STUDIES ON THE RELATIONSHIP BETWEEN FATTY ACID BIOSYNTHESIS AND ESTERIFICATION IN RAT ADIPOCYTES

A thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy in the Faculty of Science

by

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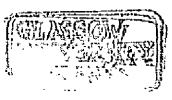

CONTENTS

LIST OF TABLES

LIST OF FIGURES

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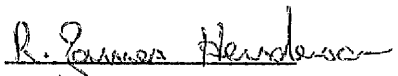
DECLARATION

I hereby declare that this thesis entitled 'Studies on The Relationship between Fatty Acid Biosynthesis and Esterification in Rat Adipocytes' is entirely my own work and has not been accepted or submitted for any degree.

Signed 

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SUMMARY

This thesis describes studies in which rat adipocytes, prepared by collagenase digestion of parametrial adipose tissue, were used to examine the relationship between the synthesis of fatty acids and their subsequent esterification into triacylglycerols.

The introductory chapter reviews the literature available to date concerning fatty acid and triacylglycerol biosynthesis in adipose tissue, and discusses the control of each separately. It is noted that although lipogenesis in adipose tissue has been studied extensively, its relationship to the synthesis of the final product, triacylglycerols, is poorly understood particularly with respect to any effect on the esterification of fatty acids into particular positions of the triacylglycerol molecule. Studies have therefore been made of the manner in which fatty acids of exogenous origin were utilised for triacylglycerol biosynthesis by the adipocyte *in vitro* in comparison to fatty acids synthesized *de novo* by the cell from low molecular weight precursors or by modification of preformed fatty acids.

A comparison was made between the rates of esterification of fatty acids supplied to the adipocytes individually or as components of a fatty acid mixture of a composition similar to that likely to be available to the cell. Whilst most of the fatty acids in each instance were taken up and esterified into triacylglycerols, a significant proportion were recovered in diacylglycerols having a slow rate of conversion to triacylglycerols. No differences in the rates of esterification into acylglycerols were obvious between palmitic,

stearic, oleic and linoleic acids when supplied individually but oleic acid had the highest rate of esterification into triacylglycerols when the same fatty acids were incubated as a mixture. Using a stereospecific analysis procedure, the positional distribution of esterified extracellular fatty acids in triacylglycerols was shown to differ from the natural distribution of fatty acids in rat adipocyte triacylglycerols and oleic acid formed by the desaturation of exogenous stearic acid was located predominantly in position sn-3. Tripalmitoylglycerol was found to be synthesized by adipocytes incubated with palmitic acid on its own at a high concentration. The rate of desaturation of stearic acid was greater when the stearic acid substrate was supplied to the fat-cells alone than in the presence of other fatty acids, and in both instances the oleic acid product was rapidly esterified into acylglycerols, predominantly triacylglycerols.

Experiments on fatty acid synthesis de novo from acetate or glucose showed that the rate of fatty acid synthesis decreased with the age of the rat from which the adipocytes were prepared. Preparative G.L.C. with subsequent measurement of radioactivity was used to study the fatty acids synthesized from ^{14}C-labelled acetate or glucose and esterified into triacyl- and diacylglycerols. The pattern of fatty acids formed from acetate was found to be dependent on age, with a higher proportion of oleic acid being formed by adipocytes isolated from young rats.

The effect of exogenous fatty acids on the synthesis and esterification of endogenous fatty acids was studied by incubating adipocytes simultaneously with a mixture of fatty acids and either ^{14}C-labelled acetate or glucose, or tritiated water. Whereas the

ncorporation of acetate and $^{3}H_2O$ into fatty acids was decreased in the presence of extracellular fatty acids, fatty acid synthesis from glucose was not inhibited. The pattern of triacylglycerol fatty acids formed from acetate was influenced by the presence of fatty acids in the incubation medium, under which circumstances the proportion of newly-synthesized fatty acids incorporated into position sn-3 of triacyl-sn-glycerols decreased.

In all experiments, diacylglycerol was the only intermediate in the sn-glycerol 3-phosphate pathway that tended to accumulate, and in particular with adipocytes from mature rats an accumulation in diacylglycerols of fatty acids newly-synthesized from acetate was observed at high acetate concentrations. However, under the same experimental conditions exogenous oleic acid was esterified predominantly into triacylglycerols. The rate of diacylglycerol conversion to triacylglycerols was stimulated when long-chain fatty acids were present in the incubation medium. It appeared, therefore, that in the intact adipocyte, diacylglycerol acyltransferase may have been the rate-limiting enzyme in triacylglycerol synthesis.

Structural analyses proved that the diacyl-sn-glycerols synthesized from acetate or glucose by the adipocytes were of the sn-1,2 configuration.

Acetate concentration did not affect the pattern of fatty acids synthesized de novo but did influence their distribution between positions sn-2 and sn-3 of triacylglycerols formed by adipocytes from younger rats. No such effect, however, was found in the positional distribution of oleic acid esterified into triacylglycerols from the incubation medium.

An investigation of the subcellular location of acylglycerols newly-synthesized from various lipogenic precursors by the fat-cells was carried out using a rapid method for the separation of adipocytes, that had been incubated with labelled substrates, into particulate and cytoplasmic components.

The final chapter discusses the results presented in this thesis in terms of triacylglycerol biosynthesis from exogenous fatty acids in comparison to that using endogenously-synthesized fatty acids. A model is proposed for the synthesis of triacylglycerols in the adipocyte in relation to the origin of the fatty acids used and the site of synthesis or esterification.

CHAPTER 1. INTRODUCTION

A. Fatty Acid Biosynthesis in Adipose Tissue

1. Adipose Tissue as a Site of Fatty Acid Synthesis

Prior to the mid-1930's, adipose tissue was generally regarded as being a connective tissue filled with fat droplets. The tissue was considered to have primarily physical functions in body insulation and in supporting certain other tissues, whilst its importance as a metabolically active tissue was largely underestimated. (For reviews of early literature see Wertheimer and Shapiro (1) and Wertheimer (2)). However, this concept was challenged when Schoenheimer and Rittenberg (3,4) clearly demonstrated the rapid turnover and continuous synthesis of body fat in mice, although for several years afterwards the liver was still believed to be the principal site of lipogenesis in animals (5). In 1942 Mirski (6) provided indirect evidence for the importance of adipose tissue as a site of fat synthesis when he found that adipose tissue of refed rats had a high R.Q. value and proposed that the tissue converted glucose into products of low oxygen content, probably fatty acids. Masoro et al (7) showed that the liver was not the sole tissue capable of fatty acid synthesis when they demonstrated the recovery of radioactivity from injected ^{14}C-labelled glucose in fatty acids extracted from the carcasses of hepatectomized rats.

More direct evidence came in 1948 from the work of Shapiro and Wertheimer (8) who found that rat adipose tissue incubated in serum enriched with deuterium oxide could incorporate deuterium into its

fatty acids. Later studies in 1955 showed (9) the synthesis of fatty acids from ^{14}C-labelled glucose, acetate and pyruvate in rat mesenteric adipose tissue. Since the ^{14}C was distributed throughout the fatty acid molecule and was not confined to the carboxyl carbon, it was concluded that a complete new synthesis had taken place. In the same year, Favager and Gerlach (10) eliminated the suggestion (11) that the newly-formed fatty acids found in adipose tissue actually came from the liver, by comparing the specific activities of newly-synthesized fatty acids in mouse mesenteric adipose tissue, liver and blood.

The synthesis of fatty acids in adipose tissue of species other than the mouse and rat has been demonstrated (for review see Rudman and Girolamo (12)).

Hausberger et al (13) suggested in 1954 that when the large difference between the lipid and protein content of liver and adipose tissue was taken into account, rat adipose tissue was more active than rat liver in fatty acid synthesis. This suggestion was later supported by Feller (14) who reported that the recovery of radioactivity from ^{14}C-labelled acetate in fatty acids of mouse adipose tissue was as great or greater than the recovery in liver slices when compared on a fat-free wet tissue basis. It has been demonstrated more recently (15) that sheep adipose tissue homogenates have a ten fold greater lipogenic rate per mg protein than homogenates of sheep liver.

The extent to which adipose tissue synthesizes fatty acids in relation to other tissues, varies with species. O'Hea and Leveille (16) found that adipose tissue played a major if not an exclusive role in the synthesis of fatty acids in the pig. Both adipose and hepatic

tissues of the mouse (17) and rat (17,18) were active in fatty acid synthesis, with adipose tissue accounting for at least 50% of the newly-synthesized fatty acids and as much as 95% in meal-fed rats. Liver did, however, appear to be the major site of fatty acid formation in pigeon (19), sparrow (20) and chicken (21). Low lipogenic activity in adipose tissue may not be restricted to avian species since Shrago et al (22) suggested that synthesis of fatty acids de novo was not an important physiological function of human adipose tissue.

In ruminant animals, both liver and adipose tissue appear to be important sites of fatty acid synthesis. Hanson and Ballard (23) observed in studies with tissue slices in vitro that aged sheep liver tissue and mesenteric adipose tissue had similar rates of fatty acid synthesis, whereas the adipose tissue of mature cows had a threefold greater lipogenic rate than liver. More recent studies on non-lactating sheep in vivo by Ingle et al (24) showed that adipose tissue was the predominant site of fatty acid synthesis with the liver, rumen, abomasum and small intestine together contributing only 8% of the total fatty acids synthesized. The greatest amount of fatty acid synthesis from ^{14}C-acetate precursor occurred in the perirenal and omental adipose tissues, a finding in agreement with that of Holdsworth et al (25).

Adipose tissue from different body depots apparently possess different rates of lipogenesis (26,27), the internal depots being more active than the subcutaneous ones.

2. Carbon Source for Fatty Acid Synthesis

The origin of carbon used for fatty acid synthesis in adipose

tissue of non-ruminant mammals under normal conditions is glucose derived from dietary carbohydrates. O'Hea and Leveille (16) demonstrated that when ^{14}C-glucose was utilised as substrate in the pig virtually all the newly-synthesized fatty acids were found in the adipose tissue in vivo. When mice were fed a diet containing (U-^{14}C)glucose (17), about 90% of the radioactivity incorporated into fat in the epididymal fat pads was present in the acylglycerol fatty acids and 10% in the glycerol moiety. Flat and Ball (28) attempted to quantitate the metabolism of glucose by adult rat adipose tissue in the absence of hormonal stimulation and concluded that 30% of the metabolised glucose carbon was converted to glyceride fatty acids.

In ruminant animals, however, little glucose is absorbed from the digestive tract due to microbial degradation of dietary carbohydrate in the rumen. The small amount of glucose available is produced by gluconeogenesis, mainly in the liver. Various early studies (25,29) suggested that products of rumen metabolism such as acetate and butyrate are the major precursors for fatty acids in ruminants. Hanson and Ballard (23) noted that with adipose tissue slices from sheep and cows more ^{14}C-acetate than ^{14}C-glucose was incorporated into lipids, whereas the converse was true with rat adipose tissue. Their results suggested that in both liver and adipose tissue of ruminants, acetate was a more important precursor of fatty acids than was glucose. A similar situation exists in ruminant mammary tissue (30,31) where it has also been shown that β-hydroxybutyrate is used for fatty acid synthesis (32).

3. Transfer of AcetylCoA from Mitochondrion to Cytoplasm for Fatty

Acid Synthesis

Since fatty acid synthesis is known to occur in the cytoplasm (33), acetylCoA produced in the mitochondrion by pyruvate dehydrogenase must be transported into the cytoplasm for use in lipogenesis. It is generally accepted that the mitochondrial membrane is not directly permeable to acetylCoA and so some indirect method for the transfer of acetyl units into the cytoplasm must operate.

Several mechanisms for this transfer have been suggested (34,35). The hydrolysis of mitochondrial acetylCoA to acetate with subsequent diffusion of the acetate through the mitochondrial membrane and resynthesis of acetylCoA in the cytoplasm has frequently been ruled out as it appeared that the rate of acetylCoA hydrolysis was low (35,36). However, Rous (37,38) has recently provided evidence for acetylCoA leaving the mitochondrion as acetate or acetoacetate in mouse adipose tissue. Acetyl-carintine has also been implicated (35) as a transporter of acetyl units as have α-ketoglutarate and glutamate (39).

The presently accepted concept for the supply of mitochondrial acetylCoA for fatty acid synthesis involves the tricarboxylic acid, citrate. Spencer and Lowenstein (34) found that citrate served as a good precursor for fatty acid synthesis in rat mammary tissue and suggested that citrate formed within the mitochondrion from the condensation of acetylCoA with oxaloacetic acid by the enzyme citrate synthetase (EC 4.1.3.7), diffused through the membrane into the cytoplasm where it was cleaved to yield oxaloacetic acid and acetylCoA for use in fatty acid synthesis. Similar studies with avian liver

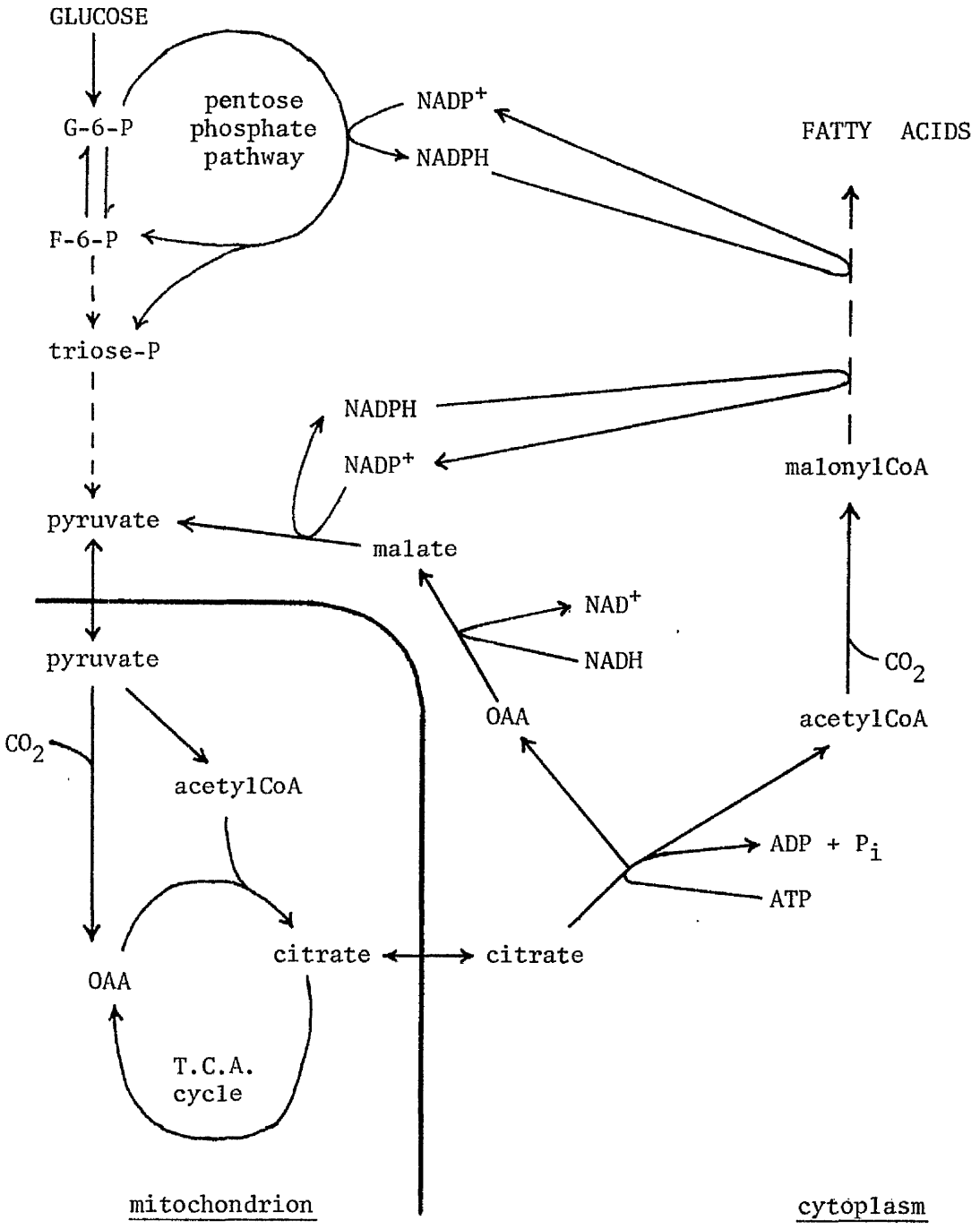


Fig. 1.1. Fatty acid synthesis in non-ruminant adipose tissue, showing the 'malate cycle'.

G-6-P, glucose 6-phosphate; F-6-P, fructose 6-phosphate; OAA, oxaloacetate; T.C.A., tricarboxylic acid.

(40) supported this suggestion and showed that the citrate cleavage enzyme, or citrate lyase (EC 4.1.3.6) was ATP and CoA dependent.

The existence of this method of acetylCoA transfer in adipose tissue was apparent after the demonstration (41) of a very active citrate cleavage enzyme which was stimulated sixfold by diets which induced lipogenesis in rat epididymal adipose tissue. In the same study, the nutritional state of the animal was found to alter the activity of NADP-dependent malate dehydrogenase or 'malic enzyme' (EC 1.1.1.40) in a manner very similar to that of citrate lyase, suggesting that the two enzymes operated together in lipogenesis in adipose tissue.

To relate the activities of the citrate cleavage and malic enzymes, Kornacker and Ball (41) proposed that oxaloacetate produced by citrate cleavage could be reduced to malate by the enzyme malate dehydrogenase (EC 1.1.1.37) using NADH generated by glycolysis. Malate so produced could then act as a substrate for malic enzyme in the conversion of malate to pyruvate, producing CO_2 and NADPH in the process. The NADPH could then be used in fatty acid synthesis, and the pyruvate could either be oxidised to acetylCoA by pyruvate dehydrogenase (EC 1.2.4.1) or carboxylated by pyruvate carboxylase (EC 6.4.1.1) to oxaloacetate. The so-called 'malate cycle' as presented in Fig. 1.1, is thereby completed. Overall, one pyruvate molecule has given rise to one acetylCoA in the cytoplasm and one NADH molecule has been converted to one NADPH. Evidence for the operation of this cycle in adipose tissue comes from the finding of a very active pyruvate carboxylase (42) and cytoplasmic malate dehydrogenase (43) in the tissue. The transfer of hydrogen from

NADH to NADPH via malate dehydrogenase and malic enzyme has also been demonstrated with mitochondrial-free fractions of rat adipose tissue (44).

The absence of citrate lyase from the cells of a tissue results in the inability of that tissue to use glucose-derived acetylCoA for fatty acid synthesis. Low citrate lyase activity in human adipose tissue led Shrago et al (22) to conclude that net synthesis de novo of fatty acids was not an important physiological process of human adipose tissue. In general, ruminants possess very low citrate lyase activity in comparison with non-ruminants (45). This feature is in keeping with the fact that ruminants do not utilise glucose to any great extent as fatty acid precursors (46,47). Since glucose is present in very low concentration in the ruminant, it is understandable that the enzyme responsible for the transfer of glucose-derived acetylCoA out of the mitochondrion is also in short supply. The net result is that all or most of the acetylCoA originating from glucose can be totally oxidised via the tricarboxylic acid cycle for energy production.

4. Source of Hydrogen for Fatty Acid Synthesis

The origin of the hydrogen used in fatty acid synthesis has been studied extensively (for review see Rous (48)) and will not be dealt with in great detail here. The NADPH required for fatty acid synthesis in rat adipose tissue is known to be generated exclusively in the cytoplasm since the amount of reduced nicotinamide nucleotides produced in the cytoplasm was found to be sufficient for all the cytoplasmic reactions dependent on them (49,50,51,52).

One source of NADPH is that produced by the dehydrogenases of the pentose phosphate pathway, namely glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) but under normal conditions it has been shown that these enzymes provide only 50-80% of the total NADPH required for fatty acid synthesis in rat adipose tissue (49,50) and isolated adipocytes (51, 52). In conditions of insulin-stimulated lipogenesis, the pentose pathway could only provide 50-60% of the required NADPH (49,51). Only when lipogenesis was very low (after 48 hours starvation) could the pentose pathway dehydrogenases supply all the reducing equivalent required for fatty acid synthesis in rat adipocytes (51). Under normal conditions and conditions of high lipogenic activity some other cytoplasmic reactions must therefore contribute to the supply of NADPH for fatty acid synthesis from acetylCoA.

As described in the previous section, one molecule of NADPH is produced by malic enzyme for each complete malate cycle. Of the two NADPH molecules required for the reduction of one acetyl unit in fatty acid synthesis, one is derived from the pentose phosphate pathway and the other from the malate cycle. Since the fat cell is capable of producing more NADPH in the cytoplasm than is actually necessary for fatty acid synthesis, a mechanism must exist whereby excess NADPH production is prevented. It has been suggested (53,54) that a net transport of malate into the mitochondrion from the cytoplasm can occur, thus avoiding the generation of NADPH in the malic enzyme of the malate cycle.

Ruminant animals differ from non-ruminants in their source of reducing equivalents for use in fatty acid synthesis. Glucose-6-

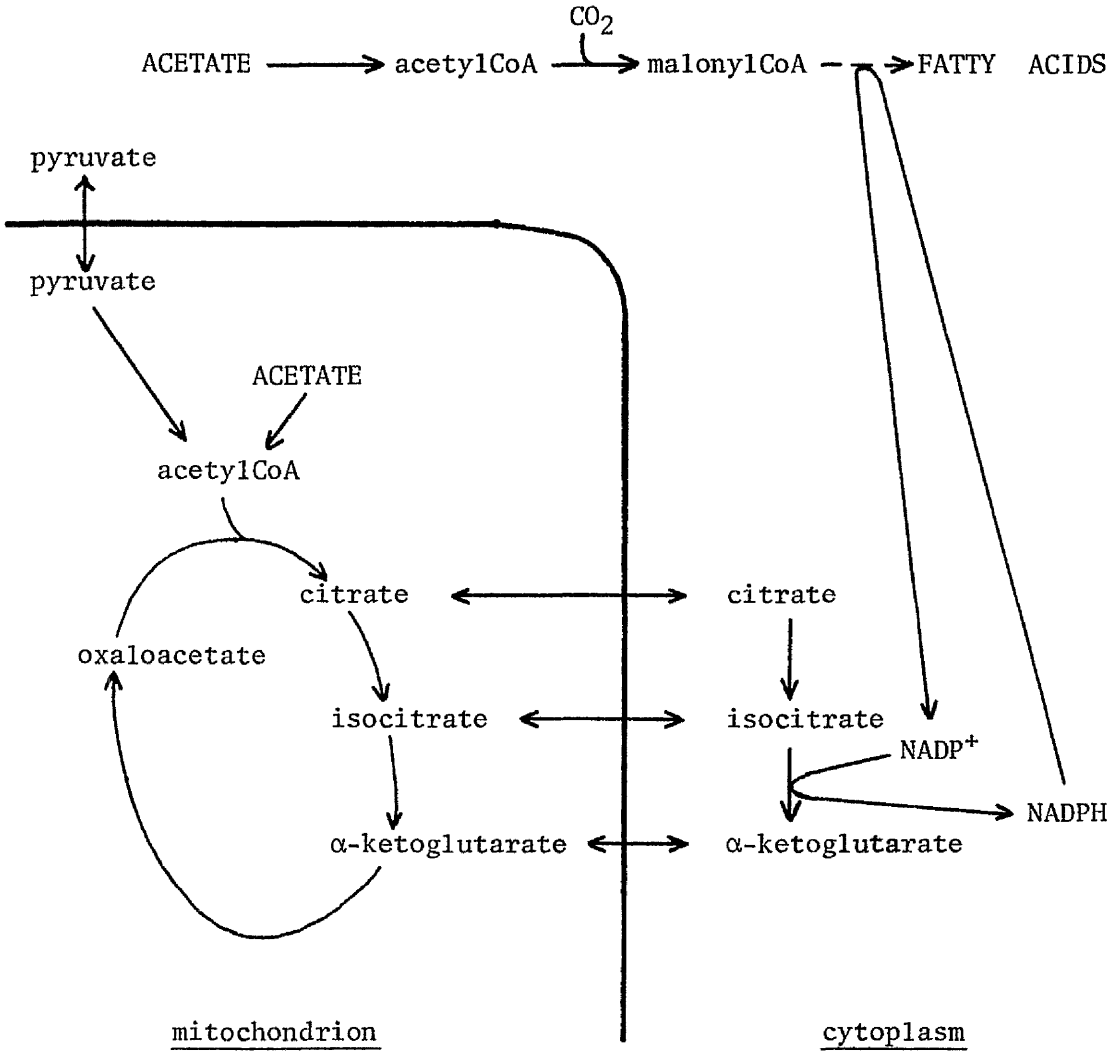


Fig. 1.2. Fatty acid synthesis in ruminant adipose tissue: operation of the 'isocitrate cycle'.

phosphate dehydrogenase and 6-phosphogluconate dehydrogenase have been shown to be active in the production of NADPH in sheep adipose tissue (55) and in isolated adipocytes from bovine adipose tissue (46) although Baldwin et al (56) found these dehydrogenases to be almost twice as active in rat adipose tissue than in bovine adipose tissue. As with non-ruminant animals, the pentose phosphate pathway dehydrogenases cannot supply all the NADPH required in ruminant adipose tissue for fatty acid synthesis from acetate. Calculations by Baldwin et al (56) showed that at least 23% of the required NADPH must originate from other sources.

Whereas in non-ruminant adipose tissue the remainder of the NADPH is generated in the malate cycle, the activity of malic enzyme in ruminant tissues is known to be very low (23,45,57). However another enzyme capable of producing NADPH, isocitrate dehydrogenase (EC 1.1.1.42) is very active in ruminant adipose and mammary tissues (55,56), being 20 fold more active than in rat mammary tissue (57). This enzyme is known to generate less NADPH than the pentose phosphate pathway dehydrogenases in rat adipose tissue (36), and is not regarded as playing a major role in the production of reducing equivalents in non-ruminant adipose tissue.

A pathway involving isocitrate dehydrogenase in the production of NADPH for fatty acid synthesis in ruminant mammary tissue was suggested by Bauman et al (57), and a very similar model was proposed by other workers (46) for bovine adipocytes. The pathway, often termed the 'isocitrate cycle' is presented in Fig. 1.2.

The operation of this pathway allows acetate, the main source of carbon for fatty acid synthesis in ruminants, to be used in the

generation of NADPH.

5. Synthesis of Fatty Acids from AcetylCoA

Detailed reviews are available concerning the enzymes of fatty acid biosynthesis and their regulation in general biological systems (33,58,59) and more specifically in adipose tissue (60).

Fatty acid synthesis de novo in mammalian adipose tissue can be discussed in general terms in relation to two main enzyme systems, acetylCoA carboxylase (EC 6.4.1.2) and fatty acid synthetase which catalyse reactions 1 and 2 respectively.

$$\text{acetylCoA} + CO_2 + \text{ATP} \rightarrow \text{malonylCoA} + \text{ADP} + P_i \quad (1)$$

$$\text{acetylCoA} + 7\text{malonylCoA} + 14\text{NADPH} + 14H^+ \rightarrow \text{palmitic acid} + 8\text{CoA} + NADP^+ + 7CO_2 + 6H_2O \quad (2)$$

Both these enzymes operate in the cytoplasm and will be discussed separately.

(i) AcetylCoA Carboxylase: Although most studies on acetylCoA carboxylase have used liver as an enzyme source, the enzyme has also been purified from rat (61,62,63) and bovine (64,65) adipose tissue.

In non-ruminants, the acetylCoA substrate is derived from glucose involving citrate as a means of transporting it out of the mitochondrion as described earlier. In ruminant tissue the acetyl-CoA arises directly from acetate since the cytoplasm is known to contain a highly active acetylCoA synthetase (66).

The overall reaction brought about by the enzyme is the carboxylation of acetylCoA to malonylCoA at the expense of one ATP molecule. Since malonylCoA has no other apparent metabolic function than its role as an intermediate in fatty acid biosynthesis, its production by acetylCoA carboxylase is the first step specifically

directed towards fatty acid synthesis.

AcetylCoA carboxylase purified from mammalian tissues, including adipose tissue, appears to contain three non-identical subunits; a biotin-binding protein, biotin carboxylase and a transcarboxylase. The molecular weight of each subunit or protomer is approximately 400,000 but the enzyme is only in an active form when the protomers aggregate into long filamentous polymers of molecular weight 4 million to 8 million. Agents favouring the aggregation of the protomers lead to an activation of the enzyme and therefore a stimulation of fatty acid biosynthesis. AcetylCoA carboxylase is therefore an important enzyme in the regulation of fatty acid biosynthesis and will be discussed further in section B of this chapter.

(ii) Fatty Acid Synthetase: MalonylCoA produced by acetylCoA carboxylase acts as substrate for fatty acid synthetase in the production of palmitic acid, as presented above in the overall reaction. Fatty acid synthetase from mammalian tissues functions as a multienzyme complex of which the individual enzymes are not easily separable. Consequently, the properties of the component enzymes are as yet generally unknown, although in recent studies (67,68) fatty acid synthetase complexes from rat, human and chicken liver were separated into two subunits each containing different enzyme activities. Fatty acid synthetase from pigeon liver has been studied extensively and is probably a representative model for the enzyme in most mammalian systems in terms of component enzymes (most likely seven in number) and their manner of action (69,70,71).

Brindley et al (72) classified fatty acid synthetases behaving as single enzymes and having molecular weights in the range 0.5 to 2.3 million as Type I synthetases. Although the molecular weight

of fatty acid synthetase from several animal tissues has been determined (73,74,75), that of adipose tissue fatty acid synthetase remains unknown. It is, however, most probably a Type I.

NADH cannot replace NADPH for the action of fatty acid synthetase purified from rat adipose tissue (76) and free palmitic acid is predominantly the product of the system. With regard to requirements and products, the fatty acid synthetase of rat adipose tissue is very similar to that of other mammalian tissues such as liver (74) and mammary gland (77) although the purified system from lactating rabbit mammary gland has been shown (75) to produce significant amounts of butyric acid, a short-chain fatty acid, but no medium-chain fatty acids which are characteristic of rabbit milk (78). It appears that the medium-chain fatty acids are produced when a cytoplasmic enzyme, acylCoA-hydrolase, acts specifically on medium-chain fatty acylCoAs associated with the fatty acid synthetase, causing their release as unesterified fatty acids (79).

The fatty acid synthetase system of adipose tissue therefore most likely involves very similar individual enzymes with protein-bound β-keto, β-hydroxy and α,β-monoene acyl intermediates as established for E. coli (33) (a Type II synthetase as classified by Brindley et al (72)). Evidence has been presented (80) for the existence in fatty acid synthetase from rat adipose tissue of a 4'-phosphopantetheine prosthetic group similar to that of acyl carrier protein and known to be important in the functioning of other mammalian fatty acid synthetases.

6. Fatty Acid Elongation

Recent studies (81) have shown that stearic and oleic acids can

be formed by fatty acid elongation in rat adipose tissue in vivo. Although the major proportion of fatty acid synthesis de novo takes place in the cytoplasm of the adipocyte, Kanoh and Lindsay (82) reported that mitochondrial and microsomal fractions of rat epididymal adipose tissue incorporated ^{14}C-labelled acetylCoA into various fatty acids by a chain elongation mechanism. With both these subcellular fractions, fatty acids containing 18 and 20 carbon atoms accounted for approximately 80% of the total synthesized fatty acids. Both fractions utilized NADH, acetylCoA and endogenous fatty acids but the mitochondrial system was not inhibited by avidin suggesting that acetylCoA was not carboxylated to malonylCoA and that acetylCoA was incorporated directly. The microsomal system did, however, apparently proceed via malonylCoA. Very similar results were obtained in studies with subcellular preparations of rat adipocytes (81), although the malonylCoA elongation system was found to be associated with the fat-cell membrane.

AcetylCoA and malonylCoA elongation systems have been described in the mitochondria (83,84) and microsomes (85,86) of liver.

7. Fatty Acid Desaturation

The formation of unsaturated fatty acids in adipose tissue can occur by the enzymic desaturation of preformed fatty acids as reported with other tissues (87,88,89). Gelhorn and Benjamin (90) demonstrated the conversion of stearic acid to oleic acid in the microsomal fraction of rat adipose tissue by an oxygenase enzyme requiring molecular oxygen and either NADH or NADPH as cofactors. The CoA thioester rather than the free fatty acid is known to be the actual substrate for the enzyme (89).

Ruminant adipose tissue also contains an active desaturase system (91,92) capable of desaturating both palmitic and stearic acids. The fact that ruminant adipose tissue contains a more active desaturase than that of non-ruminants has been attributed (92) to adaptation by the ruminant animal to the higher percentage of saturated fatty acids absorbed from the digestive tract as a result of biohydrogenation in the rumen.

The desaturase system has not yet been purified from adipose tissue but studies with liver have revealed the enzyme system to have a requirement for microsomal lipid (93). Other enzymatic components of the stearoylCoA desaturase (acylCoA desaturase EC 1.14.99.5) have been shown to include cytochrome b_5 (94), NADH-cytochrome b_5 reductase (95), a cyanide-binding protein (96) and another unidentified protein (97). Recent studies with rat liver (98) suggest that the actual stearoylCoA desaturase (cytochrome b_5 oxidase) component may be buried in the endoplasmic reticulum with the cytochrome b_5 and NADH-cytochrome b_5 reductase components positioned nearby on the surface of the membrane.

B. Regulation of Fatty Acid Biosynthesis in Adipose Tissue

It has frequently been proposed that acetylCoA carboxylase is the rate-limiting enzyme in fatty acid synthesis on the basis that its activity was lower than that of fatty acid synthetase (61,99). However, the rates of the two enzyme systems have been demonstrated to be similar under normal circumstances in rat adipose tissue (100) and consequently acetylCoA carboxylase may not be the rate-limiting

enzyme under all physiological conditions.

As stated previously, acetylCoA carboxylase is activated by agents favouring the aggregation of its inactive protomers into the active polymeric form. One such effector is the tricarboxylic acid, citrate. The competitive inhibition with respect to citrate of acetylCoA carboxylase by long-chain fatty acylCoA's is also well established and reviewed by Volpe and Vagelos (33). Dietary and hormonal factors affecting the cellular levels of citrate and fatty acid CoA's can therefore influence the activity of acetylCoA carboxylase.

1. Hormonal Control

It is well established that insulin increases the rate of fatty acid synthesis from glucose in adipose tissue (101,102). Although glucose transport into the adipocyte is stimulated in the presence of insulin (103,104), Halperin (105) showed that the hormone also exerted an effect within the cell since fatty acid synthesis from pyruvate by rat epididymal fat pads was increased in the presence of insulin. Considerable evidence exists for insulin affecting two critical enzymes of fatty acid synthesis, namely pyruvate dehydrogenase (EC 1.2.4.1) necessary for the production of glucose-derived acetylCoA in the mitochondrion, and acetylCoA carboxylase in the cytoplasm. Both these enzymes exist as interconvertible active and inactive forms. Pyruvate dehydrogenase in mammalian tissues is inactivated by phosphorylation brought about by a MgATP-linked kinase, and re-activation results when a specific phosphatase removes the phosphate group(s) (106).

In adipose tissue exposed to insulin for 10 minutes or more,

the proportion of pyruvate dehydrogenase present in its active form increases (107,108,109). This effect of insulin may result from activation of the phosphatase enzyme as suggested by Mukherjee and Jungas (110), or by inhibition of the kinase, or perhaps both. The action of the hormone could therefore either be to alter the cellular levels of cofactors known to stimulate the phosphatase or to inhibit the kinase. Since Ca^{2+} inhibits the kinase but activates the phosphatase, the effect of insulin on mitochondrial calcium levels has been studied extensively (111). As no changes in the incorporation of ^{45}Ca into the mitochondrial fraction of rat epididymal adipose tissue were found with insulin under conditions where pyruvate dehydrogenase activity was increased, Severson et al (112) concluded that activation of pyruvate dehydrogenase could not be explained by increased levels of Ca^{2+} in the mitochondrion.

Schiller et al (113) have suggested that pyruvate dehydrogenase activity is decreased by high concentrations of citrate when long-chain fatty acylCoA's prevent citrate transport out of the mitochonrion (114), and that in the presence of insulin the transport of citrate into the cytosol is increased resulting in increased activities of both pyruvate dehydrogenase and acetylCoA carboxylase.

The increased activity of acetylCoA carboxylase in rat adipose tissue incubated with insulin was found by Halestrap and Denton (115) to coincide with an increase in the proportion of the enzyme present in its active polymeric state. No evidence was found for insulin causing any phosphorylation or dephosphorylation of the enzyme, but Lee and Kim (116) have recently provided evidence that the inter-conversion of active and inactive acetylCoA carboxylase was related

to phosphorylation and dephosphorylation.

Adipose tissue from diabetic rats had a decreased capacity to synthesize fatty acids from glucose (117), acetate (90), acetylCoA (118) and malonylCoA (118) when compared to normal animals. The administration of insulin corrected these defects both in vivo (118) and in vitro (90). The desaturation of stearic acid was also impaired by diabetes in adipose tissue but was restored by insulin treatment (90).

Adrenaline is known to antagonise the insulin-induced increase in the rate of fatty acid synthesis from glucose in rat adipose tissue (101,102), but the observed effects of adrenaline on the enzymes of fatty acid biosynthesis may be less important than the increased lipolysis brought about by the hormone. The effect of insulin on pyruvate dehydrogenase is cancelled out by adrenaline (107,119), although the exact manner by which this occurs is not known. Similarly the effect of adrenaline on acetylCoA carboxylase from rat adipose tissue was found to be opposite to that of insulin (115) and corresponded with an increase in the proportion of the enzyme present in its inactive protomeric form.

Although the changes brought about by insulin and adrenaline could be mediated by alterations in the cellular concentration of c-AMP, no direct effect of c-AMP on pyruvate dehydrogenase activity in rat adipose mitochondria has been demonstrated (120,121).

Volpe and Marasa (122) have demonstrated equal decreases (65%) in both acetylCoA carboxylase and fatty acid synthetase activities when the glucocorticoid hormone, hydrocortisone, was administered in adipose tissue of fed rats, and attributed the effect on fatty

acid synthetase to a decrease in enzyme content. The same workers also found that glucagon reduced fatty acid synthetase activity further than that of acetylCoA carboxylase, and suggested that whereas inhibition of acetylCoA carboxylase could be a short-term control, the regulation of fatty acid synthetase activity might be more important in the long-term control of fatty acid synthesis.

2. Dietary Control

The influence of diet on lipogenic enzymes in animal tissues has been reviewed elsewhere (123). The rates of several steps in the production of acetylCoA from glucose for use in fatty acid synthesis are known to vary with the nutritional state of the animal. Fasting decreases the activities of hexokinase (124), phosphofructokinase (125) and aldolase (125) in adipose tissue, and the ratio of active pyruvate dehydrogenase to the inactive form is higher in adipose tissue of the fed rat than in the fasted animal (126). The production of acetylCoA from acetate by the enzyme acetylCoA synthetase is also known to increase threefold in adipose tissue of rats refed after fasting (66).

Dietary fat influences fatty acid synthesis in adipose tissue of many species (127) but the physiological significance of CoA thioesters of fatty acids originating from the diet has been questioned since Dorsey and Porter (128) showed palmitoylCoA to be a strong detergent with nonspecific effects on many enzymes in pigeon liver. However, more recent studies have demonstrated that the inhibitory effect of long-chain acylCoA's on acetylCoA carboxylase (129) and citrate synthetase (130) are independent of their

detergent properties.

The inhibition of citrate transport out of the mitochondrion by long-chain acylCoA's reported by Halperin et al (114) effectively reduces fatty acid synthesis since as well as being the substrate for acetylCoA carboxylase, citrate is also an activator of the enzyme. The decreased activity of citrate cleavage enzyme observed in the adipose tissue of rats fed a high fat diet (127) may result from high concentrations of fatty acylCoA reducing the availability of free CoA for use in the citrate cleavage (131). High levels of dietary fat also decreased the activities of the hydrogen-generating enzymes glucose-6-phosphate dehydrogenase (127) and malic enzyme (132).

The composition of dietary fat is also important in the regulation of fatty acid synthesis since unsaturated fatty acids depressed the activity of glucose-6-phosphate dehydrogenase in adipose tissue more than did saturated fatty acids when included in the diet of rats at high concentrations (127). Similarly, polyunsaturated fatty acids reduced the activity of lipogenic enzymes to a greater extent than saturated fatty acids in rat adipocytes (133). It is well known that diets having a high carbohydrate and low fat content stimulate the activity of several lipogenic enzymes (134). The effect of dietary protein on fatty acid synthesis in adipose tissue has received little study and is difficult to assess since the inclusion of high levels of protein, fat or carbohydrate in the diet corresponds with a decrease in the proportion of some other component. However, a diet of 36% protein has been reported (135) to depress overall fatty acid synthesis from glucose in rat adipose tissue.

Not only is fatty acid synthesis in adipose tissue affected by

the constitution of the diet, but it is also influenced by the pattern of food intake. The rate of fatty acid synthesis was found to be greater in adipose tissue from rats (135) and pigs (136) fed only periodically than from animals allowed to feed ad libitum on the same diet.

3. Developmental and Genetic Effects

Fatty acid synthesis in adipose tissue is dependent on the stage of development of the animal.

Although the rate of fatty acid synthesis in adipose tissue decreases with age in rats (137), the incorporation of acetate into fatty acids by adipose tissue of lambs was found to increase about tenfold during the ten days following birth (138). Since the effect of insulin on fatty acid synthesis from glucose was greater in isolated adipocytes from four-week-old rats than in those from fifteen-week-old animals (137), age-related decreases in the rate of fatty acid synthesis may actually be a reflection of reduced sensitivity to insulin.

