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PARTIAL PURIFICATION AND CHARACTERIZATION OF PIG LIVER ALKALINE LIPASE

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JOANNA HOLLAND LEDFORD

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PARTIAL PURIFICATION AND CHARACTERIZATION OF

PIG LIVER ALKALINE LIPASE

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This dissertation is dedicated, with affection, to Dow and Leigh.

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CHAPTER I

INTRODUCTION

The ability of mammalian liver to hydrolyze simple organic esters was recognized at the beginning of the century (1, 2). It was not until 1965, however, that an enzyme capable of hydrolyzing esters of long-chain fatty acids with glycerol was postulated to exist in this organ (3, 4, 5, 6, 7). It has now been established that rat liver contains at least two long-chain triglyceride lipases. One of these enzymes possesses an acidic pH optimum and is localized in lysosomes (8, 9, 10, 11, 12, 13). The second has a neutral or alkaline pH optimum and its localization has been variously reported as soluble (6), microsomal (11, 14, 15) or mitochondrial (16).

In 1970, Müller and Alaupovic (17) noted that pig liver also contained acidic and alkaline triglyceride lipases. The acidic enzyme was clearly lysosomal in origin while the alkaline lipase showed highest specific activity in microsomes. The objective of the present study has been to further characterize the long-chain triglyceride lipases of pig liver. Particular emphasis has been placed on the less well-understood alkaline activity. The subcellular distributions of both lipases have

been compared with the distributions of several marker enzymes for cellular organelles. The alkaline lipase has been purified approximately 90-fold from the soluble fraction. Optimal conditions for assay of the purified enzyme and its responses to various effectors have been determined. The properties of pig liver alkaline lipase are compared with the properties of other known mammalian lipases.

CHAPTER II

LITERATURE REVIEW

Enzymes of the mammalian liver are capable of hydrolyzing carboxylic ester linkages present in a wide range of organic compounds. The present review will discuss two groups of enzymes demonstrating this general specificity: "simple" esterases and lipases. Simple esterases have been defined as enzymes which catalyze the hydrolysis of an ester linkage between an organic acid and a monohydric alcohol. Enzymes catalyzing such reactions have been classified by the Enzyme Commission as aliphatic carboxylesterases (E.C.3.1.1.1), arylesterases (E.C.3.1.1.2), and acetylesterases (E.C.3.1.1.6) (18). Aliphatic or carboxylesterases preferentially hydrolyze esters of fatty acids with aliphatic alcohols. Arylesterases show specificity for phenol esters, and acetylesterases are characterized by preference for any ester of acetic acid. Lipases, as defined by the Enzyme Commission, are those enzymes which catalyze the hydrolysis of esters of organic acids and the trihydric alcohol glycerol (E.C.3.1.1.3) (18).

Unfortunately this classification of esterolytic enzymes has not proven entirely satisfactory. As early as 1958, Sarda and Desnuelle (19) reported that pancreatic lipase showed little or no hydrolytic activity with the triglyceride triacetin or the ester methyl butyrate when either

of these substrates was present in true solution at a concentration below its saturation point. When the saturation point of either compound was exceeded and the substrate was present in emulsified form, concentration-dependent hydrolytic activity was demonstrable. Just the reverse was true for hepatic esterase. Both triacetin and methyl butyrate were hydrolyzed in a concentration dependent fashion when in true solution, but this activity reached plateau level prior to the saturation point for each substrate, and no further increase in activity occurred when saturation was exceeded. These observations formed the basis of generally accepted alternative definitions of esterolytic enzymes according to which a lipase acts only at an oil-water interface on substrates present in aggregated or emulsified form, while an esterase cleaves only compounds present in true solution regardless of the nature of the alcohol present.

For purposes of the present study the latter definitions are preferred. The esterases to be considered are those enzymes catalyzing hydrolysis of esters of acetic acid, esters of other fatty acids with aliphatic alcohols, esters of fatty acids with aryl alcohols and soluble esters of short-chain fatty acids with glycerol. The discussion of lipases will be restricted to enzymes catalyzing the hydrolysis of insoluble esters of fatty acids with glycerol. For the purpose of clarifying the differences between lipase and esterase substrates, a discussion of the interrelating substrate specificities of the two types of enzymes will conclude the review.

Esterases in Mammalian Liver

An impressive bulk of literature exists on the ability of liver to hydrolyze simple organic esters. As early as the first decade of the

twentieth century, attempts to characterize and purify liver esterase were reported (1, 2, 20). In 1935 Baker and King (21) described a 12-fold purification of esterase from liver acetone powders utilizing a series of precipitations with acetic acid and sodium sulfate, and in 1948 Mohamed (22) claimed the preparation of liver esterase in crystalline form by acetone precipitations and crystallization from ammonium sulfate. In 1950 Connors et al. (23) achieved a 270-fold purification of liver esterase by utilizing precipitation techniques and selective heat denaturation. Burch's method (24), appearing in 1953, yielded a 140-fold purification, and involved several precipitations and adsorption on calcium phosphate gel.

At about this same time, studies designed to characterize the catalytic behavior of the partially purified esterases appeared. Substrates other than the standard methyl butyrate were tested; kinetic behavior was explored; inhibitors were described. That purified liver esterase could be strongly inhibited by diisopropyl-fluorophosphate was reported by Boursnell and Webb (25). They further established that ^{32}P-labelled inhibitor was attached tightly to the enzyme and therefore postulated that its binding occurred at the active center. Connors et al. (23) found their esterase preparation to have a pH optimum of 8.0 and a K_m of 0.022 M with methyl butyrate as substrate. Burch (24) described K_m's of 2.32×10^{-3} M for methyl butyrate and 6.2×10^{-4} M for ethyl butyrate. Her preparation was inhibited by fluorescein, eosin, rhodamine B, o-5-acridylbenzoic acid, pyronine, and N-methylacridinium chloride. Hofstee explored the kinetic behavior of liver esterase toward a series of aryl esters of straight chain fatty acids (C_2-C_{14}) with m-hydroxyl-

benzoic acid (26, 27). It was reported that for each substrate, velocity curves showed a discontinuity at higher substrate concentrations. The longer the fatty acyl chain of the substrate, the lower the concentration at which this discontinuity occurred. The discontinuity was believed to correspond to the critical micellar concentration for each substrate; the formation of micelles was thought to result in a reduced rate of activity. At low substrate concentrations, the enzyme hydrolyzed the compounds containing longer chain fatty acids more efficiently.

Since 1960, a veritable plethora of information on mammalian liver esterase has become available. This material will be discussed under several headings, including localization, purification, kinetics, inhibition and physical-chemical properties.

Localization of Liver Esterase and Occurrence of Isozymes

Krisch (28) reported in 1963 that an acetanilide deacetylase, measured at pH 8.6, showed highest specific activity in the microsomal fraction of liver from both rats and pigs. In the case of pig liver, the microsomal activity was 4 to 5 times higher than that of the homogenate, and approximately 10 times higher than that of the soluble or cytoplasmic fraction. This enzyme was also capable of cleaving simple aliphatic esters (29). With the substrate β-naphthyl acetate, Shibko and Tappel (30) described the presence of one esterase with a pH optimum of 8.5 localized in rat liver microsomes and another with a pH optimum of 5.0 present in lysosomes. The microsomal activity was strongly inhibited by E-600 (diethyl-p-nitrophenyl phosphate); the lysosomal was not.

According to Carruthers et al. (31) approximately 80% of the esterase activity of mouse liver, with β-naphthyl acetate as substrate, resided in the microsomes isolated from sucrose homogenates, while only 6% of the activity occurred in the cytoplasm. However, incorporation of glycerol into the homogenizing medium caused a shift of activity to the cytoplasmic fraction; this fraction now contained approximately 50% of the total activity with the loss reflected in the microsomes. Anion exchange chromatography of the cytoplasmic fraction and of microsomes solubilized in the non-ionic detergent Lubrol-W revealed a number of peaks of enzyme activity present in both fractions. Microsomes isolated from glycerol and sucrose homogenates contained 3 peaks in common and two which differed. The supernatant fractions from both procedures contained 4 common peaks, one of which was different from any peak present in the microsomal preparations. The possible presence in mouse liver of 6 different esterases was suggested.

In 1962, Ecobichon and Kalow (32) identified human liver esterases in the following manner. Liver tissue was homogenized in distilled water; the homogenate was subjected to freezing and thawing, and the soluble supernatant fraction was collected by centrifugation. This soluble fraction was submitted to electrophoresis in starch gel. Slices of starch gel were permitted to react with esters of α- or β-naphthol, and the released naphthol was detected within the starch block at the site of the enzyme reaction by coupling with an azo dye. It was assumed that all loosely-bound esterases were released into the soluble supernatant fraction by homogenization and freezing, and the histochemical "zymograms" obtained after electrophoresis demonstrated three major zones of activity,

each of which contained multiple bands. By use of a wide range of substrates and inhibitors, one zone was found to have the properties of an aliphatic or carboxylesterase; one zone appeared to contain several esterases with differing properties, and the third was classified as an acetylesterase. The isozyme bands within the carboxylesterase and acetylesterase zones were subsequently determined to be due to multiple forms of each enzyme differing in net electrical charge, but not molecular size or shape (33).

Ecobichon (33, 34, 35, 36, 37), Schwark and Ecobichon (38), and Holmes and Masters (39, 40) have all explored this general method of approach. Schwark and Ecobichon (38) reported that rat liver soluble esterases are multiple in number and belong to the aliphatic or carboxylesterase group (E.C.3.1.1.1.). Holmes and Masters (39) detected multiple carboxylesterases and a minor arylesterase in rat liver using vertical polyacrylamide gel electrophoresis for separation of the soluble supernatant fraction. Species-specific electrophoretic patterns of esterase activity were noted for extracts of pig, beef, sheep and horse livers by Ecobichon (34). Most of the observed esterases were classified as aliphatic esterases (E.C.3.1.1.1), but acetylesterases (E.C.3.1.1.6) were detected in pig and sheep liver, and an arylesterase (E.C.3.1.1.2) in pig liver. Species specificity was also demonstrated by Holmes and Masters (40) for liver of horse, sheep, ox, and possum. All liver tissues exhibited high aliphatic esterase activity. Horse, sheep, and possum also showed minor bands of arylesterase activity, but no acetylesterases were reported. In further studies Ecobichon (35, 36, 37) combined the zymogram approach with several types of chromatography to

elucidate the physical properties of liver esterases from large mammals. These results will be discussed later.

Isolation of esterase from liver microsomes. In 1963, Krisch (28) purified an acetanilide deacetylase approximately 75-fold from pig liver microsomes, with a recovery of 10-20% of the original microsomal activity. This represented an overall 300- to 400-fold purification from the homogenate. The enzyme was later demonstrated to be much more active on simple aliphatic esters than on acetanilide, and was therefore considered to be a carboxyl- or aliphatic esterase (29). Ultracentrifugally isolated microsomes were solubilized in 10% glycerol solution. This preparation was brought to 50% saturation with ammonium sulfate, and the inactive precipitate was removed. A second precipitation occurred when the ammonium sulfate supernate was adjusted to pH 4.2; this precipitate was likewise inactive. The supernate was readjusted to alkaline pH and brought to 80% saturation with ammonium sulfate, which yielded an active precipitate. The redissolved dialyzed enzyme preparation was chromatographed on DEAE-Sephadex A-50 with a continuous sodium chloride gradient. Several protein peaks were eluted, only one of which contained the enzymatic activity. The enzyme could then be obtained in crystalline form by further precipitation from ammonium sulfate between 60 and 67.5% saturation.

Benöhr and Krisch (41) later isolated a carboxylesterase from beef liver by a slight modification of the same procedure. The acid precipitation was carried out at pH 5.5, and the final 60-67% ammonium sulfate precipitate was dialyzed and rechromatographed on DEAE-Sephadex A-50. Purification was similar to that for the pig liver enzyme. Homogeneity of this preparation was demonstrated by column chromatography,

gel filtration, and paper electrophoresis; however, a second faster-moving esterase-active band was demonstrable in starch gel following electrophoresis.

Isolation of esterase from acetone powders of liver. Homogenization of whole liver tissue in large volumes of acetone, followed by removal of the acetone and thorough drying of the precipitate has produced the starting material of choice for a number of esterase preparations. Adler and Kistiakowsky (42) extracted pig liver acetone powders with ammonium hydroxide, and fractionated the extract by several precipitations with acetic acid. The enzyme preparation from this step was further fractionated with ammonium sulfate and acetone precipitations. The final step consisted of column chromatography on DEAE-cellulose, using phosphate and sodium chloride gradients. The active peak was considered to be a homogeneous protein on the basis of rechromatography on DEAE-cellulose, ultracentrifugation, and electrophoresis. However, no quantitative data on purification were reported. The substrate of choice was methyl n-butyrate, indicating the enzyme to be an aliphatic esterase.

A similar isolation procedure for pig liver carboxylesterase has been reported by Horgan et al. (43). The use of chloroform and acetone in the preparation of powders was recommended as a means of eliminating a red pigment, probably hemoglobin, which had previously been a rather tenacious contaminant. Powders were extracted at pH 4.5, and the soluble material was subjected to ammonium sulfate fractionation. The active fraction occurred between 45 and 70% saturation. This material was further purified by chromatography on CM-cellulose. Additional chromatography on CM-Sephadex, Sephadex G-100 or Bio-Gel P-150, and CM-

Sephadex again completed the scheme. The final preparation was 60-70-fold purified with respect to the original chloroform-acetone powder as assayed against ethyl butyrate. The enzyme showed only one band on starch gel electrophoresis when stained either for protein or esterase activity. In polyacrylamide gel electrophoresis, only one band staining for protein was observed, but the more sensitive esterase stain revealed several faster moving minor components. Two components, one major and one minor were present on analytical ultracentrifugation.

Runnegar et al. (44, 45) applied a similar procedure to ox liver preparations. The extraction from liver powder, ammonium sulfate fractionation and CM-cellulose chromatography were identical to Horgan's method (43). Further purification on CM-Sephadex and Sephadex G-100, or DEAE-Sephadex and Sephadex G-200 yielded a preparation of about 50-fold purification. Three electrophoretic variants, all stainable for esterase activity after starch gel electrophoresis, could be identified in these preparations. However, no other evidence could be obtained to indicate the presence of more than one enzyme protein.

The purification of an esterase active on aryl esters (e.g., p-nitrophenyl acetate) has likewise been reported by several groups. These methods were very similar to those discussed above for carboxylesterase. Keay and Crook (46) extracted acetone powders of pig liver with distilled water, submitted the soluble material to ammonium sulfate fractionation, and chromatographed the active precipitate on DEAE-cellulose columns utilizing first an ascending salt and descending pH gradient, and second a descending pH gradient only. The final preparation was 270-fold purified compared to the acetone powder extract when assayed

against p-nitrophenyl acetate; it showed only one peak of activity on DEAE-cellulose and one band on paper electrophoresis.

Bauminger and Levine (47) purified an arylesterase from beef liver by a method nearly identical to that of Horgan et al. (43) for carboxylesterase. Acetone powder extraction was followed by ammonium sulfate precipitation between 45 and 70% saturation and the following series of column chromatographic steps: CM-cellulose, CM-Sephadex, Sephadex G-200, and DE-52 cellulose. This enzyme preparation was 360-fold purified with a yield of 3.5%. The enzyme was immunogenic in rabbits and showed a single precipitin arc with its corresponding antiserum. Only one band was demonstrable by either protein or esterase staining after polyacrylamide gel electrophoresis.

Physical-Chemical Properties of Liver Esterase

Molecular weight and equivalent weight. Adler and Kistiakowsky (42) first estimated the molecular weight of a purified carboxylesterase, isolated from pig liver, to lie between 150-200,000. Boguth et al. (48), using pig liver microsomal esterase prepared by the method of Krisch (28), determined a more precise molecular weight of 174,000. This value was obtained by several ultracentrifugal methods (S/D and Archibald's approach to equilibrium) and by gel filtration on Sephadex G-200. Moreover, it was found that 0.2% sodium dodecylsulfate caused disaggregation of the enzyme molecule into four subunits of M.W. $\sim$ 42,000 each.

Krisch (49) found that an identical enzyme preparation catalyzed a rapid initial splitting of diethyl p-nitrophenyl phosphate (E-600 or Paraoxon). This initial reaction caused, however, inhibition of the enzyme activity toward other substrates. Stoichiometric studies showed

that 86,300 g of esterase protein released one mole of p-nitrophenol from the inhibitor, indicating the presence of two active centers per molecule of enzyme of M.W. 174,000. This finding was confirmed by Heymann and Krisch (50) with a second inhibitor: bis-[p-nitrophenyl]phosphate. In a similar study Benöhr and Krisch (51) were able to obtain two active esterolytic fractions by DEAE-Sephadex A-50 column chromatography of a purified preparation of beef liver esterase. One fraction had a molecular weight of 167,000 and contained two active centers, as determined by stoichiometric reaction with E-600. The other fraction had a molecular weight of 85,000 and contained only one active center. These two forms were considered to represent the dimer and monomer, respectively, of liver esterase.

The studies by Barker and Jencks (52) on an enzyme preparation isolated from pig liver acetone powders by the method of Adler and Kistiakowsky (42) agreed well with the above results on liver microsomal esterase. The molecular weight was found to be 167,000 by several ultracentrifugal methods. The enzyme underwent reversible dissociation to active subunits of molecular weight 75-90,000 under slightly acidic conditions (pH 4.5), in extremely dilute solution near neutrality or in the presence of salts. However, below pH 4, the enzyme was irreversibly converted to inactive half-molecules. Moreover, in 6 M guanidine hydrochloride - 0.1 M mercaptoethanol, an uncorrected molecular weight of 53,500 was obtained. This was interpreted as evidence for further dissociation of the enzyme into subunits of molecular weight 42,000 which interacted preferentially with guanidine hydrochloride. The results of Horgan et al. (53) are also in agreement with those described above for

pig liver carboxylesterase. A molecular weight of 163,000 was determined by several ultracentrifugal methods, and the presence of two subunits, each containing an active center, was confirmed. No evidence could be found, however, for further dissociation.

Runnegar et al. (44, 45) isolated two active esterolytic fractions by DEAE-Sephadex A-50 column chromatography of purified ox-liver esterase, similar to those isolated by Benöhr and Krisch (51) from pig liver enzyme. They reported, however, that these two fractions represented electrophoretic variants of the enzyme, rather than monomers and dimers. Each electrophoretic variant appeared capable of reversible dissociation upon dilution. The molecular weight of "mixed" enzyme (both variants) was ∿ 150,000; an equivalent weight estimated by titration with inhibitors was ∿ 68,000.

In contrast to most of these results, Ecobichon (36, 37) obtained considerably lower molecular weights for the cytoplasmic esterases of a number of species. In the case of bovine liver (36), DEAE-cellulose column chromatography of crude cytoplasmic liver extracts resulted in the separation of a group of electrophoretically slow and a group of electrophoretically fast esterases. Both the slow and fast esterases, however, gave molecular weight values of 55,000, as estimated by gel chromatography on Sephadex G-100. Further studies on extracts of bovine, ovine, equine, and human liver (37) gave the following results. The carboxylesterase activity in crude extracts of bovine, ovine, and equine liver each was eluted as a single peak from Sephadex G-100. Molecular weights were 55,000, 65,000, and 65,000, respectively. Human liver extracts contained two peaks of activity, one of molecular weight 65,000

and the other of molecular weight 180,000. In addition, several esterase peaks of higher molecular weight could be induced in equine extracts by prolonged freezing or concentration. The peaks of high molecular weight from both human and equine extracts could be converted to the lower molecular weight forms by incubation with 1 M NaCl or at pH 4.5, suggesting that the higher molecular weight forms were dissociable polymers. It was suggested that, in the natural state, most esterases occurred in an undissociated form, and that the high molecular weights reported by most workers for purified enzymes were a result of aggregation induced during the purification procedures.

Isoelectric point. The isoelectric point for purified pig liver esterase was reported to be pH 5.0 $\pm$ 0.5 by Adler and Kistiakowsky (42). Barker and Jencks (52) determined a value of pH 5.0-5.1, also for pig liver enzyme.

Immunochemical properties. The pig liver enzyme isolated by Barker and Jencks (52) produced a single antibody in rabbits. A single, identical antibody was also produced by a pig liver esterase preparation obtained according to the method of Horgan et al. (43). Bauminger and Levine (47) produced antibodies to both beef and pig liver esterases. Each antiserum was monospecific, and cross reactions were noted between pig esterase and anti-beef esterase as well as between beef esterase and anti-pig esterase. A similar cross reaction was found by Hain and Krisch (54) between beef liver esterase and anti-pig liver esterase. The immunoprecipitin cross reactions showed spurs with immunoprecipitin reactions between homologous antigen-antibody pairs, establishing that beef and pig liver esterases were similar but not identical.

Kinetic Properties of Liver Esterase

pH optimum. With acetanilide as substrate, Krisch (29) found that purified pig liver microsomal esterase exhibited a pH optimum of 8.6 to 8.8. With β-naphthyl acetate, Shibko and Tappel (30) observed a rat liver lysosomal esterase with pH optimum of 5.0 and a microsomal one with optimum pH 8.5. Mahadevan and Tappel (55) observed a slightly lower optimum of 3.6-4.0 for rat liver lysosomal esterase as measured against p-nitrophenyl myristate. With methyl butyrate as substrate, Albertson (56) reported a pH optimum range of 7-9 for rat liver homogenates. Barker and Jencks (57) studied the pH dependence of a purified pig liver esterase measured against p-nitrophenyl acetate and m-(n-pentanoyloxy) benzoic acid (m-C_5). The first substrate was hydrolyzed optimally near pH 8, by both associated and dissociated forms of the enzyme. The second substrate was hydrolyzed most rapidly between pH 4 and 5. As their preparation was not believed to contain two enzymes, the results with the anionic m-C_5 were interpreted to reflect activation of the substrate by protonation of its carboxylate group at lower pH, followed by formation of inhibitory micelles due to continued neutralization of net charge below pH 4.

Substrate specificity and influence of substrate on liver esterase kinetics. Histochemical and electrophoretic substrate specificity. The approach utilized by Ecobichon and co-workers in identifying the esterases present in the soluble fraction of mammalian liver has been discussed earlier (32, 34, 38). Their results demonstrated that a large number of bands capable of hydrolyzing α-naphthyl acetate occurred in zymograms of all mammalian livers. Most of these bands also reacted

with β-naphthyl acetate. Esters with long-chain fatty acids (β-naphthyl caprylate, myristate, laurate, or stearate) were also hydrolyzed but at slower rates. In several instances, a single band or a closely related group of bands exhibited specificity for either β-napthyl benzoate, indoxyl acetate or α-naphthyl butyrate. Albertson (56) reported that disc gel electrophoresis of rat liver homogenates revealed only one slow band capable of hydrolyzing methyl butyrate. This was in sharp contrast to the multiple bands observed to hydrolyze α-naphthyl acetate.

Substrate specificity of lysosomal and microsomal esterases. Mahadevan and Tappel (55) determined the relative rates of hydrolysis of p-nitrophenyl esters containing fatty acids with 8 to 18 carbon atoms by both the lysosomal (acid) esterase and the microsomal esterase from rat liver. The lysosomal esterase exhibited high activity against esters of long-chain fatty acids but only low activity against p-nitrophenyl caprylate. On the other hand, the microsomal esterase was most active toward p-nitrophenyl caprylate and displayed only negligible activity toward esters of higher fatty acids.

Influence of substrates on kinetics of purified liver esterase. It was established on the basis of substrate specificities that the acetanilide deacetylase isolated from pig liver microsomes by Krisch (29) was identical to carboxylesterase. The enzyme was capable of cleaving several acid amides, with the following turnover numbers (moles/substrate/min X 100,000 g protein): acetanilide, 50; L-leucyl-β-naphthylamine, 7; monoethylglycinexylidide, 2400; xylocaine, 70. Simple esters were cleaved generally at much higher rates: procaine, 50; L-tyrosine ethyl ester, 4260; triacetin, 5200; tributyrin, 20,900; ethylbutyrate,

21,400. K_m's for the more rapidly hydrolyzed substrates were much lower than those for the less active ones. Utilizing tyrosine ethyl ester, it was also shown that at high substrate concentration the reaction kinetics no longer followed Michaelis-Menten principles; there occurred an apparent substrate activation. Benöhr and Krisch (41) carried out a similar series of studies on beef liver esterase. This enzyme also hydrolyzed several acid amides as well as carboxyl esters but, in contrast to the pig enzyme, showed a marked preference for esters of aryl alcohols. Turnover numbers of hydrolyzed esters (based on a M.W. of 170,000) were as follows: procaine, 13, tyrosine ethyl ester, 5900; phenylalanine methyl ester, 9500; tributyrin, < 1; methyl butyrate, 3100; ethyl acetate, 750; p-nitrophenylacetate, 13,200; o-nitrophenylacetate, 1400. The enzyme preparation showed reduced activity when o- or m- methoxy-acetanilide was substituted for p-methoxyanilide, demonstrating that configuration of the alcohol moiety did affect reactivity of the acid amides. However, substitution of fluoro- or nitroacetanilide for acetanilide did not reduce activity. The enzyme was also able to catalyze the transfer of amino acid residues to form dipeptides, e.g., phenylalanyl-phenylalanine from phenylalanine methyl ester.

Adler and Kistiakowsky (58) investigated the kinetics of pig liver esterase with methyl butyrate, ethyl butyrate, and methyl chloroacetate as substrates. With all three substrates, deviation from classical kinetics was observed at high substrate concentration. At low substrate concentrations V_{max} was identical for methyl and ethyl butyrate; methyl chloroacetate was a less effective substrate. At high substrate concentration V_{max} for methyl chloroacetate was substantially higher

than that for the butyrate esters, but the K_m for the former substrate was also higher. The data were interpreted as favoring both a nucleophilic attack mechanism for liver esterase catalysis and the presence of two identical active sites which interacted so that the presence of substrate bound at one site changed the kinetic parameters of the neighboring site.

Barker and Jencks (57) also investigated kinetic properties of a pig liver esterase purified by the method of Adler and Kistiakowsky (42). The substrates of choice were p-nitrophenyl acetate, m-carboxyl-p-nitrophenyl acetate, m-(n-heptanoyloxy)benzoic acid (m-C_7) and m-(n-pentanoyloxy)benzoic acid (m-C_5). The phenomenon of substrate activation was found to occur at high substrate concentrations of both p-nitrophenyl acetate and m-C_7. The anionic substrate m-carboxyl-p-nitrophenyl acetate was hydrolyzed less than 1% as rapidly as p-nitrophenyl acetate, reflecting a low specificity of this enzyme toward charged substrates. An interesting observation made was that the activity of the enzyme was often higher toward preparations of m-C_5 which contained micelles than toward the completely dissolved substrate.

Stoops et al. (59) also reported biphasic kinetics (substrate activation) for hog liver carboxylesterase with phenyl butyrate as substrate. Phenyl and ethyl butyrates served as equally good substrates; nitro- substituted phenyl butyrates showed decreased activity, although the position of the nitro group on the benzene ring was not important. For a series of phenyl esters with fatty acids of differing chain length, the rate of hydrolysis did not seem to depend upon the acyl moiety. This was believed to indicate that binding of the substrate was not respon-

sible for the high reactivity of the systems.

