STUDIES ON THE EPINEPHRINE-SENSITIVE LIPASE
OF ADIPOSE TISSUE

by

MAS YAMAMOTO

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

In the Department
of
Pharmacology

We accept this thesis as conforming to the
required standard

THE UNIVERSITY OF BRITISH COLUMBIA

August 1964
In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the Head of my Department or by his representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Pharmacology

The University of British Columbia,
Vancouver 8, Canada

Date 28 August 1964
ABSTRACT

The study of the role of adipose tissue in the maintenance of the caloric homeostasis of organisms is currently the object of widespread research. In particular, the enzymes of lipid metabolism in adipose tissue are being extensively investigated in both intact fat pads and in broken-cell preparations. Special attention is being paid to factors which control the activity of these enzymes.

We have examined some properties of a lipase in epididymal fat pads of rats. The enzyme has been assayed by measuring the free fatty acids liberated when triglycerides are incubated with crude adipose tissue extracts. Quantitative measurements of free fatty acids were performed by (a) titrating the liberated acid with dilute alkali solution, and (b) reacting the free fatty acids with Cu$^{++}$ to form the chloroform-soluble copper soap of long chain fatty acids, then assaying the copper with diethyl-dithiocarbamate spectrophotometrically.

It is well known that lipase activity in adipose tissue decreases during incubation in a Krebs-Ringer bicarbonate medium at $37^\circ$ C. The deactivated enzyme can be activated by briefly exposing the intact tissue to epinephrine. The study of this epinephrine-sensitive lipase in adipose tissue has been the main object of this thesis.

When epinephrine was added to media containing intact epididymal fat pads, the dramatic mobilization of free fatty acids from the pads into the media was observed. When epinephrine was added directly to unfractionated homogenates, little, if any, response was elicited, indicating perhaps that some activating factor was destroyed or diluted.
out during homogenization.

When ATP, cyclic 3',5'-AMP and Mg$^{++}$ were added to unfractionated homogenates of adipose tissue, some lipase activation was observed. Similarly, when these nucleotides and Mg$^{++}$ were added to the supernatant fluid obtained from centrifuged homogenates, some activation of the lipase was observed, although the results obtained were not consistent. Other nucleotide 3',5'-cyclic phosphates generally inhibited lipase activity in the supernatant fluid.

Our data indicates that epinephrine activates adipose tissue lipase only when added to the intact fat pad before homogenization. Little or no activation occurred when the amine was added to homogenates. Cyclic 3',5'-AMP had some ability to reactivate the lipase, both in unfractionated homogenates and in the supernatant fluid prepared by centrifugation. The effects, however, were not marked. It is concluded that if epinephrine-activation of adipose tissue is mediated through cyclic 3',5'-AMP, precise conditions for showing this have not yet been achieved.

Additional experiments were performed on the epinephrine-sensitive lipase. Intact adipose tissue obtained from reserpinized rats was exposed to epinephrine after a 3-hour incubation period. The results indicated that epinephrine does not activate the lipolytic system in adipose tissue of reserpinized rats. Finally, some of the factors regulating the degree of inactivation of the epinephrine-sensitive lipase during incubation were investigated. Fat pads removed from rats which had been either anaesthetized or not anaesthetized prior to sacrifice were incubated for 3 hours. Data collected from a number of experiments indicated that there were virtually no differences in the extent of lipase inactivation between the two groups of rats.
ACKNOWLEDGEMENT

I am indebted to Dr. G. I. Drummond of the Department of Pharmacology, University of British Columbia, not only for providing the necessary equipment and facilities required for undertaking this project, but also for his valuable guidance, helpful criticism and encouragement throughout the entire course of this work. In addition, I wish to gratefully acknowledge the Medical Research Council grants which he kindly made available to me.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>B. EXPERIMENTAL PROCEDURE</td>
<td>18</td>
</tr>
<tr>
<td>Methods - Lipase Assay I</td>
<td>18</td>
</tr>
<tr>
<td>Lipase Assay II</td>
<td>20</td>
</tr>
<tr>
<td>Lipase Assay III</td>
<td>21</td>
</tr>
<tr>
<td>Materials</td>
<td>22</td>
</tr>
<tr>
<td>C. RESULTS</td>
<td>25</td>
</tr>
<tr>
<td>I. Preliminary Studies</td>
<td>25</td>
</tr>
<tr>
<td>1. Accuracy of the Titrimetric Method</td>
<td>25</td>
</tr>
<tr>
<td>2. Determination of FFA by the Copper Soap Method</td>
<td>25</td>
</tr>
<tr>
<td>3. Effect of Mg(^{++}) on the recovery of FFA by the Copper Soap Method</td>
<td>27</td>
</tr>
<tr>
<td>4. Stability of the Diethyldithiocarbamate-Copper Complex</td>
<td>27</td>
</tr>
<tr>
<td>5. Time Dependence of the Epinephrine-sensitive Lipase</td>
<td>31</td>
</tr>
<tr>
<td>6. Preparation and Use of Extracted Natural Lipids as Substrate</td>
<td>31</td>
</tr>
<tr>
<td>7. Preparation and Use of Heated Crude Homogenate as Substrate</td>
<td>34</td>
</tr>
<tr>
<td>II. Experimental Results</td>
<td>36</td>
</tr>
<tr>
<td>1. Response of Epididymal Fat Pads to Epinephrine</td>
<td>36</td>
</tr>
<tr>
<td>2. Response of Crude Homogenates to Epinephrine</td>
<td>38</td>
</tr>
<tr>
<td>3. Effect of Epinephrine on Lipolytic Activity when Added Before, During and After Homogenization</td>
<td>38</td>
</tr>
<tr>
<td>4. Search for Effects of ATP, Cyclic 3',5'-AMP, Mg(^{++}) on Crude Homogenate</td>
<td>40</td>
</tr>
<tr>
<td>(a) ATP Effects</td>
<td>42</td>
</tr>
<tr>
<td>(b) Cyclic 3',5'-AMP Effects</td>
<td>44</td>
</tr>
<tr>
<td>(c) Mg(^{++}) Effects</td>
<td>47</td>
</tr>
<tr>
<td>5. Effect of ATP, Cyclic 3',5'-AMP, Mg(^{++}) on Lipolytic Activity in Supernatant Fractions of Adipose Tissue Homogenates</td>
<td>49</td>
</tr>
<tr>
<td>6. Effect of Other Cyclic Nucleotides on Lipolytic Activity in Supernatant Fractions</td>
<td>52</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS (Cont'd)

III. Additional Experiments on Adipose Tissue Lipase

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Effect of Epinephrine on Adipose Tissues Obtained from Reserpinized Rats</td>
<td>54</td>
</tr>
<tr>
<td>2. Effect of Pentobarbital-induced Anaesthesia and Length of Incubation on Inactivation Rate of Adipose Tissue Lipase</td>
<td>56</td>
</tr>
<tr>
<td>3. Effect of Insulin on Inactivation Rate of Adipose Tissue Lipase</td>
<td>58</td>
</tr>
</tbody>
</table>

D. DISCUSSION

E. BIBLIOGRAPHY

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>67</td>
</tr>
<tr>
<td>No.</td>
<td>Title</td>
</tr>
<tr>
<td>-----</td>
<td>-----------------------------------------------------------------------</td>
</tr>
<tr>
<td>1.</td>
<td>Accuracy of the Titrimetric Method</td>
</tr>
<tr>
<td>2.</td>
<td>Effect of Mg$^{++}$ on the Extraction of Palmitic Acid by the Copper Soap Method</td>
</tr>
<tr>
<td>3.</td>
<td>Stability of the Diethyldithiocarbamate-Copper Complex</td>
</tr>
<tr>
<td>4.</td>
<td>Epinephrine Effects on Intact Epididymal Fat Pads</td>
</tr>
<tr>
<td>5.</td>
<td>Summary of ATP, Cyclic 3',5'-AMP, and Mg$^{++}$ on Lipase Activity in the Supernatant Fraction of Adipose Tissue Homogenates</td>
</tr>
<tr>
<td>6.</td>
<td>Effect of other Cyclic Nucleotides on Lipase Activity in Supernatant Fraction of Adipose Tissue Homogenates.</td>
</tr>
<tr>
<td>7.</td>
<td>Effect of Pentobarbital-induced Anaesthesia and Length of Incubation on the Inactivation Rate of the Lipolytic System</td>
</tr>
<tr>
<td>No.</td>
<td>Title</td>
</tr>
<tr>
<td>-----</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td>1</td>
<td>Pathway of Triglyceride Synthesis in Liver</td>
</tr>
<tr>
<td>2</td>
<td>Outline of Metabolic Pathways in Adipose Tissue</td>
</tr>
<tr>
<td>3</td>
<td>Activation of Phosphorylase in Skeletal Muscle</td>
</tr>
<tr>
<td>3a</td>
<td>Palmitic Acid Standard Curve</td>
</tr>
<tr>
<td>4</td>
<td>Time Dependence of the Epinephrine-sensitive Lipase</td>
</tr>
<tr>
<td>5</td>
<td>Substrate Concentration Curve (De-activated Homogenate)</td>
</tr>
<tr>
<td>6</td>
<td>Epinephrine Effect on Crude Homogenate of Adipose Tissue</td>
</tr>
<tr>
<td>7</td>
<td>Epinephrine Effects on Lipase Activity When Added Before, During, and After Homogenization</td>
</tr>
<tr>
<td>8</td>
<td>Effect of ATP, Cyclic $3',5'$-AMP and Mg$^{++}$ at Various Concentrations on Unfractionated Adipose Tissue Homogenate</td>
</tr>
<tr>
<td>9</td>
<td>ATP Concentration Curve with Respect to Lipase Activity (Unfractionated Homogenate)</td>
</tr>
<tr>
<td>10</td>
<td>Cyclic $3',5'$-AMP Concentration Curve with Respect to Lipase Activity (Unfractionated Homogenate)</td>
</tr>
<tr>
<td>11</td>
<td>Mg$^{++}$ Concentration Curve with Respect to Lipase Activity (Unfractionated Homogenate)</td>
</tr>
<tr>
<td>12</td>
<td>Effect of ATP, Cyclic $3',5'$-AMP, and Mg$^{++}$ on Lipase Activity in the Supernatant Fraction</td>
</tr>
<tr>
<td>13</td>
<td>Effect of Epinephrine on Intact Adipose Tissue Obtained from Reserpinized Rats</td>
</tr>
<tr>
<td>14</td>
<td>Effect of Insulin on the Degree of Lipase Inactivation during Incubation</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

FFA..... Free Fatty Acids
ATP..... Adenosine Triphosphate
CoA...... Coenzyme A
GMP...... Guanosine Monophosphate
CMP...... Cytosine Monophosphate
AMP...... Adenosine Monophosphate
UMP...... Uridine Monophosphate
TMP...... Thymidine Monophosphate
NADH..... Nicotinamide Adenine Dinucleotide (Reduced form)
NADPH.... Nicotinamide Adenine Dinucleotide Phosphate (Reduced form)
Tris...... Tris(hydroxymethyl)aminomethane
INTRODUCTION

Until relatively recent times, adipose tissue was assumed to be a metabolically inert fat depot. Its main physiological functions were considered simply to provide thermal insulation, mechanical padding, and to act as an energy storehouse which released its fat reserves rather sluggishly.

Perhaps the first hint that adipose tissue does, in fact, take a very active part in the dynamic processes of the body arose from the work of Schoenheimer and Rittenberg in 1937 (1-3). But in spite of their pioneering work indicating that a rapid turnover and continuous synthesis of fat did occur in the animal organism, most investigators were relatively slow to accept this new concept of adipose tissue metabolism. Few textbooks on physiology and biochemistry even now mention these depots as sites of active metabolism.

