This dissertation has been
microfilmed exactly as received 67-15,915

OLSON, Anita C., 1929-
THE LIPOLYTIC ACTIVITIES OF RAT LIVER.

The University of Oklahoma, Ph.D., 1967
Chemistry, biological

University Microfilms, Inc., Ann Arbor, Michigan

Downloaded from SHAREOK. Persistent Handle URL: https://hdl.handle.net/11244/2264

© Copyright by

Anita C. Olson

1967

THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

THE LIPOLYTIC ACTIVITIES OF RAT LIVER

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

ANITA C. OLSON

Oklahoma City, Oklahoma

1967

THE LIPOLYTIC ACTIVITIES OF RAT LIVER

APPROVED BY

P. Alaupovic

B Connor Johnson

Reagan H. Bradford

Earl G Larsen

Robert A. Patrick

DISSERTATION COMMITTEE

ACKNOWLEDGMENTS

It is with appreciation and gratitude that I acknowledge the assistance given to me by all the people with whom I have been associated during the course of this research.

I am especially indebted to Dr. Petar Alaupovic for the stimulation he provided by his knowledgeable insight of lipid metabolism, for providing an environment for scientific growth and independence, and for his friendship.

I am indebted to Dr. Robert H. Furman and the Cardiovascular Section of the Oklahoma Medical Research Foundation who provided support (grants from the U. S. Public Health Service: GM-11195, TI HE-5403 and HE-10575; the American Heart Association and the Oklahoma State Heart Association), and laboratory facilities in which I have been privileged to work. Credit goes to the personnel associated with this section, all of whom have at some time been of invaluable assistance to me. Special credit goes to Mr. Kirby Jarolim who assisted me in early research studies and to Mrs. Shirley Anderson who performed the tedious task of preparing glassware for me.

Appreciation is extended to the graduate faculty of the Departments of Biochemistry and Microbiology and especially to Dr. B. Connor Johnson, Dr. Reagan H. Bradford, Dr. Earl G. Larsen and Dr. Robert A. Patnode who served as reading committee for this dissertation.

TABLE OF CONTENTS

		Page
LIST OF TABLES		v
LIST OF ILLUSTRATIONS		vii
Chapter		
I.	INTRODUCTION	1
II.	MATERIALS AND METHODS	21
III.	RESULTS	31
IV.	DISCUSSION	73
V.	SUMMARY	89
BIBLIOGRAPHY		91

LIST OF TABLES

Table		Page
1.	The Effect of Lipopolysaccharide and Heparin on the Lipolytic Activity in Rat Serum and Liver	33
2.	The Effect of Lipopolysaccharide on the Liver Lipolytic Activity	34
3.	Comparison of Lipolytic Activities of Various Enzyme Preparations	36
4.	Comparison of Lipolytic Activities of Individual Enzyme Preparations	38
5.	Lipolytic Activities of Precipitates from Ammonium Sulfate Fractionation of BIPH	43
6.	Lipolytic Activities of Precipitates from Ammonium Sulfate Fractionation of BIPH	44
7.	Comparison of Activities Using Tris and Phosphate Buffers	48

LIST OF TABLES--Continued

Table		Page
8.	Comparison of Specific Activities Using Glycyl Glycine and Phosphate Buffers	49
9.	Substrate Specificities	55
10.	Substrate Specificities of Enzyme Fractions Obtained by Ammonium Sulfate Fractionation of BIPA	57
11.	Effects of Additives to the Substrate Media	63
12.	Effect of Inhibitors on Liver Lipolytic Activity	64
13.	Effect of Inhibitors on Liver Lipolytic Activity	66
14.	Effect of Inhibitors on Liver Lipolytic Activity	67
15.	The Effect of Desoxycholate on the Lipoprotein Lipase Assay System	69
16.	Comparison of Lipolytic and Esterolytic Activity of Subcellular Fractions	70
17.	Comparison of Activity Toward Various Substrates by Subcellular Fractions	72

LIST OF ILLUSTRATIONS

Figure		Page
1.	Fractionation of BIPA on Sephadex G-75	39
2.	Fractionation of BIPH on Sephadex G-75	40
3.	Fractionation of BIPH on Sephadex G-150	42
4.	pH Activity Curves Obtained by Using 0.1 M Acetate (pH 5.2-6.0) or Phosphate (pH 6.5-8.8) Buffer	47
5.	Relationship of the Fatty Acids Released from Various Substrates As a Function of Time	51
6.	Fatty Acids Released from Olive Oil Substrate As a Function of Protein	52
7.	Fatty Acids Released from Various Substrates As a Function of Protein Concentration of the Enzyme Preparation	53
8.	Effect of Increasing Concentrations of Methyl Butyrate Substrate	59
9.	Effect of Increasing Concentrations of Substrate	60
10.	Effect of Desoxycholate and Albumin on Lipolytic Activity	61

THE LIPOLYTIC ACTIVITIES OF RAT LIVER

CHAPTER I

INTRODUCTION

Origin of the Problem and Objectives

In previous studies (1) on the mechanism of the "lipemia-clearing" effect (2) of bacterial lipopolysaccharides a lipolytic activity was observed in rat liver which had not been previously described in the literature and appeared to be different from the so-called "post-heparin" or lipoprotein lipase and ali-esterase. The virtual absence of lipoprotein lipase in the serum and liver of lipopolysaccharide-treated animals prompted the investigation of other possible lipolytic enzymes considered to be of general importance in the removal and metabolism of long-chain triglycerides by the liver.

The objective of this research study was to demonstrate the presence of a liver triglyceride lipase, to characterize its lipolytic activity, and thus to establish criteria for the differentiation between the post-heparin lipase, esterase, and triglyceride lipase. Within the family of enzymes catalyzing the hydrolysis of esters it has long been customary to differentiate between lipases and esterases on the basis of their relative preferential substrate specificity. Lipases have been variously described as (a) enzymes hydrolyzing esters of glycerol, principally tri-

glycerides, (b) enzymes hydrolyzing esters of long chain fatty acids irrespective of the chemical nature of alcohol, and (c) enzymes hydrolyzing esters in heterogeneous systems (3). In this investigation the third definition has been used as the criterion. However, there is not a serious discrepancy between the first and the third definition, because triacetin is the only triglyceride which can be utilized in a homogeneous system (4).

In view of the central role played by the liver in triglyceride metabolism and the numerous studies which have indicated that chylomicron triglycerides (CTG) are removed intact from the circulation by the liver without appreciable intravascular lipolysis (5-9), it was anticipated that physiologically the demonstration of the triglyceride lipase might represent one of the important missing factors for understanding the mechanism of triglyceride uptake and metabolism by the liver. In liver cells the CTG are hydrolyzed and the fatty acids are oxidized and/or utilized for synthesis of lipoprotein triglycerides and phospholipids (9-11). A tacit assumption of the proposed mechanism was the presence, among others, of a triglyceride lipase in the liver. This research project was designed to test this assumption.

Absorption, Transport and Removal of Dietary Fats

With the exception of the fat soluble vitamins and certain unsaturated fatty acids, lipids are apparently not an essential component in the diet. However, the importance of fats which represent a large part of the caloric intake of animals should not be underestimated. Fats are the most concentrated source of energy, yielding per unit weight twice

as many calories as carbohydrates or proteins. The bulk of the dietary lipids are triglycerides containing long-chain fatty acids of animal or plant origin.

Digestion, Absorption and Transport of Dietary Fats

The water-insoluble character of the dietary fats requires special mechanisms for their digestion, absorption and transport. Indications are that the majority of fatty acids fed as glycerides are absorbed after hydrolysis to free fatty acids but that the majority of the glycerol of dietary glyceride is absorbed bound to one or more fatty acids (12).

The most important enzyme for the hydrolysis of triglycerides in the intestine is pancreatic lipase. This enzyme has been studied extensively (13). Hydrolysis by pancreatic lipase occurs predominantly at the α- and/or α'- positions, producing an α, β-diglyceride and fatty acid. Diglyceride is then hydrolyzed predominantly to β-monoglyceride (14-16). Hofmann found only β-monoglyceride in the intestinal contents following a triglyceride-rich meal (17).

More recently, several reports have indicated the presence of an intestinal monoglyceride lipase (18-20). A hydrolytic activity has been demonstrated for both α- and β-monoglyceride (18-20). It has been suggested that the intestinal monoglyceride may function in the intracellular completion of fat digestion (20).

There are two major postulates (21) about the mechanism of fat absorption of mucosal cells and the chemical nature of lipid components passing through the membranes: (1) "Particulate theory", according to which the products of limited triglyceride hydrolysis in combination with

bile salts emulsify the remaining triglycerides and form particles which can penetrate the mucosal cell, and (2) "Lipolytic theory" which requires a complete hydrolysis of dietary triglycerides. The fatty acids penetrate the mucosal cells and are resynthesized into triglycerides. An attractive hypothesis allowing somewhat of a merger of the two postulates has been suggested by Hofmann and Borgstrom (22). According to their hypothesis, the ingested triglycerides form an emulsion with bile salts. The emulsified triglycerides are hydrolyzed to β-monoglycerides and fatty acids which in combination with bile salts form a micellar solution. The fatty acids and monoglycerides in micellar solution penetrate the mucosal cell possibly by a diffusion process and are resynthesized intracellularly into triglycerides. Borgstrom et al. (23) have shown in the human that the absorption of fat is started in the distal part of the duodenum and is completed in the first 50-100 cm. of the jejunum.

The enzyme systems necessary for the synthesis of triglycerides from fatty acids and either glycerol or monoglycerides have been demonstrated in the intestinal mucosa. These enzymes have a specificity for fatty acids containing at least eight carbon atoms (only the long-chain fatty acids appear in the thoracic duct triglycerides whereas shorter-chain fatty acids are transported primarily via the portal circulation) (21).

In 1877, Edmonds noted the appearance of particles in the blood following fat absorption. Gage and Fish (24) introduced the term chylomicrons for these particles and established that they represent fat particles being transported from the intestine to sites of uptake via the lymphatic and circulatory systems. Very little information is available

regarding the mechanism by which chylomicrons are formed. It is now generally accepted that the chylomicrons are formed from the products of fat digestion within the cytoplasmic matrix of the epithelium of the small intestine (25). It has been suggested that the chylomicrons are discharged at the side of the epithelial cell into the extracellular spaces (26). From there they enter the lymphatic circulation via the central lacteals and then the systemic circulation via the thoracic duct.

The chylomicrons constitute a family of particles of different sizes, on the order of 0.5-1.0 μ. Their percent composition is approximately 86% triglycerides, 3% cholesterol, 8% phospholipids, and 2% protein (27).

Bierman and coworkers have demonstrated (28) two distinct groups of fat particles appearing in serum during alimentary lipemia. These particles can be separated by differential flocculation with polyvinylpyrrolidone and characterized by starch-block electrophoresis. The so-called "primary" particles seem to originate in the intestine and are identical with the lymph chylomicrons. The "secondary" particles originate presumably in liver and contain a mixture of exogenous and endogenous triglycerides. Both particles serve a vehicular role in the transport of triglycerides to tissues that metabolize and utilize long-chain fatty acids.

Removal of Chylomicron Triglycerides from the Systemic Circulation

When chylomicrons with labeled triglyceride fatty acids are injected intravenously, the fatty acids are rapidly removed with a circulating half-life of only a few minutes (6-8, 29-33). The results of

numerous investigations have indicated that, under normal physiological conditions, the liver is one of the major sites for removal of chylomicrons from the blood. From studies with injected labeled chylomicrons it has been established that about one-third of the triglyceride fatty acids is taken up by the liver, one-third by the adipose tissue and the remainder by other organs (5, 8, 6, 34).

There is little information available about the removal mechanism. It has been suggested, on the basis of electron-microscopic studies, that chylomicrons are taken into the liver cells by pinocytosis (35). Robinson has proposed that chylomicrons are trapped in the extracellular spaces, such as the spaces of Disse and the sinusoids (40).

Recently Felts and Mayes (37, 38) suggested that the liver may not be involved directly in the removal and metabolism of CTG. According to this view the CTG are taken up and hydrolyzed in the peripheral vascular beds and the liberated serum free fatty acids enter the parenchymal liver cells. Thus, not triglycerides but fatty acids seem to be the chemical form that penetrates the cell membranes.

Elovson, Olivecrona and Belfrage demonstrated (39) that the liver removed about one-third of the labeled chyle fatty acids (stearic acid) from the circulation per time unit. They considered this relatively large amount of CTG to be entrapped most probably in the extracellular spaces where it was slowly metabolized. Subsequently, increasing amounts of labeled fatty acids appeared in the liver phospholipids. Label appeared actually in oleic acid in both phospholipids and non-phospholipids (a desaturase catalyzed the conversion of stearic acid into oleic acid). Belfrage (40) further evaluated the "trapping" phenomenon. Contrary to

Felts' proposal, he found that although a portion of "trapped" CTG could be perfused from the liver, a large portion was apparently hydrolyzed by the liver and the fatty acids and glycerol were utilized for synthesis of liver lipids. However, he admitted that previous estimates of the role of the liver in the direct metabolism of CTG may have been overestimated.

Higgins and Green and Green and Webb (41, 42) have found that isolated rat liver cells are capable of binding chylomicrons and bringing about the hydrolysis of chylomicron triglycerides. Of all subcellular fractions the plasma membrane had, per unit weight, the greatest capacity for binding lipid and catalyzing triglyceride hydrolysis (41). They concluded that these findings are not necessarily in conflict with a pinocytotic mechanism as a means of uptake (41) (the plasma membrane in vivo pinches off into pinocytotic vesicles). However, some of their findings did not wholly support this mechanism (42).

In liver perfusion studies Rodbell et al. (10) have found that the chylomicrons containing C^{14}-labeled tripalmitin were taken up without de-esterification. All entry sites were saturated within five minutes. C^{14}-tripalmitin was found in both parenchymal and reticuloendothelial cells.

Very recently, Ontko and Zilversmit (43) used a combination of the liver perfusion and slice techniques to study the role of the liver in the metabolism of chylomicrons. They found a very low initial level of free fatty acids in liver following chylomicron administration. The subsequent rise in free fatty acids in the liver was interpreted as a clear indication of hepatic lipolysis of CTG, in agreement with studies of Green and Webb (42). In livers removed from intact rats which had

been given labeled chylomicrons the ratio of labeled triglycerides to phospholipids was high (12-14 to 1). On the other hand, after the intravenous administration of albumin-bound radioactive free fatty acids, the ratio of labeled triglycerides and phospholipids was close to unity (44). Therefore, Ontko and Zilversmit (43) suggested that the liver possesses the ability to remove and utilize chylomicrons directly. Contrary to the opinions of Felts and Mayes (38), they concluded that a significant portion of the fatty acids utilized by the liver of intact rats stems from the direct removal of chylomicrons rather than from the absorption of circulated free fatty acids formed by the extrahepatic lipolysis of chylomicrons.

It is generally considered that the uptake of CTG by the extrahepatic tissues involves the degradation of chylomicron structure (29). Bragdon and Gordon (5) have demonstrated that adipose tissue and the lungs, kidneys, muscles, spleen and heart take up significant amounts of labeled CTG fatty acids. They showed that the tissue distribution was dependent upon the nutritional state of the animal. Morphological evidence also indicates that chylomicrons are altered before leaving the extrahepatic circulation. The luminal surface of the blood capillaries of extrahepatic tissues is lined by a continuous layer of endothelial cells without fenestrations, and there is no evidence for a direct passage of chylomicrons across the capillary walls (45).

The hydrolysis of CTG to free fatty acids probably takes place in peripheral capillary beds by action of lipoprotein lipase (46). Hahn was the first to observe that heparin injection in dogs brings about a clearing of lipemic sera (47). Anderson and Fawcett correlated this ac-

tion with clearing factor lipase (48). Korn (49) and others have demonstrated the presence of this lipolytic activity in a large number of tissues and suggested that the corresponding enzyme be called lipoprotein lipase. Numerous studies have been performed on the role of clearing factor lipase in the distribution of triglyceride fatty acids in the extrahepatic tissues. Robinson (45) in reviewing these studies, commented about the possible special significance for the clearing factor mechanism as follows: "If the function for the clearing factor lipase in distributing triglyceride fatty acids to the extrahepatic tissues which has been outlined is substantiated, the enzyme will determine the pattern of distribution of the fatty acids in the body since the extent of uptake by any tissue will depend upon its content of clearing-factor lipase".

The Lipid Hydrolases

In broad terms the lipid hydrolases are a rather poorly defined family of enzymes which catalyze the hydrolysis of ester bonds in a variety of substrates. Within the family it is usually customary to distinguish lipases and esterases. Early workers assumed that a lipase hydrolyzes natural triglycerides and esterases hydrolyze esters. Extensive investigations of specificities, however, have shown the lipases to be very unspecific enzymes displaying a considerable overlap with equally nonspecific esterases.