When mice and rats were weaned from a high-fat milk diet onto a fat-free diet, hepatic fatty acid synthesis increased (139,140) due perhaps to an increase in the content of fatty acid synthetase (140). StearoylCoA desaturase activity decreased with age in rat liver (141) and has been shown to increase gradually in chicken liver after hatching (142). However the effect of age on the desaturase system of adipose tissue has not been studied.

Genetic factors also influence fatty acid synthesis in mammalian adipose tissue. The rate of lipogenesis is known to be greater in adipose tissue from genetically obese mice (143), rats (144) and

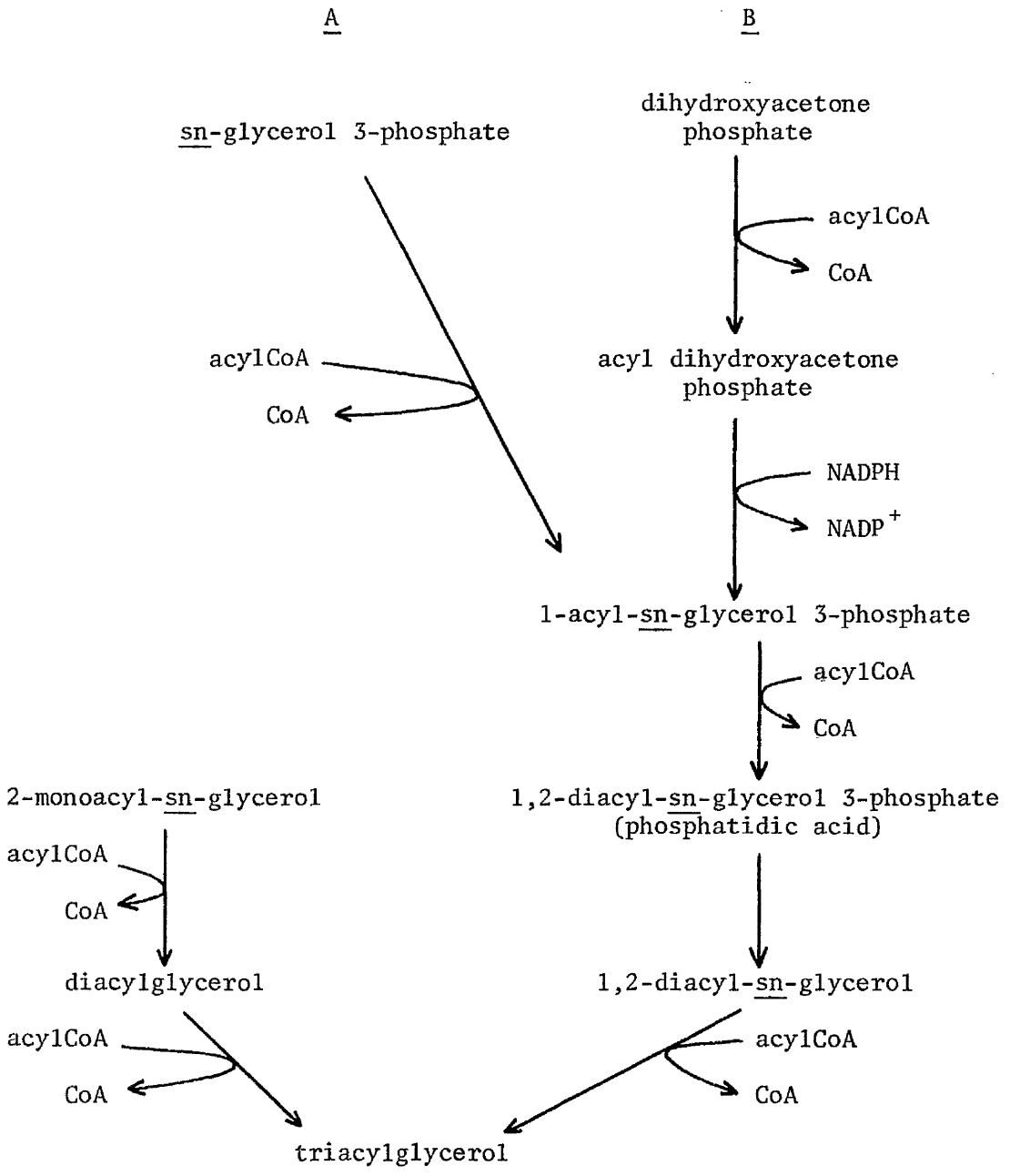


Fig. 1.3. Pathways of triacylglycerol synthesis in adipose tissue.

pigs (145) than in tissue from normal animals. Although there was no significant difference in the activities of fatty acid synthetase from normal and obese pig adipose tissues, Martin and Herbein (145) found that malic enzyme, citrate cleavage enzyme and the pentose phosphate pathway dehydrogenases were more active in tissue from the obese animal.

The increased fatty acid synthesis associated with genetic obesity may also correspond with an increased amount of acetylCoA carboxylase present in the tissue as has been found with livers from genetically obese mice (146). The synthesis of oleic acid by the desaturation of stearic acid has also been shown to proceed at a threefold greater rate in adipose tissue of obese mice in comparison to that in normal lean mice (147).

C. Biosynthesis of Triacylglycerols in Adipose Tissue

The biosynthesis of triacylglycerols in animal tissues has been reviewed comprehensively elsewhere (148,149,150,151) and for this reason only the more recent findings are described in detail here. Fig. 1.3 illustrates the three metabolic pathways that can lead to triacylglycerol synthesis in adipose tissue. A represents the sn-glycerol 3-phosphate pathway, B the dihydroxyacetone phosphate pathway and C the monoacylglycerol pathway. The pathways are described below individually.

The fatty acids esterified into triacylglycerols by each of the three pathways in adipose tissue can originate from two sources; fatty acids formed de novo within the tissue as described previously

(endogenous fatty acids), and preformed fatty acids taken up from the blood (exogenous fatty acids). The exogenous fatty acids are predominantly of dietary origin although a proportion may be synthesized in the liver. The absorption of lipids from the gut and their transport in blood as chylomicra are well characterised and are reviewed elsewhere (152), as is the uptake of fatty acids from the triacylglycerols of chylomicra mediated by the enzyme lipoprotein lipase (for reviews see Robinson and Wing (153) and Scow et al (154)).

1. sn-Glycerol 3-Phosphate Pathway

The sn-glycerol 3-phosphate pathway, which is known to operate in adipose tissue (155,156), mammary tissue (157) and other organs of the body, is the major synthetic route to triacylglycerols in most tissues although the intestinal mucosa in some non-ruminants uses predominantly the monoacylglycerol pathway (158).

The sn-glycerol 3-phosphate is derived from glucose either via glycolysis or the pentose phosphate pathway. Katz et al (49) reported that the pentose phosphate pathway contributed 14% of the sn-glycerol 3-phosphate for triacylglycerol synthesis in epididymal adipose tissue from fed rats, but the extent to which the pentose phosphate pathway contributes sn-glycerol 3-phosphate may be subject to dietary and hormonal control. A small proportion of the sn-glycerol 3-phosphate may also arise from the phosphorylation of free glycerol derived from the hydrolytic breakdown of exogenous triacylglycerols by lipoprotein lipase or from endogenous lipolysis. The phosphorylating enzyme glycerol kinase (EC 2.7.1.30) has been found to be active in adipose tissue of normal (159) and genetically obese

rats (160) although its activity in ruminant adipose tissue is somewhat less (161).

The activation of fatty acids to fatty acylCoA's is brought about by ATP-dependent acylCoA synthetase enzymes (EC 6.2.1.3) which are known to be highly active in adipose tissue (162).

Limited information is available about the esterification enzymes of the sn-glycerol 3-phosphate pathway, and of the monoacylglycerol and dihydroxyacetone phosphate pathways, since their association with membranes has prevented their solubilisation and purification to homogeneity (151). However, the present knowledge of the individual enzymes of the sn-glycerol 3-phosphate pathway is outlined below.

(i) AcylCoA: sn-Glycerol 3-Phosphate Acyltransferase. The formation of phosphatidic acid from sn-glycerol 3-phosphate by the acylation of positions sn-1 and sn-2, has been studied in many tissues other than adipose tissue.

Since two acylations are involved, the synthesis of phosphatidic acid could be catalysed by two separate enzymes or by one enzyme having either one or two active sites (149). AcylCoA: sn-glycerol 3-phosphate acyltransferase (EC 2.3.1.15), the enzyme capable of acylating sn-glycerol 3-phosphate, has been reported to be present in both the mitochondrial and microsomal fractions of rat epididymal adipose tissue (163,156). However, some disagreement exists as to the principal intracellular location of the enzyme, since Roncari and Hollenberg (164) reported that the mitochondrial fraction was more active in the esterification of (1-^{14}C)palmitic acid, whereas more recent studies (165) have shown the acyltransferase to be most active

in the microsomal fraction of adipose tissue. The high capacity of adipose tissue for triacylglycerol synthesis was reflected in the finding that adipose tissue homogenates contained much greater acylCoA: sn-glycerol 3-phosphate acyltransferase activity than homogenates of other rat tissues (163).

Phosphatidic acid has been identified as the product of acylation of sn-glycerol 3-phosphate with palmitoylCoA by mitochondrial and microsomal preparations of rat adipose (164,156) and mammary tissues (166). However in studies with rat liver mictosomes, Fallon and Lamb (167) found monoacyl-sn-glycerol 3-phosphate to be the initial product with the nature of the substrate fatty acylCoA determining whether the monoacyl-sn-glycerol 3-phosphate formed was the sn-1 or sn-2 isomer (168). The results of several other studies (169,170,171) have suggested that two distinct enzymes acting sequentially are required for the synthesis of phosphatidic acid from sn-glycerol 3-phosphate in rat liver microsomes; an acylCoA: sn-glycerol 3-phosphate acyltransferase forming 1-acyl-sn-glycerol 3-phosphate and an acylCoA: 1-acyl-sn-glycerol 3-phosphate acyltransferase to yield phosphatidic acid.

(ii) Phosphatidate Phosphohydrolase. The hydrolysis of phosphatidic acid to 1,2 diacyl-sn-glycerol is carried out by phosphatidate phosphohydrolase (EC 3.1.3.4), an enzyme which exists in both the cytoplasmic and particulate fractions of mammalian cells (149). Daniel and Rubinstein (172) found the enzyme to be equally distributed between the particulate and soluble fractions of rat adipose tissue homogenates, and to have a pH optimum of 6.0. Jamdar and Fallon (156) reported that Mg^{2+} concentration was important for

the activity of the cytoplasmic enzyme on membrane-bound phosphatidic acid and suggested that in adipose tissue Mg^{2+}-dependent and Mg^{2+}-independent phosphatidate phosphohydrolases might exist. The same workers postulated that the microsomes contained both types of enzyme while the cytoplasm contained mainly the Mg^{2+}-dependent enzyme. In liver, the cytoplasmic enzyme utilised membrane-bound phosphatidic acid preferentially as substrate, whereas microsomal phosphatidate phosphohydrolase had a higher activity on aqueous phosphatidic acid (173).

The cytoplasmic phosphatidate phosphohydrolase of hepatic tissues is generally regarded (174) as being more important in triacylglycerol synthesis than mitochondrial and microsomal species of the enzymes. In contrast, Jamdar and Fallon (156) proposed that the microsomal enzyme was more active than that of the cytoplasm for triacylglycerol synthesis in adipose tissue. Earlier studies with adipose tissue homogenates had also implicated the mitochondrion as a major site of triacylglycerol synthesis and demonstrated that the addition of the cytoplasm fraction promoted this activity possibly by activating a microsomal phosphatidate.

The role of cytoplasmic phosphatidate phosphohydrolase in rat adipocytes has recently been studied by Moller et al (175) who suggested that the enzyme may have a regulatory function in triacylglycerol synthesis.

(iii) AcylCoA: 1,2-diacyl-sn-glycerol Acyltransferase. The final step in the sn-glycerol 3-phosphate pathway is the acylation of 1,2-diacyl-sn-glycerol to yield the end product, triacylglycerol. This acylation is achieved by the enzyme acylCoA: 1,2-diacyl-sn-

glycerol acyltransferase (EC 2.3.1.20), more commonly known as diacylglycerol acyltransferase.

Although the enzyme has been studied in various other tissues (176,177), its occurrence and properties in adipose tissue have not been investigated to any great extent. However, the few studies that have been carried out revealed that the diacylglycerol acyltransferase was associated predominantly with the microsomes (172,178,179,180).

In rat liver, the enzyme was capable of acylating 2,3-diacyl-sn-glycerol (181) but evidence to date suggests that 1,2-diacyl-sn-glycerol is the preferred substrate (178,182). The specificity of diacylglycerol acyltransferase in relation to the fatty acid composition of the 1,2-diacyl-sn-glycerol substrate in rat adipocytes has been studied by Coleman and Bell (180) who found 1,2-dioleoyl-sn-glycerol to be the best substrate. Unfortunately the interpretation of such studies with microsomal preparations is hindered by a lack of knowledge of the preferred physical form of the vatious diacylglycerols presented as substrates for the enzyme.

2. Dihydroxyacetone Phosphate Pathway

The participation of the dihydroxyacetone phosphate pathway in glycerolipid biosynthesis in mammalian tissues has recently been discussed by Hajra (183). In rat adipose tissue triacylglycerol synthesis via this pathway was found to be secondary to the operation of the sn-glycerol 3-phosphate pathway (184). The acylation of dihydroxyacetone phosphate to form acyldihydroxyacetone phosphate has been shown (185) to occur in both the mitochondrial and microsomal fractions of rat adipose tissue. Although the enzyme responsible for this acylation is generally regarded (184) as being a distinct enzyme,

recent evidence (165) has suggested that the acylation of both sn-glycerol 3-phosphate and dihydroxyacetone phosphate in rat fat-cells may be carried out by the same enzyme.

Reduction of acyldihydroxyacetone phosphate to 1-acyl-sn-glycerol 3-phosphate was carried out by a membrane-bound, NADPH-reductase (EC 1.1.1.101) associated with both the mitochondria and microsomes in adipose tissue (186). Acylation of the 1-acyl-sn-glycerol 3-phosphate thus produced then proceeds as in the sn-glycerol 3-phosphate pathway.

3. Monoacylglycerol Pathway

The monoacylglycerol pathway plays only a minor role in the synthesis of triacylglycerols in rat adipose tissue, as demonstrated by the recent finding of Dodds et al (184) that 2-monoacyl-sn-glycerol was acylated at a lesser rate than sn-glycerol 3-phosphate and dihydroxyacetone phosphate in rat adipose tissue homogenates. On the other hand, in earlier studies, Schuttz and Johnston (187), using the ether analogue of 2-monooleoyl-sn-glycerol as substrate in vitro showed that hamster adipocytes had a monoacylglycerol pathway activity equal to that of the sn-glycerol 3-phosphate pathway, but that in microsomal preparations the glycerol phosphate pathway was at least twice as active. The monoacylglycerol pathway may also operate in human adipose tissue (188).

Although the actual enzymes for triacylglycerol synthesis via the monoacylglycerol pathway have never been isolated from adipose tissue, Rao and Johnston (189) found that those in hamster intestinal mucosa were present as a complex containing acylCoA: monoacylglycerol acyltransferase, acylCoA: diacylglycerol acyltransferase and acylCoA synthetase, with both products and substrates being enzyme bound.

In keeping with the fact that 2-monoacyl-sn-glycerol is the main product of lipolysis catalysed by pancreatic lipase, the 2-monoacyl-sn-glycerol was the best acyl acceptor in intestinal epithelium, and 1,2-diacyl-sn-glycerol was the principal product (190).

D. Regulation of Triacylglycerol Biosynthesis in Adipose Tissue

1. Control of Positional Distribution of Fatty Acids in Triacylglycerols

Analysis of the fatty acid composition of triacylglycerols in natural fats does not permit any conclusions to be made regarding the different molecular types of triacylglycerols present. In a natural triacylglycerol mixture containing n different fatty acid constituents, the number of possible molecular species of triacylglycerols (including enantiomers), N, can be calculated from the formula:

$$N = n^3$$

Thus, 216 molecular species of triacylglycerols can theoretically exist when six different fatty acids are present although biological material usually contains at least seven different fatty acids. Before the specific nature of fatty acid distribution in triacylglycerols was known, the idea prevailed that the fatty acids were simply distributed in a random manner. However, it is now evident that natural triacylglycerols have an asymmetric fatty acid distribution. The stereospecific distribution of fatty acids in triacylglycerols from a variety of plant and animal sources has been reviewed by Brockerhoff (191). Some general rules were deduced for the positional distribution of fatty acids in adipose tissue triacyl-

glycerols. In mammalian depot fat the triacylglycerol molecule was usually of the structure:-

position sn-1: saturated fatty acid.

position sn-2: unsaturated or short-chain fatty acid.

position sn-3: long-chain fatty acid, random distribution

where the positions refer to the sn-carbons of sn-glycerol.

However, several exceptions to these rules exist. The greatest proportion of palmitic acid present in triacylglycerols of pig adipose tissue was located in position sn-2 (192,193), although the distribution of fatty acids in triacylglycerols from the liver of the same animal conforms to the above pattern (193). In adipose tissue triacylglycerols of the elephant (194), hippopotamus (194), tree hyrax (195) and animal species closely related to the domestic pig (196), the overwhelming proportion of palmitic acid was found esterified at position sn-2. Although Brockerhoff et al (187) found the fatty acid composition of positions sn-1 and sn-3 to be almost identical in triacyl-sn-glycerols from adipose tissue of several avian species, more recent studies have shown (198) the positioning of fatty acids in triacylglycerols from depot fat of chickens to follow the rules outlined above.

The fatty acid composition of triacylglycerols in rat adipose tissue has been shown (199) to reflect that of the diet. In contrast, the pattern of fatty acids present in triacylglycerols of ruminant adipose tissue is comparatively unreflected by dietary fat since unsaturated fatty acids are extensively hydrogenated in the rumen, although the tissue fatty acid composition can be altered by feeding fats which are protected from ruminal biohydrogenation (200). The influence of diet on the fatty acid composition of various tissues has

been discussed by Garton (201).

Early studies on the incorporation of fatty acids into acylglycerols have been reviewed by Lands (202). The non-random, asymmetric manner of fatty acid esterification into acylglycerols was demonstrated by Elovson et al (203) who found that more than 90% of ^{14}C-labelled palmitic acid incorporated into diacylglycerols by rat livers *in vivo* was recovered in position *sn*-1. With studies on rat liver homogenates, other workers (204) later confirmed the preferential esterification onto position *sn*-1 and also showed oleic acid to enter predominantly position *sn*-2 of the triacylglycerols synthesized by the system. It has been suggested (170) from the results of studies with partially-purified enzymes from rat liver that in the acylation of *sn*-glycerol 3-phosphate to 1-acyl-*sn*-glycerol 3-phosphate the preferred substrate is a long-chain saturated fatty acylCoA and that an unsaturated fatty acylCoA is preferentially esterified onto position *sn*-2.

Kinsella (205) recently reported that palmitoylCoA was esterified onto monoacyl-*sn*-glycerol 3-phosphate at a higher rate than other fatty acylCoA's by bovine mammary microsomes and suggested that such a substrate preference of the acyltransferase explained the predominantly saturated fatty acid composition of position *sn*-2 in milk fat triacylglycerols (206). In earlier studies with microsomes from rat (166) and bovine (207) mammary tissue in which 1-acyl-*sn*-glycerol 3-phosphate was not detected as an intermediate product, palmitoyl-CoA was also the preferred substrate for acylCoA: *sn*-glycerol 3-phosphate acyltransferase in the production of phosphatidic acid.

Marshall and Knudsen (208) have recently demonstrated the acylation of membrane-bound 1,2-diacyl-*sn*-glycerol with butyrylCoA

by the microsomal fraction of lactating bovine mammary tissue, suggesting that the positioning of butyric and hexanoic acids exclusively in position sn-3 of ruminant milk fat (209) may be due to the ability of diacylglycerol acyltransferase to use short-chain fatty acids as substrates.

Although the distribution of fatty acids in triacylglycerols of mammary tissue (210) and adipose tissue (211,212) has been attributed to the specificity of the acyltransferases in the sn-glycerol 3-phosphate pathway, other factors may influence the positioning of fatty acids. Stokes and Tove (213) have, for example, extracted an uncharacterised factor from pig adipose tissue which apparently interacts with the acyltransferase(s) to direct palmitic acid onto position sn-2.

2. Control of Triacylglycerol Biosynthesis

The regulation of triacylglycerol synthesis in adipose tissue is not fully understood.

Since fatty acids are stored in adipose tissue as triacylglycerols, it can be expected that diets leading to increased fatty acid synthesis will also stimulate the enzymes involved in triacylglycerol formation. Although the sn-glycerol 3-phosphate pathway is the predominant route for the synthesis of triacylglycerols in adipose tissue, the results of Dodds et al (199) suggested that the composition of the diet may influence the extent to which the dihydroxyacetone phosphate pathway contributes to triacylglycerol synthesis in adipose tissue. Similarly, the activity of the dihydroxyacetone pathway in rat liver microsomes was increased when the animals were fed a high-carbohydrate diet (214).

sn-Glycerol 3-phosphate acyltransferase and monoacyl-sn-glycerol

3-phosphate acyltransferase activities in rat adipose tissue (199) and diacylglycerol acyltransferase activity in rat liver (177) have all been shown to increase in animals fed a high carbohydrate diet.

Lloyd-Davies and Brindley (215) demonstrated that the activation of fatty acids was not rate-limiting in the synthesis of phosphatidic acid by rat liver microsomes but that sn-glycerol 3-phosphate acyltransferase could be.

The hydrolysis of phosphatidic acid to 1,2-diacyl-sn-glycerol has been postulated (216) as being an important rate-controlling step in triacylglycerol synthesis in rat liver mainly because phosphatidate phosphohydrolase was found to have the lowest rate in vitro of the enzymes involved in triacylglycerol synthesis in rat liver microsomes (173). More recently, studies (212) with homogenates of rat adipose tissue have also implicated phosphatidate phosphohydrolase as a rate-limiting step in the sn-glycerol 3-phosphate pathway.

Insulin stimulation of fatty acid synthesis in adipose tissue probably corresponds with increased activity of the enzymes involved in triacylglycerol formation from the fatty acids. Jason et al (217) have recently shown the activity of acylCoA synthetase in the microsomes of rat adipocytes to be doubled in the presence of insulin, and the hormone may also exert a stimulatory effect on sn-glycerol 3-phosphate acyltransferase (218).

It has been noted (170) that divalent cations, especially Ca^{2+} are necessary for sn-glycerol 3-phosphate acyltransferase activity in liver, and the results of Jamdar and Fallon (156) suggest that in rat adipose tissue the balance between Mg^{2+} and other divalent ions may play a role in the regulation of triacylglycerol synthesis. Factors affecting the levels and distribution of such ions within the cell

may therefore influence the synthesis of triacylglycerols.

E. Present Aims

In mammalian systems, the biosynthesis of fatty acids and triacylglycerols cannot be regarded as two completely separate processes. Evidence for the existence of relationships between the synthesis de novo of fatty acids and their subsequent esterification into triacylglycerols has been obtained indirectly by several workers. Analytical studies have shown the importance of the positional distribution of fatty acids in triacyl-sn-glycerols and the unique nature of position sn-3: stereospecific analysis of triacyl-sn-glycerols from pig adipose tissue showed (219) that position sn-1 possibly contained only fatty acids formed de novo within the tissue; short- and medium-chain fatty acids of milk fat triacylglycerols are known (191,209) to be present predominantly in position sn-3 and unusual fatty acids of plant triacyl-sn-glycerols are also generally located in position sn-3 (220). The incorporation into position sn-3 of oleic acid formed de novo by the desaturation of stearic acid has been suggested (221) as a regulatory step in triacylglycerol biosynthesis in ruminant mammary tissue.

Present knowledge concerning the esterification of fatty acids into triacylglycerols and its control, is only fragmentary at the biochemical level. In particular, the manner in which fatty acids synthesized de novo within adipose tissue are esterified into triacylglycerols in comparison with exogenous fatty acids arising from the diet or hepatic synthesis, is completely unknown. The study presented

here was undertaken with the aim of examining in vitro the relationship between the synthesis of fatty acids and their subsequent esterification into triacylglycerols for comparison with the natural distribution of fatty acids in triacylglycerols of rat adipose tissue, and to determine whether such relationships are important in controlling the positional distribution of fatty acids in triacylglycerols and the rate of triacylglycerol biosynthesis.

CHAPTER 2. MATERIALS AND METHODS

A. Materials

1. Solvents

All solvents used throughout these studies were of Analar grade or were distilled before use. When solvents were evaporated under reduced pressure or in a stream of nitrogen, the temperature was maintained below 35°C.

2. Chemicals and Reagents

The following were purchased from Sigma (London) Chemical Co. (Kingston-upon-Thames, Surrey, U.K.):- insulin (25.3 I.U./mg), digitonin, bovine serum albumin (Fraction V), potassium morpholino-propane sulphonate (MOPS), highly-polymerised calf thymus DNA, snake venom (Ophiophagus hannah), pancreatin and collagenase (Type 1 from Cl. histolyticum).

The bovine serum albumin was defatted and dialysed by the method of Hanson and Ballard (222) before use.

(1-^{14}C) and (9,10-^{3}H)fatty acids, (1-^{14}C)Na acetate, (U-^{14}C)-glucose and $^{3}H_2O$ were all supplied by the Radiochemical Centre (Amersham, Bucks, U.K.).

All other chemicals were of Analar grade and were purchased from B.D.H. Chemicals (Poole, Dorset, U.K.) unless otherwise stated in the text.

3. Animals

Female Wistar rats were purchased from A. Tuck and Son Ltd. (Rayleigh, Essex, U.K.). The animals were maintained on cube diet

41B (Oxoid Ltd., Basingstoke, Hampshire, U.K.).

B. Techniques for the Preparation and Characterisation of Adipocytes

1. Preparation

Adipocytes, or fat-cells, were prepared from rat adipose tissue essentially by the method of Rodbell (223) as described by Fain (224).

Rats were killed by cervical dislocation and the parametrial adipose tissue was excised into isotonic saline maintained at 37°C. The weight of tissue removed varied with the weight of rat used; approximately 300 mg from an animal weighing 129 g, to 3.4 g from a rat of weight 295 g. The tissue was finely minced with scissors and transferred to a 30 ml polythene bottle containing 5 ml Krebs-Ringer bicarbonate buffer (225) of pH 7.4, but with half the usual calcium concentration, 200 mg defatted bovine serum albumin and 16.6 mg collagenase. The bottle was sealed with a rubber stopper after aeration with O_2:CO_2 (95:5 v/v) for 20 seconds, and incubated in a water bath at 37°C with shaking by a flask shaker for 45 minutes.

The contents of the bottle were then gently pushed by means of a plunger, through a thin layer of cheesecloth fastened across the bottom of a 10 ml plastic syringe from which the nozzle had been removed, into a polythene conical centrifuge tube of 12 ml capacity. Most of the stromal-vascular cells and any undigested tissue were retained on the cheesecloth. The tube with its contents was centrifuged at approximately 400 g for 1 minute. This resulted in

a floating layer of adipocytes, a brown internatant layer and a slight precipitate at the bottom of the tube. The sediment, mostly stromal-vascular cells, and the internatant layer were removed by means of a syringe to which a length of small-bore plastic tubing was attached, and discarded. The layer of fat cells was resuspended in 10 ml prewarmed Krebs-Ringer bicarbonate buffer with 4% albumin, centrifuged and the infranatant removed as before. This washing procedure was repeated once more when the adipocytes were finally resuspended in 16 ml Krebs-Ringer bicarbonate buffer containing 4% albumin in a 25 ml plastic beaker maintained at 37°C.

Aliquots were taken from this suspension by means of a polythene pipette after gentle swirling to ensure a homogenous distribution of the adipocytes throughout the buffer. Portions removed in this way were used in the incubation studies in vitro, or for DNA estimation and microscopic examination.

2. Microscopic Examination

A small drop of the fat-cell suspension was placed on a siliconized glass slide and an equal volume of 0.01% (w/v) acridene orange in water added as stain (226). The suspension was covered with a siliconized glass coverslip and examined under a Leitz Ortholux II microscope equipped with a Wild Photoautomat camera attachment.

Approximate measurements of fat-cell diameters were made by superimposing the photographic transparencies of the adipocytes on one taken of a 1 mm grid at the same magnification.

3. Isolation of the Particulate Components, Cytoplasm and Lipid Storage Droplet of Adipocytes

Digitonin reacts specifically with the 3β-hydroxyl group of cholesterol and can thereby cause the lysis of cholesterol-containing membranes. The plasma membrane of the rat liver cell is known (227) to contain more cholesterol than mitochondrial membranes of the same cell. A similar situation probably exists in the rat adipocyte. By incubating adipocytes with digitonin of a concentration such that the plasma membrane is ruptured while the mitochondrial membrane remains intact, it should be possible to separate the cytoplasm from the mitochondria by centrifugation. The method described by Zuurendonk and Tager (228) for the rapid separation of particulate components and soluble cytoplasm of isolated rat-liver cells was applied with only slight modifications in the volumes used, to rat adipocytes after these had been incubated with ^{14}C-labelled lipid precursors.

At the end of such incubations, the contents of the incubation bottle were decanted into a polythene centrifuge tube and centrifuged at 400 g for 30 seconds. The medium was removed by means of a syringe equipped with a length of small-bore plastic tubing, the adipocytes resuspended in 8 ml Krebs-Ringer buffer containing albumin and the suspension centrifuged again. The buffer was removed as before leaving a thick suspension of adipocytes. A portion of this adipocyte suspension (2 ml) was transferred to a glass conical centrifuge tube surrounded with ice, and to it was added 5 ml of a solution (pH 7.0) containing 0.25M sucrose, 20mM potassium morpholinopropane sulphonate (MOPS), 3mM EDTA and digitonin

at a concentration of 1 mg/ml, at 4°C. After 40 seconds the suspension was centrifuged for approximately 25 seconds at a maximum of 3,000 g on an MSE bench centrifuge.

This gave a layer of lipid (fat plug) on top of a clear internatant layer with a small white pellet at the bottom of the centrifuge tube. The pellet was quickly removed to another test-tube using a syringe equipped with a long blunted needle. Similarly, the internatant layer was transferred to a 50 ml centrifuge tube leaving the fat plug in the original tube.

Lipid was extracted immediately from the pellet, internatant layer and fat plug as described in section D.

4. Measurement of Adipocyte DNA

Extraction of DNA. DNA was extracted from adipocytes by the procedure described by Denton et al (229).

A portion of the adipocyte suspension (2 ml) was extracted for 3 minutes in a hand homogeniser with 2 ml 5% (v/v) perchloric acid and 2 ml diethyl ether at 0°C. The mixture was then transferred to a 12 ml glass centrifuge tube along with a 2 ml 5% (v/v) perchloric acid rinse of the homogeniser, and centrifuged at 3,000 g for 5 minutes. The ether was removed by Pasteur pipette and the aqueous layer carefully decanted off to leave a fat plug and precipitate in the centrifuge tube. The combined fat plug and precipitate were washed once with 5 ml acetone and three times with 5 ml diethyl ether at 4°C to remove lipid.

To the final precipitate was added 1.5 ml 5% (v/v) perchloric acid. The tube and its contents were maintained in a water bath at

70°C for 15 minutes, at the end of which time the tube was centrifuged at 3,000 g for 5 minutes to sediment undissolved residue. The supernatant layer contained the extracted DNA.

DNA assay. DNA was assayed by the method of Burton (230). 1 ml of the above supernatant was mixed with 2 ml of a diphenylamine reagent (1.5 g steam-distilled diphenylamine dissolved in 100 ml acetic acid containing 1.5 ml concentrated sulphuric acid and 0.01 ml acetaldehyde). Standard solutions of highly-polymerised calf thymus DNA and a blank containing no DNA were similarly treated. All tubes were maintained at 30°C for 16 hours to permit development of colour.

The absorbance at 600 nm was measured against the blank in a Gilford 240 spectrophotometer (Gilford Instruments, Ohio, U.S.A.) and compared with values obtained with standards.

Each estimation was performed in triplicate.

C. Incubations of Adipocytes in vitro

1. General Incubation Conditions

Experiments were carried out at least three times and each measurement in a single experiment was duplicated wherever possible.

Whereas a single rat weighing in the region of 280 g, provided a sufficient mass of adipose tissue for the preparation of adipocytes for use in a single experiment, combined adipose tissues excised from two or more rats were used in studies with younger animals.

All incubations were carried out under an atmosphere of O_2:CO_2 (95:5, v/v) in 30 ml plastic bottles sealed with Suba-Seal rubber stoppers (Gallenkamp, London, U.K.), at 37°C in a shaking water bath

(2 cycles/s).

The basic incubation medium contained adipocytes (1 ml of suspension), Krebs-Ringer bicarbonate buffer (pH 7.4) with half the original calcium concentration, glucose (5mM), insulin (20 munits/ml) and defatted albumin (40 mg/ml) in a total volume of 3 ml. Additions to this basic medium are described below or in the relevant position in chapter 3. In later studies, the basic incubation medium system was scaled up to accommodate 4 ml of fat-cell suspension in order to provide sufficient adipocyte material for separation into cytoplasmic and particulate components.

2. Incubations with (1-^{14}C)Fatty Acids

In studies of the rates of esterification of extracellular fatty acids, the incubation medium contained in addition to the basic ingredients, either a single (1-^{14}C)fatty acid of concentration 1mM, or else a (1-^{14}C)fatty acid present as the labelled component of a fatty acid mixture of total concentration 1mM. The mixture consisted of palmitic acid (30 mol %), stearic acid (10 mol %), oleic acid (40 mol %) and linoleic acid (20 mol %). The component carrying the ^{14}C label was varied according to the fatty acid under study. The amount of label per incubation bottle was always 0.3 μCi both when the (1-^{14}C)fatty acid was supplied alone or as a component of the fatty acid mixture.

To permit comparison of results obtained with adipocytes prepared from different rats, each experiment on fatty acid esterification rates had control incubations containing (1-^{14}C)palmitic acid alone. Results could therefore be corrected relative to these control incubations. Other control incubations included fat-cells incubated

in media containing no (1-^{14}C)fatty acids, and a complete incubation system except for the adipocytes. All incubations were carried out in duplicate.

In all experiments, fatty acids were added to the incubation medium as their potassium salts bound to albumin and these were prepared by dissolving the pure fatty acids in 0.1 ml of 0.1M potassium hydroxide containing 4 mg albumin.

In studies of the positional distribution of esterified extracellular fatty acids in triacylglycerols, each (1-^{14}C)fatty acid contained 0.6 μCi of radioactivity and was incubated as part of the fatty acid mixture. In experiments designed to study the effect of acetate concentration on the esterification of extracellular fatty acids into triacylglycerols, (1-^{14}C)oleic acid (0.3 μCi) was present as part of a fatty acid mixture in the basic incubation medium to which sodium acetate at several concentrations had also been added.

In order that allowance could be made for fatty acids present in the albumin, the absolute unesterified fatty acid content of the incubation medium was determined in triplicate by extracting lipids from a complete incubation or from a control incubation containing the (1-^{14}C)fatty acid but no adipocytes. 1 ml of a standard solution of pentadecanoic acid in methanol was added during this lipid extraction. The unesterified fatty acid fraction of the lipid extract was separated by T.L.C., methylated and analysed by G.L.C. By comparison with the pentadecanoin standard the amounts of the other fatty acids present were calculated (231). In this way, the overall amount of individual fatty acids available to the fat-cells from the incubation medium was determined and taken into consideration in subsequent calculation of results. Full details of the

chromatographic procedures are given in section E below.

3. Incubations with (1-^{14}C)Acetate, (U-^{14}C)Glucose and $^{3}H_2O$

In studies of the incorporation of acetate into acylglycerols, sodium (1-^{14}C)acetate was included at several concentrations in the incubation medium. The specific activities used varied and are given in the legends to the relevant Tables or Figures. Similarly, in experiments on lipogenesis from (U-^{14}C)glucose as the primary precursor, the concentration of ^{14}C-labelled glucose in the incubation medium was varied, and the initial specific activities used in each experiment are presented later along with the relevant experimental results.

In some experiments, (9,10-^{3}H)oleic acid and (9,10-^{3}H)stearic acid were incubated with fat-cells in the basic incubation medium containing either (U-^{14}C)glucose or (1-^{14}C)acetate at various concentrations. Again the initial specific activity of each ^{3}H-labelled fatty acid is given in the legends to the relevant results. In studies of the incorporation of $^{3}H_2O$ into lipids, adipocytes were incubated in the basic medium containing glucose or acetate at various concentrations to which $^{3}H_2O$ was added. The specific activity of $^{3}H_2O$ in all incubations was 1 mCi/ml.

Concurrent incubations were carried out as part of these experiments with (1-^{14}C)acetate, (U-^{14}C)glucose and $^{3}H_2O$ substrates in which the medium also contained the fatty acid mixture with the composition already described (section C.2) and of total concentration 1mM.

D. Isolation of Lipids

1. Extraction of Total Lipids from Adipocytes

Lipid was extracted from adipocytes by the method of Folch et al (232).

After incubating the adipocytes with various substrates, the reaction was stopped by the addition of 8 ml methanol, and the contents of the reaction vessel decanted into a 30 ml glass centrifuge tube fitted with a ground-glass stopper. About 1 mg of a mixture consisting of tripalmitoylglycerol, dipalmitoylglycerol, dimyristoyl lecithin and palmitic acid was added in a small volume (100 µl) of chloroform to act as a carrier. 16 ml of chloroform was then added and the tube shaken vigorously. Potassium chloride (3 ml) 1.76% (w/v) was added to the mixture resulting in a final chloroform:methanol:water ratio of 8:4:3 by volume, with the overall concentration of potassium chloride in the aqueous fraction being 0.88%. The incubation bottle was rinsed briefly with a small volume of chloroform:methanol (2:1 v/v) which was added to the extraction mixture. The mixture was shaken vigorously for two minutes and allowed to stand at room temperature for one hour with occasional shaking. At the end of this time, the tube and its contents were centrifuged for 10 minutes at 1,000 g. The upper aqueous layer which contained non-lipid materials was aspirated and discarded. The lower, organic layer, containing extracted lipid was filtered into a round-bottomed flask, and the solvent was removed under reduced pressure on a rotary film evaporator, yielding a pure lipid extract which was transferred in a small volume of chloroform to a previously weighed test-tube. The round-bottomed flask was rinsed with chloroform:

methanol (2:1 v/v) and these washings added to the test-tube. The solvent was evaporated under a stream of nitrogen and the weight of the lipid extract determined.

Lipid samples extracted in this manner were stored in solutions of 1 ml of chloroform in a refrigerator at 4°C under an atmosphere of nitrogen, prior to further analysis.

When (1-^{14}C)acetate and (U-^{14}C)glucose were the labelled substrates in studies with young rats, the 3 ml of 1.76% potassium chloride also contained 1% (w/v) sodium acetate and 1% (w/v) glucose respectively, to aid the removal of residual labelled precursors.

Extraction of total lipids from the fat plug, internatant layer and pellet formed on centrifugation of adipocytes lysed by digitonin was carried out essentially as above.

2. Separation of Lipid Classes

The separation of extracted lipid into triacylglycerols, diacylglycerols, phospholipids and unesterified fatty acids was achieved by thin layer chromatography (T.L.C.) in unlined tanks on 20 x 20 cm or 10 x 20 cm glass plates coated with Kieselgel G (E. Merck, Darmstadt, Germany) in layers 0.5 mm thick.

Up to 40 mg of lipid in solvent (usually chloroform) was applied as a band to one 20 x 20 cm plate and separated into its component lipid classes using hexane:diethyl ether:formic acid (80:20:2 by volume) as developing solvent. The developed chromatogram was sprayed with 0.1% (w/v) 2',7'-dichlorofluorescein in 96% methanol containing 0.1% (w/v) 2,6-di-tert-butyl-p-cresol (B.H.T.) as antioxidant, and was viewed under ultraviolet light to detect the bands of different lipid classes. The identity of each band was

ascertained by comparison with a similar plate on which lipid standards had been separated.

When ^{14}C-labelled substrates were used, the lipid fractions together with the adsorbent were scraped off the plate and counted directly by liquid scintillation spectrometry (see section G). Lipid fractions containing ^{3}H were eluted from the adsorbent as described below before measurements were made of their radioactivity.

Although methyl esters of triacylglycerols, diacylglycerols, phospholipids and fatty acids could be formed without prior extraction of the lipids from the silica gel adsorbent (233), the trans-esterification process was found to be more efficient if the lipids were first eluted from the adsorbent. In this instance, the bands were scraped from the developed plate into separate small chromatographic columns, and the lipids eluted with 60 ml of a suitable solvent. Triacylglycerols and diacylglycerols were eluted with chloroform or diethyl ether, phospholipids with chloroform:methanol:water (5:5:1 by volume) and fatty acids were extracted with diethyl ether:formic acid (199:1 by volume). The solvents were removed under reduced pressure to yield the relevant pure lipid class.

E. Fatty Acid Analysis

1. Methylation of Lipids

Methyl esters of the various lipid classes were formed by adding 2 ml of 1% (v/v) concentrated sulphuric acid in dry methanol to the lipid in a 15 ml ground-glass test-tube. Since triacylglycerols have only limited solubility in methanol, 1 ml of benzene

was added when this component was being methylated. Diacylglycerols, unesterified fatty acids and phospholipids did not require the addition of any solvent. The test-tube was filled with nitrogen, stoppered and maintained at 50°C for 16-18 hours in a thermostatically-controlled heating block.

The methylation was stopped by the addition of 5 ml water, and the solution extracted once with 5 ml diethyl ether and once with 5 ml hexane:ether (1:1 v/v). The organic layers were removed by Pasteur pipette and washed with 5 ml 2% (w/v) potassium bicarbonate to remove any dye before drying over anhydrous sodium sulphate (234).