During the past two decades, however, and especially so in the past few years, investigators have become keenly aware of the true role of adipose tissue in the overall physiology of the intact organism. This awakening interest in various aspects of adipose tissue metabolism, including the relative importance of fats as a source of energy, is reflected in the increasing number of fine reviews based on these topics (4-11).

The mobilization of the caloric reserve of adipose tissue occurs in the form of free fatty acids (FFA), also frequently referred to as non-esterified fatty acids (NEFA) and unesterified fatty acids (UFA). These fatty acids are known to be readily transportable and readily metabolizable substrates. Gordon and Cherkes (12), Gordon (13), and Dole (14), correlated the concentrations of FFA in serum with changes in the nutritional state of organisms, and
concluded that fats were released from adipose tissue and transported in blood as FFA-Albumin complexes. These acids, although representing a relatively small fraction of circulating plasma lipids, have a high turnover rate \((15)\). The \textit{in vitro} release of FFA from adipose tissue was soon demonstrated in a number of laboratories \((16,17,18)\). It became evident that the release of lipids as FFA was the only significant means by which fats can be liberated from adipose tissue, and that the concentration of plasma FFA closely reflected the extent of body fat utilization.

Besides releasing FFA to the circulation, adipose tissue also plays a major role in the synthesis of triglycerides. Even after the classic work of Schoenheimer and Rittenberg \((1-5)\), it was generally believed that the liver, and not adipose tissue, was the main site of lipogenesis. This notion was largely dispelled, however, when Masoro \textit{et al} \((19)\) demonstrated that heptectomized animals could readily synthesize triglycerides from glucose. It is now believed that lipogenesis in the liver probably represents only a fraction of body fat synthesis. Nevertheless, the mechanism of triglyceride synthesis in adipose tissue is similar to the pathways suggested by Weiss and Kennedy \((20)\) and also by Tietz and Shapiro \((21)\) for triglyceride synthesis in liver. The synthetic pathway in the liver is outlined in Fig. 1. One important point must be emphasized concerning triglyceride synthesis in adipose tissue. Whereas liver possesses glycerokinase activity and is therefore able to phosphorylate glycerol to \(\alpha\)-glycerophosphate, no such activity has been demonstrated in adipose tissue \((22)\). In other words, liver can readily utilize free glycerol as its source of \(\alpha\)-glycerophosphate, but adipose tissue must depend on some other source for its supply of \(\alpha\)-glycerophosphate, an essential precursor for triglyceride synthesis. For this reason, lipolytic
Fig. 1. Synthetic pathway for triglycerides in liver (20,21). No glycerokinase activity has been demonstrated in adipose tissue. (Θ indicates phosphate moiety).
activity of adipose tissue is frequently measured on the basis of free glycerol produced. Since re-esterification of FFA proceeds simultaneously with hydrolysis of triglycerides in adipose tissue, the amount of glycerol produced is therefore more indicative of the actual degree of lipolytic activity.

The source of \(\alpha\)-glycerophosphate in adipose tissue appears to be dependent on the breakdown of glucose to dihydroxyacetone phosphate and \(\alpha\)-glycerophosphate. Dihydroxyacetone phosphate can be readily reduced to \(\alpha\)-glycerophosphate by NADH. This strongly suggests that triglyceride synthesis in adipose tissue may be dependent on the availability of glucose and its breakdown in the cells, and furthermore, that any factor which affects this glycolytic scheme will similarly affect lipogenesis.

The assimilation of triglyceride and its hydrolysis and subsequent breakdown to FFA and glycerol in adipose tissue are continuous dynamic processes, and the question naturally arises as to what factor or factors control these two opposing reactions. But before the metabolic regulation of adipose tissue is discussed, a brief histological outline of adipose tissue and some emphasis on the importance of lipids as a source of energy is indicated.

The epididymal fat pads of rats have been used most extensively by investigators in the study of adipose tissue metabolism. The mesenteric and mesometrial adipose tissues of female rats have also been frequently used. In histological sections, the cells of white adipose tissue are large, nearly spherical and usually contain a single globule of lipid enclosed by a very thin film of cytoplasm (23). Each cell contains a nucleus which appears to be pressed against the inner aspect of the cell membrane (24). It is most
interesting that when the ratio of capillary surface to volume of active protoplasm is calculated, the capillary bed, relative to the amount of cytoplasm, is actually richer than it is in muscle (23,25). This observation alone demands that attention be focussed on adipose tissue concerning its important physiological function in the organism. Similarly, a rich network of nervous tissue is present in adipose tissue (4). Triglycerides comprise more than 99% of the total lipids in adipose tissue, and only traces of cholesterol and phospholipids are found (26). Gas-liquid chromatographic studies have indicated that some 22 different fatty acids occur in adipose tissue, the major acids being palmitic (20%), oleic and isomers (46%) and linoleic acid (11%).

As mentioned earlier, the importance of lipids as an energy source, not only during starvation, but also during the post-absorptive state, was suggested by Fredrickson and Gordon (15,27). It has been estimated that approximately 50% of total respiration is derived from FFA oxidation under basal conditions (15,28), which particularly emphasizes the important role of lipids in the oxidative processes of the intact organism.

Individual organs, however, vary in their ability to oxidize fatty acids for energy. It has been estimated that cardiac muscle of man, under basal conditions, oxidizes approximately 30 to 40 micromoles of fatty acids per minute (15,29). In fact, cardiac muscle appears to utilize lipids as its predominant source of energy. Skeletal muscle appears to derive its energy from the oxidation of glucose during short, intensive exercise characterized by hypoxia. But during moderate, sustained work, if the oxygen supply is adequate, lipids may be its major substrate (30,31). For example, the non-stop migration of the ruby-throated hummingbird across the
Gulf of Mexico causes a decrease in its fat content from 43% of its weight to 8% (32). In this particular instance, lipids are obviously burned directly and most efficiently by flight muscles for energy. Evidence has been presented which indicates that the pectoralis muscle of pigeons can also oxidize lipids directly (33). The comparative aspects of muscle metabolism, with special emphasis on the importance of lipid metabolism in insects, birds and fishes has been reviewed by Drummond and Black (6).

The brain depends primarily on blood glucose as its source of energy (34). No arteriovenous difference in FFA concentration across the brain under basal conditions was found by Gordon and Cherkes (12). However, it is known that the brain does possess the enzymatic machinery necessary for oxidizing lipids (35).

The liver accounts for almost one-half of the fatty acids oxidized in man under basal conditions (15). Fritz (7), however, suggests that this organ should not be considered essential for the metabolism of lipids since fatty acid oxidation and other aspects of lipid metabolism can occur at various extrahepatic sites in the absence of the liver.

Isolated adipose tissue has a relatively low rate of conversion of added fatty acids to CO$_2$, the rate being elevated in starved animals (36). No information is available regarding the utilization of lipids for energy by smooth muscle.

Using washed mitochondria from various organs, Bode and Klingenberg (56) recently demonstrated that liver, kidney, heart and diaphragm mitochondria could oxidize medium to long chain FFA at appreciable rates, whereas skeletal muscle mitochondria showed the lowest activity. They also observed that carnitine esters of fatty acids increased the rate of oxidation by
mitochondria of all organs.

The foregoing outline on the active oxidation of lipids by certain organs re-emphasizes the important role of lipids in providing a readily utilizable source of energy. It is evident that no knowledgeable investigator can any longer hold to the belief that adipose tissue is merely an inert accumulation of fat. The salient features of adipose tissue metabolism already discussed are summarized schematically in Fig. 2.

Physiological control of Adipose Tissue Metabolism.

The foregoing has pointed to the prominent role played by adipose tissue in overall energy metabolism. In recent years, many investigators have sought to understand how these metabolic reactions are controlled or altered under differing physiological conditions. That the metabolic activity of adipose tissue is markedly influenced by a number of hormones is now a firmly established fact. The effect of these various hormones are readily observed in vivo, and in vitro on intact fat pads, but the interpretation of these effects has perplexed, and continues to perplex, investigators everywhere.

Insulin.

Of all the hormones which are known to affect adipose tissue metabolism, insulin has probably been the most widely studied. Insulin has been shown by a number of workers to increase glucose uptake by epididymal fat pads in vitro (37,38,39,41,42). The oxidation of glucose to O\textsubscript{2} by isolated epididymal fat pads or by isolated fat cells is also enhanced by insulin (24,40,43). These effects are detected with as little as 10 microunits of insulin per ml of incubation medium (24,41); hence this sensitive in vitro
Fig. 2. Outline of the metabolic processes in adipose tissue cell, indicating the relationship between carbohydrate and lipid metabolism. In normal cells, fat droplet occupies almost the entire space within cell. Furthermore, there is evidence that more than one pool of FFA exists in adipose tissue cells (34,78).
responsiveness by rat epididymal fat pads has been used as the basis for the assay of insulin or insulin-like activity in biological fluids (44,45). Electron micrographic evidence indicating that insulin may enhance glucose uptake in adipose tissue by a pinocytotic process has been presented by Barrnett and Ball (58). Triglyceride synthesis is enhanced by insulin (46,47,49,50). Vaughan (49) incubated adipose tissue from fasted rats with uniformly labelled glucose, and showed that insulin in vitro increased the specific activity of adipose tissue triglycerides by approximately 400%. Raben and Hollenberg (52,53) have suggested that the action of insulin is directly on the esterification process. Evidence has also been presented indicating that insulin may increase the availability of NADPH for synthetic processes by stimulating the pentose phosphate pathway (43,54) or that the hormone may stimulate the hexokinase reaction (57). On the other hand, Jungas and Ball (51) recently suggested that insulin, in some unknown manner, inhibits the hydrolysis of triglycerides. They indicated rather strongly that insulin may have an inhibitory action, either directly or indirectly, on tissue lipase. The more recent studies by Tarrant et al (65) with animals made diabetic with anti-insulin serum indicate that insulin may, indeed, inhibit the lipolytic system. They found that lipolysis in adipose tissue of diabetic rats, as indicated by the amount of glycerol released, was higher than control tissue, and the addition of insulin to the medium reduced this lipolytic activity toward the control level. They speculated that the increased lipolytic activity observed in insulin-deficiency is due to the absence of the normal restraining action of insulin on other adipokinetic hormones, thus adding support to the suggestion made earlier by Jungas and Ball (51). Further evidence against the widely held re-esterification
hypothesis as an explanation of insulin's action on FFA release from adipose tissue has recently been presented. In vivo studies by Zierler and Rabinowitz (86) indicated that when insulin is infused at concentrations which do not have any effect on glucose uptake by adipose tissue, its inhibitory action on FFA release is still evident. They concluded that the action of insulin on FFA release was independent of glucose translocation.

The mechanism of insulin action on adipose tissue therefore remains unsettled. The difficulty in interpreting the results obtained may be due to a number of factors, and at least two are apparent. First, not all experiments have been performed under physiological conditions. Secondly, the measurement of the release of FFA from adipose tissue does not necessarily reflect actual lipolytic activity, since much of the FFA released may be immediately re-esterified. As indicated earlier, a true indication of lipolytic activity in adipose tissue is obtained by measuring glycerol released, as this product of triglyceride hydrolysis is not re-utilized in the esterification process.

Prolactin.

The in vitro effects of prolactin on adipose tissue is similar to those observed with insulin, although much higher concentrations (0.5 to 1.0 mg/ml) are necessary (59). It should be noted that although insulin corrects the defect in fatty acid synthesis from glucose in adipose tissue from diabetic rats, prolactin shows no such action (40).

Glucagon.

Glucagon has been shown to increase the rate of both FFA and glycerol release (49,60,61). It also stimulates glucose uptake (61,62) and increases
phosphorylase activity (60,61) in adipose tissue.

Serotonin.

Little information is available regarding the action of serotonin on adipose tissue. Vaughan (60) demonstrated that serotonin could increase phosphorylase activity in adipose tissue. The hormone, at a concentration of 0.4 micromole/ml of incubation medium, increased glucose uptake but did not increase FFA release by adipose tissue (49).

