A STUDY OF THE REGULATION OF HEPATIC MICROSOMAL GLYCEROL PHOSPHATE ACYLTRANSFERASE (GPAT)

A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry at MASSEY UNIVERSITY

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ABSTRACT

Experiments described in this thesis were conducted to examine the possibility that hepatic microsomal GPAT activity in rats is regulated by insulin.

Hepatic microsomal fractions were prepared by a procedure based on published methods and it was established by assay of cytochrome oxidase, monoamine oxidase and NADPH cytochrome C reductase that there was less than 11% mitochondrial impurity. A butanol extraction of GPAT assays was adopted to separate the butanol-soluble [^{14}C]-lipid products from the unreacted aqueous-soluble [^{14}C]-glycerol 3-phosphate substrate. Methods were developed to simplify the determination of [^{14}C]-radioactivity. The kinetics of the response of the assay system to changes in the concentrations of glycerol 3-phosphate and palmitoyl-CoA were similar to those published for the microsomal GPAT. The products of the assay were identified as phosphatidic acid and lysophosphatidic acid by their chromatographic properties before and after hydrolysis with chicken liver phosphatidate phosphohydrolase. These products are consistent with literature reports.

Male Sprague-Dawley rats were treated with insulin (4 i.u./kg body weight) or saline, and extracts of hind-limb muscle were prepared and fractionated on Sephadex G-25 in 50 mM formic acid. Fractions which eluted subsequent to the void volume were assayed with hepatic microsomal GPAT and the effect of insulin-treatment fractions were compared with the effect of saline-treatment fractions.

Fractions containing material of approximately 3000 and 1000 daltons molecular weight enhanced GPAT activity in an insulin-dependent manner by 0.46 and 0.64 nmol/min/mg of microsomal protein, respectively (both $P<0.01$), compared to the effect of the saline controls. Control rates were approximately 3.5 nmol/min/mg of microsomal protein. It was calculated that these insulin-dependent increases in hepatic microsomal GPAT activity would be sufficient to account for the difference between the estimated hepatic triacylglycerol production of fed and fasted rats. Furthermore, published studies suggest that insulin-dependent changes in activities of enzymes, demonstrated with _in vitro_ systems utilising low molecular weight fractions from rat muscle, may parallel sensitivity of the same enzymes to insulin _in vivo_. The low molecular weight stimulator or stimulators of hepatic microsomal GPAT have an apparent molecular weight within the range 1000-3000 daltons, appear to be heat and acid stable, are soluble in aqueous solution, have very low absorbance at 220nm (or a very high specific activity) and may be sensitive to oxygen. These properties suggest that the low molecular weight stimulator or stimulators of hepatic microsomal GPAT activity may be related to the putative insulin mediator substance (IMS).

In initial experiments, where rats were heparinised prior to treatment with insulin or saline, it was observed that some fractions were able to stimulate hepatic microsomal GPAT activity in an insulin-independent manner. Experiments to resolve this suggested that the treatment of rats with heparin alone led to the presence of low molecular weight material, in the fractions of muscle extracts, with the potential to enhance GPAT activity. It was found that low

molecular weight fractions of the saline treatment muscle extracts did not enhance GPAT activity. This supported the suggestion that heparin was responsible for the ability of low molecular weight fractions of muscle extracts to stimulate GPAT activity in an insulin-independent manner.

Experiments were also conducted in which impure hepatic plasma membranes were treated with insulin (20-1000 μunits/ml). However, when hepatic microsomal GPAT was assayed with material from these incubations a stimulator of GPAT was not detected.

The results of experiments presented in this thesis provide further evidence in favour of the hypothesis that hepatic microsomal GPAT activity can be modified by insulin and may contribute to the overall regulation of glycerolipid biosynthesis in liver.

ACKNOWLEDGEMENTS

I wish to thank my supervisor, Dr D. R. Husbands, for his advice during the course of this study and also Dr J. C. Hawke for his helpful comments on aspects of the work. The help and encouragement received from friends and colleagues was appreciated. I would also like to acknowledge the encouragement of my wife Debbie and the support of my parents, Vic & Betty Stevens.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine monophosphate
cAMP	adenosine 3',5'-cyclic monophosphate
AQC	Automatic Quench Compensation
ATP	adenosine triphosphate
BSA	bovine serum albumin
CoA	coenzyme A
cpm	counts per minute
dpm	disintegrations per minute
DG	diacylglycerol
DHAP	dihydroxyacetone phosphate
DHAPAT	dihydroxyacetone phosphate acyltransferase
DTNB	dithiobis nitrobenzoic acid
EDTA	ethylene diamine tetraacetic acid
α-GP or GP	sn-glycerol 3-phosphate
GPAT	acyl-CoA: sn-glycerol 3-phosphate acyltransferase
h	hour(s)
IMS	insulin mediator substance
i.u.	international units
LPA	lysophosphatidic acid
min	minute(s)
MG	monoacylglycerol
NEM	N-ethylmaleimide
NADH	β-nicotinamide adenine dinucleotide, reduced

LIST OF ABBREVIATIONS (continued)

NADPH	nicotinamide adenine dinucleotide phosphate, reduced
PA	phosphatidic acid
PC	phosphatidylcholine
PL	phospholipid
POPOP	1,4-bis[2-(5-phenyloxazolyl)]benzene
PPO	2,5-diphenyloxazole
sec	seconds
SD	standard deviation
SEM	standard error of the mean
TLC	thin-layer chromatography
TMPD	tetra methyl phenylene diamine
Tris	tris-(hydroxymethyl)-aminomethane
VLDL	very low density lipoprotein
v/v	volume/volume
v/w	volume/weight
vol	volume(s)

NOTE

In this thesis unless stated otherwise, < 100% = less than control enzyme activity; 100% = control enzyme activity; > 100% = stimulation of enzyme activity relative to the appropriate control.

LIST OF APPENDICIES

CHAPTER ONE

INTRODUCTION

Chapter 1. INTRODUCTION

1.1 THE CENTRAL ROLE OF GLYCEROLIPIDS IN METABOLISM

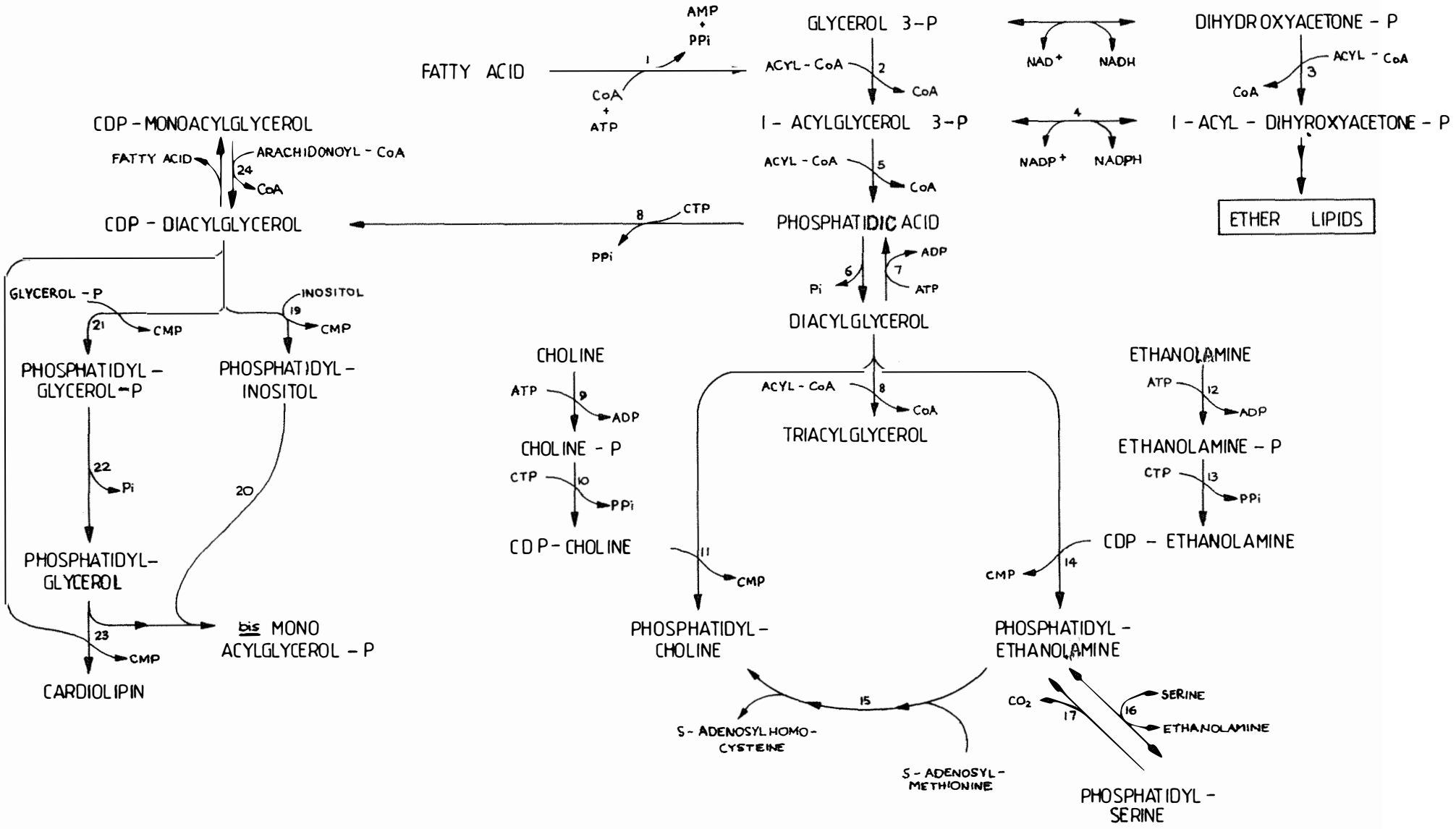
Glycerolipids are major components of eukaryotic cells, comprising between 10 and 90% of the dry weight of the cell (Bell & Coleman, 1980; Geelen et al., 1980). Compared with protein and carbohydrate, triacylglycerol-glycerolipids have a high content of energy and are the most important storage fuel in eukaryotes (Bell & Coleman, 1980; Brindley & Sturton, 1982). In adipose tissue they serve as insulation and protection. In higher animals they are incorporated into lipoproteins to transport fatty acids from the small intestine and liver to other organs (Brindley & Sturton, 1982; Mayes, 1976). The phosphoglyceride-glycerolipids are major components of all cell membranes and are critical to proper cell structure and function (Geelen et al., 1980). They form a permeability barrier and aid in the association and function of membrane proteins and enzymes. They are also important components of bile, serum lipoproteins and lung surfactant (Bell & Coleman, 1980; Van Golde, 1976).

Adipose tissue depots are the primary sites for storage of triacylglycerol (Masoro, 1977). Fatty acids, for the synthesis of triacylglycerols in adipose tissue, can come from dietary sources or be

synthesised de novo, but a significant proportion are provided by release from very low density lipoprotein (VLDL) originating from the liver. There appears to be reasonable agreement that the liver has a major role in fatty acid biosynthesis for the whole body (Masoro, 1977). Fatty acids synthesised de novo are esterified to glycerol 3-phosphate and retained in the liver or packaged into VLDL which are secreted into the blood. Fatty acids from dietary triacylglycerols and those mobilised from adipose tissue, but surplus to requirements for β-oxidation, are also esterified and either retained in the liver or exported as VLDL (Masoro, 1977). The metabolic pattern of the liver changes with fasting and, compared to the fed animal, a greater fraction of the free fatty acids that enter the liver undergo β-oxidation and a smaller fraction undergo esterification to form triacylglycerols and phospholipids (Masoro, 1977; McGarry et al., 1975; McGarry & Foster, 1980). In this transition from the fed to the fasted state, the liver switches (in a brief period of several hours) from an organ of carbohydrate utilisation and fatty acid synthesis, to one of fatty acid oxidation and ketone body production. These changes are mediated partly through changes in the tissue concentration of malonyl-CoA which inhibits β-oxidation in the fed state (McGarry & Foster, 1980). In addition to roles in fatty acid esterification and oxidation, hepatic tissues are very active in the synthesis of phospholipids for cellular membranes, VLDL and bile. Quite large amounts of phosphatidylcholine are secreted in bile (Ansell & Spanner, 1982; Van den Bosch, 1974; Van Golde & Van den Bergh, 1977).

The liver has a central role in lipid metabolism and in the past numerous studies have concentrated on pathways and regulatory mechanisms in this tissue, although similar processes occur in other tissues (Van Golde & Van den Bergh, 1977). The pathways of hepatic glycerolipid synthesis form a tightly knit, branched system with numerous end products which have a number of fates (see Fig 1.1). It is to be expected, therefore, that this complex network of reactions is governed by an equally complex set of controls (Saggerson & Bates, 1981). However, elucidation of these controls is hampered as most, but not all, of the enzymes involved in these pathways are associated with cellular membranes. Hence isolation, characterisation and investigation of their kinetic and regulatory properties suffers from difficulties generally encountered with membrane-bound enzymes (Saggerson & Bates, 1981). Complex compartmentation problems also arise at times as several enzymes are found in more than one type of cellular membrane or cell compartment (Bell & Coleman, 1980; Saggerson & Bates, 1981).

Fig 1.1: Enzymes and pathways of glycerolipid biosynthesis. 1. fatty acid CoA ligase (AMP) (EC 6.2.1.3); 2. sn-glycerol 3-phosphate acyltransferase (EC 2.3.1.15); 3. dihydroxyacetone-phosphate acyltransferase (EC 2.3.1.42); 4. acyl(alkyl)dihydroxyacetone-phosphate oxidoreductase (EC 1.1.1.101); 5. lysophosphatidic acid acyltransferase (EC 2.3.1.-); 6. phosphatidic acid phosphatase (EC 3.1.3.4); 7. diacylglycerol kinase 1; 8. diacylglycerol acyltransferase (EC 2.3.1.20); 9. choline kinase (EC 2.7.1.32); 10. choline-phosphate cytidyltransferase (EC 2.7.7.15); 11. diacylglycerol cholinephosphotransferase (EC 2.7.8.2); 12. ethanolamine kinase (EC 2.7.1.cc); 13. ethanolamine-phosphate cytidyltransferase (EC 2.7.7.14); 14. diacylglycerol ethanolaminephosphotransferase (EC 2.7.8.1); 15. phosphatidylethanolamine N-methyltransferase (EC 2.1.1.17); 16. phosphatidylethanolamine serinetransferase; 17. phosphatidylserine decarboxylase; 18. phosphatidic acid cytidyltransferase (CDP-diacylglycerol synthase) (EC 2.7.7.41); 19. phosphatidylinositol synthase; 20. bis-monoacylglycerol-phosphate synthase; 21. glycerol-phosphate phosphatidyltransferase (EC 2.7.8.5); 22. phosphatidylglycerol-phosphate phosphatase (EC 3.1.3.27); 23. cardiolipin synthetase; 24. CDP-monoacylglycerol arachidonoyl-CoA acyltransferase. (after Scheme 1. of Bell & Coleman, 1980).


FATTY ACID
AMP + PPi
CoA + ATP
1
GLYCEROL 3-P
ACYL-CoA
2
CoA
NAD+
NADH
DIHYDROXYACETONE - P
ACYL - CoA
3
CoA
1 - ACYL - DIHYROXYACETONE - P
4
NADP+
NADPH
1 - ACYLGLYCEROL 3-P
ACYL-CoA
5
CoA
ETHER LIPIDS
PHOSPHATIDIC ACID
6
Pi
7
ADP
ATP
8
CTP
PPi
CDP - DIACYLGLYCEROL
FATTY ACID
ARACHIDONOYL - CoA
24
CoA
CDP - MONOACYLGLYCEROL
DIACYLGLYCEROL
ACYL-CoA
8
CoA
TRIACYLGLYCEROL
GLYCEROL - P
21
CMP
INOSITOL
19
CMP
PHOSPHATIDYL - GLYCEROL - P
PHOSPHATIDYL - INOSITOL
22
Pi
PHOSPHATIDYL - GLYCEROL
20
23
CMP
CARDIOLIPIN
bis MONO ACYLGLYCEROL - P
CHOLINE
ATP
9
ADP
CHOLINE - P
CTP
10
PPi
CDP - CHOLINE
11
CMP
PHOSPHATIDYL - CHOLINE
ETHANOLAMINE
ATP
12
ADP
ETHANOLAMINE - P
CTP
13
PPi
CDP - ETHANOLAMINE
CMP
14
PHOSPHATIDYL - ETHANOLAMINE
15
S - ADENOSYL HOMO - CYSTEINE
S - ADENOSYL - METHIONINE
CO2
17
16
SERINE
ETHANOLAMINE
PHOSPHATIDYL - SERINE

1.2 PATHWAYS FOR THE SYNTHESIS OF GLYCEROLIPIDS

Two possible pathways exist for the formation of glycerolipids (see Fig 1.1). One involves the formation of phosphatidic acid (PA) via the acylation of sn-glycerol 3-phosphate. The other involves the acylation of dihydroxyacetone phosphate (DHAP) and its subsequent reduction to lysophosphatidic acid (LPA) which is then acylated to form phosphatidic acid. Phosphatidic acid is an important and central intermediate in the synthesis of glycerolipids (Bell & Coleman, 1980; McMurray & Magee, 1972; Thompson, 1980; Van den Bosch, 1974) and arachidonic acid metabolites (Hasegawa-Sasaki & Sasaki, 1982; Hawthorne & Pickard, 1977; Lapetina et al., 1981).

The first reaction in the formation of phosphatidic acid by the glycerol 3-phosphate route is catalysed by the enzyme sn-glycerol 3-phosphate acyltransferase (GPAT) (EC 2.3.1.15) which utilises fatty acyl-CoA thioesters to esterify a fatty acid to the 1-position of the sn-glycerol 3-phosphate, forming the compound 1-monoacyl-sn-glycerol 3-phosphate (commonly known as lysophosphatidic acid) (see Fig 1.1) (Monroy et al., 1973; Tamai & Lands, 1974; Yamashita & Numa, 1972). This is subsequently acylated by lysophosphatidic acid acyltransferase (EC 2.3.1.-) to form phosphatidic acid. The biosynthesis of phosphatidic acid via the acylation of sn-glycerol 3-phosphate was originally detected in the microsomal fraction of guinea pig liver by Kornberg & Pricer (1953) and phosphatidic acid was later shown to be the precursor of triacylglycerols and phospholipids (Smith et al., 1957; Stein & Shapiro, 1957).

The first reaction in the formation of phosphatidic acid by the dihydroxyacetone phosphate route is catalysed by the enzyme dihydroxyacetone phosphate acyltransferase (DHAPAT; EC 2.3.1.42) which also utilises fatty acyl-CoA thioesters to esterify a fatty acid to the primary alcohol group of the DHAP forming the compound acyl-dihydroxyacetone phosphate (acyl-DHAP). The acyl-DHAP is subsequently reduced to lysophosphatidic acid which is then acylated to form phosphatidic acid (see Fig 1.1) (Agranoff & Hajra, 1971; Hajra, 1968a, 1968b, 1977; Hajra & Agranoff, 1968a, 1968b; La Belle & Hajra, 1972b, 1974). The synthesis of acyl-DHAP by DHAPAT activity was first observed in the mitochondrial fractions of guinea pig liver (Bowley et al., 1973; Hajra, 1968a, 1968b; Hajra & Agranoff, 1968a, 1968b) and later in the hepatic microsomes of guinea pigs (Hajra, 1968b) and rats (La Belle & Hajra, 1972b). This activity has a preference for palmitoyl-CoA (Bates & Saggerson, 1979; Hajra, 1968b) and the phosphatidic acid formed via this route is predominantly 1-saturated, 2-unsaturated phosphatidic acid in line with the observed positional specificity of fatty acids in naturally-occuring glycerolipids (Hajra, 1968a).

There is, however, some question as to whether the acylation of DHAP is catalysed by a unique enzyme. The acyl transfer to dihydroxyacetone phosphate, detected in the microsomal fraction (Hajra, 1968b; La Belle & Hajra, 1972b), appears to be catalysed by GPAT (Schlossman & Bell, 1976b, 1977). GPAT and DHAPAT activities appear to be dual catalytic functions of a single microsomal enzyme. Support for this comes from the observations that glycerol 3-phosphate competitively inhibits DHAPAT activity and DHAP competitively inhibits

GPAT activity (Bramley & Grigor, 1982; Coleman & Haynes, 1983; Dodds et al., 1976b; Hajra, 1968b; Schlossman & Bell, 1976b, 1977). The two acyltransferase activities are also virtually identical in their pH and acyl-CoA chain length dependence, thermolability, and susceptibility to inactivation by N-ethylmaleimide (NEM), trypsin and detergents (Coleman & Bell, 1980; Coleman & Haynes, 1983; Schlossman & Bell, 1976a, 1976b, 1977). DHAPAT and GPAT activities from yeast are also similar (Schlossman & Bell, 1978). Microsomal GPAT and DHAPAT activities undergo a coordinated 30-70 fold increase during differentiation of 3T3-L1 preadipocytes to adipocytes (Bell & Coleman, 1980; Coleman & Bell, 1980). This suggests that a single enzyme with both activities is being induced by differentiation. Rat hepatic microsomal GPAT and DHAPAT activities increase in parallel 74-fold after birth (Coleman & Haynes, 1983) which also implies the induction of a single enzyme with both activities. Furthermore, the active sites of rat liver DHAPAT and GPAT are both found on the cytosolic side of microsomal membranes (Ballas & Bell, 1980, 1981; Bell et al., 1981; Coleman & Bell, 1978; Jamdar, 1977, 1979). However, there have been reports (Rock et al., 1977) of what is thought to be a microsomal DHAPAT which differs from the microsomal enzyme with dual GPAT/DHAPAT activities discussed above. This microsomal DHAPAT activity is resistant to trypsin and NEM and is not inhibited by glycerol 3-phosphate. To this extent it is similar to the mitochondrial DHAPAT activity identified initially which cannot use glycerol 3-phosphate as a substrate and is not inhibited by glycerol 3-phosphate (Bowley et al., 1973; Hajra, 1968b). The mitochondrial DHAPAT activity is either not affected by (Saggerson et al., 1980) or is stimulated by NEM (Bates & Saggerson, 1979). It appears that the mitochondrial GPAT and DHAPAT

activities are separate enzymes as the mitochondrial GPAT activity is not inhibited by DHAP (Bowley et al., 1973; Hajra, 1968b) and compared to the DHAPAT, the mitochondrial GPAT has a different response to hypolipidaemic drugs (Bowley et al., 1973). The DHAPAT activity associated with the mitochondrial fraction may actually be a peroxisomal enzyme (Bates & Saggerson, 1979; Hajra et al., 1979; Jones & Hajra, 1977, 1980).

The relative contribution of the glycerol 3-phosphate and dihydroxyacetone phosphate pathways to total glycerolipid synthesis is a matter of controversy. In yeast, the synthesis of glycerolipids from acyl-DHAP appears to be unimportant as the reduction of acyl-DHAP to LPA does not occur (Schlossman & Bell, 1978). It is not so straight foward in mammalian tissues, however, as they have a full complement of the necessary enzymes. There have been many attempts to estimate the relative contributions and importance of these two pathways. Early studies with rat liver slices utilised glycerol labelled with [^{3}H] or [^{14}C] (Manning & Brindley, 1972; Okuyama & Lands, 1970) but indications that the glycerol 3-phosphate pathway predominated were called into question as the mitochondrial sn-glycerol 3-phosphate dehydrogenase (EC 1.1.99.5) discriminates against sn-[2-^{3}H]glycerol 3-phosphate (Manning & Brindley, 1972). When this is corrected for neither of the pathways for glycerolipid synthesis appears to be clearly dominant (Manning & Brindley, 1972). Other studies with rat lung (Balint et al., 1975) and liver tissue (Rao et al., 1971a) suggest that the DHAP pathway might be more important than the glycerol 3-phosphate pathway, but another using rat liver parenchymal cells (Rognstad et al., 1974) suggests that the synthesis

of glycerolipids via the acyl-DHAP pathway is of minor importance. Other attempts to resolve this problem have endeavoured to utilise the apparent nucleotide specificity of the glycerol 3-phosphate dehydrogenase and the acyldihydroxyacetone-phosphate reductase (see Fig 1.1) for NADH and NADPH, respectively (Bell & Coleman, 1980; Hajra & Agranoff, 1968b; La Belle & Hajra, 1972a, 1972b). However, overall, results from these studies are inconclusive, largely because the nucleotide specificities are not absolute (Agranoff & Hajra, 1971; Brindley & Sturton, 1982; Chae et al., 1973; Lamb & Fallon, 1976; Pollock et al., 1975b, 1976; Rao et al., 1970; Van den Bosch, 1974; Wykle et al., 1972).

The rate of phosphatidic acid synthesis from DHAP via either acyl-DHAP or glycerol 3-phosphate in homogenates of liver and twelve other tissues has also been examined (Pollock et al., 1975a). Despite the fact that in all tissue homogenates DHAP appeared to enter phosphatidic acid more rapidly via acyl-DHAP than via glycerol 3-phosphate, the activities of glycerol 3-phosphate dehydrogenase and GPAT were much greater than the activity of DHAPAT. Hence all the tissue homogenates possessed an apparently greater capability to synthesise phosphatic acid via glycerol 3-phosphate than via acyl-DHAP, but according to these authors, did not express it. This raises questions about the ability of such assays to provide a meaningful measure of the contributions of the two pathways to glycerolipid biosynthesis. Later studies indicated that adipose tissue incorporates glycerol 3-phosphate into glycerolipids at a higher rate than DHAP (Dodds et al., 1976a, 1976b) while other studies with lung tissue suggest approximately equal contributions from both pathways (Fisher et

al., 1976; Mason, 1978; Snyder & Malone, 1975).

It is evident that research utilising radioactively labelled metabolites has not conclusively resolved the relative importance of the contributions of the two pathways to glycerolipid biosynthesis. A further consideration is whether observations suggesting a major contribution by the DHAP pathway really relate to the in vivo situation or are instead based upon results obtained through use of relatively non-physiological concentrations of substrates and metabolites in the in vitro assays. Consideration of metabolite concentrations found in vivo suggest this may be so as the physiological concentration of glycerol 3-phosphate is much greater than the physiological concentration of DHAP. In rat liver, assuming 70% of the weight is aqueous and that there is no compartmentalisation of substrates (Schlossman & Bell, 1977), the concentration of glycerol 3-phosphate is within the range of 0.185 mM to at least 1 mM (see Table 1.2), while the DHAP concentration is in the range of 0.019 to 0.057 mM. Hence the [glycerol 3-phosphate]/[DHAP] ranges from 2.1:1 to 26:1 (Bowley et al., 1973; Baquer et al., 1976; Burch et al., 1970, 1974; Faupel et al., 1972; Greenbaum et al., 1971; Hohorst et al., 1961; Kahonen et al., 1972; Kalkhoff et al., 1966; Rawat, 1968; Saggerson & Greenbaum, 1970a; Veech et al., 1970; Wieland & Loffler, 1963; Woods & Krebs, 1973; Woods et al., 1970; Zakim et al., 1967). In mouse liver the ratio is 13.4:1 (Ananda Rao & Abraham, 1973) while in rat adipose tissue the [glycerol 3-phosphate]/[DHAP] is within the range 2.5:1 to 9.2:1 (Ballard, 1972; Molaparast-Shahidsaless et al., 1979). In mouse brain and mammary gland, ratios are 4.36:1 and 14:1, respectively (Ananda Rao & Abraham, 1973; Lowry & Passonneau, 1964; Lowry et al.,

1964), and 9.1:1 in ascites cells (Garfinkel & Hess, 1964). Therefore, given the greater activity of GPAT than DHAPAT in many tissues (Pollock et al., 1975a) and a greater concentration of glycerol 3-phosphate than DHAP, it appears likely that in vivo the glycerol 3-phosphate pathway for the formation of glycerolipids is more active than the acyl-DHAP pathway (Lamb & Fallon, 1976; Pollock et al., 1975a).

Using glycerol 3-phosphate and DHAP concentration data similar to those discussed, together with apparent kinetic constants it appears that, in rat liver microsomes in vivo, the ratio of glycerol 3-phosphate acylation to DHAP acylation is greater than 84:1 (Schlossman & Bell, 1976b, 1977). In adipocyte microsomes in vivo, the ratio is between 24:1 (Schlossman & Bell, 1976b) and 30:1 (Schlossman & Bell, 1976a). Notably, the K_m of GPAT is lower than or equal to the physiological concentration of glycerol 3-phosphate, but the K_m of the DHAPAT is 2-10 times greater than the tissue concentration of DHAP (Ballard, 1972; Burch et al., 1970; Coleman & Bell, 1980; Coleman & Haynes, 1983; Dodds et al., 1976b; Greenbaum et al., 1971; La Belle & Hajra, 1972b; Molaparast-Shahidsaless et al., 1979; Schlossman & Bell, 1976b, 1977; Woods & Krebs, 1973). Therefore, it is likely that, in vivo, the concentrations of substrates and the saturation kinetics of the respective enzymes would favour the glycerol 3-phosphate pathway of glycerolipid synthesis. Furthermore, the apparent K_i value for inhibition of GPAT by DHAP is 8-46 times higher than the tissue concentration of DHAP, while the apparent K_i value for inhibition of the DHAPAT by glycerol 3-phosphate is 1.6 to 14 times lower than the tissue concentration of glycerol 3-phosphate (see the above references). Therefore, it is likely that the DHAPAT activity is

markedly inhibited at the normal cellular concentrations of glycerol 3-phosphate. In contrast, GPAT activity would be largely unaffected at the normal cellular concentrations of DHAP. Hence there is good reason to believe that the glycerol 3-phosphate pathway is the major route for the synthesis of glycerolipids, although the matter has yet to be completely resolved.

1.3 THE CONTROL OF GLYCEROLIPID BIOSYNTHESIS

1.3.1 REGULATION BY SUBSTRATE AVAILABILITY

The availability of the substrate sn-glycerol 3-phosphate has often been implicated as a regulatory factor governing the partition of fatty acids between β-oxidation and esterification (Ballard, 1972; Brindley, 1978; Christiansen, 1979; De Beer et al., 1981; Declercq et al., 1982; Exton & Park, 1967; Fellenius et al., 1973; Frank et al., 1968; Howard & Lowenstein, 1964; Lund et al., 1980; Mayes, 1976; Mayes & Felts, 1967; Sugden et al., 1980; Tubbs & Garland, 1964; Tzur et al., 1964; Van Harken et al., 1969; Van Tol, 1974; Williamson et al., 1980; Wirthensohn et al., 1980). It was suggested that in the fed state, glycerol 3-phosphate concentration is elevated and this increases esterification. Conversely, in the fasted state the glycerol 3-phosphate concentration falls leading to a decrease in the rate of esterification. However, experiments have so far failed to clearly establish that glycerol 3-phosphate concentration limits esterification. Several recent experiments (De Beer et al., 1981; Declercq et al., 1982) do, however, suggest concentrations below which glycerol 3-phosphate may limit esterification. A number of treatments and conditions appear to affect glycerol 3-phosphate concentrations (see Table 1.1). However, as Table 1.1 illustrates, there are inconsistencies in the response of glycerol 3-phosphate concentration to these treatments, e.g. both insulin treatment and diabetes appear to increase or decrease hepatic glycerol 3-phosphate concentration.

Table 1.1: Treatments reported to affect glycerol 3-phosphate concentrations in mammals.

Treatments	Reference
INCREASE GLYCEROL 3-PHOSPHATE CONCENTRATION	
↑ adrenaline (c)	Denton & Halperin, 1968 Molaparast-Shahidsaless et al., 1979;
diabetes (a)	Denton & Randle, 1967 Greenbaum et al., 1971;
fat free diet (a)	Burch et al., 1974
feeding ethanol (a)	Hawkins & Kalant, 1972 Kahonen et al., 1972; Nikkila & Ojala, 1963b; Rawat, 1968; Zakim, 1965;
↑ glycerol (a)	De Beer et al., 1981; Lund et al., 1980; Williamson et al., 1969 Woods & Krebs, 1973;
high carbohydrate diet (a)	Greenbaum et al., 1971; Veech et al., 1970; Waddel & Fallon, 1973
high fat diet (a)	Burch et al., 1974; Guynn et al., 1972 Molaparast-Shahidsaless et al., 1979;
↑ insulin (a,c,d)	Beynen et al., 1980; Molaparast-Shahidsaless et al., 1979; Saggerson & Greenbaum, 1970a; Denton & Halperin, 1968; Denton & Randle, 1967
nembutal anaesthesia (a)	Faupel et al., 1972; Threlfall & Heath, 1968
parturition (b)	Kuhn, 1967b

↑ = (increase or increased concentration of...). (a) = liver; (b) = mammary gland; (c) = adipose tissue; (d) = heart.

...../continued

Table 1.1, continued

Treatments	Reference
DECREASE GLYCEROL 3-PHOSPHATE CONCENTRATION	
clofibrate (a)	Fallon et al., 1972;
diabetes (d)	Denton & Randle, 1967; Kraupp et al., 1967;
dibutyryl cAMP (a)	Molaparast-Shahidsaless et al., 1979;
↑ glucagon (a)	Beynen et al., 1980; Christiansen, 1979; Kneer et al., 1979; Molaparast-Shahidsaless et al., 1979; Sugden et al., 1980;
high carbohydrate diet (a)	Molaparast-Shahidsaless et al., 1979;
hyperthyroid animals (a)	Rawat & Lundquist, 1968;
hypothyroid animals (a)	Baquer et al., 1976; Carnicero et al., 1972; Rawat & Lundquist, 1968;
↑ insulin (a)	Frank et al., 1968;
insulin deficiency (a)	Kalkhoff et al., 1966;
nicotinamide (a)	Lagunas et al., 1970;
vasopressin (a)	Sugden et al., 1980;
DO NOT AFFECT GLYCEROL 3-PHOSPHATE CONCENTRATION	
↑ adrenaline (c) (may ↑ α-GP)	Denton & Halperin, 1968; Molaparast-Shahidsaless et al., 1979;
lactation (a)	Zammit, 1981a.

↑ = (increase or increased concentration of...). (a) = liver; (b) = mammary gland; (c) = adipose tissue; (d) = heart.

Table 1.2: Concentrations of glycerol 3-phosphate in tissues of fed mammals.

Tissue	Glycerol 3-P conc^{n} (mM)	Reference
RAT LIVER	0.16-0.19	Greenbaum et al., 1971; Guynn et al., 1972; Faupel et al., 1972; Woods & Krebs, 1973; Woods et al., 1970;
	0.20-0.26	Burch et al., 1970, 1974; Baquer et al., 1976; Hems & Brosnan, 1970; Veech et al., 1970; Wieland & Loffler, 1963;
	0.33-0.37	Fellenius et al., 1973; Hohorst et al., 1959; Rawat, 1968;
	0.41-0.47	Ackermann et al., 1974; Bartels & Hohorst, 1963; Carnicero et al., 1972; Lagunas et al., 1970; Kahonen et al., 1972; Sestoft & Fleron, 1975; Sugden et al., 1980; Waddel & Fallon, 1973; Zakim & Herman, 1968;
	0.50-0.58	Bates et al., 1977; Kalkhoff et al., 1966; McGarry & Foster, 1971;
	0.60-0.67	Bowley et al., 1973; Threlfall & Heath, 1968; Zakim & Herman, 1968; Zammit, 1981a;
	0.71-0.76	Bowley et al., 1973; Lagunas et al., 1970; Zakim, 1965; Zammit, 1981a;
	0.86-0.99	Bortz & Lynen, 1963; Christiansen, 1979; Leva & Rapoport, 1943; Rawat & Lundquist, 1968; Robinson & Newsholme, 1969;
	1.00-1.34	Beynen et al., 1980; Tzur et al., 1964; Wieland, 1957; Zakim et al., 1967;
	1.82	Frank et al., 1968;
	2.23	Bohmer, 1967[a];
	5.14-6.00	Fallon & Kemp, 1968;
	6.43	Nikkila & Ojala, 1963a;
	8.70	Fallon et al., 1972;

[a]assuming 3 µl/mg protein (Christiansen, 1979). Where values were not reported as concentrations, the values for sn-glycerol 3-phosphate in liver have been calculated assuming 70% of the weight is aqueous and that there is no compartmentalisation of substrates (Schlossman & Bell, 1977).

...../continued

Table 1.2, continued

Tissue	Glycerol 3-P conc^{n} (mM)	Reference
RAT ADIPOSE TISSUE		
	0.01-0.03	Ballard, 1972; Denton & Halperin, 1968;
	0.17	Molaparast-Shahidsaless et al., 1979;
	0.23	Denton & Randle, 1967;
	1.04	Saggerson & Greenbaum, 1970a;
	1.30	Denton et al., 1966;
RAT HEART		
	0.09	Kraupp et al., 1967;
	0.56	Denton et al., 1966;
RAT DIAPHRAM	0.22	Denton et al., 1966;
PREGNANT RAT MAMMARY GLAND	0.27	Gumaa et al., 1971;
LACTATING RAT MAMMARY GLAND	0.80	Gumaa et al., 1971;
PREGNANT GUINEA PIG MAMMARY TISSUE	0.05	Kuhn, 1967b;
LACTATING GUINEA PIG MAMMARY GLAND	0.13	Kuhn, 1967b;
ASCITES CELLS	1.09	Garfinkel & Hess, 1964;
MOUSE LIVER	0.82	Ananda Rao & Abraham, 1973;
MOUSE BRAIN	0.09	Lowry et al., 1964;
MOUSE MAMMARY TISSUE	0.74	Ananda Rao & Abraham, 1973;

[a]assuming 3 µl/mg protein (Christiansen, 1979). Other values for rat liver = 55-99 nmol/mg DNA (Faupel et al., 1972), 430 nmol/mg DNA (Pilkis et al., 1976), and hepatocytes = 0.42 µmol/10^{8} cells (De Beer et al., 1981). Where values were not reported as concentrations, the values for sn-glycerol 3-phosphate in liver have been calculated assuming 70% of the weight is aqueous and that there is no compartmentalisation of substrates (Schlossman & Bell, 1977). When values were not reported in mM terms for adipose tissue, they were calculated assuming 30% of the weight is aqueous and there is no compartmentalisation of substrates (Schlossman & Bell, 1976b).

Table 1.3: Concentrations of glycerol 3-phosphate in tissues of fasted rats.

Length of fast	Glycerol 3-P conc^{n} (mM)	Reference
RAT LIVER		
2 h	1.81	Frank et al., 1968
4 h	1.63	Frank et al., 1968
24 h	0.56	Greenbaum et al., 1971
	0.79	Tzur et al., 1964
	0.82	Frank et al., 1968
	1.13	Bohmer, 1967[a]
	1.52	Zammit, 1981a
36 h	0.48	Zakim, 1965
48 h	0.13	Fellenius et al., 1973
	0.23	Guynn et al., 1972
	0.33	Hems & Brosnan, 1970
	0.37	Williamson et al., 1969
	0.43	Tzur et al., 1964
	0.60	Veech et al., 1970
	0.77	McGarry & Foster, 1971
	1.09	Frank et al., 1968
	3.54	Fallon & Kemp, 1968
72 h	0.18	Greenbaum et al., 1971
	1.03	Frank et al., 1968
	2.95	Fallon & Kemp, 1968
7 days	0.07	Tzur et al., 1964
undefined	0.46	Leva & Rapoport, 1943
	4.86	Nikkila & Ojala, 1963a
RAT ADIPOSE TISSUE		
24 h	0.01	Ballard, 1972
48 h	0.08	Molaparast-Shahidsaless et al., 1979

[a] assuming 3 µl/mg protein (Christiansen, 1979). Where necessary the concentrations have been calculated as outlined in the footnote to Table 1.2. 24 h fasted rat heart = 0.05 mM (Kraupp et al., 1967). Hepatocytes = 0.16 µmol/10^{8} cells (De Beer et al., 1981).

Furthermore, the role of glycerol 3-phosphate in the control of glycerolipid synthesis is called into question as a wide range of overlapping concentrations of glycerol 3-phosphate have been reported in the tissues of fed and fasted animals (see Tables 1.2 and 1.3).

Hence consideration of Tables 1.2 and 1.3 supports the view that there is not a clear distinction between the concentration of glycerol 3-phosphate in fed and fasted states. This is consistent with the apparent conflict over just how dietary and hormonal treatments affect the glycerol 3-phosphate concentration (Table 1.1). Furthermore, it has been reported that there is a poor correlation between glycerol 3-phosphate concentration and rates of fatty acid esterification and oxidation (Bohmer, 1967; Guynn et al., 1972; McGarry & Foster, 1971; Zammit, 1981a). Similarly, glycerol 3-phosphate concentration does not appear to affect hepatic fatty acid synthesis (Zakim et al., 1967). Increased accumulation of hepatic triacylglycerol is seen with acute ethanol administration (Abrams & Cooper, 1976) and, although this is associated with increases in glycerol 3-phosphate concentration (Hawkins & Kalant, 1972; Nikkila & Ojala, 1963b; Rawat, 1968; Zakim, 1965), it does not appear that glycerol 3-phosphate concentration is the causative factor (Abrams & Cooper, 1976). However, a correlation between glycerol 3-phosphate concentration and triacylglycerol synthesis in the oleate-perfused rat liver has been observed (Fellenius et al., 1973), but without oleate in the perfusion medium there was no correlation. In other studies (Mayes, 1976), esterification in livers of fed, but not fasted, rats appeared to correlate with availability of glycerol 3-phosphate. Consistent with this, rates of change of

glycerol 3-phosphate concentration appear too small and too slow to be of importance for regulation (Bohmer, 1967; Guynn et al., 1972). As would be expected, the concentration of activated fatty acids responds much more rapidly to dietary changes (Bohmer, 1967; Guynn et al., 1972;). It appears that glycerol 3-phosphate concentration increases during fasting (Guynn et al., 1972; McGarry & Foster, 1971; McGarry et al., 1973; Veech et al., 1970; Zammit, 1981a), rather than decreasing as would be expected if substrate concentration controls glycerolipid synthesis. Indeed, glycerol 3-phosphate concentration varies in proportion to the rate at which liver oxidises fatty acids (Zammit, 1981a) reflecting the increased delivery of glycerol to the liver from adipose tissue under ketogenic conditions (Zammit, 1981a). Thus, fasting for 24 h causes a 2-3 fold increase in glycerol 3-phosphate concentration (Zammit, 1981a).

In adipose tissue the rate of triacylglycerol synthesis cannot be correlated with either the tissue glycerol 3-phosphate concentration, or long-chain fatty acyl-CoA concentration (Denton & Halperin, 1968; Denton & Randle, 1967; Saggerson & Greenbaum, 1970a, 1970b). Such correlations would be expected if substrate concentrations were involved in the control of glycerolipid biosynthesis. Furthermore, there is no correlation between glycerol 3-phosphate concentration and the rate of glycolysis in adipose tissue (Halperin & Denton, 1969). This seems consistent with the probability that a high glycerol 3-phosphate concentration would antagonise glycolysis, through action on phosphofructokinase (Claus et al., 1982; Hers & Van Schaftingen, 1982; Richards & Uyeda, 1982; Uyeda et al., 1982), which provides metabolites required for glycerolipid biosynthesis.

1.3.2 REGULATION BY CONTROL OF PHOSPHATIDATE PHOSPHOHYDROLASE ACTIVITY

Phosphatidic acid, formed by the acylation of glycerol 3-phosphate or DHAP, is dephosphorylated through the action of the enzyme phosphatidate phosphohydrolase (PPH; EC 3.1.3.4). The diacylglycerol formed is utilised in the synthesis of triacylglycerols and phospholipids (see Fig 1.1) (Bell & Coleman, 1980). The study of phosphatidate phosphohydrolase has been complicated by the dependence of the amount of enzyme activity detected on the assay conditions, making it difficult to determine the significance of some reports. Hence, although PPH activity is found in virtually all cellular membranes and in the cytosolic fraction of the liver (Coleman, 1968; Hosaka et al., 1975; Jelsema & Morre, 1978; Kent & Vagelos, 1976; Sedgwick & Hubscher, 1965; Wilgram & Kennedy, 1963), the apparent subcellular distribution is greatly influenced by the type of substrate preparation used. For example, if assayed with aqueous dispersions of phosphatidic acid the particulate activities appear to predominate, but if assayed with membrane-bound phosphatidic acid the cytosolic activity appears to be greater (Lamb & Fallon, 1974a; Mitchell et al., 1971; Smith et al., 1967). However, if Ca^{2+} is removed during the preparation of phosphatidic acid, it appears that high rates of activity for the cytosolic PPH can be obtained using dispersions of phosphatidic acid (Brindley & Sturton, 1982). Despite these difficulties, it appears that the cytosolic PPH is the major activity involved in glycerolipid biosynthesis (Brindley, 1977; Smith et al., 1967). The sensitivity of enzyme specific activity to assay conditions is also illustrated by reports that the cytosolic PPH activity may be activated (Hosaka et al., 1975; Moller et al., 1977; Smith et al.,

1967) or inhibited (Lamb & Fallon, 1974a; Mitchell et al., 1971; Smith et al., 1967; Sturton & Brindley, 1977) by Mg^{2+}. Similarly, Mg^{2+} may activate (Lamb & Fallon, 1974a; Smith et al., 1967) or inhibit (Sturton & Brindley, 1977; Van Heusden & Van den Bosch, 1978) membrane-bound PPH. However, Van Heusden & Van den Bosch, (1978) suggested that Mg^{2+}-dependence is only demonstrated in microsomes prepared in the presence of EDTA. In addition to the above difficulties, controversy surrounds the method for detecting the PPH activity. Brindley & Sturton, (1982) suggest that measurement of PPH activity by phosphate release is unreliable compared to radioactive label techniques.