2. Separation of Saturated and Unsaturated Fatty Acid Methyl Esters

Silver nitrate T.L.C. was used for the separation of methyl esters of saturated fatty acids from those containing one or more double bonds (235).

Kieselgel G containing 10% (w/w) silver nitrate was used to coat 10 x 20 cm glass plates in layers 0.5 mm thick. The mixture of methyl esters to be separated was applied as a band in hexane solution to a T.L.C. plate coated with silver nitrate-impregnated silica gel and this was developed in the dark with hexane:diethyl ether (95:5 v/v) as solvent mixture. Bands of saturated, monoenoic and dienoic fatty acid methyl esters were identified by comparison with known standards after spraying the developed plate with 2',7'-dichlorofluorescein solution and viewing under ultraviolet light.

The radioactivity of fatty acid methyl esters could not be measured directly in the presence of silver nitrate-impregnated

adsorbent and separated components were therefore always extracted from the silica gel prior to counting. Two methods were adopted for this purpose:-

(i) The bands containing the methyl esters were scraped into small glass columns of ¼" diameter plugged at one end with cotton wool and containing a small layer (approximately 1 g) of Florisil. The methyl esters were then eluted with 15 ml diethyl ether directly into glass scintillation vials. The ether was evaporated to dryness under a stream of nitrogen and the silica eluted once more with diethyl ether:methanol (14:1 v/v) directly into the same vial. The solvent was again evaporated, toluene-fluor added and the sample counted by a liquid scintillation spectrometer. Trials with standard mixtures of methyl (1-^{14}C)palmitate and methyl (1-^{14}C)oleate had shown that this procedure gave about 85% recovery of ^{14}C label and had high reproduceability.

(ii) Alternatively, each silica gel band was scraped into a test-tube and 1 ml methanol was added followed by 1 ml 20% (w/v) sodium chloride and 3.5 ml hexane:diethyl ether (1:1 v/v). The contents of the tube were mixed thoroughly and the silica gel sedimented by centrifugation at 1,000 g for 5 minutes. The layer of organic solvent was transferred to another test-tube by Pasteur pipette, and the silica gel extracted twice more with 3.5 ml hexane: ether (1:1 v/v). The combined organic layers (10.5 ml) were washed with 2.5 ml 5% (w/v) potassium bicarbonate to remove 2',7'-dichloro-fluorescein dye, and finally transferred to a scintillation vial for measurement of radioactivity (234).

Recoveries of better than 90% were obtained with (1-^{14}C)fatty acid methyl esters using this method.

3. Gas Liquid Chromatography (G.L.C.)

Gas liquid chromatographic analyses were carried out on a 7' by ¼" glass column of 15% w/w EGSS-X on Chromosorb W (100-120 mesh, acid-washed and silanised) (Applied Sci. Inc., U.S.A.) at 185°C, in a Pye 104 Chromatograph (Pye-Unicam Ltd., Cambridge, U.K.) equipped with a flame ionisation detector.

The fatty acids of all lipid classes were analysed as their methyl esters. Peak areas were calculated by multiplying the height of each peak by its retention time (236). Individual ester peaks were identified with regard to chain length and number of double bonds by comparison with authentic standards. To determine molar percentages each peak area was multiplied by an appropriate factor calculated to compensate for the differences in molecular weights of the different fatty acids.

Preparative G.L.C. of lipid methyl esters containing radioactivity was carried out on a 7' by ⅜" glass column containing 15% w/w EGSS-Y on Chromosorb W (100-200 mesh, acid-washed and silanised) in a Pye 104 chromatograph. A stream-splitter situated immediately before the flame ionisation detector allowed a high proportion (nominally 99%) of the material eluting from the column to be directed through an outlet maintained at oven temperature (185°C) into traps containing glass beads (60 mesh) moistened with toluene-fluor. In this way, the esters corresponding to individual peaks could be trapped separately by using a new trap for each component as the peak emerged on the recorder chart. The trapped esters were eluted directly into glass scintillation vials with 15 ml toluene-fluor for radioactivity measurement (237).

Recoveries of 85-90% with ^{14}C-labelled fatty acid methyl esters

were consistently obtained using the above system.

4. High Pressure Liquid Chromatography (H.P.L.C.)

A stainless steel column of length 30 cm and internal diameter 0.4 cm packed with μ Bondapak C18 (particle size 10 μm) (Waters Associates, Milford, Mass., U.S.A.) was used in all analyses of fatty acid methyl esters. Samples were loaded onto the column by means of a U6K injection block and an M-600 pump (Waters Associates) was used to drive the solvent through the column. The column effluent was monitored by a refractive index detector (Varian Aerograph, Walnut Creek, California, U.S.A.) linked to a chart recorder.

All solvents were degassed before use by refluxing in a flask equipped with a water condenser for 30 minutes.

The optimum operating conditions for the analyses of fatty acid methyl standards were determined by varying the ratio of methanol to water in the solvent, and the solvent flow rate.

In an attempt to reduce active sites of the adsorbent by silanisation of the column, the polarity of the solvent passing through it was gradually decreased by pumping methanol:water (90:10 v/v), methanol and benzene through in that order each for 30 minutes. A 15% (v/v) solution (2 ml) of dimethylchlorosilane in benzene was injected onto the column and 2 minutes later the flow of the benzene solvent was stopped. The column, wrapped in metal foil, was maintained at 50°C overnight by resting it on top of a thermostatically controlled heating block. At the end of this time, benzene was pumped through the column for 30 minutes, followed thereafter by methanol for an equal length of time. Finally, methanol:water (90:10 v/v) was allowed to flow through for a further 30 minutes before separation of

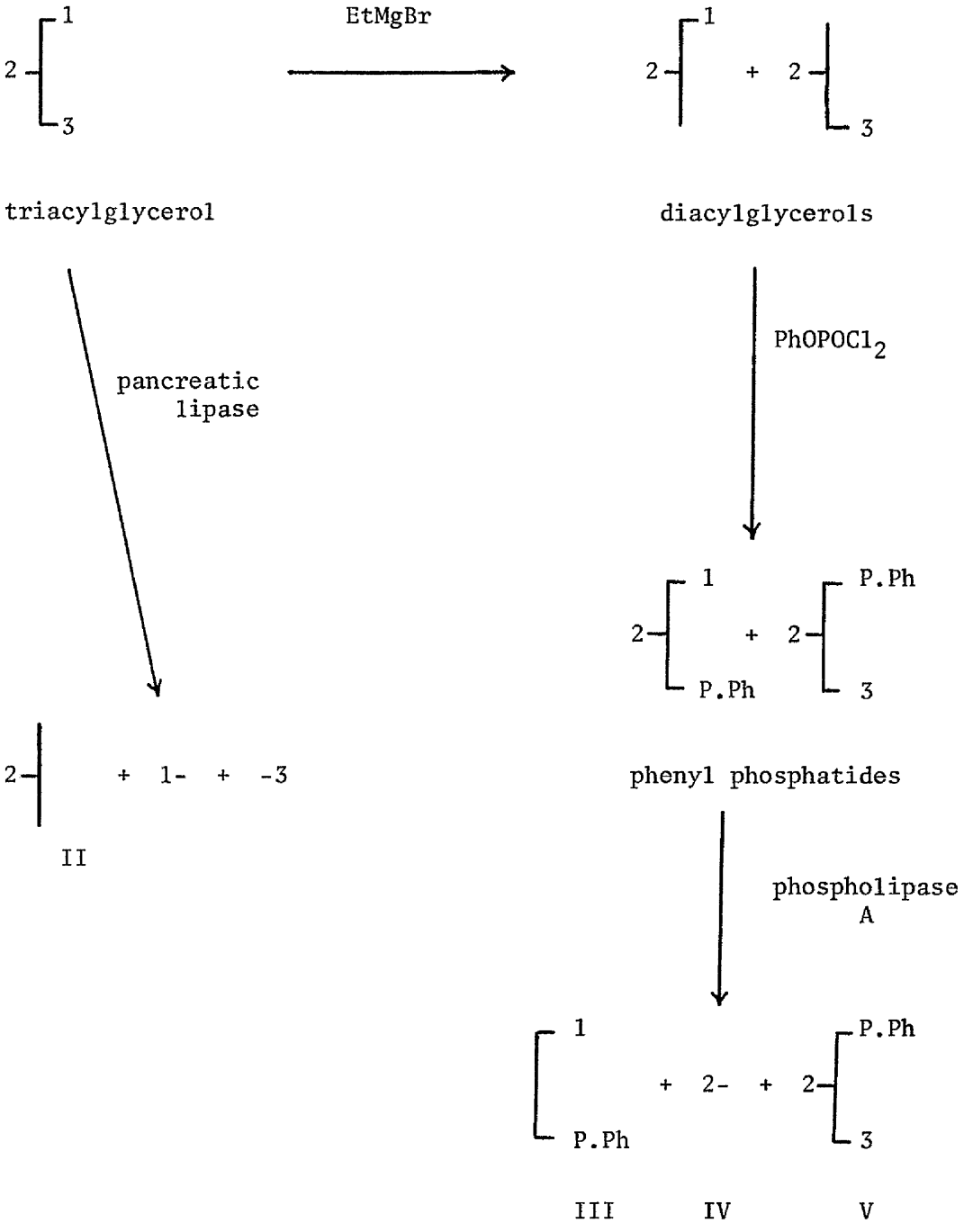


Fig. 2.1. Stereospecific analysis of triacylglycerols.

I, triacylglycerol; II, 2-monoacyl-sn-glycerol; III, lysophosphatide; IV, free fatty acid; V, phospholipid.

standard mixtures was attempted.

F. Structural Analyses

1. Stereospecific Analysis of Triacylglycerols

Stereospecific analysis of triacylglycerols allowed the composition of the fatty acids esterified to each of the three positions of the sn-glycerol molecule to be determined.

The procedure used was that developed originally by Brockerhoff (238) as modified by Christie and Moore (239).

Principles: The method involves the chemical hydrolysis of triacylglycerols to a mixture of 1,2- and 2,3-diacyl-sn-glycerols using a Grignard reagent, followed by the conversion of these diacylglycerols to phenyl phosphatides. These synthetic phospholipids are then treated with the enzyme phospholipase A (EC 3.1.1.4) which specifically hydrolyses the fatty acid present in position sn-2 of phospholipids containing the phosphate group in position sn-3 i.e. the L-phosphoglyceride. Thus, the end-products of the phospholipase hydrolysis are free fatty acids released from position sn-2 (Product IV in Fig. 2.1), and a lysophosphatide (Product III) containing only those fatty acids originally present in position sn-1. The synthetic phospholipid with the phosphate group on position sn-1 (Product V) is not hydrolysed by the enzyme and can be separated from the products of hydrolysis by T.L.C.

The fatty acid composition of position sn-2 is also determined separately by hydrolysis of the triacylglycerols with pancreatic lipase. This enzyme removes fatty acids from positions sn-1 and sn-3

yielding a 2-monoacyl-sn-glycerol (Product II), the fatty acid composition of which can be determined by G.L.C. analysis of its methyl esters.

The fatty acids present in position sn-3 can then be calculated using the following relationships:-

(position sn-3) = 3 x (triacylglycerol) - (position sn-1 + position sn-2)

and

(position sn-3) = 2 x (sn-2,3-phosphatide) - (position sn-2)

Thus the composition of both positions sn-2 and sn-3 can be determined by alternative methods, and the accuracy of the analysis can be assessed by the extent to which values obtained by the alternative methods agree.

Experimental detail: Up to 40 mg of triacylglycerols in 2 ml of dry diethyl ether was reacted for 1 minute with 1 ml of Grignard reagent (ethyl magnesium bromide, 0.5M), and the reaction stopped by the addition of 0.05 ml glacial acetic acid. To neutralise excess acid, 5 ml of 2% (w/v) potassium bicarbonate was then added. The mixture was extracted twice with 5 ml diethyl ether which was then washed with 5 ml water and dried over anhydrous sodium sulphate. After decanting into another test-tube the solvent was evaporated under a stream of nitrogen. The lipid was redissolved immediately in a small volume of chloroform, and applied to a 20 x 20 cm T.L.C. plate coated with Kieselgel G containing 5% (w/w) boric acid (240). The plate was developed using hexane:diethyl ether (1:1 v/v) as solvent, and sprayed with 0.1% (w/v) rhodamine 6G in water. (Rhodamine 6G was used as the detecting spray since 2',7'-dichloro-

fluorescein interfered with subsequent T.L.C. separations.) When the plate was viewed under ultraviolet light, a band containing both 1,2- and 2,3-diacyl-sn-glycerols was visible as well as another containing 1,3-diacyl-sn-glycerols. Any unreacted triacylglycerols were present in a third band nearer the solvent front. The band containing the 1,2- and 2,3-diacyl-sn-glycerols was scraped off the plate into a small glass chromatography column and eluted with 60 ml diethyl ether. After removal of the solvent, the diacylglycerols were redissolved in 1 ml of dry diethyl ether. Pyridine (1 ml) and 0.2 ml of phenyl dichlorophosphate were then added to convert the diacylglycerols to phospholipids.

After 90 minutes at room temperature, the reaction mixture was cooled in an ice bath, 2 ml of pyridine was added and the reaction was finally stopped by the addition of 0.5 ml water. The mixture was transferred to a separating funnel with chloroform (30 ml), methanol (30 ml), water (25 ml) and a small volume (2 ml) of triethylamine. The lower layer was collected and the solvent removed under reduced pressure. Dry diethyl ether (3 ml), 0.5 ml of 0.5M tris buffer (pH 7.5 and 2mM with respect to calcium chloride), 2 mg of B.H.T. and 1 mg of snake venom (Ophiophagus hannah) were added to the phospholipids and the mixture was shaken overnight under an atmosphere of nitrogen. The ether was then evaporated under a stream of nitrogen, and to the tube were added 8 ml chloroform, 4 ml methanol and 2.5 ml water. After vigorous shaking, the tube was centrifuged at 1,000 g for 5 minutes and the aqueous layer aspirated and discarded; the organic layer was filtered and evaporated to dryness. The lipid was redissolved in 300 µl chloroform:methanol

(2:1 v/v) and applied to a 20 x 20 cm T.L.C. plate coated with Kieselgel G, which was developed with hexane:diethyl ether:formic acid (50:50:1 by volume). The top half only of the plate was sprayed with 2',7'-dichlorofluorescein solution in order that the band of free fatty acids liberated by phospholipase A might be detected. After this band had been scraped off, the plate was re-developed with chloroform:methanol:ammonia (90:8:2 by volume) as solvent. The developed plate was sprayed with rhodamine 6G solution to locate the two bands containing lysophosphatide and phospholipid, which were methylated and the fatty acid composition of each determined by G.L.C. after elution from the adsorbent.

For pancreatic lipase hydrolysis of triacylglycerols, 3 ml of 0.5M tris buffer (pH 8.0), 0.3 ml of 2.2% (w/v) calcium chloride and 0.75 ml of 0.05% (w/v) bile salt solution were added to 10 mg of triacylglycerols in a test-tube. The mixture was equilibrated for 5 minutes in a water bath at 37°C for 5 minutes before 10 mg of pancreatin was added and the tube shaken vigorously for 4 minutes in the water bath. The reaction was stopped by the addition of a few drops of ethanol, and a small volume of dilute hydrochloric acid was added to acidify the solution. The solution was then extracted twice with 5 ml diethyl ether, the combined ether extracts washed with water and dried over anhydrous sodium sulphate. The ether was then decanted into another test-tube, the solvent removed under a stream of nitrogen and the lipids applied in a small volume of chloroform to a T.L.C. plate coated with Kieselgel G in layers 0.5 mm thick. After development with hexane:diethyl ether:formic acid (60:40:1 by volume), the plate was sprayed with 2',7'-dichlorofluorescein solution and the

2-monoacyl-sn-glycerol band scraped off. The monoacylglycerol was recovered from the adsorbent before being methylated in preparation for G.L.C. analysis.

Stereospecific analyses of triacylglycerols containing ^{14}C-labelled fatty acids was carried out as detailed elsewhere (241,237) using an internal standard of tri-(10,11-^{3}H) heptadecanoin to enable the losses of radioactivity at each stage of the stereospecific procedure to be determined. The amount of ^{14}C-labelled fatty acid originally present in each position of the triacyl-sn-glycerol could thereby be determined by relating the activity of the labelled fatty acid to that of the internal standard in each of the products of the analysis.

2. Structural Analysis of Radioactively-Labelled Diacylglycerols

For the structural analysis of diacylglycerols formed by adipocytes, the diacylglycerols had to be extracted in a manner that minimised isomerisation. Accordingly, in this instance, the extraction solvent contained no methanol and low temperatures were maintained throughout the procedure. After incubation of adipocytes with the appropriate substrate, the contents of the incubation bottle were decanted into a small hand-homogeniser surrounded by ice. Ice-cold diethyl ether (2 ml) was added plus a small amount of cold carrier. The contents of the vessel were homogenised for 3 minutes and then transferred to a test-tube maintained on ice. The homogeniser was rinsed twice with 5 ml cold diethyl ether, each time with several strokes of the homogeniser rod, and the washings added to the extraction mixture. The organic and aqueous solvents were separated by centrifugation. The upper, ether layer was transferred to another

test-tube of known weight and the solvent removed at low temperature under a stream of nitrogen. The lipid extract was weighed before being redissolved in diethyl ether, and was used immediately for subsequent analyses.

In determining whether the diacylglycerols formed by fat-cells incubated with (1-^{14}C)acetate or (U-^{14}C)glucose were the sn 1,2-stereoisomers, use was made of the fact that the enzyme phospholipase A uses specifically 1,2-diacyl-sn-glycerophosphate as a substrate, as discussed previously. Therefore, 1,2-diacyl-sn-glycerophosphophenol synthesized from 1,2-diacyl-sn-glycerols can be hydrolysed by the enzyme to a lysophospholipid and free fatty acid, whereas phospholipids originating from 2,3-diacyl-sn-glycerols do not act as substrate for the enzyme. α,β- (potentially a mixture of sn-1,2 and 2,3) diacylglycerols extracted from adipocytes were converted by chemical synthesis to phospholipids and were subjected to phospholipase A hydrolysis. The amount of radioactivity recovered as lysophospholipid and free fatty acid relative to 2,3-diacyl-sn-glycerophosphophenol gave an indication of the proportions of 1,2- and 2,3-diacyl-sn-glycerols originally present.

Up to 40 mg of lipid obtained by ether extraction as described above was applied to a 20 x 20 cm T.L.C. plate coated with boric acid-impregnated silica gel, which was developed with hexane:diethyl ether (1:1 v/v). After spraying with rhodamine 6G solution, two bands containing diacylglycerols were visible. The band containing 1,3-diacyl-sn-glycerols was scraped off and the radioactivity of this fraction was measured after extraction from the adsorbent.

The other band containing 1,2- and 2,3-diacyl-sn-glycerols was

isolated, the diacylglycerols eluted and converted to phospholipids with phenyl dichlorophosphate as described previously for the stereospecific analysis procedure. The synthetic phospholipids were then treated with phospholipase A, and the products isolated as in the stereospecific analysis procedure for measurement of the radioactivity present in each.

3. Measurement of ^{14}C and ^{3}H Incorporation into Fatty Acid and Glycerol Moieties of Acylglycerols

When acylglycerols are methylated, the fatty acids are hydrolysed from the glycerol moiety during transesterification to yield fatty acid methyl esters and free glycerol. On extraction of the methylation mixture to which water has been added, with hexane and ether, the fatty acid methyl esters are extracted into the organic solvent whereas the glycerol remains in the aqueous layer.

To determine the amounts of radioactivity incorporated into the fatty acid and glycerol moieties of acylglycerols when (U-^{14}C)glucose, (1-^{14}C)acetate and $^{3}H_2O$ were used as labelled substrates, the following procedure was employed.

Triacylglycerols, diacylglycerols, phospholipids and unesterified fatty acids separated by T.L.C. were methylated as described previously. The organic solvent extract of the methylation mixture contained the labelled fatty acid methyl esters, the radioactivity of which could be measured directly after evaporation of the solvent in a glass scintillation vial.

A known volume (4 ml) of the aqueous layer of the methyl ester extraction mixture was shaken with Unisolve I in a glass scintillation vial for radioactivity determination. The volume of

the aqueous fraction remaining was determined by means of a small measuring cylinder to permit the amount of radioactivity present in the original total aqueous layer to be calculated. The radioactivity found in the aqueous layer on extraction of the methyl esters of the free fatty acid band was subtracted from the values obtained for triacylglycerols, diacylglycerols and phospholipids to correct for background radioactivity not due to the glycerol moiety.

For the purpose of these studies, all the radioactivity recovered in the aqueous fraction was assumed to be present in glycerol.

G. Measurement of Radioactivity

In preparation for the direct measurement of radioactivity in lipid classes separated by thin layer chromatography, the appropriate bands of silica gel adsorbent were scraped directly into glass scintillation vials and a suspension was formed by the addition of 4 ml water and 10 ml Unisolve I (Koch-Light Laboratories, Colnbrook, Bucks., U.K.). Similarly, for the measurement of radioactivity present in material dissolved in water, a 4 ml portion of the solution was placed in a glass scintillation vial and 10 ml of Unisolve I added to form an emulsion.

Material dissolved in organic solvents was transferred to a glass vial, the solvent evaporated under a stream of nitrogen and 15 ml of toluene-fluor added. The toluene-fluor used throughout these studies was 0.4% (w/v) Scintimix 2 (Koch-Light Laboratories) in scintillation-grade toluene.

The radioactivity of samples prepared by the above methods was

measured either in a Tri-Carb 24025 liquid scintillation spectrometer (Packard Instruments, Reading, Berks., U.K.) or an I.C.N. Corumat 2700 (I.C.N. Instruments, Surrey, U.K.) on line to a Wang 600 programmable calculator (Wang Electronics, Middlesex, U.K.).

Quenching within the sample was corrected for by the use of an external standard. ^{14}C was counted with an average efficiency of 75% in Unisolve I, and 90% in toluene-fluor. With ^{3}H, the counting efficiencies were approximately 55% in toluene-fluor and 35% in Unisolve I.

C.p.m.'s obtained by the measurement of samples in the Tri-Carb 24025 were converted to d.p.m.'s by comparison with a standard calibration curve. This calculation was carried out automatically by the Wang 600 calculator when samples were measured by the I.C.N. Corumat.

Recovery of radioactivity from T.L.C. plates was routinely determined and was never less than 95%.

H. Expression of Results

The expression of experimental results on the basis of adipocyte protein was not adopted since the fat-cells were prepared in medium containing a high concentration of albumin. Residual albumin did not, however, interfere with the measurement of adipocyte DNA and for this reason results are expressed relative to the amount of DNA.

A loss of several samples set aside for DNA analysis in the series of preliminary experiments necessitated the expression of these results relative to the weight of lipid present in the incubation.

Wherever possible, results are expressed as mean ± standard error of the mean (s.e.m.) with the number of observations reported in the legend to the relevant Figure or Table. Differences between means were tested for significance by the Student's t-test (242).

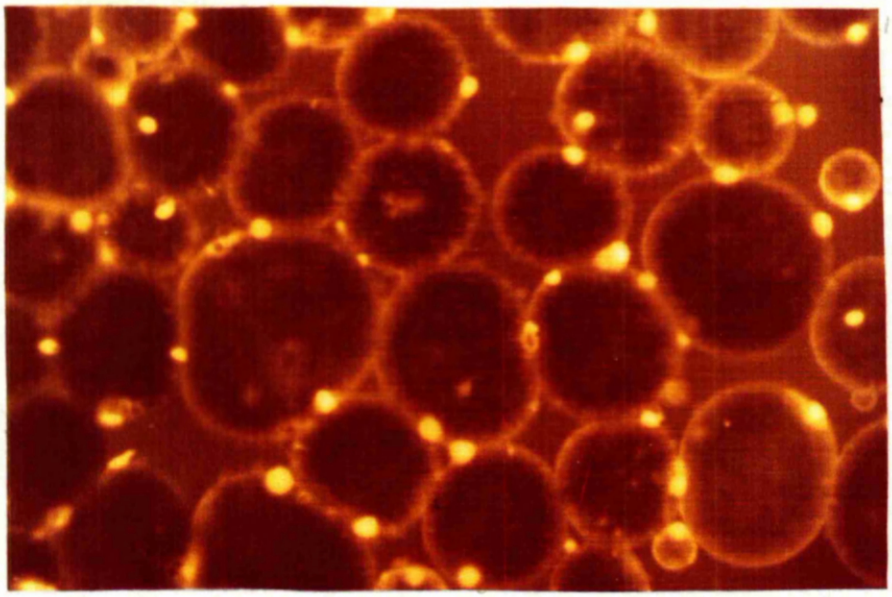


Fig. 3.1. Adipocytes, obtained from collagenase-treated parametrial adipose tissue from a 289 g rat and stained with acridene orange, at a magnification of 125x.

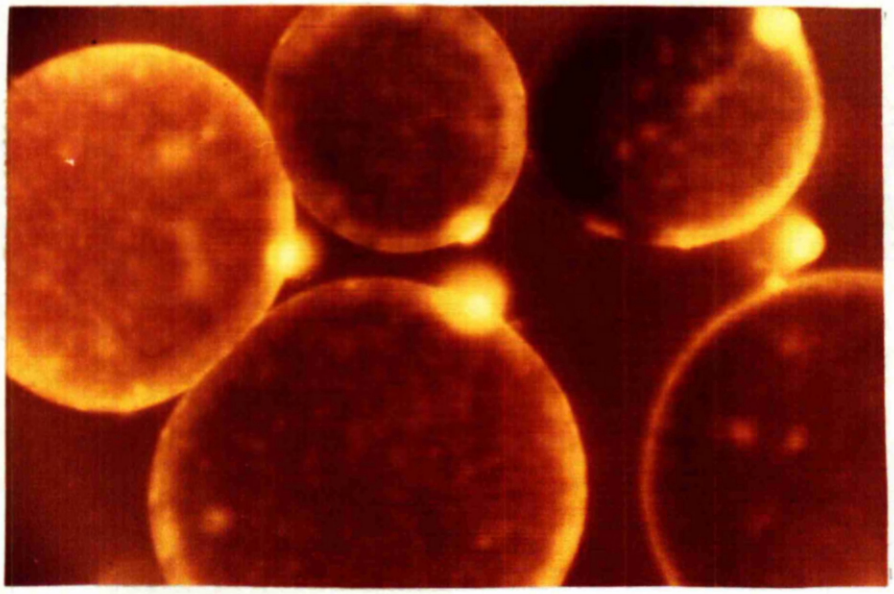


Fig. 3.2. Adipocytes prepared as described for Fig. 3.1 at a magnification of 320x.

CHAPTER 3. RESULTS

A. Examination of Adipocytes

Viable preparations of adipocytes were consistently obtained by incubation of rat parametrial adipose tissue with collagenase. On the few occasions when the preparation was not successful a visible layer of lipid on top of the fat-cell suspension was an indication of cell lysis during isolation. Adipocytes were stained with acridene orange and examined under a fluorescence microscope to assess their integrity and viability. With lysed cells, large lipid droplets were found which bore no resemblance to intact adipocytes when viewed under the microscope.

An adipocyte appeared as a spherical cell with a brightly stained nucleus, usually located at the periphery (Fig. 3.1). Apart from the nucleus, no other identifiable organelles were visible at the magnifications used, although at a magnification of 320x small patches of intensely-stained material could be seen (Fig. 3.2). These were not recognisable as specific organelles.

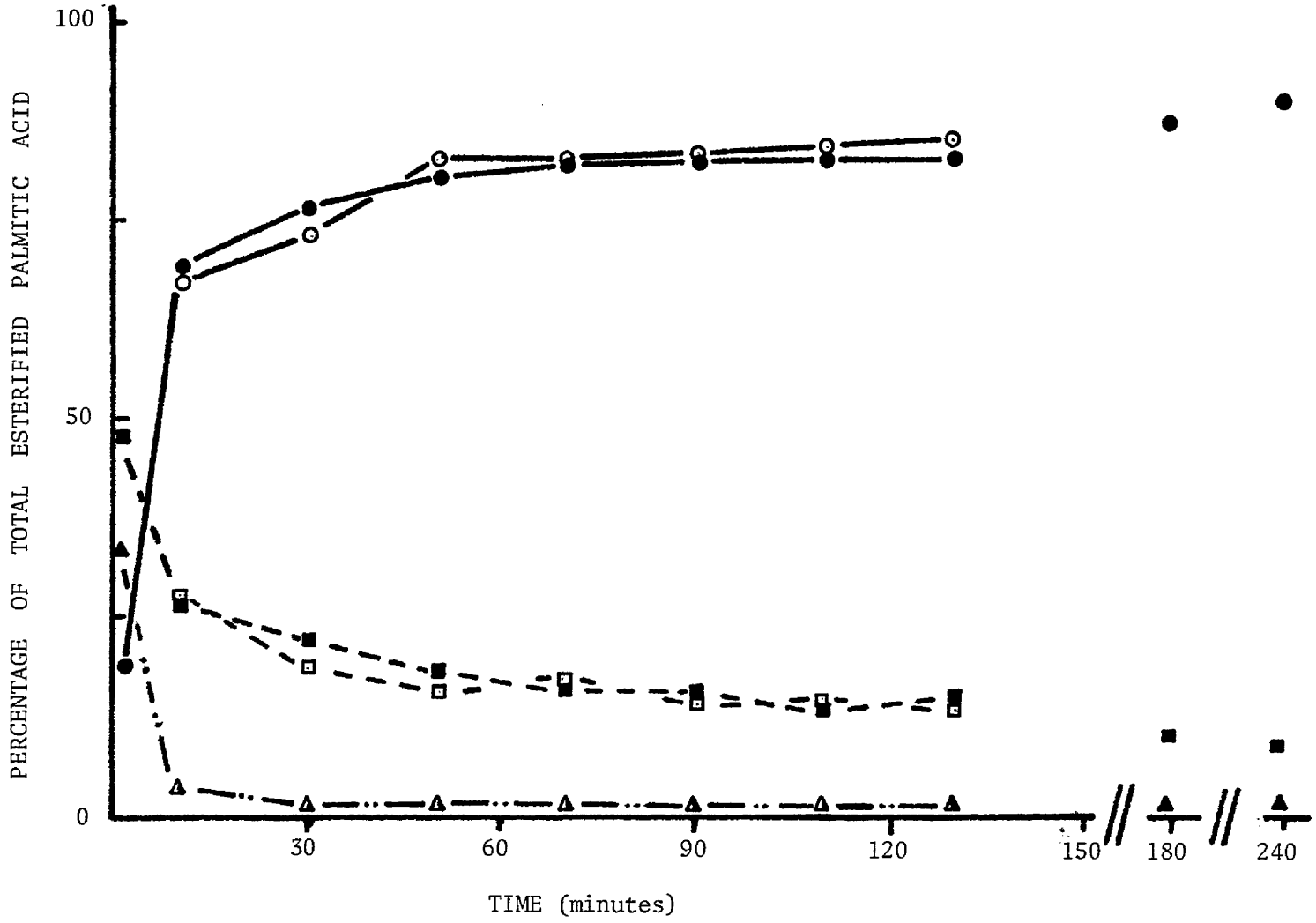
Although insufficient adipocytes were measured for a reliable value for fat-cell diameter to be obtained, 90 μ was found to be the mean diameter of 25 adipocytes originating from a rat of weight 289 g. When the diameters of 30 adipocytes isolated from a younger rat weighing 129 g were measured, the mean value was found to be 40 μ. The sizes of adipocytes were therefore apparently related to the age and weight of the animals from which they were isolated.

Adipocytes examined microscopically after a two-hour incubation at 37°C in the basic incubation medium, were found to be still intact, suggesting that isolated fat-cells remained viable throughout

Fig. 3.3. Distribution of Esterified (1-^{14}C)Palmitic Acid in Acylglycerols of Adipocytes.

(1-^{14}C)Palmitic acid (0.3 μCi) was included in the basic incubation medium either on its own at a concentration of 1mM, or as part of a fatty acid mixture of total concentration 1mM. Values are means of three experiments; s.e.m.'s were always less than 2%. Open symbols (○, □, △) refer to (1-^{14}C)palmitic acid supplied alone, shaded symbols (●, ■, ▲) to (1-^{14}C)palmitic acid supplied as part of a fatty acid mixture. ▲ was used where △ and ▲ points were too close together to distinguish.

○——○, ●——●, triacylglycerols; □— —□, ■— —■, diacylglycerols; △ — .. —▲, phospholipids.


100
50
0
PERCENTAGE OF TOTAL ESTERIFIED PALMITIC ACID
30
60
90
120
150
180
240
TIME (minutes)

incubation periods of at least 2 hours. Attempts to prepare viable fat-cells from sheep omental adipose tissue proved unsuccessful due to 'clumping' of the adipocytes during the isolation procedure.

B. Esterification of (1-^{14}C)Fatty Acids into Acylglycerols

To determine whether isolated adipocytes were capable of carrying out the metabolic activities of intact adipose tissue, the ability of the fat-cells to take up and esterify preformed long-chain fatty acids into acylglycerols was studied. Such studies were also intended to provide information on the manner in which fatty acids originating outwith the adipocyte were distributed among the three positions of triacyl-sn-glycerols when esterified.

The system used for the incubation of adipocytes with albumin-bound (1-^{14}C)fatty acids was described fully in Chapter 2, section C2.

1. Distribution of Esterified (1-^{14}C)Fatty Acids among Acylglycerols

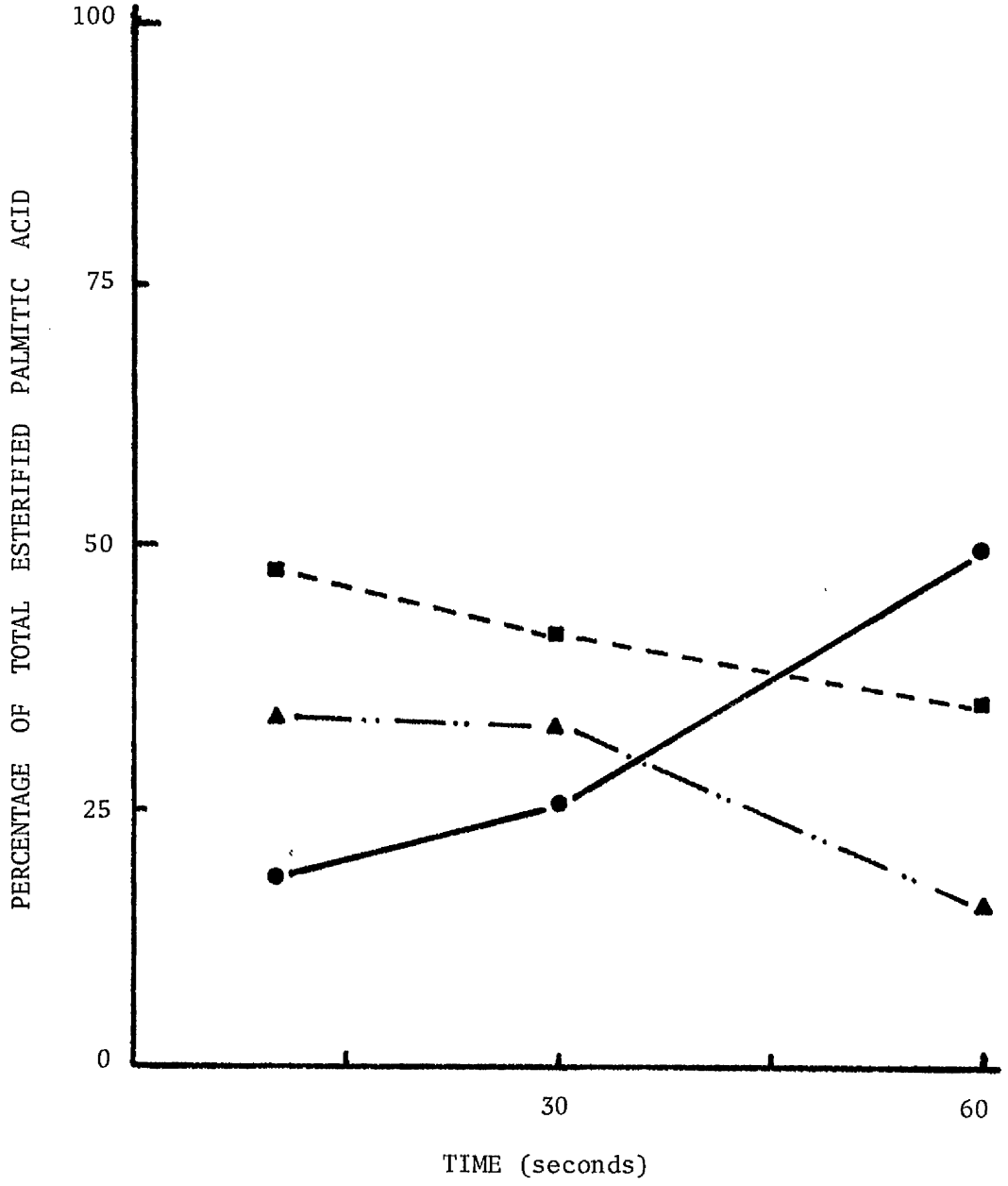
The ^{14}C-labelled fatty acids were readily esterified into acylglycerols by adipocytes prepared from rats of average weight 280 g when supplied either as the sole fatty acid in the incubation medium or as part of a fatty acid mixture designed to simulate unesterified fatty acids supplied under normal physiological conditions. After 90 minutes, less than 2.5% of the radioactivity from any (1-^{14}C)fatty acid substrate was recovered in the free fatty acid fraction.

The proportional distribution of esterified (1-^{14}C)palmitic acid among triacylglycerols, diacylglycerols and phospholipids with respect to time is presented in Fig. 3.3. Although only the results obtained with (1-^{14}C)palmitic acid are shown, similar results were obtained

Fig. 3.4. Distribution of Esterified (1-^{14}C)Palmitic Acid in Acylglycerols of Adipocytes after Short Incubation Periods.

(1-^{14}C)Palmitic acid (1 μCi) was included in the basic incubation medium as a component of a fatty acid mixture (1mM). Values are means of two experiments that agreed closely.

●——●, triacylglycerols; ■ – –■, diacylglycerols; ▲ – ·· –▲, phospholipids.


100
75
50
25
0
PERCENTAGE OF TOTAL ESTERIFIED PALMITIC ACID
30
60
TIME (seconds)

with (1-^{14}C)stearic, oleic and linoleic acids. The incorporation into monoacylglycerols was also measured but was negligible in relation to that into other acylglycerols and is not reported here.

The addition of the ^{14}C-labelled fatty acid to the basic incubation medium either alone or as a component of a fatty acid mixture had no apparent effect on its distribution among triacylglycerols, diacylglycerols and phospholipids. For (1-^{14}C)palmitic acid, this was also true in terms of absolute amounts esterified as will be discussed later. In Fig. 3.3, at 90 minutes, 100% is equivalent to 1.972 μmoles for (1-^{14}C)palmitic acid supplied alone and to 1.977 μmoles for (1-^{14}C) palmitic acid incubated as part of a fatty acid mixture.

With incubation times longer than 1 minute the largest proportion of esterified (1-^{14}C)fatty acid was recovered in the triacylglycerol fraction. From its initial high value, the percentage of ^{14}C-labelled fatty acid recovered in diacylglycerols decreased rapidly during the first 50 minutes of incubation but thereafter only gradually. Even after 4 hours, 8.5% of the total esterified (1-^{14}C)palmitic acid was found in diacylglycerols. Phospholipids accounted for nearly 4% of the total esterified (1-^{14}C)fatty acid substrate after 10 minutes' incubation, but at the end of 60 minutes only 1% was recovered as this component.

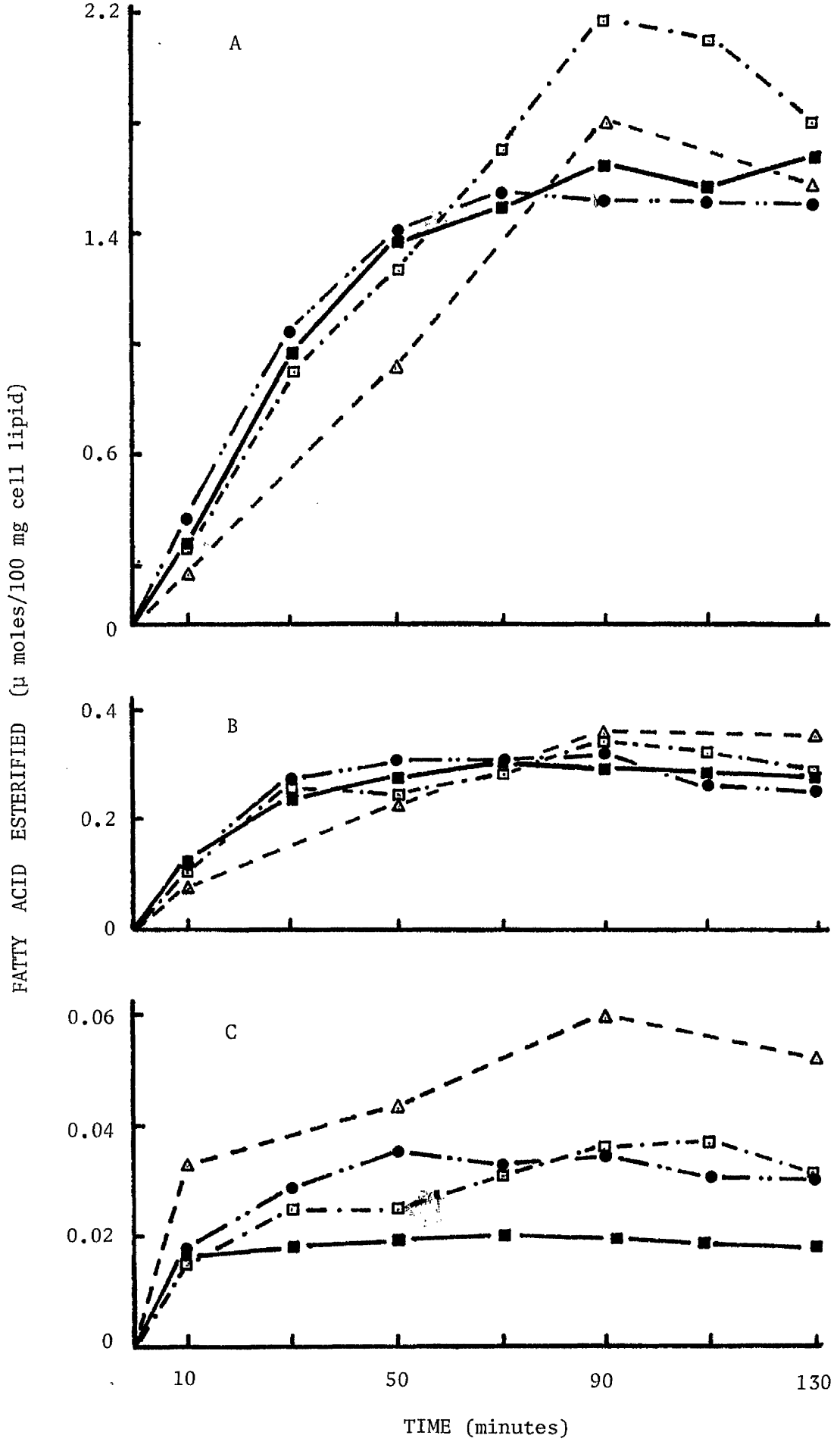
When the esterification of (1-^{14}C)palmitic acid supplied as part of a fatty acid mixture (see Chapter 2, section C2) was studied for short incubation times, differences in its distribution among the various acylglycerols were noticeable (Fig. 3.4). After 15 seconds' incubation, the highest proportion of esterified (1-^{14}C)fatty acid was recovered in diacylglycerols, with triacylglycerols containing less than phospholipids. During the next 45 seconds, the percentage of the

Fig. 3.5. Esterification of (1-^{14}C)Fatty Acids Supplied Alone into Acylglycerols by Adipocytes.