AOTH

Adrenocorticotropic hormone (AOTH) has been demonstrated by a number of investigators to cause the release of FFA by adipose tissue in vitro (60,63,64). Lipase activity is stimulated (65), as well as the uptake of glucose from the medium (41,49). Phosphorylase activity is also apparently increased by AOTH (60). The similarity in the effects of AOTH and catecholamines on adipose tissue metabolism in vitro is rather remarkable, considering the dissimilarity in the structures of these hormones. Since adipose tissue which has been depleted of catecholamines no longer responds to AOTH, it has been suggested that AOTH acts by causing the release of catecholamines, chiefly norepinephrine, in adipose tissue. One point of difference in the action of AOTH and catecholamines has been observed on adipose tissue (66); whereas epinephrine action is independent of calcium ion in the medium, this divalent cation is essential for the enhanced lipolytic effects observed with AOTH.

Other Pituitary Hormones.

Other pituitary hormones have been shown to have effects on adipose tissue metabolism, and these will be mentioned only briefly in passing.
The actions of these polypeptide hormones have been reviewed by Rudman (11), Vaughan (9), Winegrad (10) and Wertheimer (5). Growth hormone possesses a lipolytic action similar to that of ACTH and epinephrine, although higher concentrations are required (64). Similarly Thyrotropin (TSH) has been demonstrated by White and Engel (64) to stimulate the release of FFA from adipose tissue in vitro. However, both Vaughan (9) and Winegrad (10) stress the point that no conclusions about the part played by these pituitary hormones on adipose tissue metabolism can be drawn due to the difficulty of ascertaining the purity of these hormone preparations.

Adrenergic Amines.

The actions of adrenergic amines on adipose tissue metabolism have attracted widespread attention, and at the moment are being actively studied in a vast number of laboratories. Since much of the experimental work on this thesis is directly related to some of these effects observed, particularly the dramatic stimulation of lipolytic activity in adipose tissue, the actions of these catecholamines on this tissue will be discussed in some detail.

The concept that the sympathetic nervous system plays an important role in the regulation of FFA release from adipose tissue has been supported by numerous in vivo experiments. Pharmacological blockade of norepinephrine action, using ergotamine, dibenzyline, or dibenamine has been shown to decrease plasma FFA concentration (67,68), indicating decreased FFA mobilization from adipose tissue. Unilateral denervation of symmetric fat bodies results in a diminished rate of lipid depletion from the denervated side (4). Conversely, physiological stresses of various
types cause an increase in FFA levels in plasma (69,70). For example, fasting (12,14) and fear (70) elevates plasma FFA levels, all of which indicates that the release of FFA from adipose tissue is primarily due to an increased sympathetic discharge. It is interesting to note that norepinephrine, in vivo, has greater lipolytic activity than epinephrine (67,71), although the two compounds possess similar levels of activities in vitro (64).

The lipolytic action of epinephrine on intact fat pads in vitro has been adequately demonstrated by numerous investigators (16,18,46,47,71,72). This effect has been observed with very low concentrations of epinephrine (1 x 10^{-6} M) in the incubation medium. The increased release of FFA is accompanied by an increased release of glycerol into the medium (47), which would be in accord with the increased lipolysis of tissue glycerides. Dibenamine and dibenzyline both inhibit the release of FFA in vitro (75), as they have been shown to do in vivo (67,68).

In general, the observed effects of epinephrine and of insulin on adipose tissue metabolism are directly opposite. Whereas insulin appears to promote triglyceride synthesis, epinephrine enhances the breakdown of triglycerides. However, these two hormones do share at least one common feature; both increase glucose uptake by epididymal fat pads in vitro (46,49,74), although in this respect it should be stated that the effect of epinephrine is quantitatively much less than that produced by insulin (49). However, Vaughan (49) was able to demonstrate that at lower concentrations of epinephrine (0.1 microgram/ml), no measurable changes in glucose uptake was observed, while the stimulatory effect of epinephrine on FFA release was still largely evident. Similar effects were observed
with ACTH and glucagon at concentrations of 0.04 units/ml and 5 microgram/ml respectively.

Ball and Jungas (75) recently studied the effect of anaerobic conditions and diet on the response of adipose tissue to both insulin and epinephrine. They observed that epinephrine had no effect on glycerol and FFA production by adipose tissue from normally fed rats under anaerobic conditions. In the presence of oxygen, however, lipolytic activity was clearly indicated. Tissue from fasted-refed rats (whose glycogen content in adipose tissue is markedly elevated) responded to epinephrine under anaerobic conditions with a three-fold increase in glycerol production, but no increase in tissue FFA level was observed. Under aerobic conditions, glycerol production was extremely elevated, whereas tissue FFA level remained quite low, indicating that re-esterification of the hydrolysed FFA had occurred at a very rapid rate. From observations such as these, Ball and Jungas suggested that epinephrine might increase the rate of lipolysis by increasing the activity of adipose tissue lipase, the enzyme responsible for hydrolysis of triglycerides. Their data also indicated that some form of high energy phosphate might be required for activation of adipose tissue lipase. They furthermore suggested that activation and inactivation of lipase may not be unlike that seen for skeletal muscle phosphorylase. It will be recalled that epinephrine activates phosphorylase by mediating the formation of adenosine 3',5'-cyclic phosphate (cyclic 3',5'-AMP) (76), as shown schematically in Fig. 3.

Later, Rizack (77) studied lipase activity in adipose tissue homogenates and this activity did indeed appear to be epinephrine sensitive. Rizack observed that when fat pads were incubated in Krebs-Ringer medium and then
Fig. 3. Activation of phosphorylase in skeletal muscle by epinephrine, mediated by cyclic 3',5'-AMP.
homogenized, they contained much lower lipase activity than homogenates prepared from paired pads which had not been incubated. This loss in lipase activity could be regained by the addition of epinephrine to the incubation medium prior to homogenization and centrifugation. Activity could be regained by the addition of epinephrine to the sucrose extract after centrifugation only if tissue sediment from an unincubated fat pad was also added. This indicated that some factor produced by some sedimentable particulate component of the cell was necessary for epinephrine action. Furthermore, he demonstrated that ATP could replace the tissue sediment in the activating system. This evidence strongly suggested that either cyclic 3',5'-AMP or a nucleotide similar to it was involved in the activation of the lipolytic system in adipose tissue. However, prior to this, several investigators had shown that cyclic 3',5'-AMP and other nucleotides had no effect on FFA release from intact fat pads (60,78). Dole (78) observed that cyclic 3',5'-AMP at concentrations in the medium between $10^{-5}$ and $10^{-3}$ M greatly inhibited the release of FFA by intact fat pads. Similarly, he found that ATP at $1 \times 10^{-3}$ M even more strongly inhibited the release of FFA.

It is not surprising that cyclic 3',5'-AMP and other nucleotides would fail to increase FFA release from intact adipose tissue. These charged molecules are known to cross cell barriers with great difficulty. It seemed logical that if cyclic 3',5'-AMP were involved, its function could be more clearly ascertained by the use of homogenates and fractionated cellular components. When the present project was formulated, Rizack (77) had already suggested (although without data) in his first study of the epinephrine sensitive lipase in homogenates that cyclic 3',5'-AMP may not
be the activating factor.

Recently, several other 3',5'-cyclic nucleotides have become available, and it was not illogical to consider that one of these or some other nucleotide may be implicated. A project was therefore formulated and undertaken in this laboratory to examine the effects of epinephrine, ATP, cyclic 3',5'-AMP and other cyclic nucleotides on lipolytic activity of intact fat pads, and on crude and fractionated homogenates prepared therefrom. It was hoped to learn something more of the factors involved in the control of FFA production by adipose tissue. The purpose of this thesis is to record the results to date.

While this work was in progress, Rizack presented further data on the epinephrine-sensitive lipase present in adipose tissue homogenates (79). This recent data has indicated that cyclic 3',5'-AMP may indeed mediate the activation of adipose tissue lipase. He demonstrated that when supernatant fluid from incubated fat pads was pre-incubated for six minutes at 37° C with concentrations of ATP and cyclic 3',5'-AMP both at $2 \times 10^{-5}$ M, Mg$^{++}$ at $5 \times 10^{-2}$ M, in 0.25 M Tris buffer, pH 7.4, the enzyme activity in the supernate returned from 15% to 66% of its original activity.

The present report describes our studies regarding these problems of control of adipose tissue lipolysis. It may be pertinent to point out that this is the first excursion into this aspect of lipid metabolism undertaken in this laboratory. In some respects then, it must be considered preliminary and does, in fact, embody a number of preliminary and exploratory experiments. It is hoped it will be a continuing study.
EXPERIMENTAL PROCEDURE

Methods.

Epididymal fat pads were obtained from normally fed Wistar rats weighing between 275 and 325 g. Unless otherwise indicated, the rats were anaesthetized with 6 mg pentobarbital per 100 g body weight at least 30 minutes prior to sacrifice. The fat pads were quickly removed and transferred to a freshly prepared Krebs-Ringer bicarbonate buffer, pH 7.4, at room temperature. The left pad was bisected longitudinally and one of the pieces blotted, weighed on a torsion balance, and used to determine the initial lipase activity. The other half was combined with the whole right pad, rinsed, blotted, weighed, and incubated for either 90 or 180 minutes at 37° C in a solution of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 5% bovine albumin. At the end of the incubation period, the pads were assayed for lipase activity under different experimental conditions.

Lipase Assays.

Three different assay methods for the epinephrine-sensitive system were used in the course of these studies.

Assay I. This assay was performed according to Rizack (77). Extracts of fat pads were prepared by homogenizing the tissues in 3 volumes of 0.25 M sucrose in a glass chamber with a Teflon pestle at 4° C for 30 to 60 seconds. The crude homogenate was centrifuged for 10 minutes at 12,000 x g at 0° C. The hard fat cake which formed at the top was lifted and pushed aside carefully with a metal spatula, and the supernatant fluid transferred to a chilled test tube using a Pasteur pipette. The pellet remaining at the bottom of the tube was discarded, along with the fat cake.
Lipolytic activity was measured by placing 0.2 ml of the extract in a glass-stoppered 20 x 150 mm tube, with 0.1 ml of a 1:4 dilution of a 50% coconut oil emulsion as substrate, 0.5 ml of 20% extracted albumin solution, pH 6.8, 0.2 ml of 0.06 M phosphate buffer at pH 6.8, and sufficient glass-distilled water to make a final volume of 2.0 ml. After 30 minutes' incubation at 37° C, the reaction was stopped by the addition of 10.0 ml of an acidic extraction mixture of heptane and isopropanol, as described by Dole (14) for the extraction of long chain fatty acids. Control mixtures were not incubated, but extracted immediately after the tissue extracts were added. For the extraction of FFA liberated, the tubes were placed in a mechanical shaker and agitated vigorously for 10 minutes. 4.0 ml of glass-distilled water and 6.0 ml of heptane were then added, and the tubes shaken again for 10 minutes. Both blank and standard reference tubes were subjected to the same extraction procedure in every assay. The blanks contained a total of 6.0 ml of glass-distilled water, 10.0 ml of the acidic organic extraction mixture, and 6.0 ml of heptane. The standard reference tubes held 6.0 ml of glass-distilled water, 10.0 ml of extraction mixture, and 6.0 ml of heptane containing 156 microequivalents of palmitic acid per litre. The system separated cleanly into two phases upon standing for 5 minutes, and two 3.0 ml aliquots of the upper organic phase were transferred from each extraction tube to 15-ml conical centrifuge tubes, using 3-ml volumetric pipettes. 1.0 ml of an ethanol solution of thymol blue was added to each 3.0 ml aliquot, and titrations were performed with NaOH solutions of approximately 0.056 N concentration, using a Gilmont microburette ("Manostat Digi-pet"), capacity 0.1 ml. Nitrogen was delivered to the bottom of the tubes during titration to expel CO₂ from
the sample and to keep the two phases well mixed. Good fluorescent lighting was essential in order to determine the yellow-green endpoint accurately.