Balls and Matlack (50) have referred lipase specificity to enzymes splitting long-chain fatty acid esters. Included in this definition are those enzymes splitting long-chain fatty acids from monohydric alcohols. Alternately, specificity has been referred to esters of glycerol, irrespective of the chain length of the fatty acid. A further com-

plication has arisen from the use of water soluble esters, such as p-nitrophenyl acetate and Tweens (51), as lipase substrates.

Using purified pancreatic lipase as a "model lipase" Sarda and Desnuelle (3) have shown that lipases will act only in a heterogeneous system and do not act, or act very slowly on water-soluble substrates.

In 1846 Claude Bernard demonstrated lipase activity in the pancreas (52). Little further advance was achieved in studies on the lipases until Willstatter and his coworkers made significant contributions in the early 1920's. Lipases are widely distributed in animals, plants and microorganisms. Pancreatic lipase has been the more thoroughly studied and has been isolated in a relatively purified form.

Pancreatic lipase. Desnuelle and his associates have made by far the greatest advances in the study of pancreatic lipase. However, a vast amount of literature is available from many laboratories. Pancreatic lipase, similarly to other enzymes present in the pancreas secretions, is produced in the acinar cells and is probably contained in the "zymogen granules" as active lipase (13). However, very recent electron microscopic studies indicate that lipase is formed in the endoplasmic reticulum of the acinar cells and concentrated in the Golgi zone rather than in the zymogen granules (53).

The importance of this enzyme in the digestion of dietary fat was indicated earlier. The pathway of lipolysis is: triglyceride $\longrightarrow$ α,β-diglyceride $\longrightarrow$ β-monoglyceride $\longrightarrow$ glycerol. The splitting of chains in the β-position is slow and possibly occurs only after intramolecular acyl migration (13). The positional specificity of this enzyme has been used to elucidate the distribution of fatty acids in many natural triglycerides.

Wills (4) found the highest hydrolysis rates in both crude and purified pancreatic lipase to be displayed toward tripropionin and tributyrin. The rate of hydrolysis of ethyl butyrate was extremely slow.

Lipoprotein lipase (clearing factor lipase, post-heparin lipase). Whether the terms lipoprotein lipase, clearing factor lipase, and post-heparin lipase are synonomous is a moot question. They share many common characteristics and their differences may be largely due to semantics. As indicated earlier, this lipase is stimulated *in vivo* by heparin and after heparin administration is found in increased amounts in serum and in all tissues with the possible exception of the brain. This enzyme, unlike many other lipases, requires the presence of a lipoprotein complex for its maximal activity. Its normal substrate is lipemic plasma. In addition to activation by heparin, lipoprotein lipase requires the addition of cations and of a fatty acid acceptor such as serum albumin (54). The activity of the enzyme is determined by measuring the clearance of or the production of the end-products of hydrolysis of a triglyceride lipoprotein emulsion.

Studies on the characterization of lipoprotein lipase have been done without the benefit of purified enzyme preparation. The enzyme has been found to be inhibited by high concentrations of NaCl (1 M), 5% sodium taurocholate, 5% protamine sulfate (49,55), sodium fluoride, diethyl p-nitrophenyl phosphate (56), sodium desoxycholate (57) and certain polyanions (58).

Inactivation of lipoprotein lipase by bacterial heparinase led to the conclusion that heparin is an integral part of the enzyme (59). Jeffries (60) perfused post-heparin blood through the isolated rat liver

with a resultant decrease in its lipolytic activity. He concluded that this organ destroys clearing activity.

Korn (54) concluded that lipoprotein lipase of rat plasma showed no special positional specificity and that fatty acids present in all three positions in glycerides are readily hydrolyzed. The clearing factor had no differential specificity for glycerides composed of a wide range of different fatty acids. Capric, lauric, stearic, oleic and linoleic esters were split at about the same rate.

It has been suggested that heparin releases more than one enzyme, or more than one form of the enzyme, and that a stepwise degradation of the lipoprotein triglycerides occurs (61).

Several authors have reported that lipoprotein lipase is absent or present in only insignificant amounts in the liver (7, 34, 39, 49, 62, 63); more recent findings seem to indicate its presence in either relatively small amounts or in an inactive state. Constantinides and coworkers (64) found that CCl_4 poisoned or partially hepatectomized rats exhibited greater lipemia clearing activity and concluded that this represented indirect evidence for a clearing factor inactivating system in the liver.

Mayes and Felts (65) demonstrated a lipoprotein lipase in the rat liver, but in an inactivated state. However, the inactivation could be reversed by a ten-fold increase of heparin concentration in the *in vitro* assay system.

Hamilton (66) found that clearing factor lipase was released into the blood stream in dogs following a single passage of heparin through the liver vascular beds.

Some other lipases of metabolic importance. A number of lipases

which can be differentiated from lipoprotein lipase have been observed in various tissues. Mosinger, Pechar and Segova demonstrated two lipolytic activities in rat lungs, one of which was not identical to that of lipoprotein lipase (67). On the basis of dietary studies (68) and age dependence (69) they postulated that these enzymes have different functions in fat metabolism.

Various reports of lipases occurring in the intestinal mucosa have been published. In many cases the possibility of their identity with the pancreatic lipase was not excluded. However, as related earlier, several reports of an intestinal monoglyceride lipase have demonstrated clearly the presence of an independent enzyme (18, 19, 20).

The storage and mobilization of the fatty acids from the lipid depot centers has focused a special attention on the adipose tissue lipases. At least three lipolytic activities have been demonstrated in this tissue in the rat: lipoprotein lipase, a hormone-sensitive lipase activated by epinephrine and cyclic AMP (59, 70, 71) and a lipase specific for monoglycerides (72). Lynn and Perryman purified and described an adipose tissue lipase which had an apparent specificity for tributyrin (73). Two lipolytic activities in human adipose tissues were demonstrated by Schnatz, of which one was characterized by its optimum activity at an alkaline pH and an apparent specificity toward tributyrin, and the other by optimum activity at neutral pH and with olive oil as substrate (74).

The lipid hydrolases present in the liver. Mammalian liver contains several types of hydrolases which might be concerned with the hydrolysis of various liver lipids. The earlier descriptions included those termed ali-esterase (75), lipase (76, 77, 78), lecithinase or phos-

pholipase (79, 80), cholesterol esterase (81), vitamin A esterase (82), and aryl sulfatase (83). In 1942 Richter and Croft (84) described ali-esterases as enzymes hydrolyzing esters of N-free alcohols, and they subdivided this class into lipases which preferentially split fats and oils, and esterases which cleave simple esters of monohydric alcohols. The Enzyme Commission defined ali-esterase as carboxylic ester hydrolase (3.1.1.1).

In more recent studies, non-specific esterases have been demonstrated (85) by the use of water soluble substrates such as β-naphthyl acetate, a method introduced by Nachlas and Seligman (86). Keay (87) used p-nitrophenyl esters to study a purified hog liver esterase and Krisch (88) used acetanilide and other anilides to study a highly purified hog liver microsomal esterase.

Boguth, Krisch and Niemann (89) purified hog liver microsomal esterase by means of DEAE-Sephadex column chromatography and determined its molecular weight as 174,000. The enzyme was disaggregated completely into four equal subunits by sodium dodecylsulfate. From their substrate and inhibitor studies they concluded that this esterase was identical with microsomal amidase, amino acid esterase, ali-esterase and procaine esterase which had been described previously.

Only very recently have a limited number of reports indicating the presence of a liver triglyceride lipase appeared in the literature. Since some of these observations have been based on Desnuelle's definition of a lipase it is possible to compare the results.

In a preliminary note, Belfrage (90) described a rat liver monoglyceride splitting enzyme (glycerol-monoester hydrolase). The enzyme

was active only toward monoglycerides and did not hydrolyze diolein under the experimental conditions used. Utilizing Ediol as substrate, Grafnetter and Grafnetterova (91) described a lipolytic activity in rat liver homogenates. Although the rate of lipolytic activity was increased by the addition of albumin, it was not influenced by the presence of plasma or 0.01 M Ca^{++}, and it was inhibited by serum. Protamine sulfate (2 mg/ml) and 1 M NaCl caused a 20% inhibition of activity. Irwin and coworkers (92) and Biale and coworkers (93) also noted the presence of a triglyceride lipolytic activity in the liver without differentiating it from activity catalyzed by a lipoprotein lipase. However, the conditions used in their assays would not favor the latter enzyme. Higgins and Green (41) demonstrated that the plasma membrane of the liver cell has the capacity to bind chylomicrons and hydrolyze their triglyceride component. This hydrolysis was unaffected by the usual inhibitors of lipoprotein lipase.

In 1965, it was demonstrated independently by Vavrinkova and Mosinger (94) and Olson and Alaupovic (95, 96) that the rat liver does contain a triglyceride lipase. Using various artifical fat emulsions or lipemic serum, Vavrinkova and Mosinger (94) demonstrated the release of fatty acids from triglycerides by liver homogenates. Although they found no enhancement of activity with serum, the presence of albumin increased the activity. The enzyme was described as thermolabile and showed two pH optima. Maximal activity was displayed at pH 5, with a secondary activity demonstrated at pH 8. In further studies (97) they related the lipolytic activity to the nutritional state of the animal. They evaluated the intracellular lipolysis by observing an increased concentration

of fatty acids within the liver slices and the extracellular lipolysis by a similar increase occurring in the media in which the slices were incubated. They concluded that the lipolysis occurs extracellularly and that the increased content of fatty acids in liver slices could be attributed to their uptake from the media. There was an increased accumulation of fatty acids in slices derived from fasted rats. In contrast, fed rats showed a higher rate of lipolysis in the liver media. The lipolytic activity was enhanced by 0.5% protamine sulfate or 0.44 M NaCl, an effect opposite to that anticipated if the enzyme activity were due to lipoprotein lipase.

Olson and Alaupovic (95, 96) described the isolation of rat liver triglyceride lipase (glycerol-ester hydrolase, EC 3.1.1.3). Using phosphate buffer and Ediol substrate peak lipolytic activity was found at pH 7.2 with an additional smaller peak at pH 7.8-8. The lipolytic activity was increased several fold in the presence of sodium desoxycholate. However, with albumin as the sole additive, a sharp decrease in activity was observed. With Ediol substrate and phosphate buffer (pH 7.2), highest specific activity was located in the soluble fraction of subcellular preparations. Highest activity toward methyl butyrate was located in the mitochondria and microsomal fractions.

Rat liver homogenates were also recently shown by Carter (98) to have a lipolytic activity against water-insoluble glycerol esters of long-chain fatty acids. This activity was found to be very labile. Preincubating the whole homogenate for 1 hr. at 37° resulted in loss of 80% of its lipolytic activity. NaCl (0.5 M), EDTA (10^{-3} M) and protamine (300 µg/ml) had no effect on the rate of hydrolysis. The activity was inhib-

ited by Ca^{++} ions and, surprisingly, by rat serum. However, 0.05 M NH_4^+ enhanced the hydrolysis rate 20%. When either triolein or tripalmitin were used as substrates, all liver subcellular fractions showed lipolytic activity. The highest specific activity was displayed by the microsomes. The assay system contained albumin and was buffered to pH 7.4 with phosphate buffer.

The Unique Physical Chemical Considerations of the Lipase Enzyme Action

Elucidation of the mechanisms by which triglycerides are hydrolyzed poses a particular challenge. Not only is it necessary to make the usual characterization of the enzymatic reaction, but it is also equally important to resolve the added problems relating to the fact that these reactions are taking place at an oil/water interface.

Sarda and Desnuelle (3), using methyl butyrate as substrate, demonstrated that as long as it was present in concentrations below the saturation point it was hydrolyzed little, if at all, by pancreatic lipase. However, when the solubility limit was exceeded, the substrate was hydrolyzed rapidly. Thus, lipase acts only on emulsified fat globules separated from water by an interface. In a mixture of oil and water, lipase occurs neither in water nor in oil, but adheres to the interface. It is possible that the adsorption at the interface controls the enzyme activity in an "all or none" way (13). Desnuelle suggested at a time when it had not yet been verified that "a Michaelis constant can be defined for a lipase as being the interfacial area (or the number of substrate molecules located in this area) which gives to the lipolysis reaction the half of its maximal rate" (13). He has also shown that the ini-

tial rate varies with the interfacial area for a constant weight of substrate. The initial rate for a given weight of substrate is lower with a coarser emulsion than with a finer one. Two different curves were obtained when the initial rates were plotted against substrate concentration, but a single curve was obtained for both emulsions when the plot was made against interfacial area. Thus, a basis has been provided for the "interfacial enzymology" (13).

Mattson and Volpenhein (99) have pointed out the importance of the rates at which the reactants diffuse to the interface and the rates at which the products diffuse away from the interface. They found that the rate of hydrolysis of incubation mixtures shaken at a rate of 150 cycles/min. is only half of that realized at a rate of 330 cycles/min. Free fatty acids are considered by many to be inhibitors of hydrolysis. It was suggested by them that this may be due to their accumulation at the interface, thus blocking the formation of the enzyme-substrate complex. They also demonstrated that the fatty acids released from triglycerides during hydrolysis *in vitro* showed a pattern of alkaline salt to nonesterified fatty acid ratio as a function of pH. This was demonstrated by carrying out the assays at various pH values and maintaining the selected pH values by the addition of standardized KOH. At the end of 10 min. the volume of KOH was recorded (alkaline salt). The incubation mixture was then acidified and the total free fatty acids were determined. It was found that 90% of fatty acids formed during digestion were present as alkaline salts at pH 9, but only 20% were present at pH 7. Since soaps and free fatty acids do not have the same surface active properties, the hydrolysis at an oil/water interface would be influenced by a change in

the pH at which the digestion is carried out simply because of the change of the ratio of soap to nonesterified fatty acid.

Sarda et al. (100) found that the potent organo-phosphate inhibitor, diethyl p-nitrophenyl phosphate, inhibited lipase only in an emulsified state. With "dilute" emulsions a part of the activity disappeared completely and irreversibly. They postulated that an active center does not exist as long as the lipolytic enzyme is in solution and that it is formed and becomes able to interact with substrates and inhibitors when the noncovalent structure of the lipase molecule is modified by interfacial adsorption.

A very interesting phenomenon of activation and inhibition is observed with pancreatic lipase upon the addition of bile salts. Borgstrom (101) noted that when digestion was carried out at pH 8 bile salts had an inhibitory effect. At pH 6, the addition of 0.2% bile salts caused an acceleration in the rate of hydrolysis; higher concentrations did not show this acceleration.

Desnuelle and Sarda (13) showed that the addition of taurocholate to a triolein emulsion at 37° increased the initial rate of hydrolysis with pancreatic lipase by a factor of 4. They suggested that lipolysis in the absence of taurocholate was not temperature dependent between 10 and 40°, but that it became temperature dependent in the presence of this compound. Although short of a final interpretation these authors summarized their views, as follows: "enzymic catalysis occurs by the successive reactions, and the over-all rate is the rate of the slowest reaction. It may, therefore be assumed that, in the absence of taurocholate the over-all rate of lipolysis is determined by a nontemperature-

dependent reaction, that taurocholate accelerates this reaction, and that another, actually temperature-dependent reaction then becomes rate limiting." (13).

Tributyrin lipolysis is temperature-dependent in the absence of taurocholate and is not affected by its addition (13). Thus, Desnuelle (13) asserts that the step at which taurocholate appears to act is not the primary interaction of lipase with an interface, but another process which is especially slow with long-chain fatty acids. He suggests that perhaps this is a physical process such as glyceride diffusion or orientation at the interface, or free fatty acid removal from the interface.