In contrast to this study, Hofstee (60) demonstrated that the rates at which liver esterase hydrolyzed low concentrations of a series of esters of m-hydroxybenzoic acid depended on the acyl chain length. Similar behavior was shown by esterases from pig, horse, cow, rat, and rabbit. For esters containing C_4, C_5, or C_6 fatty acids, the rate increased nearly exponentially with each additional carbon atom. At high substrate concentration, however, where substrate activation occurred, specificities differed. Especially in the case of pig enzyme, there was only a relatively small rate increase between C_4 and C_5 esters, and no increase whatever between C_5 and C_6 esters. The results were interpreted as indicating that the structures of the active centers of enzymes from these various species were very similar, accounting for similar behavior at low substrate concentration. Additional substrate binding sites or modifier sites, probably involved in substrate activation at high substrate concentration, were believed to vary among species and therefore to give varying rates of activity under identical conditions.

Influence of Effectors on the Kinetics of Mammalian Liver Esterases

Inhibitors. Cholinesterases are generally inhibited by the complex carbohydrate eserine or physostigmine and by various organophosphates at low concentration. Aliphatic esterases are sensitive to the organophosphates, but not to eserine. Aromatic esterases are relatively insensitive to both these classes of inhibitors (32). Among the organophosphates which have been demonstrated to inhibit liver esterase are Malathion, Phosdrine (32), DFP (diisopropylfluorophosphonate) (29, 32,

42, 61), bis-[p-nitrophenyl] phosphate (41, 50), and E-600 (diethyl p-nitrophenyl phosphate or Paraoxon) (29, 30, 44, 49, 51, 53, 55, 56). Other inhibitors described are o- and p-nitrophenyl dimethylcarbamates (44, 53), alkoxydyrenes (62), Atoxyl (sodium arsanilate) (29), calcium oleate (29), heavy metals and high Mg^{++} concentration (46), the acid product of the esterase reaction (58), sodium fluoride (41), and sodium dodecyl sulfate (41).

As discussed earlier, it was the use of the combinations of specific substrates and differential inhibitors which permitted several groups to determine the esterase-specificity of electrophoretic bands observed in zymograms of cytoplasmic supernates from liver (32, 33, 34, 38, 39, 40, 56). Also as mentioned previously, it was the use of the active-site binders E-600, p-nitrophenyl dimethylcarbamate, and bis-[p-nitrophenyl] phosphate which permitted estimation of the number of active sites per mole of enzyme (44, 49, 50, 51). Shibko and Tappel (30) and Mahadevan and Tappel (55) reported that while rat liver microsomal esterase was quite sensitive to E-600 and DFP, the lysosomal esterase was not.

The non-classical kinetics exhibited by liver esterase have been discussed above. To explain the mechanism of substrate activation, Ocken and Levy (63) studied the inhibition of esterase with butanol and 2-butanone. Their preliminary studies confirmed that pig liver esterase contained two active sites and that the rate of hydrolysis increased with increasing acyl chain length of a series of ethyl esters. This suggested the presence of one modifier site and only one catalytic site, with the modifier site preferentially binding hydrophobic compounds and serving as an internal accelerator of the reaction. It was found that

butanol and 2-butanone, in addition to inhibiting enzyme activity toward ethyl butyrate, caused a loss of biphasic kinetics; the activity remaining after inhibition no longer showed substrate activation. It was concluded that for non-inhibited enzyme at high substrate concentration, the control site acquired a substrate molecule causing a probable modification of the reaction pathway and an observed increase in velocity. Butanol and 2-butanone were pictured as excluding substrate molecules from the control site thereby producing linear kinetics, as well as competing for the esterolytic site to produce inhibition. These results were in contrast to those of Adler and Kistiakowsky (58) who considered both sites to be identical and esterolytic.

Activators. Low levels of Ca^{++} and Mg^{++} were found to activate hydrolysis of p-nitrophenyl acetate by pig liver esterase (46). Barker and Jencks (57) reported that pig liver esterase was activated by organic solvents such as acetone and dioxane, as well as by increased substrate concentrations. Acetone increased the maximal velocity at both high and low substrate concentrations. This was interpreted as favoring a catalytic mechanism involving two active sites, one of which acted as an effector site to modify the reactivity of the second or esterolytic site. Acetone, dioxane, and substrate (at high concentrations) were postulated to bind at the effector site and produce the increased rate of esterolysis. A similar conclusion was reached by Stoops et al. (59), who found that benzene also activated pig liver esterase.

Methanol and other weakly acidic alcohols caused an increase in the rate of disappearance of phenyl acetate catalyzed by pig liver esterase according to Greenzaid and Jencks (64). This was not due to an in-

creased rate of hydrolysis, however, but to the occurrence of methanolysis. Acetate was readily transferred to the nucleophilic methanol rather than to water by the enzyme. The authors interpreted their results as favoring an acyl-enzyme mechanism, in which the acyl portion of the substrate remained bound to the enzyme after release of the alcohol and could then be transferred to either water or a nucleophile.

Lipases of Mammalian Liver

Uptake of Chylomicrons by Liver

That the liver plays an essential role in mammalian lipid metabolism is not disputed. Whether or not the liver has, however, the capability to remove and/or hydrolyze the plasma long chain triglycerides is a matter of some controversy. For a number of years it was believed that the liver constituted one of the major organs involved in the uptake of dietary triglycerides carried by lipoprotein particles known as chylomicrons. Ashworth et al. (65) subjected livers of rats ingesting a high-fat diet or receiving corn oil by stomach tube tc electron microscopy. Chylomicrons were observed in hepatic sinusoids, traced into the spaces of Disse, and detected in different stages of direct transfer across the hepatic parenchymal cell membrane. Larger cytoplasmic lipid droplets then formed within the liver cells.

Another method of approach to the problem of chylomicron uptake has been to inject radiolabeled dietary fat particles into test animals and to follow the tissue distribution of the label at various time peroids after injection. French and Morris (66) found that labeled chylomicron fat, injected into rats, was taken up principally by the liver.

The injection of heparin accelerated this uptake, and protamine sulfate and Triton WR-1339 delayed it. Bragdon and Gordon (67) found the radioactivity to be located principally in the liver when injected rats were in the fasting state. Adipose tissue took up the bulk of activity, however, when animals were fed a high carbohydrate diet. Carbohydrate feeding greatly reduced the overall oxidation of injected triglycerides. It was also believed that the chylomicrons did not undergo intravascular hydrolysis and were removed intact from the circulation. Blood plasma lipoproteins of various densities were injected into rats by Stein and Shapiro (68). In all cases, large amounts of the radioactive material (24-60%) were recovered in the liver 15 minutes after injection. Labeled triglycerides were located predominantly in the particulate fractions of liver homogenates with very little present in the floating fat fraction. Intravenous hydrolysis did not seem to be necessary for this uptake, but intrahepatic hydrolysis did occur at a later time. A similar conclusion was reached by Olivecrona (69), who found 35% of the recovered activity from triglyceride fatty acid in the liver and 10% in the adipose tissue of rats 20 minutes after chylomicron injection. According to Ashworth *et al.* (70) such chylomicron clearing is carried out by hepatic parenchymal cells rather than by Kupffer or reticuloendothelial cells.

Havel and Goldfien (71) reported that hepatectomy reduced the rate of removal of both injected chylomicron triglycerides and free fatty acids in dog. However, further studies in which chylomicrons were infused into dogs over a period of time simulating physiological fat uptake convinced Nestel *et al.* (72) that most of this lipid was removed directly by extrahepatic tissues. Nestel *et al.* (73) injected chylomicrons la-

beled in both triglyceride and cholesterol moieties. In normal dogs almost all the infused chylomicron cholesterol radioactivity but only a smaller and more variable fraction of the triglyceride radioactivity was removed by the liver. While exclusion of the liver from the circulation did result in decreased rates of removal for triglyceride, the increased half-time for removal of cholesterol under these conditions was much greater.

Perfusion of isolated livers with chylomicrons has provided a direct study of the ability of this organ to handle dietary lipid particles. Morris and French (74) reported that perfused rat livers were capable of taking up approximately 54% of a fatty acid-labeled chylomicron preparation in 3 hours. Pre-treatment of the chylomicrons with heparin-stimulated clearing factor lipase (75, 76) increased this uptake to 76%. The removed triglycerides were oxidized to carbon dioxide. Uptake proceeded more rapidly in livers from starved than from fed rats (77). Oxidation of chylomicron triglycerides also occurred more rapidly in livers of starved rats (78). It was observed in this study that free fatty acids were taken up and oxidized to carbon dioxide more rapidly than chylomicron triglycerides. Rodbell et al. (79) also suggested that chylomicron triglycerides were taken up from the blood without prior hydrolysis. In contrast to previous studies (68, 70), however, it was established that both parenchymal and reticuloendothelial cells participated in this reaction. The bulk of the recovered radioactivity was associated with the floating fat layer rather than the particulate fractions of liver homogenates.

In direct contrast to the above reports, several researchers

utilizing perfusion techniques have reported that the liver removes and oxidizes only insignificant amounts of chylomicron triglyceride. Felts (80) and Felts and Mayes (81) presented evidence that the bulk of the triglyceride taken up by rat liver during chylomicron perfusion had simply been trapped within extracellular compartments, and could be removed by retrograde perfusion. They observed that infused fatty acids were oxidized to a greater extent than chylomicrons and that the addition of heparin-stimulated clearing factor to the perfusion medium increased the uptake of fatty acids. On the basis of these experiments, the authors suggested that liver cells could not remove intact chylomicron triglyceride from the bloodstream but rather took up only free fatty acids derived from extrahepatic lipolysis.

Liver slices have been used only rarely in studies on the uptake of chylomicrons. Edgren and Zilversmit (82) reported that liver slices from fed dogs were capable of taking up labeled chylomicron lipids in vitro. However, liver slices from fed rats did not oxidize labeled chylomicrons in vitro (83).

There are only two reports describing the uptake of chylomicron fatty acids by isolated liver cells (84, 85). Rat liver cells were found to be capable of binding injected chylomicrons (40 μg lipid per mg of tissue nitrogen), and the bound chylomicrons were hydrolyzed at the plasma membrane to yield fatty acids.

Presence of Lipoprotein Lipase in the Liver

The question of whether chylomicron triglyceride is utilized directly by the liver is intimately tied to the question of whether the liver contains an enzyme with the characteristics of lipoprotein lipase.

In 1955, Korn (86, 87) detected in heart a lipase with an absolute requirement for triglyceride complexed in the form of lipoprotein. This enzyme, displaying a high specificity for chylomicron-like particles, was believed to be identical with "clearing factor" lipase. Since that time, it has been generally assumed that any organ normally involved in chylomicron uptake should contain an enzyme with characteristics of lipoprotein lipase.

Early studies on rat liver indicated the absence of clearing factor lipase in this organ. In contrast with a number of other rat organs (71, 78, 88), perfusion of rat liver with heparinized medium failed to cause release of clearing factor into the perfusate. In fact, Spitzer and Spitzer (89) observed that the rate of clearing of an emulsion by serum containing clearing factor was decreased after perfusion through the liver. Mayes and Felts (90, 91, 92) confirmed that lipoprotein lipase activity could not be measured in acetone powders of rat liver, whereas rat heart showed high levels of activity. If liver extracts and heart extracts were combined and assayed together, the liver caused suppression of the normal activity present in heart. This suppression could be overcome by the addition of very large amounts of heparin. With high heparin concentrations, lipoprotein lipase activity could be detected in both liver tissue and liver perfusates. It was postulated, therefore, that an inhibitor, possibly a heparinase, maintained the liver enzyme in an inactive form *in vivo*.

Several groups, however, have reported lipoprotein lipase-like activity in rat liver. Payza *et al.* (93) demonstrated that such activity was present in acetone powder extracts of liver although not in whole

homogenates. Heparin administered to intact rats caused a drop in the amount of activity measurable in the liver concomitant with a rise in the level observed in the serum. Activity was detected in crude rat liver extracts by Yasuoka and Fujii (94). Approximately 50% of this activity could be precipitated and inactivated by reaction with an antiserum to purified post-heparin rat plasma lipoprotein lipase. It must be noted, however, that the test emulsion used as substrate in both these reports was Ediol, a commercially available preparation of coconut oil, which is known to contain significant amounts of mono- and diglycerides as well as triglycerides (95).

The release of lipoprotein lipase from liver under the influence of heparin has also been studied in dogs and humans. The livers of both these species seem to be an excellent source of this enzyme. LeQuire et al. (96), injected heparin into the portal vein of dogs, and removed blood samples from the hepatic veins at various time periods after the injection. Twenty seconds following heparin, prior to any possible recirculation, the lipoprotein lipase activity in blood samples from the hepatic veins increased some twenty-five-fold over preheparin values. This response to heparin was significantly greater than that observed in a similar experiment with the dog hind limb. The results were considered to be consistent with a heparin-triggered release of enzyme from the vascular endothelium, the high titer of activity released by the liver being due to its extensive capillary bed. Boberg et al. (97) observed the release of lipoprotein lipase activity from isolated, perfused dog liver in response to heparin. Moreover, the released activity was inhibited by NaCl and protamine, showing identical properties

to the lipolytic activity released into the plasma of an intact animal following heparin. The release of lipolytic activity from dog livers after in vivo injection of heparin was confirmed by Condon et al. (98). The released activity behaved like lipoprotein lipase with respect to inhibitors, and hepatectomy severely hampered the heparin response. Moreover, increased levels of activity were noted in blood from the hepatic vein of two human subjects within 15 seconds of systemic injection of heparin (98). And finally, Muir (99) recorded release of lipoprotein lipase across the splanchnic bed under heparin stimulation in seven human beings. However, the possibility that the extrahepatic portions of the splanchnic bed contributed significantly to the measured release could not be eliminated.

Other Lipases in the Liver

All question of whether the liver functions as a site of uptake and hydrolysis of chylomicron-type substrates aside, the presence of other lipases in the liver might be suspected simply from the knowledge of the role of the liver in the turnover of many kinds of lipids. A number of such activities have been reported and will be described below.

Endogenous lipolysis in the liver. Incubation of rat liver slices at 37° for several hours caused a rise in free fatty acid content according to Mosinger and Vavrinkova (3). Free fatty acids increased more strikingly in livers from fasted than fed rats. It was established that the increased fatty acid release in starved livers was due to an increased rate of lipolysis rather than a decreased rate of re-esterification. Rat liver homogenates incubated in glycyl-glycine buffer at pH 6.5 showed an increase in free fatty acid content (100). Total lipid

analysis of the homogenates after incubation showed a 65% decrease in triglyceride content. The triglyceride was apparently completely hydrolyzed, as no significant accumulation of lower glycerides could be detected.

Liver lipolytic activity with Ediol as substrate. As mentioned earlier, Ediol, a commercial preparation of emulsified coconut oil, contains some mono- and diglycerides as well as triglycerides (95). The major fatty acid component of the glycerides is lauric acid (C_{12}). As it is difficult to determine whether the activity measured against such a mixed substrate is due to one or several enzymes, discussion of studies utilizing this substrate is separated from discussion of studies utilizing homogeneous substrates.

In 1965, a number of studies were reported attesting to the ability of liver preparations to hydrolyze Ediol. In all cases, the data were believed to indicate the presence of a long-chain triglyceride lipase different from lipoprotein lipase. The release of fatty acids from Ediol by rat liver homogenates was not stimulated by preincubation of the substrate with serum according to Vavrinkova and Mosinger (4). The activity was stimulated by albumin and showed two pH optima, one at pH 8, the other at pH 5. Liver slices incubated with Ediol caused a rise in free fatty acid concentration in the medium, which was not due to loss of free fatty acids from the cells (3). The activity required albumin, but was not inhibited by either NaCl or protamine. Lipolytic activity could not be released into the medium by heparin, nor was pretreated Ediol any better a substrate than untreated. In contrast to endogenous lipolysis, which occurred more readily in fasted livers, extracellular lipolytic activity was higher in fed livers. The Ediol hydrolyzing

system of rat liver homogenates studied by Grafnetter and Grafnetterova (5) was likewise enhanced by albumin, and was, in fact, inhibited by pretreatment of the substrate with serum. A sodium deoxycholate-stimulated hydrolysis of Ediol by rat liver preparations was noted by Olson and Alaupovic (6, 7). However, substitution of albumin for deoxycholate caused inhibition of activity. Subcellular fractionation of the liver homogenates showed localization of maximal specific activity in the soluble fraction. The pH optimum for this preparation was approximately 7.2.

Long chain triglyceride lipases in liver. Lipases with neutral or alkaline pH optima. In 1965, Olson and Alaupovic (7) first reported hydrolytic activity of rat liver acetone powders against olive oil emulsified in gum arabic. As olive oil is essentially free of partial glycerides and has a fatty acid composition of greater than 75% oleic acid (101), it is considered a definitive substrate for long chain triglyceride lipase. The pH optimum determined in this study was 7.2; sodium deoxycholate stimulated and albumin decreased the lipolytic activity. In the same year Biale et al. (102) noted a rather low level of activity in rat liver homogenates toward tripalmitin dispersed in gum arabic. The pH used was 7.2, and bovine albumin was included in the system. Further studies (14) showed inhibition of this lipolysis by homogenization of the tissue in the presence of ATP or divalent cations, or by preincubation of the liver tissue with uncouplers of oxidative phosphorylation. Maximal specific activity observed for this substrate was in the microsomal fraction.

Tripalmitin dispersed only in water was found by Carter (103)

to be a suitable triglyceride substrate for rat liver lipase. The pH used was 7.4, and albumin was included in the system. Products of the reaction between whole homogenates and tripalmitin were primarily free fatty acids and glycerol. The highest specific activity for this lipase also occurred in the microsomes. Additional confirmation of the presence of long chain triglyceridase activity in microsomes has come from several other groups. Guder et al. (11, 15) described maximal specific activity against triolein at pH 8.5 in rat liver microsomes. Gum arabic was used as emulsifying agent but no albumin was added. The distribution of the lipolytic activity, however, did not parallel the distribution of the microsomal marker enzyme glucose-6-phosphatase. In addition, large amounts of heparin were found to stimulate triolein hydrolysis in a crude plasma membrane preparation in the presence of Ca^{++}. Although the pH optimum for this activity was 8.5 without heparin, maximum stimulation by heparin occurred at pH 7.5. This was interpreted as providing evidence for a second, heparin-dependent lipase. Müller and Alaupovic (17) reported that the maximal lipolytic activity of pig liver subcellular fractions occurred in microsomes. The substrate was purified olive oil emulsified in sodium taurocholate solution; no albumin was present. The pH optimum was 7.5, and Triton X-100, Ca^{++}, and NH_4^+ were inhibitory to the system.

Waite and van Deenen (16) found that the release of labeled fatty acid at pH 7.4 from mixed sonicates of ^{14}C-oleic acid triglyceride with phosphatidyl ethanolamine was greatest in the mitochondrial fraction. The products of reaction were free fatty acid and diglyceride. No albumin was included in the system. The activity was sensitive to

p-chloromercuribenzoate.

Lipases with acidic pH optima. As mentioned earlier, Vavrinkova and Mosinger (4) reported Ediol hydrolyzing activity in rat liver to show a pH optimum at 5.0. Higgins and Green (104) found that isolated liver cells, when incubated in the presence of heparin, released a chylomicron lipase with pH optimum 4.0-4.5. The lipase could also be released from the cells in the absence of heparin but was inactive until heparin was added. This enzyme was believed to be located in the plasma membrane. In 1967, Stoffel and Greten (8) demonstrated the localization of a long chain triglyceride lipase in the lysosomes of rat liver. The measured pH optimum was 6.5, and the substrate, 1,2-dipalmitoyl-3-linoloylglycerol, was dispersed in sodium deoxycholate. Mahadevan and Tappel (9) isolated lysosomes from livers of normal and Triton WR-1339 treated rats and established the presence of a lipase with pH optimum of 4.2. While the preparations displayed maximum specific activity against glyceryl tridecanoate (to be discussed later), activity was also established with trilaurin, trimystin, tripalmitin, tristearin and triolein. The presence of Triton X-100 was required for optimal activity; the reaction was inhibited by NaCl, NaF, iodoacetate, and Hg^{++}. Fowler and de Duve (10) observed that a similar preparation of lysosomes hydrolyzed tripalmitin optimally at pH 4.1 and required 5% Triton X-100 for activity. Guder *et al.* (11) reported a pH optimum of 5.0 for triolein lipolysis by rat liver homogenates. Purification of Triton-filled lysosomes yielded a 300-fold purification of this lipolytic activity. The enzyme was activated by sonication or hypotonic treatment of the isolated lysosomes. Complete solubilization of the lipase could be achieved by suspending

purified lysosomes in 0.05 M phosphate buffer at pH 6.5 according to Hayase and Tappel (12). This preparation hydrolyzed triolein to oleic acid and diglyceride exclusively, and was strongly inhibited by sulfhydryl reagents. The esterase inhibitor diisopropyl fluorophosphate did not affect the activity. Kariya and Kaplan (13) were able to separate rat liver lysosomal lipase from an endogenous lipid activator. Although the activator was not purified, several preparations of phospholipid were found to increase markedly the activity of the enzyme. Lysosomal lipase has also been demonstrated in and partially purified from pig liver by Müller and Alaupovic (17). Peak activity with triolein as substrate occurred at pH 4.5; the enzyme was stimulated by low but inhibited by high concentrations of Triton X-100.

An interesting correlative to the story of acid lipase is the recently raised possibility of its absence in the inborn error of metabolism known as Wolman's disease. This disease is characterized by large accumulations of triglyceride and cholesteryl ester in the liver and spleen of young infants, and is generally fatal within the first year of life. In 1969, Patrick and Lake (105, 106) demonstrated hydrolytic activity against both tripalmitin and cholesteryl oleate at pH 4.6 in human liver homogenates. The activity against both substrates was entirely absent from tissue specimens of patients with Wolman's disease. Young and Patrick (107) further reported that greatly reduced levels of an acid esterase acting on fatty acid esters of p-nitrophenol were present in these patients, while other types of lysosomal hydrolase activities were increased. Burke and Schubert (108) observed that acid lipolytic activity against long-chain triglyceride and cholesteryl ester was also

absent from livers of patients with cholesteryl ester storage disease. In this syndrome large amounts of cholesteryl ester, but not triglyceride, accumulate in the liver; the overall course of the disease is relatively benign compared to Wolman's disease.

Short- and medium-chain triglyceride lipases in the liver. A number of reports have described the presence of liver hydrolytic activity toward emulsions of tributyrin at neutral or alkaline pH values. Unfortunately, in none of these cases were comparisons drawn between the activity toward the emulsified tributyrin and possible activity toward fully soluble tributyrin at lower substrate concentrations. A purified liver carboxylesterase preparation has already been shown to have a high degree of specificity for soluble tributyrin (29). Emulsified tributyrin was hydrolyzed by rat liver homogenates at a much greater rate than tripalmitin in the experiments of Biale et al. (102). The same situation existed for triolein according to Guder et al. (11) and Biale et al. (14). In vivo injection of n-butyl carbamic acid methyl ester (BCME) in dogs was found to inhibit strongly tributyrin hydrolysis by liver homogenates in the study of Wallach and Ko (109). Interestingly enough, the removal of injected procaine from the blood of dogs, believed to be carried out by an esterase, was inhibited to the same extent by BCME.

Hydrolysis of tricaprylin (C_8 fatty acid) at pH 7.4 by rat liver subcellular fractions was maximal in mitochondria according to Claycomb and Kilsheimer (110). The enzyme activity was solubilized by sonication in Triton X-100 and purified 77-fold by gel filtration. The purified preparation was nearly 8 times more active against emulsified tributyrin than against tricaprylin and was less than half as active against long-

chain triglycerides (tripalmitin, triolein, trilinolein, trilaurin) than against tricaprylin. As mentioned before, the acid lipase present in purified lysosomes (9) showed maximal activity toward tridecanoin. Activity decreased very strikingly with triglycerides of lower chain length, and somewhat less strikingly with higher triglycerides.

Hydrolysis of mono- and diglycerides by liver. In 1965, hydrolysis of monoolein by rat liver homogenates at neutral to alkaline pH was observed by Belfrage (111) and by Biale et al. (102). In the former case the reaction was stimulated by sodium taurodeoxycholate and serum albumin. In the latter case, hydrolysis of monoolein was approximately 37 times as efficient as hydrolysis of tripalmitin under similar conditions.

The phenomenon of higher rates of hydrolysis for mono- and diglycerides than for triglycerides at alkaline pH has been further reported by a number of workers. Biale et al. (14) established that lipolytic activities in liver showed increasing rates of cleavage from tri- to di- to monoolein. Both triolein and monoolein hydrolysis proceeded maximally in the microsomal fraction and responded similarly to the effects of ATP, divalent cations and uncouplers of oxidative phosphorylation. However, the differing effects of other inhibitors and unequal ratios of triolein to monoolein hydrolysis observed in a number of tissues studied led them to postulate that different enzymes were responsible for these two activities. The same conclusion was reached by Carter (103), who was also able to differentiate the two activities in the soluble fraction of rat liver by their response to pre-incubation at 37° and the effect of diisopropyl fluorophosphate.

The already mentioned (110) purified lipase preparation from rat liver mitochondria which demonstrated high specificity for short-chain triglycerides was also capable of hydrolyzing long-chain partial glycerides. The specific activity of this preparation toward monopalmitin, monoolein, and monolinolein was about the same as that toward tricaprylin. Diolein and dilinolein were hydrolyzed about one-half as efficiently as the corresponding monoglycerides.

In contrast to the above studies, the purified lysosomal preparation of Hayase and Tappel (12) hydrolyzed tri-, di- and monodecanoate in decreasing order of activity at acid pH. With a similar enzyme preparation, however, Fowler and de Duve (10) observed good release of fatty acid from monopalmitin, a small release from dipalmitin and no release from tripalmitin. When Triton X-100 was included in the system, the activity toward di- and tripalmitin was stimulated, but that toward monopalmitin was substantially depressed.

The presence of hormone-sensitive lipase in liver. The stimulation of the intracellular adipose tissue lipase by such hormones as epinephrine and glucagon and inhibition of its activity by insulin are well accepted phenomena. The occurrence of such an activity in liver has also been postulated. In 1966, Bewsher and Ashmore (112) observed increased ketone body production by liver slices of starved rats given glucagon *in vivo* before sacrifice. This effect could be duplicated *in vitro* by the addition of glucagon to incubating liver slices or homogenates. It was further found that free fatty acid production by liver slices was stimulated in glucagon-treated rats and that liver triglyceride concentration was decreased in these animals. The results were in-

terpreted as favoring the presence of a hormone-sensitive lipase which, after glucagon stimulation, provided increased levels of fatty acids and hence increased levels of β-oxidation and ketone body formation. In further studies, Claycomb et al. (113) observed that liver lipolytic and ketogenic activities were increased in alloxan-diabetic rats and could be reduced by injection of insulin.

