TRIACYLGLYCEROL SYNTHESIS IN RAT ADIPOSE TISSUE

A thesis

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ABBREVIATIONS

AMP - adenosine 5'-monophoshate

ATP - adenosine 5'-triphoshate

B.P. - boiling point

B.S.A. - bovine serum albumin

cAMP - adenosine 3',5'-monophosphate

Ci - curie

CoA - coenzyme A

DG - diacylglycerol

DGAT - diacylglycerol acyltransferase

DHAP - dihydroxyacetone phosphate

DHAPAT - dihydroxyacetone phosphate acyltransferase

EDTA - ethylenediaminetetraacetic acid

E.R. - endoplasmic reticulum

FFA - free fatty acid

G-3-P - sn-glycerol-3-phosphate

GPAT - sn-glycerol-3-phosphate acyltransferase

I.U. - international unit

MAGAT - monoacylglycerol acyltransferase

MAGPAT - monoacylglycerol phosphate acyltransferase

MES - 2-[N-morpholino]ethanesulfonic acid

MG - monoacylglycerol

PA - phosphatidic acid

PAP - phosphatidic acid phosphatase

PC - phosphatidylcholine

PE - phosphatidylethanolamine

PL - phospholipid

POPOP - 1,4 bis(2-(5-phenyloxazolyl))-benzene

PPO - 2,5-diphenyl oxazole

S.D. - standard deviation

sn - stereospecific numbering

TG - triacylglycerol

Tris - tri-(hydroxymethyl)methylamine

Lipid nomenclature

For the specific structural designation of complex lipids containing a glycerol moiety, the nomenclature suggested by the IUPAC-IUB Commision on Biochemical Nomenclature (European J. Biochem.(1967) 2,127). The trivial names of complex lipids have been used when it is more appropiate to do so.

Fatty acids are designated by the shorthand notation of the number of carbons atoms: number of double bonds, e.g. 16:0 refers to hexadecanoic acid (palmitic acid).

CHAPTER 1

Review of literature

1.1 Introduction

Adipose tissue consists of two types of cell; the adipocyte and the non-adipocyte (Rodbell, 1964). The adipocyte is unique amongst mammalian cell types in that one class of component (TG) comprises up to 80% of its weight. These TG's constitute the major energy storage material in higher animals. The importance of adipose tissue in mammalian metabolism lies in its ability to store free-fatty acids (FFA) as TG and to release them again according to physiological demands. It is therefore understandable that the pathways of synthesis and degradation should be under strict control. Apart from adipose tissue TG metabolism is especially active in the mammary gland during lactation and in the liver and intestinal mucosa cells during synthesis and secretion of serum lipoproteins.

The general pathway of TG synthesis in adipose tissue as proposed by Kennedy and co-workers (1961), according to Figure 1-1, involves the step wise acylation of sn-glycerol-3-phosphate (G-3-P) by acyl-CoA thioesters. This G-3-P pathway is accepted as being quantitatively the most important, but acylation of dihydroxyacetone phoshate (DHAP) (Hajra, 1972) and monoacylglycerol (MG) are also known to occur. As well as the synthesis of TG the acylation of G-3-P and subsequent dephosphorylation to diacylglycerol (DG) are steps involved in the synthesis of the glycero-phospholipids (PL), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Figure 1-2). Significant progress has been made in understanding the hormonal control of TG degradation via the cyclic 3',5' AMP-dependant activation of hormone sensitive

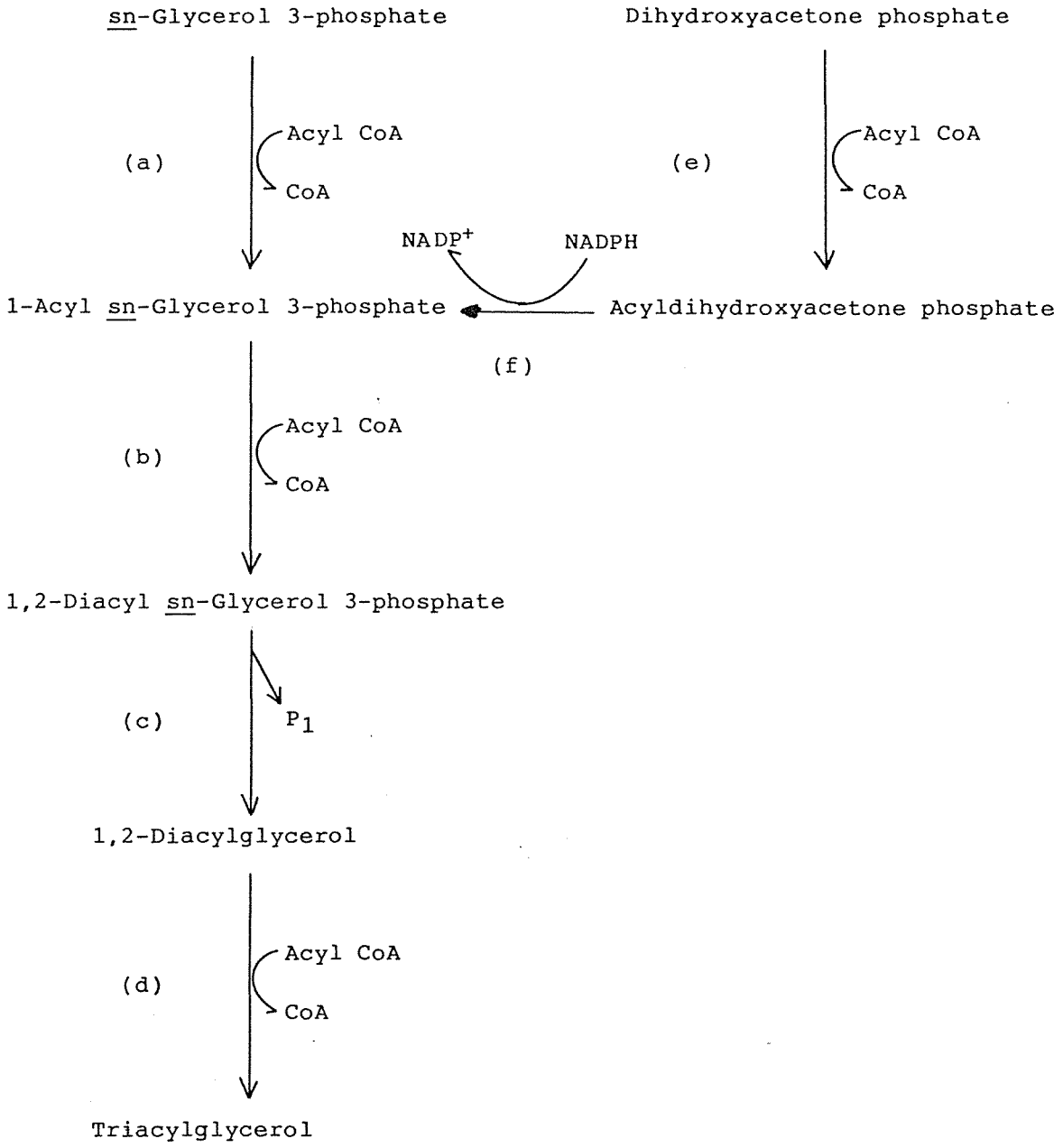


Figure 1-1. Pathways of triacylglycerol synthesis in adipose tissue. The enzymes involved are (a) sn-glycerol 3-phosphate 1-acyltransferase (GPAT), (b) 1-acylglycerol 3-phosphate acyltransferase, (c) phosphatidate phosphatase (d) diacylglycerol acyltransferase, (e) dihydroxyacetone phosphate acyltransferase (f) 1-acylglycerol 3-phosphate dehydrogenase.

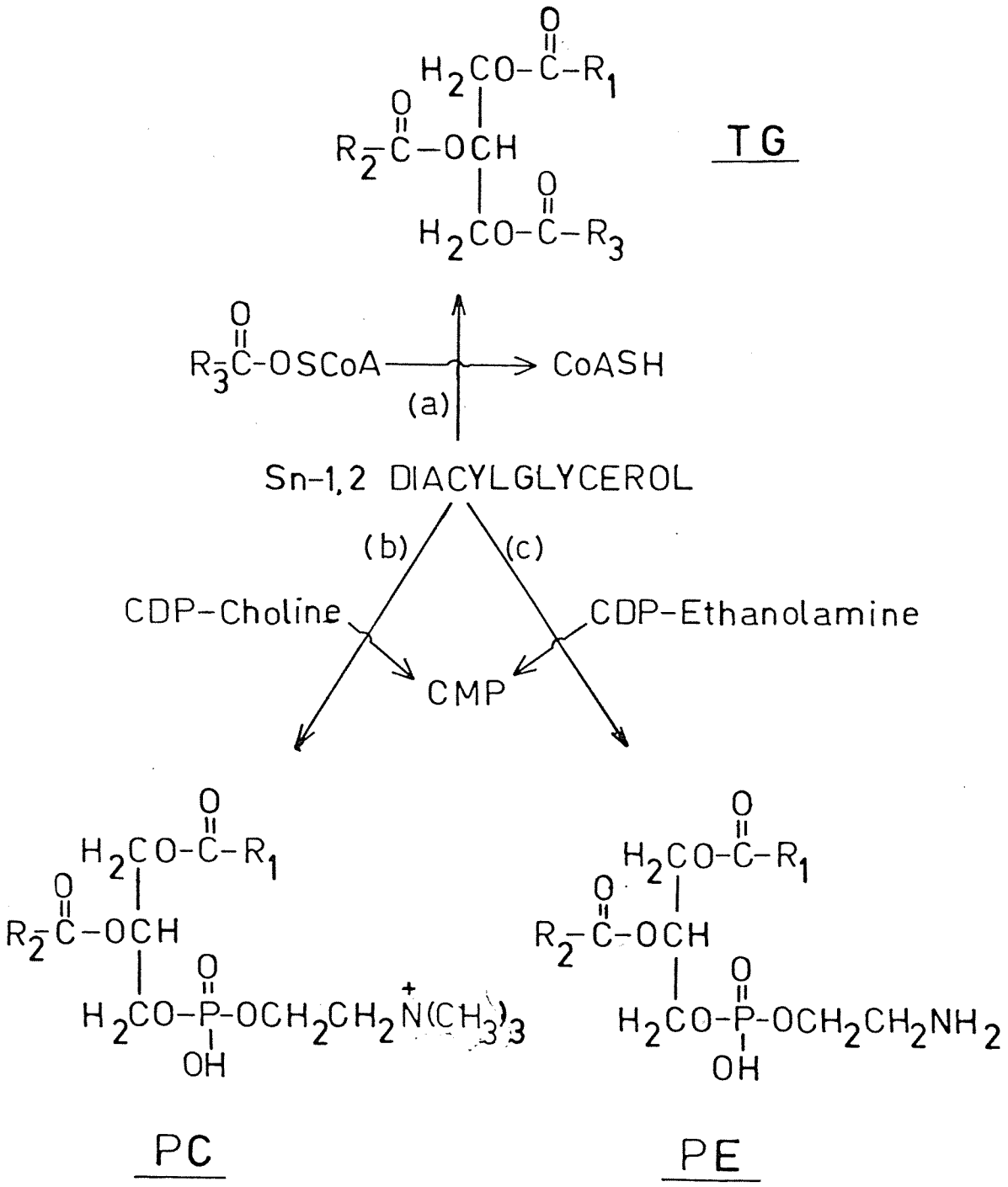


Figure 1-2. Synthesis of triacylglycerol, phosphatidylcholine and phosphatidylethanolamine from sn-1,2 diacylglycerol.

The enzymes involved are (a) diacylglycerol acyltransferase, (b) cholinephosphotransferase, (c) ethanolaminephosphotransferase. TG : triacylglycerol, PC : phosphatidylcholine, PE : phosphatidylethanolamine, CDP : cytidine 5'-diphosphate, CMP : cytidine 5'-monophosphate.

lipase (Khoo, et al. 1974). However, knowledge concerning the mechanisms regulating the synthesis of TG remain incomplete. The major question is how glycerolipid metabolism is regulated to give rise to an appropriate mixture of products to meet the demands of energy storage and utilisation, membrane biogenesis and lipoprotein biosynthesis.

1.2 Cellular location of triacylglycerol synthesis

It has been shown that the principle site of TG and PL synthesis occurs at the endoplasmic reticulum (ER). The products of these pathways are either incorporated into the adipocyte membranes (PL's) or transferred to the storage lipids (TG's). Electron microscope studies of adipocytes have shown that the smooth ER cisternae are applied to the surface of the fat droplet (Cited by Giacobino, 1979). Localisation of the enzymes at the ER has been facilitated by studies showing that microsomal vesicles maintain the orientation of the ER (DePierre and Dallmer, 1975). Coleman and Bell (1978) found that treatment of rat liver microsomes with chymotrypsin or pronase lead to the inactivation of acyl-CoA synthetase, dihydroxyacetone phosphate acyltransferase (DHAPAT), monoacylglycerolphosphate acyltransferase (MAGPAT) and diacylglycerol acyltransferase (DGAT). This would indicate that the critical regions of TG synthesis are exposed on the cytoplasmic surface. This coupled with the inability of ATP and palmitoyl-CoA (16:0 CoA) to penetrate microsomal vesicles would tend to indicate that the active sites are at the cytoplasmic surface. This orientation would allow ready access to FFA's, CoA and ATP which are required for fatty acid activation, and access to CDP-choline and CDP-ethanolamine (synthesised by soluble cytoplasmic enzymes) for PL synthesis.

1.3 Fatty acid activation

The activation of FFA's to acyl-CoA thioesters by one of several chain-length dependent acyl-CoA synthetases (Groote, et al. 1976) is required prior to acylation. TG's contain predominantly long chain FA's (Van Golde et al. 1974) involving activation by long-chain acyl-CoA synthetase (EC 6.2.1.3)

$$R\text{-}\overset{O}{\overset{\|}{C}}\text{-}OH + CoASH + ATP \longrightarrow R\text{-}\overset{O}{\overset{\|}{C}}\text{-}SCoA + AMP + PPi$$ (Khoo and Steinberg, 1974)

Detailed knowledge of acyl-CoA synthetase from work with bacterial systems is available (Coleman and Bell, Review 1980) but little information exists concerning eucaryote systems. Long-chain acyl-CoA synthetase has been solubilized from chicken adipose tissue to reveal a protein of 90,000 daltons (Banis and Tove, 1974). Jason et al. (1976) have characterised this activity using the microsomal fraction from isolated fat cells. They established that 80% of activity was associated with the microsomal fraction, they also established the kinetics for the various FA's. A two-fold increase in activity was demonstrated when isolated fat cells were incubated with physiological levels of insulin. The increase was both rapid and consistent regardless of using radioactively labelled CoA or FFA. This effect of insulin on acyl-CoA synthetase would serve to promote the capture of FFA's and hence promote TG synthesis. Also the rapid response would serve to control the distribution of catabolic fuel as requirements change. In conjunction Sooranna and Saggerson (1978c) have reported a 30-40% decrease in acyl-CoA synthetase activity in homogenates prepared from freeze-stopped adipocytes incubated with adrenalin. The effect however was relatively slow (30 mins) compared to the more rapid effects of insulin (2 mins). The effects of adrenalin could be blocked by the β blocker propanolol and by insulin. Insulin alone, however, had no

effect. It could be that the effects of adrenalin are secondary to a increase in the rate of lipolysis. The lack of stimulation by insulin is in contrast to Jason et al. (1976) but it should be remembered that different assay systems were employed.

The critical micelle concentration of acyl-CoA thioesters is 3-4 μM (Zahler, et al. 1968). Acyl-CoA's when added to incubations are usually added in excess of this concentration and it has been observed that the incorporation of 16:0 in the presence of CoA and ATP is greater than that of 16:0-CoA. Adipose tissue, as in the case of brain (De Jiminez and Cleland, 1969) and liver (Abou-Issa and Cleland, 1969), does not contain a pool of endogenous acyl-CoA thioesters.

1.4 Substrates for esterification of acyl-CoA thioesters

There are three initial acylations of three different substrates that can lead to TG synthesis in adipose tissue. They are the acylation of G-3-P, DHAP and MG. There has been some conjecture as to whether the acylation of G-3-P and DHAP are catalysed by a single enzyme. The distribution of both acylation activities in adipose tissue have been shown to be essentially the same (Schlossman and Bell, 1976). It has also been demonstrated that the acylation of each substrate is competitively inhibited by the other substrate while both acylation activities respond identically to N-ethylmaleimide, trypsin and detergent treatment (Schlossman and Bell, 1976). The failure of G-3-P to inhibit DHAPAT completely is probably due to the dual localisation of the enzyme in adipose tissue with the G-3-P-insensitive activity located in the mitochondria (Dodds, et al. 1976).

With rat liver slices it was found that 50-60% of the glycerol

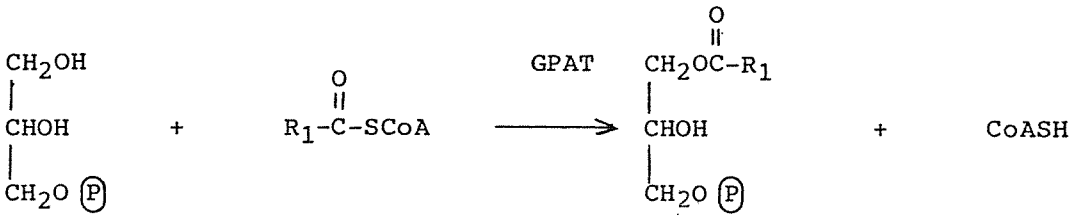
incorporated into lipid proceeded by the DHAP pathway (Manning and Brindley, 1972). In rat adipocytes the levels of G-3-P and DHAP have been estimated to be approximately 8.1 and 2.9 nmoles g^{-1} respectively (Ballard, 1972). Using these concentrations and known kinetic parameters it has been calculated that the ratio of G-3-P to DHAP acylation is greater than 12.0. The existence of a single enzyme would simplify the regulation of these committed steps of TG/PL synthesis, while the existence of two separate enzymes would complicate regulation unless they were coordinately controlled.