CHAPTER II

MATERIALS AND METHODS

Materials

Materials used in the course of these experiments were as follows: Ediol (Riker Laboratories: 50% coconut oil, 12.5% sucrose USP, 1.5% glycerol monostearate, 2% polyoxyethylene sorbitan monostearate, 0.1% sodium benzoate USP, 0.08% methyl paraben USP, 0.02% propylparaben USP, 0.01% butylate hydroxyanisole, 0.05% EDTA), olive oil (Sargent, pure imported), tricaproin, tricaprylin, trilaurin, trimyristin, and methyl stearate (K & K Laboratories, Inc.), tripalmitin (Mann Research Laboratories), triolein (Cal Biochem), dipalmitin, monopalmitin, diolein (Hormel Institute), methyl butyrate and monoolein (Eastman Organics). Organic solvents used were: n-butanol (Matheson, Coleman & Bell), acetone (Fisher), diethyl ether (Fisher, anhydrous), isopropanol (J. T. Baker Co.), heptane (Eastman Organics). Buffer components used were: Tris (Mann Research Laboratories, trihydroxy amino methane), glycyl glycine (Cal Biochem), sodium phosphate and potassium dihydrogen phosphate (Mallinckrodt). Inhibition and activation studies were carried out using: heparin (Upjohn, Heparin, sodium), albumin (Armour Pharmaceutical, Fraction V bovine plasma), atoxyl (K & K Laboratories, sodium arsanilate), Eserine (Merck, phytostigmine sulfate), E-600 (K & K Laboratories, diethyl

p-nitrophenyl phosphate), protamine (Sigma Chem. Co., Protamine Sulfate), Polybrene (Abbott Laboratories, poly (1-5 diazundeca methylene methobromide)), iodoacetic acid and sodium desoxycholate (Mann Research Laboratories, special enzyme grade), sodium chloride, sodium fluoride, calcium chloride (Fisher), Sephadex (Pharmacia) and DEAE cellulose (Applied Science Lab., Inc., washed and pretested for column chromatography).

Tissue Preparation

Tissue samples were prepared routinely from Holtzman male rats weighing approximately 300 grams. Rats were secured from the Houston Cheek Co., Houston, Texas. Animals were either fasted overnight or fed *ad libitum* on Purina rat chow. They were sacrificed under light ether anesthesia by exsanguination from the descending aorta and the livers were perfused *in situ* with ice cold 0.9% NaCl until blood color disappeared from the tissue.

Whole Homogenates

The blotted and weighed liver was cut into slices and homogenized with 0.1 M phosphate buffer (pH 7.2) by 30 strokes in a glass homogenizer designed by Drs. Caputto and McCay in the Biochemistry Section of the Oklahoma Medical Research Foundation. Both the whole homogenate and the supernatant fraction obtained by its centrifugation at 755 x g for 20 min. were utilized as starting material for enzyme preparations.

Acetone Powders

Acetone powders were prepared in a Waring blendor by mincing approximately 12 g. of liver with 160 ml. of precooled (-40^{0}) acetone for 1 min. The precipitated liver tissue was recovered by filtration, washed

with at least three volumes of cold acetone and dried by suction at room temperature. The drying procedure was aided by stretching several layers of Saran wrap across the top of the Buchner funnel. The dried powders could be stored indefinitely at -40°.

Preparation of the Powders of Butanol-Water Interfacial Layer from Liver Homogenates (BIPH)

The supernatant fraction of whole liver homogenate was treated with an equal volume of n-butanol which had been cooled to 4°. The mixture was gently shaken (100 strokes) in a separatory flask and then allowed to separate into two liquid phases and an interfacial phase at 4°. The phases were separated, and the interfacial layer was precipitated with cold acetone. The acetone precipitate was collected by suction at room temperature, washed with acetone to remove residual butanol and dried in vacuo. Traces of acetone were removed by washing the filter cake with cold anhydrous diethyl ether and by suctioning the filter cake to dryness. This preparation (BIPH) was stored at -40°.

Preparation of the Powders of Butanol-Water Interfacial Layer from Liver Acetone Powders (BIPA)

Three grams of liver acetone powder were extracted with 100 ml. of 0.1 M phosphate buffer (pH 7.2) for 30 min. at 4°. The insoluble residue was separated either by centrifugation or filtration and was discarded. The supernatant fraction was treated with an equal volume of cold n-butanol, shaken gently (100 strokes) and allowed to separate into phases in the cold. This interfacial butanol-water layer (BIPA) was then further treated as described above in the procedure for the preparation of

BIPH.

Ammonium Sulfate Fractionation

A saturated solution of ammonium sulfate was allowed to equilibrate at 4°. Three grams BIPH or BIPA were extracted with 100 ml. 0.1 M phosphate buffer (pH 7.2) for 30 min. at 4°. The extracts were centrifuged at 755 x g for 10 min. and the insoluble debris was discarded. Sequential ammonium sulfate fractionations of the extracts were performed at 4° by adding successively the required volume of saturated ammonium sulfate solution for each desired percentage of saturation. The precipitates were recovered by centrifugation at 8500 x g for 10 min. The final fraction was precipitated by the addition of solid ammonium sulfate. The individual precipitates were resuspended in cold, 0.1 M phosphate buffer (pH 7.2) and assayed.

Column Chromatography

Sephadex gel filtration. Sephadex was equilibrated with 0.1 M phosphate buffer (pH 7.2) and the fine particles were removed by several decantations with the buffer. Several grades of Sephadex (G-50, G-75, G-100 and G-150) were used. A Sephadex slurry was packed into either 5 x 100 cm. or 3 x 40 cm. columns. The efficiency of the column packing was tested by eluting a solution of blue dextran. In order to make a denser solution sucrose (approximately 50 mg./ml.) was added to 10-15 ml. of BIPH or BIPA extract. The solution was then applied to the top of the column by layering it carefully under the buffer. A portion of the original extract was retained for the assay. The column was eluted with 0.1 M phosphate buffer. Fractions (4.5 ml.) were collected with the aid of

a Gilson fraction collector (Gilson Medical Electronics, Middleton, Wisconsin). The protein content of each fraction was measured.

DEAE-cellulose column chromatography. DEAE-cellulose was equilibrated with 0.02 M phosphate buffer (pH 7.2). It was then packed to a height of 21 cm. in a 1.9 x 30 cm. column. The column was charged with 15 ml. of an enzyme preparation which had been obtained by a 50-75% ammonium sulfate precipitation of BIPH. The ammonium sulfate precipitate had been dialyzed against 0.02 M phosphate buffer overnight. The flow rate was maintained at approximately 10 ml. per hour. The column was developed with increasing concentrations of phosphate buffer (pH 7.2). Approximately 80 ml. of each of the following buffers were passed through the column: 0.02, 0.075, 0.1, 0.125, 0.175 and 0.2 M. Fractions of 4.5 ml. were collected with the aid of the Gilson fraction collector and the protein concentration of each fraction was determined.

Subcellular Fractionation

Rat livers were divided into approximately 6 g. units and homogenized with 0.25 M sucrose solution by fifteen up and down strokes of a glass homogenizer. Thirty-two milliliters of sucrose were used for each g. of liver tissue. A portion of the mixture was retained for assay and the remainder was centrifuged at 755 x g for ten minutes. The sediment represented debris. The supernatant portion was brought to a total volume of approximately 40 ml. with a 0.25 M sucrose solution and centrifuged at 8700 x g for 10 min. The supernatant fraction was separated and retained. Approximately 40 ml. of sucrose solution were added to the precipitate and it was again extracted and centrifuged at 8700 x g for 10 min. This procedure was repeated once more. The precipitate represented

the mitochondrial fraction. The pooled supernatant fractions which now amounted to approximately 120 ml./6 g. of liver were again centrifuged at 8500 x g for 10 min. and the residue was added to the mitochondrial fraction. The supernatant fraction was then centrifuged at 105,000 x g for 1 hr. The supernate from this centrifugation constituted the soluble fraction and the precipitate the microsomal fraction.

Protein Determination

The protein content of the enzyme preparations was determined by the Warburg absorbance analysis method according to Kalckar (102). Spectrophotometric determinations were performed in a 1 cm. Beckman silica cuvette in a Beckman DUR Spectrophotometer. The amount of protein contained in one ml. of solution was determined by the formula:

$$[1.45 \times O.D.(280\ m\mu)] - [0.74 \times O.D.(260\ m\mu)] = \text{mg. protein/ml.}$$

The Enzyme Assay System

Preparation of Enzyme Extracts

In order to obtain extracts of enzyme powder preparations approximately 1 g. of powder was solubilized with 7.5 ml. of appropriate buffer by stirring it in an ice bath for 30 minutes. Residue was separated from the extract by centrifugation or filtration. When fresh homogenates were used, large particles were precipitated by centrifugation at 755 x g for 10 min. and removed to facilitate pipeting and protein analysis. Subcellular fractions were resuspended in ice cold, 0.25 M sucrose to give a protein concentration of approximately 10 mg./ml.

Preparation of Substrates

All substrates with the exception of methyl butyrate, which was used as a 1% solution, were prepared in an emulsified form. The following procedure was used to prepare a stable emulsion of olive oil: One hundred and eighty milliliters of an ice cold 10% aqueous solution of gum arabic and 20 ml. of chilled olive oil were homogenized in a Waring blendor at high speed for 15 min. This emulsion was stable for at least 4 months. A stable emulsion of tributyrin was prepared in the same manner by substituting 20 ml. of tributyrin for the olive oil. Ediol (see Materials) was used as supplied by Riker Laboratories or diluted with water (1:3 v/v).

Standard substrate solutions were prepared immediately before use in the following manner:

1 ml. of appropriate emulsion

3.5 ml. 0.1 M phosphate buffer (pH 7.2)

0.5 ml. sodium desoxycholate (4% w/v in water)

Ingredients were mixed, preincubated at 37° for 30 min., and the pH was adjusted if necessary by the addition of 0.1 N HCl or 0.1 N NaOH.

Substrates to be used in characterization studies, such as purified triglycerides, monoglycerides, diglycerides or esters, were prepared in the same manner in amounts corresponding to 40 μEq of their esterified fatty acids (EFA) available per 0.5 milliliter.

Incubation

Incubations were carried out in a total volume of 1 ml. Culture tubes with teflon-lined caps were used as incubation vessels. Each sample was assayed in either duplicate or triplicate, together with its own

"zero time" control. One-half milliliter of appropriate substrate was pipeted into each tube and placed in an ice bath. Five milliliters of Dole's extraction mixture were added to tubes designated as "zero time". One-half milliliter of enzyme extract was then added to each tube and the incubation was carried out at 37-38° for 1 hour in a Dubnoff water bath shaker set for 110 oscillations per minute. The enzyme reaction was stopped by placing the tubes in an ice bath and adding 5 ml. of Dole's extraction mixture (103). In order to extract the liberated fatty acids, the tubes were capped, shaken for 1 min., allowed to stand for 10 min. and again shaken for one-half minute. After 5 min., 4 ml. of heptane and 4 ml. of water were added. The tubes were capped again, inverted 6 times and centrifuged at 755 x g for 10 min. to separate the phases. Fatty acids were present in the upper heptane layer.

Palmitic acid standards were prepared for each set of assays. Two μEq of palmitic acid dissolved in 2 ml. of heptane were pipeted into an incubation tube. Five ml. of Dole's extraction mixture (103, 104) were added and the tube was shaken in the same manner as described above. Instead of subsequently adding 4 ml. of heptane and water, 2 ml. of heptane and 5 ml. of water were added. A standard blank (zero time) was prepared by treating 2 ml. of heptane (without palmitic acid) in the same manner. Equal aliquots were taken from the heptane phases in each case. The titration values obtained at zero time were subtracted from those obtained at 1 hr. and the difference represented the liberated fatty acids.

Titration of Fatty Acids

Lipolytic activity was evaluated as the release of free fatty acids from the substrate. The free fatty acids were determined by the

method of Dole and Meinertz (103, 104). Nile Blue A was substituted for Thymol Blue as indicator because the former gave a more easily visualized end point. The titrations were made manually with the aid of a microburette and micrometer (Microchemical Specialities Co., Berkeley, Calif.).

For the bulk of the experiments the automatic titrimetric procedure described by Schnatz (105) was employed. Samples were extracted with a mixture of isopropanol, heptane and 1 N H_2SO_4 (40:10:1, v/v/v) according to the method of Dole (103). A 3 ml. aliquot of free fatty acid containing heptane layer was placed in a 50 ml. polycarbonate tube used as a titrating vessel. One ml. Thymol Blue indicator (103, 104) and 3 ml. isopropanol were added to produce a single-phase system (105). Then 0.015 N NaOH was delivered automatically to an end point reading of pH 9.8. The automatic titration was carried out by means of a Radiometer TTT 1 c Titrator, 0.5 ml. SBU 1a syringe burette, SBR 2c titragraph, and combined electrode, GK 2021C (London Company, Westlake, Ohio).

In both titration procedures a reference standard of palmitic acid in heptane solution was used.

Definition of Specific Activity

The unit of specific activity was defined as the μEq. of fatty acids released by 1 mg. of protein in 1 hour.

Lipoprotein Lipase Assay

Determination of lipoprotein lipase activity was performed according to a modification of the procedure described by Korn (49). Composition of the substrate was as follows:

8 parts 10% aqueous solution bovine serum albumin

1 part normal rat serum

1 part Ediol (diluted with H_2O 1:3 v/v)

0.5 parts 1 M $(NH_4)_2$ SO_4

Ingredients were mixed, preincubated for 30 min. at 37° and the pH adjusted to 8.5 with ammonium hydroxide.

Substrate (0.5 ml.) was incubated with 0.5 ml. of liver acetone powder extract (1 gm. powder extracted with 7 ml. 0.025 M ammonium hydroxide in the cold for 10 min.), or with 0.5 ml. of a post-heparin serum at 37-38°. Incubation and titration of fatty acids was carried out as described in assay system for triglyceride lipase.

CHAPTER III

RESULTS

Initial Observations on the Presence of a Liver Lipolytic Activity

Meir and Schuler (2) reported that the lipemia clearing effect of bacterial lipopolysaccharides exceeded that of heparin and postulated that the lipopolysaccharides do not stimulate lipoprotein lipase release. Previous studies in this laboratory (1) showed that, contrary to the results reported by Meir and Schuler, a single injection of lipopolysaccharides had little or no effect on serum triglyceride concentration. However, an increased clearing of experimentally induced hypertriglyceridemia was observed after two injections of lipopolysaccharides administered at 24 hour intervals. In order to test the possible involvement of lipases in the clearing process liver acetone powders of lipopolysaccharide-treated and control animals were assayed for lipolytic activity. It was in these experiments that a liver triglyceride-cleaving activity was detected for the first time. Consequently, it became necessary to establish (1) the effect of lipopolysaccharides on the normally occurring lipolytic activity and (2) its possible identity with the lipoprotein lipase.

Lipoprotein lipase activity may be stimulated by the administration of heparin (47, 48, 49). Therefore, the serum and liver acetone powders from control, lipopolysaccharide, and heparin (7.5 mg./kg. body

wt. injected 8 min. before sacrifice) treated animals were assayed. The lipopolysaccharide treated animals were administered intravenously 10 μg. of lipopolysaccharide per 100 g. body wt. on the first day and 100 μg. per 100 g. body wt. 24 hours later. They were sacrificed 48 hrs. after the initial injection and after a 17 hr. fast. The control group of animals was also fasted for 17 hours.

The results (Table 1) showed that only in post-heparin serum was there an elevated lipolytic activity indicative of clearing factor lipase. A slight increase in liver lipolytic activity was observed after treatment with lipopolysaccharide but none was seen following the administration of heparin. These results verified the presence of a liver lipolytic activity which seemed to be different from lipoprotein lipase, and confirmed many other reports of the absence of lipoprotein lipase in the rat liver (7, 34, 39, 49, 62, 63).

The statistical evaluation (Table 2) of a comparative study of a large number of lipopolysaccharide-treated and control animals showed that lipolytic activity was not increased in the liver as a result of lipopolysaccharide administration. In this study, the "standard" enzyme substrate using Ediol replaced the lipoprotein lipase assay system. In addition, a high degree of variability in the lipase activity among individual enzyme preparations was observed, a finding which may explain earlier discrepancies about liver lipolytic activity in the literature.

Isolation and Purification of the Liver Lipolytic Activity

Acetone Powder Preparation

Lipolytic activity in dried acetone powders appeared to be stable

TABLE 1

THE EFFECT OF LIPOPOLYSACCHARIDE AND HEPARIN ON THE LIPOLYTIC ACTIVITY IN RAT SERUM AND LIVER

Experiment no.		Lipolytic Activity[a]		
		Control	LPS treated	Heparin treated
Serum[b]	I	0.368	0.479	4.438
Serum[b]	II	2.177	1.403	10.946
Liver[c]	I	1.645	1.837	1.399

[a]Each value is the average of 3 experimental determinations carried out using the lipoprotein lipase assay system.

[b]Lipolytic activity is expressed as μEq. of fatty acids released per 0.3 ml. of serum/120 min.

[c]Lipolytic activity is expressed as μEq. of fatty acids released per 70 mg. of acetone powder/120 min.

TABLE 2

THE EFFECT OF LIPOPOLYSACCHARIDE ON THE LIVER LIPOLYTIC ACTIVITY

Treatment	No. animals	Specific Activity[a]	
		Mean (Range)	Standard error
Lipopolysaccharide treated	37	0.683 (0.168-1.416)	± 0.049
Control	45	0.781 (0.247-1.524)	± 0.040

[a]Specific activity is expressed as μEq. fatty acids released per mg. protein/hour.

indefinitely when stored at -40°. Powders which had been stored for 2 years still retained lipolytic activity.