Despite sometimes conflicting reports it has been suggested that PPH may be a point at which the glycerolipid biosynthetic pathway is regulated. The possibility that PPH activity is rate limiting and observations that PPH is influenced by dietary and hormonal stimuli are used to support this proposal (Brindley, 1977; Brindley & Sturton, 1982; Brindley et al., 1979a; Fallon et al., 1977). It has been suggested that PPH activity in rat liver is rate limiting, with the lowest rate of the enzymes involved in glycerolipid biosynthesis (Fallon et al., 1977; Lamb & Fallon, 1974a). However, this was only observed for the microsomal PPH while the rate of the cytosolic PPH was about 10 times greater (Lamb & Fallon, 1974a). Furthermore, addition of the cytosolic PPH to the microsomal fraction greatly increases the rate of phosphatidic acid dephosphorylation (Fallon et al., 1977; Lamb & Fallon, 1974a) and as the cytosolic PPH is probably the major PPH activity involved in glycerolipid synthesis (Brindley, 1977; Smith et al., 1967), PPH activity may not be rate limiting in vivo. Moreover,

the administration of [^{14}C]glycerol to animals or its addition to intact hepatocyte preparations, does not result in the accumulation of radioactivity in phosphatidic acid as might be expected if the clearance of PA was limited by the activity of PPH. Instead, the radioactive label appears promptly in the triacylglycerol and complex lipids of hepatocytes (Fallon et al., 1977; Lamb et al., 1977). The relatively low concentration of phosphatidic acid (5 nmol/mg of microsomal protein) and high concentration of diacylglycerol (20 nmol/mg of microsomal protein) found in rat liver microsomal preparations (Fallon et al., 1975) does not appear to be consistent with the effective activity of PPH being the lowest of the the enzymes involved with glycerolipid synthesis. It has been suggested that the low levels of phosphatidic acid may be due to phospholipases which could rapidly dispose of excess phosphatidic acid not converted to diacylglycerol (Brindley & Sturton, 1982). This, however, would appear to be less efficient than regulating the amount of phosphatidic acid produced, through limiting the initial acylation of glycerol 3-phosphate by GPAT. The observation that the combined cytosolic and membrane-bound PPH activity exceeds the activity of GPAT (Lamb & Fallon, 1974a) makes the need to invoke the action of such lipases seem unnecessary. Furthermore, the incubation of radioactively labelled phosphatidic acid bound to microsomal particulate preparations, at pH 8.3 in the presence of Ca^{2+} and rat liver homogenate, does not reveal any substantial conversion of phosphatidic acid into either lysophosphatidic acid or glycerol (Fallon et al., 1977). In addition, measurements of PPH activity, using both membrane-bound and exogenous phosphatidic acid suggests that PPH has a large reserve capacity beyond that needed to deal with the in vivo concentrations of phosphatidic

acid (Bell & Coleman, 1980; Thompson, 1980; Van Heusden & Van den Bosch, 1978).

Although the cytosolic PPH activity appears to change in response to dietary and hormonal stimuli (Brindley, 1977; Brindley & Sturton, 1982; Fallon et al., 1977; Moller et al., 1977, 1981), the direction of the response of PPH activity to some of these stimuli appears to be contrary to that which would be expected. It is generally accepted that during fasting, pathways in the liver favour β-oxidation of fatty acids and a reduction of esterification and triacylglycerol production, but hepatic PPH activity is increased more than 2-fold by fasting for 6-40 hours (Mangiapane et al., 1973; Vavrecka et al., 1969), although adipocyte PPH activity appears to be lowered slightly by fasting for 72 h (Moller et al., 1977). Feeding increased amounts of carbohydrate, fat and ethanol also increases hepatic PPH activity (Brindley, 1977; Fallon et al., 1977; Lamb & Fallon, 1972b, 1974b; Savolainen, 1977), but this response seems to be more appropriate than the response of the hepatic enzyme to fasting. It also seems appropriate that drugs found to decrease PPH activity in vitro appear to reduce hepatic triacylglycerol formation in vivo (Fallon et al., 1977). However, each of these drugs also caused some inhibition of GPAT activity (Fallon et al., 1977). Just as the response of the hepatic PPH to fasting was unexpected, it is also surprising that PPH activity in adipocytes is increased by up to 200% by adrenaline or dibutyryl cAMP (Moller et al., 1981). However, noradrenaline decreases adipocyte PPH activity, but this response is apparently dependent on the presence of Mg^{2+} and part of the inactivation might be due to accumulation of free fatty acids in the incubation medium (Cheng & Saggerson, 1978a). Insulin and

propranolol are both able to block the effect of noradrenaline and insulin is able to reactivate PPH in cells previously exposed to noradrenaline (Cheng & Saggerson, 1978b). Insulin has not been shown to increase the activity of PPH although a supraphysiological concentration of insulin (20 munits/ml) may cause a slight decrease in hepatocyte PPH activity (Lawson et al., 1982a). The same, very high, concentration of insulin has also been shown to inhibit the ability of corticosterone to increase hepatocyte PPH activity (Lawson et al., 1982a). Glucocorticoids in general appear to increase cytosolic PPH activity (Brindley et al., 1979a, 1979b; Fallon et al., 1975; Glenny & Brindley, 1978; Jennings et al., 1981; Knox et al., 1979; Lehtonen et al., 1979; Pritchard & Brindley, 1977; Savolainen, 1977).

Therefore, these considerations suggest that while the possibility that PPH is a point of regulation cannot be totally dismissed, the justification relies on elaborate arguments and systems which appear to ignore the simpler solution of controlling the rate of glycerolipid synthesis at the point of initial acylation of glycerol 3-phosphate.

1.3.3 REGULATION BY CONTROL OF DIACYLGLYCEROL ACYLTRANSFERASE ACTIVITY

Diacylglycerol formed by the dephosphorylation of phosphatidic acid can be utilised for the synthesis of triacylglycerols and phospholipids (Bell & Coleman, 1980). Phospholipid synthesis does not seem to be strongly affected by the quantity of diacylglycerol available to it (Thompson, 1980). It appears that diacylglycerols in excess of requirements for phospholipid synthesis are rapidly converted to triacylglycerols (Numa & Yamashita, 1974). The formation of triacylglycerol from diacylglycerol is catalysed by the enzyme diacylglycerol acyltransferase (DGAT; EC 2.3.1.20) (see Fig 1.1) (Bell & Coleman, 1980). In contrast to many of the other enzymes of the glycerolipid biosynthetic pathway, DGAT is located solely in the microsomes (Coleman & Bell, 1976; Hosaka et al., 1977; Sarzala et al., 1970; Sedgwick & Hubscher, 1965; Van Golde et al., 1971; Wilgram & Kennedy, 1963) and faces the cytosolic side of the membrane (Bell et al., 1979). DGAT is the only enzyme of the glycerolipid biosynthetic pathway that is unique to triacylglycerol synthesis (Bell & Coleman, 1980) and DGAT activities are highest in adipose tissue and liver (Coleman & Bell, 1976). Before it was discovered that ethanol could be used to disperse the diacylglycerol substrate, there were numerous problems associated with the assay of this enzyme (Bell & Miller, 1976; Coleman & Bell, 1976).

Despite suggestions that DGAT may be important in the regulation of glycerolipid biosynthesis, there is only limited evidence to support this proposal. Hepatic DGAT activity does not appear to be rate-limiting (Lamb & Fallon, 1974a) and may have a large reserve

capacity (Haagsman & Van Golde, 1981; Haagsman et al., 1982). However, DGAT activity in adipocytes may be regulated to prevent futile cycling following the activation of hormone sensitive lipase (Bell & Miller, 1976; Coleman & Bell, 1976) and, accordingly, the incubation of rat adipocytes with adrenaline reduces DGAT activity by about 50% (Sooranna & Saggerson, 1978b). A very high concentration of insulin blocks the effect of adrenaline, although insulin alone appears to have no effect on DGAT activity (Sooranna & Saggerson, 1978b). In contrast to the effect of adrenaline on adipocyte DGAT activity, fasting, surgical procedures, diabetes, high carbohydrate and high fat diets have little effect on hepatic DGAT activity (Brindley, 1977). However, observations that hepatic DGAT activity increases in both ketotic-diabetic animals and those fed a fructose-enriched diet (Fallon et al., 1975; Young & Lynen, 1969) seem inconsistent with a regulatory role.

More recently, there have been suggestions that glucagon may influence partitioning of available diacylglycerols between triacylglycerol or phospholipid formation in rat hepatocytes (Beynen et al., 1981; Geelen et al., 1978). This seems consistent with suggestions that DGAT activity may be subject to hormonal control, possibly via a phosphorylation-dephosphorylation mechanism. These suggestions are supported by the observation that incubation of rat hepatocytes with glucagon decreases DGAT activity by about half (Haagsman et al., 1981). Furthermore, hepatic DGAT activity is reduced after preincubation of hepatic microsomes with Mg^{2+}, ATP, and cytosolic fraction (Haagsman et al., 1981) and restored by washing the microsomes and adding cytosolic fraction and dithiothreitol. Fluoride, a

phosphatase inhibitor, inhibits reactivation (Haagsman et al., 1981). On the other hand, the rate of DGAT inactivation in the above system is not influenced by the addition of cAMP or by high concentrations of the catalytic subunit of cAMP-dependent protein kinase (Haagsman et al., 1982). An earlier study (Soler-Argilaga et al., 1978a) found that DGAT activity increased, rather than decreased, after perfusion of rat liver with dibutyryl cAMP. Hence these observations raise questions as to how the action of glucagon on this enzyme might be mediated (Haagsman et al., 1981, 1982). Further research is required to determine whether other hormones regulate DGAT activity.

1.3.4 REGULATION BY CONTROL OF GLYCEROL PHOSPHATE ACYLTRANSFERASE ACTIVITY

GPAT catalyses the first reaction to commit fatty acids to glycerolipid biosynthesis (see Fig 1.1) (Bell & Coleman, 1980). Experimental observations suggest that GPAT may be an important point at which glycerolipid biosynthesis is regulated (Aas & Daae, 1971; Bremer et al., 1976; Geelen et al., 1980; Kuhn, 1967a; Lund et al., 1980; Zammit, 1981a). Regulation of GPAT activity would not only affect the partition of fatty acids between esterification and oxidation, but also the flux of the whole pathway. GPAT activity is affected, either chronically or acutely, to varying extents by physiological, dietary, drug and hormonal stimuli. In mitochondrial and microsomal fractions of rat liver GPAT activity is rate-limiting for phosphatidic acid synthesis (Lloyd-Davies & Brindley, 1973, 1975; Sanchez et al., 1973; Thompson, 1980). GPAT activity may also be rate-limiting in adipose, mammary, and intestinal tissue (Brindley, 1973; Saggerson et al., 1977; Short et al., 1977).

1.3.4.1 Physiological effectors of GPAT activity

The response of GPAT to changes in the age of the animal and state of metabolism suggests that GPAT activity may be regulated in a dynamic way. Microsomal GPAT activity is very low in foetal rat liver microsomes until about 2 to 3 days before birth (Coleman & Haynes, 1983). After birth GPAT activity increases briefly to greater than adult levels, but after a month GPAT activity is approximately the same as in adult liver (Coleman & Haynes, 1983; Goldsmith, 1981). A similar trend is seen with kidney microsomal GPAT activity (Goldsmith,

1981). GPAT activity in neonatal liver, compared to adult liver, is higher in rough microsomes than smooth microsomes (Goldsmith, 1981) suggesting a strategic association between protein and lipid synthesis for the production of new membranes and packaging of proteins to be exported to other parts of the cell or the body. GPAT activity in neonatal sarcoplasmic reticulum is 6-fold greater than in adult membranes (Smith et al., 1982). In contrast, the rate of glycerolipid formation in epididymal fat pads is very low in 15-day-old rats, but is about 8.5-fold higher at 60 days (Jamdar & Osborne, 1981a). Thereafter rates tend to decline (Jamdar & Osborne, 1981a). Adipocytes from older rats esterify glycerol 3-phosphate at a lower rate than cells of a similar size from young rats (Jamdar & Osborne, 1981a). The specific activity of GPAT in guinea pig intestinal mucosa is higher after birth compared to foetal values (Short et al., 1975).

Obesity in the obese-hypoglycaemic mouse is associated with an increase in number and size of adipocytes and obese mouse adipocytes show an increased capacity for triacylglycerol formation (Jamdar et al., 1976). Consistent with this, the microsomal GPAT is 2-fold more active in adipocytes of obese mice (Jamdar et al., 1976). Similarly, rates of glycerol 3-phosphate esterification are higher in adipose tissue depots of obese rats compared to lean rats (Jamdar, 1978). As the size of the adipocytes is smaller in the tissues of lean rats some effect of cell size is implied. In support of this, adipocytes of equal sizes from lean and obese animals have similar capacities for lipid synthesis (Jamdar & Osborne, 1981b). However, larger adipocytes synthesise lipid at higher rates than small adipocytes (Jamdar & Osborne, 1981a; Jamdar et al., 1981). Consistent with this, GPAT

activity is about 3.5-fold higher (activity/10^6 cells) in large adipocytes compared to small adipocytes (Jamdar & Osborne, 1981a; Jamdar et al., 1981). Similarly, differences exist between subcutaneous adipocytes and larger gonadal or perirenal adipocytes (Durham et al., 1971; Jamdar & Osborne, 1981a; Jamdar et al., 1981; Reardon et al., 1973). In contrast to increases in the rates of enzymes of the glycerol 3-phosphate pathway, fatty acid synthesis de novo in large adipocytes appears to be lower than in small adipocytes (Francendese & Digirolamo, 1981).

The specific activity of guinea pig mammary gland microsomal GPAT increases 37-fold at parturition which probably accounts in part for the substantial increase in triacylglycerol synthesis also seen at parturition (Kuhn, 1967a, 1967b; Pynadath & Kumar, 1964). Other lipogenic enzymes parallel this marked increase in GPAT activity (Caffrey & Kinsella, 1977b; Grigor et al., 1982; Short et al., 1977). GPAT activity of rabbit mammary tissue homogenate also increases during pregnancy and early lactation (Short et al., 1977). By day 15 of lactation, GPAT activity is about 20-fold greater than during pregnancy (Short et al., 1977). Esterification of glycerol 3-phosphate by rat mammary microsomes increases 6-fold during pregnancy and rises markedly after parturition (Cooper & Grigor, 1978). In late-lactation GPAT activity is 70-fold higher than in virgin rats, but declines rapidly to pre-lactation levels after weaning (Cooper & Grigor, 1978). Hepatic GPAT activity in lactating rats is similar to that in the pregnant rat (Zammit, 1981a). However, the activity of hepatic microsomal GPAT in pregnancy and mid-lactation is higher than in virgin rats (Zammit, 1981a). Hepatic mitochondrial GPAT activity is also significantly

increased in mid-lactation compared to virgin rats (Zammit, 1981a).

Physical training of rats increases glycerol 3-phosphate esterification in adipose tissue, skeletal muscle, and heart, but not liver (Askew et al., 1973, 1975). Increased capacity to esterify glycerol 3-phosphate may enable muscular tissue of trained rats to rapidly replenish local lipid stores during inactivity after exercise. GPAT activity is also affected by some genetic conditions. Cardiomyopathic hamsters have a reduced ability to esterify glycerol 3-phosphate in heart muscle and adipose tissue (Barakat et al., 1977). Accordingly, lipid content is reduced in some tissues of the cardiomyopathic hamster. Rats with glycogen storage disease (gsd/gsd) exhibit mild hypoglycaemia, an inability to mobilise glycogen in the fasted state, and abnormalities in lipid metabolism (Saggerson & Topping, 1981). GPAT activity in the livers of these rats is reduced compared to normal rats, due mainly to a reduction in the mitochondrial GPAT activity although microsomal GPAT activity is reduced slightly too, as is DHAPAT activity (Saggerson & Topping, 1981).

1.3.4.2 Dietary effectors of GPAT activity

Triacylglycerol, but not phospholipid, synthesis is, in general, markedly affected by the dietary regime of the animal (Abrams & Cooper, 1976; Groener & Van Golde, 1977; Iritani et al., 1976) and there are corresponding changes in the activity of GPAT.

1.3.4.2.1 Fasting

The response of GPAT to fasting suggests that GPAT activity is regulated in an acute manner. There is considerable evidence that fasting reduces the rate of esterification of fatty acids, increases their rate of uptake by mitochondria (Amatruda, et al., 1978), and increases their rate of oxidation (Harper & Saggerson, 1976; McGarry & Foster, 1971; McGarry et al., 1973; Ontko, 1972; Zammit, 1980, 1981b). As discussed above (see 1.3.1), the response of glycerol 3-phosphate concentration to feeding and fasting is variable and is unlikely to be controlling the activity of GPAT, suggesting that GPAT activity must be regulated by other factors such as hormones. Fasting of rats for up to 72 h decreases total GPAT activity per liver to as low as half that in fed rat liver (Aas & Daae, 1971; Fallon & Kemp, 1968; Vavrecka et al., 1969; Weigand et al., 1973). Similarly, fasting for up to 72 h decreases the specific activity of hepatic microsomal GPAT to as low as 30% of fed controls (Mangiapane et al., 1973; Van Tol, 1974; Zammit, 1981a). However, in some cases, the effect of fasting has been minimal (Bates & Saggerson, 1979; Fallon & Kemp, 1968; Weigand et al., 1973; Zammit, 1981a). The activity of hepatic microsomal DHAPAT is also decreased to 52% after fasting for 72 h (Rao et al., 1971b). Similarly, the activity of hepatic mitochondrial GPAT is reduced by fasting for 24-48 h to as low as 57% of fed rat controls (Bates & Saggerson, 1979; Van Tol, 1974; Zammit, 1981a).

GPAT activity in homogenates of fat cells from animals fasted for 48-72 h is decreased to 30-60% of the activity in homogenates from fed animals (Angel & Roncari, 1967; Daniel & Rubinstein, 1967; Harper &

Saggerson, 1976; Jamdar & Osborne, 1982; Sooranna & Saggerson, 1979). Mitochondrial GPAT in adipose tissue of rats fasted for 48-72 h is also reduced to 50-87% of fed controls (Jamdar & Osborne, 1982; Lawson et al., 1981a), but as only a small proportion of adipose GPAT activity is mitochondrial (Bagul, 1972; Bell & Coleman, 1980; Jamdar & Fallon, 1973; Jamdar & Osborne, 1982; Saggerson et al., 1980; Schlossman & Bell, 1976a, 1976b), reductions on fasting seem unlikely to be physiologically relevant. However, the activity of microsomal GPAT, the predominant activity in adipose tissue, is decreased to 47-77% of fed controls by fasting (Jamdar & Osborne, 1982; Steffen et al., 1978).

Feeding is associated with an increase in the concentration of insulin in the circulation (Millward et al., 1974; Sinha & Caro, 1985). This would enhance anabolic activities such as triacylglycerol synthesis, but decrease catabolic activities such as β-oxidation of fatty acids. Accordingly, GPAT activity is greater in refed animals than in fasted animals, suggesting an acute response to insulin. Hepatic mitochondrial GPAT increases approximately 2-fold after refeeding rats for 10 h following 48 h fasting (Borrebaek, 1975). GPAT activity in liver homogenates increases 1.4-1.6-fold after refeeding rats for 24 h following 48 h fasting (Aas & Daae, 1971). Similarly, 2.5 h after refeeding, hepatic mitochondrial and microsomal GPAT activities are increased to approximately 112% and 121%, respectively, of the activities in 24 h fasted rats (Sugden & Munday, 1983). Mammary mitochondrial and microsomal GPAT activities also increase to 155% and 143%, respectively, in response to the same treatment (Sugden & Munday, 1983). The GPAT activity in adipose tissue of rats subjected to 96 h

fasting undergoes a 2.7-fold increase after refeeding for 48 h (Angel & Roncari, 1967); similarly, after fasting for 72 h and refeeding for 48 h, the increase is 2.7-fold (Jamdar & Osborne, 1982). Under these conditions, mitochondrial GPAT increased to 1.15-fold (non-significant), but microsomal GPAT increased 2.90-fold (Jamdar & Osborne, 1982).

1.3.4.2.2 High fat, high carbohydrate and ethanol diets

GPAT also responds to other dietary modifications which suggests a regulatory role. Variable effects on GPAT activity of feeding high fat diets, either corn oil or tallow, have been reported. These diets have variously been reported to cause large increases (Fallon & Kemp, 1968; Lawson et al., 1981a, 1981b) or decreases (Aas & Daae, 1971; Dodds et al., 1976a; Glenny et al., 1978; Iritani & Fukuda, 1980; Kako & Peckett, 1981; Rao et al., 1971b) or to have no effect (Askew et al., 1975; Kako & Peckett, 1981; Lawson et al., 1981a, 1981b; Steffen et al., 1978; Weigand et al., 1973) on GPAT activities in liver and adipose tissue. Differences in response may be attributable to differences in the fat content and composition of the diet fed (Weigand et al., 1973). Lack of common controls and differences in the duration of the treatments also makes comparison of the effects on GPAT difficult.

Hepatic microsomal and adipose tissue GPAT activities increase up to 1.75-fold in response to high carbohydrate diets (Dodds et al., 1976a; Fallon & Kemp, 1968; Lamb & Fallon, 1974b; Lawson et al., 1981b; Waddel & Fallon, 1973) and hepatic microsomal DHAPAT activity responds to these diets in a similar manner (Fallon & Kemp, 1968; Lamb

& Fallon, 1976). Ethanol administration leads to the accumulation of hepatic triacylglycerol (Abrams & Cooper, 1976; Cunningham et al., 1982; Goheen et al., 1983; Hawkins & Kalant, 1972; Lieber, 1974; Zakim, 1965) and increases in hepatic and adipose tissue GPAT activity by up to 1.3-fold have been observed under these conditions (Joly et al., 1970, 1973; Pritchard et al., 1977). Although the response of GPAT to the above dietary modifications appears complex and requires further research, it seems likely that the effects of these diets on GPAT could be mediated by the action of hormones.

1.3.4.3 Pharmacological effectors of GPAT activity

GPAT activity has also been observed to respond to a number of drugs. Total hepatic GPAT activity increases approximately 1.70-fold in animals treated with phenobarbital (Goldberg et al., 1981a, 1981b; Higgins & Barrnett, 1972), but the specific activity may decline due the proliferation of other microsomal proteins (Goldberg et al., 1980, 1981a, 1981b, 1981c; Higgins & Barrnett, 1972; Joly et al., 1973). Therefore, this response of GPAT appears to be due to an increased synthesis of GPAT enzyme protein and not enhancement of enzyme activity. Consistent with the increases in liver microsomal GPAT, phenobarbital treatment increases the rate of hepatic triacylglycerol synthesis and secretion (Goldberg et al., 1980, 1981a, 1981b).

Hypolipidaemic drugs, which reduce hepatic glyceride formation in vivo (Aoyama et al., 1982; Kritchevsky, 1974), inhibit GPAT activity directly in vitro (Bowley et al., 1973; Brindley, 1978; Brindley & Bowley, 1975; Brindley et al., 1977; Daae & Aas, 1973; Fallon et al., 1972; Laker & Mayes, 1976; Lamb & Fallon, 1972a). Despite some

reports that treatment of rats *in vivo* with clofibrate (a hypolipidemic drug) can increase GPAT activity (Burke & Hajra, 1980; Daae & Aas, 1973; Das *et al.*, 1983), it is possible that the effects of these drugs on lipid metabolism could be mediated largely by an effect on GPAT. The ability of these drugs to modify GPAT activity both *in vitro* and *in vivo* suggests that GPAT activity could be modified by other molecules, perhaps second messengers of hormones, *in vivo*.

1.3.4.4 *Control by glucagon, catecholamines and insulin*

It is generally accepted that glucagon, adrenaline, noradrenaline and insulin influence lipid metabolism and the partitioning of nutrients within the animal. Effects of glucagon and β-effects of catecholamines are mediated by cAMP (Ganong, 1983; Lehninger, 1975) and various nucleotides have, at times, been proposed as mediators of the action of insulin (Czech, 1977). Therefore, there has been interest in the effects of nucleotides and cyclic nucleotides on GPAT activity. Cytidine nucleotides markedly decrease incorporation of *sn*-[^{14}C]glycerol 3-phosphate in homogenates and subcellular fractions of rat brain (Possmayer, 1974; Possmayer & Mudd, 1971; Possmayer *et al.*, 1973). Reports indicate that CTP stimulates rat liver mitochondrial GPAT (Zborowski & Wojtczak, 1969), but inhibits rat liver microsomal GPAT (Fallon & Lamb, 1968). However, even with unphysiological concentrations (Van den Bosch, 1974), it is difficult to demonstrate consistent effects of cytidine nucleotides on hepatic GPAT activity (Davidson & Stanacev, 1971; Dils & Clark, 1962; Possmayer, 1974; Possmayer *et al.*, 1973).

Cyclic AMP and its derivatives cause a coordinated change in fat

metabolism in the liver, directing fatty acids towards β-oxidation and ketone body production, and away from esterification and triacylglycerol synthesis (Akhtar & Bloxham, 1970; Heimberg et al., 1969; Homcy & Margolis, 1973; Klausner et al., 1978; Soler-Argilaga et al., 1978a, 1978b). When rat livers are perfused with dibutyryl cAMP it seems that the inhibition of hepatic microsomal triacylglycerol synthesis occurs via GPAT inhibition (Soler-Argilaga et al., 1978a). Furthermore, studies with hepatocytes show that mitochondrial and microsomal GPAT activities are decreased to 85% and 91%, respectively, by dibutyryl cAMP (Sugden et al., 1980). However, dibutyryl cAMP does not seem to affect triacylglycerol synthesis in adipose tissue (Matsuoka et al., 1974). Regulation of several enzymes of the glycerolipid pathway, including microsomal GPAT in liver and adipose tissue, by phosphorylation-dephosphorylation via cAMP-dependent protein kinase and protein phosphatases has been proposed (Berglund et al., 1982; Haagsman et al., 1982; Nimmo, 1980; Nimmo & Houston, 1978), but it is unlikely that microsomal GPAT is regulated in this way (Nimmo & Nimmo, 1984; Rider & Saggerson, 1983).

A number of studies examining the effect of various hormones on GPAT suggest that its activity can be regulated in a dynamic manner compatible with changes in fat metabolism induced by these hormones in vivo. Glucagon depresses the production of lipid by 50% in intact liver and hepatocytes (Beynen & Geelen, 1981; Beynen et al., 1980, 1981; Geelen et al., 1978; Heimberg et al., 1969; Soler-Argilaga et al., 1978b; Williamson et al., 1980; Witters & Trasko, 1979) and GPAT activities in hepatocytes and adipocytes are reduced to 62-89% by glucagon (Lamb et al., 1982; Rider & Saggerson, 1983; Sugden et al.,

1980). GPAT activity in the perfused liver seems to be unaffected (Saggerson et al., 1981). Some of the effects of cAMP and glucagon on GPAT may arise from their effects on calcium metabolism as MgATP-dependent uptake of calcium by rat liver microsomes reduces GPAT activity to approximately 66% of control (Soler-Argilaga et al., 1977, 1978b).

The β-effects of adrenaline are also mediated by cAMP and GPAT activity in isolated adipocytes is depressed to 50-80% by adrenaline (Saggerson et al., 1979a; Sooranna & Saggerson, 1976a, 1978a, 1979). This effect is evident even with adipocytes from fasted rats (Sooranna & Saggerson, 1979). Noradrenaline treatment also decreases adipocyte GPAT activity to 30-50% (Rider & Saggerson, 1983). The effects of adrenaline and noradrenaline on GPAT are abolished by propranolol (Rider & Saggerson, 1983; Sooranna & Saggerson, 1978a) which implies that β-adrenergic receptors may mediate the action of adrenaline on GPAT (Sooranna & Saggerson, 1978a). The study of Rider & Saggerson (1983) suggests that the effect of noradrenaline on microsomal GPAT may be mediated by a mechanism involving non-covalent binding of a regulatory ligand to the GPAT.

Insulin stimulates synthesis of lipids in short-term incubations of rat hepatocytes (Beynen & Geelen, 1981; Beynen et al., 1980; Geelen et al., 1978) and adipocytes (Bagul et al., 1972; Laursen et al., 1981; Sooranna & Saggerson, 1975) and perfusion of rat livers with insulin stimulates synthesis and secretion of triglycerides (Beynen & Geelen, 1981; Beynen et al., 1981; Poledne & Mayes, 1970; Topping & Mayes, 1970, 1972, 1976). Total GPAT activity is increased (to 124%)

in livers perfused for 30 min with an insulin concentration similar to that in the blood of carbohydrate-fed rats (Bates et al., 1977). This treatment increases both mitochondrial and microsomal GPAT activity by 1.3-fold and 1.1-fold, respectively (Bates et al., 1977). Adipocyte GPAT activity is also stimulated by insulin although this appears to be slightly dependent on the assay conditions (Bagul et al., 1972; Evans & Denton, 1977; Sooranna & Saggerson, 1976a, 1976b). Incubation with insulin alone increases GPAT activity in adipocytes significantly 1.2-1.3-fold (Sooranna & Saggerson, 1976a). Insulin also abolishes the inhibition caused by adrenaline (Sooranna & Saggerson, 1978a). In addition, the stimulatory effects of refeeding after fasting on GPAT, as discussed above (1.3.4.2.1), also suggest that insulin causes increases in the activity of mitochondrial and microsomal GPAT.

Diabetes and induced insulin deficiency reduce the production of hepatic triacylglycerols and phospholipids (Chang et al., 1971; Heimberg et al., 1967; Woodside & Heimberg, 1976), but enhance fatty acid uptake by mitochondria (Amatruda, et al., 1978). Esterification of fatty acids is reduced in adipose tissue from diabetic humans (Galton et al., 1971). Therefore, it seems reasonable to expect that induction of the diabetic condition would cause a reduction in hepatic GPAT activities. However, while acute administration of anti-insulin serum and longer term streptozotocin-induced diabetes both decrease mitochondrial GPAT activity, they affect microsomal activity only slightly (Bates & Saggerson, 1977, 1979; Saggerson et al., 1979b; Whiting et al., 1977).

A number of studies therefore provide evidence that GPAT activity

can be regulated by catecholamines, insulin and possibly glucagon. However, the exact mechanisms by which these effects are mediated are still unclear and will require further study.

1.3.4.5 Control by other hormones

Other hormones such as thyroid hormones, vasopressin and glucocorticoids will affect fat metabolism. Studies have been conducted to determine whether any of the responses to these hormones can be explained by effects on GPAT activity. (a) Thyroid hormones: Alterations in thyroid status appear to bring about changes in serum triacylglycerol concentrations (Keyes & Heimberg, 1979). Depending on the procedure for inducing hypothyroidism, this state has been found to increase (Keyes & Heimberg, 1979), decrease (Dory et al., 1981), or have no effect (Laker & Mayes, 1981; Roncari & Murthy, 1975) on liver lipid output, but it decreases esterification by about half in adipose tissue (Roncari & Murthy, 1975). Conversely, treatment of animals with triiodothyronine (T_3) appears to enhance or inhibit esterifcation depending on the tissue and the study (Diamant et al., 1972; Keyes & Heimberg, 1979; Laker & Mayes, 1981; Roncari & Murthy, 1975) while thyrotrophin (TSH) appears to stimulate GPAT in the thyroid (Schneider, 1972). Clearly there is a need for more research under standardised conditions to examine and elucidate the effects of thyroid status on GPAT activities. (b) Vasopressin: Vasopressin stimulates GPAT activity in hepatocytes (Pollard & Brindley, 1984; Sugden et al., 1980; Williamson et al., 1980), but whether this hormone regulates triacylglycerol synthesis by an increase in GPAT activity is unclear because fatty acid esterification is stimulated much more than GPAT activity (Williamson et al., 1980). (c) Glucocorticoids: The role of

adrenal glucocorticoids in lipid metabolism is poorly understood (Kirk et al., 1976; Klausner & Heimberg, 1967), but they have wideranging effects on protein and carbohydrate metabolism (Ganong, 1983) and so a number of studies have sought to determine their effect on lipid metabolism. Adrenalectomy of rats depresses hepatic release of triacylglycerol (Kirk et al., 1976; Klausner & Heimberg, 1967), but while liver mitochondrial and microsomal GPAT activities are almost halved in fasted adrenalectomised rats, they are relatively unaffected in fed adrenalectomised animals (Bates & Saggerson, 1979, 1981b). Corticotrophin treatment of adipocytes halves GPAT activity (Rider & Saggerson, 1983), but its administration to rats appears to increase hepatic GPAT activity (Lawson et al., 1981b). It might reasonably be expected, therefore, that glucocorticoid treatment would produce similar effects. However, the effect of cortisol on hepatic triacylglycerol output is variable (Klausner & Heimberg, 1967) and corticosterone has little effect on hepatocyte GPAT activities (Lawson et al., 1982b; Pollard & Brindley, 1984).

1.4 GLYCEROLPHOSPHATE ACYLTRANSFERASE - CHARACTERISTICS AND PROPERTIES

1.4.1 Distribution of GPAT between mitochondrial and microsomal fractions

Early studies conflicted over whether GPAT activity is associated with only the microsomes (Davidson & Stanacev, 1972; Eibl et al., 1969; Possmayer et al., 1973) or also with the mitochondria (Roncari & Hollenberg, 1967; Stoffel & Schiefer, 1968; Zborowski & Wojtczak, 1969). This conflict arose because, initially, glucose 6-phosphatase was regarded as being solely microsomal whereas more recent studies have shown that glucose 6-phosphatase activity is also intimately associated with the outer membrane of mitochondria (Brunner & Bygrave, 1969; Davidson & Stanacev, 1972; Dallner et al., 1966; De Duve et al., 1955, 1962; Eibl et al., 1969; Possmayer et al., 1973; Sottocasa et al., 1967; Wilgram & Kennedy, 1963). It is now accepted that GPAT is located in both the mitochondrial and microsomal fractions of a number of different tissues (Bjerve et al., 1974; Bremer et al., 1976; Carroll, et al., 1982; Daae, 1973; Daae & Bremer, 1970; Davidson & Stanacev, 1974; Eibl et al., 1969; Hendry & Possmayer, 1974; Liu & Kako, 1974; Lloyd-Davies & Brindley, 1975; Monroy et al., 1972, 1973; Roncari & Hollenberg, 1967; Saggerson et al., 1980; Sanchez et al., 1973; Sarzala et al., 1970; Shepard & Hubscher, 1969; Stoffel & Schiefer, 1968; Zborowski & Wojtczak, 1969). It is probable that early conclusions arose, in part, from the use of chloroform-methanol extractions which do not recover significant amounts of lysophosphatidic acid, the main product of the mitochondrial assay (Bjerve et al., 1974; Bremer et al., 1976; Sanchez et al.,

1973). GPAT has also been reported in plasma membranes and cytosol, but contamination by microsomes cannot be ruled out (Rao et al., 1969; Smith et al., 1982; Stein et al., 1968). Not surprisingly, GPAT activity is also found in rapidly sedimenting endoplasmic reticulum, a complex between endoplasmic reticulum and mitochondria (Meier et al., 1981; Stuhne-Sekalec & Stanacev, 1982).

There is general agreement that rat liver has approximately equal proportions of mitochondrial and microsomal GPAT (Bates & Saggerson, 1977, 1979; Bremer et al., 1976; Daae & Bremer, 1970; Haldar et al., 1979; Klutymans & Raju, 1974; Lloyd-Davies & Brindley, 1975; Saggerson et al., 1980; Sanchez et al., 1973; Sarzala et al., 1970; Shepard & Hubscher, 1969). However, microsomal GPAT activity appears to predominate in lung (Garcia et al., 1976; Hendry & Possmayer, 1974), heart (Kako & Peckett, 1981), mammary gland (Kuhn, 1967a), intestinal epithelium (Bickerstaffe & Annison, 1969; Brindley & Hubscher, 1965; Hulsmann & Kurpershoek-Davidov, 1976), tumour cells (Haldar et al., 1979; Tso et al., 1976), cultured cell lines (Bell & Coleman, 1980; Coleman & Bell, 1980; Coleman et al., 1978; Grimaldi et al., 1978; Stern & Pullman, 1978), and adipocytes (Bagul, 1972; Coleman & Bell, 1980; Coleman et al., 1978; Grimaldi et al., 1978; Jamdar & Fallon, 1973; Jamdar & Osborne, 1982; Saggerson et al., 1980; Schlossman & Bell, 1976a, 1976b). Mitochondria contribute 10-20% or less of the cellular GPAT in adipocytes (see above references) and this suggests that the microsomal GPAT has a major role in triacylglycerol synthesis (Bell & Coleman, 1980; Brindley et al., 1979c). Furthermore, during differentiation of fibroblasts into adipocytes, the microsomal GPAT activity increases 30-80 fold, but

there is little change in the mitochondrial GPAT activity (Coleman & Bell, 1980; Grimaldi et al., 1978; Kuri-Harcuch & Green, 1977),

1.4.2 Location of GPAT within mitochondrial and microsomal fractions

Mitochondrial GPAT is located on the inner surface (Carroll, et al., 1982; Nimmo, 1979b) of the outer mitochondrial membrane (Daae, 1972a; Daae & Bremer, 1970; Monroy et al., 1972; Shepard & Hubscher, 1969; Stoffel & Schiefer, 1968; Zborowski & Wojtczak, 1969). Acyl-CoA synthetase, which catalyses production of acyl-CoAs from free fatty acids, is also located in the outer membrane of mitochondria and in the microsomes (Aas, 1971; Nimmo, 1979b).

Microsomal GPAT appears to be located on the cytosolic side of the microsomes (Ballas & Bell, 1980; Bell et al., 1981; Coleman & Bell, 1978; Jamdar, 1977, 1979) although some cytochemical studies (Benes et al., 1972; Higgins, 1976) suggest a luminal location for GPAT. However, controls demonstrating microsomal integrity were not performed and the 75 μM palmitoyl-CoA, without albumin has been demonstrated to disrupt microsomal vesicles (Polokoff & Bell, 1978). There is uncertainty as to whether there is greater GPAT activity in the rough or smooth endoplasmic reticulum, with age, ethanol administration and phenobarbital treatment being additional complications (Brindley & Hubscher, 1965; Goldsmith, 1981; Higgins, 1976; Higgins & Barrnett, 1972; Joly et al., 1973; Nachbaur et al., 1971; Yamashita et al., 1973b). Similar amounts of lysophosphatidic acid acyltransferase and other transferases are found in the two types of microsomes (Yamashita et al., 1973b). There have been suggestions that the GPAT may be physically separated from other enzymes involved later in the pathway

leading to the production of triacylglycerol (Tzur & Shapiro, 1976), but other work (Ballas & Bell, 1980, 1981; Bell et al., 1981) provides strong evidence that the active sites of all these enzymes face the cytoplasm. The microsomal location of most enzymes involved with triacylglycerol and phospholipid synthesis (Bell & Coleman, 1980) and the fact that adipose tissue GPAT is predominantly microsomal (see 1.4.1) suggests a key role for the microsomal GPAT. The mitochondrial fraction lacks lysophosphatidic acid acyltransferse (Eibl et al., 1969; Saggerson et al., 1980) as well as enzymes for the synthesis of most glycerolipids (Bell & Coleman, 1980) and so the role of the mitochondrial GPAT is unclear.

1.4.3 Properties of GPAT

Glycerol 3-phosphate is acylated to form lysophosphatidic acid and this is subsequently acylated to form phosphatidic acid (Bell & Coleman, 1980). Initially it was suggested that GPAT catalysed both acylation steps (Kuhn & Lynen, 1965), but it has subsequently been shown that two separate enzymic steps are involved in the formation of phosphatidic acid (Lands & Hart, 1965). The two enzyme activities involved in glycerol 3-phosphate acylation are now clearly distinguished in a number of animal tissues and in yeast (Caffrey & Kinsella, 1976, 1977a, 1978a, 1978b; Gross & Kinsella, 1974; Ishinaga et al., 1976; Miki et al., 1977; Monroy et al., 1973; Morikawa & Yamashita, 1978; Yamashita & Numa, 1972; Yamashita et al., 1972, 1973a, 1975). Further evidence is provided by the observation that the main product of esterification by the mitochondrial fraction is lysophosphatidic acid (Bremer et al., 1976; Daae, 1972a, 1972b; Grosjean & Haldar, 1982; Haldar et al., 1979; Kako & Peckett, 1981;

Liu & Kako, 1974; Monroy et al., 1973; Nachbaur et al., 1971; Sanchez et al., 1973) whereas with the microsomal fraction, which unlike the mitochondrial fraction contains lysophosphatidic acyltransferase (Eibl et al., 1969; Gross & Kinsella, 1974; Haldar et al., 1979; Okuyama et al., 1971; Saggerson et al., 1980), the main product is phosphatidic acid (Abou-Issa & Cleland, 1969; Bremer et al., 1976; Daae, 1972a, 1972b; Dodds et al., 1976a; Fallon et al., 1975; Gross & Kinsella, 1974; Hendry & Possmayer, 1974; Husbands & Lands, 1970; Jamdar & Fallon, 1973; Jamdar et al., 1976; Kuhn, 1967a; Kornberg & Pricer, 1953; Lamb et al., 1980; Lands & Hart, 1964; Lloyd-Davies & Brindley, 1975; Nachbaur et al., 1971; Okuma et al., 1973; Possmayer et al., 1969; Roncari & Hollenberg, 1967; Stansly, 1955; Stoffel et al., 1967; Tamai & Lands, 1974). Mitochondrial and microsomal cross-contamination may explain reports that lysophosphatidic acid is the main product of microsomal GPAT assays (Fallon & Lamb, 1968; Haldar et al., 1979; Lamb & Fallon, 1970, 1972a) and other reports that phosphatidic acid is the main product of mitochondrial GPAT assays (Bjerve et al., 1976; Haldar et al., 1979; Monroy et al., 1972; Zborowski & Wojtczak, 1969).

Despite an early report which suggested that GPAT acylates the 1-position of glycerol 3-phosphate when palmitoyl-CoA is the substrate but the 2-position when oleoyl-CoA is the substrate (Lamb & Fallon, 1970), it now seems to be generally accepted that 1-acyl-sn-glycerol 3-phosphate is the product of glycerol 3-phosphate acylation in microsomes in a variety of tissues and with a variety of substrates (Haldar et al., 1979; Okuyama et al., 1971; Saggerson et al., 1979a; Tamai, 1972; Tamai & Lands, 1974; Yamashita & Numa, 1972, 1981; Yamashita et al., 1972;

Zaror-Behrens & Kako, 1976). Moreover, the lysophosphatidic acid produced by mitochondrial GPAT is also exclusively 1-acyl-sn-glycerol 3-phosphate (Daae, 1972a, 1973; Haldar et al., 1979; Monroy et al., 1972, 1973; Yamada & Okuyama, 1978) and 1-acyl-sn-glycerol 3-phosphate is the product of E. coli GPAT (Ishinaga et al., 1976).

Most naturally-occurring glycerolipids are characterised by an asymmetrical distribution of their acyl constituents between the 1- and 2-position of the glycerol moiety, i.e. saturated fatty acids occur predominantly at the 1-position and unsaturated acyl groups are usually found at the 2-position (Lands, 1965; Slakey & Lands, 1968; Tove, 1961; Van Golde & Van den Bergh, 1977). Some in vitro systems reproduce this non-random acylation (Akesson, 1970; Akesson et al., 1970a, 1970b; Barden & Cleland, 1969; Elovson et al., 1969; Hill et al., 1968; Possmayer et al., 1969; Raju & Reiser, 1970; Winand et al., 1973). Despite suggestions that the acylation of glycerol 3-phosphate, by GPAT, with saturated and unsaturated fatty acids was random and not fatty acid-specific (Abou-Issa & Cleland, 1969; Barden & Cleland, 1969; Bjerve et al., 1976; Hajra, 1968a; Hendry & Possmayer, 1974; Hill & Lands, 1968; Hill et al., 1968; Husbands & Lands, 1970; Lands & Hart, 1964; Okuyama & Lands, 1972; Sanchez de Jimenez & Cleland, 1969; Snyder & Malone, 1975; Stein et al., 1963; Stern & Pullman, 1978; Stoffel et al., 1967; Yamada & Okuyama, 1978), rat liver microsomes incubated with sn-glycerol 3-phosphate and mixtures of saturated and unsaturated fatty acids give rise to predominantly 1-saturated, 2-unsaturated phosphatidic acid (Baker & Thomson, 1972; Lamb & Fallon, 1970; Okuyama & Lands, 1972; Possmayer et al., 1969; Tamai & Lands, 1974) suggesting that GPAT has a

preference for saturated fatty acids. Rat adipose tissue homogenates exhibit similar fatty acid positional specificity (Christie et al., 1976). Microsomal GPAT from a number of other sources appears to have specificities for either saturated or unsaturated fatty acyl-CoAs in keeping with the in vivo fatty acid positional specificities of the lipids found in these tissues (Bickerstaffe & Annison, 1969; Brindley & Hubscher, 1966; Cooper & Grigor, 1978, 1980; Gross & Kinsella, 1974; Husbands & Reiser, 1966; Kinsella & Gross, 1973; Kuhn, 1967a; Marshall & Knudsen, 1977a, 1977b; Raju & Six, 1975; Stokes & Tove, 1975; Stokes et al., 1975; Tanioka et al., 1974). A partially purified rat liver microsomal GPAT catalyses the acylation at the 1-position preferentially with saturated substrates to form 1-acyl-sn-glycerol 3-phosphate, but lysophosphatidic acid acyltransferase uses unsaturated acyl-CoA substrates preferentially (Yamashita et al., 1973a, 1975). Similarly, mitochondrial GPAT has a distinct preference for the saturated substrate palmitoyl-CoA (Bjerve et al., 1976; Bramley & Grigor, 1982; Bremer et al., 1976; Daae, 1972a, 1973; Haldar & Pullman, 1975; Haldar et al., 1979; Kelker & Pullman, 1979; Monroy et al., 1972, 1973; Saggerson et al., 1979a; Stern & Pullman, 1978; Yamada & Okuyama, 1978). Overall, it appears that both mitochondrial and microsomal GPAT would contribute to the patterns of fatty acids found in the naturally occuring glycerolipids.

