FATTY ACID ESTERIFICATION IN A SUBCELLULAR PREPARATION OF ADIPOSE TISSUE

by

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EXPERIMENTAL SECTION CONT'D.

ABBREVIATIONS

ACTH	Adrenocorticotrophic Hormone
ATP	Adenosine 5' triphosphate
AMP	Adenosine 5' monophosphate
CDP	Cytidinide diphosphate
CTP	Cytidine triphosphate
CoASH	Reduced Coenzyme A
DNA	Desoxyribonucleic Acid
DFP	Diisopropylfluoro Phosphate
FFA	Free Fatty Acid (s)
GH	Growth Hormone
αGP	α Glycerophosphate
GSH	Reduced Glutathione
RNA	Ribonucleic Acid
S.A.	Specific Activity
TG	Triglyceride
TGFA	Triglyceride Fatty Acids

PART I ADIPOSE TISSUE STRUCTURE AND COMPOSITION

The controversy regarding the cell of origin of white adipose tissue fat cells has not been resolved. It has not as yet been established whether a fat cell represents a modified connective tissue fibroblast which has accumulated lipid (44,35) or is derived from a specialized perivascular reticular mesenchymal cell (174).

The fat cell has been classically described as having a signet ring appearance on histological section due to the peripherally displaced flattened nucleus and the large central vacuole representing the space occupied by fat. A very thin attenuated ring of cytoplasm exists but is not readily apparent by light microscopy. The electron microscope has recently been used to characterize the intimate structure of adipose tissue cells (8,43,151,175). This technique is promising and many studies are underway in an attempt to correlate metabolic events with structural alterations. Sheldon et al (151) have examined the rat epididymal pad from fasted and diabetic animals and described microinvaginations of the cell membrane and numerous cytoplasmic processes. They have postulated that an intricate and elaborate tubular system exists within the cells that communicate with the cell membrane. This has brought forth an interesting and provocative suggestion that the lipid droplets present in the cell may, strictly speaking reside outside the cell cytoplasm and within the tubular labyrinth.

Most, if not all of the modern studies on adipose tissue metabolism have been carried out using the epididymal fat pad of the rat. Because it is readily accessible and symetrically paired it has served as a model tissue in studies on white adipose tissue physiology.

The composition of epididymal fat tissue taken from normal fed animals has been shown to be related to the pad weight (2,14). Pads weighing 1 gm or more contain 80-85% lipid whereas pads weighing less than .5 gm contain less than 50% lipid by weight. The nitrogen content ranges from 5-11 mg/gm and varies with the size of the depot being proportionally greatest in the smaller pad (2).

Greater than 99% of the extractable lipid in adipose tissue of fed animals is triglyceride (63). Small amounts of cholesterol esters, lower glycerides (170) and phospholipids are detected by thin layer chromatography (65). Free fatty acids in concentrations of 1-2 uEq/gm wet weight can be titrated in adipose tissue of fed rats (166). Although quantitatively small FFA are an exceedingly important intermediate in adipose tissue lipid metabolism.

The triglyceride present in rat adipose tissue are esters of long chain fatty acids. The composition of triglyceride fatty acid varies between species and depends on a number of factors, the most important of which is the antecedent dietary regimen (102, 152,153). Hollenberg and Douglas (66) studying the effects of fasting

and diabetes on rat epididymal TGFA compositions observed the following proportions in normal Purina Chow fed animals: myristic 2.8%, palmitic 32.0%, palmitoleic 10%, stearic 4.5%, oleic 29% and linoleic acid 24%. Small amounts of lauric and linolenic acids were also present. These acids are not randomly distributed between the α, β, and α' positions of the glycerol residue. Mattson and Lutton (109) have clearly demonstrated a preponderance of unsaturated 18 carbon fatty acids in the β ester of rat adipose tissue triglyceride. Because of the variety of fatty acids and the non random type of distribution the triglycerides must be regarded as a family of compounds or a mixture of classes. Mattson (110) has pointed out that having as few as five different fatty acids in a triglyceride mixture could mean the presence of 75 separate classes of triglyceride.

Difficulty in securing accurate class separation by simple rapid techniques has been a formidable obstacle in lipid technology (62,110). Kuksis et al (95) have recently separated natural triglyceride mixtures on the basis of chain length by gas liquid chromatography. Analysis of mammalian adipose tissue triglyceride by this technique should help in dilineating the class distribution.

PART II ADIPOSE TISSUE METABOLISM

A) HISTORICAL INTRODUCTION

Not long ago adipose tissue was regarded as an inert repository of fat without any specific metabolic activity. In 1948 Werthiemer and Shapiro (179) in their classical review on adipose tissue physiology, stressed that this view was no longer tenable in view of recently accumulated data.

The monumental studies of Schoenheimer and Rittenberg carried out in the later 1930's must be cited for their tremendous impact on the biological sciences and specifically for demonstrating the dynamic nature of adipose tissue lipid (128,142,143). Using deuterated materials as tracers in intermediary metabolism these scientists demonstrated rapid turnover of adipose tissue fat, fatty acid interconversion and the ability of this tissue to synthesize predominately long chain saturated fatty acids.

Adipose tissue was shown to have a rich capillary network (47) and an abundant nerve supply to the blood vessels and parenchymal cells (20,116). These structural endowments were regarded as some index of a tissues functional significance. Mobilization of fat from adipose stores was thought to be largely under nervous regulation. Both Werthiemer (180) and Hausberger (61) sectioned the nerve supply to adipose tissue and could demonstrate impaired mobilization of fat.

Fat metabolism was known to be markedly affected by hormonal principles obtained from extracts of the anterior pituitary gland. Extracts of this gland injected into rats or mice produced fatty infiltration of the liver (13,60). Barret et al (13) established beyond doubt that adipose tissue stores was the source of this lipid and that the extract caused excessive mobilization of depot fat. These findings suggested a direct influence of anterior pituitary on adipose tissue activity (179). Intact adrenal glands were necessary to demonstrate liver fatty infiltration as adrenalectomy was shown to inhibit the deposition of fat in the liver from various causes (73,106) and was regarded as playing a permissive role in fat mobilization.

The nature of the hormonal influence and the hormonal factors involved as well as the form in which lipid was released from adipose tissue and carried to the liver, were unknown.

By 1945 adipose tissue was recognized as an extremely active organ primarily concerned with the synthesis, storage, and release of fats. Over the next ten years interest in adipose tissue waned somewhat as greater attention was directed toward purification and identification of hormonal principals causing liver fatty infiltration (135,185). Although crude preparations rich in thyrotropic activity (37), growth hormone (176), and prolactin (125) were known to induce fatty livers it remained until Astwood et al (5,6)

developed the oxycel purification method for corticortropin that specific adipokinetic properties could be ascribed to a single hormone. In the same laboratory Raben et al (122) isolated growth hormone by a glacial acetic acid extraction method and Rosenberg (135) demonstrated the adipokinetic properties of the pure corticotropin, and growth hormone.

In 1956 Dole (38) and Gordon and Cherkes (50,52) simultaneously demonstrated a highly labile plasma lipid fraction bound to albumin called free fatty acids (FFA). In human subjects they found a consistant increase in plasma FFA concentration with fasting that could be rapidly depressed upon oral or intra venous glucose administration or insulin injection. Intravenous epinephrine resulted in an abrupt increase followed by a decrease in plasma FFA concentration. These studies indicated a strong correlation of plasma FFA concentration with changes in nutritional state and suggested that fat is released from adipose tissue and transported in the plasma as FFA. These observations were a milestone in the understanding of total body energy economy and focussed attention to adipose tissue as the central mechanism in the control of free fatty acid metabolism. No less significant was the technological development by Dole (38) of a rapid, accurate and simple method for the isolation and titration of plasma free fatty acids.

Recognition of the highly active pool of plasma FFA which fundamentally reflects the marked sensitivity of adipose tissue to various nutritional and hormonal influences provided the stimulus to many studies on the dynamic aspects of adipose tissue metabolism.

Within a short period in vitro incubation techniques for adipose tissue were devised and the direct effects of adipokinetic hormones was explored. In 1958 White and Engel (181) demonstrated that corticotropin stimulates the breakdown of adipose tissue triglyceride (lipolysis) and Gordon and Cherkes (51) reported a similar direct lipolytic effect of epeniphrine. These observations were quickly confirmed by Rabin and Hollenberg (120) and others (182) and provided the basis for understanding the mechanism whereby administration of various adipokinetic hormones to the intact animal results in liver fatty infiltration.

The free fatty acids produced in adipose tissue consequent to hydrolytic cleavage of the triglyceride molecule need not leave the tissue but instead may be resynthesized to glyceride esters by the esterification process. In vitro uptake and esterification of FFA by adipose tissue was first demonstrated by Stern and Shapiro (162) in 1954. This reaction was found to be an active process sensitive to metabolic inhibitors (148). The importance of carbohydrate for the esterification of FFA in intact adipose tissue was reported simultaneously by Raben and Hollenberg (120,121) and Balley

et al (11). These investigators demonstrated that in the presence of glucose or glucose and insulin the in vitro uptake and esterification of added FFA was augmented. The latter group postulated that the glucose increased esterification by providing the precursor of newly formed glyceride glycerol presumably as ∝ glycerophosphate.

Thus adipose tissue has been shown to contain the enzymes necessary for lipolysis of triglyceride and esterification of free fatty acids. That these processes go on simultaneously was clearly shown by Leboeuf et al (97) and Cahill et al (29,30) who described the effects of epiniphrine on carbohydrate metabolism in intact adipose tissue. An increased utilization of medium glucose was observed in the presence of epinephrine and about 70% of the glucose carbon taken up was found in glyceride glycerol. These studies demonstrated that concomitant with increased lipolysis due to epinephrine, reesterification of the FFA products is also increased resulting in accelerated turnover of tissue triglycerides. All available evidence to date indicates that adipose tissue triglyceride breakdown and resynthesis goes on continuously (53,54,63,97,160,167,169), that is to say the triglyceride stores of adipose tissue are constantly turning over and therefore exist in a dynamic equilibrium. The dynamic nature of triglyceride turnover is schematically illustrated in Fig. I. The componant reactions of the cycle are lipolysis and esterification. Adipose tissue triglyceride is constantly being broken down to FFA and glycerol by tissue lipase and the FFA products are esterified to ∝ glycerophosphate and triglyceride is reformed.

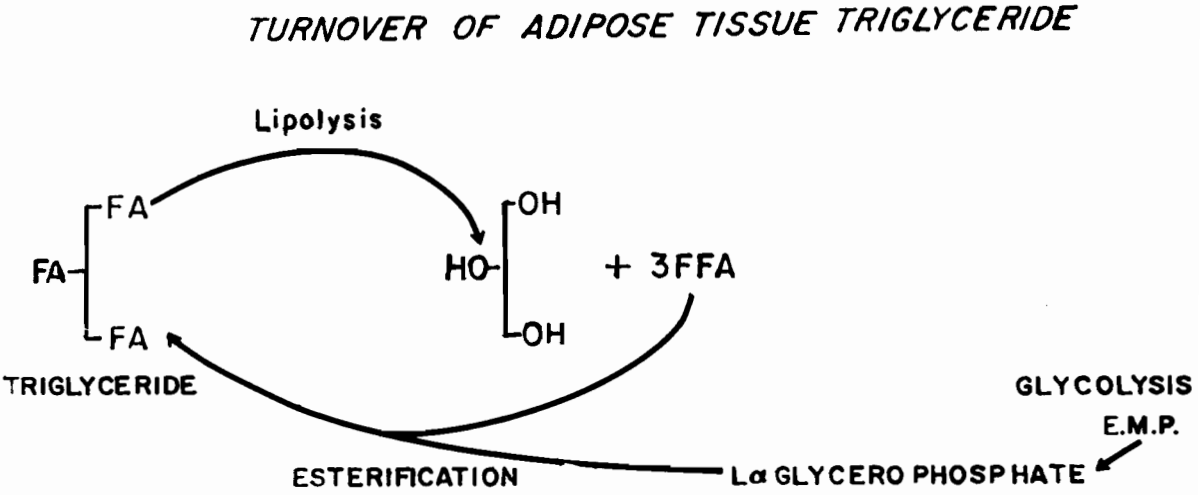

FIGURE I

Schematic representation of triglyceride turnover in adipose tissue. Triglyceride is broken down to glycerol and FFA (Free Fatty Acids). The glycerol cannot be reutilized and is lost to the tissue. The FFA may be reesterified to α glycerophosphate derived from glycolytic intermediates of the Embden Myerhoff pathway (E.M.P.) and triglyceride is reformed.

Although reversal of enzymic hydroysis of triglyceride takes place it is probably of little significance where net synthesis of ester bonds occur. This is in keeping with the general biological principal that separate pathways exist for the synthesis and degradation of complex molecules (39,178). It is apparent that an equilibrium such as this may be shifted toward net FFA production by either increasing the rate of lipolysis or decreasing the rate of esterification or altering both unequally. A net decrease in FFA production may be effected by the opposite influences.

In the following discussion a review of the literature relevant to the componant reactions of triglyceride turnover will be presented. Firstly lipolysis of adipose tissue triglyceride and fatty acid release in vitro is discussed followed by esterification of FFA and lipid assimilation by adipose tissue. Finally a review is presented of very recent work in which FFA-glycerol balance studies are used to quantitate triglyceride turnover in adipose tissue.

B) THE IN VITRO PRODUCTION OF FREE FATTY ACIDS BY RAT ADIPOSE TISSUE

Incubation of rat epididymal fat in Krebs-Ringer phosphate buffer containing albumin has been used by many investigators to study the lipolytic effects of various hormones (51,67,120,181). The presence of a fatty acid acceptor such as albumin is essential in order to demonstrate release of FFA into the medium (126). Adipose

tissue from normally fed animals release very small amounts of FFA in the absence of lipolytic hormone (121,126). Addition of ACTH (66,67,96,181), epinephrine (51,66,182) glucagon (56), T.S.H. (46) G.H. (170) and other pituitary peptides (137,138) results in an increased accumulation of FFA and glycerol (27,104) due to the hydrolytic breakdown of tissue triglyceride. The magnitude of the lipolytic response depends upon the nutritional state of the donor animal. Adipose tissue from fasted rats is more sensitive to ACTH than tissue from fed controls (67). Whether hydrolysis of triglyceride represents a direct effect of the hormone or mediated by some intra cellular agent is not entirely settled.

Hollenberg et al (67) studied the lipolytic activity of homogenates of adipose tissue which had been incubated with and without ACTH or epinephrine. Greater lipolytic activity was consistantly observed with the homogenates of hormone treated tissues. These results give strong support to the suggestion that corticotropin and epiniphrine augment FFA accumulation by activating a lipase in adipose tissue. Rizack (129) isolated a subcellular fraction of adipose tissue containing lipolytic activity. In these experiments extracts of incubated adipose tissue had reduced lipolytic activity compared to extracts of unincubated controls. Epiniphrine or epinephrine plus ATP added to the extracts increased their lipolytic activity to control levels.

The exact mechanism whereby these hormones activate tissue lipase is unknown. The possibility that epinephrine stimulation may be mediated by a system analagous to the activation of phosphorylase (47) has been explored and 3' 5' cyclic AMP, known to be involved in phosphorylase activation by epinephrine did not have a lipolytic effect on intact adipose tissue (40,168). That high energy phosphate bonds are necessary for hormone activated lipolysis has recently been emphasized by Ball and Jungas (10). These investigators re-examined the effects of anaerobiasis on hormone stimulated lipolysis. Strictly anaerobic conditions inhibited lipolysis in tissues of normal and fasted animals whereas tissues of fasted-refed rats responded to epinephrine treatment. The explanation of these findings rests on the fact that only tissues of refed animals contain significant amounts of glycogen stores that provide substrate for glycolysis from which high energy materials are derived for the lipolytic reaction.

The hormone sensitive lipolytic enzymes studied by Hollenberg et al (67) and Rizack(129) are similar to the pig adipose tissue lipase characterized by Lynn and Perryman (105) and tributyrinase activity of an acetone powder from rat adipose tissue isolated by Schnatz and Williams (140). In the aforementioned studies enzyme activity was optimal at pH 6.8-7 and strongly inhibited by sodium fluoride thus distinguishing it from another lipase system of adipose tissue called lipoprotein lipase (87,70).

It is important to realize that the physiological significance of the various lipolytic hormones in the moment to moment control of the lipolytic reaction in adipose tissue has yet to be established. Current opinion has held that the control of FFA mobilization rests less with the rate of lipolysis which is constant, but depends on the esterification rate. Thus, increases or decreases in net FFA production would be dependant on the relative availability of glucose for esterification of the FFA produced by lipolysis. The view that the lipolytic reaction may also be the site of control is strengthened by the observations of Roth et al (136) who demonstrated in man a marked sensitivity of circulating G.H. levels to fluctuations in blood glucose concentration, fasting and uncontrolled diabetes and that intravenous administration of human G.H. to man has been shown to cause a sharp rise in circulating FFA (123) presumably due to increased lipolysis in adipose tissue. Furthermore hypophysectomized diabetics on full replacement therapy do not mobilize FFA appropriately or become ketotic when insulin is withdrawn but upon the administration of minute amounts of human growth hormone FFA mobilization increases and a prolonged ketoacidosis results (103). The role of circulating epinephrine and glucagon in FFA mobilization under physiological situations like fasting must also be considered as these hormones are secreted in increased amounts and are potent lipolytic agents.

PHYSICAL CONSIDERATIONS

An appreciation of the unique mechanism of transport of

the relatively insoluble fatty acid anions is essential for accurate interpretation of in vitro studies with adipose tissue. As previously mentioned the FFA produced in vitro by hormonal stimulation accumulate in the tissue and pass into the medium only if an adequate amount of a suitable acceptor such as serum albumin is present. In the absence of albumin FFA accumulates in the tissue. The degree of FFA accumulation is limited by the toxic effects of free fatty acid anions. Excessively high concentrations of FFA within adipose tissue has been shown to inhibit further accumulation of FFA, decrease tissue oxygen consumption, depress glucose utilization and impair esterification of FFA (9,58,171). Albumin, when added to the medium accepts the FFA from the tissue thereby decreasing the tissue FFA concentration and affords a protective effect on tissue metabolism. Therefore, in any study on intermediary metabolism of adipose employing lipolytic hormones it is imperative that the effects of the hormone per se be differentiated from that of FFA.

