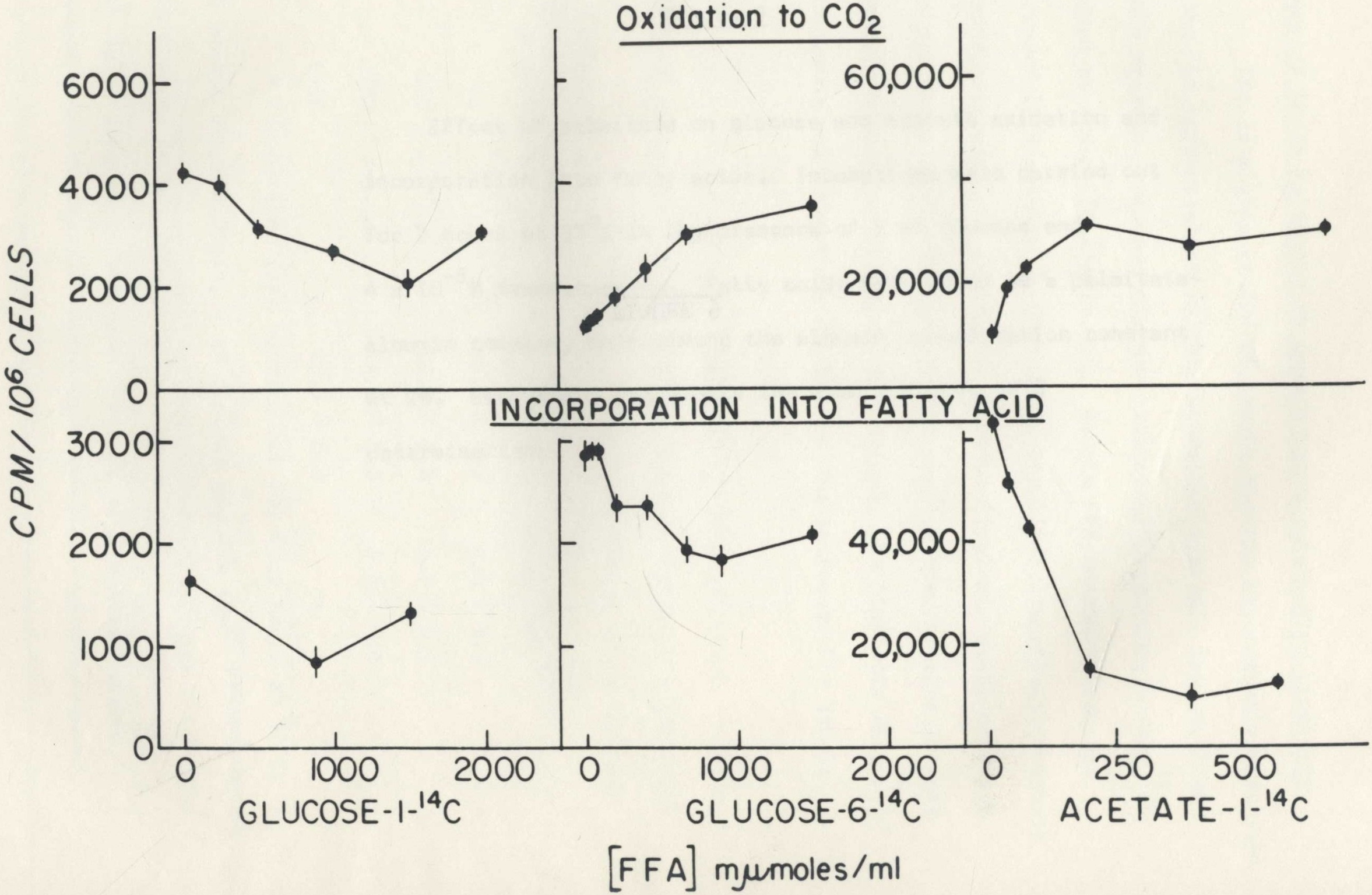


FIGURE 9

Figure 9

Effect of palmitate on glucose and acetate axidation and incorporation into fatty acids. Incubations were carried out for 2 hours at 37°C in the presence of 3 mM glucose and $4 \times 10^{-8}$M dexamethasone. Fatty acids were added as a palmitate-albumin complex, maintaining the albumin concentration constant at 2%. Each value represents the mean ± S.E.M. of 3 determinations.

Effect of Added Palmitate on Growth Hormone-Activated Lipolysis

The lipolytic action of growth hormone was markedly dependent on the FFA concentration in the incubation system. One ug.ml of growth hormone increased glycerol release 290 per cent in the absence of added FFA (Table V1). As the FFA concentration was increased (FFA/albumin $\bar{V}$ 0 to 4.2), the lipolytic effect of growth hormone progressively decreased although lipolysis still occurred at a palmitate concentration of 1400 µEq/l. When smaller amounts of growth hormone were used (100 m µg/ml), the lipolytic effect of the hormone was completely blocked by the increased FFA levels.

Table VI

Effect of Added Palmitate on Growth Hormone-Activated Lipolysis*

| Added Palmitate | Glycerol Release Above Control | |
|---|---|---|
| | 1 µg GH/ml | 0.1 µg GH/ml |
| m umoles/ml | m µmoles/$10^6$ cells/hour | |
| 10 | 19.1 ± 0.9 | 6.5 ± 0.3 |
| 350 | 16.5 ± 0.8 | 2.4 ± 0.1 |
| 700 | 14.5 ± 0.8 | 3.3. ± 0.1 |
| 1400 | 10.9 ± 0.3 | - |

* Incubations were carried out for four hours at 37°C. 2 % bovine serum albumin, 3 mM glucose and 4 x $10^{-8}$M dexamethasone were present in all incubations.

Each value represents the mean ± S.E.M. of 3 determinations.

## Human Placental Lactogen.

Human placental lactogen was prepared as described above. Isoelectric precipitation of the extract after DEAE cellulose fractionation resulted in considerable loss of protein, with little loss of HPL activity until the pH fell below 5.0. At pH 5.0, 36 per cent of the protein was precipitated with 7 per cent loss of HPL activity (Table VII). At pH 4.0, 53 per cent of the protein was precipitated but the loss of HPL activity rose to 27 per cent.

Table VII

Isoelectric Precipitation of 0.33 M Nacl DEAE-cellulose Fraction*

| Fraction | Total Protein | HPL | Specific Activity |
|---|---|---|---|
| | mg | mg | mg reference HPL/mg protein |
| 1. 300 mg DEAE HPL | 138 | 83 | 0.60 |
| 2. pH 6.0 Supernatant | 126.5 | 81.4 | 0.64 |
| Precipitate | 9.5 | 1.6 | 0.17 |
| 3. pH 5.0 Supernatant | 88.7 | 77.2 | 0.87 |
| Precipitate | 49.3 | 5.8 | 0.12 |
| 4. pH 4.0 Supernatant | 65.4 | 60.8 | 0.93 |
| Precipitate | 72.6 | 22.2 | 0.30 |

* Fractions obtained by adding 4N Hcl dropwise to DEAE-HPL in 2 ml 0.01 M TRIS buffer pH 9.0.

Values for precipitated protein at pH 6,5 and 4 represent the differences between the protein content of the original pH 9 material and the corresponding supernatant.

The elution pattern of the fraction remaining in the supernatant solution at pH 5.0 when applied to a Sephadex G-200 column is shown in Figure 10. HPL activity was mainly confined to the third protein peak (table VIII). Although there was considerable loss of HPL activity during the gel fractionation, material of high activity and low protein content was obtained.

Table VIII

HPL Activity of Eluted Fractions From Sephadex G-200 Column*

| Fraction | Protein | HPL | Specific Activity |
|---|---|---|---|
| | mg | mg | mg reference HPL/mg protein |
| FractionI | 13.14 | 0.13 | 0.01 |
| Fraction II | 13.74 | 1.1 | 0.08 |
| Fraction IIa | 8.81 | 7.1 | 0.80 |
| Fraction III | 8.72 | 15.5 | 1.78 |

* Fractions I,II,IIa and III refer to the pooled fractions indicated in Figure 10.

---

Polyacrylamide gel electrophoresis showed a dense band in the active third protein fraction (Figure II). This band was distinct from the dense retarded band seen in the second protein fraction, and both bands could be seen in the fraction between II and III. Elution of the protein from sectioned areas of the gel revealed marked activity by immunoassay in the dense band from fraction III. However, some activity was also present in the retarded band of fraction II.

FIGURE 10

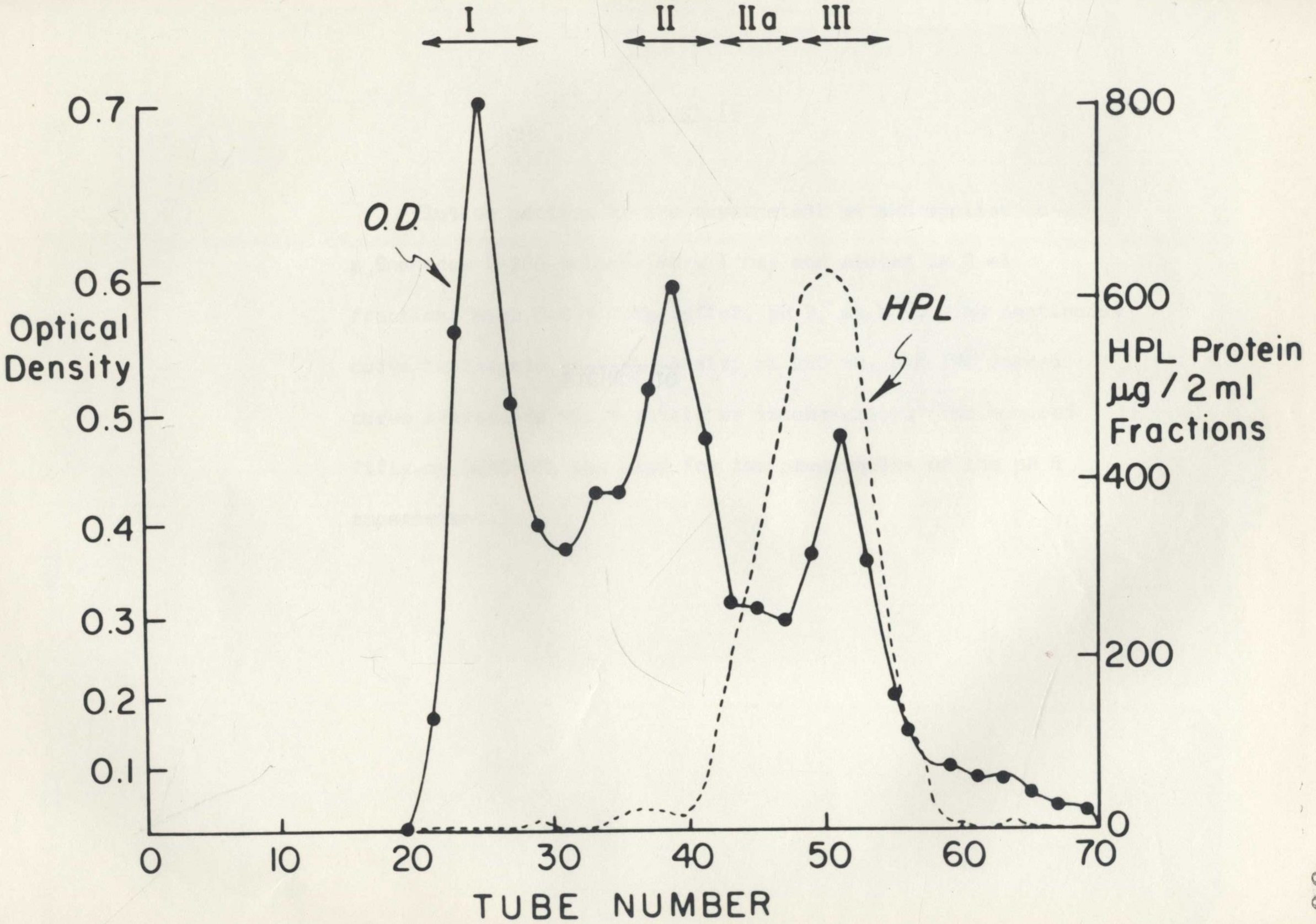

I
II
IIa
III
O.D.
HPL
0.7
0.6
Optical Density
0.5
0.4
0.3
0.2
0.1
800
600
HPL Protein µg / 2 ml Fractions
400
200
0
0
10
20
30
40
50
60
70
TUBE NUMBER

FIGURE 10

Figure IO

Elution pattern of the supernatant at pH5 applied to a Sephadex G-200 column (40 x I cm) and eluted in 2 ml fractions with O.OIM Tris buffer, pH 9, at 5°C. The continuous curve represents optical density at 280 mU, and the dashed curve represents HPL activity by immuno-assay. Two hundred fifty mg DEAE-HPL was used for the preparation of the pH 5 supernatant.

FIGURE 11

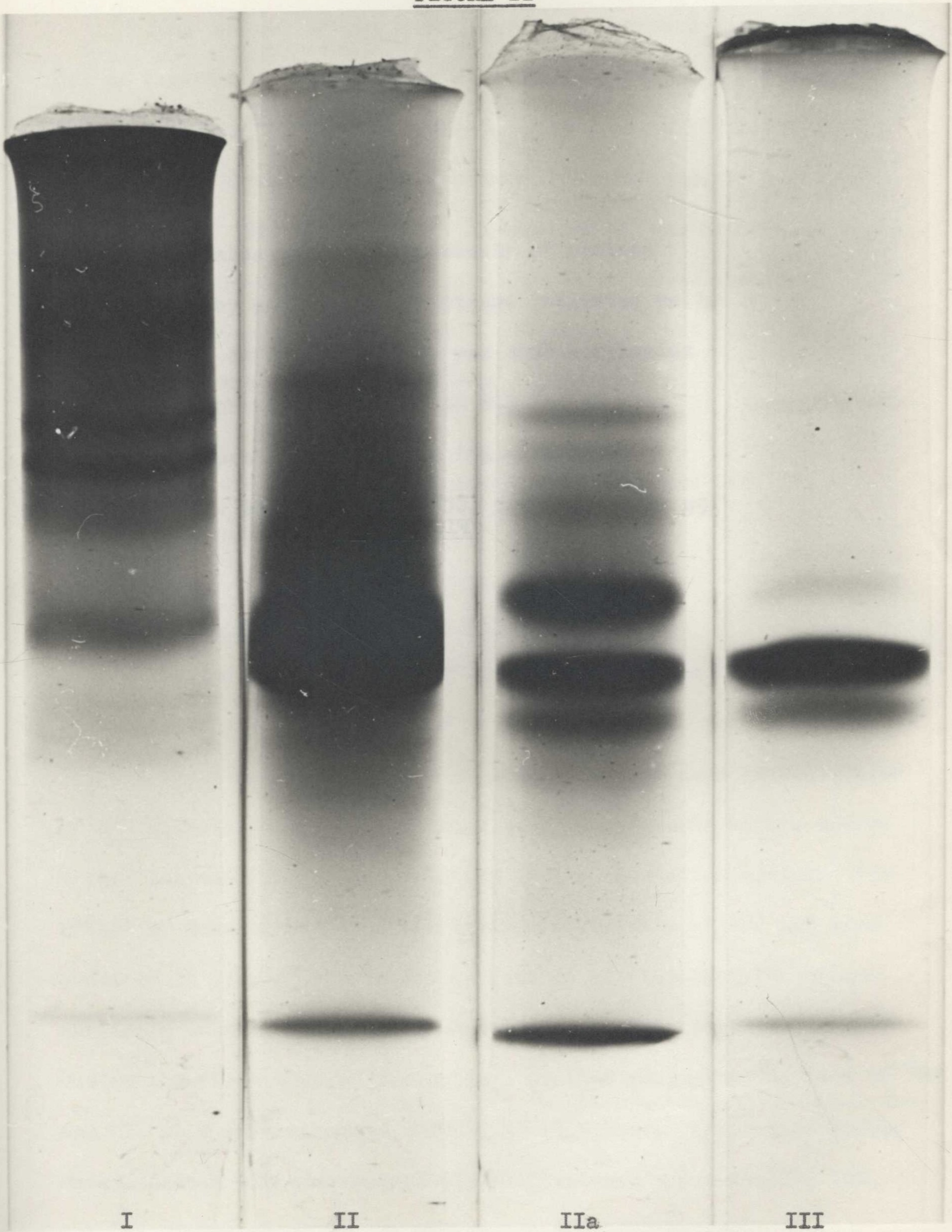


FIGURE 11

Figure II

Polyacrylamide gel electrophoresis of protein peaks obtained by pooling the fractions indicated as I, II, IIa and III in Fig. I. Seven per cent acrylamide was stacked at pH 8.9 and run at pH 9.5 for 45 min. The amounts of protein applied to the gels were:

I: 315 µg; II: 309 µg; IIa: 330 µg; III: 336 µg.

The potency of the purified HPL was compared with that of Dr. M. Grumbach's CGP 12-5, Dr. H. Friesen's HPL 67, and Growth Factor Placental Origin Human (GFPOH) produced by the Lederle Laboratories using the double antibody radioimmunoassay procedure of Beck et al (30) (Figure 12). Comparison of the bound/free ratio of HPL-$I^{131}$ produced by comparable amounts of these peptides showed the relative potencies with reference to the purified HPL to be as follows: C.G.P. 12-5 6.1%, Friesen's HPL 67 21% and GFPOH 22%.

This highly purified preparation of HPL was used for all subsequent studies on lipolysis.

Effect of HPL and Dexamethasone on Lipolysis

During the first hour of incubation, 500 µg HPL/ml did not stimulate lipolysis as measured by either glycerol or FFA release (Figure 13). After four hours of incubation the same concentration of HPL stimulated glycerol release over 200 per cent with a concomitant increase in FFA accumulation in the incubation medium. The effect of dexamethasone on the lipolytic activity of HPL was also examined in view of the report by Fain et al (26) that the glucocorticoid potentiated the adipokenetic action of growth hormone. During the first hour of incubation, neither dexamethasone ($4 \times 10^{-8}$M) nor HPL plus dexamethasone stimulated lipolysis. After four hours, dexamethasone alone increased glycerol release approximately 32%, but FFA accumulation in the medium was not accelerated.

FIGURE 12

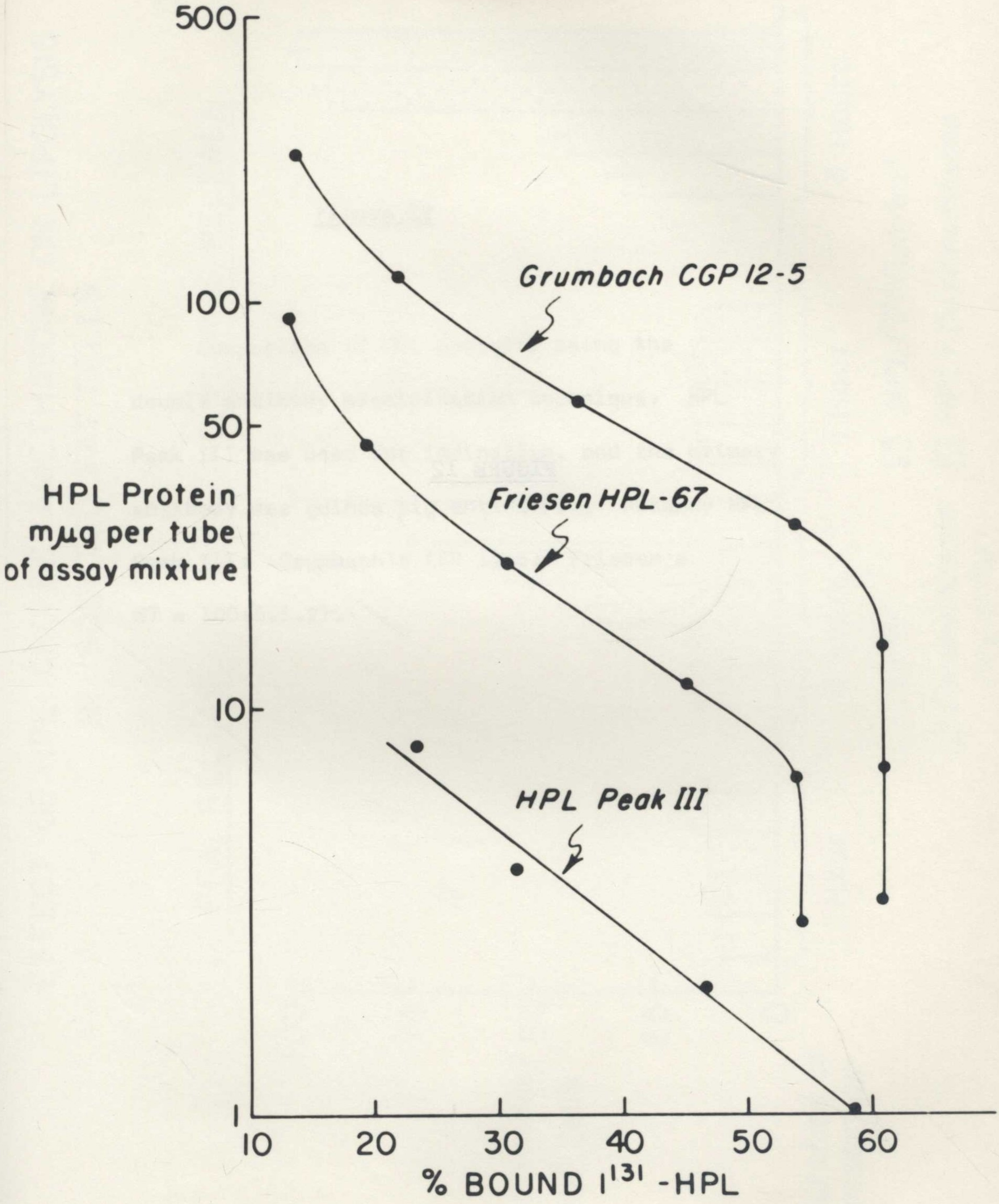

500
100
50
10
1
HPL Protein mμg per tube of assay mixture
Grumbach CGP 12-5
Friesen HPL-67
HPL Peak III
10
20
30
40
50
60
% BOUND I131 -HPL

FIGURE 12

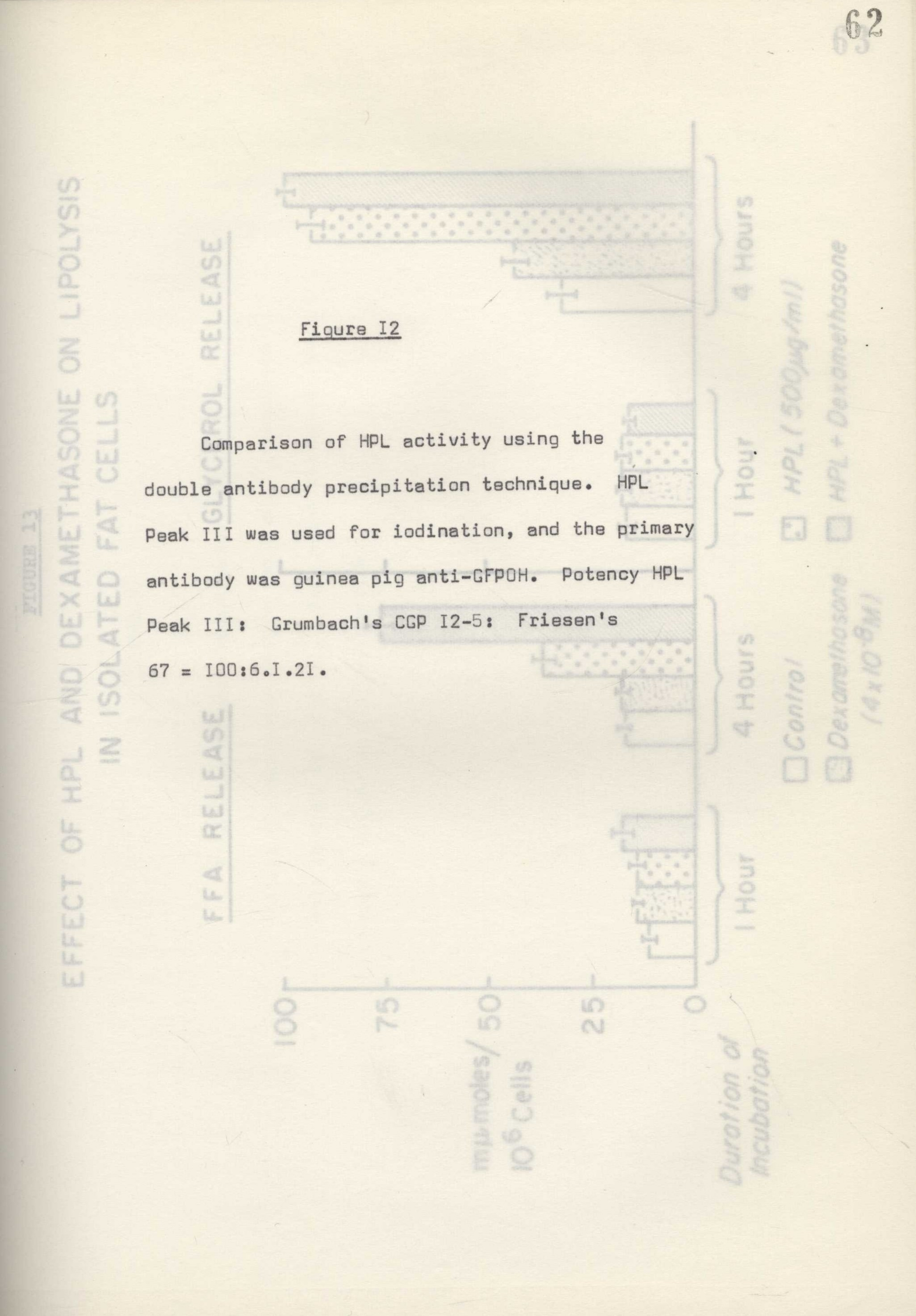



Figure I2

Comparison of HPL activity using the double antibody precipitation technique. HPL Peak III was used for iodination, and the primary antibody was guinea pig anti-GFPOH. Potency HPL Peak III: Grumbach's CGP I2-5: Friesen's 67 = I00:6.I.2I.