There appears to be considerable difference in the properties of the mitochondrial and microsomal GPAT which leads to the conclusion that they are isoenzymes (Monroy et al., 1972). The microsomal isoenzyme is almost totally and irreversibly inhibited by reagents which react with thiol groups, such as NEM, DTNB, and iodoacetamide,

while the mitochondrial isoenzyme, with the possible exception of the heart enzyme (Liu & Kako, 1974), is barely affected (Abou-Issa & Cleland, 1969; Bates & Saggerson, 1979; Bramley & Grigor, 1982; Carroll, et al., 1982; Fallon & Lamb, 1968; Haldar et al., 1979; Klutymans & Raju, 1974; Kuhn & Lynen, 1965; Lands & Hart, 1965; Monroy et al., 1972; Nimmo, 1979a; Saggerson et al., 1979a; Stern & Pullman, 1978; Yamada & Okuyama, 1978). Conversely, dithiothreitol stimulates the microsomal GPAT (Abou-Issa & Cleland, 1969; Fallon & Lamb, 1968; Monroy et al., 1972), but can inhibit the mitochondrial isoenzyme (Monroy et al., 1972). Pre-treatment with low concentrations of acyl-CoA protects the microsomal enzyme against inactivation by thiol reagents (Husbands & Lands, 1970; Kuhn & Lynen, 1965; Nimmo, 1979a). These properties were used (Husbands & Lands, 1968, 1970) to show that pigeon liver microsomes contain 0.2-0.3 nmol of GPAT per mg of microsomal protein.

The mitochondrial GPAT is insensitive to bromelain, papain, pronase and trypsin, all of which destroy the microsomal enzyme activity (Carroll, et al., 1982; Haldar et al., 1980). The resistance of the mitochondrial GPAT to these proteolytic enzymes is probably due to its location on the inner surface of the outer membrane of mitochondria (Carroll, et al., 1982; Nimmo, 1979b). The microsomal GPAT faces the cytosol (Ballas & Bell, 1980, 1981; Bell et al., 1981) and is much more thermolabile than the mitochondrial GPAT (Carroll, et al., 1982; Coleman & Bell, 1980; Coleman & Haynes, 1983; Nimmo, 1979a; Schlossman & Bell, 1976b). The microsomal GPAT is inhibited by acetone (Haldar et al., 1979) while the mitochondrial enzyme is stimulated by acetone (Grosjean & Haldar, 1982; Haldar et al., 1979).

Both enzymes are reported to have a broad pH optimum over the range pH 7.0-8.0 (Askew et al., 1971; Coleman & Bell, 1980; Coleman & Haynes, 1983; Daniel & Rubinstein, 1968; Garcia et al., 1976; Gross & Kinsella, 1974; Klutymans & Raju, 1974; Lamb et al., 1980; Nimmo, 1979a; Okuma et al., 1973; Pynadath & Kumar, 1964; Stansly, 1955; Tamai & Lands, 1974; Yamashita & Numa, 1972), although a pH optimum of 6.5 for the microsomal GPAT has been reported (Fallon & Lamb, 1968).

The microsomal enzyme is inhibited by $MgCl_2$ (Fallon & Lamb, 1968; Monroy et al., 1972, 1973) whereas the mitochondrial isoenzyme is stimulated or unaffected by $MgCl_2$ depending on the assay conditions (Monroy et al., 1972, 1973). Responses of mitochondrial and microsomal GPAT to other cations are also reported but they do not appear to differentiate the two isoenzymes (Fallon & Lamb, 1968; Jamdar & Fallon, 1973; Lamb et al., 1980; Monroy et al., 1973; Yamashita & Numa, 1972). Polymyxin B, an antibiotic known to alter bacterial membrane structure, stimulates the mitochondrial GPAT, but inhibits the microsomal isoenzyme (Carroll, et al., 1982; Grosjean & Haldar, 1982). Rat liver microsomal GPAT is inactivated both by low concentrations of deoxycholate and osmotic shock (Higgins, 1976), but pigeon liver microsomal GPAT is active in low concentrations of deoxycholate (Husbands & Lands, 1970).

Table 1.4: Hepatic mitochondrial GPAT - K_m values for glycerol 3-phosphate.

K_m value (mM glycerol 3-phosphate)	Reference
0.11, 0.17	Declercq et al., 1982;
0.18	Nimmo, 1979a;
0.24	Declercq et al., 1982;
0.30	Van Tol, 1974;
0.31	Borrebaek, 1975;
0.36	Declercq et al., 1982;
0.50	Van Tol, 1974;
0.90	Stern & Pullman, 1978;
1.00	Monroy et al., 1972;
	Daae & Bremer, 1970.

K_m for mitochondrial GPAT in Ehrlich tumour cells = 0.38 mM (Haldar et al., 1979).

Table 1.5: Microsomal GPAT - K_m values for glycerol 3-phosphate.

Tissue	K_m value[a]	Reference
LIVER		
	0.05[b]	Husbands & Lands, 1970;
	0.14	Schlossman & Bell, 1977;
	0.16	Coleman & Haynes, 1983;
	0.17-0.18	Declercq et al., 1982;
	0.20	Yamashita & Numa, 1972; Stern & Pullman, 1978;
	0.33	Nimmo, 1979a;
	0.50	Yamashita & Numa, 1972; Abou-Issa & Cleland, 1969;
	0.52-0.54	Declercq et al., 1982;
	0.67	Lamb & Fallon, 1970;
	1.00-1.20	Tamai & Lands, 1974;
	1.70	Van Tol, 1974;
	6.70	Fallon & Lamb, 1968;
ADIPOSE TISSUE		
	0.008	Schlossman & Bell, 1976b;
	0.03	Coleman & Bell, 1980;
	0.13	Jamdar & Fallon, 1973;
	0.20	Nimmo & Houston, 1978;
	0.28	Rider & Saggerson, 1983;
	0.60	Dodds et al., 1976b;
	0.86	Angel & Roncari, 1967;
MAMMARY GLAND		
	0.11	Tanioka et al., 1974;
	0.26	Gross & Kinsella, 1974; Kinsella & Gross, 1973;
	2.70	Kuhn, 1967a;
LUNG		
	0.10	Hendry & Possmayer, 1974;
	2.20	Garcia et al., 1976;
BRAIN		
	0.20	Possmayer & Mudd, 1971;
	0.40	Sannchez de Jimenez & Cleland, 1969;
	0.80	Martensson & Kanfer, 1968;
INTESTINE		
	0.20	Hulsmann & Kurpershoek-Davidov, 1976;
EHRLICH TUMOUR CELLS		
	0.21	Haldar et al., 1979;
PLATELETS		
	0.48	Okuma et al., 1973;

[a](mM glycerol 3-phosphate) [b]pigeon liver enzyme.

K_m values reported for glycerol 3-phosphate for the mitochondrial and microsomal GPAT are given in Tables 1.4 and 1.5. It has been suggested (Bell & Coleman, 1980; Brindley et al., 1979a; Nimmo, 1979a) that the two isoenzymes have different K_ms for glycerol 3-phosphate, but the overlap in reported values makes such a claim questionable (see Table 1.4 and 1.5). A meaningful apparent K_m for the acyl-CoA substrates cannot be calculated as higher concentrations of the acyl-CoA inhibit GPAT esterification (Angel & Roncari, 1967 Askew et al., 1971; Bates & Saggerson, 1979; Husbands & Lands, 1970; Jamdar, 1977; Jamdar, 1979; Kinsella & Gross, 1973; Kuhn, 1967a; Kuhn & Lynen, 1965; Monroy et al., 1972; Okuma et al., 1973; Rider & Saggerson, 1983; Van Tol, 1974; Zahler & Cleland, 1969; Zahler et al., 1967), probably through disruption of membranes by micelles which form at higher acyl-CoA concentrations (Barden & Cleland, 1969a; Dorsey & Porter, 1968; Husbands & Lands, 1970; Powell et al., 1981; Taketa & Pogell, 1966; Zahler & Cleland, 1969; Zahler et al., 1968). Other enzymes are also inhibited by high acyl-CoA concentrations (Brandes & Shapiro, 1967; Dorsey & Porter, 1968; Fujiwara et al., 1981; Halperin et al., 1972, 1975; Pande, 1973; Srere, 1965; Taketa & Pogell, 1966; Tubbs & Garland, 1964; Wieland & Weiss, 1963). The inhibition caused by high acyl-CoA concentrations is relieved by the addition of bovine serum albumin (BSA) (Gross & Kinsella, 1974; Husbands & Lands, 1970; Jamdar, 1979; Taketa & Pogell, 1966) and so BSA is routinely included in GPAT assay mixtures (Brandes et al., 1963; Daniel & Rubinstein, 1968; Dils & Clark, 1962; Fallon & Kemp, 1968; Jamdar, 1979; Jamdar & Fallon, 1973; Lamb & Fallón, 1970; Lloyd-Davies & Brindley, 1975; Monroy et al., 1972; Prottey & Hawthorne, 1967; Tzur & Shapiro, 1964; Weigand et al., 1973). Other

proteins such as β-lipoprotein, γ-globulin, β-globulin and fibrinogen, but with the exception of NEM-treated microsomal protein, are less effective (Fallon & Lamb, 1968; Husbands & Lands, 1970). BSA in the reaction mixture may bind the acyl-CoA, making it more soluble in aqueous solution and may reduce the effective concentration of the thioester below its critical micelle concentration or prevent micellar formation (Husbands & Lands, 1970; Tove et al., 1980). However, the role of serum albumin in intracellular lipid metabolism seems doubtful (Tove et al., 1980). BSA also enhances the GPAT reaction by maintaining the acyl-CoA concentration through inhibition of the acyl-CoA hydrolase (Brandes et al., 1963; Daniel & Rubinstein, 1968; Jamdar, 1979; Lamb & Fallon, 1970; Lloyd-Davies & Brindley, 1975) which is located mainly in the microsomal fraction (Berge & Farstad, 1979; Berge et al., 1981; Daniel & Rubinstein, 1968; Hulsmann & Kurpershoek-Davidov, 1976; Jamdar, 1977; Lamb et al., 1973; Lands & Hart, 1965; Lands & Merkyl, 1963) and to a lesser extent in the mitochondrial fraction (Berge & Farstad, 1979; Berge et al., 1981). The hydrolase appears to be more active towards micellar than protein-bound substrate (Barden & Cleland, 1969; Lamb et al., 1973) and so a concentration of 2 mg BSA/ml causes approximately 90% inhibition of the hydrolase (Jamdar, 1979).

Mitochondrial and microsomal GPAT appear to differ in their response to BSA so that 6.0 mg BSA/ml and 1.75 mg BSA/ml, respectively, are reported to be optimum concentrations (Bates & Saggerson, 1977, 1979). However, a concentration as low as 0.4 mg BSA/ml has been used in a mitochondrial assay (Monroy et al., 1972) while a range of 1.8-16 mg BSA/ml has been used in the assay of microsomal GPAT (Brandes et

al., 1963; Fallon & Kemp, 1968; Jamdar & Fallon, 1973; Lamb & Fallon, 1970; Lloyd-Davies & Brindley, 1975; Tzur & Shapiro, 1964; Weigand et al., 1973). Concentrations of 5 mg BSA/ml or greater can markedly reduce the activity of microsomal GPAT (Yamada & Okuyama, 1978) while a concentration of 16 mg BSA/ml appears to reduce the pH optimum of the reaction (Brandes et al., 1963). Polyamines, which appear to act in a manner similar to BSA (Bates & Saggerson, 1981a; Jamdar, 1979), also stimulate the activity of mitochondrial (Bates & Saggerson, 1981a) and microsomal GPAT (Bates & Saggerson, 1981a; Jamdar, 1977, 1979) in vitro at physiological concentrations (Bates & Saggerson, 1981a; Bethel & Pegg, 1981; Tabor & Tabor, 1976), but only with concentrations of acyl-CoA substrate which are inhibitory. Spermidine, another polyamine, stimulates the activity of GPAT from E. coli (Vallari & Rock, 1982). Z-protein (Iritani et al., 1980; Mishkin & Turcotte, 1974a, 1974b; Mishkin et al., 1972, 1975; Trulzsch & Arias, 1981) and fatty acid binding proteins (Haq et al., 1982; Ketter et al., 1976; Ockner et al., 1979, 1982; Roncari & Mack, 1975; Smith & Hubscher, 1966; Wu-Rideout et al.,1976) which bind fatty acids and fatty acyl-CoAs, appear to have an action similar to that of polyamines and BSA and as more Z-protein is found in the fed liver it may have a role in the control of triacylglycerol synthesis (Iritani et al., 1980). Fatty acid binding proteins may regulate the flux of fatty acids between mitochondria and the endoplasmic reticulum (Brindley et al., 1979c).

1.4.4 GPAT purification

Mitochondrial GPAT from rat liver has been solubilised and partially purified 5 to 6-fold using a cholate extraction and an ammonium sulphate fractionation (Monroy et al., 1973). This GPAT preparation was devoid of lysophosphatidic acid acyltransferase activity as evidenced by the production of only 1-acyl-sn-glycerol 3-phosphate. The substrate and positional specificity of the partially purified enzyme was identical with that in intact mitochondria. The purified GPAT required the presence of added phospholipid for maximum activity, asolectin and phosphatidylserine being the most effective (Kelker & Pullman, 1979; Monroy et al., 1973). However, this purification is not great when compared to those obtained for soluble enzymes. Because of the membrane-bound nature of the mitochondrial GPAT (and microsomal GPAT), success at purification is limited as most available processes usually inactivate the enzyme. Hence this hampers the full characterisation of the enzyme.

The microsomal enzyme from rat liver has also been partially purified 3-4 fold in a procedure involving Triton X-100 solubilisation, gel filtration chromatography and sucrose density gradient centrifugation (Yamashita & Numa, 1972, 1981; Yamashita et al., 1972). Like the partially purified mitochondrial GPAT, the partially purified microsomal GPAT product was 1-acyl-sn-glycerol 3-phosphate as the lysophosphatidic acid acyltransferase was removed during the purification. This microsomal GPAT preparation also required the presence of phospholipid for maximum activity. The microsomal GPAT was stimulated by phosphatidylcholine and phosphatidylethanolamine but inhibited by phosphatidylserine and phosphatidylinositol. This

contrasts with the mitochondrial enzyme which was stimulated markedly by phosphatidylserine and only slightly by phosphatidylcholine and phosphatidylethanolamine (Kelker & Pullman, 1979; Monroy et al., 1973; Yamashita & Numa, 1972, 1981; Yamashita et al., 1972). The purification procedure for microsomal GPAT led to the enzyme expressing a greater preference for palmitoyl-CoA (ahead of stearoyl-CoA) as a substrate while activities toward unsaturated acyl-CoAs such as linoleyl-CoA and oleyl-CoA, normally reasonable substrates for microsomal GPAT, were almost lost during purification. The relative activity toward stearoyl-CoA was not lost during purification. The reason for this is uncertain. It is not known whether the purification procedure caused a change in the specificity of one microsomal GPAT or whether a second enzyme with a specificity for unsaturated acyl chains was lost during the purification (Van den Bosch, 1974). Another 4-fold purification of microsomal GPAT from rat and bovine liver has also been reported (Klutymans & Raju, 1974) involving extraction with phosphate buffer and gel filtration. This gave a preparation with better specific activities than those of the purification discussed above (Yamashita & Numa, 1972). However, the GPAT activity was not separated from the lysophosphatidic acid acyltransferase activity. Other attempts to purify the enzyme have been less successful (Husbands & Lands, 1970; Martensson & Kanfer, 1968). In contrast to the lack of success in purifying GPATs from mammals and birds, a soluble GPAT from chloroplasts (Joyard & Douce, 1977) has been purified 200 (spinach) to 1000-fold (pea) (Bertrams & Heinz, 1979, 1981). In E. coli, membrane-bound GPAT has been purified to apparent homogeneity (Green et al., 1981; Ishinaga et al., 1976; Kessels & Van Den Bosch, 1982; Kessels et al., 1982, 1984) with a molecular weight of 83 000 (Green et

al., 1981).

1.5 PURPOSE AND SCOPE OF THE INVESTIGATION

As discussed above, there is evidence that hepatic microsomal GPAT is important in the regulation of glycerolipid metabolism and that its activity is affected by hormones such as insulin and adrenaline. The purpose of this investigation was to examine the possibility of showing an insulin-dependent enhancement of hepatic microsomal GPAT activity and to provide further evidence to support a regulatory role for GPAT in glycerolipid biosynthesis. The identity and the appropriate purity of the microsomal preparation used as the GPAT source was established by assaying appropriate marker enzymes. The validation of a shortened method of GPAT assay was carried out and the specificity of the assay with respect to substrates and products was confirmed. Based upon methodology used to show insulin-dependent effects on key enzymes (Jarett & Seals, 1979b; Larner et al., 1979d), experiments were conducted in which low molecular weight fractions of muscle extracts, prepared from rats treated with insulin, were assayed with hepatic microsomal GPAT. The effect of these fractions on GPAT activity, relative to the appropriate saline controls, was examined to determine whether GPAT activity could be enhanced in an insulin-dependent manner. In addition, GPAT was assayed in the presence of material from crude hepatic plasma membrane fractions treated with insulin, in order to further examine the possibility of showing an insulin-dependent stimulation of hepatic microsomal GPAT.

CHAPTER TWO

MATERIALS

Chapter 2. MATERIALS

2.1 Reagents

The reagents used and the source of supply are given in Tables 2.1 and 2.2.

2.2 Instruments & Equipment

Unicam SP 1800 and Shimadzu UV-110 spectrophotometers were used. Centrifuges used were Sorvalls, models RC2-B, RC 5B, and SS-3. The ultracentrifuges were Beckman models L2-65B, L5-75 and a Sorvall OTD-75. The liquid scintillation counter used was a Beckman LS8000.

2.3 Statistical package

Data were analysed by Student's t test with the aid of the Minitab 82.1 Statistical Package on the Prime 750 mainframe computer of Massey University.

2.4 Animals

2.4.1 Rats

Sprague-Dawley Rats were obtained from the Small Animal Production Unit, Massey University, Palmerston North. They were housed at 25^{o}C with light between 6 a.m. and 6 p.m. They were fed ad libitum a pelleted diet containing buttermilk powder (200 g/kg), barley meal (400 g/kg), lupin meal (170 g/kg), maize meal (150 g/kg), lucerne meal (20 g/kg), plain salt (5g/kg), lime (10g/kg), dried blood (35 g/kg), TVL vitamin and mineral premix 202 (10 g/kg) with fresh water. In experiments where the rats were fasted for 48-72 hours, the food was removed at 9.00am. Animals were sacrificed between 8.45-10.00am on the

day of the experiment.

2.4.2 Chickens

One day old cockerels were obtained from a local hatchery and fed *ad libitum* a commercial cereal-based chicken grower diet as a dry mash (from Massey University Poultry Research Centre) with continual access to water. Infra-red heat was provided. They were killed by cervical dislocation.

Table 2.1: Reagents used and source of supply.

REAGENT	SOURCE
acetic acid	J.T.Baker Chemical Co, N.J., USA
ADP	Sigma Chemical Co, USA
Aluminoxide 90	E. Merck, Darmstadt, Germany
AMP	Sigma Chemical Co, USA
ATP	Sigma Chemical Co, USA
benzylamine	British Drug House, Poole, England
blue dextran	Sigma Chemical Co, USA
boric acid	Koch Light Colnbrook, Bucks, England
bovine serum albumin	Sigma Chemical Co, USA
calcium chloride	Riedel De Haen AG Seelze-Hanover
coomassie blue G-250	Sigma Chemical Co, USA
cysteine	Sigma Chemical Co, USA
cytochrome C	Sigma Chemical Co, USA
DEAE-Sephadex	Pharmacia Fine Chem. Uppsala, Sweden
decapeptide	Dr D. Harding, Dept. Chem & Biochem, Massey University
2,7-dichlorofluorosceine	Riedel De Haen AG Seelze-Hanover
disodium hydrogen phosphate	May & Baker Ltd, Dagenham, England
dry ice	N.Z. Industrial Gases, Palmerston Nth
EDTA	Ajax Chemicals, Sydney, Australia
filter papers	Whatman Ltd, England
formic acid - univar grade	Ajax Chemicals, Sydney, Australia
fructose 2,6-bisphosphate	Sigma Chemical Co, USA
sn-glycerol-3-phosphate	Serva Fein Biochemica Heidelberg
sn-glycerol 3-phosphate	Sigma Chemical Co, USA
[U-^{14}C]-L-glycerol-3-phosphate	Amersham Int. Ltd, Amersham, England
[^{14}C]-hexadecane	Amersham Int. Ltd, Amersham, England
hydrindantin	British Drug House, Poole, England
hydrochloric acid	J.T.Baker Chemical Co, N.J., USA
liquid air	Dept. Chem. & Biochem. Massey Univ.
liquid nitrogen	L.I.A., Palmerston North
β-mercaptoethenol	Koch Light Colnbrook, Bucks, England
methyl cellosolve	Shell Chemical Company, N.Z.
NAD+	Sigma Chemical Co, USA
NADH	Sigma Chemical Co, USA
NADPH	Sigma Chemical Co, USA
ninhydrin	Sigma Chemical Co, USA

Table 2.2: Reagents used and source of supply.

REAGENT	SOURCE
oestradiol 17β-dipropionate	Mann Research Labs inc. New York
orcinol	British Drug House, Poole, England
palmitoyl-CoA	Sigma Chemical Co, USA
phosphorous pentoxide	Riedel De Haen AG Seelze-Hanover
POPOP	Ajax Chemicals, Sydney, Australia
porcine insulin	Novo Industri A/S, Denmark
potassium cyanide	British Drug House, Poole, England
potassium chloride	May & Baker Ltd, Dagenham, England
potassium hydroxide	British Drug House, Poole, England
PPO	Sigma Chemical Co, USA
Sephadex G-25 50-150 mesh	Pharmacia Fine Chem. Uppsala, Sweden
Silica gel DGF	Riedel De Haen AG Seelze-Hanover
Silica gel G	E. Merck, Darmstadt, Germany
sodium acetate	Riedel De Haen AG Seelze-Hanover
sodium azide	British Drug House, Poole, England
sodium dihydrogen phosphate	Riedel De Haen AG Seelze-Hanover
sodium hydroxide	May & Baker Ltd, Dagenham, England
solvents (in bulk)	Dept. Chem. & Biochem. Massey Univ.
sucrose	British Drug House, Poole, England
TMPD	British Drug House, Poole, England
Tris buffer	Koch Light Colnbrook, Bucks, England
Triton X-100	Rohm & Hass, Auckland, New Zealand
urethane	Sigma Chemical Co, USA

CHAPTER THREE

METHOD DEVELOPMENT AND VALIDATION OF GPAT ASSAY PROCEDURE

Chapter 3. METHOD DEVELOPMENT AND VALIDATION OF GPAT ASSAY PROCEDURE

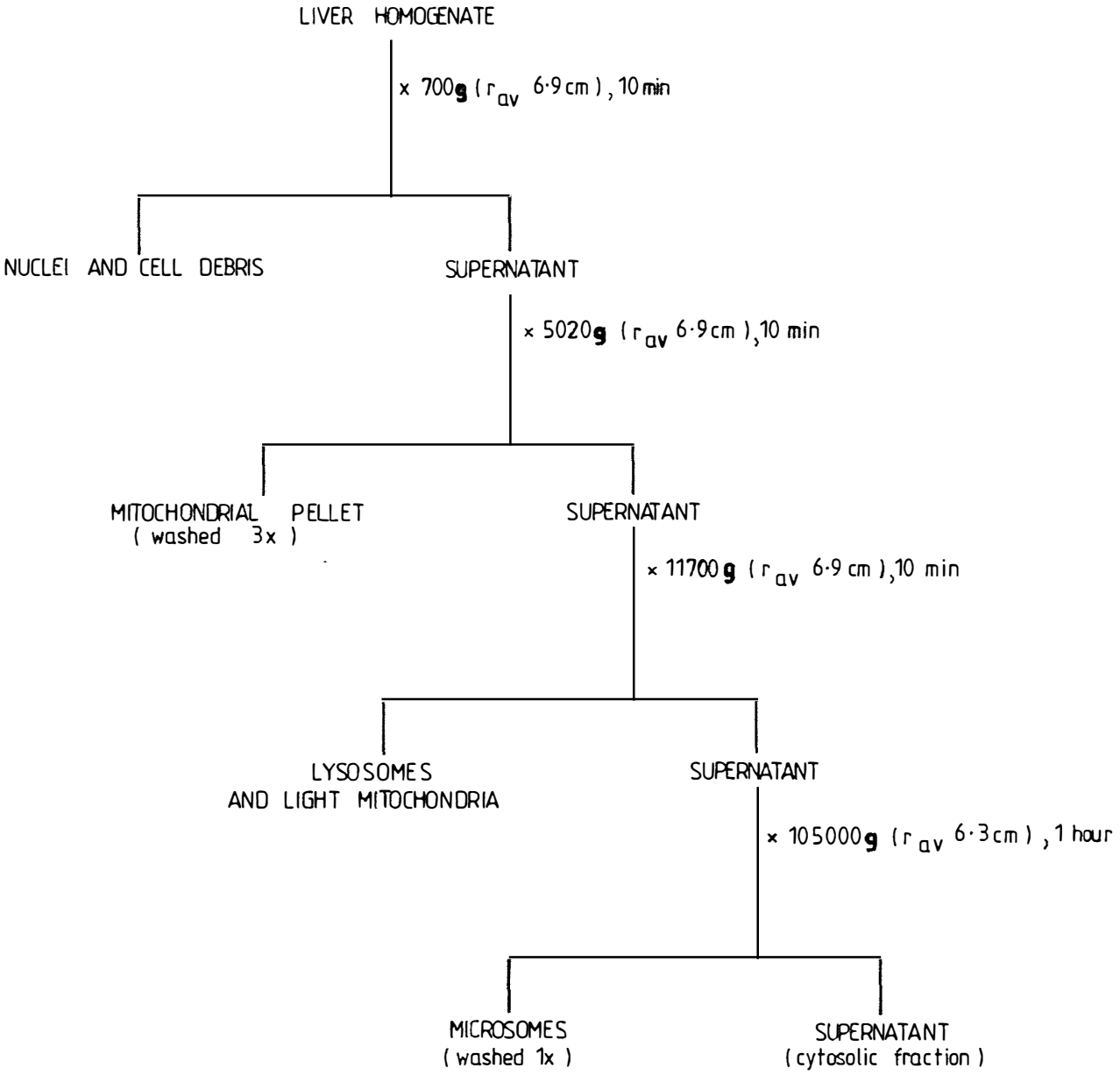
3.1 Introduction

As a first step in the verification of the procedure used to assay microsomal GPAT, marker enzyme assays were conducted to ensure that the hepatic microsomal fraction from rat liver was not significantly contaminated with mitochondrial protein. In order to assay microsomal GPAT activity with a high degree of repeatibility a procedure was required which extracted all the radioactive lipid products from assay mixtures containing unreacted [^{14}C]-glycerol 3-phosphate. A butanol extraction was preferred to a chloroform-methanol extraction (Husbands & Lands, 1970; Possmayer et al., 1969) because Bjerve et al. (1974) showed that it was much more effective in extracting lysophosphatidic acid and phosphatidic acid from the aqueous phase. As a means of increasing the rate of assay throughput, experiments were conducted to verify that the n-butanol phase of the extraction could be combined directly with the scintillant rather than evaporating it. To verify that the butanol-extractable radioactivity was due to the GPAT activity, its dependency on [glycerol 3-phosphate] and [palmitoyl-CoA] was examined. The products of the reaction were also identified as a means of further verification.

3.2 Procedure for subcellular fractionation

Rat liver was fractionated, after homogenisation, by a procedure based on methods described in the literature (Bates & Saggerson, 1979; Daae, 1972a, 1973; Fallon & Lamb, 1968; Hajra, 1968b; Liu & Kako, 1974; Monroy et al., 1972; Nimmo & Houston, 1978; Rao et al., 1969; Sarzala et al., 1970; Shepard & Hubscher, 1969; Yamashita & Numa, 1972). The Sprague-Dawley rats, approximately 200 - 400 g, were killed by cervical dislocation. All subsequent operations were carried out at 0 - 4°C. The liver was minced with scissors, rinsed briefly in homogenisation buffer (0.25 M sucrose; 1 mM EDTA, pH 7.4; 10 mM Tris-HCl, pH 7.4; 15 mM β-mercaptoethanol) and then homogenised in 5 volumes of the buffer using a Jecons Uniform glass homogeniser with a motor driven, loose fitting, teflon pestle (5 strokes at 400 rpm). It was then filtered through 4 layers of cheese cloth and fractionated by differential centrifugation according to the scheme shown in Fig 3.1. The mitochondrial pellet was washed with homogenisation buffer, but the microsomal pellet was washed with a Tris washing buffer (Depierre & Dallner, 1976) (150 mM Tris-HCl, pH 8.0; 15 mM β-mercaptoethanol). For the purpose of marker enzyme studies, aliquots of the various fractions were retained and the protein concentration of the various fractions determined (3.4).

Fig 3.1: Scheme for the subcellular fractionation of rat liver. The liver homogenate was prepared as described in the text. Where noted a shortened version of this scheme, involving homogenisation by hand with 3 strokes of the homogeniser and omission of the 700**g** and 5020**g** centrifugations, was used for the preparation of the microsomal fraction.


LIVER HOMOGENATE
× 700g (r_{av} 6·9 cm), 10 min
NUCLEI AND CELL DEBRIS
SUPERNATANT
× 5020g (r_{av} 6·9 cm), 10 min
MITOCHONDRIAL PELLET
(washed 3x)
SUPERNATANT
× 11700g (r_{av} 6·9 cm), 10 min
LYSOSOMES
AND LIGHT MITOCHONDRIA
SUPERNATANT
× 105000g (r_{av} 6·3 cm), 1 hour
MICROSOMES
(washed 1x)
SUPERNATANT
(cytosolic fraction)

3.3 Validation of subcellular fractionation procedure

Marker enzyme assays were carried out on subcelluar fractions, prepared as described above, to determine the extent of cross-contamination of the mitochondrial and microsomal fractions:

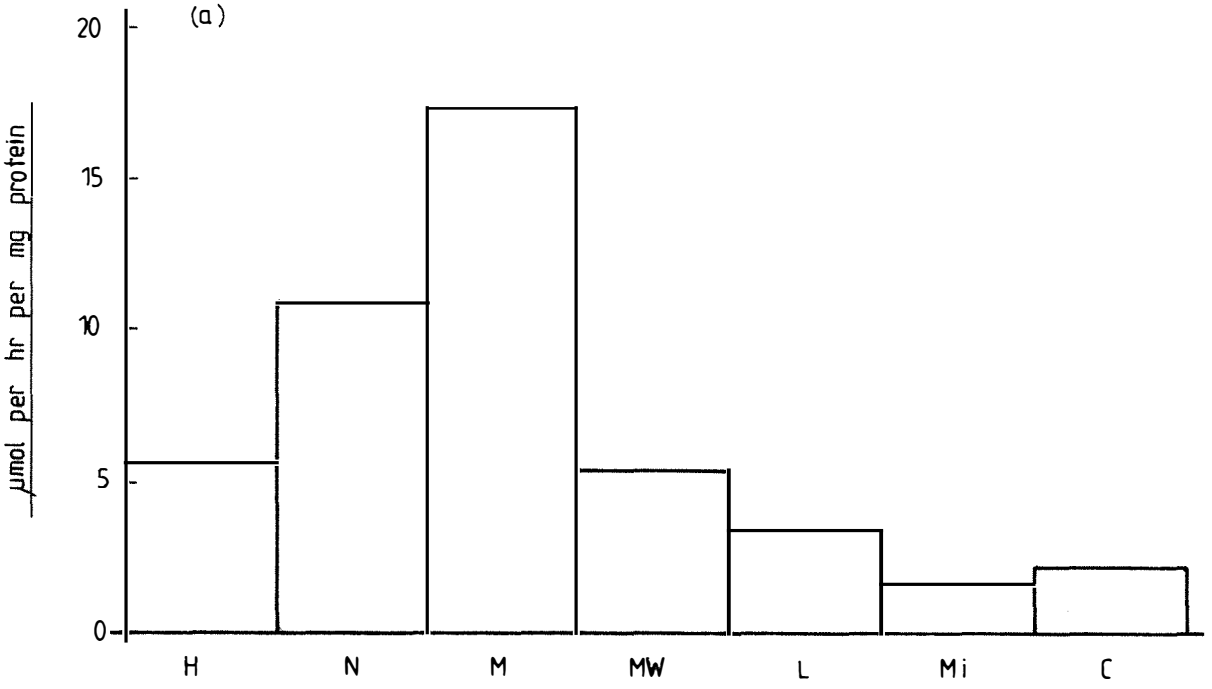
1. Assay for cytochrome oxidase (EC 1.9.3.1). Cytochrome oxidase, located on the inner membrane of mitochondria (Lehninger, 1975), was assayed according to the following method (Schnaitman et al., 1967). A Yellow Springs Instruments oxygen electrode was used with a water jacket temperature of 31°C. The assay system contained the following in a total volume of 3 ml: 75 mM phosphate buffer, pH 7.2; 0.03 mM cytochrome C; 3.75 mM sodium ascorbate; 0.3 mM TMPD; and 0.78 - 4.4 mg protein from subcellular fractions. The activity of the cytochrome C oxidase was measured in µmols O_2 taken up per mg of protein per hour.

2. Assay for monoamine oxidase (EC 1.4.3.4). The following method was used for the assay of monoamine oxidase which, along with mitochondrial GPAT, is located in the outer membrane of mitochondria (Nimmo, 1979b; Schnaitman et al., 1967). Using a final volume of 3 ml the assay contained: 2.5 mM benzylamine; 50 mM phosphate buffer, pH 7.6; and 0.23 - 0.56 mg protein from subcellular fractions. Monoamine oxidase activity was determined by following the conversion of benzylamine to benzaldehyde at 250 nm at 33°C. The formation of 0.15 µmol of benzaldehyde results in an increase in absorbance of 1 unit.

3. Assay for NADPH cytochrome C reductase (EC 1.6.2.4). NADPH cytochrome C reductase, which is specific to the microsomal fraction, was assayed as follows (Sottocasa et al., 1967). In a final volume of 3 ml, at 33°C, the enzyme activity was determined in a reaction mixture containing: 0.1 mM NADPH; 0.1 mM cytochrome C; 0.3 mM KCN; 50 mM phosphate buffer, pH 7.5; and 0.26 - 0.56 mg protein from subcellar fractions. Reduction was monitored at 550 nm using molar absorbancy of 27.7×10^3 cm^2 mol^{-1} for reduced cytochrome C.

The results of the assays of marker enzymes in the subcellular fractions are given in Table 3.1 and Figs 3.2, 3.3 and 3.4. The recovery of homogenate protein was 71.5% while the recoveries of cytochrome oxidase, monoamine oxidase and NADPH cytochrome C reductase activities were 81.3%, 112.0%, and 85.6% respectively (see Table 3.1). On the basis of the distribution of NADPH cytochrome C reductase and monoamine oxidase, the microsomal fraction contained 10.6% mitochondrial impurity and the mitochondrial fraction contained 32.3% microsomal impurity. Similarly, on the basis of the distribution of NADPH cytochrome C reductase and cytochrome oxidase, the microsomal fraction contained 5.8% mitochondrial impurity and the mitochondrial fraction contained 34.3% microsomal impurity. Cross contamination must be low in order to be able to draw meaningful conclusions about the nature or behaviour of specific isoenzymes of GPAT. Therefore, the above procedure (see Fig 3.1) was considered to give microsomes of suitable purity and was adopted in subsequent experiments except, where noted, liver was homogenised by hand with 3 strokes of the Homogeniser and the 700**g** and 5020**g** centrifugations omitted.

Fig 3.2: The specific activity and total activity of cytochrome oxidase in various subcellular fractions. Results are means of duplicate assays of cytochrome oxidase in the respective subcellular fractions prepared from the livers of two rats. Assay conditions are given in section 3.3 (1). See Table 3.1 for means of determinations. H = homogenate; N = nuclei, cell debris etc; M = mitochondrial fraction; MW = supernatants from washing of mitochondrial fraction; L = lysosomes and light mitochondria; Mi = microsomal fraction; C = cytosol.


(a)
20
15
10
5
0
µmol per hr per mg protein
H
N
M
MW
L
Mi
C

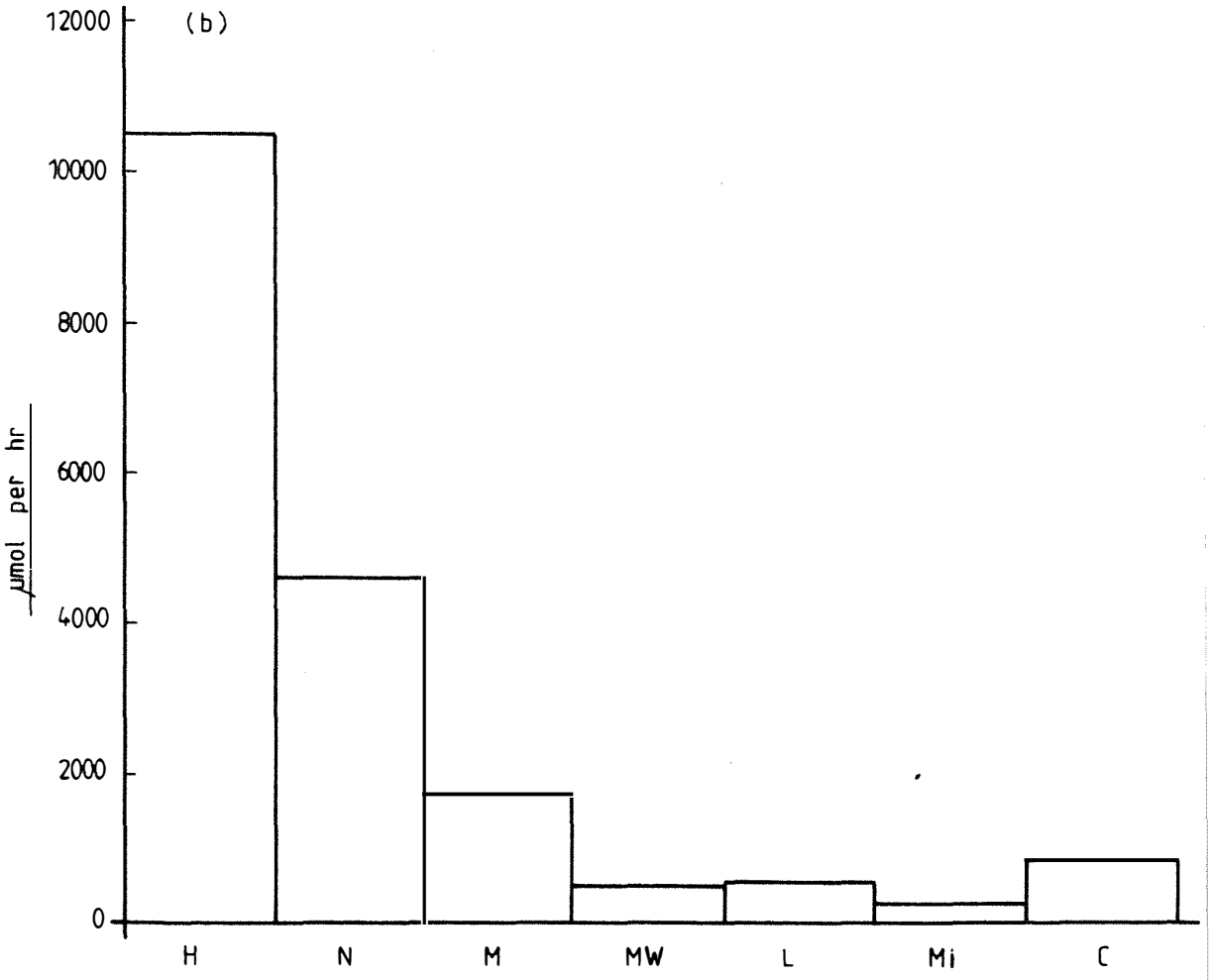
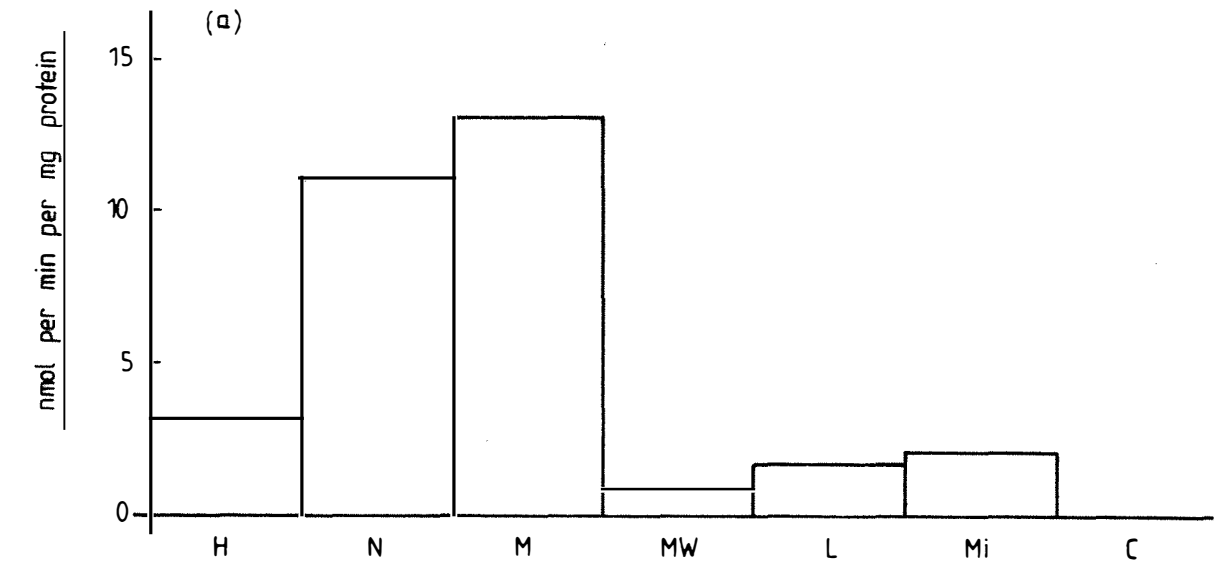

(b)
12000
10000
8000
6000
4000
2000
0
µmol per hr
H
N
M
MW
L
Mi
C

Fig 3.3: The specific activity and total activity of monoamine oxidase in various subcellular fractions. Results are the means of duplicate assays of monoamine oxidase in the respective subcelluar fractions from the livers of two rats. Assay conditions are given in section 3.3 (2). See Table 3.1 for means of determinations. H = homogenate; N = nuclei, cell debris etc; M = mitochondrial fraction; MW = supernatants from washing of mitochondrial fraction; L = lysosomes and light mitochondria; Mi = microsomal fraction; C = cytosol.