Extracts with lipolytic activity could be made from acetone powders by a variety of buffers, 0.025 N ammonia solution, 0.25 M sucrose, or distilled water. In most instances, the specific activity of acetone powders was slightly greater than that of corresponding whole homogenates (Table 3, groups I and II). Activity toward various triglyceride substrates, as well as methyl butyrate (Table 3), appeared to be retained in acetone powder preparations. On the other hand, it was not apparent from these experiments to what extent lipolytic activity toward a particular substrate might be affected by the acetone treatment.

Butanol-Water Interfacial Preparation

Two procedures were used to obtain butanol-water interfacial preparations, one of which utilized homogenates and the other extracts of acetone powders as starting materials. In all triglyceride substrate evaluations, as shown in Table 3, the butanol-water interfacial preparation (BIPH) obtained directly from liver homogenates was more active.

Occasionally (Table 3, water phase), it was noted that a minor portion of the lipase activity was not totally adsorbed onto the interface and still resided partially in the water phase. An alternate explanation could be that it may have been adsorbed, but was then re-extracted into the water phase during separation of the phases. The enzyme which catalyzed the hydrolysis of methyl butyrate was often shifted into the water phase. This procedure should be considered as a possible means for separating esterolytic activity.

Each of the three enzyme preparations shown in this study (Table

TABLE 3

COMPARISON OF LIPOLYTIC ACTIVITIES OF VARIOUS ENZYME PREPARATIONS

Preparation	Group No.[a]	Mg. protein	Specific Activity				
			Tributyrin	1% Methyl butyrate	Ediol	Olive oil	Lipoprotein lipase assay system
Fresh homogenate	I	31.1	0.60	0.25	0.08	0.03	0.04
	II	8.4	0	0.23	0.35	0.06	0.17
	III	15.9	1.14	0.34	0.18	0.03	0.10
Acetone powder	I	8.0	2.36	0.74	0.14	0.08	0.10
	II	12.6	1.40	0.19	0.27	0.08	0.13
	III	24.2	0.82	0.31	0.12	0.03	0.12
BIPA	I	2.4	2.05	0	0.33	0.09	0.14
	II	2.4	3.50	0	0.80	0.06	0.08
	III	1.5	2.30	0.28	0.76	0	0.69
BIPH	I	0.88	4.41	1.23	0.82	1.00	0.40
	II	0.37	6.50	0.13	6.20	1.10	4.30
	III	1.96	4.90	0.38	2.60	1.60	0.87
Water phase BIPA	I	0.64	0	2.30	0	0.10	0.33
	II	0.92	0.18	0.05	0.23	0	0.16
	III	1.23	0	0.61	0	0	0
Water phase BIPH	I	0.34	0	3.80	0	0.50	0.80
	II	0.81	0.02	0.58	0	0	0.17
	III	1.16	0.22	0.78	0	0	0

[a]Each group represents a pool of the livers from four animals.

3) was obtained by pooling fresh livers from four animals. Therefore, the substrate specificity noted for a group represented a composite of four activities. Under the specified assay conditions (0.1 M phosphate buffer, pH 7.2), all three preparations showed an apparent substrate specificity for tributyrin. The degree of activity toward this, as well as other substrates, varied with the preparation.

The variability of activity for different substrates was observed also in the examples presented in Table 4. One of the randomly-selected BIPH samples showed a preferential activity toward the short-chain triglycerides and the other toward the long-chain triglycerides. The BIPA sample, which displayed a high olive oil activity, was obtained by selectively assaying a number of preparations.

Column Fractionations

Initial observations indicated that gel-filtration might be a useful tool with which to attain a purification of the lipase. Figure 1 illustrates the results of a preliminary experiment using Sephadex gel-filtration. Relatively large fractions (20-30 ml.) were collected, concentrated by dialysis against dextran, and assayed for activity using Ediol as substrate. Fraction 6 contained the major portion of the protein and Fraction 11 showed the highest enzyme activity. This latter peak represented a 6-fold increase in activity above that of the BIPA applied to the column.

The elution pattern from a Sephadex G-75 column is shown in Figure 2. It was reproducible when BIPH was used as the enzyme preparation and assaying with tributyrin as substrate. Fractions were collected as 4.5 ml. aliquots. In this example, of the 136 mg. of protein applied to

TABLE 4

COMPARISON OF LIPOLYTIC ACTIVITIES OF INDIVIDUAL ENZYME PREPARATIONS

Preparation	Mg. protein	Specific Activity					
		Tributyrin	1% Methyl butyrate	Ediol	Olive oil	Tricaprylin	Monoolein
BIPH[a]	1.6	7.2	5.9	0	0	0.7	3.8
BIPH[a]	0.7	0.6	0.9	1.1	2.3	2.3	2.5
BIPA[b]	1.4	1.8	2.7	1.2	6.2	6.8	0.2

[a]BIPH represents a random selection.

[b]BIPA represents a selected preparation highly active upon olive oil as substrate.

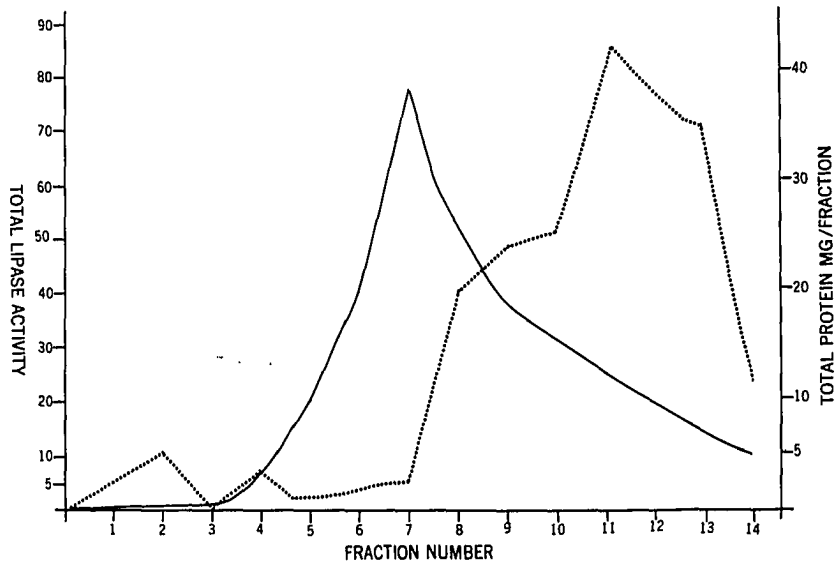

Figure 1. Fractionation of BIPA on Sephadex G-75.

The column was 40 cm. in length and elution was done with 0.1 M. phosphate buffer (pH 7.2). Assay was with Ediol substrate. Fractions were 15-20 ml. volume. Total lipase activity is defined as μEq. fatty acids released in one hour. Dotted line indicates lipase activity and solid line indicates protein.

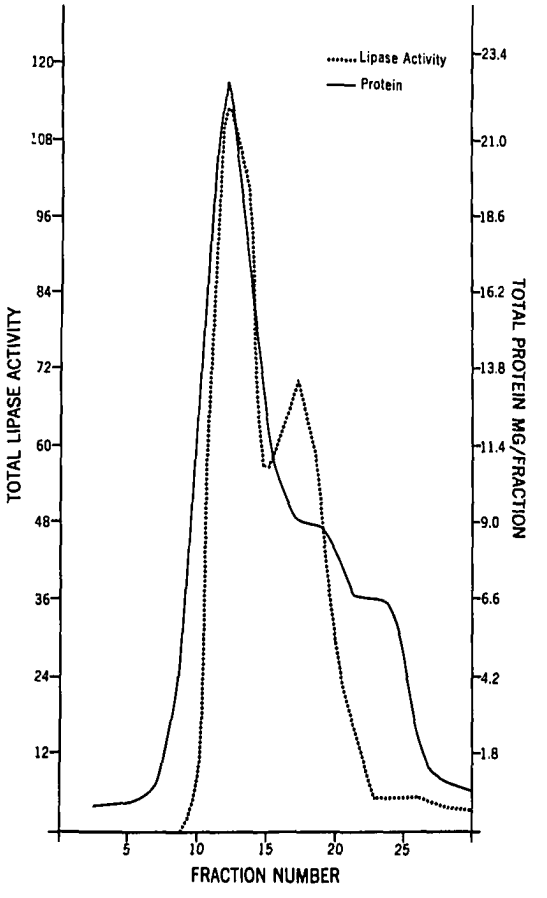

Figure 2. Fractionation of BIPH on Sephadex G-75.

The column was 40 cm. in length. Elution was with 0.1 M phosphate buffer (pH 7.2). Assay was with tributyrin substrate. Fractions were 4.5 ml. volume. Total lipase activity is defined as µEq. fatty acids released in one hour.

the column, 98% was recovered in the eluted fractions. The highest specific activity observed was 10.3, approximately a 4-fold increase over BIPH. Peak activity corresponded to peak protein concentration.

Using a 100 cm. column of Sephadex G-150, lipase activity (evaluated with tributyrin as substrate) was consistently found as a sharp peak in the area of the 60th fraction (Figure 3). This fractionation procedure separated the protein (recovery 123%) into two peaks eluted between fractions 20 and 80. Fractions were again collected as 4.5 ml. aliquots. The highest specific activity, a sharp area in the broader protein peak, was 14.7. The specific activity of the material applied to the column (a sucrose extract of BIPH) was 2.4.

Difficulties encountered in attempts to fractionate whole liver homogenates by gel-filtration indicated the impracticality of such an approach. Some eluted fractions were very dense and caused the fraction collector to operate improperly. Furthermore, instead of a clear separation, the activity was spread throughout many fractions.

DEAE-Cellulose. Attempts at fractionation of lipolytic activities by elution from DEAE-Cellulose columns with buffers of increasing ionic strength were unsuccessful. A protein precipitate was formed in buffer of low ionic strength. It either did not redissolve and pass through the column or it was bound, possibly irreversibly, to the cellulose.

Ammonium Sulfate Fractionation

Ammonium sulfate precipitation resulted in a slight increase in the activity of certain fractions (Tables 5 and 6). Although no clear separation of the activities displayed toward various substrates was ob-

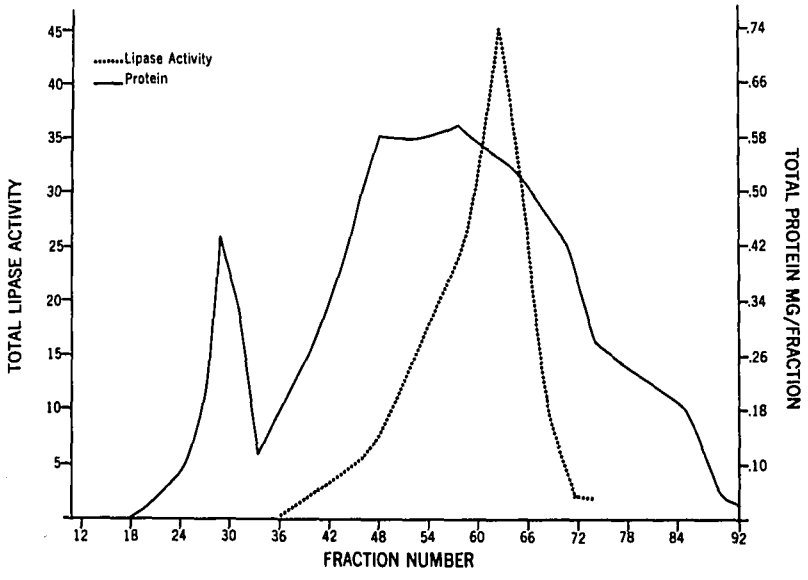

Figure 3. Fractionation of BIPH on Sephadex G-150.

The column was 80 cm. in length. Elution was with 0.1 M phosphate buffer (pH 6.8). Assay was against tributyrin substrate. Fractions were 4.5 ml. volume. Total lipase activity is defined as μEq. fatty acids released in one hour.

TABLE 5

LIPOLYTIC ACTIVITIES OF PRECIPITATES FROM AMMONIUM SULFATE FRACTIONATION OF BIPH[a]

Fractions	Mg. protein	Specific Activity			
		Tributyrin	1% Methyl Butyrate	Ediol	Olive oil
Unfractionated	3.7	1.6	6.8	0.4	0
0-25%	2.7	0	0.9	0.3	0
25-40%	1.2	0.2	0.6	1.4	0
40-50%	1.5	2.0	14.2	0.2	0
50-100%	4.4	5.7	5.9	0.9	0.2

[a]BIPH represents a random selection.

TABLE 6

LIPOLYTIC ACTIVITIES OF PRECIPITATES FROM AMMONIUM SULFATE FRACTIONATION OF BIPH[a]

Fractions	Mg. protein	Specific Activity		
		Tributyrin	1% Methyl Butyrate	Ediol
Unfractionated	0.62	6.5	10.5	1.4
0-40%	1.70	1.2	3.4	0.3
40-45%	0.02	21.0	180.0	0
45-50%	1.12	1.6	6.1	0.3
50-55%	1.12	3.0	6.1	1.7
55-60%	1.03	12.1	6.8	1.0
60-75%	2.03	5.0	3.2	0.5
75-100%	0.6	0.5	6.2	0

[a]BIPH represents a random selection.

served, it appeared advantageous to eliminate protein precipitated by 25% saturation. This fraction was almost free of lipase activity.

Characterization of Lipase Activities Using Liver Butanol Interfacial Preparations

Characterization studies were done using butanol-water interfacial preparations. These materials were used rather than the more purified fractions from the Sephadex columns because only small quantities of the latter material were obtainable. By using the more readily available butanol-water interfacial preparations it was possible to study a large number of substrates, inhibitors, pH levels, or other parameters with the same enzyme source. Thus, it was feasible to illustrate a variety of lipolytic activities with a single preparation.

Numerous liver acetone powders were prepared and were then assayed using olive oil as substrate. Acetone powders having a relatively high activity toward the long-chain triglycerides were pooled and further fractionated into butanol-water interfacial preparations (BIPA). Several preparations were made directly from liver homogenates (BIPH) to represent a random selection of long-chain or short-chain triglyceride activity. In addition to these two types of preparations which were used for the major part of the studies, preliminary observations had been made using either whole homogenates or acetone powders as enzyme sources.

pH and Buffer Studies

Initial studies using crude acetone powders as the enzyme source and Ediol as substrate had revealed at least two pH-optima for lipolytic activity. Further studies indicated that these pH optima were correlated

with a substrate preference. The pH curves obtained by using three different substrates indicated three peak areas of activity (Figure 4). The activity at pH 8 appeared to prefer long-chain triglycerides with maximal activity displayed against olive oil and there was little or no activity against the short-chain triglycerides. The optimum at pH 7 was displayed preferentially toward medium-chain triglycerides (tricaprylin at pH 6.8, specific activity 25). In addition, some activity was observed toward tributyrin and olive oil. A smaller activity peak with all three substrates was noted in the acid range (pH 5.5).

Comparison of Tris and phosphate buffers. In order to compare the relative effectiveness of buffering with Tris or phosphate buffers, substrates and the enzyme sources were prepared using the appropriate analogous buffer system.

It was observed (Table 7) that phosphate buffer served as the preferential buffering system with all substrates except olive oil. In that case the activity observed was small. However, Tris appeared to be the more adequate buffer. Therefore, Tris buffer should be considered for use in experiments designed to test the possible specificity of an enzyme preparation for olive oil as substrate.

Glycyl glycine and phosphate buffer comparison. Two enzyme preparations and three substrates prepared in the appropriate buffer systems were compared. The results of this study (Table 8) revealed that glycyl glycine was a superior buffering system for measuring activities against tributyrin and tricaprylin. It was noted, however, that at pH 8.5 activity toward tricaprylin was suppressed. Glycyl glycine was inhibitory toward olive oil activity throughout the pH range with BIPA. When glycyl

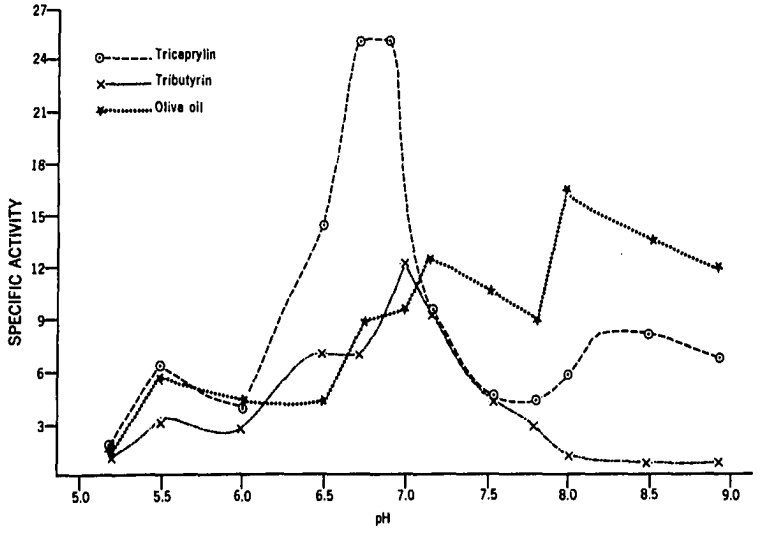

Figure 4. pH activity curves obtained by using 0.1 M acetate (pH 5.2-6.0) or phosphate (pH 6.5-8.8) buffer.