A. Triacylglycerols B. Diacylglycerols C. Phospholipids.

The (1-^{14}C)fatty acid (0.3 μCi) was included in the basic incubation medium on its own at a concentration of 1mM. Results are means of three experiments, and the s.e.m. for each value was always less than 15% of the mean.

■ —— ■, palmitic acid; □ — · — · — □, stearic acid; ● — ·· — ●, oleic acid; Δ — — Δ, linoleic acid.


A
B
C
2.2
1.4
0.6
0
0.4
0.2
0
0.06
0.04
0.02
0
10
50
90
130
TIME (minutes)
FATTY ACID ESTERIFIED (μ moles/100 mg cell lipid)

(1-^{14}C)palmitic acid recovered in diacylglycerols decreased whilst that found in triacylglycerols increased to make it the most highly labelled acylglycerol.

2. Rates of Esterification of (1-^{14}C)Fatty Acids into Acylglycerols

Because of the loss of some samples intended for DNA analysis, the results of these experiments are expressed as μmoles/100 mg cell lipid. Although the weight of lipid present in adipocytes varies with the size of the cell and does not reflect cell number, good agreement between results obtained with different rats was found even when the results were expressed in the above manner. Other workers have frequently expressed their results in terms of cell lipid (243), triacylglycerol content (223,244) and tissue weight (113,245) in studies with adipocytes or adipose tissue. To allow comparison with later experiments, 100 mg of cell lipid from adipocytes isolated from animals of approximate weight 280 g, was found to correspond to about 7.4 μg DNA.

Fig. 3.5 A, B and C illustrates the amounts of different (1-^{14}C)-fatty acids incorporated into triacylglycerols, diacylglycerols and phospholipids respectively by adipocytes isolated from 280 g rats when the (1-^{14}C)fatty acid was the sole fatty acid added to the incubation.

There were no significant differences between fatty acids in their rates of esterification into triacyl- and diacylglycerols. (1-^{14}C)-Linoleic acid was apparently esterified into phospholipids to a greater extent than the other (1-^{14}C)fatty acid substrates while (1-^{14}C)palmitic acid was incorporated into phospholipids in the lowest proportion. There was no significant difference between fatty acids in the amount incorporated into total acylglycerols in 130 minutes.

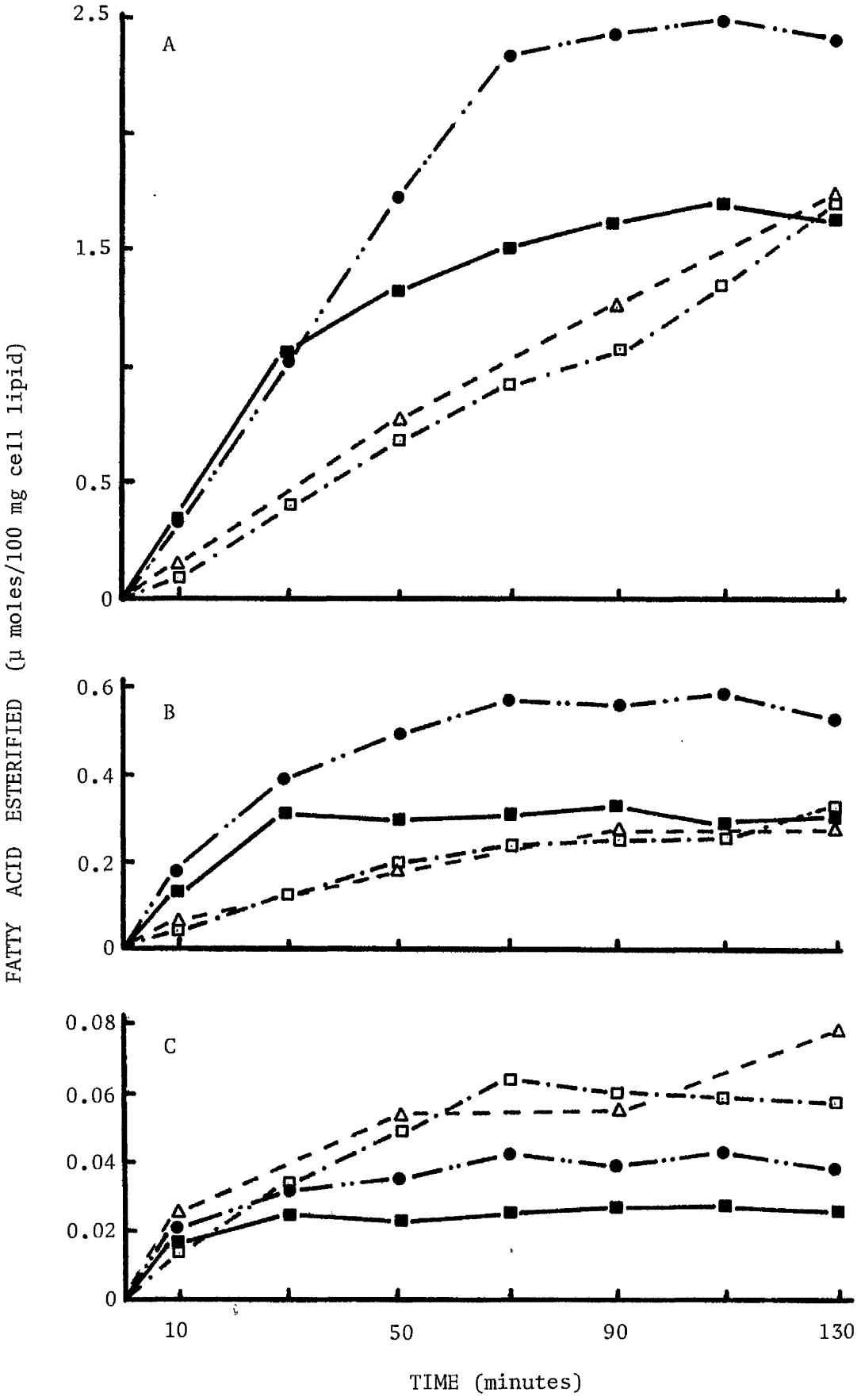
When the (1-^{14}C)fatty acid substrate was supplied as part of the

Fig. 3.6. Esterification of (1-^{14}C)Fatty Acids, Supplied as Part of a Fatty Acid Mixture, into Acylglycerols by Adipocytes.

A. Triacylglycerols B. Diacylglycerols C. Phospholipids.

The (1-^{14}C)fatty acid (0.3 μCi) was included in the basic incubation medium as a component of a fatty acid mixture of total concentration 1mM. Results are means of three experiments and the s.e.m. was always less than 15% of the mean value.

■ —— ■, palmitic acid; □ — · — · □, stearic acid; ● — ·· — ●, oleic acid; Δ — — Δ, linoleic acid.


2.5
1.5
0.5
0
A
0.6
0.4
0.2
0
B
0.08
0.06
0.04
0.02
0
C
10
50
90
130
TIME (minutes)
FATTY ACID ESTERIFIED (μ moles/100 mg cell lipid)

fatty acid mixture, there were notable differences in the rates of incorporation of the different (1-^{14}C)fatty acids into acylglycerols. The results obtained in this series of experiments were corrected so that the values presented in Fig. 3.6 represent the situation if the (1-^{14}C)fatty acid component comprised 100% of the mixture. This correction allowed the esterification of ^{14}C-labelled fatty acids supplied as a mixture to be compared with esterification of the same (1-^{14}C)fatty acids supplied to the adipocytes on their own.

When (1-^{14}C)oleic acid was included as a component of a fatty acid mixture rather than alone in the incubation medium, significantly increased incorporation in 130 minutes into triacylglycerols ($P < 0.002$) and diacylglycerols ($P < 0.01$) was obtained

The amounts of (1-^{14}C)stearic, oleic and linoleic acids esterified into total acylglycerols in 130 minutes were not affected significantly when they were incubated in mixture form. However, the esterification of (1-^{14}C)stearic acid over 50 minutes into triacylglycerols was significantly less ($P < 0.05$) when supplied in a mixture than when incubated on its own. Its esterification into diacylglycerols over the same time was not significantly different.

Whereas the four (1-^{14}C)fatty acids investigated were esterified into triacylglycerols at similar rates when incubated individually, there were obvious differences in the rates of esterification of the same fatty acids supplied as components of a mixture. The amount of (1-^{14}C)oleic acid incorporated in 90 minutes into triacylglycerols was significantly greater than that of (1-^{14}C)palmitic acid ($P < 0.02$), (1-^{14}C)linoleic acid ($P < 0.001$) and (1-^{14}C)stearic acid ($P < 0.001$). (1-^{14}C)Stearic and linoleic acids were incorporated into triacylglycerols

at lower rates than (1-^{14}C)palmitic and oleic acids. Similarly, (1-^{14}C)oleic acid was esterified into diacylglycerols at a significantly higher rate than (1-^{14}C)palmitic acid ($P < 0.002$), (1-^{14}C)stearic acid ($P < 0.01$) and (1-^{14}C)linoleic acid ($P < 0.001$). Again, (1-^{14}C)-stearic and linoleic acids had lower esterification rates than (1-^{14}C)-oleic and palmitic acids.

Although the amounts of (1-^{14}C)oleic acid recovered in triacyl- and diacylglycerols were greater than those of other (1-^{14}C)fatty acids when incubated as part of a mixture, it was incorporated into phospholipids at a lower rate than (1-^{14}C)stearic acid or (1-^{14}C)linoleic acid. The high rate of (1-^{14}C)oleic acid esterification into triacyl- and diacylglycerols therefore coincided with a low rate of esterification into phospholipids. Conversely, although (1-^{14}C)stearate and (1-^{14}C)-linoleate had low rates of incorporation into triacyl- and diacylglycerols they were quickly esterified into phospholipids in comparison with (1-^{14}C)oleic and palmitic acids. The net result was that there was no significant difference between the rates of esterification of the four (1-^{14}C)fatty acids into total acylglycerols.

These results showed that adipocytes were capable of taking up a number of different long-chain fatty acids from the incubation medium and esterifying them into acylglycerols at comparable rates. The entry of such fatty acids into the fat-cell was also thereby demonstrated not to be a rate-limiting step in their esterification into acylglycerols.

C. Esterification of (1-^{14}C)Oleic Acid formed by Desaturation of (1-^{14}C)Stearic Acid

Desaturation of (1-^{14}C)stearic acid to (1-^{14}C)oleic acid by

Fig. 3.7. Esterification of (1-^{14}C)Oleic Acid Formed by Desaturation of (1-^{14}C)Stearic Acid into Acylglycerols. A. (1-^{14}C)Stearic acid supplied alone B. (1-^{14}C)-Stearic acid supplied as a component of a fatty acid mixture

The incubation conditions for A and B were similar to those described for Figs. 3.5 and 3.6 respectively. Results are means ± s.e.m. of three experiments. Only s.e.m.'s distinguishable from the symbols are presented in this, and subsequent Figures.

●——●, triacylglycerols; □— —□, diacylglycerols; Δ— ·· —Δ, phospholipids; o— — —o, unesterified fatty acids.

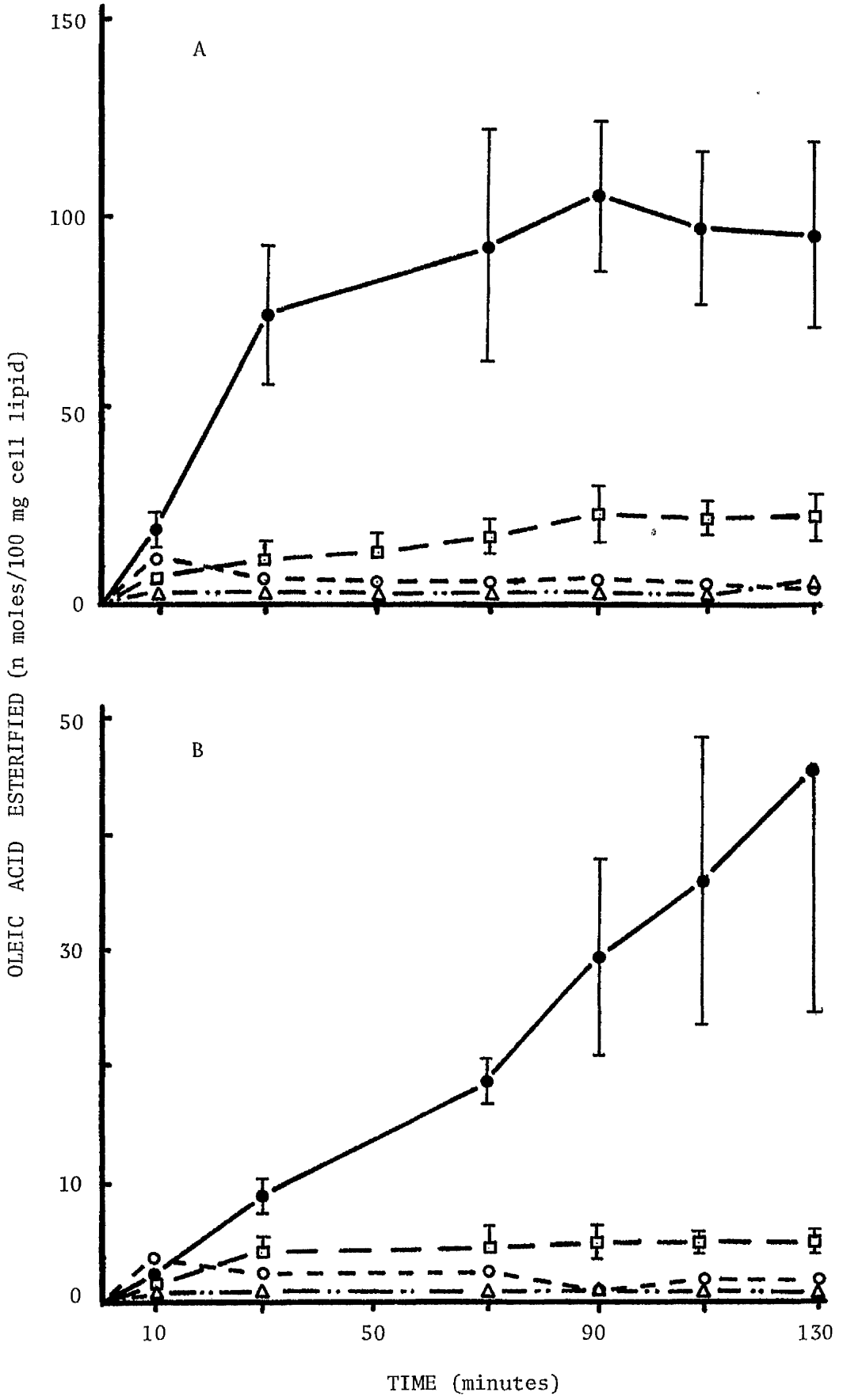

A
B
150
100
50
0
50
30
10
0
10
50
90
130
OLEIC ACID ESTERIFIED (n moles/100 mg cell lipid)
TIME (minutes)

Table 3.1. (1-^{14}C)Oleic Acid Derived from (1-^{14}C)Stearic Acid as a Proportion (%) of Total (1-^{14}C)Fatty Acid in Lipids

	(1-^{14}C)stearic acid supplied	
	alone	in fatty acid mixture
Triacylglycerols	7.0 ± 0.3	23.6 ± 0.9
Diacylglycerols	5.6 ± 0.5	18.8 ± 0.6
Phospholipids	6.1 ± 0.3	5.6 ± 0.3
Unesterified fatty acids	1.6 ± 0.4	3.4 ± 0.7

Incubation conditions were as described in the legend to Fig. 3.7. Results are means ± s.e.m. of 10, 30, 70, 90, 110 and 130 minute incubations from three experiments i.e. the mean of 18 values.

adipocytes prepared from 280 g rats occurred with (1-^{14}C)stearic acid incubated on its own and as part of the fatty acid mixture. (1-^{14}C)-Stearic acid and the (1-^{14}C)oleic acid derived from it were separated by argentation T.L.C. of the methyl ester derivatives of fatty acids prepared from the separated lipid classes. The total amount of (1-^{14}C)oleic acid produced in both cases increased steadily with time, but the desaturation rate was lower when the (1-^{14}C)stearic acid substrate was a component of the fatty acid mixture.

(1-^{14}C)Oleic acid formed from (1-^{14}C)stearic acid supplied as part of the fatty acid mixture was incorporated into triacylglycerols linearly with respect to time (Fig. 3.7). The amounts of (1-^{14}C)oleic acid recovered in diacylglycerols and phospholipids increased only slightly after 30 minutes, whereas that recovered as unesterified fatty acid actually decreased with increasing time.

At all times studied, triacylglycerols contained most of the (1-^{14}C)oleic acid formed by the desaturation of (1-^{14}C)stearic acid incubated on its own. Again, the amounts recovered in diacylglycerols and phospholipids increased slightly with time, and that found in the unesterified fatty acid fraction remained relatively constant after 30 minutes.

(1-^{14}C)Oleic acid formed as a result of desaturation remained a fairly constant proportion of the total ^{14}C-labelled fatty acid esterified in each of the acylglycerols with respect to time (Table 3.1). Triacylglycerols contained the highest ratio of (1-^{14}C)oleic acid to (1-^{14}C)stearic acid, and unesterified fatty acid the lowest.

These results demonstrate that oleic acid formed in the adipocyte by desaturation of extracellular stearic acid, is rapidly esterified into acylglycerols.

Table 3.2. Stereospecific Analysis of Triacylglycerols from Rat Adipocytes. Fatty Acid Composition (mole %) of Total Triacyl-sn-glycerols and Positions sn-1, 2 and 3.

Fatty acid	I Total triacyl-sn-glycerols	III Position sn-1	II Position sn-2 'lipase'	IV Position sn-2	V Position sn-(2 + 3)	3I - (II + III) Position sn-3	2V - II Position sn-3
16:0	30.8 ± 0.6	49.8 ± 1.3	10.8 ± 0.7	14.1 ± 1.3	19.5 ± 0.8	31.6 ± 2.9	28.2 ± 2.2
16:1	5.9 ± 0.3	5.4 ± 0.2	5.3 ± 0.3	5.6 ± 0.4	6.5 ± 0.5	7.1 ± 0.5	7.7 ± 0.8
18:0	3.7 ± 0.1	5.2 ± 0.1	1.5 ± 0.4	1.6 ± 0.2	2.0 ± 0.0	4.2 ± 0.8	2.6 ± 0.4
18:1	35.8 ± 0.2	26.9 ± 0.7	41.7 ± 1.0	40.5 ± 0.7	41.2 ± 0.8	38.6 ± 2.1	40.6 ± 2.5
18:2	22.3 ± 0.5	10.9 ± 0.4	39.4 ± 0.4	37.1 ± 0.5	28.9 ± 0.6	16.8 ± 1.3	18.4 ± 0.8
18:3	1.5 ± 0.1	1.8 ± 0.2	1.3 ± 0.1	1.1 ± 0.0	1.9 ± 0.1	1.7 ± 0.2	2.5 ± 0.2

Numerals I, II, III, IV and V refer to the products of the stereospecific analysis procedure as illustrated in Fig. 2.1. Values are means ± s.e.m. of results obtained with adipocytes from three different rats.

D. Stereospecific Analysis of Triacylglycerols

1. Natural Distribution of Fatty Acids

Before any meaningful interpretation could be made of the positional distribution of (1-^{14}C)fatty acids esterified into triacylglycerols from the incubation medium, it was first necessary to determine the natural long-chain fatty acid composition of rat adipocyte triacylglycerols and the manner in which the fatty acids are distributed within the three positions of the triacyl-sn-glycerol molecule.

Triacylglycerols extracted from non-incubated adipocytes isolated from rats of approximate weight 295 g, were subjected to stereospecific analysis. The results are presented in Table 3.2.

The shorthand nomenclature used for fatty acids in Tables throughout this study is that generally accepted, in which the number of carbon atoms and double bonds in the fatty acid are represented by the relevant numbers separated by a colon e.g. 16:0 represents the saturated, sixteen-carbon palmitic acid and 18:2 linoleic acid with its two double bonds (246).

The fatty acid composition of position sn-2 was obtained by G.L.C. analysis of the methyl esters of the monoacylglycerol produced by pancreatic lipase hydrolysis (product II in Fig. 2.1) and confirmed by analysis of the methyl esters of the free fatty acids (IV) produced during phospholipase A hydrolysis of the phospholipids synthesized from the 1,2-diacyl-sn-glycerols. G.L.C. analysis of the fatty acid methyl esters of the lysophosphatide (III) produced in the phospholipase A hydrolysis gave the fatty acid composition of position sn-1. The subtraction for each fatty acid of the sums of positions sn-1 and sn-2 from three times the total triacylglycerol allowed the fatty acid

composition of position sn-3 to be calculated. G.L.C. analysis of the methyl esters of the phospholipid (V) synthesized from 2,3-diacyl-sn-glycerols gave the total fatty acid composition of positions sn-2 and sn-3 from which the composition of position sn-3 could be obtained by subtracting that of position sn-2. The fatty acid composition of position sn-3 could therefore be obtained by two methods. The accuracy of each analysis was considered acceptable only when the results obtained for position sn-2 and position sn-3 calculated by alternative procedures, agreed within 4%.

Oleic acid was the major fatty acid present in rat adipocyte triacylglycerols, accounting for 35.8% of the total fatty acids examined. 30.8% of the fatty acids was palmitic acid. Linoleic acid also represented a considerable proportion (22.3%) of the total fatty acids whereas palmitoleic, stearic and linolenic acids together accounted for less than 12% of the fatty acids.

In position sn-1, the saturated fatty acid, palmitic acid, was predominant (49.8%), with oleic acid and linoleic acid accounting for 26.9% and 10.9% respectively of the remaining fatty acids. However, position sn-2 contained a high proportion of unsaturated fatty acids as monoenoic oleic acid and dienoic linoleic acid together made up approximately 80% of the fatty acids present. The two saturated fatty acids, palmitate and stearate, together only constituted about 15% of the fatty acids in this instance. In position sn-3, oleic acid was again the main fatty acid (approximately 40%), with palmitic acid accounting for 30%. 17.5% and 3.5% of the fatty acids in that position were linoleic and stearic acids respectively. Comparable results have been obtained by others (197).

The proportional distribution of each fatty acid in the three

Table 3.3. Proportional (%) Distribution of Fatty Acids in Positions sn-1, 2 and 3 of Triacyl-sn-glycerols from Adipocytes.

Fatty Acid	Position sn-1	Position sn-2	Position sn-3
16:0	54.1 ± 1.9	11.7 ± 0.8	34.2 ± 2.7
16:1	30.3 ± 0.8	29.6 ± 0.8	40.1 ± 1.5
18:0	47.9 ± 2.6	13.8 ± 3.6	38.3 ± 3.1
18:1	25.1 ± 0.7	38.9 ± 1.0	36.0 ± 1.8
18:2	16.2 ± 0.8	58.8 ± 0.7	25.0 ± 1.4
18:3	37.6 ± 3.5	27.8 ± 1.3	34.6 ± 2.3

Values are means ± s.e.m. of results obtained with adipocytes from three different rats.

Table 3.4. Relative Incorporation of (1-^{14}C)Fatty Acids into the Three Positions of Triacyl-sn-glycerols by Adipocytes.

Fatty Acid	Proportional (%) distribution in each position		
	Position sn-1	Position sn-2	Position sn-3
16:0	53.0 ± 1.4	13.7 ± 0.3	33.3 ± 1.2
18:0	(48.3 ± 0.9	6.7 ± 0.2	45.0 ± 0.8[a]
	(51.7 ± 0.7	3.6 ± 0.1	44.7 ± 0.6[b]
18:1	(22.4 ± 0.5	45.2 ± 0.5	32.4 ± 0.5[c]
	(21.5 ± 0.9	33.8 ± 0.8	44.7 ± 1.4[d]
18:2	21.9 ± 1.5	56.7 ± 1.2	21.4 ± 2.7

Incubation conditions were similar to those described in the legend to Fig. 3.6 except that the (1-^{14}C)fatty acid contained 0.6 μCi of radioactivity. Duplicate samples were analysed and results are means ± s.e.m. of 50, 90 and 130 minute incubations i.e. the mean of 3 values.

a. total stearic acid esterified
b. stearic acid corrected for that desaturated
c. oleic acid itself added
d. oleic acid formed from stearic acid.

positions of the triacyl-sn-glycerols was calculated from these results. The values obtained are presented in Table 3.3.

More than half the total palmitic acid of the triacylglycerols was found esterified naturally in position sn-1 with only 11.7% in position sn-2 and 34.2% in position sn-3. 40.1% of palmitoleic acid was found in position sn-3 with the remainder almost equally divided between positions sn-1 and sn-2. Stearic acid was found predominantly in position sn-1 with 38.3% in position sn-3 and only 13.8% in position sn-2. The natural distribution of linoleic acid was almost the converse of that of palmitic and stearic acids, in that most of it was located in position sn-2 with positions sn-1 and sn-2 containing only 16.2% and 25.0% respectively. The mono-unsaturated fatty acid, oleic acid, was also present in greatest proportion in position sn-2 although position sn-3 contained only 2.9% less, with the remaining 25.1% being located in position sn-1. Linolenic acid was distributed almost equally between the primary positions with 27.8% present in position sn-2.

2. Distribution of (1-^{14}C)Fatty Acids Esterified from the Incubation Medium

The stereospecific distribution of (1-^{14}C)fatty acids incorporated from the incubation medium into triacylglycerols by fat-cells isolated from rats of average weight 280 g was examined using tri (10,11-^{3}H)-heptadecanoin as internal standard during the analysis procedure (241).

Very similar results were obtained for the relative proportion of each (1-^{14}C)fatty acid esterified in the three positions after incubation periods of 50, 90 and 130 minutes, and for this reason the values presented in Table 3.4 represent the mean of duplicate samples from each of these times taken together.

Table 3.5. Relative Incorporation of (1-^{14}C)Fatty Acids into the Three Positions of Triacyl-sn-glycerols by Adipocytes in 10 Minutes.

	Proportional (%) distribution in each position		
Fatty Acid	Position sn-1	Position sn-2	Position sn-3
16:0	73.0	7.4	19.6
18:0	53.0	10.7	36.3
18:1	23.2	43.2	33.6
	19.0	41.1	39.9[a]
18:2	24.9	65.7	9.4

Incubation conditions were as described in the legend to Table 3.4. Values are means of duplicate incubations.

a. oleic acid formed from stearic acid.

With the exception of (1-^{14}C)palmitic acid, the distribution of esterified ^{14}C-labelled extracellular fatty acids did not resemble closely their natural distribution in rat adipocyte triacylglycerols. (1-^{14}C)Stearic acid (including that desaturated) was incorporated into position sn-1 in similar proportion to its natural distribution but considerably more was found in position sn-3 and less in position sn-2. A similar distribution pattern was found for (1-^{14}C)stearic acid corrected for the amount desaturated to (1-^{14}C)oleic acid.

A higher proportion of (1-^{14}C)oleic acid esterified from the incubation medium was recovered in position sn-2 than was found naturally; positions sn-1 and sn-3 each contained less than the normal relative proportions. Position sn-3 contained a high proportion of (1-^{14}C)oleic acid formed by the desaturation of (1-^{14}C)stearic acid. Correspondingly, less of this (1-^{14}C)oleic acid entered positions sn-1 and sn-2 than was found for the natural distribution of oleic acid. The proportion of (1-^{14}C)oleic acid formed by desaturation of (1-^{14}C)-stearic acid recovered in position sn-3 was significantly higher ($P < 0.002$) than that of (1-^{14}C)oleic acid incubated as such. A similar proportion of (1-^{14}C)linoleic acid was recovered in position sn-2 to that found naturally but more of the ^{14}C-labelled fatty acid entered position sn-1 and less position sn-3.

The results obtained for the positional distribution of (1-^{14}C)-fatty acids in adipocyte triacylglycerols after 10 minutes' incubation differed from those found for longer incubation times and are presented separately in Table 3.5.

73% of the (1-^{14}C)palmitic acid esterified into triacylglycerols in 10 minutes was recovered in position sn-1 with 7.4% and 19.6%

Table 3.6. Relative Incorporation of (1-^{14}C)Palmitic Acid, Supplied Alone, into the Three Positions of Triacyl-sn-glycerols by Adipocytes.

Incubation time (mins)	Proportional (%) distribution in each position		
	Position sn-1	Position sn-2	Position sn-3
50	38.9 ± 1.9	29.6 ± 0.9	31.5 ± 1.8
90	37.6 ± 1.9	30.3 ± 0.7	32.1 ± 1.4

(1-^{14}C)Palmitic acid at a concentration of 1mM was included in the basic incubation medium. Duplicate samples were analysed and results are means ± s.e.m. of four independent experiments.

in positions sn-2 and sn-3 respectively. The highest proportion of esterified (1-^{14}C)stearic acid was found in position sn-1 with position sn-2 containing only 10.7% of the incorporated fatty acid. 43.2% of esterified (1-^{14}C)oleic acid incubated as such, was recovered in position sn-2 whereas (1-^{14}C)oleic acid formed within the adipocyte by desaturation of extracellular (1-^{14}C)stearic acid was distributed almost evenly between positions sn-2 and sn-3 with only 19% in position sn-1. (1-^{14}C)Linoleic acid entered position sn-2 preferentially (65.7%).

3. Distribution of (1-^{14}C)Palmitic Acid Supplied Alone

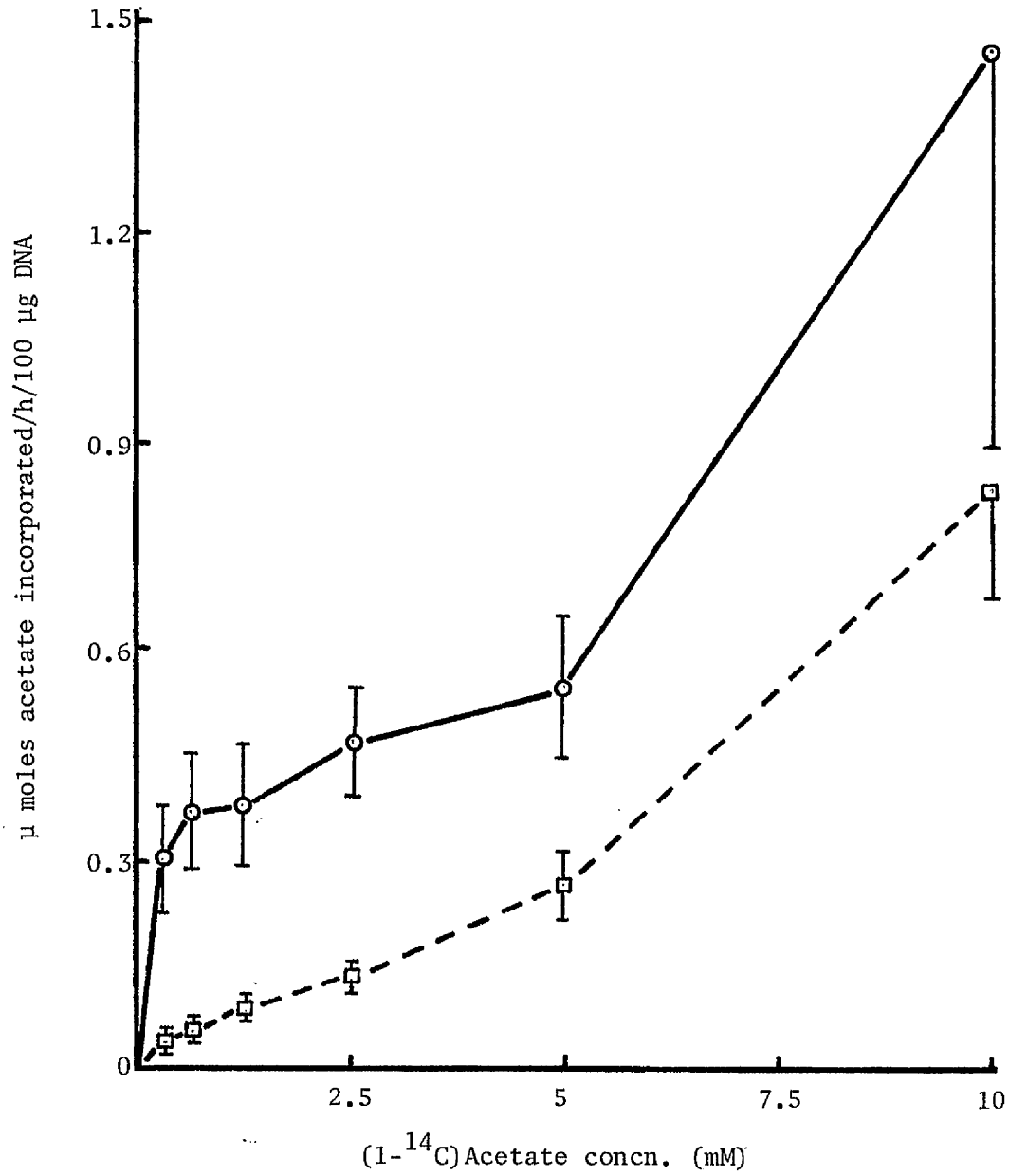
In the foregoing studies on the rates of incorporation of extracellular fatty acids into adipocyte triacylglycerols, control incubations consisted of fat-cells incubated with (1-^{14}C)palmitic acid alone. Triacylglycerols from these were subjected to stereospecific analysis to determine the positional distribution of the esterified (1-^{14}C)palmitic acid for comparison with the results obtained when (1-^{14}C)palmitic acid was supplied as part of a mixture.

The distribution in triacylglycerols of (1-^{14}C)palmitic acid supplied alone differed from both that of (1-^{14}C)palmitic acid incubated as part of a mixture and of the natural distribution of palmitic acid in rat adipocyte triacylglycerols. As Table 3.6 shows, there was little difference in the compositions of positions sn-1, 2 and 3, which probably indicates that tripalmitoyl-sn-glycerol only was synthesized when (1-^{14}C)palmitic acid was the sole fatty acid in the incubation medium. There was little difference between samples taken at 50 and 90 minutes.

Fig. 3.8. Incorporation of (1-^{14}C)Acetate into Total Lipids by Adipocytes from 280 g Rats.

In addition to the basic ingredients, the incubation medium contained (1-^{14}C)sodium acetate (10 μCi) per incubation bottle) at various concentrations, and in concurrent incubations a 1mM albumin-bound fatty acid mixture was also included. All incubations were of 1 hour's duration. Results are means ± s.e.m. of three experiments.

o —— o, (1-^{14}C)acetate alone; □ – – □, (1-^{14}C)acetate + fatty acid mixture.


1.5
1.2
0.9
0.6
0.3
0
2.5
5
7.5
10
μ moles acetate incorporated/h/100 μg DNA
(1-14C)Acetate concn. (mM)

E. Incubations with (1-^{14}C)Acetate

Since the distribution of exogenous fatty acids esterified into rat adipocyte triacylglycerols, with the exception of palmitic acid, was not identical to their natural distribution, it was desirable to establish how fatty acids synthesized within the fat-cell were esterified into triacylglycerols, with a view to comparing the distribution of fatty acids formed de novo and those exogenous to the tissue, within the triacylglycerol molecule.

Adipocytes were incubated in media containing (1-^{14}C)acetate as a labelled fatty acid precursor. The concentration of the (1-^{14}C)acetate in the incubation medium was varied in order to determine the optimum acetate concentration for fatty acid synthesis by the fat-cells in the system used. Concurrent incubations were carried out in which the incubation mixture also contained a mixture of long-chain fatty acids, thereby permitting the effect of extracellular fatty acids on fatty acid synthesis and esterification to be determined. All incubations contained glucose and insulin at the levels described in Chapter 2, section C1.

1. Studies with Adipocytes from Rats of Average Weight 280 g

The fat-cells used in this series of experiments were isolated from the adipose tissue of rats weighing in the region of 280 g.

(i) Incorporation of (1-^{14}C)Acetate into Lipids. The dependence of (1-^{14}C)acetate incorporation into adipocyte total lipid upon the initial acetate concentration in the incubation medium is shown in Fig. 3.8. The amount of (1-^{14}C)acetate used by the fat-cells in lipogenesis was related to its concentration in the incubation medium.

Fig. 3.9. Incorporation of (1-^{14}C)Acetate Supplied Alone into Lipids by Adipocytes from 280 g Rats.

Incubation conditions were the same as described in the legend to Fig. 3.8 with no fatty acids in the medium. Results are means ± s.e.m. of three experiments.

●——●, triacylglycerols; □— —□, diacylglycerols; Δ— ·· —Δ, phospholipids; O— — O, unesterified fatty acids.