(a)
nmol per min per mg protein
15
10
5
0
H
N
M
MW
L
Mi
C

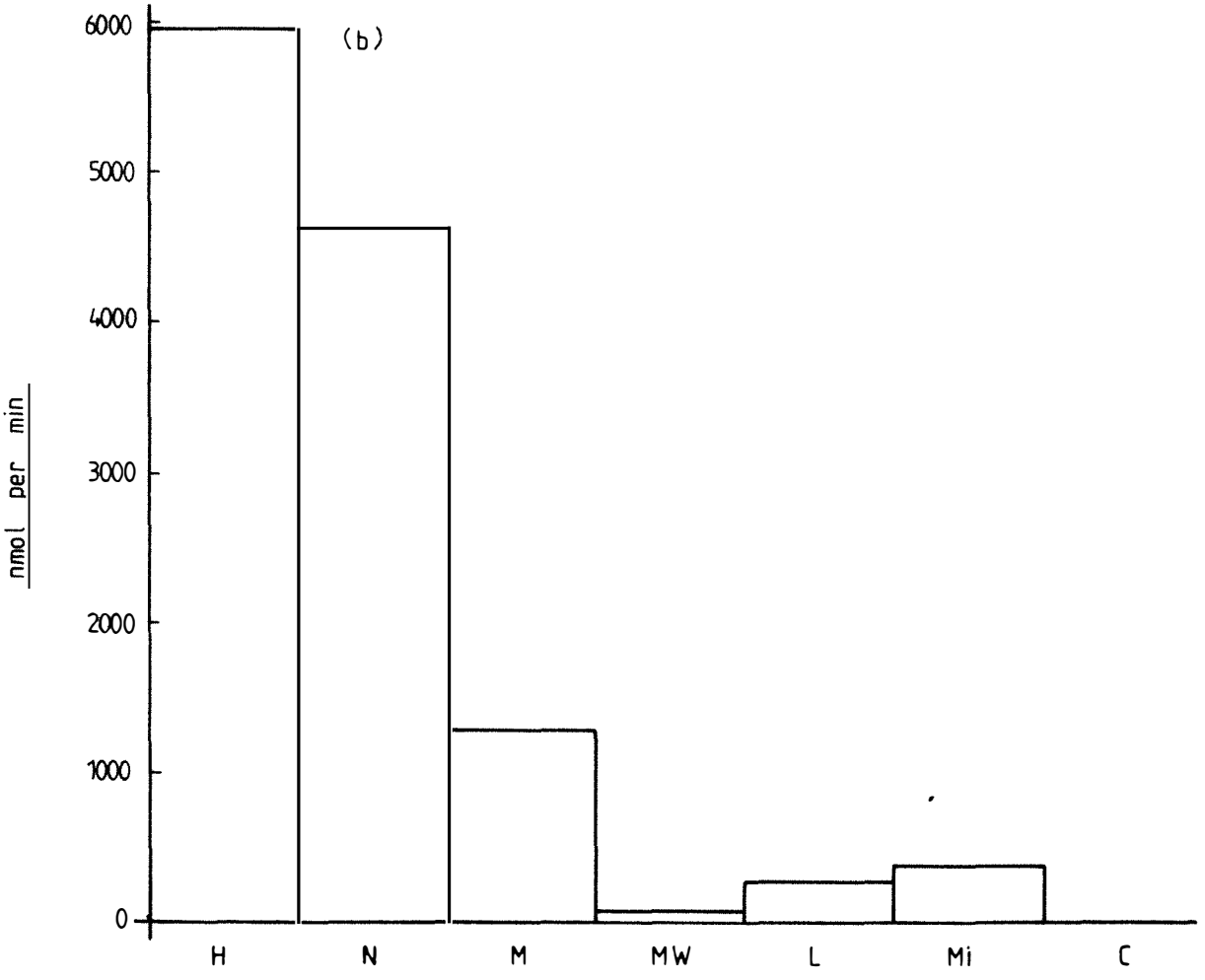
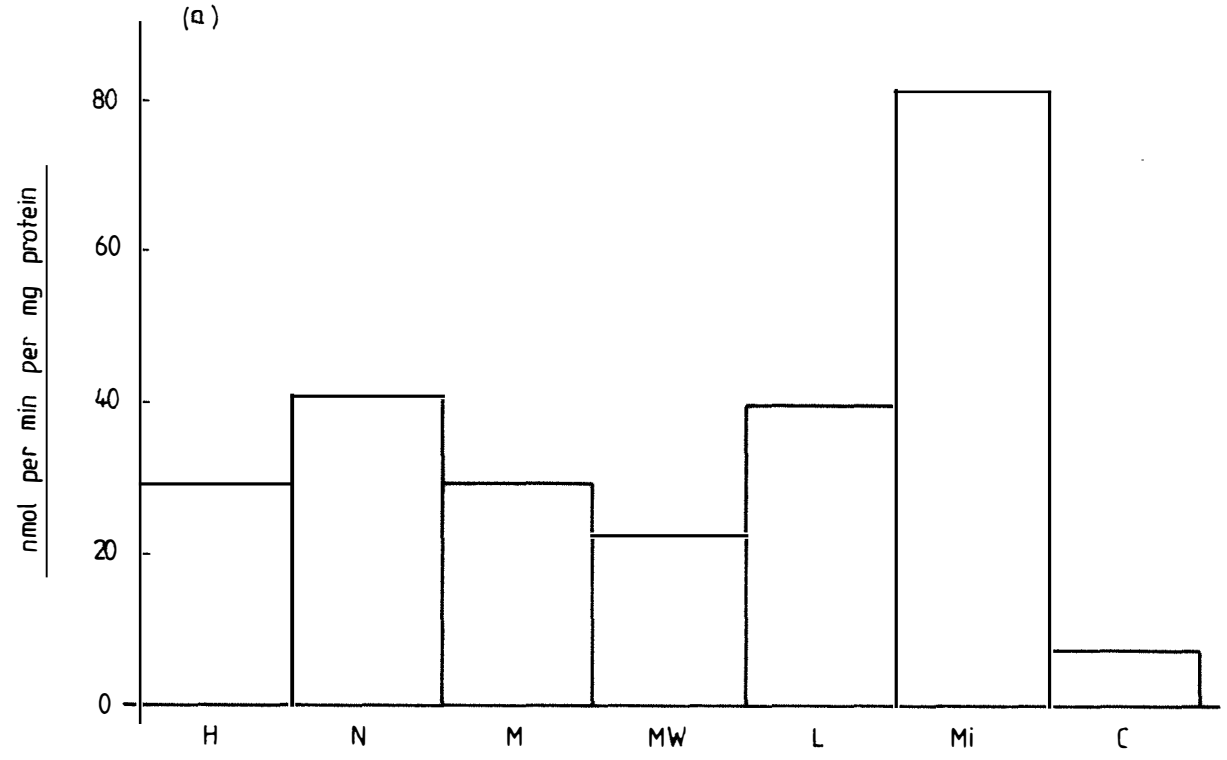

(b)
nmol per min
6000
5000
4000
3000
2000
1000
0
H
N
M
MW
L
Mi
C

Fig 3.4: The specific activity and total activity of NADPH cytochrome C reductase in various subcellular fractions. Results are the means of duplicate assays of NADPH cytochrome C reductase in the respective subcellular fractions from the livers of two rats. Assay conditions are given in section 3.3 (3). See Table 3.1 for means of determinations. H = homogenate; N = nuclei, cell debris etc; M = mitochondrial fraction; MW = supernatants from washing of mitochondrial fraction; L = lysosomes and light mitochondria; Mi = microsomal fraction; C = cytosol.


(a)
nmol per min per mg protein
80
60
40
20
0
H
N
M
MW
L
Mi
C

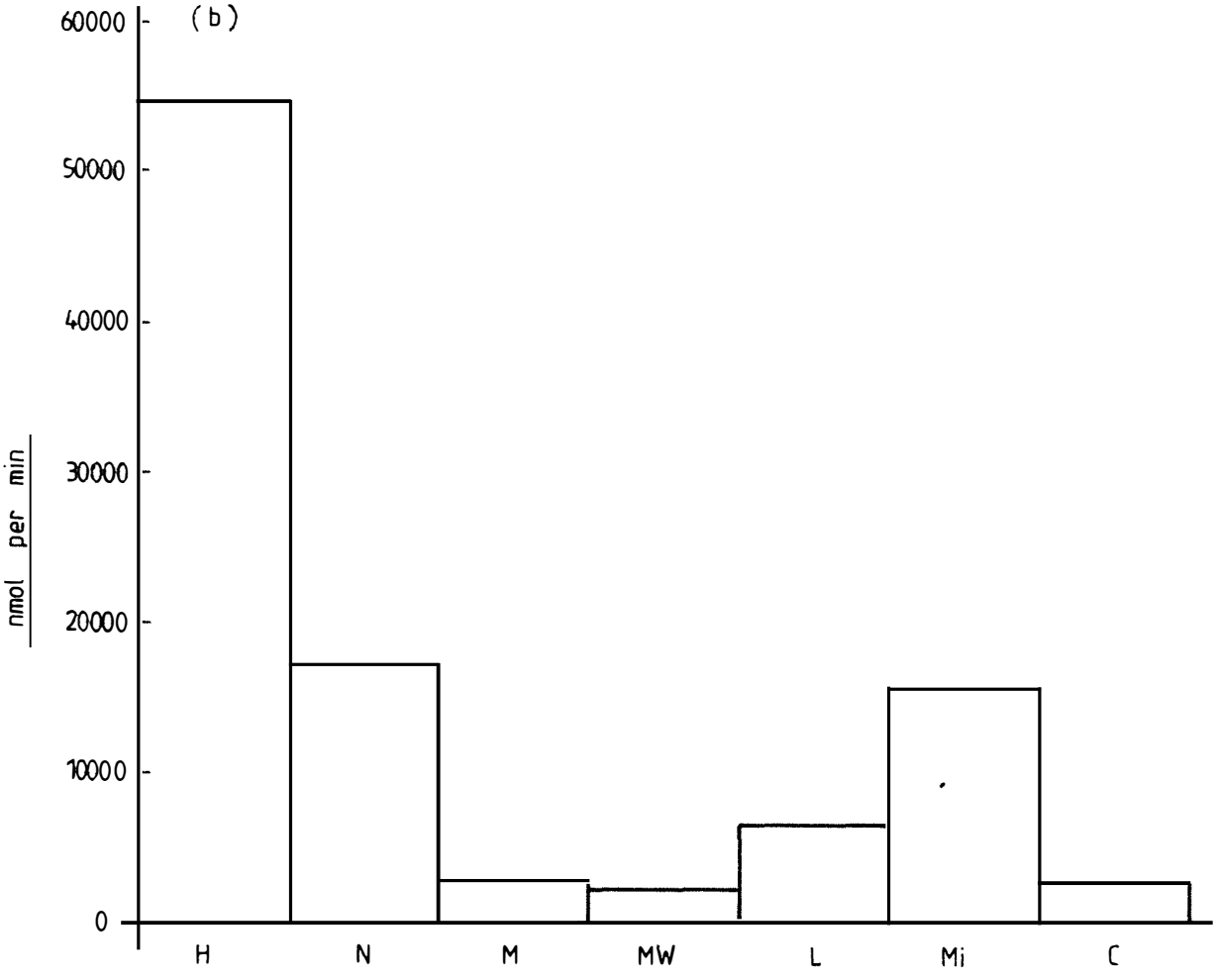

(b)
nmol per min
60000
50000
40000
30000
20000
10000
0
H
N
M
MW
L
Mi
C

Table 3.1: Protein content and marker enzyme activities in subcellular fractions of rat liver.

	H	N	M	MW	L	Mi	C
PROTEIN							
Total mg	1892.0	419.3	98.9	101.3	163.5	191.7	378.3
CYTOCHROME OXIDASE							
units[a]/mg	5.54	10.97	17.40	5.30	3.49	1.55	2.14
Total[c]	10481.7	4599.2	1720.9	536.9	567.1	297.2	809.6
MONOAMINE OXIDASE							
units[b]/mg	3.16	11.04	13.08	0.91	1.75	2.05	0
Total[c]	5978.7	4628.5	1293.6	92.2	283.6	393.0	0
NADPH CYTOCHROME C REDUCTASE							
units[b]/mg	29.04	40.62	29.52	22.05	39.63	81.33	7.47
Total[c]	54943.7	17029.9	2919.5	2233.7	6439.9	15593.4	2825.9

Keys to abbreviations: H = homogenate; N = nuclei, cell debris etc; M = mitochondrial fraction; MW = supernatants from washing of mitochondrial fraction; L = lysosomes and light mitochondria; Mi = microsomal fraction; C = cytosol. [a]units = µmol O_2 per h; [b]units = nmol per min. [c]units/mg x mg protein. Results are means of duplicate assays of fractions prepared from livers of two rats. See 3.3 (1, 2, 3) and 3.4 for assay conditions.

3.4 Method for protein determination

The protein of various subcellular fractions was determined using the Coomassie blue method of Bradford, (1976; see also Chiappelli *et al.*, 1979) except that 0.5 ml of the protein-containing solution was combined with the dye reagent. The method was standardised using BSA fraction V, the concentration of which was estimated by absorbance at 280nm (Bradford, 1976). A standard curve with a range of 10 - 50 µg of protein per tube was prepared each time and the regression line fitted by a least squares technique.

3.5 Determination of radioactivity

As discussed in the introduction to this chapter, a butanol extraction of GPAT assays was adopted as it was reported to be more efficient than chloroform-methanol extractions at recovering the radioactive products (Bjerve et al., 1974). In order to facilitate multiple assays of GPAT, the possibility of combining the butanol phase, which contains small amounts of water, directly with the scintillant was examined. Because of the aqueous contamination, a Triton-toluene scintillant (Fox, 1968) was selected and prepared by combining: 2 litres of redistilled toluene, 1 litre of Triton X-100, 12 g PPO and 0.3 g POPOP. In a preliminary experiment it was determined that butanol quenches the fluorescence of the scintillant by ^{14}C, by approximately 24%. Only one phase was present. To compensate for quenching, the Automatic Quench Compensation (AQC) facility of the Beckman LS8000 liquid scintillation counter was used after checking its applicability by comparison with the internal standard method using ^{14}C-hexadecane in aqueous butanol-scintillant mixtures (Table 3.2).

Table 3.2: The effect of using the Automatic Quench Compensation facility of the Beckman LS 8000 scintillation counter on the efficiency of counting of [^{14}C]-hexadecane in the presence of varying amounts of water-saturated butanol.

butanol added (ml)	% counting efficiency with AQC	% counting efficiency without AQC
0	44.50	41.23[a]
0.2	44.89	40.68
0.4	43.21	37.61
0.6	44.21	39.02
0.8	43.39	37.89
1.0	44.31	37.23
1.2	44.16	36.25
1.4	43.17	35.33
1.6	43.34	34.61
1.8	43.44	33.96
2.0	43.54	33.46
2.2	43.09	32.56

[a]mean of 10 determinations on 1 sample vial which contained a known weight of [^{14}C]hexadecane of known specific activity (126.91 dpm mg^{-1}); counting efficiency = cpm/dpm x 100/1.

3.6 Extraction of radioactive glycerol 3-phosphate from aqueous butanol.

A washing procedure was developed which minimised amounts of unreacted, water-soluble, [^{14}C]glycerol 3-phosphate contamination in the butanol phase and ensured that radioactivity in the butanol phase was due to non water-soluble radioactive products of the GPAT assay. The efficiency of removal of [^{14}C]glycerol 3-phosphate from the butanol phase, which contains small amounts of water, was determined by successive washings with butanol-saturated water, i.e. a known amount of [^{14}C]glycerol 3-phosphate (35 000 cpm) in 0.35 ml was mixed with 2.2 ml of butanol and this was washed 1, 2, or 3 times with 2 ml, 5 ml or 10 ml, of butanol-saturated water. The butanol and aqueous phases were separated by centrifugation at 700$\mathbf{g}_{av}$ for 10 min. Results of this examination are shown in Table 3.3. The third system involving three 10 ml aqueous washes was determined to be most suitable and adopted as standard procedure in subsequent experiments except that butanol-saturated water was replaced with butanol-saturated 0.1 M boric acid (see Kako & Peckett, 1981).

Table 3.3: The effect of the volume of the aqueous phase and number of washings on the removal of [^{14}C]glycerol 3-phosphate from aqueous butanol.

vol of aqueous phase added (ml)	no of washings	[^{14}C]radioactivity (times greater than background cpm[a])
2	1	14.0
2	2	4.9
2	3	2.0
5	1	10.8
5	2	2.1
5	3	1.3
10	1	6.0
10	2	1.8
10	3	1.2

[a]present in the butanol phase; mean of duplicate determinations; background cpm was 19.6.

3.7 Examination of microsomal esterification assay system

3.7.1 Preparation of glycerol 3-phosphate and palmitoyl-CoA

[U-^{14}C]glycerol 3-phosphate was diluted with "cold" sn-glycerol 3-phosphate to give a specific radioactivity of 1.1 x 10^5 dpm/μmol which corresponds to approximately 100 cpm/nmol of glycerol 3-phosphate. This was stored at -20°C. Palmitoyl-CoA was made up to a concentration of 707 - 726 μM and stored at -20°C.

3.7.2 Initial selection of conditions for GPAT assay

The literature was surveyed and initial conditions for the validation of the microsomal GPAT assay selected. The assay conditions used by earlier workers are within the ranges: acyl-CoA, 20-144 μM; glycerol 3-phosphate, 0.3-6 mM; microsomal protein, 0.025-4.6 mg/assay; a reaction pH of 7.0-8.0; and either dithiothreitol or β-mercaptoethanol, 3-15 mM (Abou-Issa & Cleland, 1969; Bates & Saggerson, 1977, 1979, 1981a,b; Bjerve et al., 1976; Daae, 1972a; Daae & Bremer, 1970; Eibl et al., 1969; Fallon & Lamb, 1968; Haldar et al., 1979; Hendry & Possmayer, 1974; Husbands & Lands, 1970; Joly et al., 1973; Lamb & Fallon, 1970; Lamb et al., 1977; Mangiapane et al., 1973; Martensson & Kanfer, 1968; Monroy et al., 1972, 1973; Nachbaur et al., 1971; Nimmo, 1979a; Nimmo & Houston, 1978; Possmayer & Mudd, 1971; Sanchez et al., 1973; Stern & Pullman, 1978; Weigand et al., 1973; Yamada & Okuyama, 1978; Yamashita & Numa, 1972). Therefore, the following approximate reaction conditions were selected initially: palmitoyl-CoA, 100 μM; glycerol 3-phosphate, 3 mM; microsomal protein, 1 mg; 50 mM Tris-HCl, pH 7.4; and 15 mM

β-mercaptoethanol; 2 mg (30 μM) BSA/ml (Jamdar, 1979); an assay duration of 1 min ; and an assay temperature of 30°C (Bates & Saggerson, 1979). The following procedure was also adopted at the outset:

1. The assays were allowed to eqilibrate at 30°C for at least 5 min after which time the reaction was started by the addition of the palmitoyl-CoA.
2. The reaction was terminated after 1 min by the addition of 2.2 ml butanol and 10 ml butanol-saturated water. Zero time reactions were terminated as soon as the palmitoyl-CoA was added and thereafter treated the same as the other reactions in order to estimate the [^{14}C]α-GP carried over into the washed butanol phases. The butanol phases were washed as described in section 3.6 and the radioactivity counted as described in section 3.5.

The microsomal esterification system was then examined to verify that it was an adequate assay for the microsomal isoenzyme of GPAT, which has been extensively characterised by many workers since the early 1950's (see Chapter 1).

3.7.3 Response to [palmitoyl-CoA]

The results presented in Fig 3.5 show that the rate of esterification of the [^{14}C]-α-GP with palmitoyl-CoA increased to a maximum at 100 - 125 μM palmitoyl-CoA, but above this, the rate of esterification tended to level off, probably due to inhibition by palmitoyl-CoA (Husbands & Lands, 1970; Jamdar, 1979). This dependence of the microsomal esterification assay on palmitoyl-CoA suggested that the system was assaying GPAT activity. BSA was routinely included in the assay because, as reviewed in Chapter 1, it stimulates GPAT activity through relieving the inhibition caused by high acyl-CoA concentrations and by maintaining the acyl-CoA concentration through inhibition of acyl-CoA hydrolase (Jamdar, 1979). Without BSA, it is likely that the inhibition by palmitoyl-CoA would have been more marked and occurred at a lower concentration. The inhibitory nature of higher concentrations of palmitoyl-CoA meant that Lineweaver-Burk or Eadie-Hofstee analysis could not be used to estimate K_m and V_{max} parameters, but when the data were transformed with a Hill plot (Fig 3.6) a $S_{0.5}$ parameter (similar to K_m) of 58.54 μM was calculated. This may or may not have physiological significance as it is difficult to determine the actual concentration of acyl-CoA within the liver. In the several experiments performed the slope of the Hill plot was approximately 2.3 which, in other situations, would be taken to indicate that the enzyme has two binding sites for palmitoyl-CoA with positive co-operativity. However, as the palmitoyl-CoA to BSA ratio was not kept constant it seems likely that these kinetics are due to changes in the amount of free to BSA-bound palmitoyl-CoA in the reaction volume.

3.7.4 Response to [glycerol 3-phosphate]

The results presented in Fig 3.7 show that as the concentration of glycerol 3-phosphate in the assay was increased, the rate of its esterification with palmitoyl-CoA increased in a hyperbolic manner. This dependence of the reaction on [glycerol 3-phosphate] indicated that GPAT activity was being assayed. The data were analysed by an Eadie-Hofstee transformation (Fig 3.8) and the K_m and V_{max} parameters estimated. The K_m ($\pm$ SD) was 4.02 $\pm$ 0.69 mM while the V_{max} ($\pm$ SD) was estimated to be 7.83 $\pm$ 1.01 nmol/min/mg microsomal protein. An apparent K_m of 4.02 mM for sn-glycerol 3-phosphate is within the range of K_m values reported for microsomal GPAT from liver (see Table 1.5) which provides further evidence that GPAT activity is being assayed.

Fig 3.5: Effect of [palmitoyl-CoA] on esterification of glycerol 3-phosphate with palmitoyl-CoA by a hepatic microsomal fraction. Points are the mean of assays conducted in triplicate and error bars show ± SEM. The reaction (at 30°C) contained in a total volume of 0.35 ml: 50 mM Tris-HCl, pH 7.4; 30 μM BSA; 15 mM β-mercaptoethanol; 2.93 mM [^{14}C]glycerol 3-phosphate; 1 mg microsomal protein; and variable palmitoyl-CoA. Assays were terminated after 1 min.

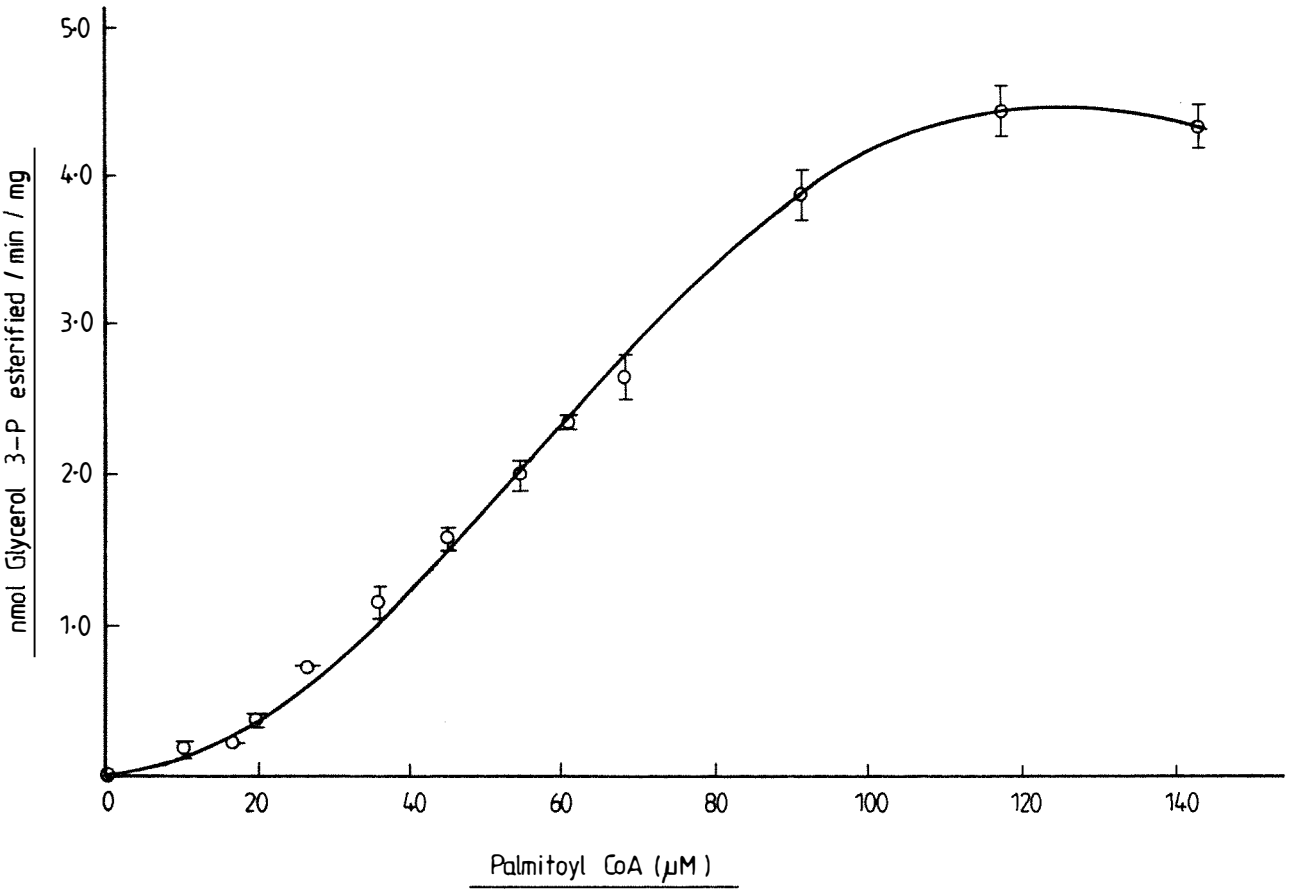

5·0
4·0
3·0
2·0
1·0
nmol Glycerol 3-P esterified / min / mg
0
20
40
60
80
100
120
140
Palmitoyl CoA (μM)

Fig 3.6: Hill plot transformation of the data for the response of esterification of glycerol 3-phosphate with palmitoyl-CoA by a hepatic microsomal fraction to varying [palmitoyl-CoA]. The slope of the Hill plot = 2.20 and the $S_{0.5}$ = 58.54 μM. The line shown was fitted by a least squares regression procedure for the "central 80%" of the data (Fersht, 1977). Assay conditions are described in the legend to Fig 3.5.

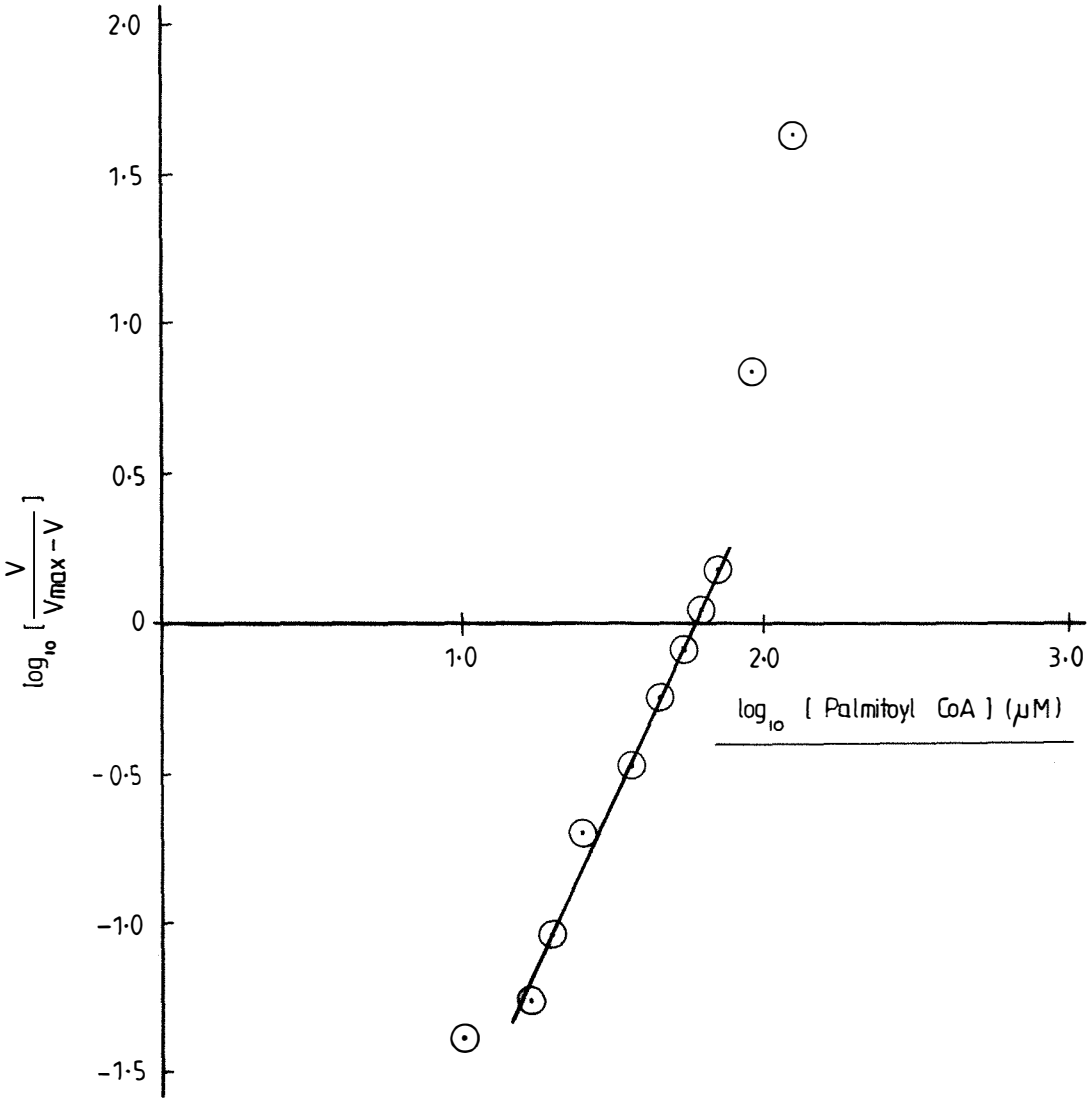

2·0
1·5
1·0
0·5
0
-0·5
-1·0
-1·5
1·0
2·0
3·0
log10 [Palmitoyl CoA] (μM)
log10 [V / Vmax - V]

Fig 3.7: Effect of [glycerol 3-phosphate] on esterification of glycerol 3-phosphate with palmitoyl-CoA by a hepatic microsomal fraction. The curve was fitted visually. Points are the means of assays conducted in triplicate and error bars show ± SEM. The reaction (at 30°C) contained in a total volume of 0.35 ml: 50 mM Tris-HCl, pH 7.4; 30 μM BSA; 15 mM β-mercaptoethanol; variable [^{14}C]glycerol 3-phosphate; 1 mg microsomal protein; and 108 μM palmitoyl-CoA. Assays were terminated after 1 min.

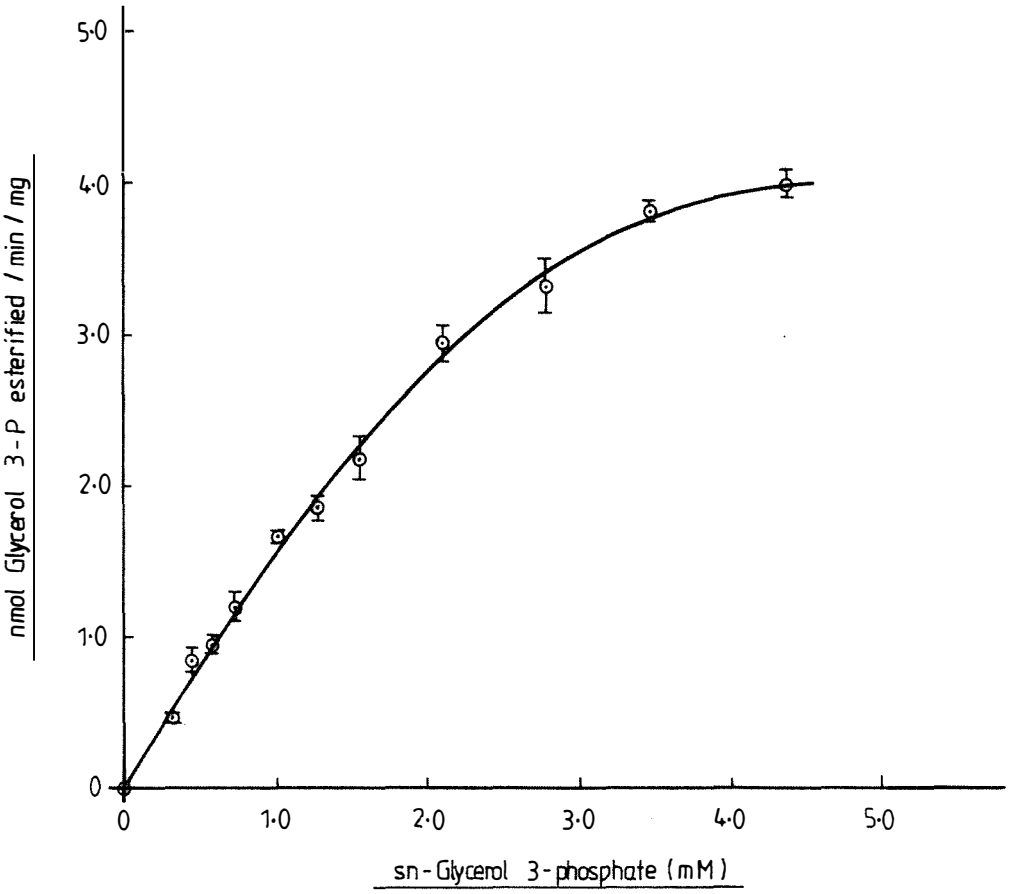
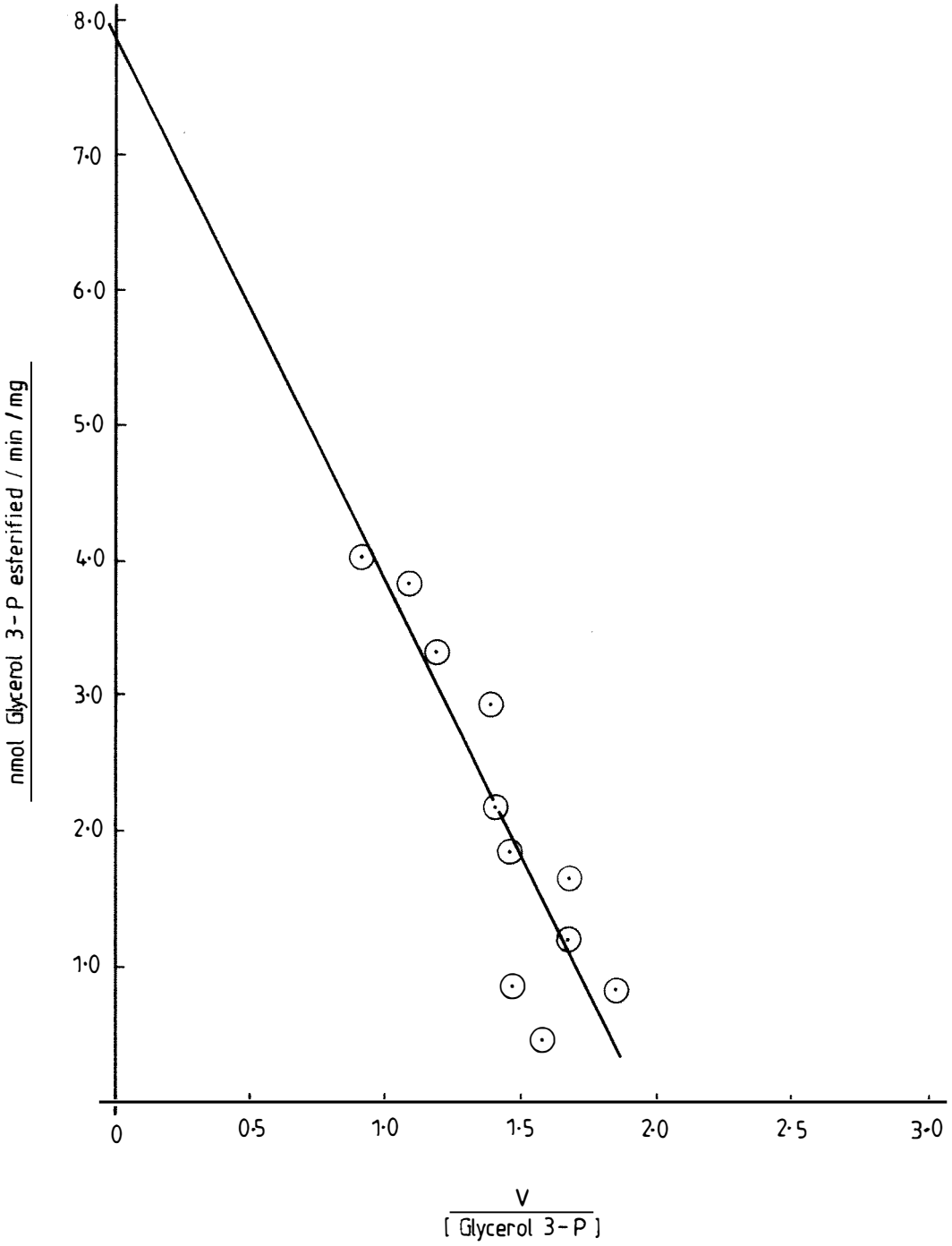

nmol Glycerol 3-P esterified / min / mg
5·0
4·0
3·0
2·0
1·0
0
0
1·0
2·0
3·0
4·0
5·0
sn-Glycerol 3-phosphate (mM)

Fig 3.8: Eadie-Hofstee transformation of data for the effect of [glycerol 3-phosphate] on esterification of glycerol 3-phosphate with palmitoyl-CoA by a hepatic microsomal fraction. The line shown was fitted by a least squares linear regression procedure. Assay conditions are described in the legend to Fig 3.7.


8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
nmol Glycerol 3-P esterified / min / mg
0
0·5
1·0
1·5
2·0
2·5
3·0
V
[Glycerol 3-P]

3.7.5 Response to amount of microsomal protein

From the results shown in Fig 3.9 it was determined that increasing the amount of microsomal protein in the assay up to approximately 1 mg gave a linear increase in the rate of esterification of glycerol 3-phosphate with palmitoyl-CoA, but with greater amounts present the rate plateaued.

3.7.6 Dependence on time

It can be seen from Fig 3.10 that the rate of esterification of glycerol 3-phosphate was linear for nearly 1.5 minutes after the reaction was started by the addition of palmitoyl-CoA.

3.7.7 Response to pH

The rate of esterification of glycerol 3-phosphate with palmitoyl-CoA was found to change when the pH of the reaction was varied over the range pH 7.0 to 8.4 (Table 3.4). At pH 8.4 the rate was 14% higher ($P<0.05$) than at pH 7.0. Reports in the literature have usually indicated that the rat liver microsomal GPAT has a broad pH optimum over the range pH 7.0 to 8.0 (Coleman & Haynes, 1983; Klutymans & Raju, 1974; Nimmo, 1979a; Tamai & Lands, 1974) although an early report put the pH optimum for the microsomal GPAT at 6.5 (Fallon & Lamb, 1968). A partially purified rat liver microsomal GPAT preparation had a broad pH optimum within the range pH 6.6 to 9.0 (Yamashita & Numa, 1972) and another preparation had a broad optimum over the range pH 7.0 to 8.7 (Klutymans & Raju, 1974). Therefore, the response to pH observed in this present study is consistent with the esterification activity being due to the action of microsomal GPAT.

Fig 3.9: Effect of amount of microsomal protein on esterification of glycerol 3-phosphate with palmitoyl-CoA by a hepatic microsomal fraction. The curve was fitted visually. Points are the means of assays conducted in triplicate and error bars show ± SEM. The reaction (at 30°C) contained in a total volume of 0.35 ml: 50 mM Tris-HCl, pH 7.4; 30 µM BSA; 15 mM β-mercaptoethanol; 2.93 mM [^{14}C]glycerol 3-phosphate; variable mg microsomal protein; and 108 µM palmitoyl-CoA. Assays were terminated after 1 min.

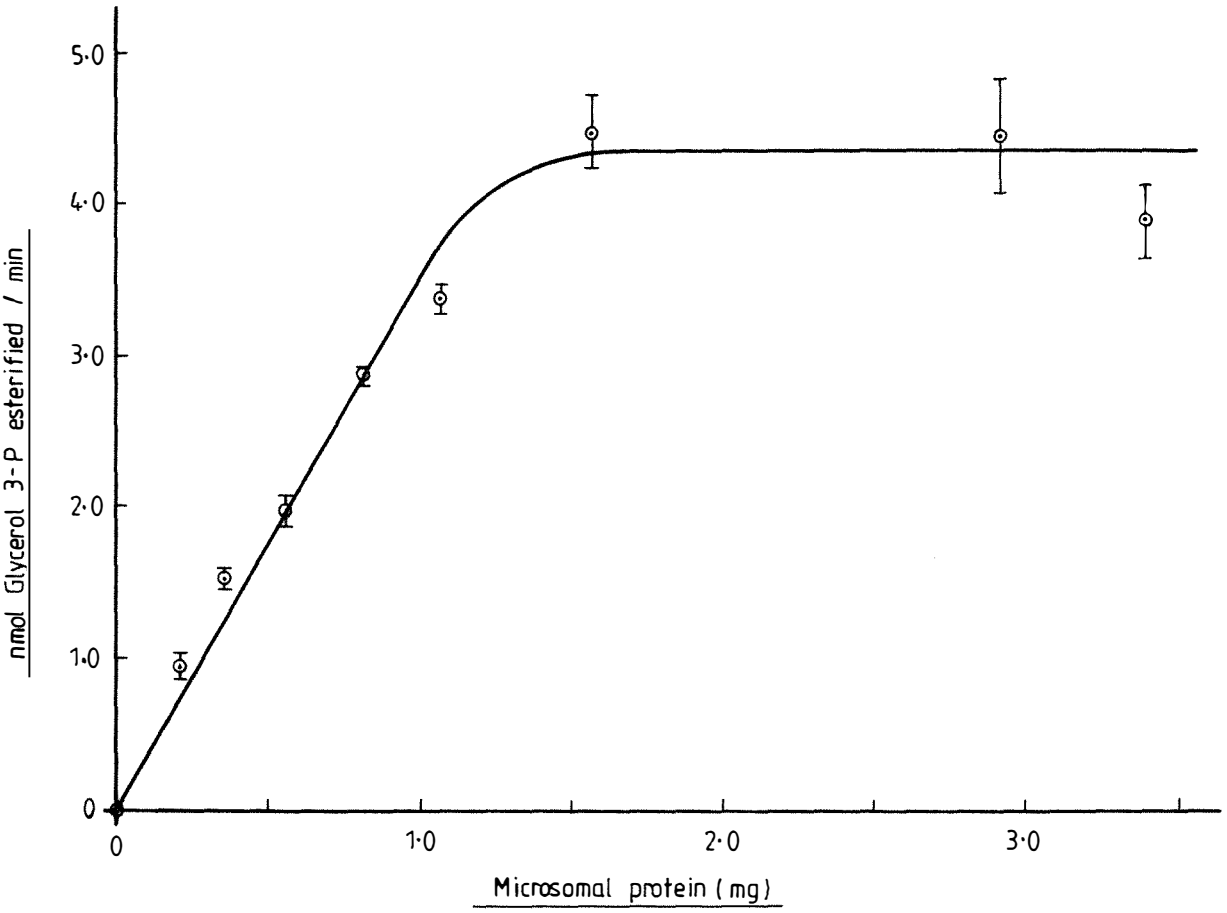

5·0
4·0
3·0
2·0
1·0
0
0
1·0
2·0
3·0
nmol Glycerol 3-P esterified / min
Microsomal protein (mg)

Fig 3.10: Effect of time on esterification of glycerol 3-phosphate with palmitoyl-CoA by a hepatic microsomal fraction. The curve was fitted visually. Points are the means of assays performed in triplicate and error bars show ± SEM. The reaction (at 30°C) contained in a total volume of 0.35 ml: 50 mM Tris-HCl, pH 7.4; 30 μM BSA; 15 mM β-mercaptoethanol; 2.93 mM [^{14}C]glycerol 3-phosphate; 1 mg microsomal protein; and 108 μM palmitoyl-CoA. Assays were terminated at various times.

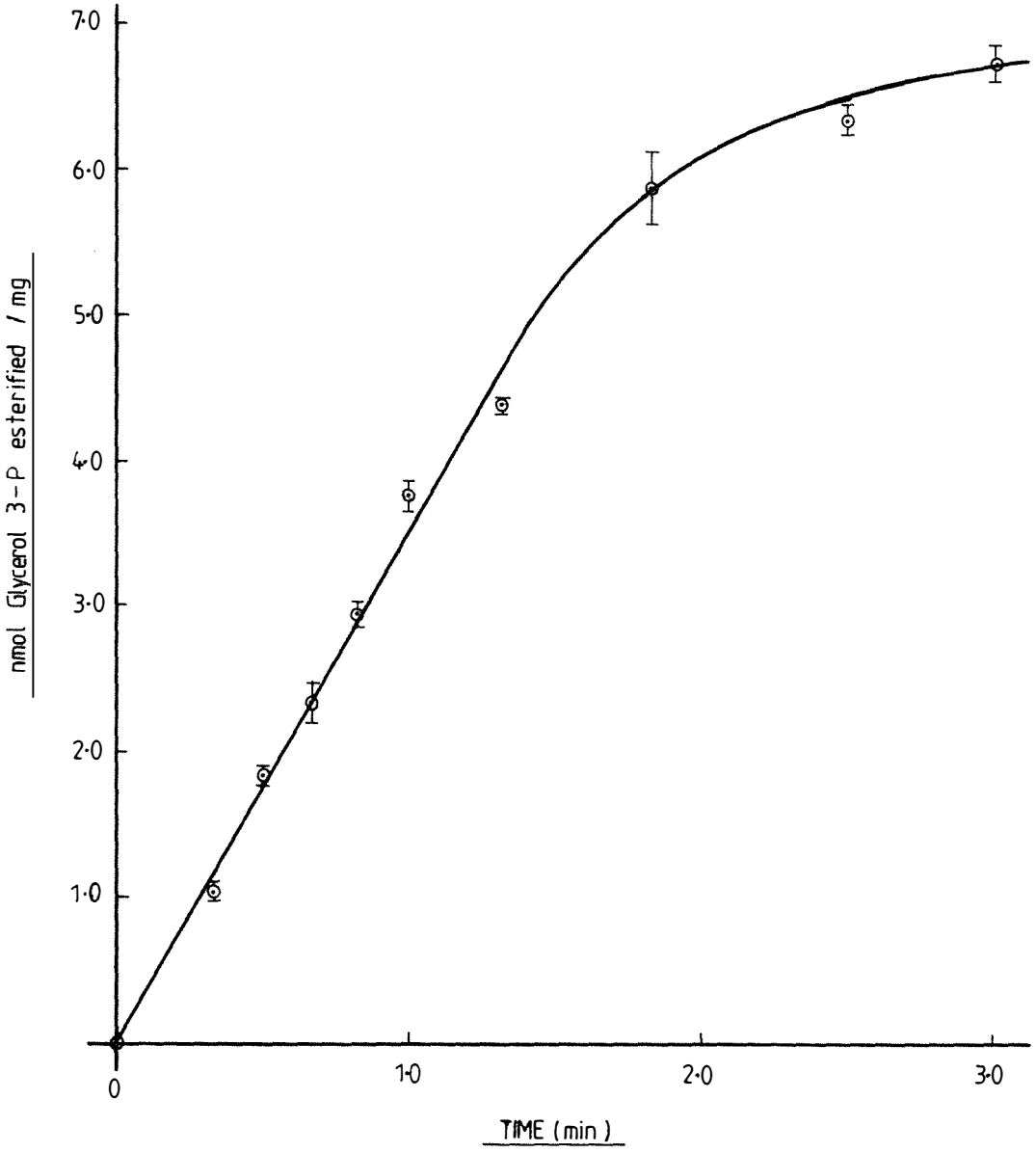

7·0
6·0
5·0
4·0
3·0
2·0
1·0
0
1·0
2·0
3·0
nmol Glycerol 3-P esterified / mg
TIME (min)

Table 3.4: The effect of pH of the reaction on esterification of glycerol 3-phosphate with palmitoyl-CoA by a hepatic microsomal fraction.

pH of reaction	GPAT activity (nmol/min/mg)
7.0	3.81 ± 0.08^{a}
7.4	4.16 ± 0.09^{ab}
8.0	4.25 ± 0.07^{ab}
8.4	4.36 ± 0.06^{b}

Means with superscripts not in common are significantly different ($P<0.05$). Data are the means of assays conducted in duplicate $\pm$ SEM. The reaction (at $30^{o}C$) contained in a total volume of 0.35 ml: 50 mM HEPES, variable pH; 30 μM BSA; 15 mM β-mercaptoethanol; 3 mM [^{14}C]glycerol 3-phosphate; 1 mg microsomal protein; and 101 μM palmitoyl-CoA. Assays were terminated after 1 min.