The role to the enzyme involved in the third acylation, MAGAT, is relatively unknown. This enzyme may play a role in the reesterification of MG's which are absorbed from the blood or it may be involved in the recycling of MG (derived from lipolysis) back into TG.

1.5 Enzymes of triacylglycerol synthesis

1.5.1 Glycerol-3-phosphate acyltransferase (GPAT)

The first step in the glycerophosphate pathway of TG synthesis is the acylation of G-3-P which is catalysed by the enzyme GPAT (EC 2.3.1.15).



G-3-P lyso-phosphatidic acid

In adipose tissue GPAT is almost exclusively located in the microsomal fraction (Schlossman and Bell, 1976) in contrast to the liver where activity is found more equally in the microsomal and mitochondrial fractions. Due to the differing responses to the thiol group reagents

iodoacetamide and N-ethylmaleimide (Bates and saggerson, 1977) it has been proposed that the two hepatic activities are due to isoenzymes of GPAT (Nimmo, 1979).

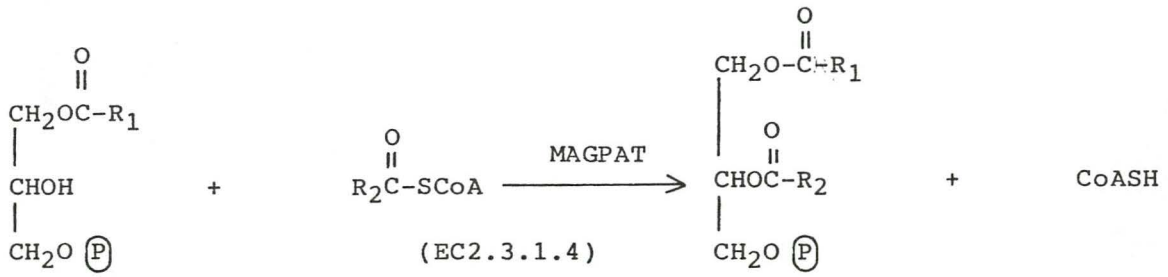
In assays where a whole tissue homogenate is used the product of G-3-P acylation is dependant on the assay conditions. Lysophosphatidic acid is not usually an isolatable intermediate of the synthetic pathway with acylation proceeding through to phosphatidic acid (PA). Increasing the amount of albumin in the medium favours formation of lysophosphatidic acid whereas longer incubations and increased amounts of microsomal protein tends to favour PA as the isolatable intermediate (Zaror-Behrens and Kako, 1976). The initial acylation of G-3-P is exclusively in the sn-1 position with little substrate specificity. This is in contrast to hepatic GPAT which preferentially utilises 16:0 CoA compared to 18:1 CoA and 18:2 CoA (Haldar and Pullman, 1975).

Being the first reaction of the TG/PL biosynthetic pathways GPAT has received considerable attention as a possible control site of the pathway. Incubation of rat adipocytes with adrenalin results in a decrease in GPAT activity (Sooranna and Saggerson, 1978b). Although there is an accompanying accumulation of FFA's it seems unlikely that the decrease in GPAT activity is due entirely to this, as there was no further decrease in GPAT activity when 16:0 was added in a concentration in excess of 3.5 mM (Sooranna and Saggerson, 1976b). Also a lower concentration of adrenalin produced significant decreases in GPAT activity while only producing modest accumulation of unesterified FFA's (Sooranna and Saggerson, 1976a). The effect of adrenalin can be abolished by propanolol implying involvement of a β adrenergic receptor.

Nimmo and Houston (1978) have reported a 87% inhibition of GPAT activity by cAMP-dependent protein kinase when added to adipose tissue microsomes. GPAT activity could be reactivated to 80% of the previous activity by addition of alkaline phosphatase. No inhibition was recorded in the absence of ATP/$MgCl_2$ indicating inhibition by phosphorylation due to cAMP-dependent protein kinase. Unlike hormone sensitive lipase, GPAT activity could not be reactivated by addition of the multifunctional protein phosphatase III from rabbit muscle. Although GPAT can be reactivated by addition of alkaline phosphatase, no fraction of adipose tissue has been shown to be capable of reversing the reaction. These results serve to strengthen the analogies between TG synthesis and glycogen synthesis.

1.5.2 Monoacylglycerol-3-phosphate acyltransferase (MAGPAT)

This enzyme catalyses the second acylation step



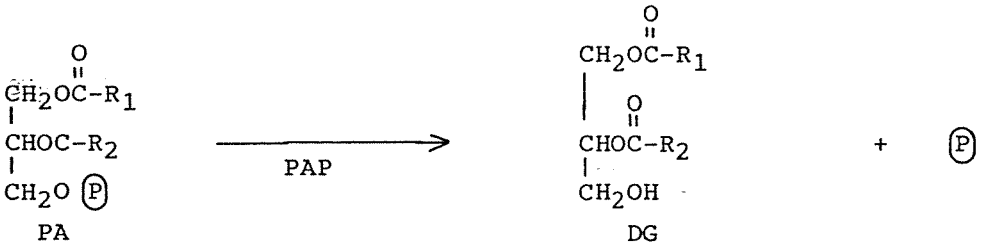
lysophosphatidic acid phosphatidic acid

An analysis of TG's of animal fats reveals that the positional distribution of FA's within the glyceride molecule is not random. There is a tendency for unsaturated FA's to occupy the sn-2 position and for saturated FA's to occupy either the sn-1 or the sn-3 position or both. The exception to this pattern is found in the pig where in all tissues, except the liver, 82% of the 16:0 is found in the sn-2 position (Mattson et al., 1964). The structure of the tissue TG's can be accounted for by the acyl donor specificity of the

microsomal acyltransferases. Although partially purified GPAT shows a preference for 16:0 compared to 18:1/18:2, MAGPAT shows no preference for unsaturated acyl-CoA donors. In conjunction the characteristic accumulation of short chain FA's (C -C) at the sn-3 position in ruminant milk TG's is most likely due to the ability of DGAT to preferentially use these short chain FA's (Marshall and Knudsen, 1977). Although acylation of G-3-P leads to PA as the major isolatable intermediate, MAGPAT is a distinct identity. All three acyltransferase activities have been partially separated from microsomes, solubilised with triton X-100, by sucrose density centrifugation (Hosaka et al., 1977).

1.5.3 Phosphatidic acid phosphatase (PAP)

Phosphatidic acid phosphatase (EC 3.1.3.4) catalyses the conversion of phosphatidic acid into diacylglycerol.

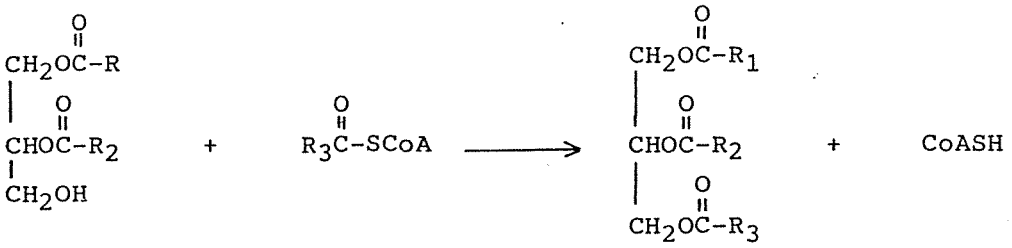


Studies of the incorporation of radioactive G-3-P into lipid show a rapid accumulation of radioactivity into PA with a much slower accumulation into neutral lipid (Fallon et al., 1975). As such PAP is considered to be the rate-limiting step in TG synthesis (Dodds et al., 1976a). Both Mg -dependent and Mg -independent forms of PAP have been shown to be present in the soluble and microsomal fractions of adipose tissue (Jamdar and Fallon, 1973). The specific activity of PAP is 20-fold higher in adipocytes compared to the liver and is the only activity of the TG synthetic pathway that does not increase with differentiation of 3T3-L1 preadipocytes

(Coleman et al., 1978). Adipocyte Mg - dependent PAP activity can be decreased by physiological concentrations of noradrenalin (Cheng and Saggerson, 1978a). This effect can be abolished by propanolol and insulin, with insulin resulting in an actual increase in PAP activity. Part of the inactivation of PAP activity by adrenalin could be due to an accumulation of FFA's, however, it cannot be considered secondary to the accumulation of FFA's (Sooranna and Saggerson, 1978c). It is therefore not known whether inhibition is due to some product of lipolysis or a more direct effect of the hormone, i.e., through a second messenger(s).

1.5.4 Diacylglycerol acyltransferase(DGAT)

The final enzyme in the TG biosynthetic pathway, DGAT (EC 2.3.1.20) catalyses the reaction



sn-1,2 diacylglycerol triacylglycerol

DGAT activity was first described from chicken adipose tissue (Weiss, et al. 1960). DGAT activity is present in a variety of tissues with greatest activity in adipose tissue and in liver (Table 1-1). The reaction catalysed by DGAT is the only reaction of the glycerolipid pathway that is exclusively concerned with TG synthesis. As the sn-1,2 DG substrate is also utilised in PL synthesis regulation of TG/PL synthesis at this branch point seems likely. Regulation of DGAT in adipose tissue seems likely since its uncontrolled action during lipolysis would form a futile cycle. It has been demonstrated by Brooks et al. (1982) that there is an increase in FA/TG cycling with the use of lipolytic hormones. DGAT activity has been documented from a

TABLE 1-1

Tissue survey of DGAT activity

Tissue	Specific activity
	nmoles min^{-1} mg $protein^{-1}$
Fat	51.3±20.1*
Liver	2.9
Brain	2.2
Lung	1.3
Intestine	1.2
Kidney	1.1
Heart	0.9
Skeletal muscle	0.4

* mean value ± S.D. of six independent microsomal preparations

Coleman and Bell (1976)

variety of tissues, namely rat liver (Hosaka, 1977; Haagsmann, et al 1982), rat adipocytes (Coleman and Bell, 1976), bovine mammary gland (Marshall and Knudsen, 1977) and pig perinephric adipose tissue (Stokes, et al. 1975). Characterisation of DGAT activity has always been impeded due to the difficulties in presenting DG substrates to the enzyme. Results from early methods have been proved unsatisfactory with the advent of an assay utilising ethanol as a dispersal medium (Coleman and Bell, 1976). The use of ethanol dispersal has revealed that detergents such as Tween 20 actually inhibit DGAT activity. There has been a 12-fold increase in DGAT activity in spinach leaves using a 0.02% solution of Zwittergent 2.08 rather than ethanol (Martin and Wilson, 1983). The use of ethanol-dispersed substrates has allowed the use of 100 to 1000-fold less protein than previous methods and results in a 100-fold increase in specific activity. This is essential as it has been shown that adipocytes contain little protein (Jarett, 1974). Adipose tissue DGAT has been characterised fully by Coleman and Bell (1976) in regard to substrate specificity. DGAT is highly specific for the sn-3 position as less than 5% of the activity present with sn 1,2 diolein as substrate was seen when using sn-1,3 diolein as the DG substrate. The dependence on the acyl-CoA substrates was also tested, with C_{10}-CoA giving the highest activity of all the acyl-CoA's tested.

1.6 Regulation of DGAT

Haagsman (1981a) has demonstrated that in hepatocytes exposed to glucagon there is a decrease in DGAT activity while cholinephosphotransferase activity is uneffected. While incubation of hepatocytes in the presence of FFA's leads to enhanced TG synthesis with

PL synthesis being less effected (Haagsman, 1981b). Hepatic DGAT activity can be reduced rapidly by incubation of microsomes in the presence of the 105,000 x g supernatant. Haagsman et al. (1982) concluded that this would tend to suggest that DGAT activity could be modulated in a reversible way, possibly by a phosphorylation-dephosphorylation mechanism similar to that proposed by Nimmo (1978) for the regulation of GPAT activity. This is supported by the fact that ATP cannot be replaced by methylene ATP, suggesting the cleavage of a phosphate group playing a role in the regulatory process. The use of fluoride ions in the homogenisation medium, which is a known inhibitor of phosphoprotein phosphatase, results in a lower DGAT activity. The activating factor has been shown to be a protein and the fact that it can be stored at $4^{o}C$ with no loss in activity eliminates soluble phosphatidic acid phosphatase which looses activity when stored at $4^{o}C$ (Haagsman, et al. 1982). This process of reversible activation/inactivation would therefore allow for rapid interconversion of DGAT between metabolically active and inactive forms. Apart from this reported control of DGAT, the enzyme is also subject to control by a number of metabolic effectors with levels of acyl-CoA substrates being inhibitory above 40 μM (Coleman and Bell, 1976). Conversion of DG to TG has been reported to be activated 15-fold by Z protein which constitutes 2% of liver proteins and is known to have different affinities for the various acyl-CoAs and is thought to actively promote the activity of DGAT.

1.7 Systems for the study of TG biosynthesis in adipose tissue

Many approaches have been taken in the course of the study of the TG biosynthetic pathway. These include use of whole adipose tissue (incubation of the fat pad), whole adipose tissue homogenates, isolated

adipocytes, subcellular fractionation (microsomal vesicles) and use of the fat cell model (3T3-L1 preadipocyte cell line). Tissue homogenates have probably been the most commonly used source of enzymes in the investigation of TG synthesis. This cell-free system, which contains the whole complement of synthetic enzymes, has been very important in determining the steps involved. Information about the possible control of the pathway and characterisation of cofactor requirements has been gained through use of this system (Christie et al., 1976). Isolated adipocytes make use of the isolated functional adipose tissue unit and allows the role of transport processes to be studied, especially the effects of hormones which normally interact at the plasma membrane. Incubation of adipocytes with hormones followed by homogenisation and study of the individual enzyme activities has lead to the elucidation of the regulatory steps of the synthetic pathway (Sooranna and Saggerson, 1976a,b; 1978a,b,c). Subcellular fractionation (Jarett, 1974) involves the isolation of the microsomal fraction which contains the complement of enzymes associated with the endoplasmic reticulum (Ballas and Bell, 1976). Higher specific activities of the enzymes are achieved, but there are difficulties in presenting the substrates to the enzyme system. The 3T3-L1 subline of the original Swiss 3T3-M fibroblasts has the capacity to differentiate into adipocyte colonies (Green and Kehinde, 1974). During the differentiation of 3T3-L1 fibroblasts to adipocytes there is a simultaneous and coordinated increase in the activity of the four microsomal enzymes of TG synthesis; namely acyl-CoA synthetase, GPAT, MAGPAT and DGAT. The specific activities of these enzymes were 30-100-fold greater compared to undifferentiated 3T3-L1 or 3T3-C2 control cells (Coleman et al. 1978). The levels of PAP activity best correlates the TG content of the cell while DGAT

activity is a sensitive indicator of 3T3-L1 differentiation into adipocytes (Grimaldi et al., 1978). The rise in enzyme activities during differentiation can be prevented by cycloheximide indicating induction due to increased enzyme synthesis.

The 3T3-L1 fat cell model can also be used to follow the development of hormone receptors and hormone responsiveness in adipocytes (Rosen et al., 1978). Whichever system is used to study TG systhesis a number of difficulties exist, these include; the tight association of enzymes with the endoplasmic reticulum resulting in difficulties in solubilisation, difficulty in fractionation of solubilized enzymes, the dependence of partially purified enzymes on PL's for activity and the delivery of amphipathic and hydrophobic substrates to membrane bound and solubilised enzyme preparations. Therefore our understanding of the regulatory process of the glycerolipid pathway has been limited due to the lack of homogenous enzymes, specific enzyme inhibitors and mutations containing defective enzymes.

1.8 The effect of adipocyte size

It is a well established fact that the size (surface area) of fat cells from man and animals is an important determinant for metabolic rates (Jamdar and Osborne, 1981). Those functions of adipose tissue which are subject to change with adipocyte cell size include lipolysis, lipoprotein lipase activity, glucose incorporation into lipid and esterification of exogenous FFA's into lipid. Glycerolipid formation is most active in rat adipose tissue taken from donors 60 days old where the mean adipocyte cell size is 66 μM, compared to 74 μM in animals 130 days old (Jamdar and Osborne, 1981). The accompanying increase in the amount of adipose tissue with age is known to be due to both hypertrophy

and hyperplasia of the adipocyte (Hirsh and Han, 1969). In age-dependent studies glycerolipid formation was shown to be low in 15 day-old animals with a 6-7-fold increase at 30 days with maximal activity at 60 days. The decline with further age has been linked with age, as adipocytes taken from animals 28 days old were twice as active in glyceride formation as adipocytes of the same size taken from 100 day-old animals (Jamdar and Osborne, 1981). It has been intimated that both PAP and DGAT activities may be higher in younger rats (30 days) compared to older rats (120 days old). Changes in enzyme activity with age could be due to variations in the PL content of the microsomal membranes; several of the TG synthetic enzymes require PL's for their activity (Coleman and Bell, 1980). In conjunction Jamdar et al., (1981) has reported an increase in GPAT activity with age (15 to 60 days) and also a 3.5-fold increase in DGAT activity, and concludes that there is an acceleration of the entire pathway with age. Also of importance is the anatomical location of the adipocytes, with subcutaneous adipocytes being significantly smaller than perirenal and epididymal adipocytes.

1.9 The effects of hormones on triacylglycerol synthesis

When assessing the effects of hormones it is difficult to decide whether the changes are due to alterations in intracellular enzyme activities or are they secondary to changes in precursor concentrations in the adipocyte. The hormonal control of TG synthesis has been summarised by Nimmo (1980) while the effects of hormones on the enzymes of lipid metabolism have been summarised by Saggerson et al. (1979).