The enzyme source was BIPA.

TABLE 7

COMPARISON OF ACTIVITIES USING TRIS AND PHOSPHATE BUFFERS

Buffer system	Enzyme[a] source	Specific Activity			
		Tributyrin	1% Methyl Butyrate	Ediol	Olive oil
pH 7.2					
0.1 M Tris	1	3.2	1.4	0.2	0
	2	6.0	2.4	1.1	3.0
	3	5.6	2.4	0.3	0
0.1 M PO_4	1	9.5	3.7	6.6	0
	2	10.1	2.5	1.0	1.0
	3	12.8	3.7	0.5	0
pH 8.0					
0.1 M Tris	1	0.5	1.3	0.2	0.2
	2	0.5	1.0	1.0	3.6
	3	0.5	2.9	0	0
0.1 M PO_4	1	2.9	2.5	3.0	0
	2	4.9	-	-	-
	3	3.1	2.7	0.3	0

[a]The enzyme sources were three individual random selections of BIPH.

TABLE 8

COMPARISON OF SPECIFIC ACTIVITIES USING GLYCYL GLYCINE AND PHOSPHATE BUFFERS

Substrate	Specific Activity			
	Glycyl glycine		Phosphate	
	BIPH	BIPA	BIPH	BIPA
Tributyrin				
pH 7.0	12.2 <<	>> 7.9	2.2	>> 1.2
7.5	5.5	5.1	7.5<<	0.5
8.0	7.0 <	4.3	1.2	>> 1.0
8.5	3.9	1.0	2.8<	0.1
Tricaprylin				
pH 7.0	4.8 <<	7.0	4.9<<	>> 7.5
7.5	3.9	>>26.5	2.3	2.0
8.0	2.6	> 25.2	1.3	> 5.7
8.5	0	0.1	3.0	0.6
Olive oil				
pH 7.0	1.4 <<	>> 8.0	2.8<<	>> 12.0
7.5	0.9	5.7	0.7	8.3
8.0	0.9	>> 7.6	1.1	> 10.5
8.5	0.6	2.8	2.2<<	8.7

>> indicates peak activity.

> indicates secondary peak activity.

glycine was used, the activity was never higher than the lowest activity noted when phosphate was the buffering system.

Time Curve

With both tributyrin and tricaprylin as substrates, the early reaction rates were similar (Figure 5). However, after the first 25 minutes the hydrolysis of tricaprylin appeared to be accelerated. The hydrolysis rate of olive oil was nearly linear throughout the 120 min. incubation period.

This study showed the selection of 60 min. as time point for comparing the activities to be adequate in the following respects: at this point the reaction rate was neither appreciably slowed nor unusually accelerated for any of the substrates, and sufficient product had been formed to make evaluation reliable. However, it should be noted that the apparent activity under these assay conditions at 60 min. was not necessarily indicative of the comparative initial velocities of the reactions.

Enzyme Concentration

The classical concept of a linear relationship between the enzyme concentration and rate of lipolysis was demonstrated in a study, the results of which are shown in Figure 6. However, that this relationship varied with each enzyme preparation as well as with the type of substrate assayed was shown in another study (Figure 7). To obtain the enzyme preparations, approximately 3 g. of BIPA were extracted with 7.5 ml. buffer. Dilutions were made by the addition of buffer. The protein concentration of each dilution was determined. From the observation that increasing the protein concentration beyond a point caused a decreased ac-

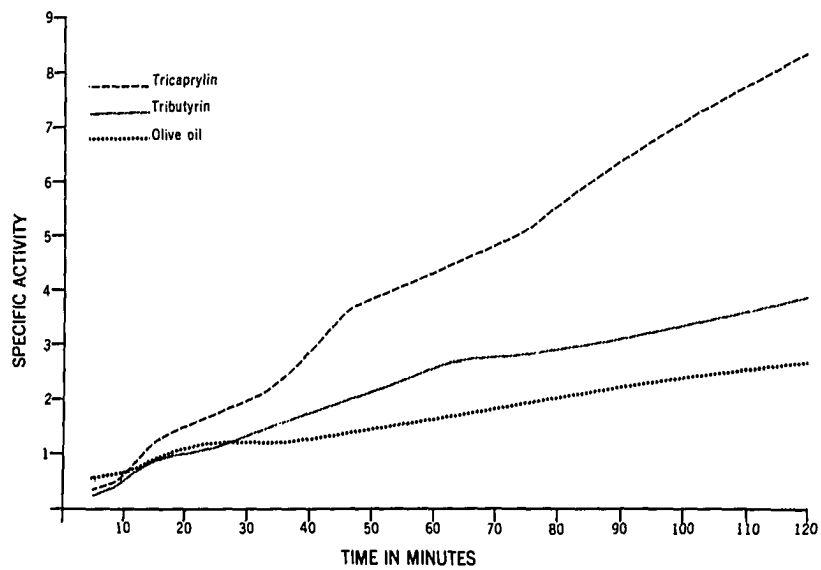

Figure 5. Relationship of the fatty acids released from various substrates as a function of time.

The buffer system was 0.1 M phosphate (pH 7.2). BIPA (.51 mg. protein/assay) was used as the enzyme source. Specific activity in this case is defined as μEq. fatty acids released per mg. protein.

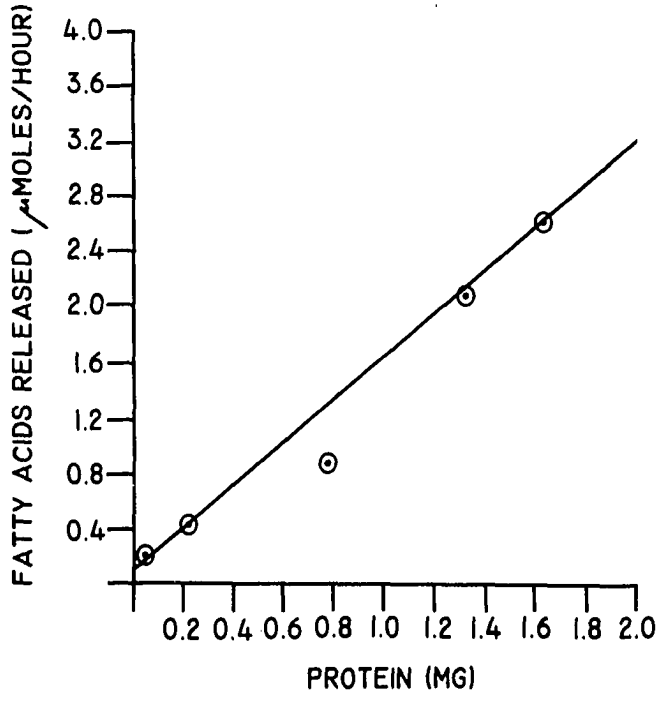

Figure 6. Fatty acids released from olive oil substrate as a function of the protein concentration of enzyme preparation.

BIPA was the enzyme source. The buffer system was 0.1 M phosphate (pH 7.2).

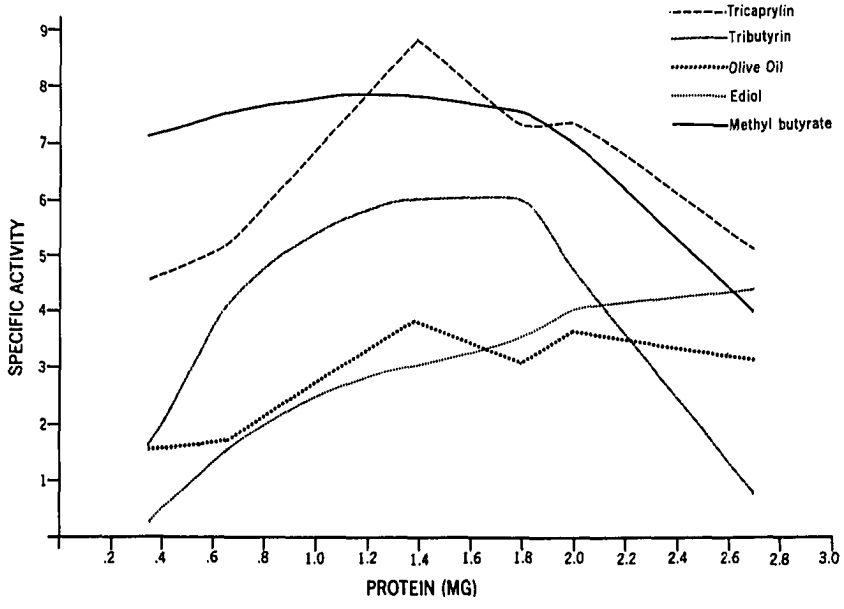

Figure 7. Fatty acids released from various substrates as a function of protein concentration of the enzyme preparation.

Incubation was carried out for 1 hr. with BIPA as enzyme source. The buffer system was 0.1 M phosphate (pH 7.2). Specific activity in this case is defined as the μEq. of fatty acids released in 1 hour.

tivity with some substrates, it was concluded that the protein concentration may effect the specific activity in a non-linear manner. Nevertheless, there were protein concentrations at which all activities could be demonstrated (from .7-1.7 mg.). The curve obtained with Ediol (Figure 7) showed the expected linear relationship between the enzyme protein concentration and rate of lipolysis. The curves for methyl butyrate, tricaprylin, and olive oil reached a point beyond which further increases of protein reduced the activity. The rate of methyl butyrate hydrolysis was very high when only small amounts of enzyme protein were present. This could have been the factor which made the specific activity of the 40-50% ammonium sulfate fraction in Table 6 appear disproportionately high.

Substrate Studies

Substrate specificities. Substrate preparations used for these specificity studies displayed a rather uniform appearance. With the exception of palmitic acid glycerides, the emulsions were stable for several days. Tri-, di-, and mono- palmitin containing emulsions appeared to be stable during the assays, but separated upon standing. With the exception of Ediol and olive oil which were used as outlined in the "standard" assay, the concentration of each substrate corresponded to 40 μEq. of esterified fatty acids. Three different enzyme preparations were used. Thus, there were three possible substrate preferences. From the results presented in Table 9 it will be seen that BIPH-1 attacked preferentially short-chain triglycerides (tributyrin, specific activity 7.2). The hydrolysis of long-chain triglyceride was either very slight or virtually absent. The utilization of monoolein indicated the possible presence of

TABLE 9

SUBSTRATE SPECIFICITIES[a]

Substrate	Specific Activity		
	BIPH-1	BIPH-2	BIPA
	1.6 mg. protein/assay	0.7 mg. protein/assay	1.4 mg. protein/assay
1% Methyl Butyrate	5.9	0.9	2.7
Methyl Stearate	-	0.6	0.5
Monoolein	3.8	2.5	0.2
Monopalmitin	-	2.0	0.9
Dipalmitin	0	-	0.2
Tributyrin	7.2	0.6	1.8
Tricaproin	2.8	1.8	9.5
Tricaprylin	0.7	2.3	6.8
Trilaurin	0	2.1	2.4
Trimyristin	0.2	2.9	4.9
Tripalmitin	0	0.5	0
Triolein	-	0.9	2.2
Ediol	0	1.1	1.2
Olive oil	0	2.3	6.2

[a]Phosphate buffer (pH 7.2) was used for this study.

a monoglyceride lipase. The specific activity obtained with 1% methyl butyrate as substrate could be explained by postulating either the presence of an esterase or the ability of lipase to split esters. BIPH-2 (Table 9) was active primarily on the medium-chain triglycerides and showed little activity toward tributyrin or esters. This medium-chain triglyceridase was also active on long-chain triglycerides and monoolein. BIPA (Table 9) was equally active toward both medium-chain triglycerides (tricaproin, specific activity 9.5 and tricaprylin, specific activity 6.8) and long-chain triglycerides (olive oil, specific activity 6.2). Activity on monoglyceride was low in contrast to the high activity seen with BIPH-1 and BIPH-2.

The enzyme protein active toward olive oil was present primarily in the fraction obtained as a precipitate from 50% ammonium sulfate saturation. This observation was made in a study of the substrate specificities of ammonium sulfate precipitates (Table 10). This precipitation increased the specific activity in all cases. Various lipolytic activities were not affected quantitatively to the same extent by a given precipitation. Tricaprylin activity, for example, was still very high in the 75% precipitate, while tricaproin activity which had initially been quite high was markedly diminished in the same fraction. If only one protein were responsible for all of the lipolytic activities, it would seem reasonable to have expected all activities to have been affected proportionately by the precipitation.

Substrate concentration. Methyl butyrate can exist either in a true, molecular solution (low concentrations) or in a supersaturated solution (high concentrations). It could thus be used as substrate for

TABLE 10

SUBSTRATE SPECIFICITIES[a] OF ENZYME FRACTIONS OBTAINED BY AMMONIUM SULFATE FRACTIONATION OF BIPA

Substrate	Specific activity of fractions			
	Unfractionated	0-50%	50-60%	60-75%
1% Methyl Butyrate	3.7	11.2	3.5	5.6
Tributyrin	0.7	7.0	1.7	0.6
Tricaproin	6.2	26.0	20.0	4.8
Tricaprylin	10.9	31.6	16.2	21.0
Trilaurin	2.8	6.1	1.8	2.1
Trimyristin	2.1	9.7	5.0	5.0
Triolein	2.9	7.0	2.9	1.9
Olive oil	4.2	11.5	1.2	0.5

[a]Phosphate buffer (pH 7.2) was used for this study.

either esterase or lipase. With acetone powder, both activities were noted (Figure 8) when evaluated with increasing concentrations of substrate. As expected, esterase was active at low substrate concentrations. With increasing concentrations of methyl butyrate the esterolytic activity decreased until the saturation point was reached and lipase activity appeared.

The effect of substrate concentration on the rate of hydrolysis of various substrates is shown in Figure 9. With tributyrin and tricaprylin a lag in the increase of activity was observed between substrate concentrations from 10 to 20 μEq. of esterified fatty acids. A reasonable appearing curve was obtained using olive oil. By comparison with a classical enzyme system, an abnormal slowing of the velocity with increasing substrate was indicated.

Studies on the effect of additives. A preliminary study on the effect of additives was carried out in order to test the suggestion that albumin (as fatty acid acceptor) would increase lipase activity. Sodium desoxycholate was also studied in view of its known effect upon pancreatic lipase. For this purpose, the alterations were made with a "standard" Ediol substrate by adding either albumin or replacing the desoxycholate solution with a corresponding portion of buffer. The results of this experiment are presented in Figure 10. The lipolytic activity increased several fold in the presence of sodium desoxycholate. The presence of albumin as the sole additive caused an inhibition of activity.

The advantage of incorporating sodium desoxycholate into the long-chain triglyceride substrate was also demonstrated again in a comprehensive study of the effect of additives upon olive oil and tributyrin

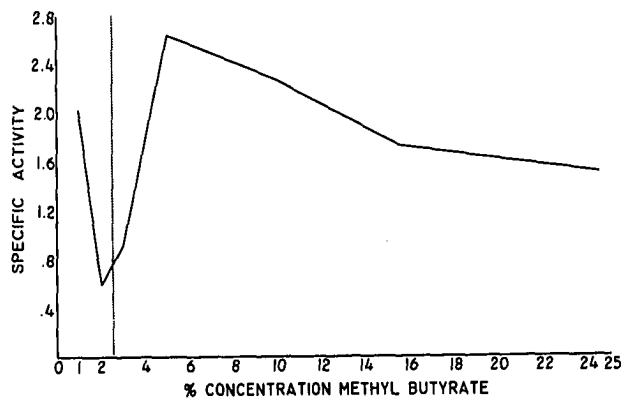

Figure 8. Effect of increasing concentrations of methyl butyrate substrate.

Esterolytic activity is noted toward unsaturated concentrations and lipase activity is observed after the substrate becomes saturated. The enzyme source was acetone powder. The buffer system was 0.1 M phosphate (pH 7.2).