3.7.8 Identification of products of the GPAT assay

3.7.8.1 Thin layer chromatography

1. Solvents

Chloroform, methanol, diethyl ether, and acetone were obtained in bulk and all were redistilled. These solvents were used in all extraction mixtures and for thin layer chromatography.

2. Preparation of TLC plates

Thin layers of silica gel G were spread with a Desaga spreader (Heidelberg, West Germany) to the desired thickness. Thin layers were spread on microscope slides with a glass rod. TLC plates were activated at 110°C - 130°C for at least 1 h and then stored over saturated $CaCl_2$ solution in a sealed container.

3. Detection of lipids

Iodine vapour and 2,7-dichlorofluorosceine (10mg/250 ml of methanol) were used as general methods for the detection of lipids on TLC. Lipids recovered from TLC were freed of dichlorofluorosceine by the method of Arvidson (1967). Phospholipids were detected using the spray reagent of Vaskovsky & Kotetsky (1968). Phosphatidylethanolamine was detected specifically by the heat-developed (110°C, 10 min), amino acid and amine spray reagent described by Husbands (1964), i.e 0.3 g of ninhydrin dissolved in 95 ml of methanol with 5'ml of 2,4,6-trimethylpyridine.

4. Preparation of phospholipid markers

Phosphatidylcholine (PC) was prepared from total egg lipids, extracted according to the method of Folch et al., (1957) from fresh egg yolk. The egg lipids were then fractionated on an a column of Aluminoxide 90 (Merck). The column was eluted initially with chloroform, to remove the neutral lipids, and then with 9:1 chloroform:methanol (by volume). Fractions eluted by chloroform:methanol were monitored using TLC and those containing PC were concentrated and rechromatographed on TLC in 65:35:3 chloroform:methanol:water (by volume), in which PC had a R_f of 0.3-0.4 compared to a R_f of 0.9-1.0 for neutral lipids, to yield pure PC. Phosphatidic acid (PA), required as a TLC standard and as a carrier in experiments to identify the products of the GPAT reaction, was prepared from the above PC through the action of phospholipase-D from fresh cabbage (Bergelson, 1980; Dawson & Hemington, 1967; Davidson & Long, 1958; Kates & Sastry, 1969). The reaction mixture was extracted with 3 volumes of 2:1 (v/v) chloroform:methanol and washed with 0.2 volumes of chloroform:methanol:0.01 M $CaCl_2$ (3:47:45, by volume). The PA was purified on TLC in chloroform:methanol:acetic acid:water 50:28:10:5 (by volume) (Skipski et al., 1967). Additional egg lipids, for use as TLC markers, were extracted from an acetone precipitation of fresh egg yolk (Brandt & Lands, 1967) by the method of Folch et al., (1957).

3.7.8.2 Protocol for identification radioactive products of the microsomal GPAT assay

After completion of the experiments in which the response to [glycerol 3-phosphate] and [palmitoyl-CoA] was shown to be characteristic of the microsomal GPAT, further verification was sought

through identification of the products of the GPAT assay. Eight replicated reactions were carried out and contained in a total volume of 0.35 ml: 50 mM Tris-HCl, pH 7.4; 30 µM BSA; 15 mM β-mercaptoethanol; 3 mM [^{14}C]glycerol 3-phosphate; 101 µM palmitoyl-CoA; and 0.82 mg and 0.46 mg of microsomal protein was used in the reactions in part 1 and 2 below, respectively, and assays were terminated after 1 min. Reactions were extracted with butanol (see section 3.6), two of the reactions counted to estimate the amount of [^{14}C] radioactivity present (section 3.5) and the extracts of the remaining 6 reactions bulked. 0.5 mg of phosphatidic acid was added to carry the ^{14}C products.

TLC of microsomal GPAT assay products- Part 1

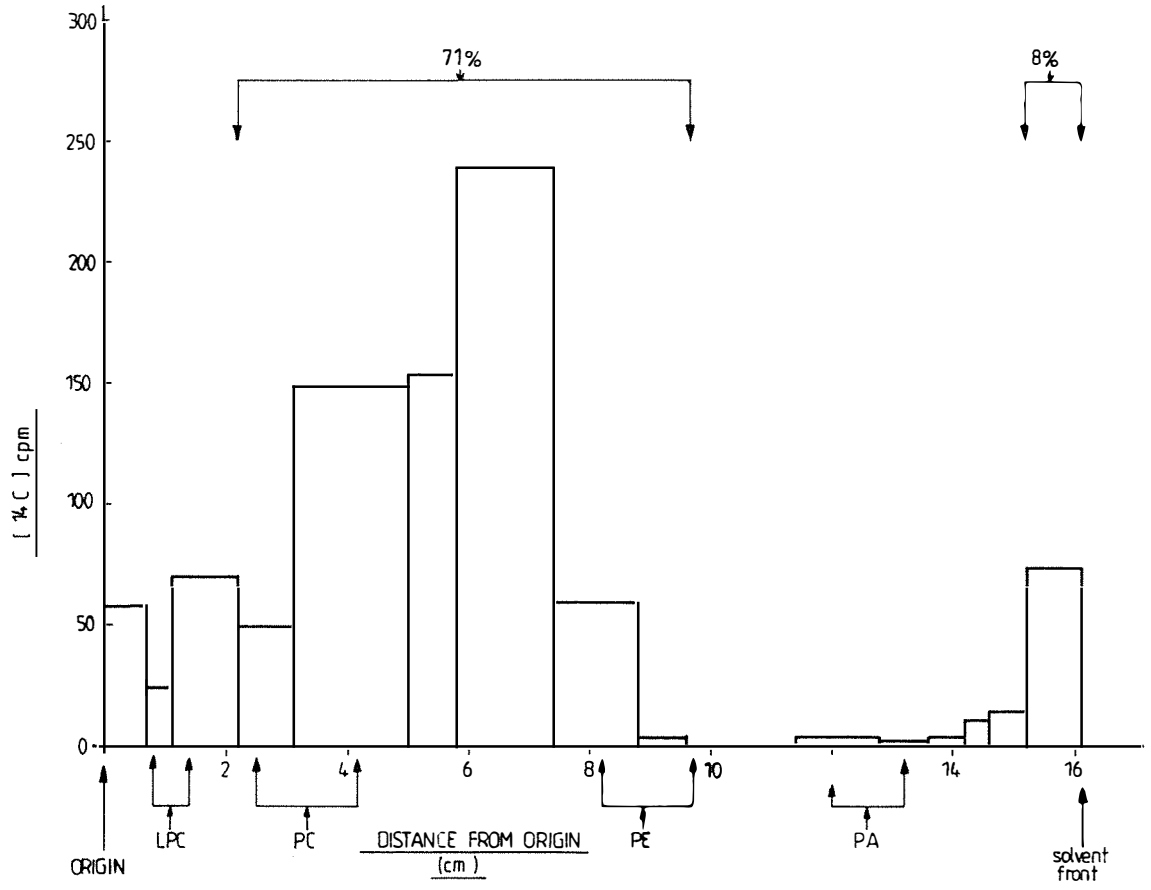
An attempt was made to identify the assay products directly. The [^{14}C]-lipids from the GPAT assay were chromatographed on a silica gel G thin layer and developed in chloroform:methanol:acetic acid:water 50:25:4:2 (by volume) (Skipski et al., 1967). Egg phospholipids and phosphatidic acid were run as markers. Lipids were visualised with iodine vapour and the plate divided into bands which were removed to determine the radioactivity in the Triton-toluene scintillant (see section 3.5).

This initial attempt to identify the [^{14}C]-lipid products of the hepatic microsomal GPAT assay was unsuccessful (Fig 3.11). Although carrier phosphatidic acid was added, very little (approximately 1%) of the [^{14}C]-lipid co-chromatographed with the phosphatidic acid marker. Approximately 71% of the products of the microsomal GPAT assay chromatographed between the phosphatidylcholine and the

phosphatidylethanolamine markers. Recovery of [^{14}C] cpm added to the plate was 79.4%. The inability of this set of experiments to resolve the nature of the microsomal GPAT assay products may have been due to different salt forms of the [^{14}C]-lipids leading to streaking in this solvent system. The failure of the [^{14}C]-lipid to co-chromatograph with the phosphatidic acid marker was unexpected as virtually all reports in the literature have identified phosphatidic acid as the main product of the microsomal GPAT assay (see Chapter 1).

Fig 3.11: Identification of microsomal GPAT assay products - Part 1.

Microsomal GPAT assay products labelled with [^{14}C] were chromatographed on silica gel G (0.4mm) in chloroform: methanol: acetic acid: water 50:25:4:2 (by volume). GPAT assay products were isolated from reactions as described in the text. Recovery of the [^{14}C] added to the plate was 79.4%. 6.2% of the recovered cpm remained at the origin while 71.2% of the recovered cpm chromatographed with and between the PC and PE markers. 8.1% of the cpm chromatographed at the solvent front. Markers were lysophosphatidylcholine (LPC), phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidic acid (PA).


300
250
200
150
100
50
0
[14C] cpm
71%
8%
2
4
6
8
10
14
16
ORIGIN
LPC
PC
DISTANCE FROM ORIGIN
(cm)
PE
PA
solvent
front

TLC of microsomal GPAT assay products - Part 2

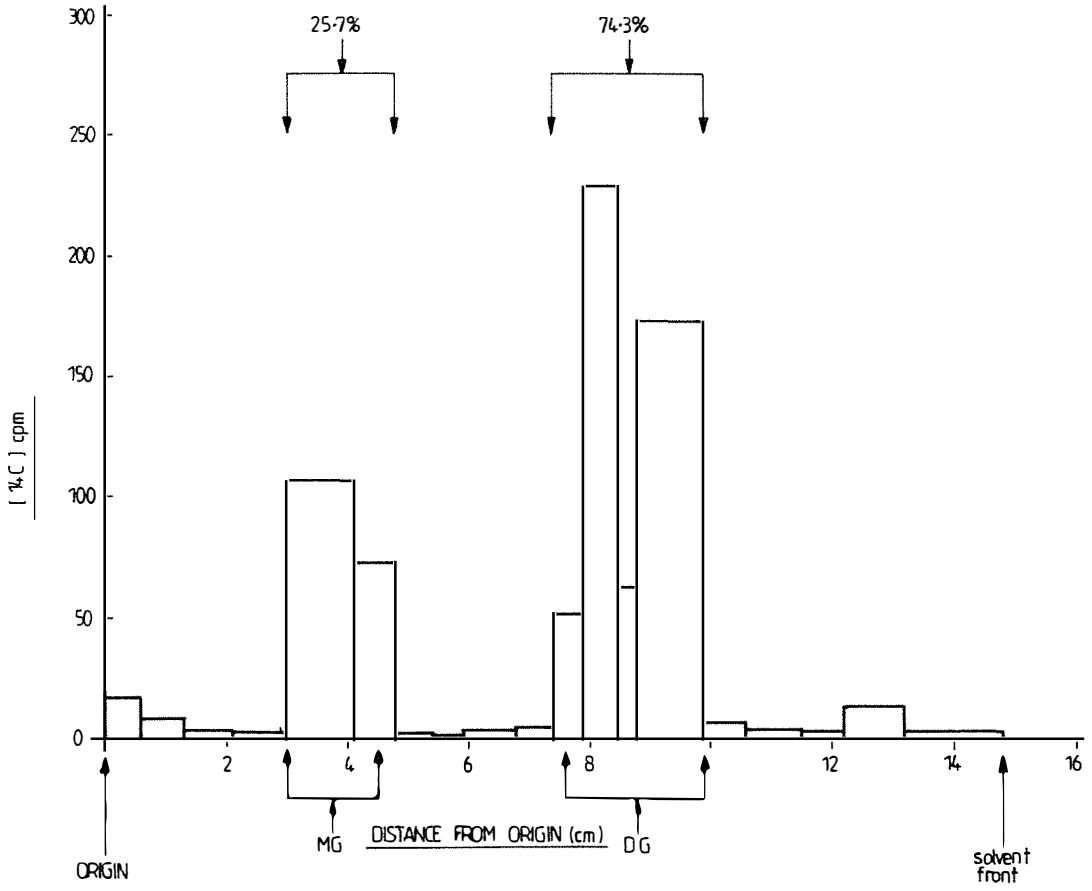
As the attempt to identify the hepatic microsomal GPAT products directly was unsuccessful, the [^{14}C]-products were subjected to hydrolysis catalysed by chicken liver phosphatidate phosphohydrolase. The phosphatidate phosphohydrolase was prepared from livers of 1 week old cockerals by the method of Smith et al. (1957) except that a microsomal fraction was prepared directly by the shortened method for preparation of microsomes from rat liver (sections 3.2 and 3.3). The radioactive lipid products, along with carrier phosphatidic acid were hydrolysed according to the method of Smith et al. (1957) for 2.5 h at 37^{o}C. The hydrolysis products were extracted according to the method of Folch et al. (1957) and carrier monoacylglycerol and diacylglycerol were added. A solvent system was developed so that monoacylglycerol and diacylglycerol had R_fs of 0.19-0.31 and 0.63-0.77, respectively. This hexane:diethyl-ether:acetic acid (50:80:1, by volume) solvent system was used to separate the hydrolysis products on TLC. Lipids on the developed plate were visualised with iodine vapour and the plate divided into sectors which were combined with Triton-toluene scintillant (section 3.5) to determine the radioactivity.

The results of the TLC of the hydrolysed GPAT assay products are shown in Fig 3.12. The recovery of [^{14}C] added to the TLC plate was 104.5%. When the [^{14}C]-acylglycerols were separated on TLC (Fig 3.12), over 90% of the total radioactivity on the plate was associated with either the MG or DG markers and of this radioactivity only 26% co-chromatographed with the MG marker while 74% co-chromatographed with the DG marker. Only 2% the [^{14}C] cpm added to the plate remained at

the origin suggesting that the hydrolysis by chicken liver phosphatidate phosphohydrolase had been complete. In Part 1 above, 8% of the radioactive products of the GPAT assay chromatographed at the solvent front in the solvent system used (see Fig 3.11). This suggested that some of the assay products may have been neutral lipids. However, an aliquot of the assay products, removed prior to the hydrolysis, did not contain any compounds that co-chromatographed with the MG or DG markers when run in the Part 2 solvent system. Instead the radioactivity remained at the origin as would be expected if the original GPAT assay products were solely phospholipids. It follows therefore that approximately 74% and 26% of the original microsomal GPAT assay products were phosphatidic and lysophosphatidic acid, respectively. This agrees well with literature reports of the products of microsomal GPAT assays (see Chapter 1). The high proportion of phosphatidic acid is due to the presence of lysophosphatidic acid acyltransferase which is predominantly located in the microsomes (Eibl et al., 1969; Haldar et al., 1979; Okuyama et al., 1971; Saggerson et al., 1980). Therefore, this examination of the GPAT assay products provided additional evidence that the microsomal GPAT was being assayed by the system used.

Fig 3.12: Identification of microsomal GPAT assay products - Part 2.

Microsomal GPAT assay products labelled with [^{14}C] were reacted with phosphatidate phosphohydrolase and chromatographed on silica gel G (0.4mm) in hexane: diethyl ether: acetic acid 50:80:1 (by volume). GPAT products were isolated from assays conducted as described in the text. Recovery of the [^{14}C] cpm added to the plate was 104.5%. Of the [^{14}C] associated with the MG and DG markers (90.3%), the percentage associated with the markers is given at the top of the figure. 2.0% of the [^{14}C] cpm recovered remained at the origin. Markers were monoacylglycerol (MG), and diacylglycerol (DG).


25·7%
74·3%
300
250
200
150
100
50
0
[14C] cpm
2
4
6
8
12
14
16
ORIGIN
MG
DISTANCE FROM ORIGIN (cm)
DG
solvent front

3.8 Summary of methodology adopted for the assay of GPAT

After verifying that the hepatic microsomal esterification system was appropriate for assaying GPAT activity the following methodology was adopted. The reaction, which was carried out in a total volume of 0.35 ml and at 30°C, contained:

50 mM Tris-HCl pH 7.4

30 μM BSA[a]

15 mM β-mercaptoethanol

3 mM sn-glycerol 3-phosphate

1 mg microsomal protein

101 μM palmitoyl-CoA

[a]reference Jamdar, 1979

and the following procedure was adopted:

1. The assays were allowed to eqilibrate at 30°C for at least 5 min after which time the reaction was started by the addition of the palmitoyl-CoA.
2. The reaction was terminated after 1 min by the addition of 2.2 ml butanol and 10 ml butanol-saturated water. Zero time reactions were terminated as soon as the palmitoyl-CoA was added and thereafter treated the same as the other reactions in order to estimate the [^{14}C]α-GP carried over into the washed butanol phases. The butanol phases were washed as described in section 3.6 and the radioactivity counted as described in section 3.5.

CHAPTER FOUR

EXPERIMENTS TO EXAMINE THE EFFECT OF INSULIN ON GPAT ACTIVITY

Chapter 4. EXPERIMENTS TO EXAMINE THE EFFECT OF INSULIN ON GPAT ACTIVITY

4.1 Introduction

As discussed in Chapter 1, there is evidence that hepatic microsomal GPAT is important in the regulation of glycerolipid metabolism and that its activity is affected by insulin. Recent reports concerned with the mechanism of action of insulin have demonstrated insulin-dependent effects on a number of key enzymes through the use of low molecular weight fractions of muscle extracts (Jarett & Seals, 1979b; Larner et al., 1979d). Using these, insulin-dependent inhibition of cAMP-dependent protein kinase from muscle and insulin-dependent stimulation of muscle glycogen synthase phosphoprotein phosphatase were shown (Larner et al., 1979d). These fractions also enhanced adipocyte mitochondrial pyruvate dehydrogenase activity in an insulin-dependent manner (Jarett & Seals, 1979b). Therefore, similar methodology was employed in examining the possibility of demonstrating an insulin-dependent enhancement of hepatic microsomal GPAT activity. Such a demonstration would provide further evidence that GPAT is regulated by insulin.

In the present study, based on the method of Larner et al. (1979d), rats were treated with insulin or saline (control). Extracts were prepared from hind limb muscle and then chromatographed on Sephadex G-25 to obtain low molecular weight fractions encompassing a molecular weight range similar to that obtained by Larner et al.

(1979d), i.e. approximately 1000-4000 daltons. Therefore, fractions eluting subsequent to the void volume and prior to the salt peak were considered to be of special interest. These fractions were assayed to detemine whether they stimulated hepatic microsomal GPAT in an insulin-dependent manner. It was assumed that the liquid nitrogen used, in the method of Larner et al. (1979d) initially for freezing and later when grinding the muscle, was to ensure rapid cooling and to prevent enzymatic degradation of potentially short-lived substances necessary for the demonstration of insulin-dependent effects on insulin-sensitive enzymes. Owing to unavailability of liquid nitrogen, liquid air was substituted for liquid nitrogen in the method adopted. The charcoal extraction step, generally accepted to be somewhat variable, but part of the method of Larner et al. (1979d) was assumed to have been included because the enzymes of their studies are affected by nucleotides. Such effects have not been demonstrated for hepatic microsomal GPAT (Chapter 1). It was considered that the initial boiling under acid conditions, during preparation of the extract, and subsequent acid conditions would degrade the nucleotides (Dawson et al., 1969).

4.2 Methods

4.2.1 Effect of insulin on blood glucose

The efficacy of the insulin was verified by examining its effect on blood glucose concentration.

1. A Sprague-Dawley rat (387 g; female) was fasted overnight to lower the concentration of endogenous insulin and anaesthetised with an intraperitoneal injection of urethane (1290 mg per kg body weight).
2. The rat was cannulated in the right jugular vein and 0.3 ml of heparinised 0.15 M-NaCl (125 units per ml) was introduced through the cannula to heparinise the whole animal.
3. Two basal blood samples of 0.15 ml were taken 10 min apart. After the second sample the animal was given insulin (1 i.u. per kg body weight) via the cannula and further blood samples were taken at 13 min, 25 min, 40 min, and 60 min.

Blood glucose was estimated according to the method of Tietz (1976). The effect of insulin administration on the concentration of glucose in the blood of the rat is shown in Fig 4.1. Insulin treatment produced a substantial reduction in blood glucose concentration consistent with the generally accepted action of insulin. Therefore, it was concluded that it was suitable for use in this study.

4.2.2 General method for preparation of rat muscle extracts

Extract from rat muscle was prepared based on the method of Larner *et al*. (1979d), but with the procedural differences noted in the introduction above. Minor deviations from this general method, where they occur, are noted in the text and figure legends. The general

procedure was as follows:

1. Male Sprague-Dawley rats (200 - 400 g) were anaesthetised by intraperitoneal administration of urethane (0.780 g/kg body weight + 0.150 g initially and then as required to maintain anaesthesia).
2. The rats were cannulated in the right jugular vein and 0.2 ml of heparinised (125 units per ml) saline (0.15 M NaCl) was given intraveneously to clear the cannula.
3. Four minutes later 4 i.u. of insulin per kg body weight was given through the cannula and washed in with 0.2 ml of heparinised saline. Control animals were given 0.3 ml of non-heparinised saline in the place of insulin (saline treatment).
4. The hind limb muscle was exteriorised during the 5 min following the insulin/saline administration.
5. Five minutes after the insulin/saline administration the muscle was removed, quickly cut into small pieces and frozen in liquid air.
6. The weighed frozen rat muscle from either the insulin- or saline-treated rats was ground to powder under liquid air with a mortar and pestle.
7. The powder was slurried with 3.65 ml of cysteine-EDTA solution (0.3 mM cysteine, 0.3 mM EDTA) per gram of muscle and acidified to pH 3.8 with 17.5 M acetic acid. The curve for the titration of resuspended powdered rat muscle with 17.5 M acetic acid is shown in Fig 4.2. The slurry was then diluted with distilled deionised water to give final cysteine and EDTA concentrations of 0.08 mM.
8. The slurry was heated to 100^{o}C, in glass with stirring, held at this temperature for 7 min, cooled on ice for 70 min to 2^{o}C and centrifuged for 10 min at 7070**g** ($r_{av.}$ 6.9 cm).
9. The supernatant from step 8 was filtered through glass wool and

then evaporated under vacuum below 50°C until dry using n-octanol as an antifoaming agent.

10. The dried extract was stored over KOH and P_2O_5 under vacuum.

4.2.3 Chromatography of extracts

Extracts, prepared as described, were chromatographed on Sephadex G-25. The general procedure for preparation of columns and chromatography of the extract is outlined below; specific details are given in appropriate figure legends.

1. An appropriate amount of dry Sephadex G-25 was weighed out and rehydrated for several hours (as recommended by Pharmacia) in 50 mM formic acid and then poured into the column.
2. Columns were characterised with blue dextran (2 000 000 daltons) DNP-aspartate (326 daltons), or KCl. Relative amounts of blue dextran and DNP-aspartate in each fraction were determined by measuring absorbance at 605nm and 360nm, respectively, while the concentration of K^+ was determined by flame emission spectrophotometry. Where specified, the Sephadex G-25 column was also characterised with porcine insulin (6 000 daltons), decapeptide (1 064 daltons), ATP (551 daltons). The relative amount of the decapeptide and insulin were estimated by their ninhydrin reactivity. ATP was located using Bial's test for pentoses. Ninhydrin reagent was prepared (Pers. Comm., Dr. C. Moore, Dept of Chemistry & Biochemistry, Massey University) by mixing 112.5 ml of methyl cellosolve with 37.5 ml of 4 M sodium acetate buffer (pH 5.5). This was then degassed by flushing with O_2-free N_2 for 10 min, after which 3 g of ninhydrin was dissolved in the solution and the solution was again flushed with N_2 for

10 min. Hydrindantin (0.45 g) was then added and the final solution was stored under N_2 at 4°C. 2 ml of the ninhydrin reagent was added to 0.25 ml aliquots of column fractions and heated at 100°C for 15 min. After cooling, 7.75 ml of ethanol : water (1:1, by volume) was added to each tube and the absorbance read at 570nm against a reagent blank. Bial's test for pentoses, based on a method described by Dische (1955) was used semi-quantitatively to identify the fractions that contained ATP. 2 ml aliquots of the column fractions were added to 1 ml of water and then 6 ml of orcinol reagent (0.05% $FeCl_3$ in concentrated HCl) was added followed by 0.4 ml of 6% alcoholic orcinol (6 g per 100 ml 95% ethanol). The tubes were then heated at 100°C 20 min and after cooling the absorbance was determined at 600nm against a reagent blank.

3. The solvent used to elute the column in all experiments was 50 mM formic acid and all column chromatography was carried out at 0 - 4°C.
4. Resuspended rat muscle extract (4mg/ml) in 50 mM formic acid was chromatographed on the Sephadex G-25 column in 50 mM formic acid at 4°C. The fractions were stored at 4°C overnight prior to assay with GPAT.
5. The eluting solvent was pumped through the column using a peristaltic pump and fractions were collected on a LKB brand fraction collector. The absorbance of column fractions was measured at 260nm, 254nm, or 220nm.
6. When not in use the column was filled with 0.05% sodium azide in 50 mM formic acid. This storage solvent was always washed out thoroughly before the column was used by eluting the column with

approximately 200 ml of 50 mM formic acid.

4.2.4 Assay of Sephadex G-25 fractions with liver microsomal GPAT

Sephadex G-25 fractions of muscle extract from rats treated with insulin or saline were assayed with microsomal GPAT. The microsomal GPAT assay (section 3.8) was modified to include Sepahdex G-25 fractions of rat muscle extracts:

1. 98 µl volumes of Sephadex G-25 fractions of insulin- or saline-treated rat muscle extracts were included in separate assays to determine their effects on liver microsomal GPAT.
2. As the fractions were 50 mM with respect to formic acid, 6.1 µmol of Tris as the N-base was included in the assay to maintain the reaction pH at 7.4.
3. The GPAT reactions were thermally equilibrated for at least 4 min, after which the microsomal enzyme protein was added.
4. After 20 min, the reaction was started by the addition of palmitoyl-CoA and the subsequent procedure was as described in section 3.8.
5. Assays were conducted in either triplicate or duplicate as described in the figure and table legends.

Fig 4.1: The effect of insulin on blood glucose concentration. A rat was fasted overnight, then cannulated in the jugular vein and given 1 i.u. insulin per kg body weight immediately after the second basal sample was withdrawn. Blood glucose concentration was measured at the times indicated.

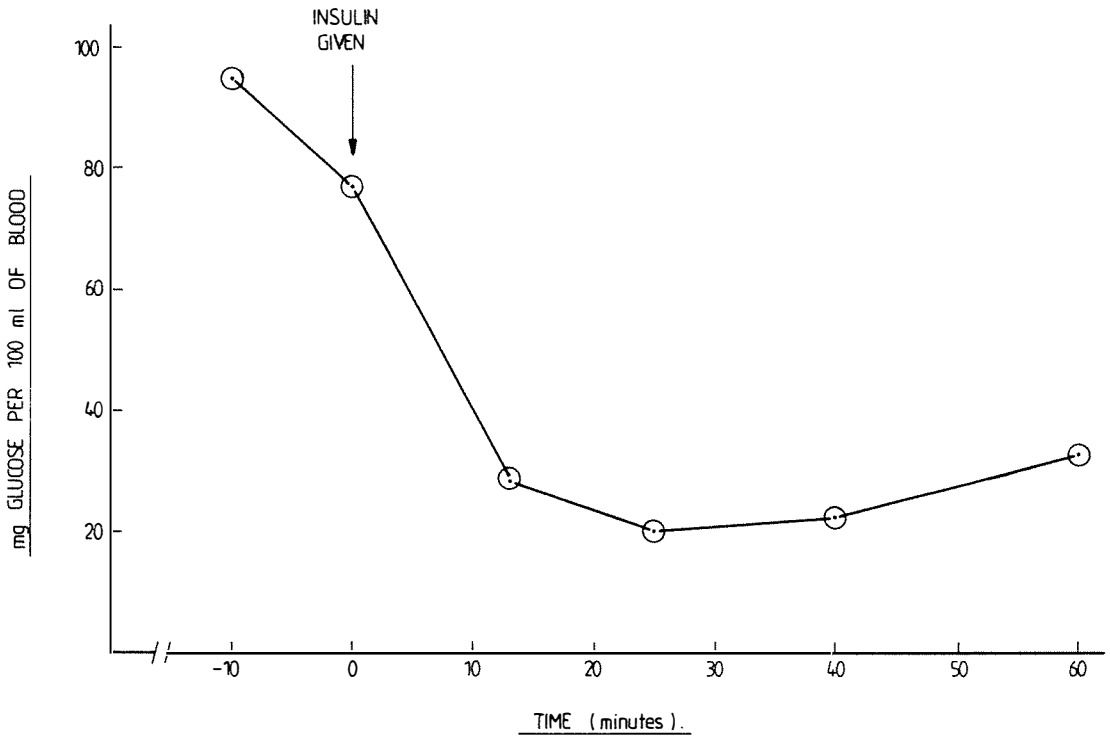
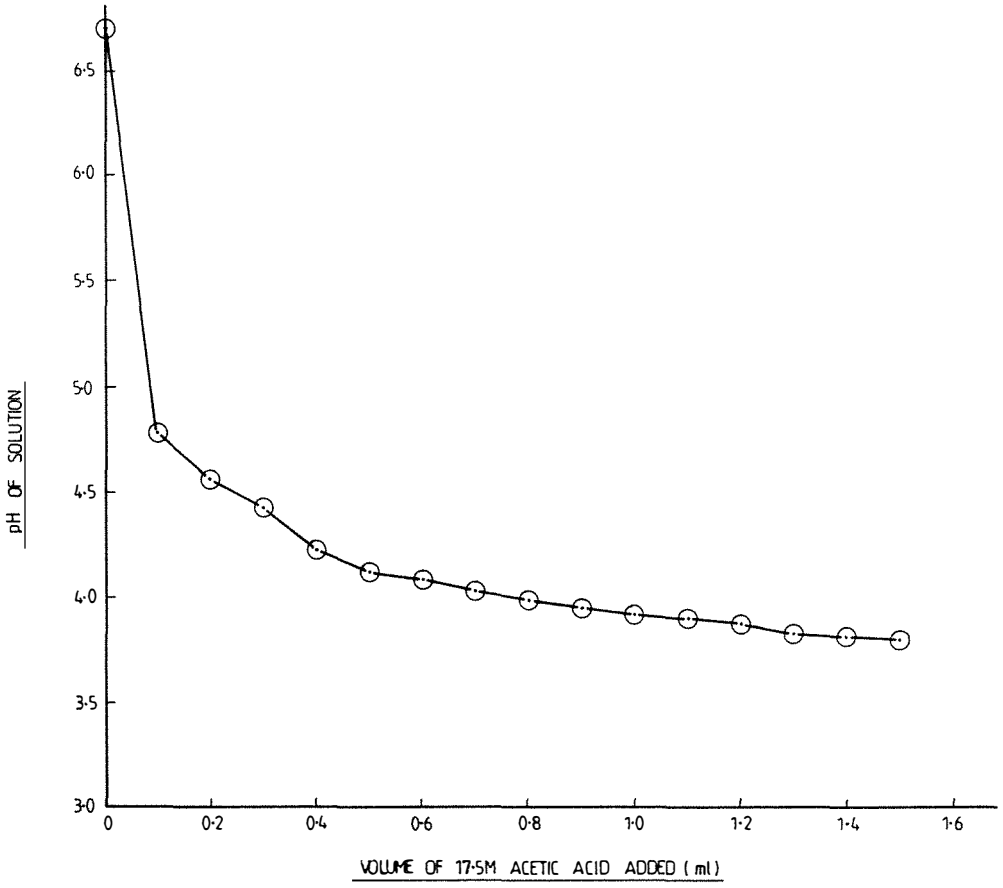

INSULIN
GIVEN
100
80
60
40
20
mg GLUCOSE PER 100 ml OF BLOOD
-10
0
10
20
30
40
50
60
TIME (minutes).

Fig 4.2: Titration curve of rat muscle solution acidified with acetic acid to pH 3.8. 16.4 g of powdered rat muscle, resuspended as described (4.2.2 (7)) was titrated with 17.5 M acetic acid as part of the procedure for the preparation of the rat muscle extract.


pH OF SOLUTION
6·5
6·0
5·5
5·0
4·5
4·0
3·5
3·0
0
0·2
0·4
0·6
0·8
1·0
1·2
1·4
1·6
VOLUME OF 17·5M ACETIC ACID ADDED (ml)

4.3 Results and Discussion

4.3.1 Section 1 - Preliminary experiments with extracts from heparinised rats treated with insulin (Extract A) or saline (Extract B), respectively: the effect of low molecular weight fractions of these extracts on GPAT activity

Initially a muscle extract (Extract A) was prepared from a heparinised rat treated with insulin and fractionated on Sephadex G-25. The effect of a number of these fractions on microsomal GPAT from fed rat liver was then determined (Experiment 1, Fig 4.3) to examine the possibility of demonstrating an insulin-dependent stimulation of GPAT. Hepatic microsomal GPAT from fed rats was used as it seemed logical that insulin-dependent effects would be evident in the fed state. It was found that the GPAT was, relative to the formic acid control, stimulated markedly by fractions eluting just prior to, with, and after the salt peak. Fractions eluting just prior to the salt peak stimulated GPAT activity to 108% of the formic acid control corresponding to an absolute increase in GPAT activity, above the control rate (stimulated GPAT rate minus formic acid control rate = absolute increase) of about +0.3 nmol/min/mg (Experiment 1, Fig 4.3, Table 4.1). The fractions eluting at or after the salt peak enhanced GPAT activity by $\leq$ 1.22 nmol/min/mg (132%, $P<0.01$) above the formic acid control.

The effect of fractions from Extract A (insulin treatment) on microsomal GPAT from 72 h-fasted rats was examined (Fig 4.3) because of

evidence in the literature that fasting reduced hepatic microsomal GPAT activity to as low as 30% of controls (Mangiapane et al., 1973; Van Tol, 1974). It seemed reasonable to suggest that GPAT from the fed rat could already be in an "enhanced" (or "partially enhanced") state due to the action of endogenous insulin in the fed rat. Hence, GPAT from livers of fasted rats might be in a "down-regulated" state and might therefore have greater potential to respond to stimulatory material. Catecholamines and cAMP inhibit microsomal GPAT and insulin abolishes the inhibitory effect of adrenaline on GPAT (see section 1.3.4.4). Also a recent report shows that treatment of anaesthetised 24 h-fasted rats with insulin (1 i.u./kg body weight) for 5 min increases the rate of adipose tissue esterification to 153% of the saline treated controls (Wilson, 1983).

Whereas GPAT from fed rats was stimulated by fractions of Extract A, GPAT from 72 h-fasted rats was inhibited by most fractions assayed (Fig 4.3, Experiment 2). Fraction 90 (Fig 4.3), however, stimulated 72 h-fasted rat liver GPAT activity by about the same amount that it stimulated GPAT activity from fed rats i.e. to about 113% of the formic acid control vs 108% of the formic acid control, respectively, corresponding to absolute increases in GPAT rates of about 0.5 ($P<0.10$) and 0.3 nmol/min/mg. Fractions eluting before and after fraction 90 (Fig 4.3) reduced the activity of microsomal GPAT from fasted rat liver by as much as 0.56 nmol/min/mg below the formic acid control rate (to about 85% of the formic acid control). These results were contrary to those expected for the reasons outlined above and also because these same fractions had previously stimulated microsomal GPAT from fed rats. For example, fractions 80, 93, (both from Expt 1, Table 4.1, Fig 4.3),

stimulated GPAT activity to 111%, 121% of formic acid controls, respectively, corresponding to absolute changes in GPAT activity of +0.41, +0.81 nmol/min/mg.

It was considered desirable to confirm the results of Experiment 1 and so the same fractions of Extract A were re-assayed with GPAT from livers of fed rats (Expt 3, Fig 4.4; Table 4.2). These fractions enhanced GPAT activity as they did in Experiment 1, but this time by $\leq$ +1.03 nmol/min/mg above the formic acid control rate ($\leq$174% of the formic acid control; fraction 104, Fig 4.4 and Table 4.2). Fractions eluting between the void and the salt peaks enhanced GPAT activity by $\leq$ +0.89 nmol/min/mg ($P<0.01$) above the formic acid control rate (164%; fraction 76, Fig 4.4 and Table 4.2).

Although fractions of Extract A stimulated GPAT activity relative to the formic acid control (i.e. just elution buffer in the GPAT assay) there was some question as to what was an appropriate control for this study. The possibility existed that effects observed with fractions of muscle extract from rats treated with insulin would be no different from the effects of the fractions of muscle extract from rats treated with saline. To address this question an extract (Extract B) was prepared from a heparinised rat treated with saline (as the insulin was carried in 0.9% NaCl). The effect of fractions of Extract A (insulin treatment) relative to the effect of Extract B (saline treatment) would provide some indication as to whether the effects of fractions of Extract A on GPAT were actually insulin dependent. GPAT from livers of fed rats was inhibited by fractions from of Extract B (Experiment 4, Fig 4.4, Table 4.2), i.e. GPAT activity was reduced by

about 0.15 nmol/min/mg below the formic acid control rate. However, fraction 113 reduced GPAT activity by 0.36 nmol/min/mg below the control rate (about 92%). This was in contrast to the effect of fractions of Extract A on GPAT from fed rat (Experiment 3). Comparison of the effects of fractions of Extracts A and B on microsomal GPAT from fed rat liver suggested an insulin-dependent enhancement of GPAT activity. Comparison of the effect of Extract B (saline treatment) on GPAT with the formic acid control suggested that fractions of the saline treatment had only a slight inhibitory effect. Therefore, the formic acid control did not appear to be entirely inappropriate for this study. However, as there was some effect of fractions from the saline treatment extract it was thought advisable to compare the effect of insulin treatment fractions with that of saline treatment fractions. Material present in the Sephadex G-25 fractions of Extract A caused quite marked stimulation of the GPAT activity from fed rats relative to the formic acid control rate (Expt 3, Fig 4.4). GPAT activity was stimulated to 164% ($P<0.01$) of the formic acid control by material present in fraction 76. The equivalent saline-treatment fraction 76 inhibited GPAT activity to about 96% of its formic acid control (Expt 4, Fig 4.4). Assuming that the percentage effects of the insulin and saline treatments are additive then there was a 68% stimulation of GPAT activity, apparently attributable to the insulin treatment. However, such a comparison of the percentage effects must rely on the rates of the formic acid controls of the insulin and saline treatment experiments not differing significantly. It was recognised that ideally the insulin and saline treatment extracts should have been prepared, fractionated, and assayed in parallel, with the same microsomal GPAT preparation. However, the time required to prepare the

extract, to fractionate it on Sephadex G-25 and to conduct the GPAT assays, prevented this from being done. Microsomal GPAT is unstable, even in the presence of β-mercaptoethanol, and so that refrigeration of the GPAT for use in the next assay was not practical. GPAT is reported to be stable at -70°C (Carrol et al., 1982), but as GPAT is membrane-bound and ice crystals disrupt membrane structure on freezing-thawing, it was considered undesirable to introduce another variable into the present study. In the above case, however, the formic acid control rates of the insulin and saline treatments differed significantly (1.39 ± 0.18 vs 4.35 ± 0.10 (SEM) nmol/min/mg, respectively; $P<0.01$). Nevertheless, when the formic acid control rates were subtracted from the GPAT rate obtained in the presence of the respective Sephadex G-25 fractions then, for example, it was found that fraction 76 of the insulin treatment caused a 0.89 nmol/min/mg increase in GPAT activity whereas the equivalent fraction from the saline treatment reduced GPAT activity by 0.16 nmol/min/mg. Again, assuming that the effects of these treatments are additive, the insulin-dependent enhancement of GPAT activity in the fed rat liver microsomes was 1.05 nmol per min per mg of microsomal protein. If the 1.05 nmol/min/mg insulin-dependent increase in GPAT rate is (with reservations) expressed as a percentage of the mean of the insulin and saline treatment formic acid controls (i.e. 2.87 nmol/min/mg) then the insulin-dependent stimulation of GPAT activity appears to be of the order of 37%. The percentage effect of the insulin treatment on the microsomal GPAT is of a similar magnitude to that reported by Larner et al. (1979d) for the inhibition of cAMP dependent protein kinase by an insulin treatment Sephadex G-25 fraction of rat muscle extract relative to the effect of the equivalent saline treatment extract fraction.

However, the 20 to 30% insulin-dependent inhibition of cAMP dependent protein kinase reported by Larner et al. (1979d) was derived in an unusual way i.e. the saline control treatment fraction caused a 45.6% inhibition of the protein kinase (in the absence of cAMP) while the insulin treatment fraction caused a 60.4% inhibition. The difference between these two figures is 14.8% (i.e. 60.4 - 45.6). Larner et al (1979d) expressed this as a percentage of the 45.6% figure (i.e. 14.8/45.6 x 100/1) to get a "Net effect due to insulin" of 33%. This procedure was followed for other calculations and magnified the differences attributable to the insulin treatment by 2 to 3 times. Standard errors and control rates for the enzymes studied were not reported. Hence as a percentage will depend on the divisor, the results were viewed with some concern. Similarly, Jarett & Seals (1979b), using the fractions of Larner et al (1979d), reported an approximately 20% insulin-dependent enhancement of pyruvate dehydrogenase activity in the presence or absence of ATP (which reduces pyruvate dehydrogenase activity from 6.28 to 2.47 nmol/min/mg). This corresponds to an absolute increase in rate of 0.54 and 1.11 nmol/min/mg. In the present study, even though the response, as a % of formic acid controls, varies from about +20% to +60%, the actual absolute changes in GPAT rate per mg of protein were relatively constant increases within the range 0.4 to 0.9 nmol/min/mg (see Fig 4.4 and compare with Fig 4.3). This also highlights the way in which the use of percentages can be misleading.

In Experiment 5 the amount of Extract A (insulin treatment) fractionated on Sephadex G-25 was doubled (8.0 mg vs. 4.0 mg in the previous experiments) to examine whether twice as much extract would

increase the potential of fractions to stimulate GPAT activity. A saline-control was not chromatographed in this instance (see Fig 4.5 and Table 4.3). As observed in the previous experiments, the microsomal GPAT (from fed rat liver) was stimulated by fractions of the extract which eluted between the void and salt peaks (compare Fig 4.5 with 4.3). The percentage stimulation observed was less than when 4.0 mg of extract was chromatographed, i.e. a maximum of 117% of control vs. 164% of control in the previous experiments. The maximum changes in GPAT activity caused by these fractions were about half those of the previous experiments i.e. about +0.45 nmol/min/mg of protein (Fig 4.5 and Table 4.3). There was an apparent lack of stimulatory material present in fractions 80 to 110 (compare Fig 4.5 with Fig 4.4). The apparent reduction in enhancement of GPAT activity to an approximate maximum of 117% of the formic acid control (an absolute increase in activity of 0.46 nmol/min/mg) and an absence of material in the very low molecular weight fractions compared to the activation seen in earlier experiments (Figs 4.3, 4.4, Tables 4.1 and 4.2) suggested that there was no advantage to be gained by loading more than 4.0 mg of extract on the column.

Although inclusion of some proteins in the GPAT assay can enhance GPAT activity (see 1.4.3), it is unlikely that this was responsible for the stimulation of GPAT activity seen in these experiments as the acidification-boiling procedure would produce an extract relatively free of protein. However, if not all of the protein was removed, the remainder would be separated from the material of interest by the gel filtration chromatography, i.e. Sephadex G-25 has an exclusion limit of approximately 5000 daltons and so the fractions to be assayed should

contain no protein. Furthermore, with 100 μM palmitoyl-CoA and optimal BSA present in the GPAT assay, if additional protein was introduced into the assay (from a Sephadex G-25 fraction) this would reduce, not increase, the rate of esterification by GPAT (Husbands & Lands, 1970). Hence, any stimulation of GPAT activity by these fractions would not be due to addition of protein.

Fig 4.3: Sephadex G-25 fractionation of muscle extract from insulin-treated, heparinised rat and absolute effect of fractions on microsomal GPAT from livers of fed or fasted rats. 4.0 mg of extract (Extract A) from a heparinised rat, treated with insulin, was chromatographed in 50 mM formic acid on a 1.6 x 91 cm Sephadex G-25 column and eluted with 50 mM formic acid. 1.7 ml fractions were collected. In Experiment 1 Sephadex G-25 fractions from Extract A were assayed with microsomal GPAT prepared from fed rats (microsomes; see Fig 3.1) while in Experiment 2 the same fractions were assayed with microsomal GPAT from livers of 72 h-fasted rats. Assays were performed in triplicate and means (± SEM) are given in Table 4.1.