Other attempts to establish the presence of a hormone-sensitive liver lipase have failed, however. Sarkar (114) found that neither the synthetic glucocorticosteroid triamcinolone diacetate nor dibutyryl cyclic AMP stimulated rat liver trioleinase activity, if injected 4 to 6 hours prior to sacrifice of the animals. No stimulation of the lipolytic activity of rat liver homogenates could be found after addition of noradrenalin to the incubation mixture by Scaria and Prabha (115). Partially purified tricaprylinase was not stimulated in vitro by either glucagon or cyclic AMP according to Claycomb and Kilshimer (110). And finally, Carter (103) reported a loss of approximately 80% of tripalmitin hydrolyzing activity when rat liver homogenates were preincubated for 1 hour at 37° before assay. Epinephrine did not restore the lost activity.

Interrelationships of lipases and esterases in the liver. The question of what type of compound represents a true esterase substrate as compared to a true lipase substrate remains somewhat uncertain. As mentioned early in this review, the classification of esterases as enzymes acting on esters of monohydric alcohols and lipases as enzymes acting on esters of glycerol is unsatisfactory. Sarda and Desnuelle (19) have adequately proven that pancreatic lipase acts on esters of glycerol only when they are presented in aggregated or emulsified form.

While this fact has not yet been proven for hepatic lipases, it has been well established (19, 29) that hepatic esterase will also hydrolyze true solutions of glycerol esters with no increase in level of activity when saturation is reached and the compound becomes aggregated. This clearly points to a division of specificities for these two types of enzymes based on the solubility of the substrate involved. Complications have arisen in the studies on liver esterolytic activities due to the fact that the substrates used have not been clearly defined as being specific either for lipases or esterases.

Further complications stem from the nature of the enzymes themselves. Lipases and esterases are both considered to be rather non-specific enzymes, and there is question of how extensive the overlap between their activities may be. Evidence was presented earlier that there were a number of different esterases in mammalian liver showing preference for various ester substrates, but specificities often overlapped widely (29, 41). On the other hand, it is believed that long-chain triglyceride lipases are probably different enzymes from long-chain monoglyceride lipases (14). However, no purified liver lipases have been studied with a wide enough range of substrates to permit any very strong conclusions concerning their specificities.

A few studies are available, however, which touch upon some of these possible interrelationships and differences, and these will be discussed now. Possibly the most comprehensive paper on this subject is the one by Hayase and Tappel (116) describing the purification of a glyceryl-monodecanoate hydrolyzing enzyme from rat liver. The enzyme was purified 254-fold with respect to this substrate. The specificity of

the preparation for other substrates was then determined. The hydrolytic rates for di- and tri-decanoate were one-third and one-hundredth of that for monodecanoate, respectively. Very little activity could be found for long-chain mono-, di- or triglycerides. In addition, the enzyme hydrolyzed p-nitrophenyl caprylate very well, but p-nitrophenyl esters of long-chain fatty acids (C_{12} to C_{18}) were hydrolyzed little, if at all. The enzyme could be inhibited by E-600, a typical esterase inhibitor. These data strongly favored designation of this enzyme as an esterase rather than a lipase, despite its ability to hydrolyze the C_{10}-monoglyceride. It was noted in the report that glyceryl 1-monodecanoate was sparingly water-soluble. It may also be mentioned here that p-nitrophenyl laurate is not water-soluble, and that Desnuelle and Savary (117) have considered it a suitable substrate for pancreatic lipase.

In contrast to this esterase, Mahadevan and Tappel (9), as mentioned earlier, purified liver lysosomes and detailed the specificity of the lipase present in these particles. Among triglycerides tested, tridecanoate was hydrolyzed most efficiently. Both shorter and longer chain triglycerides were attacked at a lesser rate. It was also found (55) that dispersions of p-nitrophenyl esters of long-chain fatty acids were hydrolyzed efficiently by the lysosomal preparation. In further studies Hayase and Tappel (12) demonstrated that the same enzyme preparation hydrolyzed tri-, di-, and monodecanoate in decreasing order of activity, with activity toward tridecanoate being 57-fold higher than that against monodecanoate. Although it was not proven that only one enzyme was present in this preparation, the system showed a clear preference for longer chain, insoluble esters of glycerol or p-nitrophenyl.

Although somewhat more difficult to interpret, the results of Okuda and Fujii (118, 119) on substrate specificities of several liver fractions also provide some interesting comparisons. When a soluble fraction from rat liver homogenates was subjected to ammonium sulfate fractionation, the distribution of Ediol-hydrolyzing activity paralleled that of methyl butyrate hydrolysis. Likewise, when the 0-30% ammonium sulfate fraction containing these activities was subjected to gel filtration on Sephadex G-200, both activities were recovered in the void volume. If the 0-30% ammonium sulfate fraction was treated with acetone prior to gel filtration, the Ediol hydrolyzing activity of the preparation was abolished and the methyl butyrate hydrolyzing peak migrated more slowly through the column. Acetone treatment of the ammonium sulfate fraction also reduced the specific activity of this preparation toward α-monoolein, α-monomyristin, and α-monolaurin. The activities toward α-monocaprylin and α- and β-monobutyrin were not reduced. Utilizing the Ediol-hydrolyzing peak from gel filtration of a crude liver extract, it was found that the α-monomyristin splitting capability of this preparation was competitively inhibited by α-monobutyrin. A 320-fold purification of liver esterase was achieved. Antibodies prepared to this preparation were found to precipitate the chromatographic fractions which exhibited hydrolytic activities against Ediol, methyl butyrate, α-monomyristin, and tributyrin. Finally, it was found that sonication of an acetone-treated liver esterase preparation with liver lipid restored Ediol hydrolyzing activity. Moreover, the esterase which had shown slow migration through a Sephadex G-200 column was partially converted to a faster void volume peak, in which the Ediol-hydrolyzing

activity resided. It was concluded from these studies that the protein moiety of the liver esterase studied was identical to that of the "lipase" active on Ediol, α-monoolein, α-monomyristin and α-monolaurin. It was believed that acetone treatment of liver fractions caused removal of a lipid component from the protein and decreased its lipolytic activities, while addition or binding of lipid to the esterase protein conferred the ability to hydrolyze lipase substrates.

CHAPTER III

MATERIALS AND METHODS

Materials

Solvents used in these studies included n-heptane, isopropanol, acetone, absolute methanol, ethyl acetate, diethyl ether, and n-hexane, all obtained from J. T. Baker Chemical Company. Absolute ethanol was purchased from U.S. Industrial Chemicals Company and methyl cellosolve (ethylene glycol monomethyl ether) from Fisher Scientific Company. Various preparatory procedures required the use of sucrose (Baker 'Analyzed'), sodium-EDTA (Calbiochem), olive oil (Sargent-Welch), sodium taurocholate (Maybridge Research Chemicals), sodium deoxycholate (Mann), Triton X-100 (Calbiochem), and Silica Gel G (Brinkmann). Buffer components included sodium acetate, maleic anhydride, potassium dihydrogen phosphate, 2-amino-2-(hydroxy-methyl)-1,3-propanediol (Tris), all obtained from Baker, and glycyl-glycine from Calbiochem. Chemicals required for analytical determinations were *l*-leucine (Mann), denatured hemoglobin (Nutritional Biochemicals), ninhydrin (Baker), sodium cyanide (Baker), trichloroacetic acid (as 40% solution from Curtin Scientific), potassium fluoride (Baker), glucose-6-phosphate (Sigma), ammonium molybdate and 1-amino-2-naphthol-4-sulfonic acid (Mallinckrodt), sodium sulfite (Matheson, Coleman and Bell), sodium bisulfite (Fisher Scientific), potassium chloride and

magnesium chloride (Baker), adenosine-5'-monophosphate (Sigma), sodium succinate (Sigma), iodonitrotetrazolium violet (INT) and INT formazan (Nutritional Biochemicals), sodium chloride and sodium carbonate (Baker), cupric sulfate and sodium tartrate (Mallinckrodt), Folin-Ciocalteau phenol reagent (Curtin), human albumin (Hoechst-Behringwerke), bovine albumin-fraction V powder-fatty acid free (Pentex), palmitic acid and triolein (Sigma), gum arabic (Fisher Scientific), and thymol blue and Nile blue A indicators (Allied Chemical). Several compounds tested for inhibitory action on lipolytic activity were eserine sulfate (Merck), iodoacetamide and protamine sulfate (Sigma), and diethyl-p-nitrophenyl phosphate (K & K).

Methods

Preparation of Tissue

Pig livers, from animals of either sex, were obtained fresh from a local meat packing firm and were held on ice for less than 1 hour before use. Fresh livers were either processed immediately or divided into pieces of approximately 100 g each and frozen at -12°. Frozen tissue was used within a 1 month period.

Homogenization. Fresh or thawed tissue, always obtained from several different lobes of the same liver, was chopped into pieces of about 1 cm^3 size. Twenty-five grams of chopped tissue were mixed with 200 ml of ice-cold 0.25 M sucrose containing 0.001 M sodium-EDTA, pH 7.2. The mixture was homogenized in a Sorvall Omnimixer (Ivan Sorvall Co.) for 30 seconds at setting 3 ("gentle" homogenization) or for an additional minute at setting 10 ("harsh" homogenization). Homogenates were filtered through 8 layers of coarse cheesecloth into a chilled container, and the

pH was adjusted to 7.2 with 5 N KOH.

Subcellular fractionation. Homogenates prepared from 50 g of fresh tissue according to the "gentle" procedure were fractionated essentially according to the method of de Duve et al. (120) and Appelmans et al. (121). The procedure is summarized in Figure 1. All steps were performed at 0°. Centrifugations were carried out either in a refrigerated Servall centrifuge (Ivan Sorvall Co.) with GSA rotor or in a Spinco Model L 2-65B ultracentrifuge with Type 42 rotor. After each centrifugation, supernates were removed by gentle suction. Sediments obtained from the initial spin at each speed were resuspended by short homogenization in 100 ml of the sucrose-EDTA solution and washed by an identical centrifugation. Final pellets were resuspended in approximately 50 ml of sucrose-EDTA solution by short homogenization, and the suspensions were frozen immediately in small aliquots until analyzed. Wash supernates were combined with those from the initial centrifugations at each step.

Purification of alkaline triglyceride lipase. For each experiment a homogenate was prepared from 50 g of fresh or frozen pig liver by "harsh" homogenization. The homogenate was centrifuged for 30 minutes at 113,000 x g in a Spinco Model L 2-65B ultracentrifuge with Type 60 rotor. The soluble fraction was decanted and frozen at -12° if not used immediately.

Binding of enzyme to lipid emulsion. An artificial lipid emulsion was prepared by mixing 3.32 g of olive oil with 21.3 ml 12 mM sodium taurocholate solution and sonicating the mixture for two 1 minute periods at setting 5 on a Bronwill Biosonik II. Assuming a molecular weight of

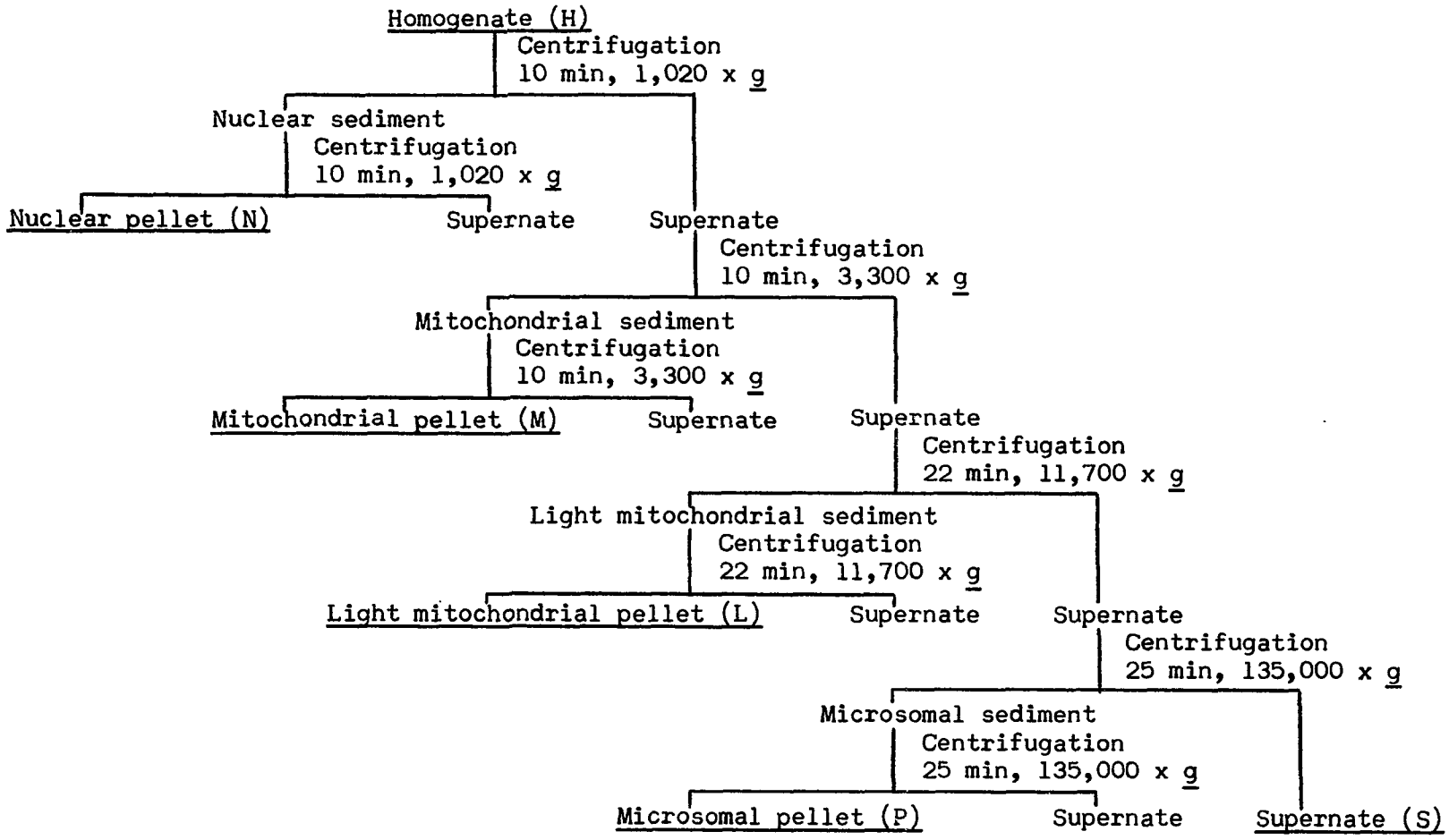

Figure 1. Subcellular fractionation of pig liver homogenate.

triolein for the olive oil, the final concentration of triglyceride in the prepared emulsions was 150 μmoles/ml. Twenty-five milliliters of emulsion were mixed with 205 ml of soluble fraction and the pH of the mixture was adjusted to 7.5 with 1 N NaOH. The mixture was held on ice for 5 minutes with stirring to permit binding of enzyme to lipid substrate.

Centrifugal isolation of emulsion-bound lipase. The mixture of lipid emulsion and soluble fraction was placed into prechilled tubes and centrifuged for 30 minutes at 93,000 x g in an L2-65B ultracentrifuge with Type SW-27 swinging bucket rotor. Rotor temperature was carefully maintained at 0°. At the conclusion of the run, the tightly packed lipid cakes were removed from the clear infranate with a small plastic spoon. The lipid cakes were suspended in a small volume of sucrose-EDTA solution, pH 7.5, by aspiration through an 18-1/2 gauge needle with syringe. The suspension was diluted to 230 ml with sucrose-EDTA solution and washed by centrifugation under identical conditions. The washing procedure was repeated once. The final lipid cakes were suspended in sucrose-EDTA solution when direct assay was desired; otherwise, they were treated as described below. The centrifugal isolation of emulsion-bound lipase is outlined in Figure 2.

Separation of bound lipase from lipid emulsion. Two procedures were used to accomplish this step. In the first procedure, the final lipid cakes were suspended in a total volume of 10 ml of sucrose-EDTA solution. The suspension was mixed with 20 ml of 0.01 M phosphate buffer, pH 7.5, containing either 0.5% sodium deoxycholate or 1 mg/ml Triton X-100. The mixture was stirred for 1.5 hours at 4°, and then centrifuged

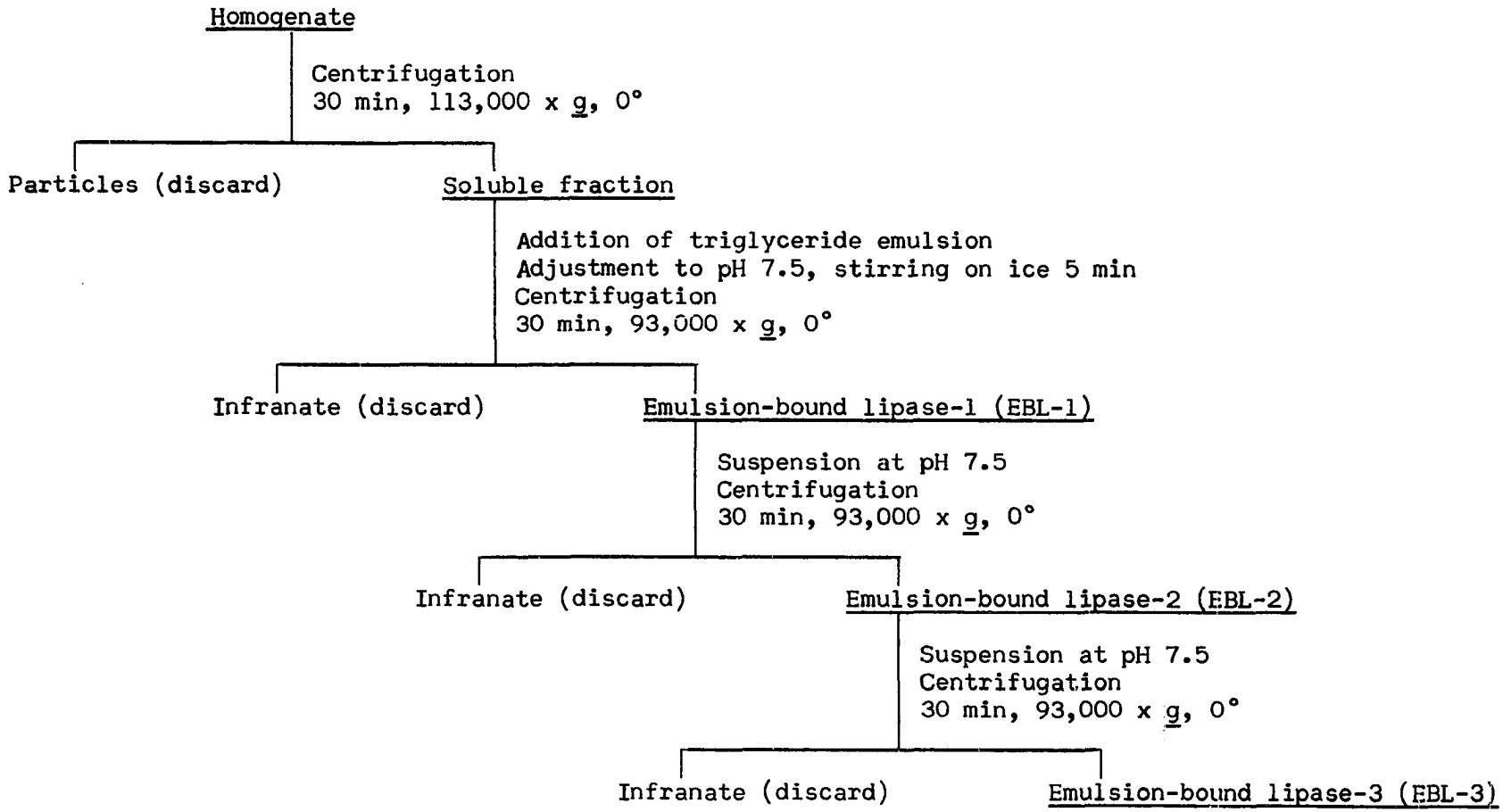

Figure 2. Centrifugal isolation of emulsion-bound lipase.

for 30 minutes at 93,000 x g in the Type SW-27 rotor. The somewhat cloudy infranate was drained from a hole punctured in the bottom of the centrifuge tube. The second procedure, a refinement of the first, consisted of density gradient centrifugation of the lipid cakes in detergent-containing media. The media were prepared by dissolving sucrose in 0.01 M phosphate buffer, pH 7.5, to give solutions of density 1.25, 1.15, 1.05, and 1.00 g/ml. To each solution was added Triton X-100 at a concentration of 3 mg/ml. The lipid cakes were suspended by aspiration in 17 ml of the solution of density 1.25 g/ml, and the suspension was stirred for 1.5 hours at 4°. Eight milliliter aliquots of this mixture were then layered, by means of a long needle with syringe, under discontinuous density gradients formed by sequential layering of 10 ml of each of the three lighter solutions in centrifuge tubes (1 x 3.5 inches). Centrifugation was carried out for 18 hours at 93,000 x g and 0° in the Type SW-27 rotor. A number of discrete bands could be visualized by their opacity, and a total of 6 fractions was collected in addition to the packed lipid cake. The lipid cake was removed by spoon and succeeding fractions by gentle suction with needle and syringe. When only the bottom fraction was desired, it was permitted to drip through a hole punctured in the bottom of the tube.

Fractions collected from either procedure were dialyzed and concentrated in a 150 ml Amicon ultrafiltration apparatus with Diaflo PM-10 filter. Each fraction was placed in the apparatus along with 3 volumes of ice-cold distilled water. Pressure was applied by a flow of 5-7 ℓ/min N_2, and dialysis was continued until the preparation was reduced to less than 10 ml in volume. The preparation was washed 3 times

with 10 ml volumes of water, the total volume being reduced to 1 or 2 ml after each wash. The final fractions, removed from the apparatus with a Pasteur pipette, were frozen at -12° in small aliquots until analyzed. The ultrafiltration apparatus was maintained in an ice-water bath during the entire filtration procedure. The separation of bound lipase from the lipid emulsion is outlined in Figure 3.

Preparation of Lipid Substrates

Purification of olive oil. Ten grams of olive oil were dissolved in acetone and slurried with 10 g of silica gel G. The mixture was dried by rotary evaporation under vacuum. The dried material was slurried in n-hexane and applied to the top of a 2.5 x 60 cm column packed with 120 g of silica gel G (adjusted to 10% water content) in n-hexane. The column was washed with n-hexane overnight. The eluting solvent was changed to 6% diethyl ether in n-hexane by volume, and 7 ml fractions were collected. This solvent was permitted to run until approximately 1 ℓ had passed through the column. Thin layer chromatography of the resulting fractions on silica gel G in the solvent system petroleum ether:diethyl ether (95:5, v:v) revealed small amounts of faster moving materials preceding the triglyceride peak from the column. Chromatography in the system n-hexane:diethyl ether:glacial acetic acid (80:20:1, v:v:v) demonstrated that the bulk of triglyceride was eluted between fractions 40 and 85 (approximately 280-600 ml of eluting solvent). Later fractions contained slower moving components and small amounts of triglyceride. The purified triglyceride fractions were combined, dried by rotary evaporation under vacuum, and stored in the dark under N_2 near 0°. The final preparation was a clear, nearly colorless liquid and contained only tri-

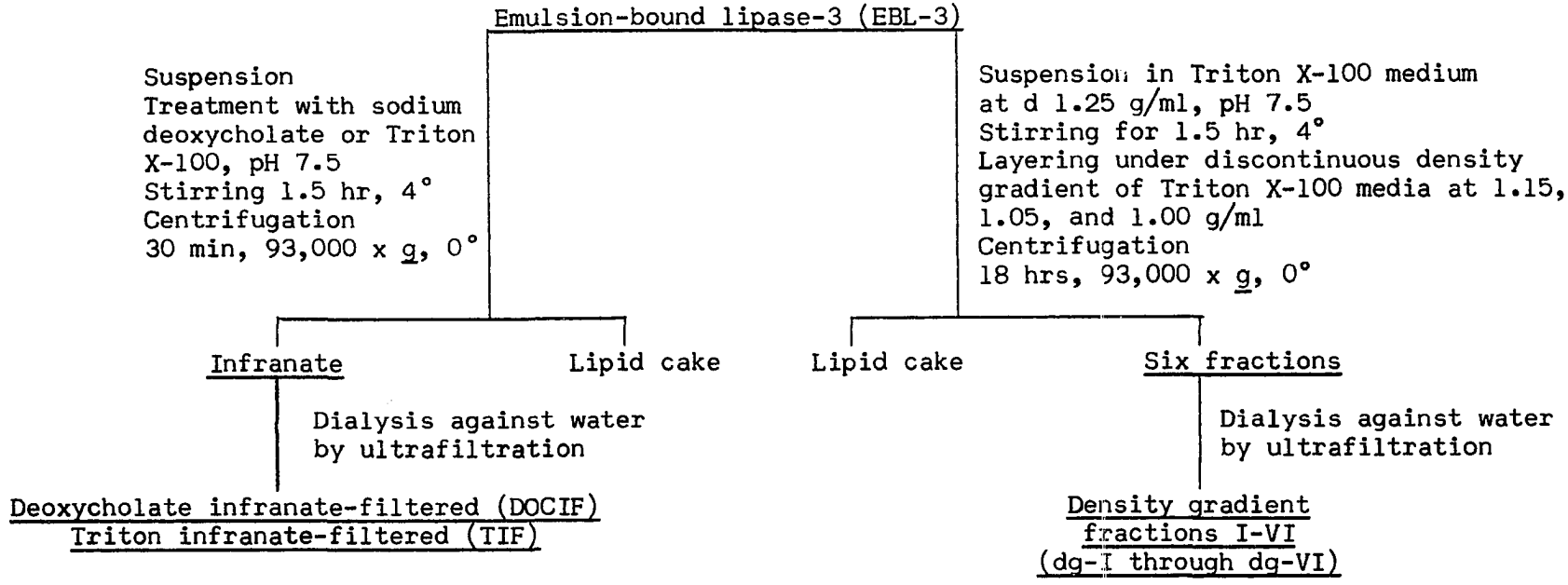

Figure 3. Separation of bound lipase from lipid emulsion.

glyceride by thin-layer chromatography. This method of purification represents a modification of the procedure of Crider et al. (122).

Chyle and plasma lipoproteins. Very-low density lipoproteins were isolated from a fasting hypertriglyceridemic subject according to the method of Gustafson et al. (123). Chyle chylomicrons were isolated from the abdominal fluid of a patient suffering from a chylous fistula. Chylous fluid was centrifuged at 93,000 x g in a Type SW-27 swinging bucket rotor for 1.5 hr at 0°. The packed lipid cake was suspended in 0.15 M NaCl containing 0.001 M sodium-EDTA at pH 7.0 and washed by recentrifugation for 1 hr. Washing was repeated a second time, and the final lipid cake was resuspended in a small volume of 0.15 M NaCl.