1.9.1 The effects of insulin

The effects of insulin appear to be initiated by the interaction of the hormone with its receptor on the target cell plasma membrane (Butcher, et al 1973). The binding of insulin to its receptor correlates with the initiation of many of the short term effects of insulin including stimulation of glucose transport, stimulation of glucose metabolism, lipogenesis and inhibition of lipolysis. Phosphorylation of proteins has been shown to be an important regulatory mechanism in metabolic pathways (Greengard, 1978). Treatment of adipocytes with insulin has been shown to alter the incorporation of ^{32}P, phosphate into several phosphoproteins (Benjamin and Clayton, 1978) (Walaas, et al. 1981). Control of membrane protein phosphorylation may be of importance in the initial steps of insulin action. Hormone control of the phosphorylation of serine residues could alter specific properties of the proteins controlling the entry of solute molecules into the channel and hence could alter the membranes transport capacity.

In isolated adipocytes insulin treatment results in an increase in TG synthesis (Sooranna and saggerson, 1975). This is probably due to an increase in glucose transport into the adipocyte and thus increasing the amount of triose phosphate available for esterification. Coupled to this is the strong effect of insulin on lipoprotein lipase which increases the supply of exogenous precursors to fat cell. Insulin also effects a 2-fold increase in acyl-CoA synthetase activity increasing the supply of acyl CoA thioesters (Jason et al., 1976).

Insulin has been shown to stimulate fatty acid synthesis at low concentrations of fatty acids, probably through activation of acetyl-CoA carboxylase and pyruvate dehydrogenase (Sooranna and Saggerson, 1975). Insulin inhibits lipolysis in adipocytes at physiological concentrations

(1 nM). The mechanism by which insulin achieves this may be due to a lowering of cAMP levels, but the decrease in lipolysis is not comparable to the increase in cAMP levels (Kono, 1973). When the levels of cAMP have been elevated by noradrenalin the relative effect of insulin to lower the nucleotide level is too small to explain the antilipolytic effect of the hormone. Treatment of rat adipocytes with insulin results in a small but significant activation of GPAT activity (Sooranna and Saggerson, 1976a). Insulin alone has no effect on PAP activity, it does however reverse the inactivation of PAP by noradrenalin (Cheng and Saggerson, 1978b). A similar effect is seen in DGAT activity where insulin alone has no effect but it can oppose the effects of catecholamines (Sooranna and Saggerson, 1978c).

1.9.2 The effects of adrenalin

In adipose tissue, adrenalin has a lipolytic effect with an increase in both the release of FFA's into the blood and the rate of their re-esterification back into TG. The majority of the effects of adrenalin can be blocked by propanolol indicating action via a β-adrenergic receptor. Adrenalin has been shown to cause; a 30-40% decrease in acyl-CoA synthetase activity (Sooranna and Saggerson, 1978), a dose-dependent decrease in GPAT activity (up to 50%) (Sooranna and Saggerson, 1978a) a decrease in the diolein dependent incorporation of 16:0 CoA into TG (Sooranna and Saggerson, 1978e). The net result of adrenalin action therefore is a decrease in the activity of the key regulatory sites of TG synthesis resulting in decreased TG synthesis. These effects have been observed in tissue that has been exposed briefly to adrenalin, freeze-stopped and then a tissue extract prepared for the enzyme assay. This method gives insight into the location of the individual steps that are altered by adrenalin, but does not reveal anything about the mechanisms involved.

1.9.3 The effects of noradenalin

Much less is known about the action of noradrenalin on adipose tissue compared to the actions of insulin and adrenalin. Incubation of adipocytes with noradrenalin causes a rapid inactivation of PAP activity. This inactivation is confined to the Mg^{++}-dependent activity which is mainly in the soluble fraction (Cheng and Saggerson, 1978a). An important governing factor appears to be the ratio of catecholamines to insulin.

1.9.4 The effects of glucagon

The concentration of glucagon, a lipolytic hormone, increases with fasting and corresponds to a decrease in FA synthesis and an increase in FA oxidation. Inhibition of glycerolipid formation by glucagon is probably best explained by an inhibition of glycolysis, this limits the supply of triose phosphate precursors. In liver glucagon has been shown to enhance the utilisation of DG's for PL synthesis, this may be important in maintaining the rate of PL synthesis at the expense of TG formation (Geelen et al., 1978a).

1.10 Effect of diet on triacylglycerol synthesis

It is expected that the rate of TG synthesis will be high in both the liver and adipose tissue of animals that consume large amounts of fat and carbohydrate. Conversely during starvation TG is broken down in adipose tissue and the FA's transported to the liver and the other organs for metabolism. It is therefore reasonable to assume that these dietary changes will be reflected by changes in the activities of the TG synthetic enzymes. The effects of these dietary modifications on the hepatic activity of the enzymes of TG synthesis have been summarised (Brindley, 1978).

1.10.1 The effect of starvation

During periods of starvation there is a decrease in the amount of adipose tissue and a reduction in the rate of TG formation by adipose tissue homogenates (Angel and Roncari, 1967). This reduced ability to synthesise TG should therefore be reflected in the reduced activity of one or more of the synthetic enzymes. A 20% reduction in GPAT activity in adipocyte homogenates has been recorded during a 48hr fast (Sooranna and Saggerson, 1979), as well as a 64% reduction in cytosolic Mg^{++}-dependent PAP activity during a 72hr fast (Moller et al., 1977). In conflict with these results Lawson et al., 1981) has reported no significant changes in GPAT, Mg^{++}- dependent PAP or DGAT activities over a 48hr fast. They did however note a 25% decrease in palmitoyl-CoA synthetase activity.

The products of esterification of G-3-P by adipocyte homogenates from animals subjected to starvation are altered in comparison to fed controls. The esterification of FA's into PA is increased with an accompanying decrease in the formation of neutral lipid, which is consistent with a decrease in PAP activity (Jamdar and Osborne, 1982). In adipose tissue from normally-fed animals the rate of oxidation of FA's is low compared to the rate of estification. During starvation there is a decrease in the ability to restrain the oxidation of FA's, both endogenous and exogenous (Harper and Saggerson, 1976). Refeeding rapidly restores lipid synthesis to the normal fed levels and frequently produces an overshoot (Etherton and Allen, 1980). The age of the adipose tissue also appears to influence the effect of starvation, with a 2-fold decrease in GPAT activity recorded in young rats (42-60 days) following a 72hr fast but no decrease recorded in older rats (120 days) even though they were fasted for 96hr. A 2-fold reduction in PAP

activity was seen regardless of tissue age (Jamdar and Osborne, 1982). Also in starvation there is a marked decrease in lipoprotein lipase activity and also a significant decrease in the supply of acyl-CoA thioesters available for esterification (Lawson et al., 1981).

1.10.2 The effect of a high lipid diet

Increasing the amount of lipid in the diet, either by the addition of corn-oil (polyunsaturated plant oil) or tallow (a more saturated animal fat), has the effect of increasing plasma levels of glucose, cholesterol and glycerol. Accompanying insulin levels are above normal, indicating perhaps a diminished sensitivity to insulin. The lipolytic effects of glucagon, adrenalin and noradrenalin are also reduced in fat-fed animals, the diminished response due to the reduced activation of the adenylate cyclase (Gorman et al., 1973). The binding of adrenalin to the receptors is not effected therefore the effect must be at a point between the receptor and the activation of adenylate cyclase. There is also a diminished response to insulin in animals on a high-fat diet. This mechanism of insulin insensitivity could be related to the effect of diet on the cell membrane (Gorman et al., 1973) and could explain the decrease in lipoprotein lipase activity in animals fed a high-fat diet (Lawson et al., 1981). These diminished cellular responses may be due to an increase in the size of the fat-cell (Gorman et al., 1972).

1.10.3 The effect of a high carbohydrate diet

Diets rich in carbohydrate increase the levels of plasma TG and can lead to triglyceridaemia (Smith et al., 1974). The greatest change in activity of the enzymes associated with TG synthesis is observed in lipoprotein lipase. A high carbohydrate diet leads to increased GPAT

activity and twice the molar flux through PAP compared to high lipid diets, this is compatible to the increased activities of PAP and DGAT (Dodds et al., 1976b). This is in agreement with a glucose/fructose diet increasing PAP/DGAT activity in the liver (Fallon et al., 1975). Generally a high carbohydrate diet leads to enhanced storage of the excess caloric material as TG.

1.10.4 The effect of a high-protein diet

The effect of a high protein diet on adipose tissue metabolism is largely unknown although its effects on hepatic glyceride formation have been documented (Holub, 1975). In the liver results indicate that the level of dietary protein can regulate the rate of lipid synthesis from G-3-P (Holub, 1975).

CHAPTER 2

The aim of the present study

The present study was undertaken to investigate the synthesis of TG, and the intermediates of the TG pathway, by parametrial adipose tissue homogenates from rats subjected to a variety of dietary and hormonal treatments. Consequently a study was undertaken to determine whether any of the differences in the ability to synthesis TG are reflected in changes in microsomal DGAT activity.

CHAPTER 3

Materials and methods

3.1 Materials

3.1.1 Reagents and Solvents

Chemicals used in the present study were obtained from the following sources: ATP, CoA, DTT, sn-1,2 diolein, sn- 1,2 dipalmitin, collagenase (C.histolyticum), insulin, alloxan, PPO, glucagon, B.S.A fraction V, DL glycerophosphate from Sigma Chemical Co. U.S.A.; Silica gel G from E. Merck A.G., Dramstadt, Germany; 1-^{14}C palmitic acid from Radiochemical Centre Ltd., Amersham, England. All other reagents were of analar grade and were obtained through BDH Chemicals Ltd, Poole, England or May and Baker Ltd., Dagenham, England. All solvents used were of analytical grade and were distilled before use.

3.1.2 Experimental animals

Female Sprague-Drawly rats were used throughout the course of this study. Animals were kept in a light-controlled room with a 12hr photoperiod (6.00am-6.00pm) with the temperature maintained at 25^{o}C. Fresh water and a pelletised food (Farm Products, Palmerston North) were available ad libitium. All animals were killed by stunning and cervical dislocation at between 9.00am-10.00am. For experiments involving infusion of hormones the animals were firstly anaesthetised with an interperotoneal injection of ethyl carbonate at a dosage of 1.5gm/kg.

3.1.3 Treatment of experimental animals

(a) Experimental diets

For the purpose of determining the effects of diet on TG synthesis, animals were divided into four groups of 10 animals. Each group received an experimental diet as described in Table 3-1. The fourth group serving as the control group received the standard lab chow (Farm Products, Palmerston North). Each diet was supplemented with a mineral and vitamin mix as described in Table 3-2. Body weights were measured individually each day throughout the experimental period. After 10 days the animals were sacrificed and the parametrial adipose tissue from each group pooled for tissue preparation.

(b) Induction of alloxan-diabetes

Alloxan-diabetes was induced in animals weighing approximately 200g, by injecting interperotoneally 8.0 mg of alloxan per 100 g of body weight, following a 24hr fast. Control animals were injected with an identical volume of saline. Alloxan-diabetes was detected by a positive reaction for glucose in the urine, animals not showing a positive reaction were excluded. The alloxan-diabetic and control animals were fed regular lab chow for a 10 day period prior to sacrifice. During this period the alloxan-diabetic animals received no insulin to alleviate the diabetic condition.

3.2 Analytical Methods

3.2.1 Thin-layer chromatography

(a) Preparation of thin-layers

Silica gel G (E. Merck A.G., Dramstadt, Germany) was slurried with water in the proportions 1:2 (w/v) and spread onto alcohol washed glass plates

TABLE 3.1

Composition of experimental diets

HIGH CARBOHYDRATE	HIGH PROTEIN
60% glucose	60% casein
7% cellulose	7% cellulose
7% vitamin/mineral	7% vitamin/mineral
5% casein	5% glucose
5% maize oil	5% maize oil
1% NaCl	1% NaCl
15% rolled oats	15% rolled oats

HIGH LIPID
40% maize oil
7% cellulose
7% vitamin/mineral
5% casein
5% glucose
1% NaCl
35% rolled oats

TABLE 3.2

Composition of mineral and vitamin mix for experimental diets

MINERAL MIX

$CaHPO_4.2H_2O$	350 g
$CaCO_3$	334 g
$Mg(OH)_2$	38 g
KCl	100 g
K_2HPO_4	211 g
$MnSO_4.H_2O$	10.7 g
$ZnSO_4.7H_2O$	12.4 g
$FeSO_4.7H_2O$	17.5 g
$CuSO_4.5H_2O$	1.2 g
KIO_3	0.5 g
$Na_2MoO_4.2H_2O$	90 mg
$CrK(SO_4)_2.12H_2O$	192 mg
$NaSeO_4$	93 mg

Burstyn and Husbands (1980)

VITAMIN MIX

per Kg of diet

Vitamin A	20,000 I.U. (palmitate ester)
Vitamin B	200 mg
Vitamin C	1,000 mg
Vitamin D_3	5,000 I.U.
Vitamin H	1 mg
Choline chloride	3,000 mg
Inositol	100 mg

Holub (1975)

(20x20cm) using a Desago applicator, to a thickness of 0.25 mm (analytical) or 0.50 mm (preparative). Thin-layers were allowed to air dry for 30 mins then activated by heating at 110°C for at least 2hr. Thin-layers impregnated with boric acid were prepared in a similar manner, the slurry consisting of silica gel G and 3% aqueous boric acid solution 1:2 (w/v) to give approximately 6% boric acid. After activation, thin-layers were allowed to cool and were stored in a dessicator before use.

(b) Conditions for thin-layer chromatography

Samples were applied about 2 cm from one edge of the thin-layer as bands approximately 2 cm long using a 100 µl syringe. Application was aided through use of a plastic template. Standard lipids were used to confirm the identity of radioactive components. Thin-layer plates were developed by the ascending method using chromatographic tanks (Desago Co, Germany) lined with filter paper to enhance vapour saturation of the atmosphere within the tank. The developing solvent was added to the tank to a depth of approximately 0.5 cm. Thin-layer plates were developed at room temperature (about 20°C) to 12-14 cm above the origin. After developing, the thin-layer chromatograms were air-dried before spraying.

(c) Detection of lipids on thin-layer chromatograms

General lipid spray: Lipids were normally detected by spraying thin-layers with a 0.1% (w/v) solution of 2,7 dichlorofluorescein in methanol and viewed under ultra-violet light. Lipid components showed as yellow fluorescent spots on an orange background.

Phosphate ester spray: A spray reagent, specific for compounds containing a phosphate ester group, was prepared according to the method

of Vaskovsky and Kostetsky (1968). Upon spraying on thin-layer chromatograms the reagent reacted immediately with phospholipids to give blue spots on a white background. The colour gradually disappeared over 2hr.

3.2.2 Elution of phospholipids from silica gel

Phospholipids, which remain at the origin after development of the thin-layer chromatogram in hexane:diethyl ether:acetic acid (50:50:1, v/v/v), were extracted by suspending each portion of silica gel removed from the thin layer in four successive 2.0 ml aliquots of chloroform:methanol:formic acid:water (97:97:4:2, v/v/v/v). The extracts were separated at each stage by centrifugation and the combined extracts evaporated to dryness under a stream of oxygen-free nitrogen. Phospholipids were then dissolved in chloroform, rechromatogramed onto silica gel and chromatographed in chloroform:methanol:acetic acid:water (85:15:10:3.5, v/v/v/v).

3.2.3 Determination of protein

Protein from whole adipose tissue homogenates was determined by the Coomassie dye binding method of Bradford (1976). Protein from the subcellular fractionation of adipose tissue was determined using the method of Lowry et al. (1951). Solutions of B.S.A. (0-100 μg) were prepared in 0.01 M phosphate buffer pH 7.2 and used to construct standard protein curves.

3.2.4 Centrifugation

Ultracentrifugations were carried out in a Beckman Model L2-65B ultracentrifuge. Centrifugations at lower speeds were preformed in a Sorval RC-3 centrifuge or a Gallenkamp Junior centrifuge. All

preparative centrifugations were carried out at 4°C.

3.2.5 Determination of radioactivity

^{14}C radioactivity was determined using a Beckman Model LS 8000 scintillation counter. Aqueous samples were counted in Triton X-100/toluene (1:2, v/v) containing 0.4% PPO and 0.01% POPOP. Non-aqueous samples were counted in toluene containing 0.4% PPO and 0.01% POPOP. Radioactivity on chromatograms was determined by removal of silica gel to a scintillation vial containing 5.0 ml of the appropriate scintillation fluid. The efficiency of the counting system was determined by including a standard sample of ^{14}C-hexadecane in scintillation fluid with each batch.

3.3 Lipid synthesis by homogenates of parametrial adipose tissue

3.3.1 Preparation of homogenate

Parametrial adipose tissue from rats killed by stunning and cervical dislocation was homogenised in 0.15 M-KCl (1:4, w/v), buffered with potassium phosphate buffer (pH7.0, 46.5 mM), in a motor driven glass homogeniser (Jencons; uniform, Type PTG 1) fitted with a teflon pestle at 4°C. The homogenate was kept on ice and centrifuged at 700xg for 12 mins, and separated into upper fat cake, a pellet (containing cell debris, nuclei and other tissue fragments) and an intermediate layer. This intermediate layer, called the fat-free homogenate, was aspirated and used directly as the enzyme source.

