SHORT TITLE:

ESTERIFICATION OF FATTY ACIDS BY CELL-FREE PREPARATIONS.

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ESTERIFICATION OF FREE FATTY ACIDS

BY SUBCELLULAR PREPARATIONS OF RAT ADIPOSE TISSUE.

BY

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A THESIS

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ABBREVIATIONS.

FFA	Free Fatty Acid
HMP	Hexose Monophosphate Shunt
C(n)	C refers to the carbon chain of a fatty acid and n (in brackets) represents the number of carbon atoms present.
UDPG	Uridine Diphosphate Glucose
AMP	Cyclic 3′ 5′ adenosine monophosphate
Acetyl CoA	Acetyl coenzyme A
E. coli	Escherichia coli
ACP-SH	Acyl carrier protein

I. REVIEW SECTION

A) HISTORICAL INTRODUCTION.

About 30 years ago, adipose tissue was considered to be metabolically inert. It was thought that its main functions were insulation of the body against heat loss and provision of mechanical support for certain organs. During the past 20 years, a surge of experimental work has changed these concepts radically. At the present time, adipose tissue is known to be the site of an intricate interplay of metabolic processes. Indeed, this tissue is now known to contain by far the largest reserve of metabolic energy in the body. It accumulates fat during periods of feeding and releases it during periods of fasting.

Even though most scientists, prior to 1940, believed that adipose tissue was metabolically inactive, a few challenged this view, and even fewer, provided experimental evidence in favour of an active physiological role of this tissue. Wasserman (1), on the basis of his pioneer work on the histology and embryology of adipose tissue, concluded that it was unique in its capacity to accumulate and mobilize fat. The classical work of Schoenheimer and Rittenberg (2, 3, 4, 5) who used fats labelled with deuterium, demonstrated the rapid turnover and continuous synthesis of body fat. Their work inspired many other investigators, and paved the way to a period of intensive research in the metabolism of adipose tissue.

Despite the rapid turnover of body fat,

Schoenheimer and Rittenberg noted that under ordinary feeding conditions, the size of the fat stores remained fairly uniform. This observation suggested a dynamic equilibrium between the deposition of fat in the adipose depots and its mobilization and utilization. It soon became apparent that this equilibrium was under complex nutritional, hormonal and neural control.

In 1926, Wertheimer (6) showed that insulin could counteract the fatty liver induced in phloridzin-treated animals. This hormone, moreover, could partially prevent the development of fatty livers resulting from the injection of anterior pituitary extracts (7). Other studies showed more directly that insulin promoted the deposition of glycerides in adipose tissue. For example, when rats on a diet rich in starch received frequent injections of protamine insulin, an excessive deposition of fat was noted in their adipose stores (8, 9). In the diabetic animal, on the other hand, fat synthesis was found to be 5% of that occurring normally (10). These studies suggested that insulin, by virtue of its effect on glucose transport and metabolism, played an important role in the deposition of fat in adipose tissue.

In 1948, Shapiro and Wertheimer (11) proved conclusively the occurrence of lipogenesis from glucose by demonstrating that deuterium was incorporated into the fatty acids of adipose tissue incubated in serum containing deuterium oxide. These findings confirmed previous evidence, which was based on the observation that the accumulation of glycogen in adipose tissue during a period of refeeding was accompanied by a rise in respiratory quotient above unity (12). Although this evidence was indirect, it suggested strongly that carbohydrate was converted into fat in the

adipose tissue itself.

The early studies concerning the mobilization of fat from adipose tissue were handicapped by the lack of information regarding the form in which lipids were transported in the blood plasma. The finding that section of the spinal cord in the vicinity of the first thoracic segment prevented the formation of fatty liver in a phloridzin-treated fasting dog, indicated that the nervous system played a role in the mobilization of fat from adipose tissue (13). In addition, a number of investigators presented evidence which suggested that certain hormones were involved in the mobilization of fat from adipose tissue. Best, Barrett and co-workers (14, 15) and Stetten and Salcedo (16) injected pituitary extracts into mice and rats and interpreted their findings as signifying a redistribution of fat from adipose tissue to the liver. The adrenal medulla was also implicated in the mobilization of fat when Clement and Schaeffer (17) showed that epinephrine was active in this regard.

In 1948, Wertheimer and Shapiro (18) wrote the first review on the physiology of adipose tissue. In this classical treatise, they stressed the fact that adipose tissue had a special structure and histology. They pointed out that it had a comparatively dense capillary net and that it was innervated by sympathetic fibres. These authors, moreover, emphasized the fact that the mobilization and deposition of fat in adipose tissue were active processes that were regulated by endocrine and neural factors.

This review stirred the interest of many scientists, and soon after it was published, many centres

throughout the world began to work in the field of adipose tissue metabolism.

In 1954, Hausberger et al. (19) and Feller (20) demonstrated, in vitro, that adipose tissue itself is the major site of conversion of carbohydrates into fat, and not the liver. These findings were confirmed in vivo by Favarger and Gerlach (21, 22).

Finally, the role of adipose tissue as a major source of energy became clear when the free fatty acid fraction of plasma lipids became recognized as the form in which fat is mobilized from this tissue (23, 24).

B) COMPOSITION OF WHITE ADIPOSE TISSUE.

White adipose tissue of land mammals usually contains 5-10% water, 2-3% proteins and 60-85% lipids, of which 90-99% are triglycerides (25, 26, 27, 28, 29).

Thus, minute amounts of other lipids are normally found in this tissue. Epididymal fat from fed rats, for example, contains about 1000 μmoles of triglyceride (30), about 29 μmoles of diglyceride, about 4 μmoles of monoglyceride (31), and 1-2 μmoles of free fatty acids (32) per gram wet weight. Alkyl and alkenyl glycerol ethers, moreover, comprise 1.3% of the neutral lipid fraction of rat adipose tissue (33). The alkenyl ethers form 82% of the total nonphosphatide glycerol ether fraction (33).

Phospholipids constitute 0.23 to, at most, 4% of the wet weight of white adipose tissue (34, 35). When their level, however, is expressed as grams per unit of nonlipid dry weight, their concentration is of the same order as for organs such as thymus or skeletal muscle (36). In white adipose tissue of adult mouse, lecithin, phosphatidylethanolamine, and sphingomyelin make up about 80% of the phospholipid fraction (35). The remainder is comprised of small proportions of ethanolamine plasmalogen, phosphatidylserine, polyglycerophosphatide, monophosphoinositide, and choline plasmalogen.

Finally, the non-saponifiable fraction comprises 0.19 - 1.3% of the wet weight of adipose tissue (20, 34, 37, 38). Cholesterol (37), various steroids (37), tocopherols (39), carotenoids (37), and squalene (40) are members of this small fraction.

It is, thus, clear that triglyceride is the predominant lipid in adipose tissue. Indeed, storage of fatty acids in

the form of triglyceride during periods of adequate nutrition appears to be the major function of white adipose tissue.

The distribution of fatty acids within the triglyceride molecule and the factors influencing the fatty acid patterns of adipose tissue glycerides will now be discussed briefly.

1) Distribution of Fatty Acids Within the Triglyceride Molecule.

It was originally thought that fatty acids were distributed within triglyceride molecules in a random fashion (40). More recently, however, experimental data obtained with more refined methods, have indicated that fatty acids are distributed in a non-random fashion (41, 42). In fact, a rather high degree of specificity in the distribution of fatty acids within the triglyceride molecule appears likely. Specific enzymes may be responsible, at least in part, for this non-random arrangement.

Mattson and Lutton (41) used pancreatic lipase in their studies because of its specificity for the 1 and 3 positions of the triglyceride molecule (43, 44). Their ingenious experiments have led to several interesting findings, which will now be summarized. Animal fats, as a group, do not have a fatty acid pattern since the proportion of any fatty acid type in any position of the triglyceride molecule differs widely among various samples of adipose tissue. Certain fatty acids, however, tend to present a degree of selectivity in some species. In fats of beef, horse, and sheep, saturated fatty acids are preferentially distributed in the 1 and 3 positions, a trend which is also observed in triglycerides of vegetable origin. There is a strong tendency

toward localization of linoleic acid at the 2 position of the triglyceride in most species. The fat of the pig is unusual because the saturated acids are predominantly in the 2 position. Other studies (44, 45) have confirmed the existence of oleic and linoleic acid predominantly in the 2 positions of triglycerides and the usual occurrence of palmitic and stearic acid in the 1 and 3 position. Thus, the selective location of the various fatty acids within triglycerides seems to be widespread and species dependent.

2) Factors Influencing Fatty Acid Patterns of Adipose Tissue Triglycerides.

Within certain limits of dietary intake, phylogenetic influences appear to be the most important factors which determine the fatty acid patterns of adipose tissue triglycerides in many species. Examples of this generalization are the existence of dissimilar depot fat composition in various species receiving similar diets (40), and the presence of similar fatty acid patterns in the chimpanzee and western man, in spite of marked dietary differences (46). Within an individual of a given species, on the other hand, adipose tissue seems to be quite homogenous and only few minor differences, if any, are noted from one site to another (29, 38, 47). Similarly, the fatty acid composition of adipose tissue triglycerides from male and female of the same species is the same (40, 48).

Table I illustrates the fatty acid composition of fat samples obtained from three mammalian species, namely, man, rabbit, and rat. As noted, adipose tissue contains almost exclusively long-chain fatty acids. Although the fatty acid composition varies somewhat between different species, a large proportion of the fatty

TABLE I

FATTY ACID COMPOSITION OF ADIPOSE TISSUE TRIGLYCERIDE *

		Man	Rabbit	Rat
Myristic Acid **	(14:0)	2.8	3.3	2.6
Palmitic Acid	(16:0)	23.0	31.7	30.4
Palmitoleic Acid	(16:1)	8.0	5.1	8.1
Stearic Acid	(18:0)	3.6	4.5	3.6
Oleic Acid	(18:1)	55.3	28.5	36.1
Linoleic Acid	(18:2)	5.4	23.7	19.2
Others		1.7	3.2	trace

* Expressed as mole percent. Data are taken by permission of Dr. Hollenberg from Hollenberg, C.H. and Roncari, D.A.K., Applied Therap. 7: 24, 1965.

** The acids are designated in brackets by the method suggested by Dole (49).

acids is always of the unsaturated variety. This preponderance of unsaturated fats is responsible for the semi-fluid consistency of adipose tissue. This physical state, in turn, may influence the rate of certain enzymatic processes in this tissue.

As previously mentioned, the fatty acid patterns in adipose tissue can be influenced to a certain extent by dietary means. Foods rich in carbohydrate increase the content of saturated fatty acids, particularly that of stearic acid (50, 51, 52). Another example of dietary influence is the difference in fatty acid composition between wild and tame rabbits (40, 53). The wild rabbit, which eats a diet containing large amounts of linolenic acid, has a high proportion of this acid in its fat depots. However, when the wild and tame rabbits are fed a low-fat diet, they assume a similar fatty acid composition, the linoleic-linolenic content becoming much lower. Dietary linoleic acid, moreover, leads to an increased proportion of linoleic, palmitic and stearic acids, and a decreased proportion of palmitoleic acid in the adipose tissue of the mouse (54). By comparison, oleic acid feeding results in an increased percentage of oleic acid, and a decreased percentage of palmitic acid, and, to a smaller extent, of myristic acid (54). Thus, dietary factors can influence the composition of fatty acids in adipose tissue triglycerides. The magnitude of this effect, however, varies between different species. It is, therefore, probable that an active regulation of these fatty acid patterns occurs in the tissue itself.

C) THE RELATIONSHIP BETWEEN CARBOHYDRATE AND LIPID METABOLISM IN ADIPOSE TISSUE.

Carbohydrate is the source of carbon atoms required for glyceride-glycerol and de novo fatty acid synthesis in adipose tissue. Oxidation of glucose also provides cofactors which are obligatory for these synthetic processes. It is, thus, clear that the metabolism of carbohydrate in this tissue, is closely related to that of lipid. Because of their importance, the pathways of carbohydrate metabolism and their close relationship to lipogenesis will now be discussed in some detail.

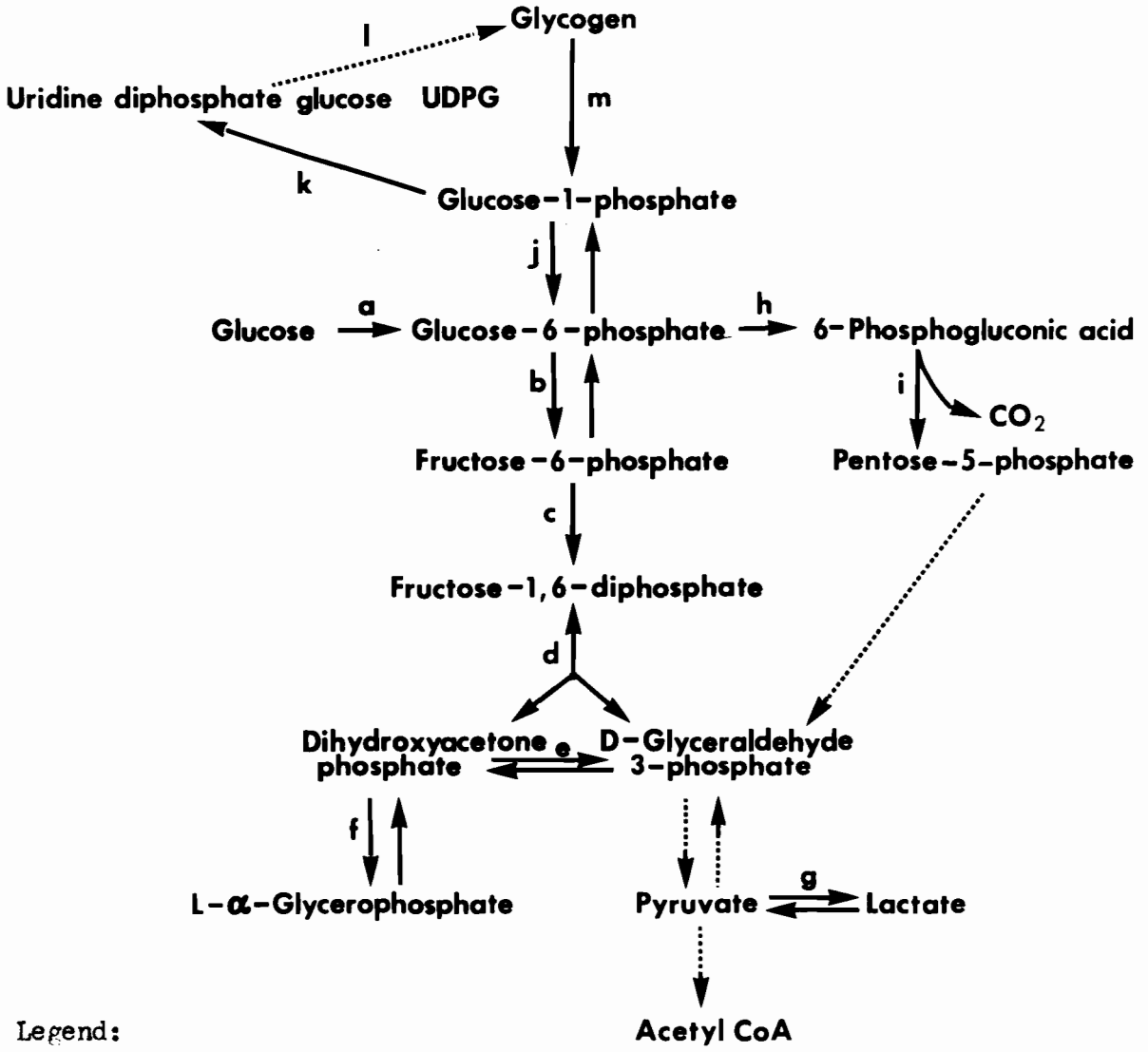
1) Pathways of Carbohydrate Metabolism in Adipose Tissue.

The major pathways of glucose metabolism in adipose tissue are the glycolytic pathway and the hexose monophosphate shunt (55, 56, 57, 58, 59, 60). The minor pathways include the glycogen cycle (61, 62, 63) and the glucuronic acid pathway (60, 64, 65, 66). The major pathways and the glycogen cycle are schematized in Fig. I.

Phosphorylation of glucose is the initial step in the metabolism of this sugar. Glucose-6-phosphate is, then, utilized in the different metabolic pathways. Adipose tissue has two enzymes that catalyze the phosphorylation of glucose (67). One has a high Km for glucose and is influenced by changes in nutritional status. It has been called glucokinase. The other has a low Km for glucose and is not affected by nutritional manipulation. It has been named hexokinase. The characteristics of the two phosphorylat-

FIG. 1

Pathways of Carbohydrate Metabolism in Adipose Tissue



Legend:

a) Glucokinase
b) Phosphohexose isomerase
c) Phosphofructokinase
d) Fructose-1,6-diphosphate aldolase
e) Triose phosphate isomerase
f) α-Glycerophosphate dehydrogenase
g) Lactic dehydrogenase
h) Glucose-6-phosphate dehydrogenase
i) 6-Phosphogluconate dehydrogenase
j) Phosphoglucomutase
k) UDPG pyrophosphorylase
l) UDPG glycogen transglucosylase
m) Glycogen phosphorylase

ing enzymes are similar to those present in liver (68, 69, 70).

In contrast to liver, kidney, and small intestinal mucosa, which have an active glucose-6-phosphatase (71, 72), adipose tissue cannot re-form free glucose from glucose-6-phosphate and hence, from glycogen, because of the virtual absence of this enzyme (73, 74).

Direct and indirect evidence for the presence of glycolytic enzymes in adipose tissue has been obtained in recent years. These enzymes are phosphohexose isomerase, phosphofructokinase, fructose-1, 6-diphosphate aldolase, triose-phosphate isomerase, α - glycerophosphate dehydrogenase (74, 75, 76), and lactic dehydrogenase (77). Fructose-1, 6-diphosphate is the immediate precursor of the two triose phosphates, dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. The former is derived from the upper three carbons of fructose-1, 6-diphosphate and the latter from the lower three carbons. The enzyme α - glycerophosphate dehydrogenase (77) is of utmost importance in adipose tissue metabolism because it catalyzes the reduction of dihydroxyacetone phosphate with the formation of α - glycerophosphate. This metabolite is the "activated" form of glycerol, which is esterified by fatty acids to form glycerides (78, 79, 80). Because of its importance, the metabolism of α - glycerophosphate will be discussed separately in greater detail.

There is good evidence, moreover, that the reactions between glyceraldehyde-3-phosphate and acetyl CoA are operative in adipose tissue, but all the enzymes have not been assayed directly. An adequate supply of acetyl CoA is essential for both the synthesis of fatty acids (81, 82, 83), and the operation of the citric acid cycle. As would be expected this cycle is active in

adipose tissue, and some of its enzymes and other associated respiratory enzymes, have been assayed in this tissue. These are isocitric dehydrogenase (84, 85, 86), succinic dehydrogenase, succinic oxidase, cytochrome C oxidase, and NADH cytochrome C reductase (84).

Finally, as opposed to liver, which has an active diphosphofructose phosphatase (87, 88). The glycolytic pathway is not completely reversible in adipose tissue because of the virtual absence of this enzyme (74). The irreversibility of the glycolytic pathway explains why Rose and Shapiro (89) found no glycogen synthesis from acetate-1-C^{14}, or from pyruvate-2-C^{14} under conditions in which uniformly labelled C^{14}-glucose was actively incorporated into glycogen. Fat tissue, furthermore, is deficient in phosphoenolpyruvate carboxykinase, another enzyme which is strategically placed at a metabolic point crucial for the reversal of glycolysis (86, 90).

Adipose tissue cannot play a role in gluconeogenesis because of the absence of this enzyme, in addition to that of glucose-6-phosphatase and diphosphofructose phosphatase.

The HMP dehydrogenases have been assayed in adipose tissue (74, 76). Even though this tissue contains only about one-seventh the amount of nitrogen found in liver, it exhibits more than five times as much glucose-6-phosphate dehydrogenase activity per mg tissue nitrogen as liver (74). It is the only enzyme of the major pathways that is more active in adipose tissue than in liver.

It is of considerable interest that the HMP dehydrogenases are active in a tissue which has a high capacity for lipogenesis. Indeed, the NADPH which is produced during the operation of the HMP is a coenzyme necessary for the reductive processes

leading to the synthesis of fatty acids (91, 92, 93, 94). A direct relationship between HMP activity and lipogenesis has,in fact, been shown by many investigators (58, 95, 96, 97, 98, 99, 100, 101).

Flatt and Ball (102) have demonstrated that, when intact adipose tissue is incubated in the absence of insulin and epinephrine, the amount of NADPH produced during the operation of the HMP, is sufficient to provide the total reducing equivalents for fatty acid synthesis. When insulin is present, the HMP makes available only 63% of such reducing equivalents, while in the presence of both insulin and epinephrine, the value falls to 53%. When these hormones are present, the NADH, which is formed during the oxidation of triose phosphates to acetyl CoA may supplement the NADPH derived from the HMP. Indeed, there is some evidence that NADH can be utilized for the synthesis of fatty acids, even though NADPH appears to be used preferentially for this purpose (103, 104). It is also possible that NADH is first converted to NADPH by trans-hydrogenation. Such a reaction has been demonstrated in adipose tissue (85, 86). Finally, malic enzyme, which links the glycolytic pathway with the citric acid cycle, adapts to changes in nutritional state in the same manner as the HMP dehydrogenases and may contribute to the supply of NADPH that is used during fatty acid synthesis. This enzyme is more active in adipose tissue than in liver.

The early attempts at quantitative estimation of the major pathways (58, 105), did not take into account the probably incomplete equilibration between the two triose phosphates (105, 106, 107) and/or the isomerization of fructose-6-phosphate back to glucose-6-phosphate, a reaction leading to recycling of the HMP (105, 108). More recently, methods that take these factors into account, have been

developed for the estimation of pathway contributions (109). By these means, 11 to 15% of total glucose metabolism in adipose tissue has been estimated to proceed via the HMP when insulin is not present (108). The contribution of the minor pathways appears to be quite small. Hence, the glycolytic pathway appears to be the major route of glucose metabolism in fat tissue.

2) Hormonal Regulation of Major Pathways.

a) Insulin.

The addition of insulin, in vitro, markedly stimulates the uptake of glucose by adipose tissue (55, 102, 110, 111, 112), and the oxidation of glucose (58, 102, 105, 110, 113). The insulin effect on the oxidation of glucose carbon 1 is greater than that on the oxidation of carbon 6 (105, 113). Recently, it has been shown that the contribution of the HMP to total glucose metabolism increases from about 13% to 23% in the presence of this hormone (108).

The direct relationship between HMP activity and lipogenesis, partly explains the enhancement of fatty acid synthesis from glucose brought about by insulin (58, 105, 110, 111, 112). Another factor is the partial shutdown of the citric acid cycle in the presence of this hormone (102). It has been suggested that this phenomenon is a compensatory mechanism to permit the reoxidation of the reduced coenzymes that are formed during the production of acetyl CoA from glucose. This reoxidation must take place to enable further acetyl CoA production and fatty acid synthesis. It may be relevant that palmityl CoA has been shown to inhibit hepatic

citrate synthase activity (114). Recently, it has been estimated that the addition of insulin to adipose tissue, in vitro, increases the synthesis of fatty acids by 560% over the control value (102).

In contrast to its marked enhancement of fatty acid synthesis, insulin increases the production of glyceride-glycerol only 2-to-3-fold (102, 105). Thus, while in unstimulated tissue the excess molar glycerol synthesis is about 50 times that of fatty acids, the excess after insulin stimulation is only about ten-fold (105). These observations are of considerable interest, especially in view of the finding that insulin, in the absence of added glucose, can inhibit the lipolytic process (115, 116, 117). Under these circumstances, adipose tissue triglyceride would not be a significant source of free fatty acids, re-esterification would not occur to any appreciable extent, and, consequently, α - glycero-phosphate would not be needed for this purpose.

Thyroid hormones, furthermore, enhance the capacity of adipose tissue to respond to insulin (118). The maximal effect of insulin in vitro on glucose uptake and on excess CO_2 production by adipose tissue from hyperthyroid rats, is twice as great as its effect on adipose tissue from normal rats (118). The action of insulin to depress free fatty acid release is about 6 times greater in tissue from hyperthyroid rats than in tissue from normal rats, and about one and one half times as great as in tissue from fasted animals (118).

White adipose tissue is considered to be a major target of insulin action. The site of this action has been a controversial subject. MacLeod et al. (119) noted that insulin causes a decrease in intracellular glucose with a concomitant rise

in glucose-6-phosphate concentration. On the basis of these findings, they have concluded that the action of insulin is to stimulate glucose phosphorylation rather than transport. Using sorbitol-C^{14} as the extracellular marker, Kahlenberg and Kalant (120) have observed that in diabetic human omentum and in normal rat fad pad, transport is the rate-limiting step in glucose utilization, while in normal human omentum, insulin stimulates both transport and utilization.

Froesch and Ginsberg (121) have observed that fructose metabolism is not inhibited by equimolar amounts of glucose except at very high concentrations and in the presence of insulin. Since the affinity of adipose tissue "hexokinase" * is thought to be much greater for glucose than for fructose, these authors have deduced that there can be little intracellular free glucose at physiological concentrations, and, therefore, that glucose uptake is regulated by glucose transport. Using different criteria, DiPietro (122) and Hernández and Sols (123) have reached a similar conclusion. Finally, Crofford and Renold (124), using sorbitol-C^{14}, have shown that the transport of glucose from the extracellular to the intracellular space, is the major rate-limiting step in glucose uptake under conditions of adequate diffusion.

b) Epinephrine, Norepinephrine and ACTH.

Epinephrine increases the uptake of glucose by

* Written in quotation marks because this term does not differentiate between the two enzymes that phosphorylate glucose, but refers to total phosphorylating activity.

adipose tissue (107, 125, 126). This effect is similar to that of insulin. This catecholamine also increases the oxidation of both carbon 1 and carbon 6 of glucose (126). In contrast to insulin, however, it enhances the oxidation of carbon 6 relative to that of carbon 1 (107, 126).

Epinephrine, moreover, brings about a marked promotion of glyceride-glycerol synthesis (107, 126). When both insulin and epinephrine are present in the incubating medium, the production of α - glycerophosphate, as measured by glyceride-glycerol formation is augmented by 70% , while the glycerol released from triglycerides increases to about the same extent (102).

The effect of epinephrine on fatty acid synthesis is not as clear. Cahill et al. (126) have reported that the hormone does not influence this process in vitro. Lynn et al. (107), however, have observed an inhibition of fatty acid synthesis, whereas Flatt and Ball (102) have found that when both insulin and epinephrine are used, this catecholamine causes an additional increment in fatty acid synthesis of 11% over that observed when insulin is used alone. These authors attribute this finding to a greater availability of coenzymes needed for the production of acetyl CoA, provided that the supply of this thiolester is a rate-limiting factor for fatty acid synthesis under these conditions. The fact that more α - glycerophosphate and lactate are formed when both insulin and epinephrine are present than when insulin is used alone (102, 126), suggests that acetyl CoA may be the rate-limiting factor in this situation. Indeed, when α - glycerophosphate and lactate are synthesized, reduced coenzymes are reoxidized, leading to a greater production of acetyl CoA (102). This metabolite may, then, be

channeled into fatty acid synthesis.

As opposed to unstimulated tissue, in the presence of epinephrine, carbon 1 of glucose is recovered almost quantitatively in glyceride-glycerol (107, 126). This observation suggests that augmentation of glycerol synthesis results in a proportionately decreased equilibration of triose phosphates (107, 126). The fact, moreover, that under the influence of epinephrine, a smaller fraction of glucose-6-phosphate is metabolized by the HMP, would favour a greater recovery of carbon 1, relative to carbon 6, in glycerol.

Other hormones, such as, norepinephrine (126), corticotropin (107), thyroid-stimulating hormone (127, 128), and glucagon (129), have qualitatively the same effect on the metabolism of glucose as epinephrine.

As will be described in section I.-(F), these hormones accelerate the hydrolysis of adipose tissue triglyceride and, thus, increase the concentration of tissue and medium free fatty acids. Since the addition of fatty acids to the incubation medium, elicits alterations in glucose metabolism qualitatively similar to those induced by the lipolytic hormones, it has been proposed that the changes in glucose metabolism observed in their presence, are secondary to the elevated fatty acid levels resulting from accelerated lipolysis (126). It has also been suggested that the increased free fatty acids act as acceptors for α - glycerophosphate, resulting in a depletion of the intracellular concentration of glucose-6-phosphate, which is an inhibitor of glucokinase (106). By this means, an accelerated phosphorylation of glucose could be achieved. An increase in glucose metabolism by adipose

tissue, however, would not be observed if the transport of this sugar from the extracellular to the intracellular space were rate-limiting, unless the entry of glucose were also altered in addition to depletion of glucose-6-phosphate (106). It has, thus, been postulated that like insulin, these lipolytic hormones may facilitate the transport of glucose.