The amount of FFA passing into the medium depends on the total amount of albumin present. Large quantities of FFA may saturate the albumin which would prevent further transfer of tissue FFA to medium resulting in accumulation of FFA within the tissue (126). More precisely, the net movement of titratable FFA between tissue and medium depends on the chemical potential of medium FFA which in turn depends upon the molar ratio of FFA/albumin in the medium. Goodman (49) has studied the interaction of long chain fatty acids with serum albumin

which had been pretreated to remove FFA. Interpretation of the data is based on the assumption that each albumin molecule bears a discrete number of sites to which fatty acids are bound; that the sites are independent and that the interaction of any one site with a fatty acid is a simple association-dissociation equilibrium that obeys the law of mass action. Each albumin molecule was found to bear three classes of binding sites. The first class consists of two sites with the greatest affinity for fatty acids; the second class has five sites with a lesser affinity for FFA and the third class has about twenty sites having the lowest affinity (45). It is apparent that the higher the FFA/Albumin molar ratio the greater the concentration of unbound FFA hence the greater its chemical potential.

Cytoplasmic binding sites for FFA in adipose cells have been postulated (166). Isolated subcellular fractionsof adipose tissue have a marked affinity for added FFA and can displace and bind fatty acids introduced in albumin bound form (127). It is possible that binding sites within adipose tissue cells have association-dissociation equilibria with FFA and the chemical potential of adipose tissue FFA would depend upon the amount of unbound free fatty acid. Furthermore, the net movement of tissue FFA into the medium may be directly dependent on the concentration gradient between tissue unbound FFA and medium unbound FFA. This could account for (a) the obligatory requirement of fatty acid acceptor in the medium in order to demonstrate significant accumulation of FFA in the medium and (b) the inhibitory

effect of high FFA/albumin ratio on release of FFA from adipose tissue.

FFA COMPOSITION

The free fatty acids produced by hormonal stimulation of adipose tissue are composed of the same major fatty acids present in the triglyceride (66). Hollenberg et al (66,68) determined the composition of FFA produced by ACTH and epinephrine stimulation and observed that palmitic and palmitoleic acid were present in higher proportions in the free acid fraction than in the triglyceride acids. It was suggested that preferential cleavage at certain ester linkages similar to the positional specificity of pancreatic lipase (111,139) could account for the results. Mienertz (113) on the other hand, concluded that hormone stimulated lipolysis exhibited fatty acid specificity as he consistently found palmitoleic acid in higher, and oleic in lower molar concentration in the free acid fraction throughout a wide range of adipose tissue triglyceride compositions. The apparent discrepancy in results was due to a basic difference in experimental design as the latter investigator analyzed only the medium FFA. Following hormone stimulated lipolysis analysis of the tissue and medium FFA composition separately, revealed disproportionate distribution of individual FFA. A significant relationship between fatty acid structure and its distribution between tissue and medium was observed. The release of individual FFA from adipose tissue into the medium was related to chain length and degree of unsaturation such that the more polar acids passed into the medium most readily (4).

C) UPTAKE OF FFA BY ADIPOSE TISSUE IN VITRO

Uptake and esterification of FFA by adipose tissue was first demonstrated by Stern and Shapiro (162). They found the uptake to be markedly affected by the nutritional state of the donor animal as tissues taken from fasted animals had a much reduced uptake of medium FFA. Refeeding of fasted animals was associated with an augmented esterifying capacity compared to tissues from normally fed control rats (148). Over 95% of the assimilated FFA-C^{14} may be recovered in esterified form as triglycerides (11,39,85,156). With tissues from fasted rats only about 60-80% of the C^{14} labelled FFA taken up is esterified presumably as a result of reduced esterifying ability. The uptake of FFA from the medium follows a first order reaction rate in that it is proportional to the FFA concentration (85). Albumin is an essential part of the medium as it serves to solubilize the fatty acid anions. In the presence of albumin the total concentration of FFA does not give a true indication of the effective concentration of the fatty acid because a portion of the FFA is bound to albumin at any given instant. The concentration that is free (unbound to albumin) depends upon the FFA/albumin molar ratio and determines the chemical potential of the medium fatty acid. A FFA/albumin molar ratio of 5 is about optimum for esterification with intact adipose tissue.

Glucose utilization has been shown to be important in esterification of FFA (11,74,121). Raben and Hollenberg (121) demonstrated that the addition of glucose alone in adequate amounts

resulted in an increased disappearance of medium fatty acid. Furthermore, the addition of glucose plus as little as 30μU/ml insulin markedly increased esterification of fatty acids into adipose tissue from both fed and fasted animals. On the basis of these results the authors concluded that the esterification of FFA in adipose tissue is promoted by carbohydrate utilization.

Bally and co-workers (11) using intact rat epididymal fat pads found that palmitic acid-1-C^{14} uptake and oxidation to CO_2 was proportional to the concentration of medium fatty acid. The presence of glucose markedly increased the incorporation of palmitate-1-C^{14} into triglyceride and decreased its oxidation to CO_2. These effects were proportional to the glucose concentration in the incubation medium and Insulin did not influence the metabolism of palmitate in the absence of glucose. They suggested that glucose utilization augments esterification of FFA in adipose tissue by providing glycerophosphate from the glycolytic intermediate dihydroxyacetone phosphate, and in the absence of adequate amounts of glucose, glycerophosphate, which is the precursor of glyceride glycerol, will not be formed and esterification is reduced. Although glycerol is constantly being produced as a product of triglyceride hydrolysis (97,167) it cannot be reutilized by adipose tissue to any significant extent (27,104,108,148) because glycerokinase, the enzyme that catalysis the formation of L∝glycerophosphate from glycerol and ATP is absent (108,183). Therefore the esterification reaction in adipose tissue is dependant upon a continuous supply of glyceryl precursors from glycolytic intermediates.

Kerpel et al (85) and Dole (39) have attempted to assess the participation of the tissue FFA pool during uptake of palmitic acid-1-C^{14}. This pool had been shown to expand with lipolytic stimuli or contract when esterification is promoted with glucose and insulin (121,31) and it was thought of interest to determine whether this represented a common FFA pool for the opposing reactions of lipolysis and esterification. The results of these studies showed general agreement in that the medium FFA C^{14} failed to enter the tissue FFA pool in significant quantity despite a substantial uptake of label into fatty esters. Dole (39) extended the earlier observations of Kerpel et al (85) and determined the incorporation of labelled FFA during epinephrine induced expansion of the tissue FFA pool. The uptake of label from the medium into esters (corrected for dilution effects) of epinephrine treated tissues averaged 95% of the uptake shown by the paired pad incubated in medium without epinephrine. These data indicate a functional separation of FFA uptake and lipolysis and suggested that the two processes may be separated anatomically. Thus, uptake might be a surface function and esterification keeping pace with entry into the cell. Lipolysis, on the other hand, takes place deeper in the cell and is independent of transport across the cell curface. The tissue FFA pool which represents the FFA products of intracellular lipolysis (and therefore called the lipolytic pool) could expand or contract independent of uptake and esterification of medium FFA. This points out a basic difference between in vitro esterification of fatty acids added to the medium and esterification of the tissue FFA lipolytic pool (40).

In order to learn more about the compartmentalization of newly esterified fatty acids Kerpel et al (85) and Stein and Stein (156) prepared subcellular fractions of adipose tissue which had been incubated in medium containing FFA-C^{14}. A particulate fraction was isolated in the 15,000 x g and 103000xg sediments and the specific activity of the glyceride esters in this fraction was found to be very high as compared to the floating free fat. An ingenious technique of *in vivo* incubation of epididymal fat, where both pads are exteriorized and placed in a specially constructed well incubator containing FFA-C^{14}, was used to determine the time for particulate fat and bulk fat to equilibrate. The specific activity of the two fractions were found to be equal four hours after an initial ten minute incubation. This indicated a two compartment system and provided objective evidence to what Hirsch (63) had anticipated. They concluded that the cytoplasmic compartment (particulate fraction) has a very rapid turnover rate as evidenced by the rapid labelling and high specific activity followed by a rapid decline of activity as the label moves into the central lipid droplet (bulk fat) which has a much longer turnover time. Experiments using the *in vivo* incubation technique were undertaken to determine whether newly esterified fatty acids are most readily mobilized. Both pads of a number of animals were incubated in palmitic acid-1-C^{14} for a brief period and one pad from each animal was removed and analysed. The second pad was replaced and the animals fasted from one to four days following which the pad was removed. The specific activity of both pads were found to be the same up to four days fasting. This indicated non-

preferential utilization of the label and argues strongly against the so-called onion skin effect or last on first off mechanism of fat mobilization.

Not all fatty acids are esterified at the same rate. Angel and Hollenberg (4) observed a relationship between fatty acid structure and esterification. Pieces of epididymal fat were briefly exposed to epinephrine in an acceptor deficient medium. Half of the pieces were then incubated in fresh medium containing glucose and insulin and half were incubated in buffer alone. The composition and amount of FFA present after the second incubation was determined and the relative rates of esterification calculated. The results indicated that the more polar acids are most readily esterified. For the saturated and monounsaturated acids the shorter the chain length the greater the esterification and at a given chain length the more unsaturated acids were most readily esterified.

A new and interesting dimension in the control of fatty acid metabolism has recently been presented by Dole (40,41) on the relationship of nucleic acid and its metabolites to the <u>in vitro</u> intermediary metabolism of rat adipose tissue. He has shown that nucleic acids (RNA,DNA), nucleotides (ATP,AMP,3'5' cyclic AMP), nucleoside (adenosine) and homogenates of tissue rich in nucleic acids, inhibit the lipolytic action of epinephrine and purine and pyrimidine bases increase lipolysis in the presence of epinephrine. RNA also inhibited

the lipolytic actions of ACTH, TSH and glucagon by causing net reestrification of fatty acids in the lipolytic pool. This effect is distinct from esterification of medium fatty acids which was not affected by RNA. Whether the reduction in net production of fatty acids by RNA represented inhibition of lipolysis or an increase in the rate of esterification could not be discerned. These results are in contrast to those of Rizack (129) who has suggested that ATP augments the lipolytic action of epinephrine in subcellular preparations of incubated adipose tissue.

Subsequent study has demonstrated insulin like actions of RNA,AMP and adenosine on intact adipose tissue in addition to their antilipolytic effects. RNA increased the labelling of lipids two to five fold when added to medium containing glucose-u-C^{14}. Seventy percent of the radioactivity was in glyceride glycerol with most of the remainder in the fatty acid components. By using specifically labelled compounds the nature of the stimulation was defined. With glucose-1-C^{14} as substrate, RNA increased the output of $C^{14}O_2$ proportionately more than it increased lipogenesis; with glucose-6-C^{14} the relative stimulation was reversed. These effects are similar to those reported for insulin (28,184).

Stimulation of fatty acid synthesis from acetate-1-C^{14} was increased by nucleic acid derivatives only with the addition of glucose to the medium. The distribution of label in tissue lipids formed

from acetate-1-C^{14} was found to be almost entirely in the fatty acid fraction. Gas chromatographic fractionation located most (70%) of the label in palmitic acid. RNA caused a striking increase in labelling of a diglyceride fraction and lesser percentage increase in triglycerides. These findings suggested that the diglycerides serve as intermediates in the esterification of fatty acids formed from acetate-1-C^{14} and that RNA may increase synthesis of this intermediate. The great difference in lipogenic potency of RNA and insulin should be noted. Stimulating effects of RNA were found at concentrations of 0.1mg/ml whereas insulin at a concentration of 1.0mu/ml (.00004mg/ml) stimulated lipogenisis more markedly.

D) LIPOPROTEIN LIPASE AND THE ASSIMILATIVE FUNCTION OF ADIPOSE TISSUE

The in vivo clearing of alimentary lipemia following intravenous injection of heparin was first described by Hahn (59) in 1943. Anderson and Fawcett (3) later demonstrated that intravenous administration of heparin resulted in the appearance in blood of a substance which was able to clear lipemic plasma in vitro and therefore called clearing factor. Following these observations it was demonstrated that such clearing was the result of hydrolysis of triglyceride in the large, light scattering, triglyceride rich lipoprotein (88,131,150). Korn (87,88,89,90) partially purified clearing factor from extracts of normal rat hearts and adipose tissue and determined some of its properties. He suggested the name lipoprotein

lipase to identify the nature of the activity of this enzyme and to distinguish it from pancreatic lipase. The enzyme catalyzes the hydrolysis of triglycerides when they are in the form of lipoproteins, either chylomicrons or low density lipoproteins. Lipoprotein lipase hydrolyses all three ester bonds of the triglyceride molecule and shows no positional specificity (91). It is sensitive to a number of inhibitors including salt solutions of high ionic strength, EDTA, protimine, and heparinase (92). Korn has suggested that the enzyme may be a mucoprotein with a heparin like prosthetic group (92). Nothing is known about the mechanism by which heparin causes lipase activity to appear in the blood, presumably spilling out from the tissues. It has been suggested that lipoprotein lipase is liberated from sites immediately accessible to circulating blood such as the capillary endothelium (132). Hollenberg (69) has shown that in vitro preparations of rat epididymal fat release lipoprotein lipase activity into the medium when heparin was present in the medium. This lipolytic activity, measured by the amount of hydrolysis of an activated coconut oil substrate was affected by the nutritional state of the animal. The activity was reduced with adipose tissue of fasted rats and refeeding was associated with an accentuated response to heparin. Furthermore, the addition of glucose and insulin to medium containing pads from fasted animals increased lipoprotein lipase activity only in the presence of heparin. The author suggested that this type of lipase activity was primarily concerned with the assimilation of circulating lipoprotein triglyceride rather than the mobilization of tissue lipid. This

mechanism could thus account for the observation that augmented carbohydrate utilization accelerated the clearing of lipemia (1,173). The observation that adipose tissue of fed rats took up a greater amount of intravenously administered labelled chylomicrons than the corresponding tissue of fasted rats (21) could also be explained. Subsequent studies with adipose tissue have demonstrated the correlation between nutrition state and lipoprotein lipase activity (19,133,140) and ability to assimilate lipoprotein triglyceride (19,55).

The in vitro uptake and metabolism of chylomicron triglyceride has been studied by Rodbell (134). He showed that chylomicron triglyceride and a synthetic tripalmitin C^{14} emulsion are taken up equally well by adipose tissue. Once in the cell a portion of the triglyceride is hydrolyzed and the FFA products which presumably mix with the endogenous pool of FFA are reesterified. By alcohol acetone precipitation he was able to recover tripalmitin C^{14} which had not undergone hydrolysis upon cell entry. The addition of DFP, a potent lipoprotein lipase inhibitor, did not affect the total uptake of medium triglyceride but markedly inhibited its subsequent metabolism. These results indicate that uptake and subsequent hydrolysis of assimilated triglycerides are functionally separate and lipoprotein lipase activity is primarily concerned with the latter. Olivecrona (117) also found that triglyceride was taken up intact and subsequently hydrolyzed and re esterified. Lipoproteins containing doubly labelled triglyceride of known glycerol-C^{14}/FA-H^3 ratio was infused into rats and the C^{14}/H^3

ratio was determined in different organs after various time intervals. The ratio fell very rapidly in adipose tissue indicating hydrolysis of the triglyceride soon after cell entry.

It is of interest to note that adipose tissue lipoprotein lipase activity is maximum in situations where esterification and lipogenesis is increased as in the refed state. This suggests that an important function for lipoprotein lipase may be the hydrolysis of recently synthesized triglyceride.

E) TRIGLYCERIDE TURNOVER IN ADIPOSE TISSUE AS MEASURED BY THE FFA-GLYCEROL BALANCE

The principle upon which this method is based rests on the observation that adipose tissue is unable to reutilize to any significant degree the glycerol released by lipolytic breakdown of triglyceride (27,104,148). Therefore, each mole of free glycerol produced during incubation represents the hydrolytic cleavage of 3 moles of FFA and is taken as a quantitative estimation of total lipolysis. The actual amount of FFA produced is measured concomitantly and the difference between it and the total lipolysis reflects the amount of FFA esterified (10,54,79,167,170). The validity of this approach depends upon the following assumptions; that glycerol production from non triglyceride sources such as lower glycerides or α glycerophosphate is negligible; that hydrolysis of triglyceride is complete; and that reesterification of FFA to partial glycerides does not occur. The extent

to which these assumptions are true determines the accuracy of FFA-glycerol balance method for quantitating triglyceride turnover in adipose tissue incubated in vitro. Certain experimental evidence supports the first two assumptions. Vaughan and Steinberg (170) found little change in di and monoglyceride content following incubation of rat epididymal fat with or without ACTH. Lynn et al (104) have demonstrated that glycerol released from adipose tissue incubated with C^{14} glucose and epinephrine contained no radioactivity which is good evidence that it did not come from α glycerophosphate. Furthermore, Margolis et al (108) and Gorin et al (54) found minimal glycerophosphate phosphatase activity in cell free preparations of adipose tissue. Although it has been shown that diglyceride is an intermediate in the synthesis of triglyceride (Fig. II) (48) it is not known whether lower glycerides produced during sequential hydrolysis of triglyceride can be esterified to triglyceride.

The glycerol content of freshly excised epididymal fat has been studied in a number of laboratories with good agreement in results. Vaughan (167) reported a glycerol content of $1.8 \pm .19$(mean $\pm$ S.E.) μM/gram wet weight by the periodate oxidation method. Gorin and Shafrir (54) found 0.8 - 2.0 μM/gram wet weight and Jungas and Ball (79) found 0.8 μM/gram wet weight; the latter two by enzymatic assay. Since this amount is small compared to that released into the bathing medium following stimulation with lipolytic hormones (up to 29 μM glycerol/gram

adipose tissue in 2 hrs. (79) the pre-existing glycerol content may be assumed to be 1 or 2 μM/gram wet weight in such studies. On the other hand, the tissue glycerol content rapidly falls during the first few minutes of incubation due to escape into the medium and a 15 minute preincubation period has been found useful in getting rid of a portion of the preformed glycerol (54). Following this preincubation period, the release of glycerol into the medium is linear with time even when large amounts are produced as with epinephrine stimulation (54).