3.3.2 Incubation procedure

The optimal assay for the biosynthesis of TG as described by Christie *et al.* (1976) was used for all experiments. The incubation medium

contained; homogenate (1 ml), K_2HPO_4 (pH 7.0, 93 µmol in total), ATP (10 µmol), Mg^{++} (5 µmol), CoA (0.12 µmol) DDT (0.5 mg), DL α-glycerophosphate (25 µmol), F^- (20 µmol), KCl (0.25 mmol in total) and 1-^{14}C palmitic acid (0.1 µCi, 1.17 µmol) bound to fatty acid-free B.S.A. (0.09 µmol) in a final volume of 2.0 ml. Incubations were preformed in triplicate with gentle agitation in a water bath at 37°C for 1hr. The reaction was terminated by the addition of chloroform/methanol (2:1, v/v) and the lipids extracted by the method of Folch et al. (1957). The mixture of lipid was evaporated to dryness under a stream of oxygen-free nitrogen and the lipid taken up in 1.0 ml chloroform. Lipids were separated into TG, FFA, DG and PL by thin-layer chromatography.

3.4 Subcellular fractionation of parametrial adipose tissue

3.4.1 Isolation of fat cells from adipose tissue

Fat cells, isolated from rat parametrial adipose tissue as described by Rodbell (1964), were used for the preparation of microsomal and other cell fractions. Plastic vessels were used during the preparation and incubation of fat cells. Parametrial adipose tissue was removed and rinsed in 0.85% NaCl. 1 gm of minced tissue was added to 3.0 ml of Krebs-Ringer bicarbonate buffer at pH 7.4 modified to contain half the usual calcium concentration, 10 mg collagenase (C.histolyticum), 3% fatty acid-poor B.S.A., 200 mg glucose per 100 ml and gassed with 95% O_2-5% CO_2. Fat cells were liberated from the tissue fragments by gentle agitation with a glass rod. The suspension of cells was centifuged at 400xg for 1 min resulting in the fat cells floating to the surface while the stromal vascular cells were sedimented.

3.4.2 Fractionation of isolated fat cells

Isolated fat cells were washed once with 10 volumes of homogenization medium (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.25 M sucrose) to rid the cells of Krebs-Ringer buffer. The cells were resuspended in 3.5 volumes of homogenization medium and transferred to a glass homogenizer (Jencons; Uniform, type PTG 1) fitted with a teflon pestle (clearance 0.006-0.009 inch). The cell suspension was homogenised with 10 up-and-down strokes by means of a variable speed motor (Heidolph, speed 10). The resulting homogenate was placed on ice and all subsequent steps carried out at $4^{o}C$. The homogenate was centrifuged at 16,000xg for 15 min to yield a pellet (P_1), a supernatant solution (S_1) and a congealed fat cake above the supernatant. S_1 was aspirated and centrifuged at 160,000xg for 70 min to precipitate the microsomal fraction. The microsomal supernatant was aspirated and the pellet gently rinsed with homogenization medium and the interior walls of the tube wiped dry. The pellet was resuspended in homogenization medium using a motor driven (Heidolph, speed 1) Kel-F-Kontes pestle which fits Beckman 5/8 x 3 inch polyallomer tubes. The supernatant was discarded and the pellet resuspended as before to yield the microsomal fraction at a protein concentration equal to approximately 1 mg/ml.

3.4.3 Assay of microsomal DGAT activity

Microsomal fractions of parametrial adipose tissue were used to assay DGAT (EC 2.3.1.20) activity by the method of Coleman and Bell (1976).

Assays were preformed in Kimex screw-capped culture tubes (13x100 mm) at $23^{o}C$ in a final volume of 0.2 ml. Each tube contained 175 mM Tris-HCl, pH 8.0, 8 mM $MgCl_2$, 1 mg/ml fatty acid-poor B.S A, 30 μM 1-^{14}C palmitoyl-CoA (2.37μ Ci/ mol) and 20 μl of 1.25 mM diolein in

absolute ethanol. The reaction was initiated by adding 5 µg of protein followed by the glyceride substrate and blending in a vortex mixer. The reaction was terminated after 10 mins by addition of 1.5 ml of propan-2-ol/heptane/water (80:20:2, v/v/v). After 5 min, 1 ml of heptane and 0.5 ml of water were added. The heptane layer was removed and washed twice with 2 ml of a solution containing 0.5 N NaOH/ethanol/water (10/50/50, v/v/v). A sample of the final heptane layer was removed and the amount of radioactivity present determined.

3.5 Preparation of fatty acid-free serum albumin

Fatty acids were removed from B.S.A. by the methods of Hanson and Ballard (1968) and Chen (1967). 25 g B.S.A. was dissolved in distilled water at 23°C. 12.5 charcoal powder was mixed into the solution and the pH adjusted to 3.0 using 0.2 N HCl. This solution was then placed on ice and stirred for 1hr. Charcoal was then removed by centrifugation at 20,200xg for 20 mins. The pH of the clarified solution was adjusted to pH 7.0 with 0.2 N NaOH. This solution was then dialysed against 0.9% NaCl for 3 days and finally dialysed against distilled water overnight. This final solution was then lyophilised and stored at 4°C.

3.5.1 Assay of free fatty acid content

The free fatty acid content of B.S.A. was measured by the procedure of Chen (1967).

1.0 ml containing 60 mg B.S.A. was shaken with 5.0 ml of extraction mixture (iso-propyl alcohol:iso-octane:1N H_2SO_4; 40:10:1, v/v/v) in a glass stoppered test tube and allowed to stand for 15 mins. 3.0 ml iso-octane and 2.0 ml 0.1 N HCl were added and again shaken. 3.0 ml of the iso-octane phase was transferred to

another stoppered tube containing 5.0 ml of 0.01 N H_2SO_4 and the contents shaken. The upper phase was transferred to another test tube containing 1.0 ml of titration mixture (0.002% Nile blue A and 90% v/v ethanol). The free fatty acid was titrated with 0.02 N NaOH to a light pink end point, mixing being effected by a stream of CO_2-free O_2. The results showed a free fatty acid content of 0.056 moles per mole of albumin.

3.6 Preparation of palmitoyl-CoA ester

Palmitoyl-CoA was synthesised under nitrogen by the procedure of Young and Lynen (1969). Approximately 20 moles of palmitic acid was dissolved in tetrahydrofuran and reacted with a 15% excess of triethylamine, after which the mixed anhydride was formed by reacting with ethylchloroformate. The CoA ester was synthesised by reacting the mixed anhydride of the acid, with free CoA. Palmitoyl-CoA was precipitated by addition of perchloric acid to 1% and the precipitate collected by centrifugation. Unreacted palmitic acid was removed by extracting three times with petroleum ether (B.P.+30-60^{o}C) with the residual petroleum removed under a stream of nitrogen. Palmitoyl-CoA was dissolved in 40 mM MES buffer and adjusted to pH 5.0 with 1 M $KHCO_3$. The palmitoyl-CoA was approximately 90% pure as determined by absorbance at 232 nm and 260 nm. Palmitoyl-CoA was stored in ampoules under nitrogen at -20^{o}C.

CHAPTER 4

Results

4.1 Esterification of palmitic acid

Fat-free homogenates of rat parametrial adipose tissue were incubated with 1-^{14}C palmitic acid according to the procedure of Christie et al. (1976). In all experiments the amount of palmitic acid esterified into lipid products (TG, DG and PL) was determined (Figure 4-1). Analysis of the PL fraction, which remained at the origin after chromatography of the lipid products, showed that the major component was PA with trace amounts of lysophosphatidic acid, PL and PE (Figure 4-2).

The DG fraction contained both the sn-1,2 and sn-1,3 stereoisomers with the sn-1,2 isomer predominating. There was also trace amounts of radioactivity in areas corresponding to authentic MG but the amounts of radioactivity present were too small to appear on the autoradiograms (Figure 4-1).

4.1.1 Lipid synthesis by homogenates of rat parametrial adipose tissue

The synthetic capabilities of parametrial adipose tissue were determined by assaying the ability of homogenates to esterify palmitic acid into lipid. The specific activities for the incorporation of palmitic acid into the various lipid classes are expressed in Table 4-1. The animals from which the tissue was removed were maintained in the fed-state up to the time of sacrifice. The results of these experiments served as a comparison to the results obtained by Christie et al., (1976).

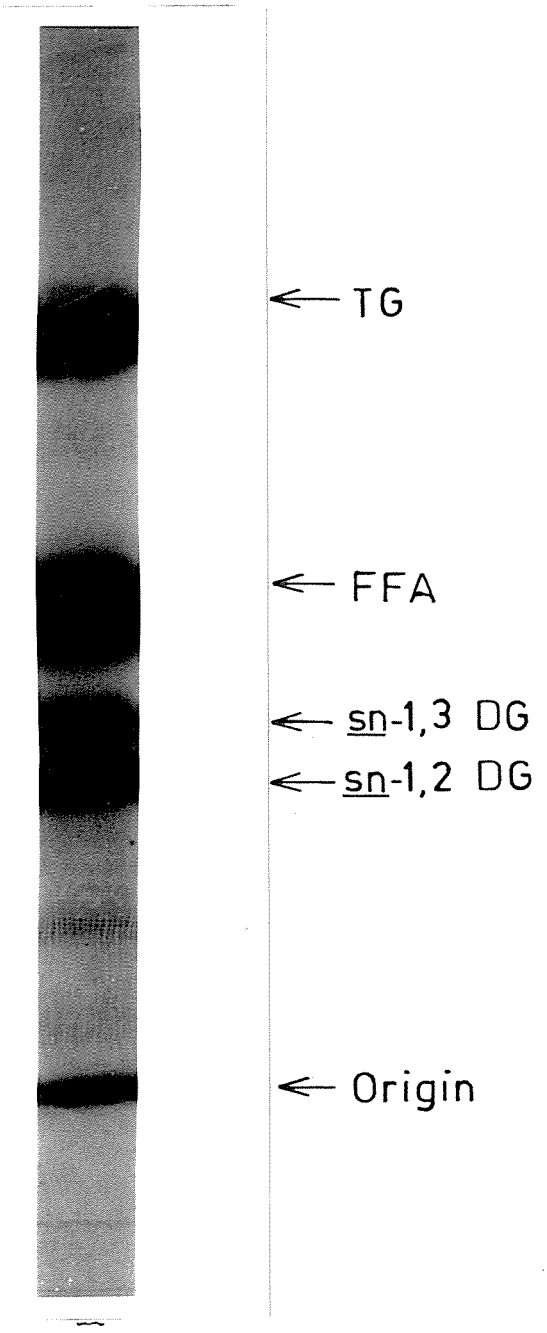


Figure 4-1. Thin-layer chromatogram of lipid products from the incubation of 1-^{14}C 16:0 with a fat-free homogenate of rat parametrial adipose tissue according to the procedure of Christie et al., (1976).

TG : triacylglycerol, FFA : free fatty acid, DG : diacylglycerol.
Solvent - hexane : diethyl ether : acetic acid (50:50:1, v/v/v).

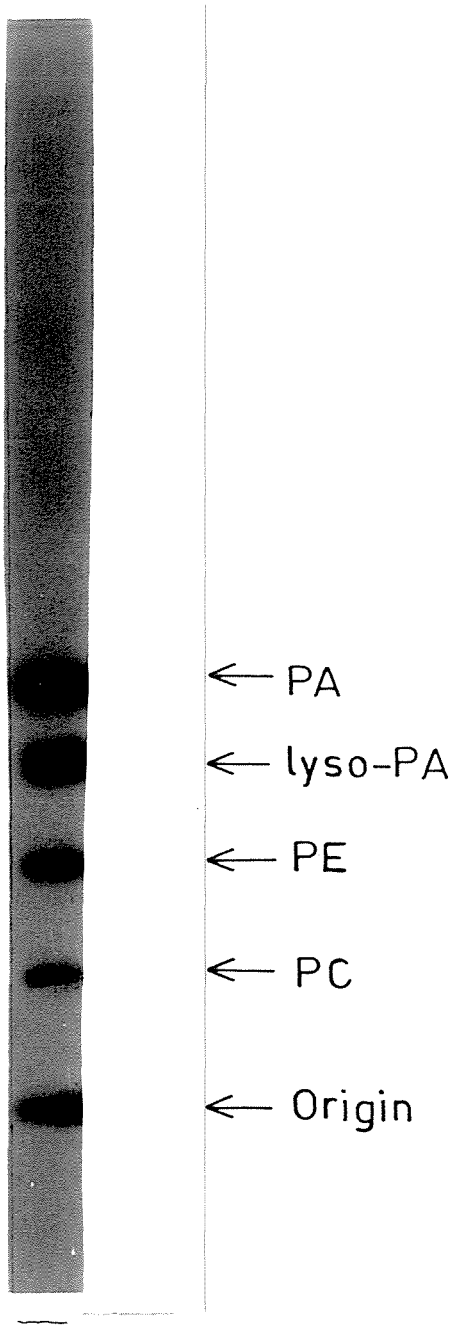


Figure 4-2. Thin-layer chromatogram of the phospholipid fraction from the incubation of 1-^{14}C 16:0 with a fat-free homogenate of rat parametrial adipose tissue.

Assayed according to the procedure of Christie et al., (1976)
Eluted from original thin-layer (figure 4-1) with chloroform:methanol:formic acid:water (97:97:4:2, v/v/v/v) and rechromatographed in chloroform:methanol: acetic acid:water (85:15:10:3.5, v/v/v/v).
PA : phosphatidic acid, PE : phosphatidylethanolamine, PC : phosphatidyl-choline.

TABLE 4-1

Esterification of 1-^{14}C palmitic acid into lipid by homogenates of parametrial adipose tissue from fed-animals.

lipid class	specific activity
	nmoles min^{-1} mg $protein^{-1}$
PA	3.35 ± 0.91
MG	0.23 ± 0.05
DG	1.22 ± 0.11
TG	6.36 ± 0.78
TOTAL	11.16 ± 1.85

Mean values ± S.D. of six independent experiments

Incubation conditions and analytical methods as described in Materials and Methods (Chapter 3)

The cofactor requirements of the assay system were confirmed by omitting the suggested cofactors and comparing levels of esterification with the levels obtained using the complete assay system (Table 4-2).

The assay system displayed a dependence on G-3-P, Mg^{++}, ATP and CoA for maximal activity while there were no significant changes when DTT and F^{-} were omitted from the incubation medium. The exclusion of G-3-P and the glycerol moiety substrate, resulted in a large decrease in both total esterification and also esterification into the individual lipid classes.

Incubation in the absense of Mg^{++} resulted in an approximate 50% reduction in total esterification. The lipid class most effected by the exclusion of Mg^{++} was the TG class where esterification was reduced from 6.36 to 0.92 nmoles min^{-1} mg $protein^{-1}$. In contrast there was an increase in esterification into PA, with the specific activity of esterification increasing from 3.35 to 4.43 nmoles min^{-1} mg $protein^{-1}$. Esterification into DG was also reduced while the amount of material corresponding to MG was reduced to an almost negligible level in comparison to the control incubations.

The omission of ATP also resulted in marked decreases in total esterification of palmitic acid and in the esterification into all the individual lipid classes. Total esterification was reduced approximately 5-fold while esterification into PA and TG was reduced approximately 7-fold and 6-fold, respectively. Esterification into DG was also reduced significantly while incorporation into MG was comparable to the control incubations.

TABLE 4-2

Cofactor requirements for the esterification of palmitic acid by homogenates of parametrial adipose tissue

lipid class	Deletion from complete assay system			
	Complete*	-G-3-P	$-Mg^{2+}$	-ATP
	nmoles min^{-1} mg $protein^{-1}$			
PA	3.35±0.91	0.15±0.02	4.43±0.41	0.46±0.14
MG	0.23±0.05	0.23±0.04	0.03±0.03	0.26±0.07
DG	1.22±0.11	0.64±0.16	0.79±0.13	0.73±0.20
TG	6.36±0.78	0.31±0.03	0.92±0.18	1.06±0.32
TOTAL	11.16±1.85	1.33±0.25	6.17±0.75	2.51±0.73

lipid class	Deletion from complete assay system		
	-CoA	$-F^{-}$	-DTT
	nmoles min^{-1} mg $protein^{-1}$		
PA	0.25±0.05	3.98±0.68	4.78±1.07
MG	0.33±0.06	0.30±0.13	0.28±0.05
DG	0.81±0.09	1.51±0.41	1.34±0.19
TG	0.83±0.07	6.82±0.40	6.15±0.76
TOTAL	2.22±0.27	12.61±1.62	12.55±2.07

mean values ± S.D of two independent experiments

*mean values ± S.D of six independent experiments

Incubation conditions and analytical methods as described in Materials and Methods (Chapter 3).

The exclusion of CoA from the incubation medium demonstrated the dependence of the assay system on this cofactor for maximal activity. Total esterification of palmitic acid was reduced approximately 5-fold while esterification into all the lipid classes, except MG, were reduced significantly. The most dramatic reduction was seen in the specific activity of incorporation into TG which was reduced from 6.36 to 0.83 nmoles min^{-1} mg $protein^{-1}$.

When F^- and DTT were omitted from the incubation medium both total esterification and the incorporation into the individual lipids showed no significant differences in comparison to the complete assay system.

4.1.2 Rate of esterification of palmitic acid

The rate of esterification of palmitic acid into the lipid classes, by homogenates of parametrial adipose tissue prepared from fed-animals, was followed over a 60 min incubation period (Figure 4-3). This revealed that initially the most rapid incorporation of palmitic acid was into PA. The accumulation of PA reached a maximal level after approximately 20 mins incubation, after which the level of PA remained relatively constant for the duration of the incubation. The rate of accumulation of palmitic acid into TG was approximately constant throughout the 60 min incubation period. After an initial rapid rise (5 min) in the amount of palmitic acid detected in DG the level of DG remained at this level throughout the incubation. The amount of MG detected was constant over the 60 min period and was low in comparison to the other lipid products.