3) Metabolism of α - Glycerophosphate.

Unlike liver (130, 131), kidney (131, 132, 133), and small intestinal mucosa (134, 135), which have an active glycerokinase, adipose tissue from normal mammals cannot use ATP to phosphorylate glycerol (75, 80, 131). Indeed, free glycerol is metabolized only very slowly by intact adipose tissue (107, 136, 137, 138), and α - glycerophosphate is an absolute requirement for triglyceride synthesis (75, 80).

As previously described, α - glycerophosphate is formed during the operation of the glycolytic pathway. It has also been demonstrated (75) that homogenates of adipose tissue can form α - glycerophosphate by a transaldolase exchange reaction. This reaction has been studied in bacteria, yeast, and mammalian tissue (139, 140, 141, 142). The particular form of the transaldolase exchange reaction which has been suggested to occur in adipose tissue (75), is as follows:

Fructose-6-P + D-Glyceraldehyde $\rightleftharpoons$ Glyceraldehyde-3-P + D-Fructose

This reaction is probably of minor quantitative significance.

α - Glycerophosphate may also be derived, to a limited extent, from non-carbohydrate sources. In fact, phosphoglyceric acid kinase and glyceraldehyde phosphate dehydrogenase

activity, as well as conversion of isotopic pyruvate into glyceride-glycerol, have been demonstrated in adipose tissue (143).

Adipose tissue homogenates, furthermore, can form α - glycerophosphate by transphosphorylation under certain in vitro conditions (75). Glycerol, has been shown to accept the phosphate moiety of phenyl phosphate with the production of α - glycerophosphate. ATP, inorganic phosphate, or glucose-6-phosphate cannot replace phenyl phosphate. The optimal pH for this transphosphorylation reaction is about 5.5. The pH characteristics and patterns of inhibition have suggested that the reaction is mediated by a microsomal α - glycerophosphate phosphatase. A transphosphorylation of glycerol by pyrophosphatase has also been noted. The optimal pH for this reaction is 5.2. Pyrophosphate is formed in adipose tissue during the activation of fatty acids. The significance of these transphosphorylation reactions, however, is not clear. Large amounts of glycerol are needed for the formation of α - glycerophosphate by these alternate pathways. Indeed, the evidence which has been accumulated up to the present time, indicates that, in adipose tissue α - glycerophosphate is formed chiefly from dihydroxyacetone phosphate, an intermediate of the glycolytic pathway. It has been suggested that under conditions of glucose deficiency, the concentration of α - glycerophosphate in adipose tissue, becomes rate-limiting for the synthesis of neutral lipids (144).

Hydrolysis of α - glycerophosphate by adipose tissue homogenates has been observed under certain in vitro conditions. An alkaline phosphatase which cleaves glycerophosphate has been found in the subcutaneous adipose tissue of man (145) and in the epididymal fat pad of the rat (146, 147). While detecting a low level of alkaline

phosphatase activity in rat adipose tissue homogenates, Margolis and Vaughan (75) have assayed a much more active acid phosphatase. Its optimal activity is at a pH of about 5.5, and it is located mainly in microsomes and in the soluble fraction. The microsomal phosphatase, unlike the soluble enzyme, hydrolyzes α - glycerophosphate more rapidly than β-glycerophosphate; in addition, it is inhibited by a concentration of linoleate that does not alter the activity of the soluble enzyme (75). While α - glycerophosphate phosphatase activity is observed under alkaline and acid conditions, α - glycerophosphate is not broken down at neutral pH, a condition necessary for the synthesis of this compound by adipose tissue homogenates (75). Furthermore, the concentration of α - glycerophosphate in adipose tissue is low, being about 0.1 to 0.2 μmole/Gm tissue wet weight. It is believed, in fact, that under physiological conditions, most of the glycerol is generated by the lipolysis of adipose tissue glycerides, rather than by hydrolysis of α -glycerophosphate. In support of this view, it has been demonstrated that when adipose tissue is incubated with isotopic glucose, the glycerol appearing in the medium has a very low specific radioactivity (107). This finding is consistent with the concept that most of the glycerol is liberated by hydrolysis of stored, unlabelled glycerides. Hydrolysis of α - glycerophosphate does not appear to be of physiological significance.

4) Glycogen Cycle.

Even though the glycogen cycle is considered to be a minor pathway of carbohydrate metabolism in adipose tissue, it is of importance because glycogen is an efficient precursor of the

glycerol and fatty acid moieties of adipose tissue triglycerides (11, 148, 149). The known pathways of glycogen synthesis and breakdown in fat tissue are the same as those described for other tissues (62, 63). The glycogen content has been reported to be about 0.2 mg/g wet weight in tissue from fed rats (61, 63). Glycogen disappears rapidly on fasting and accumulates readily on refeeding, reaching levels several times greater than those found under conditions of normal nutrition (63, 150, 151). The high concentrations of glycogen in tissues from refed animals are, in turn, associated with enhanced lipogenesis (148, 150, 152).

The enzymes of the glycogen cycle, namely, phosphoglucomutase, UDPG-pyrophosphorylase, UDPG-glycogen transglucosylase, and glycogen phosphorylase have been assayed in adipose tissue (62, 63).

Since in fed animals, UDPG-glycogen transglucosylase is much less active than the other enzymes involved in the synthesis of glycogen, it is considered to be the rate-limiting enzyme under basal conditions (62). The fact that in fed rats, glycogen phosphorylase activity is about 10-fold that of UDPG-glycogen transglucosylase (62), may be an important reason for the rapid turnover of glycogen in adipose tissue. In addition, the high ratio of enzymic activity to product, may be related to such a turnover (153), and may explain the low net incorporation of isotopic glucose into glycogen (105).

In spite of the almost total disappearance of glycogen during a period of prolonged fasting, UDPG-glycogen transglucosylase activity is not abolished (62, 63). The persistence of an appreciable level of this activity, may explain why there is no decrease, <u>in vitro</u>, in the incorporation of radioactive glucose into adipose tissue

glycogen, following a protracted period of fasting (89).

Such a nutritional manipulation, moreover, decreases the activity of all the other enzymes of the glycogen cycle. Gutman and Shafrir (63) have proposed that since the relatively prompt restoration of their activity upon refeeding, probably precedes that of the various enzymes of glycolysis and fat synthesis (89, 154), the enhanced incorporation of glucose into glycogen during this nutritional state may be partly due to decreased utilization of glucose by other metabolic pathways.

The main endocrine factor in the formation of adipose tissue glycogen is insulin (155, 156, 157). Glycogen breakdown, on the other hand, is influenced by a number of hormones. Indeed, catecholamines, corticotropin, thyroid-stimulating hormone, glucagon, and vasopressin have the ability to stimulate glycogen phosphorylase activity (73, 158). Syclic AMP is the probable mediator of the hormonal effects on the phosphorylase activity (158). The effect of vasopressin is inhibited by PGE_1 (158), a prostaglandin isolated by Bergström and Sjövall (159). Its mechanism of action remains to be elucidated.

In spite of recent information, many of the mechanisms involved in the control of the glycogen cycle are still obscure. In fact, a few investigators have postulated the existence of a pathway from glucose to glycogen, independent of UDPG-glycogen transglucosylase (160), but closely linked to inducible glucokinase levels (62). This postulate has not been confirmed by definite experimental evidence up to the present time.

5) Quantitative Assessment of the Synthesis of Lipids and Glycogen from Glucose.

When epididymal adipose tissue from fed rats is incubated with uniformly labelled glucose, 44% of the glucose carbon is recovered in glyceride-glycerol, 13% in tissue fatty acids, 1% in glycogen, and 42% in CO_2 (105). Landau and Katz (108) have reported a similarly low incorporation of isotopic glucose into glycogen. When glucose-1-C^{14} and glucose-6-C^{14} are used, the ratios of recovered glucose carbon 1 to glucose carbon 6 in glyceride-glycerol and fatty acids are approximately 0.8 (105) and 0.5 (58, 105), respectively. Cahill et al. (105) have suggested that the difference in ratios may be due to incomplete equilibration at the triose phosphate level (106, 107).

Lynn et al. (107) have also proposed that the transaldolase exchange reaction, which favours the utilization of hexose carbons 4, 5, and 6 for fatty acid synthesis, relative to carbons 1, 2, 3, may partly explain the relatively greater recovery of carbon 1 in glycerol as compared to its recovery in fatty acids (105).

Cahill et al. (105), furthermore, have emphasized the prominent role of glyceride-glycerol synthesis in adipose tissue incubated without added insulin. About three times as much glucose carbon is recovered in glycerol as in fatty acids. Assuming a mean fatty acid chain length of 17 carbon atoms, 17 moles of glycerol are produced for each mole of fatty acid. Since 1 mole of glycerol, in the form of α - glycerophosphate, can be esterified with 3 moles of fatty acid, the excess molar glycerol synthesis is theoretically 51 times that of fatty acids (105). Since the glycerol formed by lipolysis cannot be re-esterified (75, 80, 131), α - glycerophosphate

is essential for esterification with fatty acids synthesized in adipose tissue, de novo, and for re-esterification with free acids which have been hydrolyzed from adipose tissue triglyceride. In vivo, moreover, α - glycerophosphate is required for esterification of fatty acids, which are derived from plasma triglyceride. Hence, α - glycerophosphate must be supplied in excess of fatty acids in order to maintain the equilibrium between synthesis and hydrolysis of adipose tissue triglyceride.

6) Summary of the Significance of Carbohydrate Metabolism in Adipose Tissue.

In summary, the pathways of carbohydrate metabolism in adipose tissue are similar to those described in other tissues. One notable difference is the inability of fat tissue to participate in the process of gluconeogenesis.

Carbohydrate is the major source of α - glycerophosphate. In addition, it gives rise, via the glycolytic pathway, to acetyl CoA, a building block for fatty acid synthesis. Thus, the carbon atoms of glyceride-glycerol and of fatty acids synthesized de novo, are derived almost entirely from carbohydrate sources. Reduced cofactors which are obligatory for fatty acid synthesis are also formed during the oxidation of glucose.

D) ACCRETION OF LIPID IN ADIPOSE TISSUE.

Accumulation of fat in adipose tissue is the resultant of two processes. One is assimilation of plasma lipid and the other is in situ synthesis. As previously discussed, the lipid that accumulates as a result of both processes is almost entirely triglyceride.

1) Lipoprotein Lipase and the Assimilative Function of Adipose Tissue.

The process of assimilation involves the incorporation of circulating triglyceride-fatty acid into fat tissue. The quantitative importance of this process as a source of adipose tissue lipid, has not been evaluated, but it is likely that it plays a significant role in this regard. A lipase, distinct from the enzymes involved in the hydrolysis of stored adipose tissue triglyceride, is concerned with the uptake of plasma triglyceride-fatty acid, and has been termed lipoprotein lipase (161). The characteristics of this enzyme and its role, in vivo, will now be discussed briefly.

a) Properties of Lipoprotein Lipase.

As implied by its name, lipoprotein lipase hydrolyzes lipoprotein-triglyceride complexes much more readily than plain triglyceride emulsions (161, 162). Korn (161, 163) has demonstrated in a convincing manner that this enzyme is the same as clearing factor, the lipase which appears in the plasma after heparin injection and removes the lactescence caused by alimentary lipemia (164, 165, 166).

In vitro, lipoprotein lipase, which has an optimal pH of 8.5, is activated by ammonium ion, divalent ions, and low concentrations of heparin (161, 167, 168, 169, 170). This enzyme, on the other hand, is inhibited by polyanions (heparin in high concentrations), polycations (protamine), diisopropylfluorophosphate, sodium pyrophosphate, and relatively high concentrations of sodium chloride (161, 167, 168, 169, 170). The inhibition of lipoprotein lipase activity by protamine (161, 171) suggests that this enzyme may be a mucoprotein with a heparin-like prosthetic group. It is possible that the inhibition of this activity by 4-arsonophenylazoproteins, which have a structure similar to that of heparin, is due to displacement of the latter from its combination with the protein moiety of the enzyme (172).

Mammalian heart and adipose tissue are the main sites of origin of lipoprotein lipase (161, 167). Slices of these tissues release this factor when incubated in the presence of heparin (168, 169, 173, 174), a process analogous to that observed in vivo. It is possible that heparin liberates the enzyme by competing for tissue binding sites with the heparin-like moiety of the lipoprotein lipase molecule.

The almost instantaneous appearance of lipolytic activity in plasma after in vivo heparin injections, has led some investigators to suggest that this enzyme is located, at least in part, inside the walls of blood vessels (175, 176, 177). After separating adipose cells from stromal-vascular cells with collagenase, Rodbell (178) has found all of the lipoprotein lipase activity in rat parametrial adipose tissue, to be associated with the fat cells, and has suggested that the enzyme is synthesized by them.

He has proposed, however, that these findings do not rule out the possibility that it is secreted or released by adipose cells, and that it hydrolyzes lipoprotein-triglyceride at the surface of capillary cells.

Actinomycin D at concentrations that inhibit deoxyribonucleic acid synthesis, causes a marked and sustained rise of lipoprotein lipase activity (179). This effect is observed both in vitro and in vivo, and is not abolished by fasting. Since puromycin prevents this activation, it has been suggested that the enhanced activity is due to formation of new enzyme. It has also been proposed that the synthesis of lipoprotein lipase occurs on a stable ribonucleic acid template, and its destruction is mediated through the action of an unstable, actinomycin-sensitive form of ribonucleic acid.

b) Assimilation of Plasma Lipid by Adipose Tissue.

A number of investigators have studied the process of assimilation by injecting triglyceride, labelled in both the glycerol and fatty acid moieties, into experimental animals (180, 181, 182). The results of these experiments have shown that, while most of the triglyceride leaves the circulation without prior hydrolysis, rapid splitting of this molecule occurs within adipose tissue and heart. The fatty acids which are liberated in this way, are retained by adipose tissue and re-esterfied with α - glycerophosphate formed in situ. Glycerol, on the other hand, is lost from this tissue because of the absence of glycerokinase activity.

The incorporation of plasma triglyceride-fatty acid by adipose tissue is profoundly influenced by the nutrit-

ional state of the animal. This process is enhanced by refeeding and decreased by fasting (183, 184). It is of considerable interest that these changes parallel alterations in lipoprotein lipase activity (168, 169, 170). In fact, Bezman (185) has demonstrated, in vitro, a striking correlation between the ability of rabbit adipose tissue slices to incorporate triglyceride-fatty acid during different nutritional states, and the activity of lipoprotein lipase.

In contrast to adipose tissue, when chylomicrons, labelled in the triglyceride moiety with palmitic acid-1-C^{14}, are injected intravenously into rats, a greater amount of radioactivity is recovered in heart muscle during fasting than during refeeding (183). This observation is consonant with the finding that during fasting, a period during which the myocardium derives most of its energy from oxidation of fatty acids (186, 187), the activity of rat heart lipoprotein lipase is increased (173, 188, 189), while that in adipose tissue is decreased (168, 169, 177, 185). In addition, induction of diabetes mellitus, enhances this activity in heart (190), and depresses it in adipose tissue (190, 191).

In view of the specificity of lipoprotein lipase for lipoprotein-triglyceride, it is tempting to assign to this enzyme, the rate-limiting role during the process of assimilation. It is possible, however, that this process is controlled at the stage of esterification of the fatty acids liberated by the hydrolytic reaction catalyzed by lipoprotein lipase. Indeed, the rate of esterification in adipose tissue is also reduced by fasting and enhanced by refeeding (136). Regardless of which of these two processes is rate-limiting, the fact that they change accordantly, leads to the appropriate distribution of energy during different nutritional

states.

Hence, during the process of assimilation lipoprotein-triglyceride interacts with lipoprotein lipase, which is possibly siturated at or near capillary walls in adipose tissue. The fatty acids which are released by hydrolysis, create a localized area of high FFA concentration, and can traverse readily the basement membrane of the endothelial cells. After reaching the adipose cells, the free acids are esterified to form, to a major extent, triglyceride.

2) Synthesis of Fatty Acids in Adipose Tissue.

The second process by which fat accumulates in adipose tissue involves the in situ synthesis of α - glycerophosphate and fatty acids. These fatty acids, and those derived from plasma lipoproteins, are, then, esterified to form new triglyceride. The synthesis of α - glycerophosphate has been discussed in Section I - (C). The synthesis of fatty acids in adipose tissue will be dealt with briefly in this section.

Adipose tissue is capable of synthesizing fatty acids more efficiently than liver (19, 20, 22). This conclusion can be reached by calculating the results with respect to wet weight and tissue protein content. In fact, in vivo experiments have shown that the quantity of fatty acids synthesized by mammalian adipose tissue probably exceeds that formed by all other tissues combined (22, 192).

a) Mechanisms of Fatty Acid Synthesis.

The enzymes that synthesize fatty acids are present in the "particle-free" supernatant of homogenates of adipose tissue (81, 193, 194, 195, 196, 197). The recognition that malonyl coenzyme A is the critical intermediate in the synthesis of long-chain fatty acids from acetyl CoA (198, 199, 200) has confirmed previous evidence that the pathways of synthesis and oxidation of fatty acids are different. The over-all biosynthetic sequence of fatty acid synthesis may be resolved into two constituent reactions:

(1) $$\text{Acetyl CoA} + \text{ATP} + CO_2 \xrightarrow{Mn^{++}} \text{Malonyl CoA} + \text{ADP} + P_i$$

(2) $$\text{Acetyl CoA} + 7 \text{ moles Malonyl CoA} + 14 \text{ NADPH} + 14H^+ \longrightarrow \text{Palmitate} + 14 \text{ NADP}^+ + 7 CO_2 + 8 \text{ CoA-SH} + 6H_2O$$

The first reaction, the carboxylation of acetyl CoA to form malonyl CoA, is catalyzed by the biotin enzyme, acetyl CoA carboxylase. It has been isolated from a variety of plants and animal tissues (198, 200, 201, 202). Acetyl CoA carboxylase from adipose tissue has been purified 70-fold (202, 203). The activity of this enzyme, moreover, is depressed by avidin, which is a specific inhibitor of biotin (202). The carboxylation of acetyl CoA to form malonyl CoA has been found to be the rate-limiting reaction in the synthesis of fatty acids from acetyl CoA (81, 82, 202).

In E. coli the responsible enzyme system has been separated into a heat-stable and a heat-labile fraction, both of which are required for the synthesis of palmitic and cis-vaccenic acids (204, 205, 206). The activity of the heat-stable fraction resides in a protein with a molecular weight of about 9400 (207, 208, 209).

This protein has a 2-mercaptoethylamine residue, and, presumably, it is covalently linked to acyl groups of the substrates for the fatty acid synthesizing enzymes through the sulfhydryl group of this moiety (209). Hence, it has been named acyl carrier protein (ACP-SH).

Present evidence indicates that the biosynthetic sequence leading to long-chain fatty acids in E. coli is as follows:

$$\underset{\text{Acetyl CoA}}{CH_3COSCoA} + ACP\text{-}SH \rightleftharpoons \underset{\text{Acetyl-S-ACP}}{CH_3COSACP} + CoA\text{-}SH$$

$$\underset{\text{Malonyl CoA}}{HOOCCH_2COSCoA} + ACP\text{-}SH \rightleftharpoons \underset{\text{Malonyl-S-ACP}}{HOOCCH_2COSACP} + CoA\text{-}SH$$

$CH_3COSCoA$ + $HOOCCH_2COSACP \rightleftharpoons CH_3COCH_2COSACP + ACP\text{-}SH + CO_2$
Acetyl-S-ACP Malonyl-S-ACP Acetoacetyl-S-ACP

$CH_3COCH_2COSACP + NADPH + H^+ \rightleftharpoons CH_3CHOHCH_2COSACP + NADP^+$
Acetoacetyl-S-ACP D(-)-β-Hydroxybutyryl- S-ACP

$CH_3CHOHCH_2COSACP \rightleftharpoons CH_3CH=CHCOSACP + H_2O$
D(-)-β-Hydroxybutyryl-S-ACP Crotonyl-S-ACP

$CH_3CH=CHCOSACP + NADPH + H^+ \longrightarrow CH_3CH_2CH_2SCOACP$
Crotonyl-S-ACP Butyryl-S-ACP

Initially, the acyl groups of acetyl CoA and malonyl CoA are transfered by specific transacylases to this protein, and, then, the protein-bound intermediates condense to form acetoacetyl-S-ACP with the concurrent loss of CO_2. The protein-bound acetoacetate is reduced in the presence of NADPH by β-ketoacyl-ACP reductase, an enzyme which is relatively specific for ACP-SH thiolesters, to form D(-)-β-hydroxybutyryl-S-ACP (210). This compound is, then, acted upon by an enoylhydrase, which is specific for acyl carrier protein thiolesters, to form crotonyl-S-ACP (210, 211). The latter is, then, reduced in the presence of NADPH to form butyryl-S-ACP (208, 209). The sequential steps leading to this compound have been shown above.

It is thought that this thiolester condenses, in turn, with another molecule of malonyl-S-ACP to start six more identical cycles (208, 209). Thus, a total of 14 carbon atoms are added to the initial two carbons of acetyl CoA to form, eventually, palmitic acid. All of the reactions in which the fatty acyl chain is elongated, reduced, dehydrated, and, again reduced, appear to occur while the chain is in acyl linkage to acyl carrier protein (209). Indeed, the intermediates β-hydroxydecanoyl-S-ACP, β-hydroxylauryl-S-ACP, β-hydroxymyrystyl-S-ACP, and palmityl-S-ACP have been isolated (208). The latter is presumably

hydrolyzed by a specific deacylase to form palmitic acid and ACP-SH (209). An additional cycle of reactions is believed to lead to cis-vaccenic acid.

ACP-SH has been isolated from E. coli, but not from avian or mammalian systems. Similar reactions, however, seem to occur in pigeon liver (212, 213, 214). In fact, protein-bound acetate, malonate, acetoacetate, and butyrate have been isolated. In addition, protein-bound β-ketohexanoate, laurate, myristate, palmitate, and stearate have been isolated during the formation of free palmitic and stearic acids in this avian system.

Intact rat adipose tissue, furthermore, is capable of desaturating stearic acid with the formation of oleic acid (215). While synthesis of fatty acids de novo occurs in the "particle-free" supernatant of adipose tissue homogenates, the site of the desaturation reaction has been localized to the microsomes (216). This oxygen-dependent reaction is negligible in adipose tissue from alloxan-diabetic rats, whereas de novo synthesis of saturated acids is only partially depressed (215, 217). The defect in desaturation of stearic to oleic acid is corrected by the administration of insulin to the animals before sacrifice.

Thus, the "particle-free" supernatant of adipose tissue can synthesis fatty acids de novo, and the microsomes can desaturate stearic, and probably, palmitic acid, to their monoenoic analogues. There are no reports, however, to indicate that adipose tissue mitochondria have a separate enzyme system for elongation, or de novo synthesis of fatty acids. Such a system has been described in mitochondria from avian and mammalian liver (218, 219, 220).

b) Control of Fatty Acid Synthesis.

Certain tricarboxylic acid cycle intermediates, notably citrate and isocitrate stimulate fatty acid synthesis in avian and mammalian tissues (92, 202, 221, 222, 223, 224, 225, 226). In adipose tissue, citrate is the most effective intermediate (202). It stimulates acetyl CoA carboxylase, but has no effect on palmitate synthetase. When the former is pretreated with citrate, its activity is enhanced when the other components of the reaction are added. Using the technique of sucrose gradient centrifugation (227), Vagelos et al. (203) have observed a difference in sedimentation constant between citrate-pretreated and untreated enzyme. These findings have suggested that, with activation of the enzyme, monomers aggregate to form trimers. The physiological significance of the effect of citrate on fatty acid synthesis is unknown.

It is well known, moreover, that fatty acid synthesis from both glucose and acetate in intact mammalian adipose tissue is depressed by a period of fasting (18, 89, 228). It has been proposed that NADPH may become limiting under these conditions (92, 93, 99, 229, 230). The correlation between the activity of the hexose monophosphate shunt dehydrogenases, an important source of NADPH, and the synthesis of fatty acids, has been discussed in Section I.-(C).

Decreased activity of acetyl CoA carboxylase occurs in subcellular preparations of liver from fasted (82) and diabetic animals (83, 231). The depressed activity of this enzyme may be due to a deficiency of citric acid cycle intermediates under these conditions. In fact, the concentration of these intermediates is greatly lowered in livers of diabetic rats (232). Acetyl CoA carboxylase from rat liver, moreover, is inhibited by palmityl CoA,

in vitro (233), and it is possible that this mechanism has some physiological significance. It is conceivable, moreover, that the levels of citric acid cycle intermediates are lowered and those of long-chain acyl CoA derivatives are elevated in adipose tissue of fasted rats. Conditions such as these may be responsible, at least in part, for the depression of fatty acid synthesis which has been noted in intact tissue.

Thus, adipose tissue is an active site of de novo synthesis of fatty acids. It can also desaturate stearic acid with the formation of oleic acid. While acyl carrier protein plays an important role in bacterial fatty acid synthesis, it has not been isolated from mammalian systems up to the present time. The carboxylation of acetyl CoA to form malonyl CoA is the rate-limiting step in the synthesis of fatty acids. Acetyl CoA carboxylase, the enzyme that catalyzes this reaction, is activated by citric acid cycle intermediates.

In this section, two aspects of fat accretion in adipose tissue were discussed briefly. The first part dealt with the assimilation of plasma lipid by this tissue. The second summarized the mechanisms and control of the in situ synthesis of fatty acids.

E) ESTERIFICATION OF FATTY ACIDS AND BIOSYNTHESIS OF TRIGLYCERIDE.

The final biochemical process in fat accretion involves the esterification of free fatty acids and α - glycerophosphate to form, almost exclusively, triglyceride. The free acids that are esterified are derived from plasma triglyceride and from de novo synthesis in adipose tissue.

The pathway of triglyceride biosynthesis was first elucidated in liver (235, 236, 237, 238, 239, 240); subsequently, a similar pathway was delineated in adipose tissue (79, 80). The enzymatic reactions leading to the formation of triglyceride and certain phospholipids, are schematized in Fig. II. Because of the relevance to the experimental work carried out in the present study, each reaction will now be described in detail.

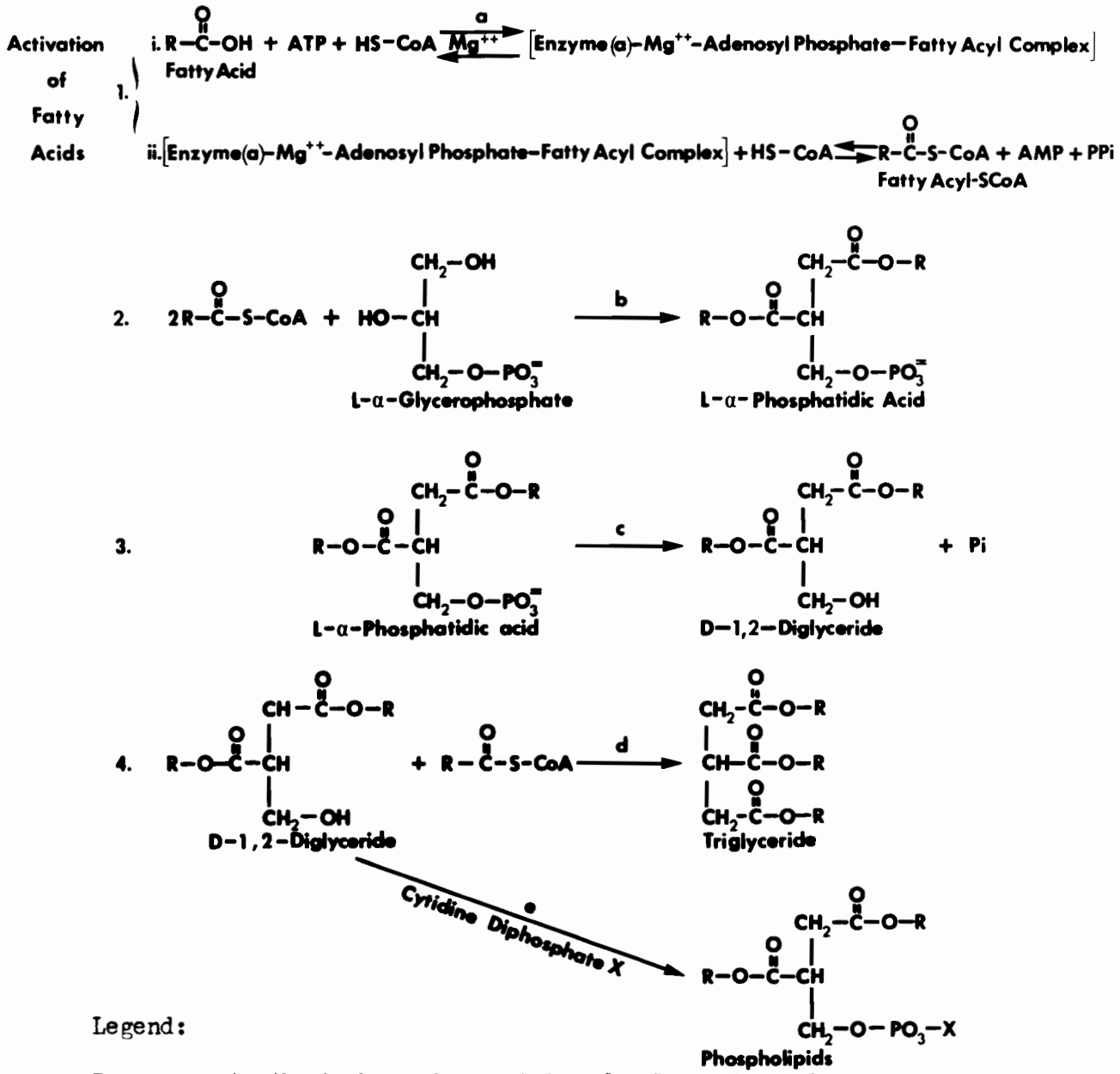
1) Pathways of Triglyceride Synthesis.

a) Activation of Fatty Acids.