Adipose tissue from normal fed rats incubated in buffered medium containing albumin produces 1.3 μM glycerol/gram tissue/hr. (167) indicating basal lipolytic activity. During this period no net accumulation of FFA occurs and is interpreted as signifying FFA reesterification thus demonstrating triglyceride turnover in unstimulated adipose tissue. Experiments with tissues from 18 hr. fasted animals revealed an increased turnover of triglyceride as glycerol release was greater than normal controls with no net change FFA production. In tissues of 42 hr. fasted animals the turnover was still greater but the balance favoured net lipolysis as FFA accumulated (167). Similar observations with adipose tissue of fasted rats have been reported by Gorin and Shafrir (54) and Ball and Jungas (10). These findings are important as they cast doubt on the theory that net production of FFA in adipose tissue is solely a function of depressed esterification and strengthens the possibility that net FFA production is partly determined by variations in the rate of lipolysis.

Addition of lipolytic hormones not only increases the rate of glycerol production (lipolysis) but also augments the rate of esterification as less FFA are found than might be expected on the basis of the amount of glycerol released (79,167,170). Maximum glycerol production and FFA esterification occurs in adipose tissue incubated with epinephrine plus glucose and insulin (79). These findings confirm the earlier observations of Lebouf et al (97) and Cahill et al (30) that lipolytic hormones augment adipose tissue triglyceride turnover.

In the absence of glucose, insulin has been shown to inhibit the epinephrine stimulated release of glycerol in adipose tissue (79). This phenomenon has been called the antilipolytic effect of insulin since both FFA and glycerol production are depressed (79). The exact mechanism and the physiologic significance of this observation is not known. Dole (40) has reported similar antilipolytic properties of RNA which also has other insulin-like effects on adipose tissue metabolism (41). It is possible that the antilipolytic effect of insulin may in fact represent the stimulation of lower glyceride esterification or the activation of a latent glycerolkinase, both of which would manifest as a decreased production of glycerol and FFA. These possibilities must be differentiated from a true inhibition of the lipolytic reaction before this effect can be regarded as antilipolytic.

PART III THE ESTERIFICATION OF FREE FATTY ACIDS AND TRIGLYCERIDE SYNTHESIS

The original experiments delineating the multienzyme biosynthetic pathway of triglyceride formation were carried out in tissue preparations other than adipose tissue. Recently Steinberg and coworkers (161) investigated fatty acid esterification and glyceride synthesis in adipose tissue homogenates and subcellular preparations. Their findings were entirely in keeping with the pathway previously described in liver homogenates (94,157,158,159,177).

In this section the general scheme of triglyceride biosynthesis from precursors will be presented followed by a more detailed analysis of individual reactions. Finally, the deviations from the general scheme and reactions and intermediates peculiar to certain tissues, will be discussed.

The first good evidence for the operation of a pathway of triglyceride synthesis clearly different from reversal of lipase action (17,75) was obtained by Teitz and Shapiro (164) who found that the incorporation of labelled fatty acid into neutral lipid of liver homogenates requires ATP and some heat stable cofactor. Earlier work on the biologic synthesis of phosphorus containing lipids demonstrated the importance of α glycerol phosphate and activated fatty acid as precursors for phosphatidic acid synthesis (80,93,94). When it became apparent that phosphatidic acid and 1,2 diglyceride are essential intermediates in the biosynthesis of glycerolphosphatides, Weiss and

Kennedy (177,178) tested the hypothesis that these compounds are also involved in the synthesis of triglyceride and found that a particulate enzyme from chicken liver catalyzes the net synthesis of triglyceride from palmityl-CoA and 1,2 diglyceride.

A schematic representation of the various enzyme catalyzed reactions whereby long chain fatty acids are coupled with glycerophosphate and its derivatives to form triglyceride is shown in Fig. II. The condensation of L-α glycerophosphate with thiolesters of long chain fatty acids is catalyzed by a transacylase to form successively lysophosphatidic acid and phosphatidic acid. A phosphatase hydrolyses the terminal phosphate of phosphatidic acid yielding 1,2 diglyceride which serves as a common intermediate in subsequent triglyceride and phosphatide synthesis. The third acyl moiety is transferred from acyl-CoA under the influence of a tranacylase to 1,2 diglyceride forming triglyceride. Alternatively, 1,2 diglyceride may react with CDP-choline or CDP-ethanolamine to form lecithin and phosphatidyl ethanolamine respectively.

A) ACTIVATION OF FREE FATTY ACID

In earlier studies on complex lipid formation Kornberg and Pricer (93) using a partially purified enzyme from rat liver found that phosphatidic acid P^{32} was synthesized when α GP^{32}, ATP, CoA and stearic acid were present. In the absence of ATP the amount of label incorporated was reduced by 95% and in the absence of CoA it was

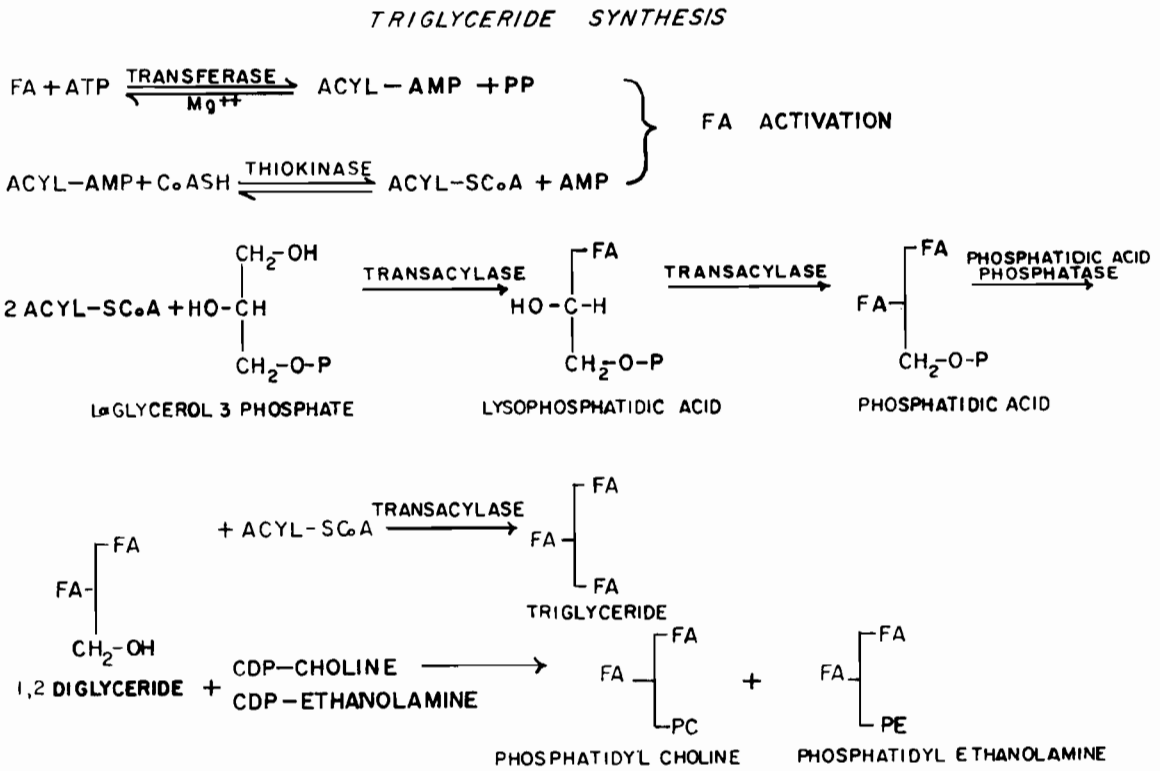

FIGURE II

Biosynthetic pathway for triglyceride in adipose tissue and liver. 1,2 diglyceride is a common intermediate to triglyceride and certain phospholipids. It is not known whether the latter reaction occurs in adipose tissue.

reduced by 85%. In view of the participation of ATP and CoA in this system a mechanism analagous to that proposed for the ATP-CoA activation of acetate (72,31) was suggested. The overall reaction was thought to be the resultant of two distinct steps, activation (equation 1) and esterification (equation 2).

$$\text{FATTY ACID} + \text{CoA} \xrightleftharpoons{\text{ATP}} \text{ACYL - CoA} \qquad (1)$$

$$2\ \text{ACYL - CoA} + \alpha\ \text{GP} \rightleftharpoons \text{PHOSPHATIDIC ACID} + 2\ \text{CoA} \qquad (2)$$

Further evidence for activation of long chain fatty acid by conversion to acyl-CoA derivatives was obtained by the same investigators (93) using soluble and insoluble guinea pig liver preparations. Pure enzymically synthesized palmityl-CoA was isolated from a reaction mixture and quantitative correspondence between ATP and CoA consumed and the acyl-CoA, AMP and PP formed was demonstrated. Divalent cation Mg^{++} stimulated the rate of synthesis up to 30% but was not an absolute requirement. Straight chain saturated fatty acids C_5-C_{22} and mono, di, and trienoic C_{18} served as substrate. Maximum velocity of fatty acid activation was seen with lauric acid (C_{12}) and marked inhibitory effects were observed with high concentrations of oleic and linoleic acid. The activation reaction was shown to proceed according to equation 3 in which inorganic pyrophosphate is split off ATP and a thiol ester linkage between CoA and fatty acid is formed (93,157).

$$\text{FATTY ACID} + \text{CoA-SH} + \text{ATP} \xrightleftharpoons{Mg++} \text{ACYL-S-CoA} + \text{AMP} + \text{PP} \qquad (3)$$

Finally, chemically synthesized acyl-CoA substituted for fatty acid, ATP, and CoA in α GP esterification, indicating that the thiol ester form was an intermediate. Continued interest in the acetate activating system resulted in the demonstration of adenyl-acetate as an intermediate in acetyl-CoA synthesis (15). An entirely analogous activation sequence thought to exist for higher fatty acids is shown on Fig. II. Vignais and Zabin (172) prepared synthetic adenyl palmitate, incubated it with guinea pig liver enzyme plus CoA and demonstrated the formation of palmityl-CoA at a velocity twice that using an ATP, + CoA, + palmitate mixture. Adenyl palmitate may be split by liver enzymes to palmitic and adenylic acids (23,76,172).

Shapiro and coworkers (148,149) have demonstrated the presence of long chain fatty acid activating systems in adipose tissue using the hydroxamate method. To date, details of their results have not been published.

B) DEACYLASE AND TRANSACYLASE ACTIVITY

Acyl-CoA derivatives are subject to hydrolytic disruption due to deacylase activity in most cellular extracts (22,146,155). The transacylase and deacylase activity of rat and guinea pig liver microsomal and mitochondrial preparations has been studied in detail by Brandes et al (22). They observed that albumin altered the relative activity of tranacylation and deacylation. At pH 6.5 in the absence of albumin about half of the CoA liberated from palmityl CoA was due to

deacylase activity. Upon the addition of albumin the pH optimum of transacylation shifted and deacylation was markedly reduced so that at pH 6.5 80% of the CoA liberated was due to transacylation. Deacylase activity in adipose tissue preparations has to date not been the subject of any report. Vaughan, in a review (166), quoting unpublished observations mentioned the presence of deacylase activity in adipose tissue microsomes.

C) ESTERIFICATION OF L ∝GLYCEROPHOSPHATE

L ∝glycerophosphate is esterified with 2 acyl-CoA to form phosphatidic acid (93,178). The order in which the fatty acid residues attach to form 1 or 2 lysophosphatidic acid as an intermediate is not known. The enzyme catalyzing the esterification of ∝GP is called transacylase because it facilitates transfer of the acyl portion of the thioacyl ester to a primary alcohol group of ∝GP. Glycerophosphate has been shown to be an obligatory intermediate in phosphatidic acid synthesis (80,93,94) as no direct acylation of free glycerol has been observed in any tissue thus far studied (158). Before glycerol can be incorporated into phospholipid or neutral fat it must be phosphorylated by ATP in the presence of glycerokinase (24,25,81). Glycerokinase activity is found primarily in liver, kidney and heart (25) and in small gut mucosa of certain species (26). No glycerokinase activity has been observed in normal adipose tissue (27,104,108,148). Therefore, glycerol freed due to triglyceride breakdown cannot be reutilized to any appreciable extent and is released into the medium

(56,97,167) or into the serum *in vivo* (57).

Lochaya *et al* (100) have postulated the presence of glycerokinase in adipose tissue of obese hyperglycemic mice. By this mechanism an abundance of glycerophosphate augments esterification thereby decreasing net release of FFA following epinephrine stimulation, thus accounting for the diminished ability of epinephrine to promote free fatty acid release in this tissue (98).

In normal adipose tissue the glycerol portion of newly synthesized glycerides is derived from glycolytic intermediates as shown in Fig. III. Margolis and Vaughan (108) in an exhaustive study of αGP synthesis and breakdown in homogenates of rat adipose tissue, have shown that all the intermediates shown in Fig. III (except fructose-6-P which was not tested) will substitute for αGP in neutral fat synthesis. Glucose, glucose-1-P and glycogen could not be substituted for αGP. Glycerophosphate phosphatase has been demonstrated in adipose tissue (108). Its activity could be inhibited by sodium fluoride and omission of Mg^{++} ions.

D) PHOSPHATIDIC ACID PHOSPHATASE

1,2 Diglyceride and inorganic phosphate is formed following hydrolytic cleavage of the phosphate residue of phosphatidic acid (64,154). This highly specific phosphatase, called phosphatidic acid phosphatase is widely distributed in mammalian tissues (36,154) and

L-α-GLYCEROPHOSPHATE SYNTHESIS FROM GLYCOLYTIC INTERMEDIATES

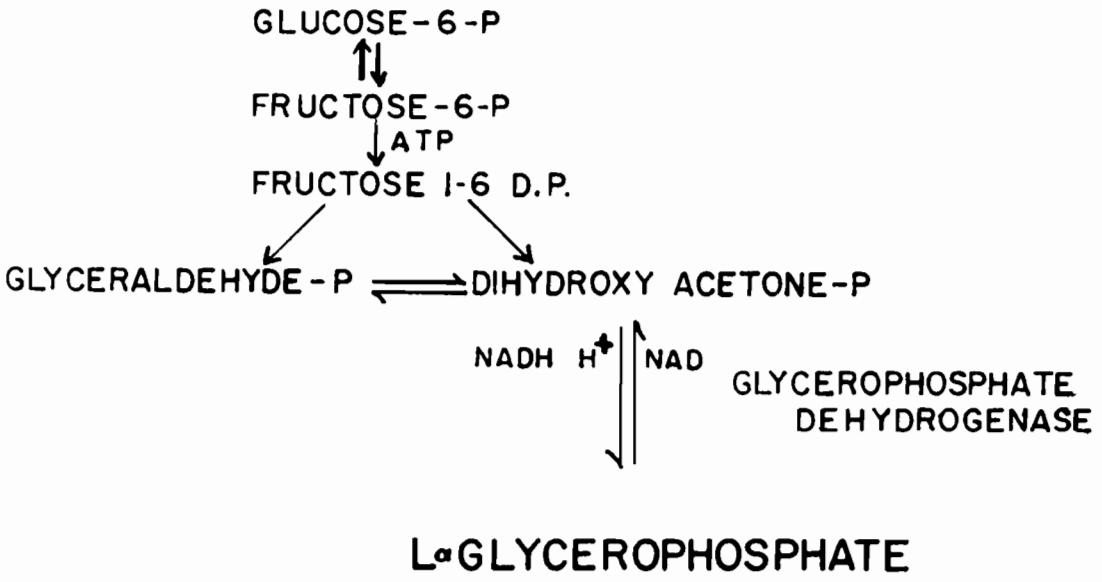

FIGURE III

α Glycerophosphate synthesis from glycolytic intermediates in rat adipose tissue.

accounts for the failure of phosphatidic acid to accumulate in tissues. The major portion of this activity is associated with microsomes and is sensitive to divalent cations (154). Indeed the accumulation of phosphatidic acid in the experiments of Kornberg and Pricer (93) and Kennedy (80) may be explained in part by the inhibition of phosphatidic acid phosphatase by added magnesium ions. Coleman and Hübscher (36) demonstrated phosphatidic acid phosphatase activity in liver, brain, intestine and kidney of rabbit, cat, guinea pig, ox and pig. The Km for pig kidney enzyme pH 7.4, 37° was 2.2×10^{-4}. pH optimum was 6.

The activity of this enzyme in adipose tissue has been studied but not reported. Vaughan (166) has quoted unpublished observations of Margolis who observed little effect of varying pH from 6.5 - 8.0 and no inhibitory effect of Mg^{++} on phosphatidic acid phosphatase activity in rat adipose tissue.

E) ESTERIFICATION OF 1,2 - DIGLYCERIDE

The third fatty acid is transferred from acyl-CoA to 1,2 diglyceride forming triglyceride (177). The transacylase catalysing this reaction is thought to be distinct from the enzyme involved in the esterification of αGP (166). The evidence for this is suggestive but inconclusive.

Goldman and Vagelos (48) have investigated the specificity of triglyceride synthesis from various diglyceride emulsions in a

particulate enzyme system of chicken adipose tissue. Their results indicate that diglycerides containing myristate were more reactive than those containing palmitate, which in turn were more reactive than those containing stearate and 1,2 diglycerides with at least one unsaturated fatty acid are more reactive in general than saturated diglycerides. Interpretation of these data is made difficult because of the physical state in which these water insoluble substrates are presented to the enzyme. The greater reactivity of the diglycerides with shorter chain fatty acids or with at least one unsaturated fatty acid might be expected from these physical factors. To what extent these physical factors play a role in vivo is not known.

F) PHOSPHOLIPID SYNTHESIS FROM 1,2 DIGLYCERIDE

It is well known that 1,2 diglycerides serve as intermediates in phosphatidyl choline and phosphatidyl ethanolamine synthesis. Cytidine coenzymes are required for this reaction. The detailed chemical steps in phosphatide synthesis has been worked out almost exclusively by Kennedy and Weiss (81,82,83,84). Briefly, phosphocholine reacts with CTP to form the coenzyme CDP-choline. The phosphocholine is then transferred from CDP choline to 1,2 diglyceride forming phosphatidyl choline (lecithin) equation (4). The enzyme

$$\text{CDP-CHOLINE} + \text{1,2 DIGLYCERIDE} \rightleftharpoons \text{PHOSPHATIDYLCHOLINE} + \text{CMP} \qquad (4)$$

catalyzing the reaction has been named phosphocholine glyceride transferase.