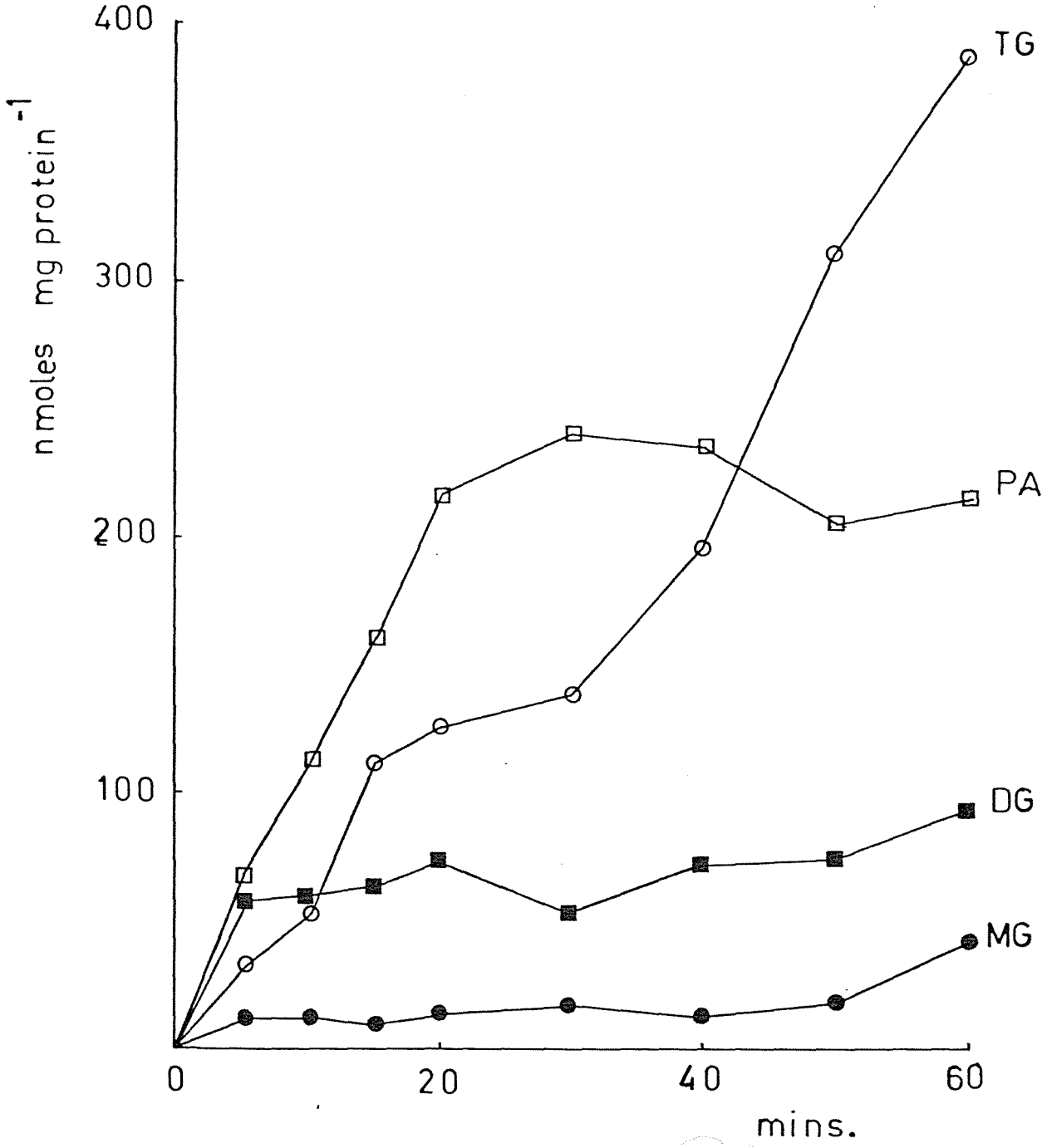


Figure 4-3. The rate of incorporation of 1-^{14}C plamitic acid into lipid by homogenates of parametrial adipose tissue.

Incubation conditions and analytical methods as described in Materials and Methods (Chapter 3).

TG : triacylglycerol, PA : phosphatidic acid, DG : diacylglycerol, MG : monoacylglycerol.

Mean values of two independent experiments.

4.1.3 Relationship between the products of esterification of palmitic acid and the levels of homogenate protein

The amount of homogenate protein, in the range 0-4 mg, present in the incubation medium influenced both the extent of incorporation of palmitic acid into lipid and also the nature of the lipid products (Figure 4-4). When the amount of homogenate protein was 0.5 mg or less, the major product of esterification was PA. As the amount of protein was increased to 1.0 mg or more then the major product produced was TG. With 0.5 mg -2.0 mg protein the incorporation of palmitic acid into PA decreased while the incorporation into TG increased with both reaching constant levels at 2.0 mg. The incorporation palmitic acid into DG increased as the amount of protein was increased to 1.5 mg. At 2 mg protein and above the incorporation of palmitic acid into both PA and DG was approximately constant.

4.2 The effect of diet on the estification of palmitic acid

The influence of diet on the ability of parametrial adipose tissue to esterify palmitic acid was assessed by maintaining groups of animals on the diets described in Materials and Methods (Chapter 3). A comparison between animals in the fed-state and animals that had been subjected to various periods of starvation was made in conjunction with changes in the nature of the diet.

4.2.1 The effect of starvation

The major physical result of fasting is the decrease in the amount of sub-cutaneous parametrial adipose tissue. The ability of homogenates of parametrial adipose tissue obtained from these animals, to esterify palmitic acid is expressed in Figure 4-5.

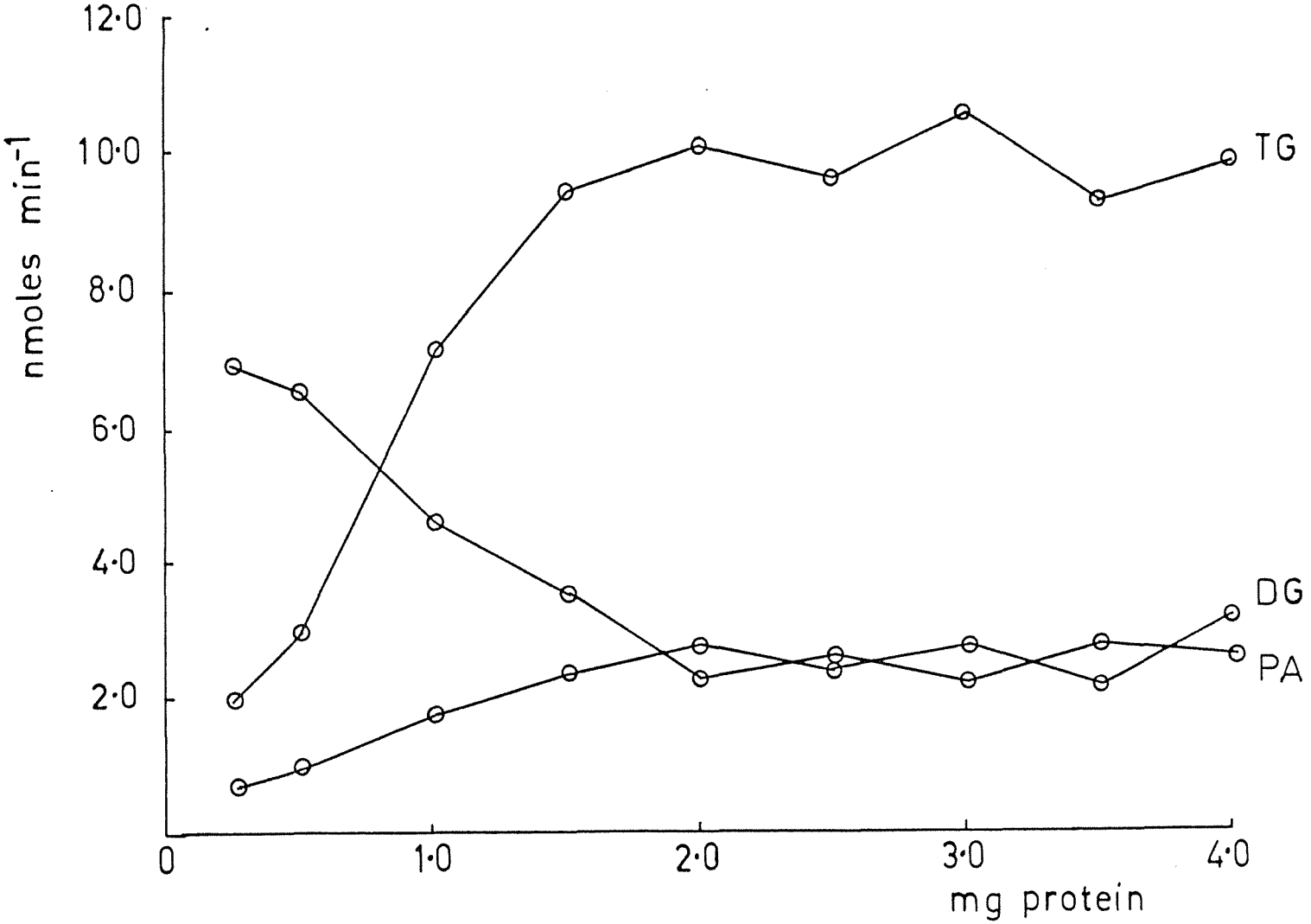


Figure 4-4. The effect of the amount of homogenate protein in the incubation medium on the esterification of palmitic acid into lipid.

Incubation conditions and analytical procedures as described in Materials and Methods (Chapter 3).

TG : triacylglycerol, DG : diacylglycerol, PA : phosphatidic acid.

Mean values of two independent experiments.

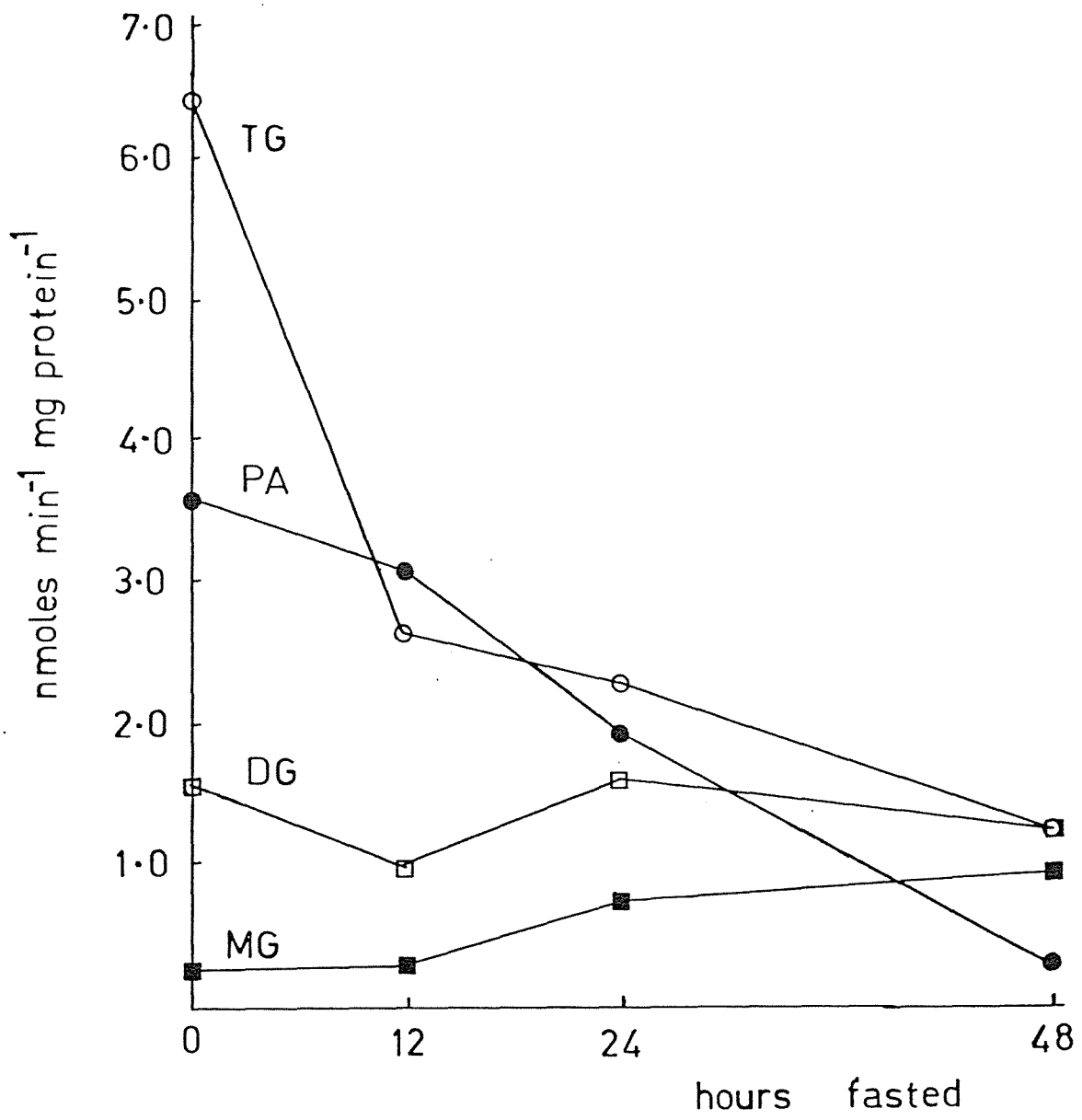


Figure 4-5. The effect of starvation on the ability of homogenates of parametrial adipose tissue to esterify palmitic acid into lipid.

Treatment of animals, incubation conditions and analytical methods as described in Materials and Methods (Chapter 3).

TG : triacylglycerol, PA : phosphatidic acid, DG : diacylglycerol, MG : monoacylglycerol.

Mean values of two independent experiments.

The most striking feature of these results in the large decrease in the ability of homogenates from animals fasted overnight (12hr) to esterify palmitic acid into TG. The specific activity of esterification into TG decreased from 6.45±0.96 nmoles min^{-1} mg $protein^{-1}$ in the fed group to 2.6±0.37 nmoles min^{-1} mg $protein^{-1}$ with homogenates from animals fasted overnight. Total esterification of palmitic acid was decreased 57% from 12.34±1.75 to 7.09±1.12 nmoles min^{-1} mg $protein^{-1}$ due largely to the dramatic decrease in the incorporation into TG, and to a lesser extent the reduction in incorporation into PA. The reduction in incorporation into TG and PA between 12hr and 48hr starvation was less dramatic. There was also a significant increase (3.6-fold) in the incorporation of palmitic acid into MG by animals starved for 48hr compared with fed animals. There were no significant differences in the levels of incorporation into DG between fed and fasted animals.

4.2.2 Alteration of diet

The ability of homogenates of parametrial adipose tissue prepared from animals maintained on the experimental diets, to esterify palmitic acid is given in Table 4-3. The specific activities for the estification of palmitic acid into total lipid were approximately the same for the four experimental diets. In general the specific activities of esterification into the individual lipid classes were also approximately equal. A notable exception was the lower esterification of palmitic acid into PA with the high protein diet compared to the other diets. Also there was unusually high activities of esterification into MG with both the high carbohydrate and high lipid diets. Esterification into both DG and TG were essentially the same for the four diets.

TABLE 4-3

The effect of diet on the esterification of palmitic acid by homogenates of parametrial adipose tissue

Lipid Class	Normal	60% Protein	60% Carbohydrate	40% Lipid
	nmoles min^{-1} mg $protein^{-1}$			
PA	3.59±0.34	1.51±0.14	3.42±0.63	2.14±0.52
MG	0.23±0.04	0.24±0.03	1.05±0.42	1.60±0.62
DG	1.57±0.29	1.66±0.06	1.68±0.35	2.27±0.54
TG	6.45±0.96	6.76±0.32	6.10±1.50	5.41±0.86
TOTAL	11.84±1.63	10.17±0.55	12.25±2.90	11.42±2.54

Mean values ± S.D. of two independent experiments

	Normal	Protein	Carbohydrate	Lipid
wt adipose tissue(g)	1.4±0.4	0.4±0.3	2.1±0.3	1.6±0.4
wt gain/rat/10 days(g)	30.0±5.6	-22.4±7.6	12.0±3.6	-7.6±4.6

Mean values ± S.D. of two independent experiments

Treatment of experimental animals, incubation conditions and analytical methods as described in Materials and Methods (Chapter 3)

Probably of more interest than the specific activities of esterification of palmitic acid were the effects of the four diets on the amount of parametrial adipose tissue and the changes in body weight (Table 4-3). The amount of parametrial adipose tissue in animals on the high protein diet was less compared to animals on the other three diets. In contrast the amount of parametrial adipose tissue in animals fed on the high carbohydrate diet was greater than the amount present in animals fed on the standard chow diet. The animals on the three unbalanced diets (high protein, high carbohydrate and high lipid) all recorded lower weight gains than those maintained on the normal balanced diet; animals on the high protein and high lipid diets actually lost weight over the 10 day period.

4.3 The effect of hormones on the ability of parametrial adipose tissue homogenates to esterify palmitic acid into lipid

4.3.1 The effect of insulin treatment

It has been demonstrated that homogenates of parametrial adipose tissue obtained from fasted animals, have a lower ability to esterify palmitic acid than homogenates obtained from fed animals. It was therefore of interest to determine if esterification could be enhanced by the administration of insulin. Insulin was injected into the aorta of anaesthetised rats that had been previously fasted, at a dosage of 1 I.U./Kg. A control group of fasted animals were injected with a similar volume of saline. Animals were sacrificed 5 min after the hormone had been injected. The results obtained using these two groups of animals are expressed in Figure 4-6 and Table 4-4.