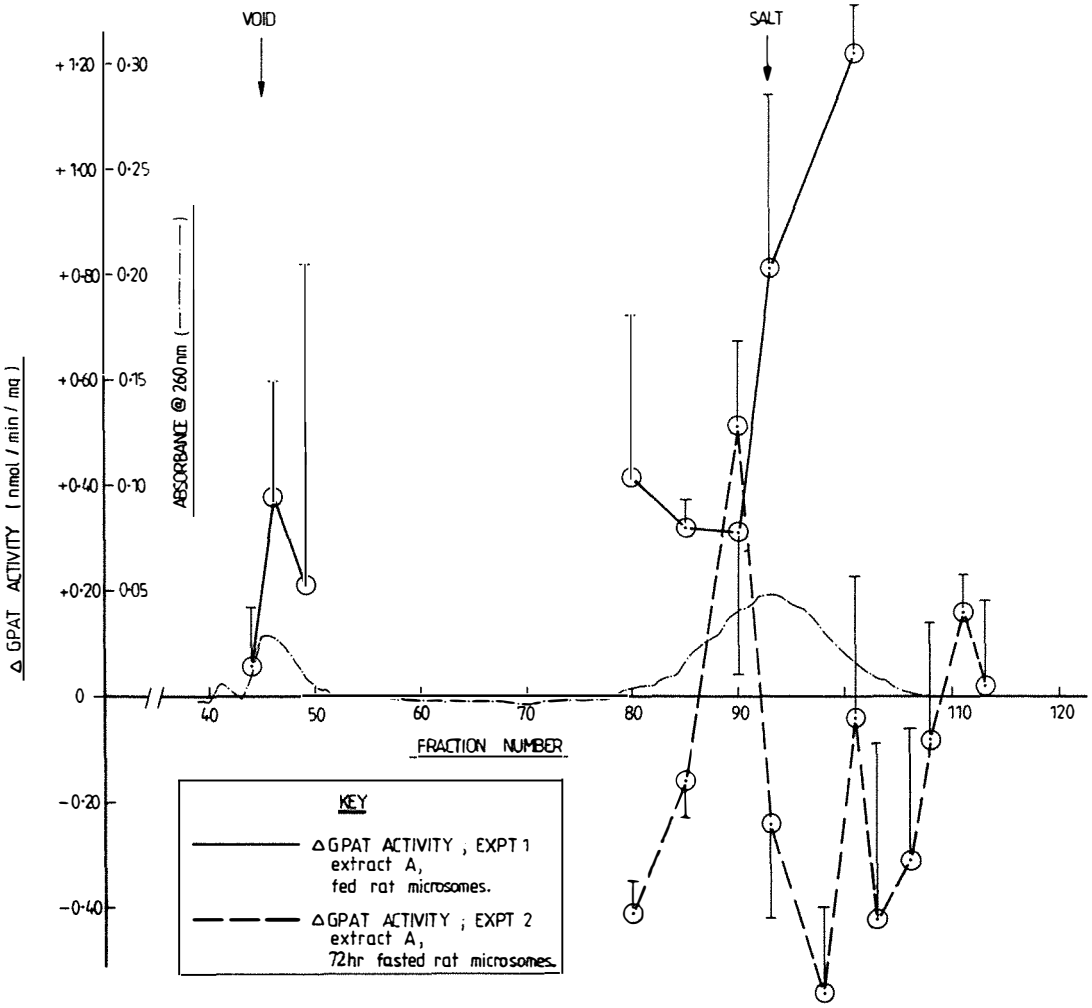
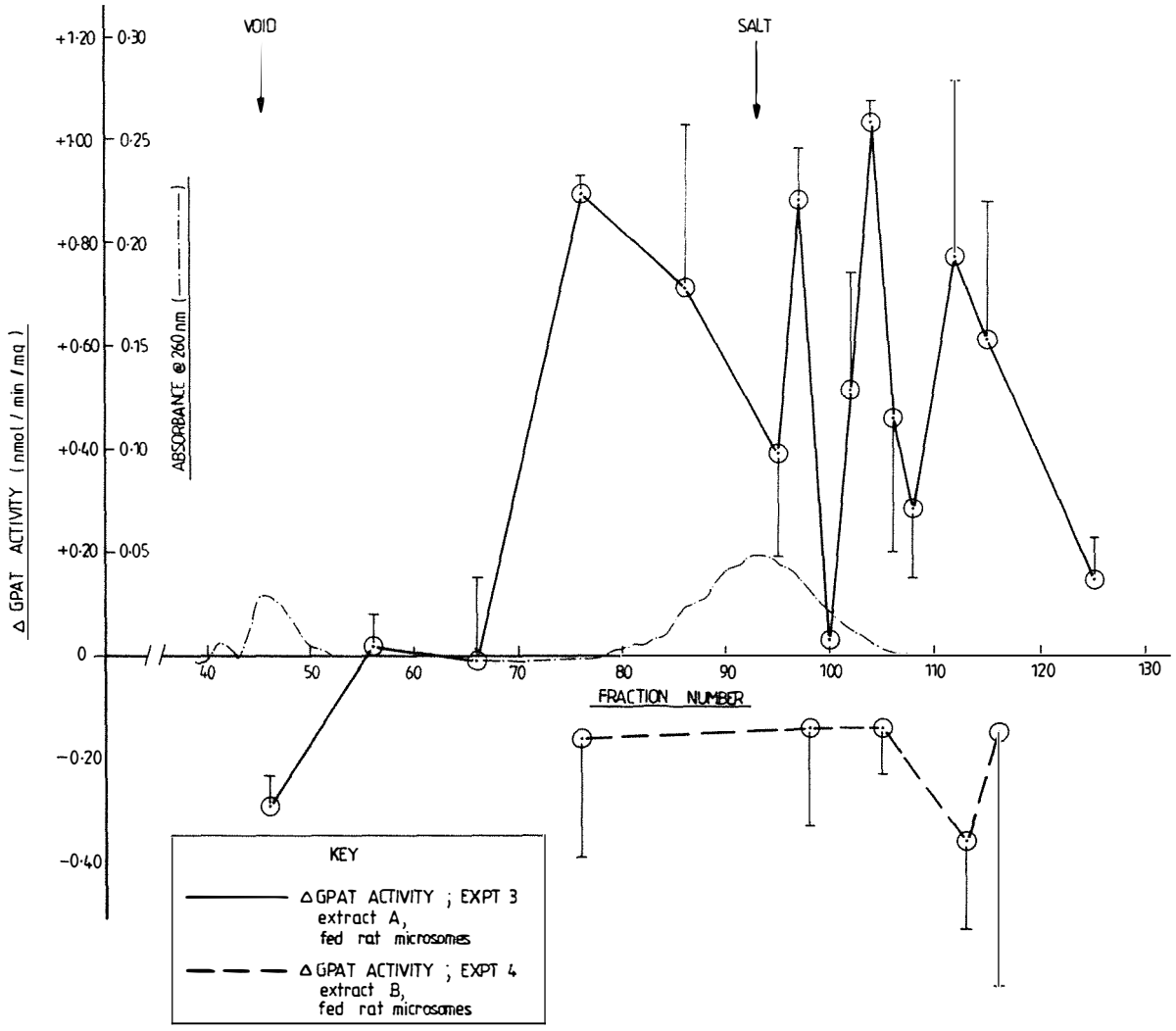

VOID
SALT
+1·20
+1·00
+0·80
+0·60
+0·40
+0·20
0
-0·20
-0·40
0·30
0·25
0·20
0·15
0·10
0·05
ABSORBANCE @ 260nm (——·——)
Δ GPAT ACTIVITY (nmol / min / mg)
40
50
60
70
80
90
110
120
FRACTION NUMBER
KEY
Δ GPAT ACTIVITY ; EXPT 1
extract A,
fed rat microsomes.
Δ GPAT ACTIVITY ; EXPT 2
extract A,
72hr fasted rat microsomes.

Table 4.1: The effect of Sephadex G-25 fractions of muscle extract (A) from heparinised rat treated with insulin on liver microsomal GPAT from fed rats or 72 h-fasted rats.

Fraction number	GPAT activity nmol/min/mg	deviation[d]	% of control
Experiment 1. - insulin-treatment[e], fed rat GPAT			
Control	3.79 ± 0.14		100.0
44	3.85 ± 0.11	+0.06	101.7
46 *	4.17 ± 0.22	+0.38	109.9
49 *	4.00 ± 0.61	+0.21	105.5
80 *	4.20 ± 0.31	+0.41	110.9
85 *	4.11 ± 0.05	+0.32	108.4
90 *	4.10 ± 0.27	+0.31	108.3
93 *	4.60 ± 0.33	+0.81	121.4
101	5.01 ± 0.09[c]	+1.22	132.2
Experiment 2. - insulin-treatment[e], 72 h-fasted rat GPAT			
Control	3.87 ± 0.11		100.0
80 *	3.46 ± 0.06[b]	-0.41	89.4
85 *	3.71 ± 0.07	-0.16	95.9
90 *	4.38 ± 0.16[a]	+0.51	113.2
93 *	3.63 ± 0.18	-0.24	93.9
98	3.31 ± 0.16[b]	-0.56	85.6
101	3.83 ± 0.27	-0.04	99.1
103	3.45 ± 0.33	-0.42	89.2
106	3.56 ± 0.25	-0.31	92.0
108	3.79 ± 0.22	-0.08	97.9
111	4.03 ± 0.07	+0.16	104.2
113	3.89 ± 0.16	+0.02	100.4

* Fractions eluting between void and salt peaks. Values are the means of triplicate assays ± SEM. Experimental details are described in the caption to Fig 4.3. Means (± SEM) with superscripts differ from controls [a]P<0.10, [b]P<0.05, [c]P<0.01, [d]GPAT rate minus GPAT formic acid control rate. [e]Extract A

Fig 4.4: Sephadex G-25 fractionation of muscle extract from heparinised rats treated with insulin and saline and absolute effect of fractions on microsomal GPAT from livers of fed rats. 4.0 mg of muscle extract, from heparinised rat treated with either insulin or saline (Extract A or B, respectively), in 50 mM formic acid was chromatographed on a 1.6 x 91 cm Sephadex G-25 column and eluted with 50 mM formic acid. 1.7 ml fractions were collected. In Experiment 3 fractions from Extract A (insulin treatment) were assayed with microsomal GPAT prepared from fed rats (microsomes; see Fig 3.1) while in Experiment 4 fractions from Extract B (saline treatment) were assayed with liver microsomal GPAT from fed rats. Assays were performed in triplicate and means (± SEM) are given in Table 4.2.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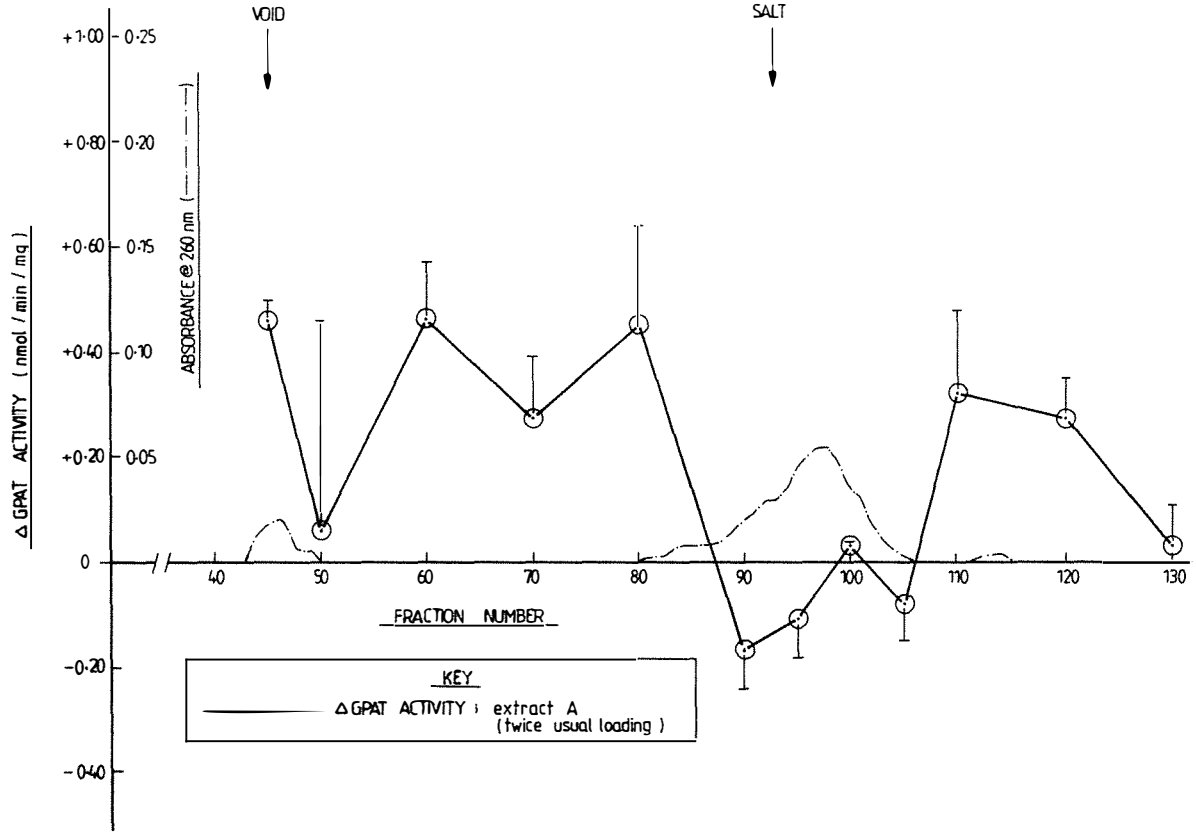
0·30
0·25
0·20
0·15
0·10
0·05
Δ GPAT ACTIVITY (nmol / min / mg)
ABSORBANCE @ 260 nm (—·—·—)
40
50
60
70
80
90
100
110
120
130
FRACTION NUMBER
KEY
Δ GPAT ACTIVITY ; EXPT 3
extract A,
fed rat microsomes
Δ GPAT ACTIVITY ; EXPT 4
extract B,
fed rat microsomes

Table 4.2: The effect of Sephadex G-25 fractions of muscle extract from heparinised rats treated with insulin (Extract A) or saline (Extract B) on liver microsomal GPAT from fed rats.

Fraction number	GPAT activity nmol/min/mg	deviation[c]	% of control
Experiment 3. - insulin-treatment[d], fed rat GPAT			
Control	1.39 ± 0.18		100.0
46 *	1.10 ± 0.06	-0.29	79.1
56 *	1.41 ± 0.06	+0.02	101.4
66 *	1.38 ± 0.16	-0.01	99.3
76 *	2.28 ± 0.04[b]	+0.89	164.0
86 *	2.10 ± 0.32	+0.71	151.1
95	1.78 ± 0.20	+0.39	128.1
97	2.27 ± 0.10[a]	+0.88	163.3
100	1.42 ± 0.12	+0.03	102.2
102	1.90 ± 0.23	+0.51	136.7
104	2.42 ± 0.04[b]	+1.03	174.1
106	1.85 ± 0.26	+0.46	133.1
108	1.67 ± 0.13	+0.28	120.1
112	2.16 ± 0.34	+0.77	155.4
115	2.00 ± 0.27	+0.61	143.9
125	1.54 ± 0.08	+0.15	110.8
Experiment 4. - saline-treatment[e], fed rat GPAT			
Control	4.35 ± 0.10		100.0
76 *	4.19 ± 0.23	-0.16	96.3
98	4.21 ± 0.19	-0.14	96.8
105	4.21 ± 0.09	-0.14	96.8
113	3.99 ± 0.17	-0.36	91.7
116	4.20 ± 0.49	-0.15	96.6

* Fractions eluting between void and salt peaks. Values are the mean of triplicate assays ± SEM. Experimental procedures are described in the caption to Fig 4.4. Means (± SEM) with superscripts differ from controls [a]$P<0.05$, [b]$P<0.01$. [c]GPAT rate minus GPAT formic acid control rate. [d]Extract A, [e]Extract B.

Fig 4.5: Sephadex G-25 fractionation of muscle extract (twice usual loading on column) from heparinised rat treated with insulin and absolute effect of fractions on microsomal GPAT from livers of fed rats. 8.0 mg of muscle extract (Extract A), from a heparinised rat treated with insulin, in 50 mM formic acid, was chromatographed on a 1.6 x 91 cm Sephadex G-25 column and eluted with 50 mM formic acid. Fractions of approximately 1.7 ml were collected and assayed with liver microsomal GPAT (microsomes; see Fig 3.1) from 2 fed rats. Points are means of assays performed in triplicate. Means (± SEM) are given in Table 4.3.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0·20
0·15
0·10
0·05
ABSORBANCE @ 260 nm (——·——·——)
Δ GPAT ACTIVITY (nmol / min / mg)
40
50
60
70
80
90
100
110
120
130
FRACTION NUMBER
KEY
Δ GPAT ACTIVITY ; extract A
(twice usual loading)

Table 4.3: The effect of Sephadex G-25 fractions of muscle extract (A) from a heparinised rat treated with insulin on liver microsomal GPAT from fed rats.

Fraction number	GPAT activity nmol/min/mg	deviation[a]	% of control
Experiment 5. - insulin-treatment[b], fed rat GPAT			
Control	2.67 ± 0.21		100.0
45 *	3.13 ± 0.04	+0.46	117.2
50 *	2.73 ± 0.40	+0.06	102.2
60 *	3.13 ± 0.11	+0.46	117.2
70 *	2.94 ± 0.12	+0.27	110.1
80 *	3.12 ± 0.19	+0.45	116.9
90 *	2.50 ± 0.07	-0.17	93.6
95	2.56 ± 0.07	-0.11	95.9
100	2.70 ± 0.01	+0.03	101.1
105	2.59 ± 0.07	-0.08	97.0
110	2.99 ± 0.16	+0.32	112.0
120	2.94 ± 0.08	+0.27	110.1
130	2.70 ± 0.08	+0.03	101.1

* Fractions eluting between void and salt peaks. Values are the means of assays performed in triplicate ± SEM. [a]GPAT rate minus GPAT formic acid control rate. [b]Extract A. Experimental procedures are described in the caption to Fig 4.5.

4.3.2 Section 2 - Experiments with further extracts from heparinised rats treated with insulin (Extracts C, D, E, & G) or saline (Extracts F & H): reconsideration of role of liquid air versus liquid nitrogen in the method for preparing extracts.

In Section 1 (4.3.1), preliminary experiments with extracts from heparinised rats treated with insulin or saline provided evidence that hepatic microsomal GPAT, from fed rats, could be stimulated in an insulin-dependent manner. At the completion of these experiments, a considerable amount of Extracts A and B (about 900 mg of each) was still available. However, rather than continuing to rely on just two extracts, a decision was made to prepare additional extracts from rats treated with insulin or saline. This was done to ensure that the extract procedure and the effect of the Sephadex G-25 fractions was repeatable and not subject to variation between individual rats used. Some difficulties encountered with these subsequent extracts led to reconsideration of the role of liquid air versus liquid nitrogen in the method for preparation of extracts.

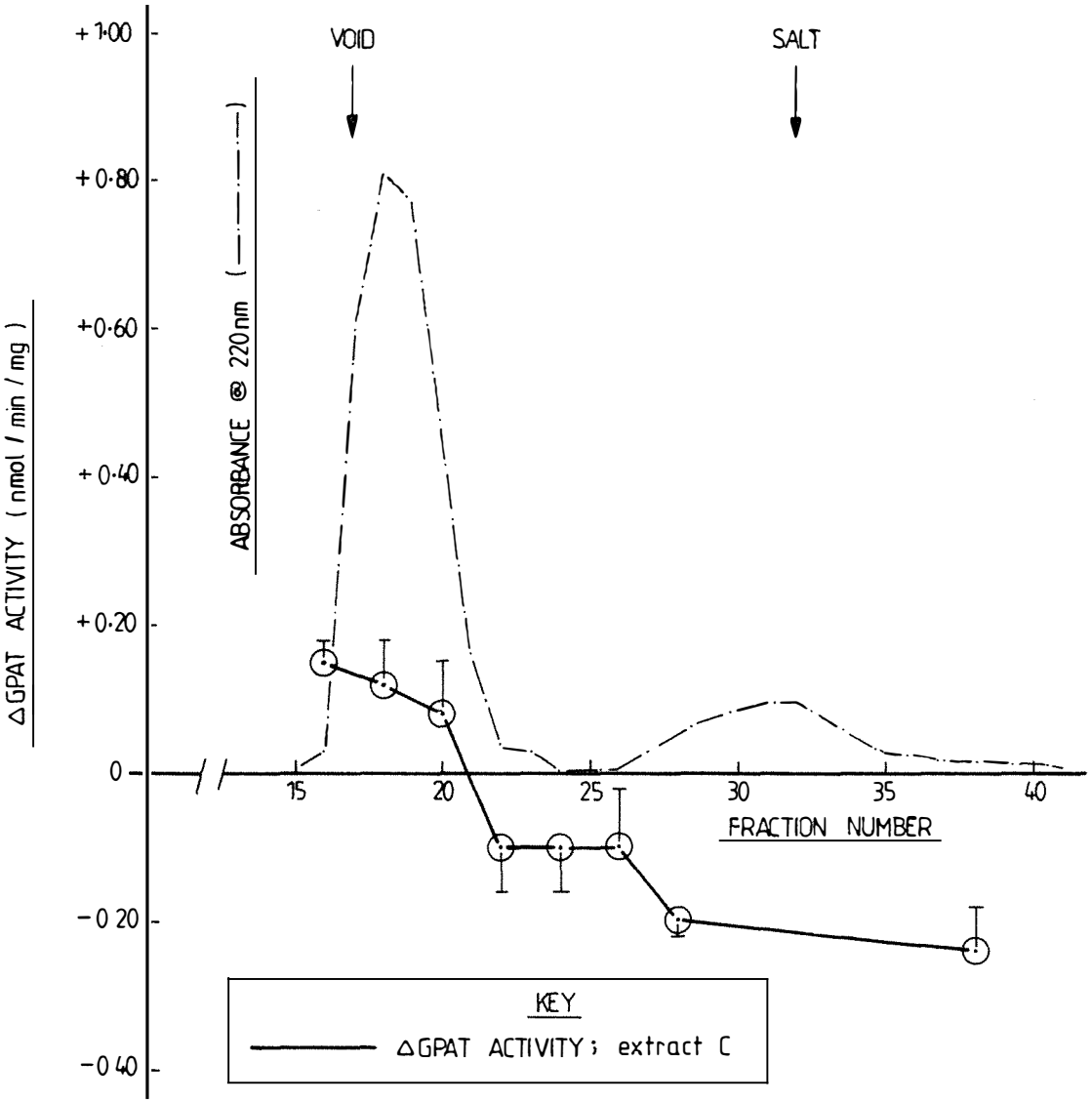
The results presented in Fig 4.6 (Experiment 6; see Table 4.4) show that the effect of Sephadex G-25 fractions of Extract C, prepared from a heparinised rat treated with insulin, on microsomal GPAT were not the same as the effect of fractions of Extract A, the initial insulin treatment extract, on microsomal GPAT from livers of fed rats. Fractions of Extract C that eluted subsequent to the void volume inhibited GPAT relative to the formic acid control (Fig 4.6, Table 4.4). It seemed possible that the negative effect of these fractions

on GPAT, relative to the formic acid control, could just be peculiar to this extract. However, when two further extracts (Extracts D and E) were prepared from heparinised rats treated with insulin, the fractions assayed also inhibited GPAT activity relative to the formic acid controls (Experiments 7 and 8, Fig 4.7, Table 4.5). Based on the results of Experiments 6, 7 and 8, it appeared that the methodology being used was in some way faulty, despite the fact that the extract used in experiments in Section 1 had been entirely satisfactory. An extract was prepared from a heparinised rat treated with saline (Extract F), to determine whether the problem was with the whole system or whether the relativity of the insulin and saline treatments was maintained. When fractions of Extract F (saline treatment) were assayed with GPAT (from livers of fed rats) these also inhibited GPAT activity relative to the formic acid control (Fig 4.7, Table 4.5). However, in comparison to the effect of the insulin treatment fractions (Experiments 7 and 8, Fig 4.7), fractions 5, 7 and 8 of Extract F, the saline treatment, inhibited GPAT activity to a greater extent. Comparison of the mean effects of fractions from the two insulin treatment extracts (D and E) with equivalent fractions from the saline treatment extract (F) (Fig 4.8, Table 4.6) illustrated this more clearly. Comparison of the effects of fractions 5, 7, and 8 from both treatments (Fig 4.8) on GPAT activity suggested GPAT activity was enhanced in an insulin-dependent manner. When the effects of fraction 7 from both treatments were compared, the difference in favour of the insulin treatment was about 13% of the formic acid controls. This corresponded to an absolute insulin-dependent increase in GPAT rate of 0.39 nmol/min/mg ($P<0.01$; assuming the effects of the treatments are additive). This was similar to results obtained with Extracts A and B

in Experiments in Section 1 (4.3.1).

Therefore, despite the fact that extracts from both the insulin and saline treated rats (Extracts D and E vs. F) inhibited GPAT activity compared with formic acid controls, relativity between the insulin and saline treatments was maintained. However, the problem of the negative effect of the fractions on GPAT, relative to the formic acid controls, was not resolved. Therefore, the method for preparation of the extracts was re-examined and the role of liquid air versus liquid nitrogen in the method reconsidered. Recently published papers indicated that the presence of oxygen might be disadvantageous in systems for demonstrating insulin-dependent effects (Larner, 1982; Larner et al., 1982c).

Fig 4.6: Sephadex G-25 fractionation of muscle extract from a heparinised rat treated with insulin and absolute effect on GPAT activity: inhibition of GPAT activity relative to the formic acid control GPAT activity. 3.2 mg of muscle extract (Extract C) from a heparinised rat treated with insulin, prepared using liquid air, was chromatographed on a Sephadex G-25 column (2.54 x 27 cm) in 50 mM formic acid. 3.8 ml fractions were collected. The effect of these on liver microsomal GPAT (microsomes; see Fig 3.1, shortened) from fed rats is shown. Points are the means of assays performed in triplicate. Means (± SEM) are given in Table 4.4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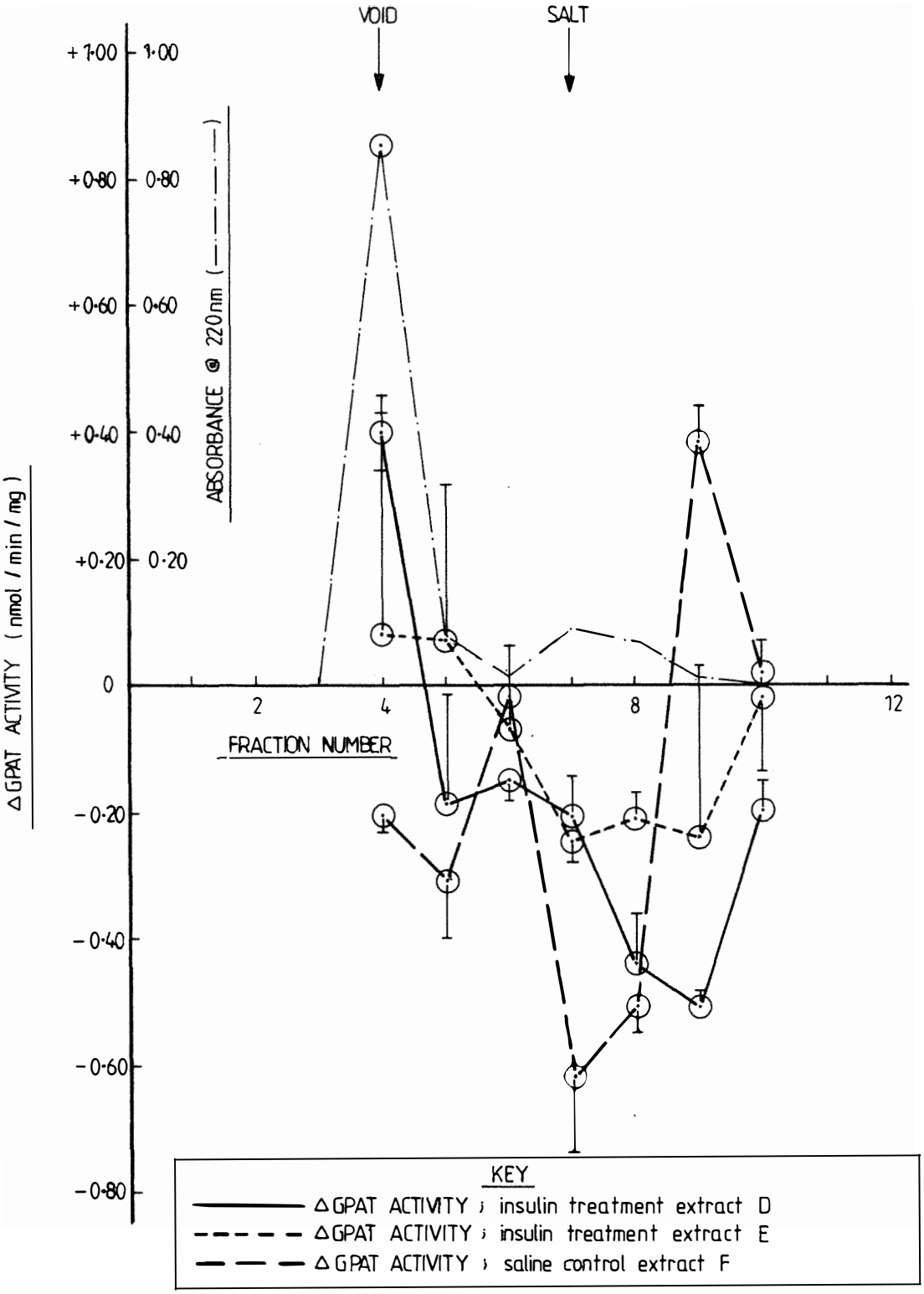
-0 20
-0 40
VOID
SALT
ABSORBANCE @ 220nm (——·——·——)
ΔGPAT ACTIVITY (nmol / min / mg)
15
20
25
30
35
40
FRACTION NUMBER
KEY
ΔGPAT ACTIVITY; extract C

Table 4.4: The effect of Sephadex G-25 fractions of muscle extract (C) from a heparinised rat treated with insulin, prepared using liquid air, on liver microsomal GPAT from fed rat (Experiment 6).

Fraction number	GPAT activity		
	nmol/min/mg	deviation[a]	% of control
Control	1.31 ± 0.10		100.0
16	1.46 ± 0.03	+0.15	115.5
18 *	1.43 ± 0.06	+0.12	109.2
20 *	1.39 ± 0.07	+0.08	106.1
22 *	1.21 ± 0.06	-0.10	92.4
24 *	1.21 ± 0.06	-0.10	92.4
26 *	1.21 ± 0.08	-0.10	92.4
28 *	1.11 ± 0.02	-0.20	84.7
38	1.07 ± 0.06	-0.24	81.7

* Fractions eluting between void and salt peaks. Values are the means of assays performed in triplicate ± SEM. [a]GPAT rate minus GPAT formic acid control rate. Experimental procedures are as outlined in the caption to Fig 4.6.

Fig 4.7: Sephadex G-25 fractionation of muscle extract from heparinised rats treated with insulin or saline and absolute effect on GPAT: inhibition of GPAT activity relative to formic acid controls. Muscle extracts were prepared from two heparinised rats treated with insulin (Extracts D and E) and from a heparinised rat treated with saline (Extract F), using liquid air. 3.2 mg amounts were chromatographed separately on the same Sephadex G-25 column (2.54 x 27 cm) in 50 mM formic acid. 16 ml fractions were collected and the effect of these fractions on liver microsomal GPAT (microsomes; see Fig 3.1, shortened) from fed rats is shown (Experiments 7, 8 and 9). Points are the means of assays performed in triplicate. Means (± SEM) are given in Tables 4.5 and 4.6.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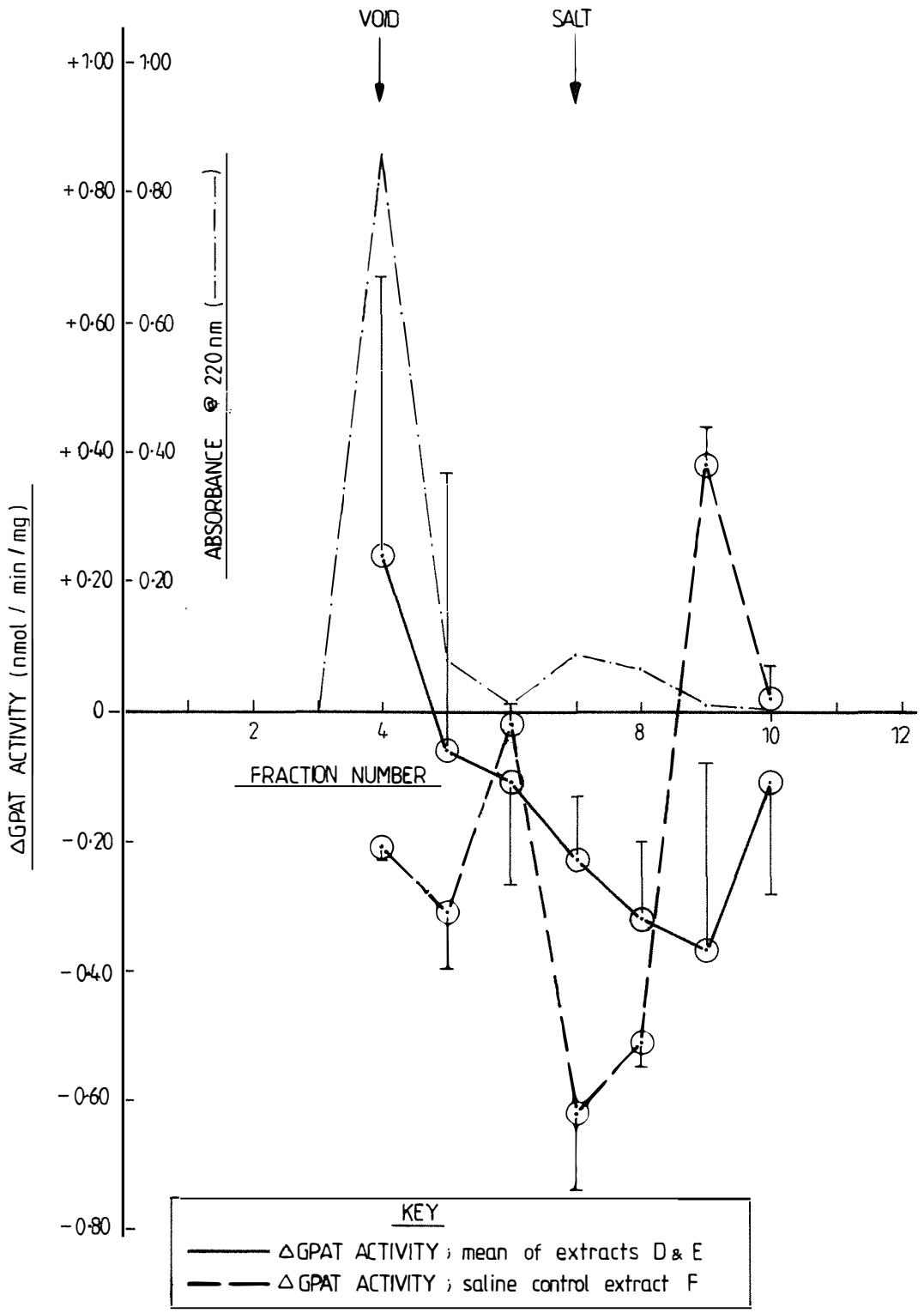
-0·40
-0·60
-0·80
1·00
0·80
0·60
0·40
0·20
ABSORBANCE @ 220nm (——·——·——)
ΔGPAT ACTIVITY (nmol / min / mg)
2
4
8
12
FRACTION NUMBER
KEY
ΔGPAT ACTIVITY ; insulin treatment extract D
ΔGPAT ACTIVITY ; insulin treatment extract E
ΔGPAT ACTIVITY ; saline control extract F

Fig 4.8: Sephadex G-25 fractionation of muscle extract from heparinised rats treated with insulin or saline and absolute effect on GPAT: inhibition of GPAT activity relative to formic acid controls. Muscle extracts were prepared from two heparinised rats treated with insulin (Extracts D and E) and from a heparinised rat treated with saline (Extract F), using liquid air. 3.2 mg amounts were chromatographed separately on the same Sephadex G-25 column (2.54 x 27 cm) in 50 mM formic acid. 16 ml fractions were collected and the mean of the effect of fractions of the two insulin treatments (Extracts D and E) and the effect of the fractions of the saline treatment (Extract F) on liver microsomal GPAT (microsomes; see Fig 3.1, shortened) from fed rats are shown (Mean of the results of Experiments 7 and 8; results of Experiment 9). Points are the means of assays performed in triplicate. Means (± SEM) are given in Table 4.6.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-0·40
-0·60
-0·80
1·00
0·80
0·60
0·40
0·20
ABSORBANCE @ 220 nm (—·—·—·—)
ΔGPAT ACTIVITY (nmol / min / mg)
2
4
8
10
12
FRACTION NUMBER
KEY
ΔGPAT ACTIVITY ; mean of extracts D & E
ΔGPAT ACTIVITY ; saline control extract F

Table 4.5: The effect of Sephadex G-25 fractions of muscle extracts from heparinised rats treated with insulin (Extracts D & E) or saline (Extract F), prepared using liquid air, on liver microsomal GPAT from fed rats.

Fraction number	GPAT activity nmol/min/mg	deviation[c]	% of control
	Expt 7 - insulin treatment[d], fed rat GPAT		
Control	2.83 ± 0.05		100.0
4 *	3.23 ± 0.06[a]	+0.40	114.1
5 *	2.64 ± 0.18	-0.19	93.3
6 *	2.68 ± 0.03	-0.15	94.7
7 *	2.62 ± 0.07	-0.21	92.6
8	2.39 ± 0.08[a]	-0.44	84.5
9	2.32 ± 0.03[b]	-0.51	82.0
10	2.63 ± 0.05	-0.20	92.9
	Expt 8 - insulin treatment[e], fed rat GPAT		
Control	2.81 ± 0.13		100.0
4 *	2.89 ± 0.35	+0.08	102.9
5 *	2.88 ± 0.25	+0.07	102.5
6 *	2.74 ± 0.13	-0.07	97.5
7 *	2.56 ± 0.03	-0.25	91.1
8	2.60 ± 0.04	-0.21	92.5
9	2.57 ± 0.27	-0.24	91.5
10	2.79 ± 0.12	-0.02	99.3
	Expt 9 - saline treatment[f], fed rat GPAT		
Control	3.33 ± 0.22		100.0
4 *	3.12 ± 0.02	-0.21	93.7
5 *	3.02 ± 0.09	-0.31	90.7
6 *	3.31 ± 0.03	-0.02	99.4
7 *	2.71 ± 0.12	-0.62	81.4
8	2.82 ± 0.04	-0.51	84.7
9	3.71 ± 0.06	+0.38	111.4
10	3.35 ± 0.05	+0.02	100.6

* Fractions eluting between void and salt peaks. Values are the means of assays performed in triplicate ± SEM. Experimental procedures are outlined in the caption to Fig 4.7. Means (± SEM) with superscripts differ from controls [a]$P<0.05$, [b]$P<0.01$. [c]GPAT rate minus GPAT formic acid control rate. [d]Extract D, [e]Extract E, [f]Extract F.

Table 4.6: The effect of Sephadex G-25 fractions of muscle extracts from heparinised rats treated with insulin[d] (means of values in Table 4.5) or saline[e], prepared using liquid air, on microsomal GPAT from fed rats.

Fraction number	GPAT activity (nmol/min/mg) Mean insulin treatment	Saline treatment
Control	2.82 ± 0.18	3.33 ± 0.22
4 *	3.06 ± 0.43	3.12 ± 0.02
5 *	2.76 ± 0.43	3.02 ± 0.09
6 *	2.71 ± 0.16	3.31 ± 0.03
7 *	2.59 ± 0.10	2.71 ± 0.12
8	2.50 ± 0.12	2.82 ± 0.04
9	2.45 ± 0.29	3.71 ± 0.06
10	2.71 ± 0.17	3.35 ± 0.05

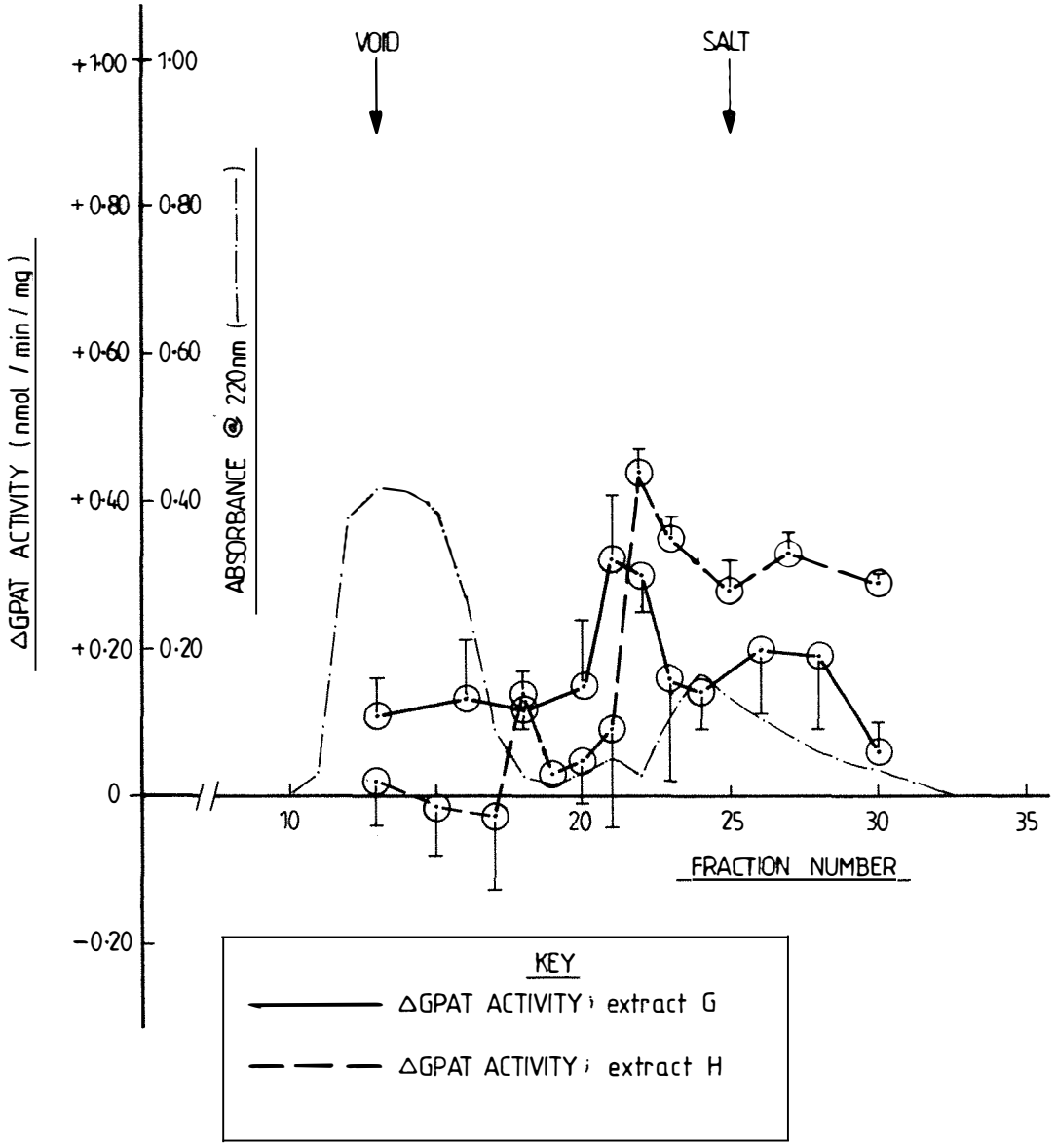
	Insulin[a] deviation	Saline deviation	Difference	Insulin[b] effect(%)
4 *	+0.24	-0.21	+0.45	+14.6
5 *	-0.06	-0.31	+0.25	+ 8.1
6 *	-0.11	-0.02	-0.09	- 2.9
7 *	-0.23[c]	-0.62[c]	+0.39	+12.7
8	-0.32	-0.51	+0.19	+ 6.2
9	-0.37[c]	+0.38[c]	-0.75	-24.4
10	-0.11	+0.02	-0.13	- 4.2

* Fractions eluting between void and salt peaks. [a]Mean insulin-treatment means (± SEM) minus mean insulin-treatment control (nmol/min/mg). [b]Difference divided by mean of mean insulin- and saline-control (i.e. 3.08 nmol/min/mg). Deviations within rows that have the same superscript differ, [c]$P<0.01$. Experimental procedures are described in the caption to Fig 4.8. [d]Extracts D and E, [f]Extract F.