FIGURE 13

# EFFECT OF HPL AND DEXAMETHASONE ON LIPOLYSIS IN ISOLATED FAT CELLS

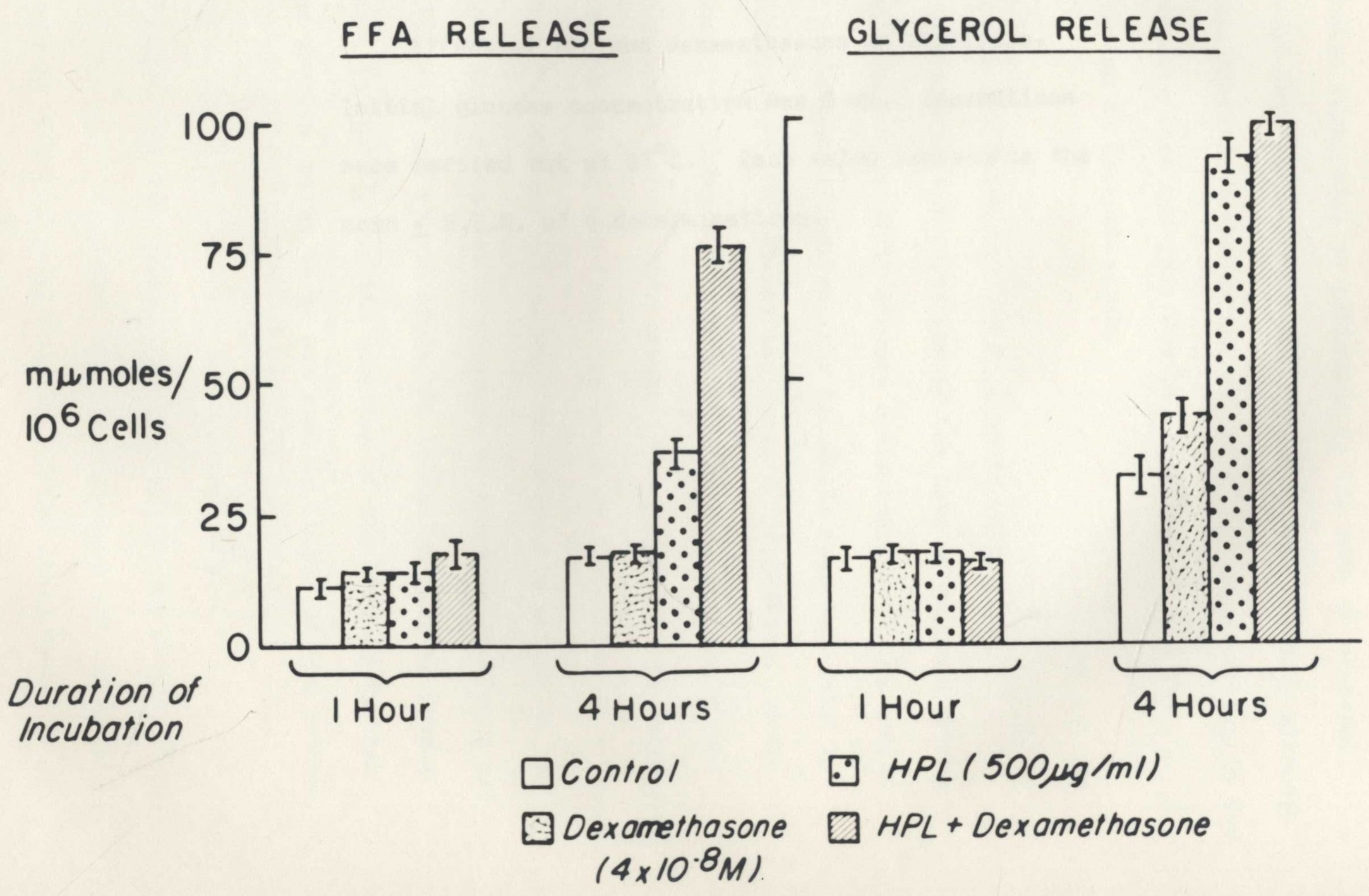


Figure I3

Effect of HPL and dexamethasone on lipolysis.

Initial glucose concentration was 3 mM. Incubations were carried out at 37°C. Each value represents the mean ± S.E.M. of 4 determinations.

HPL activated lipolysis, as reflected by the rate of glycerol release, was not significantly influenced by the steroid, although under these conditions the concentration of FFA accumulating in the medium was more than doubled, due to impairment of FFA re-esterification by dexamethasone.

Effect of HPL and Dexamethasone on Glucose Uptake.

In order to avoid changes in the rate of glucose uptake resulting from a decreasing glucose concentration in the medium during a prolonged incubation, the fat cells were suspended in fresh medium after the initial two hours of incubation. Under these conditions glucose uptake by the fat cell suspension was linear throughout a four hour incubation. Dexamethasone decreased glucose uptake within the first two hours of incubation by 27 per cent ($p < 0.01$), whereas HPL exerted no detectable effect at this time either in the absence or presence of the steroid (Table IX).

During the third and fourth hours HPL inhibited glucose uptake 24 per cent ($p < 0.01$), and the addition of the steroid further decreased glucose uptake to 54 per cent ($p < 0.01$) of the control rats.

Table IX

The Effect of HPL and Dexamethasone on Glucose Uptake by Isolated Fat Cells*

| Additions | Glucose Uptake | |
|---|---|---|
| | Period I | Period 2 |
| | m μmoles/$10^6$ cells/hour | |
| Control | 82.2 ± 4.2 | 88.6 ± 3.4 |
| Dexamethasone | 60.4 ± 3.1 | 70.7 ± 2.8 |
| HPL | 84.6 ± 4.8 | 62.6 ± 3.2 |
| Dexamethasone + HPL | 62.2 ± 5.2 | 47.8 ± 3.6 |

* Initial glucose concentration 3 mM. Incubations were carried out at 37°C. Period I represents the first two hours of incubation and period 2 the subsequent two hours. Following period I, the cells were resuspended in fresh incubation medium containing glucose 3 mM and the same hormone mixture present during period I. Dexamethasone concentration $4 \times 10^{-8}$ M and HPL concentration 500 μg/ml. Each value represents the mean ± S.E.M. of 6 determinations.

Time Sequence of Appearance of HPL Activated Lipolysis

Glycerol and FFA release continued at a linear rate (glycerol 7 m μmoles/$10^6$ cells/hour, FFA 3 m μmoles/$10^6$ cells/hour) throughout a five hour period of incubation (Figure 14). HPL activated lipolysis was preceded by a 60 minute lag period following which glycerol and FFA release rapidly increased and continued at an accelerated rate (glycerol 32 m μmoles/$10^6$ cells/hour, FFA 22 m μmoles/$10^6$ cells/hour) for the next three hours.

FIGURE 14

TIME SEQUENCE FOR APPEARANCE OF HPL-STIMULATED LIPOLYSIS IN ISOLATED FAT CELLS

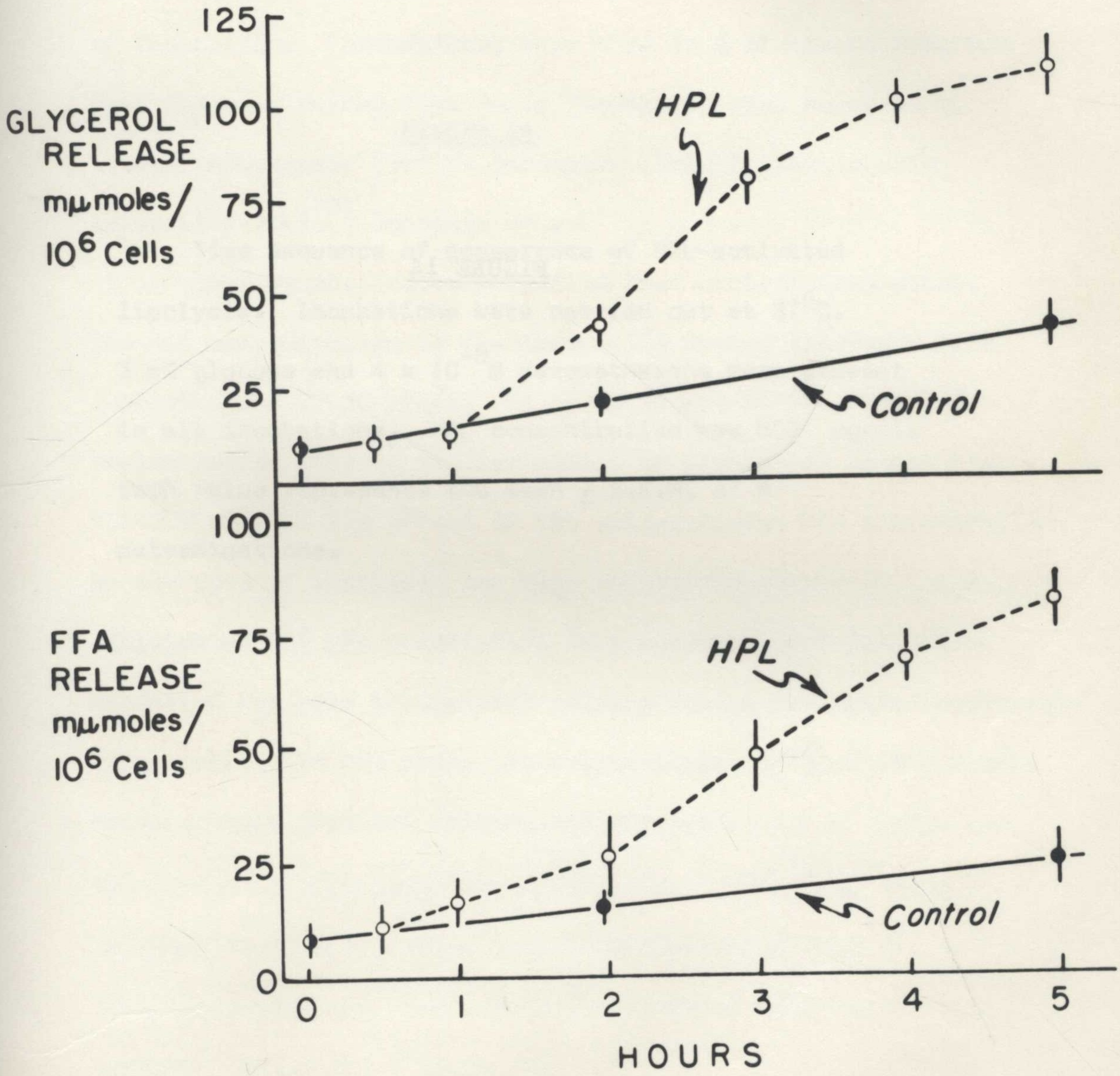


FIGURE 14

Figure I4

Time sequence of appearance of HPL-activated lipolysis. Incubations were carried out at 37°C. 3 mM glucose and 4 x $10^{-8}$ M dexamethasone were present in all incubations. HPL concentration was 500 µg/ml. Each value represents the mean ± S.E.M. of 4 determinations.

During the fifth hour of incubation, lipolysis, as reflected by glycerol release, returned to the control rate. This slowing of lipolysis was not secondary to destruction of HPL during the course of incubation, since it was not prevented by the addition of 500 μg HPL/ml during the second and third hours of incubation. Furthermore, less than 32 % of the hormone had been degraded during five hours incubation when measured by either immunoassay (30) or increased trichloracetic acid solubility of $I^{131}$ labelled HPL.

During the course of a five hour control incubation, the FFA concentration in the incubation medium increased from 10 μEq/l to 60-80 μEq/l. In the presence of HPL, the FFA concentration after a similar period of incubation ranged from 250-300 uEq/l. The effect of the extracellular FFA concentration on the rate of lipolysis was also determined (Table X). The addition of 250 μEq palmitate/l to a suspension of fat cells decreased the rate of glycerol release during two hours incubation approximately 50 per cent. At extracellular [FFA] > 1000 μEq/l, no detectable glycerol release was observed.

Table X

Effect of Added Palmitate on Glycerol Release by Isolated Fat Cells*

| Palmitate | $\frac{\text{Palmitate}}{\text{Albumin}}$ Ratio | Glycerol Release |
|---|---|---|
| uEq/l | V | m μmoles/$10^6$ cells/hour |
| 10 | 0.03 | 12.6 ± 1.6 |
| 250 | 0.76 | 7.8 ± 0.5 |
| 500 | 1.52 | 5.6 ± 0.4 |
| 1100 | 3.34 | N.D. |

* Incubations were carried out for two hours at 37°C. 2% bovine serum albumin and 3 mM glucose were present in all incubations. Each value represents the mean ± S.E.M. of 6 determinations.

N.D. - not detectable.

---

Effect of Actinomycin D and Puromycin on HPL-Activated Lipolysis

Actinomycin D ($1.5 \times 10^{-6}$M) and puromycin ($1 \times 10^{-4}$M) when added at the beginning of the incubation, completely inhibited the stimulation of lipolysis by HPL (Table XI, Figure 15). If these inhibitors were added after the first hour of incubation, HPL-Activated lipolysis was not blocked (Table XI). It is of interest that in one experiments the release of both FFA and glycerol into the medium was lower in the presence of Actinomycin D than in control incubations (Figure 15).

FIGURE 15

# EFFECT OF ACTINOMYCIN D ON HPL STIMULATED LIPOLYSIS

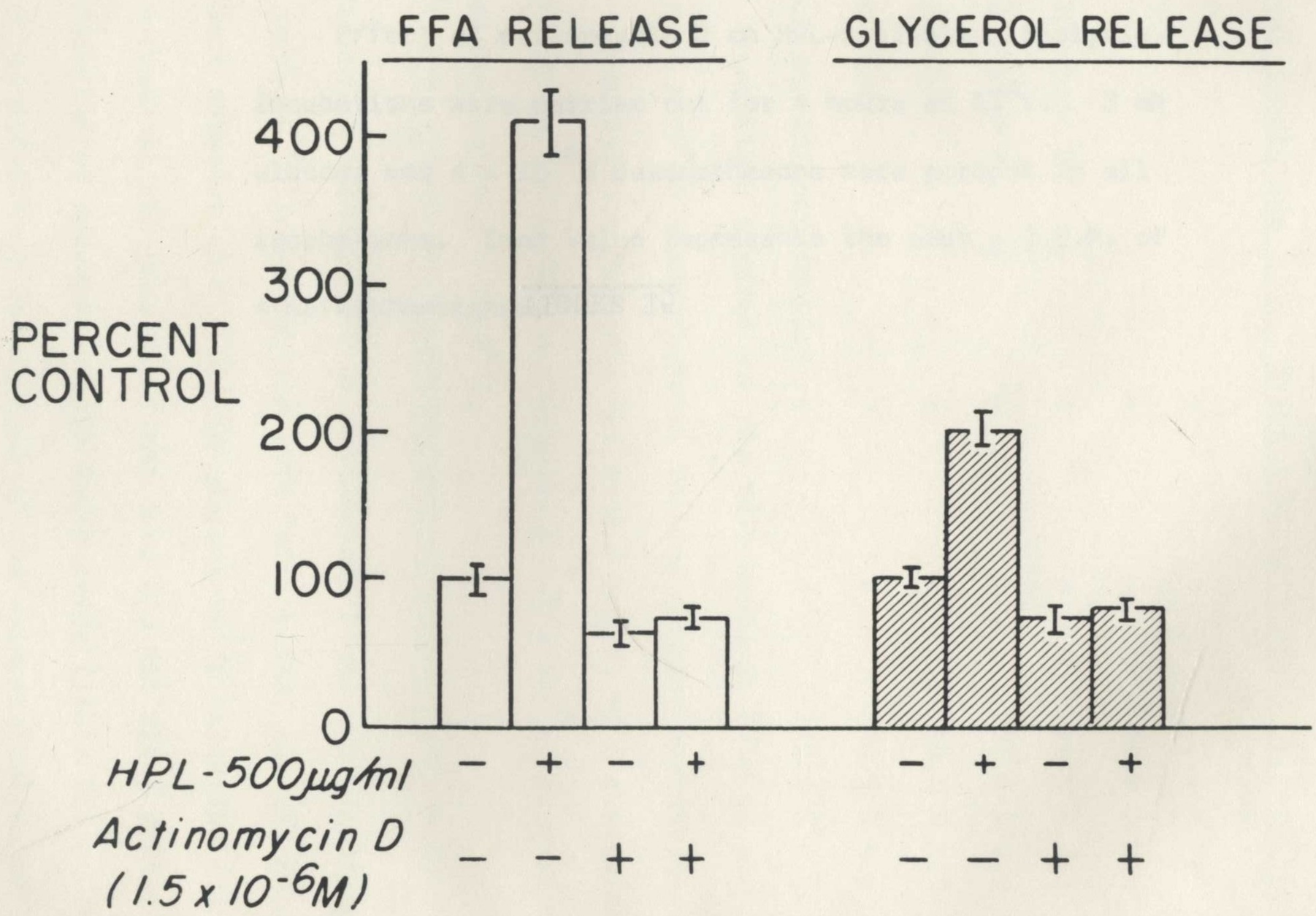


FIGURE 15

Figure I5

Effect of actinomycin D on HPL-activated lipolysis. Incubations were carried out for 4 hours at 37°C. 3 mM glucose and 4 x $10^{-8}$M dexamethasone were present in all incubations. Each value represents the mean ± S.E.M. of 4 determinations.

Table XI

Effect of Actinomycin D and Puromycin on HPL-Activated Lipolysis*

| Experimental Conditions | Inhibitor Added: Start | Inhibitor Added: After 1 hour | Glycerol Release: m μmoles/$10^6$ cells/hour.. |
|---|---|---|---|
| Control | - | - | 12.5 ± 1.4 |
| HPL | - | - | 34.8 ± 2.8 |
| HPL + Actinomycin D | + | - | 14.3 ± 2.3 |
| HPL + Actinomycin D | - | + | 37.6 ± 3.8 |
| HPL + Puromycin | + | - | 10.8 ± 3.7 |
| HPL + Puromycin | - | + | 32.9 ± 4.4 |
| HPL + Puromycin Aminonucleoside | + | - | 30.4 ± 4.2 |

* Incubations carried out for four hours at 37°C in the presence of $4 \times 10^{-8}$ M dexamethasone, initial glucose concentration was 3 mM. Each value represents the mean ± S.E.M. of 5 determinations. HPL concentration 500 μg/ml, actinomycin D $1.5 \times 10^{-6}$ M, puromycin and its aminonucleoside $1 \times 10^{-4}$ M.

---

Similar concentrations of actinomycin D did not significantly affect glucose-6-$^{14}$C incorporation into fatty acids, but did cause a 25 per cent increase ($p < 0.01$) in glucose-1-$^{14}$C oxidation (Table XII). Puromycin at the concentrations used did not alter either glucose oxidation or conversion into fatty acids (Table XII). The addition of comparable concentrations of the aminonucleoside of puromycin at the beginning of the incubation did not alter glucose oxidation or conversion to fatty acids (Table XII) and did not affect HPL-activated lipolysis (Table XI).

Table XII

Effect of Actinomycin D and Puromycin on Glucose Oxidation and Incorporation into Fatty Acids by Isolated Fat Cells*

| Substrate | Inhibitor | $^{14}C$ Glucose Conversion to $CO_2$ | Fatty Acids |
|---|---|---|---|
| | | cpm/$10^6$ cells/hour | |
| Glucose-1-$^{14}C$ | -- | 1352 ± 65 | 537 ± 23 |
| | Actinomycin D | 1701 ± 70 | 541 ± 37 |
| | Puromycin | 1462 ± 84 | 572 ± 48 |
| Glucose-6-$^{14}C$ | -- | 650 ± 36 | 2460 ± 280 |
| | Actinomycin D | 710 ± 48 | 2610 ± 188 |
| | Puromycin | 628 ± 52 | 2525 ± 204 |

* Incubations were carried out for three hours at 37°C. The initial glucose concentration was 3 mM. Actinomycin D concentration $1.5 \times 10^{-6}$M, puromycin $1 \times 10^{-4}$M. Each value is the mean ± S.EM. 5-6 determinations.

Effect of HPL on Growth Hormone-Activated Lipolysis

The lowest concentrations of HPL and Growth Factor Placental Origin - Human (GFPOH) stimulating lipolysis in fat cell suspensions as measured by glycerol release, were 20 and 100 µg/ml, respectively (Figure 16). The five-fold greater biological activity of the purified HPL preparation compared to GFPOH is similar to the relative immunological potencies of the two preparations (Figure 12). At these concentrations increased free fatty acid accumulation was not demonstrable. Human growth hormone, 10 m µg/ml, produced the same degree of stimulation of lipolysis as measured by glycerol release, as that produced by 20 µg HPL/ml.

FIGURE 16

# EFFECT OF HPL ON LIPOLYTIC ACTIVITY

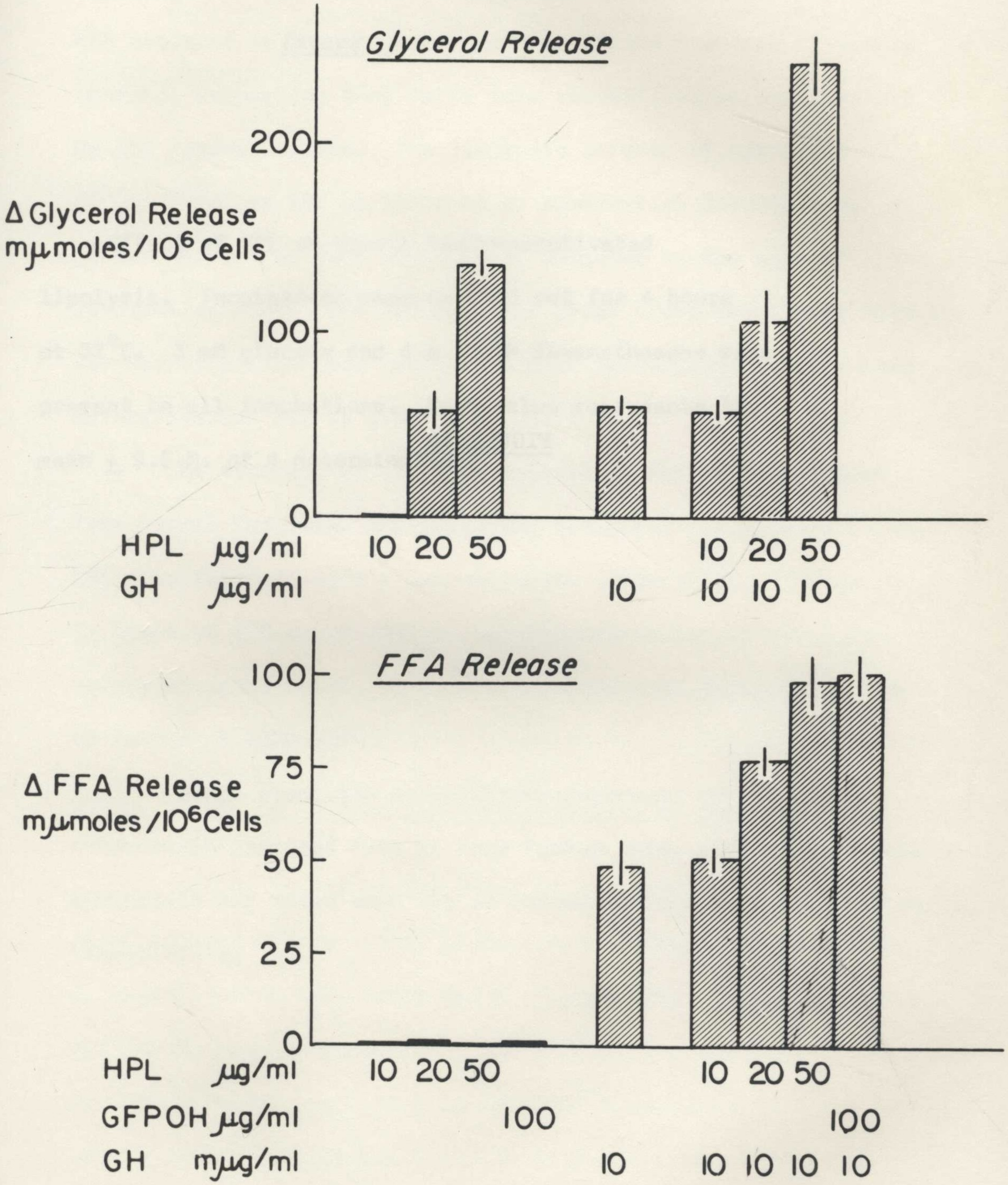


FIGURE 16

Figure I6

Effect of HPL on growth hormone-activated lipolysis. Incubations were carried out for 4 hours at 37°C. 3 mM glucose and $4 \times 10^{-8}$ M dexamethasone were present in all incubations. Each value represents the mean ± S.E.M. of 4 determinations.

In contrast to the effect of HPL, considerable accumulation of FFA occurred in the incubation medium in the presence of growth hormone, suggesting that fatty acid esterification was impaired by the latter hormone. The lipolytic effects of either 20 or 50 μg HPL/ml or 100 μg GFPOH/ml in combination with 10 m μg growth hormone/ml were additive as measured by the rate of glycerol release. FFA accumulation under these conditions however, was markedly enhanced, consistent with inhibition of fatty acid esterification due to growth hormone.