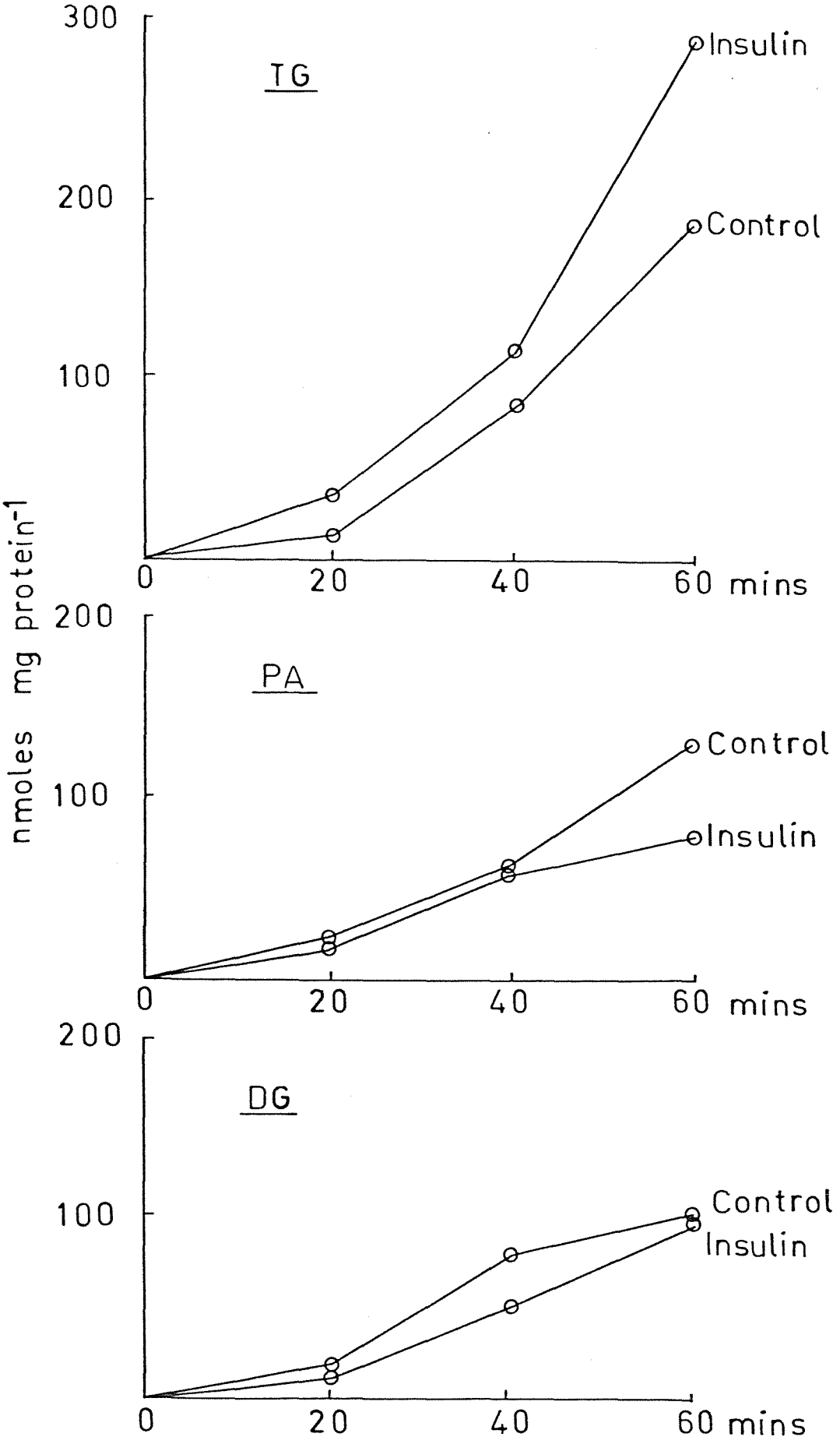
There was a significant increase (53%) in the esterification of palmitic

Figure 4-6. The effect of insulin administration (1 I.U./kg) to rats fasted for 24 hr on the incorporation of palmitic acid by homogenates of parametrial adipose tissue.

Treatment of animals, incubation conditions and analytical methods as described in Materials and Methods (Chapter 3).

TG : triacylglycerol, PA : phosphatidic acid, DG : diacylglycerol.

Mean values of two independent experiments.


300
200
100
TG
Insulin
Control
0
20
40
60 mins
nmoles mg protein-1
200
100
PA
Control
Insulin
0
20
40
60 mins
200
100
DG
Control
Insulin
0
20
40
60 mins

acid into TG in comparison to the control group. Esterification into DG and PA was not greatly affected by the insulin treatment, except that with esterification into PA at 60 mins was lower in the insulin treated group compared to the control group. This decrease in the insulin group was not present with the shorter incubation periods.

Esterification of palmitic acid was also enhanced when insulin was administered to rats that had been fasted for 48hrs. The rates of esterification were also higher in the saline treated group in comparison to the group that had been fasted only (Table 4-4). Total esterification was approximately the same for the insulin and saline control groups but the main difference with the insulin group was the decrease in esterification into PA and a corresponding increase in esterification into TG.

In contrast to the increased esterification obtained with the in vivo infusion of insulin to fasted animals no increase could be demonstrated when insulin was added to the incubation medium. The esterification of palmitic acid into all the lipid classes was approximately the same between the control incubations and those containing insulin at a level of 1 I.U. (Table 4-5). It should be noted that these results were obtained using homogenates prepared from fed animals unlike the in vivo experiments where homogenates were prepared from animals which had been previously fasted for varying periods.

4.3.2 The effect of glucagon

As insulin administration increased the esterification of palmitic acid into TG it was of interest to determine if glucagon (a lipolytic

TABLE 4-4

The effect of insulin administration to fasted rats on the esterification of palmitic acid

48 hour Starvation			
Lipid Class	Control	Saline Control	1 I.U/kg Insulin
	nmoles min^{-1} mg $protein^{-1}$		
PA	0.32±0.16	2.21±0.04	1.22±0.08
MG	0.19±0.12	0.20±0.06	0.21±0.06
DG	1.23±0.24	1.56±0.29	1.48±0.04
TG	1.26±0.36	3.42±0.47	5.73±0.40
TOTAL	3.00±0.36	7.39±0.86	8.64±0.58

mean values ± S.D. of two independent experiments

12 hour Starvation			
Lipid Class	Control	Saline Control	1 I.U./kg Insulin
	nmoles min^{-1} mg $protein^{-1}$		
PA	3.18±0.27	2.21±0.04	1.2±0.08
MG	0.27±0.05	0.23±0.01	0.31±0.12
DG	0.98±0.43	1.70±0.37	1.68±0.07
TG	2.66±0.37	3.10±0.46	4.76±0.43
TOTAL	7.09±1.12	7.24±0.88	7.97±0.70

mean values ± S.D. of two independent experiments

Incubation conditions and analytical methods as described in Materials and Methods (Chapter 3).

TABLE 4-5

The effect of insulin added to the incubation medium on the esterification of palmitic acid into lipid

Lipid Class	Control	1I.U. Insulin
	nmoles min^{-1} mg $protein^{-1}$	
PA	1.83±0.44	2.23±0.45
MG	0.14±0.05	0.19±0.03
DG	0.85±0.08	1.10±0.14
TG	5.63±0.42	5.60±0.33
TOTAL	8.45±0.99	9.12±0.95

Mean values ± S.D. of two independent experiments

Incubation conditions and analytical methods as described in Materials and Methods (Chapter 3)

hormone) has the opposite effect. Anaesthetised animals which had been maintained in the fed-state were injected with glucagon at a level of 1 mg/Kg. The glucagon was allowed to circulate for 5 mins prior to sacrifice. A control group of fed animals were injected with a comparable volume of saline.

There was a 33% reduction in total esterification of palmitic acid in the treated group compared to the control (Table 4-6). Contributing to this lower incorporation of 16:0 were decreases in esterification into PA and TG. Esterification into PA was reduced 54% while esterification into TG was reduced 29%. Esterification into MG and DG was not affected by the glucagon treatment.

4.3.3 The effect of adrenalin

Adrenalin was administered to fed animals at a dosage of 200 μg/kg by injection into the aorta of anaesthetised animals. The specific activities for the esterification of palmitic acid by homogenates of parametrial adipose tissue from both the adrenalin treated group and also a saline treated group are expressed in Table 4-7. The levels of total esterification and also the specific activities of esterification into the individual lipid classes are appproximately identical between the two groups. The dosage level of adrenalin was lower than the level of glucagon as levels above 200 μg/kg resulted in severe trauma in the experimental animals which led to inconsistent results.

4.4 The effect of alloxan treatment on the esterification of palmitic acid

Groups of animals were rendered alloxan-diabetic by the administration of alloxan as described in Materials and Methods (Chapter 3). After 10

TABLE 4-6

The effect of glucagon on the esterification of palmitic acid into lipid

lipid Class	Saline Control	+Glucagon(1mg/kg)
	nmoles min^{-1} mg $protein^{-1}$	
PA	3.35±0.54	1.52±0.16
MG	0.17±0.05	0.15±0.06
DG	1.64±0.16	1.51±0.10
TG	6.10±1.07	4.32±0.41
TOTAL	11.26±1.82	7.50±0.73

Mean values ± S.D. of two independent experiments

Incubation conditions and analytical methods as described in Materials and Methods (Chapter 3)

TABLE 4-7

The effect of adrenalin on the esterification of palmitic acid

Lipid Class	Control	+Adrenalin(200μg/kg)
	nmoles min^{-1} mg $protein^{-1}$	
PA	1.83±0.21	1.49±0.29
MG	0.15±0.01	0.16±0.02
DG	1.57±0.10	1.73±0.10
TG	5.37±0.56	5.78±0.35
TOTAL	8.92±0.88	9.16±0.76

Mean values ± S.D. of two independent experiments.

Incubation conditions and analytical methods as described in Materilals and Methods (Chapter 3).

days the animals were sacrificed and the ability of the homogenates of parametrial adipose tissue to esterify palmitic acid into lipid was examined (Table 4-8). Total esterification of palmitic acid was reduced by 41% while esterification into PA and TG was reduced by 30% and 57%, respectively in comparison to the control group. No significant differences in the incorporation into MG and DG were detected between the two groups. The amount of parametrial adipose tissue recovered at the end of the 10 day period was also less in comparison to the control group. During the 10 day experimental period, the animals received no insulin to counteract the effects of the alloxan-diabetes.

4.5 DGAT activity in parametrial adipose tissue

DGAT activity in microsomal preparations of parametrial adipose tissue was assayed by the procedure of Coleman and Bell (1976) which involved monitoring the formation of TG using 1-^{14}C palmitoyl-CoA and diolein Analysis of the final heptane phase by thin-layer chromatography revealed that the major radioactive component migrated with authentic TG with a small amount of radioactivity corresponding to FFA. There was also a small amount of radioactive material remaining at the origin (figure 4-7).

The specific activity of microsomal DGAT obtained from parametrial adipose tissue and the dependence of the assay system on various cofactors is expressed in Table 4-9. The true value of DGAT activity was obtained by deducting the small amount of activity present in the heptane phase of incubations containing no added microsomal protein.

TABLE 4-8

The effect of alloxan treatment on the esterification of palmitic acid

Lipid Class	Control	Alloxan-Diabetic
	nmoles min^{-1} mg $protein^{-1}$	
PA	3.68±0.42	2.56±0.22
MG	0.18±0.04	0.10±0.04
DG	1.83±0.37	1.78±0.34
TG	6.72±0.84	2.84±0.46
TOTAL	12.41±1.67	7.28±1.06

	Control	Alloxan-Diabetic
wt of adipose tissue(g)	1.4	0.8

Incubation conditions and analytical methods as described in Materials and Methods (Chapter 3)

Mean values ± S.D. of two independent experiments.

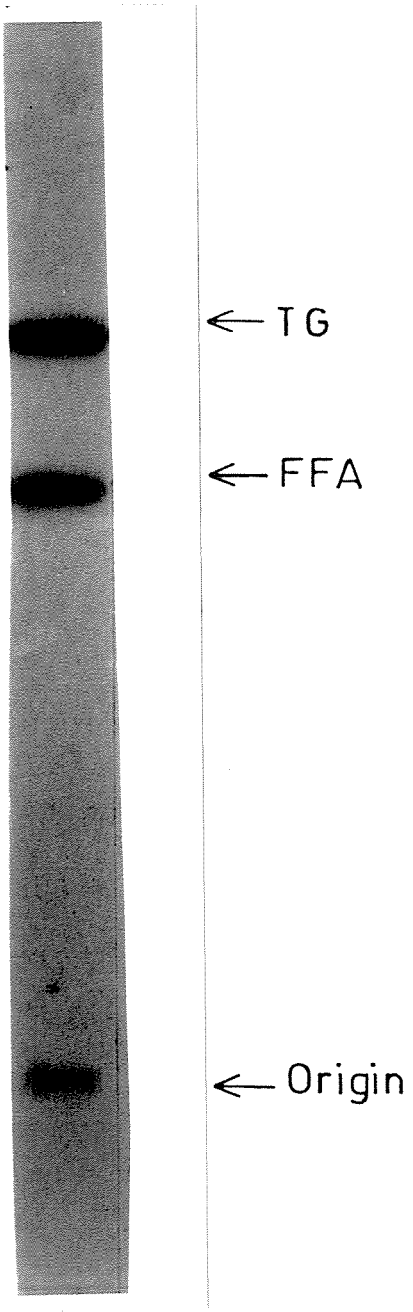


Figure 4-7. Thin-layer chromatogram of the final heptane phase from the incubation of 1-^{14}C palmitoyl-CoA with the microsomal fraction of parametrial adipose tissue according to the procedure of Coleman and Bell (1976).

Incubation conditions and analytical methods as described in Materials and Methods (Chapter 3).

TG : triacylglycerol, FFA : free fatty acid.

Solvent - hexane : diethyl ether : acetic acid (50:50:1, v/v/v).

TABLE 4-9

DGAT activity in parametrial adipose tissue

Additions/Deletions	DGAT specific activity
	nmoles min^{-1} mg protein
Complete System	44.2±10.4
-microsomal protein	(0.02 nmoles/10 min)
$-MgCl_2$	28.3±7.4
-B.S.A.	17.2±9.6
+DDT	4.9±3.6
Diolein in Tyloxapol	0.9±0.6
Dipalmitin in ethanol	19.5±6.3

Mean values ± S.D. of two independent experiments

Incubation conditions and analytical methods as described in Materials and Methods (Chapter 3).

The dependence of the enzyme on $MgCl_2$ and B.S.A. was demonstrated by a 36% and a 61% decrease in DGAT activity, respectively when these two components were omitted. Addition of the sulphydryl reducing agent DDT, inhibited the enzymes activity by 90%. Using Tyloxapol as an alternative to ethanol as dispersant for the diolein resulted in a considerably lower specific activity for DGAT. The specific activity obtained using Tyloxapol, a nonionic surfactant, was 0.9 ± 0.6 nmoles min^{-1} mg $protein^{-1}$ which is considerably less than the 44.2 ± 10.4 nmoles min^{-1} mg $protein^{-1}$ recorded when the diolein was dispersed in ethanol. Substitution of diolein, which is more readily dispersed, with dipalmitin resulted in a 56% reduction in specific DGAT activity.

Further confirmation of the characterisation of DGAT activity was obtained by monitoring DGAT activity in relation to time of incubation. The production of TG, as a measure of DGAT activity, was approximately linear over a 14 min incubation period (figure 4-8).

4.6.1 The effect of starvation on DGAT activity in parametrial adipose tissue

In earlier experiments, it was demonstrated that the ability of homogenates of parametrial adipose tissue from fasted animals to esterify palmitic acid was much lower than corresponding homogenates from fed animals. The most pronounced effect of this starvation was the marked decrease in the incorporation of palmitic acid into TG, while synthesis of DG was approximately the same in fed and fasted animals. It was therefore decided to determine the levels of DGAT activity in the parametrial adipose tissue of animals subjected to various periods of starvation.

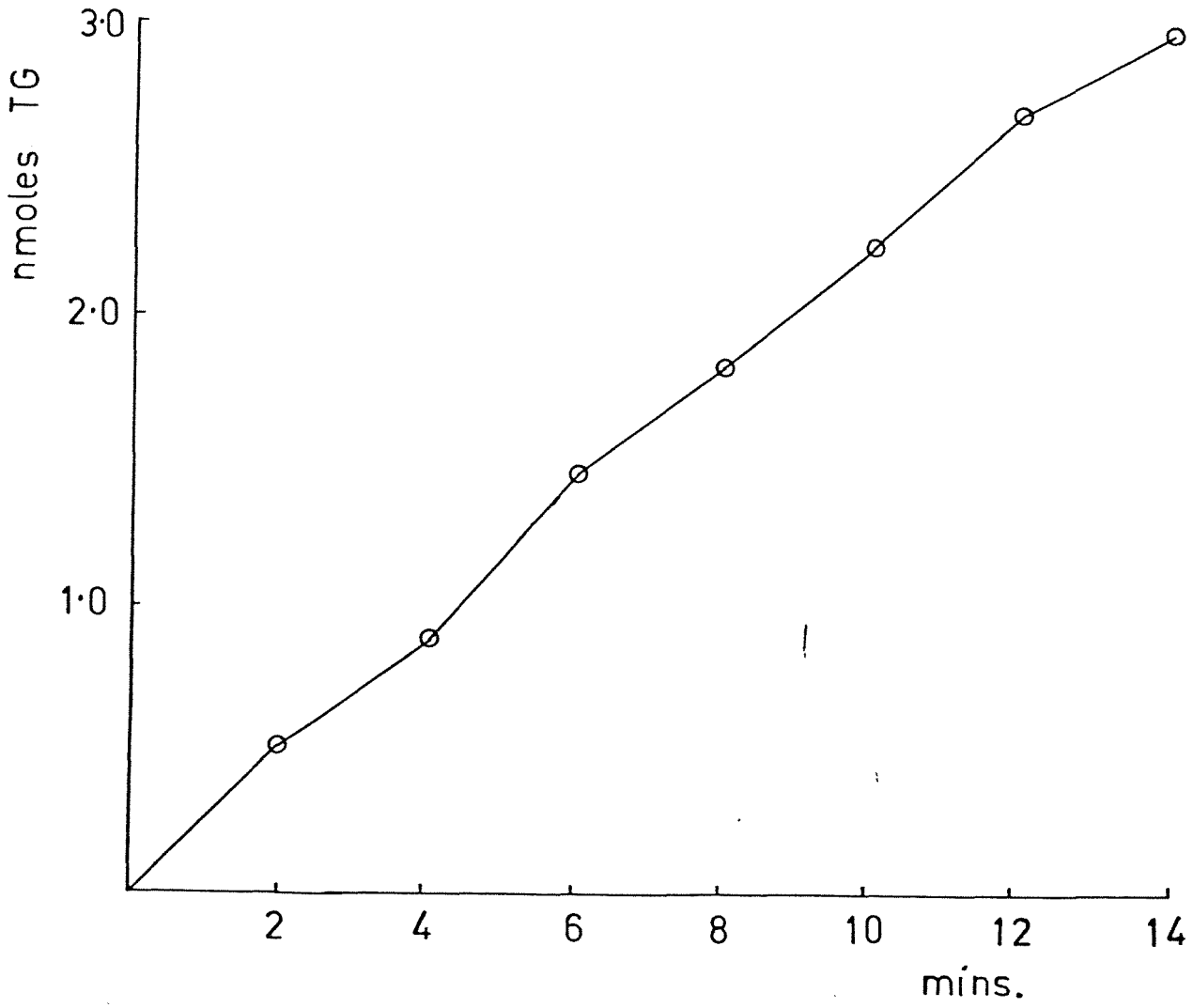


Figure 4-8. Rate of triacylglycerol production by the microsomal fraction of rat parametrial adipose tissue.