Analytical Methods

Protein determination. Protein was determined by the method of Lowry et al. (124). Samples showing mild turbidity due to the presence of lipid or detergent were cleared by shaking with a small volume of diethyl ether after development of the color reaction. Phases were separated by brief centrifugation at room temperature and the clear infranates were analyzed spectrophotometrically. Samples containing excess lipid were delipidized prior to the protein determination. One-half milliliter of sample was injected into 20 ml of ethanol:diethyl ether (3:1, v:v) or chloroform:methanol (2:1, v:v). The residue was collected by centrifugation, washed once with diethyl ether, and air-dried. The residue was redissolved in 0.25 ml of 0.1 N NaOH, neutralized with the same amount of 0.1 HCl, and analyzed.

Marker enzyme assays. Succinic dehydrogenase. Succinic dehydrogenase was used to monitor the presence of mitochondria during sub-

cellular fractionation procedures; it was determined according to the method of Pennington (125). The oxidation of succinate was coupled to the reduction of the dye iodonitrotetrazolium violet (INT). The reduction product of the dye, a colored formazan, was determined spectrophotometrically at 490 nm.

Cathepsin D. This protease releases amino acids from denatured hemoglobin at acid pH and is a marker for the presence of lysosomes. The methodology of Beck et al. (126) was followed, and released amino acids were quantitated by reaction with ninhydrin according to the method of Rosen (127) with the modification of Grant (128).

Glucose-6-phosphatase. Removal of inorganic phosphate from glucose-6-phosphate is catalyzed by this microsomal enzyme. The assay was carried out by the method of Hübscher and West (129).

5'-Nucleotidase. Occurring primarily in plasma membranes, this enzyme releases inorganic phosphate from 5'-adenosine monophosphate. The method of Emmelot et al. (130) was used. Inorganic phosphate resulting from 5'-nucleotidase and glucose-6-phosphatase assays was determined according to Fiske and Subbarow (131).

Lipase assays. Preparation of lipid emulsions. A weighed amount of purified olive oil was mixed with the necessary volume of 0.012 M sodium taurocholate to bring the final concentration of triglyceride to approximately 120 μmoles/ml in an even dispersion, assuming a molecular weight of triolein for the olive oil. Dispersion was accomplished by 3 successive sonications of 20 seconds each at setting 5 on a Bronwill Biosonik II equipped with catenoidal probe. To prevent overheating, sonications were interrupted by one minute periods of stirring.

The final milky emulsion was chilled on ice and used immediately for assay. Commercially obtained triolein (99%) was emulsified in sodium taurocholate by an identical procedure except that final concentration of the triglyceride was 90 μmoles/ml. An alternative procedure for emulsifying triolein consisted of substituting a 10% solution of gum arabic for the sodium taurocholate; all other steps were identical.

Alkaline lipase assay systems. Preparations resulting from the subcellular fractionation of liver tissue were assayed in a system comprised of 0.5 ml 0.1 M glycyl-glycine buffer, pH 7.5, 0.5 ml of purified olive oil emulsion containing 60 μmoles triglyceride, and 0.5 ml of enzyme preparation diluted in 0.25 M sucrose to contain 1.0-2.0 mg of protein. Control assays contained 0.5 ml of 0.012 M sodium taurocholate in place of emulsion. Reaction components were placed in chilled 16 x 150 mm culture tubes, capped with Teflon-lined screw caps under N_2, mixed briefly, and incubated for 15 minutes at 37° in a shaking water bath. Reactions were stopped by injection of 5 ml of a mixture of isopropanol:n-heptane:1 N H_2SO_4 (4:1:0.1, v:v:v); fatty acids were extracted and quantitated as described below. All reactions were run in duplicate; assays lacking enzyme were included periodically to check for breakdown of triglyceride during emulsification, non-enzymic hydrolysis, and aging of substrate. This method is identical to that of Müller and Alaupovic (17).

Assays of intermediate and final fractions obtained during purification of liver lipase were carried out with a few minor differences. Protein concentrations were reduced to a level necessary to keep total fatty acid release within limits of the standard curve. Denatured hemoglobin was dissolved in the assay buffer at a concentration of 4 mg/ml

to stabilize the emulsion during incubation. Control assays of fractions containing high levels of endogenous triglyceride (EBL's) were not incubated but were stopped at zero time to avoid subtraction of fatty acids released from endogenous substrate from those released from exogenous substrate.

The effects of various compounds on the activity of purified alkaline lipase were tested in a slightly different assay system. The reaction mixture was composed of 0.5 ml 0.02 M phosphate buffer, pH 7.25, containing 2 mg of fatty acid-free albumin, 0.5 ml of triolein emulsion prepared in 10% gum arabic solution, pH 7.25, and containing 45 μmoles of lipid, and 0.5 ml of enzyme preparation diluted in 0.25 M sucrose, pH 7.25, to contain 0.01-0.02 mg protein. Control assays contained 0.5 ml of 10% gum arabic solution in place of emulsion. Handling was identical to that described above except that incubations were carried out for 30 minutes. Compounds added to the basic system were dissolved in the assay buffer, and the pH corrected if necessary.

Acidic lipase assay system. The method for assay of acid lipase was identical to that of Müller and Alaupovic (17). The reaction mixture was comprised of 0.5 ml 0.1 M glycyl-glycine buffer, pH 4.5, containing 2 mg Triton X-100, 0.5 ml of purified olive oil emulsion containing 60 μmoles triglyceride, and 0.5 ml of enzyme preparation appropriately diluted in 0.25 M sucrose. Crude preparations were diluted to provide approximately 1.0 mg protein per test; purified lipase was diluted to provide approximately 0.01 mg protein per test. Handling was identical to that described above except that incubation was carried out for 60 minutes.

Quantitation of fatty acids. Fatty acids were extracted from reaction mixtures essentially according to the method of Dole (132). After addition of extracting solvent as described above, samples were recapped and mixed by inversion for 10 minutes. Phases were formed by addition of 4 ml of water and 4 ml of n-heptane containing 1 μmole palmitic acid. Further mixing was carried out for 30 minutes, and samples were permitted to stand for at least 90 minutes prior to analysis of the upper heptane phase for fatty acid. Standard solutions of palmitic acid in n-heptane were extracted and analyzed in identical fashion.

Two methods were used for measurement of fatty acids. In the first procedure, 3 ml of upper heptane phase were transferred to the sample cup of a Radiometer TTT-1C titrator equipped with TTA31 titration assembly and SBR-2 recorder. After addition of 4 ml of a titration mixture consisting of isopropanol:ethanol:0.1% aqueous thymol blue indicator (3:0.9:0.1, v:v:v), each sample was titrated to an end point of pH 9.8 with standardized 0.02 N NaOH. The volume of NaOH required to effect the titration served as a measure of fatty acid present in the sample. For this procedure, standard samples of palmitic acid in n-heptane containing from 1 to 3 μmoles of fatty acid were used.

For the alternative method of fatty acid quantitation, 3 ml aliquots of upper heptane phase were transferred to 12 ml conical centrifuge tubes equipped with ground glass stoppers. To each tube were added 1.5 ml of a freshly prepared mixture of ethanol:0.02% aqueous Nile Blue A sulfate:0.02 N NaOH (9:1:0.2, v:v:v). Individual tubes were stoppered, shaken rapidly by hand for 10 seconds, and the phases were permitted to separate. The lower ethanolic phase was transferred by Pasteur

pipette to a 1.5 ml quartz cuvette and its absorbance at 640 nm was determined spectrophotometrically against a solvent blank. Standard solutions of palmitic acid in n-heptane containing from 1.0 to 1.6 μmoles fatty acid were used as references. Stock solutions of 0.02% aqueous Nile Blue A sulfate were washed with several volumes of n-heptane after preparation to remove extractable impurities and stored at room temperature.

CHAPTER IV

RESULTS

Colorimetric Assay for Long-Chain Fatty Acids

Gordon et al. (133) first recommended the use of Nile Blue indicator in manual titration of free fatty acids to a visual end point. A variation of this procedure has been in use in our laboratories for a number of years. A small volume of ethanolic Nile Blue indicator (ethanol:0.02% aqueous Nile Blue 9:1, v:v) is added to an aliquot of fatty acid extracted into heptane by the Dole (132) procedure, and the resultant two-phase system is titrated with dilute NaOH. Change of the blue indicator to its pink end point indicates the completion of titration. The amount of NaOH required to effect titration provides a measure of the fatty acid present in the sample.

Attempts to utilize this method in the present study were not considered satisfactory due to the difficulties in visual detection of the end point. However, the fact that a color change was involved in this reaction suggested the possibility that the assay might be adapted to spectrophotometric quantitation. The ethanolic Nile Blue indicator taken to its pink colored point did not display an absorption maximum within the wavelength range 220-800 nm. In the absence of base, however, the blue indicator solution was found to absorb maximally at 640 nm. It was reasoned that samples of fatty acids could be mixed with indicator

solution previously taken to its alkaline end point, producing a reverse titration of the indicator and a return to the color blue which could be quantitated spectrophotometrically. Accordingly, experiments were set up to determine conditions necessary to produce a reverse titration and to establish whether there was a direct relationship between color yield and amount of fatty acid used. Known amounts of fatty acid, dissolved in 4 ml n-heptane, were assayed as described in Methods, utilizing experimental color reagents in which the volume ratios of ethanol, 0.02% aqueous Nile Blue, and 0.02 N NaOH were varied. The results of two representative assays are shown in Figure 4. Development of blue color was sigmoidal with respect to fatty acid concentration. A range of linearity occurred in each plot; this range was found to be determined primarily by the ratio of NaOH to Nile Blue present in the color reagent.

On the basis of these results, the method was considered to be quite satisfactory for quantitation of long-chain fatty acids released by lipolysis and was adopted for use under the following specific conditions. Standard solutions of palmitic acid in n-heptane contained from 1.0 to 1.6 μmoles of fatty acid; all assay samples contained 1.0 μmoles fatty acid, added in n-heptane during the extraction procedure, and the enzyme concentration of assay samples was adjusted to maintain fatty acid release at less than 0.6 μmole.

Subcellular Fractionation

Subcellular fractionation of fresh pig liver resulted in quantitative isolation of five subcellular fractions: the nuclear (N), the mitochondrial (M), the light mitochondrial (L), the microsomal (P), and the soluble cytoplasm (S). The homogenate and each of the isolated

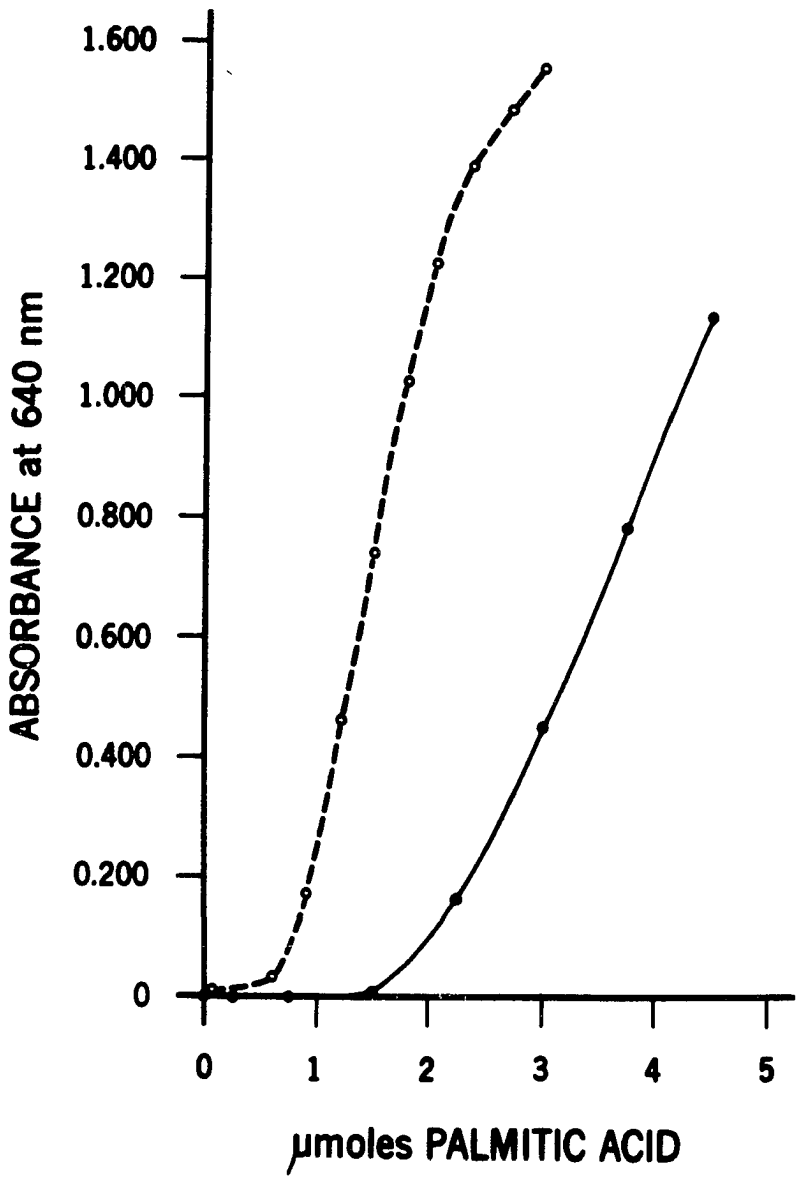

Figure 4. Relationship of color yield to fatty acid concentration with several experimental color reagents. Color reagent mixtures contained absolute ethanol: 0.02% aqueous Nile Blue: 0.02 N NaOH in volume ratios of 9.0:1.0:0.5 (•——•) and 9.0:1.0:0.2 (o---o).

fractions were assayed for their protein content and activities of six different enzymes. The entire experimental scheme was carried out on livers of three different animals, and the data reported are based on the means of the three experiments.

The absolute values of protein content and enzyme activities of whole homogenate are shown in Table 1. As the data for the various subcellular fractions will be presented in relative rather than absolute figures, this table provides the baseline data for each assay to which the corresponding data from the subcellular fractions will be compared.

The percent distribution of protein and enzyme activities in all subcellular fractions are shown in Table 2. An overall recovery for each assay has also been included. An almost quantitative recovery of protein and the enzyme activities cathepsin D, glucose-6-phosphatase, and triglyceride lipase at pH 7.5 was achieved. The recovery of succinic dehydrogenase, lipase at pH 4.5, and 5'-nucleotidase activities was somewhat less satisfactory. The latter enzyme, especially, appears rather labile under the experimental conditions. Vertical scanning of the distribution pattern for each enzyme points out those fractions in which the activity appears to concentrate. Succinic dehydrogenase, a marker for mitochondria, was distributed equally in the nuclear, mitochondrial, and light mitochondrial fractions; it was essentially absent from the soluble cytoplasm (S). Relatively small and equal amounts of cathepsin D, a marker for lysosomes, were present in all particulate fractions; by far the largest bulk of activity was located in the cytoplasm. Glucose-6-phosphatase concentrated heavily in the microsomal fraction, for which it is classically a marker. The 5'-nucleotidase activity, specific to

TABLE 1

ABSOLUTE VALUES OF PROTEIN AND ENZYME ACTIVITIES IN PIG LIVER HOMOGENATE

Assay	Specific Activity[a]	Total Activity[b]
Protein	19.042[c] (17.250 - 20.250)	7189 (6124 - 7850)
Succinic Dehydrogenase	0.816 (0.707 - 1.031)	5941 (4331 - 8093)
Cathepsin D	0.660 (0.489 - 0.825)	4842 (2995 - 6476)
Glucose-6-Phosphatase	6.141 (5.510 - 6.804)	44,517 (33,741 - 53,411)
5'-Nucleotidase	0.884 (0.702 - 0.988)	6058 (5162 - 7503)
Lipase, pH 4.5	1.049 (0.671 - 1.619)	7710 (4109 - 12,295)
Lipase, pH 7.5	0.916 (0.640 - 1.458)	6649 (3852 - 11,072)

[a]Expressed as mg/ml for protein, μmoles product/mg protein/time of incubation for enzymes.

[b]Expressed as mg/ml x total volume for protein, specific activity x total protein for enzymes.

[c]Figures are means of three experiments; numbers in parentheses give range of values.

TABLE 2

PERCENTAGE DISTRIBUTION OF PROTEIN AND TOTAL ENZYME ACTIVITIES IN SUBCELLULAR FRACTIONS OF PIG LIVER

Fraction	Percent of Total Present[a]						
	Protein	Succinic Dehydrogenase	Cathepsin D	Glucose-6-Phosphatase	5'-Nucleotidase	Lipase at pH 4.5	Lipase at pH 7.5
H	100	100	100	100	100	100	100
N	13[b] (7 - 18)	24 (10 - 35)	12 (5 - 19)	13 (9 - 21)	16 (7 - 27)	11 (3 - 18)	9 (5 - 13)
M	9 (4 - 13)	20 (12 - 27)	7 (4 - 9)	9 (1 - 17)	12 (0.4 - 25)	8 (5 - 11)	6 (2 - 8)
L	7 (4 - 9)	23 (10 - 33)	10 (9 - 12)	4 (3 - 6)	2 (0.6 - 3)	10 (6 - 13)	5 (4 - 7)
P	16 (15 - 17)	12 (7 - 18)	8 (3 - 11)	61 (51 - 76)	28 (23 - 34)	9 (8 - 10)	17 (16 - 19)
S	47 (41 - 51)	1 (0.2 - 1.3)	51 (43 - 66)	3 (1 - 4)	10 (5 - 17)	42 (21 - 56)	58 (51 - 61)
Recovery	92 (90 - 94)	79 (73 - 85)	89 (80 - 95)	91 (83 - 95)	68 (63 - 74)	80 (64 - 89)	95 (87 - 102)

[a] $\frac{\text{mg/ml x volume of fraction}}{\text{mg/ml x volume of homogenate}} \times 100$, for protein and $\frac{\text{specific activity x total protein of fraction}}{\text{specific activity x total protein of homogenate}} \times 100$, for enzyme activities.

[b] Figures are means of three experiments; numbers in parentheses give range of values.

plasma membrane fragments, localized mainly in the microsomal fraction, but was also present in the heavier nuclear and mitochondrial fractions. The acidic triglyceride lipase was distributed in a pattern nearly identical to that of the acidic lysosomal marker cathepsin D. Finally, the alkaline triglyceride lipase, like the acidic lipase and the lysosomal marker, showed distinct preference for the soluble fraction. Unlike these two enzymes, however, the portion of activity associated with the particles was present primarily in the microsomal fraction. No other activity tested showed a distribution similar to that of alkaline triglyceride lipase.

The ratios of the specific activity of each subcellular fraction to the specific activity of the homogenate for each enzyme assayed are presented in Table 3. A ratio greater than 1.0 indicates a certain degree of purification of the enzyme in that fraction with respect to the homogenate; a ratio less than 1.0 indicates the converse. The data are comparable to those presented in Table 2: in general, the fractions demonstrating greatest percentage of total activity of a given enzyme are the ones in which greatest purification has been achieved. Several exceptions should be noted, however. While the greatest percentage of total activities of the lysosomal marker cathepsin D and the acidic triglyceride lipase were present in the soluble fraction, greatest purification of these enzymes occurred in the light mitochondrial fraction. The highest specific activity ratio for alkaline triglyceride lipase was present in the soluble fraction. Unlike all other enzymes tested, essentially no purification of this activity was achieved in any particulate fraction.

TABLE 3

DISTRIBUTION OF ENZYME SPECIFIC ACTIVITY RATIOS IN SUBCELLULAR FRACTIONS OF PIG LIVER

Fraction	Specific Activity Ratios[a]					
	Succinic Dehydrogenase	Cathepsin D	Glucose-6-Phosphatase	5'-Nucleotidase	Lipase at pH 4.5	Lipase at pH 7.5
H	1.000	1.000	1.000	1.000	1.000	1.000
N	1.779[b] (1.424-1.966)	0.891 (0.745-1.065)	1.093 (0.694-1.367)	1.498 (1.071-1.937)	0.776 (0.424-1.036)	0.663 (0.597-0.739)
M	2.418 (2.051-2.964)	0.846 (0.559-0.998)	0.8676 (0.217-1.279)	1.060 (0.085-1.839)	0.987 (0.787-1.116)	0.613 (0.502-0.708)
L	3.217 (2.609-3.862)	1.629 (1.194-2.185)	0.701 (0.640-0.817)	0.290 (0.160-0.385)	1.430 (1.345-1.508)	0.807 (0.622-1.022)
P	0.756 (0.425-1.195)	0.528 (0.166-0.711)	3.799 (3.343-4.380)	1.773 (1.499-2.255)	0.671 (0.472-0.896	1.097 (0.971-1.250)
S	0.016 (0.005-0.025)	1.093 (0.838-1.325)	0.054 (0.022-0.081)	0.206 (0.139-0.339)	0.987 (0.835-1.089)	1.223 (0.998-1.477)

[a] $\frac{\text{specific activity of fraction}}{\text{specific activity of homogenate}}$

[b] Figures are means of three experiments; figures in parentheses give range of values.

As noted above, the overall recoveries of activity were variable from enzyme to enzyme. To correct for this variability, the amount of protein and activity of each enzyme which were actually recovered in the five subcellular fractions have been summed up and these figures taken as 100%. The amount of protein or total activity which occurred in a given fraction has then been expressed as a percentage of this value. The effect of this treatment is to provide all enzymes with a common baseline of 100% activity so that the percentages of individual enzyme activities in any subcellular fraction may be compared with one another. These data, presented in Table 4, were based on the assumption that a proportionate amount of each enzyme activity was lost in each subcellular fraction. The correctness of this assumption was not tested.

If the percentage of total enzyme activity in a fraction is divided by the percentage of total protein in that fraction, the resulting figure is defined as a relative specific activity. It provides a measure of purification similar to that of the specific activity ratio. The relative specific activities, calculated from the data corrected for loss of protein and activity, are shown in Table 5.

The data in Tables 4 and 5 are presented in a more illustrative fashion in Figure 5. The enzyme profiles presented in these graphs have been obtained by plotting the mean relative specific activity of the fractions against their mean percentage of total protein. The area under each block is therefore proportional to the total percentage of activity recovered in that fraction, and the height of each block is a measure of the relative purification of the enzyme. The trends described earlier for the localization of each enzyme are clearly evident here. The mito-

TABLE 4

PERCENTAGE DISTRIBUTION OF PROTEIN AND TOTAL ENZYME ACTIVITIES IN SUBCELLULAR FRACTIONS OF PIG LIVER, BASED ON ACTUAL RECOVERIES OF ACTIVITY

Fraction	Percent of Total Present[a]						
	Protein	Succinic Dehydrogenase	Cathepsin D	Glucose-6-Phosphatase	5'-Nucleotidase	Lipase at pH 4.5	Lipase at pH 7.5
N	14[b] (8 - 19)	30 (13 - 44)	13 (6 - 21)	15 (10 - 23)	22 (12 - 36)	15 (3 - 28)	10 (5 - 13)
M	10 (4 - 15)	26 (15 - 35)	8 (4 - 12)	10 (1 - 18)	19 (0.5 - 37)	11 (5 - 16)	6 (2 - 8)
L	7 (4 - 9)	27 (13 - 39)	12 (10 - 15)	5 (4 - 6)	3 (1 - 5)	12 (9 - 15)	6 (4 - 8)
P	17 (17 - 18)	14 (8 - 20)	9 (3 - 13)	67 (55 - 80)	42 (35 -54)	11 (9 - 13)	18 (16 - 20)
S	52 (45 - 57)	1 (0.3 - 2)	58 (50 - 69)	3 (1 - 5)	14 (8 - 23)	51 (33 - 63)	61 (59 - 64)

[a] $\frac{\text{total protein of fraction}}{\text{total protein of five subfractions}} \times 100$, for protein and $\frac{\text{total activity of fraction}}{\text{total activity of five subfractions}} \times 100$, for enzymes.

[b] Figures are means of three experiments; numbers in parentheses give range of values.

TABLE 5

DISTRIBUTION OF ENZYME RELATIVE SPECIFIC ACTIVITIES IN SUBCELLULAR FRACTIONS OF PIG LIVER

Fraction	Relative Specific Activity[a]					
	Succinic Dehydrogenase	Cathepsin D	Glucose-6-Phosphatase	5'-Nucleotidase	Lipase at pH 4.5	Lipase at pH 7.5
N	2.055[b] (1.752-2.258)	0.919 (0.845-1.062)	1.113 (0.687-1.472)	1.666 (1.008-2.455)	0.940 (0.431-1.474)	0.683 (0.659-0.726)
M	2.770 (2.355-3.285)	0.894 (0.557-1.135)	0.882 (0.215-1.238)	1.478 (0.108-2.524)	1.128 (1.075-1.191)	0.594 (0.558-0.663)
L	3.731 (3.000-4.280)	1.689 (1.177-2.181)	0.707 (0.141-0.792)	0.394 (0.219-0.551)	1.841 (1.609-2.045)	0.787 (0.583-0.912)
P	0.799 (0.488-1.193)	0.558 (0.163-0.805)	3.842 (3.238-4.332)	2.425 (1.983-3.233)	0.609 (0.504-0.668)	1.068 (0.954-1.170)
S	0.018 (0.006-0.030)	1.122 (0.949-1.305)	0.055 (0.021-0.087)	0.268 (0.174-0.430)	0.972 (0.733-1.108)	1.182 (1.110-1.318)

[a] $\frac{\text{corrected percentage of total enzyme activity in fraction}}{\text{corrected percentage of total protein in fraction}}$

[b] Figures are means of three experiments; numbers in parentheses give range of values.