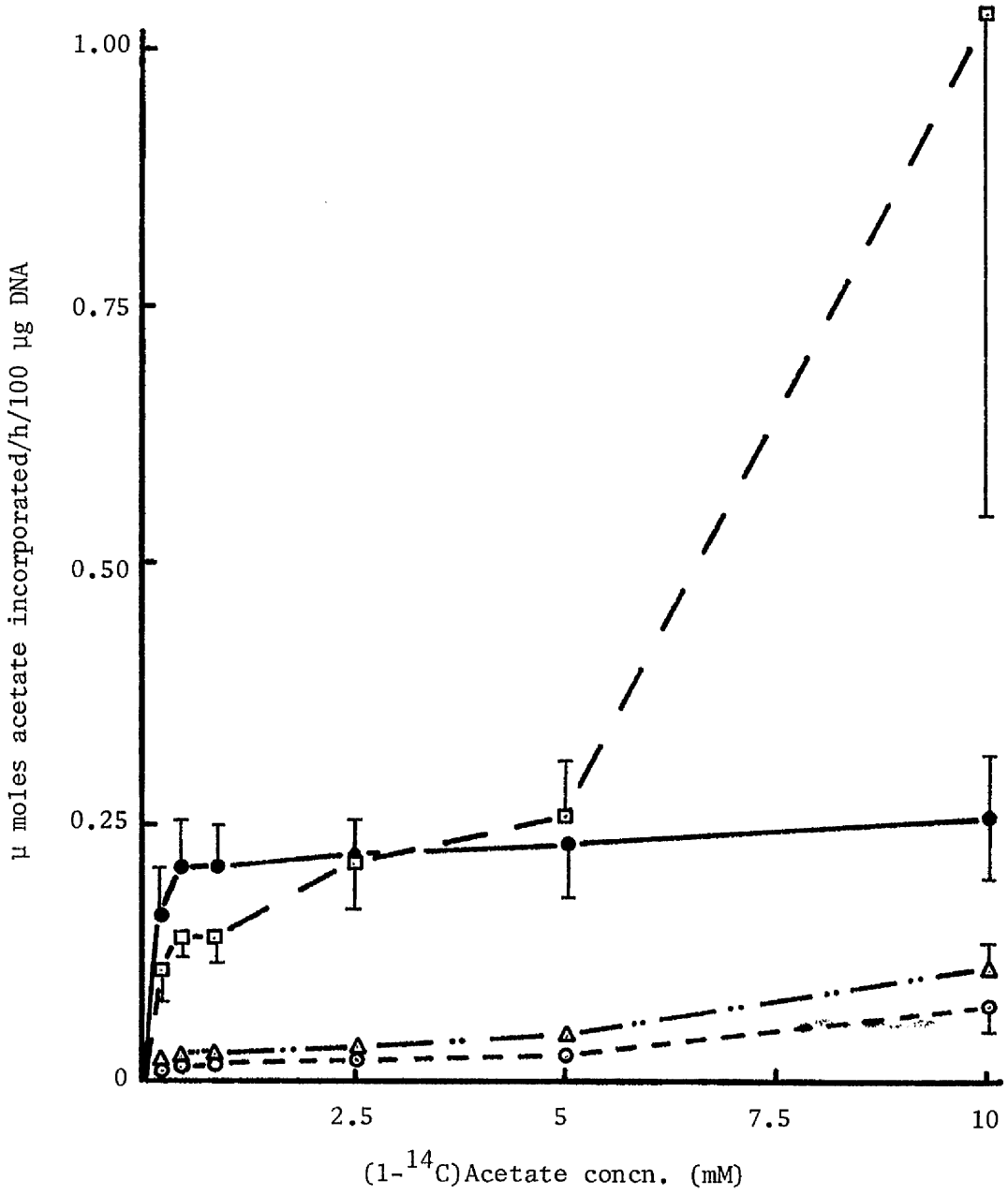
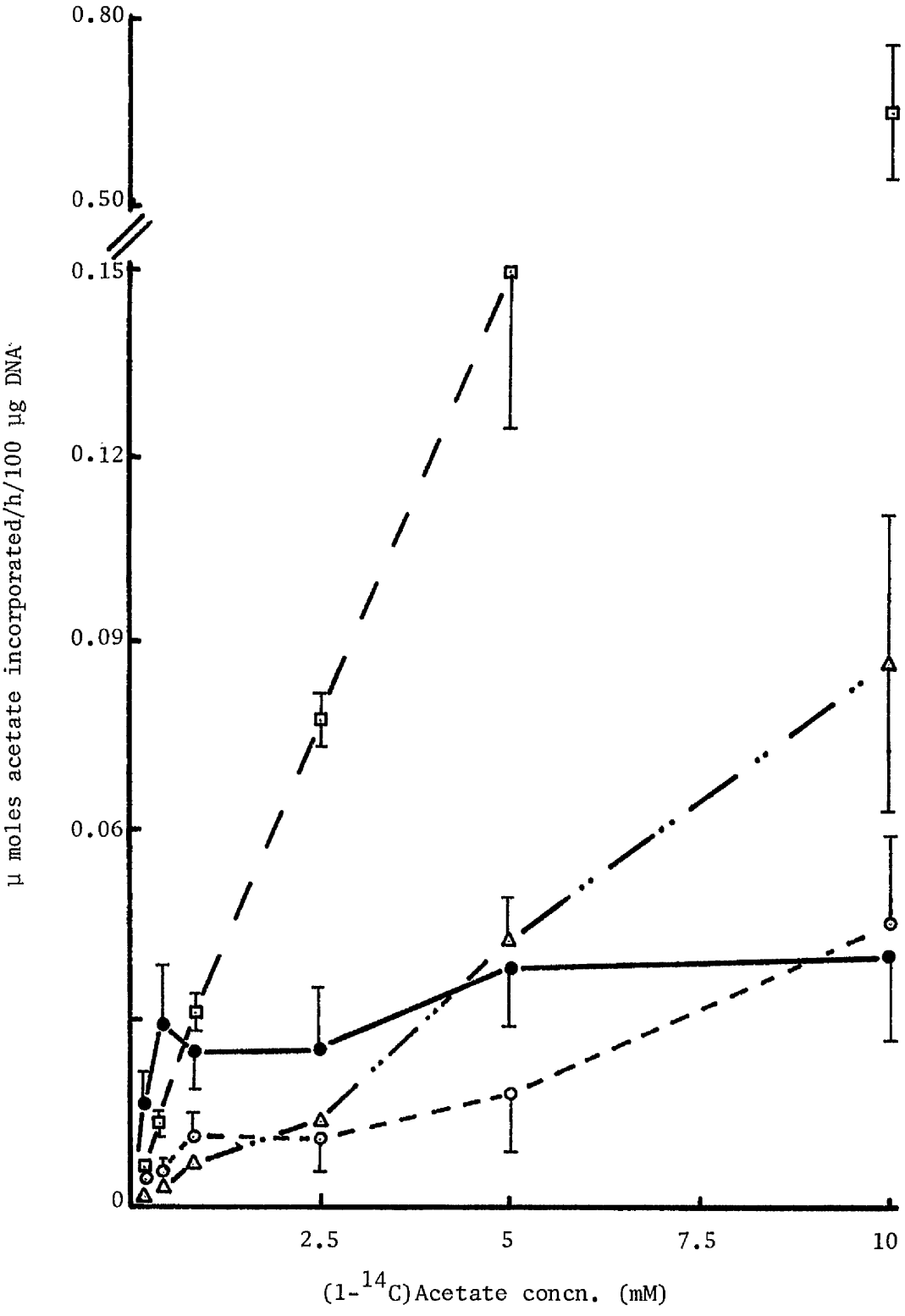

1.00
0.75
0.50
0.25
0
2.5
5
7.5
10
µ moles acetate incorporated/h/100 µg DNA
(1-^{14}C)Acetate concn. (mM)

Fig. 3.10. Incorporation of (1-^{14}C)Acetate into Lipids by Adipocytes from 280 g Rats in the Presence of Extracellular Fatty Acids.

The conditions of incubation were the same as those described in the legend to Fig. 3.8, with the medium containing a 1mM fatty acid mixture. Results are means ± s.e.m. of three experiments.

●——●, triacylglycerols; □— —□, diacylglycerols; Δ— ·· —Δ, phospholipids; O— —O, unesterified fatty acids.


0.80
0.50
0.15
0.12
0.09
0.06
0
μ moles acetate incorporated/h/100 μg DNA
2.5
5
7.5
10
(1-^{14}C)Acetate concn. (mM)

The presence of long-chain fatty acids in the incubation medium decreased lipogenesis from (1-^{14}C)acetate appreciably; the incorporation of (1-^{14}C)acetate into lipids at a concentration of 5mM was halved, whereas at the lower, more physiological, concentration of 0.5mM, its incorporation was decreased by 83%.

Fig. 3.9 illustrates the manner in which the (1-^{14}C)acetate incorporated was distributed among triacylglycerols, diacylglycerols, phospholipids and unesterified fatty acids when the adipocytes were incubated with the (1-^{14}C)substrate on its own. At acetate concentrations of less than 2.5mM, triacylglycerols contained the highest proportion of incorporated (1-^{14}C)acetate. The amount of (1-^{14}C)-acetate incorporated into triacylglycerols, phospholipids and unesterified fatty acids was apparently independent of the concentration of acetate in the incubation medium, whereas the amount recovered in diacylglycerols rose substantially as the acetate concentration exceeded 1mM. Diacylglycerols were the most highly-labelled acylglycerols when the fat-cells were presented with very high acetate concentrations of 5 and 10mM.

The presence of fatty acids in the incubation medium influenced the distribution of incorporated (1-^{14}C)acetate among the individual lipid classes (Fig. 3.10). At low acetate concentrations of 0.25 and 0.5mM, triacylglycerols contained most of the (1-^{14}C)acetate incorporated into lipid. Whereas approximately equal amounts of incorporated ^{14}C-labelled substrate were recovered in triacylglycerols at all acetate concentrations greater than zero, the amounts recovered in phospholipids and unesterified fatty acids rose steadily with increasing acetate concentration in the incubation medium. Incorporation of (1-^{14}C)acetate into diacylglycerols increased almost linearly with increasing acetate concentration. When the incubation medium

contained 10mM (1-^{14}C)acetate, diacylglycerols accounted for most of the incorporated ^{14}C-labelled substrate with phospholipids containing only 10.5%, unesterified fatty acids 5.5% and triacylglycerols only 5%.

The results presented in Figs. 3.9 and 3.10 could also have arisen if unincorporated (1-^{14}C)acetate, not removed during the lipid extraction procedure, co-chromatogrammed with diacylglycerols in T.L.C. separations, in which case the amount of radioactivity recovered in diacylglycerols would also increase as (1-^{14}C)acetate concentration increased. To examine this possibility, 0.1 μCi (1-^{14}C)acetate mixed with a little lipid cold carrier was applied to a T.L.C. plate and developed with the same solvent used for the separation of total lipid into its constituents. After identification, the radioactivity of the separated lipid classes was measured. Triacylglycerols, diacylglycerols and unesterified fatty acids were all found to contain the same small proportion (< 1%) of the total radioactivity applied to the chromatogram, as did a sample of the adsorbent taken from between the diacylglycerol and unesterified fatty acid bands. By far the major part of the (1-^{14}C)acetate applied to the plate was recovered along with phospholipids on the origin.

The accumulation of incorporated (1-^{14}C)acetate in diacylglycerols at high acetate concentrations was not due therefore to a simple experimental artefact.

Of the (1-^{14}C)acetate incorporated into each lipid, all was recovered in the fatty acid moiety of triacyl- and diacylglycerols both in the absence and presence of unesterified fatty acids in the incubation buffer and at all substrate concentrations. The water-soluble moiety of the phospholipids, however, contained up to 15% of

Table 3.7. Relative Incorporation of (1-^{14}C)Acetate into the Three Positions of Triacyl-*sn*-glycerols by Adipocytes from 280 g Rats. Effect of Extracellular Fatty Acids.

	Proportional (%) distribution in each position		
	Position *sn*-1	Position *sn*-2	Position *sn*-3
(1-^{14}C)acetate alone	51.0 ± 1.7	12.6 ± 2.4	36.5 ± 1.5
(1-^{14}C)acetate + fatty acid mixture	54.9 ± 4.9	15.8 ± 5.3	29.3 ± 0.7

Incubation conditions were as described in the legend to Fig. 3.8. Results are means ± s.e.m. of three experiments.

the total radioactivity present in that lipid class (data not shown).

These results confirmed that the adipocytes could take up acetate from the incubation medium for use in fatty acid synthesis, and that there was a direct relationship between the amount of acetate available and total fatty acid synthesis.

(ii) Stereospecific Distribution of Total Incorporated (1-^{14}C)-Acetate in Triacylglycerols. Triacylglycerols formed by adipocytes incubated in buffer containing 0.5mM (1-^{14}C)acetate were subjected to stereospecific analysis with tri (9,10-^{3}H)heptadecanoin as internal standard in the analytical procedure, to determine the distribution of total incorporated (1-^{14}C)acetate (and therefore of total newly-synthesized fatty acids) over positions sn-1, 2 and 3. Triacylglycerols isolated from fat-cells incubated in a similar medium but to which a long-chain fatty acid mixture had been added, were analysed similarly.

Fatty acids formed de novo by the adipocytes were preferentially esterified into position sn-1 since approximately half the total incorporated (1-^{14}C)acetate was recovered in that position both in the absence and presence of extracellular fatty acids (Table 3.7). The inclusion of fatty acids in the incubation medium resulted in a significant ($P < 0.02$) decrease in the proportion of (1-^{14}C)acetate recovered in position sn-3, suggesting that the exogenous fatty acids might have reduced the incorporation of newly-synthesized fatty acids into that position.

(iii) Incorporation of (1-^{14}C)Acetate into Fatty Acids. To study the pattern of fatty acids synthesized by adipocytes from (1-^{14}C)acetate, samples of triacylglycerols and diacylglycerols formed

Table 3.8. Percentage Distribution of Radioactivity in Triacylglycerol Fatty Acids of Adipocytes from 280 g Rats after Incubation with Various Concentrations of (1-^{14}C)Acetate.

Fatty acid	(1-^{14}C)Acetate concentration (mM) 0.5	2.5	10.0
(1-^{14}C)Acetate alone		a	
14:0	7.9 ± 0.3	6.6	10.0 ± 1.2
16:0	81.4 ± 0.7	82.8	76.2 ± 3.2
16:1	3.8 ± 0.5	3.8	2.7 ± 0.5
18:0	2.9 ± 0.1	2.0	4.1 ± 0.9
18:1	4.0 ± 0.3	4.8	7.0 ± 0.9
total saturated	92.2 ± 0.5	91.4	90.3 ± 1.2
total unsaturated	7.8 ± 0.5	8.6	9.7 ± 1.2
(1-^{14}C)Acetate + Fatty Acid Mixture		a	
14:0	20.3 ± 1.3	15.9	17.1 ± 2.4
16:0	65.4 ± 1.6	72.8	53.4 ± 4.6
16:1	0.5 ± 0.2	0.1	0.0
18:0	6.7 ± 0.2	4.5	13.6 ± 1.9
18:1	6.7 ± 2.1	6.7	15.9 ± 2.4
total saturated	92.4 ± 2.2	93.2	84.1 ± 2.4
total unsaturated	7.6 ± 2.2	6.8	15.9 ± 2.4

Incubation conditions were as described in the legend to Fig. 3.8. Results are means ± s.e.m. of three experiments. a, mean of two experiments.

at three concentrations of acetate were subjected, after methylation, to preparative G.L.C. with subsequent measurement of the radioactivity present in each fatty acid component. The composition of labelled fatty acids in phospholipids was not determined due to the low levels of radioactivity present in that component.

The results obtained for triacylglycerols are presented in Table 3.8. The composition of labelled fatty acids in triacylglycerols formed by adipocytes in the presence of extracellular fatty acids differed from that formed when the incubation medium contained no such fatty acids. Lauric acid (12:0), linoleic acid (18:2) and longer-chain fatty acids were found to contain negligible amounts of ^{14}C and are therefore not reported. Palmitic acid accounted for most of the radioactivity from (1-^{14}C)acetate in triacylglycerols formed at all acetate concentrations both in the absence and presence of extracellular fatty acids. The shorter-chain fatty acid, myristic acid, also contained marked proportions of the total radioactivity.

When the ^{14}C-labelled substrate was supplied alone, the proportions of total ^{14}C label recovered in oleic acid was greater than that in stearic acid at all acetate concentrations. In the presence of extracellular fatty acids, the proportion of radioactivity from (1-^{14}C)acetate recovered in stearic and myristic acids was increased whilst that recovered in palmitic and palmitoleic acids was decreased. Triacylglycerols formed at the high acetate concentration of 10mM contained more oleic acid formed de novo than did triacylglycerols synthesized by adipocytes incubated with 0.5 and 2.5mM acetate.

Palmitic acid also contained the highest proportion of radioactivity recovered in the fatty acids of diacylglycerols formed under

Table 3.9. Percentage Distribution of Radioactivity in Diacylglycerol Fatty Acids of Adipocytes from 280 g Rats after Incubation with Various Concentrations of (1-^{14}C)Acetate.

Fatty acid	(1-^{14}C)Acetate concentration (mM) 0.5	2.5	10.0
(1-^{14}C)Acetate alone			
14:0	2.1 ± 2.0	1.1 ± 0.6	1.6 ± 0.7
16:0	91.9 ± 2.6	89.0 ± 2.3	80.0 ± 3.6
16:1	2.1 ± 0.2	3.1 ± 0.8	1.9 ± 0.7
18:0	2.1 ± 0.1	3.7 ± 0.6	12.2 ± 2.7
18:1	1.8 ± 0.1	3.1 ± 0.7	4.3 ± 0.8
total saturated	96.4 ± 0.5	93.8 ± 1.5	93.8 ± 1.4
total unsaturated	3.6 ± 0.5	6.2 ± 1.5	6.2 ± 1.4
(1-^{14}C)Acetate + Fatty Acid Mixture			
14:0	1.7 ± 0.9	11.2 ± 4.5	11.7 ± 2.2
16:0	87.5 ± 3.1	59.5 ± 9.9	52.5 ± 4.4
16:1	4.1 ± 1.9	6.5 ± 2.7	7.6 ± 4.1
18:0	3.7 ± 0.4	11.4 ± 1.9	18.2 ± 0.7
18:1	3.0 ± 0.4	11.3 ± 3.4	10.0 ± 1.6
total saturated	92.9 ± 2.3	82.1 ± 6.1	82.4 ± 2.6
total unsaturated	7.1 ± 2.3	17.9 ± 6.1	17.6 ± 2.6

Incubation conditions were as described in the legend to Fig. 3.8. Results are means ± s.e.m. of three experiments.

all incubation conditions examined (Table 3.9). The percentage of the total ^{14}C label present in palmitic acid decreased when the (1-^{14}C)-acetate substrate was incubated along with fatty acids in the medium. Correspondingly, the proportions recovered in the other fatty acids generally increased. Acetate concentration apparently affected the pattern of diacylglycerol fatty acids synthesized from (1-^{14}C)acetate since the proportion of total radioactivity recovered in palmitic acid decreased, whilst that in stearic and oleic acids increased, as acetate concentration increased whether extracellular fatty acids were present or not. When ^{14}C-labelled acetate was supplied on its own, a higher proportion of the diacylglycerol fatty acids synthesized at all acetate concentrations was saturated in comparison with fatty acids formed in the presence of exogenous fatty acids. This finding was not so obvious with the results obtained for the composition of fatty acids synthesized de novo in triacylglycerols.

Triacylglycerols synthesized by the adipocytes at all acetate concentrations contained a smaller proportion of palmitic acid synthesized de novo and relatively more oleic acid than diacylglycerols, when fatty acids were omitted from the incubation medium.

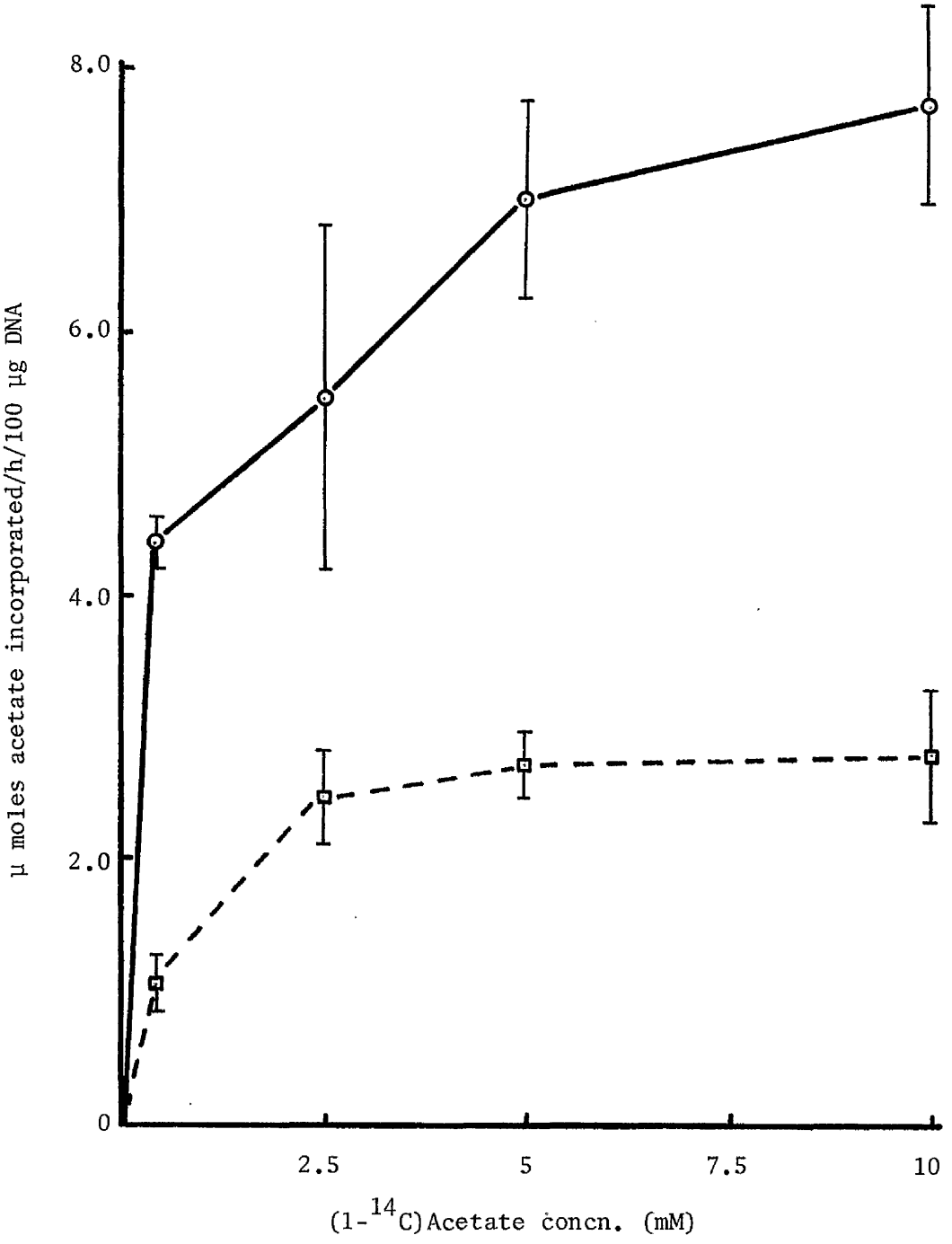
2. Studies with Adipocytes from Rats of Average Weight 150 g

The above studies with adipocytes from 280 g rats provided useful information on the total amounts of fatty acids formed de novo from (1-^{14}C)acetate and esterified into triacylglycerols and diacylglycerols. It was desirable to investigate the manner in which each newly-synthesized fatty acid in triacyl-sn-glycerols was distributed over the three positions with a view to establishing whether there was a preferential esterification of fatty acids formed de novo into any

Fig. 3.11. Incorporation of (1-^{14}C)Acetate into Total Lipid by Adipocytes from 150 g Rats.

Various concentrations of (1-^{14}C)acetate (5 μCi in 0.5 and 2.5mM, 10 μCi in 5 and 10mM incubations) were included in the basic incubation medium. Concurrent incubations contained in addition a 1mM fatty acid mixture of which (9,10-^{3}H)stearic acid (1 μCi) was a component. All incubations were carried out for 1 hour. Results are means ± s.e.m. of three experiments.

o——o, (1-^{14}C)acetate alone; □— — —□, (1-^{14}C)acetate + fatty acid mixture.


8.0
6.0
4.0
2.0
0
μ moles acetate incorporated/h/100 μg DNA
2.5
5
7.5
10
(1-^{14}C)Acetate concn. (mM)

one particular sn-position, and to compare the distribution of newly-synthesized fatty acids with their natural distribution. However, the specific activities of individual fatty acids formed de novo from (1-^{14}C)acetate by fat-cells prepared from 280 g rats were too low to permit the stereospecific distribution of each in the triacylglycerols to be determined.

Since the rate of fatty acid synthesis in rat adipose tissue is known to decrease with age (247), it was decided to carry out studies with adipocytes from younger rats in order that the increased recovery of radioactivity in fatty acids might enable a closer study to be made of the esterification of individual newly-synthesized fatty acids in triacylglycerols.

The incubations with adipocytes isolated from rats of approximate weight 150 g, with (1-^{14}C)acetate, were very similar to those carried out with fat-cells from older rats. However, in those incubations containing a fatty acid mixture, (9,10-^{3}H)stearic acid was present as a component of the mixture to permit the esterification of an exogenous fatty acid mixture to be measured in relation to fatty acid synthesis de novo and esterification.

(i) Incorporation of (1-^{14}C)Acetate into Lipids. (1-^{14}C)Acetate incorporation into lipids was ten fold greater when adipocytes from the younger rats were used, in comparison with the results obtained previously with fat-cells prepared from 280 g animals.

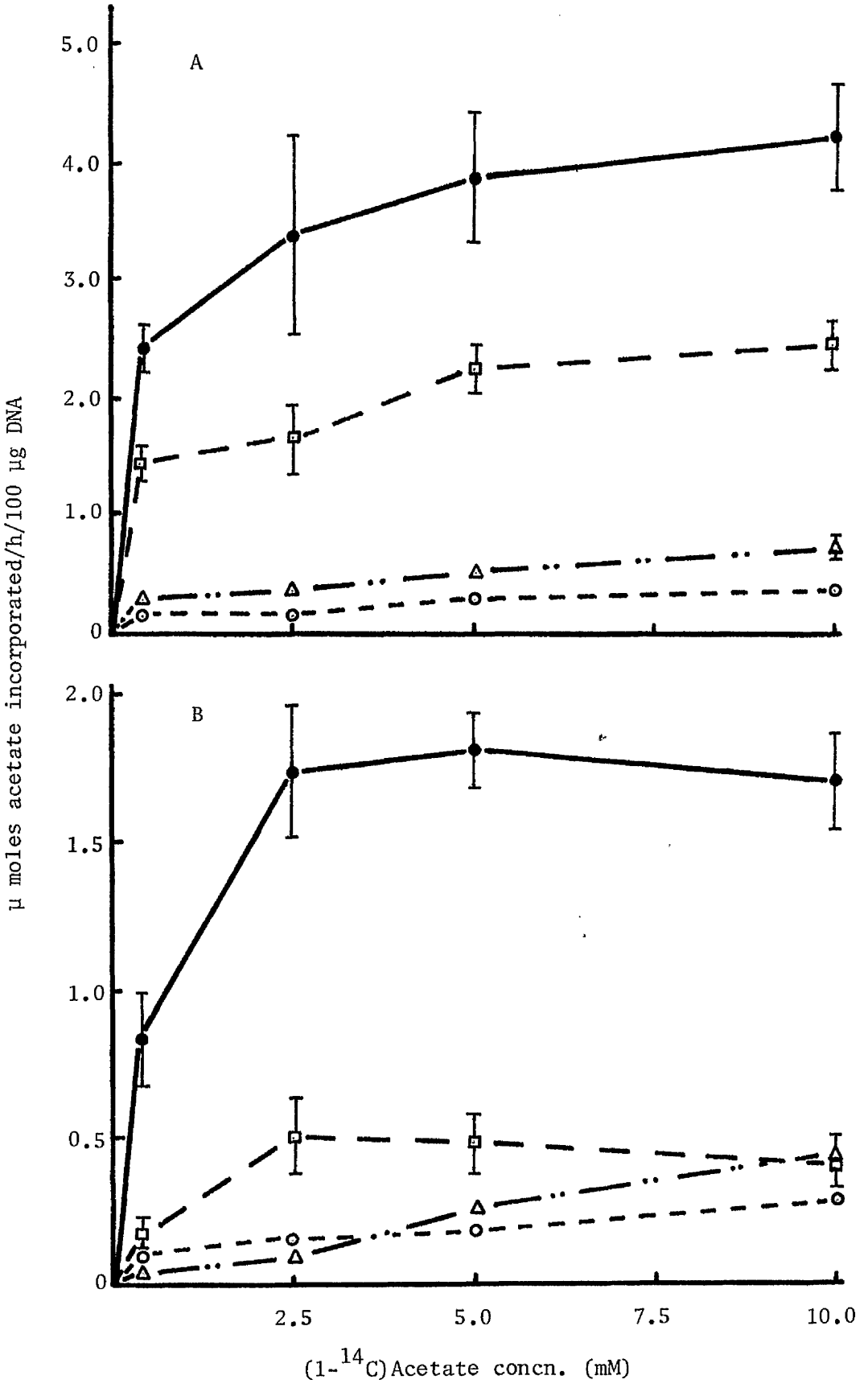
The relationship between (1-^{14}C)acetate incorporation into total lipid and its initial concentration in the incubation medium is illustrated in Fig. 3.11. The amount of (1-^{14}C)acetate, supplied on its own, incorporated into lipids by the adipocytes rose gradually

Fig. 3.12. Incorporation of (1-^{14}C)Acetate into Lipids by Adipocytes from 150 g Rats.

A. (1-^{14}C)Acetate supplied alone B. (1-^{14}C)-Acetate supplied in presence of fatty acid mixture.

Incubation conditions were the same as described in the legend to Fig. 3.11. Results are means ± s.e.m. of three experiments.

●——●, triacylglycerols; □— —□, diacylglycerols; Δ— ·· —Δ, phospholipids; o— — o, unesterified fatty acid.


A
B
5.0
4.0
3.0
2.0
1.0
0
2.0
1.5
1.0
0.5
0
2.5
5.0
7.5
10.0
μ moles acetate incorporated/h/100 μg DNA
(1-^{14}C)Acetate concn. (mM)

as its concentration increased. In the presence of extracellular fatty acids, the maximum rate of (1-^{14}C)acetate utilisation was reached at a concentration of 2.5mM. Extracellular fatty acids apparently inhibited lipogenesis from acetate since in their presence the amount of (1-^{14}C)acetate incorporated into lipid was more than halved at every substrate concentration.

Very little incorporated (1-^{14}C)acetate was recovered as unesterified fatty acid in any incubation, suggesting rapid esterification of newly-synthesized fatty acids into acylglycerols (Fig. 3.12). Only at an acetate concentration of 10 mM in the presence of extracellular fatty acids did phospholipids contain a significant proportion (12%) of incorporated ^{14}C-labelled acetate. When incubated either on its own or in the presence of a fatty acid mixture, (1-^{14}C)acetate of all concentrations was predominantly incorporated into triacylglycerols by the fat-cells. The proportion of total incorporated ^{14}C-labelled substrate recovered in diacylglycerols was influenced by extracellular fatty acids; in their absence, diacylglycerols contained approximately 33% of the total incorporated acetate whereas their inclusion in the incubation medium halved the proportion recovered in diacylglycerols.

Unlike the results obtained with fat-cells from older rats, the amount of ^{14}C-labelled acetate incorporated into diacylglycerols never exceeded that recovered in triacylglycerols.

All the radioactivity from (1-^{14}C)acetate recovered in triacylglycerols and diacylglycerols formed under all incubation conditions, was found to be present in the fatty acid moiety. On average, 2% of the total radioactivity present in phospholipids was associated with the water-soluble portion (data not shown). The results presented in

Table 3.10. Percentage Distribution of Radioactivity in Triacylglycerol Fatty Acids of Adipocytes from 150 g Rats after Incubation with Various Concentrations of (1-^{14}C)Acetate.

Fatty acid	(1-^{14}C)Acetate concentration (mM)		
	0.5	2.5	10.0
(1-^{14}C)Acetate alone			
12:0	2.2 ± 0.3	1.2 ± 0.4	0.1[a]
14:0	4.9 ± 0.5	5.2 ± 0.6	5.0
16:0	52.0 ± 3.1	55.2 ± 0.5	63.5
16:1	13.8 ± 0.8	11.6 ± 0.4	10.2
18:0	5.9 ± 1.1	5.5 ± 1.2	1.6
18:1	20.4 ± 1.5	19.1 ± 0.7	19.5
18:2	0.8 ± 0.3	2.4 ± 0.5	0.2
(1-^{14}C)Acetate + Fatty Acid Mixture			
12:0	0.4 ± 0.1	0.8[a]	-[b]
14:0	11.0 ± 0.7	10.6	-
16:0	65.4 ± 2.3	67.0	-
16:1	2.7 ± 0.1	2.0	-
18:0	5.1 ± 0.7	4.6	-
18:1	14.4 ± 1.6	14.6	-
18:2	1.0 ± 0.4	0.4	-

Incubation conditions were as described in the legend to Fig. 3.11. Results are means ± s.e.m. of three experiments. a, means of two experiments; b, not measured.

Fig. 3.12 therefore represent the incorporation of (1-^{14}C)acetate into acylglycerol fatty acids.

Overall, the effect of exogenous fatty acids on fatty acid synthesis de novo was greater than that of acetate concentration.

(ii) Incorporation of (1-^{14}C)Acetate into Fatty Acids of Triacylglycerols and Diacylglycerols. To determine whether the concentration of a fatty acid precursor and the presence of extracellular fatty acids had any influence on the pattern of fatty acids synthesized de novo by the adipocyte, the distribution of radioactivity from (1-^{14}C)acetate in the fatty acids of triacylglycerols and diacylglycerols formed at three acetate concentrations was examined.

Methyl esters prepared from purified triacylglycerols and diacylglycerols were isolated by preparative G.L.C. for subsequent measurement of the radioactivity present in each fatty acid component.

(a) Triacylglycerols. Table 3.10 shows the effects of acetate concentration and of exogenous fatty acids on the distribution of radioactivity from (1-^{14}C)acetate in the fatty acids of the triacylglycerols. The concentration of acetate in the incubation medium had no obvious effect on the pattern of fatty acids synthesized de novo by the adipocytes from ^{14}C-acetate. Although the values reported for palmitic and stearic acids at 10mM acetate were slightly different from those obtained at 0.5 and 2.5mM, less emphasis can be attached to them since they represented the mean of only two experiments.

Palmitic acid contained more than half the total radioactivity in fatty acids at all three acetate concentrations, with oleic acid accounting for approximately 20%. Palmitoleic acid also contained a significant proportion of the incorporated ^{14}C-labelled acetate.

The effect of including long-chain fatty acids in the incubation

Table 3.11. Percentage Distribution of Radioactivity in Diacylglycerol Fatty Acids of Adipocytes from 150 g Rats after Incubation with Two Concentrations of (1-^{14}C)Acetate.

Fatty acid	(1-^{14}C)Acetate concentration (mM)	
	0.5	2.5
(1-^{14}C)Acetate alone		
12:0	0.0[a]	0.0
14:0	2.8 ± 0.7	3.0
16:0	77.2)	74.2
	) 80.7 ± 0.1	
16:1	3.6)	4.9
18:0	4.3 ± 0.4	4.4
18:1	12.1 ± 0.4	13.5
18:2	0.1 ± 0.1	0.0
(1-^{14}C)Acetate + Fatty Acid Mixture		
12:0	0.0	0.4
14:0	3.2	4.2
16:0	75.5	70.2
16:1	1.3	1.9
18:0	)	12.3
	) 20.0	
18:1	)	11.0
18:2	0.0	0.0

Incubation conditions were as described in the legend to Fig. 3.11. Results are means of two experiments. a, mean of three experiments.

medium along with (1-^{14}C)acetate was to increase the proportion of ^{14}C label recovered in the saturated fatty acids, palmitic and myristic, whilst reducing that incorporated into the monoenoic fatty acids, oleic and palmitoleic. The resulting triacylglycerols therefore contained a higher proportion of newly-synthesized saturated fatty acids.

(b) Diacylglycerols. The distribution of radioactivity from (1-^{14}C)acetate in the fatty acids of diacylglycerols, is presented in Table 3.11.

The acetate concentration of the incubation medium had no obvious effect on the pattern of ^{14}C-labelled fatty acids recovered in diacylglycerols. Palmitic acid was the most highly labelled fatty acid found in diacylglycerols, with oleic acid containing approximately three times the proportion of radioactivity recovered in stearic acid at both 0.5 and 2.5 mM acetate.

The proportion of incorporated (1-^{14}C)acetate found as palmitic acid was not greatly affected when the incubation medium contained a mixture of long-chain fatty acids. However, under such conditions, the proportion recovered as oleic and palmitoleic acids decreased with a corresponding increase in the synthesis of stearic acid from ^{14}C-labelled acetate.

Triacylglycerols formed in the absence of exogenous fatty acids was richer proportionally in oleic acid formed de novo from (1-^{14}C) acetate than diacylglycerols synthesized under the same conditions.

(iii) Stereospecific Distribution in Triacylglycerols of Fatty Acids Formed from (1-^{14}C)Acetate. The proportional distribution of total fatty acids formed de novo from (1-^{14}C)acetate over the three positions of triacyl-sn-glycerols at different acetate concentrations

Table 3.12. Relative Incorporation of (1-^{14}C)Acetate into the Three Positions of Triacyl-*sn*- glycerols by Adipocytes from 150 g Rats. Effect of Acetate Concentration

(1-^{14}C)Acetate concn. (mM)	Position *sn*-1	Position *sn*-2	Position *sn*-3
0.5	50.3 ± 1.0	31.3 ± 2.5	18.4 ± 1.8
2.5	51.1 ± 5.1	27.8 ± 2.9	21.1 ± 2.3
5.0	51.0 ± 1.3	23.7 ± 2.8	25.3 ± 1.5

Incubation conditions were as described in the legend to Fig. 3.11, with no fatty acid mixture in the incubation medium. Results are means ± s.e.m. of three experiments.

was determined by stereospecific analysis.

As Table 3.12 shows, approximately half the fatty acids newly-synthesized from ^{14}C-labelled acetate were esterified into position sn-1 at all acetate concentrations. The percentage of total ^{14}C-labelled fatty acids recovered in position sn-2 decreased as the concentration of acetate in the incubation medium increased, whereas the converse was true of position sn-3. The proportion recovered in position sn-3 was significantly ($P < 0.001$) greater at 5 mM than at 0.5 mM acetate.

The initial concentration of acetate in the incubation medium did not therefore apparently affect the esterification of newly-synthesized fatty acids into position sn-1 but it did influence their distribution between positions sn-2 and sn-3.

The stereospecific distribution in triacylglycerols of (1-^{14}C)-acetate incorporated into fatty acids could not be determined since the fatty acid mixture in such incubations contained a ^{3}H-labelled component. The use of tri (9,10-^{3}H)heptadecanoin as the internal standard necessary for the estimation of losses in the stereospecific analysis procedure was therefore prevented and consequently no accurate stereospecific analyses could be carried out on triacylglycerols formed in the presence of exogenous fatty acids by adipocytes from younger rats.

The methyl esters of stereospecific analysis products were subjected to preparative G.L.C. with subsequent measurement of the radioactivity present in each fatty acid component. This allowed the newly-synthesized fatty acid composition of each position in triacyl-sn-glycerols to be determined. The results obtained for the distribution of radioactivity in the fatty acids present in positions sn-1, 2 and 3 of triacylglycerols formed when the incubation medium

Table 3.13. Percentage Distribution of Radioactivity in Fatty Acids of Positions sn-1, 2 and3 of Adipocyte Triacyl-sn-glycerols after Incubation with Various Concentrations of (1-^{14}C)Acetate.

Adipocytes were prepared from 150 g rats and incubated as described in the legend to Fig. 3.11, in medium which contained no fatty acids. Results are means ± s.e.m. of three experiments.
a, denotes values which are means of two experiments
b, determined by pancreatic lipase (product II in Fig. 2.1)
c, calculated from 2 x position sn-(2 + 3) - position sn-2.

Fatty acid	(1-^{14}C)Acetate concentration (mM) 0.5	2.5	5.0
Position sn-1			
12:0	0.7 ± 0.2	0.0	2.3[a]
14:0	4.4 ± 0.1	3.7 ± 0.8	3.4
16:0	80.0 ± 1.2	83.6 ± 2.6	79.4
16:1	8.4 ± 0.4	5.2 ± 0.5	7.2
18:0	2.8 ± 0.6	2.9 ± 0.8	4.8
18:1	3.5 ± 0.4	4.4 ± 0.8	4.0
18:2	0.2 ± 0.1	0.3 ± 0.2	0.0
Position sn-2[b]			
12:0	0.8[a]	0.0	0.4 ± 0.3
14:0	4.8	5.0 ± 1.0	3.0 ± 1.5
16:0	36.4	32.4 ± 1.4	29.3 ± 1.5
16:1	13.2	17.7 ± 0.8	22.5 ± 1.7
18:0	2.1	2.1 ± 0.4	4.0 ± 0.4
18:1	41.2	42.7 ± 1.7	40.0 ± 0.4
18:2	1.5	0.1 ± 0.1	0.7 ± 0.2
Position sn-3[c]			
12:0	0.0[a]	1.2 ± 0.6	1.1 ± 0.6
14:0	8.4	1.1 ± 0.6	8.3 ± 1.5
16:0	35.4	44.4 ± 0.4	53.9 ± 2.4
16:1	22.8	16.4 ± 4.1	9.2 ± 1.9
18:0	8.9	6.9 ± 1.2	7.0 ± 1.2
18:1	21.6	29.0 ± 2.6	20.5 ± 1.3
18:2	3.1	1.2 ± 0.8	0.0

Table 3.14. Relative Incorporation of Fatty Acids Formed de novo by Adipocytes Incubated with Various Concentrations of (1-^{14}C)Acetate into the Three Positions of Triacyl-sn-glycerols.

(1-^{14}C)Acetate concn. (mM)	Fatty acid	Proportional (%) distribution in each position		
		Position sn-1	Position sn-2	Position sn-3
0.5	16:0	65.5	19.2	15.3
	16:1	33.8	44.0	22.2
	18:0	34.2	14.4	51.4
	18:1	12.4	57.6	30.0
2.5	16:0	69.6	15.0	15.4
	16:1	23.9	44.7	31.4
	18:0	41.2	16.6	42.2
	18:1	11.0	57.9	31.1
5.0	16:0	65.6	12.6	21.8
	16:1	30.7	50.2	19.1
	18:0	48.6	20.6	30.8
	18:1	9.0	63.5	27.5

Incubation conditions were as described in the legend to Fig. 3.11, with no fatty acids in the incubation medium.

contained different concentrations of (1-^{14}C)acetate, are presented in Table 3.13.

Of the newly-synthesized fatty acids present in position sn-1, palmitic acid was by far the most predominant, accounting for approximately 80% of the incorporated (1-^{14}C)acetate in that position. The relative distribution of radioactivity among the fatty acids of this position was independent of acetate concentration.

In position sn-2, the amount of newly-synthesized oleic acid present exceeded that of palmitic acid at all acetate concentrations studied. Palmitoleic acid also accounted for an appreciable proportion of the total newly-synthesized fatty acids. As acetate concentration increased, the amount of newly-synthesized palmitic acid present in position sn-2 apparently decreased in relation to the other fatty acids.

This finding was in contrast to the situation in position sn-3 where the proportion of radioactivity recovered in palmitic acid actually increased with increasing acetate concentration.

The change in the distribution of total incorporated (1-^{14}C)acetate in triacylglycerols noted in Table 3.12 may therefore have been related to an alteration in the distribution of newly-synthesized palmitic acid between positions sn-2 and sn-3.

The proportional distribution of each newly-synthesized fatty acid over triacylglycerols formed by fat-cells at different concentrations was calculated using the results shown in Table 3.13, and is presented in Table 3.14. Palmitic acid formed from (1-^{14}C)acetate was preferentially esterified into position sn-1 at all acetate concentrations, but as acetate concentration increased there was a decrease in the proportion recovered in position sn-2 with a corresponding increase in that entering position sn-3. Newly-synthesized palmitoleic acid

was esterified preferentially into position sn-2 at all concentrations of acetate. The proportion of stearic acid, formed de novo from (1-^{14}C)acetate recovered in position sn-1 rose considerably as the acetate concentration of the incubation medium increased, with a corresponding decrease in that found in position sn-3. Position sn-2 contained the highest proportion of oleic acid synthesized from (1-^{14}C)acetate and position sn-1 the smallest, at all concentrations of the ^{14}C-labelled substrate.