The synthesis of phosphatidyl ethanolamine is identical substituting phosphorylethanolamine instead of phosphocholine. Stereospecificity for certain diglycerides has been shown in the synthesis of lecithin (178) with preference of D-1,2 diglyceride over the L-enantiomorph. DL-1,2 dilaurin and DL 1,2 dioctanoin were not incorporated into lecithin but would form triglyceride indicating substrate specificity of phospholipid synthesizing enzymes.

Phospholipid synthesis undoubtedly occurs in adipose tissue but to date has not been reported.

G) MONOGLYCERIDE TRANSACYLASE AND MONOGLYCERIDE LIPASE

Synthesis of higher glycerides has been shown to occur by direct esterification of monoglycerides in subcellular preparations of small gut mucosa (32,33,71,77,146). The reaction is distinct from a glycerol phosphate - phosphatidic acid system of Weiss et al (178) and is catalysed by monoglyceride transacylase (34) according to equation (5). Diglyceride is the principal product of this reaction (77,34). By this mechanism

$$1 \text{ or } 2 \text{ MONOGLYCERIDE} + \text{ACYL-CoA} \longrightarrow 1,2 \text{ DIGLYCERIDE} + \text{CoA} \qquad (5)$$

$$1 \text{ or } 2 \text{ MONOGLYCERIDE} \longrightarrow \text{glycerol} + \text{FFA} \qquad (6)$$

monoglyceride produced during triglyceride digestion may be directly esterified in the mucosal cell without further degradation. The quantitative significance of this pathway is not known.

The monoglyceride esterifying system has been found in soluble preparations of pig kidney, rabbit pancreas, rat intestinal mucosa and liver (72), and in microsomal preparations of chicken and rat liver (158,178).

Attempts to demonstrate monoglyceride transacylase activity in rat adipose tissue have failed (161). This may have been due to a specific lipolytic enzyme called monoglyceride lipase. This enzyme ruptures the solitary ester linkage of monoglycerides liberating free glycerol and FFA as products (equation 6), (16,146,147,161). The presence of monoglyceride transacylase in adipose tissue has not been excluded and awaits definitive analysis.

EXPERIMENTAL SECTION

I INTRODUCTION

It is apparent that adipose tissue is of central importance in the energy balance of the mammalian organism. The FFA released by this organ provides the peripheral tissues with a continuous supply of matabolic substrate during periods of fasting. During carbohydrate feeding FFA release is curtailed due to augmented esterification in adipose tissue and storage of lipid is favoured. Thus output of fatty acid by adipose tissue is co-ordinated with nutritional requirements. That is to say the equilibrium of adipose tissue triglyceride turnover alternates from one favouring net lipolysis to one favouring net esterification depending on the nutritional state.

As lipolysis and esterification are separate enzyme catalysed reactions the control of FFA accumulation in adipose tissue may be through agents affecting lipase activity and/or by factors that modify esterification.

The esterification of FFA for triglyceride synthesis in adipose tissue homogenates has been found to follow the general sequence observed in liver (161,178). However, the multienzyme biosynthetic process of triglyceride formation in adipose tissue is not well characterized.

The present study was undertaken to define more fully the FFA esterifying system of rat adipose tissue with the view that its

elucidation would undoubtedly provide a better idea of regulating mechanisms.

II MATERIALS AND METHODS

A) CHEMICALS AND REAGENTS

The inorganic chemicals were reagent grade. DL-α glycerophosphate 95% pure, adenosine-5'-triphosphate disodium salts 99% pure, Coenzyme A-from yeast 91% pure, cytidine-5' diphosphocholine 98% pure, and glutathione were purchased from Sigma Chemical Co. and used directly. Glycerol and glucose were reagent grade. The fatty acid substrates used for this study were certified 99% pure by the distributors.

Unlabelled fatty acids were obtained from Hormel Foundation; palmitic acid-1-C^{14} and myristic acid-1-C^{14} from Nuclear-Chicago Corp. and stearic acid-1-C^{14}, oleic acid-1-C^{14} and linoleic acid-1-C^{14} from Volk Radiochemical Corp. Concentrated aqueous stock solutions of the potassium salts were made up by adding about 1/3 excess KOH to the free acid and stored in the deep freeze to minimize decomposition. Deionized water redistilled in glass was used throughout the study.

Bovine albumin fraction V was obtained from Brickman and Co. Montreal and used directly. Human serum albumin (Connaught Med. Res. Lab. Toronto) was defatted according to the method previously described (69) dissolved in water and adjusted to pH 7.0 with NaOH.

B) ENZYME PREPARATION AND ANALYSIS

Male wistar rats (200-260 G) were killed by a blow to head and subsequent manipulations carried out in a cold room at 4°C. For each experiment epididymal pads of three rats were pooled and gently homogenized with 0.15M KCl 1:4 weight to volume in a Potter-Elvehjem type glass homogenizer by a motor driven tephlon pestle. The homogenate was centrifuged for 12 minutes at 700xG in a refrigerated centrifuge at 4°C. As shown in Fig. IV three distinct layers separated: (1) the bulk fat remained as a semisolid supernatant; (2) an intermediate, slightly pink, opalescent aqueous layer; and (3) red blood cells nuclei and dense stroma gravitated to the bottom as a pellet. Without disrupting the bulk fat, the intermediate layer was aspirated into a chilled syringe through a long 18 gauge needle. This fraction served as the enzyme preparation and was assayed within 5 minutes of its isolation.

The triglyceride content of the enzyme preparation was determine by the method of Van Handel and Zilvershmitt (165) and the protein content was determined by the standard micro Kjeldahl technique (163).

The reaction mixture, consisting of 1 or 2cc enzyme preparation plus various cofactors and substrates was made up to a final volume of 3cc with water in 25 ml conical flasks and incubated in a Dubnoff metabolic shaker with air as the gas phase.

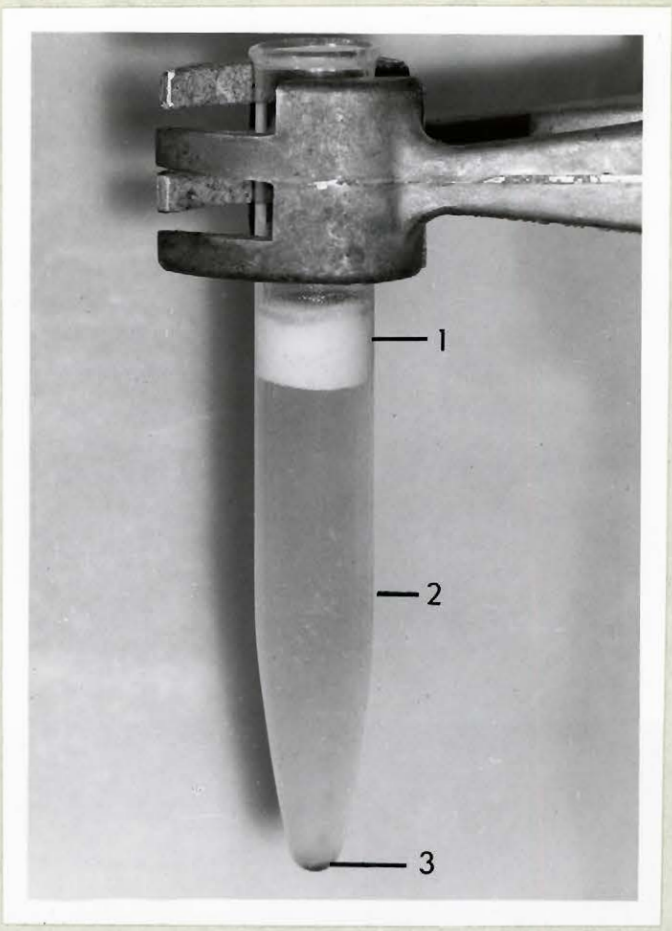

FIGURE IV

Three distinct layers are seen following centrifugation (700xgx12 min. at 4°C) of the 4:1 (.15M KCl:GM Tissue) homogenate of rat epididymal fat. (1) Bulk fat. (2) Aqueous supernatant which served as the enzyme preparation and (3) Pellet containing RBC, fibrous debris and nuclei.

C) ELECTRON MICROSCOPY

Freshly prepared defatted homogenate was fixed in 1% osmium tetroxide buffered to pH 7.4 in isotonic veronal acetate solution (186) and the percipitate isolated by centrifugation. Following dehydration in graded alcohols, the precipitate was embedded in Epon 812 (187). This sections were cut with a glass knife on a Porter-Blum microtome, mounted on carbon coated formvar films, stained with lead hydroxide (188) and examined in an RCA EMU 3E electron microscope.

D) LIPID EXTRACTION

Following incubation the entire content of each flask was extracted with 15 ml isopropyl alcohol: heptane: water (H_2SO_4) according to the method of Dole and Mienertz (42) in a 120 ml separatory funnel. With the further addition of heptane and water this system resolves into an upper "heptane" phase containing virtually all the triglycerides, diglycerides, free cholesterol and cholesterol ester (161) and over 90% of the FFA, and a lower aqueous isopropyl alcohol phase containing most of the phospholipid and about 10% of the FFA. The lower phase was removed and the upper phase remaining in the separatory funnel was washed twice with an equal volume of alkaline ethanol according to the method of Bergström (18) to extract all the FFA. The radioactivity present in the washed heptane phase was taken to represent the esterification of isotopically labelled FFA in glyceride esters. The

free fatty acids could be recovered from the alkaline ethanol by acidifying and extracting with 2 volumes of petroleum ether (BP 30-60°) and the FFA remaining in the original lower phase was recovered by extraction with pure solvents upper phase (isoproponal: heptane: water (.03N H_2SO_4) (36:36:28) which was then pooled with the petroleum ether wash of the acidified ethanol.

E) SILICIC ACID CHROMATOGRAPHY

Fractionation of lipid mixtures into their component classes was accomplished by silicic acid column chromatography on a semi micro scale. Reagent grade silicic acid (Mallenkrodt #100 mesh) was activated overnight at 100°C and placed in 10cc syringe barrels. A photograph of the column arrangement is shown in Fig. V. Two grams silicic acid was tapped into the plugged column containing about 5cc pet ether and agitated with a spatula to remove trapped air bubbles. A small wad of glass wool supported the silicic acid and prevented its loss. Following a preliminary wash with 30cc petroleum ether the lipid to be fractionated was added in amounts not exceeding 15 mg lipid per gram silicic acid in a small volume of petroleum ether. Eluting solvents were redistilled in glass prior to use and applied to the columns in reservoirs. The apparatus was set up in a fume chamber at room temperature and positioned so that the eluates could be collected in beakers resting on a heated sand bath. The fume chamber window was kept partially open so that a continuous flow of air was drawn across the open face of the collection beakers providing simultaneous collection and

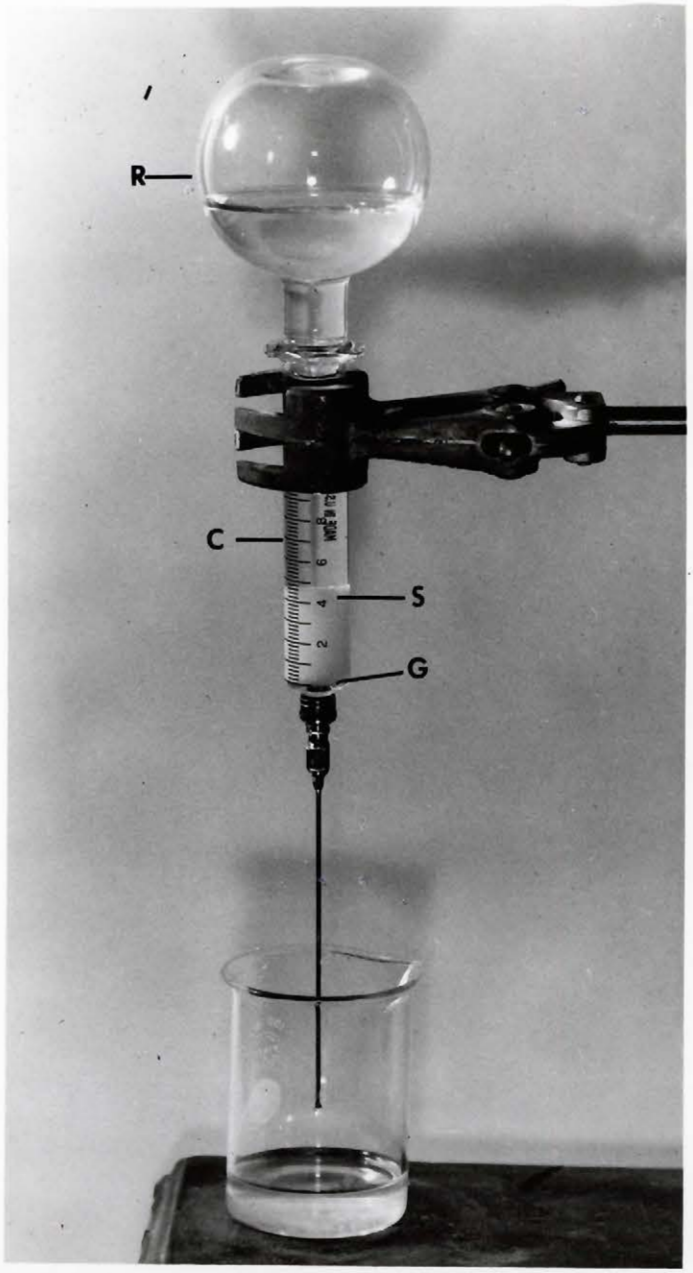

FIGURE V

The semi micro silicic acid column arrangement. R-reservoir containing the eluting solvent, C-10cc syringe barrel which served as a column, S-2 gm silicic acid, G-glass wool plug to prevent loss of adsorbant.

evaporation of solvents without boiling. The step-wise elution scheme selected, resolved the complex lipid mixture into four fractions as follows.

FRACTION	SOLVENT	VOL.	LIPID CLASS
I	1% V/V ETHYL ETHER in Pet. ETHER (BP 30-60°C)	75cc	CHOLESTEROL ESTERS
II	4% "	200cc	TRIGLYCERIDE
	8% "	200cc	FREE CHOLESTEROL
III	100% ETHYL ETHER	100cc	DIGLYCERIDE MONOGLYCERIDE
IV	100% METHANOL	100cc	PHOSPHOLIPIDS

Thin layer chromatography on silicic acid coated glass plates was carried out according to the method of Mangold (107).

Free fatty acids were titrated by the method of Dole and Mienertz (191).

F) DETERMINATION OF RADIOACTIVITY

The various extracts or fractions isolated from the silicic acid columns were evaporated in 20 ml counting vials after which 5 ml scintillation mixture consisting of 0.6% 2,5-Diphenyloxazole (PPO) and 0.02% P-BIS 2-(5-Phenyloxazolyl) Benzene (POPOP) in toluene was added. Radioactivity was determined in a Packard Tricarb liquid scintillation spectrometer at 5°C. Counting efficiency with standard C^{14} toluene was 37%. Corrections for quenching and self-absorption were not necessary.

PART III RESULTS

A) ENZYME PREPARATION

(i) Electron Microscopy

Representative electron photomicrographs illustrating the structural components of the enzyme preparation is shown in Fig. VI and VII. Two distinct types of organelles are apparent. Firstly, a loose array of shapeless membrane components (E) predominate and probably represents disrupted endoplasmic reticulum. The second type of organelle is vesicular (V) in conformation bounded by an intact electron-dense membrane. The dark material within some of the vesicles is osmium fixed lipid. Noteworthy, is the apparent absence of free structurless lipid. Mitochondria could not be identified in the sections studied. This may be the effect of homogenization in salt solution. Schneider (141) has pointed out that in salt solution mitochondria are strongly agglutenated and consequently sediment with the nuclei. Further studies on the pellet are required to prove this point.

The enzyme preparation was free of intact adipose tissue cells, red blood cells and nuclei.

(ii) Protein, FFA, and Triglyceride Content

Representative analysis of the enzyme preparation for protein, FFA, and triglyceride are shown in Table 1. One cc of the enzyme preparation corresponds to about 250 mg intact tissue. The preparative

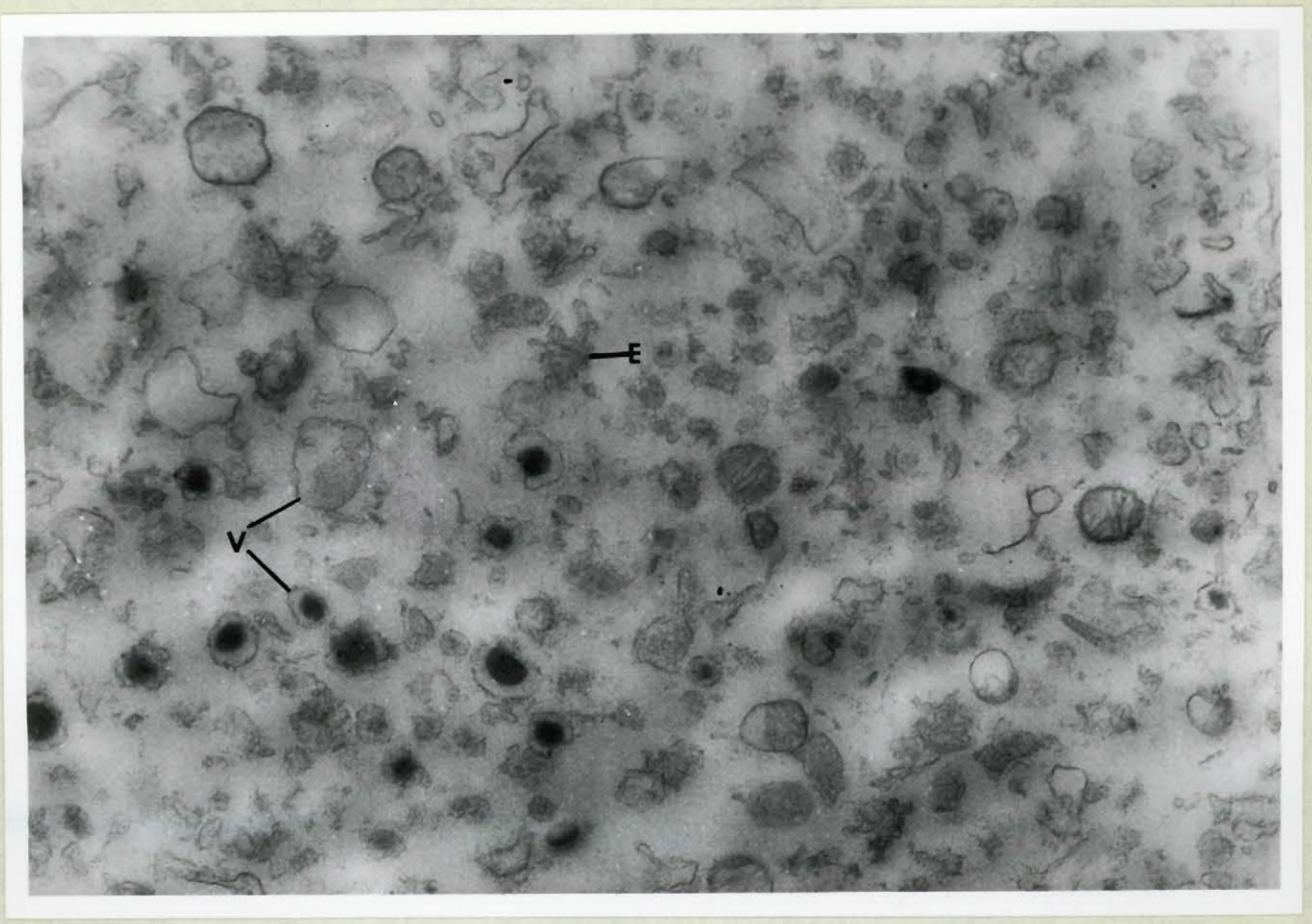

FIGURE VI ELECTRON PHOTOMICROGRAPH I

Osmium tetroxide fixed enzyme preparation. The organelles are of two main types. Endoplasmic reticulum-E and vesicles-V. Some vesicles contain dark staining lipid. Notice the absence of intact cells, RBC, nuclei, and mitochondria. Magnification x 15,095.