As measured by glycerol release in fat cells prepared from normal fed rats, HPL-activated lipolysis exhibited a linear log dose response over a concentration range of 20 - 500 μg/ml. In terms of FFA accumulation, hormone-activated lipolysis was rarely observed below 100 μg/ml, but increased progressively up to levels of 1000 μg/ml.

Basal lipolysis was markedly increased in fat cell suspensions prepared from 24 hour fasted rats, and no detectable activation was noted with HPL at concentrations less than 100 μg/ml (Table XIII).

Table XIII

Effect of HPL on Lipolysis of Fat Cell Suspensions Prepared from Fed, Fasted and Hypophysectomized Rats*

| Animal Preparation | HPL | Glycerol Release |
|---|---|---|
| | | m μmoles/$10^6$ cells/hour |
| Normal Fed | - | 11.4 ± 1.2 |
| | 1 | 10.8 ± 1.6 |
| | 10 | 12.6 ± 1.8 |
| | 20 | 16.8 ± 1.6 |
| Normal Fasted | - | 22.4 ± 2.2 |
| | 1 | 20.2 ± 3.6 |
| | 20 | 22.8 ± 3.4 |
| | 100 | 32.6 ± 3.2 |

* Incubations carried out for four hours at 37°C, initial glucose concentration was 3 mM and dexamethasone $4 \times 10^{-8}$ M. Each value represents the mean ± S.E.M. of four determinations.

---

Effect of HPL on Glucose and Acetate Oxidation and Incorporation into Fatty Acids

During the first two hours of incubation, HPL did not significantly affect either glucose and acetate oxidation to $CO_2$ or incorporation into fatty acids (Figure 17). During the third and fourth hour of incubation, HPL decreased glucose-1-$^{14}$C oxidation 30 - 35 per cent, increased glucose-6-$^{14}$C oxidation 30 - 35 per cent and increased acetate oxidation 50 - 55 per cent (Figure 18). Furthermore, incorporation of all precursors into fatty acids was decreased uniformly to 25 - 45 per cent of control levels (Figure 18).

FIGURE 17

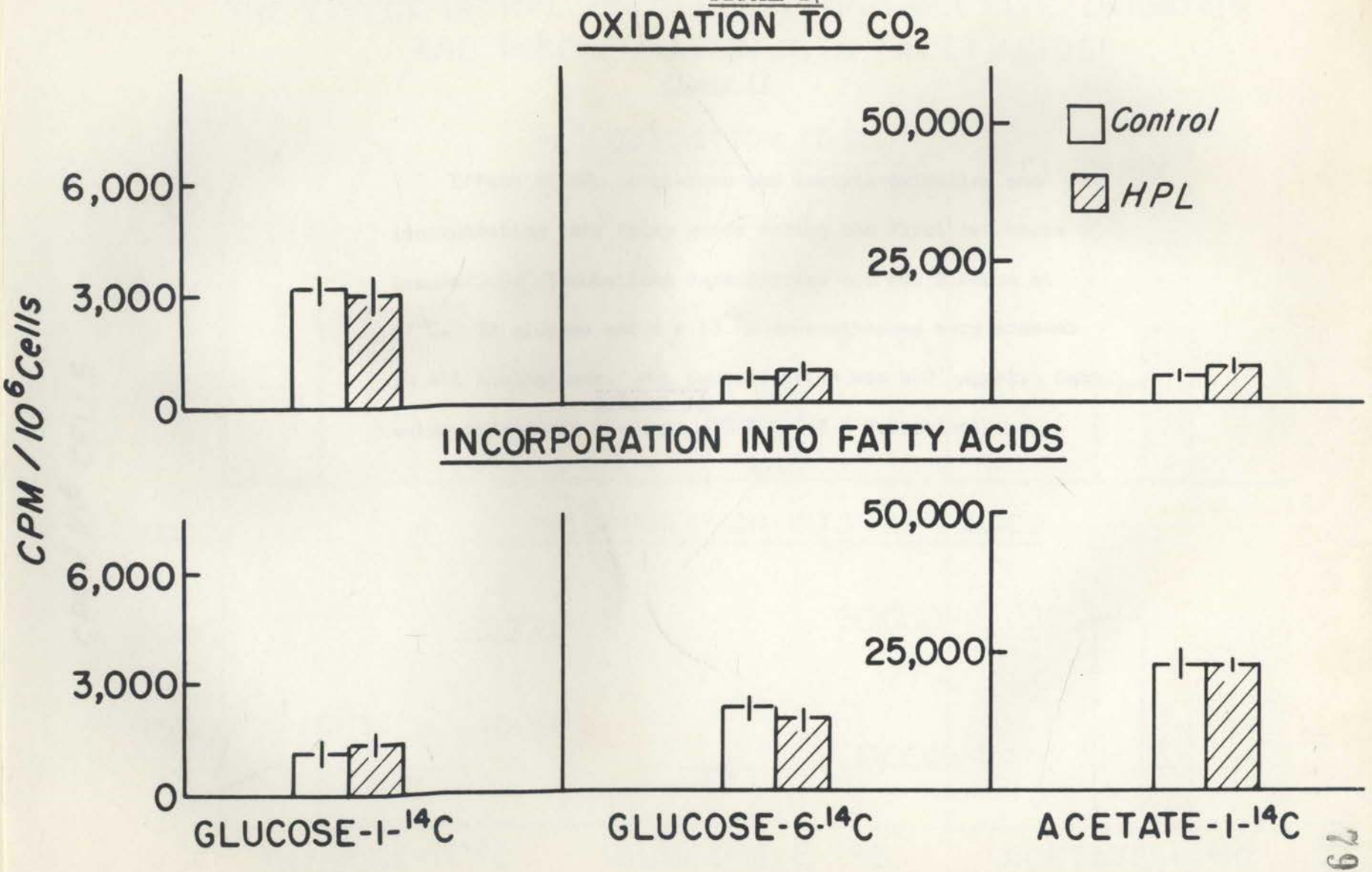
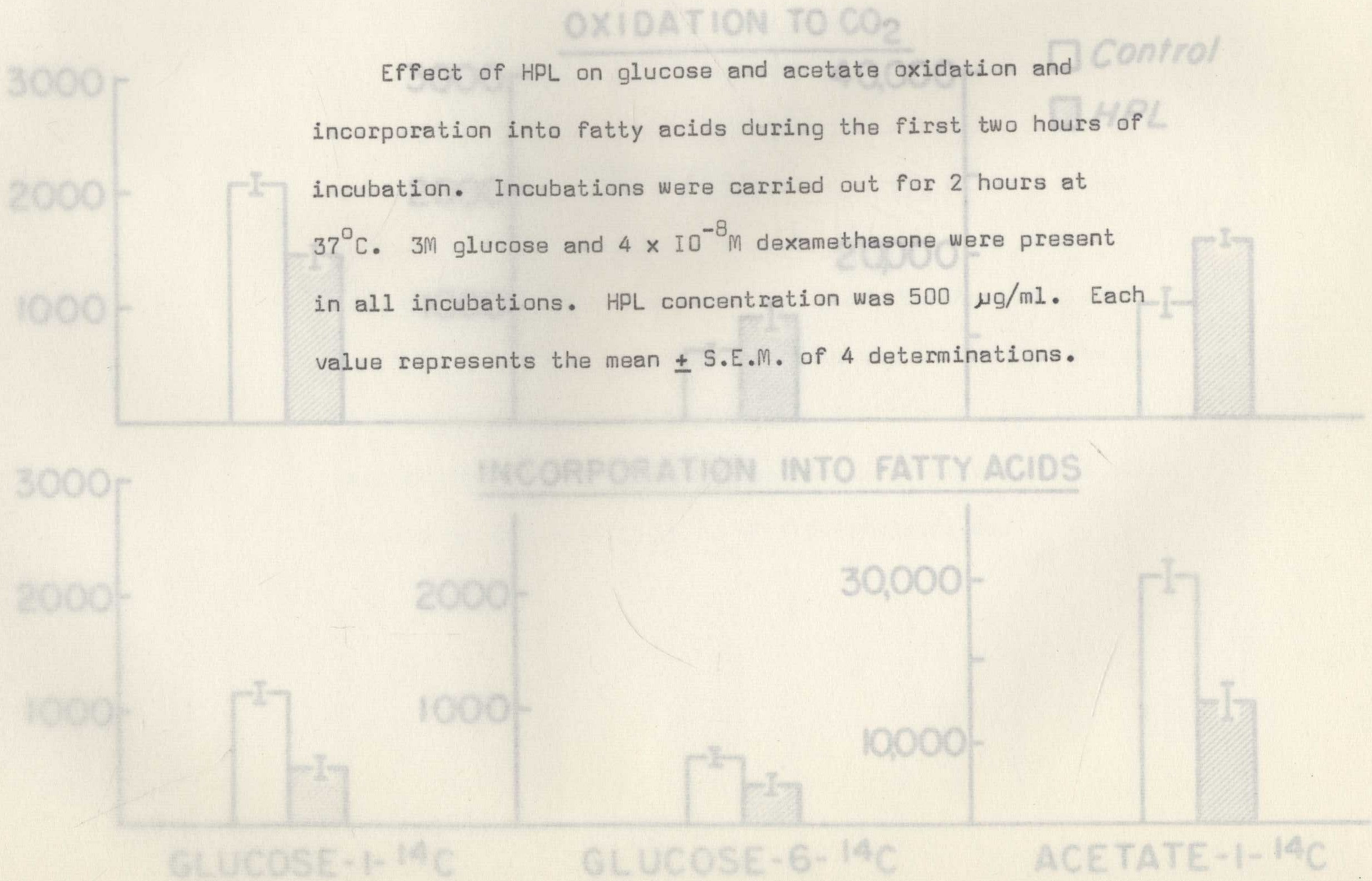

OXIDATION TO $CO_2$
Control
HPL
50,000
25,000
6,000
3,000
0
CPM / $10^6$ Cells
INCORPORATION INTO FATTY ACIDS
50,000
25,000
6,000
3,000
0
GLUCOSE-1-$^{14}$C
GLUCOSE-6-$^{14}$C
ACETATE-1-$^{14}$C

FIGURE 17

FIGURE 18

# THE EFFECT OF HPL ON GLUCOSE AND ACETATE OXIDATION AND INCORPORATION INTO FATTY ACIDS

Figure I7




Effect of HPL on glucose and acetate oxidation and incorporation into fatty acids during the first two hours of incubation. Incubations were carried out for 2 hours at $37^{o}$C. 3M glucose and $4 \times 10^{-8}$M dexamethasone were present in all incubations. HPL concentration was 500 µg/ml. Each value represents the mean ± S.E.M. of 4 determinations.

FIGURE 18

# THE EFFECT OF HPL ON GLUCOSE AND ACETATE OXIDATION AND INCORPORATION INTO FATTY ACIDS

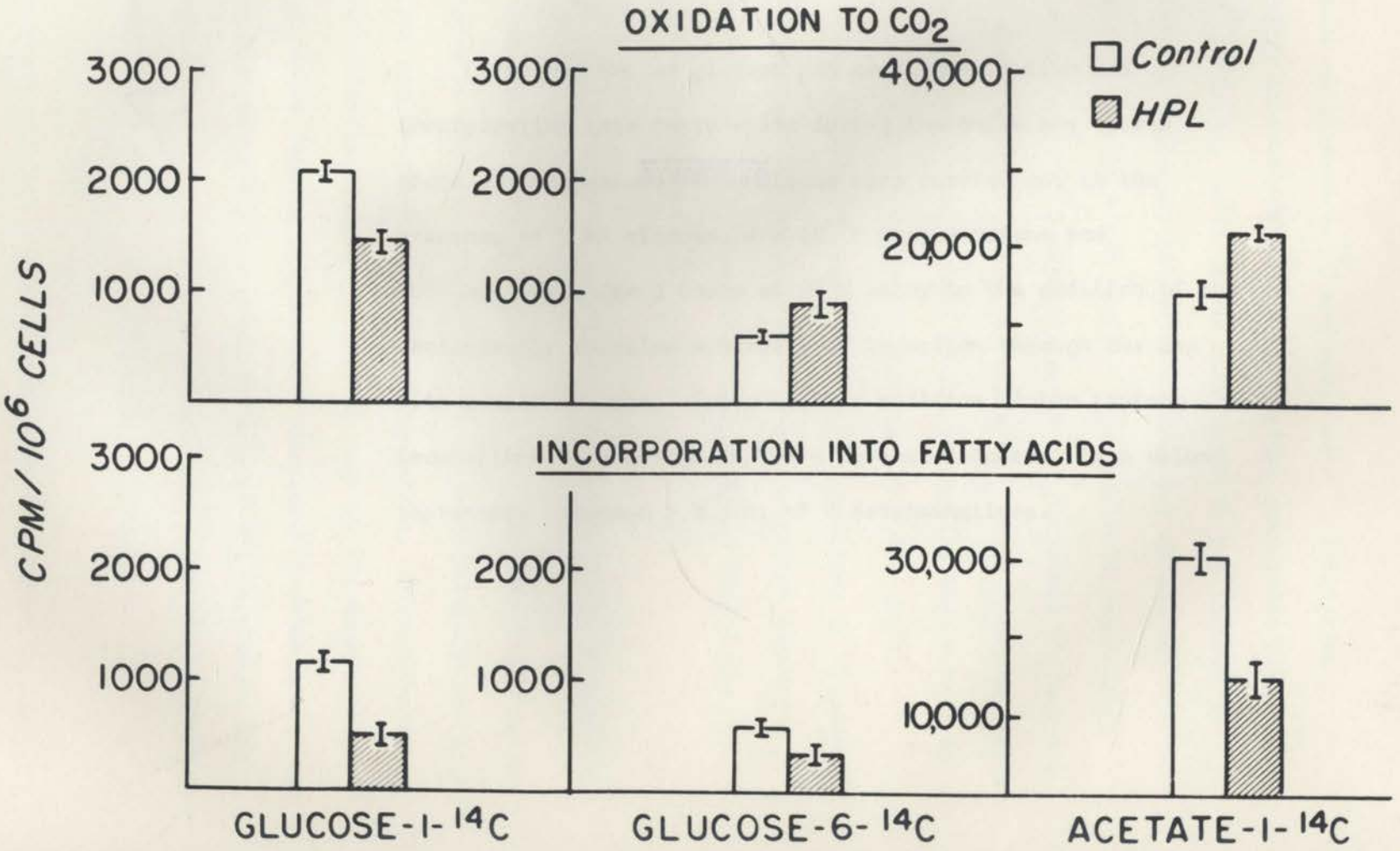


FIGURE 18

Figure I8

Effect of HPL on glucose and acetate oxidation and incorporation into fatty acids during the third and fourth hours of incubation. Incubations were carried out in the presence of 3 mM glucose, $4 \times 10^{-8}$ M dexamethasone and 500 µg HPL/ml for 3 hours at 37°C prior to the addition of the isotopically labelled substrate by injection through the cap with a microsyringe. Following the addition of the isotope incubations were continued for a further 2 hours. Each value represents the mean ± S.E.M. of 4 determinations.

The effects on glucose-6-$^{14}$C and acetate-1-$^{14}$C metabolism were readily observed with cell suspensions containing 2.5 - 3.5 x $10^6$ cells/ml; under these conditions in the incubation medium the final glucose concentration was 2.1 mM and the FFA concentration was 200 - 250 μEq/l. To demonstrate significant effects on glucose-1-$^{14}$C metabolism, it was necessary to increase the cell count to 5.0 - 5.5 x $10^6$ cells/ml; under these conditions the final glucose concentration in the incubation medium fell to 1.5 mM and the FFA concentration rose to 350 - 400 μEq/l.

These results suggested that the alterations in glucose and acetate metabolism associated with HPL activated lipolysis were secondary to the accumulation of FFA either within the fat cell, the incubation medium, or both.

Effect of HPL on Glucose and Acetate Metabolism in the Presence of a High Glucose Concentration.

Incubations were carried out in the presence of 300 mg per cent glucose in order to accelerate FFA re-esterification and prevent both intra- and extra-cellular FFA accumulation. HPL-activated lipolysis was still readily observed as seen by the accelerated rate of glycerol release (Figure 19). However FFA accumulation was prevented and the previously noted effects of HPL on glucose and acetate oxidation and incorporation into fatty acids were completely abolished.

FIGURE 19

THE EFFECT OF HPL ON LIPOLYSIS AND GLUCOSE AND ACETATE OXIDATION AND INCORPORATION INTO FATTY ACIDS IN THE PRESENCE OF HIGH GLUCOSE CONCENTRATION (300mg%)

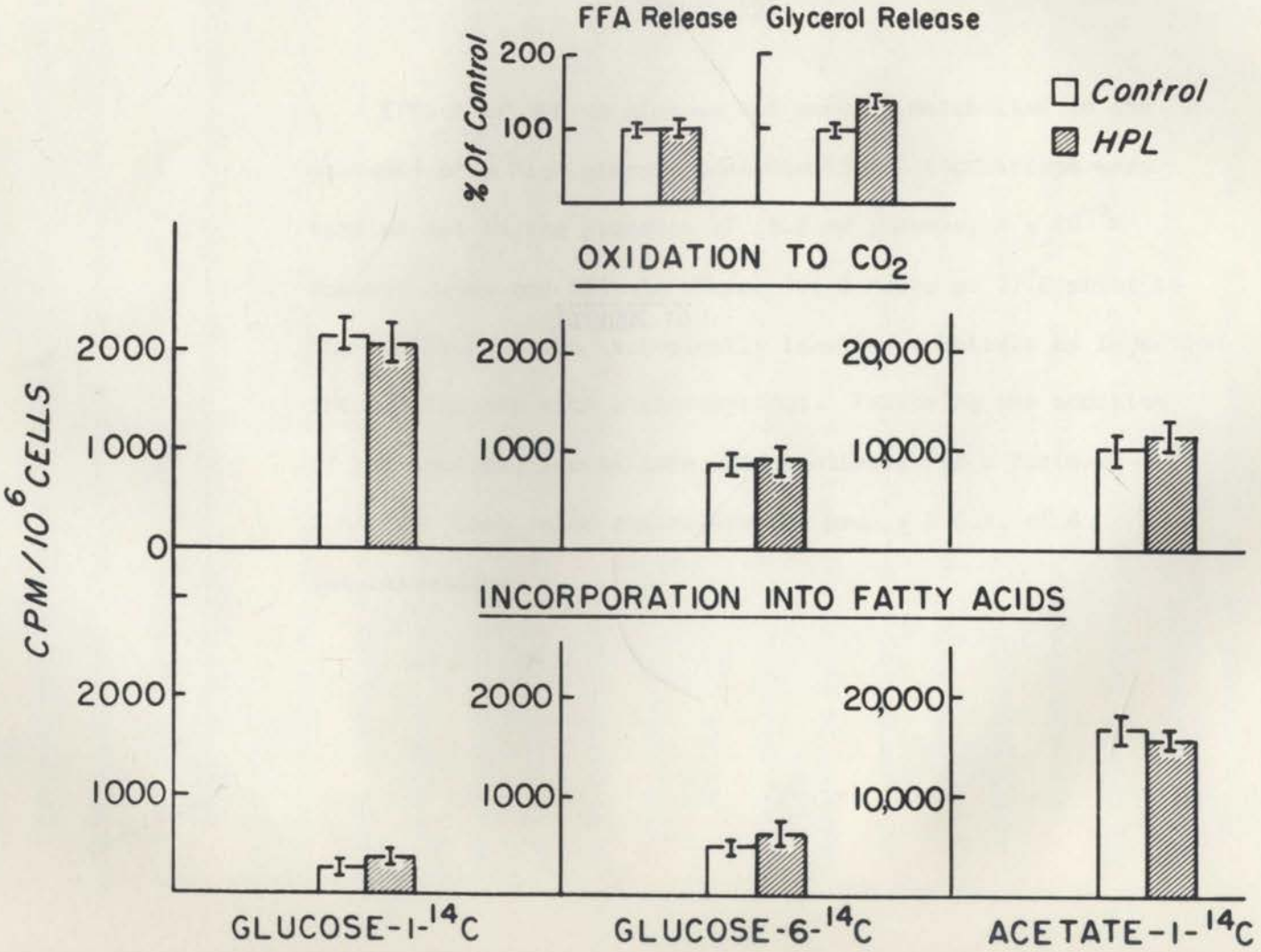


FIGURE 19

Figure 19

Effect of HPL on glucose and acetate metabolism in the presence of a high glucose concentration. Incubations were carried out in the presence of 16.6 mM glucose, $4 \times 10^{-8}$ M dexamethasone and 500 µg HPL/ml for 3 hours at 37°C prior to the addition of the isotopically labelled substrate by injection through the cap with a microsyringe. Following the addition of the isotope, incubations were continued for a further 2 hours. Each value represents the mean ± S.E.M. of 4 determinations.

Effect of Palmitate on Glucose and Acetate Oxidation and Incorporation into Fatty Acids.

The pattern of delayed effects of HPL on glucose-1-$^{14}$C, glucose-6-$^{14}$C and acetate-1-$^{14}$C oxidation and incorporation into fatty acids was identical with that of growth hormone (Figure 5), and could be reproduced by the addition of palmitate complexed to albumin by the method of Bowman (61) to the incubation medium (Figure 20). To reproduce the effects of HPL almost twice as much FFA had to be added as the concentration of FFA observed to accumulate in the incubation medium during the course of hormone-activated lipolysis. This suggests that the intracellular FFA concentration, rather the extracellular level, is the primary factor influencing glucose and acetate oxidation and fatty acid synthesis. In order to demonstrate effects of added palmitate on glucose-1-$^{14}$C oxidation and incorporation into fatty acids, it was necessary to use concentrations of 900-1000 μEq/l. Higher concentrations of palmitate (> 1500 μEq/l) stimulated both glucose-1-$^{14}$C oxidation and incorporation into fatty acids as originally reported by Leboeuf and Cahill (3) using the epididymal fat pad.

B. A Mechanism for the Hormonal Control of Lipolysis in Vivo.

The findings of Rizack in 1964 (47) suggested that in a cell-free system, the hormone-sensitive lipase from adipose tissue could be converted from an inactive to an active form by cyclic 3'5' AMP. The observation that the principal effect of both human growth hormone and HPL on adipose tissue was activation of lipolysis,

FIGURE 20

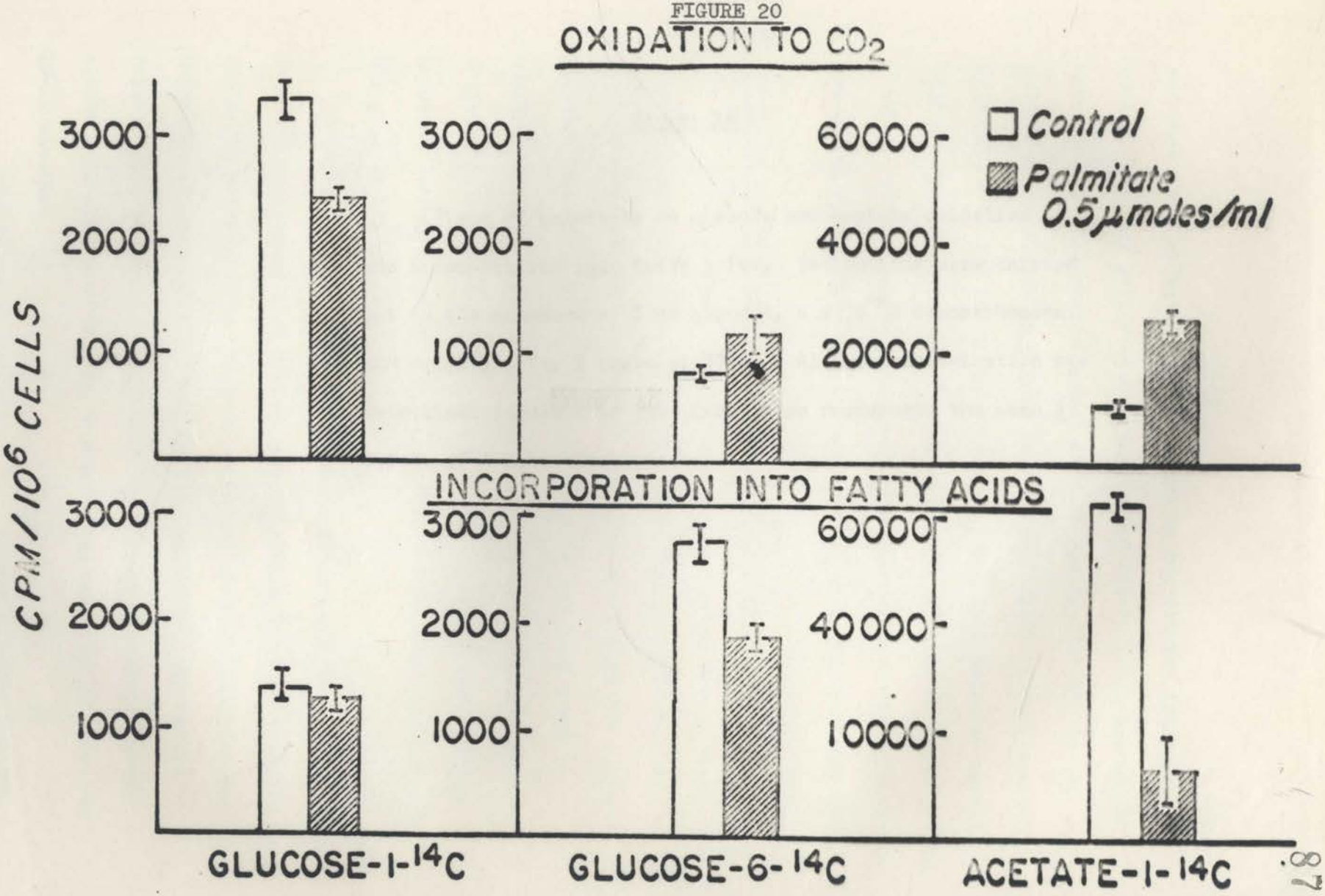

OXIDATION TO $CO_2$
Control
Palmitate
0.5 μ moles/ml
CPM/$10^6$ CELLS
3000
2000
1000
60000
40000
20000
INCORPORATION INTO FATTY ACIDS
10000
GLUCOSE-1-$^{14}$C
GLUCOSE-6-$^{14}$C
ACETATE-1-$^{14}$C

FIGURE 20

Figure 20

Effect of palmitate on glucose and acetate oxidation and incorporation into fatty acids. Incubations were carried out in the presence of 3 mM glucose, $4 \times 10^{-8}$ M dexamethasone and palmitate for 2 hours at 37°C. Albumin concentration was maintained constant at 2%. Each value represents the mean ± S.E.M. of 4 determinations.

and that other effects were secondary to the primary lipolytic action, together with the observations of Rizack (47), led to an assessment of the physiological side of cyclic 3'5' AMP in the control of lipolysis *in vivo*. As the penetration of cell membranes by cyclic 3'5' AMP is probably poor (62) the intracellular cyclic 3'5' AMP concentration was increased by the infusion of theophylline, a methyl xanthine which inhibits cyclic nucleotide phosphodiesterase, the enzyme responsible for the degradation of cyclic 3'5' AMP (63).