To determine whether use of liquid nitrogen in the extract preparative procedure might overcome the difficulties encountered in Experiments 6-9 with Extracts C-F, liquid nitrogen was obtained. Muscle extracts from heparinised rats treated with insulin (Extract G) or saline (Extract H) were prepared and fractionated on Sephadex G-25 according to the original method except that liquid nitrogen was substituted for liquid air. The effect of these fractions on hepatic microsomal GPAT (from fed rats) was examined. In contrast to results with fractions of Extracts C-F it was found that the majority of the fractions from Extracts G and H stimulated GPAT activity relative to the formic acid controls (Fig 4.9, Tables 4.7 and 4.8). Once again many of the saline treatment fractions did not alter GPAT activity significantly relative to the formic acid control. However, in further contrast to earlier experiments a number of fractions of Extract H (saline treatment) stimulated GPAT activity relative to the formic acid control. In Experiment 9 (Fig 4.7, Table 4.5) several fractions of Extract F (saline treatment; prepared using liquid air) stimulated GPAT activity slightly relative to the effect of the equivalent fractions of the Extracts D and E (insulin treatment; Expts 7 and 8). However, a larger number of fractions of Extract H (saline treatment; prepared using liquid nitrogen) stimulated GPAT activity relative to fractions of Extract G (insulin treatment; liquid N_2). The reason for this was unclear. This apparently non insulin-dependent stimulation was not evident in Experiment 4 with fractions of Extract B (Section 1, (4.3.1), Fig 4.4). However, despite this insulin-independent phenomenon, a number of low molecular weight fractions of Extract G (insulin treatment), as in earlier experiments (Section 1; 4.3.1), stimulated GPAT activity relative to the effect of the equivalent

fractions of Extract H (saline treatment; see Fig 4.9, Tables 4.7 and 4.8). Fraction 21 of Extract G enhanced GPAT activity to 130% of the formic acid control, an increase of 0.32 nmol/min/mg in GPAT rate. In comparison fraction 21 of Extract H enhanced GPAT activity to 107% of the formic acid control, an increase of 0.09 nmol/min/mg in GPAT rate. When the percentage figures of the effects of the two treatments (for fraction 21) were compared, an insulin-dependent increase in GPAT activity of 23% was evident. Similarly, when absolute figures for the two treatments were considered, then an insulin-dependent increase in GPAT activity of 0.23 nmol/min/mg was apparent. Expressed as a percentage of the mean of the formic acid controls for the insulin and saline treatments, this corresponded to a 19.4% stimulation of GPAT, in favour of the insulin treatment. The 0.23 nmol/min/mg insulin-dependent increase in GPAT activity was less than in experiments in Section 1 (4.3.1), but consistent with these earlier results. Therefore, substitution of liquid nitrogen for liquid air in the method for preparing the extracts removed the inhibitory effects of the fractions on GPAT relative to the formic acid controls. It is unclear why the earlier extract (Extract A), prepared with liquid air, was successful in comparison to the later ones, also prepared using liquid air (Extracts C-F; Experiments 6-9). It may have been that the liquid air used in the preparation of the earlier extract had just been freshly condensed, in which case its concentration of oxygen would have been lower than if it had been stored for 1-2 weeks. The nitrogen tends to evaporate at a faster rate than the oxygen, leaving liquid air that is enriched with oxygen, as evidenced by a light blue colouration.

Fig 4.9: The absolute effect of Sephadex G-25 fractions of muscle extracts from heparinised rats treated with insulin or saline, prepared using liquid nitrogen, on microsomal GPAT from livers of fed rats. Muscle extract was prepared from heparinised rats treated with insulin (Extract G) or saline (Extract H), using liquid nitrogen. 3.2 mg amounts were chromatographed on a Sephadex G-25 column (2.54 x 27 cm) in 50 mM formic acid and fractions of approximately 4.7 ml were collected. The absorbance values in this experiment differ from the 220nm absorbances in other experiments as it was later found that a glass cuvette had been substituted for one of the quartz cuvettes. The effect of these fractions on liver microsomal GPAT (microsomes; see Fig 3.1, shortened) activity from fed rat is shown. Points are the means of assays performed in duplicate. Means (± SEM) are given in Tables 4.7 and 4.8.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0·40
0·20
ΔGPAT ACTIVITY (nmol / min / mg)
ABSORBANCE @ 220nm (——·——·——)
10
20
25
30
35
FRACTION NUMBER
KEY
ΔGPAT ACTIVITY ; extract G
ΔGPAT ACTIVITY ; extract H

Table 4.7: The effect of Sephadex G-25 fractions of muscle extracts, from heparinised rats treated with insulin (Extract G) or saline (Extract H), using liquid nitrogen, on liver microsomal GPAT from fed rats.

Fraction number	GPAT activity nmol/min/mg	deviation[c]	% of control
	Experiment 10 - insulin treatment[d], fed rat GPAT		
Control	1.06 ± 0.03		100.0
13 *	1.17 ± 0.05	+0.11	110.4
16 *	1.19 ± 0.08	+0.13	112.3
18 *	1.18 ± 0.03	+0.12	111.3
20 *	1.21 ± 0.09	+0.15	114.2
21 *	1.38 ± 0.09	+0.32	130.2
22 *	1.36 ± 0.05[a]	+0.30	128.3
23 *	1.22 ± 0.14	+0.16	115.1
24 *	1.20 ± 0.05	+0.14	113.2
26	1.26 ± 0.09	+0.20	118.9
28	1.25 ± 0.10	+0.19	117.9
30	1.12 ± 0.04	+0.06	105.7
	Experiment 11 - saline treatment[e], fed rat GPAT		
Control	1.31 ± 0.02		100.0
13 *	1.33 ± 0.06	+0.02	101.5
15 *	1.30 ± 0.07	-0.01	99.2
17 *	1.28 ± 0.10	-0.03	97.7
18 *	1.45 ± 0.03[a]	+0.14	110.7
19 *	1.34 ± 0.01	+0.03	102.3
20 *	1.36 ± 0.06	+0.05	103.8
21 *	1.40 ± 0.13	+0.09	106.9
22 *	1.75 ± 0.03[b]	+0.44	133.6
23 *	1.66 ± 0.03[b]	+0.35	126.7
25 *	1.59 ± 0.04[b]	+0.28	121.4
27	1.64 ± 0.02[b]	+0.33	125.2
30	1.60 ± 0.01[b]	+0.29	122.1

* Fractions eluting between void and salt peaks. Control and fraction assays were performed in triplicate and duplicate (± SEM), respectively. Experimental procedures are described in the caption to Fig 4.9. Means (± SEM) with superscripts differ from controls, [a]$P<0.05$, [b]$P<0.01$. [c](Treatment - control rate). [d]Extract G, [e]Extract H.

Table 4.8: The absolute and percentage effect of Sephadex G-25 fractions of muscle extracts from heparinised rats treated with insulin (Extract G) or saline (Extract H), prepared using liquid nitrogen, on liver microsomal GPAT from fed rats.

Fraction number	Insulin deviation[a]	Saline deviation[a]	Difference	Insulin[b] effect(%)
13 *	+0.11	+0.02	+0.09	+ 7.6
18 *	+0.12	+0.14	-0.02	- 1.7
20 *	+0.15	+0.05	+0.10	+ 8.4
21 *	+0.32	+0.09	+0.23	+19.4
22 *	+0.30	+0.44	-0.14	-11.8
23 *	+0.16	+0.35	-0.19	-16.0
30	+0.06	+0.29	-0.23	-19.4

* Fractions eluting between void and salt peaks. For further details see Table 4.7 and the caption to Fig 4.9. [a]treatment minus control (nmol/min/mg), [b]Difference divided by mean of insulin and saline formic acid controls (=1.19 nmol/min/mg) and expressed as a percentage.

4.3.3 Section 3 - Experiments with extracts from 48 h-fasted heparinised rats treated with insulin (Extract K) or saline (Extracts I & J), and with Extracts L and M from fed or 48 h-fasted non-cannulated rats, respectively: consideration of the possible influence of cannulation and heparin.

The apparently non insulin-dependent stimulation of GPAT activity by fractions of the saline treatment extracts, observed in experiments in Section 2 (4.3.2), was of concern as it might have confounded the detection of insulin-dependent stimulation of GPAT activity. As the rat muscle extracts had been prepared from rats fed ad libitum, it was postulated that the non insulin-dependent enhancement of GPAT activity might be due to endogenous insulin in the circulation. This suggestion seemed somewhat unsatisfactory though because, if this was the case, one would expect similar to greater amounts of stimulation by extract fractions from the 4 i.u. insulin/kg body weight treated rats. Nevertheless, to address this possibility, extracts were prepared from heparinised rats that had been fasted for 48 h and then treated with saline (and insulin). The results of experiments with fractions of these extracts led to the consideration of the influence of cannulation and subsequently to the consideration of the effect of heparin.

To address the problem of stimulation of GPAT activity by fractions of saline treatment extracts, an extract was prepared from a 48 h-fasted heparinised rat treated with saline (Extract I). It was considered that concentration of endogenous insulin in the circulation would be reduced to very low levels after fasting for 48 h (Millward et

al., 1974; Sinha & Caro, 1985). Hence, it might be reasonably expected that, if this stimulation of GPAT activity by saline treatment fractions was due to endogenous insulin present in the circulation of fed rats, there would be an absence of stimulation of GPAT by fractions of Extract I. Fractions of this extract were, however, found to stimulate GPAT activity relative to the formic acid control by $\leq$ +0.58 nmol/min/mg (see Expt 12, Fig 4.10 and Table 4.9). This was not expected as Extract I had been prepared from muscle of a rat that had been fasted and not treated with insulin. Enhancement of GPAT activity by these saline treatment fractions was very similar to the extent of insulin-dependent enhancement seen in earlier experiments. When the results of this experiment (Expt 12, Fig 4.10) were compared with the results of the experiments with the first extracts prepared with liquid nitrogen (Expts 10 and 11; Fig 4.9), it was evident that fractions of the saline treatment extracts (H and I) which stimulated GPAT activity in a non insulin-dependent manner were equivalent (with respect to elution volume and hence apparent molecular weight).

In order to assess the repeatability of this phenomenon, another muscle extract (Extract J) was prepared from a 48 h-fasted heparinised rat treated with saline. Fractions of Extract J also stimulated GPAT activity to almost the same extent as fractions of Extract I (Expt 13, Fig 4.11 and Table 4.9). Higher molecular weight fractions, that had previously been associated only with insulin-dependent stimulation of GPAT, also stimulated GPAT activity. However, one fraction (fraction 24; Fig 4.11, Table 4.9) reduced GPAT activity to 0.21 nmol/min/mg below the formic acid control activity. This at least was consistent with the effects of similar low molecular weight fractions of earlier

saline treatment extracts.

To determine whether the stimulation of GPAT activity by these fractions was truly independent of insulin, a 48 h-fasted heparinised rat was treated with insulin and an extract prepared (Extract K) and fractionated. The possibility existed that insulin treatment of this fasted rat might give rise to extract fractions that enhanced GPAT activity to a far greater extent than did the fractions from the previous saline treatment extract. If this occurred, it would indicate that, despite stimulation of GPAT by saline treatment fractions, it was still possible to demonstrate an insulin-dependent enhancement of GPAT activity using this system. Conversely, if the fractions from the insulin treatment extract only stimulated GPAT activity to the same or to a lesser extent than did the saline treatment fractions (above the formic acid controls), then this would indicate non insulin-dependent stimulation of GPAT activity by the system.

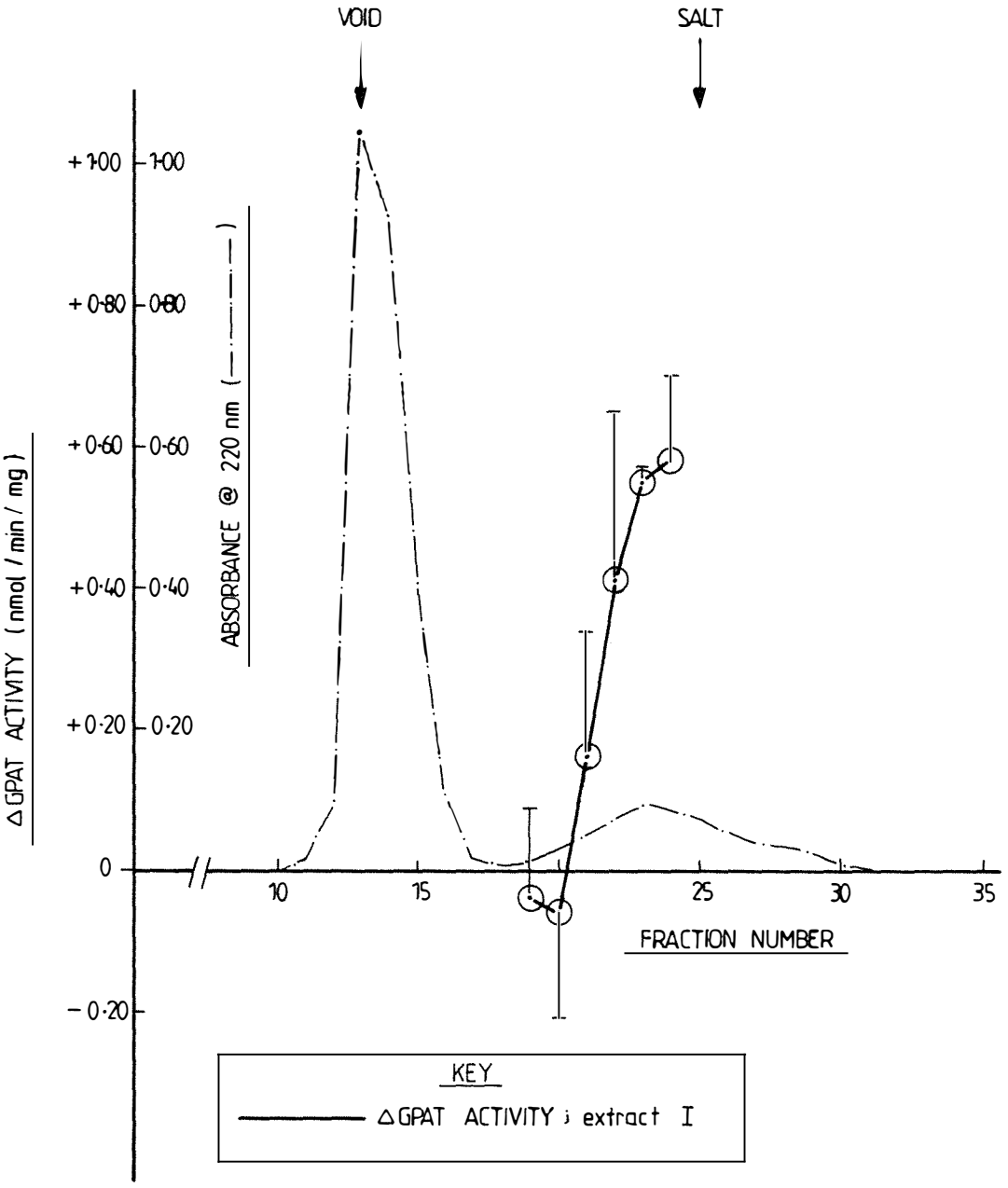
Comparison of the effects on GPAT activity of the insulin and saline treatment fractions of extracts from fasted rats, would show whether the relativity between these effects was the same as that observed in Experiments 10 and 11 with extracts from fed rats (see Fig 4.9). It was found, however, that many fractions of Extract K (fasted rat; insulin treatment) stimulated GPAT activity to a lesser extent than did the equivalent fractions of Extract J (fasted rat; saline treatment; Expts 13 and 14; Fig 4.11). A number of fractions of Extract K did, however, enhance GPAT activity slightly more than fractions of Extract J. Fraction 24 of Extract K enhanced GPAT activity by about +0.48 nmol/min/mg above the formic acid control.

Fraction 24 of Extract J, however, depressed GPAT activity by -0.21 nmol/min/mg below the formic acid control. There was therefore an apparent insulin-dependent stimulation of GPAT activity of about 0.7 nmol/min/mg ($P<0.10$) evident. However, fraction 24 of Extract I (fasted rat; saline treatment) had enhanced GPAT activity by about +0.58 nmol/min/mg above the formic acid control. Hence, if the insulin treatment effect (+0.48 nmol/min/mg) had been compared with this, there would have been no insulin-dependent stimulation of GPAT activity apparent.

As these extracts had been prepared from rats that had been fasted for 2 days, it seemed unlikely that concentrations of insulin in the circulation would have been significant (Millward et al., 1974; Sinha & Caro, 1985). Furthermore, if the insulin-dependent GPAT stimulatory activity was produced in response to endogenous insulin then increased concentrations of circulating insulin (exogenous) would have been expected to heighten the production of stimulatory material further so that still greater enhancement of GPAT activity would have been observed with the insulin treatment fractions. Fasting of rats for 48 h, even though it would have reduced the concentration of insulin in the blood, did not lead to a reduction in the extent to which saline treatment fractions enhanced GPAT activity relative to either the formic acid controls or the effect of the equivalent insulin treatment fractions. Hence, there seemed little support for the suggestion that endogenous insulin was responsible for the presence of the exogenous insulin-independent stimulation of GPAT activity by low molecular weight fractions of Extract H (saline treatment; Expt 11). It seemed that the ability of these fractions to enhance GPAT activity was not

dependent on insulin. If anything, fasting for 48 h before preparation of the extract intensified the problem. As a result of these experiments, the method for preparation of the extracts was again re-examined.

Fig 4.10: The absolute effect of Sephadex G-25 fractions of muscle extract from 48 h-fasted heparinised rat treated with saline on microsomal GPAT from fed rat. 3.2 mg of muscle extract (Extract I), prepared with liquid nitrogen from 48 h-fasted heparinised rat treated with saline was chromatographed on a Sephadex G-25 column (2.54 x 27 cm) in 50 mM formic acid and fractions of approx. 4.7 ml collected. Absorbance was measured at 220nm. The absolute effect of these fractions on liver microsomal GPAT (microsomes; see Fig 3.1, shortened) from fed rat is shown (Experiment 12). Points are the means of assays performed in duplicate. Means (± SEM) are given in Table 4.9.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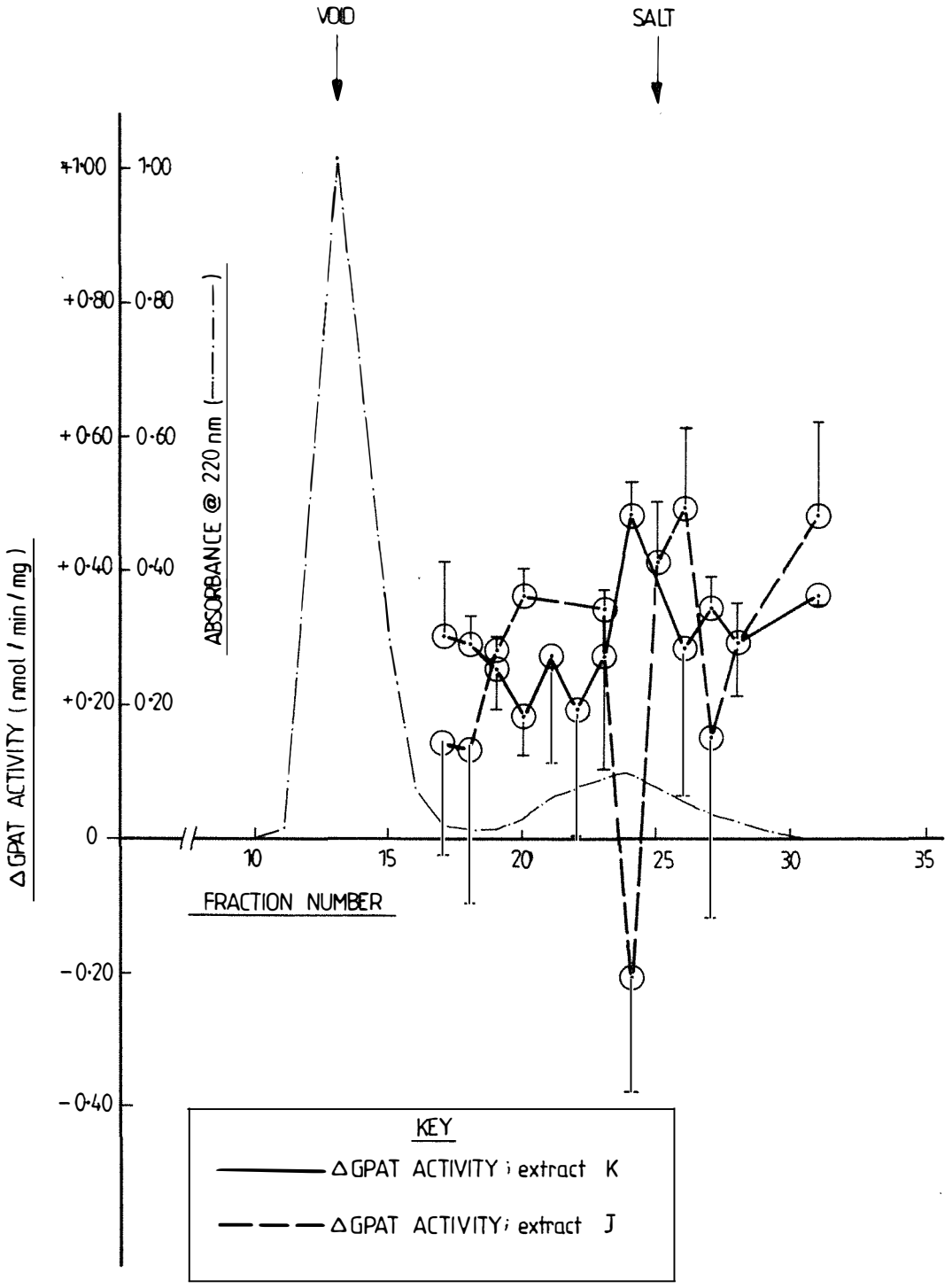
0·40
0·20
ΔGPAT ACTIVITY (nmol / min / mg)
ABSORBANCE @ 220 nm (——·——·——)
10
15
25
30
35
FRACTION NUMBER
KEY
ΔGPAT ACTIVITY ; extract I

Fig 4.11: The absolute effect of Sephadex G-25 fractions of muscle extract from 48 h-fasted heparinised rats treated with saline or insulin on microsomal GPAT from fed rats. Muscle extracts were prepared from 48 h-fasted heparinised rats treated with saline or insulin (Extracts J and K, respectively), using liquid nitrogen. 3.2 mg amounts were chromatographed on a Sephadex G-25 column (2.54 x 27 cm) in 50 mM formic acid and fractions of approx. 4.7 ml collected. Absorbance was measured at 220nm. The absolute effect of these fractions on liver microsomal GPAT (microsomes; see Fig 3.1, shortened) from fed rats is shown (Experiments 13 and 14). Points are the means of duplicate assays. Means (± SEM) are given in Tables 4.9 and 4.10.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+0·60
+0·40
+0·20
0
-0·20
-0·40
1·00
0·80
0·60
0·40
0·20
ABSORBANCE @ 220 nm (—·—·—)
ΔGPAT ACTIVITY (nmol / min / mg)
10
15
20
25
30
35
FRACTION NUMBER
KEY
ΔGPAT ACTIVITY ; extract K
ΔGPAT ACTIVITY; extract J

Table 4.9: The effect of Sephadex G-25 fractions of muscle extracts from 48 h-fasted heparinised rats treated with saline (Extracts I & J) on liver microsomal GPAT from fed rats.

Fraction number	GPAT activity nmol/min/mg	deviation[c]	% of control
	Experiment 12 - 48 h-fasted saline-treatment[d]		
Control	2.21 ± 0.05		100.0
19 *	2.17 ± 0.13	-0.04	98.2
20 *	2.15 ± 0.15	-0.06	97.3
21 *	2.37 ± 0.18	+0.16	107.2
22 *	2.62 ± 0.24[a]	+0.41	118.6
23 *	2.76 ± 0.02[b]	+0.55	124.9
24 *	2.79 ± 0.12	+0.58	126.2
	Experiment 13 - 48 h-fasted saline-treatment[e]		
Control	2.61 ± 0.11		100.0
17 *	2.75 ± 0.17	+0.14	105.4
18 *	2.74 ± 0.23	+0.13	105.0
19 *	2.89 ± 0.02	+0.28	110.7
20 *	2.97 ± 0.04	+0.36	113.8
23 *	2.95 ± 0.03	+0.34	113.0
24 *	2.40 ± 0.17	-0.21	92.0
25 *	3.02 ± 0.09	+0.41	115.7
26	3.10 ± 0.12	+0.49	118.8
27	2.76 ± 0.27	+0.15	105.7
28	2.90 ± 0.06	+0.29	111.1
31	3.09 ± 0.14	+0.48	118.4

* Fractions eluting between void and salt peaks. Control and fraction assays were performed in triplicate and duplicate, respectively (± SEM). Means (± SEM) with superscripts differ from controls [a]$P<0.01$, [b]$P<0.05$. [c]GPAT rate minus GPAT formic acid control rate. [d]Extract I, [e]Extract J. Experimental procedures are described in the captions to Figs 4.10 and 4.11.

Table 4.10: The effect of Sephadex G-25 fractions of muscle extracts from 48 h-fasted heparinised rats treated with saline (Extracts I & J) or insulin (Extract K) on liver microsomal GPAT from fed rats.

Fraction number	GPAT activity nmol/min/mg	deviation[a]	% of control
	Experiment 14 - 48 h-fasted insulin-treatment[e]		
Control	2.99 ± 0.05		100.0
17 *	3.29 ± 0.11	+0.30	110.0
18 *	3.28 ± 0.04	+0.29	109.7
19 *	3.24 ± 0.06	+0.25	108.4
20 *	3.17 ± 0.06	+0.18	106.0
21 *	3.26 ± 0.16	+0.27	109.0
22 *	3.18 ± 0.19	+0.19	106.4
23 *	3.26 ± 0.17	+0.27	109.0
24 *	3.47 ± 0.05	+0.48	116.0
26	3.27 ± 0.22	+0.28	109.4
27	3.33 ± 0.05	+0.34	111.4
28	3.28 ± 0.08	+0.29	109.7
31	3.35 ± 0.01	+0.36	112.0

	Insulin deviation[a]	Saline deviation[a]	Difference	Insulin[b] effect(%)
17 *	+0.30	+0.14	+0.16	+ 5.7
18 *	+0.29	+0.13	+0.16	+ 5.7
19 *	+0.25	+0.28	-0.03	- 1.1
20 *	+0.18	+0.36	-0.18	- 6.4
23 *	+0.27	+0.34	-0.07	- 2.5
24 *	+0.48[c]	-0.21[c]	+0.69	+24.6
26	+0.28	+0.49	-0.21	- 7.5
27	+0.34	+0.15	+0.19	+ 6.8
28	+0.29	+0.29	0	0
31	+0.36	+0.48	-0.12	- 4.3

* Fractions eluting between void and salt peaks. Control and fraction assays (± SEM) were performed in triplicate and in duplicate, respectively. [a]Treatment minus control (nmol/min/mg). [b]Difference divided by mean of insulin (Expt 14) and saline treatment formic acid controls (i.e. 2.80 nmol/min/mg). [c]Superscripted values differ at P<0.10. [d]Extract K. [e]For experimental details see captions to Figs 4.10 and 4.11.

Experiments with Extracts I-K did not resolve the problem of apparently non insulin-dependent stimulation of GPAT activity by fractions of extracts from heparinised rats treated with saline. The method for preparation of the extracts was therefore re-examined. After due consideration of results of experiments with Extracts I-K, it seemed reasonable to suggest that cannulation, or some procedure associated with cannulation, might have led to fractions of saline treatment extracts having the potential to enhance GPAT activity in an insulin-independent manner.

In an attempt to answer this question, muscle extracts were prepared from a fed rat and a 48 h-fasted rat, neither of which was cannulated and using liquid nitrogen, i.e. Extracts L and M, respectively. The only compound these rats were injected with was urethane anaesthetic (intraperitoneally). Low molecular weight fractions of Extract L (from fed, non-cannulated rat muscle) did not stimulate GPAT activity relative to the formic acid control, but some fractions inhibited GPAT activity slightly (see Fig 4.12 and Table 4.11). These results were consistent with the suggestion that cannulation was in some way associated with the fractions of the saline treatment extracts (of previous experiments) having the potential to stimulate GPAT in a non insulin-dependent manner.

Several low molecular weight fractions of Extract M (from non-cannulated 48 h-fasted rat) stimulated GPAT activity to about 110% of the formic acid control. This corresponded to an increase of $\leq$ +0.10 nmol/min/mg (see Fig 4.13 and Table 4.11). However, the amount

of stimulation, in absolute terms and as a percentage of control was less than in previous experiments with extract prepared from fed or fasted rats which had been cannulated and infused with saline. Again, this seemed to indicate that the cannulation was associated with the potential of fractions from saline treatment extracts (from fed or fasted rats) to stimulate GPAT activity. It also seemed possible that fasting might have increased the potential for non insulin-dependent stimulation of GPAT activity.

Heparin was the only compound, other than NaCl and anaesthetic, being injected into rats treated with insulin or saline. Therefore, it seemed possible that heparin might have been responsible for fractions of saline treatment extracts having the potential to stimulate GPAT activity in an insulin-independent manner. Further weight was added to this suggestion when it became apparent that heparin, present in the saline used in the insulin and saline treatment protocols, had other properties besides the more widely recognised role as a anticoagulant.

Chemically, heparin is a highly sulphated mucupolysaccharide composed mainly of D-glucuronic acid, L-iduronic acid and D-glucosamine with 1.6 to 3 sulphates per disaccharide unit and a molecular weight inthe range of 6000 to 25,000 daltons depending on the method of isolation (Lindahl & Hook, 1978). Medically, heparin is a drug that is used universally as an essential aid in surgery of the heart and blood vessels, with organ transplants and artificial organs, for cardiovascular diagnostic techniques and for the control and prevention of thromboembolism following surgical operations and childbirth (Jaques, 1980). Similarly, in physiological investigations it is often

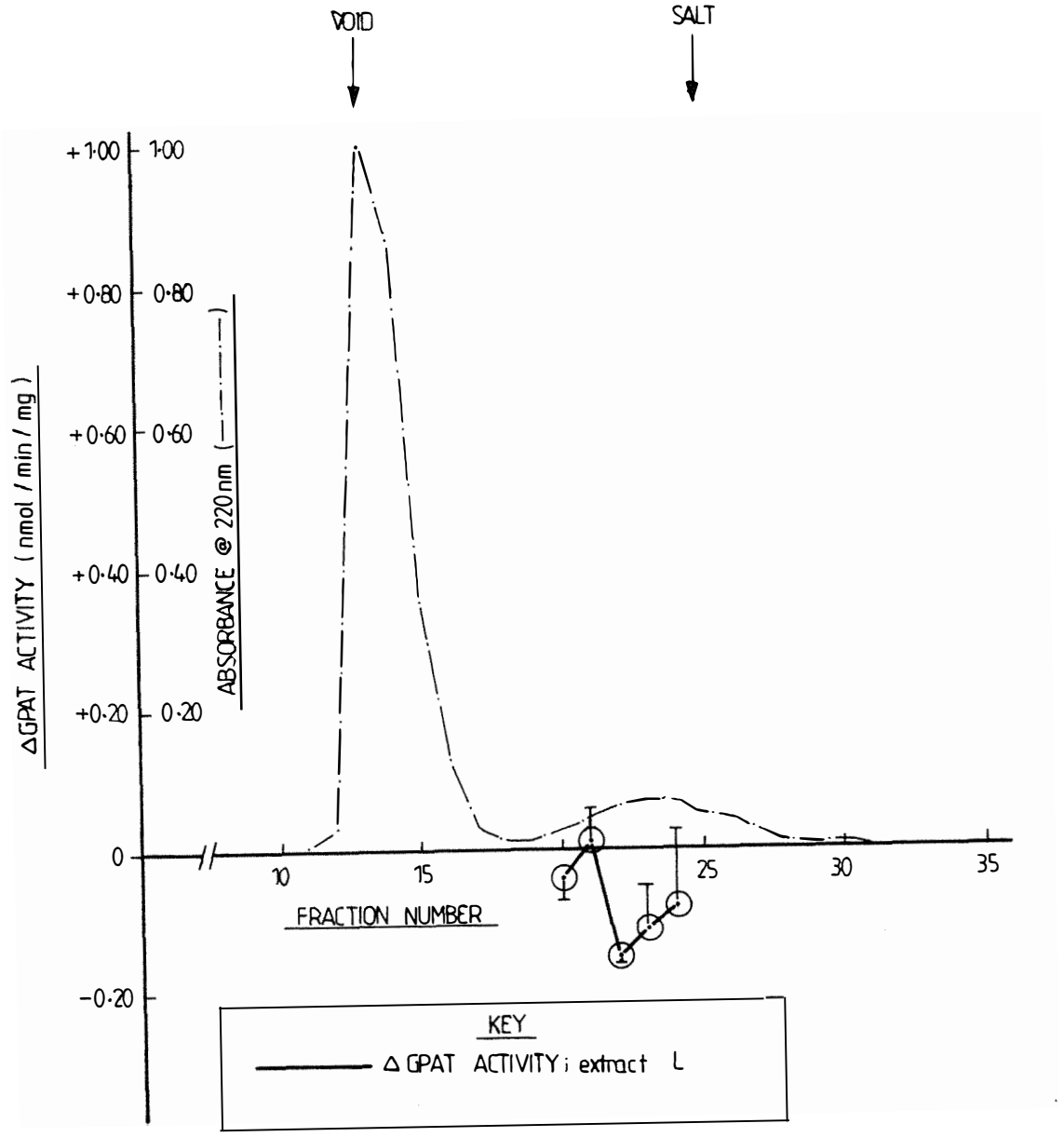
routinely used to keep cannula patent for blood sampling operations.

Biochemically however, the effects of heparin are far more wideranging than sometimes realised. In vivo, heparin liberates the following enzymes: lipoprotein lipase, lecithinase, diamine oxidase, acid ribonuclease, β-glycerolphosphatase, and procollagenase from bone explants (Jaques, 1980). Heparin has also been shown to activate the following enzymes: lipoprotein lipase, brain tyrosine hydroxylase, trehalose phosphate synthetase, pepsinogen, human skin esterase, blood arginine esterase, phosphorylase kinase, DNA polymerase, and factor XII to produce kallikrein (Chrisman et al., 1981; Jaques, 1980). Similarly, heparin has been shown to inhibit the following enzymes: adenylate deaminase, hyaluronidases, procollagenase, acid phosphatase, alkaline phosphatase, myosin ATPase, β-amylase, β-glucuronidase, lysozyme, catalase, fumarase, pyruvate kinase, alcohol dehydrogenase, renin, glutamic acid dehydrogenase, glutathione reductase, succinic dehydrogenase, 18-hydroxylase, ribonucleases, RNA polymerases, reverse transcriptase, cathepsin B_1, neutral protease, pepsin, trypsin, casein kinase type I, casein kinases, nuclear protein kinase NII, and protein phosphatase I (Feige et al., 1980; Gergely et al., 1984; Hara et al., 1981; Hathaway et al., 1980; Jaques, 1980; Meggio et al., 1982; Rigobello et al., 1982; Rose et al., 1981). Heparin also exerts a stimulatory effect on the phosphorylation rate of pyruvate kinase and phosphorylase kinase by the catalytic subunit of cAMP-dependent protein kinase (Meggio et al., 1983). It appears that heparin may be involved in the initiation of RNA synthesis in sea urchin embryos (Kinoshita, 1971, 1974). Furthermore, endogenous heparin has been found in rat blood (Horner, 1974) and time-dependent, reversible, and saturable

binding of heparin to rat liver cells has been shown (Kjellen et al., 1977) suggesting it may have a physiological role.

Therefore, it appeared that heparin might have been a confounding factor in some of the earlier experiments. It seemed reasonable to suggest that if heparin was omitted from the saline, so that rats were not heparinised, then this might reduce, or completely remove the potential for non insulin-dependent stimulation of GPAT activity by fractions from saline-treated rats.

Fig 4.12: The absolute effect of Sephadex G-25 fractions of muscle extract from fed, non-cannulated rat, on microsomal GPAT from fed rat. 3.2 mg of muscle extract from fed rat (Extract L), prepared using liquid nitrogen, was chromatographed on a Sephadex G-25 column (2.54 x 27 cm) in 50 mM formic acid. Fractions of approximately 4.7 ml were collected. Absorbance was measured at 220nm. The absolute effect of these fractions on liver microsomal GPAT (microsomes; see Fig 3.1, shortened) from fed rat is shown (Expt 15). Points are the means of assays performed in duplicate. Means (± SEM) are given in Table 4.11.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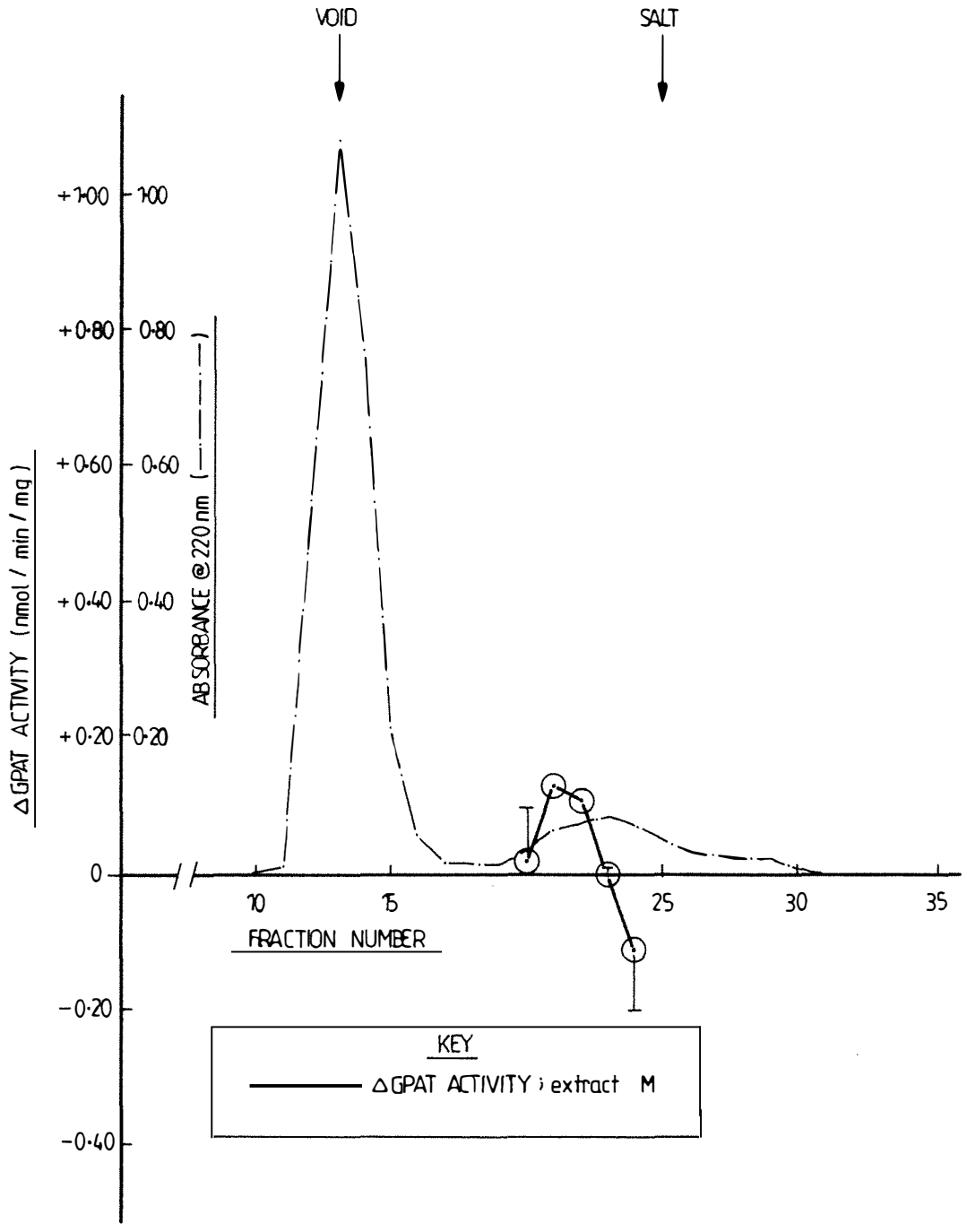
0·40
0·20
ΔGPAT ACTIVITY (nmol / min / mg)
ABSORBANCE @ 220 nm (——·——·——)
10
15
25
30
35
FRACTION NUMBER
KEY
Δ GPAT ACTIVITY ; extract L

Fig 4.13: The absolute effect of Sephadex G-25 fractions of muscle extract from 48 h-fasted, non-cannulated rat on microsomal GPAT from fed rat. 3.2 mg of muscle extract from 48 h-fasted rat (Extract M), prepared using liquid nitrogen, was chromatographed on a Sephadex G-25 column (2.54 x 27 cm) in 50 mM formic acid. Fractions of approximately 4.7 ml were collected. Absorbance was measured at 220nm. The absolute effect of these fractions on liver microsomal GPAT (microsomes; see Fig 3.1, shortened) from fed rat is shown (Expt 16). Points are the means of assays performed in duplicate. Actual means (± SEM) with standard error values are given in Table 4.11.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+0·20
0
−0·20
−0·40
1·00
0·80
0·60
0·40
0·20
ΔGPAT ACTIVITY (nmol / min / mg)
ABSORBANCE @ 220 nm (———)
10
15
25
30
35
FRACTION NUMBER
KEY
ΔGPAT ACTIVITY ; extract M

Table 4.11: The effect of Sephadex G-25 fractions of muscle extracts from fed or 48 h-fasted, non-cannulated rat on liver microsomal GPAT from fed rats.

Fraction number	GPAT activity		
	nmol/min/mg	deviation[a]	% of control
	Experiment 15 - Fed rat muscle extract[b]		
Control	1.31 ± 0.04		100.0
20 *	1.27 ± 0.03	-0.04	97.0
21 *	1.32 ± 0.05	+0.01	100.8
22 *	1.16 ± 0.01	-0.15	88.6
23 *	1.20 ± 0.06	-0.11	91.6
24 *	1.23 ± 0.11	-0.08	93.9
	Experiment 16 - 48 h-fasted rat muscle extract[c]		
Control	1.17 ± 0.05		100.0
20 *	1.19 ± 0.08	+0.02	101.7
21 *	1.30 ± 0.003	+0.13	111.1
22 *	1.28 ± 0	+0.11	109.4
23 *	1.17 ± 0.01	0	100.0
24 *	1.06 ± 0.09	-0.11	90.6

* Fractions eluting between void and salt peaks. Control assays were performed in triplicate and fraction assays were performed in duplicate ± SEM. Experimental procedures are described in the captions to Figs 4.12 and 4.13. [a]GPAT rate minus GPAT formic acid control rate. [b]Extract L, [c]Extract M.

4.3.4 Section 4 - Experiments with extracts from non heparinised rats treated with insulin (Extracts N, P & R) or saline (O, Q, & S): insulin-dependent stimulation of hepatic microsomal GPAT activity.

Experiments in Section 3 (4.3.3), with muscle extracts prepared from fasted heparinised rats treated with saline or insulin, suggested heparin might have been partly or wholly responsible for the potential of saline treatment fractions to stimulate GPAT activity in a non insulin-dependent manner. To address this suggestion, muscle extracts were prepared from fed rats treated with either normal saline (0.15 M NaCl) or insulin (Extracts N-S). Heparinised saline was replaced with normal saline and the extracts were fractionated on Sephadex G-25. It was hoped that the low molecular weight fractions from these insulin and saline treatment extracts might constitute a better system with which to demonstrate an insulin-dependent stimulation of GPAT activity. Such a demonstration would provide further evidence in favour of the hypothesis that GPAT, at a key position in the pathway for biosynthesis of glycerolipids, could be regulated by insulin.

The low molecular weight fractions of muscle extracts from fed rats treated with normal saline (Extracts O, Q and S) did not stimulate GPAT activity. Some fractions, however, caused quite marked inhibition of GPAT activity relative to the formic acid control or the insulin treatment fractions (see Fig 4.14). This lack of stimulation of GPAT activity by the saline treatment extracts seemed to confirm that heparin had been at least partly responsible for the potential of fractions of extracts, from fed heparinised rats treated with saline,

to stimulate GPAT activity. The results of Experiments 18, 20 and 22 (Fig 4.14), which involved fractionation of extract prepared from fed rats infused (i.v.) with normal saline, were consistent with the results of Experiment 15 (Fig 4.12) where fractions of Extract L, from a non-cannulated fed rat, were assayed with GPAT. In both cases, muscle extract was prepared from fed rats which were not treated with insulin, and fractions of these extracts did not stimulate GPAT activity relative to the formic acid controls. Fractions from these extracts did inhibit GPAT activity (see Figs 4.12 and 4.14). These results indicated that when fed rats treated with saline were used as a source of muscle, low molecular weight fractions of extracts did not possess the potential to stimulate GPAT activity. Saline treatment fractions did, however, inhibit GPAT activity to varying extents (Fig 4.14, Table 4.12).

Muscle Extracts N, P and R were prepared from rats treated with 4 i.u. insulin per kg body weight. The low molecular weight fractions of these extracts stimulated microsomal GPAT activity relative to both the formic acid controls and the corresponding fractions of the saline treatment (Fig 4.14). The material which eluted in fractions 17 and 19 (see Fig 4.14) increased GPAT activity, relative to the effect of saline treatment fraction, by 0.46 and 0.64 nmol/min/mg (both $P<0.01$), respectively (mean of 3 experiments). Expressed as a percentage of the mean of the formic acid controls for the insulin and saline treatments, this corresponded to 12.7% and 17.8% enhancement of GPAT activity, respectively (see Table 4.12). The GPAT stimulatory factor in fraction 19 eluted in the same volume as a 1064 dalton marker decapeptide while the GPAT stimulatory factor in fraction 17 eluted in a volume

intermediate between the void volume and the marker decapeptide. Hence, it would most probably have a molecular weight within a range of 2000 to 4000 daltons. It appeared, therefore, that insulin-dependent low molecular weight factor(s), which were capable of enhancing GPAT activity in microsomes prepared from fed rats, were present in muscle extracts prepared from insulin-treated rats and all stimulation of liver microsomal GPAT was insulin-dependent.