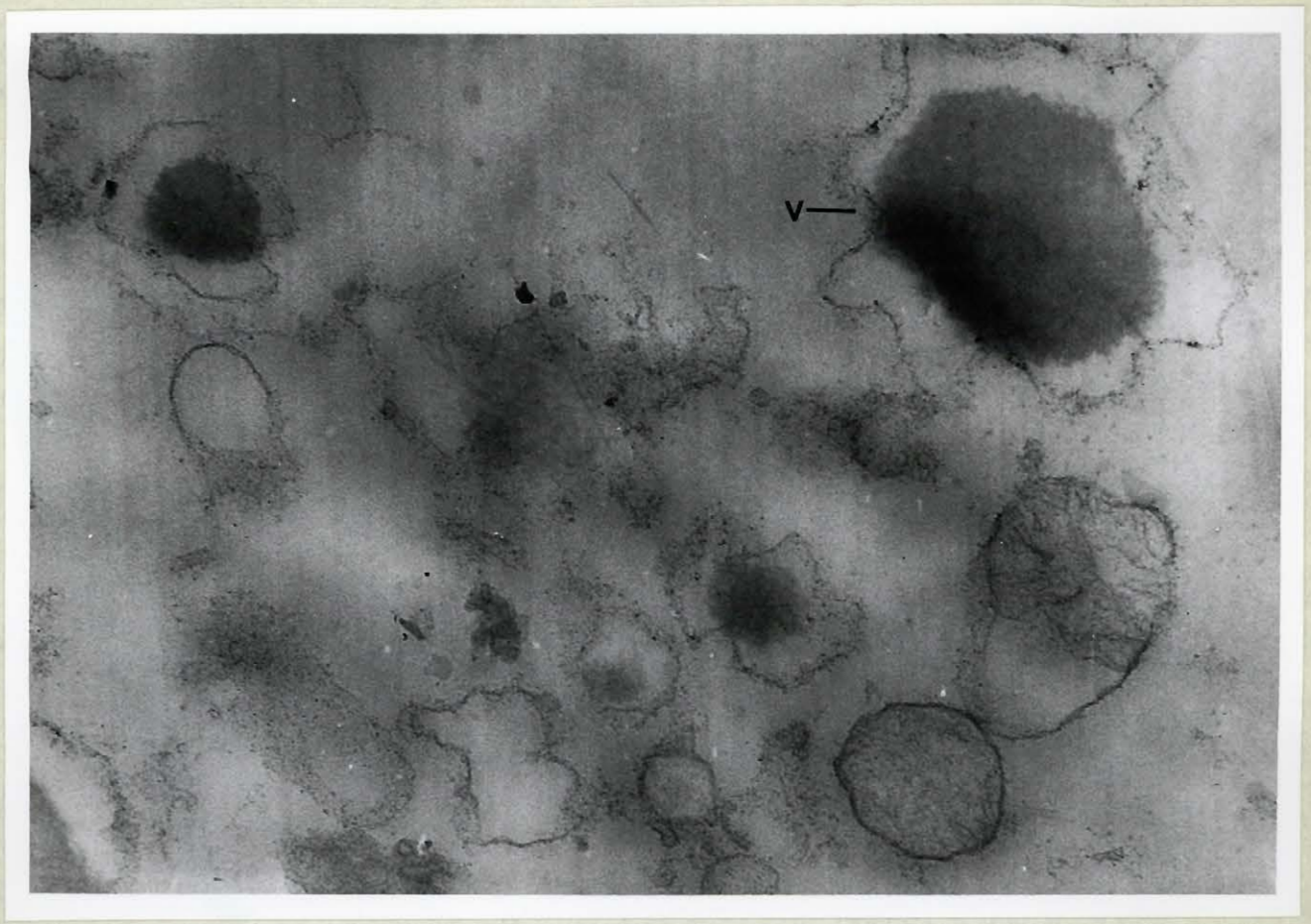

FIGURE VII ELECTRON PHOTOMICROGRAPH II

Higher power view of lipid containing vesicles showing intact membrane structure surrounding the lipid droplet. No structureless lipid was observed indicating adequate isolation methods. Magnification x 28,972.

TABLE I

ENZYME PREPARATION

PROTEIN (mg/cc)	FFA (μM/cc)	TG (μM/cc)
1.21	.274	.215
1.32	.156	.209
1.22	.226	
1.60	.335	

Chemical analysis of enzyme preparation. FFA - free fatty acid. TG - triglyceride.

procedure effectively removed, most of the bulk fat as less than 0.1% of the tissue triglyceride remained in the extract. This very small amount of triglyceride is not unexpected in view of the electron microscopic studies and may represent structural (vesicular) triglyceride. The FFA content of the extract was comparatively large and represents 40% of the pre existing tissue FFA if it assumed that no hydrolysis of triglyceride occurred during homogenization.

The protein content of 18 enzyme preparations was found to be quite constant: 1.38$\pm$ .24 mg/cc (mean $\pm$S.D.). The varience in potency of different enzyme preparations was largely eliminated when velocity was expressed in terms of protein content.

B) FFA ESTERIFICATION

In this section the results of the studies carried out to establish the optimum condition for fatty acid esterification by the cell free preparation of adipose are presented. For the initial experiments 2cc enzyme preparation was added to flasks containing various cofactors and substrates in amounts shown in Table II. The entire incubation mixture is designated the complete system. Zero time was taken upon the addition of enzyme and following 20 minutes incubation at 37^{o}C the reaction was stopped by adding solvents for lipid extraction.

(i) Cofactor Dependance

Very rapid and almost complete (92%) incorporation of C^{14} palmitic acid into glyceride esters was found within 20 minutes

TABLE II

COFACTORS

ATP	10. μM
CoA	0.25 μM
GSH	2.5 μM
NaF	20.0 μM
Mg^{++} ($MgCl_2$)	3.0 μM
K phosphate buffer pH 7.0	140. μM

SUBSTRATES

DL -α GP	10. μM
Palmitic acid-1-C^{14}	0.25 μM (10,500 CPM)

The amount of each cofactor and substrate to which 2cc of the enzyme preparation was added. A final volume of 3cc was made up with deionized distilled water. The entire incubate is designated the complete system.

incubation of the complete system (Table III). In the absence of added ATP, esterification of isotopic fatty acid was reduced to 1.4% of the total. The obligatory ATP requirement for triglyceride synthesis in this system differentiates it from a lipase catalysed exchange phenomenon. Excluding coenzyme A from the reaction mixture decreased incorporation to 9.4% suggesting the presence of small amounts of endogenous CoASH in the enzyme preparation. Fluoride was included as a cofactor because of its ability to inhibit ATP ase. A very slight reduction in esterification results in the fluoride deficient system. Reduced glutathione was arbitrarily added as a preservative for CoASH.

Boiling the enzyme preparation for 5 minutes completely abolished the esterifying capacity undoubtedly due to enzyme denaturation. In the absence of added enzyme only trace amounts of radioactivity was found in the washed heptane phase of the lipid extract demonstrating the absence of spontaneous condensation of fatty acid with α glycerophosphate.

(ii) Time Course of Esterification

For the time course study a series of flasks each containing the complete system were incubated from 1-20 minutes at 37°C. The results are graphically illustrated in the upper curve of Fig. VIII where percent of total C^{14} palmitic acid esterified is plotted as a function of time. Immediately apparent is the rapidity and degree to which esterification takes place. Within 5 minutes over 70% of the isotope is esterified and by 10 minutes the reaction is almost complete.

TABLE III

COFACTOR REQUIREMENTS

	PALMITIC ACID-1-C^{14} ESTERIFIED % of TOTAL
Complete System	92%
" Minus ATP	1.4%
" Minus CoASH	9.4%
" Minus NaF	87.0%
" Minus Enzyme	< 1 %
" Boiled Enzyme	< 1 %

The effect on esterification of omitting various cofactors is shown. The complete system is described in Table II. Boiled enzyme - the enzyme preparation was immersed in a boiling water bath for 5 min. prior to addition. The fraction of the total radioactivity in the ethanol washed heptane phase represents percent C^{14} palmitic acid esterified. Incubation at 37°C for 20 min.

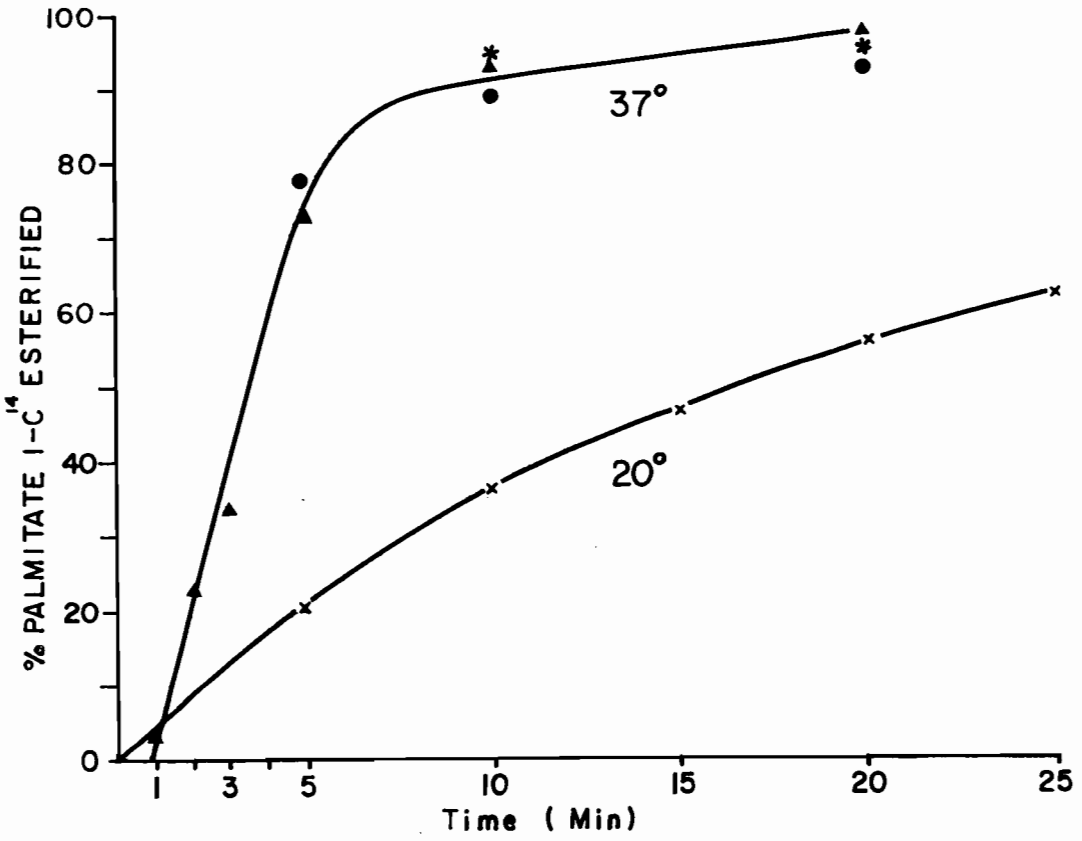

FIGURE VIII

Time course and the effect of temperature on FFA esterification. The complete system as described in the legend to Table II was incubated for the time indicated by the symbols at 37°C (▲ ● * separate experiments) and at 20°C.-×. Incubation at 20°C reduced velocity of esterification to about 1/5 that at 37°C. and therefore easily monitored.

Day to day reproducibility was obtained as good agreement in results of three such experiments was observed. The rapid componant of the curve does not pass through origin. This lag is probably a temperature effect as the enzyme preparation was stored at $4^{o}C$ prior to use. In order to characterize the kinetic events accurate measurement of initial velocities is mandatory. The rapid velocity of esterification observed would require incubation periods of 1 to 2 minutes for this purpose. Apart from the technical difficulties considerable error would result due to the lag phase. Attempts to decrease the velocity of esterification by reducing the enzyme concentration resulted in a marked inhibitory effect which could not be explained. An alternative method employing the well known effect of temperature on an enzyme catalyzed reaction was used to control the esterification rate.

(iii) Effect of Reduced Temperature on Esterification

Incubation of the complete system at $20^{o}C$ resulted in a reduction in the velocity of esterification to about 1/5 that at $37^{o}C$. The lower curve in Fig. VIII depicts the time course of C^{14} palmitic acid esterification at $20^{o}C$ and has the appearance of a first order type reaction. Extrapolation of the slope projects the curve through the origin indicating the elimination of a significant lag phase in the reaction.

Thus by reducing the temperature of incubation the rate of esterification was reduced sufficiently to enable accurate measurement of initial velocities. Subsequent experiments designed to characterize

the kinetic of the reaction were therefore carried out at 20°C.

(iv) Distribution of Radioactivity: Silicic Acid Chromatography

Aliquots of the ethanol washed lipid extracts obtained from the time course experiments (Fig. VIII) were chromatographed on silicic acid columns to determine the distribution of label in the various lipid componants. The results of these fractionations are shown in Figs. IX and X. Most of the radioactivity was found in the triglycerides in all the specimens analysed. At 37°C about 95% of the radioactivity was present in triglyceride and 4.5% was in the diglyceride fraction after 10 minutes incubation (Fig. IX). The corresponding distribution at 20°C and 25 minutes incubation was 89% and 10% (Fig. X). Less than 1% of the activity was found in the cholesterol ester and phospholipid fractions (not shown) at these times. Greater amounts of radioactivity were found in the cholesterol ester fraction after short periods of incubation. (3 and 5 minute 37°, and 5 minute 20°C). The radioactive moiety in this fraction has not been conclusively identified. Preliminary observations by thin layer chromatography on silicic acid coated plates revealed a radioactive componant with Rf close to but less than cholesterol esters.

The fractional distribution of radioactivity between tri- and di-glycerides at 20°C (Fig. X) over the 20 minute period lookslike a horizontal expansion of the initial 5 minutes of 37° experiment (Fig. IX).

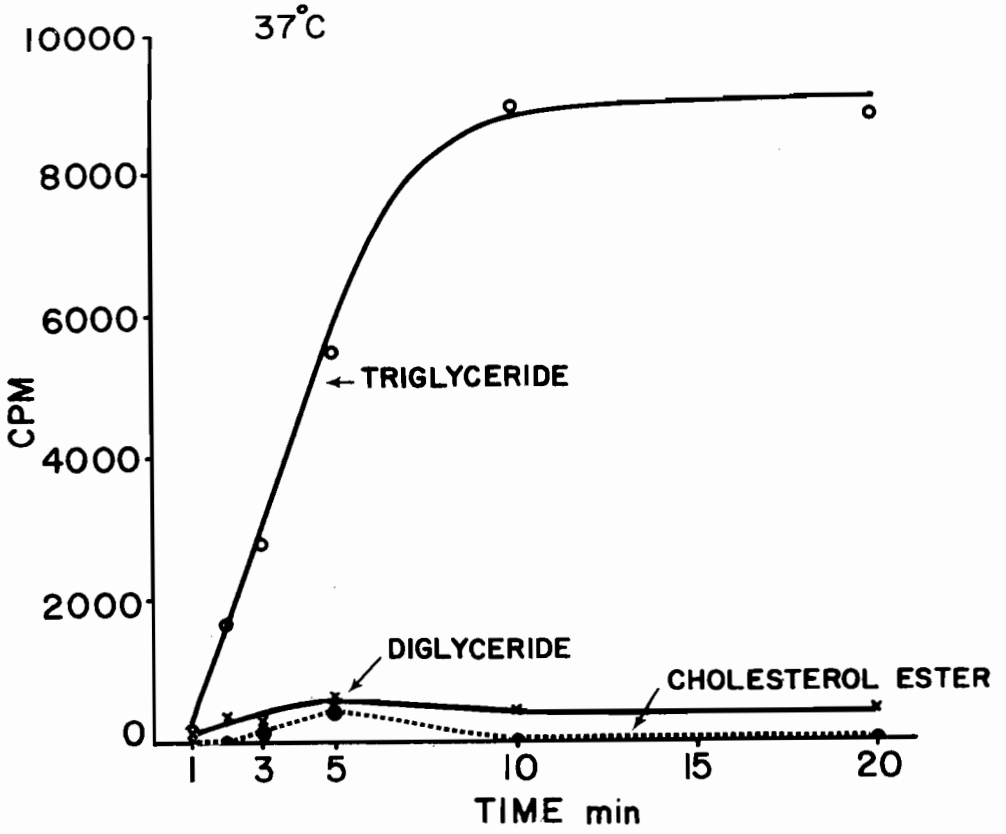

FIGURE IX

Distribution of radioactivity in washed heptane phase of time course experiment (Fig. VIII ▲) at 37°C as determined by silicic acid column chromatography. Less than 1% of the radioactivity was found in the phospholipid fraction and is not plotted.