B. Effect of Theophylline on Lipolysis and Insulin Release in Vivo.

In the acutely adrenalectomized rat fasted for 48 hours, theophylline (15mg in 0.5ml physiological saline administered intravenously within 10 seconds) increased the plasma FFA concentration from 605 ± 37 μEq/l to a maximum of 815 ± 49 μEq/l in eight rats within three minutes. This moderate increase was followed by a prompt fall to 490 ± 21 μEq/l, levels below those obtained before injection, and occurring within three minutes after the peak of FFA concentration.

This fall in plasma FFA concentration occurred concomitantly with the appearance of a marked rise in plasma insulin, measured by radio-immunoassay. The increase in plasma insulin following the injection of theophylline could be detected within one minute (Figure 21), it rose to 10-13 times as high as the basal value within 5-10 minutes after the injection and remained elevated for 20-30 minutes.

FIGURE 21

EFFECT OF INTRAVENOUS GLUCOSE AND THEOPHYLLINE ON PLASMA INSULIN IN ADRENALECTOMISED FASTED RATS

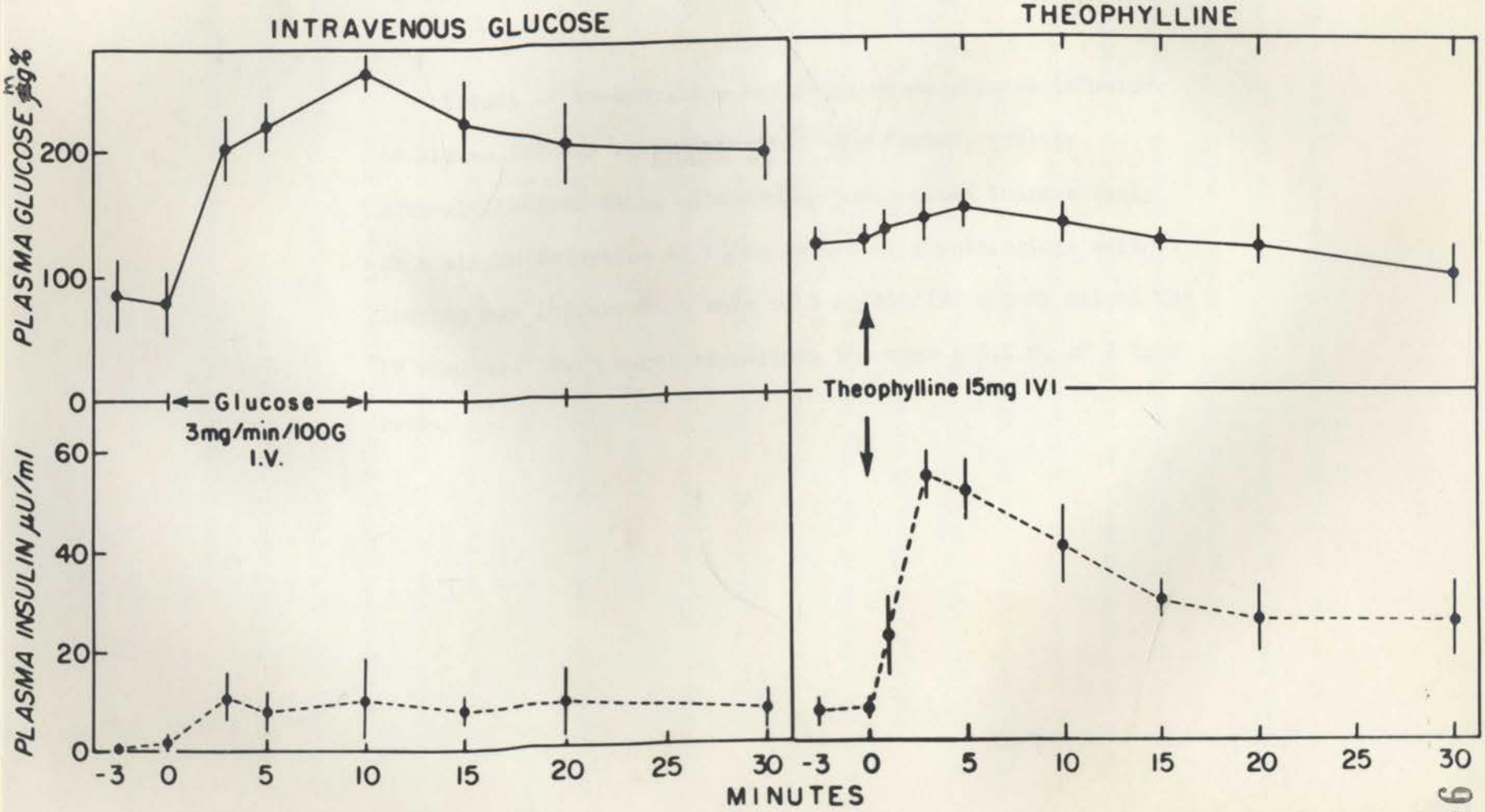


FIGURE 21

Figure 2I

Effect of theophylline and intravenous glucose infusion on plasma insulin responses of 48 hour fasted, acutely adrenalectomized rats. Theophylline was given intravenously in a single injection of I5 mg in 0.5 ml physiological saline. Glucose was infused at a rate of 3 mg/min/I00 g body weight for I0 minutes. Each point represents the mean ± S.E.M. of 4 to 8 rats.

The plasma insulin response following theophylline was not a consequence of the associated slight rise in blood glucose because raising blood glucose to greater levels by glucose infusion elicited only a minimal insulin response (Figure 21). Furthermore the increase in plasma insulin produced by theophylline persisted even after the blood glucose had returned to control concentrations.

The half-life of plasma insulin did not differ significantly between control animals (33 ± 2.8 min) indicating that this effect of theophylline was not due to inhibition of insulin disposal. When insulin secretion is expressed as the area circumscribed by the plasma insulin response curve, expressed as $\mu$ units-minutes $ml^{-1}$, theophylline stimulated insulin secretion (2881 ± 327 $\mu$ units-minutes $ml^{-1}$) more than three times the secretion seen in response to glucose administration (931 ± 164 $\mu$ units-minutes $ml^{-1}$), even though the blood sugar levels attained during glucose infusion were almost twice those seen after the injection of theophylline. Furthermore, if insulin secretion is expressed in terms of the rise in blood glucose above control levels during the 30 minute experimental period, the insulin glucose ratio is 101.8 $\mu$ units-minutes $ml^{-1}$ glucose following glucose infusion. This ratio indicates that the insulin increase due to theophylline is 69 times greater than that expected from the observed rise in plasma glucose.

Effect of Theophylline on Portal Venous Insulin

Within one minute of the injection of theophylline, portal venous insulin increased from 136 ± 16 to 328 ± 28 μU/ml (Figure 22). This prompt response followed by a subsequent fall and more prolonged increase in plasma insulin is consistent with an immediate effect of an initial bolus of theophylline which then underwent uniform mixing in the plasma compartment.

Effect of Epinephrine, Adrenergic Blocking Agents and Theophylline on Insulin Secretion

Recent studies by Porte (64) suggest that alpha adrenergic receptors mediate the inhibitory effect of epinephrine on insulin secretion, whereas beta adrenergic receptor stimulation increases insulin release. As reported in man (65) and pig (66), plasma insulin levels remained low in fasted rats during infusion with epinephrine (Figure 23). The failure to demonstrate a significant rise in blood sugar in these animals reflects the depletion of liver glycogen characteristic of the 48 hour fasted rat.

Theophylline (15mg) injected 10 minutes after beginning an epinephrine infusion still provoked a prompt 8-fold rise in plasma insulin, but less than the 10-13 fold rise following theophylline alone. When the alpha adrenergic receptors were blocked by phentolamine during the course of an epinephrine infusion, theophylline provoked an extraordinary increase in plasma insulin (30-40 fold rise to 348 ± 15 μ units/ml) which continued throughout

FIGURE 22

EFFECT OF THEOPHYLLINE ON PLASMA INSULIN IN ADRENALECTOMIZED, FASTED RATS

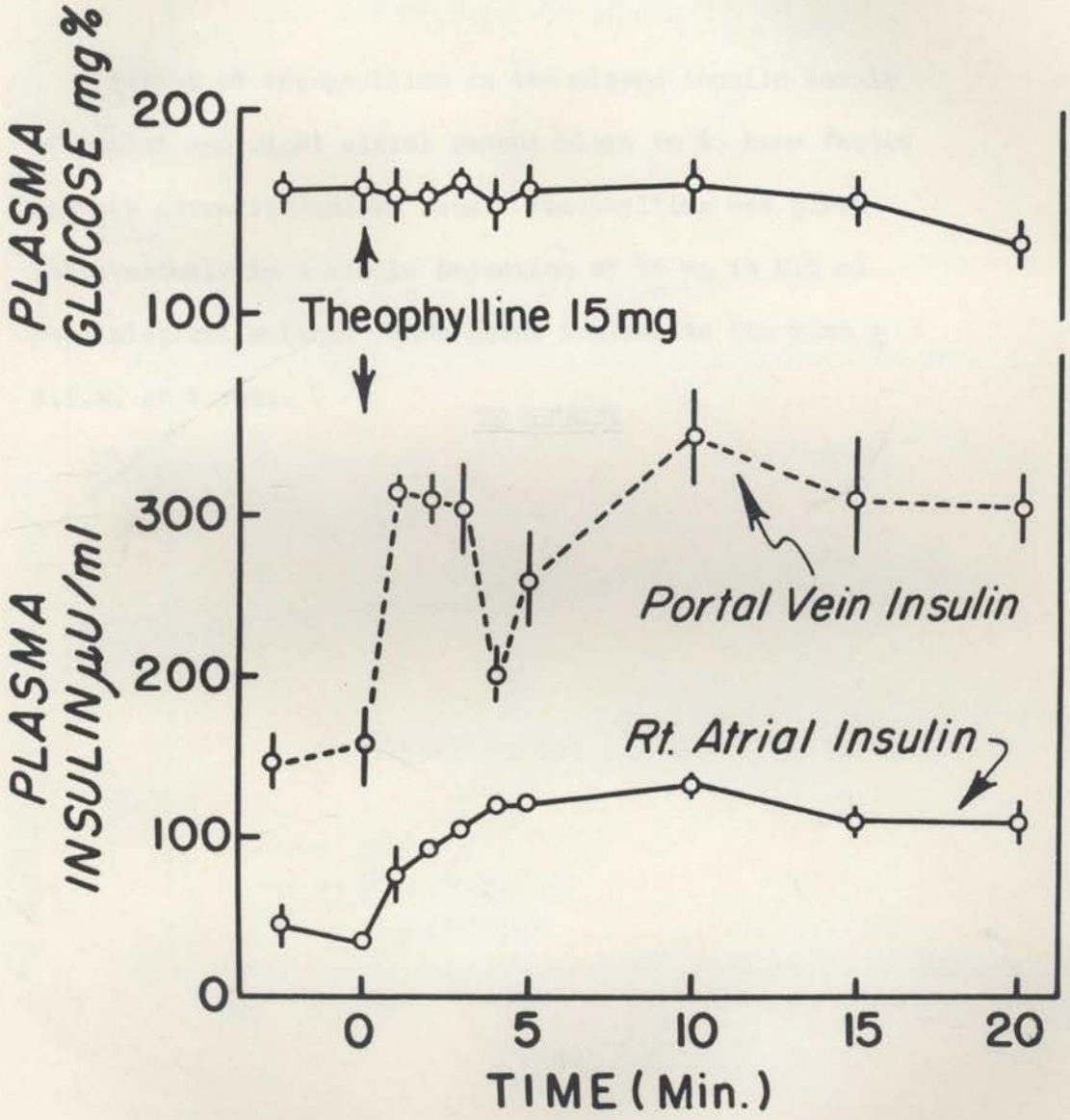


FIGURE 22

FIGURE 23

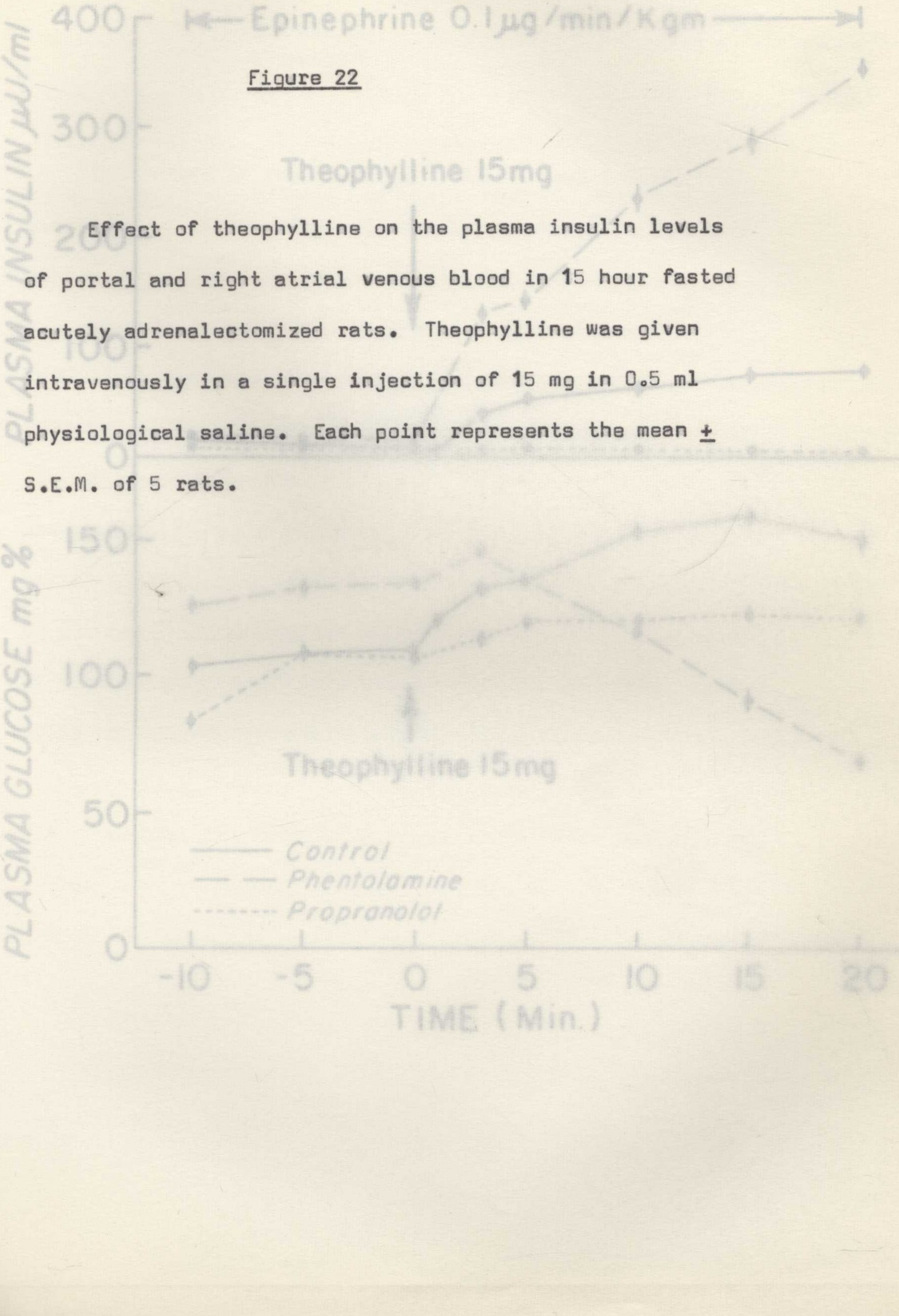



Figure 22

Effect of theophylline on the plasma insulin levels of portal and right atrial venous blood in 15 hour fasted acutely adrenalectomized rats. Theophylline was given intravenously in a single injection of 15 mg in 0.5 ml physiological saline. Each point represents the mean ± S.E.M. of 5 rats.

FIGURE 23

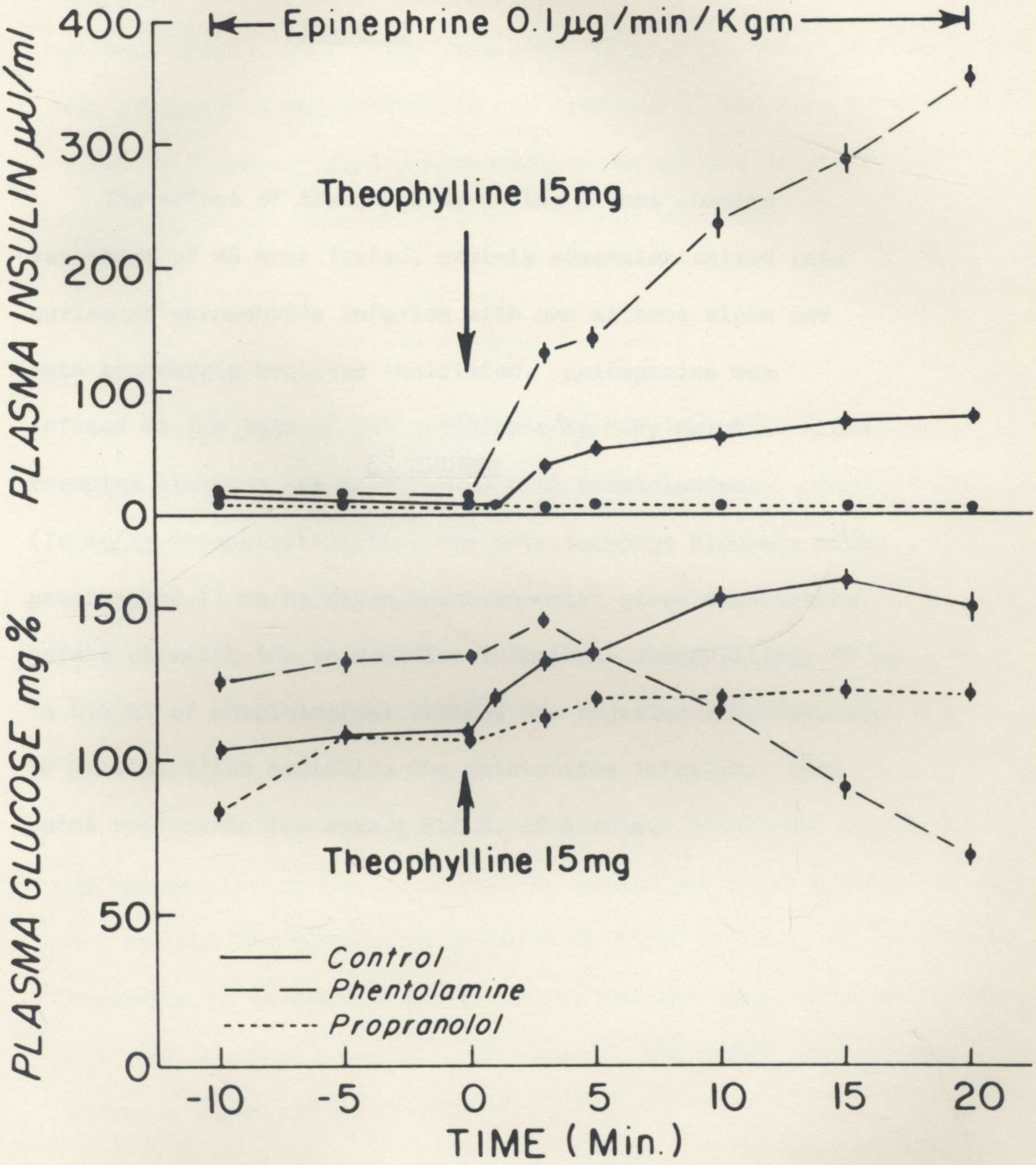

PLASMA INSULIN μU/ml
400
300
200
100
0
PLASMA GLUCOSE mg%
150
100
50
0
← Epinephrine 0.1 μg/min/Kgm →
Theophylline 15mg
Theophylline 15mg
Control
Phentolamine
Propranolol
-10
-5
0
5
10
15
20
TIME (Min.)

FIGURE 23

Figure 23

The effect of theophylline on the plasma insulin responses of 48 hour fasted, acutely adrenalectomized rats during an epinephrine infusion with and without alpha and beta adrenergic receptor inhibition. Epinephrine was infused at the rate of 0.I μg/minute/kg body weight. Alpha receptor blockade was established with phentolamine (Io mg/kg intraperitoneally) and beta receptor blockade with propranolol (I mg/kg given intravenously) given immediately before starting the epinephrine infusion. Theophylline, 15 mg in 0.5 ml of physiological saline, was injected intravenously 10 minutes after beginning the epinephrine infusion. Each point represents the mean ± S.E.M. of 5 rats.

the experimental period and resulted in a rapid fall in plasma glucose (Figure 23). When only alpha adrenergic receptor stimulation was produced by epinephrine in the presence of the beta adrenergic receptor blocking agent propranolol, theophylline injection did not produce any detectable plasma insulin response.

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The results above suggest that the alpha and beta adrenergic receptors in the pancreatic islet beta cell mediate divergent affects of epinephrine on cyclic 3'5' AMP synthesis; alpha receptors inhibiting and beta receptors increasing the generation of the cyclic nucleotide. Furthermore they suggest that the intracellular concentration of cyclic 3'5' AMP in the pancreatic islet determines the rate of insulin secretion.

In order to test these observations definitely and to determine whether the intracellular concentration of cyclic 3'5' AMP may mediate the effects of hormones in other tissues, it was necessary to develop an assay for the precise measurement of cyclic 3'5' AMP in micro quantities of tissue. The development of this assay is described in Part II.

## PART II

## DEVELOPMENT OF A NEW ASSAY FOR CYCLIC 3'5' ADENOSINE MONOPHOSPHATE IN TISSUES

Cyclic 3'5' adenosine monophosphate (cyclic 3'5' AMP) is involved in many biological processes, from activation of enzyme systems to the reproduction of specific tissue responses to target hormones. In 1960 Sutherland and Rall first reported cyclic 3'5' AMP-activation of hepatic UDPG glycogen alpha-4-glucosyltransferase (67), followed by Murad et al who showed activation of hepatic phosphorylase (68) and Rosell-Perez and Larner in 1964 reported activation of tryptophan pyrrolase (69).

In addition to enzyme activation, cyclic 3'5' AMP will produce the effects of hormones on specific tissue response patterns, such as:- 1. Activation of lipolysis in cell-free systems - Chytil and Skrivanova (70) and Rizack (47), 2. Increasing the permeability of the toad bladder to water - Orloff and Handler (71), 3. Increasing uterine protein synthesis - Creange and Roberts (72), Szego (73) and 4. Stimulation of steroid hydroxylation - Butcher et al (74).

Murad et al in 1963 reported that epinephrine stimulates the synthesis of cyclic 3'5' AMP in heart muscle by a direct action on adenyl cyclase, the enzyme controlling the synthesis of cyclic 3'5' AMP from ATP (68). Similar effects of epinephrine on cyclic 3'5' AMP have been reported in other tissues (62). The studies of the effects of epinephrine and theophylline on insulin secretion

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reported above in Part I, suggest still another function for the cyclic nucleotide, that is, mediation of the adrenergic effects on insulin secretion. To define precisely the effects of hormones on the cellular concentration of cyclic 3'5' AMP and the role of this cyclic nucleotide in mediating specific tissue response it was necessary to establish a new sensitive assay for cyclic 3'5' AMP.

Evaluation of Published Methods for Cyclic 3'5' AMP Determination

Two techniques have been described for the assay of cyclic 3'5' AMP.

I. Activation of Phosphorylase

Variations of this procedure have been described by Posner et al in 1964 (75), Kye et al in 1964 (76) and Butcher et al in 1965 (74).

Essentially the method involved :-

1. Separation of cyclic 3'5' AMP from other nucleotides by column chromatography on Dowex. This requires two separate fractionations with precise collection of eluted fractions, monitored by pH and volume of the effluent.
2. Conversion of phosphorylase b to phosphorylase a by cyclic 3'5' AMP activation of phosphorylase kinase.
3. Estimation of glycogenolysis using a glycogen substrate and comparison of blanks with cyclic 3'5' AMP standards or chromatographed tissue extracts.

The assay of cyclic 3'5' AMP in tissue extracts was limited by poor separation of the cyclic nucleotide from other nucleotides, together with 60% loss of cyclic 3'5' AMP during the column chromatography. Furthermore non-specific glycogenolysis occurred in the system, giving high blank readings (77). Due to these limitations a minimum of 100 mg of tissue was required for measurement of cyclic 3'5' AMP.