The difference in the fatty acid content of the non-incubated (control) mixture and the incubated mixture was taken as the lipolytic activity of the tissue extract, expressed in milliunits, where 1.0 milliunit = 1.0 millimicroequivalents of FFA released per minute per ml of supernatant fluid.

Assay II. This assay was performed according to Vaughan's modification (80) of the method of Duncombe (82). Fat pads were homogenized for 30 to 60 seconds at room temperature, using 4 volumes of 0.154 KCl in a glass chamber with a Teflon pestle. The assays were performed in suitable glass-stoppered tubes in a total volume of 1.0 ml containing 30 mg bovine serum albumin, pH 7.0, 20 micromoles of sodium phosphate buffer, pH 7.0, and 0.2 ml of the crude homogenate which had been filtered through two layers of cheesecloth. The tubes were incubated at 37° C for either 10 or 15 minutes. The reaction was stopped at the desired time by the addition of 1.0 ml of a mixture containing 0.9 M triethanolamine, 0.1 N acetic acid, and 5% cupric nitrate·3H2O. The purpose of this treatment is to convert the FFA formed to the chloroform-soluble copper soaps. Chloroform, 4.0 ml was added, the tubes placed horizontally in a mechanical shaker and agitated vigorously for 30 minutes. After brief centrifugation, as much as possible of the upper aqueous phase and precipitated protein was removed by suction. Approximately 5% of the tubes did not yield a clear separation of the phases even after centrifugation. In such cases, a
glass rod was used to break up the thick, gelatinous protein precipitate in the chloroform layer, and the tubes re-centrifuged. 2.0 ml aliquots of the chloroform layer were carefully transferred with a special long-tipped 2-ml pipette to appropriate test tubes containing 0.25 ml of 0.1% sodium diethyldithiocarbamate in n-butanol. After mixing, the optical density was read at 440 millimicrons in a Beckmann DU spectrophotometer, using a light path of 1.0 cm. The colour yield was proportional to the various quantities of added palmitic acid tested; i.e., from 0 to 300 millimicroequivalents.

In this assay, no exogenous substrate was added, since the homogenate itself provided sufficient triglycerides. Lipolytic activity, expressed as microequivalents of FFA produced per gram wet weight of tissue per 10 minutes, was taken as the difference in the fatty acid content of the non-incubated mixture and the incubated mixture.

**Assay III.** This assay was both a combination and modification of the methods used in Assays I and II. The incubation of the enzyme system was performed according to Assay I and the determination of FFA so formed was carried out using the cupric nitrate method of Assay II. The reaction mixture contained 50 mg extracted bovine albumin, pH 6.8, 6 micromoles sodium phosphate buffer, pH 6.8, 0.1 ml of supernatant fluid, 0.05 ml of a 1:4 dilution of a 50% coconut oil emulsion (or 0.15 ml of inactivated adipose tissue homogenate), and sufficient glass-distilled water to make a final volume of 1.0 ml. Incubation of the mixture was performed at 37° C for 30 minutes, and the reaction stopped by the addition of 1.0 ml of the cupric nitrate solution as described for Assay II. Lipolytic activity in
the supernatant fluid was expressed in milliunits, as for Assay I.

Materials.

Bovine serum albumin (Fraction V, lot A23 B-70) was purchased from Sigma Chemical Company and purified according to the method of Goodman (81) to remove FFA. 50 g of the crude albumin was dissolved in 200 ml of glass-distilled water by simply placing the albumin powder over the water and allowing it to dissolve overnight. The resultant dark amber solution was then lyophilized, powdered with mortar and pestle, covered with anhydrous 2,2,4-trimethylpentane containing 5% acetic acid, and placed in the coldroom overnight. As much as possible of the acetic acid-trimethylpentane extraction solvent was then aspirated and the albumin washed twice with anhydrous trimethylpentane. Agitation of the albumin suspension during the extraction process with organic solvents was kept to a minimum to reduce the extent of protein denaturation. After aspiration of the trimethylpentane, the albumin was covered again with the anhydrous 5% acetic acid-trimethylpentane mixture and stored in the coldroom overnight. The removal of the acetic acid-trimethylpentane mixture and washing with anhydrous trimethylpentane was repeated. The organic solvent was removed under vacuum, and the powder obtained was taken up in a suitable volume of glass-distilled water. To remove the last trace of acetic acid, the albumin solution was dialyzed continuously for 5 days against a total volume of 60 litres of demineralized water, followed by 20 litres of glass-distilled water. The solution was then lyophilized and the extracted albumin stored in the deep-freeze until required.

The commercially prepared albumin was found to contain 0.60 eq FFA.
25- per mole. After extraction by the method of Goodman (81) just described, the content of FFA was reduced to 0.14 eq/mole in one of the extractions and to 0.18 eq/mole in another. The extent of purification approached that of Goodman, who reduced the FFA concentration to 0.10 eq/mole. The recovery of albumin after extraction of FFA was approximately 90%.

The 20% albumin solutions used for the assays were prepared by sprinkling 10 g of finely powdered lyophilized albumin over 40 to 45 ml of glass-distilled water in a large Erlenmeyer flask, and allowing it to dissolve overnight in the coldroom. The pH of the solution, usually around 4.6, was adjusted to either pH 6.8, 7.0 or 7.4 with 5 N NaOH, and the solution made up to a final volume of 50 ml with glass-distilled water.

Palmitic acid (Eastman Organic Chemicals) was re-crystallized twice before use as a reference standard.

The 50% coconut oil emulsion was prepared by homogenizing equal parts by weight of coconut oil and water, using Brij 76® , a brand of polyoxyethylene fatty alcohol ether, as the emulsifying agent. Before use, the commercial coconut oil was extracted with either chloroform : methanol (2:1) or ether and bicarbonate solution to reduce the amount of endogenous FFA.

The FFA extraction mixture of Dole (14) described for Assay I consisted of isopropanol, heptane and 1.0 N sulfuric acid in the ratio 40:10:1 by volume respectively.

Sodium diethyldithiocarbamate was re-crystallized twice before use. Its butanol solution was stored in a refrigerator, and was kept no longer than one week due to the relative instability of the reagent.

The alkaline solution used in the titrimetric method of Assay I was
prepared daily by adding 0.05 ml of a saturated solution of NaOH to 25.0 ml of freshly boiled, glass-distilled water. The thymol blue indicator solution was prepared as follows: 0.1 g thymol blue was dissolved in 21.5 ml 0.01 N NaOH solution, then diluted to 250 ml with carbon dioxide-free water. 10.0 ml of this solution was further diluted to 100.0 ml with freshly distilled ethanol, and the pH adjusted so that 1.0 ml of the indicator required five to 10 microlitres of base for neutralization.

ATP (disodium salt) was purchased from Nutritional Biochemical Corporation. Cyclic 3',5'-AMP was also a commercial preparation. Cyclic 3',5'-GMP, cyclic 3',5'-GMP and cyclic 3',5'-UMP were available in this laboratory (84). The deoxyribonucleoside-3',5'-cyclic phosphates, namely cyclic 3',5'-deoxy-AMP, cyclic 3',5'-deoxy-GMP, cyclic 3',5'-deoxy-GMP, and cyclic 3',5'-TMP, were prepared by the method of Drummond et al (85) and were donated by Dr. M. Smith.
RESULTS

I. Preliminary Studies

1. Accuracy of the Titrimetric Method. The determination of FFA in Assay I depends upon the titration of FFA with alkali using a microtitrator (See Experimental). Since the yellow-green endpoint was rather difficult to detect when titrating microquantities of FFA, a series of replicate tubes were titrated to determine the degree of accuracy of this method under the conditions of the assay. The subjective aspect of the method was minimized by titrating the final two or three microlitres with the metre-face covered. The 0.056 N NaOH used as the base was prepared under very rigid conditions in order to exclude CO₂. Data obtained for a series of two experiments are shown in Table 1.

2. Determination of FFA by the Copper Soap Method. The determination of FFA in Assay II and Assay III depends upon the formation of the copper soap of FFA, extraction of these into chloroform, and subsequent reaction of the copper with diethyldithiocarbamate to form a coloured complex absorbing at 440 millimicrons. An experiment was performed to ensure that the colour yield was proportional to FFA present. From 0 to 500 milli-micro equivalent of palmitic acid was added to a series of reaction tubes and the extraction of the FFA performed according to Assay II. The tubes contained all components of Assay II except the homogenate. Optical density was read in a Beckmann DU Spectrophotometer at 440 millimicrons, using a light path of 1.0 cm. Triplicate determinations yielded excellent agreement. Under the conditions of the assay, the colour yield was
Table 1. Accuracy of the titrimetric method. In series A, five experimental tubes, each containing all the components (except the supernate) used in Assay I, were extracted and titrated. In series B, five reference standard tubes, each containing 936 millimicroequivalents of palmitic acid only, were extracted and titrated.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Aliquot</th>
<th>Microlitres of base req'd</th>
<th>Tube</th>
<th>Aliquot</th>
<th>Microlitres of base req'd</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>(a)</td>
<td>15.85</td>
<td>1</td>
<td>(a)</td>
<td>8.85</td>
</tr>
<tr>
<td></td>
<td>(b)</td>
<td>15.45</td>
<td></td>
<td>(b)</td>
<td>8.92</td>
</tr>
<tr>
<td>2</td>
<td>(a)</td>
<td>15.14</td>
<td>2</td>
<td>(a)</td>
<td>8.46</td>
</tr>
<tr>
<td></td>
<td>(b)</td>
<td>15.32</td>
<td></td>
<td>(b)</td>
<td>8.82</td>
</tr>
<tr>
<td>3</td>
<td>(a)</td>
<td>15.10</td>
<td>3</td>
<td>(a)</td>
<td>8.50</td>
</tr>
<tr>
<td></td>
<td>(b)</td>
<td>15.20</td>
<td></td>
<td>(b)</td>
<td>8.43</td>
</tr>
<tr>
<td>4</td>
<td>(a)</td>
<td>15.17</td>
<td>4</td>
<td>(a)</td>
<td>8.57</td>
</tr>
<tr>
<td></td>
<td>(b)</td>
<td>15.30</td>
<td></td>
<td>(b)</td>
<td>8.63</td>
</tr>
<tr>
<td>5</td>
<td>(a)</td>
<td>15.47</td>
<td>5</td>
<td>(a)</td>
<td>8.41</td>
</tr>
<tr>
<td></td>
<td>(b)</td>
<td>15.43</td>
<td></td>
<td>(b)</td>
<td>8.74</td>
</tr>
</tbody>
</table>

Mean = 15.34
Deviation = 0.51
Standard Deviation = 0.25
Standard Error of Mean = 0.08

Mean = 8.63
Deviation = 0.29
Standard Deviation = 0.20
Standard Error of Mean = 0.06
directly proportional to the concentration of palmitic acid added, as indicated in Fig. 3a.

3. Effect of Mg** on the Recovery of Palmitic Acid by the Copper Method of Assay II. The determination of FFA concentration by Assay II (and III) depends on the formation of cupric salts of long chain fatty acids and subsequent extraction with chloroform. A strong possibility existed that high concentrations of Mg+++ in the reaction mixture might interfere with the formation of cupric salts of long chain fatty acids. Hence a series of tubes containing 200 millimicroequivalents of palmitic acid and Mg+++ concentrations ranging from 0 to 5 x 10^-2 M was extracted and assayed according to Assay II. The data obtained (Table 2) indicated that Mg+++ had little, if any, effect on the extraction of palmitic acid when the copper soap method was used for the determination of long chain FFA.