Although acetate concentration had no effect on the pattern of fatty acids in triacylglycerols synthesized from ^{14}C-labelled acetate, it did apparently influence their distribution, palmitic and stearic acids in particular, over the three positions.

The positional distribution of fatty acids synthesized de novo from acetate did not resemble their natural distribution (Table 3.3) in rat adipocyte triacylglycerols. At all acetate concentrations, the proportion of newly-synthesized palmitic acid recovered in position sn-1 was higher, and that in position sn-3 lower, than found naturally. Although the proportion of palmitoleic acid formed de novo from (1-^{14}C)-acetate in position sn-1 was quite similar to its natural distribution, position sn-2 contained a higher proportion and position sn-3 a lower proportion than its natural distribution in triacylglycerols. In agreement with its natural distribution, the smallest proportion of newly-synthesized stearic acid was present in position sn-2, but at an acetate concentration of 0.5mM the proportion recovered in position sn-3 was far in excess of its normal distribution. In comparison with its natural distribution, the proportion of oleic acid formed de novo from ^{14}C-labelled acetate in position sn-2 was very high, whilst that in position sn-1 was only approximately half the normal.

Fig. 3.13. Incorporation of (U-^{14}C)Glucose into Triacylglycerols, Diacylglycerols and Unesterified Fatty Acids by Adipocytes from 280 g Rats.

A. (U-^{14}C)Glucose supplied alone B. (U-^{14}C)-Glucose supplied in presence of a fatty acid mixture.

(U-^{14}C)Glucose (0.17 μCi/μmole) of various concentrations was added to the basic incubation medium, and in a set of concurrent incubations a 1mM fatty acid mixture, of which (9,10-^{3}H)oleic acid (1 μCi) was a component, was also included. All incubations were of 1 hour's duration. Results are means ± s.e.m. of four experiments.

●——●, triacylglycerols; □— —□, diacylglycerols; o— —o, unesterified fatty acids.

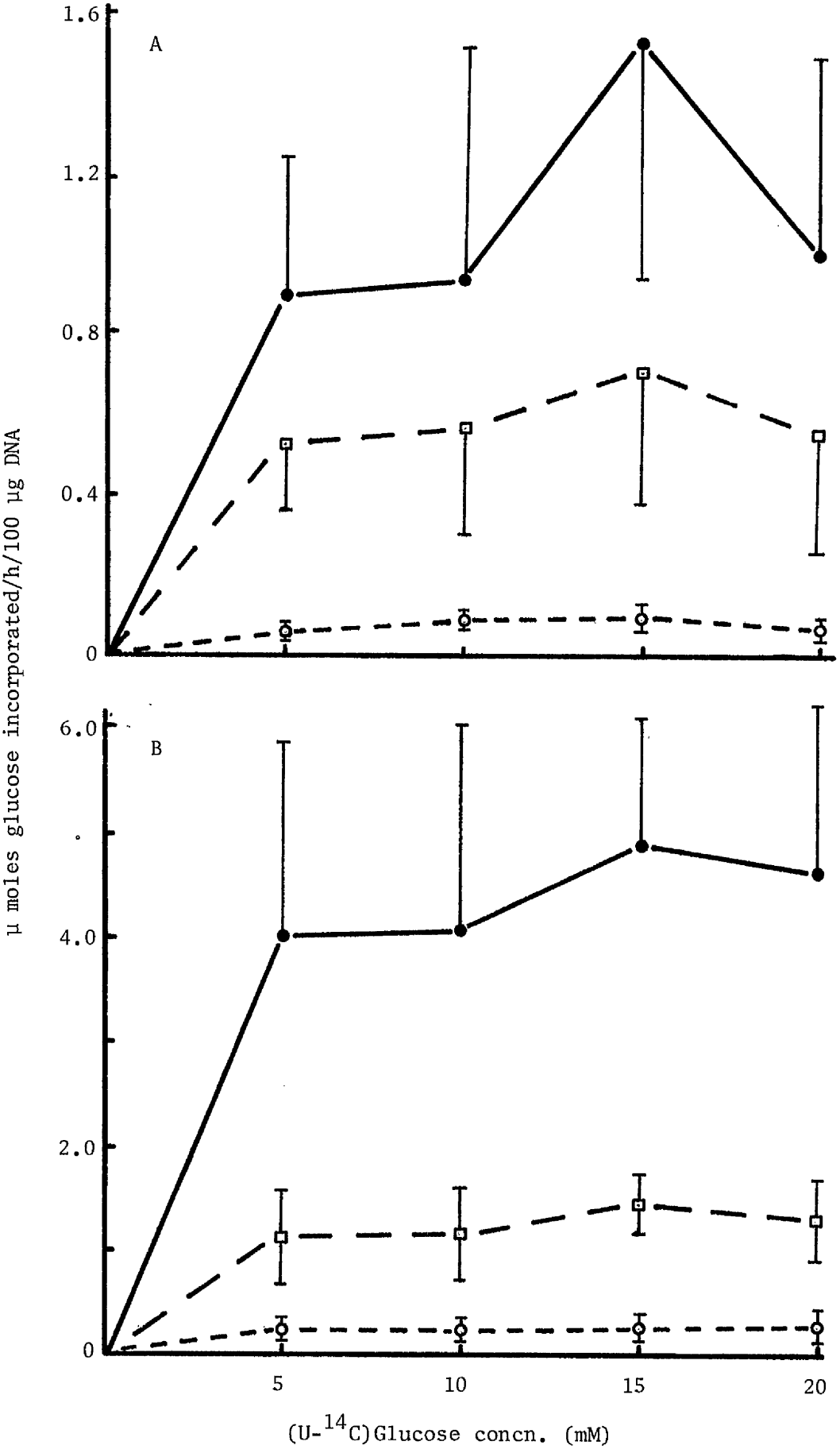
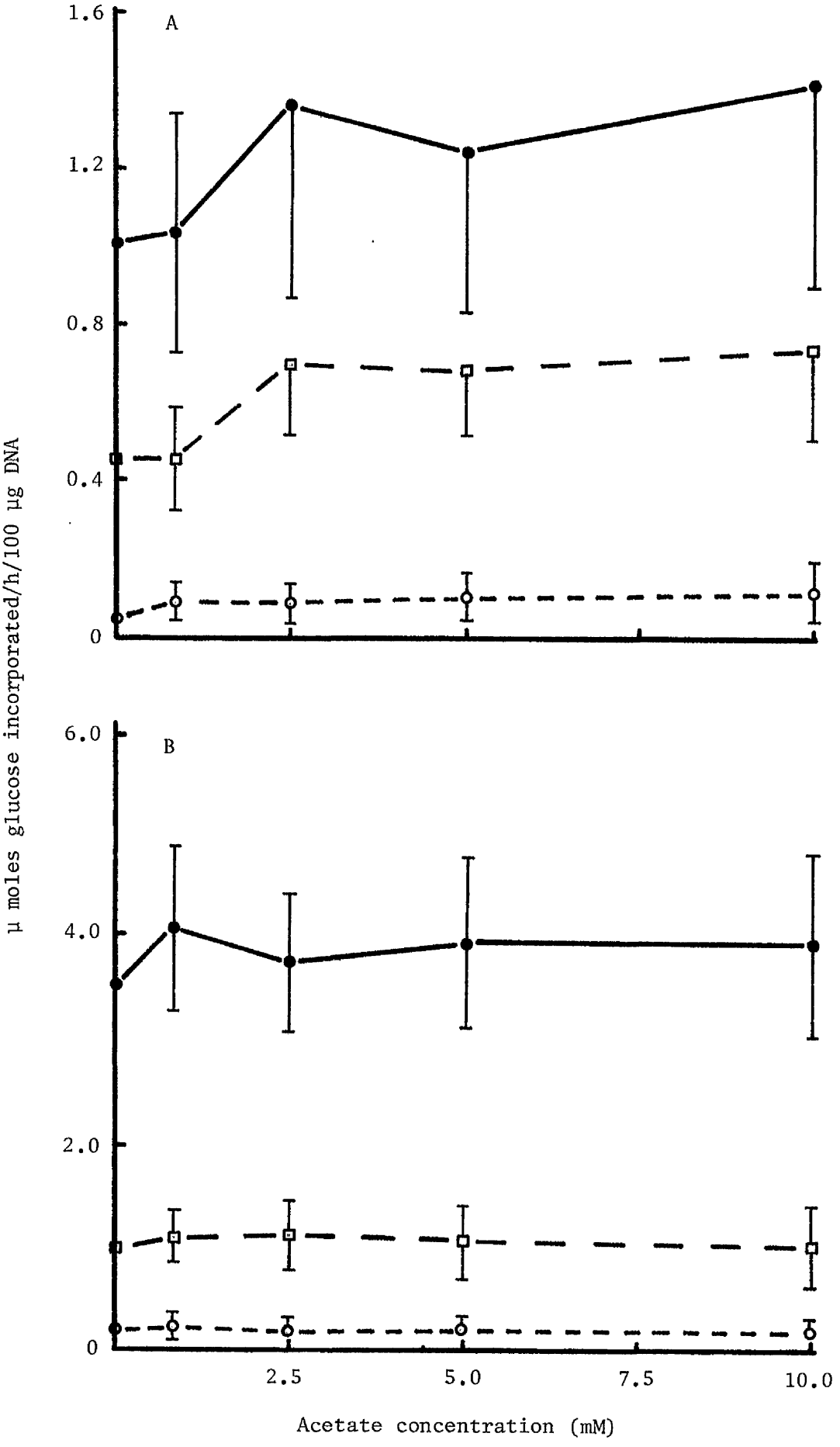

A
B
1.6
1.2
0.8
0.4
0
6.0
4.0
2.0
0
5
10
15
20
μ moles glucose incorporated/h/100 μg DNA
(U-14C)Glucose concn. (mM)

Fig. 3.14. Incorporation of (U-^{14}C)Glucose into Triacylglycerols, Diacylglycerols and Unesterified Fatty Acids by Adipocytes from 280 g Rats. Effect of Acetate Concentration. A. (U-^{14}C)Glucose supplied alone. B. (U-^{14}C)Glucose supplied in presence of a fatty acid mixture

5mM (U-^{14}C)glucose (0.17 µCi/µmole) and sodium acetate of various concentrations were added to the basic incubation medium. A series of concurrent incubations contained in addition a 1mM albumin-bound mixture of fatty acids of which (9,10-^{3}H)oleic acid (1 µCi) was a component. All incubations were carried out for

●——●, triacylglycerols; □— —□, diacylglycerols; o — — o, unesterified fatty acids.


A
B
1.6
1.2
0.8
0.4
0
6.0
4.0
2.0
0
2.5
5.0
7.5
10.0
μ moles glucose incorporated/h/100 μg DNA
Acetate concentration (mM)

F. Incubations with (U-^{14}C)Glucose

The synthesis and esterification of fatty acids formed de novo from (U-^{14}C)glucose by adipocytes incubated with glucose at several concentrations was examined to determine whether the accumulation of newly-synthesized fatty acids in diacylglycerols also occurred when glucose was the fatty acid precursor.

Adipocytes were incubated in the basic incubation medium containing (U-^{14}C)glucose of concentrations in the range 0 to 20mM. Concurrent incubations were carried out in which the incubation medium also contained an albumin-bound fatty acid mixture.

1. Studies with Adipocytes from Rats of Average Weight 280 g

Fat-cells were prepared from adipose tissue of rats having a mean weight of approximately 280 g and used in a series of experiments, the results of which are reported below.

(i) Incorporation of (U-^{14}C)Glucose into Lipids. The manner in which the adipocytes incorporated the ^{14}C-labelled glucose into the individual acylglycerols was examined by separating a portion of each lipid extract into its constituent lipid classes by T.L.C. and measuring the radioactivity present in each fraction.

The fat-cells readily used the (U-^{14}C)glucose for lipogenesis, with triacylglycerols and diacylglycerols becoming heavily labelled. The measurements of radioactivity recovered in phospholipids proved inaccurate since the lipid fraction was later found to be contaminated by residual (U-^{14}C)glucose not removed in the aqueous layer during the lipid extraction procedure. For this reason, Figs. 3.13 and 3.14 show only the incorporation of (U-^{14}C)glucose into triacylglycerols,

diacylglycerols and unesterified fatty acids. In subsequent experiments in which (U-^{14}C)glucose was used, the aqueous wash in the lipid extraction procedure contained 1% (w/v) unlabelled glucose.

Fig. 3.13 illustrates how the concentration of glucose and the presence of fatty acids in the incubation medium affected the incorporation of (U-^{14}C)glucose into three lipid classes. Glucose concentration had no significant effect on the amount of ^{14}C-labelled glucose incorporated into triacylglycerols, diacylglycerols or unesterified fatty acids. At all concentrations of glucose, triacylglycerols contained most of the labelled substrate incorporated when it was supplied in the absence of extracellular fatty acids. Under the same incubation conditions, the amount of ^{14}C-labelled glucose incorporated into diacylglycerols was fairly constant with respect to glucose concentration, being about 53-60% of that incorporated into triacylglycerols. Unesterified fatty acids contained only a small fraction of the total (U-^{14}C)glucose incorporated into lipid.

When a fatty acid mixture was present in the incubation medium, the overall incorporation of glucose into total lipid was increased three fold. This increased use of ^{14}C-labelled glucose in lipogenesis was due mainly to a four fold increase in the amount of (U-^{14}C)glucose used in triacylglycerol synthesis. Incorporation of the ^{14}C-substrate into unesterified fatty acids was increased almost three fold whereas its incorporation into diacylglycerols was only doubled by the presence of fatty acids in the incubation medium. Extracellular fatty acids therefore increased the rate of diacylglycerol acylation to triacylglycerols.

The effect of acetate concentration on the adipocyte's use of

(U-^{14}C)glucose for lipogenesis was examined with the aim of establishing whether high concentrations of extramitochondrial acetate might influence the use of glucose-derived acetyl units for fatty acid synthesis and subsequent esterification.

Fig. 3.14 shows how (U-^{14}C)glucose was incorporated into triacylglycerols, diacylglycerols and unesterified fatty acids by fat-cells incubated in media containing 5mM (U-^{14}C)glucose and various concentrations of acetate. The values obtained for the incorporation of ^{14}C-labelled glucose into triacylglycerols and diacylglycerols at zero acetate concentration both in the absence and presence of extracellular fatty acids, were similar to those obtained previously for the incorporation of 5mM (U-^{14}C)glucose (Fig. 3.13).

Acetate concentration had no significant effect on the incorporation of labelled glucose into any of the lipid classes studied, with triacylglycerols containing the highest proportion of incorporated radioactivity at all acetate concentrations. As found previously (Fig. 3.13), the inclusion of a fatty acid mixture in the incubation medium almost trebled the incorporation of (U-^{14}C)glucose into lipid, due mainly to increased incorporation into triacylglycerols.

These results suggest that fatty acid synthesis using acetylCoA derived from glucose is independent of the concentration of acetate available in the cytoplasm.

(ii) Incorporation of (U-^{14}C)Glucose into Fatty Acids and Glycerol. Since glucose is a precursor of both the fatty acid and glycerol moieties of acylglycerols, the incorporation of (U-^{14}C)-glucose into both was investigated.

When ^{14}C-labelled glucose was supplied on its own, more

Table 3.15. Incorporation of (U-^{14}C)Glucose (μmoles/h/100 μg DNA) into Fatty Acid and Glycerol Moieties of Acylglycerols by Adipocytes from 280 g Rats.

	(U-^{14}C)Glucose concn. (mM)	Fatty acid mixture[a]	Acylglycerol moiety	
			Fatty acid	Glycerol
triacylglycerols	10	-	0.34 ± 0.11	0.60 ± 0.03
		+	0.40 ± 0.11	3.54 ± 0.72
	20	-	0.46 ± 0.15	0.54 ± 0.10
		+	1.00 ± 0.60	3.48 ± 0.62
Diacylglycerols	10	-	0.11 ± 0.04	0.42 ± 0.05
		+	0.05 ± 0.02	1.06 ± 0.21
	20	-	0.09 ± 0.04	0.46 ± 0.12
		+	0.09 ± 0.03	1.12 ± 0.19
Phospholipids	10	-	0.09 ± 0.02	-
		+	0.25 ± 0.18	-
	20	-	0.13 ± 0.02	-
		+	0.45 ± 0.25	-

Incubation conditions were as described in the legend to Fig. 3.13. Results are means ± s.e.m. of four experiments.
a, the inclusion of a fatty acid mixture in the incubation is denoted by +

Table 3.16. Incorporation of (U-^{14}C)Glucose (μmoles/h/100 μg DNA) into Fatty Acid and Glycerol Moieties of Acylglycerols by Adipocytes from 280 g Rats in the Presence of Acetate.

	Acetate concn. (mM)	Fatty acid mixture	Acylglycerol moiety: Fatty acid	Acylglycerol moiety: Glycerol
Triacylglycerols	2.5	-	0.51 ± 0.21	0.86 ± 0.04
		+	0.59 ± 0.20	3.12 ± 0.63
	10.0	-	0.60 ± 0.25	0.81 ± 0.04
		+	0.55 ± 0.16	3.31 ± 0.69
Diacylglycerols	2.5	-	0.15 ± 0.08	0.55 ± 0.08
		+	0.06 ± 0.02	1.04 ± 0.20
	10.0	-	0.16 ± 0.08	0.59 ± 0.11
		+	0.05 ± 0.02	0.97 ± 0.12
Phospholipids	2.5	-	0.08 ± 0.03	-
		+	0.22 ± 0.16	-
	10.0	-	0.10 ± 0.03	-
		+	0.23 ± 0.15	-

Incubation conditions were the same as those described in the legend to Fig. 3.14. Results are means ± s.e.m. of four experiments.

radioactivity was recovered in the glycerol moiety than in the fatty acid moiety of triacyl- and diacylglycerols formed at glucose concentrations of 10 and 20mM (Table 3.15). The glycerol portion of the diacylglycerols contained 4 to 5 times as much incorporated ^{14}C-glucose as the fatty acid moiety, whereas in triacylglycerols the glycerol contained less than twice that recovered in the fatty acids.

The presence of fatty acids in the incubation medium apparently increased (U-^{14}C)glucose incorporation into the fatty acid moieties of triacylglycerols and phospholipids, but had no effect on diacylglycerol fatty acids. Glucose incorporation into the glycerol moiety was greatly increased, most notably into that of triacylglycerols. These results are consistent with extracellular fatty acids increasing the rate of triacylglycerol synthesis from diacylglycerols as suggested previously.

High extramitochondrial acetate concentrations did not appear to inhibit fatty acid synthesis from glucose-derived acetyl units, since the presence of acetate in the incubation medium at the concentrations examined did not significantly affect the amount of ^{14}C-labelled glucose incorporated by the adipocytes into either the fatty acid or glycerol moieties of triacylglycerols and diacylglycerols (Table 3.16). The incorporation of glucose into the glycerol moiety of total acylglycerols was increased when the incubation medium contained long-chain fatty acids, and these extracellular fatty acids only decreased the recovery of fatty acids formed de novo from glucose in diacylglycerols.

Overall, the distribution of incorporated ^{14}C-labelled glucose between the glycerol and acyl moieties of triacyl- and diacylglycerols was independent of glucose and acetate concentrations, and the presence of exogenous fatty acids corresponded to a net increase in glucose

Table 3.17. Incorporation of (U-^{14}C)Glucose (μmoles/h/100 μg DNA) into Total Lipid by Adipocytes from 150 g Rats. Effect of Glucose and Acetate Concentrations.

	(U-^{14}C)Glucose Alone	(U-^{14}C)Glucose + Fatty Acid Mixture
(U-^{14}C)Glucose concn. (mM)		
5	4.12 ± 0.25	10.44 ± 1.38
10	4.74 ± 0.43	9.31 ± 0.89
20	4.20 ± 0.06	12.95 ± 1.80
Acetate concn. (mM)		
0.5	4.81 ± 0.28	10.46 ± 0.65
2.5	5.02 ± 0.47	8.51 ± 1.16
10.0	6.93 ± 1.28	10.75 ± 1.74

The basic incubation medium contained either (U-^{14}C)glucose (0.17 μCi/μmole) at various concentrations, or 5mM (U-^{14}C)glucose of the same specific activity and various concentrations of sodium acetate. Other concurrent incubations contained in addition a 1mM fatty acid mixture of which (9,10-^{3}H)stearic acid (1 μCi) was a component. All incubations were of 1 hour's duration. Results are means ± s.e.m. of three experiments.

incorporation into the glycerol moiety of total acylglycerols. Glucose incorporation into the fatty acid moiety was not significantly affected by extracellular fatty acids. Fatty acids formed from (U-^{14}C)glucose at high acetate concentrations were located mainly in triacylglycerols, unlike those synthesized de novo from (1-^{14}C)acetate (Figs. 3.9 and 3.10).

2. Studies with Adipocytes from Rats of Average Weight 150 g

As in the above studies with adipocytes from 280 g rats, fat-cells isolated from younger animals were incubated in media containing (U-^{14}C)glucose of various concentrations, or with 5mM (U-^{14}C)glucose and acetate of various concentrations. In incubations where extracellular fatty acids were present, (9,10-^{3}H)stearic acid was added as part of the fatty acid mixture.

(i) Incorporation of (U-^{14}C)Glucose into Lipids. ^{14}C-Labelled glucose was used for lipogenesis by adipocytes from 150 g rats at a rate more than double that found previously with fat-cells from older rats.

The incorporation of carbon from (U-^{14}C)glucose into total lipid was independent of concentration, both when supplied on its own in the incubation medium and in the presence of a mixture of long-chain fatty acids (Table 3.17). Similarly, when the incubation medium contained acetate of various concentrations the incorporation of 5mM ^{14}C-glucose into total lipid was not significantly affected.

As was found in the studies with adipocytes from older animals, there was an increased incorporation of glucose into total lipid at all glucose and acetate concentrations when extracellular fatty acids were present in the medium.

Table 3.18. Incorporation of (U-^{14}C)Glucose (μmoles/h/100 μg DNA) into Lipids by Adipocytes from 150 g Rats. Effects of Fatty Acid Precursor Concentration and Extracellular Fatty Acids.

	(U-^{14}C)Glucose alone				(U-^{14}C)Glucose + Fatty Acid Mixture			
	TG	DG	PL	FFA	TG	DG	PL	FFA
(U-^{14}C)Glucose concn. (mM)								
5	1.89 ± 0.14	1.53 ± 0.15	0.55[a]	0.15 ± 0.03	7.85 ± 1.86	1.79 ± 0.36	0.44[a]	0.36 ± 0.05
10	2.32 ± 0.31	1.79 ± 0.10	0.50	0.13 ± 0.05	6.92 ± 0.83	1.71 ± 0.26	0.35	0.34 ± 0.05
15	1.66 ± 0.16	1.29 ± 0.11	1.10	0.16 ± 0.01	9.17 ± 1.02	2.86 ± 0.04	0.56	0.36 ± 0.07
Acetate concn. (mM)								
0.5	2.34 ± 0.18	1.61 ± 0.11	0.69	0.17 ± 0.01	7.60 ± 0.47	2.02 ± 0.37	0.51	0.33 ± 0.05
2.5	2.61 ± 0.35	1.57 ± 0.11	0.66	0.18 ± 0.01	5.41 ± 0.84	1.20 ± 0.06	0.40	0.31 ± 0.06
10.0	3.64 ± 0.99	2.32 ± 0.18	0.76	0.21 ± 0.03	8.21 ± 1.54	1.90 ± 0.10	0.31	0.33 ± 0.07

Incubation conditions were as described in the legend to Table 3.17. Results are means of three experiments. a, means of two experiments.

TG, triacylglycerols; DG, diacylglycerols; PL, phospholipids; FFA, unesterified fatty acids.

When the total lipids from incubations at two different glucose concentrations were separated into their constituent classes and their radioactivity measured, it was found that triacylglycerols contained most of the (U-^{14}C)glucose incorporated under the incubation conditions employed (Table 3.18). When supplied alone, the amount of ^{14}C-labelled glucose incorporated into triacylglycerols and diacylglycerols was independent of those glucose concentrations examined, with diacylglycerols containing almost as much incorporated glucose as triacylglycerols.

The inclusion of fatty acids in the incubation medium led to a large increase in the amount of ^{14}C-labelled glucose incorporated into triacylglycerols. However, the increase in glucose incorporation into diacylglycerols under the same conditions was much less noticeable and incorporation into phospholipids was actually diminished. The amount of unesterified fatty acids formed from (U-^{14}C)glucose doubled in the presence of extracellular fatty acids at all glucose concentrations. Very similar results were observed in incubations with 5mM (U-^{14}C)-glucose at various concentrations of acetate.

The presence of acetate at all concentrations studied in the incubation medium raised the amount of ^{14}C-glucose incorporated into triacylglycerols slightly in the absence of extracellular fatty acids, the labelled glucose concentration being 5mM.

(ii) Incorporation of (U-^{14}C)Glucose into Fatty Acids and Glycerol. The distribution of radioactivity from (U-^{14}C)glucose in fatty acid and glycerol moieties of triacylglycerols, diacylglycerols and phospholipids formed at various concentrations of glucose and acetate was determined. In contrast to the results obtained using

Table 3.19. Incorporation of (U-^{14}C)Glucose (μmoles/h/100 μg DNA) into Fatty Acid and Glycerol Moieties of Acylglycerols by Adipocytes from 150 g Rats. Effects of Glucose Concentration and Extracellular Fatty Acids.

	(U-^{14}C)Glucose concn. (mM)	Fatty acid mixture	Acylglycerol moiety	
			Fatty acid	Glycerol
Triacylglycerols	5	-	1.48	0.41
		+	3.00	4.84
	20	-	1.26	0.40
		+	4.31	4.86
Diacylglycerols	5	-	1.03	0.50
		+	0.68	1.12
	20	-	0.81	0.48
		+	1.10	1.76
Phospholipids	5	-	0.23	0.32
		+	0.09	1.01
	20	-	0.24	0.20
		+	0.15	0.41

Incubation conditions were as described in the legend to Table 3.17. Results are means of two experiments.
TG, triacylglycerols; DG, diacylglycerols; PL, phospholipids.

Table 3.20. Incorporation of (U-^{14}C)Glucose into Fatty Acid and Glycerol Moieties of Acylglycerols by Adipocytes from 150 g Rats. Effects of Acetate Concentration and Extracellular Fatty Acids.

	Acetate concn. (mM)	Fatty acid mixture	Acylglycerol moiety	
			Fatty acid	Glycerol
Triacylglycerols	0.5	-	1.87	0.48
		+	2.90	4.70
	10.0	-	3.01	0.63
		+	3.13	5.08
Diacylglycerols	0.5	-	1.12	0.49
		+	0.68	1.35
	10.0	-	1.69	0.63
		+	0.67	1.23
Phospholipids	0.5	-	0.25	0.44
		+	0.07	0.44
	10.0	-	0.33	0.43
		+	0.09	0.22

Incubation conditions were as described in the legend to Table 3.17. Results are means of two experiments.
TG, triacylglycerols; DG, diacylglycerols; PL, phospholipids.

older rats, the fatty acid moiety of triacyl- and diacylglycerols formed in the absence of extracellular fatty acids, contained more radioactivity incorporated from (U-^{14}C)glucose than did the glycerol moiety (Tables 3.19 and 3.20). A higher rate of fatty acid synthesis in adipocytes from younger rats was therefore evident.

The inclusion of long-chain fatty acids in the medium resulted in a greatly increased incorporation of ^{14}C-labelled glucose into the glycerol moiety of all acylglycerols especially triacylglycerols. In each instance the glycerol moiety contained more incorporated labelled substrate than the fatty acid moiety. However, although the amount of (U-^{14}C)glucose incorporated into the fatty acids of diacylglycerols and phospholipids was decreased in the presence of extracellular fatty acids, the amount of incorporated glucose recovered in triacylglycerol fatty acids increased. The overall synthesis of fatty acids de novo from glucose was increased when long-chain fatty acids were added to the incubation medium.

If the phospholipid is assumed to be phosphatidic acid, then the ratio of glucose incorporated into fatty acid to that incorporated into glycerol of phospholipids would be expected to be very similar to that obtained for diacylglycerols since the only difference between the two acylglycerols is a phosphate group. However, in the absence of extracellular fatty acids, the ratio for diacylglycerols was higher than that for phospholipids. The lipid extraction procedure used for these incubations involved washing with 1% (w/v) unlabelled glucose to aid the removal of any unincorporated (U-^{14}C)glucose which would contaminate the phospholipid fraction on T.L.C. of the lipid extract. However, glucose incorporation into other water-soluble products was still

Table 3.21. Percentage Distribution of Radioactivity from 5mM (U-^{14}C)-Glucose in Fatty Acids of Triacylglycerols and Diacylglycerols formed by Adipocytes from 150 g Rats.

	Fatty acid	(U-^{14}C) Glucose alone	(U-^{14}C) Glucose + fatty acid mixture
Triacylglycerols	12:0	0.3 ± 0.1	1.0[a]
	14:0	5.5 ± 0.7	10.7
	16:0	67.2[a])	)
		) 67.1 ± 1.3	) 64.7
	16:1	1.6[a])	)
	18:0	0.9 ± 0.3	3.2
	18:1	23.7 ± 3.8	17.9
	18:2	2.5 ± 2.9	2.5
Diacylglycerols	12:0	0.0	0.0
	14:0	4.2 ± 0.4	6.7 ± 0.6
	16:0	69.6 ± 1.5	64.5 ± 5.7
	16:1	7.1 ± 1.1	2.7 ± 0.6
	18:0	3.4 ± 0.3	14.5 ± 1.7
	18:1	15.1 ± 0.6	10.2 ± 1.2
	18:2	0.6 ± 0.4	1.4 ± 1.9

Incubation conditions were as described in the legend to Table 3.17. Results are means ± s.e.m. of three experiments.
a, means of two experiments.

possible and therefore the values obtained for phospholipid glycerol are liable to be less than those reported in Tables 3.19 and 3.20.

Fatty acid synthesis from (U-^{14}C)glucose in the absence of extracellular fatty acids was apparently greatest when the incubation medium contained 10mM acetate.

(iii) Distribution of Radioactivity from (U-^{14}C)Glucose in the Fatty Acids of Triacylglycerols and Diacylglycerols. The spectra of fatty acids formed from (U-^{14}C)glucose and found in the triacyl- and diacylglycerols were determined by preparative G.L.C. analysis of their methyl esters with subsequent measurement of the radioactivity present in each fatty acid component. This permitted comparison of the nature and composition of the fatty acids formed by adipocytes from glucose or acetate as precursors.

The pattern of fatty acids in triacyl- and diacylglycerols formed from (U-^{14}C)glucose present at a concentration of 5mM in the incubation medium, is presented in Table 3.21. In triacylglycerols, the fatty acids synthesized from glucose were broadly similar to those formed when 0.5mM (1-^{14}C)acetate was the precursor (Table 3.10), although in the absence of extracellular fatty acids, more stearic acid was apparently synthesized from (1-^{14}C)acetate. When a fatty acid mixture was present in the incubation medium an increased proportional incorporation of glucose into myristic and stearic acids with decreased incorporation into oleic acid resulted. Any effect on the relative proportions of palmitic and palmitoleic acids could not be distinguished clearly because of practical difficulties. These findings differed from the results obtained with rats of the same weight when (1-^{14}C)acetate was used as ^{14}C-labelled substrate, in which instance, the presence of

extracellular fatty acids increased acetate incorporation into palmitic but not stearic acid, in the triacylglycerols.

In diacylglycerols, fatty acids originating from (U-^{14}C)glucose were again rather similar to those synthesized from (1-^{14}C)acetate. However, less palmitic acid and more oleic acid was formed than when acetate was the precursor.

Free fatty acids added initially to the incubation medium had a similar effect on the pattern of fatty acids synthesized de novo from (U-^{14}C)glucose and found in diacylglycerols as they had in the related studies with ^{14}C-labelled acetate, namely to increase the proportion of newly-synthesized myristate and stearate whilst decreasing that of oleate.

G. Incubations with $^{3}H_2O$

Whereas fatty acid synthesis from (1-^{14}C)acetate was found to be inhibited when long-chain fatty acids were present in the incubation medium, the incorporation of (U-^{14}C)glucose into fatty acids by adipocytes from 280 g rats was not significantly affected under similar circumstances. To establish the overall effects of exogenous fatty acids and fatty acid precursor concentrations on fatty acid synthesis de novo, experiments were carried out in which adipocytes, prepared from rats weighing in the region of 280 g, were incubated with $^{3}H_2O$ which is believed to be a more reliable indicator of lipogenesis (248).

1. Incorporation of $^{3}H_2O$ into Lipids

When fat-cells were incubated in the basic medium containing $^{3}H_2O$

Table 3.22. Incorporation of ^3H_2O (μmoles/h/100 μg DNA) into Lipids by Adipocytes from 280 g Rats.

	^3H_2O alone					^3H_2O + Fatty acid mixture				
	TG	DG	PL	FFA	TOTAL	TG	DG	PL	FFA	TOTAL
Glucose concn. (mM)										
0	0.34	0.31	0.14	0.01	0.80	1.17	0.51	0.18	0.05	1.91
10	0.58	0.53	0.29	0.03	1.43	2.67	1.06	0.17	0.07	3.97
20	0.62	0.63	0.18	0.03	1.46	2.91	1.16	0.30	0.12	4.49
Acetate concn. (mM)										
0	0.64	0.59	0.27	0.06	1.56	2.76	1.09	0.22	0.07	4.14
2.5	0.77	0.68	0.29	0.08	1.82	2.76	1.03	0.45	0.11	4.35
10.0	0.65	0.55	0.27	0.04	1.51	2.52	0.98	0.26	0.06	3.82

The basic incubation medium, to which various concentrations of glucose or acetate had been added, contained ^3H_2O (1 mCi/ml). In incubations containing acetate, glucose was also present at a concentration of 5mM. Concurrent incubations contained in addition a 1mM mixture of unlabelled fatty acids. All incubations were carried out for 1 hour. Results are means of two experiments which agreed closely.

TG, triacylglycerols; DG, diacylglycerols; PL, phospholipids; FFA free fatty acids.

Table 3.23. Incorporation of $^{3}H_2O$ (μmoles/h/100 μg DNA) into Fatty Acid and Glycerol Moieties of Total Lipid.

Glucose concn. (mM)	Fatty acid mixture	Fatty acid	Glycerol
0	-	0.08	0.72
	+	0.05	1.86
10	-	0.23	1.20
	+	0.11	3.86
20	-	0.32	1.15
	+	0.11	4.38
Acetate concn. (mM)			
0	-	0.22	1.34
	+	0.13	4.00
2.5	-	0.40	1.42
	+	0.13	4.22
10.0	-	0.29	1.21
	+	0.17	3.65

Incubation conditions were as described in the legend to Table 3.22. Results are means of two experiments.

on its own, the diacylglycerols produced contained almost as much incorporated ^{3}H as triacylglycerols at all glucose and acetate concentrations (Table 3.22). The presence of fatty acids in the incubation medium led to an increase in the amount of $^{3}H_2O$ incorporated into total acylglycerols. This overall increase could be attributed mainly to a four fold increase in $^{3}H_2O$ incorporation into triacylglycerols, coupled with a doubling of that incorporated into diacylglycerols.

When glucose was omitted from the incubation medium, the incorporation of $^{3}H_2O$ into lipids was poor in comparison with its incorporation at 10 and 20mM glucose. Acetate concentration had no appreciable effect on $^{3}H_2O$ incorporation into lipid, although its incorporation at 2.5mM was greater than at the other two concentrations of acetate examined, in the absence of extracellular fatty acids.

2. Incorporation of $^{3}H_2O$ into Fatty Acids and Glycerol

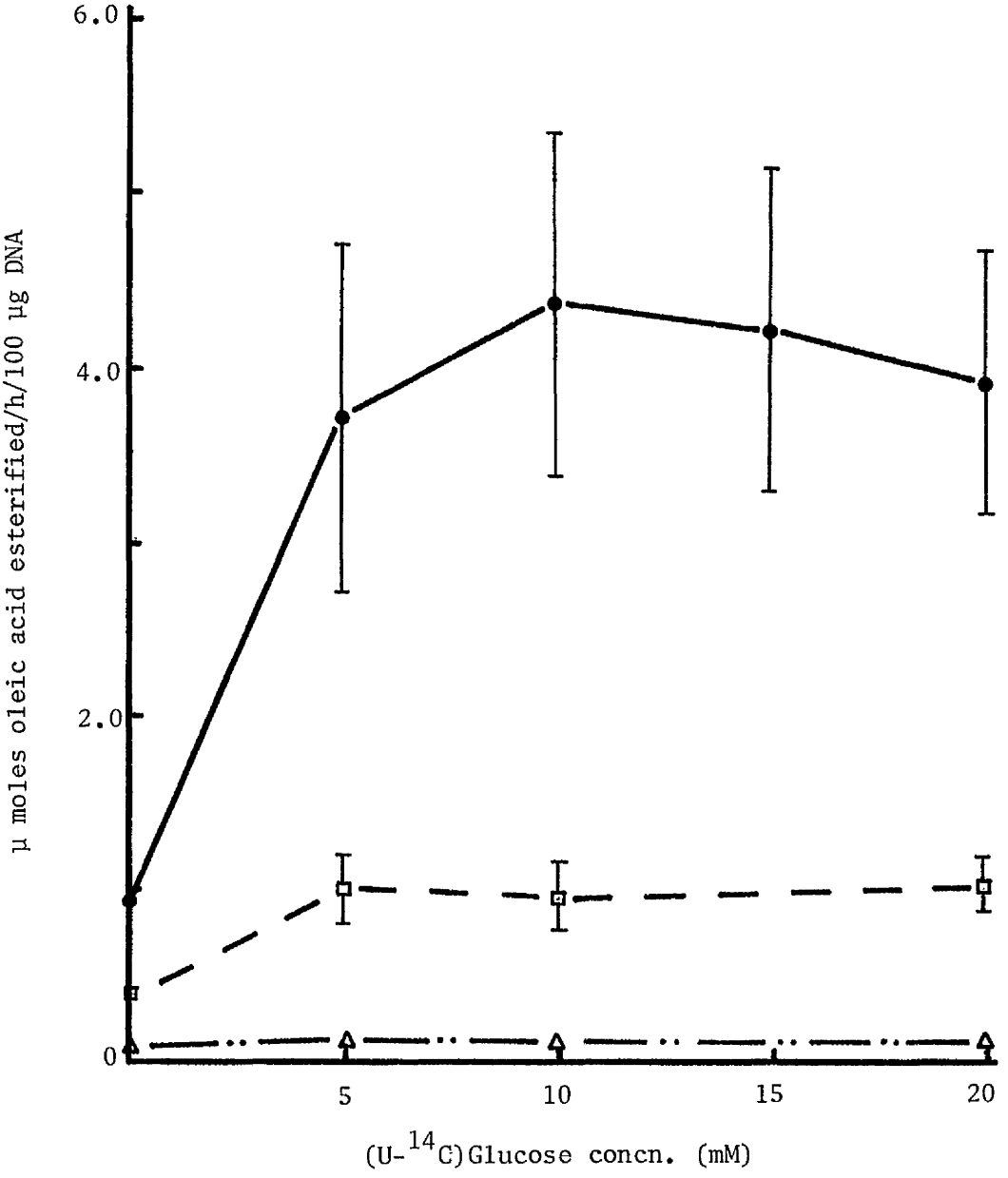
10 mg of the lipid extract from each incubation was methylated. On extraction of the methyl esters, the radioactivity of the organic and aqueous layers were measured to determine how much $^{3}H_2O$ had entered the fatty acid and glycerol moieties of acylglycerols.

A diminished $^{3}H_2O$ incorporation into acylglycerol-fatty acids was noticeable in all incubations containing extracellular fatty acids, suggesting the inhibition of fatty acid synthesis de novo within the fat-cell by exogenous fatty acids. Conversely, under the same incubation conditions, the incorporation of $^{3}H_2O$ into glyceride-glycerol was at least doubled (Table 3.23). Fatty acid synthesis, as reflected by $^{3}H_2O$ incorporation, was apparently independent of acetate concentration. However, in the absence of extracellular fatty acids, the incorporation of $^{3}H_2O$ into fatty acids at 20mM glucose was higher than

Fig. 3.15. Esterification of (9,10-^{3}H)Oleic Acid into Acylglycerols by Adipocytes from 280 g Rats. Effect of Glucose Concentration.

Experimental conditions were as described in the legend to Fig. 3.13B, with (9,10-^{3}H)oleic acid (1 μCi) present in the incubation medium. Results are means ± s.e.m. of four experiments.

●——●, triacylglycerols; □— —□, diacylglycerols; Δ— ·· —Δ, phospholipids.


6.0
4.0
2.0
0
μ moles oleic acid esterified/h/100 μg DNA
5
10
15
20
(U-^{14}C)Glucose concn. (mM)

that at 10mM, and when the incubation medium contained no glucose, very little $^{3}H_2O$ was used in fatty acid synthesis.

The amount of $^{3}H_2O$ recovered in glycerol increased as glucose concentration increased when the incubation medium contained long-chain fatty acids. No such effect was noticeable with acetate concentration. This might indicate that the production of glycerol 3-phosphate for use in the esterification of exogenous fatty acids may be related to the amount of glucose available to the fat-cell.