Free fatty acids must be activated to their coenzyme A derivatives (Figure II, Reaction 1) before being able to participate in such metabolic reactions as chain lengthening, oxidation, or esterification. Having observed that rat liver homogenates require ATP and CoA-SH for the esterification of α - glycerophosphate and fatty acids to produce phosphatidic acids, Kornberg and Pricer (241) postulated that the first step in this process is an activation of fatty acids to their acyl CoA derivatives, analogous to the acetate activation, which had been reported earlier (242, 243). Then, the same investigators found an enzyme in guinea

FIG. 11

Pathways of Triglyceride Biosynthesis



Legend:

R represents the hydrocarbon moiety of a long-chain fatty acid.

a) Fatty acid: CoA ligase (AMP) [6.2.1.3.]
b) Acyl CoA: L-glycerol-3-phosphate acyl transferase [2.3.1.15.]
c) L-α-phosphatidate phosphohydrolase [3.1.3.4.]
d) Acyl CoA: diglyceride acyl transferase
e) Phosphoryl(choline) or (ethanolamine)-diglyceride transferase

x represents one of either choline or ethanolamine

pig liver microsomes and soluble fraction which could catalyze the conversion of long-chain saturated and unsaturated monocarboxylic acids to their CoA thiolesters (244). The latter derivatives were isolated from the reaction mixture along with inorganic pyrophosphate and adenosine monophosphate, which were split from ATP by the enzyme. The acyl CoA derivatives were converted to the hydroxamic acid derivatives of the fatty acids (245), treated with the ferric chloride Reagent A of Hill (246), and estimated spectrophotometrically.

As shown in Fig. II, activation of fatty acids proceeds in two stages. At first, an acyl adenylate derivative is formed. This compound is, then, believed to react with CoA-SH to produce an activated acyl CoA thiolester. The over-all activation reaction is reversible, requires magnesium ions to bind phosphate groups, and in the analogous case of acetate, synthetic acetyl adenylate can replace ATP and acetate (247, 248). During the reaction, however, free acetyl adenylate cannot be shown to accumulate in the absence of CoA-SH, so that an intermediate enzyme complex with the reactants has been postulated (249, 250). These points have been reviewed by Cornforth (251) and are believed to be equally operative during the activation of long-chain fatty acids. In addition, a portion of the energy liberated by the pyrophosphate cleavage of ATP is used to form the fatty acyl CoA derivative, which is chemically much more reactive than the fatty acid and is water-scluble at neutral pH because of the many polar groups on the large CoA-SH moiety (252).

The enzyme which activates fatty acids to their acyl CoA derivatives has been termed fatty acyl-CoA synthetase (253), acyl-CoA thiokinase (254), and, officially, fatty acid: CoA

ligase (AMP) (6.2.1.3) (255).

The presence of an ATP-dependent activating system for long-chain fatty acids has also been demonstrated directly in adipose tissue (79, 256). By measuring the quantity of acyl hydroxamate formed, Rose and Shapiro (79) have found that palmitate and oleate are activated more rapidly than stearate and myristate. Activation of fatty acids with a chain length shorter than C14 is negligible. The fact that only higher fatty acids are activated, exemplifies the specialization of adipose tissue in long-chain fatty acid metabolism. Rose and Shapiro (79), furthermore, have observed that activation of fatty acids in rat adipose tissue, takes place almost entirely in a subcellular fraction which they have termed "acid particles" because their preparation takes place at pH 5.6-5.8. "Acid particles" are probably a microsome-rich fraction.

Activation of fatty acids has also been demonstrated in particulate fractions of small intestinal mucosa (257, 258, 259, 260). The highest specific activity of the fatty acid: CoA ligase is in the microsomal fraction (258, 260). This enzyme, moreover, is more active in microsomes from duodenum and jejunum than in those from the ileum, and it is much less active toward fatty acids of less than 12 carbons (258, 259).

Thus, the first step in glyceride biosynthesis is the activation of fatty acids to fatty acyl CoA derivatives. ATP provides the energy required for this process.

b) Acylation of α -Glycerophosphate.

The next process in the biosynthesis of triglyceride is the acylation of α -glycerophosphate to form diacyl glycerophosphate (phosphatidic acid) (Fig. II, Reaction 2). The

enzyme which catalyzes this reaction has been officially named acyl CoA:L-glycerol-3-phosphate acyl transferase (2.3.1.15). In their pioneer work, Kornberg and Pricer (241) found that a particulate enzyme preparation from rat liver catalyzes the formation of "phosphorus-containing lipid substances" from α - glycerophosphate and stearate, in the presence of ATP and CoA-SH. They tentatively designated these compounds as monostearylphosphatidic acid and distearylphosphatidic acid. These investigators, moreover, postulated that the former might be produced by the acylation of α - glycerophosphate with one mole of stearyl CoA; the newly formed compound would, in turn, be acylated with another mole of stearyl CoA to yield the diacylglycerophosphate. Kennedy (261) also obtained preliminary evidence for the synthesis of phosphatidic acid by rat liver mitochondria. Subsequently, Kornberg and Pricer (262), using guinea pig liver microsomes, confirmed the fact that diacylglycerophosphate is synthesized from α - glycerophosphate and 2 moles of a higher fatty acid. They were unable, however, to obtain convincing evidence for the formation of the monoacyl derivative, and ascribed the presence of the latter in their previous experiments to the acid lability of these phosphatides. In spite of the lack of definitive evidence, however, Kornberg and Pricer were of the opinion that monoacylglycerophosphate was most probably an intermediate. The diacyl derivative was purified by precipitation of the sodium salt from alcohol and subsequent chromatography on a Dowex anion exchange resin. Proof was also obtained for the presence of two ester bonds per mole of phosphate. Palmitic, margaric (17:0), stearic, oleic, and linoleic acid were esterified more rapidly than other long-chain fatty acids. Finally, palmityl-CoA could substitute for palmitate, ATP, and CoA-SH.

Stansly (263), moreover, has confirmed the synthesis of dipalmityl phosphatidic acid from ∝ - glycerophosphate and palmityl CoA by guinea pig liver microsomes; in addition, he has devised a simple assay system for the transacylase reaction. This assay is based on the spectrophotometric determination of the acid-soluble CoA-SH which is liberated during the reaction. The distinction between the CoA-SH formed by this reaction and that resulting from the enzymic deacylation of palmityl CoA is made possible by working at pH 7.5, which is optimal for synthesis of diacylglycerophosphate. Hydrolysis of palmityl CoA proceeds optimally at pH 8.75. Sulfhydryl compounds such as glutathione or cysteine are used because they stimulate the synthetic reaction, but do not affect the hydrolytic breakdown of a thiolester. Under these conditions, an increment in the CoA-SH liberated is due to transacylation of ∝ - glycerophosphate and the formation of phosphatidic acid.

While the method described by Stansly, is an excellent and simple means of assaying acyl CoA:L-glycerol-3-phosphate acyl transferase activity when guinea pig liver microsomes are used, it is difficult to apply when the increment of CoA-SH formed, in the presence of ∝ - glycerophosphate, is very small. Such a situation exists when rat liver microsomes and mitochondria from many sources are the enzyme preparations (264). Brandes et al. (264) have improved the assay system by adding serum albumin to the reaction mixture. This addition lowers the optimal pH from 7.5 to 6.5, depresses palmityl CoA hydrolase activity and prevents inactivation of the transacylase. In the absence of albumin, the transacylase is inactivated only when palmityl CoA is present, i.e., when free fatty acids are liberated. Thus, it seems plausible that albumin protects the enzyme

from the inhibitory action of fatty acids by its well-known ability to bind these acids. The concentrations of fatty acids, however, required for inactivation, when added to the reaction mixture, exceed about tenfold those released by the transacylase. This discrepancy may be due to inadequate dispersion of the fatty acids in the absence of albumin (264).

More recently, Lands and Hart (265) have reported that monoacylglycerophosphate, produced by phosphorylating monoolein, is acylated more rapidly than α - glycerophosphate by microsomal preparations from rat or guinea pig liver. This phenomenon may be due in part to enzyme selectivity and possibly also to easier absorption of the lipoidal acceptor onto the hydrophobic membranes that contain the enzymes. One consequence of this phenomenon is that monoacylglycerophosphate would not be expected to accumulate under physiological conditions, but would be acylated further to form diacylglycerophosphate (Phosphatidic acid). The fact that monoacylglycerophosphate is acylated more readily than α - glycerophosphate may explain why Kornberg and Pricer (262) could not isolate the monoacylated derivative, and why guinea pig liver cytoplasmic particles show very little monoacylglycerophosphate accumulation (266).

In addition, while microsomes from rat liver are less effective than those from guinea pig liver in acylating α - glycerophosphate, they exhibit comparable activity when monoacylglycerophosphate is used (265). Thus, it has been suggested that the first acylation of α - glycerophosphate may be catalyzed by an enzyme different from that concerned with the second acylation and that the first enzyme may be present at a lower concentration in rat liver than in guinea pig liver (265). Up to the present time, however, there has

been no definitive evidence for the existence of two different enzymes. Linoleate, moreover, is more effective than stearate in acylating monoacylglycerophosphate. This finding may be due in part to some relatively indirect rate-limiting aspects such as solubility and the activity of acyl-CoA thiokinase (265).

The problem of whether different fatty acids are esterified more rapidly at the 1 - or 2-position of α - glycerophosphate has been studied using guinea pig liver microsomes by noting the degree of competition for these sites as indicated by the distribution of radioactive acids. Crotalus adamanteus venom phospholipase A, which liberates selectively the fatty acid esterified at the 2- position of diacylglycerophosphate has been a useful tool in exploring this problem (265). The results of these investigations have indicated that linoleate and stearate are incorporated at both the 1- and 2-position in an almost random fashion (265). Thus, the specificities of the transacylase reactions leading to phosphatidic acid are not adequate to provide the specific pattern of fatty acids that is known to occur in tissue glycerolipids.

In adipose tissue, acylation of α - glycerophosphate to produce phosphatidic acid takes place in a manner similar to that found in liver (80). Using subcellular preparations of rat epididymal adipose tissue, phosphatidic acid is the major labelled phospholipid formed when palmitate-1-C^{14} is the radioactive precursor (80). Phosphatidic acid is the only labelled phospholipid detected when either α - glycerophosphate-1-C^{14} or α - glycerophosphate-P^{32} is the radioactive precursor. After three minutes of incubation with α - glycerophosphate-1-C^{14} at 37°C, more than half of the radioactivity in ester form is present in phosphatidic acid. After 30 minutes of

incubation, when the total amount of C^{14} in ester form is much greater, more than 60% of it is in the neutral lipid fraction. These findings are compatible with the sequence of reactions, which is outlined in Fig. II. Equimolar quantities of glycerol, moreover, cannot replace α - glycerophosphate (75, 79, 80).

Thus, α - glycerophosphate is acylated by two moles of fatty acyl CoA to produce phosphatidic acid. In adipose tissue, α - glycerophosphate is the only acceptor of fatty acids. Monoglyceride cannot be acylated directly (80). In some other tissues, however, direct acylation of monoglyceride, a shunt pathway, takes place in addition to the sequence involving α - glycerophosphate and phosphatidic acid. In this way, diglyceride and triglyceride may be formed in the absence of phosphorylated intermediates. Direct esterification of monoglyceride has been demonstrated in small intestinal mucosa (257, 267, 268, 269, 270, 271, 272, 273), kidney (274), liver (274), aorta (275), and lactating mammary gland (276). With the exception of small intestinal mucosa, the monoglyceride acyl transferase system is much less active than the pathway involving α - glycerophosphate and phosphatidic acid.

c) Enzymatic Dephosphorylation of Phosphatidic Acid.

The next step in the biosynthesis of triglyceride is the production of D-1, 2-diglyceride from phosphatidic acid (Fig. II, Reaction 3). This reaction is catalyzed by a specific phosphatase, which has been referred to as phosphatidic acid phosphatase, and officially named, L- α - phosphatidate phosphohydrolase (3.1.3.4).

This enzyme is widely distributed in mammalian tissues. It has been found in the liver, brain, small intestine,

and kidney of rabbit , rat, guinea pig, ox, and pig (277). Its presence has also been demonstrated in the heart and skeletal muscle of rat (237), in the salt gland of the albatross (278), and in erythrocyte membranes (279).

Phosphatidic acid phosphatase was first demonstrated in mitochondrial preparations of rat liver (280). Subsequently, some of its properties were studied using preparations of chicken liver (237), pig kidney (277), and rat adipose tissue (75, 79).

Phosphatidic acid phosphatase is mainly located in subcellular particles. In rat adipose tissue, it is confined almost entirely to the particles (75). Enzymatic activity can be assayed by determining the amount of inorganic phosphate that is liberated (237). In addition, Smith et al. (237) have isolated the D-1, 2-diglyceride which is produced by the action of this enzyme on phosphatidic acid. The optimal pH of the activity derived from chicken liver and pig kidney is around 6.0. Phosphatidic acid phosphatase from rat adipose tissue has an optimal pH of 7.6.

This enzyme is specific for phosphatidic acid. It does not dephosphorylate to any appreciable extent α - glycerophosphate, β-glycerophosphate, glucose-6-phosphate, lecithin, diphosphatidyl glycerol, and phosphatidyl inositoldiphosphate (237, 277).

The chicken liver enzyme is inhibited by such divalent ions as magnesium, calcium, manganese, and barium. The inhibition is believed to be due to formation of insoluble salts at alkaline conditions (237). Pig kidney phosphatidic acid phosphatase, moreover, is inhibited by mercuric, zinc, ferrous, and fluoride ions, but not by calcium and barium ions. Magnesium ions

give only a 25% inhibition at a higher concentration than that required for a complete inhibition of the chicken liver enzyme. These ions do not inhibit adipose tissue phosphatidic acid phosphatase at concentrations that decrease the activity of the chicken liver enzyme. Since p-chloromercuribenzoate inhibits phosphatidic acid phosphatase and excess glutathione reverses this effect, it is probable that thiol groups are essential for enzymatic activity (277).

Coleman and Hübscher (277), moreover, have been unable to obtain soluble phosphatidic acid phosphatase by freezing and thawing, autolysis, or digestion with ribonuclease. They have achieved partial solubilization of the enzyme with deoxycholate and certain butanols. These observations have led them to suggest that the enzyme may be a lipoprotein. In fact, the purified preparation still contains significant amounts of lipid. In addition, enzymatic activity is inhibited by surface-active agents (237, 277). It remains to be shown, however, that the lipid is a constituent of the enzymic structure.

Thus, phosphatidic acid phosphatase catalyzes a rather specific dephosphorylation of phosphatidic acid resulting in the production of D-1, 2-diglyceride. The latter is a key compound in lipid metabolism as it is the immediate precursor of certain phospholipids (237, 280, 281, 282), and of triglyceride (235, 283, 284).

d) Esterification of D-1, 2-Diglyceride.

The final step in the biosynthetic sequence leading to triglyceride is the acylation of D-1, 2-diglyceride (Fig. II, Reaction 4). The enzyme which catalyzes this reaction has been referred to as diglyceride acyl transferase. It has not been purified,

nor officially named. It is probably different from acyl CoA: glycerol-3-phosphate acyl transferase, but the separate nature of the two enzymes has not been definitively demonstrated.

The synthesis of triglyceride from D-1,2-diglyceride and fatty acyl CoA was originally studied using particulate enzyme fractions from chicken liver (235, 283). In these experiments, the newly-formed triglycerides were identified by column chromatography according to the method of Borgström (285). Net formation of triglyceride, rather than an exchange reaction, was proven by the demonstration of an increase in total ester bonds, which were measured as hydroxamates.

The study, in vitro, of the specificity of the transacylase toward diglycerides is made difficult by the insolubility of these lipids in water. For this reason, the available data are only semiquantitative. Microsomal diglyceride acyl transferase catalyzes the synthesis of triglyceride twice as actively when D-1,2-diolein is the substrate than when L-1,2-diolein is present (283). By comparison, phosphorylcholine-glyceride transferase, the enzyme which catalyzes the synthesis of phosphatidyl choline from D-1,2-diglyceride and fatty acyl CoA, is much more specific for the D-enantiomorph. The triglyceride-synthesizing enzyme, moreover, is most active, in vitro, when diglycerides containing fatty acids with shorter or unsaturated chains are used. These findings probably reflect the greater ease of emulsification of these diglycerides. In contrast to this enzyme, phosphorylcholine-glyceride transferase is not active when diglycerides such as DL-1,2-dilaurin and DL-1,2-dioctanoin are tested under the same experimental conditions. These findings suggest that the enzymes catalyzing the synthesis of neutral fat and phospho-

lipids may be specific for diglycerides of different fatty acid composition. Thus, even though D-1, 2-diglyceride is a common intermediate of both triglyceride and certain phospholipids, the fatty acid composition of these two lipid groups, in a given tissue, may be different.

Calcium ions, moreover, do not inhibit the chicken liver diglyceride acyl transferase (283) under conditions in which the synthesis of phosphatidyl choline is completely stopped (282). It is possible that the control of the calcium concentration within the intracellular particles in which these biosynthetic processes take place, may be of physiological significance in channelling diglycerides towards either triglyceride or phospholipid formation. In addition, magnesium and manganous ions stimulate triglyceride synthesis from D-1, 2-diglyceride and fatty acyl-CoA. Calcium and cobaltous ions are less effective in this respect.

The synthesis of triglyceride from diglyceride and acyl-CoA has also been studied in chicken adipose tissue (284). All the enzyme activity is present in the pellet obtained when the defatted homogenate of adipose tissue is centrifuged at 67,000 X g. In general, the best substrates are diglycerides with at least one unsaturated fatty acid, regardless of the position of the acid. In addition, as found using chicken liver preparations (283), diglycerides containing shorter, saturated fatty acid chains are more effective than those with longer, saturated chains (284). More specifically, DL-1, 2-dioleyl glyceride is the best acceptor of 1-C^{14} -palmityl CoA, and DL-1-oleyl-2-palmityl glyceride is the best acceptor for 1-C^{14}-oleyl-CoA. For either thiolester, moreover, DL-1-oleyl-2-palmityl glyceride is more effective than DL-1, 2-dimyristyl glyceride,

which, in turn, is a better substrate than DL-1, 2-dipalmityl glyceride. On the other hand, DL-1, 3-dioleyl glyceride is not an acceptor for either palmityl CoA or oleyl CoA. This finding supports the belief that 1, 2-diglyceride is the precursor of triglyceride. Except for the situation in which D-1-stearyl-2-oleyl glyceride is used, palmityl CoA is a better acyl donor than oleyl CoA.

These results, however, should be interpreted with caution, because, as has been pointed out in the case of the chicken liver system, the degree of emulsification of the various diglycerides may determine the amount of substrate available to the enzyme system. These physical factors, on the other hand, may be operative to a certain extent, in vivo.

Thus, the particulate enzyme system from chicken adipose tissue has a broad range of specificity in the synthesis of triglyceride from acyl CoA derivatives and various 1, 2-diglycerides. The fact that such a wide spectrum of diglycerides may be utilized for the synthesis of the different triglyceride classes is consistent with nutritional experiments in which the depot triglycerides of animals can be considerably altered by varying the composition of the fatty acids in the diet (54). Many other factors, however, including the specificity of other enzymatic systems concerned with fatty acid and diglyceride synthesis will influence the composition of stored triglycerides. In adipose tissue, the synthesis of phospholipids is quantitatively not significant.

Thus, in the biosynthetic pathway leading to triglyceride, the final reaction involves the acylation of D-1, 2-diglyceride by an acyl CoA derivative.

2) Evidence for Active Triglyceride Synthesis vs. an Isotope Exchange Reaction.

It has been shown, in vitro, that lipases can catalyze an exchange reaction between glyceride fatty acids and free fatty acids (286, 287, 288). Experimental evidence will now be briefly discussed indicating that this exchange process is not involved in net glyceride synthesis.

A correlation exists between the ability of intact adipose tissue to take up and esterify FFA, and the nutritional status of the donor animal (136). Esterification is most rapid with tissue from fasted-refed animals and least with tissue from fasted animals. This pattern may be correlated with that of carbohydrate metabolism during these different nutritional states. Glycogen, which disappears from adipose tissue during fasting, accumulates excessively during a period of refeeding. Thus, during this period the supply of α - glycerophosphate is increased both, from glucose directly, and from glucose derived from glycogen. More direct evidence for the role of α - glycerophosphate as an intermediate in glyceride synthesis has been obtained. By the use of pancreatic lipase, which splits triglycerides mainly in the 1 or 3 position, it has been observed that when intact rat adipose tissue is incubated with isotopic FFA, the three fatty acid positions in the newly-formed triglyceride molecule are labelled equally (78). This observation suggests formation of the whole triglyceride molecule rather than an exchange of fatty acids. It also excludes direct esterification or re-esterification of pre-formed tissue diglyceride as an important mechanism of triglyceride synthesis.

The synthesis of triglyceride by the pathway which has been described in the preceding sections, requires energy.

It is therefore, of interest, that cyanide almost abolishes the uptake and esterification of fatty acids by intact adipose tissue (136). When such tissue is incubated anaerobically with fluoride added to inhibit glycolysis, the rate of incorporation of radioactive FFA is greatly reduced (289, 290). Under these conditions, the generation of energy is blocked at the same time as the synthesis of α - glycerophosphate is inhibited. In homogenates of adipose tissue, moreover, in the absence of ATP, there is no incorporation of radioactive FFA into neutral lipids (80). Such incorporation is greatly reduced when α - glycerophosphate is not added (80). The small amount of residual neutral lipid synthesis is believed to be due to the endogenous α - glycerophosphate, which is present in the enzyme preparation. Thus, exchange incorporation does not seem to be quantitatively important, and most of the triglyceride is synthesized de novo by the reaction sequence which has been discussed.

3) Regulation of Triglyceride Synthesis in Adipose Tissue.

a) Glucose and Insulin.

Addition of glucose to the incubation medium decreases FFA release without altering the production of glycerol (30, 125, 291). At the same time, the uptake of medium FFA by isolated rat adipose tissue is enhanced (144, 292, 293) and the concentration of tissue FFA is diminished (292, 294, 295, 296). The effects of glucose appear to be due to stimulation of triglyceride synthesis in the face of an unchanged rate of lipolysis. The stimulating effect of glucose is probably due to the greater quantity of α - glycerophosphate that it makes available. The extra energy

generated by oxidation of carbohydrate may also play a role.

Addition of insulin to a glucose-containing medium further increases the uptake and esterification of radioactive FFA (107, 144). At the same time, the tissue FFA content is diminished to a greater extent (292, 295). The effects of insulin on glucose metabolism can be simulated by high concentrations of glucose alone (111, 293). It is, thus, clear that one reason for the enhanced fatty acid esterification brought about by insulin is the increased availability of α - glycerophosphate, which is derived from glucose. Insulin, however, has been shown to decrease both FFA and glycerol release in the absence of added glucose (115). Consequently, it is possible that one mechanism responsible for the apparent promotion of esterification brought about by insulin, is inhibition of lipase activity. Up to the present time, this postulate is not supported by any experimental evidence. Ribonucleic acid, adenylic acid and adenosine, moreover, have insulin-like effects on intact adipose tissue (297). Most of these effects can be explained by an increased utilization of exogenous glucose. The synthesis of glyceride-glycerol is enhanced. In addition, ribonucleic acid brings about a marked stimulation of diglyceride synthesis. The mechanisms and physiological significance of these effects remain to be elucidated.

Furthermore, the stimulation of fatty acid esterification observed upon the addition of glucose indicates that the supply of α - glycerophosphate derived from the breakdown of endogenous carbohydrate precursors is not adequate to bring about optimal rates of fatty acid esterification for a given FFA concentration. On the other hand, the comparatively small stimulation which

is brought about by increasing the medium glucose concentration or by adding insulin, and the lack of effect of insulin at high concentrations of glucose, suggest that the concentration of FFA in the pool or pools, active in the esterifying process, may become limiting when the supply of α - glycerophosphate is increased.

b) Free Fatty Acids.

High medium concentrations of FFA, moreover, have effects on adipose tissue metabolism, which appear to be related to enhanced fatty acid esterification. High medium FFA concentrations increase glucose uptake (299) and selectively promote the oxidation of carbon 6 of this sugar as compared to carbon 1, indicating a more important participation of the glycolytic pathway, and oxidation in the citric acid cycle as compared to the hexose monophosphate shunt (127, 298). Such a preferential utilization of glucose via the glycolytic pathway would lead to increased availability of α - glycerophosphate, which would, then, be esterified by the readily available fatty acids. Rapid fatty acid esterification is associated with a marked decrease in the insulin-stimulated incorporation of glucose carbon into glycogen and fatty acids (299). Finally, oxygen consumption is promoted in the presence of high medium fatty acid concentrations (127) indicating, in agreement with the data on the oxidation of isotopic glucose, augmented substrate oxidation to provide additional energy for enhanced fatty acid esterification. Thus, fatty acid esterification has an important influence on many other metabolic activities of adipose tissue.

c) Lipolytic Hormones.

In vitro, certain lipolytic hormones,

increase the rate of esterification (30, 31). Since FFA production by lipolysis is accelerated under the effect of these hormones, FFA titration data have to be supplemented by the determination of the quantity glycerol released in order to evaluate any change in esterification. Using these calculations, it has been shown that epinephrine (30, 31) corticotropin, glucagon, and thyroid-stimulating hormone increase the rate of esterification (31). Since lipolysis is stimulated to a greater extent than esterification, net release of FFA is observed (31). Hence, the lipase-activating hormones accelerate the over-all turnover of triglyceride. Their effect on glyceride synthesis may be secondary to an increased FFA concentration at the site of esterification. It must be borne in mind, however, that in these in vitro experiments, artificially high concentrations of fatty acids are present, and, thus, the findings may not reflect the physiological processes of the living organism.

4) Mechanisms of FFA Uptake and Esterification.

Adipose tissue contains a minute quantity of free fatty acids located, in large part, within cells in the intact tissue (290, 300). This FFA fraction consists almost entirely of long-chain fatty acids (290). The number of free fatty acid pools in adipose tissue is unknown.

Dole (300) found no radioactivity in the tissue FFA fraction of fat pads incubated with isotopic palmitate, and concluded that FFA from the medium were incorporated into glycerides without mixing with tissue FFA. The incubation medium, however, contained glucose (2 mg./ml.) and the tissue was obtained from fed rats. Vaughan et al. (290), using a smaller concentration of medium

glucose (0.5 mg./ml.) have recovered less than 1% of the total radioactivity in the FFA fraction of adipose tissue, while in tissues from fasted rats incubated without glucose, they have found more than 25% of the radioactivity in this fraction.

Kerpel et al. (293) have found that the specific radioactivity of the medium free fatty acids and of the labelled glycerides that are formed, is much greater than that of the tissue free acids. Thus, it would seem that one or more minute intracellular pools of FFA exist in adipose tissue, and that they provide the source of fatty acids for triglyceride synthesis. It would also appear that the free acids which are active in esterification constitute only a small fraction of the total tissue FFA, that their specific radioactivity exceeds that of the bulk intracellular free acid fraction, and that they can readily interchange with medium fatty acids.