4. Stability of the Diethyldithiocarbamate-copper Complex. During the course of these experiments, it was frequently noted that when the optical density of the chloroform solutions were re-read after 15 to 30 minutes, the readings gave slightly higher values. Therefore, after one of the experiments, the solutions were re-read in the same sequence every 20 minutes and the optical densities recorded as indicated in Table 3. The changes in optical density with each subsequent reading showed an increase of .004 to .012 O.D. units, usually around .008 units. However, since the rate of change was constant for all the tubes, and furthermore, since only the difference in O.D. between the zero time control and the experimental tubes was of any importance, it was concluded that the observed O.D. changes
Fig. 3a. Palmitic acid standard curve. 100, 200, and 300 millimicroequivalents of twice re-crystallized palmitic acid, dissolved in chloroform, were pipetted into glass-stoppered tubes. The chloroform was evaporated to dryness, albumin solution was added, and the tubes heated to 50°C and swirled until no trace of the palmitic acid residue was visible. Phosphate buffer and water were added as described in Assay II before extraction was performed.
<table>
<thead>
<tr>
<th>Mg++ Concentration in Reaction Mixture</th>
<th>Optical Density at 440 millimicrons (d = 1.0 cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>.570 ± .010</td>
</tr>
<tr>
<td>2.0 x 10^{-5} M</td>
<td>.529 ± .001</td>
</tr>
<tr>
<td>5.0 x 10^{-3} M</td>
<td>.574 ± .001</td>
</tr>
<tr>
<td>5.0 x 10^{-2} M</td>
<td>.553 ± .007</td>
</tr>
</tbody>
</table>

Table 2. Effect of Mg++ on the extraction of added palmitic acid, using Assay II. Each tube contained 200 millimicroequivalents of palmitic acid, 30 mg bovine serum albumin, phosphate buffer, 6.0 x 10^{-3} M in a final volume of 1.0 ml. The experiment was performed in duplicate, and excellent agreements of values were obtained.
<table>
<thead>
<tr>
<th>Tube No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
<th>17</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.D. at 10 min.</td>
<td>266</td>
<td>381</td>
<td>255</td>
<td>383</td>
<td>280</td>
<td>341</td>
<td>308</td>
<td>355</td>
<td>249</td>
<td>293</td>
<td>260</td>
<td>297</td>
<td>251</td>
<td>285</td>
<td>252</td>
<td>293</td>
<td>258</td>
</tr>
<tr>
<td>0.D. at 30 min.</td>
<td>276</td>
<td>390</td>
<td>262</td>
<td>392</td>
<td>288</td>
<td>352</td>
<td>318</td>
<td>364</td>
<td>258</td>
<td>300</td>
<td>265</td>
<td>306</td>
<td>258</td>
<td>294</td>
<td>260</td>
<td>302</td>
<td>266</td>
</tr>
<tr>
<td>Δ O.D.</td>
<td>010</td>
<td>009</td>
<td>007</td>
<td>009</td>
<td>008</td>
<td>011</td>
<td>010</td>
<td>009</td>
<td>009</td>
<td>007</td>
<td>005</td>
<td>009</td>
<td>007</td>
<td>009</td>
<td>008</td>
<td>009</td>
<td>008</td>
</tr>
<tr>
<td>0.D. at 50 min.</td>
<td>285</td>
<td>402</td>
<td>269</td>
<td>400</td>
<td>297</td>
<td>357</td>
<td>325</td>
<td>371</td>
<td>265</td>
<td>306</td>
<td>274</td>
<td>310</td>
<td>266</td>
<td>302</td>
<td>267</td>
<td>312</td>
<td>270</td>
</tr>
<tr>
<td>Δ O.D.</td>
<td>009</td>
<td>012</td>
<td>007</td>
<td>008</td>
<td>009</td>
<td>006</td>
<td>007</td>
<td>007</td>
<td>007</td>
<td>006</td>
<td>009</td>
<td>004</td>
<td>008</td>
<td>008</td>
<td>007</td>
<td>010</td>
<td>004</td>
</tr>
<tr>
<td>0.D. at 70 min.</td>
<td>292</td>
<td>412</td>
<td>276</td>
<td>408</td>
<td>303</td>
<td>365</td>
<td>233</td>
<td>380</td>
<td>272</td>
<td>313</td>
<td>280</td>
<td>320</td>
<td>272</td>
<td>308</td>
<td>273</td>
<td>318</td>
<td>277</td>
</tr>
<tr>
<td>Δ O.D.</td>
<td>007</td>
<td>010</td>
<td>007</td>
<td>008</td>
<td>006</td>
<td>008</td>
<td>008</td>
<td>009</td>
<td>007</td>
<td>007</td>
<td>006</td>
<td>010</td>
<td>006</td>
<td>006</td>
<td>006</td>
<td>006</td>
<td>007</td>
</tr>
<tr>
<td>0.D. at 90 min.</td>
<td>300</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δ O.D.</td>
<td>008</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Observed changes in optical density on subsequent readings when FFA concentration was determined by the copper technique of Assay II. Optical densities were read 10 minutes after mixing 2.0 ml chloroform extract with 0.25 ml diethyldithiocarbamate, then re-read in the same sequence at 20 minute intervals.
were of little consequence insofar as the accuracy in the assay for FFA was concerned. A most likely explanation for these observed changes in optical density is that during and after the process of transferring the coloured chloroform solutions between the tubes and cuvette, sufficient chloroform evaporates to cause a slight increase in the concentration of the diethyldithiocarbamate-copper complex.

5. Time Dependence of Epinephrine-sensitive Lipase. At the outset it was necessary to determine the effect of time of incubation on lipolytic activity in homogenates in order to ensure a valid assay. Fat pads were homogenized in 3 volumes of 0.25 M sucrose at 4°C and centrifuged at 12,000 x g for 10 minutes at 0°C. 0.2 ml of the supernate was incubated at 37°C with coconut oil emulsion as substrate, and albumin and phosphate buffer as described for Assay I. Reactions were stopped every 10 minutes by the addition of 10.0 ml of acidified organic extraction mixture. The results of two such experiments are shown in Fig. 4. In contrast to Rizack (77), who initially incubated his reaction mixture for 60 minutes, it was immediately apparent that the reaction approached the upper limits of linearity at 60 minutes' incubation only when lipolytic activity was low. At elevated levels of activity, the reaction proceeded in a linear manner for about 40 minutes only. Therefore, our reaction mixtures were incubated in Assays I and III for only 30 minutes. It was noted that Rizack, in his later studies (79), also reduced his incubation time from 60 to 30 minutes, thus adding support to our observations.

6. Lipids Extracted from Adipose Tissue as Substrate for the Lipolytic Enzyme System. Reference has already been made to the wide variety of
Fig. 4. Time course curves for the epinephrine-sensitive lipase. Two experiments were performed, using the supernatant fraction of adipose tissue homogenates obtained from two different rats. Assay I was used to determine FFA produced.
methods, tissue preparations, and substrates used by various investigators in this field. Selecting the most suitable substrate in particular has been a major problem. Most investigators use a commercial coconut oil emulsion, Ediol®. Rubenstein et al (88) used lipids extracted from fat pads as substrate. We also attempted to employ this more "physiological" material in our work.

A total of 13.37 g of epididymal adipose tissue was obtained from 4 rats weighing between 460 and 490 grams and homogenized in a Servall Omnimixer using 17 volumes of chloroform : methanol (2:1) mixture as described for the extraction of lipids by Folch et al (87). An additional 3 volumes of the chloroform : methanol mixture was used to rinse out the cup and flask. After filtration through fat-free paper into a separatory funnel, the organic solvent was shaken with 0.2 volumes of glass-distilled water. The lower organic phase was collected and the solvent removed under vacuum in a rotary evaporator. The clear, yellow, viscous fluid obtained was subjected to further treatment under a mechanical vacuum pump to remove the last traces of organic solvent in the lipid extract. The yield of oil obtained was 73%. A 20% emulsion was then prepared by homogenizing 1.0 ml of the oil with 4.0 ml of glass-distilled water, using 0.1 g of Brij 76® as the emulsifying agent. The emulsion was diluted with an equal volume of water before being used as substrate. Preliminary experiments indicated that virtually no lipolytic activity was evident with this preparation. Despite its great potential as a substrate for adipose tissue lipase, no further attempt was made to improve the quality of this substrate, in view of the limited time available.
7. **Heated Crude Homogenate of Adipose Tissue as Substrate.** Since our preliminary attempt to use lipids extracted from fat pads as substrate failed to give adequate reaction rates, an attempt was made to utilize the triglycerides available in a homogenate of fat pads without extraction with organic solvents. In this case, enzymatic activity in the homogenate was destroyed by heat.

Epididymal fat pads were homogenized in 5 volumes of 0.25 M sucrose at room temperature, then immediately filtered through two layers of cheesecloth into a test tube immersed in a boiling water bath. After the heated extract was re-homogenized in a Potter-Elvehjem homogenizer, a rather smooth, creamy preparation resulted. Preliminary experiments using this simple, natural substrate yielded highly encouraging results. Although lipolytic activities observed were about equal to that obtained with coconut oil emulsion, the major advantage over the latter artificial substrate was that the preparation, if immediately deactivated by heat, gave much lower blanks. In fact, the level of endogenous FFA found in this substrate was negligible.

An experiment was performed to determine the minimum volume of this substrate required for the lipolytic system under study, and the results are shown in Fig. 5. The substrate concentration curve indicated that 0.15 ml of the heated crude homogenate was required in order to saturate the enzyme. Although this material shows considerable promise as a substrate, it was not widely used in the present studies.
Fig. 5. Substrate concentration curve, using triglycerides present in a boiled extract as substrate. (See text for preparative procedure). Incubation was performed at 37° C for 30 minutes, using Assay III.
II. Experimental Results

1. Response of Epididymal Fat Pads to Epinephrine. The dramatic response of adipose tissue to epinephrine is most readily demonstrated when fat pads are incubated for a few minutes with this drug. In a series of experiments with adipose tissues obtained from normal fed 200 g rats, the addition of epinephrine to the incubation medium caused a marked increase in the release of FFA into the medium, presumably by stimulating the hormone-sensitive lipolytic system (Table 4). When epinephrine was injected into rats before removal of fat pads, the response was approximately 1/6 that obtained when epinephrine was added directly to the intact pad. Untreated control pads showed a high degree of variation in their ability to release or remove FFA from the medium. For instance, adipose tissues from rats 1 and 2 (Table 4) indicated levels of lipolytic activity equivalent to those from epinephrine-injected rats, whereas adipose tissues from the remaining controls suggested an uptake of FFA from the medium. The extreme variation found in these control tissues might be explained on the basis of later experiences; that is, the handling of rats just prior to sacrifice probably causes a high degree of sympathetic stimulation with the result that endogenous catecholamine levels are increased. This, in turn, will cause a marked increase in lipolytic activity, as probably was the case of adipose tissues obtained from rats 1 and 2. These results are typical of many available in the literature and serve here to emphasize the marked ability of epinephrine to mediate the release of FFA from intact adipose tissue. Clearly the nature of such action can only be completely understood by a study of events at a subcellular level.
Table 4. Effect of epinephrine on net release or uptake of FFA by terminal sections of rat epididymal fat pads. Epinephrine, 25 micrograms/kg, was injected into rats No. 7 and 8 approximately 5 minutes prior to removal of fat pads. Pads from rats No. 9 and 10 were incubated in a medium containing 2 micrograms epinephrine per ml. Incubations were performed under 95% O₂/5% CO₂ in a Dubnoff metabolic shaker for 1 hour at 37°C in Krebs-Ringer bicarbonate buffer, pH 7.4, containing 5% extracted bovine serum albumin. 2.0 ml aliquot of the medium were removed for the assay of FFA by the extraction and titrimetric methods of Dole (14) as described in Assay I. The activities are expressed as millimicroequivalents FFA/g wet weight/hr. Either the left or the right pad served as zero time control for the other side in each experiment. (-) indicates FFA uptake by tissue and (+) indicates FFA released.
2. **Epinephrine Effects on Crude Homogenates of Adipose Tissues.** As indicated in the foregoing section, the lipolytic effect of epinephrine on intact fat pads is a particularly outstanding feature of this hormone's action. This is presumably due to increased activity of adipose tissue lipase. It might be reasonable to assume that epinephrine could have a similar effect in homogenates assayed directly for lipase activity. Thus fat pads were removed and incubated as previously described to reduce the activity of the lipolytic system. Thereafter they were homogenized and assayed for lipase activity with and without epinephrine added to the homogenate. Another portion of the fat pad was homogenized without incubation to serve as the pre-incubation control. In the presence of epinephrine, only slight activation of the lipolytic system was observed (Fig. 6). At higher, and admittedly even less physiological concentrations of epinephrine, the activities increased only slightly. This relatively small increase in lipase activity observed with epinephrine on crude homogenates has also been documented elsewhere (88). It is pertinent to note that the substrate used in this experiment was the natural triglycerides present in an unfractionated homogenate. The results indicate that although epinephrine causes a marked release of FFA from intact fat pads, it does not cause activation of lipase present in an unfractionated homogenate.