H. Effect of Glucose and Acetate Concentrations on the Esterification of Extracellular Fatty Acids

1. Esterification of Oleic Acid into Acylglycerols

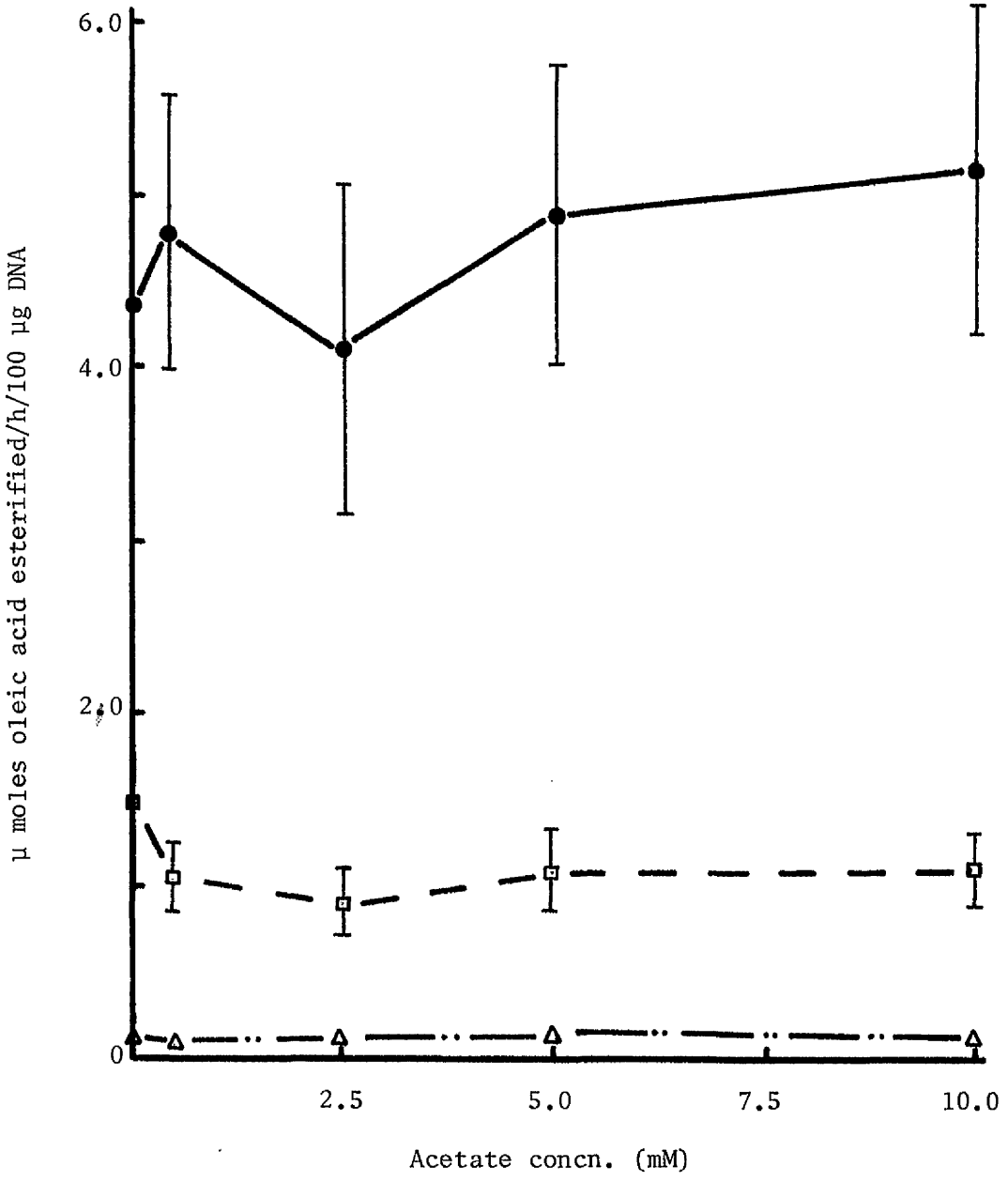
In experiments with adipocytes prepared from rats of average weight 280 g on lipogenesis from (U-^{14}C)glucose, the fatty acid mixture present in the concurrent incubations contained (9,10-^{3}H)oleic acid as a component. This allowed the esterification of oleic acid from the incubation medium to be assessed in the presence of glucose at several concentrations, thereby giving an indication of the fat-cell's ability to esterify exogenous fatty acids into acylglycerols in the presence of high concentrations of a precursor of endogenous fatty acids and acylglycerol-glycerol.

The amount of ^{3}H-labelled oleic acid esterified into total acylglycerols in the absence of glucose from the medium was only 25% of that recovered in acylglycerols at all other glucose concentrations (Fig. 3.15), showing the adipocyte's requirement for glucose in the uptake and esterification of extracellular fatty acids.

Fig. 3.16. Esterification of (1-^{14}C)Oleic Acid into Acylglycerols by Adipocytes from 280 g Rats. Effect of Acetate Concentration.

The incubation medium contained in addition to the basic ingredients sodium acetate at various concentrations and (1-^{14}C)-oleic acid (0.6 μCi) as part of an albumin-bound fatty acid mixture of concentration 1mM. All incubations were of 1 hour's duration. Results are means ± s.e.m. of three experiments.

●——●, triacylglycerols; □— —□, diacylglycerols; Δ— ·· —Δ, phospholipids.


6.0
4.0
2.0
0
μ moles oleic acid esterified/h/100 μg DNA
2.5
5.0
7.5
10.0
Acetate concn. (mM)

The results presented earlier in Fig. 3.10 suggested that conditions of high acetate concentration in the presence of exogenous fatty acids could perhaps reduce the activity of diacylglycerol acyltransferase since incorporated ^{14}C-labelled acetate accumulated in the fatty acids of diacylglycerols under such conditions. In an attempt to establish whether such an explanation was valid, adipocytes were incubated with (1-^{14}C)oleic acid supplied as part of a fatty acid mixture in media containing acetate at various concentrations. The incorporation of the labelled fatty acid into acylglycerols was measured.

As Fig. 3.16 demonstrates, the uptake and esterification of (1-^{14}C)oleic acid was unaffected by the concentration of acetate in the incubation medium. Triacylglycerols contained approximately 80% of the oleic acid esterified at all concentrations of acetate with diacylglycerols and phospholipids accounting for smaller proportions (19% and 15% respectively on average). The absolute values at zero acetate concentration compare well with those presented in Fig. 3.15 for the rate of esterification of oleic acid in the presence of 5mM glucose on its own.

Therefore, unlike fatty acids formed de novo within the adipocyte from acetate, oleic acid taken up by the fat-cell from the incubation medium did not accumulate in diacylglycerols at high acetate concentration but was esterified predominantly into triacylglycerols.

2. Stereospecific Distribution of Esterified (1-^{14}C)Oleic Acid in Triacylglycerols

The distribution of esterified (9,10-^{3}H)oleic acid among the three positions of triacyl-sn-glycerols formed at various concentrations of glucose, was not determined since the incubation medium had contained

Table 3.24. Relative Incorporation of (1-^{14}C)Oleic Acid into the Three Positions of Triacyl-sn-glycerols by Adipocytes. Effect of Acetate Concentration.

Acetate concn. (mM)	Proportional (%) distribution in each position		
	Position sn-1	Position sn-2	Position sn-3
0[a]	22.3	45.2	32.5
0.5	27.5	40.5	32.0
2.5	29.5	40.7	29.8
10.0	27.6	38.9	33.5

Incubation conditions were the same as described in the legend to Fig. 3.16. Results are means of two experiments.
a, values taken from Table 3.4.

(U-^{14}C)glucose which was also incorporated into triacylglycerols. The use of a ^{14}C-labelled synthetic triacylglycerol as an internal standard in the stereospecific analysis procedure was therefore impossible. The stereospecific distribution of esterified exogenous oleic acid in the triacylglycerols formed by fat-cells in the presence of 5mM glucose had already been determined (Table 3.4).

Table 3.24 shows the manner in which (1-^{14}C)oleic acid taken up from incubation media containing various concentrations of acetate, was distributed over the triacylglycerol molecule. Acetate concentration had no appreciable effect on the positioning of extracellular oleic acid within triacylglycerols.

3. Esterification of (9,10-^{3}H)Stearic Acid into Acylglycerols

In the studies on lipogenesis from glucose and acetate using adipocytes prepared from rats of approximate weight 150 g, the incorporation of ^{3}H-labelled fatty acid into acylglycerols was also investigated. In those incubations containing a fatty acid mixture, (9,10-^{3}H)stearic acid was added as a component for the purpose of measuring the esterification of extracellular fatty acids by fat-cells, at various concentrations of glucose and acetate. In addition, it was intended to study the desaturation of the labelled stearic acid, and the esterification of the resulting ^{3}H-labelled oleic acid into triacylglycerols. However, since half the ^{3}H label from (9,10-^{3}H)stearic acid would be removed from the fatty acid by the desaturase enzyme resulting in lower levels of radioactivity than desired, the formation of ^{3}H-labelled oleic acid from ^{3}H-stearic acid was not investigated to any great extent. In the few instances where ^{3}H-labelled oleic acid was separated and measured (20mM glucose and 10mM acetate), the

Table 3.25. Esterification of (9,10-^{3}H)Stearic Acid (μmoles/h/100 μg DNA) into Acylglycerols. Effect of Glucose and Acetate Concentrations

	Acylglycerol		
	Triacylglycerols	Diacylglycerols	Phospholipids
Glucose concn. (mM)			
5	1.19 ± 0.26	0.23 ± 0.03	0.06 ± 0.02
10	0.97 ± 0.19	0.20 ± 0.02	0.02 ± 0.01
20	1.25 ± 0.27	0.21 ± 0.04	0.04 ± 0.01
Acetate concn. (mM)			
0.5	1.04 ± 0.18	0.21 ± 0.02	0.05 ± 0.01
2.5	1.12 ± 0.24	0.21 ± 0.03	0.08 ± 0.01
5.0	1.14 ± 0.26	0.21 ± 0.03	0.07 ± 0.03
10.0	1.13 ± 0.27	0.21 ± 0.04	0.05 ± 0.01

Experimental conditions were as described in the legend to Table 3.17 with the incubation medium containing (9,10-^{3}H)stearic acid (1 μCi). Results are means ± s.e.m. of 3 experiments.

proportions of total ^{3}H-oleic acid recovered in acylglycerols were very similar to those obtained previously (Fig. 3.7) after 1 hour's incubation.

The amount of (9,10-^{3}H)stearic acid taken up by the fat-cells from the incubation medium and esterified into acylglycerols was independent of glucose and acetate concentrations over the range examined (Table 3.25).

At all concentrations of glucose or acetate, the amount of stearic acid esterified into all acylglycerols was less than half that recorded for oleic acid in similar incubation conditions but with adipocytes prepared from older rats. This could indicate that either the esterification rate of extracellular fatty acids in general was lower in fat-cells from younger rats, or that stearic acid was esterified into total acylglycerols by adipocytes at a lower rate than oleic acid, regardless of the animal's age. From the kinetic studies described in section B of this chapter, it appeared that the former conclusion was more likely.

I. Analysis of Diacylglycerols formed by Adipocytes

The possibility existed that some biosynthetic or degradative route other than the sn-glycerol 3-phosphate pathway was responsible for the production of the highly-labelled diacylglycerols in adipocytes from older rats incubated with high concentrations of (1-^{14}C)acetate as seen in Fig. 3.10. Use was therefore made of the fact that the sn-glycerol 3-phosphate pathway of triacylglycerol biosynthesis involves 1,2-diacyl-sn-glycerols exclusively as intermediates. Diacylglycerols formed in adipocytes incubated with (U-^{14}C)glucose or (1-^{14}C)acetate

Table 3.26. Percentage Distribution of Radioactivity from (U-^{14}C) Glucose and (1-^{14}C)Acetate in Diacyl-sn-glycerols formed by Adipocytes from 190 g Rats.

		Diacyl-sn-glycerols			
	Fatty acid mixture	1,3	2,3	1,2	
				position sn-1	position sn-2
(U-^{14}C) Glucose concn. (mM)					
5	-	5.8	2.5	68.3	23.4
	+	3.3	3.1	84.7	8.9
20	-	2.7	5.2	71.2	20.9
	+	3.7	2.9	86.9	6.5
(1-^{14}C) Acetate concn (mM)					
0.5	-	3.6	6.8	56.8	32.8
	+	4.8	6.0	70.7	18.5
10.0	-	2.6	6.5	59.1	31.8
	+	6.2	9.2	64.9	19.7

Adipocytes were prepared from three rats of mean weight 190 g. (1-^{14}C)Acetate (10 μCi) was included in the basic incubation medium at concentrations of 0.5 and 10mM, or (U-^{14}C)glucose (0.5 μCi/μmole) at 5 and 20mM. Similar concurrent incubations contained in addition an unlabelled fatty acid mixture of concentration 1mM. All incubations were of 1 hour's duration, and the results are means of duplicate incubations.

Table 3.27. Percentage Distribution of Radioactivity from (U-^{14}C)-Glucose and (1-^{14}C)Acetate in Diacyl-sn-glycerols formed by Adipocytes from 290 g Rats.

		Diacyl-sn-glycerols			
	Fatty acid mixture	1,3	2,3	1,2	
				position sn-1	position sn-2
(U-^{14}C)Glucose concn. (mM)					
5	-	2.3	3.7	66.8	27.2
	+	2.1	3.1	92.4	2.4
20	-	3.8	1.9	71.5	22.8
	+	3.2	4.1	84.1	8.6
(1-^{14}C)Acetate concn. (mM)					
0.5	-	2.6	2.8	72.7	21.9
	+	4.8	2.8	71.4	21.0
10.0	-	1.6	5.1	72.2	21.1
	+	5.9	9.4	61.8	22.9

Adipocytes were prepared from three rats of mean weight 290 g, and used in incubations as described in the legend to Table 3.26. Results are means of duplicate incubations.

were extracted and analysed as described in Chapter 2, section F2, to determine which two positions of the sn-glycerol moiety contained the incorporated ^{14}C-labelled substrate.

The radioactivity incorporated into diacylglycerols from (U-^{14}C)-glucose and (1-^{14}C)acetate was recovered predominantly in 1,2-diacyl-sn-glycerols under all incubation conditions examined, with adipocytes isolated from both 190 and 290 g rats (Tables 3.26 and 3.27). The proportion of radioactivity recovered in 1,3- and 2,3-diacyl-sn-glycerols was small in comparison with that in the 1,2- species and possibly arose from isomerisation of the 1,2-diacyl-sn-glycerols during the lipid extraction procedure, although the extraction solvent contained no methanol and the temperature was maintained below 4°C in an attempt to minimize such rearranging of fatty acids. Incomplete hydrolysis of the phospholipid intermediate by phospholipase A during the stereospecific analysis procedure could also contribute to the radioactivity assumed to be associated with the 2,3-diacyl-sn-glycerols.

In every instance, the presence of a fatty acid mixture in the incubation medium led to an increase in the proportion of incorporated ^{14}C-labelled precursor recovered in position sn-1 of the 1,2-diacyl-sn-glycerols. This was in keeping with the earlier results showing increased incorporation of ^{14}C-labelled fatty acid precursors into the saturated fatty acids of diacylglycerols coupled with decreased incorporation into oleic acid, in the presence of extracellular fatty acids, since position sn-1 of rat adipocyte triacylglycerols normally contains a high proportion of saturated fatty acids.

The proportion of fatty acids synthesized from (1-^{14}C)acetate supplied on its own and recovered in position sn-1 of 1,2-diacyl-sn-

glycerols formed by adipocytes from 290 g rats, was higher than that recovered in the same position of diacyl-sn-glycerols formed under similar conditions by adipocytes from younger rats. Again this agreed with the earlier result that diacylglycerols formed by adipocytes from 280 g rats contained a higher proportion of saturated fatty acids newly-synthesized from (1-^{14}C)acetate than did diacylglycerols formed by fat-cells from younger animals.

J. Distribution of Newly-Synthesized Lipids between Cytoplasmic and Particulate Components of Adipocytes

Results obtained with adipocytes from older rats showed that diacylglycerols accumulated fatty acids formed de novo from acetate, but not exogenous fatty acids, when the incubation medium contained a high concentration of acetate. One possible explanation was that diacylglycerols could exist within the fat-cell in association with particular organelles. Diacylglycerols in one particular site within the cell could contain entirely fatty acids formed de novo and diacyl-glycerols in another separate location could be involved in the esterification of exogenous fatty acids.

To examine the possible intracellular location of diacylglycerols, adipocytes were incubated either with ^{14}C-labelled glucose or acetate, or with (1-^{14}C)palmitic acid. After one hour's incubation the adipocytes were treated with digitonin in MOPS buffer and centrifuged as detailed in Chapter 2, section B3. The distribution of radioactivity incorporated into the lipids associated with the cytoplasm and particulate components was examined. The success of this method depended

Table 3.28. Distribution of Radioactivity from Incorporated (1-^{14}C)-Acetate in Lipids of Adipocytes after Treatment with Digitonin and Centrifugation.

Adipocytes were prepared from three rats of mean weight 293 g. The basic incubation medium, scaled up to accommodate 4 ml of adipocyte suspension, contained either 0.5 or 5.0mM (1-^{14}C)acetate (40 µCi). In concurrent incubations a 1mM unlabelled fatty acid mixture was also present, and all incubations were for 1 hour. Results are those obtained with individual incubations.

TG, triacylglycerols; DG, diacylglycerols; PL, phospholipids; FFA unesterified fatty acids.

	(1-^{14}C)Acetate concn. (mM)	Fatty acid mixture	Total radioactivity in lipid (d.p.m.)	Proportion (%) of Total Radioactivity in			
				TG	DG	PL	FFA
FAT PLUG	0.5	-	140,548	60.9	34.6	4.0	0.5
		+	142,125	76.1	21.4	1.9	0.6
	5.0	-	236,259	60.4	33.9	5.0	0.7
		+	92,881	65.5	30.5	3.4	0.6
INTERNATANT LAYER	0.5	-	21,516	60.3	30.9	7.5	1.3
		+	15,605	78.5	15.2	3.9	2.5
	5.0	-	14,981	64.8	28.4	5.4	1.3
		+	12,545	61.5	30.4	6.4	1.8
PELLET	0.5	-	1,150	57.0	23.0	15.8	4.2
		+	1,478	64.8	20.2	9.9	5.1
	5.0	-	1,458	58.4	22.1	13.3	6.2
		+	1,678	53.5	25.3	12.2	9.0

Table 3.29. Distribution of Radioactivity from Incorporated (U-^{14}C)-Glucose in Lipids of Adipocytes after Treatment with Digitonin and Centrifugation.

Experimental conditions were the same as described in the legend to Table 3.28 with the exception that the medium contained either 5 or 20mM (U-^{14}C)glucose (40 μCi). Results are values obtained with single incubations.

TG, triacylglycerols; DG, diacylglycerols; PL, phospholipids; FFA, unesterified fatty acids.

	(U-^{14}C)Glucose concn. (mM)	Fatty acid mixture	Total radioactivity in lipid (d.p.m.)	Proportion (%) of Total Radioactivity in			
				TG	DG	PL	FFA
FAT PLUG	5	-	158,954	55.0	39.7	4.9	0.4
		+	388,371	67.5	30.0	2.1	0.4
	20	-	126,112	55.2	39.0	5.3	0.5
		+	129,019	66.3	31.0	2.5	0.3
INTERNATANT LAYER	5	-	8,549	56.1	32.6	9.6	1.7
		+	32,488	69.4	25.3	3.7	1.6
	20	-	4,221	55.8	28.7	13.3	2.2
		+	6,064	68.3	23.5	6.4	1.8
PELLET	5	-	1,002	36.2	30.2	23.9	9.7
		+	1,815	62.0	24.1	9.3	4.6
	20	-	3,870	57.7	16.1	6.6	19.6
		+	2,006	51.9	27.2	6.3	14.6

on newly-synthesized acylglycerols remaining in association with their intracellular site of synthesis during fractionation of the fat-cell. The procedure used was chosen because it did not involve any vigorous homogenisation that might enhance the removal of newly-synthesized lipids from their place of formation, and was completed in a very short time (approximately 1 $^{1}/2$ minutes).

Table 3.28 shows that diacylglycerols containing fatty acids synthesized from (1-^{14}C)acetate were not associated specifically with either the fat plug, internatant layer or pellet. A similar result was found when (U-^{14}C)glucose was the precursor for endogenous fatty acid synthesis (Table 3.29). Most of the radioactivity incorporated into lipid from both ^{14}C-labelled glucose and acetate was recovered in the fat plug with the internatant layer containing much less. The pellet contained in general only about 1% of the radioactivity present in the fat plug.

In all three fractions, triacylglycerols accounted for the highest proportion of radioactivity incorporated into lipid, and similar relative distributions among lipid classes were found in each fraction, although with (1-^{14}C)acetate as precursor the pellet did apparently contain a slightly lower proportion of labelled triacylglycerols. The proportion of radioactivity recovered in phospholipids and unesterified fatty acids was consistently higher in the pellet fraction than in the internatant layer and fat plug, when the adipocytes were incubated with (1-^{14}C)acetate. A very similar pattern was found when (U-^{14}C)-glucose was the fatty acid precursor.

Contrary to the results obtained previously in studies with adipocytes from rats of similar weight, diacylglycerols did not contain as high a proportion of incorporated 5mM (1-^{14}C)acetate as triacyl-

Table 3.30. Distribution of Incorporated (1-^{14}C)Palmitic Acid in Lipids of Adipocytes after Treatment with Digitonin and Centrifugation.

	Total radioactivity in lipid (d.p.m.)	Proportion (%) of total radioactivity in			
		TG	DG	PL	FFA
FAT PLUG	213,548	74.6	22.9	2.1	0.5
INTERNATANT LAYER	4,923	68.7	19.2	5.8	6.3
PELLET	861	60.9	25.3	9.4	4.4

(1-^{14}C)Palmitic acid (0.83 μCi/μmole), included in the basic incubation medium as a component of a 1mM fatty acid mixture, was incubated with 4 ml of an adipocyte suspension prepared from three rats of mean weight 293 g, for 1 hour. Results are values obtained with a single incubation.

TG, triacylglycerols; DG, diacylglycerols; PL, phospholipids; FFA, unesterified fatty acids.

Table 3.31. Distribution of Radioactivity from (1-^{14}C)Acetate and (U-^{14}C)Glucose in Lipids of Adipocytes after Treatment with Digitonin and Centrifugation. Effect of Short Incubation Times.

	Time (s)	Total radioactivity in lipid (d.p.m.)	Proportion (%) of total radioactivity in TG	DG	PL	FFA
(1-^{14}C)Acetate						
Fat plug + internatant layer	30	25,454	54.0	32.2	8.7	5.1
	60	43,508	71.1	18.8	5.8	4.3
Pellet	30	1,467	31.3	34.0	8.2	26.5
	60	2,232	19.1	43.1	19.9	17.8
(U-^{14}C)Glucose						
Fat plug + internatant layer	30	27,307	63.8	19.2	11.6	5.4
	60	26,104	62.8	23.2	8.4	5.6
Pellet	30	2,354	21.6	44.8	18.8	14.8
	60	1,590	26.2	29.6	23.9	20.3

4 ml of an adipocyte suspension, prepared from two rats of mean weight 129 g, were incubated in a scaled-up basic medium with either 5mM (1-^{14}C)acetate (0.67 μCi/μmole) or 5mM (U-^{14}C)glucose (1.3 μCi/μmole) for 30 or 60 seconds. Results are values obtained with single incubations.

TG, triacylglycerols; DG, diacylglycerols; PL, phospholipids; FFA, unesterified fatty acids.

Table 3.32. Distribution of Radioactivity from (1-^{14}C)Palmitic Acid in Acylglycerols of Adipocytes after Treatment with Digitonin and Centrifugation. Effect of Short Incubation Times.

	Time (s)	Total radioactivity in lipid (d.p.m.)	Proportion (%) of total radioactivity in TG	DG	PL
Fat plug + internatant layer	30	15,332	72.3	22.2	5.5
	60	68,093	80.5	16.5	3.0
Pellet	30	936	42.8	43.1	14.1
	60	1,547	62.4	29.1	8.5

(1-^{14}C)Palmitic acid (1.2 μCi/μmole), included in a scaled-up basic medium as a component of a fatty acid mixture, was incubated for 30 or 60 seconds with 4 ml of adipocytes prepared from 129 g rats. Results are those obtained with single incubations.

TG, triacylglycerols; DG, diacylglycerols; PL, phospholipids.

glycerols, even in the presence of extracellular fatty acids, although the proportion of radioactivity recovered in the diacylglycerols was still considerable. However, since these results were obtained with fat-cells from a single rat, the significance of this finding is uncertain.

Most of the (1-^{14}C)palmitic acid taken up by the adipocytes was found in the fat plug on centrifugation (Table 3.30). The internatant layer contained only about 2% of the total radioactivity recovered as cell lipid, and the pellet an almost negligible 0.4%. Again, triacylglycerols always contained the highest proportion of incorporated ^{14}C-labelled substrate.

Whether the small values obtained for the amounts of radioactivity present in the lipids of the pellet and internatant layer were truly representative or simply a reflection of contamination by radioactivity from the fat plug is uncertain. Conversely, the fat plug may also have been contaminated by lipids from the pellet and internatant layer.

In a similar experiment, adipocytes prepared from two rats of mean weight 129 g were incubated with the same ^{14}C-labelled substrates used in the previous experiment, for only 30 or 60 seconds. When such fat-cells were treated with digitonin and centrifuged, only a very thin fat plug, which was physically inseparable from the internatant layer, was obtained because of the low lipid content of adipocytes from rats of this weight. For this reason, the fat plug and internatant layer were taken together for lipid extraction. This combined lipid extract contained most of the radioactivity incorporated from all three ^{14}C-labelled substrates, the highest proportion being recovered in triacylglycerols (Tables 3.31 and 3.32). In the pellet, the proportion of

incorporated (1-^{14}C)acetate and (U-^{14}C)glucose recovered in diacylglycerols exceeded that in triacylglycerols. However, since the total amount of radioactivity recovered in the pellet was low, the significance of this finding is uncertain.

The results of these adipocyte-fractionation experiments did not warrant further characterisation of the fat plug, internatant layer and pellet fractions by enzyme marker studies. It was assumed, however, that the pellet contained mitochondria and perhaps microsomes, with the adipocyte's soluble cytoplasm being present in the internatant layer. The fat plug undoubtedly represented the fat-cell's lipid storage droplet and probably contained newly-synthesized diacylglycerols and phospholipids dislodged from their intracellular site of synthesis.

K. High Pressure Liquid Chromatography

As an alternative to silver nitrate T.L.C. with subsequent elution of bands for scintillation counting, reversed-phase H.P.L.C. with a μ Bondapak C-18 column was investigated as a rapid means of separating (1-^{14}C)stearic acid and (1-^{14}C)oleic acid formed from it preparatively.

Preliminary trials in which the ratio of methanol to water in the mobile phase and the solvent flow rate were varied, revealed that methanol: water (90:10, v/v) with a flow rate of 2 ml/minute was the most suitable solvent system for the separation of standard mixtures of fatty acid methyl esters. A higher proportion of water in the solvent resulted in poor resolution of the components, whereas a solvent containing only 5% water gave good resolution but only at very slow flow rates resulting in impractical long retention times.

Fig. 3.17. H.P.L.C. of (1-^{14}C)Fatty Acid Methyl Esters. Radioactivity in Column Eluent Superimposed on Mass Trace.

H.P.L.C. of a standard mixture of (1-^{14}C)methyl oleate and (1-^{14}C)methyl stearate. The solvent was methanol:water (90:10 v/v) at a flow rate of 2 ml/min.

I denotes the point of sample injection. Peaks are 1, methyl oleate and 2, methyl stearate.

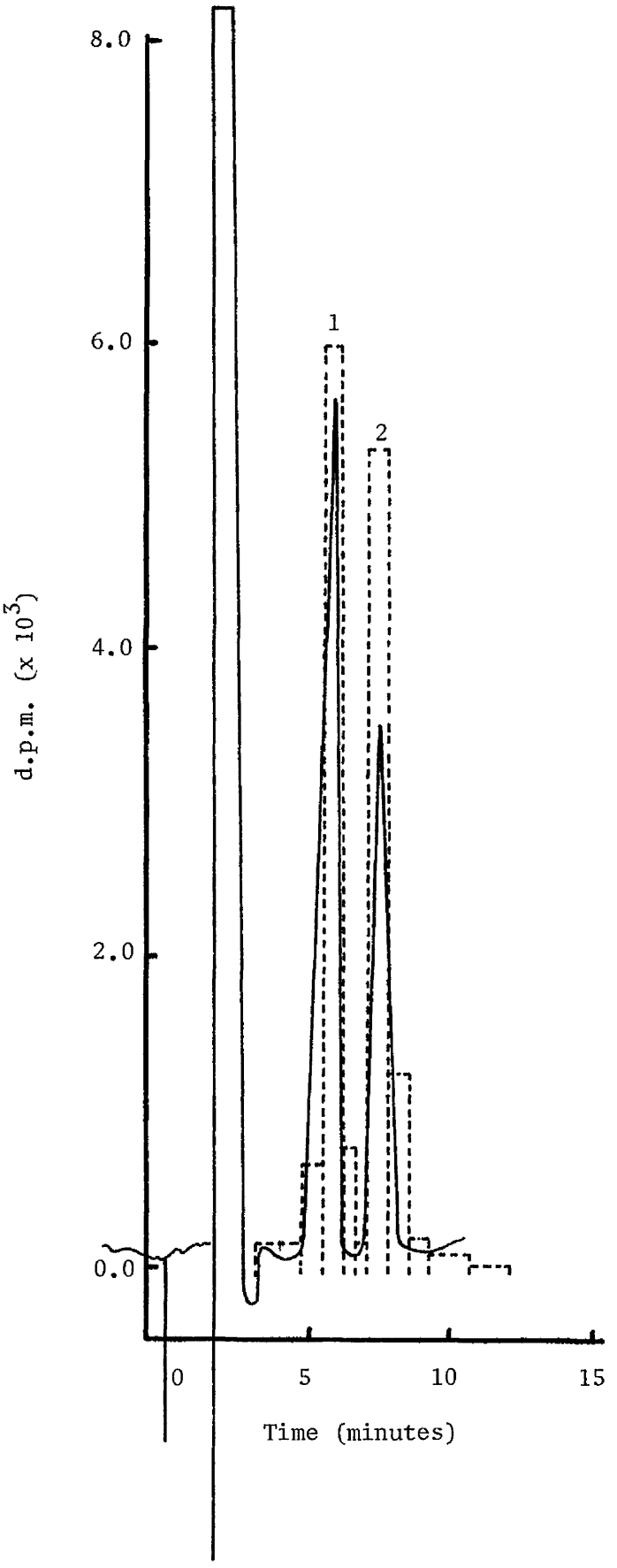
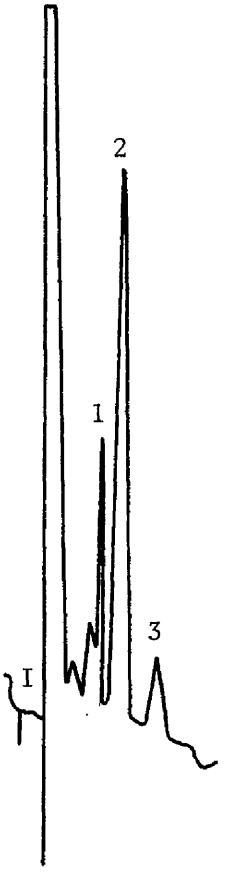

8.0
6.0
4.0
2.0
0.0
d.p.m. (x 10^3)
1
2
0
5
10
15
Time (minutes)

Fig. 3.18. H.P.L.C. Analysis of Methyl Esters of Triacylglycerols Isolated from Adipocytes



10 µl (2 mg) of triacylglycerol methyl esters were applied to the column and methanol:water (90:10 v/v) was used as solvent with a flow rate of 2 ml/min.

I denotes point of sample injection. Peaks are 1, methyl linoleate; 2, methyl palmitate + methyl oleate; 3, methyl stearate.

Consistent recoveries of over 90% of applied radioactivity were obtained when mixtures of (1-^{14}C)fatty acid methyl esters were analysed. Standards and samples, less than 1 mg, were injected into the column in 10 µl chloroform.

Fig. 3.17 shows how peaks of radioactivity corresponded with those of mass when a standard mixture of methyl (1-^{14}C)stearate and methyl (1-^{14}C)oleate was applied to the column. There was very close agreement between mass and radioactivity with only a slight 'tailing' of activity from the methyl stearate peak. Injection of an unlabelled standard methyl stearate/methyl oleate mixture twenty minutes after a ^{14}C-labelled mixture of similar composition resulted in the recovery of some radioactivity in the solvent and fatty acid peaks. Although the amount of radioactivity recovered in each unlabelled peak was low (approximately twice background), the absorption of labelled material onto active sites in the column packing was undesirable when analysing samples of low specific activity. Attempts to remove possible active sites by silanization did little to reduce the absorption of some radioactive material onto the column packing.

When methyl esters of rat adipocyte triacylglycerols were injected onto the column, a recorder trace as shown in Fig. 3.18 was obtained. The column effluent corresponding to each peak was trapped and subjected to G.L.C. analysis. This enabled the three peaks examined to be identified. Stearic and linoleic acid methyl esters were eluted as distinct components, but methyl oleate and methyl palmitate were found to be eluted from the column together. Since the basic purpose of the studies with H.P.L.C. was to separate (1-^{14}C)stearic acid from (1-^{14}C)-oleic acid, the presence of unlabelled palmitic acid in the same peak

as oleic acid was not detrimental to its use in the study of (1-^{14}C)-stearic acid desaturation. However, because of the difficulties encountered with absorption of radioactive material onto the column packing, the use of H.P.L.C. for the routine preparative separation of (1-^{14}C)stearic acid and (1-^{14}C)oleic acid was not pursued.

CHAPTER 4. DISCUSSION

A. The Use of Adipocytes in Studies of Lipid Biosynthesis in vitro: General Considerations

Isolated adipocytes provide a convenient system for the study of lipogenesis in adipose tissue *in vitro*, and have the advantage over tissue fragments in that, since adipocyte preparations ideally contain no stromal-vascular cells, the metabolism of the functional unit of adipose tissue can be studied without interference from the other cell-types normally present in the tissue. Furthermore, the more reproducible entry of substrate into isolated fat-cells contributes to their suitability for use in metabolic studies. Although most past studies on lipogenesis involving adipocytes have been concerned mainly with hormonal control, isolated fat-cells were employed in this present study as a means of investigating triacylglycerol synthesis and its relation to fatty acid synthesis.

The size of adipocytes, measured as described in Chapter 2, section B2, was found to increase with the weight, and hence in this instance the age, of the rats from which they were isolated. A similar relationship between rat adipocyte size and age has been reported in other studies in which fat-cell size was calculated on the basis of lipid weight per cell (249,250). The value found for the mean diameter of adipocytes isolated from a 129 g rat in this study compared favourably with that reported by Di Girolamo *et al* (251) for cells prepared from a rat of similar weight. More accurate measurements of fat-cell diameter, although not required for the purpose of this study, could have been made using a micrometer disc

fitted in the focussing eyepiece of the microscope. Since adipocyte numbers were not measured, no conclusions can be made as to whether the number of fat-cells per unit weight of adipose tissue also increased with the age of the animal. However, present knowledge suggests that in adipose tissue of the rat, the number of adipocytes present increases until the fifteenth week of life (249,252).

For the comparison of the metabolic activities of adipocytes from different animals, values obtained with an equal number of fat-cells are probably the most reliable although this presents technical difficulties in practice. As an alternative, the DNA content of an adipocyte preparation is directly related to the number of cells present and is independent of the amount of lipid in the cell (252). Thus, the results in the present study obtained with rats of two different weight ranges could be compared directly when expressed relative to the amount of DNA present and could be examined in relation to other studies with adipocytes in which results were expressed in terms of cell numbers, by assuming the generally accepted value of 7×10^{-6} µg DNA per cell (253). When the results of the initial experiments were expressed as per 100 mg cell lipid (Chapter 3, sections B and C) good agreement was found between different rats, but the correlation of such results with those obtained in later experiments with younger animals, where results were expressed in terms of DNA, was difficult. The disadvantages of expressing results obtained in experiments with adipose tissue in terms of tissue or cell lipid weight are obvious; no reliable comparison can be made between the metabolic activities of adipose tissue from animals of different ages or physiological status as neither tissue weight nor

lipid weight reflects the number of adipocytes present. However, the results of past studies on lipogenesis in adipose tissue have frequently been expressed in terms of tissue (113,254,255) and lipid (243,256) weight.

The components of the basic incubation medium and their concentrations were chosen as those generally considered optimum for the synthesis of triacylglycerols by adipocytes. The superiority of Krebs-Ringer bicarbonate buffer over phosphate buffer in studies on lipogenesis by adipose tissue in vitro has been demonstrated elsewhere (257). Respiration, and hence the viability of the fat-cells, was maintained by the inclusion of glucose in the medium at a physiological concentration (5mM) at which Rodbell (223) has shown the maximum synthesis of acylglycerols to occur in rat adipocytes. Although the concentration of insulin (20 m units/ml) employed in all experiments was in excess of the normal physiological range (258,259), similar and even higher concentrations have been used by other workers in studies on lipogenesis in mammalian adipose tissue (113,260,261,262). Insulin served a dual purpose, namely to reduce the release of fatty acids by the adipocytes (263) and to ensure a high rate of lipogenesis. Saggerson (264) showed that the rate of lipogenesis from glucose in isolated fat-cells was unaffected by increasing the concentration of insulin above 1.25 m units/ml, an observation which was probably explained by the fact that once the binding of insulin to receptor sites had reached equilibrium, the rate of lipogenesis in rat adipocytes was independent of insulin concentration (265).

Natural conditions were emulated further by the presence of albumin in all incubations and by supplying fatty acids bound to

albumin to represent the transport of exogenous fatty acids in plasma (263).

Although the extracellular fatty acid composition used throughout these studies (1mM) was higher than that employed by several other workers (243,255,264), it was still within the physiological range of 0.3 to 1.7mM known to occur in rat plasma (263). The high concentration of fatty acids served to swamp the effects of adipocyte endogenous unesterified fatty acids, residual fatty acids present in the albumin and fatty acids released from the fat-cells in the presence of albumin (266), as well as permitting better control of the composition of the exogenously added fatty acids. The proportions of the various components in the fatty acid mixture were chosen as being similar to the fatty acid composition of rat adipose tissue triacylglycerols on the assumption that triacylglycerols formed by fat-cells presented with such a mixture would be similar to those occurring naturally.

B. Triacylglycerol Biosynthesis from Exogenous Fatty Acids

The almost complete uptake and esterification of exogenously added fatty acids by adipocytes in the initial experiments (Chapter 3, section B) was in keeping with there being unrestricted entry of exogenous fatty acids into the fat-cell across the plasma membrane. Previous studies (260,267,268) with adipose tissue fragments had indicated that the rates of incorporation of long-chain fatty acids into tissue lipid *in vitro* were proportional to their concentrations in the incubation medium. In view of this evidence, the entry of

exogenous fatty acids into the adipocyte was therefore unlikely to be rate-limiting in esterification into cellular acylglycerols. Although the mechanism whereby fatty acids cross the plasma membrane into the fat-cell is unknown, recent studies (269) with isolated hepatocytes have implicated a protein found in the cytoplasm and capable of binding fatty acids, whilst Wright and Green (270) have suggested that a rapid deacylation-reacylation cycle of phospholipids in the plasma membrane may be important. Support for the latter comes from the recent demonstration by Giacobino and Chmelar (271) that the plasma membrane or adipocytes can actively esterify fatty acids into phospholipids and triacylglycerols.

The results obtained in this present study were consistent with the operation of the sn-glycerol 3-phosphate pathway as the principal mode of esterification of exogenous fatty acids into triacylglycerols in rat adipocytes. In agreement with the results of previous studies with rat adipocytes (243), adipose tissue fragments (256,272) and homogenates *in vitro* (155,212,273) and adipose tissue *in vivo* (274), triacylglycerols contained most of the esterified fatty acids, with the amount recovered in diacylglycerols exceeding that in phospholipids. The results presented in Chapter 3, section B suggest that the hydrolysis of phosphatidic acid to diacylglycerol by phosphatidate phosphohydrolase proceeded at a high rate in the intact adipocyte since phospholipids, and therefore phosphatidic acid, never contained a significant proportion of the total esterified fatty acids. It is noteworthy that in studies with adipose tissue homogenates in which the integrity of the fat-cell was lost, the proportion of fatty acids esterified into phospholipids was generally relatively high in

comparison with diacylglycerols although still less than the proportion recovered in triacylglycerols under optimum conditions for triacylglycerol synthesis (164,212,275). The control of phosphatidate phosphohydrolase activity may therefore be dependent, at least in part, on compartmentation effects within the cell. Since both the substrate and product of the enzyme are possibly membrane-bound within the adipocyte, and as both microsomal and mitochondrial forms of the enzyme exist, it could be expected that disruption of the cell would lead to changes in the activity of the enzymes. Throughout this study, phosphatidic acid never accumulated, regardless of the nature of lipogenic precursor and of the general incubation conditions. Although this could have been simply due to inefficient extraction of the phospholipids from the adipocytes (276), such a possibility was unlikely since practically all the radioactivity from the labelled fatty acids added to the incubation media could be accounted for in the lipid extract. It can therefore be concluded that in the intact rat adipocyte under conditions of insulin-stimulated lipogenesis, the hydrolysis of phosphatidic acid by phosphatidate phosphohydrolase may not be the rate-limiting step in triacylglycerol synthesis, but rather, in view of the evidence presented below, diacylglycerol acyltransferase. This aspect will be discussed further in the following section.