The specific activity of the newly-formed glycerides, which are associated with the subcellular particles, is much greater than that of the glycerides, which constitute the central fat droplet of the adipose cell (293, 301). Thus, a small triglyceride compartment with a rapid turnover is found in the particles, while a large triglyceride compartment with a slow turnover is present in the central fat droplet. Such observations, moreover, suggest a relatively slow movement of glycerides from the particles to sites of storage (293).

Once the triglycerides are stored, intermolecular rearrangement of triglyceride fatty acids occurs very slowly (302). The small degree of "reshuffling" which is observed is probably due to lipolysis of triglyceride and re-esterification of fatty acids, rather than extensive transesterification. Hence, the distribution of

the various triglyceride species in adipose tissue is determined by other factors, such as the composition of the fatty acids presented to the esterifying system, the interconversion of fatty acids and, perhaps, differences in fractional turnover rates between triglyceride types (302). The particular structure of a newly formed triglyceride will also be influenced by differences between free fatty acids in rates of release from and esterification in adipose tissue. Indeed, among the saturated and monoenoic acids, the shorter chain components are esterified more readily, while at a given chain length, the more unsaturated acids are esterified to a greater extent in intact adipose tissue (301, 303). Varying rates of fatty acid release are related in a similar way to their chain length and degree of saturation (303). Differences between the various free acids in aqueous solubility, binding to cellular sites, membrane transport systems, and affinity of various enzymes may be responsible for the varying rates of esterification and release (303).

The lack of information regarding the number of anatomically or functionally discrete FFA and triglyceride pools in adipose tissue, compounds the difficulty of studies dealing with FFA uptake and esterification by this tissue.

In summary, the pathway of triglyceride biosynthesis in adipose tissue is similar to that described for liver. Initially, free fatty acid are activated to form acyl CoA derivatives. α - glycerophosphate is, then, esterified by two thiolesters to produce phosphatidic acid. This lipid is acted upon by a specific phosphatase to form D-1, 2-diglyceride, which is esterified by a third acyl CoA derivative to produce triglyceride. The most important physiological mechanism which regulates this synthesis is the availability of glucose and insulin.

F) THE MOBILIZATION OF FAT FROM ADIPOSE TISSUE.

1) The FFA Fraction of Plasma Lipids.

The mobilization of fat from adipose tissue is under complex nutritional and hormonal control. Older studies were handicapped by the lack of information of the chemical nature of the lipid released. In 1956, Dole (23) and Gordon (24, 304), working independently, described the isolation and measurement of a hitherto neglected plasma lipid fraction, free fatty acid, that has proved to be the form in which fat is released from the fat depots. Since adipose tissue is the major, if not the only, source of plasma free fatty acids, it is not surprising that their composition is similar to that found in the fat stores, where they are stored almost entirely as triglyceride (29, 40).

Once released from adipose tissue, free fatty acids are bound to plasma albumin (305, 306, 307, 308). This binding greatly facilitates their solubilization in the aqueous plasma environment (308, 309). The association between FFA and albumin, is not a covalent bond, but is strong enough to transport the FFA to their sites of utilization (308, 309). Goodman (310) has studied the interaction between FFA and serum albumin, in vitro. Interpretation of the data is based on the assumption that each albumin molecule has a discrete number of sites to which fatty acids are bound, and that the interaction with a fatty acid at each site, is a simple association-dissociation equilibrium that obeys the law of mass action (310). Goodman has found that each albumin molecule has 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 lesser affinity for FFA. Finally, the third class has about twenty sites which have the least affinity for these acids. Hence, it is probable that tissues, such as heart and muscle, remove fatty acids from the plasma more readily when the FFA/albumin molar ratio is high.

Although the plasma concentration of FFA is low (307), they are potentially more effective substrates for energy production than other nutrients, such as glucose, because of their rapid turnover (half-life of minutes) and (309, 311, 312, 313) the length of their carbon chain. Indeed, free fatty acids are the major energy source for organs such as heart and muscle during periods of carbohydrate deprivation, and they may also be a substantial energy source under conditions of adequate nutrition (183, 186, 304, 314, 315, 316, 317).

In adipose tissue, free fatty acids are liberated from glycerides through the intervention of a group of enzymes, lipases, which split ester bonds. These free fatty acids are, then, either released from the adipose cell, or re-esterified with α - glycerophosphate forming again triglyceride. In the rat, under ordinary feeding conditions, only 2% of the glyceride ester bonds are split over a 24 hour period (30). While in vitro experiments have indicated that most of the freed fatty acids are retained in the tissue by re-esterification (30), more recent experiments have indicated that, in vivo, this process may not occur to a great extent and that an appreciable fraction of these acids is mobilized from adipose tissue (318). Hence, the FFA content in adipose tissue is the resultant of two opposing reactions, namely, the hydrolysis of triglyceride and the re-esterification of the fatty acid products of lipolysis. The rate of release of free fatty acids from adipose

tissue is determined by the quantity of FFA present in the fat cells. It is, thus, evident that the rate of fat mobilization, and, hence the plasma concentration of free fatty acids, will be increased by circumstances which accelerate the lipolytic rate, or retard the rate of re-esterification. Conversely, if the latter process is enhanced, the tissue FFA content will decrease, the rate of release of fatty acids into plasma will fall, and the plasma level of FFA will decrease, since the rate of entry of free acids into the circulation will fall below the rate of peripheral utilization. A variety of nutritional and hormonal factors can alter the mobilization of fat by influencing either lipolysis or re-esterification in adipose tissue. The enzymes concerned with lipolysis of endogenous glyceride and these nutritional and hormonal factors will now be discussed.

2) Adipose Tissue Lipases.

Adipose tissue has lipases, distinct from lipoprotein lipase, that hydrolyze stored glycerides. The mobilization of fat is believed to be mediated via alterations in the activity of these enzymes. The first direct demonstration of the presence of these enzymes was provided by the observation that the lipolytic activity of crude or centrifuged homogenates of rat adipose tissue can be enhanced by brief preincubation of the intact tissue with epinephrine or corticotropin (319, 320). More recent studies have confirmed the original reports, and have demonstrated that the activity of this hormone-sensitive lipase in centrifuged

homogenates of rat adipose tissue can also be stimulated by brief preincubation of the intact tissue with glucagon, thyroid-stimulating hormone, norepinephrine (321), and vasopressin (158). The pH characteristics and patterns of inhibition of this hormone-sensitive lipase are different from those of lipoprotein lipase (319, 320, 321). The hormone-sensitive lipase has a pH optimum between 6 and 7. It is inhibited by sodium fluoride, but not by protamine.

Adipose tissue, moreover, has at least one more lipase. It is much more active toward lower glycerides, and can be dissociated from hormone-sensitive lipase and lipoprotein lipase (321, 322). While this lipase can act on both monoglyceride and diglyceride, there is no clear difference between monoglyceride lipase and diglyceride lipase activity (322). It may, therefore, be better to call this enzyme, lower glyceridase. In contrast to hormone-sensitive lipase, lower glyceridase activity is not enhanced by prior incubation with such fat-mobilizing hormones as epinephrine and corticotropin (322). Hormone-sensitive lipase, moreover, appears to have a sharper pH optimum (6.5) than lower glyceridase, which exhibits maximal activity at a pH around 6.5-7.0. Both activities are inhibited by sodium fluoride, but hormone-sensitive lipase activity is inhibited by concentrations of isopropanol and tris buffer that have no effect on lower glyceridase activity. Thus, when the lipolytic process is enhanced by epinephrine, corticotropin, glucagon, thyroid-stimulating hormone and norepinephrine, the hydrolysis of adipose tissue triglyceride seems to be initiated by the hormone-sensitive lipase. The lower glycerides that are formed in this way, are hydrolyzed, in turn, by another lipase(s) which is not influenced by fat-mobilizing hormones.

The relationship between these enzymes and the lipase activity extracted from pork adipose tissue (323) and the tribut-

yrinase activity found in rat adipose tissue (324) remains to be clarified.

While lipolysis in adipose tissue can be enhanced by various hormones, a certain degree of triglyceride hydrolysis occurs when they are not added and this basal rate increases in tissues derived from fasted animals (76, 320). Although it is likely that one or more of the lipases that have been described above are responsible for the free fatty acid production under these circumstances, direct proof is lacking.

3) Nutritional Factors.

The original studies of Dole (23) and Gordon (24) have provided the first evidence that the mobilization of fat from adipose tissue is under nutritional control. Indeed, fasting of normal human subjects is constantly associated with an elevation of plasma FFA levels, while glucose administration promptly lowers these values. In addition, in uncontrolled diabetes mellitus, the concentration of plasma FFA is markedly elevated (325, 326, 327). In this situation, administration of insulin is required to bring this concentration back to the normal range.

Unequivocal evidence has been provided from in vitro studies that the elevation of plasma free fatty acids produced by fasting or insulin deficiency is due to increased release of these acids from adipose stores (328, 329, 330). In these experiments, intact adipose tissue from fasted or diabetic rats has been found to release free acids at a rate far in excess of that seen when tissue from normal animals is employed. Addition of glucose and insulin to the medium greatly reduces the release of these acids from carbohydrate- or insulin-deprived tissues.

It is now clear that alterations in the availability

of glucose and/or insulin alter the rate of fat mobilization by primarily affecting the rate of esterification of free acids within adipose tissue. When carbohydrate metabolism is impaired, the availability of α - glycerophosphate becomes limited, free fatty acids produced by hydrolysis accumulate within the tissue, and the rate of migration of these acids into plasma rises. Administration of glucose or insulin, if necessary, reverses this process.

4) Hormonal Factors.

a) Insulin.

As previously discussed, insulin reduces free fatty acid release from adipose tissue by facilitating the uptake of glucose by this tissue, and thus, increasing the metabolism of this sugar and the supply of α - glycerophosphate, which is required to esterify free fatty acids (331). Recent studies, furthermore, suggest that insulin may have an effect quite apart from its role in carbohydrate metabolism. Indeed, insulin has been shown to inhibit lipase activity in adipose tissue (115, 117, 332). Jungas and Ball (115) have suggested that insulin may inhibit lipolysis in adipose tissue by inhibiting ATP cyclase (333) and, thereby, reducing the conversion of ATP to cyclic AMP. The possible significance of this cyclic nucleotide in the lipolytic process will be discussed. Thus, the reduced free fatty acid levels that follow insulin administration may be due not only to enhanced esterification of free fatty acids, but also to a reduction in the rate at which triglycerides are hydrolyzed in adipose tissue.

b) Epinephrine and Norepinephrine.

Administration of both epinephrine and

norepinephrine to a variety of mammalian species, including man, causes a marked elevation in plasma FFA levels (23, 24, 57, 334, 335, 336). Epinephrine brings about a prompt, but relatively brief, rise in the plasma FFA concentration, while the effect of norepinephrine is more sustained (334, 335, 337). Plasma levels of FFA are not depressed in adrenalectomized dogs maintained on cortisone (334). This observation suggests that the catecholamines secreted by the adrenal medulla do not play an important part in the mobilization of FFA under ordinary conditions. The autonomic nervous system, on the other hand, appears to play a significant role in this regard. Indeed, norepinephrine secreted at post-ganglionic sympthetic nerve endings in adipose tissue, seems to be active in fat mobilization under basal conditions, since a variety of pharmacological blocking agents can lower resting FFA levels (334, 335, 338, 339, 340, 341, 342, 343).

In vivo, the lipolytic effect of epinephrine is dependent, in some way, on normal thyroid and adrenocortical function. In hypophysectomized animals, administration of epinephrine fails to evoke a normal free fatty acid response; this is restored by prior therapy with triiodothyronine (344). Studies in dogs have indicated that adrenalectomy will reduce this effect of epinephrine and that cortisone therapy will be followed by a normal response (334, 345). The mechanism by which triiodothyronine and cortisone exert this "permissive" effect is not known.

In vitro, the adrenal medullary hormones exhibit striking lipolytic activity in tissues from a variety of species (57, 107, 298, 329, 346, 347, 348, 349, 350). Considering the data obtained with both in vivo and in vitro experiments, epinephrine and norepinephrine show a marked lipolytic effect in the dog, rat, mouse,

hamster, monkey, and man, but little or no activity in the rabbit, guinea pig, and pig. Addition of glucose and insulin abolishes the effect noted in sensitive species when the concentration of epinephrine, and certain other fat-mobilizing hormones is not too large (351). Epinephrine has also been shown to release FFA from a homogenous preparation of fat cells separated from stromal-vascular cells with collagenase (352).

As previously discussed, the lipolytic effect of epinephrine and norepinephrine is now known to be due to stimulation of hormone-sensitive lipase activity in adipose tissue (319, 320, 321, 322). The percentage increments in this activity are comparable to those in the rate of glycerol release from intact adipose tissue induced under similar conditions by the lipolytic hormones (31).

Little information is available concerning the mechanism by which epinephrine and norepinephrine, stimulate hormone-sensitive lipase activity. The process is inhibited by anaerobiasis (57, 76). Rizack (353) has found that cyclic AMP activates lipolytic activity in cell-free extracts of adipose tissue when incubated with ATP and magnesium ions. Caffeine increases the degree of activation. Calcium produces the same degree of activation as cyclic AMP when added to tissue extracts along with ATP and Mg^{++}. It is possible that the mechanism by which epinephrine, norepinephrine and other lipolytic hormones activate hormone-sensitive lipase, involves increased production of the cyclic nucleotide (353), and, hence, may be basically similar to the process by which epinephrine, corticotropin and glucagon activate phosphorylase, the enzyme responsible for glycogen breakdown.

c) Glucagon.

Glucagon has been shown to increase the release of FFA and glycerol from rat adipose tissue in vitro (73, 129). As previously described, it activates hormone-sensitive lipase activity (321). The physiological significance of this pancreatic polypeptide in fat mobilization is unknown.

d) Corticotropin.

Corticotropin has an indirect effect on mobilization of fat through its action on the adrenal cortex. In addition, as previously described, it exerts a direct influence on the adipose tissue of certain species by stimulating hormone-sensitive lipase activity (319). Indeed, this hormone causes an immediate and striking elevation of plasma FFA in the rabbit, guinea pig, hamster, rat, and mouse, but it has little or no activity in pig or man. The same species differences are found in vitro (57, 319, 350, 354, 355, 356, 357, 358). When tested in rats and mice or upon isolated adipose tissue from these species, corticotropin is the most potent lipolytic agent derived from the pituitary (354, 355). In vitro, it is more active, by weight, than the catecholamines. This hormone is inactive in a calcium-depleted system (294). The stimulating effect of corticotropin on hormone-sensitive lipase and other possible mechanisms of action have been discussed.

Numerous attempts have been made to show an action of the endogenously secreted hormone upon release of storage fat, but without success. Since the lipolytic response of adipose tissue requires the "permissive" effect of corticoids, it is not practical to resort to adrenalectomy in order to evoke the release of corticotropin.

In addition, the use of a stressful stimulus to increase its secretion is accompanied by so many other responses, some mediated by the autonomic nervous system and the adrenal medulla, that any possible lipolytic response to corticotropin is obscured.

e) Growth Hormone.

Administration of human growth hormone to normal, fasted human subjects markedly increases the levels of plasma free fatty acids (359, 366). The pattern of this FFA response is different from that seen following administration of epinephrine and corticotropin. Growth hormone injection induces an initial fall in plasma fatty acids followed by a slow rise, and a sustained elevation that lasts several hours. Dogs (359) and rhesus monkeys (360, 361) show similar responses. The response to growth hormone is greatest during fasting and is largely abolished by food. Growth hormones may play a physiological role during fasting in certain mammalian species including man. Indeed, it is known that the plasma level of growth hormone, as measured by an immunochemical method, rises during fasting (362). Under these conditions, the plasma free fatty acid concentrations in hypophysectomized rhesus monkeys and human subjects do not attain normal fasting levels and the rise is more delayed (360, 363). Hypophysectomized patients or experimental animals on maintenance doses of thyroxine and cortisone also have an impaired response to fasting. Administration of growth hormone restores a normal response to fasting in hypopituitary animals or human subjects. The mechanism of the *in vivo* effect of growth hormone has not been elucidated.

In some *in vitro* studies, this hormone has

been shown to increase FFA release from adipose tissue (299, 346, 364). In rats, hypophysectomy markedly reduces the release of FFA from this tissue, in vitro, and when these animals are given a single injection of growth hormone prior to sacrifice, the in vitro release from fat tissue is greatly enhanced (365). These in vitro effects, however, are produced by large amounts of the hormone and may be attributable to other factors in the preparation. In addition, other studies failed to demonstrate an effect of growth hormone on the release of FFA from intact adipose tissue when it was added to the medium, or administered to the animal prior to sacrifice (366).

Thus, while it seems reasonably certain that growth hormone has physiological significance during a period of fasting, the mechanism by which it brings about mobilization of fat from adipose tissue remains to be clarified.

f) Other Pituitary Factors.

A variety of additional pituitary factors can increase the release of FFA from adipose tissue both in vivo and in vitro. Some of these factors may play a physiological role in certain species. Their importance, however, has not been established, and they will be dealt with briefly.

Thyroid-stimulating hormone has an indirect influence on the metabolism of adipose tissue through its effect on the thyroid gland. The "permissive" effect of thyroid hormones on the lipolytic effect of epinephrine has been mentioned previously. In addition, in vivo and in vitro studies have shown that thyroid-stimulating hormone has a direct, lipolytic action in guinea pig, rat, mouse, and dog, but has little or no effect in the hamster, rat or pig (127, 349, 367). It has also been shown to release FFA from isolated fat

cells prepared with collagenase (352). As with corticotropin, no evidence has been obtained to prove that endogenous thyroid-stimulating hormone exerts an adipokinetic effect in the living organism.

In vitro studies have indicated that two substances, peptide I and peptide II (Fraction H), which can be purified from crude pituitary extracts, (368, 369, 370, 371) exhibit appreciable lipolytic activity only upon rabbit adipose tissue (349, 372). The in vivo significance of these peptides is unknown.

In vivo and in vitro studies, have indicated that α - and β- melanocyte-stimulating hormones exhibit lipolytic activity in the rabbit, guinea pig, and dog, but little or no activity in the hamster, rat, or pig (356, 373, 374).

A posterior pituitary hormone, vasopressin, has been shown to stimulate the release of FFA from adipose tissue of rabbit and guinea pig (356, 364), and, contrary to previous reports, Vaughan (158) has recently demonstrated its activity in rat adipose tissue. Arginine vasopressin is probably the polypeptide that possesses adipokinetic properties. Vasopressin, stimulates the activity of hormone-sensitive lipase and phosphorylase (158). It is of interest that oxytocin, which has a structure only slightly different from that of vasopressin, has no effect on the release of FFA from adipose tissue (158, 356). Thus, fraction H, peptide I, the melanophore hormones and vasopressin are particularly active as fat-mobilizing agents in the rabbit. It is possible that one or another of these polypeptides plays a physiological role in this species which is so insensitive to the adipokinetic action of the catecholamines.

The effect of vasopressin on glycerol release from isolated rat adipose tissue, is inhibited by prostaglandin E_1 (158)

a pharmacologically active, acidic lipid present in seminal fluid (159). This compound also inhibits lipolytic effect of epinephrine, norepinephrine, corticotropin, thyroid-stimulating hormone, and glucagon (375, 376). The mechanism(s) of action and physiological role of the prostaglandins are unknown at the present time.

Chalmers and coworkers (377, 378, 379), extending the original observation of Weil and Stetten (380) have reported adipokinetic activity in urine obtained from fasting man, horse, dog, goat and sheep as indicated by the effect of subcutaneous injection of urine fractions upon the liver fat of the mouse. In vitro, this fat-mobilizing substance has been shown to accelerate the release of FFA from adipose tissue of non-obese mice, but not from that of their obese-hyperglycemic litter mates (381). The reported absence of adipokinetic activity in the urine of fasting, hypopituitary man (377, 378) suggests that the active material may be derived from the pituitary or hypothalamus. Preliminary characterization has shown that the fat-mobilizing substance is not a polypeptide and is, thus, different from the other pituitary factors.

g) Inactivation of Adipokinetic Pituitary Peptides.

Recently, a search for the mechanisms which might be responsible for species differences in the responsiveness of adipose tissue to various adipokinetic hormones has led to the observation that homogenized adipose tissue from the rat has factors which can inactivate corticotropin, fraction H, α-melanocyte-stimulating hormone, β-melanocyte-stimulating hormone, and vasopressin (382, 383). Adipose tissue homogenates from the rabbit, on the other hand, inactivate only α-melanocyte-stimulating hormone,

whereas those from the guinea pig do not inactivate any of these peptides (382). The process of inactivation appears to be enzymatic, and, more specifically, the factors have the properties of peptidases (382, 383).

These findings may explain why rabbit adipose tissue is more responsive to corticotropin, fraction H, β-melanocyte-stimulating hormone, and vasopressin than rat adipose tissue. Inactivation of adipokinetic hormones may be a general mechanism for the control of fat mobilization in certain species.

h) Adrenal Cortical Hormones.

In addition to their "permissive" effect on the lipolytic action of epinephrine, adrenal cortical hormones have a direct influence on adipose tissue incubated in a glucose-containing medium. Indeed, the FFA release of such tissue is increased when a variety of corticoids are added to the incubation medium (328, 384, 385).

The effect of the adrenal cortical hormones appears to be secondary to decreased glucose transport and metabolism. Cortisol, corticosterone, and desoxycorticosterone decrease the uptake of this sugar by isolated adipose tissue (386, 387, 388). In addition, the conversion of isotopic glucose to glycogen, carbon dioxide, total lipid, fatty acids, and glyceride-glycerol is decreased by corticoids. All these effects on glucose metabolism may be secondary to decreased uptake of glucose by adipose tissue, and are quite different from those of the lipolytic hormones (384, 385). Hence, the increased release of FFA from adipose tissue may be secondary to decreased esterification. It is possible that glucocorticoids act in other ways. Indeed, Mahler and Stafford (389) have reported augmented glycerol release following

exposure of rat adipose tissue to cortisol in the absence of added glucose.

Thus, while the physiological role of the corticoids in fat mobilization has not been established, their in vitro effects appear to be, at least partly, secondary to their influence on carbohydrate metabolism.

i) Androgens and Estrogens.

Androgens and estrogens have recently been added to the long list of adipokinetic hormones. Testerone and its less androgenic derivative, 19-norandrostenolone, bring about a marked increase in plasma FFA levels in starved female rats (390, 391). In view of the fact that during puberty, the subcutaneous adipose tissue decreases in boys, as opposed to girls, and that eunuchs have a tendency to be obese, it has been suggested that androgens play an active role in adipose tissue metabolism (391).

Injections of estradiol lead to a striking elevation in the plasma FFA concentration of starved rats of both sexes (392). The effect of estradiol is similar to or greater than that of testosterone. The mechanism(s) by which these hormones elevate the FFA levels is not known.

5) Summary of Factors Controlling Fat Mobilization.

It is evident from the preceding discussion that nutritional and hormonal factors control the rate of lipid mobilization from adipose tissue. Although the situation in the living organism is undoubtedly complicated, it is apparent that all these factors alter free fatty acid release from fat tissue by either changing the rate of triglyceride breakdown (e.g., epinephrine) or by affecting the rapidity with which free acids are esterified to form adipose tissue triglyceride (e.g., glucose and insulin).

Despite the complexity of the situation, one can attempt to offer an integrated picture of the control of fat mobilization in certain mammals. Norepinephrine, secreted at sympathetic nerve endings in or near adipose tissue, activates a lipase system within the tissue, leading to a basal rate of triglyceride breakdown. The free fatty acid products of this lipolysis are largely re-esterified when glucose and insulin are available, and are rapidly released into the circulation when glucose metabolism in adipose tissue is reduced. In man, and probably in primates, monkeys, dogs and certain other mammals, the fat-mobilizing effect of growth hormone is added, during fasting, to that induced by deficiency of glucose and insulin. The adipose tissue of man, monkey, dog, rat, mouse, and hamster responds markedly to catecholamines. Thus, the sudden excretion of adrenal medullary hormones that occurs in emergency situations, leads, in these mammals, to enhanced triglyceride breakdown with consequent release of high concentrations of free fatty acids. In rabbit and guinea pig, corticotropin, the melanophore-stimulating hormones, and arginine vasopressin may assume this role under emergency conditions.

Of all the factors involved, the most important

hour by hour regulatory influence is that exerted by variations in plasma glucose and insulin concentrations.

II. EXPERIMENTAL SECTION.

A) INTRODUCTION.

The elucidation by Rose and Shapiro (79), and by Steinberg et al. (80) of the pathways of triglyceride synthesis in adipose tissue, was a major advance in the understanding of the mechanisms controlling the equilibrium between lipid deposition and mobilization. The pathways and requirements of neutral lipid synthesis by subcellular preparations of adipose tissue, turned out to be similar to those described earlier for cell-free systems of liver (235, 236, 237, 238, 239).

Alterations in nutritional state have been shown to influence the process of esterification by intact mammalian adipose tissue. In fact, esterification of fatty acids by such tissue is decreased during fasting (30, 136, 290, 393), and enhanced during refeeding (30, 136, 115). These observations have been ascribed to decreased availability of α - glycerophosphate during fasting, and an increased supply of this metabolite during refeeding.

It was considered possible that changes in the activity of the FFA esterifying enzymes might play a role in the reduction of esterification noted in intact adipose tissue during fasting and the promotion of this process during refeeding. Hence, in the work to be described, this problem was explored by comparing the initial rates of esterification using enzymes form fed, fasted, and refed rats.

Previous studies have suggested that the process of fatty acid esterification occurs in subcellular particles of adipose tissue (32, 284, 293). These investigations, however, did not

distinguish between the mitochondrial and microsomal fractions, nor did they evaluate the role of the soluble fraction in the esterifying process.

In the current work, a study was undertaken to localize the subcellular site of esterification. In particular, it was considered of interest to determine whether the mitochondrion-rich fraction and the microsome-rich fraction could esterify FFA independently, and whether the 109,000 X g. supernate (soluble fraction) could participate in this reaction.

Little is known about the kinetics of the FFA esterifying enzymes. Steinberg et al. (80) used an unfractionated cell-free preparation, which was obtained by centrifuging homogenates of adipose tissue at 500 X g. for 10 minutes. This defatted homogenate was able to stimulate the esterification of FFA and α - glycerophosphate, in the presence of ATP, CoA-SH, and Mg^{++}. Goldman and Vagelos (284) described the properties of a particulate enzyme system prepared by centrifuging the defatted homogenate at>67,000 X g. for 40 minutes. Steinberg et al. and Goldman and Vagelos used an incubation temperature of 37°C. Accurate kinetic measurements were difficult to perform at this temperature because of the rapidity with which the reactions took place.

Using a subcellular preparation similar to that described by Steinberg et al. (80), Angel (394) retarded the rate of FFA esterification by lowering the incubation temperature to 20°C. He found that, while this manipulation did not alter the cofactor requirements of the FFA esterifying enzymes present in defatted homogenate, it permitted rather accurate measurement of the overall reaction of neutral lipid synthesis from palmitate and α - glycerophosphate.

It was considered of interest to extend these investigations by studying the effects of duration of incubation and progressive increase in enzyme concentration on the rate of the esterifying reaction, using subcellular particles in the presence and absence of the soluble fraction.

It was also felt that additional information was needed regarding characterization of reaction products from different subcellular fractions. In their elegant work, Steinberg et al. (80) found that the esterifying enzymes present in defatted homogenate, stimulated the production of two major isotopic esters, namely, triglyceride and diglyceride. In addition, appreciable amounts of labelled phosphatidic, a triglyceride precursor, were formed. It was considered possible that subcellular particles of adipose tissue would stimulate the production of a different pattern of isotopic esterified products from that formed in the presence of defatted homogenate. In fact, under certain conditions, subcellular particles of liver have been shown to stimulate predominantly the synthesis of phospholipids (220). To explore this problem in adipose tissue, the ester fraction formed in the presence of mitochondria, microsomes, and a combination of these particles, was characterized in the present study. The effect of the soluble fraction on the pattern of esterified products was also determined.

Recently, Tzur and Shapiro (395) have shown that the synthesis of neutral lipids and phospholipids by liver microsomes could be promoted by a variety of lipoproteins. Since these observations suggested a new mechanism of lipid synthesis, namely, the formation of lipoprotein, in situ, rather than the production of free lipid, it was considered of importance to determine whether lipoproteins had any effect on the rate of FFA esterification or the pattern of esters

synthesized by subcellular preparations of adipose tissue.

The studies that will now be described in detail were carried out in the hope to acquire a better understanding of the mechanisms involved in the deposition of fat in adipose tissue.

B) MATERIALS AND METHODS.

1) Animals.