3. **Effect of Epinephrine on Lipolytic Activities of Adipose Tissues When Added Before, During and After Homogenization.** The relatively small effect of epinephrine on lipase activity observed when it is added to crude homogenates contrasted so sharply with the large effects observed when epinephrine was added to a medium containing intact fat pads that further probing into the matter was clearly necessary. Hence fat pads which had been
Fig. 6. Effect of epinephrine on lipolytic activity when added to crude homogenate of adipose tissue. Epinephrine was added (5 to 30 microgram/ml reaction mixture) and lipolytic activity determined according to Assay II. Results were averaged from duplicate assays using homogenate obtained from one rat. One determination was made for the post-incubation activity. Activity is expressed as microequivalents FFA produced/g/10 minutes.
incubated for 3 hours were assayed for lipase activity by (a) pre-treating the pads with epinephrine 10 minutes before homogenizing, (b) homogenizing the pads with epinephrine, and (c) adding epinephrine after homogenizing was complete. The averaged results of experiments with 4 rats are shown in Fig. 7.

The average lipolytic activity after the 3-hour incubation was approximately 50% of the original activity indicating the degree of loss of lipase activity. When epinephrine was added directly to the intact pad prior to homogenizing, a large increase in lipase activity was observed in the assay. Epinephrine added during homogenization caused only a slight increase in activity, and when added after homogenization, no effect on lipase was obtained. Again, in this experiment, the lipase assay employed the triglycerides present in the unfractionated homogenate. The data indicate that epinephrine can activate the lipase only when the tissue is intact. It might be reasoned that during homogenization or assay by the technique used, some necessary factor is either destroyed or diluted out, or that epinephrine itself may be destroyed.

4. Search for Effect of Cyclic 3',5'-AMP, ATP and Mg++ on Lipase Activity of Unfractionated Homogenates. The data previously recorded indicated that epinephrine, when added to crude homogenates, was unable to increase lipase activity. If cyclic 3',5'-AMP were involved in epinephrine-induced lipolysis, homogenization may have destroyed some factor or disrupted some cellular component necessary for its formation. It is less likely that epinephrine itself would have been destroyed, but if this occurred, the conversion of endogenous ATP to cyclic 3',5'-AMP by adenylyl cyclase would
Fig. 7. Effect of epinephrine on lipolytic activity in 3-hour incubated pads when added before, during and after homogenization. Bars "A" and "B" represent initial and post-3-hour incubation lipolytic activities respectively. Epinephrine (medium concentration 17 microgram/ml) was added to the medium containing pad "C" 10 minutes before homogenizing. The 0.25 M sucrose homogenizing solution used for pad "D" contained epinephrine (17 micrograms/ml). "E" represents lipolytic activity when epinephrine (17 micrograms/ml) was added to the reaction mixture. Lipolytic activity, expressed as microequivalents FFA produced/g/10 minutes, was determined according to Assay II. Data shown were averaged from experiments on 4 animals.
not occur. The addition of cyclic 3',5'-AMP and ATP directly to the tissue homogenate was clearly indicated.

A series of experiments was therefore undertaken to determine whether these nucleotides had any effect on lipase activity in crude homogenates, and if so, to determine the concentrations of each which gave maximal activation. A preliminary experiment was performed in which the concentrations of both ATP and cyclic 3',5'-AMP were increased from $3.2 \times 10^{-7}$ M to $1.0 \times 10^{-5}$ M in the reaction mixture, while Mg$^{2+}$ concentrations were varied from $1.6 \times 10^{-5}$ to $5.0 \times 10^{-2}$ M as indicated in Fig. 8. Lipolytic activity was again determined according to Assay II and is expressed as microequivalents of FFA produced per gram wet weight of tissue per 10 minutes. The results indicate that supplementation of the extract with ATP and cyclic 3',5'-AMP in the range of $1.0 \times 10^{-6}$ M and $1.0 \times 10^{-4}$ M may have some ability to increase lipase activity. At these concentrations, the lipolytic activity was increased from 2.6 to 3.2 microequivalents/g/10 minutes. The degree of activation nevertheless failed to attain the initial lipase activity of 3.9 microequivalent/g/10 minutes. At higher concentrations of nucleotides and Mg$^{2+}$, definite inhibition of lipase activity occurred.

(a) Effect of Varying the Concentration of ATP. The concentrations of cyclic 3',5'-AMP, ATP and Mg$^{2+}$ used in the preceding preliminary study indicated that cyclic 3',5'-AMP might indeed mediate a lipase-activating system at least to a small degree. Since the concentrations of the components used in the preliminary study were chosen arbitrarily, a systematic search for the optimal concentrations of each component was considered worthwhile in order to settle the matter. Thus, in a series of assays, the concentration
(Upper Line = ATP, Cyclic 3',5'-AMP concentrations)

(Lower Line = Mg++ concentration)

Fig. 8. Effect of various concentrations of ATP, cyclic 3',5'-AMP, and Mg++ on lipolytic activity, determined according to Assay II. Concentrations of nucleotides and Mg++ were varied as indicated, and represent the final concentration in the 1.0 ml reaction mixture. The initial and post-2-hour incubation activities were 3.9 and 2.6 microequivalents/g/10 minutes respectively.
of one of the three components was varied in turn, while the concentrations
of the other two components were held constant. First, ATP concentration
was varied from $6.4 \times 10^{-8}$ M to $2.0 \times 10^{-4}$ M in the reaction mixture, while
the concentration of cyclic $3',5'-AMP$ and $Mg^{++}$ were held constant at
$8.0 \times 10^{-6}$ M and $4.0 \times 10^{-4}$ M respectively. The result of an experiment
under these conditions is shown in Fig. 9, and indicates that the optimal
concentration of ATP was around $3.2 \times 10^{-7}$ M. At these concentrations of
ATP, cyclic $3',5'-AMP$ and $Mg^{++}$, lipase activity increased from 2.9 to 5.4
microequivalent/g/10 minutes, but again failed to reach the initial lipase
activity of 6.4 microequivalent/g/10 minutes. Again, it is pertinent to
emphasize that the natural triglyceride present in the homogenate was the
substrate used (Assay II).

(b) Effect of Varying the Concentration of Cyclic $3',5'-AMP$. When the
concentration of cyclic $3',5'-AMP$ was varied from $12.8 \times 10^{-9}$ M to $1.0 \times
10^{-5}$ M, while the concentrations of ATP and $Mg^{++}$ were held constant at
$3.2 \times 10^{-7}$ M and $4.0 \times 10^{-4}$ M respectively, the optimum activation occurred
at cyclic $3',5'-AMP$ concentration of $3.2 \times 10^{-7}$ M (Fig. 10). It was
interesting to note that equimolar concentrations of both ATP and cyclic
$3',5'-AMP$ were apparently required. The experiment was repeated with adipose
tissue homogenates obtained from another rat, and the resulting concentration
curve was virtually identical in form to the first, except that the level of
lipase activities in the two homogenates differed (Fig. 10). In one of the
experiments, lipase activity was increased from 3.7 to 4.4 microequivalents/
g/10 minutes, but again failed to attain the initial activity of 5.6 micro-
equivalents/g/10 minutes. In the other experiment, lipolytic activity was
Fig. 9. Effect of various concentrations of ATP on lipolytic activity. The final ATP concentrations in the 1.0 ml incubation mixture were varied from $6.4 \times 10^{-6}$ M to $2.0 \times 10^{-4}$ M. Cyclic 3',5'-AMP and Mg$^{++}$ concentrations were held constant at $8.0 \times 10^{-6}$ M and $4.0 \times 10^{-5}$ M respectively. The initial and post-90-minute incubation activities were 6.4 and 2.9 microequivalents/g/10 minutes respectively. Lipase activity was determined according to Assay II.
Fig. 10. Effect of various concentrations of cyclic 3',5'-AMP on lipase activity. The final concentrations of cyclic 3',5'-AMP were varied from $12.8 \times 10^{-9}$ M to $1.0 \times 10^{-5}$ M in the 1.0 ml incubation mixture. The concentrations of ATP and Mg++ were held constant at $3.2 \times 10^{-7}$ M and $4.0 \times 10^{-5}$ M respectively. The initial and post-90-minute incubation activities were 5.6 and 3.7 microequivalents/g/10 minutes for experiment 1 (lower curve, circles), and 5.7 and 4.3 microequivalents/g/10 minutes for experiment 2 (upper curve, triangles) respectively. Lipase activity was determined according to Assay II.
increased from 4.3 to 4.9 microequivalents/g/10 minutes when the initial lipase activity had been 5.7 microequivalents/g/10 minutes.

(c) Effect of Mg\textsuperscript{++} at Various Concentrations. The results obtained so far indicated that the optimum concentrations of ATP and cyclic 3',5'-AMP were $3.7 \times 10^{-7}$ M. Therefore, in another experiment, this particular concentration of the nucleotides was used, while the Mg\textsuperscript{++} concentration was varied from $9.6 \times 10^{-7}$ M to $7.5 \times 10^{-2}$ M (Fig. 11). A marked inhibition of lipase activity in the homogenate was evident when the Mg\textsuperscript{++} concentration exceeded $3.0 \times 10^{-5}$ M. On the other hand, little activation of the lipolytic system was observed at lower Mg\textsuperscript{++} concentrations, despite the presence of what were considered optimal concentrations of ATP and cyclic 3',5'-AMP. The experiment was repeated with a homogenate obtained from another rat, and an identical concentration curve was obtained (Fig. 11). The possibility that Mg\textsuperscript{++} at high concentrations may interfere with the copper method of Assay II was eliminated (see Preliminary Results), hence the inhibition of lipase activity in adipose tissue homogenates by high Mg\textsuperscript{++} concentrations must be considered real.