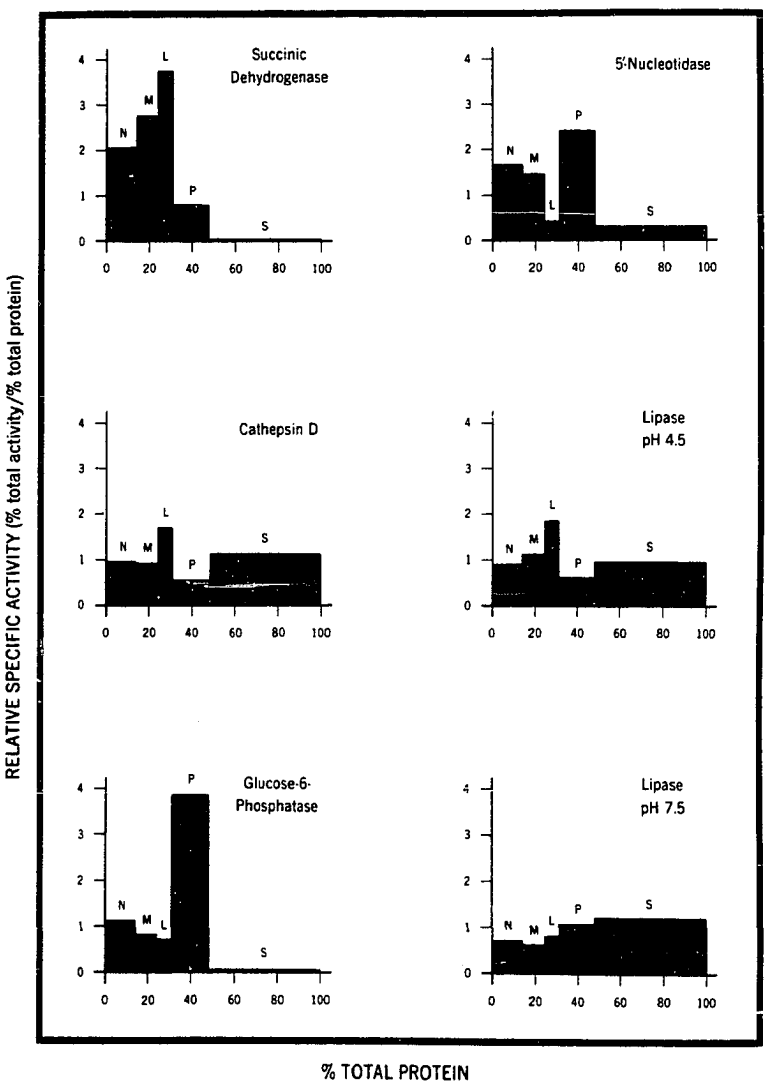

Figure 5. Distribution profiles of enzymes in pig liver subcellular fractions.

chondrial marker, succinic dehydrogenase, demonstrates specificity for the heavier particulate fractions, with maximum purification in the light mitochondria (L). The lysosomal marker, cathepsin D, and the acidic triglyceride lipase are characterized by a high percentage of total activity in the S fraction but peak purification in the light mitochondria (L). Glucose-6-phosphatase, the microsomal marker, shows classic specificity for that fraction (P), while the plasma membrane enzyme, 5'-nucleotidase, distributes somewhat erratically in both heavy (N and M) and light (P) particles. Similarly to the acidic enzymes, the alkaline triglyceride lipase is characterized by a high percentage of total activity in S but fails to parallel their distribution in the particulate fractions. In fact, this enzyme does not show clear evidence of purification in any subcellular fraction.

Purification of Alkaline Triglyceride Lipase

Subcellular fractionation of pig liver homogenates displayed a rather striking inability to yield useful information concerning the alkaline triglyceride lipolytic activity. No subcellular localization could be assigned to the activity, and it was considered possible that several enzyme activities were actually being observed. The large percentage of total activity present in the soluble fraction was of especial interest; therefore, pH curves were run on several preparations of this fraction to determine whether its pH optimum differed noticeably from that reported by Müller and Alaupovic (17) for pig liver microsomal lipase. The pH optima of soluble fractions from different livers varied from 7.25 to 7.50, but no clear distinction could be made between these curves and that presented by the above authors for the microsomal enzyme.

In one experiment, pH curves were run on both the soluble fraction and the particulate fraction isolated by centrifugation of a homogenate at 113,000 g for 30 minutes. As may be seen in Figure 6, the shapes of the curves were nearly identical.

Purification of the alkaline triglyceride lipolytic activity seemed the next logical step in attempting to obtain information about this enzyme or enzymes. Accordingly, the purification scheme presented in Methods and outlined in Figures 2 and 3 was developed. The basic concepts of this approach, isolation of the enzyme bound to a lipid substrate and removal of the enzyme from the lipid-enzyme complex with a detergent, were derived from the work of Anfinsen and Quigley (134) and Fielding (135, 136) on the purification of post-heparin plasma lipoprotein lipase. The soluble fraction obtained by centrifugation of liver homogenates was chosen as starting material because of its high percentage of total lipase activity and its lack of membranous particles.

Isolation of Emulsion-Bound Lipase

Table 6 shows that the initial centrifugation of the mixture of soluble fraction (S) and lipid emulsion, yielding EBL-1 as product, resulted in a substantial purification of alkaline lipase compared to the starting material and eliminated more than 98% of the protein of this starting fraction. Successive centrifugations, yielding EBL-2 and EBL-3 as products, removed further small amounts of extraneous protein with minimal loss of activity and resulted in moderate increases in purification. Recovery and purification of enzymatic activity were improved in a second set of eight experiments in which activity was measured only on EBL-3, the final product of centrifugation. These figures are presented

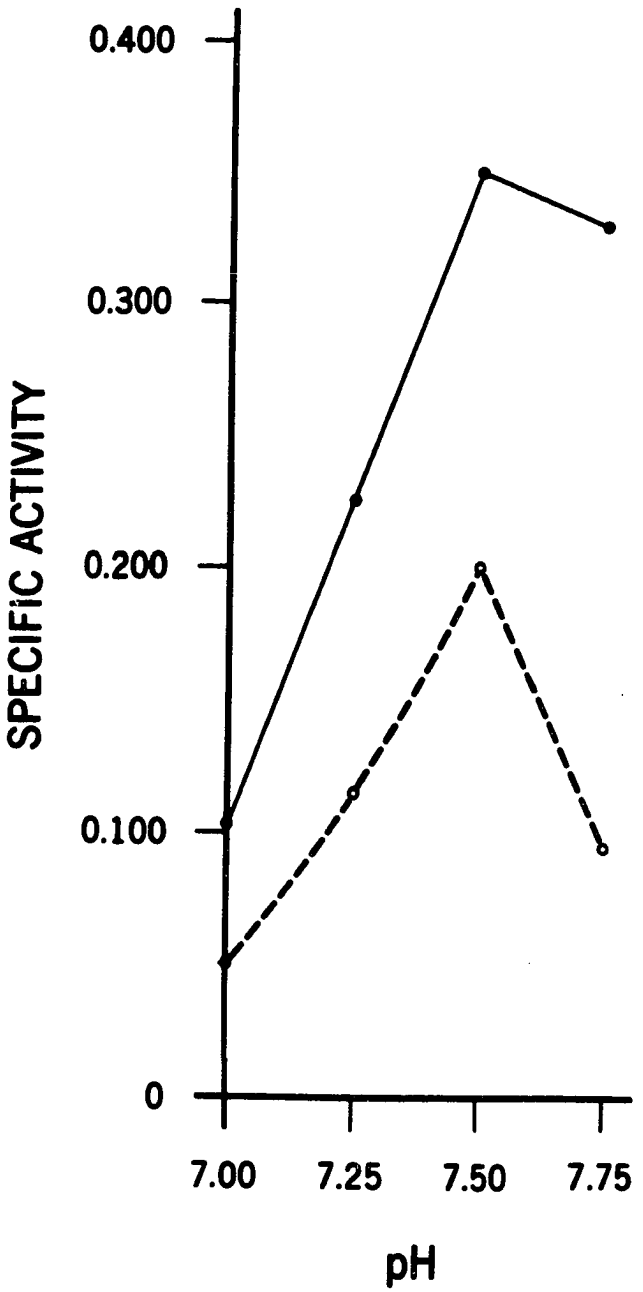

Figure 6. Alkaline range pH curves for pig liver soluble (.—.) and particulate (o---o) fractions. Glycyl-glycine buffer was adjusted to correct pH values with NaOH. Specific activity is defined as μmoles fatty acids released/mg protein/15 minutes.

TABLE 6

PURIFICATION AND RECOVERY OF EMULSION-BOUND LIPASE[a]

Fraction	Percent Recovery of Protein[b]	Percent Recovery of Lipase Activity[c]	Purification[d]
S	100	100	1
EBL-1	2[e] (1 - 2)	28 (26 - 31)	17 (15 - 19)
EBL-2	60 (57 - 62)	80 (60 - 100)	22 (18 - 26)
EBL-3	86 (80 - 92)	92 (87 - 98)	27 (22 - 34)

[a]Activity was measured against purified olive oil at pH 7.5.

[b]Expressed as $\frac{\text{total protein in fraction}}{\text{total protein in previous fraction}} \times 100$.

[c]Expressed as $\frac{\text{total activity in fraction}}{\text{total activity in previous fraction}} \times 100$.

[d]Defined as $\frac{\text{specific activity of fraction}}{\text{specific activity of S}}$

[e]Figures are means of three experiments; numbers in parentheses give range of values.

in Table 7.

The development of the isolation procedure for emulsion-bound lipase was fraught, of course, with technical difficulties, and numerous variations in the procedure were required to define optimal conditions for the system. Brief mention of some of these parameters should be made.

In early experiments, it was observed that when isolated EBL fractions were assayed for alkaline lipolytic activity, coagulation of the assay substrate (purified olive oil emulsified in sodium taurocholate) occurred. This phenomenon did not occur when the soluble fraction, S, served as enzyme source. Coagulation was defined as the formation of large particles of emulsified substrate, which rose to the top of the assay mixture. This contrasted with the smooth, creamy, and uniformly dispersed appearance of the substrate under optimal assay conditions. As the major difference between assay of purified EBL fractions and assay of crude S fractions was the greater amount of protein routinely used as enzyme source in the latter case, it was hypothesized that some minimal level of protein might be required for stabilization of the emulsion during incubation. To test this hypothesis, a preparation of soluble fraction was assayed for alkaline lipolytic activity at "high" and "low" protein concentrations. Visual observation was made of the appearance of the emulsions following incubation, and specific activities were measured. In addition, two extraneous proteins, denatured hemoglobin and bovine serum albumin, were tested for their effects in the system. The results presented in Table 8 show that when the amount of soluble protein assayed was greater than 2 mg, the emulsion remained smooth and a good specific

TABLE 7

PURIFICATION AND RECOVERY OF ALKALINE LIPOLYTIC ACTIVITY OF EBL-3

Fraction	Percent of Protein	Percent of Lipase Activity	Purification[a]
S	100	100	1
EBL-3	1[b] (0.4 - 1.3)	37 (22 - 49)	41 (26 - 56)

[a]Defined as $\frac{\text{specific activity of EBL-3}}{\text{specific activity of S}}$

[b]Figures are means of eight experiments; numbers in parentheses give range of values.

TABLE 8

DEPENDENCE OF ALKALINE TRIGLYCERIDE LIPASE ACTIVITY OF SOLUBLE FRACTION ON QUALITY OF EMULSION AND PRESENCE OF PROTEIN

Amount of Protein Assayed	Extraneous Protein Added	Specific Activity[a]	Appearance of Emulsion after Assay
2.275 mg	None	1.454	Smooth
2.275 mg	Albumin, 2 mg	1.472	Smooth
2.275 mg	Hemoglobin, 2 mg	1.639	Smooth
0.758 mg	None	0.488	Severely coagulated
0.758 mg	Albumin, 2 mg	0.699	Mildly coagulated
0.758 mg	Hemoglobin, 2 mg	2.335	Smooth
0	Albumin, 2 mg	0	Smooth
0	Hemoglobin, 2 mg	0	Smooth

[a]Defined as μmoles fatty acids released/mg protein/15 minutes.

activity was observed. Neither albumin nor denatured hemoglobin had any effect under these conditions. When the enzyme protein assayed was reduced to less than 1 mg, however, severe coagulation of the emulsion and loss of activity occurred. Hemoglobin, but not albumin, was able to overcome these effects, and, in fact, appeared to stimulate the activity. Therefore, the routine assay system used to monitor the purification of alkaline lipase included denatured hemoglobin, dissolved in the assay buffer at a level of 2 mg/test.

The success of the isolation of emulsion-bound lipase also depended on maintenance of a smooth, unbroken emulsion. Partial breaking of the emulsion, leading to the presence of free olive oil at the surface of the packed lipid cakes after centrifugation, interfered with handling of the lipid cakes and reduced the efficiency of the system. This occurred when emulsions prepared in 10% gum arabic solution were substituted for those prepared in sodium taurocholate. Sodium taurocholate stabilized emulsions, however, tended to coagulate under certain conditions of isolation, in a fashion similar to that observed during lipase assays. Coagulation led to the isolation of lipid cakes which were extremely difficult to suspend by aspiration. The suspended EBL fractions were lumpy, and when diluted with wash medium, the lumps rose rapidly to the surface of the solution without centrifugation. Mild coagulation resulted in the binding of extraneous protein to the emulsion; protein recovery was increased and purification decreased. Severely coagulated emulsions failed to bind protein at all. Coagulation occurred either when ions were present in the soluble fraction or when the temperature of the isolation system was permitted to vary by more than a degree

from 0°. In the former case, preparation of the soluble fraction in salt or buffer solutions rather than sucrose, or prior treatment of the soluble fraction with salts such as ammonium sulfate introduced sufficient amounts of ions into the starting material to cause coagulation of the emulsion. In the latter case, the precise temperature required could be maintained only by use of an ultracentrifuge in which rotor temperature, rather than chamber temperature, was controlled.

Separation of Bound Lipase from Lipid Emulsion

To free the lipase from the triglyceride emulsion, extraction of EBL-3 preparations with organic solvents was first tried. Total lipid extractions were carried out with mixtures of ethanol-diethyl ether or chloroform-methanol. Partial extraction was performed either with n-heptane or diethyl ether. Regardless of the conditions employed, solvent extraction of EBL-3 preparations led to the recovery of totally insoluble and enzymatically inactive protein residues.

The next approach was to treat EBL-3 preparations with detergent solutions and to test for protein and lipase activity in the infranates isolated after removal of the emulsion by centrifugation. In accordance with the method of Fielding (136), the first detergent employed was sodium deoxycholate. The procedure has been presented in Methods; average results for a set of six experiments are presented in Table 9. It will be noted that a portion of the bound protein present in EBL-3 could be stripped from the emulsion by this method. However, in only two of the six experiments was any lipolytic activity measurable in the filtered deoxycholate infranates (DOCIF). In one case the activity represented no apparent purification over the starting material; in the other case

TABLE 9

RECOVERY OF PROTEIN AND PURIFICATION OF FILTERED DEOXYCHOLATE INFRANATES (DOCIF)

Fraction	% Recovery of Protein	Purification[a]
EBL-3	100	41[b] (28 - 56)
DOCIF	27	[1; 5][c]

[a]Expressed as $\frac{\text{specific activity of fraction}}{\text{specific activity of soluble fraction (S)}}$

[b]Figures are means of six experiments; numbers in parentheses give range of values.

[c]Values from individual experiments.

purification was minimal. These poor results stand in contrast to the excellent purification observed for the parent EBL-3 fraction.

Severe technical difficulties also attended the use of the detergent sodium deoxycholate. Dialysis and concentration of the isolated deoxycholate infranates by ultrafiltration were marked by extremely slow flow rates and the development of excessive turbidity in the filtered preparations. When DOCIF fractions were permitted to stand, white sediments appeared in the tubes concomitant with partial clearing of the solutions. The sediments were identified as precipitated sodium deoxycholate rather than protein by their solubility in chloroform:methanol (2:1, v:v). Attempts to remove excess sodium deoxycholate from DOCIF preparations by centrifugation, gel filtration on Sephadex G-200 or gradient elution from hydroxylapatite gel failed. In no case could an enzymatically active fraction be recovered.

In one experiment a solution of 0.5% sodium taurocholate was substituted for the sodium deoxycholate. While handling of this system was much easier, sodium taurocholate failed to strip protein from the emulsion. The trace amount of protein recovered in the filtered taurocholate infranate, however, was enzymatically highly active.

The third detergent tested for its ability to split the lipid-protein complex was Triton X-100. Average results for three experiments are presented in Table 10. This detergent was capable of releasing approximately 50% of the bound protein and an even greater percentage of lipolytic activity. Filtration of Triton infranates proceeded rapidly, although TIF preparations retained some turbidity.

The process of separation of enzyme protein from its complex

TABLE 10

PURIFICATION AND RECOVERIES OF PROTEIN AND LIPASE ACTIVITY IN FILTERED TRITON INFRANATES (TIF)

Fraction	Percent Recovery of Protein	Percent Recovery of Activity	Purification[a]
EBL-3	100	100	37[b] (31 - 50)
TIF	56 (41 - 66)	76 (55 - 105)	53 (35 - 83)

[a]Expressed as $\frac{\text{specific activity of fraction}}{\text{specific activity of soluble fraction (S)}}$

[b]Figures are means of three experiments; numbers in parentheses give range of values.

with lipid was refined even further in experiments in which Triton X-100-treated EBL-3 preparations were subjected to density gradient centrifugation. The details of procedure have been presented in Methods; after centrifugation, six fractions, dg-I through dg-VI, were removed from tubes as shown in Figure 7. Lipid cakes were discarded due to technical difficulties in handling them. The combined recoveries of protein and lipolytic activity for all six density gradient fractions are presented in Table 11. The results are averaged from two experiments. Table 12 shows the purification, percentage of recovered protein and percentage of recovered enzyme activity present in each of the six fractions based upon data from the same experiments. The highest percentage of recovered activity and highest purification occurred in fraction dg-VI. The results of a series of five further experiments in which assays were performed only on the starting material (S) and the final fraction (dg-VI) are averaged in Table 13. Purification of the alkaline long-chain triglyceride lipolytic activity of pig liver was terminated at this point.

The purification figures calculated throughout the isolation scheme for the alkaline triglyceride lipolytic activity have been based on the starting material S. As may be seen by referring to the subcellular fractionation data (Table 3), the specific activity of the soluble fraction was quite similar to that of the homogenate. Therefore, calculation of purification figures based on the homogenate would give similar values to those presented.

Characterization of Alkaline Triglyceride Lipase

Presence of Acidic Triglyceride Lipase

Although the purification scheme was developed for the purpose

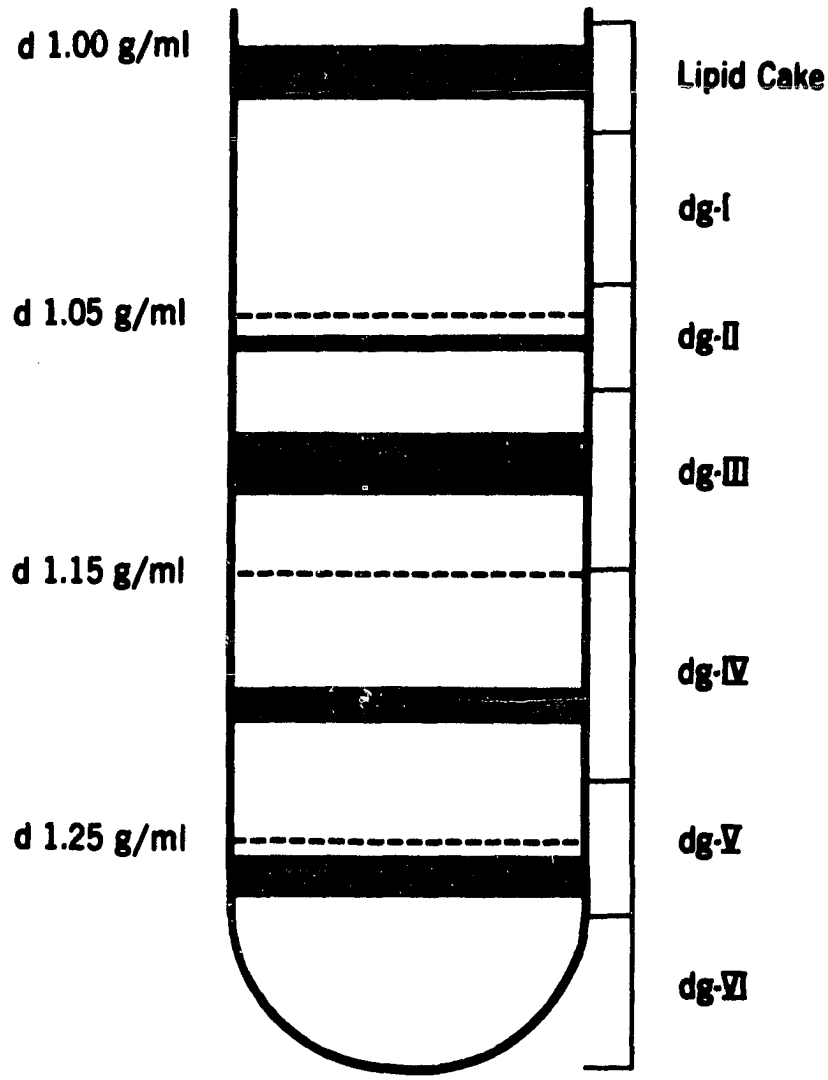

Figure 7. Density gradient centrifugation of Triton X-100 treated EBL-3.

TABLE 11

RECOVERIES OF PROTEIN AND LIPOLYTIC ACTIVITY IN SIX DENSITY GRADIENT FRACTIONS

Fraction	Percent Recovery of Protein	Percent Recovery of Activity
EBL-3	100	100
dg-I through dg-VI	62[a] (58 - 65)	63 (49 - 77)

[a]Figures are means of two experiments; numbers in parentheses give range of values.

TABLE 12

PURIFICATION AND RECOVERY OF PROTEIN AND LIPOLYTIC ACTIVITY IN DENSITY GRADIENT FRACTIONS

Fraction	Percent of Recovered Protein	Percent of Recovered Activity	Purification[a]
dg-I	8[b] (8 - 8)	1 (1 - 2)	3 (3 - 3)
dg-II	4 (4 - 4)	1 (1 - 1)	6 (4 - 7)
dg-III	9 (6 - 12)	4 (4 - 5)	13 (6 - 19)
dg-IV	22 (12 - 33)	19 (17 - 20)	29 (9 - 48)
dg-V	41 (30 - 52)	28 (23 - 33)	16 (11 - 21)
dg-VI	15 (13 - 18)	47 (44 - 49)	68 (59 - 77)

[a]Expressed as $\frac{\text{specific activity of fraction}}{\text{specific activity of soluble fraction (S)}}$

[b]Figures are means of two experiments; numbers in parentheses give range of values.

TABLE 13

PURIFICATION AND RECOVERY OF ALKALINE LIPOLYTIC ACTIVITY OF dg-VI

Fraction	Percent of Protein	Percent of Lipase Activity	Purification[a]
S	100	100	1
dg-VI	0.2[b] (0.1 - 0.2)	16 (12 - 26)	92 (84 - 111)

[a]Defined as $\frac{\text{specific activity of fraction}}{\text{specific activity of S}}$

[b]Figures are means of six experiments; numbers in parentheses give range of values.

of isolating the alkaline triglyceride lipolytic activity, it was considered important to determine the behavior of the acidic lipase during the procedure. It will be remembered that a large percentage of the acidic activity was also present in the soluble fraction, the starting material for the isolation of alkaline lipase. In four experiments, the acidic and alkaline lipolytic activities were measured on both starting material (S) and the final fraction dg-VI. Comparison of the purifications achieved for each enzyme is shown in Table 14. Some purification of the acidic lipase occurred in all experiments, but in only one case was it purified to the same extent as the alkaline activity. The purification of the acidic enzyme appeared to vary independently from that of the alkaline enzyme.

Determination of Optimal Assay Conditions for Alkaline Triglyceride Lipase

Among the first characterization studies carried out on purified dg-VI fractions were those designed to define or redefine optimal conditions for the lipolytic assay. Preparations were tested for pH dependence, comparative behavior toward purified olive oil and triolein as substrates, time dependence, and substrate concentration dependence.

Determination of pH optimum. The pH curve for a dg-VI preparation is shown in Figure 8. Based on these results, all further characterization studies were carried out at pH 7.25 in 0.02 M phosphate buffer. The use of glycyl-glycine buffer was discontinued because of its lack of buffering capacity at this pH.

Comparative behavior toward purified olive oil and triolein. The amounts of fatty acid released by a dg-VI preparation from purified

TABLE 14

COMPARATIVE PURIFICATIONS OF ALKALINE AND ACIDIC TRIGLYCERIDE LIPASES

Experiment Number	Purification of Alkaline Lipase[a]	Purification of Acidic Lipase[b]
1	59	20
2	77	7
3	77	41
4	103	93

[a] $\frac{\text{Specific activity of dg-VI at pH 7.5}}{\text{Specific activity of S at pH 7.5}}$

[b] $\frac{\text{Specific activity of dg-VI at pH 4.5}}{\text{Specific activity of S at pH 4.5}}$

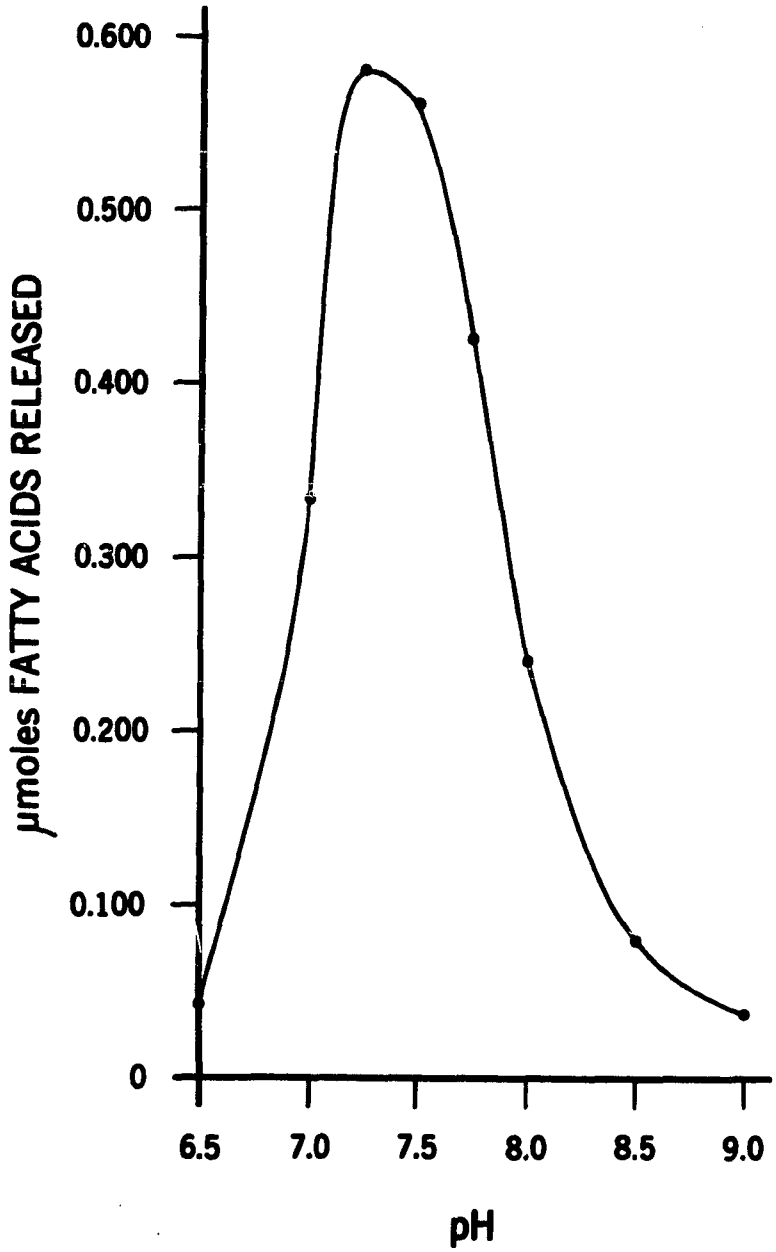

Figure 8. pH dependence of alkaline triglyceride lipase. Enzyme source was dg-VI preparation; enzyme concentration was 14 µg protein/test. Substrate was purified olive oil at concentration of 60 µmoles/test based on MW of triolein. Buffer system was 0.02 M phosphate -0.1 M glycyl-glycine. Incubations were carried out for 15 minutes.

olive oil and commercially purified triolein (99+%) are shown in Table 15. The release from triolein was lower than that from purified olive oil but was still quite satisfactory. While olive oil preparations contained only long-chain triglyceride, approximately 25% of their fatty acid content consisted of fatty acids other than oleate (101). Triolein was considered the substrate of choice for further characterization studies because of its fatty acid homogeneity.