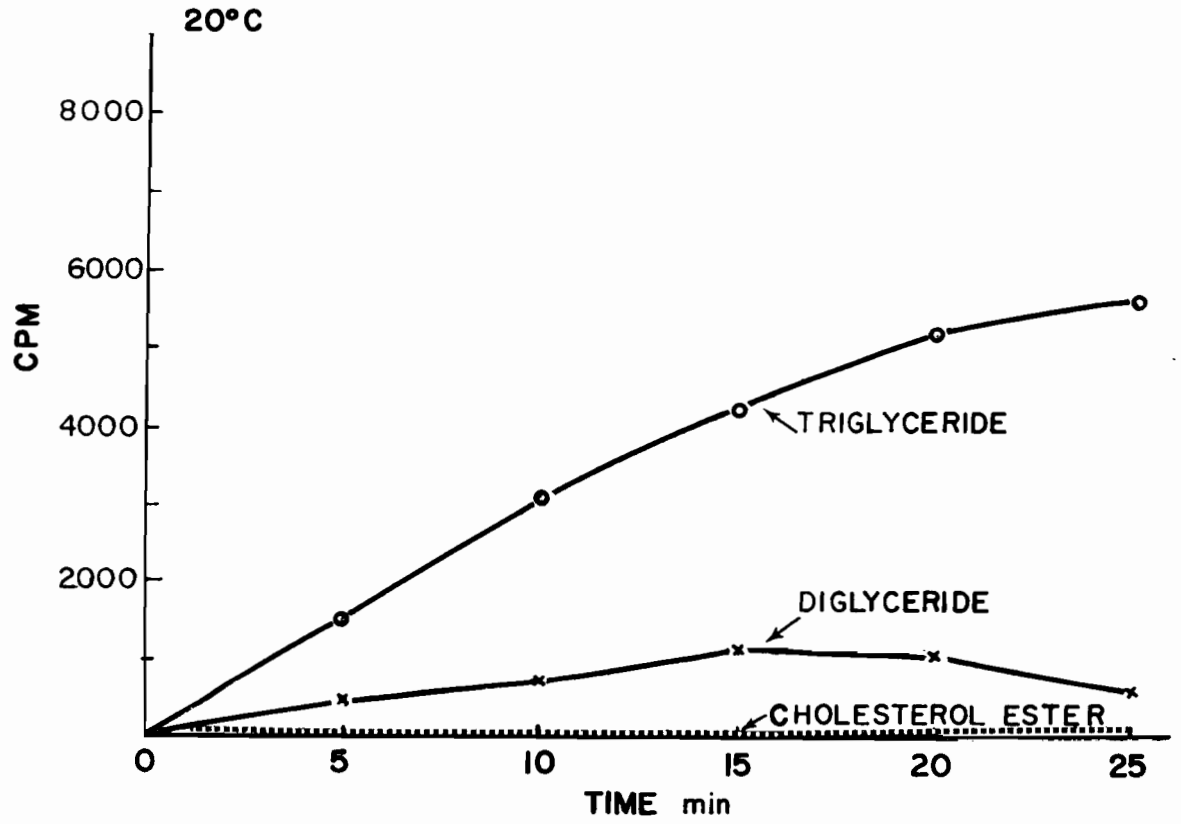

FIGURE X

Distribution of radioactivity in the washed heptane phase of time course experiment (Fig. VIII ×) at 20°C as determined by silicic acid column chromatography. Less than 1% of the radioactivity was found in the phospholipid fraction and is not plotted.

These studies demonstrated that over 98% of the radioactivity in the washed heptane phase was present as neutral esters of which about 90% is triglyceride and the remainder diglyceride and that a reduction in termperature of incubation did not significantly alter the quality or distribution of synthesized products.

(v) Enzyme Product Relationship

Characteristic of most enzyme catalyzed reactions is the linear relationship between enzyme concentration and product of the reaction or velocity of reaction. Experiments were carried out to determine whether this relationship obtained with the enzyme preparation under study. For this purpose the complete system was modified only with respect to enzyme content. Into four separate flasks 0.5, 1.0, 1.5, and 2.0 cc of enzyme preparation was added which corresponds to 0.6, 1.2, 1.8 and 2.4 mg protein respectively. The final volume of 3 cc was made up by the addition of 0.15 M KCL. Following a twenty minute incubation period at 20°C the contents of each flask was extracted and processed in the usual manner.

The results of the experiment shown in Fig. XI have been plotted for palmitic acid-1-C^{14} esterified (μM/hr) bottom curve, total palmitic acid esterified (μM/hr) middle curve and total FFA esterified (μM/hr) top curve. As the S.A. of the added palimitic acid is known the amount and velocity of palmitic acid-1-C^{14} esterification was determined. The velocity of palmitic acid-1-C^{14} esterification with respect to enzyme

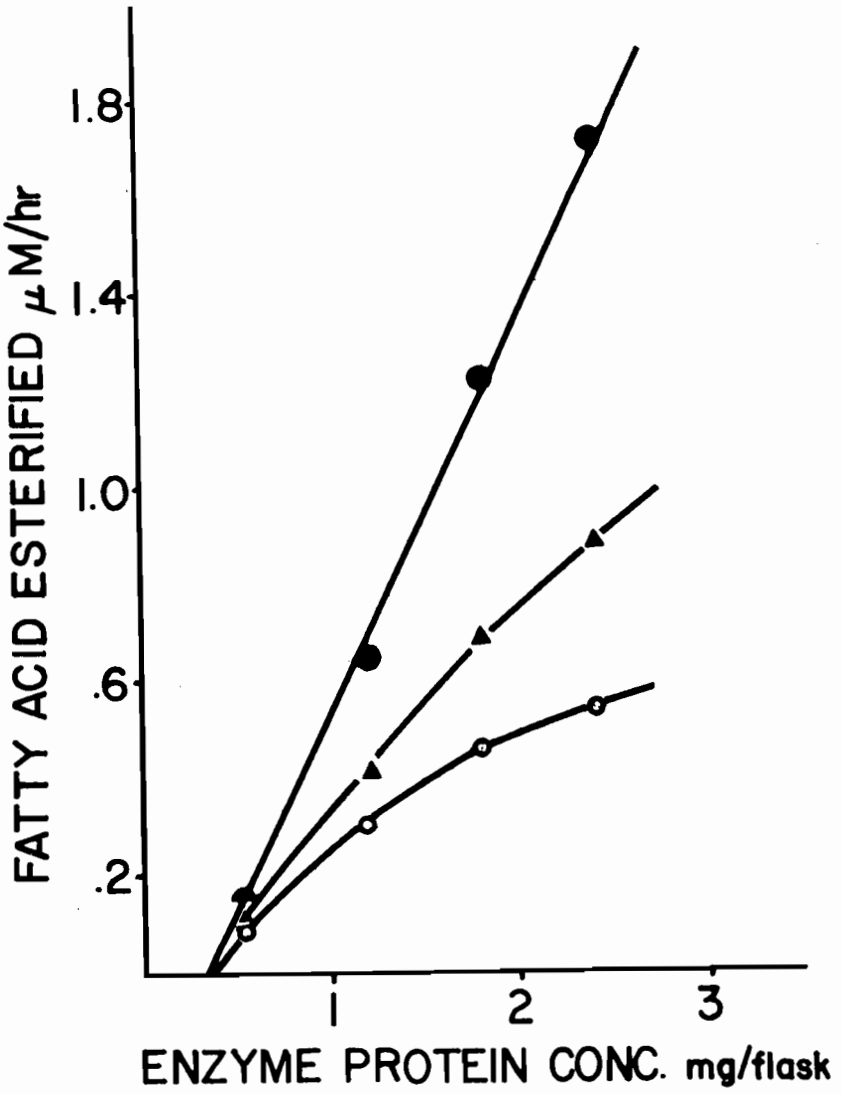

FIGURE XI

The relationship between enzyme concentration and velocity of esterification. Four flasks containing the complete system as described in legend of Table II was modified with respect to enzyme content. 0.6, 1.2, 1.8 and 2.4 mg. protein corresponds to .5, 1.0, 1.5, and 2cc enzyme preparation respectively. Incubation for 20 min. at 20°C. Results analyzed in terms of palmitic acid-1-C^{14} esterified -o-o-, palmitic acid esterified (endogenous + added)—▲-▲— , and total FFA esterified (endogenous + added)-●-●- . See text for method of calculation.

protein concentration is non linear (Fig. XI bottom curve) and is understandable when it is realized that increasing amounts of enzyme necessarily increases the concentration of non-radioactive endogenous FFA (.274 μM/cc enzyme preparation) thereby diluting the specific activity of the added C^{14} palmitic acid. Assuming that 30% of the endogenous FFA is palmitic acid, the SA of total palmitic acid in each flask was calculated as follows:

$$\text{SA (Total palmitic acid)} = \frac{\text{CPM added } C^{14} \text{ palmitic Acid}}{\text{ENDOGENOUS palmitic acid + ADDED } C^{14} \text{ Palmitic acid}} \quad (7)$$

and the velocity of total palmitic acid esterified could be determined by the following equation

$$\underline{\text{Velocity of Glyceride Synthesis (CPM/hr)}} = \frac{\text{Velocity of palmitic acid esterified}}{\text{SA of total palmitic acid}} \quad (8)$$

The velocity of total palmitic acid esterified with respect to enzyme concentration is also not quite linear (Fig. XI middle curve). This suggests that the non palmitic endogenous FFA which is present in proportionately higher concentration than palmitic acid in each successive flask may compete with palmitic acid in the esterifying reaction.

When the specific activity of the total free fatty acid pool (endogenous + added) was calculated and the velocity of the total FFA esterified compared to enzyme concentration the relationship was found to be linear (Fig. XI top curve). This would suggest that added isotope and

the endogenous FFA pool were handled in a quantitatively similar fashion. Evidence confirming this suggestion was obtained in subsequent studies in which the specific activity of the total free fatty acids did not change significantly during continued synthesis of glyceride esters. (Table VI).

It is of interest to note that reduction of enzyme concentration at 20°C did not result in an inhibition of esterification noted in previous experiments carried out at 37°C. In later experiments 1cc of enzyme preparation was used for each incubate in order to conserve the material for multiple analysis.

(vi) Optimum Cofactor Concentrations

At this point it was necessary to establish optimum conditions for esterification with regard to certain of the cofactor. With the complete system incubated at 20°C the effect of double concentration of various cofactors on esterification was examined. Listed in table IV are the results obtained when twice the usual amounts of ATP, CoA, GSH, and NaF were added. Little effect was noticed with twice the concentration of CoA, GSH, and NaF indicating that optimal concentrations were being used. A striking inhibitory effect resulted with the increase in ATP content. The mechanism whereby excess ATP is inhibitory may be due to complex formation with divalent cations especially magnesium (130,189). The possibility that $[ATP] : [Mg^{++}]$ of the complete system was sub-obtimal prompted a more detailed analysis of its influence on fatty acid activation as reflected by glyceride ester synthesis.

TABLE IV

EFFECT OF INCREASED COFACTOR CONCENTRATION

	Glyceride Esters % of Complete System
Complete System	100
" + 10. μM ATP	64
" + 0.25 μM CoA	96
" + 2.5 μM GSH	94
" + 20. μM NaF	98

Effect of double concentration of various cofactors on esterification. The complete system is described in Table II. The results are expressed as a percentage of the complete system which is taken to represent 100%. Incubation at 37°C for 20 min.

The effect of ATP and Mg^{++} on the velocity of fatty acid esterification is shown in Fig. XII A and B. In this study parallel incubations with the same enzyme preparations were used. In one group of flasks, the ATP concentration was varied and Mg^{++} was maintained at 1mM/L (Fig. XII A); in the second series of flasks the Mg^{++} concentration was varied and ATP concentration fixed at 3.3mM/L (Fig. XII B). Appropriate corrections for endogenous FFA were made and velocity is expressed in terms of total fatty acid esterified. The obligatory requirement of ATP for this reaction and the inhibitory effect of 5mM/L ATP are clearly evident. The optimal ATP concentration was between 1.5-3mM/L in the presence of 1mM/L Mg^{++}. The optimum Mg^{++} concentration was 2mM/L in the presence of 3.3mM/L ATP (Fig. XII B) and corresponds to the conditions where velocity of esterification was greatest. Similar experiments with several enzyme preparations gave identical results.

(vii) Effect of Substrate Concentration: Km FFA and Km αGP

The experiments thus far detailed, have defined the optimal physical and cofactor milieu whereby a subcellular enzyme preparation of rat adipose tissue esterifies added FFA and αGP at a velocity that is easily monitored. For these experiments αGP and C^{14} FFA concentrations had been kept constant at 3.3mM/L and 0.83mM/L respectively thus limiting interpretation of the velocity measurements. In order to obtain the necessary measurements for kinetic analysis the effect of varying substrate concentrations on the initial velocity of esterification were determined.

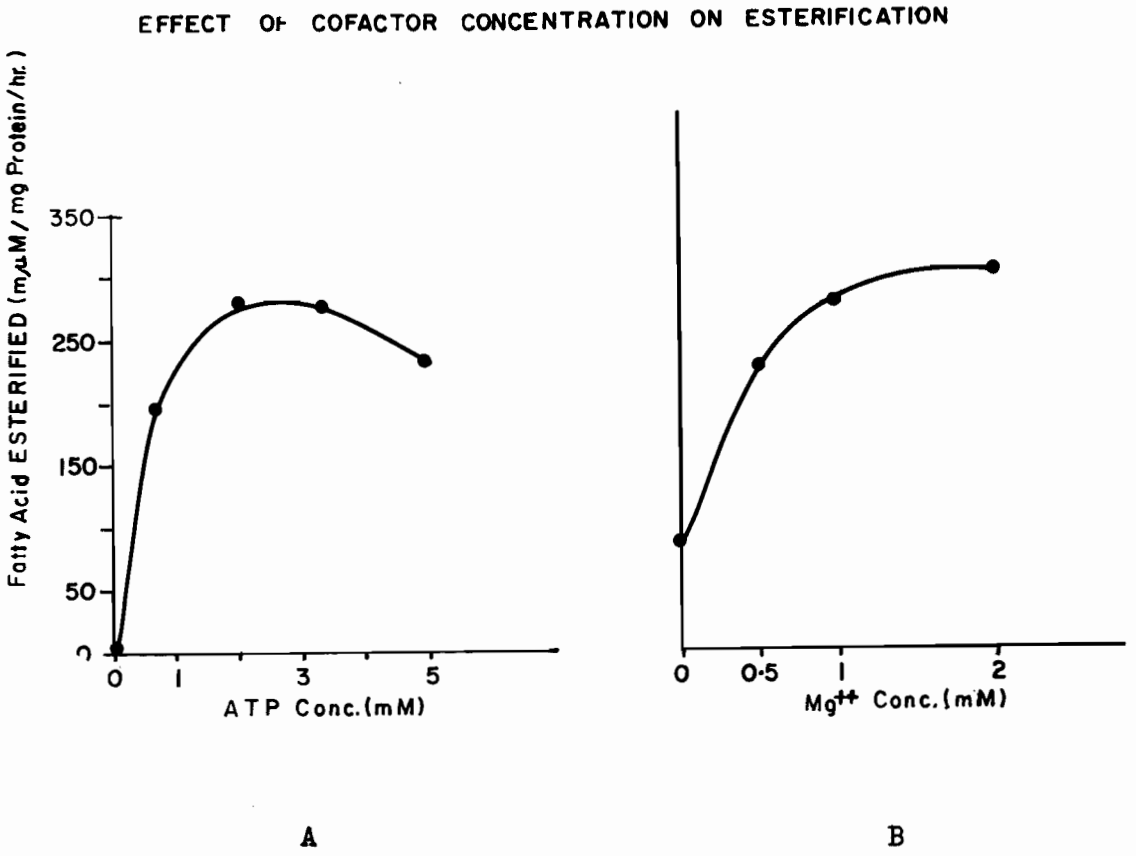

FIGURE XII

Effect of cofactor concentration on esterification. The complete system as described in Table II was modified in that 1cc enzyme preparation was used and final volume of 3cc made up with .15 M KCL. Parallel experiments with the same enzyme preparation where A. [ATP] varied and [Mg++] constant at 1 mM/L. B. [Mg++] varied and [ATP] constant at 3.3 mM/L. Twenty minutes incubation at 20°C. Corrections for endogenous FFA were made and velocity expressed as total FFA esterified/mg protein/hr. Optimum [ATP] : [Mg++] was found to be 3.3:2.

For these experiments the incubation system consisted of lcc enzyme preparation plus the optimum cofactor requirements as described in the legend to Fig XIII. Initial velocity of esterification was determined after 15 minutes incubation at 20°C. The effect of ∝ GP concentration on initial velocity of esterification was determined by adding increasing amounts of the substrate to a series of flasks in which the palmitic acid-1-C^{14} concentration was fixed. Corrections for endogenous FFA were made and the velocity was expressed as mμM free fatty acid esterified/mg enzyme protein/hr.

The relationship between ∝GP concentration and initial velocity of esterification was found to be typical of an enzyme catalyzed reaction (Fig. XIII). With lower concentrations of substrate a linear relationship to velocity is seen followed by a plateau effect at ∝GP concentrations above 3.3 mM/L and maximum velocity of esterification was obtained with 6.7 mM/L∝GP. These data were analyzed by the method of Michaelis and Menten (114) and the K_m of the overall reaction was 1.9×10^{-3}M GP (pH 7.0, 20°C). A similar experiment was carried out to determine the effect of palmitic acid concentration on initial velocity of esterification. Conditions and cofactors were identical but now increasing concentrations of C^{14} palmitic acid were added and ∝GP concentration was kept constant at 6.7mM/L.