Male Wistar rats were used throughout and were fed on a standard Purina Chow diet. The animals fed ad libitum were sacrificed when they weighed 190-200 g. The rats that were fasted for 24 hours weighed 180-190 g., while those fasted for 72 hours weighed 150-160 g. The animals that were refed for 48 hours after a fast of 96 hours weighed 160-170 g. Each rat was kept in a separate cage.

2) Chemicals and Reagents.

All inorganic chemicals and organic solvents were reagent grade. DL-glycerophosphate (95-100% α isomer), adenosine-5′ - triphosphate (from equine muscle, disodium salt, 99% pure), and coenzyme A (from yeast, 90% pure) were obtained from Sigma Chemical Company, St. Louis, Missouri.

Palmitic acid - 1-C^{14} , as purchased from Merck, Sharpe, and Dohme of Canada Limited, was about 96% pure. In most studies, it was purified before use by two successive applications on thin-layer chromatography. Following this procedure, 99.8-99.9% of the total radioactivity was associated with a material that travelled with an R_f identical with that of palmitic acid. This degree of purification permitted more accurate characterization of the isotopic esterified products, even when the percentage of radioactivity incorporated into the esterified fraction was relatively small. The purified palmitic acid was, then, converted to its potassium salt with a slight molar excess of potassium hydroxide. On gentle heating, potassium palmitate became soluble in water and could, thus, be used in

the incubation mixture.

Cholesterol palmitate, cholesterol oleate, tripalmitin, triolein, palmitic acid, oleic acid, linoleic acid, dipalmitin, diolein, dilinolein, monopalmitin, and monoolein were purchased from Hormel Foundation, Austin, Minnesota, and used as standards for thin-layer chromatography. Lecithin, phosphatidyl serine, and phosphatidylethanolamine were obtained from Applied Science Laboratories, State College, Pennsylvania. Phosphatidic acid was made available through the courtesy of Dr. B. Leboeuf, Notre Dame Hospital, Montreal. These phospholipids were also used as standards for thin-layer chromatography.

Cohn's bovine albumin fraction V and human serum fractions II, III, IV-1, and IV-4 were purchased from Brickman and Company, Montreal, and human serum albumin from Connaught Medical Research Laboratories, Toronto. Human serum albumin was the only fraction that was defatted before use (168). Deionized, redistilled water was used throughout the study.

3) Enzyme Preparations.

An enzyme preparation that contained mitochondria, microsomes, and soluble fraction, but was free of intact cells, nuclei, and dense stroma, was obtained by the method described by Steinberg (80), as modified by Angel (396). Each rat was sacrificed by a blow to the head, and all subsequent manipulations that preceded incubation, were carried out at 0-4°C. Both epididymal fat pads of three to seven animals were pooled and homogenized with 0.15 M potassium chloride, 1:4, weight to volume, in a Potter-Elvehjem glass homogenizer, (397) with the aid of a motor-driven tephlon pestle. The

homogenate was, then, centrifuged in an International Centrifuge, Model PR-2, at 700 X g. for 12 minutes. This procedure resulted in the separation of three distinct layers. The bulk fat was at the top, intact cells, nuclei, and dense stroma formed a pellet at the bottom of the centrifuge tube, and a straw-coloured aqueous liquid comprised the intermediate layer (396). Without disrupting the bulk fat, the intermediate layer was aspirated with a long 18 gauge needle. This crude enzyme preparation has been called defatted homogenate (80). It was used in those experiments in which the total activity of the FFA esterifying enzymes, derived from fed, fasted, and refed rats was compared. In addition, mitochondria, microsomes, and the soluble fraction were prepared from the defatted homogenate. The fractions that were derived from the crude homogenate, were used in the series of experiments designed to determine the subcellular site of the esterifying process, to characterize the reaction products,and to study the kinetics of the esterifying enzymes.

The mitochondrial fraction was prepared by centrifuging the defatted homogenate at 14,000 X g. for 40 minutes in a Beckman model L preparative ultracentrifuge. After decanting the 14,000 X g. supernate, the sediment was washed by stirring it with a thin glass rod in 0.15 M potassium chloride and by centrifuging again at 14,000 X g. for 40 minutes. The washing solution was, then, decanted and the sediment was resuspended by stirring in an appropriate volume of 0.15 potassium chloride. Suspension of the particles in the aqueous solution was facilitated by swirling the container gently on a Vortex Jr. Mixer.

The microsomal fraction was prepared by centrifuging the 14,000 X g. supernate at 109,000 X g. for 35 minutes in the Beckman ultracentrifuge. The 109,000 X g. supernate, which was also

called soluble fraction, was, then, decanted and the particles were washed and resuspended in a manner similar to that described for the mitochondrial preparation.

The mitochondrial and microsomal fractions were examined separately by electron microscopy. The enzyme preparations were fixed in osmium tetroxide for two hours. The precipitates were, then, isolated by centrifugation, resuspended in distilled water, dehydrated in graded alcohols, embedded in Epon 812 (398), and sectioned. Finally, these preparations were examined in an RCA EMU 3E electron microscope (396).

A representative electron microgram of the sediment obtained when the defatted homogenate was centrifuged at 14,000 X g. for 35 minutes is shown in Fig. III. Although it is impossible to quantitate the degree of contamination on the basis of a few micrograms, it is clear that only few elements of endoplasmic reticulum were interspersed admidst the mitochondria. For this reason, this enzyme preparation has been named mitochondrion-rich fraction.

A representative electron microgram of the pellet which was formed when the 14,000 X g. supernate is centrifuged at 109,000 X g. for 35 minutes is shown in Fig. IV. In this case, since there was only a small degree of contamination of the endoplasmic reticulum with mitochondria, the preparation has been termed microsome-rich fraction.

A fraction consisting of both mitochondria and microsomes, was obtained by centrifuging the defatted homogenate at 109,000 X g. for 40 minutes. The 109,000 X g. supernate was, then, decanted and the particles were washed and re-suspended in potassium chloride, as previously described.

In a few experiments, the epididymal fat

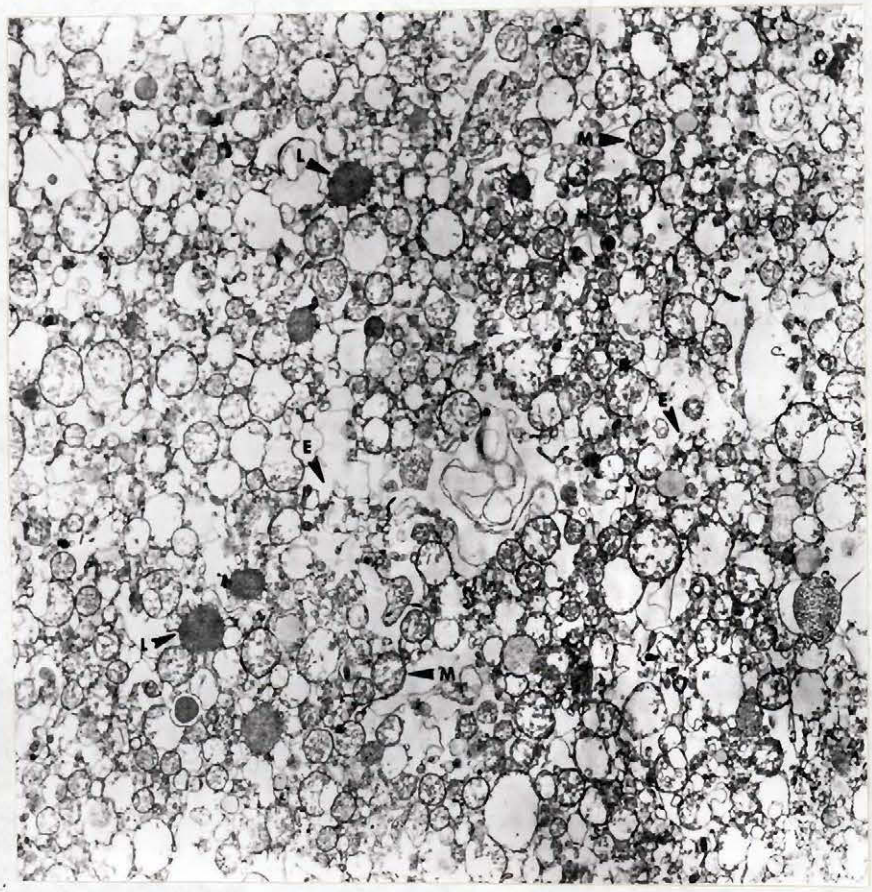


FIG. III. Electron micrograph of the 14,000 X g. sediment (Mitochondrion-rich fraction). The preparation consists almost entirely of mitochondria (M). A few elements of endoplasmic reticulum (E) are interspersed amidst the mitochondria. The dark circular structures are probably lysosomes (L). (Osmium tetroxide fixation) X 10,000.

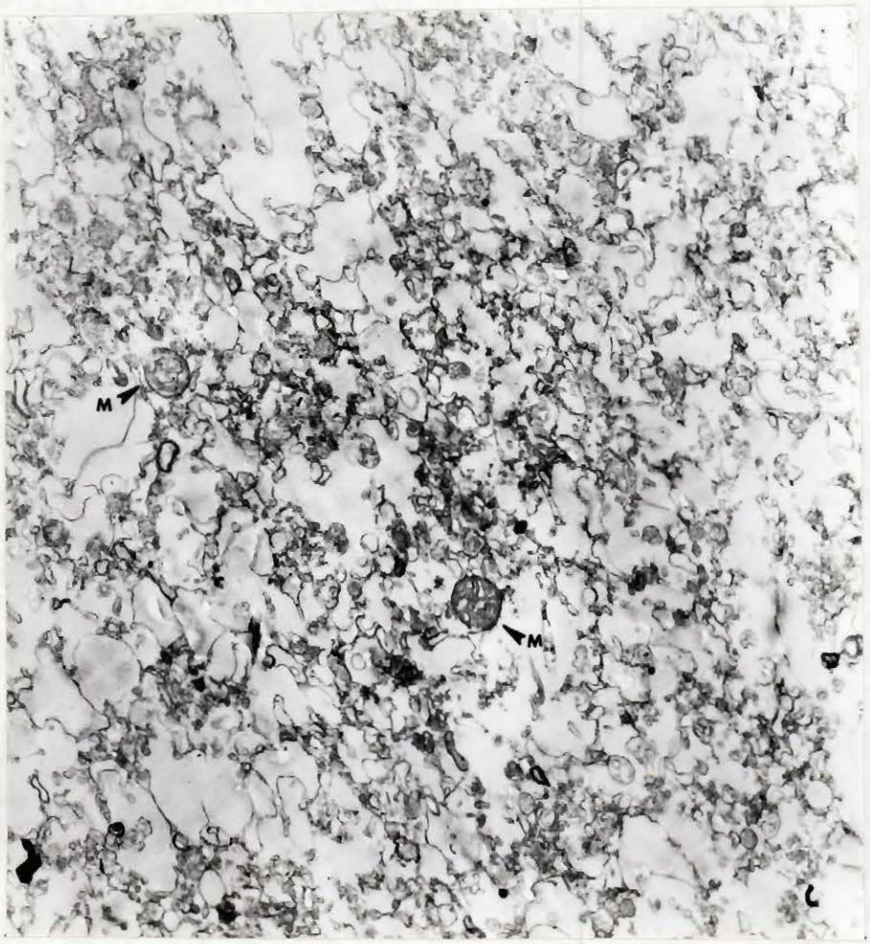


FIG. IV. Electron micrograph of the 109,000 X g. sediment. (Microsome-rich fraction). The preparation consists in great part of endoplasmic reticulum. Two mitochondria (M) are interspersed amidst the endoplasmic reticulum. (Osmium tetroxide fixation) X 16,000.

pads were homogenized in 0.25 M sucrose. All the other steps of homogenization and preparation of the various subcellular fractions were the same as detailed in the preceding paragraphs.

Dialysis of the soluble fraction was carried out using 3/4" dialyzer tubing consisting of seamless cellulose (Fisher Scientific Company, Montreal). According to the manufacturers' specifications, substances with a molecular weight of less than 10,000, can diffuse through this dialyzing membrane. Three to fifteen ml. of soluble fraction were dialyzed against either an equal volume of 0.15 M potassium chloride, or an excess of this solution (399). Dialysis was carried out at 4°C for 24 hours on an oscillating plate.

Finally, in every experiment, the protein content of each enzyme preparation was determined by the micro-Kjeldahl technique (400). Titration of the FFA present in the defatted homogenate will be described in an ensuing sub-section.

4) Conditions of Incubation.

Two or three ml. of reaction mixture, consisting of enzyme preparation, substrates, cofactors, and buffer were incubated in 25 ml. Erlenmeyer flasks in a Dubnoff metabolic shaker with air as the gas phase. Water replaced the enzyme preparation in the control flask. In most experiments, the incubation temperature was 20°C In a few instances, the temperature was 37.5°C.

5) Lipid Extraction.

In the experiments in which the total activity of the FFA esterifying enzymes from fed, fasted, and refed rats was compared, lipids were extracted by the method of Dole

(23, 401). By this procedure, virtually all the triglycerides and diglycerides, and about 90% of the FFA were present in the organic phase. The FFA were separated from glycerides by washing the organic phase with alkaline ethanol according to the method of Borgström (402). Alkaline ethanol consisted of 5% ($^{v}/_{v}$) 1N sodium hydroxide, 50% ($^{v}/_{v}$) ethanol in water, and the indicator, thymol blue. While the exact distribution of phosphatidic acid in Dole's biphasic system has not been determined, this phospholipid was removed quantitatively from the upper phase with alkaline ethanol (80). Hence, the lipids remaining in the upper phase, consisted almost exclusively of diglycerides and triglycerides. The fatty acids were recovered from alkaline ethanol by acidification with 5 N hydrochloric acid, and extraction of the free acids with petroleum ether. Calculations of recovery of radioactivity were corrected for the 10% loss of FFA in the aqueous phase. Using this correction, the recovery of total radioactivity using the methods of Dole and Borgström was 95-100%.

The method of Folch et al. (403) was used in all the experiments in which the more polar lipids, i.e., phospholipids and monoglycerides, were examined. The main steps were lipid extraction with chloroform: methanol (2:1), followed by shaking, filtering, and washing with 0.88% ($^{w}/_{v}$) potassium chloride. Following this wash, the two phases were allowed to separate completely over a period of 12 hours. The upper phase was, then, removed, and the interphase rinsed repeatedly with pure solvents upper phase in order to get rid of any remaining water-soluble substances. Finally, an aliquot of lower phase, which contained the lipids, was taken for radioactive counting and another for thin-layer chromatography. The recovery of total radioactivity using the method of Folch et al. was 95-100%. The recovery using this method followed by thin-layer chromatography was

90-95%.

6) Titration of FFA.

The concentration of FFA in aliquots of defatted homogenate and in the reaction mixtures were determined by the method of Dole (401). After the addition of 0.7 ml. or 1.0 ml. of 0.005% thymol blue to 2 or 3 ml., respectively, of Dole's upper phase, the FFA were titrated against 0.0077 N sodium hydroxide, which was released dropwise from a microburette. It was considered possible that the phospholipids associated with mitochondria and microsomes might have contributed slightly to the titratable acidity of extracts of these preparations. For this reason, after the initial extraction of the FFA by the method of Dole, the upper phase was re-extracted with pure solvents lower phase (401). This procedure did not result in titration values significantly lower than those obtained after a single extraction.

7) Thin-Layer Chromatography.

The technique of thin-layer chromatography described by Stahl was applied (404, 405, 406, 407, 408). Silica Gel H (E. Merck Ag., Darmstadt, Germany) was used instead of Silica Gel G because the latter has been shown to cause appreciable quenching of radioactivity due to its content of the binder, calcinated calcium sulphate (409). A slurry of adsorbent was made by thoroughly mixing 55 g. of Silica Gel H with 150 ml. of deionized, redistilled water. This mixture was, then, applied to glass plates. Lipids were dissolved in a mixture of chloroform: methanol (2:1) and applied on a plate with the aid of a template. Three to four samples were plated

between standards. The chromatoplates were, then, developed by ascending solvents in a covered jar. The developing solvent system consisted of petroleum ether: diethyl ether: glacial acetic acid, 190: 55: 5 (ml.). A representative thin-layer chromatogram is shown in Fig. V. The average R_f values were as follows: cholesterol ester, 1.0; triglyceride, 0.81; FFA, 0.68; 1, 3-diglyceride, 0.33; 1, 2-diglyceride, 0.22. Monoglyceride, phosphatidic acid, phosphatidyl ethanolamine, phosphatidyl serine, lecithin, and lysolecithin failed to migrate to any appreciable extent. The two classes of diglyceride were recovered together.

In a few experiments, monoglyceride was separated from phospholipids, by use of the solvent system hexane: diethyl ether: methanol: acetic acid, 180: 40: 6: 4 (ml.) (410). The average R_f values observed for this system were triglyceride, 0.92; FFA, 0.67; 1, 3-diglyceride, 0.46; 1, 2-diglyceride; 0.37; monoglyceride, (average of 1- and 2-monoglyceride, which were close together) 0.12; phosphatidic acid, phosphatidyl ethanolamine, phosphatidylserine, lecithin, and lysolecithin failed to migrate.

In most experiments, the lipids were visualized directly by spraying with a 1% ($^{w}/_{v}$) solution of resublimed iodine in methanol (411, 412). After marking the appropriate areas with a fine needle, the iodine was sublimed by heating. The portions of Silica Gel H containing the lipids were, then, scraped and placed directly into counting vials.

In some experiments, phospholipids were eluted from Silica Gel H with chloroform; methanol (2:1), prior to resolution into their component classes. Spraying was not needed prior to elution because the phospholipids remained at the origin, which is readily visualized. The developing solvent system used to

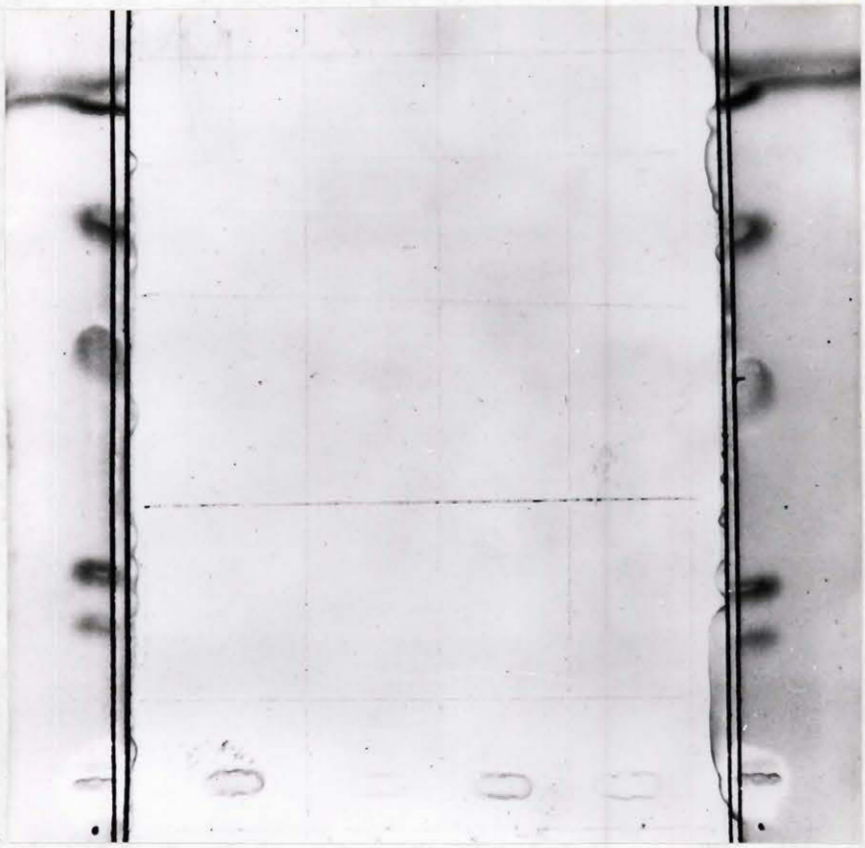


FIG. V. Thin-layer chromatogram showing usual separation of lipid classes. The developing solvent system consisted of petroleum ether: diethyl ether: acetic acid, 185: 90: 5. The standards are located on each side of the test samples. They have been sprayed with bromothymol blue. The cholesterol ester fraction is located at the solvent front area (top). Then, proceeding towards the bottom of the figure, are the triglyceride, free fatty acid, 1, 3-diglyceride, 1, 2-diglyceride, and the superimposed monoglyceride and phospholipid areas (at the origin).

separate the different phospholipids, consisted of chloroform: methanol: acetic acid: water, 125: 75: 20: 10 (ml.) (413). A representative thin-layer chromatogram of this separation is shown in Fig. VI. The R_f values of the different phospholipids were as follows: phosphatidic acid, 8.8-1.0; phosphatidyl ethanolamine, 0.77; phosphatidyl serine, 0.65; lecithin, 0.42; lysolecithin, 0.08.

In a few experiments, after separation from other lipids, the triglyceride fraction was eluted from Silica Gel H with diethyl ether. Prior to elution, the lipid standards were sprayed with bromothymol blue (0.04 mg.% in 0.01 N aqueous sodium hydroxide), while the unknown samples were covered with a template. Iodine was not used for staining prior to elution, because this halogen alters the physical and chemical properties of unsaturated fatty acids because of iodination of their double bonds (414).

The triglyceride fraction was, then, subfractionated into classes differing in degree of unsaturation. A modification of the method of Barrett et al. was used (415). The glass plates were coated with a slurry of Silica Gel H, 55 g. in 150 ml. of 12.5% aqueous silver nitrate solution. A mixed triglyceride sample obtained from adipose tissue was used as carrier for the radioactive triglycerides and as reference for thin-layer chromatography. The developing solvent system consisted of chloroform: absolute ethanol: acetic acid, 250: 2.5: 1.25 (ml.). The test samples were, then, covered while the references were sprayed with a 1% methanolic solution of 2′, 7′- dichlorofluorescein. The triglycerides appeared as bright yellow fluorescent spots when viewed under ultra-violet light. The areas of silica between the reference spots, were, then, scraped, eluted with diethyl ether and transferred to counting vials.

The free fatty acid fraction was also

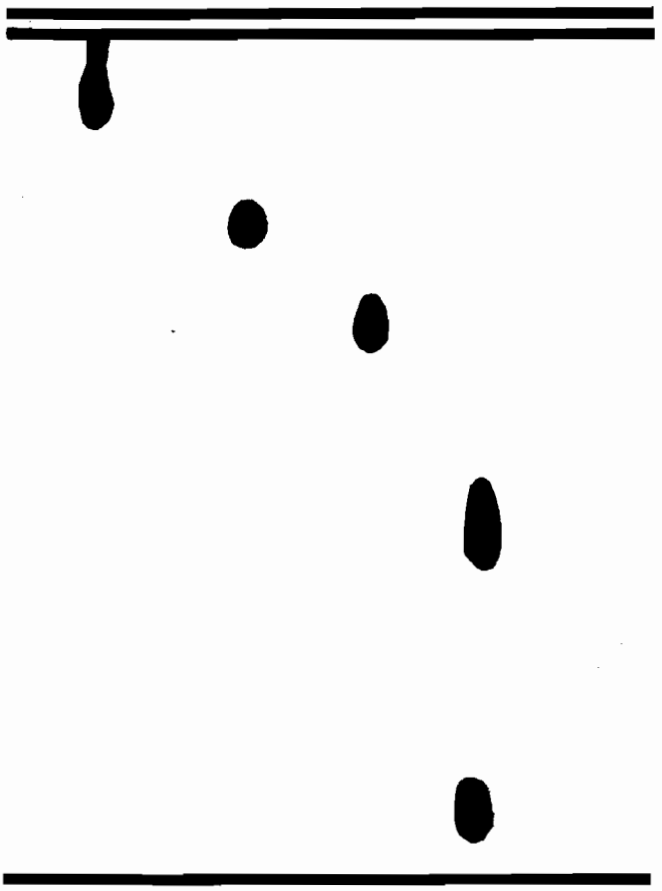


FIG. VI. Photograph of a thin-layer chromatogram tracing showing separation of the phospholipid fraction into its component classes. The developing solvent system consisted of chloroform: methanol: acetic acid: water, 125: 75: 20: 10. In this example, only standards were used. They were sprayed with bromothymol blue. Proceeding from left to right, phosphatidic acid is at the top, near the solvent front area, followed by phosphatidyl ethanolamine and phosphatidyl serine. Lecithin and lysolecithin (close to the origin) are in the last row.

separated into saturated, monounsaturated, and diunsaturated acids on silver nitrate impregnated silica gel. Methyl esters of the fatty acids formed by the method of Stoffel were used in these studies (416). The other experimental steps were similar to those described for the separation of the triglyceride fraction except for the fact that the developing solvent system consisted of petroleum ether: diethyl ether: acetic acid, 225: 25: 5 (ml.).

8) Specific Radioactivity Measurements.

The initial specific radioactivity of the FFA present in each reaction mixture was determined throughout the study by dividing the value for the total counts per minute by the concentration of FFA, which was determined on an aliquot of reaction mixture removed before incubation.

In several experiments, the specific radioactivity of FFA was determined both before and after incubation. The final specific radioactivity of FFA was determined in a similar manner as the initial value except for the fact that glycerides were first removed from FFA with alkaline ethanol, prior to radioactive counting.

9) Counting of Radioactivity.

Radioactivity was counted in a Packard Tri-carb Liquid Scintillation Spectrometer, Model 500B(417). The efficiency of this instrument was about 60% for carbon - 14. The scintillation mixture consisted of 250 mg. of 2,5 - diphenyloxazole, 10 g. of P-Bis (5-phenyloxazolyl)- 1 benzene, and 133.5 g. of naphthalene, dissolved in a mixture of 770 ml. dioxan and 230 ml.

absolute ethanol (418). Ten ml. of this mixture were added to dry counting vials which contained the various lipid fractions. A blank, consisting of 10 ml. of scintillation mixture was used to correct for background radioactivity. For each experimental system, moreover, internal standards were used periodically to check for quenching (419, 420, 421). This phenomenon was not observed at any time. When the scintillation mixture was added to counting vials which contained lipids which were adsorbed on Silica Gel H, the vials were whirled on a Vortex Jr. Mixer to insure complete elution of the lipids.

C) RESULTS.

1) Enzyme Preparations.

a) Defatted Homogenate.

The defatted homogenate from fed animals was a straw-coloured, slightly opalescent solution. The same enzyme preparation from rats fasted for 72 hours, or from those fasted for 96 hours, and, then, refed for 48 hours was light pink. The protein and FFA concentrations of defatted homogenates from animals exposed to various nutritional manipulations are shown in Table II.

The preparative procedure was effective in removing the bulk fat, as less than 0.1% of the tissue triglyceride remained in the defatted homogenate (394). Part of the endogenous FFA that were present in this enzyme preparation, were probably produced by hydrolysis of the tissue triglyceride during the experimental procedures. Electron microscopic studies of Sheldon and Angel (396) established that the defatted homogenate was free of intact cells and nuclei. There was also an apparent absence of free, structureless lipid.

b) Particulate Enzyme Preparations.

Particulate enzyme preparations were only obtained from fed rats. When they were prepared in 0.15 M potassium chloride, the particulate fractions sedimented as small, circular, yellowish-gray pellets. The mitochondrion-rich fraction formed a pellet (8 mm. in diameter) about four times as large as that formed by the microsome-rich fraction. When the particulate fractions were prepared in 0.25 M sucrose, the pellets were larger. In fact, Schneider (422) demonstrated that in salt solutions, mitochondria were strongly agglutinated. Sucrose, however, was not generally used because when the subcellular fractions were prepared

TABLE II

CHEMICAL ANALYSIS OF DEFATTED HOMOGENATE.

No. of Experiments	Nutritional State	Mean Protein Concentration (mg./ml.)	Mean FFA Concentration (mμEq./ml.)
13	Fed	2.233 ± 0.082	105 ± 9
8	Fasted 24 hrs.	2.559 ± 0.157	129 ± 18
10	Fasted 72 hrs.	3.811 ± 0.298	219 ± 23
9	Fasted-Refed 96 hrs.-48 hrs.	4.281 ± 0.252	259 ± 26

Mean values and standard errors are shown.

using a 0.25 M solution of this compound, esterifying activity was negligible.

When the mitochondria and microsomes were sedimented together, and, then, re-suspended in an appropriate volume of 0.15 M potassium chloride, the protein concentration was about one eighth that of the defatted homogenate. The protein concentration of the microsome-rich fraction was about one third that of the mitochondrion-rich fraction.

2) Kinetics of FFA Esterification Using Defatted Homogenate.

a) Substrate and Cofactor Requirements.