Time dependence. The release of fatty acids from triolein by purified lipase as a function of time is shown in Figure 9. The reaction proceeded rapidly for the first 15minutes; leveling off occurred thereafter. The choice was made to increase the standard incubation time of assays to 30 minutes. Although the reaction rate slowed during this period, the increment in fatty acid released was felt to justify the extension in time.

Substrate concentration dependence. In Figure 10 the release of fatty acids by a preparation of purified lipase is plotted as a function of the concentration of the triolein substrate. The substrate optimum was 45 μmole/test; less activity occurred at concentrations both below and above this value. Further characterization of the purified lipase has been carried out at this concentration of substrate.

Proofs of Enzymic Nature of Lipase Reaction

Heat inactivation. Pretreatment of a purified dg-VI fraction at 60° or 100° resulted in loss of all lipolytic activity toward triolein. The preparation was quite stable, however, to preincubation at 37°. These results are presented in Table 16.

Release of fatty acids as a function of protein concentration.

TABLE 15

RELEASE OF FATTY ACIDS FROM PURIFIED OLIVE OIL AND TRIOLEIN BY ALKALINE TRIGLYCERIDE LIPASE

Substrate[a]	μmole Fatty Acid Released[b]
Purified olive oil	0.452
Triolein	0.377

[a]Concentration of olive oil: 60 μmoles/test based on MW of triolein. Triolein concentration: 60 μmoles/test.

[b]As assayed at pH 7.5 with dg-VI fraction at concentration of 11 μg protein/test.

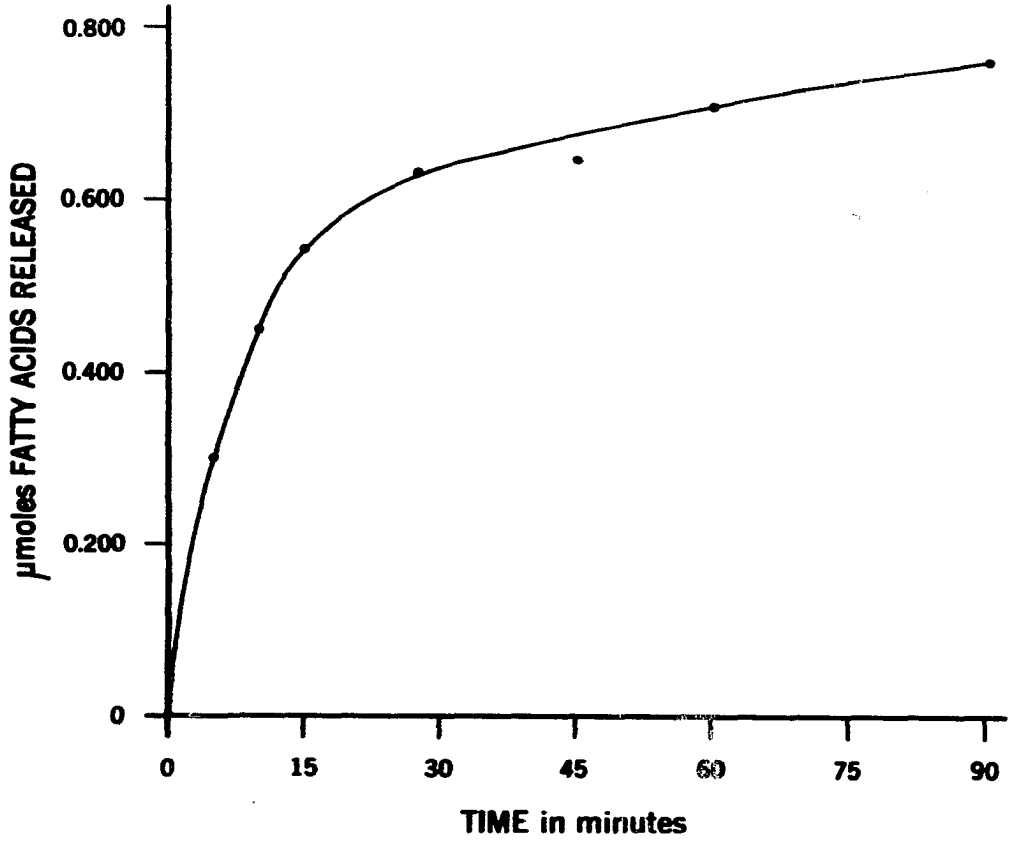

Figure 9. Time dependence of alkaline triglyceride lipase reaction. Buffer was 0.02 M phosphate at pH 7.25; substrate was 60 µmoles triolein/test. Enzyme source was dg-VI fraction at concentration of 14 µg protein/test.

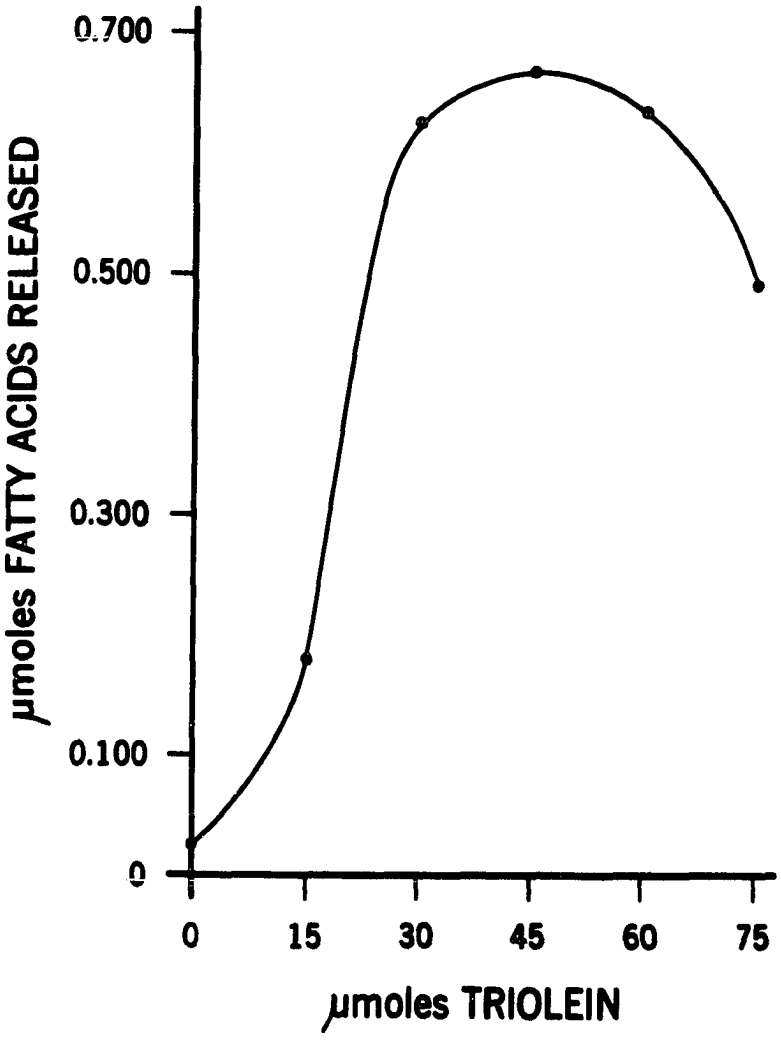

Figure 10. Substrate concentration dependence of alkaline triglyceride lipase. Incubation was carried out for 30 minutes in 0.02 M phosphate buffer at pH 7.25. Enzyme source was dg-VI fraction at concentration of 23 µg/test.

TABLE 16

LIPOLYTIC ACTIVITY REMAINING IN HEAT TREATED ALKALINE LIPASE PREPARATIONS[a]

Pretreatment of Enzyme Source	Percent Activity Remaining
None	100
100°, 5 minutes	0
60°, 10 minutes	5
37°, 15 minutes	94
37°, 30 minutes	99
37°, 60 minutes	89

[a]Assay of treated preparations was carried out in phosphate buffer at pH 7.25 with 45 μmoles/test triolein as substrate. Incubations were for 30 minutes at 37°.

The lipolytic response was linear over a 4-fold range in enzyme protein concentration, as shown in Figure 11.

Development of Alternative Assay System for Alkaline Lipase

In preliminary studies on the effects of various additives on triolein lipolysis by purified lipase, carried out under the optimal conditions detailed above, it was noticed that the substrate emulsion coagulated when ionic compounds such as NaCl or protamine sulfate were used as additives. Decreased activities were invariably present when coagulation occurred. This was reminiscent of the problems encountered during purification and during assay of purified fractions. The coagulation appeared to be caused by the presence of ions but was not counteracted by the denatured hemoglobin routinely present in the assay system. It was decided to investigate the possibility of substituting a 10% solution of gum arabic for the 12 mM sodium taurocholate normally used as emulsifying agent in an attempt to overcome the problem of coagulation. While it was observed that emulsions of triolein in gum arabic were indeed stable in the presence of ions, they were poor substrates for the lipase. Nor could activity with these emulsions be stimulated by the inclusion of denatured hemoglobin. The experiments were then extended to include the effects of several other proteins on both types of emulsions in an attempt to find either a second protein which might stimulate activity with taurocholate stabilized emulsions and provide protection against ions or a protein capable of stimulating activity with gum arabic stabilized emulsions. Results of these experiments are presented in Table 17. The stimulatory effect of denatured hemoglobin on activity with emulsions

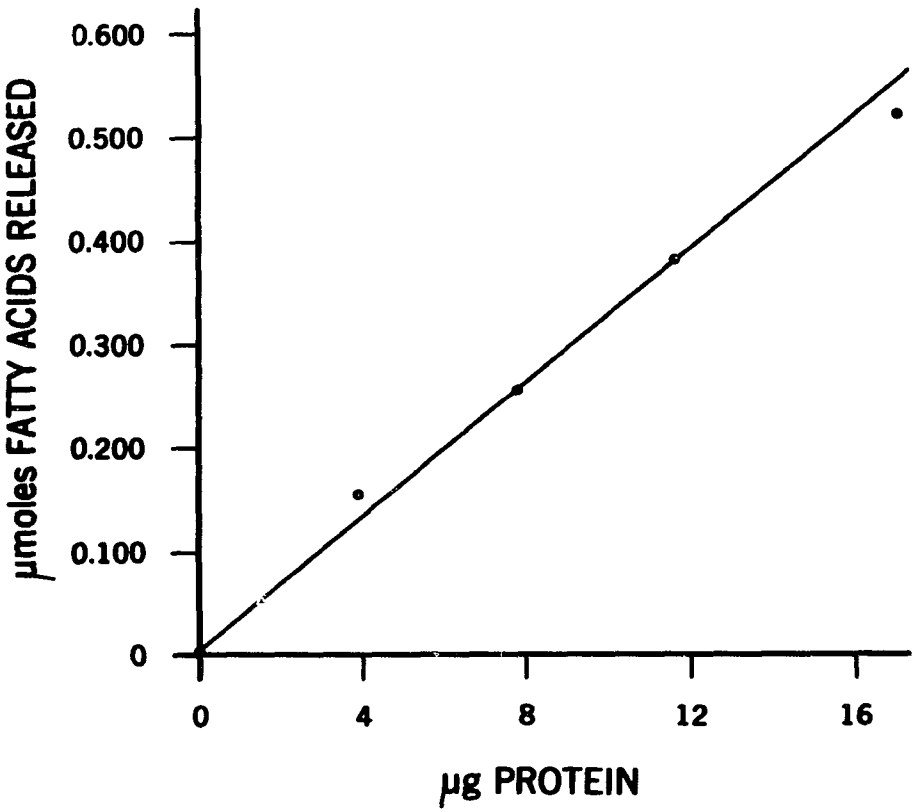

Figure 11. Release of fatty acids as a function of enzyme protein concentration. Assay was performed in phosphate buffer at pH 7.25 with a dg-VI preparation as enzyme source. Substrate was 45 µmoles triolein/test; incubation was for 30 minutes at 37°.

TABLE 17

EFFECTS OF PROTEINS ON LIPOLYSIS BY PURIFIED ALKALINE LIPASE OF TRIOLEIN EMULSIFIED IN SODIUM TAUROCHOLATE AND GUM ARABIC[a]

Protein	mg Protein Added[b]	Emulsifying Agent	Specific Activity
None	-	Sodium taurocholate	13.778
Denatured hemoglobin	2	Sodium taurocholate	56.667
Bovine serum albumin	2	Sodium taurocholate	22.444
Human plasma A-I polypeptide	0.84	Sodium taurocholate	7.333
Human plasma A-II polypeptide	0.54	Sodium taurocholate	11.556
None	-	Gum arabic	4.000
Denatured hemoglobin	2	Gum arabic	14.778
Bovine serum albumin	2	Gum arabic	65.667
Human plasma A-I polypeptide	0.84	Gum arabic	51.889
Human plasma A-II polypeptide	0.54	Gum arabic	3.444

[a]Preparation of dg-VI.

[b]All amounts represent an approximate molar concentration of 2×10^{-5} in the final mixture.

in taurocholate could not be duplicated by any other protein tested. On the other hand, emulsions in gum arabic were stimulated very well by both albumin and A-I polypeptide of human apolipoprotein A. It should be mentioned that none of these proteins exhibited intrinsic lipolytic activity. On the basis of these results, it was decided to carry out studies on the effects of additives in a system which included bovine serum albumin and triolein emulsions prepared in 10% gum arabic. Sodium taurocholate and denatured hemoglobin were omitted. Other conditions of assay (pH, substrate concentration, time of incubation) remained at their previously determined optimal values. A check of the fatty acids released by purified lipase from an emulsion prepared in gum arabic as a function of the amount of albumin present in the system showed that the value of 2 mg per test, selected previously in an arbitrary fashion, was appropriate (Figure 12).

Effects of Additives

Table 18 lists the effects of a number of compounds on albumin-stimulated lipolysis by purified dg-VI fractions of triolein emulsified in gum arabic. Compounds proving inhibitory included NaCl, Triton X-100, iodoacetamide, and higher concentrations of EDTA and sodium taurocholate. Non-inhibitors included protamine sulfate, E-600, eserine sulfate, Ca^{++} and sodium taurocholate at lower concentration. The lower concentration of EDTA tested appeared stimulatory. Of interest was the fact that Ca^{++} ions were not able to substitute for albumin in this system.

Comparison of Liver Lipase and Lipoprotein Lipase

The starting material (S) and purified lipase (dg-VI) failed to

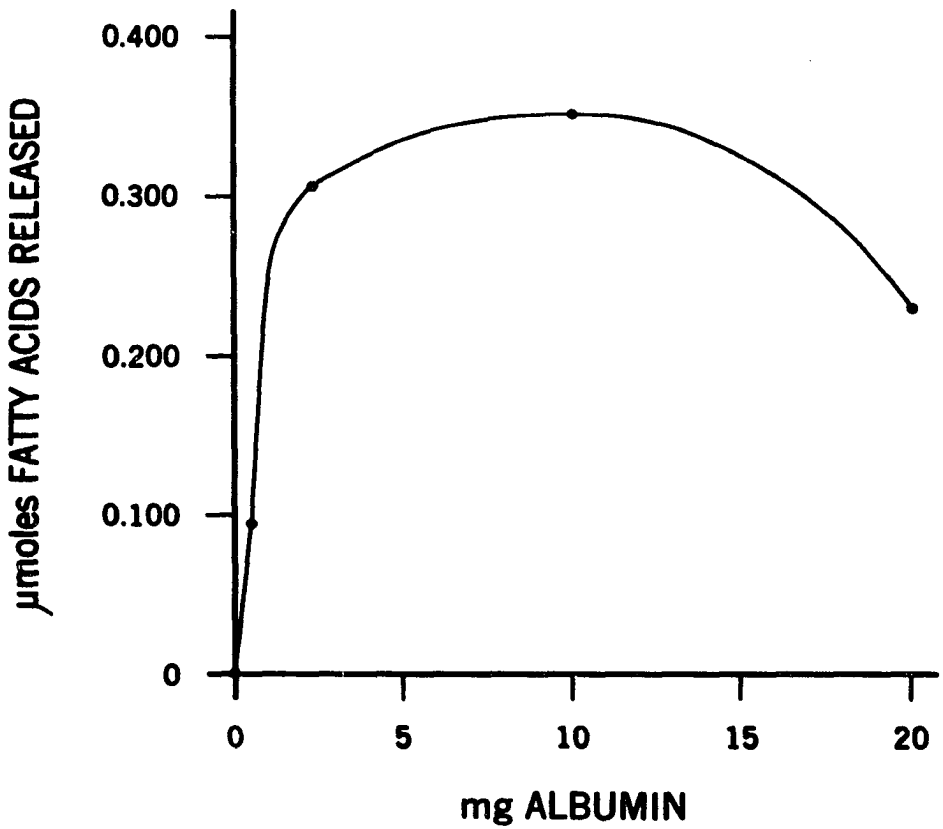

Figure 12. Release of fatty acids from triolein emulsified in gum arabic as a function of added bovine serum albumin. Enzyme source was a dg-VI fraction at a concentration of 16 µg protein/test. Triolein concentration was 45 µmoles/test; incubation was for 30 minutes at 37°.

TABLE 18

EFFECTS OF ADDITIVES ON LIPOLYSIS OF TRIOLEIN BY PURIFIED ALKALINE LIPASE

Compound	Concentration	Percent of Control Activity
NaCl	5.00×10^{-1} M	66
NaCl	1.00 M	45
Protamine sulfate	0.10 mg/ml	90
Protamine sulfate	1.00 mg/ml	109
NaF	2.00×10^{-1} M	35
Triton X-100	0.07 mg/ml	27
Triton X-100	0.67 mg/ml	3
Sodium taurocholate	4.00×10^{-3} M	109
Sodium taurocholate	12.00×10^{-3} M	63
Sodium deoxycholate	4.00×10^{-3} M	0
E-600	1.00×10^{-5} M	98
Eserine sulfate	5.00×10^{-4} M	98
Iodoacetamide	5.00×10^{-2} M	19
EDTA	1.00×10^{-2} M	150
EDTA	1.00×10^{-1} M	80
Ca^{++}	1.00×10^{-3} M	104
Ca^{++} (albumin omitted)	1.00×10^{-3} M	14
(Albumin omitted)	-	8

release fatty acid from triolein when measured in a system specific for the detection of lipoprotein lipase (137, 138), as shown in Table 19. The converse was also true: a preparation of human post-heparin plasma lipoprotein lipase, purified according to Ganesan and Bradford (139), did not release fatty acid from triolein in the liver lipase assay system.

Lipoprotein lipase has been repeatedly demonstrated to release fatty acids from human chylomicrons and plasma lipoproteins (86, 87, 140, 141). Attempts were made to demonstrate hydrolysis of human chyle chylomicrons and plasma lipoproteins by purified dg-VI preparations under a number of experimental conditions. As may be seen in Table 20, release of fatty acids from all substrates was minimal or absent. The amount of enzyme protein tested against these substrates had, in each case, previously been demonstrated to release 0.3-0.4 μmole of fatty acid from artificial triolein emulsions. In contrast, amounts of purified human post-heparin lipoprotein lipase (139) which were observed to release 2.0 μmoles of fatty acid in the lipoprotein lipase assay system, released as much as 5.8 μmoles of fatty acid from chyle chylomicrons and 4.9 μmoles of fatty acid from very low density lipoproteins when the substrates contained 2.0 mg triglyceride per test (142).

TABLE 19

BEHAVIOR OF LIVER LIPASE IN LIPOPROTEIN LIPASE ASSAY SYSTEM

Enzyme Source	Specific Activity in Lipoprotein-Lipase Assay System[a]	Specific Activity in Liver Lipase Assay System[c]
S	0	0.632
dg-VI	0.041	13.780
LPL[b]	135	0

[a]Expressed as μmoles fatty acids/mg protein/hour.

[b]Purified human post-heparin plasma lipoprotein lipase.

[c]Expressed as μmoles fatty acids/mg protein/30 minutes.

TABLE 20

RELEASE OF FATTY ACIDS FROM NATURAL SUBSTRATES BY PURIFIED ALKALINE LIPASE

Substrate	mg Triglyceride/test	Additives to Basic System[a]	μmole Fatty Acid Released
Chyle chylomicrons	2.136	None	.054
Chyle chylomicrons	2.136	4×10^{-3} M sodium taurocholate	.056
Chyle chylomicrons	2.136	2 mg denatured hemoglobin	.019
Chyle chylomicrons	2.136	2 mg albumin	.023
Chyle chylomicrons	2.136	20 mg albumin	.024
Chyle chylomicrons	2.136	10^{-3} M Ca^{++}	.031
Chyle chylomicrons	2.136	2 mg albumin, 10^{-3} M Ca^{++}	.058
Chyle chylomicrons	2.136	2 mg albumin, 10^{-3} M Ca^{++}, 10^{-3} M Mg^{++}	.057
Plasma VLDL[b]	0.585	2 mg albumin	0

[a]The basic system consisted of phosphate buffer at pH 7.25, lipoprotein preparation (in water), and enzyme preparation (in 0.25 M sucrose).

[b]Very low density lipoproteins.

CHAPTER V

DISCUSSION

Development of Spectrophotometric Assay for Long-Chain Fatty Acids

Three types of methods are in general use for the quantitation of long-chain fatty acids resulting from lipolysis: radioactivity analysis, colorimetry, and titrimetry. Radioactivity analyses are highly sensitive but often prohibitively costly. Most of the colorimetric methods for quantitation of long-chain fatty acids depend on the formation of copper or cobalt soaps (143) followed by spectrophotometric quantitation of the metal in the presence of complexing reagents such as diethyldithiocarbamate (144, 145) or diphenylcarbohydrazide (146). The principal drawback associated with the colorimetric methods is their technical complexity; a large number of steps is required to produce the final colored product.

For ease of handling, titrimetric methods have proven most satisfactory in routine quantitation of long-chain fatty acids. Extracted fatty acids may be titrated with dilute base either automatically to a fixed endpoint near pH 10 (147) or manually in the presence of an indicator solution to a visual endpoint near the same pH value (132, 133). The method developed for assay of fatty acids in the present study offers the simplicity common to other titrimetric methods. In addition, as

quantitation is carried out spectrophotometrically, the human bias of visual endpoint titration is eliminated. The method is superior to automatic titration in speed; the time required for quantitation of the fatty acids released during an average lipase assay is 1-1.5 hours, rather than 3-4 hours. The principal drawback in this method is the relatively narrow range (1.0-1.6 μmole of fatty acid) of linear color response. However, adjustment of the amount of enzyme protein incubated serves to maintain fatty acid release within the necessary limits.

Subcellular Fractionation

The presence of two long-chain triglyceridases in pig liver was described by Müller and Alaupovic (17). One of these enzymes possessed a pH optimum of 4.5 and was localized in lysosomes. The other enzyme, exhibiting a pH optimum of 7.5-7.8, displayed highest specific activity in microsomes. In the present study, preliminary experiments failed to demonstrate any increase in the specific activity of the alkaline lipase in microsomes compared to crude homogenates. For this reason quantitative subcellular fractionations were performed, and the distributions of both triglyceride lipases were compared to the distributions of several marker enzymes. The subcellular fractionation was admittedly somewhat crude, but as judged by the results for the mitochondrial, lysosomal and microsomal marker enzymes, partial separation of these organelles was achieved. The distribution of the acidic lipase, measured at pH 4.5, followed very closely that of the lysosomal marker, cathepsin D. This confirms the lysosomal origin of this lipolytic enzyme. Numerous reports on the presence of an acidic triglyceride lipase in rat liver lysosomes are available (8, 9, 10, 11, 12, 13, 148); an acidic lipase also occurs

in human liver (105, 106) but its localization has not been studied.

The occurrence of a large percentage of the activities of the lysosomal marker enzyme and the acidic lipase in the soluble fraction seems at variance with the reported tendency of lysosomes to concentrate in the light mitochondrial fraction (120). It is believed that the blade-type homogenizer used for preparing homogenates and resuspending centrifugally isolated sediments in the present study was too harsh a procedure to permit optimal recovery of lysosomal enzymes in particulate form. The fragility of lysosomes is well recognized (149), and treatment of lysosomes in a Waring Blendor is a known method of releasing enzymic activities into soluble form (120).

A precise subcellular localization could not be assigned to the alkaline triglyceride lipolytic activity on the basis of these experiments. The enzyme failed to show a distinct increase in specific activity in any subcellular fraction or to parallel the distribution of any marker enzyme. The high percentage of total activity occurring in the soluble fraction was striking. There are several explanations for this unusual distribution. The observed pattern might reflect the presence of two or more enzymes, one of which is localized in the cytoplasm and the other in particulate organelles. Guder et al. (11) observed a similar distribution of the triolein-hydrolyzing activity at pH 8.5 in rat liver. The activity displayed a slight tendency to localize in the microsomal fraction, but failed to parallel the distribution of the microsomal marker enzyme, glucose-6-phosphatase. Approximately 40% of the total activity resided in the soluble fraction. These authors suggested the presence of two particulate lipases with alkaline pH optima, one specific to

microsomes and the other to plasma membranes. The soluble activity was considered to represent either a third enzyme or activity washed off the microsomes during isolation.

The observed distribution of alkaline activity could also be explained by assuming the presence of only one enzyme, localized in the soluble fraction, and lipophilic in nature. A free lipophilic enzyme might be expected to adhere to the lipoprotein membranes of all subcellular particles rather non-specifically, producing a smeared distribution during subcellular fractionation. That the soluble enzyme is lipophilic has been adequately proven by the success of the isolation procedure based on the formation of an enzyme-substrate complex. The identity of the pH curves determined for the particulate and soluble fractions from a single liver supports the one enzyme hypothesis.

Other investigators have reported that hydrolysis of long-chain triglycerides by rat liver at alkaline pH proceeds maximally in the microsomes (14, 103) and the mitochondria (16). Hydrolysis of Ediol has been found to occur principally in the soluble fraction (7), while the hydrolysis of tricaprylin has been localized in mitochondria (110). In other mammalian organs, including pig aorta (150), rat pancreas (151), and pig intestine (152), long-chain triglyceride lipases with neutral or alkaline pH optima show greatest total activity in the soluble fraction, although in the latter case specific activities were higher in mitochondria and microsomes. The hormone-sensitive long-chain triglyceride lipase of rat adipose tissue has also been found to concentrate in the soluble or "fat poor supernatant" fraction derived from homogenates prepared in sucrose solution (157).