Assays were preformed according to the procedure of Coleman and Bell (1976) as described in Materials and Methods (Chapter 3).

TG : triacylglycerol

Mean values of two independent experiments.

The levels of DGAT activity in those groups of fed and fasted animals as well as the levels in the fed controls are expressed in Figure 4-9. There was a decrease in the mean value of DGAT activity from 44.2 to 34.7 nmole^{-1} mg protein^{-1} during the 48hr fast, with the majority of this decrease occuring with the first 12hr of fasting.

4.6.2 The effect of diet on DGAT activity in rat parametrial adipose tissue

Groups of animals were maintained on the experimental diets as described in Materials and Methods (Chapter 3). The levels of DGAT activity present in the parametrial adipose tissue of these animals are expressed in Table 4-10. Although these diets result in variations in the amount of parametrial adipose tissue the dietary changes are not reflected by changes in the specific activity of DGAT. There are differences in the mean values of DGAT activity but the magnitude of S.D's does not make these differences statistically significant. These results are consistent with the results obtained using whole tissue homogenates, where no significant changes in the esterification of 16:0 could be related to the different diets.

4.7 The effect of hormones on the DGAT activity in rat parametrial adipose tissue

Anaesthetised rats were injected, via the aorta, with either insulin (1 I.U./kg), adrenalin (200 µg/kg) or glucagon (1 mg/kg) according to the procedure outlined in Materials and Methods (Chapter 3). DGAT activity in the parametrial adipose tissue was determined after the hormones had been allowed to circulate for 5 min. The levels of DGAT activity in these animals as well as the levels in control animals infused with saline are expressed in Table 4-11. The mean value of DGAT

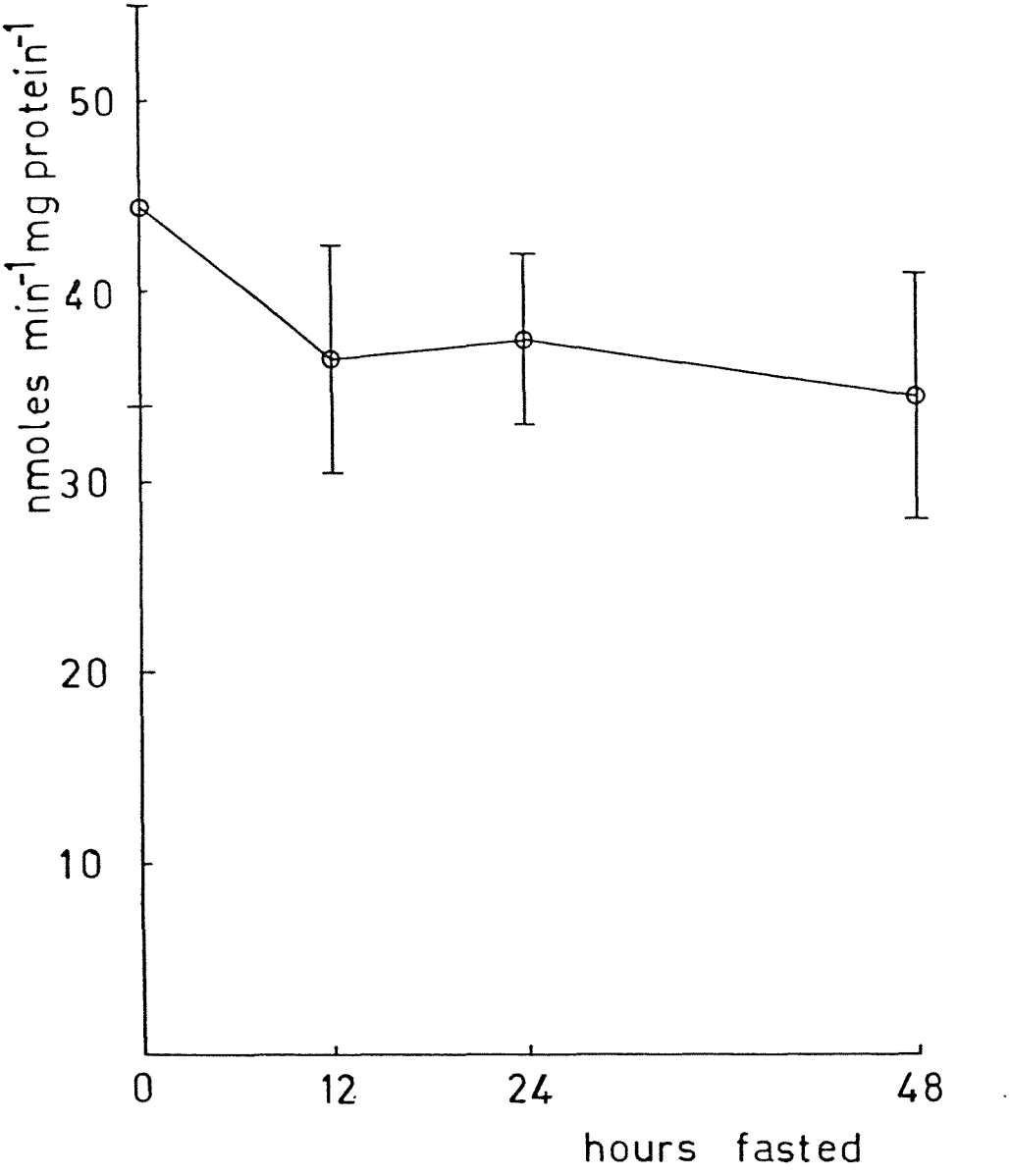


Figure 4-9. The effect of starvation on DGAT activity in rat parametrial adipose tissue.

Treatment of animals, incubation conditions and analytical methods as described in Materials and Methods (Chapter 3).

Mean values ± S.D. of two independent experiments.

TABLE 4-10

The effect of diet on the level of DGAT activity in parametrial adipose tissue

Diet	DGAT activity
	nmoles min^{-1} mg $protein^{-1}$
Normal	44.2±10.4
60% Casein	30.3± 8.1
60% Glucose	41.9± 7.4
40% Maize oil	28.7±11.1

Mean values ± S.D. of two independent experiments

Incubation conditions and analytical methods as described in Materials and Methods (Chapter 3).

TABLE 4-11

The effect of hormone administration on DGAT activity in parametrial adipose tissue

Control	Insulin) (1 I.U/kg)	Adrenalin (200μg/kg)	Glucagon (1mg/kg)
	nmoles min^{-1} mg $protein^{-1}$		
36.4±7.1	40.1±5.9	32.9±4.6	29.9±6.2

Mean values ± S.D. of two independent experiments

Incubation conditions and analytical methods as described in Materials and Methods (Chapter 3).

activity in the animals injected with the lipolytic hormones, adrenalin and glucagon were lower than the level of DGAT in the control animals.

In contrast the mean value of DGAT activity in the animals injected with insulin are higher than the levels in the control group. However due to the magnitude of experimental error no statistical significance can be placed on these differences.

4.8 The level of DGAT activity in the parametrial adipose tissue of alloxan-diabetic rats

Alloxan-diabetic animals were obtained by the administration of alloxan as described in Materials and Methods (Chapter 3). The mean value of DGAT in the alloxan-diabetic group was 29% lower than the mean value of DGAT activity in the control group (Table 4-12). This difference is not statistically significant however due to the magnitude of experimental error associated with the specific activities.

TABLE 4-12

The level of DGAT activity in the parametrial adipose tissue of alloxan-diabetic rats

Controls	Alloxan-diabetic
nmoles min^{-1} mg $protein^{-1}$	
34.7±7.3	24.3±6.2

Mean values ± S.D. of two independent experiments

Incubation conditions and analytical methods as described in Materials and Methods (Chapter 3).

CHAPTER 5

Discussion

5.1 TG synthesis in parametrial adipose tissue

This study confirms that fat-free homogenates of rat parametrial adipose tissue contain an active enzyme system for the synthesis of TG from G-3-P and FFA. The isolatable intermediates of TG synthesis were confirmed to be predominantly PA and DG (Christie et al., 1976). The small amount of PC and PE present in the mixture of lipid products is due to the presence of endogenous CDP-choline and CDP-ethanolamine in the fat-free homogenate (Van den Bosch, 1974). The presence of trace amounts of lyso-phosphatidic acid is consistent as it is an isolatable intermediate of the step-wise acylation of G-3-P (figure 1-1). The accumulation of lyso-phosphatidic acid is not favoured due to the duration of the incubation (60 mins) and also the amount of homogenate protein (1.5 mg) added to the incubation medium (Zaror-Behrens and Kako, 1976).

The concentration of cofactors in the assay procedure of Christie et al. (1976) allows for the optimal synthesis of TG rather than maximal esterification of FFA. The optimum cofactor concentrations for TG synthesis and total esterification of FFA differ appreciably. The decrease in TG synthesis in the absence of ATP and CoA results from decreased FFA activation. Both these cofactors are essential for maximal acyl-CoA synthetase activity (Khoo and Steinberg, 1974). The small amount of TG synthesis recorded in the absence of ATP and CoA is probably due to the endogenous amounts of these two cofactors. The concentration of palmitic acid in this study was 0.625 mM; rat adipose tissue acyl-CoA synthetase activity is only inhibited by FFA at levels

above 4 mM (Daniel and Rubenstein, 1968).

The increased incorporation of palmitic acid into PA and the corresponding decrease in TG accumulation in the absence of Mg^{2+} results from a decrease in Mg^{2+}-dependent PAP activity (Jamdar and Fallon, 1973). This results in a decrease in the availability of the sn-1,2 DG substrate for further acylation to TG. This also confirms that the Mg^{2+}-dependent activity of PAP predominates over the Mg^{2+}-independent activity in rat adipose tissue (Cheng and Saggerson 1978a). In the absence of exogenous G-3-P the small amount of esterification recorded is probably due to endogenous glycerol moiety substrates in the fat-free homogenate. No significant change in the esterification of palmitic acid was recorded with the presence or absence of NaF. It has been reported that NaF is an inhibitor of PAP activity (Lawson et al., 1981).

The rapid accumulation of PA during the esterification of palmitic acid supports the conclusion that PAP activity is the rate-limiting step of TG synthesis (Christie et al., 1976). This has also been demonstrated for TG synthesis in rat liver cells (Lamb and Fallon, 1974). As the amount of homogenate protein in the incubation medium is increased the predominant product changes from PA to TG. This may be due to the relative distribution of the enzyme activities within the fat cell.

TG synthesis in adipose tissue has been shown to exhibit a high degree of variability depending on several factors. These include; the age of adipose tissue (Jamdar et al., 1981), the adipocyte cell size (Jamdar and Osborne, 1981) and the anatomical location of the adipose

tissue. In order to reduce variability due to the first two factors animals of the same age and approximate weight were used throughout the course of this study. The tissue source in each instance was the parametrial adipose tissue mass of female Sprague-Drawly rats.

5.2 The effect of diet on the esterification of palmitic acid.

5.2.1 The effect of starvation

Homogenates of parametrial adipose tissue from animals subjected to various periods of fasting displayed a marked decrease in their ability to esterify palmitic acid compared to homogenates obtained from fed animals. Esterification also decreased as the duration of fasting increased. The nature of the products did not appear to be effected by starvation, except for an increase in the amount of MG accumulated. This 3.6-fold increase in MG is most likely due to increased lipase activity, especially hormone-sensitive lipase (figure 5-1). Jamdar and Osborne (1982) have reported an increase in PA and a decrease in the amount of neutral lipid (DG and TG) formed from G-3-P in cases of starvation. However, in the present study PA accumulation decreased significantly in comparison to the fed controls. This is consistent with the results of Lawson et al. (1981) who recorded no significant changes in soluble PAP activity. Moller et al. (1977) however found that soluble PAP activity decreased as the length of starvation increased.

The assay system for TG synthesis used in this study is dependent on the prior activation of palmitic acid to palmitoyl-CoA by acyl-CoA synthetase. Therefore, changes in TG synthesis produced by periods of starvation may be reflections of changes in fatty acid activation. Lawson et al., (1981) have reported a 25% decrease in acyl-CoA

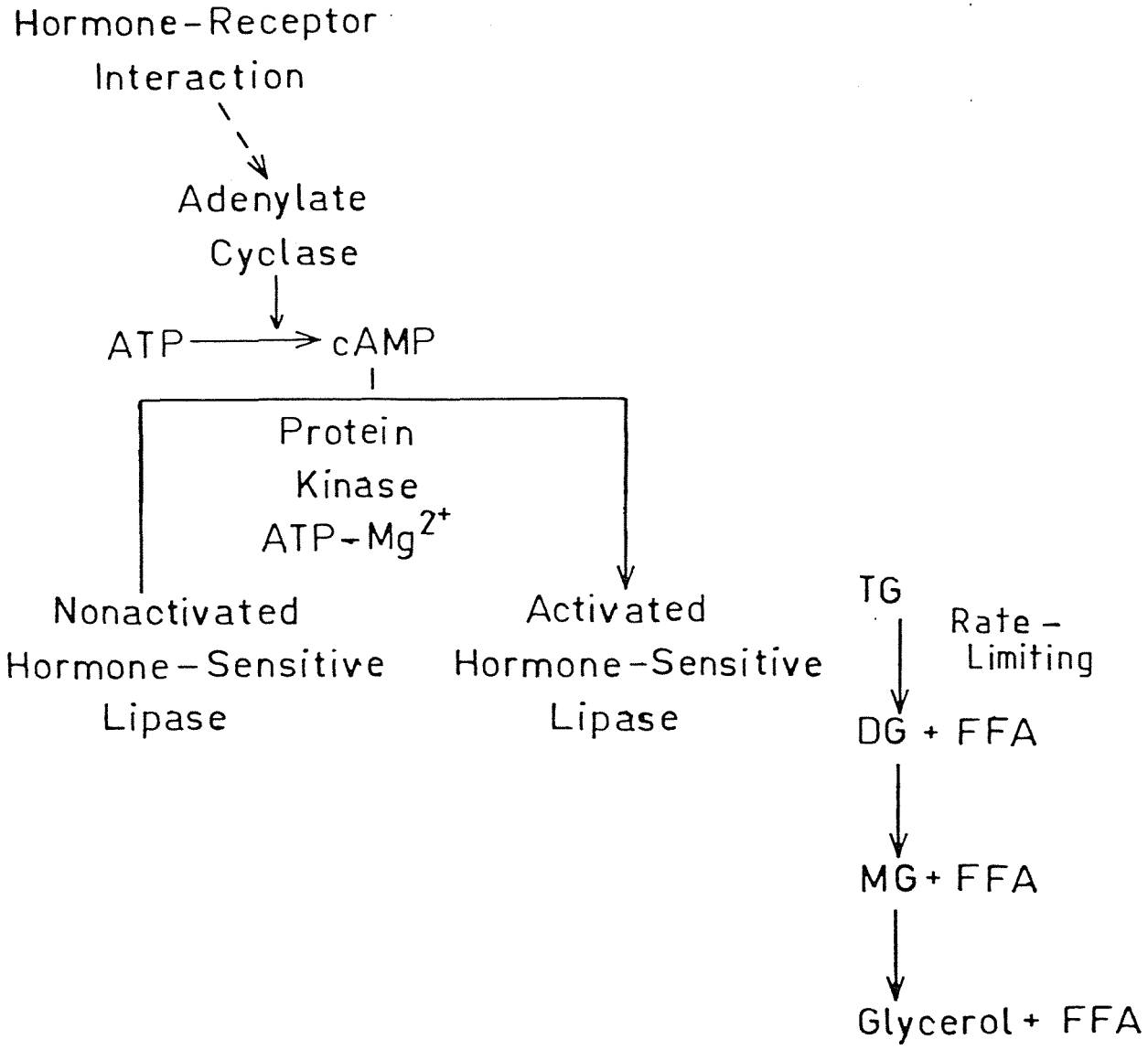


Figure 5-1. Activation of hormone-sensitive lipase in adipose tissue.