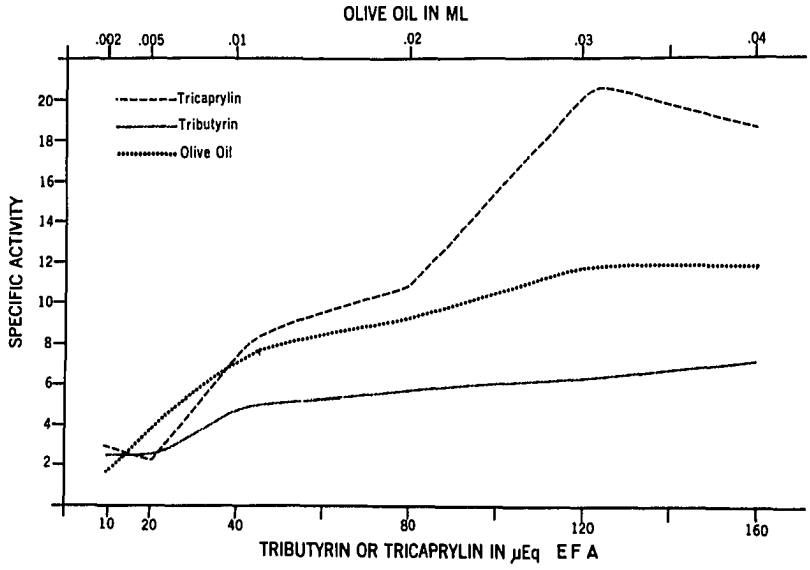

Figure 9. Effect of increasing concentrations of substrate.

The enzyme source was BIPA (0.96 mg. protein/assay). The buffer system was 0.1 M phosphate (pH 7.2).

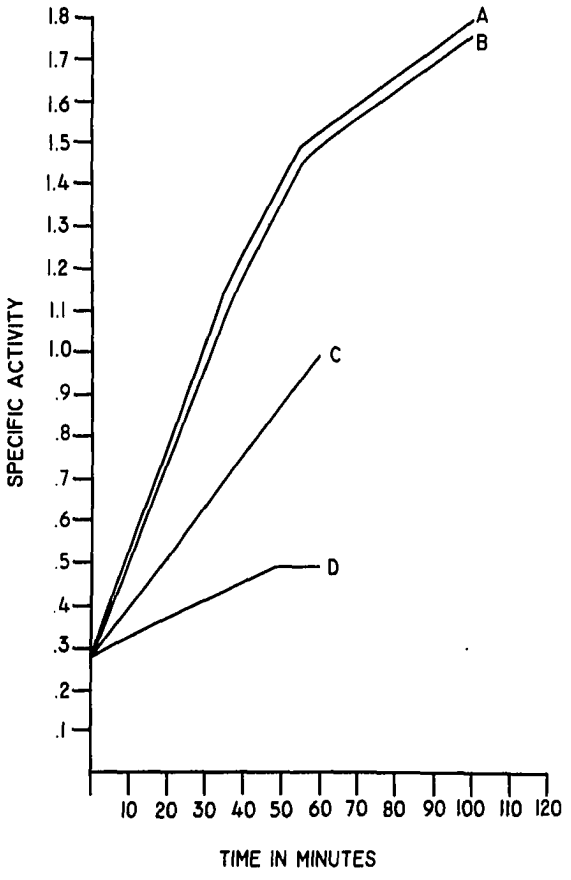

Figure 10. Effect of desoxycholate and albumin on lipolytic activity.

The incubation mixture contained 1 part acetone powder enzyme preparation, 1 part Ediol (1:3 by vol.), 7 parts 0.1 M phosphate buffer (pH 7.2) and one of the following: A, 1 part 4% sodium desoxycholate; B, 1 part 4% sodium desoxycholate and defatted albumin to a final concentration 10%; C, 1 part phosphate buffer, and D, 1 part phosphate buffer and defatted albumin to a final concentration of 10%. Specific activity in this case is defined as μEq. fatty acids released per mg. protein.

substrates (Table 11). The additions were made in quantities sufficient to give the indicated final concentrations. The activity toward olive oil was increased almost two-fold by the addition of desoxycholate. Apparently, the concentration was not critical. The activity toward tributyrin was not enhanced by this addition. The presence of albumin as the sole additive strongly inhibited the olive oil activity (Table 11). On the other hand, tributyrin activity was not affected by albumin at the highest concentration used. Desoxycholate reversed partially the inhibitory effect of albumin on the hydrolysis of olive oil. Tributyrin activity in the presence of albumin was not altered further by the addition of desoxycholate. The addition of $CaCl_2$ produced only slight effect upon activities. The effect of a simultaneous addition of $CaCl_2$ and desoxycholate on the activity toward olive oil was to decrease the activity somewhat from that observed with desoxycholate alone. This effect was concentration dependent. Conversely, the combination of $CaCl_2$ and desoxycholate caused a pronounced increase in activity toward tributyrin. This activation was increased three-fold with the higher concentration of $CaCl_2$.

The opposite effects observed with certain additives, and with the substrates studied suggests again that separate enzymatic activities might be involved.

Inhibitor Studies

Some inhibition of activity was effected by adding sulfhydryl inhibiting agents. For these studies, shown in Table 12, the inhibitor in the indicated quantities was added to the enzyme preparation and the mixture allowed to stand for 30 min. in an ice bath before assaying with

TABLE 11

EFFECTS OF ADDITIVES TO THE SUBSTRATE MEDIA[a]

Additive	Specific Activity	
	Olive oil	Tributyrin
No additions	4.1	1.2
Sodium desoxycholate (2 mg./ml.)	7.4	0.5
Sodium desoxycholate (4 mg./ml.)	7.6	1.1
Sodium desoxycholate (8 mg./ml.)	7.4	0.4
Albumin (80 mg./ml.)	0.2	1.2
Albumin (40 mg./ml.)	0	0.5
Albumin (80 mg./ml.) + Desoxycholate (4 mg./ml.)	3.5	1.0
Albumin (40 mg./ml.) + Desoxycholate (4 mg./ml.)	5.9	0.6
$CaCl_2$ (0.1 M)	4.9	0.6
$CaCl_2$ (0.01 M)	4.3	0.5
$CaCl_2$ (0.1 M) + Desoxycholate (4 mg./ml.)	5.2	3.8
$CaCl_2$ (0.01 M) + Desoxycholate (4 mg./ml.)	6.6	1.2

[a]Phosphate buffer (pH 7.2) was used for this study. The tissue preparation was BIPA.

TABLE 12

EFFECT OF INHIBITORS ON LIVER LIPOLYTIC ACTIVITY[a]

Inhibitor	Concentration	% Inhibition
p-Chloro-mercuri benzoic acid	1×10^{-2} M	18%
	1×10^{-3} M	26%
Iodine	1×10^{-3} M	36%
	1×10^{-4} M	11%
Rhodamine B	5×10^{-4} M	10% activation
	5×10^{-2} M	10% activation
Iodoacetic acid	1×10^{-2} M	5%
	1×10^{-1} M	5%

[a]Phosphate buffer (pH 7.2) was used for this study. The tissue preparation was acetone powder.

"standard" Ediol substrate. In further experiments, a number of compounds routinely studied as inhibitors of lipolytic activity were tested. Some of the results are shown in Tables 13 and 14. Different BIPA enzyme preparations were used for each of the two studies. Substrates were prepared in the standard manner (phosphate buffer, pH 7.2 and desoxycholate). Lipoprotein lipase substrate and lipoprotein lipase assay system were prepared using ammonium sulfate buffer (pH 8.5). The quantity of inhibitor to give the indicated final concentration was added to the substrate and the mixture was preincubated for 30 min. at 37°. The control substrates were incubated without inhibitor.

As shown in Table 13, methyl butyrate and tributyrin activities were enhanced in the presence of protamine sulfate or NaCl. The hydrolysis of olive oil was inhibited markedly by protamine sulfate and slightly by NaCl. Conversely, Atoxyl and Eserine caused an inhibition of methyl butyrate and tributyrin activities, but had no effect on the hydrolysis of olive oil. Activities toward monoolein and tricaprylin were inhibited by all agents tested. NaF and E-600 were potent inhibitors for all the activities.

Results presented in Table 14 show that the degree of increasing activation of tributyrin is a function of an increasing concentration of protamine sulfate or NaCl. Similarly, the increasing inhibition of activity toward olive oil and Ediol by protamine sulfate is proportional to an increasing concentration of inhibitor. However, the inhibition of olive oil hydrolysis by NaCl is effected in a reverse manner with small concentrations being more effective inhibitors than high concentrations.

Protamine sulfate and NaCl (higher concentrations) were inhibi-

TABLE 13

EFFECT OF INHIBITORS ON LIVER LIPOLYTIC ACTIVITY

Inhibitor	% Activity				
	1% Methyl Butyrate	Tributyrin	Tricaprylin	Olive Oil	Monoolein
No Inhibitor[a]	0.3 (100%)	1.0 (100%)	2.9 (100%)	3.1 (100%)	1.8 (100%)
Protamine sulfate 5 mg./ml.	300	325	23	8	55
NaF 2×10^{-1} M	0	9	12	35	0
NaCl 0.5 M	117	115	86	94	54
E 600 1 mg./ml. (sat.)	0	0	0	8	0
E 600 1×10^{-3} M (unsat.)	0	12	0	4	17
Atoxyl 5×10^{-3} M	79	33	49	105	25
Eserine 5×10^{-3} M	55	54	63	105	71

[a]Expressed as specific activity.

TABLE 14

EFFECT OF INHIBITORS ON LIVER LIPOLYTIC ACTIVITY

Inhibitor	% Activity				
	Tributyrin	Olive oil	Ediol	Lipoprotein lipase assay system	Lipoprotein lipase assay system + desoxycholate (4 mg./ml.)
No inhibitor[a]	0.8 (100%)	5.1 (100%)	2.0 (100%)	0.6 (100%)	4.1 (100%)
Protamine sulfate					
200 µg.	118	33	30	22	22
400 µg.	127	33	20	22	22
1 mg.	172	40	17	45	18
5 mg.	207	6	10	22	22
NaCl					
0.1 M	131	73	118	3	54
0.5 M	170	81	113	-	32
1 M	202	83	62	23	23
2 M	211	89	67	14	22

[a]Expressed as specific activity.

tory to the hydrolysis of Ediol used in "standard" assay system as well as when it was incorporated in the lipoprotein lipase assay system. The specific activity for Ediol in "standard" medium was 2.0. In the lipoprotein lipase assay system it was reduced to 0.6. Addition of sodium desoxycholate to the lipoprotein lipase assay system increased the activity to 4.1. This was a six-fold increase in activity above that displayed in the lipoprotein lipase assay system and a two-fold increase in activity toward Ediol in "standard" medium.

Sodium desoxycholate has been considered to be an inhibitor of lipoprotein lipase (57). As shown in Table 15 lipolytic activity in post-heparin serum was decreased 76% when desoxycholate was added to the lipoprotein lipase assay system. The results of this experiment also provided evidence that the lipolytic activity in the liver was not due to lipoprotein lipase. Instead of a decrease, the addition of desoxycholate resulted in a six-fold increase of the lipolytic activity of liver preparations.

Localization of Lipolytic Activity

Subcellular fractionation of liver homogenates was carried out to determine if such methods could be used as a means of enzyme purification and as a technique for locating the site of enzyme activity within the cell.

Initial studies were done using Ediol and methyl butyrate as substrates. The results are shown in Table 16. Although the specific activities of the different fractions varied widely the localization with Ediol as substrate was fairly consistent. Although not apparent in this experiment, the soluble fraction occasionally displayed esterase activity.

Many subcellular fractionation experiments were performed in

TABLE 15

THE EFFECT OF DESOXYCHOLATE ON THE LIPOPROTEIN LIPASE ASSAY SYSTEM

	Specific Activity		
	Normal serum	Post-heparin serum	Liver[a] preparation
Lipoprotein lipase assay system	0.80	14.00	0.60
Lipoprotein lipase assay system + sodium desoxycholate (4 mg./ml.)	0.66	3.40	4.10

[a]Ammonia extract of BIPA.

TABLE 16

COMPARISON OF LIPOLYTIC AND ESTEROLYTIC ACTIVITY
OF SUBCELLULAR FRACTIONS

Fractions	Mg. protein	Specific Activity	
		Ediol	1% Methyl Butyrate
Whole homogenate	10.5	0.15	0.87
Mitochondria	6.7	0.23	1.37
Microsomes	7.3	0.21	1.25
Soluble fraction	0.96	2.64	0

which lipolytic activity was evaluated as activity toward tributyrin. In five experiments, using fifteen animals, the microsomal fraction displayed consistently the highest specific activity. Still, appreciable activity was present in the mitochondrial fraction and lesser activity in the soluble fraction. The information provided in Table 17 was obtained in a comparative study of the subcellular fractions from livers of 3 animals. Activity toward tributyrin was high in both the microsomal fraction and the mitochondrial fraction. Tricaprylin activity also appeared in both mitochondrial and microsomal fractions, while the activity toward olive oil was too low to be evaluated.

TABLE 17

COMPARISON OF ACTIVITY TOWARD VARIOUS SUBSTRATES
BY SUBCELLULAR FRACTIONS

Fraction	Mg. protein	Specific Activity			
		Olive oil	Tricaprylin	Tributyrin	1% Methyl Butyrate
Whole homogenate					
1	3.7	0	-	2.0	1.7
2	4.1	0.11	0.45	1.7	1.6
3	4.2	0.06	0.31	1.6	1.5
Mitochondria					
1	2.6	0	0.65	1.6	1.9
2	2.7	0	0.52	1.7	1.8
3	2.3	0.1	0.58	2.0	2.1
Microsomes					
1	5.7	0	0.26	1.5	1.0
2	5.1	0.05	0.43	1.7	1.1
3	1.8	0	1.2	3.9	2.9
Soluble fraction					
1	2.6	0	0.12	0.15	0.72
2	2.7	0.1	0.12	0.17	0.91
3	2.8	0	0.13	0.14	0.70

CHAPTER IV

DISCUSSION

> "As long as an enzyme cannot be obtained in a pure state, it must be defined by its catalytic activity that is to say, by the over-all reaction in which it is catalyst. The situation is quite clear when its specificity is absolute. But when several substrates have to be considered, a somewhat embarrassing choice must be made."
> P. Desnuelle (13)

Demonstration and Isolation of Liver Triglyceride Lipase

In this research effort an enzyme(s) which fulfills the proposed criteria for triglyceride lipase (glycerol-ester hydrolase, EC 3.1.1.3) was isolated for the first time from rat liver (95, 96). The observations in this study have been related to a criterion according to which the classification of enzymes catalyzing hydrolysis of various esters is based on the physical-chemical state of substrates. According to the proposal of Sarda and Desnuelle (3), lipases are enzymes which hydrolyze esters in a heterogeneous system.

This lipolytic activity was first observed during our studies on the lipemia clearing effect of bacterial lipopolysaccharides (1). Histochemical studies showed that lipopolysaccharide-treated rats accumulated intravenously injected emulsified triglycerides preferentially in the liver parenchymal cells while untreated rats accumulated them primarily in

the liver Kupffer cells. It has been hypothesized that the lipopolysaccharides might cause the increased triglyceride uptake by hepatic parenchymal cells through a mechanism involving a change in the cellular permeability and a greater metabolic requirement during the stress produced by lipopolysaccharide injection. The virtual absence of lipoprotein lipase in the serum and liver of lipopolysaccharide-treated animals prompted an investigation of other possible lipolytic enzymes which could be responsible for the removal and metabolism of long-chain triglycerides in the liver.

The mammalian liver contains several types of enzymes which might be concerned with the hydrolysis of liver lipids. Unfortunately, the differences between liver ali-esterase and lipase have not been clearly defined in the literature. It had been assumed that a liver triglyceride lipase was virtually absent and/or due to one and the same enzyme.

In the present studies, it was demonstrated (Table 1 and 2) that the observed lipolytic activity was not due to lipopolysaccharide treatment but that it was present also in many normal animals. The degree of activity ranged from negligible values to values sufficient to be readily evaluated. The very low levels of lipolytic activity detected in many animals may account for the previous failures to demonstrate it.

Recently, the results of several independent investigations have confirmed the presence of a liver lipase (41, 42, 91-98, 106). Although the definition of lipase in these reports was consistent, direct comparison of results seemed almost impossible. The indication in our studies of the presence of more than one liver lipolytic activity may possibly explain some discrepancies in the results. Vavrinkova and Mosinger (94,

97) suggested, on the basis of two pH optima, the presence of two lipolytic enzymes. However, differences they noted with respect to the effect of albumin and inhibitors (97) make further comparison virtually impossible. On the basis of differences in heat inactivation and response to diisopropyl fluorophosphate, Carter (98) suggested that different enzymes may be acting on triglycerides and monoglycerides. The triglyceride activity in soluble cellular fractions was more heat labile than monoglyceride activity. Diisopropyl fluorophosphate inhibited triglyceride hydrolyzing activity but it enhanced the rate of monoglyceride hydrolysis.