The results presented in Fig. 3.3 resemble those of other workers (243,272) who have likewise demonstrated the presence in adipose tissue of diacylglycerols having a low turnover rate. However, the synthesis of triacylglycerols, presumably via the sn-glycerol 3-phosphate pathway, from exogenous fatty acids proceeded at a fast

rate (Fig. 3.4) suggesting that diacylglycerols which are quickly acylated to triacylglycerols must also exist within the adipocyte. The existence of more than one pool of diacylglycerols is not impossible since the synthesis of diacylglycerols in both the mitochondria and microsomes (164), and more recently (271) in the plasma membrane of adipose tissue, has been demonstrated. As well as this possible segregation of diacylglycerols by their synthesis within different organelles, a pool of diacylglycerols could originate from a portion of newly-synthesized triacylglycerols which is highly labile and susceptible to lipolysis (277). However, from studies of the structure of the diacylglycerols formed from newly-synthesized fatty acids (Chapter 3, section I) this hypothesis does not seem tenable. Alternatively, Winand et al (243) have suggested that the high specific activity of diacylglycerols formed by rat adipocytes incubated with ^{14}C-labelled acetate or palmitate, may be due to the synthesis of diacylglycerols which are not used for triacylglycerol synthesis but which are rapidly broken down by lipolysis. Angel (278,279,280) advocated that approximately 20% of newly synthesized diacylglycerols was not acylated immediately to triacylglycerols but was transferred to the lipid storage droplet. Furthermore, diacylglycerols formed within the fat-cell may be segregated on the basis of the origin of the fatty acids used in their synthesis, with diacylglycerols formed from exogenous fatty acids being separated from those formed using endogenously-synthesized fatty acids. The fact that extracellular fatty acids may not mix with intracellular fatty acids in adipose tissue (243,268) lends support to such a concept.

It would therefore appear that although diacylglycerols account

for less than 1% of the total lipid in the fat-cell (281), they may be of more metabolic importance than is implied in the role usually ascribed to them of a short-lived intermediate in triacylglycerol synthesis.

In many previous studies on the uptake and esterification of long-chain fatty acids by adipose tissue both *in vivo* (274) and *in vitro* (267,268,282) the incorporation of single fatty acids, supplied individually or at best in the presence of only one other fatty acid, into total neutral lipid was examined. The true rates of fatty acid esterification into different acylglycerols are not obvious from such studies, and since more than two different fatty acids are normally available to the fat-cell from the plasma, the results obtained in these studies may not truly reflect the natural situation. For this reason, the esterification of fatty acids presented to the adipocytes as components of a mixture of four fatty acids of a composition designed to simulate that found naturally was compared with that of the same fatty acids supplied individually. By correcting the results obtained with labelled fatty acids supplied as part of a fatty acid mixture to the situation if the labelled fatty acid comprised 100% of the mixture, differences due to the initial amount of fatty acid supplied were removed, allowing the effect of the presence of other fatty acids on the rate of esterification of a particular fatty acid to be studied.

The determination of the true rate of exogenous fatty acid esterification by adipose tissue is complicated by the fact that fatty acids released from the fat-cells dilute the extracellular fatty acids and thus conceal the actual concentrations of fatty acids available

for esterification. The contribution of unesterified fatty acids released by the adipocytes would be small in relation to the 1mM fatty acid concentration employed throughout this study. Saggerson (283) showed that, as a result of dilution of exogenous substrate, the amount of extracellular palmitic acid, of initial concentration 0.1mM, incorporated by adipocytes into acylglycerols per 100 µg DNA in any given time, varied with the concentration of cells present. The results of past studies in which low concentrations of exogenous fatty acid substrates were employed for the study of lipogenesis in adipose tissue may therefore be unreliable.

The finding that incubating a fatty acid in the presence of others made no difference to its rate of incorporation into total lipid suggested that fatty acids did not compete for esterification or entry into the adipocyte. However, the differences observed between fatty acids in their rates of esterification separately into triacylglycerols, diacylglycerols and phospholipids when supplied as a mixture (Fig. 3.6) suggested that the acyltransferase enzymes did have some substrate preferences which would not be evident when the labelled fatty acid was supplied alone at high concentration. A single fatty acid at a high concentration in the medium could be expected to swamp any endogenous competitive substrates for acyltransferases and lead to the formation of unnatural triacylglycerols. The finding that essentially tripalmitoylglycerol was synthesized by adipocytes when palmitic acid was supplied on its own (Table 3.6) supports this statement and emphasizes that a mixture of fatty acids should be used in preference to a single fatty acid in studies on triacylglycerol synthesis. The high rate at which exogenous oleic acid was esterified

into triacylglycerols when supplied in the presence of other fatty acids was in keeping with its being the principal fatty acid in rat adipocyte triacylglycerols (Table 3.2). The extent to which the rapid incorporation of oleic acid into triacylglycerols relative to other fatty acids (Fig. 3.6) was attributable to the substrate preferences of diacylglycerol acyltransferase is uncertain since Coleman and Bell (180) showed that oleoyl CoA was apparently a poor substrate for the enzyme purified from adipose tissue. However, studies *in vitro* with diacylglycerol acyltransferase may not be wholly representative of the situation *in vivo* since the composition and conformation of the natural diacylglycerol substrate for the enzyme are unknown and the physical properties of both diacylglycerols and acylCoA's *in vitro* may not resemble those when associated with subcellular membranes in the natural state. The low rate of esterification of stearic acid into triacylglycerols (Fig. 3.6) is in keeping with the results of other studies *in vivo* (274) and *in vitro* (284) with adipose tissue in which stearic acid was found to be esterified into neutral lipid at a lower rate than were other fatty acids.

Differences in the rates of esterification of fatty acids into triacylglycerols may have been due to competition between fatty acids to act as substrate for acylCoA synthetase. Apart from the fact that it had greatest activity towards palmitic acid in homogenates of the tissue (172), little is known of the substrate specificity of the fatty acid activation enzyme in adipose tissue. Conflicting information is available concerning the specificity of acylCoA synthetase in rat liver microsomes. Bar-Tana *et al* (285) found that palmitic and stearic acids served equally well as substrates with the

enzyme having a much lower specificity towards oleic and linoleic acids whereas other workers (286) have reported that the rate of activation of stearic acid was only half that of palmitic acid.

The low rate of esterification of exogenous fatty acids in the absence of glucose (Fig. 3.15) reflected the long-established requirement for glucose-derived sn-glycerol 3-phosphate for triacylglycerol synthesis via the sn-glycerol 3-phosphate pathway in the fat-cell. Other workers have also noted a reduced uptake and esterification of fatty acids by adipose tissue in vitro (267,287) when the medium contained no glucose, and adipocytes isolated from fasted rats are known (275) to esterify long-chain fatty acids at a lower rate than those from fed rats. Whereas Bally et al (260) found that the amount of palmitic acid esterified increased with increasing glucose concentration up to 5mM, the rate of fatty acid esterification in the results now reported was independent of glucose concentrations in excess of 5mM. Although this might indicate that at high glucose concentrations the production of glycerol 3-phosphate might be rate-limiting in the synthesis of triacylglycerols from exogenous fatty acids, Denton and Halperin (288) concluded that the rate of triacylglycerol synthesis was not determined solely by the availability of glycerol 3-phosphate or fatty acids in rat adipose tissue in vitro.

The rate of esterification of exogenous fatty acids into triacylglycerols by rats of both ages studied was found to be independent of acetate concentration (Fig. 3.16 and Table 3.25) and might indicate that no competition for CoA exists between fatty acids and acetate. Although the activity of cytoplasmic acetylCoA synthetase is known (66)

to increase in adipose tissue of rats fed a high carbohydrate diet, the effect of dietary fat on the enzyme has not been investigated.

The results presented in Chapter 3, section C demonstrated that the Δ9-desaturase activity known to be present in intact rat adipose tissue (90,256) was also a property of isolated fat-cells. The extent to which stearic acid supplied as a component of a fatty acid mixture was desaturated was comparable with that found by Christie and Vernon (256) in studies with rat adipose tissue slices. The higher rate of desaturation found with stearic acid supplied alone (Fig. 3.7) was in keeping with the evidence provided by other workers (289,290) that the desaturation of stearic acid is related to the amount of substrate available and that unsaturated fatty acids, in particular those containing two or more double bonds, are effective inhibitors of the desaturase system.

It is noteworthy that practically all the oleic acid resulting from the desaturation of stearic acid was found esterified in acylglycerols (Fig. 3.7) with very little present as unesterified fatty acid. As well as suggesting that acylCoA hydrolase had low activity under the conditions employed, this might also be taken as an indication of a close relationship between the desaturase system and the acyltransferases, probably attributable to their cellular locations.

The fact that the newly-synthesized oleic acid was predominantly esterified into position sn-3 (Table 3.4), the position which is the last to be acylated in triacylglycerol synthesis via the sn-glycerol 3-phosphate pathway, might indicate that the rate of stearoylCoA desaturation influenced the rate of triacylglycerol synthesis by acting at the diacylglycerol acyltransferase step. The results of Wahle (291)

supported this suggestion in that sheep liver had low rates of both desaturation and triacylglycerol synthesis whereas in hen liver a high desaturation activity corresponded with a high rate of triacylglycerol synthesis. A direct relationship between the rates of stearic acid desaturation and triacylglycerol synthesis has also been demonstrated in bovine mammary tissue by Kinsella (221) who found oleic acid formed by the desaturation of stearic acid to be esterified preferentially into the primary positions which he suggested might be position sn-3 in particular.

A further possible connection between desaturation and triacylglycerol synthesis may be their mutual control by non-enzymic protein factors. Roncari and Mack (292) have demonstrated in the cytoplasm of rat adipose tissue the existence of two proteins, the presence of which increased the rate of formation of triacylglycerols from diacylglycerols by microsomes. Similarly a protein factor which is loosely associated with the microsomes in rat liver, has been shown (97) to be necessary for the desaturation of stearic acid. It is noteworthy that this latter protein, like one of those implicated in triacylglycerol synthesis by Roncari and Mack (292), was inactivated by heat and by trypsin digestion. This evidence, although somewhat tenuous, permits a speculation that closely-related protein factors might influence both the desaturation of stearic acid and the incorporation of the resulting oleic acid into triacylglycerol by diacylglycerol acyltransferase. However, the manner in which such protein factors could exert control over these processes is uncertain, but could feasibly involve the binding of fatty acids or fatty acylCoA's. Wu-Rideout et al (269) have suggested that a protein, capable of

binding fatty acids, enhances the esterification of long-chain fatty acids into triacylglycerols in rat hepatocytes. Specific binding of fatty acylCoA's might perhaps remove problems which would otherwise be encountered by the formation of micelles (293). Caffrey and Kinsella (294) have recently pointed out the difficulties caused by micelle formation and the non-specific binding of substrates, in the interpretation of results obtained with studies *in vitro* of membrane-bound acyltransferases.

Desaturation of exogenous stearic and the subsequent esterification of the resulting oleic acid into position *sn*-3 predominantly, altered markedly the composition of that position and might conceivably play some part in controlling the rate of triacylglycerol synthesis in rat adipocytes. Further studies with adipocytes using labelled glucose as a precursor of glyceride-glycerol, an exogenous fatty acid mixture and sterculic acid, a cyclopropene acid which does not affect the synthesis *de novo* of endogenous oleic acid from low molecular weight precursors but does inhibit its formation by the desaturation of preformed stearic acid (295,296,297), may yield information on the relationship between stearic acid desaturation and triacylglycerol synthesis.

The fatty acid composition of adipocyte triacylglycerols found in this study (Table 3.2) agreed reasonably closely with that of triacylglycerols extracted from intact rat adipose tissue by others (78).

The natural pattern of fatty acids present in each position of triacyl-*sn*-glycerols from adipocytes (Table 3.2) differed slightly from that reported by Brockerhoff *et al* (197) for intact rat adipose

tissue, in that palmitic acid apparently accounted for a much higher proportion of the fatty acids in position sn-1 of the former. This discrepancy might have been related to the fact that the adipose tissue analysed by Brockerhoff and co-workers (197) was taken from subcutaneous and intestinal sites in the animal, which contained lower proportions of palmitic acid than the parametrial tissue used here. In ruminants, saturated fatty acids of exogenous origin were known to be preferentially assimilated in the internal adipose depots such as parametrial adipose tissue, whereas the fatty acids of subcutaneous adipose tissue were derived largely by endogenous synthesis (298). A similar relationship may exist in the rat.

The non-random distribution of fatty acids over the three positions of natural triacyl-sn-glycerols from adipocytes with position sn-1 being the most saturated and position sn-2 containing a preponderance of unsaturated fatty acids (Table 3.3), was typical of triacylglycerols from the adipose tissue of most animal species (191). Similar proportional distributions of fatty acids over the three positions were found in triacyl-sn-glycerols extracted from rat adipose tissue slices (256).

The results obtained for the positional distribution of exogenous fatty acids in triacylglycerols formed in vitro by adipocytes (Table 3.4) resembled those reported for similar studies with adipose tissue slices (256) and homogenates (212).

The extent to which the positional distributions of exogenous fatty acids in the triacylglycerols were attributable solely to the specificities of the acyltransferases is uncertain. Although there were general similarities between the positioning of exogenous fatty acids found here and their natural distribution in rat adipocyte

triacylglycerols (Table 3.4 cf. Table 3.3), the fact that they did not agree exactly would suggest that some factor in addition to the specificities of the acylation enzymes played a role in the formation of the asymmetric structure of triacylglycerols. Stokes et al (211) demonstrated that incorporation of palmitic and stearic acids into position sn-2 of triacyl-sn-glycerols by combined microsomal-cytoplasmic preparations of adipose tissue from several species was comparable with their natural distribution in that position, and suggested that the non-random distribution of fatty acids in triacylglycerols was governed by the specificity of microsomal acyltransferases towards the acylCoA substrate. However, it is noticeable from their data that, although the incorporation of oleic acid into position sn-2 by microsomes from rat adipose tissue was identical to its natural occurrence in that position, the percentage of esterified palmitate in position sn-2 was almost half the normal value. Furthermore, their studies did not establish whether the fatty acids were esterified into positions sn-1 and sn-3 in proportions resembling their natural distribution. In contrast, the same workers (213) have provided evidence that in pig adipose tissue a factor may exist which can control the specificity with which palmitic acid is esterified into triacylglycerols by the acyltransferases.

However, the differences between the distribution of exogenous fatty acids and the natural distribution of fatty acids in adipocyte triacylglycerols may also be associated with the fact that the concentration of individual fatty acids, even when supplied as a mixture, may not have been optimum for their esterification into triacylglycerols. Benson and Emery (299) found that with homogenates of bovine adipose

tissue the concentration of a fatty acid required for its maximum rate of esterification into neutral lipid was different for each fatty acid studied, and more recent studies with homogenates of rat adipose tissue (212) revealed that the optimum concentration of palmitic acid for its esterification into total acylglycerols was actually higher than that required for maximum incorporation into triacylglycerols. Bjerve et al (300) demonstrated that different acyltransferases existed for the synthesis of phosphatidic acid in mitochondria and microsomes of rat liver, and that the concentration of a fatty acylCoA may be more important in controlling its distribution in phosphatidic acid formed by mitochondria than in phosphatidic acid formed by microsomes. The concentration of the acylCoA acceptor also influenced the specificity of the fatty acid distribution in phosphatidic acid formed by rat liver microsomes (301). A similar concentration-dependent effect might occur in adipose tissue which is also known to be capable of producing phosphatidate in both mitochondria and microsomes (156).

A closer agreement between the positional distribution of exogenous fatty acids and the natural distribution of fatty acids in adipocyte triacylglycerols could possibly have been attained by incubating the fat-cells with a fatty acid mixture of which the components and their proportions were identical to those unesterified fatty acids supplied by the plasma; this data was not readily accessible and in any case would probably be highly variable. Effects mediated by the two pools of albumin-bound unesterified fatty acids which have been shown (302) to exist in rat plasma might also not be susceptible to control.

The results of Table 3.4 suggest that the acyltransferases

involved in triacylglycerol synthesis make a distinction between oleic acid of exogenous origin and oleic acid formed within the adipocyte by the desaturation of exogenous stearic acid. Christie and Vernon (256) have observed a similar feature in rat adipose slices although in their studies oleic acid resulting from exogenous stearic acid was preferentially esterified into position sn-2. However, their results were obtained with only one animal whereas the measurements in the present study were carried out with three different rats and were therefore probably more accurate. Diacylglycerol acyltransferase and its diacylglycerol substrate in the adipocyte are probably bound to microsomal membranes (151). Brockman (303) has shown that the rate of microsomal 1-acyl-sn-glycerol 3-phosphate acyltransferase (also membrane-bound) is controlled by the concentration of fatty acylCoA substrate on the membrane immediately adjacent to the enzyme, and diacylglycerol acyltransferase may be under similar control. If the desaturase system, which is apparently associated with microsomal membranes (98), is situated in close proximity to the diacylglycerol acyltransferase then it is conceivable that the oleoylCoA product of the desaturase is presented directly as substrate to the acyltransferase. Thus it could be expected that a high proportion of oleic acid formed by desaturation of stearic acid would be esterified into position sn-3 and that the desaturase system could influence the rate of triacylglycerol synthesis simply by determining the availability of substrate for diacylglycerol acyltransferase. Similarly, as discussed in the next section, the manner in which fatty acids newly synthesized from low molecular weight precursors were esterified may play an important part in controlling

the ultimate composition of the three positions of the triacylglycerol.

The results presented in Table 3.6 indicated that the normal substrate preferences of acyltransferases were overridden when the fat-cell was presented with a single fatty acid, and that when only a simple fatty acid is supplied to the cell it can synthesize a comparatively unusual triacylglycerol such as tripalmitoylglycerol. Similarly, rat adipose tissue slices incubated with ^{14}C-labelled palmitic acid were shown by silver nitrate T.L.C. to esterify most of the labelled fatty acid into tripalmitoylglycerol (Hunter, M.L., personal communication).

C. Triacylglycerol Biosynthesis from Endogenous Fatty Acids

The results presented in Chapter 3, section E demonstrated clearly that isolated rat adipocytes were capable of using acetate for lipogenesis. Although acetate may be regarded as a more physiological substrate for fatty acid synthesis in ruminant tissues than in those of non-ruminant animals, the presence in the plasma of the rat of acetate at a concentration of 0.2mM (304) with a high turnover rate (305), indicated that the incubation of rat adipocytes with acetate did not represent a wholly unnatural situation. Previous workers have likewise established the ability of rat adipose tissue (23,279,306) and isolated adipocytes (243) to utilise acetate for fatty acid synthesis.

In agreement with the results of Saggerson (307), the incorporation

of acetate into total lipid by adipocytes (Figs. 3.8 and 3.11) did not increase linearly with acetate concentration. AcetylCoA synthetase is known to be capable of high activity in rat adipose tissue (304) and Winand et al (273) found that the enzyme was not rate-limiting in the synthesis of fatty acids by homogenates of rat adipose tissue. The rate-controlling step in fatty acid synthesis from acetate must therefore be either acetylCoA carboxylase or fatty acid synthetase.

The results now reported suggested that the rate of fatty acid synthesis de novo in rat adipose tissue decreased with the age of the animal, since a tenfold difference was obvious between the rates of acetate incorporation into lipid by fat-cells prepared from 150 g and 280 g rats. Similarly, although the use of glucose for lipogenesis did not decrease with age to the same extent as that of acetate, it was noteable that the amount of glucose incorporated into acylglycerol fatty acids did decrease whilst the proportion incorporated into the glycerol moiety actually increased (Tables 3.15 and 3.16 cf. 3.19 and 3.20). The ratio of glucose incorporation into fatty acids to that into glycerol was therefore characteristic of the age of the rat from which the adipocytes were prepared, being highest with young animals.

Although the incubating conditions were similar, the maximum rates of acetate and glucose incorporation into fatty acids found in this present study were considerably lower than those reported by Saggerson (264,307) using adipocytes prepared from rats of comparable weight.

The slight increase in the rate of fatty acid synthesis from glucose observed in the presence of acetate (Tables 3.16 and 3.20 cf.

3.15 and 3.19) was in agreement with that noted by others (306,308), but less than that reported by Saggerson (307). This increased rate of fatty acid synthesis could be explained by the suggestion of Flatt and Ball (308) that the conversion of acetylCoA to fatty acids was not the rate-limiting factor in lipogenesis from glucose. Therefore, although the production of acetylCoA from glucose was proceeding at or near its maximum rate, as shown by the fact that increasing glucose concentration above 5mM did not result in an increase in glucose incorporation into fatty acids (Tables 3.15 and 3.19), added acetate could still have been incorporated into fatty acids producing an increase in the rate of total fatty acid synthesis and a smaller increase in the rate of glucose incorporation into fatty acids.

A reduction in the rate of fatty acid synthesis de novo by the adipocyte in the presence of extracellular fatty acids was demonstrated by the observation that the incorporation of acetate into lipids was always decreased when the incubation medium contained long-chain fatty acids (Figs. 3.8 and 3.11). This reduced lipogenesis from acetate may have been a result of the direct inhibition of acetylCoA carboxylase and other lipogenic enzymes by exogenous fatty acids (see Chapter 1, section B2). Alternatively, competition between fatty acids and acetate for the available CoA could provide an explanation since long-chain fatty acylCoA esters also inhibited acetylCoA carboxylase. This latter suggestion could explain why the inhibition of fatty acid synthesis from acetate in the presence of extracellular fatty acids was always less at 10mM than at 0.5mM acetate. If this were the case, however, the esterification rate of exogenous fatty acids into acyl-glycerols would be expected to be lower at high concentrations of

acetate because of the diminished availability of CoA. As Fig. 3.16 and Table 3.25 illustrate, the rates of esterification of exogenous oleic and stearic acids were independent of acetate concentration, thereby suggesting that fatty acid and acetate activation were non-competitive for CoA. Furthermore, inhibition of acetylCoA synthetase by long-chain fatty acids could not be excluded as a possible control in the rate of fatty acid synthesis from acetate.

Whereas the presence of exogenous fatty acids had a definite inhibitory effect on lipogenesis from acetate, they did not inhibit, and may even have stimulated, fatty acid synthesis from glucose in adipocytes from both young and mature rats (Tables 3.15, 3.16, 3.19 and 3.20). This effect of exogenous fatty acids on endogenous fatty acid synthesis from glucose might be explained by the suggestion of Flatt (54, 309) that the energy state of the cell, in other words the cellular levels of ATP and NADH, might restrain the rate of fatty acid synthesis from glucose which is a net energy-yielding process. Saggerson (264) has suggested that fatty acids might uncouple oxidative phosphorylation in the adipocyte resulting in a decrease in ATP synthesis, thereby easing the restraint on fatty acid synthesis by the high-energy state and subsequently allowing the conversion of glucose to fatty acids to proceed further. However, although Saggerson (264) found that total fatty acid synthesis, as measured by ^3H_2O incorporation, increased in the presence of exogenous fatty acids, the results of the present study indicated the opposite, namely that ^3H_2O incorporation into acylglycerol fatty acids and therefore total fatty acid synthesis, decreased under such conditions (Table 3.23). The decreased use of glucose for fatty acid synthesis corresponded

with an increase in the amount of glucose incorporated into glycerol when exogenous fatty acids were presented to the adipocytes (Tables 3.15, 3.16, 3.19 and 3.20). This increased incorporation of glucose into acylglycerol-glycerol represented a definite increase in the rate of synthesis of triacylglycerols de novo under such conditions; a feature in keeping with the established ability of adipose tissue to take up preformed fatty acids from the plasma and store them as triacylglycerols.

From the experiments with ^{14}C-labelled acetate, it was concluded that the pattern of fatty acids synthesized de novo from acetate was dependent on the age of the rat from which the adipocytes were isolated. The results presented in Tables 3.8 and 3.10 agreed closely with the pattern of fatty acids synthesized from 3.8mM acetate by adipose tissue slices from rats of comparable weights to those used in this study, as reported by Gellhorn et al (310), although they were somewhat different from the results obtained by Winand et al (243) with adipocytes in vitro.

Fat-cells from young animals synthesized more oleic acid from acetate than did cells from older rats, suggesting that desaturase activity might decrease with age. However, Gellhorn and co-workers (310,311) showed that the administration of insulin to an old rat from which adipose tissue was subsequently isolated for studies on fatty acid synthesis, resulted in a pattern of newly-synthesized fatty acids more similar to those found with a younger animal. Insulin, therefore, by its effect on desaturation, was important in determining the pattern of fatty acids synthesized in adipose tissue. Although a

constant concentration of insulin was used throughout these studies with fat-cells from both young and old animals, the age-dependent patterns of fatty acids synthesized from acetate may be associated with a decrease in the sensitivity of the adipocyte to insulin with age (137).

As expected, the fatty acids synthesized from glucose by fat-cells from younger rats (Table 3.21) were broadly similar to those formed when acetate was the lipogenic precursor (Table 3.10). The somewhat greater synthesis of stearic acid from acetate than from glucose (Table 3.10 cf. 3.21) may have been related to the acetylCoA formed in the cytoplasm being used in fatty acid elongation systems of the microsomes or plasma membrane (81) whilst glucose-derived acetylCoA might be used preferentially for mitochondrial fatty acid elongation.

Although the results presented in Tables 3.10, 3.11 and 3.21 might suggest at first sight that rat adipocytes were capable of forming linoleic acid de novo, such a finding would be contrary to the well-established fact that linoleic acid is an essential dietary fatty acid for the rat. Consideration of the statistical variation showed that the amount of radioactivity recovered in linoleic acid was almost negligible in each instance, and that found might have arisen by partial β-oxidation of unlabelled endogenous linoleic acid followed by resynthesis using labelled acetylCoA (312).

The results of the present study were also consistent with the esterification of endogenously-synthesized fatty acids into triacylglycerols proceeding via the sn-glycerol 3-phosphate pathway in the adipocyte. The accumulation of newly-synthesized fatty acids in

diacylglycerols at high acetate concentration (Figs. 3.9 and 3.10) was peculiar to older rats and synthesis from acetate since the effect was not obvious with cells prepared from younger animals, or when glucose was the fatty acid precursor (Figs. 3.13 and 3.14). For the reasons discussed previously, the rate of total fatty acid synthesis was probably greater in the studies with ^{14}C-labelled acetate since glucose was also present, than when ^{14}C-labelled glucose was the sole precursor of fatty acids and therefore the two situations are not directly comparable. However, fatty acids synthesized from glucose in the presence of acetate by adipocytes from older rats were esterified predominantly into triacylglycerols regardless of acetate concentration (Fig. 3.14). This may have indicated that glucose-derived acetylCoA generated in the mitochondrion and acetylCoA produced in the cytoplasm were used differently for lipogenesis. Rao and Abraham (313) have provided evidence from studies with mouse mammary tissue that acetylCoA derived from glucose may not mix with that produced by β-oxidation of fatty acids, within the mitochondrion, and that the two may be used at different rates for fatty acid synthesis in the cytoplasm. However, such a situation is dependent on the acetate units derived from the two sources leaving the mitochondrion at different rates, whereas in this present study acetylCoA produced by the activation of acetate was already in the cytoplasm.

Since the rate of incorporation of fatty acids arising from acetate into triacylglycerols was almost independent of acetate concentration, whereas their esterification into diacylglycerols was elevated by high acetate concentrations (Fig. 3.9), the possibility existed that the acylation of diacylglycerols to triacylglycerols proceeded at a slow

rate in relation to the previous steps in triacylglycerol biosynthesis via the sn-glycerol 3-phosphate pathway. Furthermore, in the presence of extracellular fatty acids, the acylation of diacylglycerols containing newly-synthesized (Fig. 3.10) fatty acids to triacylglycerols was apparently decreased even further in relation to the other esterification steps, or, alternatively, the rate of diacylglycerol formation from phosphatidic acid was increased. In view of this finding it is possible that diacylglycerol acyltransferase may be a rate-limiting enzyme in the esterification of endogenously-synthesized fatty acids into triacylglycerols by adipocytes from older rats. The importance of diacylglycerol acyltransferase could also be seen from the fact that its activity was apparently increased in the presence of exogenous fatty acids, as demonstrated by the decrease in the amount of labelled substrate recovered in diacylglycerols relative to that in triacylglycerols when either glucose (Fig. 3.13 and Table 3.18), acetate (in young animals only) (Fig. 3.12) or tritiated water (Table 3.22) was the labelled lipogenic precursor. This might suggest that diacylglycerol acyltransferase had a preference for exogenous fatty acids. The decreased proportion of endogenously-synthesized fatty acids that were incorporated into position sn-3 of triacyl-sn-glycerols in the presence of exogenous fatty acids (Table 3.7) supported this suggestion.

It was noteworthy, however, that the esterification of exogenous oleic acid into triacylglycerols was unaffected by the high acetate concentrations at which fatty acids newly-synthesized from acetate accumulated in diacylglycerols, thereby suggesting that acetate concentration did not directly inhibit the total diacylglycerol acyltransferase activity of the cell. The finding that the diacyl-sn-

glycerols formed by adipocytes from glucose or acetate at all concentrations were of the sn-1,2- structure confirmed that the diacylglycerols which contained high amounts of endogenously-synthesized fatty acids arose as intermediates in the synthesis of triacylglycerols via the sn-glycerol 3-phosphate pathway, since most triacylglycerol lipases would produce 2,3-diacyl-sn-glycerols as readily as 1,2-diacyl-sn-glycerols (314) and none has yet been isolated that is specific for position sn-3.

The presence of exogenous fatty acids did appear to influence the pattern of endogenously-synthesized fatty acids esterified in triacyl-glycerols. For example, the proportion of newly-synthesized oleic acid relative to that of stearic acid was decreased in fat-cells from rats of both ages studied (Tables 3.8, 3.10 and 3.21). In agreement with the results of Winand et al (243) the diacylglycerols contained a higher proportion of saturated fatty acids than did the triacyl-glycerols. This did not indicate, however, that endogenously-synthesized oleic acid was preferentially esterified into position sn-3 of newly-formed triacylglycerols since, as Table 3.14 shows, oleic acid formed de novo was predominantly located in position sn-2 of triacyl-sn-glycerols although the proportion in position sn-3 was still considerable. The natural positional distribution of oleic acid in triacylglycerols extracted from adipocytes is a product of the overall balance between different positional specificities for exogenous oleate, oleate produced from stearate and oleate synthesized from acetate. As stated previously, the presence of exogenous fatty acids appeared to reduce the incorporation of endogenous fatty acids into position sn-3. Since the content of newly-synthesized oleate in

triacylglycerols was reduced in the presence of extracellular fatty acids it was possible that under such circumstances, it was mainly oleic acid which was prevented from entering position sn-3 by a preference for oleic acid formed by the desaturation of exogenous stearic acid which is found preferentially esterified in that position as discussed previously. However, oleoylCoA has been shown (180) to be a poor substrate for microsomal diacylglycerol acyltransferase from rat adipocytes. Although this finding might be true of endogenously-synthesized oleic acid or oleic acid taken up by the cell, oleoylCoA produced by the desaturation of exogenous stearic acid might be a more favourable substrate as discussed previously. Related arguments can be advanced to account for the overall distributions of palmitic acid and other fatty acids in adipocyte triacylglycerols as discussed below. The proportion of another monoenoic fatty acid, palmitoleic acid, recovered in triacylglycerols, was also reduced in the presence of exogenous fatty acids whereas the production of myristic acid was increased. No explanation can be offered for the latter observation.

The finding that adipocytes from the older rats synthesized a smaller proportion of 18-carbon fatty acids than those isolated from young animals (Table 3.8 cf. Table 3.10) suggested that fatty acid elongation activity decreased with age. In the presence of exogenous fatty acids a greater proportion of 18-carbon fatty acids synthesized from acetate were esterified in triacylglycerols. However, this effect, which increased with acetate concentration, was only obvious in the studies with fat-cells from older rats. Conflicting reports exist (81,243) on the activity of the fatty acid elongation systems in rat adipocytes, and the results observed in this study might be

attributed to the mitochondrial elongation system, which employs acetylCoA, being more active in adipose tissue of young animals although no information is available from the literature.

Although the concentration of acetate did not affect the pattern of fatty acids formed de novo from acetate by adipocytes from young rats (Table 3.10), the distribution of newly-synthesized fatty acids between positions sn-2 and sn-3 was apparently influenced by acetate concentration (Table 3.12). In contrast, the positional distribution of esterified exogenous oleic acid in triacylglycerols was not affected by acetate concentration (Table 3.24). The effects may be due to differing concentrations of oleic acid at various sites of triacylglycerol synthesis in the cell or, alternatively, due to different acyltransferases being involved in the esterification of exogenous and endogenously-synthesized fatty acids. Bjerve et al (300), as mentioned in section B, showed that high palmitoylCoA concentration had opposite effects on the incorporation of palmitic acid into position sn-1 of phosphatidic acid formed by either mitochondria or microsomes of rat liver, with the esterification in the mitochondria being more specific. The specificity of linoleate esterification into position sn-2 by both subcellular fractions was independent of the linoleoylCoA concentration. However, Okuyama and Lands (301) demonstrated, also with rat liver microsomes, that as the concentration of 1-acyl-sn-glycerol 3-phosphate increased the degree of specificity with which fatty acids were esterified into position sn-2 decreased. Since palmitic acid synthesized de novo was found to be esterified predominantly in position sn-1 of triacyl-sn-glycerols (Table 3.14), and this was the main fatty acid synthesized by the adipocyte, it can

be expected that the higher rate of fatty acid synthesis associated with 5mM acetate in relation to 0.5mM acetate would correspond with an increase in 1-acyl-sn-glycerol 3-phosphate production and perhaps thereby influence the distribution of fatty acids between positions sn-2 and sn-3. However, this suggestion depends on the acylation of position sn-2 occurring at a slower rate than that of position sn-1 to permit the concentration of 1-acyl-sn-glycerol 3-phosphate to increase. Although the acylation of 1-acyl-sn-glycerol 3-phosphate might be rate-limiting in the production of phosphatidic acid in rat liver (315), the situation in adipose tissue is less clear. However, the demonstration by Jamdar and Fallon (156) that phosphatidic acid was the only isolatable product of acylation of sn-glycerol 3-phosphate by both mitochondrial and microsomal fractions of rat adipose tissue suggests that the above situation might not exist in rat adipocytes.

From the stereospecific analyses, it can be seen that fatty acids formed de novo within the adipocyte were esterified into triacylglycerols in a non-random manner (Table 3.14). The high proportion of newly-synthesized palmitic acid esterified in position sn-1 was in agreement with the results of Anderson and Tove (316) who found fatty acids synthesized from acetate or glucose by mouse adipose tissue to be positioned primarily at the α-positions of triacylglycerols extracted from the tissue. The overall manner in which the newly-synthesized fatty acids were esterified into triacylglycerols was generally in keeping with the natural distribution of fatty acids in rat adipose tissue triacyl-sn-glycerols, with position sn-2 containing high proportions of the monoenes oleic and palmitoleic acids, and position sn-1 containing the highest proportion of saturated fatty acids. The high

proportion of newly-synthesized stearic acid relative to oleic acid in position sn-3 might be due in part to the substrate preferences of diacylglycerol acyltransferase. Coleman and Bell (180) showed that the enzyme from rat adipocyte microsomes utilised stearoylCoA at more than twice the rate with oleoylCoA. Similarly, palmitoylCoA is known to be esterified preferentially into position sn-1 and oleoylCoA into position sn-2 by sn-glycerol 3-phosphate acyltransferase(s) from rat liver microsomes (168).

If the natural distribution of fatty acids in rat adipocyte triacylglycerols is governed simply by the specificity of the acyltransferase enzymes, then it can be expected that fatty acids taken up by the adipocyte and those synthesized de novo within the cell should be esterified into the three positions of triacylglycerols in proportions similar to the natural distribution of fatty acids in adipocyte triacylglycerols. However, since the positional distribution of exogenous (Table 3.4) and newly-synthesized (Table 3.14) fatty acids were not identical to each other or to the natural distribution in rat adipocyte triacylglycerols (Table 3.3), it can be concluded that factors other than the specificity of the acyltransferases contribute to the control of the manner in which fatty acids are esterified into the three positions of the triacylglycerols. One such factor could be the origin of the fatty acids, with those exogenous to the adipocyte being esterified differently from those synthesized within the cell as found in this study, thereby implying that endogenous and exogenous fatty acids do not mix but form separate substrate pools for esterification. This might arise if triacylglycerol synthesis from exogenous fatty acids occurred in a different subcellular

compartment from that with newly-synthesized fatty acids. The acyltransferases of each compartment could conceivably have different specificities, or the differing concentrations of the various fatty acid substrates in different compartments might affect their specificities. The possibility also exists, although it was not possible to explore it in this study, that the acyltransferases of the alternative minor pathways of triacylglycerol biosynthesis in the rat adipocyte, namely the monoacylglycerol and dihydroxyacetone phosphate pathways (184), utilise fatty acids from specific sources or have somewhat different specificities from the related enzymes of the sn-glycerol 3-phosphate pathway.

D. Subcellular Location of Newly-Synthesized Lipids and a Proposed Model for Triacylglycerol Biosynthesis in the Adipocyte

As discussed previously, the results presented in Figs. 3.10 and 3.16 suggest that endogenous and exogenous fatty acids may be used for the synthesis of two different pools of diacylglycerols which might have separate locations within the fat-cell. Previous workers, using subcellular preparations, have shown that both the mitochondria and microsomes were involved to varying degrees in the synthesis of phosphatidic acid (156,168), diacylglycerols (317) and triacylglycerols (280). More recently, the plasma membranes and microsomes of rat adipocytes were shown (271) to be equally active in triacylglycerol synthesis whereas in the hepatocyte, the esterification capability of the plasma membrane was less than that of the microsomes (318). Since adipose tissue is more active than the liver in taking up fatty acids

from the plasma for storage, this may be an adaptation in the adipocyte for the use of exogenous fatty acids in triacylglycerol synthesis. On the basis of the foregoing evidence it is possible that different acylglycerols may be synthesized in different compartments within the adipocyte depending on the origin of the fatty acids used in their synthesis. For example, exogenous fatty acids originating outwith the cell may be esterified predominantly at the plasma membrane whereas fatty acids synthesized de novo in the cytoplasm may be the preferred substrate for microsomal triacylglycerol synthesis. However, this hypothesis could not be verified in the studies with subcellular fractionations (Chapter 3, section J). The main reason for this failure was probably the method of study adopted. Although the use of digitonin followed by rapid centrifugation has proved effective for the separation of particulate and cytoplasmic components of hepatocytes, no reports on its use with adipocytes were available. The conditions employed were perhaps not entirely optimum; the concentration of digitonin used may have been too low to completely lyse the plasma membrane, resulting in the fat-cells remaining intact or, alternatively, the digitonin may have caused the lysis of both plasma and mitochondrial membranes. The optimum concentration of digitonin and the optimum incubation time could be determined by further studies involving the assay of marker enzymes in the separated fractions. It was also possible that the lipid storage droplet acted as a solvent to strip the comparatively small amounts of newly-synthesized lipids from their sites of synthesis.

Nevertheless, the proportions of incorporated radioactivity recovered in phospholipids and unesterified fatty acids were always

highest in the particulate pellet regardless of the nature of labelled precursor used and the age of the animal from which the cells were isolated. However, the significance of this observation is uncertain since the pellet contained small amounts only of radioactivity. For the same reason, no emphasis can be attached to the finding that the proportions of total incorporated acetate, glucose and palmitic acid in diacylglycerols were apparently higher in the particulate fraction than in the cytoplasmic and lipid storage fractions after short incubation periods (Tables 3.31 and 3.32). Similar results to those found in this present study for the subcellular location of newly-synthesized lipids were reported by Angel (279) who used more conventional time-consuming methods for the preparation of subcellular fractions of rat adipocytes which had been incubated with ^{14}C-labelled glucose or acetate.

The present results were consistent with the suggestion (279,280) that a major portion of the newly-synthesized diacylglycerols was transferred to the lipid storage droplet whilst the remainder was acylated to triacylglycerols. On this basis, it is possible that the diacylglycerols which accumulated newly-synthesized fatty acids at high acetate concentrations (Figs. 3.9 and 3.10) were compartmentalised within the lipid storage droplet. Alternatively, these diacylglycerols may have been associated with small droplets of newly-synthesized lipid and perhaps played a role in the transport of these droplets from the organelles of synthesis to the main storage droplet. Since the accumulation of endogenously-synthesized fatty acids in diacylglycerols was only observed with adipocytes prepared from older rats, the size of the lipid storage droplet may influence the rate at which any

diacylglycerols present in the droplet were released for further acylation.

The diacylglycerols which accumulated the newly-synthesized fatty acids in this study, might have been those formed from the degradation of an 'active' pool of triacylglycerols into which fatty acids were esterified but which equilibrated slowly with storage triacylglycerols, being predominantly turned over to diacylglycerols and unesterified fatty acid. The existence of such a triacylglycerol has been demonstrated by other workers in rat adipocytes (243,277) and protozoa (319). The hormone-insensitive 'triacylglycerol lipase' recently described by Matsumura et al (320) in pig adipose tissue might, if present in rat adipose tissue, have been involved in the rapid breakdown of this lipid. The structures of the diacylglycerols found (Tables 3.26 and 3.27) render this hypothesis unlikely, however.

In view of the foregoing discussion and the fact that different organelles in the mammalian cell may be closely related topographically with possible membrane-junctions between them (321), the synthesis of acylglycerols in the adipocyte should not be considered as being restricted to any one subcellular fraction. A more representative picture of the natural situation may be obtained by paying due regard to the origin of the fatty acids used in acylglycerol synthesis and the topographical relationships between enzymes, substrates and products.

The results of this study in general, were consistent with a model of the adipocyte in which a high proportion of exogenous fatty acids taken up by the cell were esterified predominantly at or near the plasma membrane, whereas fatty acids synthesized endogenously

from low-molecular weight precursors or by the modification (desaturation or chain elongation) of preformed fatty acids were esterified mainly in the endoplasmic reticulum or mitochondria. The rate-limiting step may be at the level of the diacylglycerol acyltransferase; diacylglycerols produced as intermediates may equilibrate with intracellular lipid storage droplets and the rate of esterification of these may be governed by the relative rate at which diacylglycerols, synthesized at various locations in the cell, are taken up and released by the lipid droplets. The positional distributions of fatty acids in triacylglycerols of the whole cell will be a mean of their distributions in triacylglycerols synthesized at various sites in the cell and may be dependent on the origin of the fatty acids, which would influence the composition of fatty acids available to the acyltransferases at the various points in the cell.

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