The initial concentration of palmitic acid was calculated by summing the amount added and the amount present as endogenous free palmitic acid. The endogenous free palmitic acid was assumed to represent

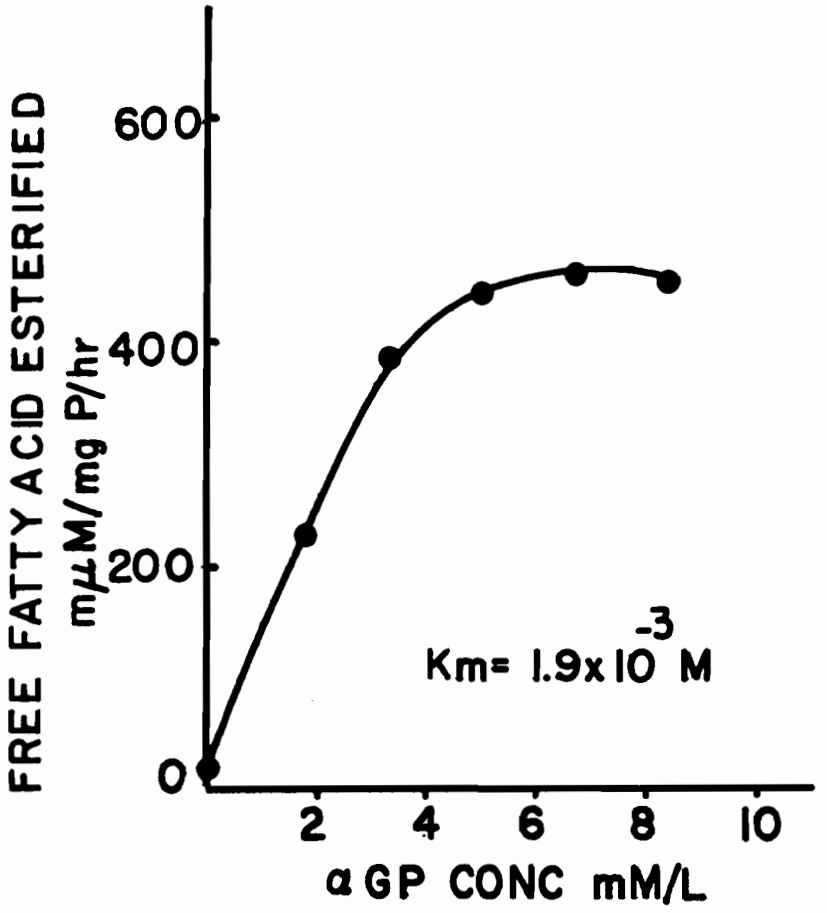

FIGURE XIII

Effect of substrate (α GP) concentration on initial velocity of esterification. The incubation mixture consisted of 1cc enzyme preparation plus 1cc of the optimal cofactor system: ATP 10 μM, CoASH 0.25 μM, $MgCl_2$, 6.0 μM, NaF 20.0 μM, GSH 2.5 μM, and buffered with K phosphate 140 μM pH 7.0. Final volume of 3cc made up with 0.15 M KCl. Incubated for 15 min. at 20°C. palmitic acid-1-C^{14} concentration was kept constant at 250 mμM (SA .05 mc/mM) / Flask and DL-α GP concentration varied as shown. Km = 1.9×10^{-3} M α GP (20°C, pH 7.0) after Michaelis and Menten (114).

30% of the total endogenous FFA concentration. The SA of the total palmitic acid and the initial velocities of palmitic acid esterified was calculated as previously described (equations 7 and 8).

The results in Fig. XIV indicate that increasing concentrations of palmitic acid increases the initial velocity of esterification. A reciprocal plot by the method of Lineweaver and Burk (99) is shown in Fig. XV. The line of best fit was calculated and the K_m was found to be 1.68×10^{-4}M palmitic acid (20°C, pH 7.0).

(viii) Kinetic Considerations. Maximum Velocity

Conventionally, the kinetics of an enzyme catalyzed reaction is described by the K_m constant which is defined as the substrate concentration at one half the maximum velocity. The enzyme system under study requires two substrates (FFA and GP) for esterification, each of which can independantly affect the velocity. Therefore the entire reaction for palmitic acid esterification into glycerides is embodied in the K_m palmitic acid and K_m GP together. Enzyme catalyzed reactions requiring two substrates for one product are defined by the following equation which relates velocity to K_m and substrate concentration (145).

$$\frac{V\ max}{v_o} = \left[1 + \frac{K_m S_1}{[S_1]}\right]\left[1 + \frac{K_m S_2}{[S_2]}\right] \qquad (9)$$

Where $V\ max$ = Maximum velocity

v_o = Initial velocity observed

$K_m S_1$ = K_m for palmitic acid

$K_m S_2$ = K_m for ∝GP

$[S_1]$ = Palmitic acid concentration at v_o

$[S_2]$ = ∝GP concentration at v_o

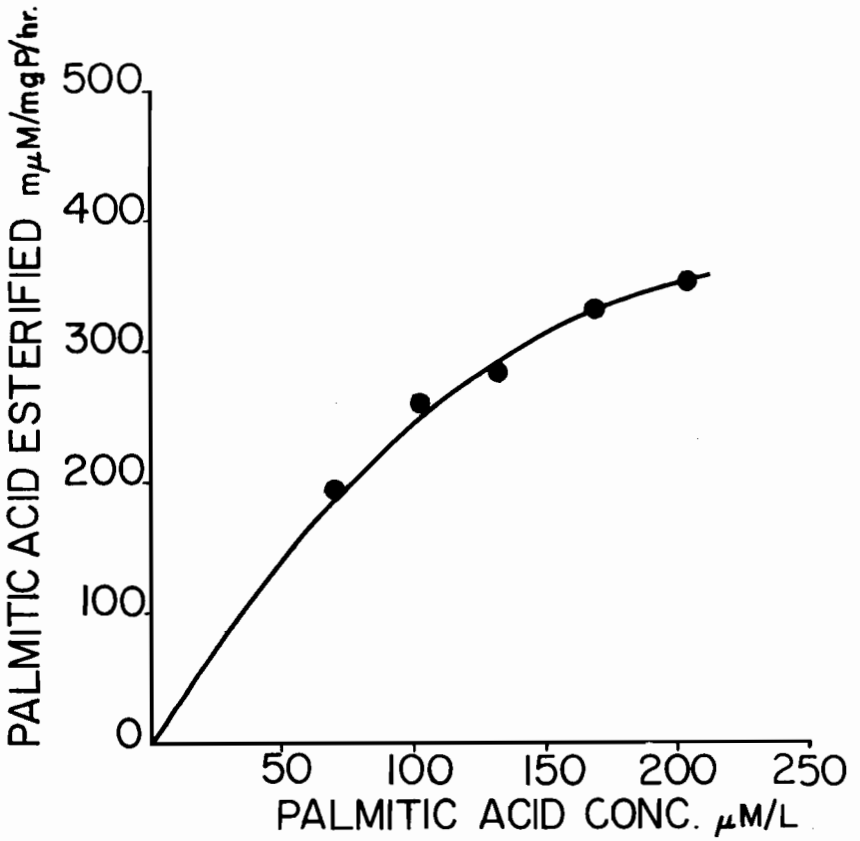

FIGURE XIV

Effect of substrate (palmitic acid) concentration on initial velocity of esterification. Incubation mixture and conditions as detailed in legend of figure XIII. DLαGP 6.7 mM/L in each flask and palmitic acid-1-C^{14} concentration varied. Palmitic acid concentration is the sum of endogenous and added. Initial velocity of total palmitic acid esterified with respect to total palmitic acid concentration is shown.

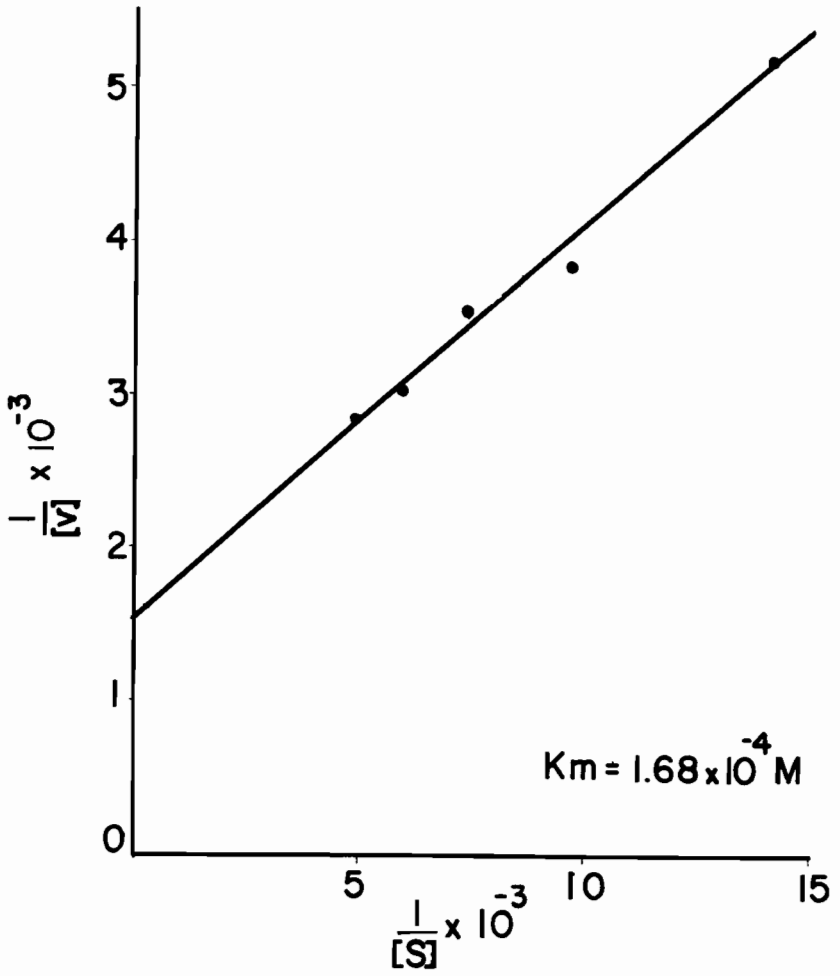

FIGURE XV

Lineweaver and Burke analysis of data in Fig. XIV relating the effect of palmitic acid concentration on initial velocity of esterification. [V] = initial velocity of esterification, [S] = substrate concentration. Km = 1.68 x 10^{-4}M palmitic acid (20°C pH 7.0).

Substituting known values for v_o, K_mS_1, K_mS_2, $[S_1]$, and $[S_2]$, the maximum velocity of palmitic acid esterification was calculated to be 809 mμM/mg enzyme protein/hr. (pH 7.0, 20°C). This value multiplied by 5 corrects for the temperature effect giving a theoretical V max for palmitic acid esterification of about 4 μm/mg enzyme protein/hr (pH 7.0, 37°C).

C) LIPOLYTIC ACTIVITY OF ENZYME PREPARATION

The subcellular enzyme preparation contains considerable amounts of esterified fatty acids in the form of triglyceride (Table 1) and phospholipid. Significant lipolytic activity during incubation could result in continuous generation of unlabelled FFA thereby diluting the specific activity of the added isotope. Should this be the case the velocity measurements would be an underestimation and recycling of FFA could not be quantitated. If no lipolysis occurs during the incubation period the velocity measurements of fatty acid esterification may be regarded as absolute.

In order to determine whether FFA accumulation occurs from endogenous sources the enzyme preparation was incubated in buffer with and without added cofactors. FFA did not accumulate during incubation of the enzyme preparation alone or with added cofactors Table V. Exp. 1 and 3.

In view of the cofactor and substrate dependance for esterification of FFA in adipose tissue homogenates it cannot be argued that failure of FFA accumulation was due to re-esterification of liberated free

TABLE V

LIPOLYTIC ACTIVITY OF ENZYME PREPARATION

EXP. NO.	ADDITIONS	CHANGE IN FFA CONTENT µEq
1.	2cc Enzyme + Buffer	-.087
2.	2cc Enzyme + Coc.Oil + Albumin	+.270
3.	1cc Enzyme + Cofactors	-.020
4.	1cc Enzyme + Coc.Oil + Albumin	+.267
5.	Coc. Oil + Albumin. (Control)	-.030

Lipolytic activity of the enzyme preparation incubated with phosphate buffer (pH 7.0). Coconut Oil emulsion (Ediol ^{R}) 0.5% and Bovine albumin 4.6% final concentrations. The cofactors were added in amounts listed in Table II. Final volume of 3cc. The change in FFA content is the difference in FFA concent between 1cc of incubate titrated at zero time and after 20 min. incubation at 20°C.

fatty acids. Thus the absence of FFA accumulation suggests that either the enzyme preparation is free of lipase activity or contained insufficient and/or inaccessible glyceride substrate.

To explore the latter possibility the enzyme preparation was incubated with a coconut oil emulsion plus albumin. With the addition of artificial substrate, lipase activity was demonstrated as appreciable accumulation of FFA occured (Table V Exp. 2 and 4). Finally, the specific activity of the FFA was determined at intervals during incubation of the complete system i.e. during continued glyceride synthesis. The results of two such experiments are shown in Table VI. Very small differences in FFA specific activity were observed while increasing amounts of radioactivity appear in the glyceride fraction. In experiment I the 10' and 30' are the same, the lower value at 20' could well be due to error in titration as the quantity of FFA was small. In Exp. II the FFA SA was constant over 30 minutes incubation and fell by about 30% in the 40' specimen. This late fall in SA could have been due to lipolytic dilution of the labelled FFA as an apparent reduction in the rate of glyceride synthesis was observed.

Thus no clear cut evidence for endogenous triglyceride breakdown during the incubation periods ordinarily used could be obtained.

The relatively constant FFA SA would suggest that the endogenous pool of mixed FFA and the added C^{14} palmitic acid are being handled in a quantitatively identical manner.

TABLE VI

MEDIUM FFA SPECIFIC ACTIVITY (CPM/mμM) DURING GLYCERIDE SYNTHESIS

	EXP. I		EXP. II	
TIME	GLYCERIDES (CPM)	FFA S.A.	GLYCERIDES (CPM)	FFA S.A.
10'	1233	15	1628	18
20'	2212	10	3310	19
30'	2519	15	4932	20
40'			5747	14

Each flask contained 1cc enzyme preparation, cofactors as described in legend of Fig. XIII plus 10 μM αGP and .25 μM palmitic acid-1-C^{14} in a final volume of 3cc and incubated for times shown at 20°C. The entire incubate was extracted and FFA isolated as described in methods; an aliquot was titrated and the remainder analyzed for radioactivity. The radioactivity in the washed heptane phase represents glyceride synthesis. The difference in FFA S.A. and glyceride activity between Exp. I and Exp. II is due to differences in endogenous FFA content of the enzyme preparations (475 mμM/CC and 335 mμM/CC respectively).

D) GLYCEROPHOSPHATE DEPENDANCE

The glyceryl moiety of adipose tissue glycerides is derived almost exclusively from ∝GP which is generated from glycolytic intermediates according to the scheme shown in Fig. III (108). Free glycerol will not serve as a precursor of glyceride glycerol due to the absence of a phosphorylating system (148,161,183).

Experiments demonstrating ∝GP dependance in our esterifying system are in accord with observations reported by others. In the absence of ∝GP only 1.2% of the added C^{14} palmitic acid was esterified compared to 28-38% in the complete system (Table VII). Glycerol, glucose, and glucose plus insulin would not substitute for∝GP. It is of interest that in the absence of ∝GP a very small but quite constant amount (1-1.5%) of the radioactive fatty acid is found in glycerides and could be due to small amounts of endogenous ∝ glycerophosphate (108).

E) INHIBITORY EFFECTS OF HIGH FFA CONCENTRATION AND ALBUMIN

Accumulation of FFA in intact adipose tissue has been shown to have deleterious effects upon oxidative metabolism (58,78). Ball and Jungas (9) have suggested that high tissue FFA concentrations may inhibit the esterification process by uncoupling oxidative phosphorylation (119,144) thereby limiting the availability of high energy phosphate bonds required for fatty acid activation.

TABLE VII

DL- GLYCEROPHOSPHATE DEPENDANCE

	PALMITIC ACID-1-C^{14} ESTERIFIED % OF TOTAL
Complete System	28-38
" Without DL-αGP	1.2
" " + Glycerol 10 μM	1.3
" " + Glucose 5 μM	1.4
" " + Glucose 5 μM + Insulin 4U	1.3
" " Boiled Enzyme	1.0

The dependence on αGP for FFA esterification in a cell free preparation of adipose tissue. The complete system as described in Table II was modified in that lcc enzyme preparation was used. Incubation at 20°C for 20 min.

Experiments were carried out to see whether high concentrations of palmitic acid would have a similar effect on esterification with the cell free preparation of adipose tissue. The data is shown in Table VIII Exp. 1 and 3. Whereas 250 and 500 mμM C^{14} palmitic acid/flask gave the usual amount of esterification the addition of 3000 mμM C^{14} palmitic acid/flask inhibited esterification. Addition of 10mg bovine or defatted human albumin likewise resulted in reduced esterification of FFA to glycerides (Table VIII Exp. 2 and3). Albumin, being a potent FFA binder could cause inhibition of esterification in this system by reducing the effective concentration of the FFA substrate. If this were the mechanism one would expect to block the inhibitory effect of high FFA concentrations by concomitant addition of albumin. To test this hypothesis increasing concentrations of isotopic palmitic acid were added to flasks containing the fortified enzyme preparation and to a parallel series 10mg defatted human albumin was also added. The results are plotted in Fig. XVI. The inhibitory effect of high FFA concentration is quite evident (solid line). Maximum esterification occurred at a palmitic acid concentration of 196 μM/L followed by an abrupt decrease in glyceride synthesis with 363 μM/L palmitic acid. The inhibitory effects of further increases in concentration of palmitic acid (696 and 1029 μM/L) are only slightly greater than at 363 μM/L.

The effect of albumin (broken line) was inhibitory at palmitic acid concentrations of 196 μM/L and 1029 μM/L. With the intermediate fatty acid concentrations a slight increase in esterification

TABLE VIII

EFFECT OF HIGH FFA CONCENTRATION AND ALBUMIN ON ESTERIFICATION

EXP. NO.	ADDITIONS PALMITIC ACID-1-C^{14} mμM/Flask		ALBUMIN mg/Flask	INCUBATION TIME 20°C	GLYCERIDES CPM
1.	Complete System 250			15	2583
	" 3000			"	1383
2.	" 250			"	2364
	" 250	+	10(Bovine)	"	1232
3.	" 500			20	4581
	" 500	+	10(Human)	"	2977
	" 3000			"	1964

Effect of high FFA concentration and albumin on esterification. Complete system as described in Fig. XIII with 6.7 mM/L αGP. Radioactivity in alkaline ethanol washed heptane phase or Dole extract represents glyceride synthesis. Human defatted albumin used. Note inhibitory effects of 3000 mμM fatty acid and both bovine and human albumin.