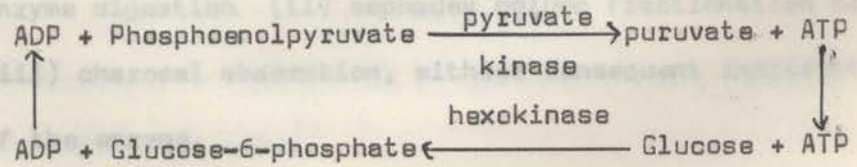
II Enzymatic Cycling Procedure

This technique was described by Breckenridge in 1964 (78). This method is summarized as follows:-

1. Enzymatic digestion of a tissue extract with apyrase and alkaline phosphatase to remove contaminating nucleotides (ATP, ADP and AMP). Cyclic 3'5' AMP resisted this digestion.
2. Conversion of cyclic 3'5' AMP to 5' AMP with cyclic 3'5' nucleotide phosphodiesterase.
3. Conversion of 5' AMP to ADP wit h myokinase, initiated with a low concentration of ATP.

$$AMP + ATP \xrightarrow{\text{myokinase}} 2\ ADP$$

4. An enzymatic cycling reaction with phosphoenolpyruvate, puruvate kinase, glucose and hexokinase as follows;



5. The accumulation of pyruvate was measured fluorimetrically by the lactic dehydrogenase reaction.

An attempt was made to use the Breckenridge procedure for tissue cyclic 3'5' AMP assay. It was not possible to measure cyclic 3'5' AMP in less than 100 mg of tissue, and frequently at these levels contamination occurred. Two difficulties were encountered making the technique unsuitable for assay of cyclic 3'5' AMP in micro quantities of tissue :

1. The initial digestion procedure with apyrase and alkaline phosphotase was incomplete, leaving contaminating adenine nucleotides which interfered in the subsequent cycling reactions and produced a high blank reading.

11. The enzymes myokinase and pyruvate kinase were contaminated with nucleotides which reacted in the cycling procedure and produced a high blank reading. The principal contaminant in myokinase was AMP and in pyruvate kinase both ATP and ADP were present. Numerous enzyme preparations from several commercial sources were all found to be contaminated. Although the AMP could be satisfactorily removed from myokinase by a procedure to be described later, the pyruvate kinase could not be satisfactorily decontaminated by (i) enzyme digestion (ii) sephadex column fractionation or (iii) charcoal absorption, without consequent inactivation of the enzyme.

An attempt to use the following reaction:

$$\text{ADP} + \text{Creatine phosphate} \xrightarrow[\text{Kinase}]{\text{Creatine}} \text{Creatine} + \text{ATP}$$

in place of the PEP-PK reaction in the cyclic procedure, was also unsatisfactory due to contamination of creatine kinase with ADP and ATP.

Thus in both procedures the assay of cyclic 3'5' AMP in tissue extracts was limited by poor separation of the cyclic nucleotide from other adenine nucleotides and limited sensitivity secondary to either interference from contaminant nucleotides in the enzyme used, or non-specific stimulation of glycogenolysis (77) Due to these limitations a minimum of 100 mg of tissue was required for measurement of cyclic 3'5' AMP.

The New Assay for Cyclic 3'5' AMP

This method permits measurement of the cyclic nucleotide in 1 mg of tissue. The procedure is summarized as follows:

I Isolation and separation of cyclic 3'5' AMP from other nucleotides by thin layer chromatography.

II Conversion of cyclic 3'5' AMP to 5' AMP with 3'5' cyclic nucleotide phosphodiesterase.

$$\text{3'5' AMP} \longrightarrow \text{5' AMP}$$

III Conversion of 5' AMP in the presence of $\gamma$-labelled ATP and myokinase to $ADP^{32}$.

$$\text{Adenosine}\sim P + \text{Adenosine}\sim P\sim P\sim P^{32} \longrightarrow 2\ \text{Adenosine}\sim P\sim P^{32}$$

IV Separation and isolation of $ADP^{32}$ by thin layer chromatography.

Experimental Procedure

Male Charles River rats weighing 150 g were used. Alkaline phosphatase (E.coli Type III), cyclic 3'5' AMP, ATP, ADP, cysteine and TRIS were purchased from Sigma Chemical Company. 3 phosphoglycerate (tricyclohexyl-ammonium salt), yeast phosphoglycerate kinase, muscle glyceraldehyde-3-P-dehydrogenase and rabbit skeletal muscle myokinase were purchased from Boehringer-Mannheim Corporation. Cyclic 3'5' AMP-$^{3}H$ (2.3 curies/mM) and ADP-$^{3}H$ (4 curies/mM) were obtained from Schwarz Bio-Research and carrier-free orthophosphoric acid $P^{32}$ from New England Nuclear Corporation. The purity of all nucleotides used was greater than 99% when chromatographed in at least two solvent systems. MN cellulose powder 300 HR and MN cellulose powder 300 DEAE (Macheley, Magel & Co.) were purchased from Brinkmann Instruments, Incorporated.

Buffer. The buffer (hereafter referred to as the TRIS-phosphate-$Mg^{++}$ buffer) used throughout for the enzymatic nucleotide conversion had the following composition: 5 mM TRIS, 10 mM sodium phosphate and 3.4 mM magnesium chloride pH 8.0. $Mg^{++}$ was required for both the cyclic nucleotide phosphodiesterase (63,79) and the myokinase reactions (80). Sodium phosphate was added to inhibit contaminating ATPase activity in the enzyme (81).

3'5' Cyclic Nucleotide Phosphodiesterase. This enzyme was prepared according to the method of Butcher and Sutherland (79) from fresh calf hearts and stored in small aliquots at -65°C as a solution capable of hydrolyzing a solution of 1 mM cyclic 3'5' AMP at the rate of 220 millimoles/litre/hour at pH 8.0 and 37°C. Activity was assessed by the hydrolysis of cyclic 3'5' AMP to adenosine with measurement of the liberated Pi in the presence of cyclic 3'5' nucleotide phosphodiesterase and 5' nucleotidase from Crotalus Atrax venom (79).

In the preparative procedure highly active material was obtained in the final isolation step by eluting protein from calcium phosphate gel first with 6% then 10% ammonium sulphate. The enzyme was confined to the 6-10% fraction. This fraction was dialyzed against $10^{-3}$ M $MgSO_4$ and $10^{-3}$ M imidozole pH 7.5, and concentrated against Pevikon C-870 (Fosfatbolaget, Stockholm). The concentrated enzyme solution was stable at -65°C for prolonged periods (at least six months), and was diluted 1:10 with the TRIS-phosphate-$Mg^{++}$ buffer immediately before use. This dilute solution lost 10% of its activity per month at 5°C. At the concentrations used in the assay procedure, no detectable contamination with nucleotides was demonstrable.

$ATP^{32}$ Labelled Specifically in the $\gamma$ Phosphorus. $ATP^{32}$ was prepared by a modification of the method of Glynn and Chappell (82). Using the modification described, $ATP^{32}$ with a specific activity of 5-6 curies per millimole was prepared. The following

materials were pipetted into a small stoppered centrifuge tube in the order listed:

50 µl 1M TRIS pH 8.0

6 µl 1M $MgCl_2$

102 µl 0.1N NaOH

2 µl 1M cysteine

3 µl 1M ATP

10 µl 100mM 3-phosphoglycerate

10 µl (0.1mg) muscle glyceraldehyde-3-P-dehydrogenase

1 µl yeast phosphoglycerate kinase

To this was added 10 millicuries of carrier-free $H_3P^{32}O_4$ in 1 ml of water. The mixture was incubated at 37°C for one hour, then 300 µl trichloracetic acid (55%) were added, the tube was shaken and centrifuged at 0°C for 30 minutes at 18,000g. The supernatant solution was extracted three times with eight volumes of diethyl ether saturated with water. The aqueous phase was lyophilized to 200 ul and the $ATP^{32}$ was separated by thin layer chromatography on MN cellulose 300HR, using the solvent system employed in the cyclic 3'5' AMP assay. The ATP spot was eluted in 2 ml of water and stored in small aliquots at -65°C as a $10^{-5}$M solution with a specific activity of 5-6 curies per millimole. Greater than 99% of the $P^{32}$ was in the $\gamma$ position when assayed by incubation with glucose and hexokinase, then subsequent thin layer chromatography to isolate the ADP. The procedure of Lamprecht and Trautschold (83) was used to convert $ATP^{32}$ to ADP + $Pi^{32}$ with glucose-hexokinase.

Myokinase. It is essential in the assay procedure that all enzymes are free of contaminating nucleotides to avoid elevation of the blank readings. No significant contamination with ATP, ADP or AMP was present in the 3'5' cyclic nucleotide phosphodiesterase prepared as described above. Myokinase obtained commercially from a variety of sources was contaminated to a variable degree with AMP in concentrations ranging from $10^{-4}$ to $10^{-7}$ M. No significant contamination with ADP or ATP was detected using the glucose-hexokinase reaction of Lamprecht and Trautschold (85), or the luciferin-luciferase reaction of Strehler (84). The contaminating AMP could be removed by digesting commercial myokinase with alkaline phosphatase, then inactivating the alkaline phosphatase by boiling in acid solution. The extraordinary stability of myokinase at acid pH and 100°C (85) permitted this procedure without loss of enzyme activity. 250 ul of a suspension of myokinase in 3.2M ammonium sulphate, containing 1.25mg of myokinase, were centrifuged at 20,000g, for 30 minutes and the supernatent fluid was removed. To the myokinase crystals were added 20 µl 0.03N NaOH, 10 µl 1M ammediol and 15 µl (150 µg) alkaline phosphatase. The mixture was incubated for one hour at 37°C, then 0.01N HCL was added to bring the mixture to pH 4.0. The tightly stoppered tube was boiled for two minutes to inactivate the alkaline phosphatase and the precipitate was removed by centrifuging for 30 minutes at 20,000g and 0°C. The supernatant myokinase solution was stored at 4°C at a concentration of 5mg per ml and used within two weeks.

Thin Layer Chromatography Procedure. 15g MN cellulose 300HR were homogenised with 90 ml water in a Virtis 45 homogenizer at low speed for five minutes, then the homogenate was applied in a 250 μ layer to five 20 x 20 glass plates. The plates were dried at room temperature and then heated to 105°C for 30 minutes before use. The solvent system used throughout was 1-butanol : acetone : glacial acetic acid : 14.8N ammonium hydroxide : water in a ratio of 90:30:20:1:60 (v/v). The purity of all nucleotides was checked in this system as well as on MN cellulose 300 DEAE plates developed in isobutyric acid : 0.5 M ammonium hydroxide in a ratio of 5:3 (v/v).

## Analytical Procedure

Preparation of tissues. Rats were anaesthetized with sodium pentobarbitone (4mg per 100g) injected intraperitoneally. Either the liver, quadriceps muscle or epididymal fat pad were exposed, frozen between aluminium clamps colled in liquid nitrogen and transferred to dry ice. Tissue fragments ranging from 1-50 mg were weighed at -20°C and then rapidly homogenized in 1 ml cold 5.6% trichloracetic acid to which 5 x 10-13 moles of cyclic 3'5' AMP-$^{3}$H had been added for calculation of recovery. The homogenates were centrifuged at 8,000g for 30 minutes at 0°C and the supernatant fractions were removed. Trichloracetic acid was removed from the supernatant fractions by rapid extraction three times with eight volumes of diethyl ether saturated with water. The fractions were evaporated to dryness at room temperature in an air stream, then dissolved in 100 μl water.

Isolation of cyclic 3'5' AMP. The extracts were applied one inch from the lower border of a 20 x 20 cm MN cellulose 300HR plate and dried in an air stream at room temperature. Six samples were applied to each plate with a marker of $10^{-8}$ moles of cyclic 3'5' AMP on each side. The development of the chromatogram was completed within four hours at room temperature. The cyclic 3'5' AMP marker was detected by ultraviolet light absorption. The corresponding areas of the tissue extract chromatograms were removed and the cyclic 3'5' AMP was eluted from the cellulose with three washes of 1 ml, 0.5 ml and 0.5 ml of 50% ethanol. The ethanol extracts were pooled, evaporated to dryness in a stream of air at room temperature and dissolved in 100 ul of the TRIS-phosphate-$Mg^{++}$ buffer pH 8.0. 50 ul of this colution was removed and counted by liquid scintillation in 10 ml Bray's solution (86).

Conversion of Cyclic 3'5' AMP to $ADP^{32}$. Cyclic 3'5' AMP standard solutions containing $5 \times 10^{-13}$ moles to $2\text{-}5 \times 10^{-11}$ moles in 10 µl, or comparable aliquots of purified tissue extracts, were pipetted in duplicate into 5 x 50 mm tubes. TRIS-phosphate-$Mg^{++}$ buffer was added to bring the total volume to 20 µl. $1\text{-}5 \times 10^{-11}$ moles of $ATP^{32}$ in a volume of 10 ul were added to each tube and the reaction was started by the addition of 1 µl cyclic nucleotide phosphodiesterase (a solution capable of hydrolyzing cyclic 3'5' AMP at the rate of 22 millimoles/litre/hour) and 1 µl myokinase (5 µg). The tubes were capped and incubated at 37°C for one hour.

At the end of the incubation, the tubes were placed in ice and 10 µl of cold 6N perchloric acid containing 10 mM sodium EDTA and 5 mM sodium fluoride were added. These concentrations of EDTA and NaF were required to inactivate the myokinase, which otherwise maintained some enzyme activity in 2N perchloric acid (85). After standing in ice for 30 minutes, 10 µl of an aqueous solution containing $10^{-12}$ moles of ADP-$^{3}H$ were added, followed immediately by 30 µl of cold 2N potassium bicarbonate. The $KClO_4$ was removed by centrifugation at 0°C.

Isolation of $ADP^{32}$. 50 µl of the supernatant solution was applied one inch from the lower border of a 20 x 20 cm MN cellulose 300HR plate and dried in an air stream at room temperature. Six samples were applied to each plate with a marker of $10^{-8}$ moles ADP on each side. The chromatogram was developed at room temperature, twice in the same direction. The area corresponding to the position of the ADP marker (detected by ultraviolet light absorption) was removed and the $ADP^{32}$ eluted with 1 ml water. After centrifugation to remove the cellulose, 0.5 ml of the aqueous extract was counted by liquid scintillation in 10 ml Bray's solution.

A standard curve of incorporation of $P^{32}$ into ADP was constructed. The cyclic 3'5' AMP content of the extracts was read from the curve and corrected for recovery of cyclic 3'5' AMP-$^{3}H$.

## Results

Cyclic 3'5' AMP separated clearly from other adenine nucleotides on cellulose-coated thin layer chromatography plates with butanol:acetone:glacial acetic acid:ammonium hydroxide:water as the developing solvent (Table XIV).

Table XIV

Rf Values of Adenine Nucleotides*

| | |
|---|---|
| Cyclic 3'5' AMP | 0.30 |
| AMP | 0.15 |
| ADP | 0.07 |
| ATP | 0.03 |

*Rf values of nucleotides on 250 u layers of MN cellulose 300 HR at room temperature, developed in butanol: acetone:glacial acetic acid:14.8N ammonium hydroxide:water = 90:30:20:1:60 (V/V) for four hours.

---

The minimum concentration of cyclic 3'5' AMP detected by ultraviolet light absorption was $10^{-9}$ moles, approximately 50-100 times the concentration of the cyclic nucleotide in the tissue extracts. For this reason it was necessary to use a marker of $10^{-8}$ moles cyclic 3'5' AMP developed adjacent to the tissue extract chromatograms.

Recovery of Cyclic 3'5' AMP from Tissues. Cyclic 3'5' AMP-$^{3}$H, $5 \times 10^{-13}$ moles was added to the tissue extract fluid (ie 5.6 per cent TCA) before the tissues were homogenized.

Greater than 92 per cent of the cyclic nucleotide was recovered with the first trichloracetic acid extraction; the remaining 4-5 per cent was found in the second extraction (Table XV). In view of the completeness of recovery, a single trichloracetic acid extraction was performed for the isolation of cyclic 3'5' AMP from tissues, thereby avoiding further dilution of the nucleotide.

Table XV

Recovery of Cyclic 3'5' AMP-$^{3}$H from Adipose Tissue*

| | Sample A | Sample B |
|---|---|---|
| | cpm | |
| 3'5' AMP-$^{3}$H added | 293 | 293 |
| Extraction 1 | 272 | 270 |
| Extraction 2 | 15 | 12 |
| Extraction 3 | 1 | 3 |
| Pooled ether washes | 9 | 7 |

*Recovery of $5 \times 10^{-13}$ moles of cyclic 3'5' AMP-$^{3}$H added to adipose tissue. Extractions were performed with 1 ml of 5.6 per cent TCA. Each extract was washed with 8 volumes of water-saturated diethyl ether and the ether washes were pooled and dried. All fractions were counted in 10 ml Bray's solution. Sample A weighed 47 mg, sample B 52 mg.

The subsequent removal of trichloracetic acid during the ether washing procedure caused the loss of 2-3 per cent of cyclic 3'5' AMP-$^{3}$H (Table XV). Isolation of the cyclic 3'5' AMP by chromatography of the tissue extract gave a recovery of 81-92 per cent of the cyclic 3'5' AMP-$^{3}$H added (Table XVI). Comparable recoveries to those reported for adipose tissue were obtained with extracts of skeletal muscle and liver.

Table XVI

Recovery of Cyclic 3'5' AMP-$^{3}$M from MN Cellulose 300HR*

| 3'5' AMP-$^{3}$H Added | | Recovery after Extraction and Chromatography | |
|---|---|---|---|
| uumoles | cpm | cpm | % recovery |
| .375 | 180 | 147 | 81.7 |
| .75 | 346 | 300 | 86.7 |
| 1.125 | 509 | 454 | 89.2 |
| 1.50 | 664 | 6I0 | 9I.8 |

*50 mg of adipose tissue with added cyclic 3'5' AMP-$^{3}$H was extracted and ether-washed as described in Table XV and then chromatographed. The cyclic 3'5' AMP was eluted in I ml of water and counted in 10 ml Bray's solution.

---

Conversion of cyclic 3'5' AMP to 5' AMP. When incubated for one hour at 37°C in the TRIS-phosphate-$Mg^{++}$ buffer containing I ul of 3'5' cyclic nucleotide phosphodiesterase solution, 90-97 per cent of cyclic 3'5' AMP-$^{3}$H in amounts ranging from $IO^{-I3}$ to $IO^{-IO}$ moles in 35 µl of buffer was converted to 5' AMP (Table XVII). In other experiments it was observed that similar conversion was obtained after only 15 minutes incubation.

Table XVII

Conversion of 3'5' AMP-$^{3}H$ to 5' AMP-$^{3}H$ with 3'5' Cyclic Nucleotide Phosphodiesterase*

| Cyclic 3'5' AMP | Recovery in 5' AMP | |
|---|---|---|
| µµmoles | cpm | cpm | % |
| 0.I | 30 | 27 | 90 |
| I.0 | 334 | 321 | 96.I |
| I0.0 | 3,I48 | 3,073 | 97.6 |
| I00.0 | 34,724 | 32,749 | 94.4 |

*Incubations were carried out in a volume of 35 ul of TRIS-phosphate-$Mg^{++}$ buffer containing cyclic 3'5' AMP-$^{3}H$ for one hour at 37°C. The reaction was started by the addition of I µl of 3'5' cyclic nucleotide phosphodiesterase with activity capable of hydrolyzing I mM cyclic 3'5' AMP at the rate of 22 mmoles/litre/hr. cyclic 3'5' AMP from control incubations and 5' AMP-$^{3}H$ from incubations with phosphodiesterase were eluted in I ml of water and counted in I0 ml Bray's solution.

---

Conversion of AMP to ADP. The conversion of AMP to $ADP^{32}$ in the presence of $ATP^{32}$ and myokinase reached equilibrium after 30-60 minutes incubation under the conditions of the assay procedure - 30 µl of TRIS-phosphate-$Mg^{++}$ buffer, containing $I0^{-I3}$ to $2.5 \times I0^{-II}$ moles of 5' AMP, $2.5 \times I0^{-II}$ moles of $ATP^{32}$ and 3.4 mM $MgCl_2$ (Figure 24). It is of interest that over this range

FIGURE 24

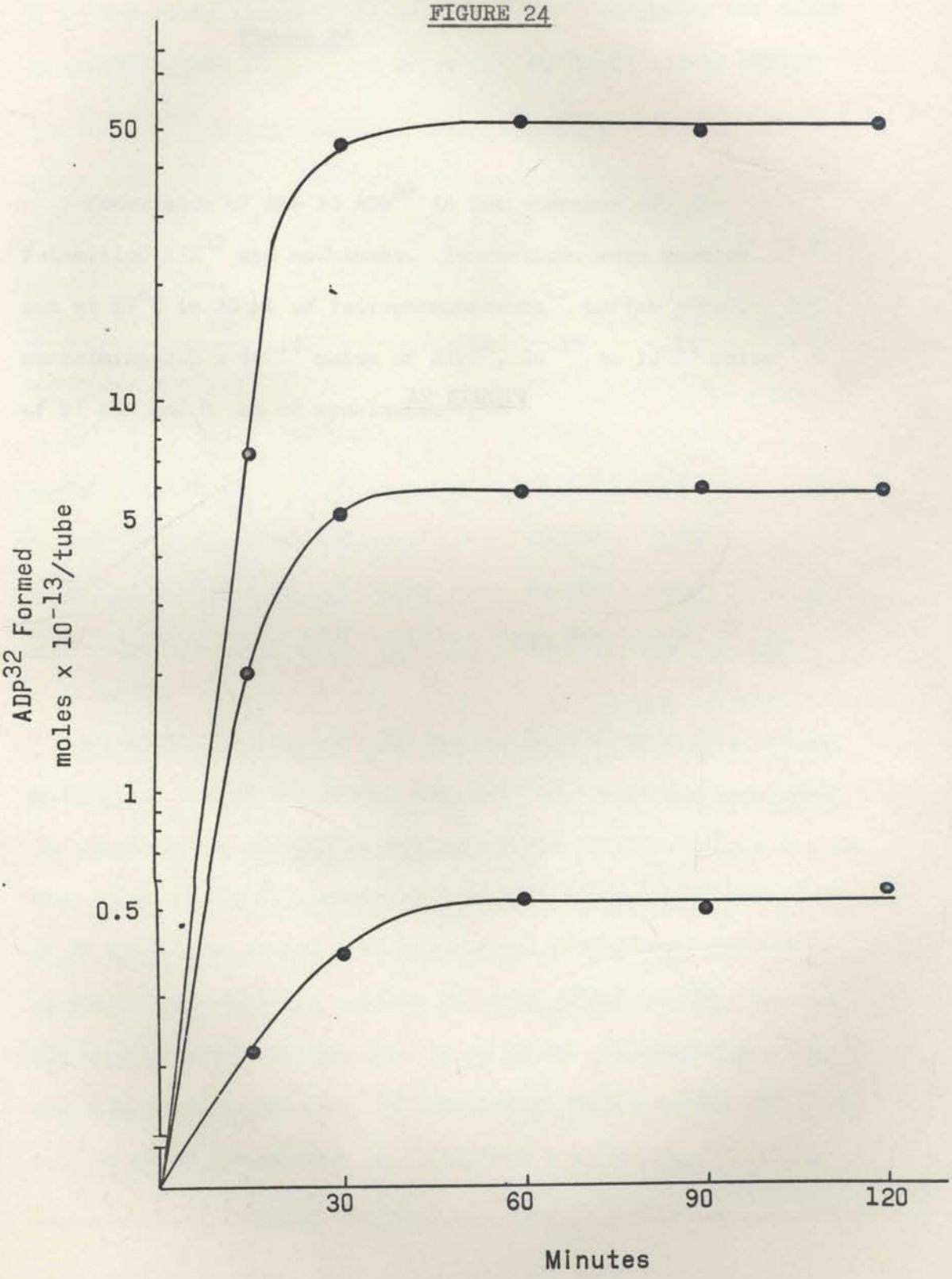


FIGURE 24

Figure 24

Conversion of AMP to $ADP^{32}$ in the presence of $\gamma$-labelled $ATP^{32}$ and myokinase. Incubations were carried out at 37°C in 30 µl of Tris-phosphate-$Mg^{++}$ buffer pH8.0, containing $2.5 \times 10^{-11}$ moles of $ATP^{32}$, $10^{-13}$ to $10^{-11}$ moles of 5' AMP and 5 ug of myokinase.

of 5' AMP concentrations the amount of $ATP^{32}$ converted was equal to approximately 50 per cent of the 5' AMP level (Table XVIII).