All of these studies have clearly demonstrated that the liver lipolytic activity is different from that of lipoprotein lipase. In our experiments, the same conclusion was based on the following findings: (1) the lack of any stimulatory effect of the in vivo administration of heparin on activity in liver preparations (Table 1) and (2) the marked differences in in vitro response to activators and inhibitors of heparin-stimulated serum lipase and liver lipase (Tables 14 and 15). Sodium desoxycholate was a potent inhibitor of post-heparin serum lipolytic activity but an activator toward long-chain triglycerides. Similarly, albumin, considered a lipoprotein lipase activator (107, 108), was inhibitory to long-chain triglyceride lipase activity in the liver (Table 11). Mayes and Felts (65) suggested the presence in rat liver of an inactive lipoprotein lipase generally considered to be virtually absent in this organ (7, 34, 39, 49, 62, 63).

Isolation and Purification

Attempts to isolate or purify the enzyme responsible for this

liver lipolytic activity have not been described previously. The isolation of the enzyme as a butanol-water interfacial preparation gave a product of increased activity. It appears that the esterase activity is excluded from the interfacial preparations. Benzonana and Desnuelle (109) found that there was an actual adsorption of pancreatic lipase at an oil/water interface and a depletion of the enzyme from the bulk aqueous phase. The affinity of lipase for an interface has been utilized by Baskys, Klein and Lever as the initial step in purifying adipose tissue lipase (110), pancreatic lipase (111), and lipoprotein lipase (112) by adsorbing the enzyme at an ether/water interfacial layer.

Although it did not separate the individual enzyme activities, the ammonium sulfate fractionation technique used in the present studies removed a portion of the inert protein thus allowing an enrichment of activity. The finding that all lipolytic activities were not affected proportionately by a given ammonium sulfate precipitation (Table 10) suggested the involvement of more than one lipolytic enzyme. A seven-fold increase in the activity of enzyme protein recovered from Sephadex columns suggested that gel-filtration might be an useful purification step. It is probable that fractionation on Sephadex columns should be preceded by either butanol-water treatment or ammonium sulfate fractionation in order to exclude as much inert protein as possible and allow for a sharper separation.

Characterization Studies

Although separation of discrete lipolytic activities has not yet been accomplished, variations in activity in response to changes in experimental conditions suggested the presence of more than one lipolytic

activity. Although such variations in the lipolytic activities could be explained by factors other than the enzyme protein, some appear to be valid evidence for the presence of several enzymes. The three discrete pH optima displayed with olive oil and tricaprylin as substrates represented an indication for more than one enzymatic activity. These substrates showed optima at approximately pH 5.5, pH 7.0 and pH 8.0. The optimum at pH 8.0 was absent when tributyrin was used. Vavrinkova and Mosinger (94) found an optimal lipolytic activity toward Ediol in liver homogenates at pH 5.0 with a secondary activity at pH 8.0. In our preparations the highest specific activity for tricaprylin was at pH 7.0, while that for olive oil was at pH 8.0 (Figure 4). Additional studies with buffering systems found to be preferential for each particular substrate are needed in order to evaluate the significance of these activity optima. It was found (Tables 7, 8), for example, that the olive oil activity was generally higher with Tris than with phosphate buffer; the reverse was true for tributyrin activity. Glycyl glycine buffer was somewhat inhibitory to olive oil activity but accentuated the activity of either tributyrin or tricaprylin.

Lipolytic activity was demonstrated toward a wide range of substrates. However, some preparations seemed devoid of activity against certain substrates, suggesting again, that more than one activity was responsible for the overall triglyceride hydrolysis. The degree of activity toward a substrate could be accentuated or depressed by varying the conditions (buffers or additives) of the assay system. Such manipulations could alter the apparent specificity of a preparation. Individual preferences for short-chain, medium-chain, and long-chain triglycerides were

noted in different preparations. For example, the highest activity in BIPH-1 (Table 9) appeared to be displayed by a short-chain triglyceridase (a tributyrinase); the activity in BIPH-2 (Table 9), by a predominantly medium-chain triglyceridase (tricaprylin as substrate); and that in BIPA (Table 8, phosphate buffer), by a long-chain triglyceridase (olive oil as substrate). Since many factors influence the degree of lipase action upon a given substrate, both the physical state and the chemical nature of the substrate must be considered when specificities are defined. For this reason it would be unwise to cite apparent substrate specificities also as a valid evidence for the presence of more than one enzyme.

Bile salts have long been known to serve as activators for pancreatic lipase (77). This activation was thought to be limited to hydrolysis of long-chain triglycerides. Glick and King (113) found, for example, that bile salts inhibited rather than enhanced lipase activity on a saturated solution of tributyrin. The role of bile salts in these reactions was thought to be merely to aid in the emulsification of insoluble substrates. For this reason, Wills (114) suggested that the activation could be non-specific and that any detergent might serve equally well. However, his experiments showed that sodium dodecyl sulfate was inhibitory to the hydrolysis of triolein by lipase. In contrast, sodium taurocholate was a powerful activator. Borgstrom (101) observed that the activating effect of taurocholate was dependent upon its concentration and upon pH value. It is now generally accepted that the bile salts act in some capacity other than as emulsifying agents.

Desnuelle (13) demonstrated that pancreatic lipase activity toward triolein was temperature dependent only in the presence of bile

salts. By using a purified pancreatic lipase, Fritz and Melius (115) found that hydrolysis of both triolein and tributyrin was temperature dependent regardless of the addition of bile salts. They also found that both substrates were activated by the addition of taurocholate. The energy of activation was almost twice as great when lipolysis took place in the absence of taurocholate. The following mechanisms for the hydrolysis were proposed by them:

(a) in the absence of bile salt,

$$E+TG \overset{(fast)}{\longleftrightarrow} E.TG \xrightarrow[-FA]{(fast)} E.DG \xrightarrow[-FA]{(slow)} E.MG \xrightarrow[-FA]{(slow)} E.G \xrightarrow{(slow)} E+G.;$$

(b) in the presence of bile salt,

$$E+TG \overset{(fast)}{\longleftrightarrow} E.TG \xrightarrow[-FA]{(fast)} E.DG \xrightarrow[(fast)]{(taurocholate)} E+DG$$

In the presence of bile salt the observed rate was considered to be essentially proportional to the rate of hydrolysis of triglyceride to diglyceride. Experimentally, the reaction rate was linear with time. In the absence of taurocholate more than one hydrolytic reaction occurred and the rate curve represented a complex relationship of the rates of all steps. The products of the hydrolysis were isolated and, indeed, the ratios of monoglycerides/diglycerides indicated that the first step, $E.TG \overset{(fast)}{\underset{-FA}{\longleftrightarrow}} E.DG \xrightarrow[(fast)]{(taurocholate)} E+DG$, was favored when taurocholate was present. They observed that the effectiveness of the bile salt was increased at alkaline pH, suggesting that the bile salt anion was the active agent. Reports in the literature which support the idea that cholic

acid derivatives are capable of splitting lipoprotein complexes led Fritz and Melius (115) to suggest that taurocholate cleaves the enzyme-substrate complex, thereby freeing the enzyme for further reaction with substrate, and that the cholic acid derivatives could be activators for any lipolytic enzyme with an alkaline pH optimum. Glycocholate and taurocholate were found to be more effective activators than desoxycholate. This finding was explained by the ability of desoxycholate to rapidly break down not only enzyme-diglyceride, but also enzyme-triglyceride complexes.

The results of experiments on the effect of sodium desoxycholate on lipolytic activities (Table 11) seemed to indicate the presence of more than one enzyme. However, the findings may represent merely differing effects of bile salts upon the equilibrium constants of the individual enzyme-triglyceride complexes. Desoxycholate activated the hydrolysis of long-chain triglycerides but it inhibited the hydrolysis of short-chain triglycerides. This apparently paradoxical finding should be explored further by studing the effect of other bile salts on triglycerides of various chain lengths at various pH optima and with various buffering systems.

Vavrinkova and Mosinger (94) found that liver lipase activity is increased by the addition of albumin to the assay medium, the optimum concentration being 2-5%. Carter (98) also used albumin in his complete assay system. In our present studies, albumin was found to be inhibitory, especially toward the hydrolysis of long-chain triglycerides. This effect was not observed when tributyrin was used as substrate (Table 11). In the lipoprotein lipase assay system it has been shown that albumin plays an important role as the acceptor of released long-chain fatty acids

which, if allowed to accumulate, inhibit the enzyme (107, 108). Conflicting reports have appeared concerning the effects of albumin on pancreatic lipase. DiNella, Meng and Park (18) found that bovine serum albumin increased the rate of this enzyme; however, Fraser and Nicol (116) noted that it was a potent inhibitor of the enzyme. Almost complete inhibition occurred at a concentration of 2.0 mg./ml.

Calcium chloride has occasionally been substituted advantageously for albumin as a free fatty acid acceptor in the lipoprotein lipase assay system (66). Wills (4) concluded that Ca-ions play a dual role in pancreatic lipase action, serving as a metal (divalent ion) stabilizer and as an activator of the enzyme when long-chain triglycerides are hydrolyzed. Borgstrom (101) observed that the addition of ions to pancreatic juice accelerated the rate of hydrolysis of triglycerides (olive oil as substrate, at all pH values). At the same time a decreased rate of resynthesis of triglycerides was observed. Calcium chloride increased slightly the olive oil activity of liver lipase (Table 11) but it decreased the tributyrin activity. However, when it was added to an assay system containing sodium desoxycholate, calcium chloride inhibited the expected olive oil activity and activated the tributyrin hydrolysis.

Whether all of these effects of additives could be attributed to either "favorable" or "unfavorable" alterations of the enzyme-substrate and enzyme-product equilibria, as postulated by Fritz and Melius (115) for cholic acid derivatives, could not be ascertained from these experiments. The activation or inhibition produced by the additives was different for short-chain and long-chain triglycerides. This is perhaps a reflection of the relative ease with which a triglyceride emulsion can be

either made or broken depending upon the fatty acid chain length. The importance of the state of the emulsion upon the rate of lipolysis has been demonstrated succinctly by Desnuelle and coworkers (3, 13). By mixing different concentrations of substrate in emulsions, they showed that for a constant weight of substrate, the initial rates vary with the interfacial area. The initial rate for a certain substrate concentration was lower with a less dispersed emulsion than with a highly dispersed one. According to their observations anything which would alter the emulsified state and thus the available interface would affect the observed rate of activity.

Wills (4) found that pancreatic lipase could be inhibited by certain SH-reagents but was resistant to others. The inhibition varied with the substrate used. He asserted that it is unlikely that sulfhydryl groups are part of the active center but that they may be adjacent to it. In the present studies a similar inhibition by SH-reagents was observed for liver lipase (Table 12). Desneuelle (13) assumed, by analogy to esterases, that lipases contain an active serine which should be blocked by organophosphates. He and his associates showed that pancreatic lipase was not inhibited by diethyl p-nitrophenyl-phosphate (E-600) in solution (100). With emulsions containing low concentrations of inhibitor a part of the activity disappeared. With more concentrated emulsions of inhibitor, the activity disappeared completely and irreversibly. In the studies reported here liver lipase was also completely inhibited by an emulsion of E-600 (Table 13).

The degree of inhibition of liver lipase activity by Atoxyl and Eserine, which are considered to be esterase inhibitors (84), varied de-

pending upon the substrate (Table 13). With short-chain and medium-chain triglycerides (tributyrin and tricaprylin) as substrates, lipolytic activity was inhibited as much as fifty percent by the Atoxyl and Eserine while with olive oil as substrate there was a slight activation. The inhibitory effect was reversed in the case of protamine sulfate and NaCl. Protamine sulfate and NaCl increased the tributyrin activity, but they decreased the olive oil activity. These studies were not designed to determine what factors in the liver lipase assay system are affected by the inhibitors. Therefore, it was not possible to conclude whether the additives influenced the enzyme-substrate complex, or the availability of interfacial area, or the enzyme protein; all are possible. However, it was observed that the degree of inhibition or activation by protamine sulfate and NaCl was dependent upon the concentration of inhibitor.

At least two conclusions can be drawn from the observations of an inverse relationship of inhibition/activation by Atoxyl and Eserine versus protamine sulfate and NaCl on tributyrin or olive oil hydrolysis. The inhibitors could possibly (1) block the enzyme responsible for one activity, but not for another and (2) cause different effects upon the substrate emulsion or enzyme-substrate complex, in one case producing alterations favorable to long-chain triglyceride lipase and, in the other, producing alterations favorable to short-chain lipase. The presence of at least two individual enzymes is indicated in both cases.

Kinetic Studies

Since the natural substrates (long-chain triglycerides) for lipases are not water soluble, the classical concepts of enzymology must be modified in the case of these compounds. Lipases act not on soluble

substrates but on emulsified triglyceride droplets separated from water by an interface (13).

Mathematical representations are not yet available which would make possible the definition of Michaelis constants for "interfacial enzymology". Benzonana and Desnuelle (109) have approached recently this problem for pancreatic lipase. They have made the following observations. The lipase is adsorbed by its emulsified substrate and the initial rate of the reaction is a function of the number of enzyme molecules adsorbed at the interface. By varying the components of the assay system, a linear Lineweaver-Burk representation could be obtained. However, the abscissae of the representation and the K_m values remained undefined. Thus, the usual K_m concept is of little value when the substrate is in the insoluble state. By using two emulsions containing particles of different sizes they showed that the initial velocity depends on the area of the interface rather than directly upon the weight of the insoluble substrate; the rate of lipolysis was inversely proportional to the total volume of the emulsion. In the case of lipase, the interfacial area (area of interface/total volume of emulsion) - the volume of emulsion unit - plays the role normally ascribed to the substrate concentration. The K_m (emulsion) of lipase corresponds to a certain value of the interfacial concentration.

Obviously, kinetic evaluation would be of little value for liver lipase at this point. It is still not certain how many individual enzyme activities are present in the preparations. Furthermore, the most suitable emulsifying and stabilizing components of the liver lipase assay system necessary for achieving a linear rate relationship are not yet known. However, it seems possible that the recently developed concepts

of interfacial enzymology will make the kinetic evaluation of heterogeneous catalysis feasible.

Experimentally obtained rate curves (Figures 5, 7, 9) indicated a very complex relationship of the rates of several hydrolytic steps. This complex rate relationship might be the result of the action of more than one enzyme, or it could represent a complicated lipolytic pattern involving activation and/or inhibition of intermediate reaction steps. The enzyme preparations used in these experiments contained not only three possible lipolytic activities showing different triglyceride substrate and activator preferences, but also monoglyceride activity. Also, the assay system used contained bile salt which has been shown to affect the thermodynamic equilibrium of individual substrates to different degrees (115). The effect of gum arabic, used as an emulsifier, upon the equilibrium is not known.

The various factors we have been discussing may cause complicated rate relationships and thereby influence specific activities and apparent specificities. However, they do not fully explain unusual relationships such as those shown in Figure 7, where increasing concentrations of protein virtually inhibited the lipolysis. Perhaps this problem will be resolved when pure enzymes become available. Although no experimental evidence is available, the following speculations are suggested as possible explanation for the inhibition of lipolysis at higher protein concentrations: (1) a reverse lipolysis might take place causing the observed activity to be smaller (such a resynthesis of triglyceride was noted by Borgstrom (101) with pancreatic lipase), (2) lipolysis might occur with a particular complex formed only with appropriate amounts of enzyme and

substrate, and (3) increasing concentrations of protein might alter adversely certain triglyceride emulsions (different effects were noted with the individual substrates). Until this phenomenon is adequately understood the effect of protein concentration on the rate of lipolysis must be evaluated separately for each enzyme preparation and substrate.

Localization of Activity

In the present studies, all liver subcellular fractions showed activity. With tributyrin and tricaprylin as substrates the highest specific activity was present in the microsomes. However, with Ediol as substrate the highest activity was present in the soluble fraction. Ediol contains monoglycerides as well as triglycerides. What portion of the activity is related to monoglyceride lipolysis was not determined. Although Carter (98) found the highest specific activity toward triolein and tripalmitin in microsomes some lipolytic activity was present in all subcellular fractions. Higgins and Green (41) found the plasma membrane to have the greatest capacity per unit weight for binding lipid and for catalyzing triglyceride hydrolysis.