TG : triacylglycerol, DG : diacylglycerol, MG : monoacylglycerol, FFA : free fatty acid.

synthetase activity in homogenates from animals subjected to a 24 hr fast, which is consistent with the proposed role of insulin in maintaining acyl-CoA synthetase activity (Jason et al., 1976). Sooranna and Saggerson (1979) have reported a decrease in GPAT activity in adipocytes from rats subjected to a 48 hr fast. This decrease in GPAT activity appears to be a function of the age of tissue and of adipocyte size (Jamdar and Osborne, 1982). In contrast Lawson et al., (1981) have reported no significant changes in the levels of activity of GPAT, soluble PAP and DGAT in animals subjected to various periods of starvation. They did report a significant decrease in lipoprotein lipase activity with starvation. This enzyme has a vital role in supplying endogenous acyl precursors to the fat cell. Lipoprotein lipase activity is promoted by insulin (Ashby et al., 1979).

In the present study the overall TG biosynthetic pathway rather than the individual enzyme activities was assayed. Therefore conclusions cannot be made as to the exact enzyme(s) that are being effected by the periods of starvation. It is likely that the enzymes which are regulated by hormonal action are playing a major part in reducing the amount of TG synthesis.

5.2.2 The effect of diet

Generally the effect of altering the dietary intake of carbohydrate, lipid and protein has resulted in no significant changes in the esterification of palmitic acid. Diets rich in carbohydrate and lipid generally provide large amounts of caloric material surplus to the animals immediate needs. It is therefore expected that in these animals the level of TG synthesis should be increased. Lawson et al.

(1981) have reported no significant differences in the activities of acyl-CoA synthetase, DHAPAT and soluble PAP in adipose tissue from animals maintained on carbohydrate and lipid diets. They did report an increase in the level of total GPAT activity in rats fed on a lipid diet compared to animals maintained on a carbohydrate diet.

Of interest is the ability of homogenates from animals fed the high protein diet to esterify palmitic acid. Even though these animals recorded a loss in body weight and a decrease in the amount of parametrial adipose tissue the specific activities for the esterification of palmitic acid into the lipid classes were equal to those recorded from animals on the other diets. The failure of the animals on the high protein diet to record an increase in weight over the 10 day experimental period is probably linked to the unusually high protein content (60%). Holub (1975) has reported increased body weights in groups of animals fed diets containing up to 30% casein over a 26 day period.

5.3 The effect of hormones on the esterification of palmitic acid

5.3.1. The effect of insulin

The administration of insulin (1 I.U./kg) to animals, which had been previously fasted, resulted in an increase in the esterification of palmitic acid to the approximate levels recorded in fed animals. This effect of insulin was not seen when insulin was added to the incubation medium, indicating the involvement of the intact cell membrane in insulin action (Butcher et al., 1973). Insulin is considered an anabolic hormone in that it promotes lipid synthesis while also inhibiting the degradation of lipid. This study, although demonstrating an increase in TG synthesis due to insulin, does not reveal any

information as to the mechanism of the insulin action (Review: Czech, 1977).

The levels of esterification of palmitic acid by homogenates from the saline control group were in all cases greater than the levels from the untreated group which was starved only. Total esterification of palmitic acid for the saline control group and the insulin treated group were approximately the same. The main difference with the insulin treated group was the decrease in the amount of PA accumulated with a corresponding increase in TG production. This would tend to suggest changes in the levels of PAP and or DGAT activity.

Insulin is known to enhance the activity of several enzymes involved in the esterification of FFA to glycerolipid, one of these is the effect on acyl-CoA synthetase. Jason et al. (1976) have reported a rapid (2 mins) 2-fold increase in acyl-CoA synthetase activity; this increase in activity would serve to increase the supply of acyl-CoA thioesters. Other studies however have failed to confirm this result with no increase in activity recorded in homogenates prepared from freeze-stopped adipocytes after insulin treatment (Sooranna and Saggerson, 1978c). Insulin treatment of adipocytes results in a significant increase in GPAT activity while also opposing the inhibitory effects of adrenalin (Sooranna and Saggerson; 1976a, 1978b). Insulin also reverses the inactivation of PAP by noradrenalin, but insulin alone has no effect on PAP activity (Cheng and Saggerson, 1978b). A similar result is seen with DGAT actvity where insulin alone has no effect on the level of activity, but can oppose the effects of the catecholamine (Soler-Argilaga et al., 1978).

In the intact animal it is likely that the effect of insulin action on lipoprotein lipase activity (Ashby et al., 1979) and glucose transport (Sooranna and Saggerson, 1975) plays an important role in determining the rate of TG synthesis. Stimulation of these two factors would result in the increased supply of both acyl and glycerol moiety precursors.

In the present study therefore, it would appear that the increase in TG synthesis due to insulin may be caused by either; an increase in the activity of one or more of the TG synthetic enzymes or insulin's ability to oppose the inhibitory effects of the catecholamines that may have accumulated during the starvation period.

5.3.2 The effect of adrenalin

In contrast to the results obtained with the administration of insulin, the infusion of adrenalin resulted in no significant change in the ability of homogenates from fed animals to esterify palmitic acid. The levels of total esterification were approximately equal between the saline control and adrenalin-treated groups. In turn these levels were approximately the same as those recorded with fed animals which had not been infused with either adrenalin or saline. This apparent lack of effect by adrenalin may be due to the length of time between administration of the hormone and sacrifice of the animals. Sooranna and Saggerson (1978b) reported that the effect of adrenalin on GPAT activity was slow in onset (15-20 mins) and increased with time. Therefore, unlike the more rapid effects of insulin, adrenalin may be more slow acting than insulin and to obtain its effects on TG synthesis longer infusion periods should be allowed. The level of adrenalin administered (200 μg/kg) should have been sufficient to result in elevated plasma

levels of adrenalin (Geigy, Scientific Tables).

It has been reported by other workers that adrenalin can influence the activity of several enzymes of the TG synthetic pathway. Sooranna and Saggerson have reported decreases in; acyl-CoA synthetase (1978c), GPAT (1976a) (1978b) and DGAT (1978e) activities in response to adrenalin. With this apparent broad spectrum of effect it would be expected that TG synthesis should be affected by adrenalin administration. Coupled to these effects of adrenalin, it is also expected that the stimulatory effects of insulin should be depressed. In adipose tissue adrenalin has a lipolytic action, with an increase in both the release of FFA's into the blood and the rate of their re-esterification to TG's. Therefore in the in vitro procedure used in this study the lipolytic effects of adrenalin may not be apparent due to the lack of removal of the FFA's which leads to their re-esterification to TG.

5.3.3. The effect of glucagon

The administration of glucagon (1 mg/kg) to animals previously maintained in the fed state resulted in a decrease in both the total esterification of palmitic acid and the esterification of palmitic acid into TG. These decreases in esterification are consistent with the lipolytic effects of glucagon. The levels of esterification by homogenates from the saline control group were approximately equal to the levels of activity obtained from normal fed animals. The effects of glucagon on the individual enzymes of TG synthesis in adipose tissue do not appear to have been reported in such detail as the effects of adrenalin and insulin. TG synthesis by liver slices has been demonstrated to be rapidly responsive to glucagon (Geelen et al., 1978b). This may lead to a decrease in the supply of acyl precursors

available for esterification in the adipose tissue of the intact animal. In the assay procedure used in this study the concentration of acyl precursors is adequate for optimal TG synthesis therefore the effect of glucagon may be due to the regulation of one or more of the enzymes of esterification. Although little information exists on the effect of glucagon on the individual enzymes of TG synthesis it is likely that they are similar to the effects of adrenalin. It is also likely that glucagon has the ability to oppose the lipogenic effects of insulin. In past studies it has been difficult to determine to what extent any of these hormone induced changes were attributable to alterations in enzyme activities or were secondary to changes in the rate of entry of precursors (Sooranna and Saggerson, 1975). In this study the administration of glucagon has resulted in a decrease in TG synthesis but it is not possible to determine which enzymes are being effected by the hormone. Due to these rapid effects of glucagon and insulin it is likely that these two hormones play an integral part in the regulation of TG synthesis in adipose tissue to meet the physiological demands of the animal.

5.4 The effect of alloxan-diabetes on TG synthesis in parametrial adipose tissue

The administration of alloxan rapidly and selectively destroys the pancreatic β cells which produce insulin (Czech, 1977). It has been reported that insulin levels decrease from 49.8±3.8 mU/ml, in control animals, to 12.1±2.0 mU/ml within 5 days of alloxan treatment, resulting in the level of plasma sugars increasing from 83±7 mg/100ml, in the control group, to 372±25 mg/100 ml in alloxan-diabetic animals (Morenkova and Karelin, 1979). This decrease in insulin is followed by a decrease in glucose utilisation and changes in both hepatic and

peripheral lipid metabolism. Triglyceridemia, which is commonly associated with the diabetic state, is probably due to an increase in the hepatic activity of enzymes associated with the TG synthetic pathway. The activity of DGAT, the terminal enzyme in the TG synthetic pathway, is increased in the ketotic state (Young and Lynen, 1969). Therefore the observed increase in plasma TG levels may be due to either; increased production by the liver or decreased clearance of the plasma TG's by the peripheral tissues like adipose tissue. Young and Lynen (1969) have demonstrated that hepatic homogenates from alloxan-induced diabetic rats incorporate sn[1,3-^{14}C]G-3-P into TG at rates 2-4-fold higher than non- diabetic animals. It has also been demonstrated that increases in plasma TG's can be reversed by administering insulin to diabetic animals (Woods et al, 1981).

The uptake of plasma TG's by adipose tissue is dependent on lipoprotein lipase which in the main is promoted by insulin action (Ashby, et al 1979). In this study homogenates of parametrial adipose tissue from alloxan-diabetic animals displayed a reduced ability to esterify palmitic acid into lipid in general and into TG in particular. As well as effecting lipoprotein lipase activity insulin is also known to effect the TG synthetic pathway at several key sites. (Chapter 5.3.1) In conjunction with the decrease in insulin levels the ratio of insulin to the catecholamines will also be reduced. This ratio is important in determining the rate of net TG synthesis (Nimmo, 1980). Therefore the decrease in TG synthesis is probably due to an inactivation of the TG synthetic enzymes, similar to that which occurs during periods of starvation.

The observed decrease in the amount of parametrial adipose tissue is

probably due to the removal of insulin's inhibitory effects on hormone-sensitive lipase activity (Khoo, et al., 1974) coupled with the decrease in net TG synthesis.

5.5 DGAT activity in rat parametrial adpose tissue

Consistent with other work the predominant product formed following the incubation of diolein and palmitoyl-CoA with the microsomal fraction of parametrial adipose tissue was TG (figure 4-7) (Coleman and Bell, 1976). The small amount of radioactivity corresponding to FFA probably arises from degradation of 1-^{14}C palmitoyl-CoA. The production of ethyl palmitate as reported by Bakken et al, (1979) was not routinely investigated in this study, although no area of radioactivity with an R_F similar to ethyl palmitate was present during t.l.c. of the final heptane phase (Lawson et al., 1981).

The specific activity of DGAT in parametrial adipose tissue of 44.2±10.4 nmoles min^{-1} mg $protein^{-1}$ is in the same order of magnitude to the DGAT activity (55.0 nmoles min^{-1} mg $protein^{-1}$) reported by Coleman and Bell (1976). The figure for the specific activity of DGAT may not be a true value as it is probably effected by contamination of collagenase from the isolation of the fat cells (Rodbell, 1964). Dispersal of the sn-1,2 DG substrate in ethanol gave greater specific DGAT activity than dispersal in the non-ionic surfactant Tyloxapol. The problem of DG solubility in the intact adipocyte is probably overcome by the formation of an enzyme-DG complex during the formation of sn-1,2 DG from PA or its association with a carrier protein. Greater DGAT activity is recorded using diolein as the substrate rather than dipalmitin. This is probably due to the greater

solubility of diolein in ethanol resulting from the presense of unsaturated oleic acid moieties. An attempt to monitor DGAT activity using an acyl-CoA generating system (Jason et al., 1976) proved unsuccessful as DGAT activity was severely inhibited by Tyloxapol and DTT, two components of the acyl-CoA synthetase system. Decreased DGAT activity in the absence of B.S.A and $MgCl_2$ and the approximately linear production of TG with time indicated that these results were comparable to those obtained by Coleman and Bell (1976).

5.6 The effect of starvation on DGAT activity in parametrial adipose tissue

The decrease in TG synthesis by homogenates of parametrial adipose tissue from fasted animals cannot be explained by a decrease in DGAT activity. No significant differences in the level of DGAT activity between fed and fasted animals were recorded. This is in agreement with the results obtained by Daniel and Rubenstein (1968) and Lawson et al. (1981). This would indicate that the decreased TG synthesis present in homogenates from fasted animals is due to regulation at some point other than the terminal enzyme of TG synthesis. Inhibition of both lipoprotein lipase and acyl-CoA synthetase activities have been recorded in adipose tissue homogenates from fasted animals (Lawson et al., 1981). In the intact adipocyte both these reductions in activity would result in decreased TG synthesis, due to a reduction in precursor supply. It is however possible that DGAT may be inactivated during starvation by a phosphorylation reaction (Nimmo and Houston, 1978) and that post-mortem procedures alter the extent of this phosphorylation.

5.7 The effect of diet on DGAT activity

There were no significant differences in the level of microsomal DGAT activity in parametrial adipose tissue of animals maintained on the different experimental diets. This is in agreement with the findings of Lawson *et al*. (1981) who found no significant differences in DGAT activity in fat-free homogenates from animals maintained on high carbohydrate or high lipid diets. As there were no changes in the specific activity of DGAT it is likely that the amount of enzyme present plays a major role in determining the amount of TG synthesis. Therefore with animals maintained on the high carbohydrate diet their ability to synthesis TG would be greater due to an increase in the amount of parametrial adipose tissue. Conversely the synthetic capabilities of animals mantained in the high protein will be less due to the reduced amount of adipose tissue. Therefore, it appears likely that any effects of diet on TG synthesis are not due to changes in the specific activities of the TG synthetic enzymes (Lawson *et al*, 1981).

5.8 The effect of hormones on DGAT activity

No significant changes in microsomal DGAT activity were recorded between animals injected with insulin (1 I.U./kg), adrenalin (200 μg/kg) or glucagon (1 mg/kg). The level of DGAT activity in these groups was comparable to the level of activity in the control group. DGAT is the only enzyme activity of the glycerolipid synthetic pathway which is exclusively concerned with TG synthesis. Hormonal control at this branch point between TG and PL synthesis could therefore regulate the fate of the *sn*-1-2 DG substrate.

Incubation of rat adipocytes with adrenalin results in a decrease in the diolein-dependent incorporation of palmitoyl-CoA (Sooranna and

Saggerson, 1978e). Once again the action of adrenalin was slow in onset (20 mins); while insulin could oppose the effects of the catecholamine, alone it had no effect on DGAT activity. Elucidation of any hormonal effects on DGAT activity seems likely to depend on improved methods for the solubilization and purification of microsomal bound enzymes.

5.9 The effect of alloxan-diabetes on DGAT activity

Young and Lynen (1969) have reported a 46% increase in hepatic DGAT activity in alloxan-diabetic anmals. In this study however, the levels of DGAT activity were similar in alloxan-diabetic animals and in the control animals. There was a 29% decrease in the mean value of DGAT activity but this was within the limits of experimental error. The increase in hepatic DGAT activity, as reported by Young and Lynen (1969), may account for the increased production of plasma TG's in alloxan-diabetic animals. Conversely the elevated levels of plasma TG's may be due to decreased clearance by the peripheral tissues. If this is true then a reduction in DGAT activity in adipose tissue would have been expected. As DGAT is the terminal enzyme in the TG biosynthetic pathway it is likely that control of the pathway is being effected at an earlier step. It is likely however, that some form of control is being asserted at this branch point to channel the sn-1,2 DG substrate between TG and PL synthesis. This control may be due to precursor supply with a decrease in the availability of acyl-CoA thioesters in the alloxan-diabetic state (Young and Lynen, 1969).

SUMMARY

1. Fat-free homogenates of parametrial adipose tissue contain an active enzyme system for the synthesis of triacylglycerol from sn-glycerol-3-phosphate and palmitic acid. The major isolatable intermediates of the pathway were phosphatidic acid and sn-1,2-diacylglycerol. The assay system was shown to be dependent on the cofactors Mg^{++}, ATP and CoA for optimal triacylglycerol synthesis.

2. The esterification of palmitic acid into triacylglycerol by adipose tissue homogenates, prepared from fasted animals, was less than the esterification by homogenates prepared from fed animals. Alteration of the levels of dietary protein, carbohydrate and lipid had no effect on the levels of esterification of palmitic acid by the adipose tissue homogenates.

3. The administration of insulin, prior to sacrifice, to fasted animals resulted in an increase in the level of palmitic acid esterification. The levels of esterification were approximately equal to the levels recorded with adipose tissue homogenates from fed animals. Conversely the administration of glucagon to fed animals resulted in a decrease in the level of palmitic acid esterification. No change in the levels of esterification were recorded when adrenalin was administered to fed animals.

4. The level of triacylgylcerol synthesis by homogenates of parametrial adipose tissue obtained from alloxan-diabetic rats was significantly

less than the level obtained with homogenates from non-diabetic rats.

5. The microsomal fraction of rat parametrial adipose tissue was shown to be capable of synthesising triacylglycerol from palmitoyl-CoA and diolein.

6. Starvation and the alteration of diet resulted in no significant change to the level of DGAT activity in parametrial adipose tissue. Also no significant change in the level of DGAT activity was recorded due to the administration of insulin, glucagon or adrenalin.

7. No significant difference in the level of DGAT activity in parametrial adipose tissue was recorded between alloxan-diabetic and non-diabetic rats.

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