The limited time available prevented a more thorough investigation into the effects of ATP, cyclic 3',5'-AMP and Mg\textsuperscript{++} on lipolytic activity in crude homogenates. These preliminary experiments appear to indicate that ATP and cyclic 3',5'-AMP can activate the lipolytic system in homogenates to a limited extent, but we were unable to raise the levels of activity in any experiment to that of the unincubated controls. If cyclic 3',5'-AMP is involved, optimal conditions have not yet been achieved. It might be mentioned, however, that increase in activity obtained was greater than that achieved by adding epinephrine to the homogenates.
**Fig. 11.** Effects of various concentrations of Mg**++** on lipase activity in unfractionated adipose tissue homogenates. The concentration of Mg**++** was varied from $9.6 \times 10^{-7}$ M to $7.5 \times 10^{-2}$ M in the reaction mixture. Cyclic 3',5'-AMP and ATP concentrations were held constant at $3.2 \times 10^{-7}$ M. The initial and post-90-minute incubation activities were 4.6 and 2.5 microequivalents/g/10 minutes for experiment 1 (upper curve, circles) and 4.6 and 2.7 microequivalents/g/10 minutes for experiment 2 (lower curve, triangles) respectively. Lipase activity was measured according to Assay II.
5. Study of Lipolytic Activity in Supernatant Fluid of Adipose Tissue Homogenates. All the experiments described so far utilized unfractionated homogenates in which the endogenous triglycerides served as the substrate (Assay II). It was during the course of these experiments that Rizack's paper (79) appeared, in which he showed that cyclic 3',5'-AMP caused an activation of the lipase present in the supernatant obtained by centrifugation of an adipose tissue homogenate at low velocity. In his studies, commercial coconut oil emulsion, Ediol®, served as the substrate. The lipase system described by Rizack (79) was then studied to determine whether it responded in our hands to cyclic 3',5'-AMP better than the unfractionated homogenates. Fat pads were pre-incubated as necessary, homogenized and centrifuged at 12,000 x g for 10 minutes at 0° C. The supernatant solution was removed and served as the source of enzyme (see Experimental). Our preliminary studies involved the titrimetric method (Assay I) with coconut oil emulsion as substrate, and the spectrophotometric method (Assay III) in which coconut oil emulsion again served as the substrate. The results of two such experiments are shown in Fig. 12. They appear to indicate that full lipolytic activity was restored when the supernatant fluid was pre-incubated with ATP, cyclic 3',5'-AMP and Mg++. However, it must be emphasized at this point that the activation of the enzyme system under these conditions was not always reproducible. A summary of the effects of these components on the activation of the lipolytic enzyme system in the supernatant fraction is presented in Table 5. It is evident that the degree of activation varies considerably from one experiment to another. Significant activation was obtained in 6 of 11 experiments, but little or no activation was obtained in the remaining 5 experiments.
Fig. 12. Activation of the epinephrine-sensitive lipase in the supernatant fraction of adipose tissue with cyclic 3',5'-AMP. Bars "A" and "B" represent initial and post-incubation lipase activities respectively. Bar "O" represents activity observed when the supernate obtained from the incubated fat pad was pre-incubated at 37° C for 6 minutes with final concentrations of cyclic 3',5'-AMP and ATP, both at 2.0 x 10^-5 M, and Mg++ at 5.0 x 10^-2 M. In addition, Tris buffer 0.25 M final concentration, pH 7.4, was used in experiment "a" (Assay I) and phosphate buffer, 6.0 x 10^-3 M, pH 7.4, was used in experiment "b" (Assay III). The data represents results from experiments on adipose tissues from two different rats, both performed in duplicate.
<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Supernatant A</th>
<th>Supernatant B</th>
<th>Supernatant B + Cyclic 3',5'-AMP</th>
<th>Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>36</td>
<td>26</td>
<td>36</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>62</td>
<td>36</td>
<td>47</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>51</td>
<td>28</td>
<td>28</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>18</td>
<td>42</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>48</td>
<td>20</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>31</td>
<td>22</td>
<td>31</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>46</td>
<td>35</td>
<td>38</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>50</td>
<td>34</td>
<td>40</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>41</td>
<td>11</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>31</td>
<td>5</td>
<td>11</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>59</td>
<td>20</td>
<td>18</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 5. Summary of the effects of ATP and cyclic 3',5'-AMP at 2.0 x 10^{-5} M, and Mg^{++} at 5.0 x 10^{-2} M. Experiments 1 to 5 were performed according to Assay I, and experiments 6 to 11 according to Assay III. Refer to legend, Fig. 12, for details of activation method. Supernatants "A" and "B" were obtained from non-incubated and incubated pads respectively.
Although our procedure used in these experiments is closely similar to that of Rizack (79), our results are considerably less impressive than his. Our data indicate that cyclic 3',5'-AMP can activate the lipase present in the supernatant fluid obtained from centrifuged homogenates of adipose tissue. It does so in an inconsistent manner; some extracts were restored to full activity, some partially restored, and some were unaffected by the nucleotides. Rizack, however, makes no mention as to how consistent his results were or whether his data represent the mean of a series of experiments. We feel that more work is warranted before definite conclusions can be drawn regarding the role of cyclic 3',5'-AMP. Perhaps we have failed to achieve the precise conditions necessary for activation.

6. Possible Effect of Other Cyclic Nucleotides on Lipase from the Supernatant Fluid. A number of other cyclic nucleotides were tested at concentrations of 2.0 x 10^{-5} M and 2.0 x 10^{-4} M. ATP and Mg^{2+} concentrations were held at 2.0 x 10^{-5} M and 5.0 x 10^{-2} M respectively. The results are presented in Table 6. Cyclic 3',5'-GMP and cyclic 3',5'-deoxy-GMP at both concentrations, and cyclic 3',5'-TMP at 2.0 x 10^{-5} M appeared to cause some inhibition of lipolytic activity. Very slight increases in lipolytic activities may have occurred with cyclic 3',5'-deoxy-AMP at both concentrations, with cyclic 3',5'-deoxy-GMP at 2.0 x 10^{-5} M, and also with cyclic 3',5'-UMP at 2.0 x 10^{-5} M. Generally, most of the nucleotides tested caused decreased lipase activities at the higher of the two concentrations used, namely 2.0 x 10^{-4} M.
<table>
<thead>
<tr>
<th>Activities Supernatant A</th>
<th>Activities Supernatant B</th>
<th>Cyclic Nucleotides</th>
<th>Final Concentrations of Nucleotides</th>
<th>Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>39</td>
<td>33</td>
<td>3',5'-GMP</td>
<td>2 x 10^{-5} M</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 x 10^{-4} M</td>
<td>24</td>
</tr>
<tr>
<td>50</td>
<td>34</td>
<td>3',5'-d-AMP</td>
<td>2 x 10^{-5} M</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 x 10^{-4} M</td>
<td>38</td>
</tr>
<tr>
<td>41</td>
<td>11</td>
<td>3',5'-d-GMP</td>
<td>2 x 10^{-5} M</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 x 10^{-4} M</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3',5'-d-CMP</td>
<td>2 x 10^{-5} M</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 x 10^{-4} M</td>
<td>8</td>
</tr>
<tr>
<td>31</td>
<td>5</td>
<td>3',5'-AMP</td>
<td>2 x 10^{-5} M</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3',5'-CMP</td>
<td>2 x 10^{-5} M</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 x 10^{-4} M</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3',5'-UMP</td>
<td>2 x 10^{-5} M</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 x 10^{-4} M</td>
<td>6</td>
</tr>
<tr>
<td>59</td>
<td>20</td>
<td>3',5'-TMP</td>
<td>2 x 10^{-5} M</td>
<td>17</td>
</tr>
</tbody>
</table>

Table 6. Effect of various cyclic nucleotides on lipolytic activity in the supernatant fluid obtained from incubated fat pads. The supernatant fluid was pre-incubated for 6 minutes with the nucleotides indicated, plus ATP 2.0 x 10^{-5} M and Mg^{2+} 5.0 x 10^{-2} M in phosphate buffer 6.0 x 10^{-3} M at pH 7.4. Lipase activity was determined by Assay III and is expressed in milliunits.
Additional Experiments on Adipose Tissue Lipase

1. Effect of Epinephrine on Adipose Tissue Obtained from Reserpinized Rats. Throughout our work, we have observed varying degrees of inactivation of lipase upon incubation of fat pads in Krebs-Ringer medium. Other investigators have observed this also. We have considered this may be due to trauma or excitement induced upon the animal prior to sacrifice, resulting in the endogenous release of catecholamines.

It is common knowledge that reserpine depletes the storage sites of endogenous catecholamines in various tissues. It was considered of interest to examine the rate of inactivation of adipose tissue lipase from fat pads of reserpinized animals, and to determine the effect of epinephrine when added to the medium containing intact fat pads which had been so depleted of endogenous catecholamines.

Three rats weighing 500 g each given subcutaneous injections of 0.5 mg reserpine (Reserpine Injection, U.S.P. Ciba) daily for 4 days and sacrificed on the fifth day. The fat pads were bisected longitudinally and one of the pieces assayed immediately for initial lipase activity by Assay III. The remaining pieces were incubated separately in the usual manner for 3 hours in Krebs-Ringer bicarbonate-albumin medium. To one of the pieces, epinephrine (17 micrograms/ml medium) was added 10 minutes before the end of the incubation period. The incubated tissues were then homogenized, centrifuged and the supernatant fluid assayed for lipase activity.

The results of two of the experiments are shown in Fig. 15. Two noteworthy observations were made. Surprisingly, the extent of lipase
Fig. 13. Effect of epinephrine on fat pads obtained from reserpinized rats. Bars "A" and "B" represent initial and post-3-hour incubation lipolytic activities respectively. Bar "C" represents lipase activity of pads to which epinephrine (17 micrograms/ml final concentration) was added 10 minutes prior to homogenizing and assaying for lipase activity by Assay III. Experimental results from 2 rats are shown. Lipase activity is expressed in milliunits.
inactivation after 3 hours' incubation was very small. Furthermore, epinephrine appeared to have little effect on activating the lipolytic system even when added to the intact pad. The average initial level of lipase activity in adipose tissues from normal rats, as determined during the course of these studies, is approximately 47 milliunits. Hence the initial lipase activities in adipose tissue from these reserpine-treated rats were probably elevated to some extent. The results are interesting but, at the moment, difficult to interpret.

2. Effect of Pentobarbital-induced Anaesthesia and Length of Incubation on the Inactivation Rate of the Lipolytic System. It has been mentioned that throughout this project we noted that the extent of inactivation of the lipolytic system varied widely from experiment to experiment. In our experiences, the degree of inactivation varied from 17% to 86% of the initial pre-incubation level. Furthermore, it became apparent that pre-treatment of rats with pentobarbital did not always cause a large decline in post-incubation lipase activity. Even more discerning was the observation that when lipolytic activities were determined by Assay II, in which incubation periods were in many cases for only 90 minutes, many tissues showed a high degree of lipase inactivation.

Therefore, data was collected from a large proportion of the experiments performed during the course of this study, and tabulated under three categories; namely, adipose tissue from rats which were (a) anaesthetized and incubated for 3 hours, (b) not anaesthetized and incubated for 3 hours, and (c) anaesthetized and incubated for 90 minutes, (Table 7).

It is most interesting to note from the averages computed that there is very little difference in the degree of inactivation of the 3-hour
<table>
<thead>
<tr>
<th>Anaesthetized with Pentobarbital</th>
<th>Not Anaesthetized</th>
<th>Anaesthetized with Pentobarbital</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pre-incubation Activity</strong>*</td>
<td><strong>Post-incubation Activity</strong>*</td>
<td><strong>Pre-incubation Activity</strong>*</td>
</tr>
<tr>
<td>(milliunits)</td>
<td>(milliunits)</td>
<td>(milliunits)</td>
</tr>
<tr>
<td>62</td>
<td>43</td>
<td>54</td>
</tr>
<tr>
<td>59</td>
<td>51</td>
<td>54</td>
</tr>
<tr>
<td>31</td>
<td>22</td>
<td>37</td>
</tr>
<tr>
<td>46</td>
<td>35</td>
<td>47</td>
</tr>
<tr>
<td>70</td>
<td>58</td>
<td>36</td>
</tr>
<tr>
<td>50</td>
<td>34</td>
<td>115</td>
</tr>
<tr>
<td>41</td>
<td>11</td>
<td>62</td>
</tr>
<tr>
<td>31</td>
<td>5</td>
<td>51</td>
</tr>
<tr>
<td>22</td>
<td>10</td>
<td>60</td>
</tr>
<tr>
<td>59</td>
<td>20</td>
<td>39</td>
</tr>
<tr>
<td>Mean 47</td>
<td>Mean 29</td>
<td>Mean 55</td>
</tr>
<tr>
<td>% Initial Activity</td>
<td>62%</td>
<td>% Initial Activity</td>
</tr>
</tbody>
</table>

*Assay III  **Assay I  ***Assay II

Table 7. Effect of pretreatment of rats with pentobarbital and the length of incubation time of fat pads on the degree of lipase inactivation. Lipolytic activities of the 3-hour incubated pads are expressed as milliunits of activity, and were determined according to Assay I or Assay III as indicated. The lipase activities of the 1-1/2-hour incubated pads are expressed as microequivalents FFA produced/g wet weight of tissue/10 minutes, and were assayed according to Assay II.
incubated pads whether or not the rats had been anaesthetized prior to sacrifice. Even more surprising is the fact that the extent of deactivation was greater in pads which had been incubated for only 90 minutes, as compared with the 3-hour incubated pads. It would seem that some unknown factors determine the rate or degree of inactivation of the enzymes during incubation, and perhaps the handling of the animal, or the degree of trauma produced by the removal of the pad, is not as important as believed by several investigators.