Purification of Alkaline Triglyceride Lipase

The use of an enzyme-substrate complex as a means for purifying post-heparin lipoprotein lipase was first described by Anfinsen and Quigley (134) in 1953. Fielding (135, 136) developed a procedure for removal of the purified enzyme from the complex by detergent treatment. Further modifications were made by Ganesan and Bradford (139). The present study represents the first attempt to use this approach for purification of a lipase other than lipoprotein lipase. Both alkaline liver lipase and lipoprotein lipase bind to the oil-water interface of a substrate emulsion. Pancreatic lipase is also adsorbed to its emulsified substrate according to Benzonana and Desnuelle (153) and Schoor and Melius (154). To confer maximum simplicity on the isolation system, a sodium taurocholate-stabilized emulsion of olive oil was substituted for the commercially prepared emulsions of coconut or soybean oil used in lipoprotein lipase purifications. The volume ratio of emulsion to soluble fraction used for isolation was high enough to permit the formation of packed lipid cakes during centrifugation; no attempt was made to determine whether binding was optimal under these conditions. Similarly, the pH of 7.5 employed for all operations was approximately the pH optimum for the enzyme activity, but the possibility that more effective binding might have occurred at another pH was not explored. Incubating the soluble fraction-substrate mixture at 37° prior to centrifugation at 0° did not improve binding of the enzyme. The interesting problem of coagulation of the emulsion under certain experimental conditions and the comparative behavior of the three detergents (sodium deoxycholate, sodium taurocholate and Triton X-100) which were used to split the enzyme-sub-

strate complex will be discussed later.

Density-gradient centrifugation of the detergent-treated enzyme-substrate complex was attempted for several reasons. Short centrifugations of the detergent-treated complex had failed to remove all lipid from the enzyme-containing infranate. It was considered possible that the enzyme protein was still lipid-bound in these infranates and that density gradient centrifugation might result in the isolation of some specific lipid-protein complex containing the enzymatic activity. Alternatively, it was felt that simply maintaining contact between the complex and detergent for a longer period of time while applying centrifugal force might result in more efficient enzyme removal. The experimental results were interesting in that the enzymatic activity occurring in any fraction collected after centrifugation appeared to be inversely related to the amount of triglyceride (or density) of the fraction. Less dense fractions, containing more lipid, demonstrated lower specific activities and purifications that did heavier fractions; the most highly purified enzyme was that remaining in the density 1.25 g/ml solution, essentially free of triglyceride. It must be argued either that the lipase protein is less tenaciously bound to lipid than are contaminant proteins and is, therefore, more easily removed by detergent or that the removal of lipid from the enzyme tends to activate it. If this were the case, density gradient centrifugation would not be producing an actual purification of the enzyme protein but would be producing an apparent purification by the removal of excess lipid.

Characterization of Alkaline Triglyceride Lipase

Optimal Assay Conditions

pH Optimum. The measured pH optimum of 7.25 for purified alkaline lipase is within physiological range and is quite similar to the value of 7.5 reported by Müller and Alaupovic (17) for pig liver microsomal lipase. Reported alkaline pH optima for hydrolysis of long-chain triglycerides by crude rat liver preparations include 7.2 and 8.0 (155), 7.2 (14), and 8.5 (11). The pH optimum of hormone-sensitive lipase from rat adipose tissue has been reported to lie between 6.5 and 7.0 (156-159) and at 7.4 (160). A hormone-sensitive lipase with pH optimum near 7.0 also resides in rat heart (161). Similar long-chain triglyceride lipases from adipose tissue whose relationship with hormones has not been established show optima between 6.5 and 7.5 (162) and at 7.5 (163). Pancreatic lipase activity is maximal at pH 8.0-8.5 (164, 165) or in the range 7.5-9.5 (166). Strongly alkaline pH optima, near 9.0, have been reported for lipases of intestine (152) and aorta (167). Post-heparin plasma lipoprotein lipase functions optimally at pH 8.2 (135, 168) as do lipoprotein lipase from adipose tissue (158) and heart (161). On the basis of pH optimum, the alkaline pig liver lipase shows possible similarity to hormone-sensitive lipase and the lipases of adipose tissue whose hormone sensitivity is undetermined. Its pH profile is distinctly different from those of pancreatic lipase and lipoprotein lipase.

Time dependence. The release of fatty acids by alkaline liver lipase plateaued between 15 and 30 minutes in the presence of apparently non-limiting amounts of substrate. This feature is a common characteristic of long-chain triglyceride lipases. Similar curves have been re-

ported for liver lipase (11, 17, 103), for adipose tissue lipases (157, 160, 169) and for lipoprotein lipase (86, 138, 168). Since purified liver alkaline lipase was stable to pretreatment at 37° for at least one hour, simple denaturation due to temperature could not account for this phenomenon. Pancreatic lipase has been shown to denature rapidly at the oil-water interface of a substrate emulsion (170) and also to be inhibited by the liberated fatty acids or their soaps. However, since bile salts were found to reduce both denaturation and product inhibition (170, 171), it is uncertain whether these mechanisms were operating in the assay system for liver lipase which contained sodium taurocholate.

Substrate concentration. The tendency for inhibition of lipolytic activity to occur above the optimal substrate concentration has been reported for long-chain triglyceridases of liver (9, 11) and aorta (167), and may be inferred from data presented for lipoprotein lipase (138) and adipose tissue lipase (163). This phenomenon, which also occurred in the present study, has never been explained.

The effective substrate concentration for a lipase is actually the interfacial area in a unit volume of emulsion rather than the molar concentration of lipid (153). Therefore, the relatively high concentration of triglyceride (45 μmoles/test or 30 mM) required for optimal activity of alkaline liver lipase probably reflects the preponderance of large emulsified particles with low surface area in the experimental emulsion rather than any lack of specificity of the enzyme for its substrate.

Development of Alternative Assay System

The coagulation of sodium taurocholate-stabilized triglyceride

emulsions was believed to be caused by electrolytes, since the phenomenon occurred in EBL preparations isolated from soluble fractions prepared in salts or buffer solutions and in assays of purified alkaline lipase to which compounds such as NaCl or protamine sulfate were added. Coagulation also occurred in assays of crude or partially purified fractions when the enzyme source contained less than 1 or 2 mg of protein, suggesting sensitivity of the emulsion to even low levels of ions stemming from the enzyme preparation. In this case, however, the ionic effect could be reversed, or protected against, by the addition of extraneous protein. It was not too surprising, then, to find that coagulation no longer occurred when denatured hemoglobin was omitted from the assays of dialyzed purified alkaline lipase. But it was most surprising to find that denatured hemoglobin was still required for activity of the enzyme, while having no lipolytic activity itself. This effect remains unexplained. Denatured hemoglobin probably was not serving as a fatty acid acceptor since albumin was not able to replace it fully. In fact, the micellar bile salt solution present in this assay system probably served to solubilize released fatty acids (171), making an additional fatty acid acceptor unnecessary. Denatured hemoglobin behaves, in this system, remarkably like the recently reported "colipase" present in pancreas and pancreatic juice (172, 173). This appears to be a low molecular weight protein (173) which is required for optimal activity of purified pancreatic lipase when the enzyme is assayed in a system containing bile salts (172). If bile salts are absent from the system, optimal activity occurs without the addition of colipase.

When gum arabic-stabilized triolein emulsions were utilized for

assay of purified liver lipase, albumin was required for activity. This alternative system seems more easily explicable than the sodium taurocholate system. An exogenous fatty acid acceptor is probably required; albumin is able to perform this function while denatured hemoglobin is not. A requirement for albumin in assays for lipolytic activity has been repeatedly demonstrated (4, 5, 161, 169, 174, 175). It is most intriguing that human plasma A-I polypeptide, but not A-II polypeptide, was capable of mimicking the effect of albumin in this system. It appears that A-I polypeptide, only, is capable of binding long-chain fatty acids while both are known to be constitutive polypeptides of human plasma apolipoprotein A (176).

Effects of Additives

Sodium chloride and protamine sulfate are classic inhibitors of lipoprotein lipase activity from post-heparin plasma (87, 168), adipose tissue (158, 174) and heart (86, 161). Sodium chloride concentrations adequate for complete inhibition of lipoprotein lipase (86) inhibited alkaline liver lipase only partially. The liver enzyme was not affected by protamine sulfate, whereas lipoprotein lipase is strongly inhibited at similar concentrations (168). Rat adipose tissue hormone-sensitive lipase is affected minimally by NaCl (157, 177). However, a similar enzyme from human adipose tissue whose hormone sensitivity is not yet established is partially inhibited by this salt (163, 169); the extent of inhibition is nearly identical to that observed for the liver lipase. The adipose tissue lipases and a hormone-sensitive lipase from rat heart have in common an insensitivity to protamine and a high degree of sensitivity to NaF (158, 161, 163, 169, 177). Both characteristics were also

observed for liver alkaline lipase. In the present study, NaF was more inhibitory at a concentration of 0.2 M than was NaCl at 1.0 M.

While the results of subcellular fractionation and lipase purification indicated that acidic and alkaline liver lipases were different enzymes, the clearest difference was their behavior when assayed in the presence of the detergent Triton X-100. This compound has long been known to increase the activities of a number of lysosomal hydrolases, possibly through the mechanism of solubilizing membrane-bound enzymes (120). Routine assay systems for acidic liver lipase frequently contain this detergent (13, 148, present study), and even an absolute requirement for it has been reported (10). It was established by Müller and Alaupovic (17) that nearly complete inhibition of liver alkaline microsomal lipase occurred at a concentration of Triton X-100 which doubled the activity of lysosomal lipase. In the present study, purified alkaline lipase was inhibited nearly 75% by a concentration of Triton X-100 which corresponded to only 10% of the amount routinely used in acid lipase assays. Hormone-sensitive adipose tissue lipase is also inhibited by this detergent (157, 159).

In addition to its extreme sensitivity to nonionic Triton X-100, alkaline liver lipase was completely inhibited by a 4 mM concentration of the anionic bile salt sodium deoxycholate. Sodium taurocholate, however, was not at all inhibitory at the same concentration (although some inhibition was observed at a 3-fold higher concentration). It is interesting to recall at this point the behavior of these three detergents in experiments in which they were tested for their ability to separate the enzyme from the enzyme-substrate complex. The strongly inhibitory com-

pounds, Triton X-100 and sodium deoxycholate, were both capable of removing protein from the complex, while the relatively non-inhibitory sodium taurocholate was not able to accomplish this. It is believed possible that the phenomena of inhibition and separation of protein from the complex are closely related. Triton X-100 and sodium deoxycholate may act at the interface of a triglyceride emulsion and, in some fashion, render its surface properties unfavorable for binding of the enzyme. In such a situation, both separation of the enzyme from its substrate and inhibition of catalytic activity would be expected to result. Sodium taurocholate, lacking this particular interfacial effect, would not be expected to affect either binding or catalytic ability. As the isolation of emulsion-bound lipase was accomplished with the aid of sodium taurocholate-stabilized emulsions, binding can obviously occur in the presence of this detergent. Schoor and Melius (154) have likewise reported that pancreatic lipase is adsorbed to emulsified triglyceride in the presence of sodium taurocholate and independently of the concentration of bile salt. No comparable data are available, however, for Triton X-100 or sodium deoxycholate.

Infranates isolated from emulsion-bound lipase preparations by sodium deoxycholate treatment were enzymatically inactive, while those resulting from Triton X-100 treatment exhibited excellent activity. It is believed that this was due to the fact that Triton X-100 could be removed from the infranates by ultrafiltration while the poorly soluble sodium deoxycholate could not. As mentioned earlier, large amounts of sodium deoxycholate were identified in the washed infranate preparations. It is likely that the enzyme preparation contained enough detergent to

cause inhibition of lipolytic activity.

The observed correlation between removal of the enzyme from the complex and inhibition of lipolytic activity is believed due to differing interfacial effects of detergents. A satisfactory explanation of these effects is beyond the scope of this study. Numerous reports are available on the effects of bile salts on the activity of pancreatic lipase. Under certain conditions they appear to be stimulatory (154, 170, 178), inhibitory (179), or both (171, 172). While surfactant properties of these detergents are generally invoked as an explanation of the experimental facts, no situation analogous to that in the present study has been reported. In the several studies, however, in which two or more bile salts have been compared (171, 172) each salt exhibited unique properties of stimulation and/or inhibition. It is therefore unnecessary to assume that sodium deoxycholate and sodium taurocholate should behave similarly under the present experimental conditions.

Alkaline liver lipase was not inhibited by eserine sulfate or the organophosphate E-600. These characteristics differentiate it from cholinesterases (32) and liver aliphatic or carboxylesterases (e.g., 49, 51).

Purified pig liver lipase was inhibited by iodoacetamide, in contrast to the results obtained by Müller and Alaupovic (17) for the microsomal enzyme. Sensitivity to this compound is usually interpreted as an indication of the presence of an -SH group at the active center of an enzyme. However, it has been reported that pancreatic lipase is sensitive to some -SH binding reagents but not to others (180). This was believed to indicate the presence of an -SH group near, but not at the

active center. In the absence of comparative data on the effect of other -SH reagents on alkaline liver lipase, no firm conclusion can be drawn.

The activity of purified alkaline lipase was enhanced by 10 mM EDTA but was not affected by 1 mM Ca^{++}. The data indicate that the enzyme does not possess a requirement for cations such as Ca^{++}, although the stimulatory behavior of EDTA is not understood. Waite and van Deenen (16) and Müller and Alaupovic (17) have also observed stimulation of liver lipase by EDTA. Again, pig liver lipase seems to be similar to human adipose tissue lipase (163) and rat adipose tissue hormone-sensitive lipase (157) in its lack of requirement for Ca^{++}. Lipoprotein lipase, on the other hand, is inhibited by EDTA (181) and possesses a distinct requirement for Ca^{++} (174, 182). Pancreatic lipase is stimulated by Ca^{++}, and it has been proposed that this is due to removal of fatty acids as soaps from the interface at which lipolysis occurs (164). In effect, this would suggest that Ca^{++} could replace albumin as a fatty acid acceptor. However, Ca^{++} was not able to substitute for albumin in the present study. The slight degree of inhibition observed with the relatively high (0.1 M) concentration of EDTA in our results is unexplained.

Comparison of Liver Lipase and Lipoprotein Lipase

The differences in the pH optima and responses to inhibitors exhibited by liver lipase and lipoprotein lipase have already been noted. The finding that each enzyme releases fatty acids from triolein only in its own specific assay system is incontestable proof of their non-identity. Moreover, liver lipase does not possess the characteristic specificity of lipoprotein lipase for chylomicron and plasma lipoprotein sub-

strates (86, 87, 140, 141). The fact that the lipoprotein preparations used in the present studies were human rather than porcine is probably immaterial; rat lipoprotein lipase hydrolyzes human lipoproteins very well (87).

Physiological Significance of Alkaline Liver Lipase

In view of its lack of specificity for chylomicrons and plasma very-low density lipoproteins, it seems unlikely that pig liver alkaline lipase could participate directly in the removal of such particles from the bloodstream *in vivo*, even assuming that the lipoproteins came into contact with the enzyme. The lipase is apparently not localized in plasma membranes, suggesting that it probably does not have direct contact with circulating lipoproteins. The highly controversial problem regarding the capacity of the liver to take up dietary triglyceride has been presented in the Review of Literature. Alkaline liver lipase does not seem to meet the requirements for an enzyme which could participate in this process, if it does occur.

On the other hand, the repeatedly noted similarities between alkaline liver lipase and lipases generally believed to be stimulated by hormones such as epinephrine and glucagon invites speculation as to a possibly similar physiological role for this enzyme. Some evidence that catabolism of fats is stimulated by these hormones (112, 113) has been reported for liver; however, a relationship of this sensitivity with the activity of a triglyceride lipase has not been established. It is generally recognized that liver satisfies a large part its energy needs from the oxidation of fatty acids. However, it has been assumed that the in-

creased amounts of fatty acids required by the liver during periods of stress or starvation are supplied to it by the hormone-sensitive lipase of adipose tissue depots. The presence of a hormone-sensitive lipase in liver itself "makes sense" in view of the dependence of the organ on free fatty acids. Such an enzyme would also make the liver more independent metabolically, by enabling it to utilize endogenously synthesized or stored triglycerides during stress situations rather than to depend on exogenously supplied fatty acids.

Future Areas of Study of Alkaline Liver Lipase

The favored hypothesis, that pig liver alkaline lipase may be a hormone-sensitive enzyme, requires immediate experimental verification. Such studies are presently in the planning stage and will be undertaken soon.

Alkaline liver lipase has been purified some 80 to 100-fold in the present studies. The product is an essentially lipid-free and relatively stable enzyme preparation. It is believed that further significant purification of the enzyme may be achieved through the techniques of protein chemistry.

Characterization of the substrate specificity of alkaline liver lipase is presently underway. Evidence available in the literature suggests that the enzymes responsible for lipolysis of long-chain triglycerides are different from those catalyzing the hydrolysis of long-chain monoglycerides in liver (102, 103), adipose tissue (159, 175, 183), plasma (95, 184) and pancreas (185). None of the studies concerning liver, however, have been carried out on purified enzyme preparations. Alkaline

lipase will therefore be tested for its ability to hydrolyze partial glycerides of oleic acid. Specificity for full and partial glycerides with shorter chain length fatty acids will also be explored. Alkaline liver lipase was not affected by several esterase inhibitors. Final proof of its identity or non-identity with liver esterase, however, will depend on its specificity toward esterase substrates.

CHAPTER VI

SUMMARY

A titrimetric method of assay for long-chain fatty acids, based on the color change of Nile Blue indicator, has been adapted for spectrophotometric quantitation. The method eliminates the human bias of manual endpoint titrations and provides rapid determination of 0 to 0.6 μmoles of fatty acids released from long-chain glycerides by lipolysis.

The subcellular distributions of acidic (pH 4.5) and alkaline (pH 7.5) long-chain triglyceride lipases of pig liver have been studied. The acidic lipase demonstrated highest relative specific activity in the light mitochondrial fraction, and its distribution closely paralleled that of the lysosomal marker enzyme, cathepsin D. The lysosomal origin of this lipase was confirmed. Approximately 60% of the alkaline lipolytic activity resided in the soluble fraction, but a clearcut peak of relative specific activity was not observed for any subcellular fraction. The distribution of this activity failed to parallel that of marker enzymes for mitochondria, lysosomes, microsomes or plasma membranes. The observed distribution might reflect the presence of several lipases, but evidence suggests that only one enzyme is present, localized primarily in the cytoplasm and exhibiting a tendency to bind to membranous particles, possibly due to its lipophilic nature.

Further information on the nature of the alkaline lipase has been sought through purification of the enzyme from the soluble fraction. Ultracentrifugal isolation of the enzyme bound to a substrate emulsion and separation of the enzyme from the emulsion by density-gradient centrifugation in detergent-containing media provided an approximate 90-fold purification with recovery of 16% of the initial activity.

Purified alkaline lipase has a pH optimum of 7.25, requires 45 μmoles of emulsified triolein for optimal activity, and ceases to liberate fatty acids after 15 to 30 minutes of incubation. Fatty acid release is linear with respect to enzyme concentration, and the activity is destroyed by heating at 60° or 100°.

Two assay systems are capable of supporting optimal activity of the lipase. One system employs sodium taurocholate as emulsifying agent for the triglyceride substrate and requires denatured hemoglobin for activation. The activating capacity of the denatured hemoglobin is not understood. The second system employs gum arabic-stabilized emulsions and requires albumin, probably as fatty acid acceptor, for maximal activity. The sodium taurocholate-stabilized emulsions of the first system are coagulated by electrolytes, while gum arabic stabilized emulsions are not. For this reason the second system is preferred for studying the effect of additives.

Alkaline liver lipase is inhibited by NaCl, NaF, Triton X-100, sodium deoxycholate and iodoacetamide. It is not inhibited by protamine sulfate, E-600, eserine sulfate, Ca^{++} and lower concentrations of sodium taurocholate and EDTA. Higher concentrations of sodium taurocholate and EDTA are mildly inhibitory. Ca^{++} cannot replace albumin as fatty acid

acceptor in this system. The responses of alkaline liver lipase to various additives serve to differentiate it from acidic liver lipase, pancreatic lipase, and lipoprotein lipase. Moreover, the enzyme fails to exhibit activity in a system specific for the detection of lipoprotein lipase and shows little or no capacity to hydrolyze chylomicrons or plasma very-low density lipoproteins. The effects of additives on liver lipase suggest, on the other hand, a close similarity between this enzyme and other mammalian lipases generally believed to be hormone-sensitive.

BIBLIOGRAPHY

1. Dakin, H. D., Hydrolysis of Esters by Liver, J. Physiol., 32, 199 (1905).

2. Loevenhart, A. S., Are the Animal Enzymes Concerned in the Hydrolysis of Various Esters Identical?, J. Biol. Chem., 2, 427 (1906-07).

3. Mosinger, B., and Vavrinkova, H., The Effect of Starvation on the Lipolytic Systems Catalysing the Hydrolysis of Intracellular and Extracellular Lipids in Rat Liver, Physiol. Bohemoslov., 14, 439 (1965).

4. Vavrinkova, H., and Mosinger, B., Liver Lipase, Physiol. Bohemoslov., 14, 46 (1965).

5. Grafnetter, D., and Grafnetterova, J., Comparative Investigations on the Lipolytic Activity of Rat Heart and Liver Homogenates, Med. Pharmacol. exp., 12, 274 (1965).

6. Olson, A. C., and Alaupovic, P., Studies of the Rat Liver Lipolytic Activity, Circulation 32, Suppl. II, 25 (1965).

7. Olson, A. C., and Alaupovic, P., Rat Liver Triglyceride Lipase, Biochim. Biophys. Acta 125, 185 (1966).

8. Stoffel, W., and Greten, H., Studies on Lipolytic Activities of Rat Liver Lysosomes, Hoppe-Seylers Z. Physiol. Chem., 348, 1145 (1967).

9. Mahadevan, S., and Tappel, A. L., Lysosomal Lipases of Rat Liver and Kidney, J. Biol. Chem., 243, 2849 (1968).

10. Fowler, S., and de Duve, C., Digestive Activity of Lysosomes, J. Biol. Chem., 244, 471 (1969).

11. Guder, W., Weiss, L., and Wieland, O., Triglyceride Breakdown in Rat Liver. The Demonstration of Three Different Lipases, Biochim. Biophys. Acta 187, 173 (1969).

12. Hayase, K., and Tappel, A., Specificity and Other Properties of Lysosomal Lipase of Rat Liver, J. Biol. Chem., 245, 169 (1970).

13. Kariya, M., and Kaplan, A., Stimulation of Rat Liver Lysosomal Lipase by Lipid and by Acidic Phospholipids, Biochem. Biophys. Res. Commun., 44, 1252 (1971).

14. Biale, Y., Gorin, E., and Shafrir, E., Characterization of Tissue Lipolytic and Esterolytic Activities Cleaving Full and Partial Glycerides, Biochim. Biophys. Acta 152, 28 (1968).

15. Guder, W., Weiss, L., and Wieland, O., Experimente zur Charakterisierung der Triglyceridhydrolyse der Rattenleber, Hoppe-Seylers Z. Physiol. Chem., 349, 7 (1968).

16. Waite, M., and van Deenen, L. L. M., Hydrolysis of Phospholipids and Glycerides by Rat-Liver Preparations, Biochim. Biophys. Acta 137, 498 (1967).

17. Müller, L., and Alaupovic, P., Long Chain Triglyceride Lipases of Pig Liver, FEBS Letters 10, 117 (1970).

18. Enzyme Nomenclature: Recommendations 1964 of the International Union of Biochemistry, Amsterdam, Elsevier Publishing Company, 1965, pp. 126-128.

19. Sarda, L., Desnuelle, P., Action de la Lipase Pancreatique sur les Esters en Emulsion, Biochim. Biophys. Acta 30, 513 (1958).

20. Peirce, C., The Partial Purification of the Esterase in Pig's Liver, J. Biol. Chem., 16, 1 (1913).

21. Baker, Z., King, C. G., The Purification, Specificity, and Inhibition of Liver Esterase, J. Am. Chem. Soc., 57, 358 (1935).

22. Mohamed, M., The Crystallization of Liver Esterase, Acta Chem. Scand., 2, 90 (1948).

23. Conners, W., Pihl, A., Dounce, A., and Stotz, E., Purification of Liver Esterase, J. Biol. Chem., 184, 29 (1950).

24. Burch, J., The Purification and Properties of Horse Liver Esterase, Biochem. J., 58, 415 (1954).

25. Boursnell, J., and Webb, E., Reaction of Esterases with Radioactive Di-isopropyl Fluorophosphonate, Nature 164, 875 (1949).

26. Hofstee, B., Specificity of Esterases. III. Critical Concentrations of the Substrate, J. Biol. Chem., 207, 211 (1954).

27. Hofstee, B., Specificity of Esterases. IV. Behavior of Horse Liver Esterase Towards a Homologous Series of n- Fatty Acid Esters, J. Biol. Chem., 207, 219 (1954).

28. Krisch, K., Isolierung einer Esterase aus Schweinelebermikrosomen, Biochem. Z., 337, 531 (1963).

29. Krisch, K., Eigenschaften und Substratspezifitat einer Esterase aus Schweineleбermikrosomen, Biochem. Z., 337, 546 (1963).

30. Shibko, S., and Tappel, A. L., Distribution of Esterases in Rat Liver, Arch. Biochem. Biophys., 106, 259 (1964).

31. Carruthers, C., Heins, E., and Baumler, A., Fractionation of Mouse Liver Microsomal Esterases, Can. J. Biochem., 43, 97 (1965).

32. Ecobichon, D., and Kalow, W., Properties and Classification of the Soluble Esterases of Human Liver, Biochem. Pharmacol., 11, 573 (1962).

33. Ecobichon, D., Multiple Forms of Human Liver Esterases, Can. J. Biochem., 43, 595 (1965).

34. Ecobichon, D., Characterization of the Liver Carboxylesterases of Large Domestic Animals, Comp. Biochem. Physiol., 23, 925 (1967).

35. Ecobichon, D., Some Physical Properties of the Esterases of Bovine Liver, Can. J. Biochem., 46, 1131 (1968).

36. Ecobichon, D., Bovine Hepatic Carboxylesterases: Chromatographic Fractionation, Gel Filtration, and Molecular Weight Estimation, Can. J. Biochem., 47, 799 (1969).

37. Ecobichon, D., Cytoplasmic Carboxylesterases of Human and Domestic Animal Liver: Aggregation, Dissociation, and Molecular Weight Estimation, Can. J. Biochem., 50, 9 (1972).

38. Schwark, W., and Ecobichon, D., Characterization of Rat Liver and Kidney Esterases, Can. J. Biochem., 45, 451 (1967).

39. Holmes, R., and Masters, C., The Developmental Multiplicity and Isoenzyme Status of Rat Esterases, Biochim. Biophys. Acta, 146, 138 (1967).

40. Holmes, R., and Masters, C., A Comparative Study of the Multiplicity of Mammalian Esterases, Biochim. Biophys. Acta 151, 147 (1968).

41. Benöhr, H., and Krisch, K., Carboxylesterase aus Rinderlebenmikrosomen, I. Hoppe-Seylers Z. Physiol. Chem., 348, 1102 (1967).

42. Adler, A., and Kistiakowsky, G., Isolation and Properties of Pig Liver Esterase, J. Biol. Chem., 236, 3240 (1961).

43. Horgan, D., Stoops, J., Webb, E., and Zerner, B., Carboxylesterases (E.C.3.1.1). A Large Scale Purification of Pig Liver Carboxylesterase., Biochemistry 8, 2000 (1969).