Initial experiments were designed to determine the optimal concentrations of substrates and cofactors that were required by the enzymes involved in the synthesis of glycerides from α - glycerophosphate and FFA. In this series of experiments, the extraction method of Dole (401) was used and free fatty acids were separated from glycerides by the method of Borgström (402). In each experiment, the concentration of one of the following compounds was varied over a wide range: palmitate-1-C^{14}, DL-α-glycerophosphate, ATP, and CoA-SH. The concentrations of substrates and cofactors that produced maximal rates of FFA esterification, as well as the other constituents of the reaction mixtures are shown in Table III. Sodium fluoride was used in these experiments because it has been reported to inhibit ATP-ase and lipase activity (80, 319) in cell-free preparations. The values for the optimal concentrations of substrates and cofactors that were obtained in this study were the same as those reported by Angel (394), and did not differ when preparations from fasted or fasted-refed animals were used.

TABLE III

INCUBATION SYSTEM.

Substrates		Cofactors			NaF	Potassium Phosphate Buffer (pH = 7.0)	Defatted Homogenate
Potassium Palmitate-1-C^{14}	DL-α-Glycerophosphate	ATP	CoA-SH	Mg^{++} ($MgCl_2$)			
0.083	6.7	3.3	0.083	2.0	6.7	46.8	0.5-1.0 ml.

The optimal concentrations of the added substrates and cofactors are given in mM/L. Deionized, redistilled water was added in such amounts as to make the final volume of each reaction mixture 3 ml.

The effect of omitting either α - glycerophosphate or one of the cofactors is shown in Table IV. It is evident that α - glycerophosphate and ATP were obligatory requirements for glyceride synthesis. The fact that a small degree of esterification proceeded in the absence of CoA-SH, suggested the presence of this cofactor in the enzyme preparation.

b) Time Course Studies.

The effect of duration of incubation on the rate of esterification of FFA and α-glycerophosphate was studied using defatted homogenates from fed, fasted and refed rats. An incubation temperature of 20°C was used in most experiments. The results of typical time courses are depicted in Fig. VII. As shown, the rate of esterification was approximately linear, in each case, during the first 15 minutes of incubation. Since optimal concentrations of substrates and cofactors were used, these experiments were performed at zero order kinetics.

In a few experiments, the incubation temperature was 37.5°C. Time courses using enzymes from fed and fasted rats at this temperature are illustrated in Fig. VIII. The rate of esterification was linear between 2 and 6 minutes of incubation. The initial delay before attaining the maximal reaction rate was present even when the enzyme preparations were warmed to 37.5°C before incubation. This delay was also observed on numerous occasions when the incubation temperature was 20°C. While it is possible that it represents the time during which FFA are activated, the reasons for this lag are not apparent at the present time. The reaction rate at 20°C was about 1/5 that at 37.5°C.

TABLE IV

DEPENDANCE OF ESTERIFICATION REACTION ON ∝ -GLYCEROPHOSPHATE AND COFACTORS.

	Relative Amount of Palmitate -1-C^{14} Radioactivity Incorporated into Glyceride Fraction
Complete System	100
Complete System Minus ∝ -Glycerophosphate	2
Complete System Minus ATP	1
Complete System Minus CoA-SH	29

Incubations were carried out at 20°C for 15 minutes.

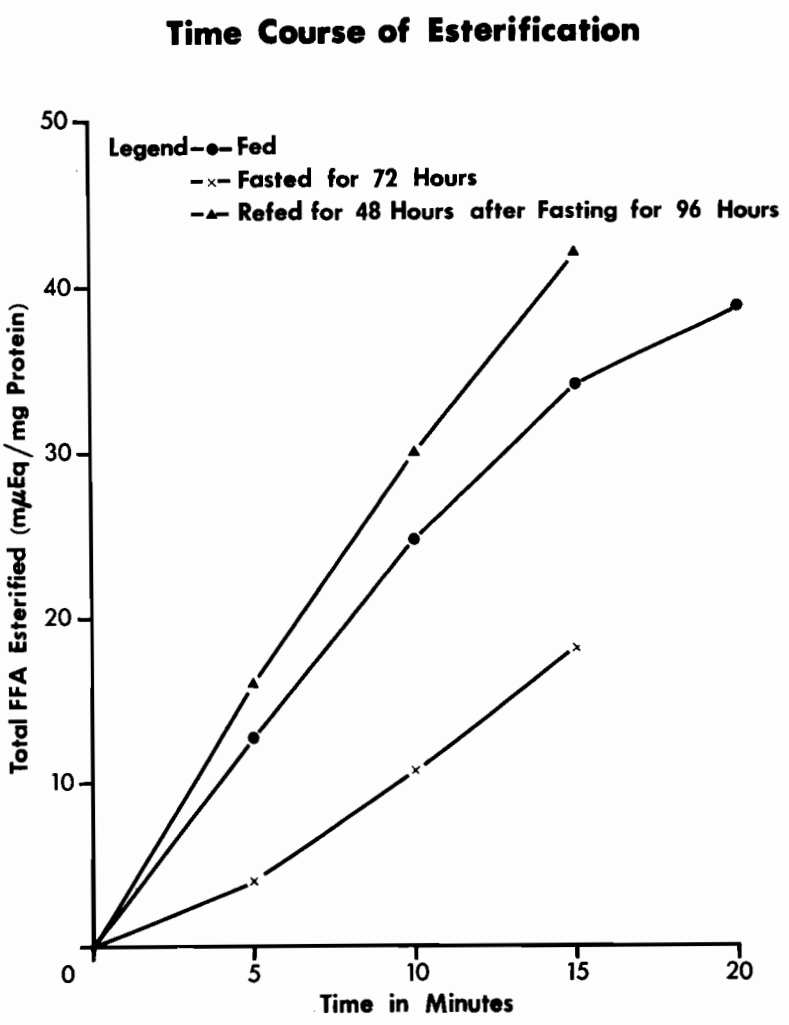


FIG. VII. Effect of duration of incubation on the rate of FFA esterification using enzymes from fed, fasted, and refed rats. Incubations were carried out at 20°C. Total FFA refers to the sum of the concentration of endogenous FFA present in defatted homogenate plus that of the added palmitate-1-C^{14}. In this group of experiments, the initial specific radioactivity of FFA in each reaction mixture was 20-30 counts per minute per mμ Eq FFA. Calculations were based on the initial specific radioactivity.

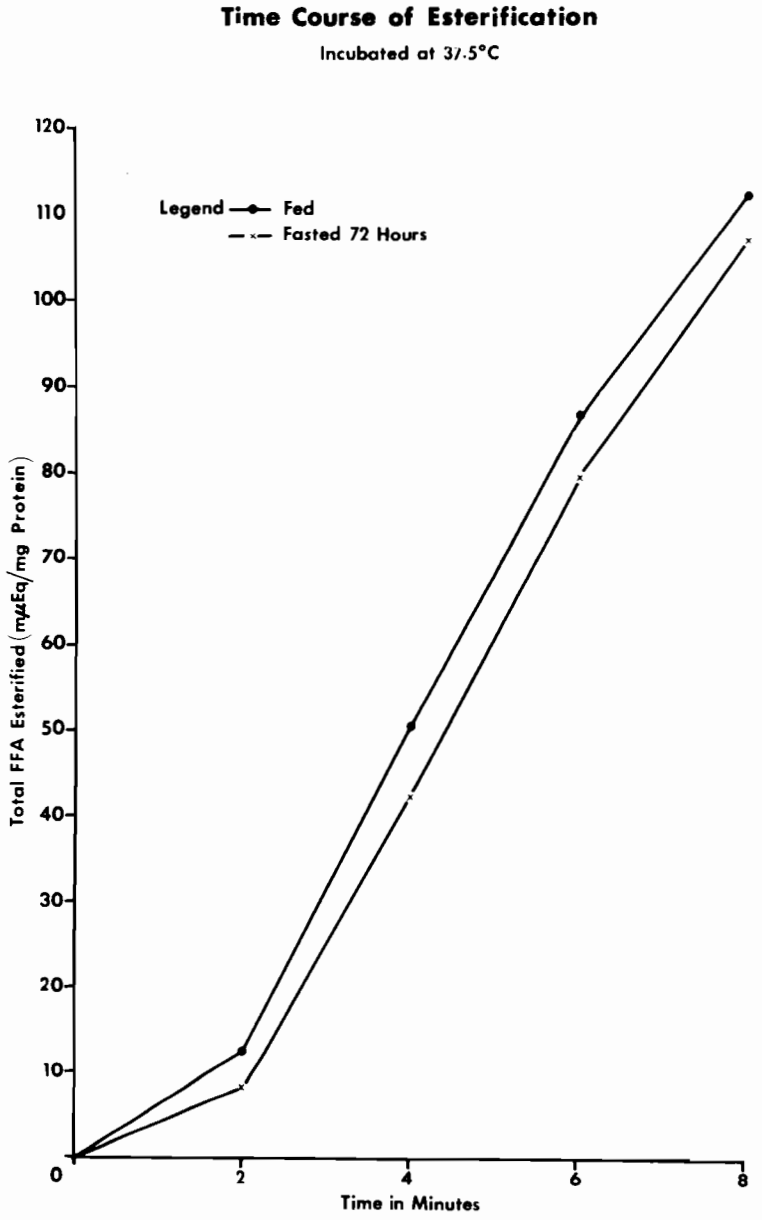


FIG. VIII. Effect of duration of incubation on the rate of FFA esterification using enzymes from fed and fasted rats. As shown, incubations were carried out at 37.5°C.

3) Activity of Enzymes From Fed, Fasted, and Refed Rats.

The subcellular system described in the preceding sub-sections was considered suitable to compare the activity of the glyceride-synthesizing enzymes, from fed, fasted, and refed rats. The defatted homogenate, an unfractionated enzyme preparation, was used. It should be emphasized again, that, since the method of Dole was used, the product that was extracted and quantitated, consisted almost entirely of diglyceride and triglyceride. Since the cofactor requirements did not vary when the enzymes were derived from fasted rats, the total activity of the glyceride-synthesizing enzymes was limiting. In order to compare initial rates, incubations were carried out at 20°C for 15 minutes. The results are illustrated in Figs. IX and X. Calculations were based, in each case, on the initial specific radioactivity. As shown, there was no significant difference between enzyme preparations obtained from rats fasted for the relatively brief period of 24 hours and fed rats. Similar results were obtained with defatted homogenate from rats fasted for 48 hours. When enzyme preparations, however, were obtained from rats fasted for 72 hours, there was a significant depression in total enzymatic activity. This activity, on the other hand, was significantly increased when the enzymes were derived from animals that had been refed for 48 hours after a fasting period of 96 hours.

4) Lipolytic Activity of Defatted Homogenate.

In a number of experiments, the specific radioactivity of the FFA present in the reaction mixture was determined at zero time and after 15 minutes of incubation. These experiments gave a measure of the degree of breakdown of endogenous glycerides and, thus, of

Percentage Incorporation of Palmitate-1-C^{14} into Glyceride by Subcellular Preparations from Fed, Fasted and Refed Rats

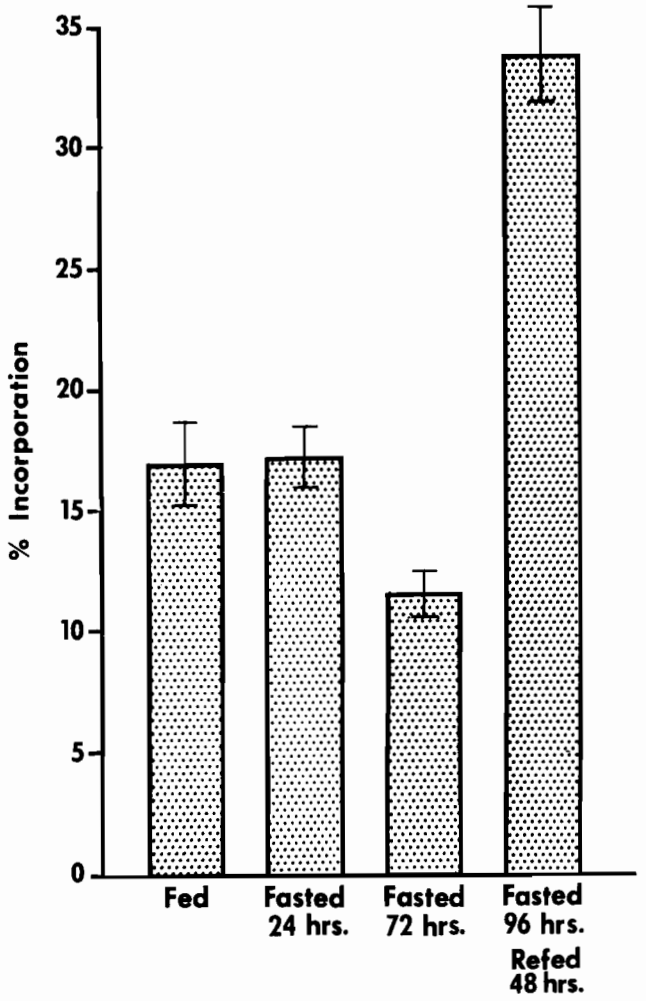


FIG. IX. Histogram compares the total activity of the FFA esterifying enzymes from rats of different nutritional states. Incubations were carried out at 20°C for 15 minutes. There is no significant difference between fed and fasted 24 hours. The difference between fed and fasted 72 hours is significant at $P = 0.005$. The difference between all other groups is significant at $P = 0.001$.

Fatty Acid Esterification by Subcellular Preparations from Fed, Fasted and Refed Rats

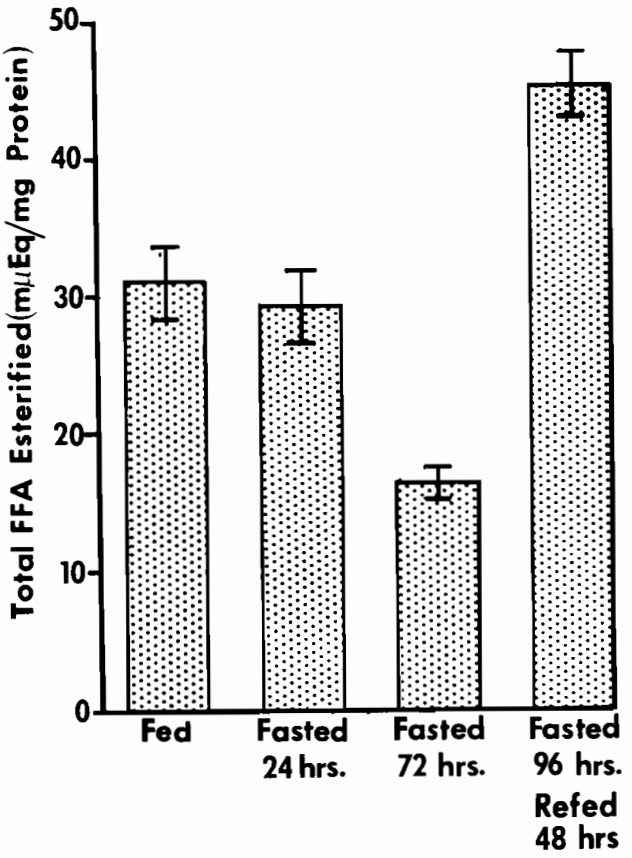


FIG. X. Histogram compares the total activity of the FFA esterifying enzymes from rats of different nutritional states. Incubations were carried out at 20°C for 15 minutes. There is no significant difference between fed and fasted 24 hours. The difference between fed and refed is significant at $P = 0.005$. The difference between all the other groups is significant at $P = 0.001$.

the lipolytic activity present in the defatted homogenate during incubation. The fall in specific radioactivity was calculated as a percentage of the intial value. The results are summarized in Table V. As noted, the specific activity of the FFA fell during incubation in most cases. As previously reported by Steinberg et al. (80), however, the degree of lipolysis was quite variable. When the mean percentage fall in specific radioactivity that occurred during incubation was compared, there was no significant difference between the three groups of animals. The trend towards increased lipolysis that was observed with enzyme preparations from refed rats would, if anything, underestimate the esterifying activity of this group. Thus, the highly significant differences noted between the refed and the fed, or fasted groups, may, in actual fact, be even greater.

5) Characterization of Isotopic Reaction Products.

The extraction method of Folch et al. (403) was used in all the experiments in which the isotopic products were characterized. The labelled esterified products obtained with defatted homogenate from fed rats are shown in Table VI. The ester fraction consisted almost entirely of triglyceride, diglyceride, and phospholipids. It was shown in a number of other experiments that either cholesterol ester or monoglyceride, comprised less than 1% of the radioactivity found in the ester fraction. When the reaction products obtained with defatted homogenate from rats fasted for 72 hours were characterized, the proportions of triglyceride, diglyceride, monoglyceride, and phospholipids were the same as those found with enzymes from fed rats.

TABLE V

LIPOLYTIC ACTIVITY OF DEFATTED HOMOGENATE.

No. of Experiments	Nutritional State	Fall in % Specific Activity of FFA
6	Fed	17.1 ± 11.7
6	Fasted 72 hrs.	12.7 ± 9.3
4	Fasted-Refed 96 hrs.-48 hrs.	41.0 ± 10.5

In each case, incubations were carried out at 20°C for 15 minutes.

The specific radioactivity of FFA was determined at 0 and 15 minutes of incubation.

The results were calculated by subtracting the final specific radioactivity from the initial specific radioactivity and by dividing the difference by the initial value.

The means and their standard errors are shown. There is no significant difference between any of the groups.

TABLE VI

CHARACTERIZATION OF REACTION PRODUCTS.

Reaction Products	% of Total Radioactivity Present in Ester Fraction
Triglyceride	68
Diglyceride	13
Phospholipids	19

Incubations were carried out at 20°C for 15 minutes.

Defatted homogenate from fed rats was the enzyme preparation used.

6) Subcellular Localization of the Esterifying Process.

The subcellular site of esterification was localized by removing successively the mitochondrion-rich and the microsome-rich fractions. The results of two experiments are outlined in Table VII. It is clear that when the mitochondrion-rich fraction was removed by centrifugation at 14,000 X g., the esterification of FFA was decreased considerably. When the microsome-rich fraction was, in turn, removed by centrifugation at 109,000 X g., the remaining soluble fraction did not stimulate esterification. Since palmitate-1-C^{14} was the initial labelled substrate, it is possible that the soluble fraction is only deficient in the FFA activating enzyme. It is probable, however, that all the enzymes involved in the synthetic reactions leading to triglyceride are located in the subcellular particles. The reasons for this proposal will be presented in the discussion.

The esterifying activity of the fractionated and washed mitochondrion-rich and microsome-rich preparations, was compared and related to the reconstituted particles and to that of the defatted homogenate. The results of two experiments are summarized in Table VIII. As shown, each particulate fraction could esterify FFA. When the mitochondrion-rich and microsome-rich preparations were reconstituted, their effects appeared to be additive, but the total value was less than that observed using defatted homogenate. In other experiments, the soluble fraction was added to the reconstituted particles. Although this addition resulted, in most cases, in a relatively small increase in the incorporation of FFA into the ester fraction, the total quantity of FFA incorporated was still lower than that observed with defatted homogenate. The effect of the soluble fraction will be described in detail in succeeding

TABLE VII

EFFECT OF SUCCESSIVE REMOVAL OF PARTICULATE FRACTIONS ON ESTERIFICATION.

Subcellular Fraction	Total mμEq FFA Incorporated into Esterified Fraction.	
	Experiment 1	Experiment 2
Defatted Homogenate (700 X g.)	13	11
14,000 X g. Supernatant	4	2
109,000 X g. Supernatant (Soluble Fraction)	0	0

Incubations were carried out at 20°C for 15 minutes.

In these and all future experiments, unless otherwise specified, the particulate and soluble subcellular fractions present in the reaction mixtures were derived from 1 ml. of defatted homogenate. The enzyme preparation was obtained from fed rats.

The specific radioactivity of the FFA in the reaction mixture was kept at 100 cpm/mμEq. Sodium fluoride was omitted from these experiments.

TABLE VIII

ESTERIFICATION OF FREE FATTY ACIDS BY DIFFERENT SUBCELLULAR FRACTIONS.

Subcellular Fraction	Total mμEq FFA Incorporated into Esterified Fraction.	
	Experiment 1	Experiment 2
Defatted Homogenate	18	13
Mitochondrion-Rich Preparation	12	8
Microsome-Rich Preparation	2	1
Mitochondrion-Rich Plus Microsome-Rich Preparations	11	9

Incubations were carried out at 20°C for 15 minutes.

The results were not expressed per mg of protein because the enzymes involved in esterification appeared to be confined to the particles. Thus, fractions containing the soluble fraction, which has the greatest protein concentration, would have spuriously low values if they were compared to those obtained with the particles on the basis of their protein content.

paragraphs. It is probable that the additional experimental steps required to obtain the mitochondrion-rich, microsome-rich, and soluble fractions could account for the fact that the reconstituted subcellular fractions exhibited less esterifying activity than the defatted homogenate. While the two particulate fractions were not entirely free of reciprocal contamination, they were fairly homogeneous. Thus, it seems probable that each can esterify FFA independently. The relative activities, however, are difficult to assess because the additional manipulations required to isolate the microsome-rich fraction may depress the enzymatic activity of this enzyme preparation.

In these experiments, the values obtained with defatted homogenate, were lower than those observed when fed, fasted, and refed groups were compared. This discrepancy is due to differences in experimental procedure. In the present experiments, the defatted homogenate was kept at 0-4°C for about 90 more minutes, while the other fractions were being prepared. The enzymatic activity was reduced by about 40% during this period of time. The composition of the esterified products, however, was not altered. In addition, in the current experiments the quantity of defatted homogenate that was used was one third of that present in those studies in which the esterifying activity of enzymes from rats of different nutritional states was compared.

In some experiments, mitochondria and microsomes were incubated with a solution of potassium palmitate-1-C^{14} at 0°C for 30 minutes. When about 2,000 µGm. of particulate protein were incubated with about 200 mµEq of palmitate-1-C^{14} (specific radioactivity 0.15 mc./mg.), 73% of the radioactivity became associated with mitochondria and microsomes. Subcellular particles containing bound radioactive fatty acid were collected by centrifugation at 109,000 X g., washing with 0.15 potassium chloride, and re-centrifugation. These particles were, then, incubated with α - glycerophosphate and

cofactors in the presence and absence of soluble fraction. Finally, the particles were separated from their supernates by centrifugation at 109,000 X g., washing, and re-centrifugation. As shown in Table IX, most of the radioactivity present in the ester and FFA fraction was recovered from mitochondria and microsomes.

7) Kinetics of FFA Esterification Using Subcellular Particles.

a) Time Course Studies.

Time courses of esterification using mitochondrion-rich and microsome-rich fractions are illustrated in Fig. XI. The variability that was encountered with these preparations was probably due to the failure of obtaining uniformly homogeneous suspensions when the particles were re-suspended in potassium chloride. In the case of the mitochondrion-rich fraction, the reaction rate was rather slow up to 15 minutes of incubation; it, then, accelerated up to 45 minutes, and declined thereafter. The findings obtained with the microsome-rich fraction were more difficult to explain because of the small degree of incorporation. In any event, the increase in incorporation of palmitate-1-C^{14} radioactivity into the esterified fraction appeared to be approximately linear up to at least 30 minutes of incubation. It was considered possible, moreover, that the small degree of incorporation noted with the microsome-rich fraction might have been due to an excessive concentration of FFA or their thiolesters. In the presence of mitochondria, a substantial fraction of FFA would probably be bound, while the microsomal binding sites might become rapidly saturated by the same concentration of FFA. The possibility that incorporation of FFA into the ester fraction using the microsome-rich

TABLE IX

CENTRIFUGAL SEPARATION OF LABELLED LIPIDS.

Subcellular Fraction	Percentage of Radioactivity Associated with Particles.	
	Ester Fraction	FFA Fraction
Mitochondria + Microsomes	89	83
Mitochondria + Microsomes + Soluble Fraction	83	83

Palmitate-1-C^{14}(S.A. 0.15mc./mg.) was bound by mitochondria and microsomes during incubation at 0°C for 30 minutes. The particles (about 500 µGm. of particulate protein in each reaction mixture) were, then, incubated with α -glycerophosphate and cofactors at 20°C for 30 minutes, in the presence and absence of soluble fraction. At the end of incubation, the particulate fractions and their supernates were separated by centrifugation, washing, and re-centrifugation. They were, then, extracted and the radioactivity, in each, was determined.

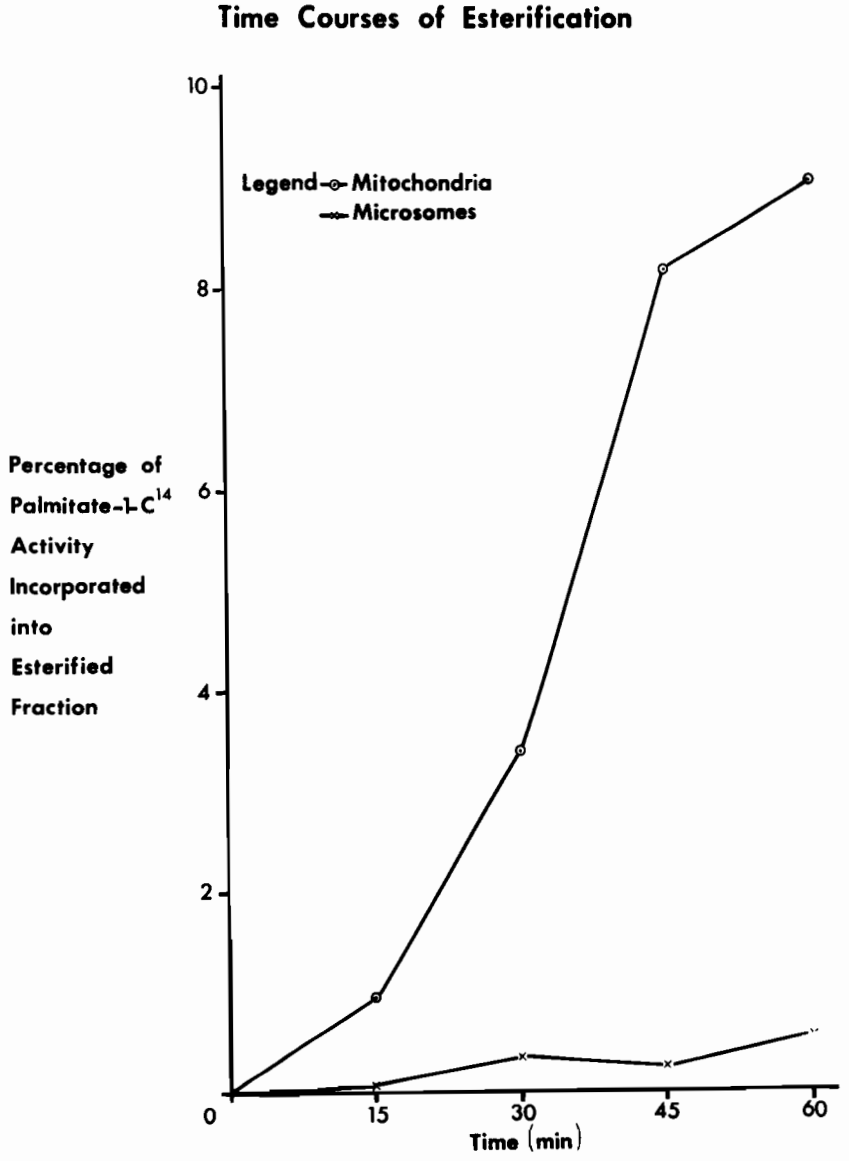


FIG. XI. Effect of duration of incubation on the rate of FFA esterification using mitochondrion-rich and microsome-rich preparations. Incubations were carried out at 20°C. The mitochondrial protein in each reaction mixture was about 700 μ Gm. The content of microsomal protein in each reaction mixture was about 500 μ Gm.

fraction was inhibited by excessive concentrations of FFA, was excluded by demonstrating that the quantity of FFA incorporated was not reduced when the concentration of added palmitate-1-C^{14} was augmented from a very small to the usual value.

Time course studies were also done using mitochondria and microsomes which had been sedimented together. The combined particulate fractions were incubated in the presence and absence of soluble fraction. The results are depicted in Fig. XII. In the absence of soluble fraction, the reaction rate accelerated up to about 90 minutes of incubation, and, then, declined. In the presence of soluble fraction, a plateau was not reached by 120 minutes of incubation. Except for the data obtained at 90 minutes, the amount of palmitate-1-C^{14} that was esterified in the presence of soluble fraction was about 30% greater than the amount esterified in the absence of the 109,000 X g. supernate. This difference could be explained by the fact that for each molecule of phosphatidic acid that was eventually converted to triglyceride, an additional molecule of fatty acid had to be incorporated into the ester fraction.

b) Effect of Increasing Amounts of Particles.