3. Effect of Insulin on the Rate of Inactivation of the Epinephrine-sensitive Lipase System. The suggestion that insulin may inhibit a lipolytic system in adipose tissue prompted a brief investigation into the effect of insulin on the rate of inactivation of the lipolytic system when fat pads are incubated for given periods of time.

Epididymal fat pads were obtained from normal fed rats, and assayed for initial and post-incubation activities by Assay II. Insulin was added to both mediums containing the experimental pads. Glucose was added to one of the insulin-treated pads. The lipase activities observed under these conditions are shown in Fig. 14. Incubation of the pads for 90 minutes caused the lipolytic activity to fall to approximately 60% of its initial level. However, when insulin was present in the medium (with or without glucose), the rate of depression of lipolytic activities was delayed. If insulin does have an inhibitory effect on an adipose tissue lipase, the presence of insulin would have been expected to accelerate the rate of lipase inactivation, rather than decrease the rate, as suggested by this experiment.
Fig. 14. Effect of insulin on the deactivation rate of the epinephrine-sensitive lipase. Bars "A" and "B" represent lipase activities before and after 90 minutes' incubation respectively. Both pads represented by "C" and "D" were incubated in a medium containing 2 units/ml insulin. "D" also contained glucose, 1.5 mg/ml. Experiment was performed in duplicate from adipose tissue obtained from one rat. Lipolytic activities were determined by Assay II and are expressed as microequivalents FFA produced/g/10 minutes.
DISCUSSION

In the past, most of the studies on adipose tissue metabolism have been performed either in vivo or in vitro on isolated, intact fat pads. The in vivo studies consisted largely of measuring the metabolic products of lipolysis in the circulation, and although this method has yielded, and continues to yield, much information on lipid metabolism, particularly with respect to the utilization of lipids for energy, it has contributed little towards elucidating the nature of the intracellular metabolic regulation of lipids in adipose tissues. In vitro studies of isolated, intact adipose tissue have contributed considerably more to our knowledge of the metabolic processes in these tissues. In this respect, epididymal fat pads of rats are particularly suitable for incubation studies, not only because they are easily isolated as discrete organs, but also because of their extremely thin, leaf-like structure, requiring no further manipulation before incubating in a suitable medium.

However, in order to seek answers to the problem of metabolic regulation in adipose tissue, (particularly to study the enzymes of this tissue), investigators have recently turned to crude homogenates and supernatant fluids of adipose tissue. Presently, only a relatively small number of workers are using broken-cell preparations of this tissue, and the reasons for this will become apparent when the problems of working with adipose tissue extracts is discussed. In our studies, we have examined the activity of the hormone- or epinephrine-sensitive lipase system, mainly in crude homogenates and in the supernatant fractions of rat epididymal fat pads.

The problems of working with enzyme systems in adipose tissue
Homogenates will be discussed to some extent, since the interpretation of some of the results obtained may be better understood on the basis of the techniques used.

Unlike other tissues such as skeletal or cardiac muscle, adipose tissue yields greasy homogenates (at temperatures near 0°C) which are virtually impossible to pipette. Only by centrifugation at low temperatures and removal of the fat cake accumulated at the top is it possible to work with this type of preparation. On the other hand, homogenization at room temperature yields a creamy suspension which, upon filtering through two layers of cheesecloth to remove pieces of the tough outer membrane, allows the preparation to be pipetted. However, unless this suspension is constantly agitated, a difference in composition between different aliquots of the same homogenate may result.

The assay of lipase activity naturally involves the use of a suitable substrate, and the hormone-sensitive lipase is known to be specific for triglycerides of long chain fatty acids. The question then arises as to what substrate to use. A brief examination of some recent work by various investigators on enzyme systems of adipose tissue indicates that there are almost as many substrates being used as there are investigators working in this particular field. For instance, Rizack (77) has used Ediol, a commercial preparation of a 50% coconut oil emulsion. Vaughan (80) used no exogenous substrate since in her assay system the crude homogenate itself supplied sufficient substrate. Rubinstein et al (88) obtained lipid extracts from adipose tissues and prepared a 20% emulsion of lipids in Krebs-Ringer bicarbonate medium. Gorin and Shafrir (89) used commercial tripalmitin in a 5% solution of gum acacia. In our laboratory
we have used our own preparation of a 50% coconut oil emulsion, Vaughan's method (80), and homogenates which were heated to 100° C, as substrates. We found that the deactivated homogenate was most suitable for our assays. Other workers have occasionally used Tween 20 as an emulsifying agent, despite the fact that this agent itself serves as a substrate for the lipase in adipose tissue. Vaughan (80) has indicated that commercially prepared Ediol® contains a considerable amount of monoglyceride (about 10 micromoles of monostearin per ml). Since monoglycerides are rapidly hydrolysed by adipose tissue extracts, results obtained with Ediol® as substrate must therefore be interpreted with this fact in mind.

The extent of decrease in lipase activity during incubation was extremely variable from fat pads of one animal to another. Our data indicate that in every case inactivation did occur, but the degree of inactivation varied widely. Vaughan (80) has indicated that in her studies, the activity remaining after a 5-hour incubation was usually about 20 to 30% of the original activity, although in some cases little or no detectable decrease was observed. In our experience, the average activity remaining after a 5-hour incubation was about 60% of the original activity. On the other hand, when fat pads were incubated for only 1-1/2 hours rather than 5 hours, the average post-incubation activity was approximately 47% of the original activity. Rubenstein et al (88) have indicated that in their work no incubation was necessary to inactivate the enzyme system, and suggest that this may be due to the fact that they prepared their homogenates in a bicarbonate-containing medium. They indicated that this particular medium may have inactivated the endogenous-activated lipase, or perhaps oxidized any endogenous epinephrine present.
A number of factors could be involved to account for the variable degree of inactivation. For example, the nutritional and/or emotional state of the animal, or the age of the animal at the time of sacrifice, would most certainly have an effect on the rate of inactivation. Furthermore, it is even possible that injury to the central nervous system during decapitation or cervical dislocation may cause widespread sympathetic discharge, resulting in the release of catecholamines in all tissues, including adipose tissue. The fact that the degree of inactivation in fat pads from pentobarbital-anaesthetized rats was no different from that of unanaesthetized animals might suggest that other factors are involved in determining the degree of inactivation.

The marked release of FFA from adipose tissue mediated by epinephrine is consistent with the idea of activation of tissue lipase. Our results obtained with unfractionated homogenates would indicate that epinephrine can cause activation of the enzyme only in intact fat pads. When epinephrine was added during or after homogenization, little or no activation of lipase occurred. These results agree with those of Rubinstein et al (88) who used triglycerides extracted from adipose tissue as substrate. They are not in accord with the findings of Rizack (77), who used commercial coconut oil, Ediol®, as substrate and a supernatant fluid obtained from a centrifuged homogenate as the enzyme source.

The present experiments suggest that cyclic 3',5'-AMP has some ability to activate the lipase present in unfractionated homogenates of adipose tissue. It must be emphasized that the effects of the nucleotides are not marked, and in no case did the activation approach that of the control level present in the homogenate prepared from unincubated tissue.
Cyclic 3',5'-AMP seemed to have a better ability to activate the lipase present in centrifuged supernatant fluid prepared from adipose tissue homogenates when coconut oil was used as substrate. However, the degree of activation was variable; some extracts responded well, some not at all. Our data is not as positive as that of Rizack, although the system we have used seems virtually identical to his. Clearly more work is necessary to settle this matter. Results of one investigator are exceedingly difficult to compare with that of another, since each seems to use a different system and different conditions to study lipase activity. This is in part due to the technical difficulties inherent in working with adipose tissue homogenates, and in obtaining preparations of highly lipophilic materials such as triglycerides in a form suitable for enzyme studies.

It is clear from the published material at hand that the study of factors governing lipid metabolism in broken cell preparations is still in an embryonic state. The problem, however, because of its physiological importance, is now attracting widespread attention. It is hoped that some effort will be made to unify the techniques and preparations used in different laboratories so as to clarify the situation and facilitate a solution to the problem.

The re-activation of an enzyme system in intact fat pads by epinephrine indicates that the triglyceride-splitting lipase exists in two forms; that is, an active and an inactive form. Our data strongly suggests that a lipase system in crude homogenates of adipose tissue was activated by ATP and cyclic 3',5'-AMP at $3.2 \times 10^{-7}$ M, and $\text{Mg}^{++}$ at $4 \times 10^{-4}$ M, despite the fact that epinephrine used under similar conditions failed to produce any
significant degree of activation. We have also demonstrated that ATP and cyclic 3',5'-AMP at $2 \times 10^{-5}$ M and Mg$^{++}$ at $5 \times 10^{-2}$ M could also activate a lipase in the supernatant fraction of adipose tissue homogenates, thus supporting Rizack's latest findings (79). The sharp inhibition of lipolytic activity in crude homogenates at Mg$^{++}$ concentrations exceeding $3 \times 10^{-5}$ M is difficult to interpret, especially in view of the optimum concentration of $5 \times 10^{-2}$ M required for the activation of the enzyme system in the supernatant fraction.

The synthetic analogues of cyclic 3',5'-AMP, with the exception of cyclic 3',5'-deoxy-AMP, showed no particular indication of activating the lipolytic system in the supernatant fluid under the conditions of the assay system.

The almost total lack of lipase activation with epinephrine, observed in our studies on adipose tissues from reserpinized rats, is difficult to understand. Since reserpine causes the depletion of catecholamines in tissues, it might be expected that the extent of decrease in lipase activity upon incubation would be minimized and, in fact, this was observed. On the other hand, if the tissues were depleted of catecholamines, their response to added epinephrine might be expected to be highly exaggerated. However, even a normal response to epinephrine was not elicited in our studies. It was observed that the rats had lost an average of 15% of their body weight, their epididymal fat pads weighed about 1/3 that of untreated rats and, in addition, the initial levels of lipase activities were elevated above the normal level. Therefore, in view of these observations, we suggest that either the dose of reserpine given (1.5 mg/kg/daily for 4 days) was inadequate to cause a marked depletion of catecholamines or, alternatively,
that the catecholamines were, in fact, depleted and that the high level of lipase activity observed was mediated through some mechanism other than that involving catecholamines.

Whether working *in vivo* with intact organisms or *in vitro* with isolated tissues and broken cell preparations, the ultimate aim of most scientists has been to seek the answer to the question of metabolic control in the normal physiological processes of the intact organism. Since adipose tissue is practically the only source of FFA (which are subsequently oxidized by the heart and other organs for energy) the answer to the question of metabolic regulation of lipids must be sought in this tissue. In fact, it would not be unreasonable to consider adipose tissues (in particular, the epididymal fat pads of rats) as true organs, having physiological functions no less important than those of the heart, lung or brain, at least from the standpoint of the regulation of the caloric homeostasis of most organisms.
REFERENCES


45. Biegelman, P.M., Metabolism 2, 580 (1960).