Table XVIII

Conversion of AMP to $ADP^{32}$ with Myokinase and $ATP^{32}$*

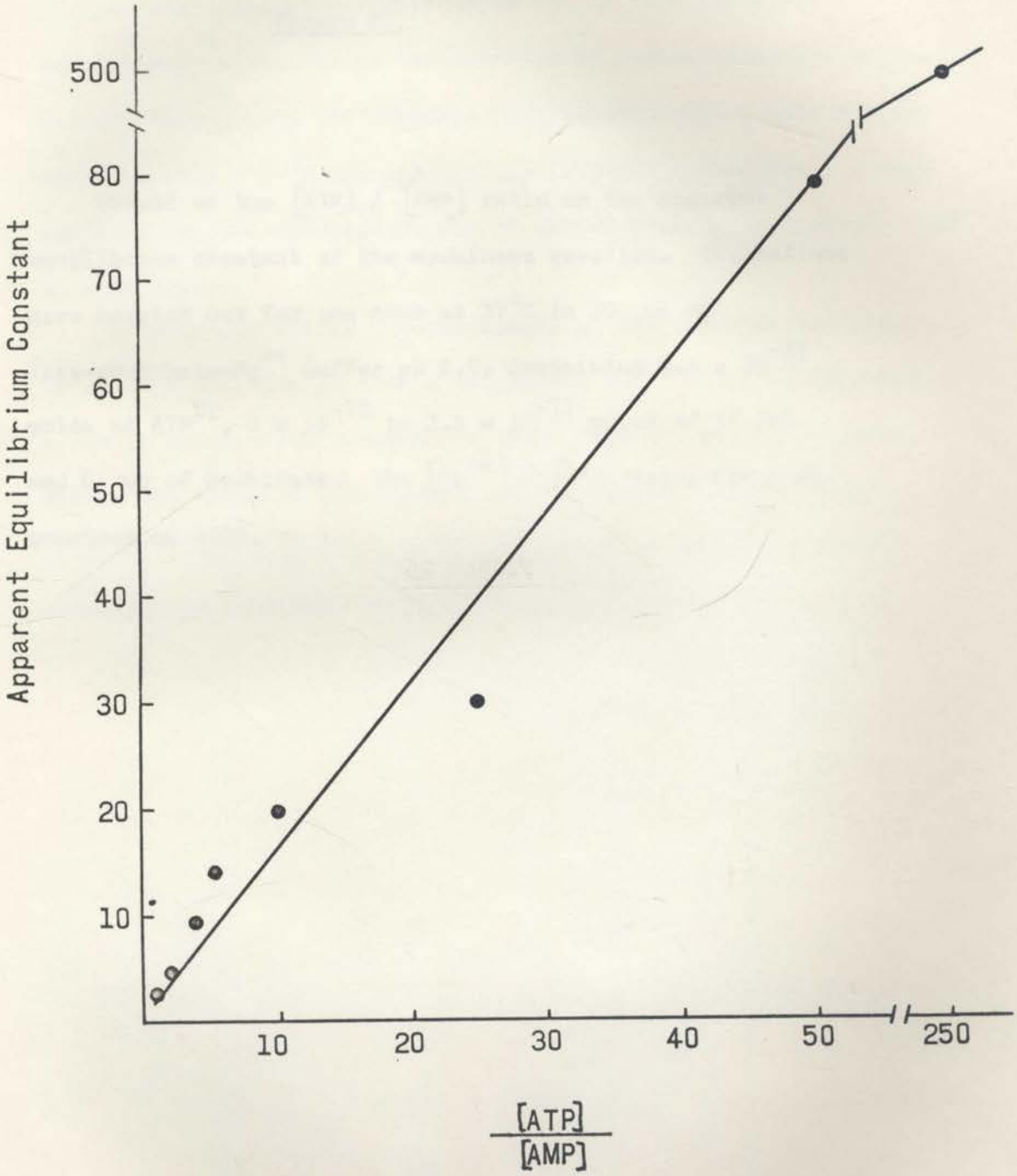
| AMP | $ATP^{32}$ | | $ATP^{32}$ converted to $ADP^{32}$ | |
|---|---|---|---|---|
| µµmoles | uumoles | cpm | cpm | µµmoles |
| 0.I | 25 | 300,000 | 648 | 0.053 |
| I.0 | " | " | 7,II6 | 0.58 |
| 5.0 | " | " | 29,568 | 2.48 |
| IO | " | " | 6I,520 | 5.05 |
| 25 | " | " | 147,752 | 12.1 |

* Incubations were carried out for one hour at 37°C in a volume of 30 µl of buffer containing AMP, $ATP^{32}$ and purified myokinase (5 µg). The reaction was stopped with IO ul of the perchloric acid reagent, then IO µl of ADP-$^{3}H$ containing I x $IO^{-I2}$ moles of ADP and 30 µl of 2N $KHCO_3$ were added. The mixture was centrifuged and the supernatant solution was applied to an MN cellulose 300 HR plate. The chromatogram was developed twice in the one direction, then the area corresponding to the position of an ADP marker was eluted in I ml water and counted in Io ml Bray's solution.

The constancy of this ratio of conversion indicates that the apparent equilibrium constant of the myokinase reaction under the conditions of these experiments varies at different 5' AMP levels (Figure 25), decreasing from 500 at the lowest nucleotide level ($I \times 10^{-13}$ moles) to approximately 2.8 at a nucleotide level of $2.5 \times 10^{-11}$ moles. Although comparable studies of the equilibrium characteristics of the myokinase reaction have not previously been reported, it has been noted by Bowen and Kerwin that the equilibrium constant increases (in the direction of ADP) as the $[ATP] / [AMP]$ and $[Mg^{++}] / [ATP]$ concentration ratios increase (80). Under the conditions of this assay procedure, the $[Mg^{++}] / [ATP]$ ratio is 3000 - 4000 and the $[ATP] / [AMP]$ ratio varies from I to 250. At a $[Mg^{++}] / [ATP]$ and $[ATP] / [AMP]$ concentration of unity, the equilibrium constant is approximately 0.44-0.48 as was reported by Eggleston and Hems (87). A value of 0.48 has been obtained under these conditions using the procedure described above.

The cyclic nucleotide phosphodiesterase preparation used in these studies does exhibit ATPase activity sufficient to hydrolyze about 10 per cent of the $ATP^{32}$ during a one hour incubation. Since the $ATP^{32}$ is labelled exclusively in the $\gamma$ position, hydrolysis will not result in labelling of any ADP. When this assay procedure was being developed, ATP-$^{3}H$ (labelled on the adenine base) was initially used and its conversion to ADP-$^{3}H$ determined as a measure of the

FIGURE 25


Apparent Equilibrium Constant
500
80
70
60
50
40
30
20
10
10
20
30
40
50
250
[ATP]
[AMP]

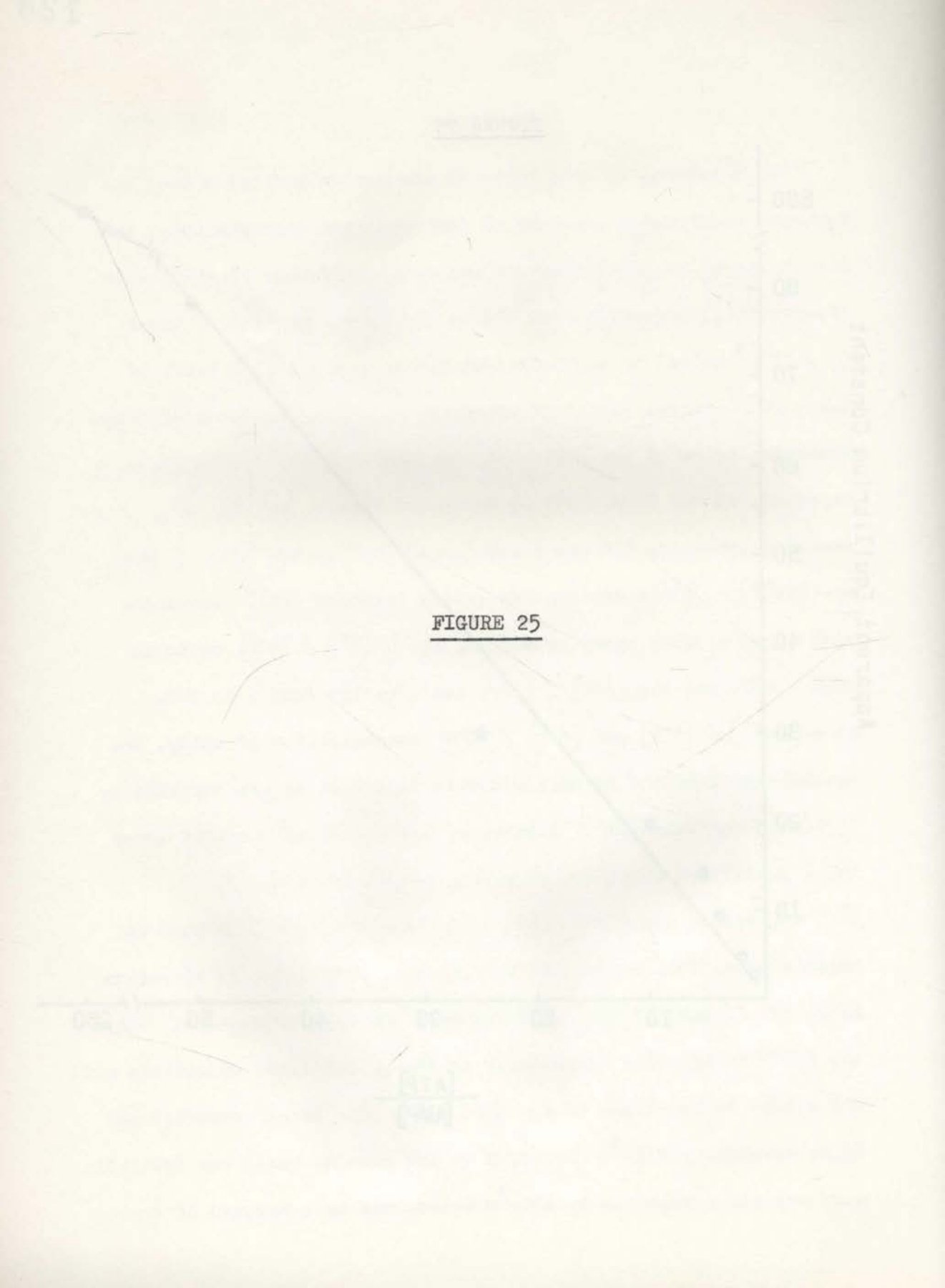


FIGURE 25

Figure 25

Effect of the $[ATP] / [AMP]$ ratio on the apparent equilibrium constant of the myokinase reaction. Incubations were carried out for one hour at 37°C in 30 µl of Tris-phosphate-$Mg^{++}$ buffer pH 8.0, containing $2.5 \times 10^{-11}$ moles of $ATP^{32}$, $5 \times 10^{-13}$ to $2.5 \times 10^{-11}$ moles of 5' AMP and 5 µg of myokinase. The $[Mg^{++}] / [ATP]$ ratio was held constant at 4100.

5' AMP concentration. However, the ATPase activity in the phosphodiesterase preparation could not be completely and consistently inhibited by the addition of sodium phosphate to the buffer, thus a high and variable blank incorporation into ADP was produced when ATP-$^{3}H$ was used as the labelled nucleotide. ATPase activity was not detected in any of the myokinase preparations used in this study.

Immediately before chromatography of the reaction mixture, IO ul of a solution of ADP-$^{3}H$ containing $IO^{-I2}$ moles of ADP were added to correct for recovery of $ADP^{32}$ from the cellulose plate. Within the range of concentrations of cyclic 3'5' AMP assayed ($5 \times IO^{-I3}$ moles to $2.5 \times IO^{-II}$ moles) the recovery of ADP-$^{3}H$ added ranged from 26.5 to 52.0 per cent (Table XIX).

Table XIX

Recovery of $ADP^{32}$ from MN Cellulose 300 HR*

| Cyclic 3'5' AMP converted to $ADP^{32}$ | $ADP-^{3}H$ added | | Recovery of $ADP-^{3}H$ | |
|---|---|---|---|---|
| μ μmoles | μ μmoles | cpm | cpm | % |
| 0.5 | I.0 | I,605 | 799 | 49.8 |
| I.0 | " | " | 835 | 52.0 |
| 5.0 | " | " | 707 | 44.0 |
| I0.0 | " | " | 558 | 34.8 |
| 25.0 | " | " | 424 | 26.5 |

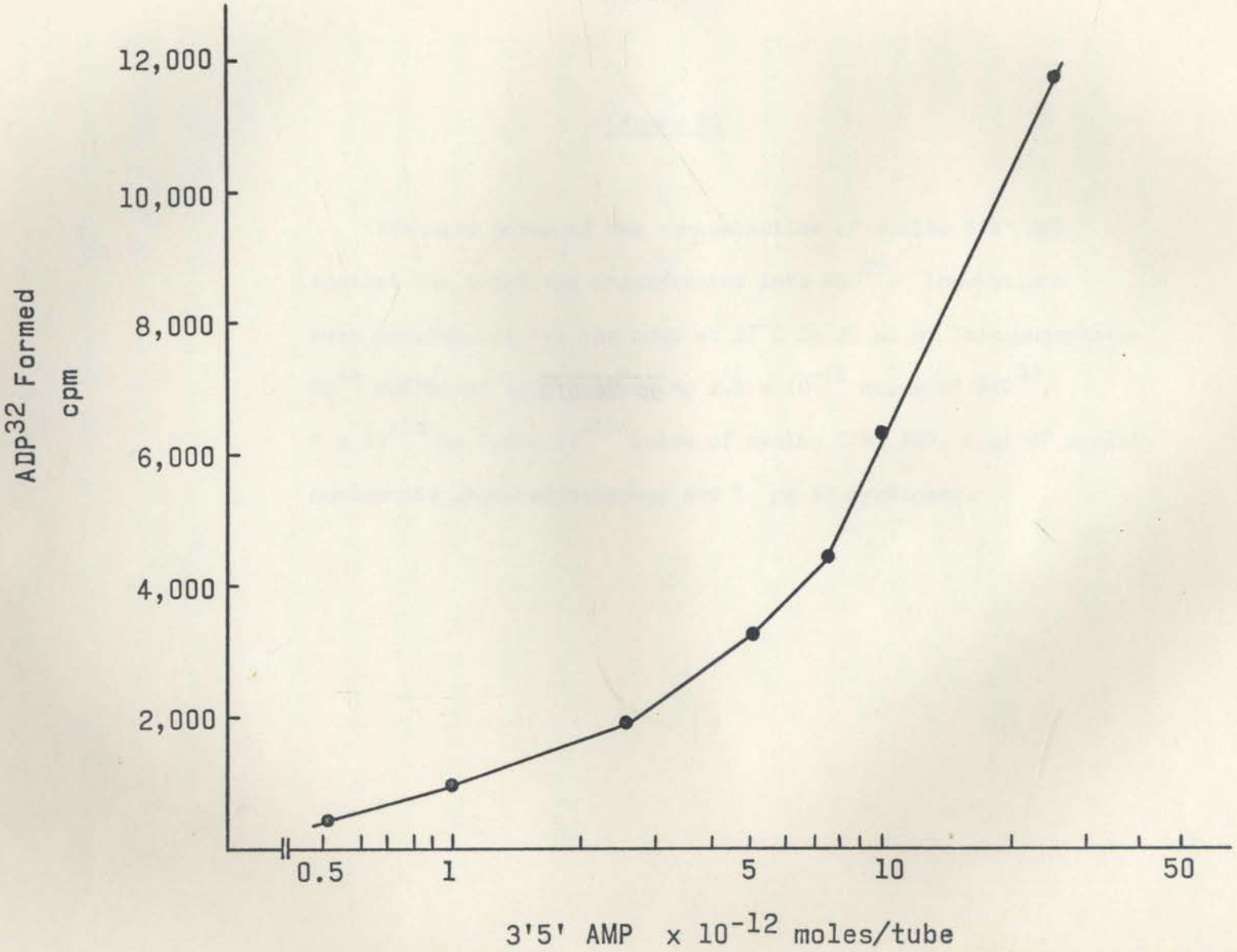
*Incubations were carried out in a volume of 30 ul of buffer containing 2.5 x $I0^{-II}$ moles $ATP^{32}$, cyclic 3'5' AMP, I μl cyclic nucleotide phosphodiesterase and 5 μg myokinase. The reaction was stopped with I0 μl of the perchloric acid reagent after one hour at 37°C, then I0 ul of $ADP-^{3}H$ containing I x $I0^{-I2}$ moles and 30 μl of 2N potassium bicarbonate were added. The mixture was centrifuged and the supernatant solution was applied to a MN cellulose 300 HR plate. The chromatogram was developed twice in the same direction, then the area corresponding to the ADP marker was eluted with I ml of water and counted in I0 ml Bray's solution.

Estimation of Cyclic 3'5' AMP Concentration in Tissues. The total cpm incorporated into $ADP^{32}$ in the reaction mixtures of both standard solutions and tissue samples were calculated by dividing the cpm recovered in $ADP^{32}$ by the per cent recovery of the added ADP-$^{3}H$. A standard curve was drawn plotting the cyclic 3'5' AMP content of the reaction mixture (containing $5 \times 10^{-13}$ moles to $2.5 \times 10^{-11}$ moles of cyclic 3'5' AMP) against the total cpm incorporated into $ADP^{32}$ (Figure 26). The cyclic 3'5' AMP content of the reaction mixture containing the tissue samples was then read from the standard curve. The tissue cyclic 3'5' AMP concentration was then calculated using the following formula:

$$\text{cyclic 3'5' AMP/g of tissue} = \frac{\text{cyclic 3'5' AMP content of the reaction mixture}}{\text{tissue weight (g)} \times \%\text{ recovery of cyclic 3'5' AMP-}^{3}H}$$

The cyclic 3'5' AMP concentration was assayed in the livers, skeletal muscle and epididymal fat pads obtained from 6-8 rats (Table XX).

FIGURE 26


12,000
10,000
8,000
6,000
4,000
2,000
ADP32 Formed
cpm
0.5
1
5
10
50
3'5' AMP x 10-12 moles/tube

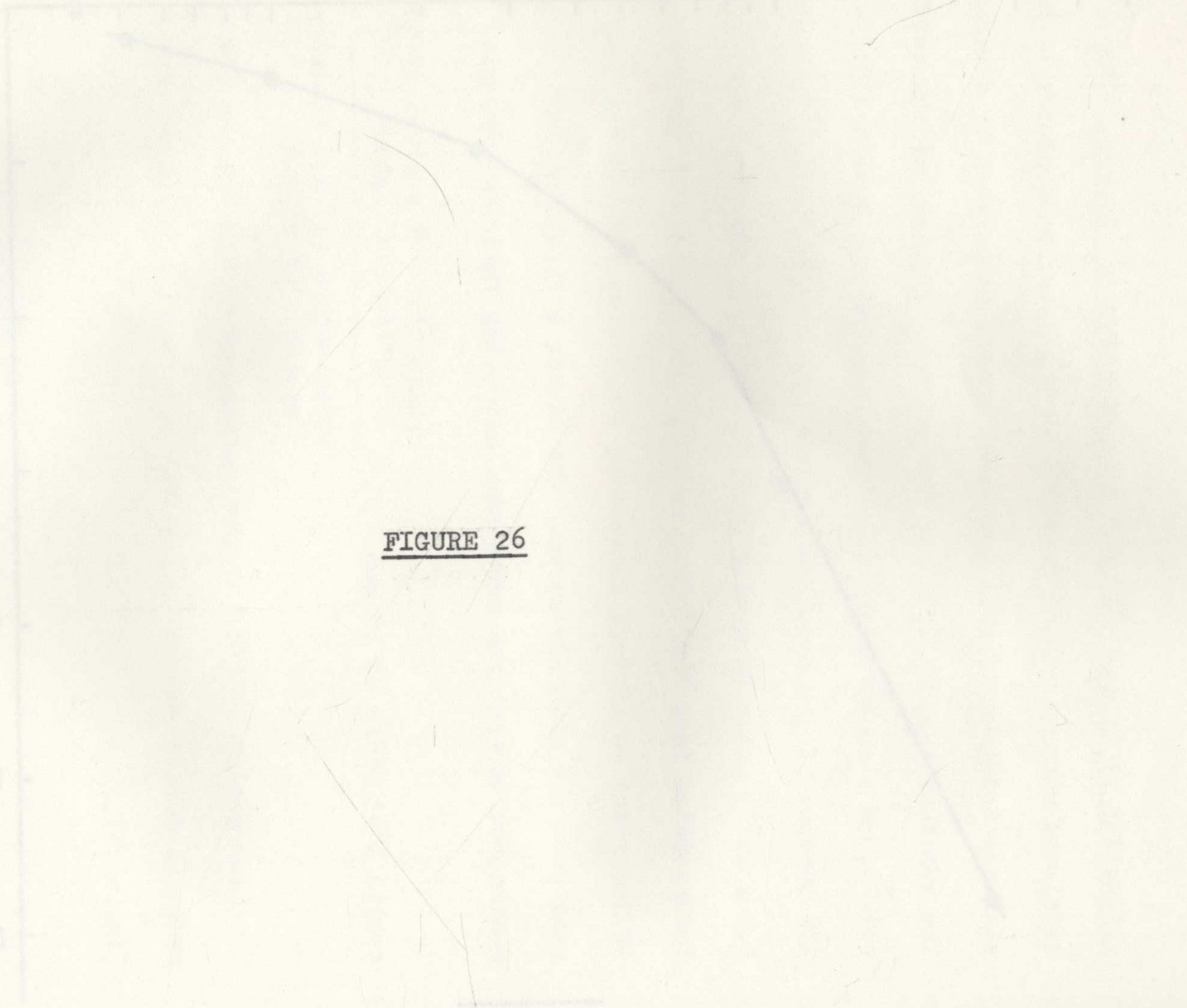


FIGURE 26

Figure 26

Standard curve of the concentration of cyclic 3'5' AMP against the total cpm incorporated into $ADP^{32}$. Incubations were carried out for one hour at 37°C in 30 µl of Tris-phosphate-$Mg^{++}$ buffer pH 8.0, containing $2.5 \times 10^{-11}$ moles of $ATP^{32}$, $5 \times 10^{-13}$ to $2.5 \times 10^{-11}$ moles of cyclic 3'5' AMP, I µl of cyclic nucleotide phosphodiesterase and 5 µg of myokinase.

Table XX

Cyclic 3'5' AMP Content of Tissues*

| | moles / g wet weight |
|---|---|
| Liver | $4.9 \pm 1.3 \times 10^{-9}$ |
| Skeletal Muscle | $1.05 \pm 0.2 \times 10^{-9}$ |
| Adipose Tissue | $5.05 \pm 0.5 \times 10^{-10}$ |
| **Isolated Fat Cells** | **moles / $10^6$ cells** |
| Control | $2.3 \pm 0.3 \times 10^{-11}$ |
| Epinephrine 0.05 ug/ml | $9.5 \pm 0.5 \times 10^{-11}$ |

*Tissues from pentobarbitone anaesthetized rats were immediately frozen in liquid nitrogen as described in the text. Aliquots of 40-50 mg liver and skeletal muscle and 60-70 mg epididymal fat were extracted. Isolated fat cells were prepared by the procedure of Rodbell (25) as described in Part I and incubated for 30 minutes at 37°C in the presence of 2% bovine serum albumin and 3 mM glucose.

The values for liver, muscle and adipose tissue represent the means ± S.E.M. of 6-8 determinations and the values for isolated fat cells represent the means ± S.E.M. of 4 determinations.

The results obtained are similar to the levels reported previously for these tissues by Posner et al (75), Butcher et al (74) and Sutherland and Rall (63). The cyclic 3'5' AMP concentration in isolated fat cells prepared by the method of Rodbell (25) was $2.3 \pm 0.3 \times 10^{-11}$ moles/$10^6$ cells. Estimation of fat cell and epididymal fat pad DNA content by the method Kissane and Robins (53), as described in Part I, has shown that the DNA content of rat epididymal adipose tissue is $0.20 \pm 0.01$ mg of DNA/g and that the DNA content per cell is $7.9 \pm 0.2 \times 10^{-12}$ g. When the cyclic 3'5' AMP content of the epididymal fat pad is expressed in terms of the concentration per $10^6$ cells, a value of $2.01 \times 10^{-11}$ moles/$10^6$ cells is obtained, identical with the cyclic 3'5' AMP concentration as measured in the isolated fat cell preparation.

In the presence of 0.05 ug of epinephrine/ml, the cyclic 3'5' AMP concentration in isolated fat cells increased over 400 per cent from $2.3 \pm 0.3 \times 10^{-11}$ moles/$10^6$ cells to $9.5 \pm 0.5 \times 10^{-11}$ moles/$10^6$ cells.

The results obtained with this new assay are consistent with those previously reported and as little as $5 \times 10^{-3}$ moles of cyclic 3'5' AMP can be measured. This would be the expected cyclic nucleotide concentration in 0.1 to 1.0 mg of tissue. The assay is specific and adequately sensitive for the estimation of cyclic

3'5' AMP in micro micro quantities of tissue.

Studies using this assay are reported in Part III.

## PART III

## A MECHANISM CORRELATING THE CONTROL OF CYCLIC 3'5' AMP SYNTHESIS WITH THE SPECIFIC PATTERN OF METABOLIC RESPONSE IN TISSUES

The marked release of pancreatic insulin and the rise in the plasma free fatty acid concentration which occurred after the injection of theophylline into rats suggested that the concentration of cyclic 3'5' AMP in the pancreatic islet and the adipose tissue cell determined the specific response pattern of these tissues (Part I). Furthermore, the studies with epinephrine, adrenergic blocking agents and theophylline suggested that, in the pancreatic islet, alpha and beta adrenergic receptors mediated divergent effects of epinephrine on cyclic 3'5' AMP synthesis; alpha receptors inhibited and beta receptors increased the generation of the cyclic nucleotide.

The response of tissue to catecholamines was first reported as being dependent on the presence of adrenergic receptor sites classified as alpha and beta on the basis of their response to sympathomimetic amines and adrenergic blocking agents in 1948 (88). In 1963, Murad et al (68) reported that epinephrine stimulated the synthesis of cyclic 3'5' AMP in heart muscle by a direct action on adenyl cyclase and that this stimulatory effect was prevented by beta adrenergic blockade with dichloroisoproterenol. Sutherland and Robinson (62) have reported similar findings in other tissues. In 1965 Butcher et al (74) reported that the metabolic effects of epinephrine on the rat epididymal fat pad were modified by alpha

and beta adrenergic blockade, then Handler et al in 1967 described similar effects in the toad bladder (89).