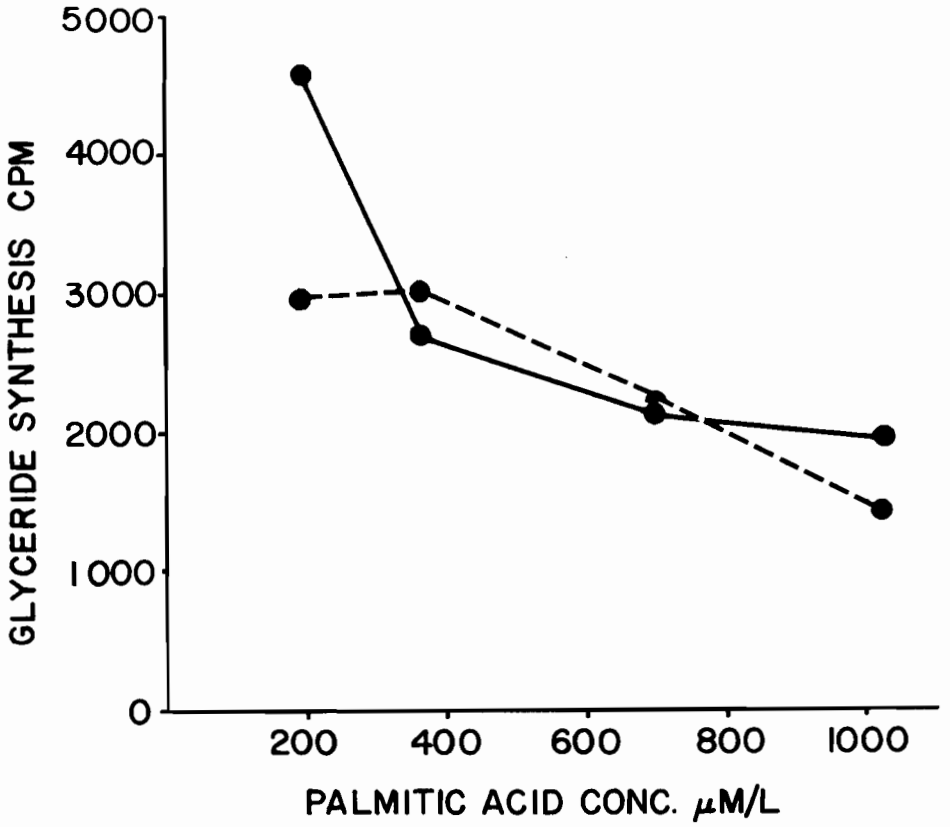

FIGURE XVI

Inhibition of esterification by high FFA concentration and the effect of albumin thereon. Esterification by the complete system as described in the legend to Fig. XIII plus 6.7 mM/L « GP to which increasing amounts of palmitic acid-1-C^{14} was added and incubated for 20 min. at 20°C. (Solid line). 10 Mg defatted human albumin buffered to pH 7.0 was added to a parallel series of vessels (broken line). The radioactivity in the synthesized glyceride is plotted with respect to total (endogenous plus added) palmitic acid concentration.

was obtained in the presence of albumin. It appears that the inhibitory effect of albumin and high FFA concentration (1029 μM/L palmitic acid) are addative. This suggests that the mechanism of albumin inhibition of esterification may be in part a direct effect unrelated to fatty acid binding. Thus inhibition of esterification by the intermediate concentrations of palmitic acid was only partially blocked because of the superimposed direct inhibitory effect of albumin.

F) ESTERIFICATION AND INTERACTION OF VARIOUS FFA

The relative velocity of esterification of various free fatty acids was determined by incubating equimolar amounts of isotopic lauric, myristic, palmitic, and stearic acids in separate flasks for 20 minutes at 20°C, following which the proportion of the label incorporated into glycerides was measured. Representative results are shown in Table IX. Lauric acid was esterified to the greatest extent followed by palmitic then myristic. Stearic acid was least well esterified. Except for myristic acid esterification increased with free fatty acids of successively shorter chain length. Repeated studies with new myristic acid-1-C^{14} gave identical results.

Interaction of fatty acids with regard to esterification was suggested by the fact that increasing concentration of enzyme and therefore endogenous FFA resulted in a non-linear increase in palmitic acid esterification (Fig. XI). Interaction between palmitic acid and a number of individual FFA was manifest when equimolar amounts of unlabelled lauric,

TABLE IX

ESTERIFICATION OF EQUIMOLAR AMOUNTS OF VARIOUS FFA-1-C^{14}

FLASK NO.		FATTY ACID ESTERIFIED % OF TOTAL
1.	LAURIC ACID-1-C^{14}	48.5
2.	MYRISTIC ACID-1-C^{14}	34.5
3.	PALMITIC ACID-1-C^{14}	38.8
4.	STEARIC ACID-1-C^{14}	6.7

The esterification of equimolar amounts of different saturated FFA. Each flask contained the complete system as described in the legend to Table II. 250 mμM of the indicated isotope was used as the fatty acid substrate. Following 20 min. incubation at 20°C the proportion of the total radioactivity in the glyceride fraction was determined.

TABLE X

EFFECT OF VARIOUS FFA ON PALMITIC ACID-1-C^{14} ESTERIFICATION

FLASK NO.		PALMITIC ACID -1-C^{14} ESTERIFIED % OF TOTAL
1.	PALMITIC ACID-1-C^{14} 250 mμM	25
2.	" + LAURIC ACID 250 mμM	6
3.	" + MYRISTIC ACID 250 mμM	2
4.	" + STEARIC ACID 250 mμM	18
5.	" + PALMITOLEIC ACID 250 mμM	2

The effect of various unlabelled FFA on the esterification of Palmitic acid -1-C^{14}. Each flask contained 1cc of the enzyme preparation plus cofactors as in Fig. XIII. The αGP concentration was 6.7 μM/L. Each flask contained 250 mμM palmitic acid-1-C^{14}. (SA .04 μc/μM). A further addition of 250 mμM unlabelled fatty acid to flasks 2-5 was made. Following 15 min. incubation at 20°C the fraction of the total activity in the glyceride fraction was determined.

myristic, stearic and palmitoleic acid were added to vessels containing palmitic acid-1-C^{14}. The results are shown in Table X. The esterification of palmitic acid-1-C^{14} was nearly abolished in the presence of an equivalent amount of myristic or palmitoleic acids. Lauric and stearic acids reduced C^{14} palmitic acid esterification by 85% and 28% respectively.

PART IV DISCUSSION

The requirements for triglyceride synthesis by the subcellular preparation of adipose tissue used in this study was very similar to that reported by Steinberg et al (161) for triglyceride synthesis in defatted homogenates of rat adipose tissue and by Weiss et al (177) and by Teitz et al (164) for triglyceride synthesis in liver homogenates and by Neptune et al (115) for glyceride synthesis in skeletal muscle preparations.

The potency of the enzyme preparation is noteworthy as almost complete incorporation of added C^{14} palmitic acid into triglyceride was obtained within 10 minutes incubation at 37°C. Over 90% of the radioactivity was found in the triglyceride fraction indicating the absence of any significant block in the synthetic sequence shown in Fig. II. This is in clear distinction to other studies on glyceride synthesis from precursors by cell free preparations of adipose tissue (161), gut (32) and liver (159), where from 50-80% of the incorporated fatty acid was found in lower glycerides.

Lowering the temperature of incubation did not alter the cofactor requirements or affect the distribution of radioactivity in the neutral lipid products to any significant degree. Apart from reducing the rate of esterification (glyceride synthesis) so that initial velocities could be easily and reproducibly obtained, incubation at 20°C seemed to protect the esterifying system or at least make it less susceptible to inhibitory influences observed at 37°C. On occasion esterification at

37°C with the complete system was markedly reduced for no apparent reason and was likewise affected by a reduction in enzyme concentration. The erratic behaviour of this esterifying system when incubated at 37°C has been noted previously by Steinberg et al (161) and Meinertz (112) but to date remains unexplained. Once the temperature of incubation was maintained at 20°C the velocity of esterification varied directly with enzyme concentration and unexplained failures with the complete system did not occur. That inhibitory effects of added FFA may be temperature dependent cannot be excluded as a possible explanation for these results.

Kinetic definition of the overall reaction sequence was simplified by virtue of the fact that little or no lipolysis of preformed tirglyceride occurred during incubation. The data reported here was based on the esterification of C^{14} palmitic acid used in chemical amounts. Since the added palmitic acid and the endogenous FFA were handled similarily the kinetic data probably approximates the behaviour of a mixed pool of FFA of the type ordinarily encountered in rat adipose and for this reason the $Km \propto GP$ was calculated on the basis of the total FFA esterified. For the calculation of Km palmitic acid it was necessary to know the total initial concentration (endogenous plus added) of this substrate. As it was not possible to determine the composition of endogenous FFA the concentration of pre-existing palmitic acid was assumed to represent 30% of the total endogenous FFA. This amount was based on compositional studies of the FFA pool in intact tissue (65). The extent to which any error in this value would effect the results is small since relatively large amounts of isotopic palmitic acid was added.

It should be pointed out that the biosynthetic evolution of the triglyceride molecule from FFA and ∝GP precursors is a resultant of at least 5 enzyme catalyzed reactions involving 4 intermediates. Therefore, kinetic measurements based on the rate of triglyceride synthesis from precursors must be regarded as the net result of all intermediate reactions.

The velocity of esterification at 20°C was dependent on the concentration of ATP, CoA, Mg^{++}, ∝GP and FFA and could be altered at will by appropriate manipulations. This gives some indication of the complex system of controls which could theoretically obtain in the intact organism. The exact interplay of all hormonal or other influences which directly or indirectly affect any of these parameters remains to be elucidated.

As might be expected of an enzyme catalyzed reaction the velocity of esterification in the cell free system was sensitive to the concentration of both substrates, ∝GP and FFA.

These findings are in keeping with the previous observations of Lebouf et al (96) and Cahill et al (29) and suggest that the FFA concentration in intact tissue may participate in the control of fatty acid esterification. In the aforementioned studies, addition of lipolytic hormones (epinephrine. G.H. and ACTH) to the incubation medium stimulated glucose uptake and incorporation of glucose carbon into glyceride glycerol signifying an increased rate of esterification. This

was attributed to the FFA which accumulated due to lipolysis as the effects of these hormones on glucose metabolism by adipose tissue were mimicked by a high medium FFA concentration.

Addition of graded concentrations of palmitic acid to the incubation system resulted in a biphasic effect on velocity of esterification. At lower concentrations the velocity of esterification increased with increments in palmitic acid concentration (Fig. XIV) and at concentrations above 200 μM/L a distinct inhibitory effect on the rate of glyceride synthesis was observed (Fig. XVI). The extent to which the endogenous pool of mixed FFA participated in this inhibitory effect is unknown. Inhibition of esterification due to added FFA has been noted by Steinberg et al (161) with adipose tissue homogenates and by Kornberg and Pricer (43) in guinea-pig liver homogenates and by Longcope and Williams (101) in studies on long chain fatty acid esterification of cholesterol by homogenates of adrenal gland. With intact adipose tissue the inhibitory effects of high tissue FFA concentrations on the esterification process as well as other aspects of tissue metabolism has been amply documented (9, 58,171). Uncoupling of oxidative phosphorylation by fatty acid resulting in a limited supply of ATP for FFA activation has been implicated (9,10). This mechanism could not apply in our cell free system as optimum amounts of ATP were supplied. The possibility remains that FFA in high concentration may alter the availability of certain coenzymes or ions which on the one hand could inhibit oxidative phosphorylation in intact tissue or reduce esterification in the cell free system. The precipitation of magnesium ions

as undissociated salts of long chain fatty acids would fulfill a common mechanism for the observed in both intact and cell free preparations of adipose tissue.

Exactly what determines the critical concentration at which FFA become inhibitory remains unanswered. It is attractive to speculate that FFA binding sites exist in both intact and cell free preparations of adipose tissue (127) which limit the amount of free (dissociated) FFA, and, as the total amount of FFA increases these binding sites become progessively more saturated. During this period the increase in dissociated FFA is relatively small and augments esterification. FFA concentrations exceeding the capacity of these binding sites would then show inhibitory effects due to the marked increase in the unbound portion of FFA. The protective effect observed with added albumin lends some support to this hypothesis. Alternative explanations for the observations include the inhibitory effect of excess substrate on an enzyme catalyzed reaction and the possibility that excessive amounts of acyl-CoA are generated which may inhibit its own further metabolism (7).

To assume that these events have an in vivo counterpart is treacherously presumptive. Nevertheless, the inhibitory effects of high FFA levels on esterification as observed in vitro would be teleologically purposeful in a situation like fasting by promoting release of FFA into the circulation and preventing the seemingly wasteful expenditure of energy required for the re-esterification process. Insofar as contined net FFA production perpetuates itself, a positive feedback

mechanism is envisaged. Its initiation could be the result of both decreased esterification due to lack of carbohydrate, and, increased lipolysis due to activation of tissue lipase by adipokinetic hormones such as growth hormone or epinephrine. Augmented release of FFA would continue until the ingestion of a carbohydrate meal reversed the process to one which favoured esterification. At this point the antilipolytic effect of insulin observed in vitro (79,118) may have its physiological expression. Thus increased insulin secretion in response to a carbohydrate load after fasting may decrease net FFA production not only by promoting glucose entry and glycerophosphate generation for esterification, but also perhaps by inhibiting the lipolytic effect of growth hormone, epinephrine or glucogen (if in fact these hormones stimulate lipolysis in fasting) thereby breaking the positive feedback cycle.

Inhibitory effects of albumin on glyceride synthesis in adipose tissue homogenates has been noted previously (161) and is confirmed in the present study. The fact that it occurs in the presence of inhibitory concentrations of FFA (Fig. XII) suggests that it is at least in part unrelated to FFA binding. As a result the protection afforded by albumin binding against the inhibitory effects of FFA is masked by the superimposed albumin inhibition. Brandes et al (22) have shown that albumin shifts pH optimum of deacylase activity in a guinea-pig liver mitochondrial and microsomal preparation. It is possible that a similar effect occurs with the adipose tissue preparation resulting in deactivation of fatty acid thiol esters.

The variations between different saturated fatty acids in the degree to which they are esterified is not surprising as it has been shown that esterification of FFA in intact adipose tissue is related to fatty acid structure (56). With the exception of myristic acid the degree of esterification increased as the chain length decreased. The effect of various FFA on the esterification of C^{14} palmitic acid was variable (Table X). Stearic acid, itself poorly esterified, had the least effect as would be expected. Lauric acid which is most readily esterified decreased palmitic acid esterification by 85%. Virtually complete inhibition of C^{14} palmitic acid esterification by equimolar amounts of myristic and palmitoleic acids was unexpected. It is conceivable that fatty acids differ with respect to their inhibitory effects and that myristic and palmitoleic acids depress triglyceride synthesis at a lower concentration that palmitic acid. This would also explain the observation that myristic acid was out of phase with the other saturated fatty acid with respect to rate of esterification. No attempt has as yet been made to differentiate between competitive, non-competitive, and excess substrate types of inhibition in this study.

Interpretation of the results obtained for esterification of different acids and the interaction of acids is further complicated by the fact that the enzyme preparation contains a considerable amount of FFA of mixed composition. If it is realized that various fatty acids differ in rates of activation and condensation with glycerophosphate to form phosphatidic acid (93), and esterification of diglyceride to form triglyceride exhibits structural specificity (48) it will be appreciated

that the reactivity of any one acid in a mixed precursor pool of FFA is probably dependent on the compositional makeup of the intermediates in which it arises and may bear no relationship to the behaviour of that acid if it alone made up the precursor pool.

PART V SUMMARY

(1) A subcellular preparation of rat epididymal adipose tissue capable of esterifying α glycerophosphate and C^{14} long chain fatty acids was obtained by homogenization in aqueous salt solution and centrifugation at 700xgx12 min. Electron microscopic study of the aqueous supernatant which served as the enzyme preparation demonstrated vescicular organelles characteristic of adipose tissue, endoplasmic reticulum and the absence of intact adipose tissue cells.

(2) Optimum cofactor requirements for the esterification reaction were determined. The reaction was totally dependant on the presence of ATP and partially dependant on added CoASH, Mg^{++} and F^{-} ions. α Glycerophosphate was an obligatory substrate requirement for FFA esterification in this system and could not be replaced by glycerol or glucose.

(3) The rapid velocity of esterification at 37°C prevented accurate measurement of initial velocity for kinetic characterization of the overall reaction. The difficulties were overcome by lowering the temperature of incubation to 20°C. This reduced the velocity of esterification to about 1/5 that at 37°C without affecting the cofactor requirements or the neutral lipid products. About 90% of the isotopic FFA incorporated into neutral lipid was found in triglyceride and most of the remainder in lower glycerides after 20 min. incubation at 20°C.

(4) The effect of substrate concentration (αGP and C^{14} palmitic acid) on initial velocity of esterification was studied. Kinetic analysis showed Km GP = 1.9×10^{-3}M (pH 7.0, 20°C) and Km palmitic acid = 1.68×10^{-4}M (pH 7.0, 20°C). The theoretical V max for palmitic acid esterification in this system corrected for temperature was 4µM/mg extract protein/hr (pH 7.0, 37°C).

(5) Lipolysis of preformed triglyceride during FFA esterification was negligable. Addition of coconut oil substrate plus albumin to the enzyme preparation was necessary in order to demonstrate lipolytic activity.

(6) Addition of graded concentrations of palmitic acid resulted in a biphasic effect on velocity of esterification. Increments in palmitic acid concentrations up to 200 µM/L were found to increase the velocity of esterification while concentrations greater than 363 µM/L were distinctly inhibitory. Simultaneous addition of albumin had a slight protective effect against fatty acid inhibition. This protective effect was masked by a superimposed inhibitory effect of albumin.

(7) Saturated fatty acids differing in chain length varied in their rates of esterification. Lauric acid was esterified most readily followed by palmitic then myristic acids. Stearic acid was poorly esterified. Interaction of various fatty acids was demonstrated. Myristic and palmitoleic acids virtually abolished esterification of palmitic acid. Lauric acid and stearic acid also decreased palmitic acid esterification but to a lesser extent.

(8) A mechanism for the inhibition of esterification by high FFA concentrations was suggested and a possible role for FFA in the physiologic control of fatty acid esterification in adipose tissue was discussed.

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