A number of experiments were designed to study the effect of increasing concentrations of subcellular particles. A typical example is illustrated in Fig. XIII. As depicted, when the quantity of mitochondria and microsomes, which had been sedimented together, was increased beyond a certain concentration, the reaction rate did not increase in a linear fashion, but it accelerated rapidly. This phenomenon will be dealt with in greater detail in the discussion.

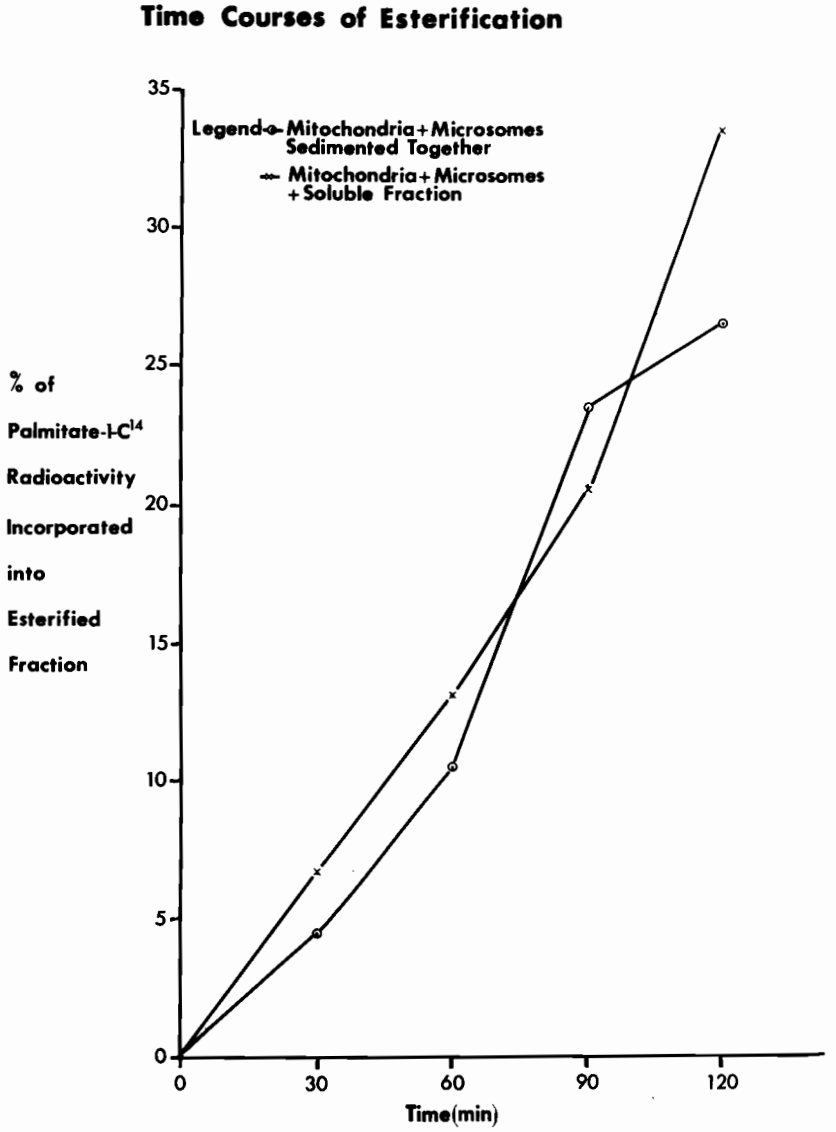


FIG. XII. Effect of duration of incubation on the rate of FFA esterification using the combined mitochondrial and microsomal fraction in the presence and absence of soluble fraction. Incubations were carried out at 20°C. The content of mitochondrial and microsomal protein in each reaction mixture was about 250 μ Gm.

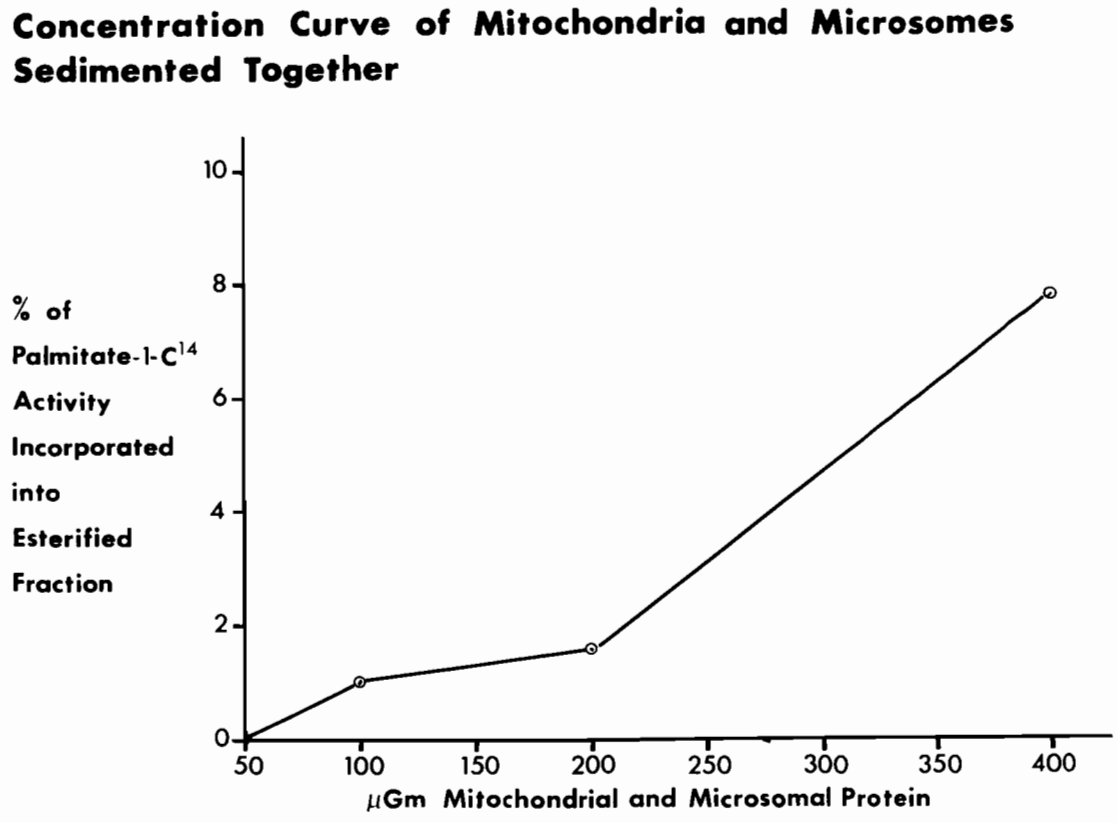


FIG. XIII. Effect of increasing concentrations of mitochondria and microsomes on the rate of FFA esterification. Incubations were carried out at 20°C for 30 minutes. 200 μ Gm. of mitochondrial and microsomal protein were derived from 1 ml. of defatted homogenate.

c) Characterization of Products Using Subcellular Particles.

The reaction products were characterized in each experiment. The typical distribution of radioactivity that was found in the esterified fraction when the combined mitochondrial and microsomal fraction was incubated for different periods of time, is illustrated in Fig. XIV. It is evident that phospholipids were the major isotopic products. Since the total amount of radioactivity present in the cholesterol ester and monoglyceride fractions was less than 1% of the label present in the ester fraction, these lipids were disregarded in most cases. As depicted in Fig. XIV, moreover, when incubation was prolonged from 30 to 120 minutes, the proportion of total phospholipid radioactivity decreased progressively, but only to a small extent. Most of the corresponding increase in the glyceride fraction was recovered in triglyceride. In other experiments, it was demonstrated that the pattern of reaction products was similar to that shown in Fig. XIV when the mitochondrion-rich and microsome-rich fractions were used separately. The labelled phospholipids which were formed using the combined mitochondrial and microsomal preparation, were fractionated further. As shown in Table X, most of the radioactivity present in the phospholipid fraction, was recovered from the phosphatidic acid area. Similar results were obtained when the enzyme preparation consisted of defatted homogenate.

The effect of increasing amounts of mitochondria and microsomes on the pattern of isotopic products is depicted in Fig. XV. When the mitochondrial and microsomal protein was increased from about 145 µGm. to about 1160 µGm., the proportion of phospholipid radioactivity was progressively decreased with a corresponding increase in the glyceride fraction. The effect of increasing concentrations of particulate protein on the pattern of isotopic esterified products was

Characterization of Reaction Products Using Mitochondria and Microsomes Sedimented Together

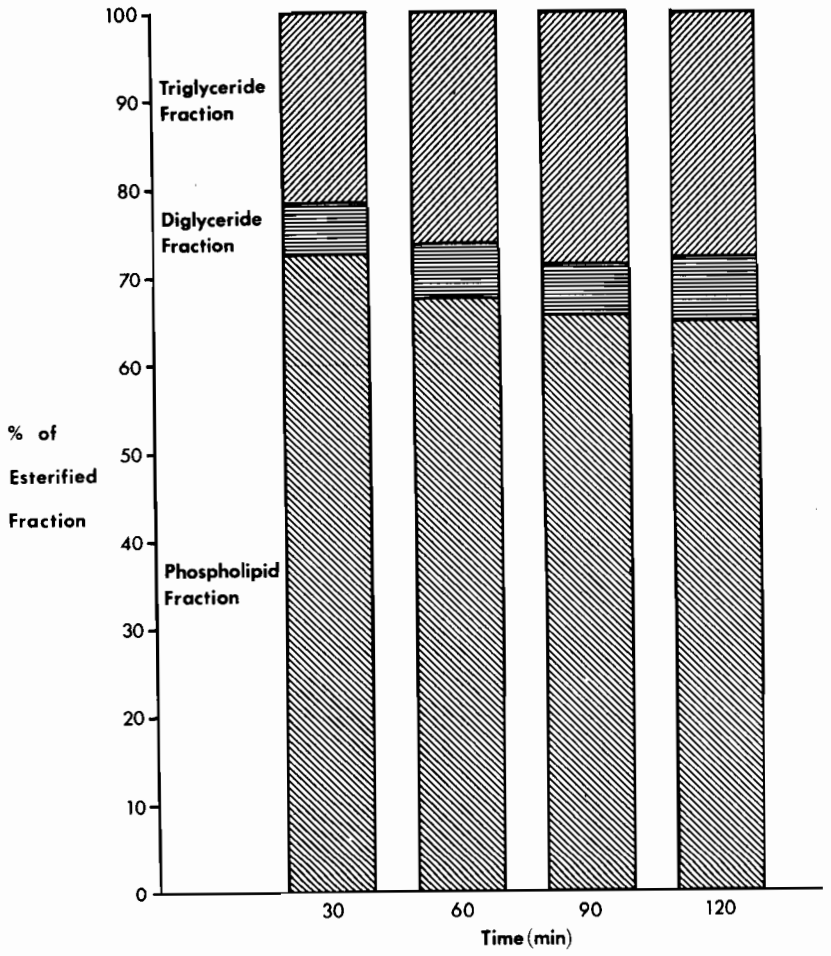


FIG. XIV. Effect of duration of incubation on the distribution of radioactivity in the ester fraction using the combined mitochondrial and microsomal preparation. Incubations were carried out at 20°C. Triglyceride, diglyceride, and phospholipids are illustrated in each bar as percentages of the total radioactivity recovered from the esterified fraction. About 250 μ Gm. of mitochondrial and microsomal protein were present in each reaction mixture.

TABLE X.

CHARACTERIZATION OF PHOSPHOLIPIDS.

Phospholipid	% of Total Radioactivity Present in Phospholipids Fraction
Phosphatidic Acid	94
Phosphatidyl Ethanolamine	1
Phosphatidyl Serine	3
Lecithin	1
Lysolecithin	1

Incubations were carried out at 20°C for 60 minutes. The enzyme preparation consisted of mitochondria and microsomes (washed 109,000 X g. sediment).

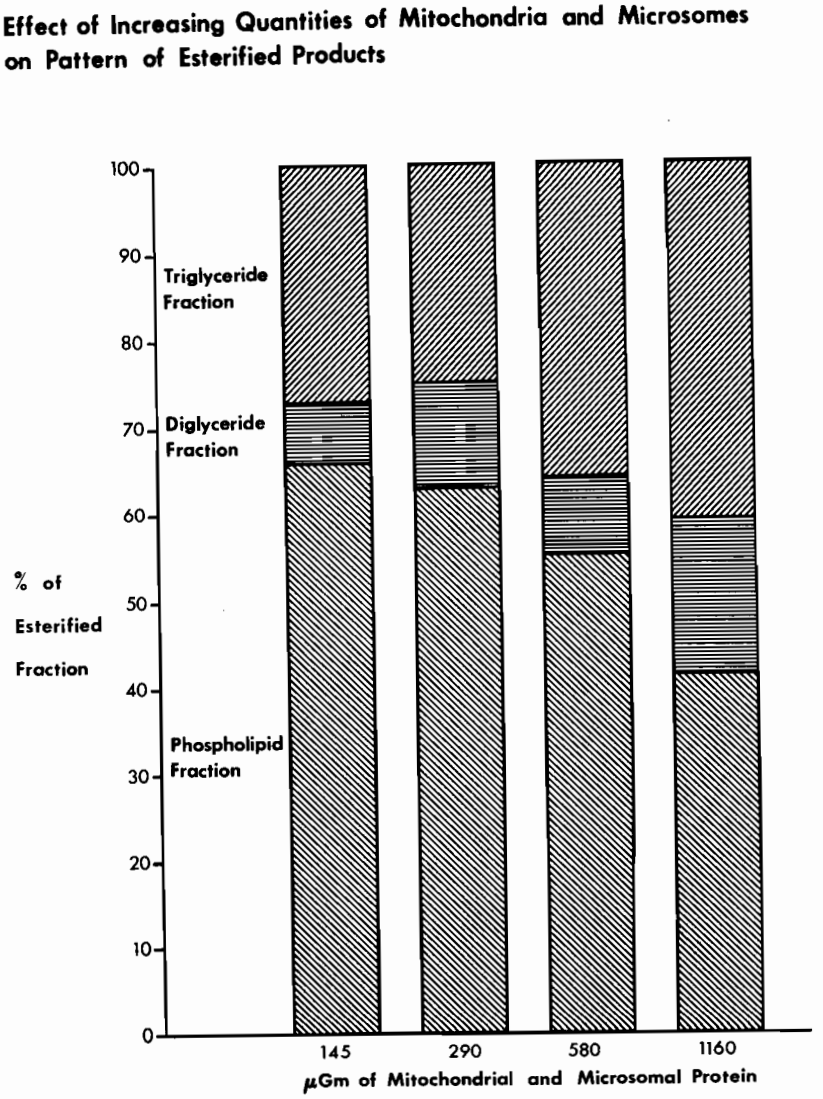


FIG. XV. Effect of increasing concentrations of mitochondria and microsomes on the distribution of radioactivity in the ester fraction. Incubations were carried out at 20°C for 30 minutes. Each bar graph shows percentage of triglyceride, diglyceride and phospholipid as explained in subscript for FIG. XIV. About 290 μ Gm. of mitochondrial and microsomal protein were derived from 1 ml. of defatted homogenate.

greater than that of prolongation of incubation.

Thus, the proportion of radioactivity recovered in the glyceride fraction was augmented by lengthening the incubation time, and by increasing the concentration of mitochondrial and microsomal protein.

8) Effect of Soluble Fraction on Pattern of Products.

While the soluble fraction was incapable of stimulating FFA esterification when used alone, it had a marked and consistent effect on the pattern of the esterified products that were formed in the presence of the particulate fractions. This effect is shown by the representative data in Table XI. The soluble fraction had a similar effect on the mitochondrion-rich and on the microsome-rich fractions. In each case, the proportion of isotopic phospholipids was strikingly reduced with a corresponding increase in labelled glycerides, most marked in the triglyceride fraction. The effect of the soluble fraction was reproduced in the 21 experiments in which it was sought.

The soluble fraction, moreover, had a similar influence on the pattern of products when the enzyme preparation consisted of the combined mitochondrial and microsomal fraction. As illustrated in Fig. XVI, the effect on this enzyme preparation was progressively enhanced when increasing amounts of soluble fraction were used. In addition, when the quantity of mitochondria and microsomes was decreased, while the volume of soluble fraction was kept at 1.0 ml., an increased incorporation of radioactivity into the glyceride fraction was observed.

The effects of lengthening the incubation time in the presence and absence of soluble fraction are depicted in

TABLE XI

EFFECT OF SOLUBLE FRACTION ON COMPOSITION OF ESTERIFIED FRACTION.

Percentage of Total Esterified Fraction	Mitochondrion-rich Fraction	Mitochondrion-rich Fraction + Soluble Fraction	Microsome-rich Fraction	Microsome-rich Fraction + Soluble Fraction
Triglyceride	39	75	33	66
Diglyceride	9	12	14	19
Phospholipids	52	13	53	15

Incubations were carried out at 20°C for 30 minutes.

About 300 µGm. of mitochondrial protein and about 130 µGm. of microsomal protein were present in separate reaction mixtures.

Effect of Increasing Quantities of Soluble Fraction on Pattern of Esterified Products

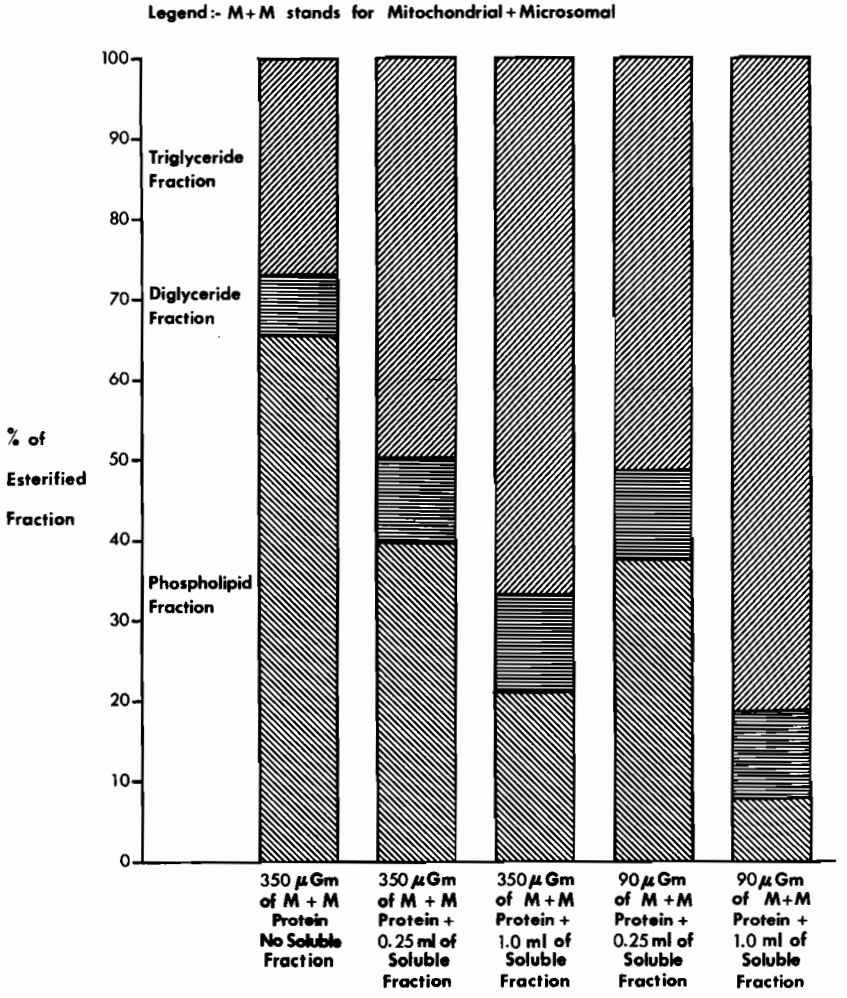


FIG. XVI. Effect of increasing quantities of soluble fraction on the distribution of ester radioactivity. Incubations were carried out at 20°C for 30 minutes. 180 μ Gm. of mitochondrial and microsomal protein and 1 ml. of soluble fraction were derived from 1 ml. of defatted homogenate.

Fig. XVII. As shown, the effect of the soluble fraction on the pattern of radioactive esterified products was more pronounced when incubation was prolonged from 30 to 120 minutes.

It was, then, considered of interest to determine whether the site of action of the soluble fraction was inside the subcellular particles or on their surface, or, alternatively, whether the soluble fraction acted at a locus other than the particles. For this reason, mitochondria and microsomes were pre-incubated with an aliquot of soluble fraction at 20°C for 30 minutes. Following incubation, discrete subcellular particles were prepared by centrifugation, washing with 0.15 M potassium chloride, and re-centrifugation. Aliquots of these particles were, then, incubated with substrates and cofactors. As shown in Table XII, a definite increase in the proportion of radioactivity found in the glyceride fraction was observed using mitochondria and microsomes that had been pre-incubated with soluble fraction.

Having established that a factor or factors responsible for the effect of the soluble fraction acted either inside the mitochondria and microsomes, or on their surface, it was considered pertinent to establish whether they exerted their effect before or after the fatty acids were bound to the particles. To explore this problem, mitochondria and microsomes containing bound palmitate-1-C^{14} were prepared as described previously. These particles were, then, incubated with α- glycerophosphate and cofactors in the presence or absence of soluble fraction. As outlined in Table XIII, the soluble fraction brought about an increase in the proportion of radioactive glycerides. These results suggest that the factor or factors present in the soluble fraction act after FFA have been bound to the particles. In this experiment, the proportion of isotopic glycerides formed in the absence of soluble fraction was unusually high. While this finding

Characterization of Reaction Products Using Mitochondria and Microsomes in Presence and Absence of Soluble Fraction

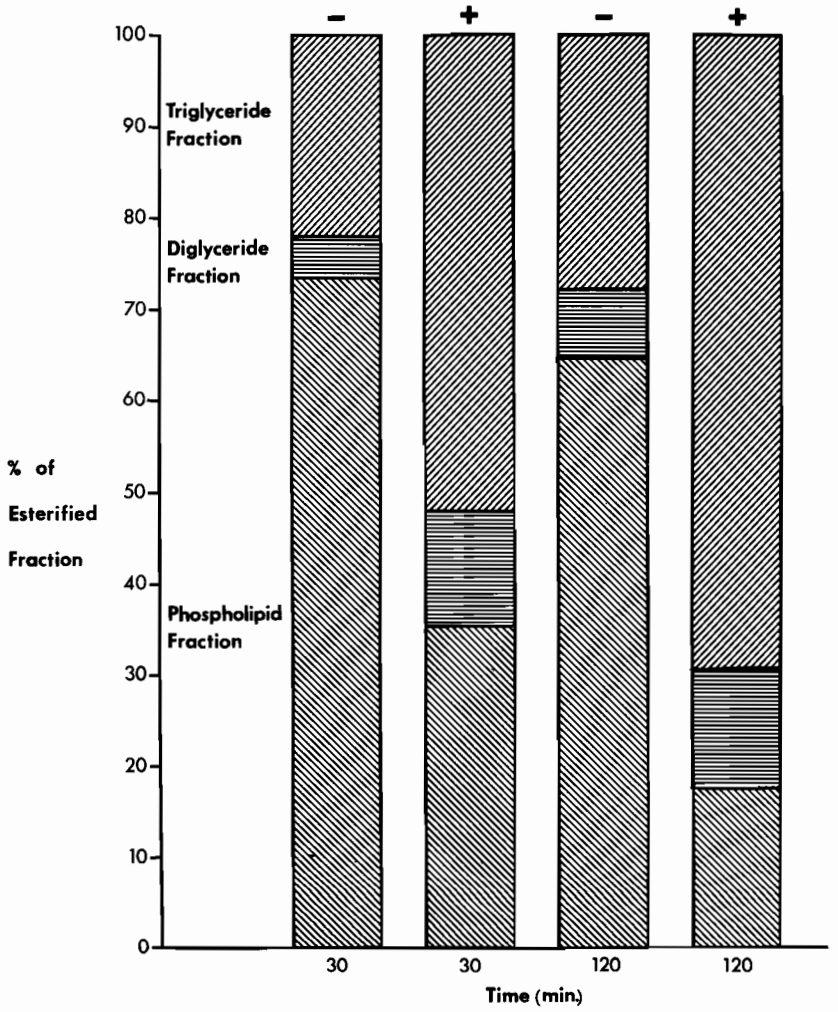


FIG. XVII. Effect of prolongation of incubation time on the distribution of radioactivity in the ester fraction, in the presence and absence of soluble fraction. Incubations were carried out at 20°C. The content of mitochondrial and microsomal protein was about 250 μ Gm. + refers to the presence of 1 ml. of soluble fraction. - refers to its absence.

TABLE XII

EFFECT OF PRE-INCUBATION OF SUBCELLULAR PARTICLES WITH SOLUBLE FRACTION.

Percentage of Total Esterified Fraction	Mitochondria and Microsomes Pre-Incubated with 0.15 M Potassium Chloride	Mitochondria and Microsomes Pre-Incubated with Soluble Fraction
Triglyceride	28	48
Diglyceride	5	5
Phospholipids	67	47

The subcellular particles were pre-incubated with 0.15 M potassium chloride or soluble fraction at 20°C for 30 minutes. The particles were sedimented at 109,000 X g., washed, and re-centrifuged. Incubations were, then, carried out at 20°C for 30 minutes with substrates and cofactors, as usual.

TABLE XIII

EFFECT OF PRE-INCUBATION OF PALMITATE-1-C^{14} WITH MITOCHONDRIA AND MICROSOMES ON COMPOSITION OF ESTERIFIED PRODUCTS.

Percentage of Total Esterified Fraction	Mitochondria + Microsomes + 0.15 M Potassium Chloride	Mitochondria + Microsomes + Soluble Fraction
Triglyceride	43	69
Diglyceride	22	20
Phospholipids	35	11

Palmitate-1-C^{14} was bound by mitochondria and microsomes during incubation at 0°C for 30 minutes. The particles (about 460 µGm. of particulate protein in each reaction mixture) were, then, incubated with α -glycerophosphate and cofactors at 20°C for 30 minutes in the presence and absence of soluble fraction.

might have been related to the fact that palmitate was bound by the particles before incubation, the reason for this observation is not known at the present time.

It was also considered of interest to determine whether the soluble fraction exerted its effect only at the beginning of esterification, or whether it could still act after most of the reactions stimulated by the particles had been completed. This problem was investigated by pre-incubating mitochondria and microsomes with substrates and cofactors at 20°C for 75 minutes. Discrete particles were collected by centrifugation at 109,000 X g., washing with 0.15 M potassium chloride, and re-centrifugation. As summarized in Table XIV, incubation of these mitochondria and microsomes with soluble fraction resulted in an increase in the proportion of radioactive glycerides. Hence, the soluble fraction could act even after most of the esterification reactions stimulated by the particles had been completed.

9) Attempts at Characterization of Soluble Fraction Factor.

Preliminary experiments were performed in an attempt to characterize the factor or factors present in the soluble fraction which were responsible for such a consistent effect on the pattern of isotopic esterified products formed in the presence of subcellular particles. The effect of heating the soluble fraction is shown in Table XV. Even though the effect of the soluble fraction was reduced by heating at 58°C for 10 minutes, the change in the pattern of the products was still clear. On the other hand, the activity of the mitochondria and microsomes was abolished by the same treatment. In another experiment, heating the soluble fraction at 53°C for 5 minutes influenced the effect of the soluble fraction to a much smaller extent, even though

TABLE XIV

EFFECT OF SOLUBLE FRACTION ON PATTERN OF RADIOACTIVE PRODUCTS USING PARTICLES PRE-INCUBATED WITH SUBSTRATES AND COFACTORS.

Percentage of Total Esterified Fraction	Mitochondria + Microsomes + 0.15 M Potassium Chloride	Mitochondria + Microsomes + Soluble Fraction
Triglyceride	28	43
Diglyceride	18	17
Phospholipids	55	40

Mitochondria and microsomes were pre-incubated with substrates and cofactors at 20°C for 75 minutes. Then, discrete particles were collected by centrifugation at 109,000 X g., washing with 0.15 M potassium chloride, and re-centrifugation. Subsequently, aliquots (containing about 40 µGm. of mitochondrial and microsomal protein) were incubated at 20°C for 30 minutes with either potassium chloride or soluble fraction. The particles in each reaction mixture were derived from 0.25 ml. of defatted homogenate.

TABLE XV

EFFECT OF HEATING SOLUBLE FRACTION ON PATTERN OF RADIOACTIVE PRODUCTS.