These findings, together with the observations on insulin release and lipolysis described in Part I, suggested that the metabolic responses of several tissues to the action of epinephrine was dependent on the presence of alpha and beta adrenergic receptors. Furthermore, they suggest that the metabolic response patterns were dependent on the concentration of cyclic 3'5' AMP in the tissues and that the level of the cyclic nucleotide was controlled by a direct effect of alpha and beta adrenergic receptors on adenyl cyclase. To test this hypothesis, a sensitive assay was developed for cyclic 3'5' AMP as has been described in Part II. Using this assay, the effects of epinephrine, in the presence and absence of alpha and beta adrenergic blocking agents, were examined on the cyclic 3'5' AMP concentration in three tissues - isolated pancreatic islets, isolated fat cells and toad bladders. The changes in the cyclic 3'5' AMP levels were correlated with the metabolic response patterns in these tissues. Evidence is presented that stimulation of the alpha adrenergic receptor decreases cyclic 3'5' AMP synthesis, whereas stimulation of the beta adrenergic receptor produces an increase in the cyclic 3'5' AMP level. Furthermore, the pattern of metabolic response in these tissues correlates with the cyclic 3'5' AMP concentration and is reproduced by the addition of the cyclic nucleotide in vitro (47, 71, 90, 91).

Experimental Procedure

Alpha adrenergic blockade was maintained with phentolamine (Ciba) and beta adrenergic blockade with propranolol (Ayerst).

Isolated rat pancreatic islets were prepared by the method of Lacy (92). Male Charles River rats weighing 500-600g were anaesthetized with sodium pentobarbitone 3 mg / 100 g body weight, injected intraperitoneally. The pancreas was distended by the injection of 10 ml of Hank's solution into the pancreatic duct as described by Lacy (92). The pancreas was removed, cut into fragments and incubated in Krebs Ringer bicarbonate buffer pH 7.4, containing 2 per cent bovine serum albumin and collagenase 10 mg/ml. After incubation for 15 minutes at 37°C, the islets were dissected free under 10x magnification. Using this procedure 200-300 islets were isolated from each rat pancreas. Vials containing 25-50 islets in 2 ml of albuminbicarbonate buffer pH 7.4 were incubated with epinephrine, adrenergic blocking agents and theophylline for one hour at 37°C under 95% $O_2$ - 5% $CO_2$. Cyclic 3'5' AMP was measured in duplicate on 200-300 islets, and the deoxyribonucleic acid content was determined by the fluorometric procedure of Kissane and Robins (53).

Isolated rat fat cells were prepared from male Charles River rats weighing 180-200 g, using the method of Rodbell (25) with the variations described in Part I. Cell counts were performed in a

siliconized Neubauer haemocytometer chamber. Lipolysis was measured by determining the rate of free fatty acid release. Free fatty acids were extracted by the procedure of Dole (50) and determined colourimetrically (51).

Toad Bladders. Bufo marinus toads were pithed immediately before use and the bilobed bladders were removed. These were washed and preincubated in Bentley's buffer for 10 minutes (93). They were then transferred to fresh flasks containing 20 ml of Bentley's buffer pH 8.0 at 23°C, together with the tesy substances. At the end of the incubation period, the tissue was removed and rapidly homogenized in 5 ml of 5.6 per cent trichloracetic acid, then the cyclic 3'5' AMP content was measured.

## Results

Isolated pancreatic islets. Theophylline (180 µg/ml) produced a 1500 per cent increase in the cyclic 3'5' AMP concentration from 355 ± 50 µmoles/g DNA to 5570 ± 302 umoles/g DNA (Table XXI). Epinephrine (10 µg/ml) almost completely inhibited this effect of theophylline, reducing the cyclic nucleotide level to 415 ± 12 µmoles/g DNA. This action of epinephrine reflected alpha adrenergic receptor stimulation since it was completely abolished by phentolamine. When residual beta adrenergic receptor activity was blocked with propranolol, the addition of epinephrine produced cyclic 3'5' AMP levels less than those in the control. Under these conditions the alpha adrenergic receptor was

stimulated alone. The addition of glucagon (I μg/ml) also stimulated the accumulation of cyclic 3'5' AMP to levels higher than those seen with epinephrine and alpha adrenergic blockade. These changes in pancreatic islet cyclic 3'5' AMP concentration correlate well with the pattern of insulin release observed in vivo in the rat in response to theophylline, epinephrine and adrenergic blocking agents (Figure 22). An increase in pancreatic islet cyclic 3'5' AMP occurs with beta adrenergic receptor stimulation and is associated with insulin release, whereas a decrease in cyclic 3'5' AMP occurs with alpha adrenergic receptor stimulation, together with reduced insulin secretion (Table XXI).

Table XXI

Effects of Theophylline, Glucagon, Epinephrine and Adrenergic Blocking Agents on Pancreatic Cyclic 3'5' AMP Content and Insulin Release in Rats*

| Experimental Conditions | Theophylline | Cyclic 3'5' AMP Concentration in Pancreatic Islets | Insulin Secretion in vivo |
|---|---|---|---|
| | | umoles/g DNA | μunits-minutes $ml^{-1}$ |
| Control | - | 355 ± 50 | 0 |
| | + | 5,570 ± 302 | 487 ± 42 |
| Epinephrine | + | 415 ± 12 | 208 ± 29 |
| Epinephrine phentolamine | + | 4,450 ± 363 | 1,272 ± 36 |
| Epinephrine propranolol | + | 305 ± 20 | 0 |
| Glucagon | + | 11,950 ± 431 | 1,023 ± 97 |

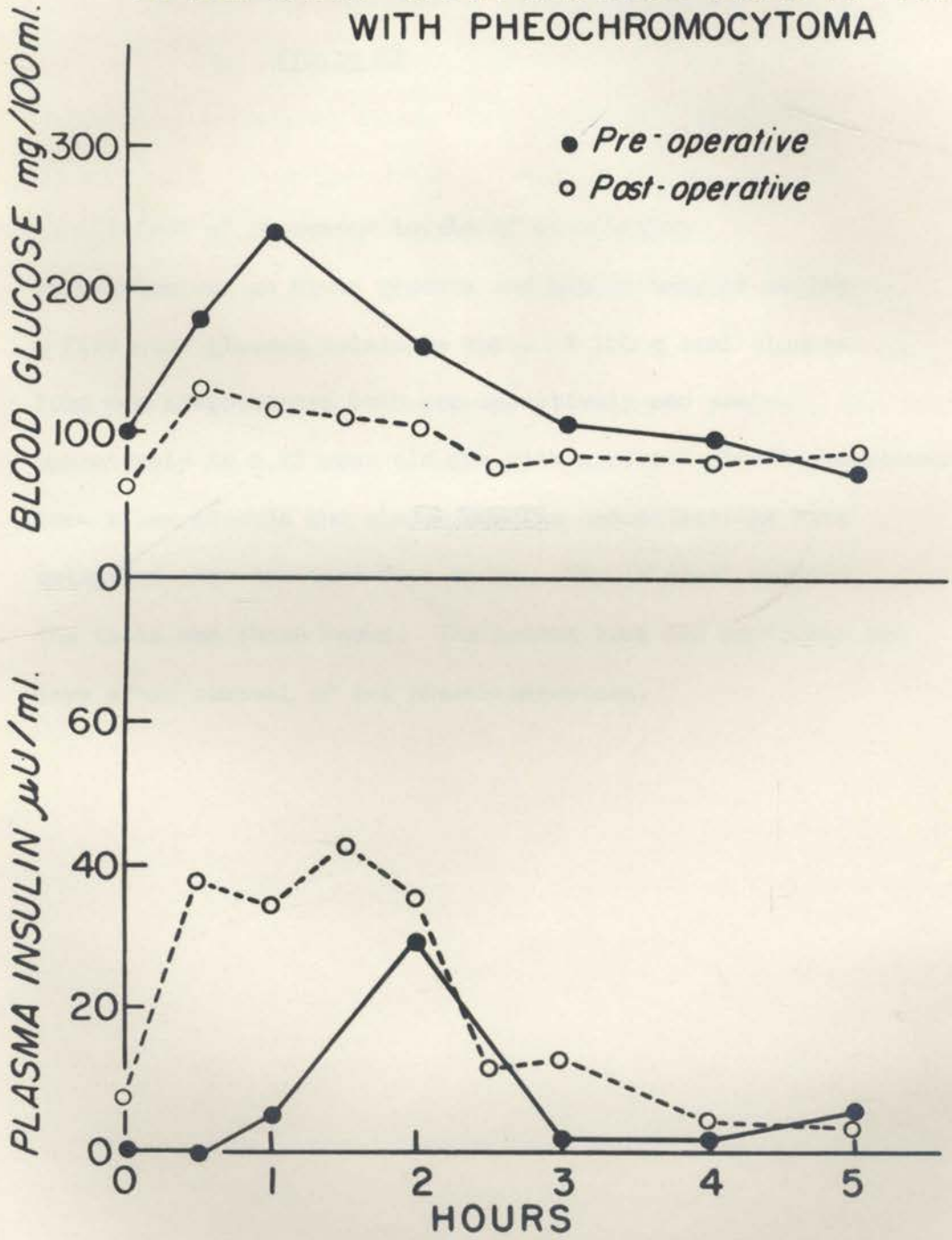
*Isolated rat islets prepared from six male Charles River rats weighing 500-600 g were incubated for one hour at 37°C in vials containing 25-50 islets in 2 ml of albumin-bicarbonate buffer pH 7.4 and 5.6 mM glucose. Epinephrine (10 μg/ml), phentolamine (10 μg/ml), glucagon (1 μg/ml) and theophylline (180 μg/ml) were added as indicated in the table. The cyclic 3'5' AMP concentration was measured in duplicate on 200-300 islets obtained by pooling the contents of eight incubation vials. The insulin secretion in vivo in the rat was calculated from the data presented in Figure 22, and represents the area circumscribed by the plasma insulin response curve.

To determine whether epinephrine inhibited insulin secretion in the human in physiological situations, a glucose tolerance test was performed in a 22 year old man with a diagnostically proven phaeochromocytoma. The blood glucose response to an oral glucose load was diabetic in type and the plasma insulin concentrations were low at all points. After the removal of the tumour the blood glucose response to an oral glucose load returned to normal and the plasma insulin concentrations at each point were within the normal range (Figure 27). These data suggest that in physiological systems epinephrine may inhibit pancreatic insulin secretion and that this effect is mediated via an alteration in the pancreatic islet concentration of cyclic 3'5' AMP.

Isolated fat cells. In the presence of epinephrine (0.05 μg/ml), cyclic 3'5' AMP increased fourfold from 23 ± 3 μmoles/$10^{12}$ cells to 95 ± 7 μmoles/$10^{12}$ cells, and lipolysis was correspondingly accelerated (Table XXII). Lipolysis was slightly accelerated by propranolol (5 ug/ml), free fatty acid release increasing from 11.4 ± 0.8 mmoles/$10^{12}$ cells/30 minutes to 15.0 ± 0.5 mmoles/$10^{12}$ cells/30 minutes. When beta adrenergic blockade was established with propranolol, epinephrine (0.05 μg/ml) decreased the cyclic 3'5' AMP concentration to 13 ± 2 μmoles/$10^{12}$ cells, a level below that in the control. There was a corresponding inhibition in the release of free fatty acids to 70 per cent of the

FIGURE 27

PRE- AND POST-OPERATIVE INSULIN RESPONSES TO 100 GRAM ORAL GLUCOSE LOAD IN A PATIENT WITH PHEOCHROMOCYTOMA



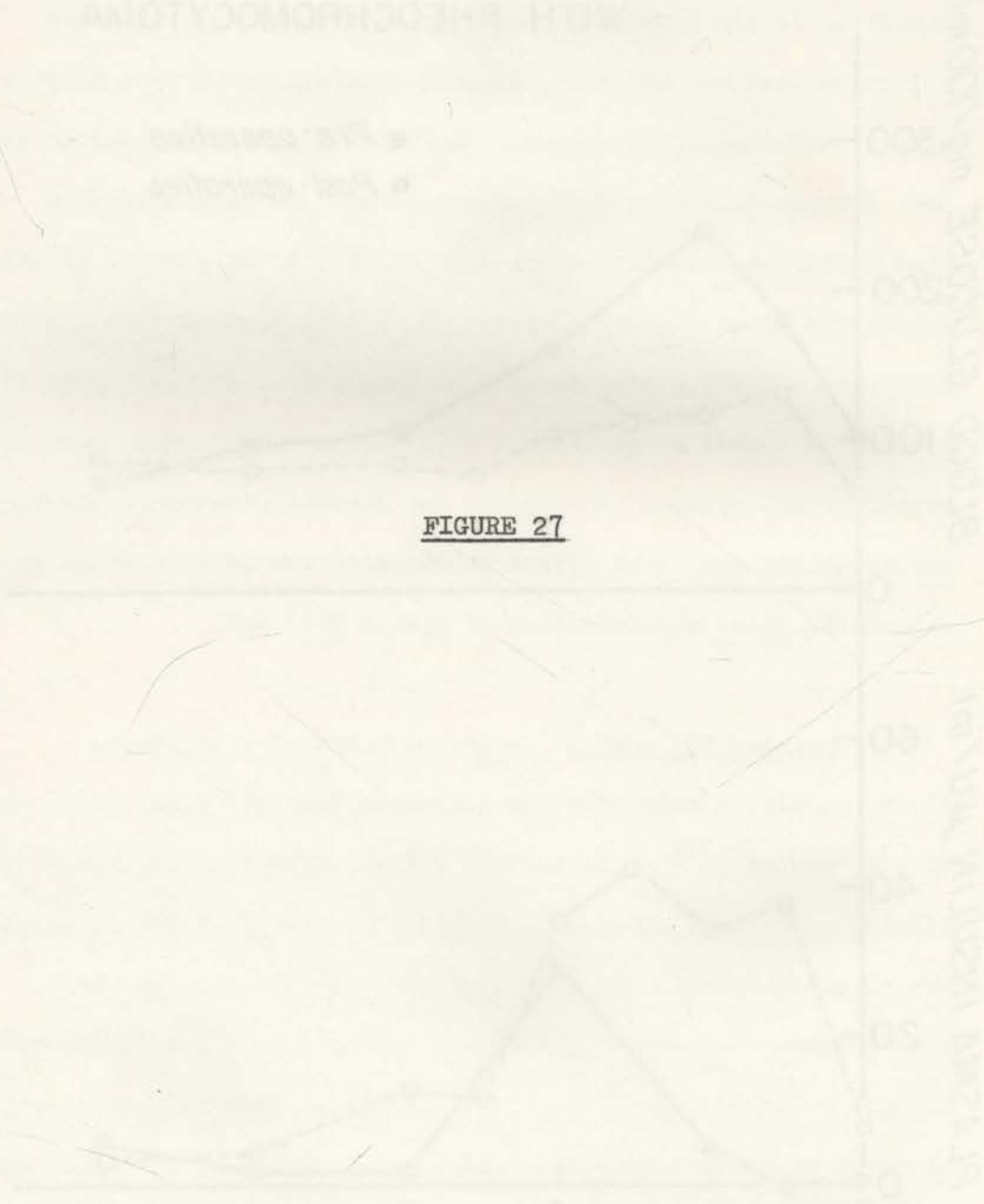


FIGURE 27

Figure 27

Effect of increased levels of circulating catecholamines on blood glucose and plasma insulin during a five hour glucose tolerance test. A 100 g oral glucose load was administered both pre-operatively and post-operatively to a 22 year old man with a proven pheochromocytoma, then blood glucose and plasma insulin concentrations were estimated over the next five hours. The interval between the tests was three weeks. The second test was performed ten days after removal of the pheochromocytoma.

control level. Furthermore, when compared with the free fatty acid release in the presence of propranolol alone (I5.0 ± 0.5 mmoles/$IO^{12}$ cells/30 minutes), epinephrine decreased the free fatty acid release by 45 per cent (8.0 ± 0.2 mmoles/$IO^{I2}$ cells/ 30 minutes). When the effect of epinephrine on the alpha adrenergic receptor was blocked with phentolamine (5 µg/ml), cyclic 3'5' AMP levels (70 ± 8 µmoles/$IO^{I2}$ cells) comparable to those with epinephrine alone (95 ± 5 µmoles/$IO^{I2}$ cells) were observed. Under these conditions epinephrine increased the free fatty acid release by I500 per cent from I3.I ± I.I mmoles/$IO^{I2}$ cells/ 30 minutes in the presence of phentolamine alone to 200 mmoles/$IO^{I2}$cells/ 30 minutes in the presence of phentolamine plus epinephrine.

Table XXII

Effects of Epinephrine and Adrenergic Blocking Agents on the Cyclic 3'5' AMP Concentration and the Rate of Lipolysis in Isolated Fat Cells*

| Experimental conditions | Cyclic 3'5' AMP Concentration $\mu$moles/$10^{12}$ cells | FFA Release mmoles/$10^{12}$ cells /30 minutes |
|---|---|---|
| Control | 23 ± 3 | II.4 ± 0.8 |
| Epinephrine | 95 ± 5 | 200 |
| Epinephrine + Propranolol | I3 ± 2 | 8.0 ± 0.2 |
| Propranolol | - | I5.0 ± 0.5 |
| Epinephrine + Phentolamine | 70 ± 8 | 200 |
| Phentolamine | - | I3.I ± I.I |

* Isolated fat cells prepared from 8 rats were incubated in I ml of albumin-bicarbonate buffer pH 7.4, containing 2% bovine serum albumin and 3 mM glucose for 30 minutes at 37°C. Epinephrine (0.05 $\mu$g/ml), propranolol (5 $\mu$g/ml) and phentolamine (5 $\mu$g/ml) were added as indicated in the table. Cell counts ranged from 2.8 to 3.I x $10^6$ cells per ml. The cyclic 3'5' AMP concentration was determined on approximately 3 x $10^6$ cells. Each value represents the mean ± S.E.M. of 4 determinations.

Toad Bladders. Theophylline (180 µg/ml) increased the cyclic 3'5' AMP concentration from 1.40 ± 0.07 m µmoles/g to 9.30 ± 0.5 m µmoles/g, an increase of over 600 per cent (Table XXIII). These values are higher than the levels reported by Handler et al (94) in 1965, who found a cyclic 3'5' AMP concentration of 0.45 m µmoles/g with an oncrease of 170 per cent in the presence of theophylline (1.8 mg/ml). These workers, however, preincubated the bladders for 20-120 minutes, a manouvre known to decrease the cyclic 3'5' AMP concentration (94). Epinephrine (1 µg/ml) alone did not significantly change the cyclic 3'5' AMP concentration. In the presence of theophylline (180 µg/ml), epinephrine decreased the cyclic 3'5' AMP concentration from 9.30 ± 0.5 m µmoles/g to 2.38 ± 0.3 m µmoles/g. Furthermore, this inhibition of cyclic 3'5' AMP accumulation by epinephrine was completely reversed by alpha adrenergic blockade with phentolamine (10 µg/ml). Under these conditions stimulation of the beta adrenergic receptor alone by epinephrine increased the cyclic 3'5' AMP concentration by 920 per cent from 2.38 ± 0.3 m µmoles/g to 21.95 ± 3.9 mµmoles/g. These changes in the concentration of the cyclic nucleotide correlate well with the changes in permeability to water in the toad bladder produced by theophylline, epinephrine and adrenergic blocking agents (71, 89).

Table XXIII

Effects of Epinephrine, Theophylline and Phentolamine on the Cyclic 3'5' AMP Concentration in Toad Bladders*

| Experimental Conditions | Theophylline | Cyclic 3'5' AMP Concentration | Permeability to Water |
|---|---|---|---|
| | | m µmoles/g | % of control |
| Control | - | I.40 ± 0.07 | I00 |
| | + | 9.30 ± 0.5 | +950 |
| Epinephrine | - | 2.0 ± 0.I | - 78 |
| | + | 2.38 ± 0.3 | - |
| Epinephrine Phentolamine | + | 2I.95 ± 3.6 | + 47 ≠ |

*Toad bladders were incubated at 23°C for 30 minutes in 20 ml Bentley's buffer pH 8.0, containing 5.6 mM glucose. Epinephrine (I µg/ml), phentolamine (I0 µg/ml) and theophylline (I80 ug/ml) were added to the incubation medium as indicated in the table. Each incubation vial contained I70-270 mg of tissue on which the cyclic 3'5' AMP was determined. Each value represents the mean ± S.E.M. of 4 determinations. The data on permeability to water were taken from the data of Orloff and Handler (7I) and Handler et al (89) for comparison of the metabolic changes under similar experimental conditions.

≠ In these studies theophylline was not used with the combination of epinephrine and blocking agent.

## Discussion

The effects of growth hormone on adipose tissue metabolism can be clearly divided into two categories:

A. Immediate responses which appear promptly after exposure to the hormone, stimulate the effects of insulin and cannot be blocked by inhibitors of nucleic acid and protein synthesis, and

B. Delayed responses which are observed only after 90-120 minutes exposure to the hormone and are blocked by inhibitors of nucleic acid and protein synthesis.

When the actions of growth hormone are examined in these terms, many of the apparently contradictory and conflicting reports on the metabolic effects of the hormone on adipose tissue can be explained.

The immediate responses are characterized by an accelerated rate of glucose uptake, increased oxidation of glucose-1-$C^{14}$, glucose-6-$C^{14}$ and acetate-1-$C^{14}$ and increased lipogenesis from all three precursors. During this early phase growth hormone has no effect on the rate of lipolysis. Similar insulin-like effects of growth hormone were originally reported with the rat epididymal fat pad, using large concentrations of growth hormone, by Jungas and Ball in 1960 (2) and Leboeuf and Cahill in 1961 (3). It does appear that this is an intrinsic property of growth hormone and is not due to a contaminant, as there is species specificity with regard to this effect (6). It was suggested by Huggins and

Ottaway (7) that the insulin-like effects could be due to a hypoglycaemia-producing peptide which they claimed could be isolated from purified growth hormone preparations. These suggestions, however, have not been confirmed. More recently Goodman (4, 5), using fat pads removed from hypophysectomized rats maintained on a high carbohydrate, fat free diet, has shown that as little as IO m ug of growth hormone per ml accelerated hexose and amino acid transport, glucose uptake, oxidation and incorporation into fatty acids. In all of these studies *in vitro*, incubations were of one to three hours duration, which is insufficient time for hormone activated lipolysis to become established. In other *in vitro* systems, for example the perfused rat heart (95) and the rat diaphragm (96) growth hormone has been shown to have a similar stimulatory effect on hexose transport and uptake of glucose.

These immediate *in vitro* insulin-like responses may account for the transient early fall in blood glucose which has occasionally been seen after the injection of growth hormone *in vivo* (6, 7). Coinciding with the early hypoglycaemia, a prompt although evanescent fall in the plasma free fatty acid level has been reported by Engel et al (97). In support of this early effect of growth hormone are the forearm perfusion experiments of Zierler and Rabinowitz in I963 (98) in which growth hormone produced a prompt stimulation of uptake of free fatty acids by skeletal muscle. It is unlikely that this effect is due to a

decreased rate of peripheral adipose tissue lipolysis, as this could not be demonstrated in the isolated fat cell preparation; nor was it observed by Fain et al (26) using either the fat pad or the isolated fat cells. The findings of Zierler and Rabinowitz (98), together with the immediate fall in respiratory quotient (RQ) observed by Brown and Bennett (99) after the injection of growth hormone, suggest that the decrease in plasma free fatty acid concentration seen in vivo after administration of the hormone reflects an increase in free fatty acid utilization.

In the experiments undertaken with human placental lactogen (HPL), no significant changes on glucose-I-$^{14}$C, glucose-6-$^{14}$C, or acetate-I-$^{14}$C oxidation or incorporation into fatty acids were seen during the early phase of incubation unless high concentrations of the hormone ( I mg/ml) were used. However in vivo experiments by Friesen (34) did show hypoglycaemia in rats after the injection of 10-20 mg of HPL by the intraperitoneal route. Riggi et al (42) did not find hypoglycaemia after HPL injection into fasted mice, rats or alloxam diabetic mice. In the latter experiments, however, the plasma free fatty acid levels were elevated and under these conditions a mild insulin-like hypoglycaemia action of HPL might not be seen.

The delayed phase of growth hormone action is characterized by the appearance of activated lipolysis. As originally reported by Fain (26), the lipid-mobilizing effect of growth hormone is

preceded by a 90-120 minute lag period, and can be readily demonstrated with low concentrations of the hormone. The lipolytic action of growth hormone is dependent on DNA-RNA mediated protein synthesis as it can be prevented by either actinomycin D or puromycin (26). In contrast to the immediate response pattern, the delayed phase of hormone action is characterized by decreased glucose uptake and oxidation of glucose-I-$^{14}$C, and a marked inhibition of lipogenesis from all three precursors. These changes are secondary to the primary lipolytic effect of the hormone and, more specifically, to the accumulation of free fatty acids released as a consequence of activated lipolysis. The delayed phase characteristics of glucose and acetate metabolism are abolished when the accumulation of free fatty acids resulting from hormone activated lipolysis is prevented by blocking the lipolytic effect with actinomycin D, puromycin or nicotinic acid. Furthermore, incubation in a medium containing a high glucose concentration stimulates free fatty acid re-esterification and prevents the accumulation of free fatty acids in the medium, although lipolysis has still occurred as reflected by glycerol release. Under these conditions the delayed phase pattern of glucose and acetate metabolism does not occur.

The addition of free fatty acids to the incubation medium reproduced all the delayed phase patterns of growth hormone effects on glucose and acetate metabolism. When palmitate was added to the medium, the amount required to duplicate the delayed

effects of growth hormone was greater than the free fatty acid concentration which accumulated in the incubation medium as a consequence of activated lipolysis. This suggests that either the extracellular and intracellular free fatty acid pools are not in equilibrium, or that the intracellular pool is compartmentalized, with variable degrees of equilibration between these compartments and the extracellular free fatty acid level. Recent kinetic studies by Bally et al (100) with the rat fat pad, and Rodbell (101) with isolated rat fat cells, have suggested that the intracellular free fatty acid pool is not homogeneous. In the intact rat diaphragm Schonfeld and Kipnis (102) have shown that the intracellular free fatty acid pool is functionally heterogeneous and not in equilibrium with the extracellular free fatty acid pool. Furthermore, they presented evidence that the intracellular free fatty acid level rather than the extracellular free fatty acid concentration is the critical determinant regulating glucose-fatty acid interactions in that tissue. These observations may also be relevant to the isolated fat cell, since it was not possible to depress glucose uptake with exogenous free fatty acids, although slight depression occurred during activated lipolysis.