44. Runnegar, M., Scott, K., Webb, E., and Zerner, B., Carboxylesterases (E.C. 3.1.1). Purification and Tritration of Ox Liver Carboxylesterase, Biochemistry 8, 2013 (1969).

45. Runnegar, M., Webb, E., and Zerner, B., Carboxylesterases (E.C. 3.1.1). Dissociation of Ox Liver Carboxylesterase, Biochemistry 8, 2018 (1969).

46. Keay, L., and Crook, E., Effect of Metal Ions on Hog Liver Esterase, Arch. Biochem. Biophys., 111, 626 (1965).

47. Bauminger, S., and Levine, L., Some Immunochemical Properties of Beef Liver Esterase, Biochim. Biophys. Acta 236, 639 (1971).

48. Boguth, W., Krisch, K., and Niemann, H., Das Molkeulargewicht der Mikrosomalen Leberesterase, Biochem. J., 341, 149 (1965).

49. Krisch, K., Reaction of a Microsomal Esterase From Hog-Liver with Diethyl p-Nitrophenyl Phosphate, Biochim. Biophys. Acta 122, 265 (1966).

50. Heymann, E., and Krisch, K., Phosphorsaure-bis-[p-nitro-phenyl ester], ein neuer Hemmstoff mikrosomaler Carboxylesterasen, Hoppe-Seylers Z. Physiol. Chem., 348, 609 (1967).

51. Benöhr, H., and Krisch, K., Carboxylesterase aus Rinderlebermikrosomen, II., Hoppe-Seylers Z. Physiol. Chem., 348, 1115 (1967).

52. Barker, D., and Jencks, W., Pig Liver Esterase, Physical Properties, Biochemistry 8, 3879 (1969).

53. Horgan, D., Dunstone, J., Stoops, J., Webb, E., and Zerner, B., Carboxylesterases (E.C. 3.1.1). The Molecular Weight and Equivalent Weight of Pig Liver Carboxylesterase, Biochemistry 8, 2006 (1969).

54. Hain, P., and Krisch, K., Immunchemische Untersuchungen an Carboxylesterasen, Z. Klin. Chem. Klin. Biochem., 6, 313 (1968).

55. Mahadevan, S., and Tappel, A., Hydrolysis of Higher Fatty Acid Esters of p-Nitrophenol by Rat Liver and Kidney Lysosomes, Arch. Biochem. Biophys., 126, 945 (1968).

56. Albertson, D. F., Comparison of Rat Liver, Testicular, and Adipose Tissue Esterases, Ph.D. Dissertation, University of Illinois, 1971.

57. Barker, D. L., and Jencks, W. P., Pig Liver Esterase. Some Kinetic Properties, Biochemistry 8, 3890 (1969).

58. Adler, A. J., and Kistiakowsky, G. B., Kinetics of Pig Liver Esterase Catalysis, J. Am. Chem. Soc., 84, 695 (1962).

59. Stoops, J. K., Horgan, D. J., Runnegar, M. T. C., de Jersey, J., Webb, E. C., and Zerner, B., Carboxylesterases (E.C. 3.1.1). Kinetic Studies on Carboxylesterases, Biochemistry 8, 2026 (1969).

60. Hofstee, B. H. H., On the Substrate Activation of Liver Esterase, Biochim. Biophys. Acta 258, 446 (1972).

61. Heymann, E., Krisch, K., and Pahlich, E., Zur Struktur des Aktiven Zentrums von Mikrosomalen Carboxylesterasen aus Schweineniere und-Leber, Hoppe-Seylers Z. Physiol. Chem., 351, 931 (1970).

62. Mendoza, C. E., Wales, P. J., and Hatina, G. V., Alkoxy Derivatives of Dyrene: Identification and Carboxylesterase Inhibition, J. Agr. Food Chem., 19, 41 (1971).

63. Ocken, P. R., and Levy, M., The Nature of the Modifier Site of Pig Liver Esterase, Biochim. Biophys. Acta 212, 450 (1970).

64. Greenzaid, P., and Jencks, W. P., Pig Liver Esterase. Reaction with Alcohols, Structure-Reactivity Correlations, and the Acyl-Enzyme Intermediate, Biochemistry 10, 1210 (1971).

65. Ashworth, C. T., Stembridge, V. A., and Sanders, E., Lipid Absorption, Transport and Hepatic Assimilation Studied with Electron Microscopy, Am. J. Physiol., 198, 1326 (1960).

66. French, J. E., and Morris, B., The Tissue Distribution and Oxidation of ^{14}C-Labelled Chylomicron Fat Injected Intravenously in Rats, J. Physiol., 140, 262 (1958).

67. Bragdon, J. H., and Gordon, R. S., Jr., Tissue Distribution of C^{14} after the Intravenous Injection of Labeled Chylomicrons and Unesterified Fatty Acids in the Rat, J. Clin. Invest., 37, 574 (1958).

68. Stein, Y., and Shapiro, B., Uptake and Metabolism of Triglycerides by the Rat Liver, J. Lipid Res., 1, 326 (1960).

69. Olivecrona, T., Metabolism of Chylomicrons Labeled with C^{14}-Glycerol-H^3-Palmitic Acid in the Rat, J. Lipid Res., 3, 439 (1962).

70. Ashworth, C. T., DiLuzio, N. R., and Riggi, S. J., A Morphologic Study of the Effect of Reticuloendothelial Stimulation upon Hepatic Removal of Minute Particles from the Blood of Rats, Exp. Mol. Pathol., Suppl. 1, 83 (1963).

71. Havel, R. J., and Goldfien, A., The Role of the Liver and of Extrahepatic Tissues in the Transport and Metabolism of Fatty Acids and Triglycerides in the Dog, J. Lipid Res., 2, 389 (1961).

72. Nestel, P. J., Havel, R. J., and Bezman, A., Sites of Initial Removal of Chylomicron Triglyceride Fatty Acids from the Blood, J. Clin. Invest., 41, 1915 (1962).

73. Nestel, P. J., Havel, R. J., and Bezman, A., Metabolism of Constituent Lipids of Dog Chylomicrons, J. Clin. Invest., 42, 1313 (1963).

74. Morris, B., and French, J. E., The Uptake and Metabolism of ^{14}C Labelled Chylomicron Fat by the Isolated Perfused Liver of the Rat, Quart. J. Exp. Physiol., 43, 180 (1958).

75. Anderson, N. G., and Fawcett, B., An Antichylomicronemic Substance Produced by Heparin Injection, Proc. Soc. Exp. Biol. Med., 74, 768 (1950).

76. Brown, R. K., Boyle, E., and Anfinsen, C. B., The Enzymatic Transformation of Lipoproteins, J. Biol. Chem., 204, 423 (1953).

77. Heimberg, M., Weinstein, I., Klausner, H., and Watkins, M. L., Release and Uptake of Triglycerides by Isolated Perfused Rat Liver, Am. J. Physiol., 202, 353 (1962).

78. Morris, B., The Metabolism of Free Fatty Acids and Chylomicron Triglycerides by the Isolated Perfused Liver of the Rat, J. Physiol., 168, 564 (1963).

79. Rodbell, M., Scow, R. O., and Chernik, S. S., Removal and Metabolism of Triglycerides by Perfused Liver, J. Biol. Chem., 239, 385 (1964).

80. Felts, J. M., The Metabolism of Chylomicron Triglyceride Fatty Acids by Perfused Rat Livers and by Intact Rats, Ann. N. Y. Acad. Sci., 131, 24 (1965).

81. Felts, J. M., and Mayes, P. A., Lack of Uptake and Oxidation of Chylomicron Triglyceride to Carbon Dioxide and Ketone Bodies by the Perfused Rat Liver, Nature 206, 195 (1965).

82. Edgren, B., and Zilversmit, D. B., Uptake of Doubly Labeled Chylomicrons by Dog Liver Slices and Adipose Tissue, Federation Proc., 22, 517 (1963).

83. Ontko, J. A., and Zilversmit, D. B., Utilization of Chylomicrons by the Liver, Federation Proc., 25, 209 (1966).

84. Green, C., and Webb, J. A., The Uptake of Chylomicron Fatty Acids by Isolated Liver Cells, Biochim. Biophys. Acta 84, 404 (1964).

85. Higgins, J. A., and Green, C., The Uptake of Lipids by Rat Liver Cells, Biochem. J., 99, 631 (1966).

86. Korn, E. D., Clearing Factor, a Heparin-Activated Lipoprotein Lipase. I. Isolation and Characterization of the Enzyme from Normal Rat Heart, J. Biol. Chem., 215, 1 (1955).

87. Korn, E. D., Clearing Factor, a Heparin-Activated Lipoprotein Lipase. II. Substrate Specificity and Activation of Coconut Oil, J. Biol. Chem., 215, 15 (1955).

88. Jeffries, G. H., The Sites at which Plasma Clearing Activity Is Produced and Destroyed in the Rat, Quart. J. Exp. Physiol., 39, 261 (1954).

89. Spitzer, J. A., and Spitzer, J. J., Effect of Liver on Lipolysis by Normal and Postheparin Sera in the Rat, Am. J. Physiol., 185, 18 (1956).

90. Mayes, P. A., and Felts, J. M., Demonstration of Lipoprotein Lipase in Rat Liver, Biochem. J., 99, 43P (1966).

91. Mayes, P. A., and Felts, J. M., The Functional Status of Lipoprotein Lipase in Rat Liver, Biochem. J., 108, 483 (1968).

92. Felts, J. M., and Mayes, P. A., Release of Lipoprotein Lipase from the Perfused Liver of the Rat, Nature 214, 620 (1967).

93. Payza, A. N., Eiber, H. B., and Walters, S., Studies with Clearing Factor V. State of Tissue Lipases after Injection of Heparin, Soc. Exp. Biol. Med., 125, 188 (1967).

94. Yasuoka, S., and Fujii, S., Relationship between Clearing Factor Lipase and Lipases in Tissues, J. Biochem., 70, 749 (1971).

95. Greten, H., Levy, R. I., and Fredrickson, D. S., Evidence for Separate Monoglyceride Hydrolase and Triglyceride Lipase in Post-Heparin Human Plasma, J. Lipid Res., 10, 326 (1969).

96. LeQuire, V. S., Hamilton, R. L., Adams, R., and Merrill, J. M., Lipase Activity in Blood from the Hepatic and Peripheral Vascular Beds Following Heparin, Proc. Soc. Exp. Biol. Med., 114, 104 (1963).

97. Boberg, J., Carlson, L. A., and Normell, L., Production of Lipolytic Activity by the Isolated, Perfused Dog Liver in Response to Heparin, Life Sci., 3, 1011 (1964).

98. Condon, R. E., Tobias, H., and Datta, D. V., The Liver and Postheparin Plasma Lipolytic Activity in Dog and Man, J. Clin. Invest., 44, 860 (1965).

99. Muir, J. R., The Regional Production of Lipoprotein Lipase in Man, Clin. Sci., 34, 261 (1968).

100. Irwin, R. J., Alpern, R. J., and Webster, G. R., Observations on Lysophosphatide and Triglyceride Hydrolysis by Rat Liver Preparations, Biochim. Biophys. Acta 125, 600 (1966).

101. Personal observation.

102. Biale, Y., Gorin, E., and Sahfrir, E., Characterization of Lipolytic Activity in Rat Tissues, Israel J. Chem., 3, 112P (1965).

103. Carter, J. R., Jr., Hepatic Lipase in the Rat, Biochim. Biophys. Acta 137, 147 (1967).

104. Higgins, J. A., and Green, C., Properties of a Lipase of Rat-Liver Parenchymal Cells, Biochim. Biophys. Acta 144, 211 (1967).

105. Patrick, A. D., and Lake, B. D., An Acid Lipase Deficiency in Wolman's Disease, Biochem. J., 112, 29P (1969).

106. Patrick, A. D., and Lake, B. D., Deficiency of an Acid Lipase in Wolman's Disease, Nature 222, 1067 (1969).

107. Young, E. P., and Patrick, A. D., Deficiency of Acid Esterase Activity in Wolman's Disease, Arch. Disease Childhood 45, 664 (1970).

108. Burke, J. A., and Schubert, W. K., Deficient Activity of Hepatic Acid Lipase in Cholesterol Ester Storage Disease, Science 176, 309 (1972).

109. Wallach, D. P., and Ko, H., Studies of the Inhibition of Lipolytic Enzymes: I. The Inhibition of a Canine Kidney and Liver Lipase *In Vitro* and *In Vivo* by n-Butyl Carbamic Acid Methyl Ester (U-14641), Biochem. Pharmacol., 12, 1117 (1963).

110. Claycomb, W. C., and Kilsheimer, G. S., Purification and Properties of a Lipase from Rat Liver Mitochondria, J. Biol. Chem., 246, 7139 (1971).

111. Belfrage, P., A Monoglyceride-Splitting Enzyme of Rat and Hog Liver, Biochim. Biophys. Acta 98, 660 (1965).

112. Bewsher, P. D., and Ashmore, J., Ketogenic and Lipolytic Effects of Glucagon on Liver, Biochem. Biophys. Res. Commun., 24, 431 (1966).

113. Claycomb, W. C., Bynagle, J. K., and Kilsheimer, G. S., Hormonal Influence on Hepatic Lipolysis and Ketogenesis, Federation Proc., 28, 707 (1969).

114. Sarkar, N. K., The Effects of Synthetic Glucocorticosteroid (Triamcinolone Diacetate) and Dibutyryladenosine-3',5'-(Cyclic) Monophosphate on Hepatic Lipase Activities in Rats, FEBS Letters 10, 301 (1970).

115. Scaria, K. S., and Prabha, K., Effect of Cold Exposure and Noradrenaline Treatment on the Lipolytic Activity of Liver and Leg Muscle of Rat, Indian J. Exp. Biol., 5, 255 (1967).

116. Hayase, K., and Tappel, A. L., Microsomal Esterase of Rat Liver, J. Biol. Chem., 244, 2269 (1969).

117. Desnuelle, P., and Savary, P., Specificities of Lipases, J. Lipid Res., 4, 369 (1963).

118. Okuda, H., and Fujii, S., Lipase and Esterase in Adipose Tissue and Liver, J. Biochem., 61, 313 (1967).

119. Okuda, H., and Fujii, S., Relationship between Lipase and Esterase, J. Biochem., 64, 377 (1968).

120. de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., and Appelmans, F., Tissue Fractionation Studies 6. Intracellular Distribution Patterns of Enzymes in Rat-Liver Tissue, Biochem. J., 60, 604 (1955).

121. Appelmans, F., Wattiaux, R., and de Duve, C., Tissue Fractionation Studies 5. The Association of Acid Phosphatase with a Special Class of Cytoplasmic Granules in Rat Liver, Biochem. J., 59, 438 (1955).

122. Crider, Q. E., Alaupovic, P., Hillsberry, J., Yen, C., and Bradford, R. H., Separation of Lipids by Silica Gel G Column Chromatography, J. Lipid Res., 5, 479 (1964).

123. Gustafson, A., Alaupovic, P., and Furman, R. H., Studies of the Composition and Structure of Serum Lipoproteins. Separation and Characterization of Phospholipid-Protein Residues Obtained by Partial Delipidization of Very Low Density Lipoproteins of Human Serum, Biochemistry 5, 632 (1966).

124. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., Protein Measurement with the Folin Phenol Reagent, J. Biol. Chem., 193, 265 (1951).

125. Pennington, R. J., Biochemistry of Dystrophic Muscle. Mitochondrial Succinate-Tetrazolium Reductase and Adenosine Triphosphatase, Biochem. J., 80, 649 (1961).

126. Beck, C., Mahadevan, S., Brightwell, R., Dillard, C. J., and Tappel, A. L., Chromatography of Lysosomal Enzymes, Arch. Biochem. Biophys., 128, 369 (1968).

127. Rosen, H., A Modified Ninhydrin Colorimetric Analysis for Amino Acids, Arch. Biochem. Biophys., 67, 10 (1957).

128. Grant, D. R., Reagent Stability in Rosen's Ninhydrin Method of Analysis for Amino Acids, Anal. Biochem., 6, 109 (1963).

129. Hübscher, G., and West, G. R., Specific Assays of Some Phosphatases in Subcellular Fractions of Small Intestinal Mucosa, Nature 205, 799 (1965).

130. Emmelot, P., Bos, C. J., Benedetti, E. L., and Rümke, P. H., Studies on Plasma Membranes I. Chemical Composition and Enzyme Content of Plasma Membranes Isolated from Rat Liver, Biochim. Biophys. Acta 90, 126 (1964).

131. Fiske, C. H., and Subbarow, Y., The Colorimetric Determination of Phosphorus, J. Biol. Chem., 66, 375 (1925).

132. Dole, V. P., A Relation between Non-Esterified Fatty Acids in Plasma and the Metabolism of Glucose, J. Clin. Invest., 35, 150 (1956).

133. Gordon, R. S., Jr., Cherkes, A., and Gates, H., Unesterified Fatty Acid in Human Blood Plasma. II. The Transport Function of Unesterified Fatty Acid, J. Clin. Invest., 36, 810 (1957).

134. Anfinsen, C. B., and Quigley, T. W., Purification of Clearing Factor by Substrate-Complex Formation, Circulation 8, 435 (1953).

135. Fielding, C. J., Purification of Lipoprotein Lipase from Rat Post-Heparin Plasma, Biochim. Biophys. Acta 178, 499 (1969).

136. Fielding, C. J., Human Lipoprotein Lipase: I. Purification and Substrate Specificity, Biochim. Biophys. Acta 206, 109 (1970).

137. Ganesan, D., Bradford, R. H., Alaupovic, P., and McConathy, W. J., Differential Activation of Lipoprotein Lipase from Human Post-Heparin Plasma, Milk, and Adipose Tissue by Polypeptides of Human Serum Apolipoprotein C, FEBS Letters 15, 205 (1971).

138. Schotz, M. C., Garfinkel, A. S., Huebotter, R. J., and Stewart, J. E., A Rapid Assay for Lipoprotein Lipase, J. Lipid Res., 11, 68 (1970).

139. Ganesan, D., and Bradford, R. H., Isolation of Apolipoprotein-Free Lipoprotein Lipase from Human Post-Heparin Plasma, Biochim. Biophys. Res. Commun., 43, 544 (1971).

140. Robinson, D. S., The Clearing Factor Lipase and Its Action in the Transport of Fatty Acids between the Blood and the Tissues. In Advances in Lipid Research, Vol. 1, ed., Paoletti, R., and Kritchevsky, D., New York, Academic Press, 1963, p. 149.

141. Borensztajn, J., Otway, S., and Robinson, D. S., Effect of Fasting on the Clearing Factor Lipase (Lipoprotein Lipase) Activity of

Fresh and Defatted Preparations of Rat Heart Muscle, J. Lipid Res., 11, 102 (1970).

142. Ganesan, D., personal communication.

143. Ayers, C. W., Estimation of the Higher Fatty Acids C_7-C_{18}, Anal. Chim. Acta 15, 77 (1956).

144. Barreto, R. C. R., and Mano, D. B., A Colorimetric Method for the Assay of Some Higher Fatty Acids in Blood and Serum, Clin. Chim. Acta 6, 887 (1961).

145. Duncombe, W. G., The Colorimetric Micro-Determination of Long-Chain Fatty Acids, Biochem. J., 88, 7 (1963).

146. Mahadevan, S., Dillard, C. J., and Tappel, A. L., A Modified Colorimetric Micro Method for Long-Chain Fatty Acids and Its Application for Assay of Lipolytic Enzymes, Anal. Biochem., 27, 387 (1969).

147. Schnatz, J. D., Automatic Titration of Free Fatty Acids, J. Lipid Res., 5, 483 (1964).

148. Bar-on, H., Stein, O., and Stein, Y., Acid Lipase in Fatty Liver in Rats, Israel J. Med. Sci., 7, 568 (1971).

149. Ragab, H., Beck, C., Dillard, C., and Tappel, A. L., Preparation of Rat Liver Lysosomes, Biochim. Biophys. Acta 148, 501 (1967).

150. Patelski, J., and Tipton, K. F., The Subcellular Localization of Lipolytic Enzymes in Pig Aorta, Atherosclerosis 13, 288 (1971).

151. Ramachandran, S., Yip, Y. K., and Wagle, S. R., Studies on Lipase: Isolation and Purification of a High Molecular Weight Pancreatic Lipase, Europ. J. Biochem., 12, 201 (1970).

152. Di Nella, R. R., Meng, H. C., and Park, C. R., Properties of Intestinal Lipase, J. Biol. Chem., 235, 3076 (1960).

153. Benzonana, G., and Desnuelle, P., Etude Cinetique de l'Action de la Lipase Pancreatique sur des Triglycerides en Emulsion. Essai d'une Enzymologie en Milieu Heterogene, Biochim. Biophys. Acta 105, 121 (1965).

154. Schoor, W. P., and Melius, P., The Influence of Sodium Taurocholate on the Pancreatic Lipase-Substrate Adsorption and Activity, Biochim. Biophys. Acta 212, 173 (1970).

155. Olson, A. C., The Lipolytic Activities of Rat Liver, Ph.D. Dissertation, University of Oklahoma, 1967.

156. Huttunen, J. K., Aquino, A. A., Steinberg, D., A Purified Triglyceride Lipase, Lipoprotein in Nature, from Rat Adipose Tissue, Biochim. Biophys. Acta 224, 295 (1970).

157. Huttunen, J. K., Ellingboe, J., Pittman, R. C., and Steinberg, D., Partial Purification and Characterization of Hormone-Sensitive Lipase from Rat Adipose Tissue, Biochim. Biophys. Acta 218, 333 (1970).

158. Björntorp, P., and Furman, R. H., Lipolytic Activity in Rat Epididymal Fat Pads, Am. J. Physiol., 203, 316 (1962).

159. Gorin, E., and Shafrir, E., Lipolytic Activity in Adipose Tissue Homogenate toward Tri-, Di-, and Monoglyceride Substrates, Biochim. Biophys. Acta 84, 24 (1964).

160. Froesch, E. R., Oelz, O., Zapf, J., and Waldvogel, M., Lipase Activity in the Fat Cake and Aqueous Phase of Adipose Tissue Homogenate of Fed, Fasted, and Fasted-Refed Rats, Europ. J. Clin. Invest., 1, 204 (1970).

161. Björntorp, P., and Furman, R. H., Lipolytic Activity in Rat Heart, Am. J. Physiol., 203, 323 (1962).

162. Schnatz, J. D., Lipolytic Activity in Human Adipose Tissue, Life Sci., 3, 851 (1964).

163. Boyer, J., LePetit, J., and Giudicelli, H., L'Activite Lipolytique du Tissu Adipeux II. L'Activite Triglyceride-Lipase du Tissu Adipeux Humain, Biochim. Biophys. Acta 210, 411 (1970).

164. Borgström, B., On the Mechanism of Pancreatic Lipolysis of Glycerides, Biochim. Biophys. Acta 13, 491 (1954).

165. Baskys, B., Klein, E., and Lever, W. F., Lipases of Blood and Tissues. III. Purification and Properties of Pancreatic Lipase, Arch. Biochem. Biophys., 102, 201 (1963).

166. Mattson, F. H., and Volpenhein, R. A., Carboxylic Ester Hydrolases of Rat Pancreatic Juice, J. Lipid Res., 7, 536 (1966).

167. Hayase, K., and Miller, B. F., Lipase Activity in the Human Aorta, J. Lipid Res., 11, 209 (1970).

168. Greten, H., Levy, R. I., and Fredrickson, D. S., A Further Characterization of Lipoprotein Lipase, Biochim. Biophys. Acta 164, 185 (1968).

169. Schnatz, J. D. Neutral and Alkaline Lipolytic Activities in Human Adipose Tissue, Biochim. Biophys. Acta 116, 243 (1966).

170. Brockerhoff, H., On the Function of Bile Salts and Proteins as Cofactors of Lipase, J. Biol. Chem., 246, 5828 (1971).

171. Benzonana, G., and Desnuelle, P., Action of Some Effectors on the Hydrolysis of Long-Chain Triglycerides by Pancreatic Lipase, Biochim. Biophys. Acta 164, 47 (1968).

172. Morgan, R. G. H., and Hoffman, N. E., The Interaction of Lipase, Lipase Cofactors and Bile Salts in Triglyceride Hydrolysis, Biochim. Biophys. Acta 248, 143 (1971).

173. Maylie, M. F., Charles, M., Gache, C., and Desnuelle, P., Isolation and Partial Identification of a Pancreatic Colipase, Biochim. Biophys. Acta 229, 286 (1971).

174. Korn, E. D., and Quigley, T. W., Studies on Lipoprotein Lipase of Rat Heart and Adipose Tissue, Biochim. Biophys. Acta 18, 143 (1955).

175. Vaughan, M., Berger, J. E., and Steinberg, D., Hormone-Sensitive Lipase and Monoglyceride Lipase Activities in Adipose Tissue, J. Biol. Chem., 239, 401 (1964).

176. Kostner, G., and Alaupovic, P., Studies of the Composition and Structure of Plasma Lipoproteins. Separation and Quantification of the Lipoprotein Families Occurring in the High Density Lipoproteins of Human Plasma, Biochemistry 11, 3419 (1972).

177. Rizack, M. A., An Epinephrine-Sensitive Lipolytic Activity in Adipose Tissue, J. Biol. Chem., 236, 657 (1961).

178. Fritz, P. J., and Melius, P., Mechanism of Activation of Hog Pancreatic Lipase by Sodium Taurocholate, Can. J. Biochem. Physiol., 41, 719 (1963).

179. Mattson, F. H., and Volpenhein, R. A., Behavior of Lipids at Interfaces and in Biological Membranes. Enzymatic Hydrolysis at an Oil/Water Interface, J. Am. Oil Chem. Soc., 43, 286 (1966).

180. Wills, E. D., The Relation of Metals and -SH Groups to the Activity of Pancreatic Lipase, Biochim. Biophys. Acta 40, 481 (1960).

181. Korn, E. D., and Quigley, T. W., Lipoprotein Lipase of Chicken Adipose Tissue, J. Biol. Chem., 226, 833 (1957).

182. Posner, I., and Morales, A., Mechanisms of Enzyme and Substrate Activation by Lipoprotein Lipase Cofactors I. A Specific Requirement of Physiological Concentrations of Calcium for Enzyme Activity, J. Biol. Chem., 247, 2255 (1972).

183. Katocs, A. S., Jr., Gnewuch, T., Lech, J. J., and Calvert, D. N., Further Studies of Monoolein Lipase from Rat Adipose Tissue, Biochim. Biophys. Acta 270, 60 (1972).

184. Nilsson-Ehle, P., and Belfrage, P., A Monoglyceride Hydrolyzing Enzyme in Human Postheparin Plasma, Biochim. Biophys. Acta 270, 60 (1972).

185. Morgan, R. G. H., Barrowman, J., Filipek-Wender, H., and Borgström, B., The Lipolytic Enzymes of Rat Pancreatic Juice, Biochim. Biophys. Acta 167, 355 (1968).