Percentage of Total Esterified Fraction	Mitochondria + Microsomes	Mitochondria + Microsomes + Soluble Fraction	Mitochondria + Microsomes + Soluble Fraction Heated at 58°C for 10 minutes.
Triglyceride	28	78	40
Diglyceride	4	5	8
Phospholipids	68	17	51

Incubations were carried out at 20°C for 30 minutes.

the particles were, again, completely inactivated.

Albumin and a number of lipoproteins have been shown to enhance both glyceride and phospholipid synthesis by rat liver microsomes (395). Bovine and human albumin, and Cohn's human serum fractions II, III, IV-1, and IV-4 (423), however, had no effect on either the total quantity of FFA incorporated into the esterified fraction or on the pattern of labelled esters formed by subcellular preparations of rat adipose tissue.

In some experiments, an aliquot of soluble fraction was dialyzed against excess 0.15 M potassium chloride. As shown in Table XVI, dialysis resulted in a decreased effect of the soluble fraction. After an aliquot of soluble fraction was dialyzed against an equal volume of 0.15 M potassium chloride, the dialyzing solution was capable of altering the pattern of radioactive products in a way qualitatively similar to that of the soluble fraction. Since the factor or factors responsible for the effect of the soluble fraction could be recovered from the dialyzing solution, they probably diffused through the dialyzing membrane, and they were not adsorbed by it to any appreciable extent. The effect of the solution inside the bag was usually greater than that of the dialyzing solution, an observation that suggested incomplete equilibration of the factor or factors between the two sides of the dialyzing membrane.

The possibility that the effect of the soluble fraction was due to a compound with an active sulfhydryl group was also investigated. The reactions were allowed to proceed for 60 minutes in the presence of mitochondria and microsomes. After this period of incubation, a large proportion of the esterification reaction had taken place. An aliquot of soluble fraction, which had been preincubated with a thiol inhibitor, was then, added, and incubation carried

TABLE XVI

EFFECT OF DIALYSIS OF SOLUBLE FRACTION ON PATTERN OF RADIOACTIVE PRODUCTS.

Percentage of Total Esterified Fraction	Mitochondria + Microsomes + Soluble Fraction	Mitochondria + Microsomes + Dialyzed Soluble Fraction
Triglyceride	56	41
Diglyceride	13	9
Phospholipids	32	50

Incubations were carried out at 20 C for 30 minutes. The aliquot of soluble fraction that was not dialyzed was kept at the same temperature for the same length of time as the sample that was dialyzed against excess 0.15 M potassium chloride.

on for 30 more minutes. Such inhibitors as 10^{-2} M sodium iodoacetate and 10^{-3} M sodium arsenate failed to alter the effect of the soluble fraction.

Since the effect of the soluble fraction was decreased by heating at 58 and 53°C, it was considered unlikely that the responsible factor or factors were simple ions. It is possible, however, that heating alters the properties of a larger compound, such as a polypeptide. The latter might have a functional, small, charged moiety. A variety of ions were added to the particulate enzyme preparations in an attempt to reproduce, perhaps through association with a larger substance, the effect of the soluble fraction. The results of these experiments are summarized in Table XVII. As shown, divalent cobalt ions, at concentrations of 2 and 6 mEq./L, were the only ones that had a definite effect on the pattern of the isotopic esters formed. At a concentration of 0.02 mEq./L, the effect of cobalt ions was not convincing, even though there appeared to be a trend toward a greater proportion of radioactive glyceride in the ester fraction.

As shown in Table XVII, divalent nickel and manganese ions, at a concentration of 2 mEq./L, brought about a slight increase in the proportion of radioactive glyceride present in the ester fraction. Although these results may show a trend, they are not significant. Iron, sodium and citrate ions did not reproduce the effect of the soluble fraction to any extent. Calcium ions, on the other hand, at a concentration of 2 mEq./L, resulted in a small decrease in the proportion of radioactive glyceride. Hence, divalent cobalt ions were the only ones that had a definite effect on the distribution of radioactivity in the ester fraction. While this effect was similar to that of the soluble fraction, it was not as pronounced at any of the concentrations that were used.

TABLE XVII

EFFECT OF IONS ON PROPORTION OF RADIOACTIVE GLYCERIDE PRESENT IN ESTER FRACTION.

Ion	Concentration of Ion (mEq/L)	% of Radioactive Glyceride		
		Mitochondria and Microsomes (None of the listed ions nor soluble fraction were added)	+ Ion	+ Soluble Fraction (None of the listed ions were added)
Co^{++}	0.02	25	32	49
Co^{++}	2	28	45	61
Co^{++}	6	55	68	79
Ni^{++}	2	28	34	61
Mn^{++}	2	28	37	61
Fe^{++}	2	28	27	61
Ca^{++}	2	25	18	49
Na^{+}	8	25	29	49
Citrate	10	25	25	49

Incubations were carried out at 20°C for 30 minutes.
All cations were in the form of chlorides before the solutions were prepared. Citrate was in the form of its sodium salt.
The term glyceride refers to triglyceride and diglyceride.

The possibility was also considered that the soluble fraction might contain diglycerides, which could act as acceptors of free fatty acids. Such a process would result in a greater percentage of isotopic triglyceride. This possibility was ruled out by the fact that esterification was negligible when α - glycerophosphate was omitted from the reaction mixture. Furthermore, the effect of the soluble fraction persisted after extraction of its lipids with diethyl ether.

Thus, these preliminary experiments suggest that the effect of the soluble fraction is due to one or more dialyzable and partially heat-stable factors.

10) Attempts at Isolation of FFA Pools Active in Esterification.

In some experiments, the newly formed isotopic triglycerides were fractionated further on the basis of their degree of unsaturation. As outlined in Table XVIII, 67% of the radioactivity present in triglyceride formed using defatted homogenate was recovered, in saturated and monounsaturated classes. These observations were consistent with the fact that saturated acids comprised 60-70% of the total FFA present in each reaction mixture. As shown in Table XIX, the percentage of radioactive saturated and monounsaturated triglycerides using the mitochondrion-rich preparation was also 67%. Since it was shown in a previous sub-section that FFA were esterified exclusively in subcellular particles, the presence of 68% of the total triglyceride radioactivity in classes containing one or more double bonds suggested two possible mechanisms. The first was desaturation of radioactive palmitate and the second was mixing of the labelled saturated acid with unlabelled, unsaturated fatty acids.

TABLE XVIII

DISTRIBUTION OF RADIOACTIVITY AMONGST TRIGLYCERIDE CLASSES (DEFATTED HOMOGENATE).

Number of Double Bonds	% of Total Radioactivity Present in Triglyceride Fraction
0	30
1	37
2	24
3 and 4	9
5 or more	1

Incubations were carried out at 20°C for 15 minutes.

Defatted homogenate was used to obtain the triglyceride fraction.

Average values of two experiments are shown.

TABLE XIX

DISTRIBUTION OF RADIOACTIVITY AMONGST TRIGLYCERIDE CLASSES

(MITOCHONDRION-RICH PREPARATION).

Number of Double Bonds	% of Total Radioactivity Present in Triglyceride Fraction
0	32
1	35
2	22
3 +	11

Incubations were carried out at 37.5°C for 60 minutes.

The mitochondrion-rich preparation was used to obtain the triglyceride fraction.

Desaturation of palmitate-1-C^{14} by mitochondria could account for the presence of unsaturated triglyceride classes. This mechanism, however, was considered unlikely in view of the fact that at least the conversion of saturated acids to their monoenoic analogues would have most probably required NADPH, (216), a cofactor that was absent in the present study. In addition, such a reaction has been described to take place in microsomes rather than mitochondria (216). In order to definitively exclude the possibility that palmitate-1-C^{14} was desaturated by subcellular particles, the FFA were isolated at the end of incubation, methylated, and separated into classes on the basis of their degree of unsaturation. As shown in Table XX, using the combined mitochondrial and microsomal fraction, radioactivity was associated almost entirely with the saturated class. The minute amount of radioactivity that was recovered in other areas might have been due to free saturated acids which were not methylated. Similar results were obtained when the soluble fraction was present in addition to mitochondria and microsomes. Hence, the added radioactive palmitate was not desaturated to any appreciable extent.

It was, thus, certain that unsaturated, unlabelled fatty acids had mixed with the radioactive palmitate during triglyceride formation stimulated by the mitochondrion-rich enzyme preparation. The unsaturated fatty acids could have been derived in two ways. The first possible consideration is that mitochondria might have one or more FFA pools, which participate actively in the esterifying process. The unsaturated fatty acids present in these hypothetical pools would mix with the added saturated, radioactive acid during esterification. The second possibility was that endogenous free fatty acids from other subcellular sites were introduced into the particles during

TABLE XX

DISTRIBUTION OF RADIOACTIVITY AMONGST FFA CLASSES.

Number of Double Bonds	% of Total Radioactivity Present in FFA Fraction
0	96
1	0
2 +	4

Incubations were carried out at 20°C for 30 minutes.

The combined mitochondrial and microsomal fraction was the enzyme preparation used to obtain the FFA fraction. Fatty acids were methylated and, then, separated on thin-layer chromatography.

such experimental steps as homogenization and differential centrifugation. Some of these free fatty acids might have been liberated from glycerides during these procedures.

To explore the second possibility, palmitate-1-C^{14} was added at the beginning of homogenization, and a model experiment was carried through in the absence of any exogenous substrates or cofactors. The results are shown in Table XXI. It is clear that an appreciable proportion of radioactivity was introduced into the particles during the experimental manipulations. Hence, while it is still possible that some of the unsaturated fatty acids have been derived from FFA pools, located in the mitochondria and active in the esterifying process, this method cannot be used to determine whether, indeed, the particles have such pools. Any future attempts to explore this problem using similar methods should be regarded as hazardous in view of these findings.

TABLE XXI

INTRODUCTION OF LABELLED PALMITATE INTO PARTICLES DURING EXPERIMENTAL PROCEDURE.

Subcellular Fraction	% of Total Radioactivity
Mitochondria and Microsomes Sedimented Together	13
Soluble Fraction	8
Fat Cake	79

There were no incubations.

Substrates and cofactors were not added.

All procedures were carried out at 4°C.

D) DISCUSSION.

In this study, the requirements that were determined for glyceride synthesis by subcellular preparations of rat adipose tissue, were the same as those found by Angel (394), and resembled those found by Steinberg et al. (80). These requirements, moreover, were similar to those which had been reported earlier for rat liver microsomes (238, 240).

As previously observed by Angel(394), the retardation of the reaction rate brought about by lowering the incubation temperature from 37.5°C to 20°C, allowed accurate kinetic studies to be performed. Since periods of fasting and refeeding did not alter the cofactor requirements for glyceride synthesis, the total activity of the FFA esterifying enzymes was limiting. Hence, the system used in this study was considered suitable for comparing the initial rates of FFA esterification by enzymes from fed, fasted, and refed animals. When the rate of glyceride formation from FFA and α - glycerophosphate was determined, the enzymes involved were fatty acid: CoA ligase, acyl CoA:L-glycerol-3-phosphate acyl transferase, L-α-phosphatidate phosphohydrolase (phosphatidic acid phosphatase) and acyl CoA:diglyceride acyl transferase. As previously stated, the total activity of these enzymes was assayed in this study.

A significant depression of total enzymic activity was observed only after a prolonged fasting period of 72 hours. Hence, the decreased esterification of fatty acids noted by others in intact rat adipose tissue during relatively short periods of fasting (30, 290), was probably due to deficiency of α - glycerophosphate, and possibly, decreased responsiveness to insulin (424). During protracted fasting, however, the total activity of the enzymes leading to

the formation of glyceride from FFA and α- glycerophosphate, was significantly reduced. Hence, the reduction of esterification observed by others in intact rat adipose tissue during prolonged fasting (136), was probably due to depressed total activity of these enzymes, in addition to decreased availability of α- glycerophosphate, and, perhaps, depressed responsiveness to insulin.

A number of possible mechanisms could explain the decreased total activity of the FFA esterifying enzymes. Prolonged fasting may result in decreased enzyme formation because of an inadequate supply of the constituents of nucleic acids and proteins, namely, nucleotides and amino acids. Decreased enzyme formation may also be due to repression of enzyme synthesis. This process has been studied in bacteria and it is recognized as a general regulatory mechanism which contributes to the control of the formation of numerous inducible bacterial enzyme systems (425, 426). The third possible explanation for the depressed enzymic activity is the presence of enzyme inhibitors. Other considerations are enzyme instability, or an increased breakdown during prolonged fasting. Characterization of products, moreover, when enzymes from both fed and fasted rats were used, did not reveal any difference. The lack of accumulation of any intermediate in the biosynthetic sequence leading to triglyceride, suggests two possible explanations for the decreased total activity of the FFA esterifying enzymes noted during a period of prolonged fasting. The first is that one or more of the mechanisms described above, such as decreased supply of nucleic acid or protein precursors, repression of enzyme synthesis, or enzyme inhibition, affect simultaneously the enzymes involved in all four reactions leading to triglyceride (Fig. II). These reactions are activation of FFA, acylation of α- glycerophosphate with two acyl CoA derivatives, dephosphorylation of phosphatidic acid, and acylation of 1,2-diglyceride to form triglyceride. The second possible

explanation for the depressed total activity of the FFA esterifying enzymes during prolonged fasting is that one or more of the responsible mechanisms decrease the rate of the first reaction, i.e., the initial activation of FFA. If this were the case, the velocity of all the ensuing reactions would also be retarded.

One important reason for the enhanced esterification and re-esterification of FFA, noted in intact tissue during a period of refeeding (30, 115, 136) is the abundant supply of α - glycerophosphate, which is derived, in great part, from the expanded stores of glycogen that are present at this time (115, 151). Another factor may be the greatly increased ability of adipose tissue from animals refed a low-fat, high-carbohydrate diet to respond to insulin (424). This increased responsiveness to insulin would result in a greater uptake and utilization of glucose, and, thus, an increased availability of α - glycerophosphate. The present findings indicate that enhanced activity of the enzymes leading to glyceride may play an important role in the promotion of esterification noted in intact tissue during a period of refeeding. The enhanced enzymic activity observed during this period, may be due to increased synthesis of new enzyme, removal of inhibitors, increased enzyme stability, or decreased breakdown. Increased synthesis of new enzyme, in turn, could be due to greater availability of precursors needed for nucleic acid and protein synthesis, or to the mechanism of induction of enzyme synthesis (426).

The changes in enzymatic activity that were observed during different nutritional states, paralleled those of other adipose tissue enzymes. These are lipoprotein lipase (168), and several enzymes involved in carbohydrate metabolism. The enzymes which belong to the latter group are "hexokinase" (62, 76), glucose-6-phosphate dehydrogenase (76, 86), 6-phosphogluconate dehydrogenase (76), malic enzyme (86),

phosphoglucomutase, UDPG-pyrophosphorylase, glycogen phosphorylase (62, 63), and UDPG-glycogen transglucosylase (63).

These changes in enzymatic activity may be of physiological significance. During prolonged fasting, the general result of such changes would be the diversion of fuel, in the form of fatty acids, to vital organs. More specifically, the depression of lipoprotein lipase activity would lead to a reduced uptake of circulating triglyceride fatty acid. In addition, the decreased activity of the enzymes involved in the biosynthesis of triglyceride, could contribute to the release of FFA from adipose tissue. On the other hand, the end result of the enhanced enzymatic activity observed during a refeeding period, would be storage of triglyceride. This is a major function of adipose tissue.

The variable degree of lipolytic activity in homogenates of adipose tissue is in agreement with the observations of Steinberg et al. (80). Even though there was no significant difference when the enzymes were derived from fed, fasted, and refed rats, a trend toward increased lipolysis was noted with the latter group. It may be relevant that in the absence of insulin, intact adipose tissue from refed rats exhibits a high rate of lipolysis (30, 76, 149).

The finding that most of the phospholipid radioactivity was present in phosphatidic acid, confirmed the observations of Steinberg et al. (80). Phosphatidic acid, however, was not found to any appreciable extent in intact white adipose tissue (35). The fact that under certain conditions using cell-free systems, phosphatidic acid comprised a high proportion of the radioactivity present in the esterified fraction, was probably a reflection of its role as precursor in triglyceride synthesis. This concept is in agreement with certain kinetic considerations. Indeed, the data of Steinberg et al. (80), and the present findings indicated that with prolongation of incubation, the proportion of radio-

activity present in phosphatidic acid, decreased progressively, and that in glyceride, mainly triglyceride, increased to a corresponding extent. When the reaction rate was accelerated by using increasing concentrations of mitochondria and microsomes, the proportion of radioactive phosphatidic acid was considerably decreased, and that of labelled glyceride was correspondingly increased. Hence, when the over-all reaction was closer to its completion as a result of prolongation of incubation, or the use of greater concentrations of particles, the proportion of isotopic phosphatidic acid decreased and that of radioactive triglyceride increased in a manner indicative of a precursor product relationship.

It must be borne in mind that using the system described by Skipski et al. (413, 427), polyglycerophosphatides and phosphatidic acid travel with the solvent front. In these studies, however, it was assumed that all of the radioactivity recovered from the solvent front area was in phosphatidic acid. Such an assumption was probably valid because, as indicated in Section I.-(B), only minute amounts of polyglycerophosphatides have been found in adipose tissue. In addition, when the phospholipids were separated by chromatography on silicic acid-impregnated paper (428), Steinberg et al. (80) found that phosphatidic acid was the major radioactive component.

The localization of the esterifying site to mitochondria and microsomes confirmed previous observations (32, 293). Since palmitate, rather than palmityl CoA, was the initial labelled substrate used in this study, it was considered possible that the soluble fraction was deficient only in the FFA activating enzyme. If this speculation had been correct, substitution of palmityl CoA for palmitate would have enabled the soluble fraction to stimulate triglyceride synthesis. This possibility was made unlikely, however, by the report of Rose and

Shapiro, indicating that the FFA activating enzyme was located almost entirely in the particulate fraction of adipose tissue homogenates (79).

The present findings suggested that the mitochondrion-rich fraction and the microsome-rich fraction from rat adipose tissue could synthesize glycerides independently. In addition, the biosynthetic pathways in the two particulate fractions appeared to be identical. The presence of a complete enzyme system for triglyceride synthesis in each particulate fraction had been previously suggested for liver (238).

Tzur and Shapiro (395) reported recently that the use of increasing concentrations of rat liver microsomes, led to a disproportionately large increment in phospholipid and triglyceride formation from FFA and labelled α - glycerophosphate. These investigators also observed that a variety of lipoproteins promoted the synthesis of esters at both low and high concentrations of microsomes. They proposed that the presence of a protein acceptor might be necessary for optimal synthesis or removal of esterified lipids. Lipoproteins might, therefore, act either by supplying the protein moiety during the synthetic process, or by providing this moiety after the esters have already been formed. In the latter case, the lipids could, thus, be removed as lipoproteins. The synthetic enzymes would, then, be free to catalyze the formation of new products. In other words, rat liver microsomes might form lipoproteins de novo, or they might, at first, synthesize free lipids, which would, then, be transported as lipoproteins. Tzur and Shapiro proposed that the disproportionate increment in ester formation noted using increasing quantities of liver microsomes, might have been due to a greater supply of a protein acceptor, or, alternatively, to increased stabilization of the dilute enzyme system by higher concentrations of particulate protein. They,

in fact, suggested that albumin, unlike other proteins, acted by stabilizing dilute microsomal preparations. Their suggestion was based on the observation that this protein enhanced esterification only in dilute enzyme systems. In addition, albumin was shown to exert such an effect in the case of L-glycerol-3-phosphate acyl transferase (264).

In the present study, albumin and various lipoproteins did not enhance the synthesis of esters in the presence of subcellular preparations of rat adipose tissue. It is possible that in these systems, the disproportionately large increment in ester formation observed using increasing concentrations of mitochondria and microsomes, may be due to the greater stability of these preparations. Other unknown mechanisms, however, may be operative.

The great capacity of subcellular particles, mitochondria and microsomes, from adipose tissue to bind FFA is in agreement with the observations of Reshef and Shapiro (429, 430, 431). Binding of FFA probably precedes their esterification. These investigators, moreover, found that adipose tissue particles had a much higher avidity for FFA per mg. of particulate protein than those derived from liver and kidney. The FFA were bound tightly to a protein component by a process that appeared to be non-enzymatic (431). The ability to bind FFA, moreover, was abolished when lipids were extracted from the mitochondria and microsomes (431). Incubation of the particles with a potassium chloride-tris (hydroxymethyl) aminomethane solution led to considerable regeneration of activity by an apparently enzymatic process (430). Thus, complex mechanisms preceded the process of esterification.

As described in the last sub-section, phosphatidic acid was the major labelled ester formed by mitochondria and microsomes. When the soluble fraction was added, it brought about an alteration in the pattern of isotopic esters formed by the particles. Indeed,

the soluble fraction led to an increase in the proportion of isotopic glyceride and to a decrease in that of radioactive phosphatidic acid. The proportion of labelled diglyceride was low both in the presence and absence of soluble fraction. The latter, however, led to a small, but consistent rise in the amount of radioactivity found in this lipid. The change in distribution of the radioactivity present in the esters, suggested that the factor or factors responsible for the effect of the soluble fraction influenced specifically the reaction catalyzed by phosphatidic acid phosphatase. Indeed, they might have stimulated this enzyme. The fact that an effect was evident using particles that had been pre-incubated with soluble fraction, indicated that the unknown factor or factors were taken up by the mitochondria and microsomes before influencing the pattern of products. The soluble fraction, moreover, exerted its effect when particles containing bound palmitate-1-C^{14} were used. This observation suggested that mitochondria and microsomes were the sites of action of the responsible factor or factors. Finally, addition of the soluble fraction at a time when most of the esterification reaction stimulated by the particles, had been completed, led to the usual alteration in the pattern of the radioactive esters. Hence, the unidentified factor or factors did not have to act at the beginning of esterification, but were still effective when most of this process had been completed.

Previous studies using subcellular preparations from rat liver, indicated that mitochondria needed two factors which were contained in a supernatant fraction containing microsomes and soluble fraction (236, 238, 239, 240). One, was heat-stable and could not be replaced by α - glycerophosphate and coenzyme A. The other was heat-labile and was needed in minute amounts. Triglyceride was the predominant ester formed by this system. Using a microsomal preparation, however, the boiled supernatant could be replaced by α - glycerophosphate and

coenzyme A. All the esterifying activity present in the native supernatant could be recovered in the microsomal fraction. Diglyceride was the predominant ester formed by this subcellular system. It is not clear whether the heat-stable factor described by these investigators bears any relation to the effect of the soluble fraction prepared from adipose tissue. The factor in liver was derived from a fraction containing both soluble fraction and microsomes. Hence, it could have been associated with these particles. In addition, it was stable to boiling for 10 minutes. On the other hand, when the soluble fraction that was prepared in this study from adipose tissue, was heated between 50 and 60°C, the magnitude of its effect was decreased considerably.

The effect of various lipoproteins and albumin on lipid synthesis by rat liver microsomes (395) was different from that of the soluble fraction prepared from adipose tissue. A 20-to 40-fold increase of ester yield was obtained when liver microsomes were supplemented by these compounds. The addition of inactivated microsomes brought about a similar increase in neutral lipid and phospholipid synthesis. An effect on the composition of the esterified fraction, however, has not been reported. In this study, on the other hand, it was shown that in the presence of subcellular particles from adipose tissue, the soluble fraction changed the composition of the esterified products without increasing the total number of moles formed. In this system, furthermore, albumin and a number of lipoproteins did not enhance the synthesis of ester, nor did they duplicate the effect of the soluble fraction.

Shapiro (430) has alluded to the possibility that lipoprotein formation might participate in the transport of glyceride from their site of synthesis on the adipose tissue particles to their site of storage. While the lipoproteins and albumin that were used in the present study, did not have any effect, it was considered possible that adipose

tissue particles, unlike liver microsomes might have required specific protein acceptors.

The synthesis and secretion of lipoproteins is an important function of the liver (184, 432, 433, 434, 435, 436, 437, 438, 439). In adipose tissue, synthesis of lipoproteins probably occurs to a much lesser extent. Consequently, this process may be more difficult to demonstrate in vitro.

The possibility that the effect of the soluble fraction was due to a large protein, was excluded by the finding that the unknown factor or factors were dialyzable. Preliminary results, moreover, suggested that they could be recovered from the dialyzing solution. Hence, they were not inactivated during dialysis, and were not adsorbed, at least to any appreciable extent, by the dialyzing membrane. In addition, they did not appear to be enzymatic in nature. In fact, the effect of the soluble fraction persisted at a temperature that inactivated all the mitochondrial and microsomal enzymes involved in glyceride synthesis. An additional argument against the enzymatic nature of the factor or factors responsible for the effect of the soluble fraction was provided by the reports of Rose and Shapiro (79) and Margolis and Vaughan (75), who found that phosphatidic acid phosphatase, the only enzyme which could have conceivably accounted for the observed alteration in the pattern of isotopic esters, was associated almost entirely with subcellular particles.

Of those tested, divalent cobalt ions were the only ones that definitely simulated the effect of the soluble fraction to a certain extent. The concentrations of cobalt used in this study, however, were much larger than those probably present in the soluble fraction. Adult rat liver contains 0.3 μM of cobalt per Kg. dried weight (440). If the cobalt content of adipose tissue were at all

similar to that of liver, the amount present in the soluble fraction would be much smaller than the lowest concentration of exogenous cobalt used in this study. A firm conclusion cannot be drawn, however, without determining directly the concentration and form of cobalt in the soluble fraction of adipose tissue. Phosphatidic acid phosphatase of chicken liver (237) and of pig kidney (277) did not seem to require any metal ions. Leal (441) was unable to show any definite effect of sodium, potassium, lithium, rubidium, and cesium on phospholipid and triglyceride synthesis from palmitate by rat liver slices. On the other hand, a number of divalent ions inhibited chicken liver and pig kidney phosphatidic acid phosphatase (237, 277). No such inhibition has been previously reported for the adipose tissue enzyme. In the present study, only calcium ions appeared to have an inhibitory effect.

The possibility that the soluble fraction might have acted by providing endogenous acceptors of fatty acids, in the form of diglyceride, was considered unlikely. Wadström (442) reported an accumulation of lower glycerides in rabbit subcutaneous tissue during the enhanced lipolysis that was brought about by the administration of epinephrine. These lower glycerides, however, did not necessarily act as substrates for esterification. In fact, calculations based on simultaneous measurement of FFA and glycerol release during intense lipolysis in intact rat adipose tissue, did not correlate with appreciable re-esterification of diglyceride (30). In addition, all three fatty acids of triglyceride formed by adipose tissue from refed rats were equally labelled (78). Thus, endogenous diglyceride could not have been an important acceptor of fatty acids in these rats. In the present study, moreover, the incorporation of radioactive palmitate into the ester fraction was negligible when α- glycerophosphate was omitted from the reaction mixture. Finally, the effect of the soluble fraction persisted after its lipids had been

extracted with diethyl ether. Hence, the effect of the soluble fraction was not due to endogenous diglyceride, but it was probably due to one or more dialyzable and partially heat-stable substance or substances.

E) SUMMARY.

1) The optimal requirements for the esterification of α - glycerophosphate and long-chain free fatty acids by subcellular preparations of rat epididymal adipose tissue were determined. α - Glycerophosphate was an obligatory substrate. The esterification reaction was also totally dependent on ATP, and partially dependent on added CoA-SH and Mg^{++}. Periods of fasting and refeeding did not alter these requirements.

2) The total activity of the enzymes involved in the synthesis of glyceride from α - glycerophosphate and FFA was markedly depressed when the enzymes were derived from rats fasted for 72 hours, and it was greatly enhanced when they were obtained from rats refed for 48 hours following a fast of 96 hours.

3) Esterifying activity was confined to subcellular particles.

4) Attempts at isolation of FFA pools, active in esterification, failed because of artifactual introduction of fatty acids into particles during the procedure. Thus, the methods used to explore this problem are hazardous, and any future attempts using such experimental steps should be carried out with caution in view of these findings.

5) The greatest proportion of radioactivity present in esters formed by mitochondria and microsomes was found in the phospholipid fraction. Isotopic phosphatidic acid was the major

component of this fraction.

6) The proportion of radioactivity present in the phospholipid fraction could be decreased and that in the glyceride fraction could be correspondingly increased by prolongation of incubation or by acceleration of the reaction rate using increasing concentrations of subcellular particles.

7) Although the soluble fraction was incapable of stimulating the esterification of FFA when used alone, it consistently altered the pattern of the isotopic esters formed by the particles so that the proportion of glyceride, mostly triglyceride, increased and that of phospholipid decreased.

8) The factor or factors present in the soluble fraction and responsible for this effect appeared to be dialyzable and partially heat stable.

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