Growth hormone-activated lipolysis was detected by glycerol release at low concentrations ($< 100$ m $\mu g/ml$), but free fatty acid release did not occur unless dexamethasone was present in the incubation medium. The glucocorticoid alone did not

significantly effect lipolysis. It did inhibit the uptake of free fatty acids added to the incubation medium and the esterification of fatty acids released by endogenous lipolysis. The primary effect of the steroid was to inhibit esterification of free fatty acids for any given rate of lipolysis.

If the delayed growth hormone-induced changes in glucose and acetate metabolism are secondary to free fatty acid accumulation rather than lipolysis per se, then physiological conditions associated with increased lipolysis without increased free fatty acid release may exhibit a mixture of immediate and delayed responses. This situation was seen in the absence of dexamethasone where hormone-activated lipolysis still occurred as reflected by glycerol release, but free fatty acids were rapidly re-esterified and did not accumulate in the incubation medium. Under these conditions the hormone stimulated both glucose uptake and glucose-I-$C^{14}$ oxidation during the immediate and delayed phases. One might predict that in a fat cell totally depleted of lipid, the effect of growth hormone after prolonged incubation may continue to be characterized by an immediate response pattern. In this regard it will be of interest to examine the growth hormone effect in the isolated rat fat cell membrane preparation recently developed by Rodbell (unpublished).

During the fourth to fifth hours of incubation, when the extracellular free fatty acid level had reached 600-900 μEq/l, hormone-activated lipolysis returned to, or below, control levels,

and the lipolytic system was no longer responsive to additional hormone. These studies confirm the original report of Rodbell (101) that an increase in the free fatty acid level inhibited the rate of endogenous lipolysis. When growth hormone was added to fat cells in the presence of increasing concentrations of exogenous palmitate, the lipolytic effect of the hormone was progressively decreased and could in fact be completely abolished if small amounts of the hormone were used (100 m μg/ml). These studies further demonstrate that the extracellular free fatty acid concentration markedly influences the responsiveness of the lipolytic system to growth hormone. This reciprocal interaction with the free fatty acid level, either within the fat cell or the extracellular fluid, and with the lipolytic process, may account for the failure to demonstrate the adipokinetic activity of growth hormone on the rat epididymal fat pad *in vitro*. The basal rate of free fatty acid release by the epididymal fat pad ranges from 2-4 μEq/g/hour which, expressed in terms of activity per cell, 150-300 m $\mu Eq/10^6$ cells/hour*, is 10-20 times greater than the rate of lipolysis in the fat cell preparation used in this study. Under the experimental conditions commonly used with the *in vitro* rat epididymal fat pad preparation (i.e. 100 mg tissue incubated in 1-2 ml medium containing 2 per cent serum albumin), free fatty

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* Footnote. Calculated from a DNA concentration of $7.9 \times 10^{-12}$ g/cell. From a calculation of the DNA content of fat cells and whole fat pad, 1 g of adipose tissue contains approximately $2.5 \times 10^6$ cells

acid levels ranging from 400-900 μEq/l (FFA/albumin $\bar{v}$ - I.2-2.7) will accumulate during the first two hours of incubation - the induction period for the lipolytic action of growth hormone. At these free fatty acid levels endogenous lipolysis is markedly depressed and, as a consequence, the adipokinetic activity of growth hormone, which requires a 60-90 minute induction period, will be either abolished or severely impaired.

This complication can be circumvented by administering the hormone to the rat and removing the fat pad at a later time. Thus if the hormone is injected in vivo and the fat pad is removed 2-3 hours later, by which time the lipolytic effect has been induced, accelerated lipolysis should be readily demonstrable during the first hour of incubation before the free fatty acid concentration in the incubation medium has risen to inhibitory levels. Using this type of experimental procedure, Knobil (20) and White and Engel (21) demonstrated a lipolytic effect of growth hormone on adipose tissue from rats and mice. With the same experimental approach it would be expected that it should also be possible to distinguish between immediate and delayed patterns of growth hormone response. When the data of Goodman (22) is examined in this fashion such was found to be the case - glucose uptake, glucose oxidation and lipogenesis were all increased in fat pads removed within 30-60 minutes after the injection of growth hormone in the intact animal. However, if the fat pads are removed 3½ hours after hormone administration, glucose uptake,

oxidation and incorporation into fatty acids are all markedly depressed (22).

The preparation of HPL used in the studies was highly purified and at least four times the potency of HPL produced by other methods. The experiments clearly demonstrate that highly purified HPL exhibits significant lipolytic activity at concentrations approximating physiological levels (30, I03). As in the case of growth hormone-activated lipolysis, the lipolytic activity of HPL is preceded by a lag period of 60-90 minutes. HPL-activated lipolysis is also inhibited by actinomycin D and puromycin when these inhibitors are present from the start of the incubation, but not if they are added 60-90 minutes after previous exposure of the fat cells to the hormone. These characteristics suggest that HPL-activated lipolysis is dependent on new protein synthesis, a conclusion supported by the failure to demonstrate inhibition of lipolysis with the aminonucleoside of puromycin.

These results differ from those of Friesen (34) who used a purified placental protein fraction which cross-reacted immunochemically with human growth hormone and also exhibited growth-promoting activity. Even in concentrations of HPL up to I mg/ml, Dr. Friesen did not observe any lipolytic effect on the rat epididymal fat pad <u>in vitro</u>. However, in that study lipolytic activity was assessed in terms of free fatty acid release, an event which reflects the net balance between the rates of lipolysis

and free fatty acid re-esterification. Significant changes in the rate of lipolysis may go undetected using this parameter rather than glycerol release as an index of lipolytic activity, due to the rapid re-esterification of free fatty acids by adipose tissue from the rat. This proved to be the case in the present study in which it was noted that 20 µg HPL/ml produced a twofold increase in the rate of glycerol release without any concomitant free fatty acid accumulation in the incubation medium.

As with growth hormone, the free fatty acid level to which the fat cell is exposed markedly influences its responsiveness to HPL. At physiological concentrations of free fatty acids (FFA/albumin molar ratio - 0.75), endogenous lipolysis was reduced to 50 per cent of the rate observed in cells incubated in the absence of added exogenous free fatty acids. At free fatty acid levels comparable to those seen under conditions of maximal lipid mobilization in vivo (FFA/albumin $\bar{v}$ - 3.0), endogenous lipolysis was completely blocked. The reciprocal interaction between the free fatty acid level in or around the fat cell and the lipolytic process, may explain the findings on the adipokinetic activity of HPL. The adipokinetic activity of 500 µg HPL/ml was not demonstrable when the extracellular free fatty acid level reached 250-300 µEq/l, whereas significant stimulation was readily observed with one twentieth this concentration of hormone if the extracellular free fatty acid level was reduced. Thus, when the steady-state free fatty acid level is depressed, one might

reasonably expect that HPL-activated lipolysis would be readily demonstrable. Consistent with this interpretation is the recent demonstration by Grumbach et al (40) that HPL given by intramuscular injection to hypopituitary children provoked a marked adipokinetic response after a 2-4 hour delay, whereas Beck and Daughaday (4I) were unable to demonstrate significant free fatty acid mobilization in normal volunteers given 2-5 times the dose of HPL by either a single injection or by prolonged intravenous infusion.

HPL also markedly modified glucose and acetate metabolism in isolated fat cells after the onset of hormone-activated lipolysis. Glucose-I-$C^{I4}$ oxidation was decreased, glucose-6-$C^{I4}$ and acetate-I-$C^{I4}$ oxidation was increased and lipogenesis from all three precursors was impaired. All these changes are similar to those observed with growth hormone, are secondary to the accumulation of free fatty acids as a consequence of activated lipolysis, and can be abolished by stimulating the re-esterification of free fatty acids with a high glucose concentration.

The evidence obtained suggested that the primary effect of both growth hormone and HPL on adipose tissue was activation of lipolysis. The mechanism of activation was dependent on DNA-RNA mediated protein synthesis as it could be blocked by actinomycin D and puromycin. This differed from the lipolytic action of ACTH, TSH, epinephrine and glucagon, which is not dependent on new protein synthesis and occurs immediately adipose tissue is exposed to the hormone (3, 25, I04, I05). The activation of lipolysis

by this wide spectrum of hormones and the observations by Vaughan (106) that norepinephrine, glucagon and ACTH increased free fatty acid release and activated phosphorylase in adipose tissue, indicated that a common mechanism may be responsible for the adipokinetic activity of many hormones in vivo. Epinephrine had been implicated in stimulating the synthesis of cyclic 3'5' adenosine monophosphate (cyclic 3'5' AMP) in adipose tissue (67, 106) as well as activating lipolysis (12, 104) and phosphorylase in many tissues (62, 104). Furthermore, several of the other adipokinetic hormones such as ACTH, TSH, glucagon and vasopressin were known to stimulate the accumulation of cyclic 3'5' AMP in tissues with which they were usually associated (107).

For these reasons the role of cyclic 3'5' AMP was assessed as a mediator for the lipolytic action of hormones in vivo. The enzymatic synthesis and degradation of cyclic 3'5' AMP is summarized:

$$\text{ATP} \xrightarrow[Mg^{++}\ F^{I}]{\text{Adenyl cyclase}} \text{cyclic 3'5' AMP} \xrightarrow[Mg^{++}]{\text{cyclic 3'5' nucleotide phosphodiesterase}} \text{5' AMP}$$

Cyclic 3'5' AMP is formed from ATP by adenyl cyclase, an enzyme with widespread distribution (108), and is hydrolyzed to 5' AMP by cyclic 3'5' nucleotide phosphodiesterase (79). As cyclic 3'5' AMP probably does not penetrate the cell membrane in low concentrations (62), the intracellular concentration of the cyclic nucleotide could not be satisfactorily increased by infusing it directly into animals. Providing a concentration of at least

$10^{-4}$ molar is maintained, the injection of theophylline competitively inhibits the 3'5' cyclic nucleotide phosphodiesterase (79), and increases the tissue level of cyclic 3'5' AMP. Another methyl xanthine, caffeine, is also potent as an inhibitor of the specific phosphodiesterase, but its potency is only approximately one third that of theophylline.

The injection of 15 mg of theophylline into rats produced a prompt increase in the circulating plasma free fatty acid level, but this promptly fell to levels below those found during the control period. At the same time there was a moderate increase in the plasma glucose level. When fasted, acutely adrenalectomized rats were used, the injection of theophylline caused a smaller increase in the plasma free fatty acid level, probably due to the elimination of acute variations in adrenal catecholamine secretion. Under these conditions of hepatic glycagon depletion in the fasted animal there was only a minimal rise in plasma glucose. The prompt fall in the plasma free fatty acid level occurring after an initial rise indicated probable insulin release, confirmed by radioimmunoassay. This insulin release was not due to the slight increase in plasma glucose as it could not be reproduced by glucose infusion. It thus appeared that the injection of theophylline not only activated lipolysis in vivo, but also caused a prompt outpouring of pancreatic insulin.

The infusion of epinephrine inhibited insulin release in the rat. Theophylline injected during an epinephrine infusion still

produced insulin release, though this was less marked than in rats given theophylline alone. This response was dependent on adrenergic receptors, as theophylline injected during an epinephrine infusion in the presence of beta adrenergic receptor blockade produced no insulin release whatever, whereas theophylline injection during alpha adrenergic receptor blockade and epinephrine infusion, produced a marked accentuation of insulin release. Since epinephrine had been shown to stimulate cyclic 3'5' AMP synthesis in many tissues (62), it seemed that its effect on pancreatic insulin secretion might be mediated via an effect on adenyl cyclase, the enzyme controlling cyclic 3'5' AMP synthesis. Murad et al (68) demonstrated in 1962 that in a dog heart preparation epinephrine stimulated adenyl cyclase, cyclic 3'5' AMP synthesis and produced a positive inotropic effect, and that these effects were blocked by dichloroisoprotenol, a beta adrenergic receptor blocking agent. No specific studies of alpha adrenergic receptor stimulation on cyclic 3'5' AMP synthesis have been reported. The results reported here suggest that in the pancreatic islet, the effects of epinephrine on the insulin secretory mechanism were mediated via alpha and beta adrenergic receptors which controlled the synthesis of cyclic 3'5' AMP by adenyl cyclase. The two receptors mediated divergent effects -- the alpha adrenergic receptor inhibiting cyclic nucleotide generation and insulin secretion, and the beta adrenergic receptor increasing cyclic nucleotide generation and stimulating insulin secretion.

To investigate this further it was necessary to evolve a

suitable assay for cyclic 3'5' AMP which would permit estimation of the cyclic nucleotide concentration in 0.1-1.0 mg of tissue, or approximately 20-100 isolated pancreatic islets containing $10^{-13}$ to $10^{-12}$ moles of cyclic 3'5' AMP. The assays of Butcher et al (74), Posner et al (75) and Breckenridge (78), involved idfficult procedures for the isolation of cyclic 3'5' AMP which were then followed by either the measurement of phosphorylase activation or an enzymatic cycling procedure. All methods are limited by poor separation of the cyclic nucleotide from other adenine nucleotides, extensive losses and contamination of the enzymes used in the assay technique. The sensitivity of these methods is limited to approximately 100 mg of tissue, rendering them unsatisfactory for cyclic 3'5' AMP assay in isolated pancreatic islets.

The development of a new assay for cyclic 3'5' AMP permitted measurement in less than 1 mg of tissue. Separation of cyclic 3'5' AMP from other adenine nucleotides using cyclic 3'5' AMP-$^{3}H$ for recovery purposes was readily accomplished by this-layer chromatography on cellulose plates. The isolated cyclic nucleotide was then hydrolyzed to 5' AMP and reacted with $ATP^{32}$ ($\gamma$ labelled) and myokinase to give $ADP^{32}$ ($\beta$ labelled). ADP-$^{3}H$ was added for recovery purposes, and the $ADP^{32}$-ADP-$^{3}H$ mixture separated from $ATP^{32}$ by thin layer chromatography on cellulose. The cpm in $ADP^{32}$ were directly proportional to the cyclic 3'5' AMP concentration in the original mixture. This assay permitted the measurement of as

little as $5 \times 10^{-13}$ moles of cyclic 3'5' AMP, thus increasing the sensitivity of previous assays by 100 fold. Considerable difficulty was encountered in purifying the enzymes and $ATP^{32}$, but meticulous attention to each substrate and enzyme, using the purification procedures described, eliminated contaminating nucleotides.

The divergent effects of alpha and beta adrenergic receptors on insulin secretion suggested that the insulin secretory response to epinephrine may be mediated via cyclic 3'5' AMP. Furthermore, the effects of epinephrine on adipose tissue lipolysis could be partially inhibited with adrenergic blocking agents (109). These findings and the recent abstract of Handler et al (89) reporting that the response of the toad bladder to epinephrine was affected by alpha and beta adrenergic blockade, indicated that the effects of epinephrine may be mediated via adrenergic receptors controlling cyclic 3'5' AMP synthesis in a variety of tissues, and may not be confined to the pancreatic islet. In fact, an alpha and beta adrenergic receptor mechanism for the control of cyclic 3'5' AMP synthesis and subsequent metabolic response may be more generalized and be responsible for the epinephrine effects in many tissues. Furthermore, the metabolic responses of tissues to other hormones may be mediated via a direct effect on the synthesis (or, possibly, the degradation) of cyclic 3'5' AMP.

Studies on the effects of epinephrine, adrenergic blocking agents, theophylline and glucagon (in one case), were undertaken

in the isolated rat pancreatic islet, the isolated rat fat cell, and the toad bladder. These representative tissues were selected because epinephrine had been shown to produce a specific metabolic response in each case. These responses were correlated with the cyclic 3'5' AMP concentration during adrenergic blockade and theophylline. In each of the three tissues studied, the patterns of metabolic response correlated accurately with the tissue concentration of cyclic 3'5' AMP.

Although the blocking agents per se had a slight stimulatory effect on lipolysis in the concentrations used, this was minimal when compared with the effects of dichloroisoproterenol alone in high doses (74). In each tissue the cyclic 3'5' AMP level was dependent on alpha and beta adrenergic receptor activity controlling adenyl cyclase. Alpha adrenergic receptor stimulation decreased cyclic 3'5' AMP and inhibited the tissue response, whereas beta adrenergic receptor stimulation increased cyclic 3'5' AMP and accelerated the tissue response. The inhibitory effect of epinephrine-stimulation of the alpha adrenergic receptor was most marked when the baseline level of cyclic 3'5' AMP was increased with theophylline. In the absence of theophylline, it was frequently difficult to demonstrate inhibition of cyclic 3'5' AMP accumulation during alpha adrenergic receptor stimulation, a factor which may well account for the failure to observe these divergent effects on lipolysis in the past.

These findings, that the effects of epinephrine on cyclic 3'5' AMP and tissue response are mediated via alpha and beta

adrenergic receptors, are consistent with the reports of Sussman et al (90), Rizack (47) and Orloff and Handler (71). These investigators reproduced the specific metabolic response pattern by the direct addition of cyclic 3'5' AMP to the tissues. In each case the effects of cyclic 3'5' AMP reproduced the effects of epinephrine when alpha adrenergic receptor activity was blocked.

The data presented demonstrates clearly that:

I) the lipolytic response of adipose tissue,

II) the pancreatic islet secretion of insulin,

III) the permeability of the toad bladder to water,

were all controlled by adrenergic mechanisms which altered the tissue concentration of cyclic 3'5' AMP and concomitantly altered the tissue metabolic response. Furthermore, it demonstrates that the tissue responses could be reproduced by cyclic 3'5' AMP, or theophylline. In all cases the intracellular concentration of cyclic 3'5' AMP correlated accurately with the metabolic response pattern of the tissues. For example, in the pancreatic islet glucagon markedly increased the cyclic 3'5' AMP concentrations and stimulated insulin release. Many hormones have been shown to activate tissue metabolic responses, and thin effects have been reproduced by cyclic 3'5' AMP -- probably most of these activate cyclic 3'5' AMP synthesis, via adenyl cyclase, although recently prostaglandin E (110) and thyroid hormones (109) have been shown to change cyclic 3'5' AMP breakdown by an action on 3'5' cyclic

nucleotide phosphodiesterase. Further study is required to determine whether the intracellular level of cyclic 3'5' AMP provides a generalized mechanism of action for all hormones.

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## Summary

The temporal characteristics of the actions of growth hormone and the relationship of hormone-activated lipolysis to changes in glucose and fatty acid metabolism have been examined in isolated fat cells. The actions of growth hormone could be divided into two categories:

(a) Immediate responses, which were insulin-like, appeared promptly after exposure to the hormone, and were not blocked by actinomycin or puromycin. During this phase glucose uptake, oxidation and lipogenesis were accelerated.

(b) Delayed responses, which occurred after 90-I20 minutes exposure to the hormone, and were blocked by actinomycin and puromycin. This phase was characterized by the appearance of accelerated lipolysis. In contrast to the immediate response pattern, the delayed phase of hormone action resulted in a decrease in glucose uptake and glucose-I-$^{I4}$C oxidation, an increase in glucose-6-$^{I4}$C and acetate-I-$^{I4}$C oxidation, and a marked inhibition of lipogenesis from all three precursors. These changes in glucose and acetate metabolism were secondary to the lipolytic action of the hormone, and specifically, to the accumulation of free fatty acids. They were inhibited by preventing the accumulation of free fatty acids and could be reproduced by the addition of palmitate to the incubation medium.

A method was described for the preparation of highly purified human placental lactogen (HPL), using a modification of a previously reported procedure. Highly purified HPL in physiological concentrations exhibited significant lipolytic activity in isolated fat cells. As with growth hormone, HPL-activated lipolysis was preceded by a 60-90 minute lag period and was inhibited by the addition of actinomycin or puromycin to the incubation medium. During the first two hours of incubation, HPL did not significantly effect glucose or acetate oxidation or incorporation into fatty acids. Following the onset of activated lipolysis HPL decreased glucose-I-$^{14}$C oxidation 30-35 per cent, increased glucose-6-$^{14}$C oxidation 30-35 per cent and increased acetate-I-$^{14}$C oxidation 50-55 per cent. The incorporation of all precursors into fatty acids was decreased to 25-45 per cent of control levels. These effects on glucose and acetate metabolism were abolished by incubation in the presence of high glucose concentration in order to accelerate FFA re-esterification and prevent FFA accumulation. Furthermore, the changes observed were similar to those seen with growth hormone and could be reproduced in the absence of HPL by the addition of palmitate to the incubation medium.

Some observations have suggested that changes in the intracellular levels of cyclic 3'5' adenosine monophosphate (cyclic 3'5' AMP) may be a generalized mechanism for the action of hormones. The role of this cyclic nucleotide was assessed

in the control of lipolysis in vivo. Administration of the methyl xanthine theophylline to fasted adrenalectomized rats caused a moderate increase in the plasma free fatty acid level with a prompt fall to pre-injection levels coinciding with a marked outpouring of pancreatic insulin. The plasma insulin response was greater than 69 times that expected from the concomitant increase in plasma glucose. Insulin degradation was not affected by theophylline. During an epinephrine infusion, beta adrenergic receptor blockade (propranolol) inhibited, and alpha adrenergic receptor blockade (phentolamine) markedly accentuated, the theophylline induced insulin secretion. Since theophylline decreases the rate of cyclic 3'5' AMP degradation by competitive inhibition of cyclic 3'5' nucleotide phosphodiesterase, these results indicated that the cyclic nucleotide was involved in the activation of lipolysis. They further suggested that the alpha and beta adrenergic receptors in the beta islet cells mediated divergent effects on cyclic 3'5' AMP synthesis - alpha receptors inhibiting, and beta receptors increasing, the generation of the cyclic nucleotide.

To investigate these findings further it was necessary to develop a new assay which would be suitable for the measurement of cyclic 3'5' AMP in micro quantities of tissue, e.g. isolated pancreatic islets and isolated fat cells. A sensitive assay was developed which permitted measurement of the cyclic nucleotide in as little as I mg of tissue. Cyclic 3'5' AMP in tissue

extracts was first separated from other nucleotides by thin layer chromatography. The isolated cyclic 3'5' AMP was then converted to 5'AMP with 3'5' cyclic nucleotide phosphodiesterase. In the presence of $\gamma$-labelled $ATP^{32}$ and myokinase, the AMP was converted to $ADP^{32}$ which was then separated by thin layer chromatography. Using this method, the cyclic 3'5' AMP concentration was measured in rat liver ($4.9 \pm 1.3 \times 10^{-9}$ moles/g), skeletal muscle $1.05 \pm 0.2 \times 10^{-9}$ moles/g) and adipose tissue ($5.05 \pm 0.6 \times 10^{-10}$ moles/g). In isolated rat fat cells the cyclic 3'5' AMP concentration increased from $2.3 \pm 0.3 \times 10^{-11}$ moles/$10^6$ cells to $9.5 \pm 0.5 \times 10^{-11}$ moles /$10^6$ cells in the presence of epinephrine. These concentrations were similar to those expected from the data of other investigators using earlier assays.

This method was used to examine the effects of epinephrine, in the presence and absence of alpha and beta adrenergic blocking agents, on the cyclic 3'5' AMP concentration, and this was correlated with the metabolic response pattern in three tissues - isolated pancreatic islets, isolated fat cells and toad bladders. In all three tissues stimulation of the alpha adrenergic receptor decreased cyclic 3'5' AMP synthesis and stimulation of the beta adrenergic receptor increased the cyclic 3'5' AMP level. Furthermore, the pattern of metabolic response in these tissues correlated with the cyclic 3'5' AMP concentration and could be reproduced by the addition of the cyclic nucleotide in vitro. These data indicated that the effects of epinephrine

on cyclic 3'5' AMP accumulation and specific tissue metabolic responses were mediated via alpha and beta adrenergic receptor sites. Whether alteration in tissue cyclic 3'5' AMP levels is the mechanism for generalized hormone action, remains a subject for future study.

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## Addendum

In August 1967 Chase and Aurbach* published a method for the measurement of cyclic 3'5' AMP in urine. The method is summarized as follows:

1. Tracer cyclic 3'5' AMP-$^{3}H$ was added to I ml of urine. The cyclic 3'5' AMP was isolated by barium hydroxide-zinc sulphate precipitation of the urine, followed by column fractionation of the supernatant solution.
2. Cyclic 3'5' AMP was converted to 5' AMP with cyclic 3'5' nucleotide phosphodiesterase and then to ATP with myokinase-pyruvate kinase.
3. ATP was measured by the $Pi^{32}$ - ATP exchange reaction, catalyzed by the coupled enzyme system triosephosphate dehydrogenase-phosphoglycerate kinase. (This reaction is to be described in detail in a later publication.)
4. Inorganic radiophosphate was removed with a 'precipitating mixture', consisting of perchloric acid, ammonium molybdate, triethylamine, ATP and inorganic phosphate.
5. After centrifugation, the supernatant solution contained $ATP^{32}$ in proportion to the amount of cyclic 3'5' AMP in the initial reaction mixture.

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*Chase, L.R. and Aurbach, G.D. - Proc. Nat. Acad. Sci. 58: 518, 1967

The range of sensitivity in this assay system was 6 to 250 uumoles of cyclic 3'5' AMP in the reaction mixture.

Although this technique appears to be satisfactory for urine, it has not been applied to measurement of the cyclic nucleotide in tissues. Furthermore, the sensitivity achieved by Chase and Aurbach (6 uumoles) was only one tenth that obtained with the new method described in this thesis (0.5 uumoles).

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