Demonstration of Individual Lipolytic Activities

The results presented here strongly suggest that the liver contains either two, or possibly three, separate lipases or one enzyme with relatively distinct activities or active sites for short-chain, medium-chain, and long-chain triglycerides. The presence of these activities is asserted on the basis of preferences for substrate, pH, buffer, and additives displayed by various liver enzyme preparations, as well as on the basis of segregation of activities by protein fractionation procedures.

The "standard" assay system used in this study appears adequate for the detection of a liver triglyceride lipolytic activity. In order to differentiate between specific lipolytic activities the following alterations should be introduced in the assay system: (1) for short-chain triglyceridase activity, tributyrin emulsion should be utilized as substrate, glycyl glycine should be used as buffer (pH 7) and albumin or a combination of $CaCl_2$ and desoxycholate should be used as additives, (2) for medium-chain triglyceridase activity, tricaprylin emulsion, glycyl glycine buffer (pH 7.4) and desoxycholate as additive should be used, (3) for long-chain triglyceridase activity olive oil emulsion as substrate, Tris buffer (pH 8.0) and desoxycholate as additive should be used.

Physiological Significance

The results of recent studies of several groups of workers (5-11) have suggested that chylomicron triglyceride-fatty acids are probably removed from the circulation without previous hydrolysis by liver or by extrahepatic tissues. Whereas removal of triglycerides by adipose tissue and heart depends most probably on the local lipoprotein lipase activity (48, 49, 50), in the liver the chylomicrons can be removed from the circulating blood as intact molecules and, after an initial lag period, triglycerides are hydrolyzed (39, 40, 43, 44). The latter hypothesis is based on two assumptions: first, the uptake of unchanged triglycerides and second, the presence of lipolytic activity in liver cells.

The detection and characterization of the lipolytic activities described in this study demonstrate the presence of a hepatic triglyceride-lipase and thus provide a heretofore "missing link" in the hypothesis on the mechanism of triglyceride uptake by the liver.

The unusually low levels of liver lipolytic activity in a relatively large number of animals and the finding of a hormone-sensitive lipase in adipose tissue by several groups of investigators (70, 71), suggests the possibility of a similar hormonal activator or system may be involved in the activation of liver lipase. Studies should be made to determine the effects of nutritional factors, such as fasting, high fat and high carbohydrate diets. Hormonal effects should be studied utilizing fat-mobilizing hormones such as epinephrine, ACTH and glucagon, or agents such as nicotinic acid and prostaglandins.

CHAPTER V

SUMMARY

Triglyceride lipase (glycerol-ester hydrolase, EC 3.1.1.3) has been isolated from rat liver for the first time (95, 96). This enzymatic activity has been related to the criterion proposed by Sarda and Desnuelle (3) that lipases are defined as enzymes which hydrolyze esters in a heterogeneous system. Enzyme preparations which catalyzed the hydrolysis of emulsified triglycerides were isolated from rat liver homogenates and were partially purified by butanol-water treatment, ammonium sulfate precipitation and Sephadex gel-filtration.

The liver lipolytic activity was shown to be different from lipoprotein lipase on the basis of (1) the lack of any stimulatory effect of the *in vivo* administration of heparin, and (2) marked differences in *in vitro* response to activators and inhibitors by heparin-stimulated serum lipase and liver lipase.

The possibility that more than one liver lipase is involved is suggested by the following evidence: (1) the presence of three discrete pH optima, (2) the fact that some preparations are devoid of activity against certain substrates, (3) preference of various preparations for short-chain, medium-chain or long-chain triglycerides, (4) the activating effect of specific buffers, (5) the finding that activities are not af-

fected proportionately by ammonium sulfate precipitation of enzyme preparations, and (6) an inverse relationship of inhibition/activation of tributyrin or olive oil activities when inhibitors or additives were added to their assay systems.

The problems of "interfacial enzymology" were discussed. The fact that lipase does not act on soluble substrates but, rather, on emulsified fat droplets separated from water by an interface requires considerations and treatments not necessary with enzymes whose substrates are water soluble.

All liver subcellular fractions possessed lipolytic activity. The highest activity toward Ediol was demonstrated in the soluble fraction of the liver cell. However, highest activities toward tributyrin and tricaprylin were found in the microsomes and mitochondria.

Assay systems which should be adequate to demonstrate the individual lipolytic activities were described.

The isolation of a liver triglyceride lipase has provided a heretofore "missing link" in the current hypothesis concerning the mechanism of triglyceride uptake by the liver.

BIBLIOGRAPHY

1. Young, A, C., Masters' Thesis submitted to Graduate College, University of Oklahoma (1963).

2. Meir, R., and Schuler, W., Experientia, 13, 249 (1957).

3. Sarda, L., and Desnuelle, P., Biochim. Biophys. Acta, 30, 513 (1958).

4. Wills, E. D., in Enzymes of Lipid Metabolism, P. Desnuelle, ed., Pergamon Press, New York, 1961, p. 13.

5. Bragdon, J. H., and Gordon, R. S., Jr., J. Clin. Invest., 37, 574 (1958).

6. Olivecrona, T., J. Lipid Res., 3, 439 (1962).

7. French, J. E., and Morris, B., J. Physiol., 140, 262 (1958).

8. Borgstrom, B., and Jordan, P., Acta Soc. Med. Upsalien, 64, 185 (1959).

9. Stein, Y., and Shapiro, B., J. Lipid Res., 1, 326 (1960).

10. Rodbell, M., Scow, R. O., and Chernick, S. S., J. Biol. Chem., 239, 385 (1964).

11. Olivecrona, T., and Belfrage, P., Biochim. Biophys. Acta, 98, 81 (1965).

12. Borgstrom, B., in Lipide Metabolism, K. Block, ed., John Wiley, New York, 1960, p. 128.

13. Desnuelle, P., in Advances in Enzymology, F. F. Nord, ed., Vol. 23, Interscience Publishers, New York, 1961, p. 129.

14. Schonheyder, F., and Volqvartz, K., Biochim. Biophys. Acta, 8, 407 (1952).

15. Borgstrom, B., Acta Physiol. Scand., 28, 279 (1953).

16. Savary, P., and Desnuelle, P., Biochim. Biophys. Acta, 21, 349 (1956).

17. Hofmann, A. F., J. Lipid Res., 3, 391 (1962).

18. DiNella, R. R., Meng, H. C., and Park, C. R., J. Biol. Chem., 235, 3076 (1960).

19. McPherson, J. C., Askins, R. E., and Pope, J. L., Proc. Soc. Exptl. Biol. Med., 110, 744 (1962).

20. Senior, J. R., and Isselbacher, K. J., J. Clin. Invest., 42, 187 (1963).

21. Johnston, J. M., in Advances in Lipid Research, R. Paoletti and D. Kritchevsky, eds., Vol. I, Academic Press, New York, 1963, p. 105.

22. Hofmann, A. F., and Borgstrom, B., Federation Proc., 21, 43 (1962).

23. Borgstrom, B., Dahlqvist, A., Lundk, G., and Sjovall, J., J. Clin. Invest., 36, 1521 (1957).

24. Gage, S. H. and Fish, P. A., Am. J. Anat., 34, 1 (1924).

25. Fredrickson, D. S., and Gordon, R. S., Jr., Physiol. Rev., 38, 585 (1958).

26. Palay, S. L., and Karlin, L. J., J. Biophys. Biochim. Cytol., 5, 363 (1959).

27. Laurell, C. B., Acta Physiol. Scand., 30, 289 (1953).

28. Bierman, E. L., Gordis, E., and Hamlin, J. T., III, J. Clin. Invest., 41, 2254 (1962).

29. Havel, R. J., and Fredrickson, D. S., J. Clin. Invest., 35, 1025 (1956).

30. Bates, M. W., Am. J. Physiol., 194, 446 (1958).

31. Fredrickson, D. S., McCollester, D. L., and Ono, K., J. Clin. Invest., 37, 1333 (1958).

32. Bierman, E. L., and Hamlin, J. T., III, Proc. Soc. Exptl. Biol. Med., 109, 747 (1962).

33. Nestel, P. J., Denborough, M. A., and O'Dea, J., Circulation Res., 10, 786 (1962).

34. Morris, B., and French, J. E., Quart. J. Exp. Physiol., 43, 180 (1958).

35. Ashworth, C. T., Stembridge, V. A., and Sanders, E., Am. J. Physiol., 198, 1326 (1960).

36. Robinson, D. S., in Lipid Transport, H. C. Meng, ed., Thomas, Springfield, Ill., 1964, p. 195.

37. Felts, J. M., Ann. N. Y. Acad. Sci., 131, 24 (1965).

38. Felts, J. M., and Mayes, P. A., Nature, 206, 195 (1965).

39. Elovoson, J., Olivecrona, T., and Belfrage, P., Biochim. Biophys. Acta, 106, 34 (1965).

40. Belfrage, P., Biochim. Biophys. Acta, 125, 474 (1966).

41. Higgins, J. A., and Green, C., Biochem. J., 99, 631 (1966).

42. Green, C., and Webb, J. A., Biochim. Biophys. Acta, 84, 404 (1964).

43. Ontko, J. A., and Zilversmit, D. B., J. Lipid Res., 8, 90 (1967).

44. Olivecrona, T., Acta Physiol. Scand., 54, 295 (1962).

45. Robinson, D. S., in Metabolism and Physiological Significance of Lipids, R. M. C. Dawson and D. N. Rhodes, eds., John Wiley, New York, 1964, p. 275.

46. Robinson, D. S., and Harris, P. M., Quart. J. Exp. Physiol., 44, 80 (1959).

47. Hahn, P. F., Science, 98, 19 (1943).

48. Anderson, N. G., and Fawcett, B., Proc. Soc. Exptl. Biol. Med., 74, 768 (1950).

49. Korn, E. D., J. Biol. Chem., 215, 1 (1955).

50. Balls, A. K., and Matlack, M. B., J. Biol. Chem., 123, 679 (1938).

51. Bier, M., in Methods in Enzymology, S. P. Colowick and N. O. Kaplan, eds., Vol. 1, Academic Press, New York, 1955, p. 627.

52. Sumner, J. B., and Somers, G. F., Chemistry and Methods of Enzymes, Academic Press, New York, 1953, p. 80.

53. Seligman, M. L., Ueno, H., Hanker, J. S., Kramer, S. P., Wasserkrug, H., and Seligman, A. M., Exp. Mol. Path., Sup. 3, 21 (1966).

54. Korn, E. D., in The Enzymes of Lipid Metabolism, P. Desnuelle, ed., MacMillan, New York, 1961, p. 231.

55. Engleberg, H., Appl. Physiol., 12, 292 (1958).

56. Zemplenyi, T., and Graffnetter, D., Arch. Intern. Pharmacodyn., 122, 57 (1959).

57. Grossman, M. I., Standler, J., Cushing, A., and Palm, L., Report #140 Army Med. Nutr. Lab., Oct. 1, 1954.

58. Bernfeld, P., and Kelley, T. F., J. Biol. Chem., 238, 1236 (1963).

59. Korn, E. D., J. Biol. Chem., 226, 827 (1957).

60. Jeffries, G. H., Quart. J. Exp. Physiol., 39, 261 (1954).

61. Shore, B., and Shore, V., Nature, 193, 163 (1962).

62. Connor, W. E., and Eckstein, J. W., J. Clin. Invest., 38, 1746 (1959).

63. Spitzer, J. A., and Spitzer, J. J., Am. J. Physiol., 185, 18 (1956).

64. Constantinides, P., So, Y., and Johnstone, F. R. C., Proc. Soc. Exptl. Biol., 100, 262 (1959).

65. Mayes, P. A., and Felts, J. M., Biochem. J., 99, 43P (1966).

66. Hamilton, R. L., Jr., Ph.D. Dissertation, Vanderbilt University, 1964, p. 101.

67. Mosinger, B., Pechar, J., Segova, E., Physiol. Bohemoslov., 13, 300 (1964).

68. Pechar, J., Mosinger, B., and Segova, E., Physiol. Bohemoslov., 13, 446 (1964).

69. Pechar, J., Segova, E., and Mosinger, B., Physiol. Bohemoslov., 15, 14 (1966).

70. Bjorntorp, P., and Furman, R. H., Am. J. Physiol., 203, 316 (1962).

71. Rizack, M. A., J. Biol. Chem., 236, 657 (1961).

72. Vaughan, M., Berger, J. E., and Steinberg, D., J. Biol. Chem., 239, 401 (1964).

73. Lynn, W. S., Jr., and Perryman, N. C., J. Biol. Chem., 235, 1912 (1960).

74. Schnatz, J. D., Biochim. Biophys. Acta, 116, 243 (1966).

75. Baker, Z., and King, C. G., J. Am. Chem. Soc., 57, 358 (1935).

76. Loevenhart, A. S., J. Biol. Chem., 2, 427 (1906).

77. Willstatter, R., and Memmen, F., Hoppe-Seyl. Z., 138, 216 (1924).

78. Sobotka, H., and Glick, D., J. Biol. Chem., 105, 199 (1934).

79. King, E. J., Biochem. J., 25, 799 (1931).

80. Belfanti, S., Contardi, A., and Ercoli, A., Ergebn. Enzymforsch., 5, 213 (1936).

81. Kondo, K., Biochem. Z., 26, 243; 27, 427 (1910).

82. McGugan, W. A., and Laughland, D. H., Arch. Biochem. Biophys., 35, 428 (1951).

83. Roy, A. B., Biochem. J., 53, 12 (1953).

84. Richter, D., and Croft, P. G., Biochem. J., 36, 746 (1942).

85. Carruthers, C., Heins, E., and Baumler, A., Canadian J. Biochem., 43, 97 (1965).

86. Nachlas, M. M., and Seligman, A. M., J. Biol. Chem., 181, 343 (1949).

87. Keay, L., and Crook, E. M., Arch. Biochem. Biophys., 111, 626 (1965).

88. Krisch, K., Biochem. Z., 337, 546 (1963).

89. Boguth, W., Krisch, K., and Niemann, H., Biochem. Z., 341, 149 (1965).

90. Belfrage, P., Biochim. Biophys. Acta, 98, 660 (1965).

91. Grafnetter, D., and Grafnetterova, J., Med. Pharmacol. Exp., 12, 274 (1965).

92. Irwin, R. J., Alpern, R. J., and Webster, G. R., Biochim. Biophys. Acta, 125, 600 (1966).

93. Biale, Y., Gorin, E., and Shafir, E., Israel J. Chem., 3, 112p. (1965).

94. Vavrinkova, H., and Mosinger, B., Physiol. Bohemoslov., 14, 46 (1965).

95. Olson, A. C., and Alaupovic, P., Circulation, 32 II, 25 (1965).

96. Olson, A. C., and Alaupovic, P., Biochim. Biophys. Acta, 125, 185 (1966).

97. Mosinger, B., and Vavrinkova, H., Physiol. Bohemoslov., 14, 439 (1965).

98. Carter, J. R., Jr., Biochim. Biophys. Acta, 137, 147 (1967).

99. Mattson, F. H., and Volpenhein, R. A., J. Am. Oil Chemists' Soc., 43, 286 (1966).

100. Sarda, L., Ailhaud, G., and Desnuelle, P., Biochim. Biophys. Acta, 37, 570 (1960).

101. Borgstrom, B., Biochim. Biophys. Acta, 13, 149 (1954).

102. Kalckar, H. M., J. Biol. Chem., 167, 461 (1947).

103. Dole, V. P., J. Clin. Invest., 35, 150 (1956).

104. Dole, V. P., and Meinertz, H., J. Biol. Chem., 235, 2595 (1960).

105. Schnatz, J. D., J. Lipid Res., 5, 483 (1964).

106. Okuda, H., and Fujii, S., J. Biochem., 61 No. 3, 313 (1967).

107. Robinson, D. S., and French, J. E., Quart. J. Exp. Physiol., 38, 233 (1953).

108. Gordon, R. S., Boyle, E., Brown, R. K., Cherkes, A., and Anfinsen, C. B., Proc. Soc. Exptl. Biol. Med., 84, 168 (1953).

109. Benzonana, G., and Desnuelle, P., Biochim. Biophys. Acta, 105, 121 (1965).

110. Baskys, B., Klein, E., Lever, W. F., and Stone, N., Federation Proc., 20, 237 (1961).

111. Baskys, B., Klein, E., and Lever, W. F., Arch. Biochim. Biophys., 102, 201 (1963).

112. Baskys, B., Klein, E., and Lever, W. F., Arch. Biochim. Biophys., 99, 25 (1962).

113. Glick, D., and King, C. G., J. Biol. Chem., 97, 675 (1932).

114. Wills, E. D., Biochem. J., 57, 109 (1954).

115. Fritz, P. J., and Melius, P., Can. J. Biochem. Physiol., 41, 719 (1963).

116. Fraser, G. P., and Nicol, A. D., Biochem. J., 96 No. 2, 35P (1965).