Fig 4.14: The absolute effect of Sephadex G-25 fractions of muscle extract from fed non heparinised rats treated with either insulin or saline, on microsomal GPAT from livers of fed rats. Muscle extracts were prepared from fed rats treated with either insulin (Extracts N, P and R) or saline (Extracts O, Q and S), using liquid nitrogen and normal saline. 3.2 mg amounts were chromatographed separately on a Sephadex G-25 column (2.54 x 27 cm) in 50 mM formic acid. Fractions of approximately 4.7 ml were collected, and absorbance was measured at 220nm. The absolute effect of these fractions on liver microsomal GPAT (microsomes; see Fig 3.1, shortened) from fed rat is shown (Experiments 17-22). Points are means of data from 3 experiments (3 insulin, 3 saline). Mean GPAT activity of the formic acid controls for insulin-treatments was 3.31 $\pm$ 1.32 (SEM) nmol/min/mg. The mean GPAT activity of formic acid controls for saline treatments was 3.90 $\pm$ 0.63 (SEM) nmol/min/mg. Insulin and saline treatment formic acid controls do not differ significantly ($P>0.10$). Molecular weight markers eluted at the positions shown: B = blue dextran (M_r 2 000 000), I = insulin (M_r 6 000), P = decapeptide (M_r 1 064), A = ATP (M_r 551) and S = KCl. Means ($\pm$ SEM) are given in Table 4.12.

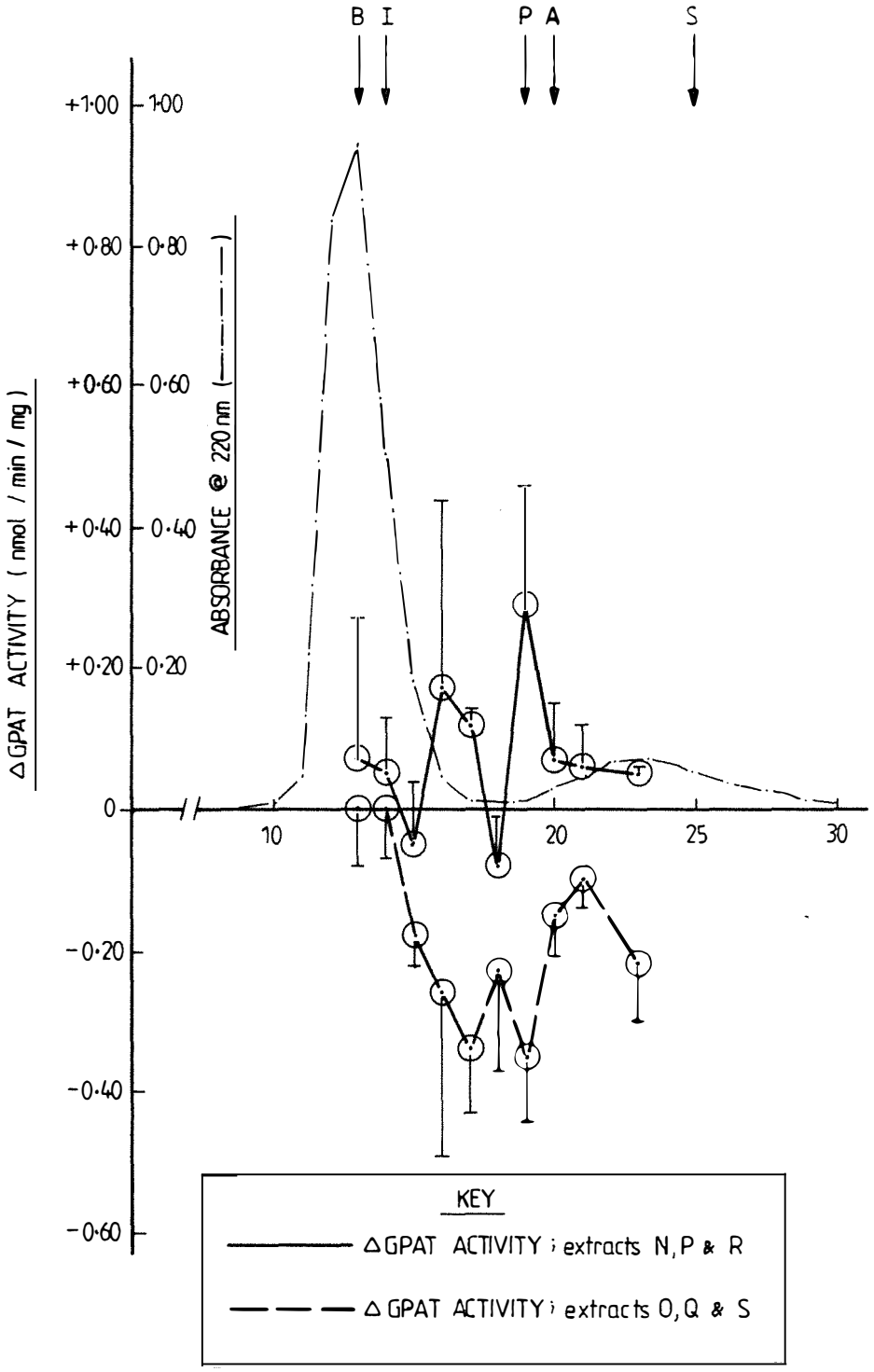

B
I
P
A
S
+1·00
+0·80
+0·60
+0·40
+0·20
0
−0·20
−0·40
−0·60
1·00
0·80
0·60
0·40
0·20
10
20
25
30
ΔGPAT ACTIVITY (nmol / min / mg)
ABSORBANCE @ 220 nm (—·—·—)
KEY
ΔGPAT ACTIVITY ; extracts N, P & R
ΔGPAT ACTIVITY ; extracts O, Q & S

Table 4.12: The absolute and percentage effects of Sephadex G-25 fractions of muscle extracts from fed rats treated with either insulin or saline, on microsomal GPAT from livers of fed rats.

Fraction number	Insulin deviation	Saline deviation	Difference	Insulin[b] effect (%)
13 *	+0.07 ± 0.20	0.00 ± 0.08	+0.07	+ 1.9
14 *	+0.05 ± 0.08	0.00 ± 0.07	+0.05	+ 1.5
15 *	-0.05 ± 0.09	-0.18 ± 0.04	+0.13	+ 3.6
16 *	+0.17 ± 0.27	-0.26 ± 0.23	+0.43	+12.0
17 *	+0.12 ± 0.02[a]	-0.34 ± 0.09[a]	+0.46	+12.7
18 *	-0.08 ± 0.07	-0.23 ± 0.14	+0.15	+ 4.2
19 *	+0.29 ± 0.17[a]	-0.35 ± 0.09[a]	+0.64	+17.8
20 *	+0.07 ± 0.08	-0.15 ± 0.06	+0.22	+ 6.1
21 *	+0.06 ± 0.06	-0.10 ± 0.04	+0.16	+ 4.3
23 *	+0.05 + 0.01	-0.22 + 0.08	+0.27	+ 7.4

* Fractions eluting between void and salt peaks. Values are the GPAT rates (nmol/min/mg), in the presence of each fraction less the formic acid control rate, averaged over three experiments (3 insulin - Expts 17, 19, 21; 3 normal saline - Expts 18, 20, 22). Mean formic acid controls for insulin and saline treatment experiments were 3.31 ± 1.32 and 3.90 ± 0.63 nmol/min/mg ($P>0.10$, NS), respectively. [a]means (± SEM) within rows differ $P<0.01$. [b]Difference divided by the mean of the insulin and saline treatments' formic acid controls (=3.61 ± 0.29 nmol/min/mg). For experimental details see caption to Fig 4.14.

CHAPTER FIVE

THE EFFECT OF SUPERNATANTS FROM LIVER PARTICULATE FRACTIONS ON GPAT

Chapter 5. THE EFFECT OF SUPERNATANTS FROM LIVER PARTICULATE FRACTIONS ON GPAT

5.1 Introduction

Following on from the experiments with extracts prepared from muscle of rats treated with insulin or saline (Chapter 4) it was considered that crude plasma membrane preparations treated with insulin could be another means by which it might be possible to demonstrate the effect of insulin on hepatic microsomal GPAT. Experiments in Chapter 4 utilised preparations from muscle to show an insulin-dependent enhancement of microsomal GPAT activity. Presumably, interaction of insulin with its receptors on the plasma membranes of the muscle cells was essential to the potential of insulin treatment fractions to stimulate GPAT activity relative to saline treatment fractions. As GPAT was isolated from liver, the next logical step was to attempt to show that GPAT activity could be enhanced in an insulin-dependent manner by a system with all components isolated from liver.

It has been shown that insulin, when added to a system consisting of plasma membranes and mitochondria, enhances the activity of mitochondrial pyruvate dehydrogenase (Jarett et al., 1981; Seals & Jarett, 1980). The presence of plasma membranes was required in the system in order to demonstrate this insulin-dependent effect. It appeared that the interaction of insulin with its receptors on the plasma membranes generated a factor which enhanced the activity of pyruvate dehydrogenase. Insulin-dependent effects on this and other

insulin-sensitive enzymes have also been demonstrated with systems in which plasma membranes or particulate fractions from rat liver were treated with insulin (Amatruda & Chang, 1983; Begum et al., 1983; Larner et al., 1979a; Saltiel et al., 1981, 1982a,c, 1983; Suzuki et al., 1984). Similarly, systems involving treatment of adipocyte plasma membranes with insulin have also been reported (Kiechle et al., 1981; McDonald et al., 1981; Seals & Czech, 1980b, 1981b). Unfractionated supernatants from plasma membranes or particulate fractions treated with insulin have been utilised in many of these experiments (Begum et al., 1982b, 1983; Kiechle et al., 1981; McDonald et al., 1981; Seals & Czech, 1980b, 1981b). Therefore, a decision was made to prepare particulate fraction from rat liver to examine the possibility of using supernatant from insulin-treated particulate fraction to demonstrate an insulin-dependent enhancement of GPAT activity.

Methods for preparation of particulate fraction from rat liver were examined. It could be inferred that several of the methods reported in the literature were suitable by virtue of the fact that experiments utilising them were successful. However, it was not evident from these reports just what proportion of the particulate fraction was actually plasma membranes. The references cited above indicate that particulate fractions are enriched with plasma membranes but, considering the procedures for their preparation, it is almost certain that they also contain microsomal membranes and mitochondrial components. It appeared that the interaction of insulin with its receptors on the plasma membranes was critical to the ability of these systems to demonstrate insulin-dependent effects on various insulin-sensitive enzymes. Therefore, it was thought advisable to

determine the proportion of plasma membranes in liver particulate fraction. It seemed logical that the higher the proportion of plasma membranes, the greater the probability of being able to demonstrate an insulin-dependent effect on GPAT activity. The proportion of plasma membrane in liver particulate fractions was estimated through assay of the plasma membrane-specific marker enzyme 5'-nucleotidase and a method selected. Experiments were then conducted in which liver particulate fractions were exposed to insulin (in phosphate buffer) or phosphate buffer (control). After a brief centrifugation of these treated fractions, aliquots of the supernatants were assayed to determine whether GPAT could be stimulated in an insulin-dependent manner.

5.2 Methods

5.2.1 Selection of method for preparation of liver particulate fraction enriched in plasma membranes.

Three methods for preparation of liver particulate fraction were compared and the extent to which the fractions were enriched with plasma membranes estimated by assay of 5'-nucleotidase (Aronson & Touster, 1974; Leloir & Cardini, 1957) which has a specific activity of 990 nmol phosphate released/min/mg (Fleischer & Kervina, 1974) in purified plasma membranes. The method described by Saltiel *et al.* (1981) and Begum *et al.* (1983) was examined and using it, a liver particulate fraction with approximately 6% plasma membrane protein was prepared. Despite the success of Begum *et al.* (1983) and Saltiel *et al.* (1981) in showing insulin-dependent effects, the proportion of plasma membrane present appeared to be relatively low. Approximately 40% of the the total 5'-nucleotidase activity sedimented with the particulate fraction, but approximately 50% of the activity sedimented with the 1 100**g** pellet which suggested incomplete homogenisation. Therefore, using the same method, but incorporating a more thorough homogenisation, a liver particulate fraction containing 9.4% plasma membrane protein was prepared. The proportion of plasma membrane protein in the fraction was still low and nearly 50% of the total 5'-nucleotidase activity still sedimented in the low speed pellet although 70% of the total activity was now found in the particulate fraction. With both methods the recovery of 5'-nucleotidase activity was slightly in excess of 100% which suggested that activity in the homogenate was not measured fully. These studies suggested that Begum

et al. (1983) and Saltiel et al. (1981) had used particulate fractions not highly enriched with plasma membranes, which is surprising as the plasma membranes are thought to be a critical component of the systems. The reason for the low proportion of plasma membrane protein in the particulate fraction may have been due to failure of the method to specify use of a 0.25 M sucrose-based buffer. The hypotonic buffer used would have caused the mitochondria to fragment, so increasing the non plasma membrane protein in the particulate fraction.

Therefore, the method of Saltiel et al. (1983) for preparation of a liver particulate fraction enriched with plasma membranes was examined. This used an isotonic sucrose buffer until the mitochondria were removed. Using this method a liver particulate fraction that contained 14.0% plasma membrane protein was isolated (Table 5.1). There was, however, still a large proportion of the total 5'-nucleotidase activity in the low speed fraction, probably partly due the gentle homogenisation used to minimise disruption of the mitochondria. This method had been used successfully by Saltiel et al. (1983) in the demonstration of insulin-dependent effects on acetyl-CoA carboxylase and the initial method considered had also been used successfully with pyruvate dehydrogenase (Begum et al., 1983; Saltiel et al., 1981). Therefore, the final method for preparation of liver particulate fraction was adopted in subsequent experiments and is outlined fully in the text following Table 5.1.

Table 5.1: Results for 5'-nucleotidase marker study using Method 3 for preparation of liver particulate fraction.

	Protein			5'-nucleotidase activity	
Fraction[a]	mg/ml	mg	%	nmol/min/mg	%
1	18.57 ± 0.21	1095.63 ± 12.39	100.0	24.4 ± 0.5	100.0
2	10.00 ± 0.35	371.30 ± 13.00	33.9	45.3 ± 1.9	62.9
3	10.20 ± 0.11	89.25 ± 0.96	8.2	37.3 ± 0	12.5
4	5.03 ± 0.08	496.31 ± 7.89	45.3	6.7 ± 0	12.3
5	11.04 ± 0.04	67.35 ± 0.24	6.2	138.4 ± 1.0	34.8
6	0.93 ± 0.01	4.73 ± 0.05	0.4	99.0 ± 4.8	0.9

Enzyme assays and protein determinations conducted in duplicate. [a]Key: 1. Homogenate; 2. 1 100**g** pellet resuspended; 3. 10 300**g** pellet resuspended; 4. 45 000**g** supernatant; 5. 45 000**g** pellet (-glycogen) resuspended (liver particulate fraction); 6. glycogen pellet resuspended.

The method adopted for the preparation of rat liver particulate fraction was based on the method of Saltiel et al. (1983):

1. Male Sprague-Dawley rats (300-400 g) were killed by exsanguination.
2. The liver was removed, weighed, and placed in ice cold homogenisation buffer (0.25 M sucrose, 10 mM HEPES, pH 7.4). It was minced using a razor blade and homogenised in 3.3 volumes (v/w) of the sucrose-HEPES homogenisation buffer using 3 strokes of a Jecons uniform glass homogeniser with a loose fitting teflon pestle.
3. The homogenate was filtered through 4 layers of cheese cloth and centrifuged at 1 100**g** (r_{av} 6.9 cm) for 10 min.
4. The 1 100**g** supernatant was centrifuged at 10 300**g** (r_{av} 6.9 cm) for 10 min. The subsequent supernatant was decanted, diluted 1:1 (v/v) with 10 mM HEPES buffer and centrifuged at 45 000**g** (r_{av} 6.47 cm) for 25 min.
5. The 45 000**g** supernatant was aspirated leaving the crude plasma membrane pellet (liver particulate fraction). The pellet was freed from the clear glycogen pellet, which firmly adhered to the side of the centrifuge tube, and resuspended in about 6 ml of 10 mM HEPES, pH 7.4 using a small glass homogeniser.

5.2.2 Incubation of particulate fraction with insulin or buffer

1. Stock insulin, 100 i.u./ml, was diluted to 10 munits/ml or 20 munits/ml in phosphate buffer (pH 7.4, 4°C) immediately prior to the experiment.
2. The liver particulate fraction, enriched with plasma membranes, was used in the incubation which contained the following in a final volume of 1 ml: 4.9-6.6 mg of liver particulate fraction in 10 mM HEPES, pH 7.4; insulin (20 to 1000 μunits) in 10 mM phosphate buffer, pH 7.4; and an appropriate volume of 10 mM phosphate buffer, pH 7.4, to make the volume up to 1 ml. The control incubation received an equivalent volume of phosphate buffer in place of the insulin. The incubations were carried out in 1.8 ml capped plastic "Eppendorf" micro centrifuge tubes.
3. Immediately after the addition of insulin or phosphate buffer (test and control respectively) the incubations were placed in a shaking water bath at 37°C. After 10 min the test and control tubes were briefly placed on ice before being centrifuged (15 600**g** at 15 000 rpm) in an "Eppendorf" micro centrifuge (model 5414S) for 15 min at 4°C.
4. Two sterile 1 ml syringes were used to aspirate 0.58 ml of the supernatant from each tube. These aliquots were placed in two clean micro centrifuge tubes and kept on ice.

5.2.3 Assay to examine the effect of particulate fraction supernatant on GPAT activity

1. Approximately 0.5 mg of liver microsomal protein (see Fig 3.1, shortened) was added to the GPAT reaction mixture which was as described in section 3.8 except that the 50 mM Tris HCl buffer, pH

7.4, was replaced with 50 mM phosphate buffer, pH 7.4.

2. The enzyme - GPAT reaction mixture was incubated in a water bath at 30°C for 5 min and varying volumes of supernatants from particulate fractions treated with insulin or buffer were added to the assay incubations and left for 10 min.
3. The GPAT reactions were started, as usual, by the addition of palmitoyl-CoA and stopped after 1 min by the addition of n-butanol (see 3.8).

5.3 Results and Discussion - The effect of supernatants from liver particulate fractions treated with insulin

The experiments conducted in the present study utilised 4.9 to 6.6 mg of liver particulate fraction protein per ml, corresponding to 0.69 to 0.92 mg of plasma membrane protein per ml (at 14% purity of the liver particulate fraction) in the incubations with or without insulin. Consideration of reports in the literature suggested that, within limits, the amount of particulate fraction was not critical to the demonstration of insulin-dependent effects. For example, Saltiel et al. (1981) and Begum et al. (1983), who both used the same method to prepare liver particulate fraction, had incubation conditions with 3 mg/ml and 15-20 mg/ml of particulate fraction (protein basis), respectively. Examination of the method, as discussed above, suggested that this fraction would have contained about 6% plasma membrane protein. Hence their incubations would have contained about 0.18 mg/ml and 0.9-1.2 mg/ml of plasma membrane protein, respectively. Saltiel et al. (1983) used 9 mg/ml of particulate fraction protein in their experiments. Examination of this method, that adopted for present experiments, suggested that this fraction would have contained about 14% plasma membrane protein and so their incubations would have contained about 1.26 mg/ml of plasma membrane protein. Other experiments involving rat liver plasma membranes (Suzuki et al., 1984) have used 0.2 mg/ml plasma membrane protein and experiments with adipocyte plasma membranes have been successful with concentrations of plasma membrane protein within the range of 0.1-3 mg/ml (Begum et al., 1983; Kiechle et al., 1981; McDonald et al., 1981; Newman et al,

1985; Seals & Czech, 1980b, 1981b; Zhang et al., 1983). Similarly, incubation of insulin with particulate fractions for 5-15 min had been used successfully to produce a supernatant able to stimulate enzymes in an insulin-dependent manner (Begum et al., 1983; Saltiel et al., 1981, 1983; Suzuki et al., 1984). The 10 min incubation time of the present study was within this range.

Experiments were conducted in which particulate fraction was incubated with 20, 100, 500, 1000 μunits of insulin/ml which was similar to the range reported for experiments utilising particulate fractions or plasma membranes in the literature (Begum et al., 1983; Kiechle et al., 1981; Newman et al, 1985; Saltiel et al., 1981, 1983; Seals & Czech, 1980b, 1981b; Suzuki et al., 1984; Zhang et al., 1983). In the fed rat the concentration of insulin in plasma, depending on nutrition and time after feeding, can rise to about 120 μunits/ml (Millward et al., 1974; see also Sinha & Caro, 1985).

Three different volumes (10, 40, 112 μl) of the subsequent insulin-treated or control supernatants were assayed to determine whether or not the insulin treatment elicited stimulation of microsomal GPAT activity (Table 5.2). Results were not expressed per mg of protein as the liver particulate fraction added to the GPAT assay also contains microsomal protein with GPAT activity. 0.45 mg of actual microsomal protein was added prior to the addition of the supernatant. GPAT rate with no supernatant additions was 2.72 ± 0.03 (SEM) nmol/min. In these experiments supernatants from insulin-treated fractions containing plasma membranes had no significant stimulatory effect on GPAT activity in comparison to the buffer-control supernatants. GPAT

activity appeared to be stimulated slightly, to 106% (+0.18 nmol/min/mg) of the control, when 10 µl of supernatant from particulate fraction, treated with 500 µunits insulin/ml, was assayed with GPAT (Table 5.2). However, when greater volumes of the supernatant were assayed with GPAT, a slight inhibition of GPAT activity was observed (see Table 5.2).

To examine the possibility that there was insulin-dependent stimulation of GPAT activity by the supernatant of the liver particulate fraction, treated with 500 µunits insulin/ml (Table 5.2), the experiment in which 10 µl of supernatant was added to the GPAT assay was repeated. However, GPAT activity was inhibited marginally by this volume of supernatant in the assay compared to the control (Table 5.3). A 5 µl volume of supernatant was also assayed to determine its effect on GPAT and to examine whether this might enhance GPAT activity (Table 5.3). This too caused a slight inhibition of GPAT activity relative to the control.

Particulate fraction was also treated with 1000 µunits insulin/ml to address the possibility that an even higher concentration of insulin (supra-physiological) might be needed to elicit an insulin-dependent stimulation of GPAT activity (Table 5.3). No stimulation of GPAT was found when GPAT was assayed with supernatant from this fraction. Therefore, use of high concentrations of insulin in this system also proved ineffective in stimulating GPAT activity in an insulin-dependent manner.

Overall, with increasing volume of particulate-fraction added to

the assay, GPAT activity was increased. This appeared to be due to the presence of microsomal GPAT activity in the particulate fraction. In one experiment the microsomal GPAT activity in the particulate fraction alone was determined to be 7.85 ± 0.23 (SEM) nmol/min/mg of protein. The possibility that the slight differences in effects of insulin treatment and control supernatants on GPAT activity might have been due to differences in the amount of protein in the supernatants appeared unlikely as the mean protein concentration in the supernatants was found to be 52.9% ± 1.4% and 53.3% ± 1.3% (SEM), respectively, of the original particulate fraction protein concentration.

The results of these experiments do not rule out the possibility that insulin treatment of particulate fraction or plasma membranes can be used to demonstrate an insulin-dependent stimulation of GPAT activity. It is possible that the insulin treatment did give rise to material in the supernatant which was capable of stimulating GPAT activity, but other material present in the supernatant might have masked this. It might be possible to demonstrate insulin-dependent stimulation of GPAT activity if some form of extraction and/or fractionation procedure were applied. Use of ultrafiltration or gel filtration techniques may be appropriate. The presence of microsomal, and possibly mitochondrial, GPAT activity in liver particulate fraction might have interfered with the detection of an insulin-dependent stimulation of GPAT activity. Recently a very rapid (3 h) method for the preparation of plasma membranes from rat liver has been reported (Armstrong and Newman, 1985). Use of a plasma membrane fraction with minimal microsomal and mitochodrial contamination may make it possible to demonstrate an insulin-dependent stimulation of GPAT activity.

Table 5.2: The effect of supernatant from liver particulate fraction treated with 20, 100 or 500 μunits of insulin/ml on GPAT.

Vol. of supernatant[a] added to assay (μl)	GPAT activity nmol/min	I/C x 100/1
20 μunits insulin/ml		
C10	2.86 ± 0.01	
I10	2.54 ± 0.19	88.8%
C40	3.43 ± 0.03	
I40	3.55 ± 0.09	103.5%
C112	5.08 ± 0.18	
I112	4.98 ± 0.08	98.0%
100 μunits insulin/ml		
C10	3.15 ± 0.12	
I10	3.18 ± 0.04	101.0%
C40	3.78 ± 0.01	
I40	3.57 ± 0.04	94.4%
C112	4.95 ± 0.08	
I112	5.01 ± 0.03	101.2%
500 μunits insulin/ml		
C10	2.88 ± 0.02	
I10	3.06 ± 0.10	106.3%
C40	3.70 ± 0.09	
I40	3.64 ± 0.05	98.4%
C112	4.81 ± 0.12	
I112	4.60 ± 0.34	95.6%

Incubations contained 6.61 ± 0.05 (SEM) mg of particulate fraction protein. Values (± SEM) are the means of duplicate assays. [a]C and I prefix denote control (-insulin) and insulin-treatment supernatants.

Table 5.3: The effect of supernatant from liver particulate fraction treated with 500 or 1000 μunits insulin per ml on liver microsomal GPAT.

Vol. of supernatant[a] added to assay (μl)	GPAT activity nmol/min	I/C x 100/1
500 μunits insulin/ml		
C5	1.99 ± 0.01	
I5	1.91 ± 0.04	96.0%
C10	2.14 ± 0.08	
I10	2.00 ± 0.02	93.5%
1000 μunits insulin/ml		
C5	2.03 ± 0.06	
I5	1.85 ± 0.16	91.1%
C10	2.02 ± 0.05	
I10	1.99 ± 0.07	98.5%

Incubations contained 4.91 ± 0.06 (SEM) mg of particulate fraction protein. Values are the means of duplicate assays ± SEM. [a]For key to superscript see footnote to Table 5.2. 0.58 mg of actual microsomal protein was added prior to the addition of the supernatant. GPAT rate with no supernatant additions was 1.87 ± 0.09 nmol/min.

CHAPTER SIX

DISCUSSION AND CONCLUSIONS

Chapter 6. DISCUSSION AND CONCLUSIONS

Evidence in the literature (Chapter 1) suggested that hepatic microsomal GPAT is an important point at which glycerolipid metabolism is regulated by hormones such as noradrenaline and insulin. During this study the hypothesis that GPAT activity is regulated by insulin was specifically addressed by experiments conducted using methodology described in the literature and involving the use of low molecular weight fractions of muscle extracts from rats treated with insulin or saline.

In the initial experiments (4.2) GPAT activity was stimulated by low molecular weight fractions of muscle extract from insulin-treated rat, compared to both formic acid controls and the effect of saline treatment fractions. Subsequently, fractions of extracts from insulin-treated rats failed to enhance GPAT activity relative to the formic acid controls (4.3) even though the relativity between the effect of insulin and saline treatment fractions was preserved. In the initial experiments liquid air was used to stop biological activity in the belief that this coolant would be just as appropriate as the liquid nitrogen referred to in the method of Larner et al. (1979d). Recent publications (Larner, 1982; Larner et al., 1982c), however, drew attention to the sensitivity of similar muscle extract systems to oxygen and it appears that the muscle extract system of the present study is also sensitive to O_2 since substitution of liquid nitrogen for liquid air in the method restored the potential of insulin-treatment low molecular weight fractions to enhance GPAT activity relative to the

formic acid control. Therefore, in all subsequent experiments liquid nitrogen was used.

In the experiments (4.3) utilising liquid nitrogen, it became apparent that certain fractions of the extract from the saline treatment muscle enhanced GPAT activity relative to both the formic acid controls and the effect of insulin treatment fractions. Furthermore, experiments with extracts prepared from fasted rats reinforced the conclusion that the potential of saline treatment fractions to enhance GPAT activity was not dependent on insulin (4.4). In contrast with these experiments in which the insulin or saline was given through a cannula, extract fractions from non-cannulated rats had little potential to stimulate GPAT activity. It was postulated therefore that the heparin used to keep the cannula patent might be associated with the ability of certain fractions to stimulate GPAT activity independent of insulin treatment. Complete omission of heparin from the protocol abolished the insulin-independent ability of saline-treatment low molecular weight fractions to enhance GPAT activity. Hence it appears that heparin treatment of rats (approximately 50 units per 300 g rat) was able to impart insulin-independent GPAT-stimulatory potential to low molecular weight fractions of muscle extracts. As endogenous heparin is detectable in the blood of rats (Horner, 1974) and binds to liver cells in a time-dependent and reversible manner (Kjellen, et al., 1977), it may be that heparin has an active role in metabolism. Its ability to interact with and activate lipoprotein lipase (Jaques, 1980) suggests that endogenous heparin might be able to enhance the entry of fatty acids into cells. If the interaction of heparin with liver cells in vivo was

also able to stimulate GPAT activity, either directly or indirectly, then esterification of the increased supply of fatty acids would be enhanced.

Using a system freed of these confounding factors, evidence was obtained that hepatic microsomal GPAT activity is enhanced in an insulin-dependent manner by low molecular weight fractions of rat muscle extracts. Fractions of muscle extracts from rats treated with insulin stimulated GPAT activity by 0.46 and 0.64 nmol/min/mg ($P<0.01$) in comparison to the effect of equivalent fractions of extracts from muscles of rats treated with saline. These fractions would have contained material of approximately 3000 and 1000 daltons, respectively. Whether there are actually one or two stimulators of hepatic microsomal GPAT cannot be resolved until it is possible to determine the chemical identity of the material or materials. However, the insulin-dependent stimulation of liver microsomal GPAT by these low molecular weight fractions is consistent with the thesis that GPAT, at a key position in the pathway for the biosynthesis of glycerolipids, is regulated by insulin. However, there was some question whether stimulating GPAT activity to this extent would have any physiological relevance.

As was discussed in Chapter 1, GPAT is the first of two enzymes involved in the provision of phosphatidic acid for phospholipid and triacylglycerol biosynthesis. The majority of the enzymes of the pathways for synthesis of phospholipids and triacylglycerols are microsomal in location and so it seems likely that microsomal GPAT has a major role in the biosynthesis of both classes of compounds (Bagul,

1972; Bell & Coleman, 1980; Coleman & Bell, 1980; Coleman et al., 1978; Jamdar & Fallon, 1973; Jamdar & Osborne, 1982; Saggerson et al., 1980; Schlossman & Bell, 1976a, 1976b). Hence GPAT assayed in microsomal preparations may be the expression of a heterogeneous population of GPATs, part of which is involved in supplying phosphatidic acid for phospholipid biosynthesis and part concerned with providing phosphatidic acid for triacylglycerol biosynthesis. Synthesis of phospholipids in rat liver, in contrast to triacylglycerol synthesis, occurs at a virtually constant rate despite nutritional status (Iritani et al., 1976). This probably ensures maintenance of membrane integrity and competency, equally as important in the fed or fasted state. It would therefore be reasonable to expect that a subpopulation of GPAT concerned with phospholipid biosynthesis would not be subject to short term regulation in response to nutritional status and energy requirements. Production of triacylglycerol by the liver declines during fasting in association with mobilisation of free fatty acids which are oxidised to provide energy. Hence, in order to facilitate shifts in metabolic emphasis, it would be logical to expect that a subpopulation of GPAT, involved with synthesis of triacylglycerols, would be subject to short term regulation through the action of hormones such as noradrenaline, adrenaline, and insulin (and possibly glucagon; Lamb et al., 1982; Rider & Saggerson, 1983; Saggerson et al., 1981; Sugden et al., 1980). With these considerations in mind, the metabolic relevancy of a 0.46-0.64 nmol/min/mg insulin-dependent stimulation of microsomal GPAT was assessed as follows with data drawn from the literature where appropriate.

Consider the following rat (Bates & Saggerson, 1977):

body weight.....194 g

liver weight.....7.9 g

total liver protein.....1.6 g

mitochondrial protein.....592 mg

microsomal protein.....584 mg

The body and liver weights of this model rat, drawn from a paper by Bates & Saggerson (1977), are similar to those observed in experiments reported in this thesis. Using additional data from this paper, it was estimated that mitochondrial and microsomal GPAT would esterify glyerol 3-phosphate, with 3 mM sn-glycerol 3-P in the assay, at approximately 3.6 and 4.5 nmol/min/mg protein, respectively. The estimated activity of the microsomal GPAT is similar to that obtained in the present study in which a 3mM concentration of glycerol 3-phosphate was also used. 3 mM sn-glycerol 3-phosphate is within the physiological range for both fed and fasted rats (see Tables 1.2 and 1.3). Using these data the potential acylating capabilities of the GPATs were estimated to be 2,131 and 2,628 nmol/min/liver for the mitochondrial and microsomal GPATs, respectively. Therefore, there would be a total potential acylating capacity of 4,759 nmol/min/liver.

The insulin-dependent stimulations of microsomal GPAT activity of +0.46 and +0.64 nmol/min/mg by low molecular weight fractions of rat muscle extracts were applied to this example. These translated into increases in esterification of 269 and 374 nmol/min/liver, respectively.

Wise & Elwyn (1965) reported that the the major phospholipid species and their quantities in the rat liver are: phosphatidylcholine, 19.6 μmol/g fresh liver or 154.8 μmol/liver; phosphatidylethanolamine, 7.4 μmol/g fresh liver or 58.5 μmol/liver; and phosphatidylserine 1.06 μmol/g fresh liver or 8.4 μmol/liver. Data of Wise & Elwyn (1965) also indicated that the turnover times for phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine were respectively 72, 127, and 442 min. When these data were applied to the 7.9 g rat liver, it was calculated that the following amount of phospholipids would be synthesised: phosphatidylcholine 2,150 nmol/min/liver; phosphatidylethanolamine 461 nmol/min/liver; phosphatidylserine 19 nmol/min/liver. Hence the total rate of synthesis of phospholipids would be about 2,630 nmol/min/liver. This is approximately 2,129 nmol/min less than the sum of the total potential acylating activity of the mitochondrial and microsomal GPATs of the liver, but comparable to the estimated potential acylating activity of the microsomal GPAT (2,628 nmol/min/liver). This is compatible with the fact that, as discussed in Chapter 1, the majority of enzymes involved in synthesis of phospholipids are located in the microsomes. However, although the above data were considered to be the most appropriate, the rate of phospholipid synthesis may have been overestimated as other workers have found turnover times ranging from 360 min to 660 min for rat liver phospholipids depending on the precursor used and its route of administration (Arvidson, 1968; Bygrave, 1969; Campbell & Kosterlitz, 1952; Popjak & Muir, 1950; Tolbert & Okey, 1952).

The data of Baker & Schotz (1964) indicated that the rate of

triacylglycerol synthesis in the liver of Sprague-Dawley rats, fed a 10% glucose solution ad libitum for 24 h prior to measurement, was approximately 0.27 mg/min per 100 g body weight. Therefore, the liver of a 194 g model rat would produce approximately 0.52 mg of triacylglycerol per min. In the absence of knowledge of the composition of the triacylglycerol, a molecular weight of 850 g/mol was assumed. Therefore, triacylglycerol output by the liver of the glucose-fed rat would be approximately 610 nmol/min. Similarly, the data of Schotz et al (1964) indicated that the rate of synthesis of triacylglycerol by the liver of 24 h fasted Sprague-Dawley rats was approximately 0.15 mg/min per 100 g body weight. For the 194 g rat this would equate to the liver producing approximately 0.29 mg/min. Hence, assuming a molecular weight of 850 g/mol, the hepatic triacylglycerol output of the 24 h fasted rat would be approximately 342 nmol/min.

If the calculated rates for the synthesis of phospholipids and triacylglycerols, in the fed state, are summed for this particular liver (2,630 nmol/min/liver and 610 nmol/min/liver), then approximately 3,240 nmol of lipid/min/liver would be synthesised. This would account for some 68% of the estimated potential acylating capacity of the mitochondrial and microsomal GPATs. Similarly, addition of the rate of triacylglycerol synthesis in the liver of the fasted rat to the rate of synthesis of phospholipids would account for 63% of the potential acylating capacity. Therefore, it appears that the various data and estimates used are compatible with that which might be expected in vivo.

The +0.46 and +0.64 nmol/min/mg insulin-dependent increases in GPAT rate were calculated above to equal 269 and 374 nmol/min/liver for the 7.9 g liver. These insulin-dependent changes in GPAT activity observed in the present study can therefore account for 44% and 61% of the predicted rate of triacylglycerol synthesis in the fed rat. The rates of triacylglycerol output by the livers of the fed and starved rat were calculated above to be 610 and 342 nmol/min, respectively. The difference between the two rates of triacylglycerol synthesis, 268 nmol/min/liver, is similar in magnitude to the calculated insulin-dependent increases in GPAT activity, which would arise if GPAT activity was enhanced by +0.46 or +0.64 nmol/min/mg of microsomal protein, i.e. 269 and 374 nmol/min/liver. Therefore, insulin-dependent increases in GPAT activity of the magnitude observed in this study could account for the difference between the estimated hepatic triacylglycerol production of fed and fasted rats and so appear to be of sufficient magnitude to have physiological significance. This assumes, as discussed above, that the rate of phosphatidic acid production for phospholipid biosynthesis is constant and not affected by insulin so that insulin-dependent increases in GPAT activity would be targeted to triacylglycerol synthesis.

The experiments described in this thesis provide further evidence that hepatic microsomal GPAT is an important point for the regulation of glycerolipid biosynthesis by insulin. The evidence is indirect in that the technique utilised to demonstrate an insulin-dependent enhancement of hepatic microsomal GPAT activity involved isolation of low molecular weight material from extracts of muscles from rats treated with insulin or saline. Clearly, <u>in vivo</u>, intracellular

factors from muscle are unlikely to exert any direct influence on a hepatic enzyme system. Instead, effects of insulin on hepatic microsomal GPAT activity would have to be mediated by a hepatic subcellular system. Hence there is some question whether an insulin-dependent enhancement of GPAT activity, demonstrated with a muscle-liver system, is relevant to the situation *in vivo*. Evidence was not accumulated in this study to show that *in vivo* administration of insulin increases the activity of hepatic microsomal GPAT. Despite this, as discussed in Chapter 1 (1.3.4.4), others have presented evidence that insulin treatment *in vivo* stimulates esterification. Thus, perfusion of rat liver with insulin for 30 min increased total liver GPAT activity (mitochondrial + microsomal) to approximately 120% of controls (+0.14-0.30 nmol/min/mg) (Bates *et al.*, 1977). It appears that this was mainly due to an increase in the activity of the mitochondrial GPAT which increased by 30% (+0.2 nmol/min/mg) whereas the microsomal GPAT activity increased by only 10% (+0.03 nmol/min/mg). Similar results were obtained by Sugden & Munday (1983) who showed that hepatic mitochondrial and microsomal GPAT activities increased by 12% (+0.13 nmol/min/mg) and 21% (+0.17 nmol/min/mg), respectively, 2.5 h after refeeding following a 24 h fast. It is probable that this increase in GPAT activity was in response to an increased insulin concentration in the circulation. The magnitude the increases reported by Bates *et al.* (1977) and Sugden & Munday (1983) are small relative to those measured in the present experiments. These workers, however, did not measure GPAT activities in the subcellular fractions directly but rather used crude homogenates and differentiated between the mitochondrial and microsomal GPAT activities by employing assay conditions favouring one or other isoenzyme. In addition to these

data, which indicate that insulin stimulates the production of an activator of hepatic microsomal GPAT activity, perhaps similar to the activator present in muscle extract fractions reported in Chapter 4, there are also parallels with other enzymes systems. For example, adipocyte pyruvate dehydrogenase (PDH) activity, which is enhanced in an insulin-dependent manner *in vitro* by muscle extract fractions (Jarett and Seals, 1979b; Larner *et al.*, 1979d), is also activated *in situ* by brief exposure of adipocytes to insulin (Denton & Hughes, 1978; Mukherjee & Jungas, 1975). Glycogen synthetase phosphoprotein phosphatase (which activates glycogen synthase) is, like PDH activity, stimulated in an insulin-dependent manner *in vitro* by low molecular weight fractions of muscle extracts (Larner *et al.*, 1979d). Accordingly, glycogen synthesis and glycogen synthase are enhanced as a result of insulin treatment of muscle (Larner *et al.*, 1978, 1982c; Smith & Lawrence, 1985). Moreover, the activity of cAMP-dependent protein kinase from muscle is decreased in an insulin-dependent manner by low molecular weight fractions from rat muscle *in vitro* (Larner *et al.*, 1979d) and, measured in the absence of cAMP, the activity of this enzyme is decreased after treatment of rats with insulin *in vivo* (Larner *et al.*, 1979c). Hence there are a number of instances in which enzymes that have their activity altered by insulin *in vivo* can also have their activity altered *in vitro* in an insulin-dependent manner by low molecular weight fractions of muscle extracts. Therefore, published studies suggest that insulin-dependent changes in activities of enzymes, demonstrated with *in vitro* systems utilising low molecular weight fractions from rat muscle, may parallel sensitivity of enzymes to insulin *in vivo*.

In the future it will be necessary to test the hypothesis that that GPAT activity is regulated by insulin more directly, perhaps by conducting experiments in which insulin is administered by liver perfusion or intravenous injection *in vivo*, followed by isolation and assay of the hepatic microsomal GPAT. The *in vivo* study of Bates *et al*. (1977), discussed above did examine the effect of insulin-perfusion on liver GPAT but did not fractionate the liver homogenate. Microsomal GPAT activity was deduced by difference assuming that NEM inhibited only the microsomal GPAT activity and so it would be advantageous to obtain direct measurement of microsomal GPAT activity after similar treatment or intravenous injection. However, such an approach is dependent upon the effect of insulin on the enzyme persisting through subcellular fractionation prior to assay, i.e. it is difficult to prepare and assay microsomal GPAT in less than 2 h which, even at 2-3^{o}C, is probably sufficient time for potentially detrimental post-treatment effects to occur. It is probable therefore that an effect of insulin will only persist through subcellular fractionation if it causes some form of covalent modification of the enzyme, or if there is a tight association of the enzyme with a regulatory molecule. Alternative mechanisms are unlikely to be detected by this method because "loosely" associated regulatory molecules or ligands will be diluted or completely lost during the the fractionation procedure. In this connection, it has been shown that the inhibitory effects of adrenaline and noradrenaline on adipocyte GPAT persist through homogenisation (Rider & Saggerson, 1983; Sooranna & Saggerson, 1978a), but only the effect of noradrenaline on the microsomal GPAT persists through subcellular fractionation (Rider & Saggerson, 1983). The experiments of Rider & Saggerson, (1983)

indicated that noradrenaline does not inactivate microsomal GPAT by covalent modification and suggested a "tight" association of GPAT with a regulatory ligand. Hence, if hepatic microsomal GPAT was regulated by insulin in a similar manner it may be possible to demonstrate a stimulatory effect on the enzyme after insulin administration by perfusion of the liver or intravenous injection. However, because of the potential difficulties with perfusion or in vivo approaches, the approach taken in experiments in this thesis may have more merit because potential stimulators of GPAT are added to the already prepared microsomal fraction.

Another approach to the problem of determining whether hepatic microsomal GPAT is regulated by insulin would be to use an in vitro system, such as that described in Chapter 5 of this thesis, in which all components are isolated from liver. It is generally accepted that insulin exerts its effect on metabolism by a series of events beginning with the binding of insulin to a receptor molecule on the surface of the cell membrane. Subsequent events are poorly understood, but insulin-dependent effects on insulin-sensitive enzymes have been demonstrated through use of systems comprised of plasma membranes, insulin and the target enzyme(s) (Begum et al., 1982b, 1983; Jarett et al., 1981; Kiechle et al., 1981; McDonald et al., 1981; Seals & Czech, 1980, 1981b; Seals & Jarett, 1980). In these systems treatment of plasma membranes with insulin is thought to cause the release of a soluble factor from the membranes, similar in nature to the factor in muscle extracts from insulin-treated rats, with the capacity to modify the activity of insulin-sensitive enzymes in a manner consistent with the action of insulin in vivo (Larner, 1982). This was the basis for

experiments described in Chapter 5, in which hepatic microsomal GPAT was incubated with supernatant from a hepatic plasma membrane-containing fraction which had been treated with insulin. Although attempts to demonstrate an insulin-dependent enhancement of GPAT activity using this system were not successful, this does not preclude the possibility that hepatic microsomal GPAT activity is regulated by insulin. As discussed in Chapter 5, the relative impurity of the hepatic plasma membrane-containing fraction supernatant may have masked the presence of a stimulatory factor. Therefore, a logical direction for future research would be to fractionate the supernatant further. The method employed for muscle extracts in Chapter 4 or ultrafiltration techniques could be tried. These would have the added advantage of removing the microsomal contamination in the supernatant so that additional GPAT and protein was not added to the GPAT assay. Therefore, modification of this type of in vitro approach may lead to the provision of additional evidence that hepatic microsomal GPAT is regulated by insulin.

The question remains as to the exact chemical nature of the factor or factors in the fractions of rat muscle extract, demonstrated in experiments described in Chapter 4, which caused significant insulin-dependent stimulation of hepatic microsomal GPAT activity. It is possible, however, to deduce some properties of the factor(s) from the experiments conducted in Chapter 4:

-an apparent molecular weight within the range 1000-3000 daltons (as judged by Sephadex G-25 chromatography).

-heat stable (by virtue of survival of the heat treatment in the isolation procedure).

-acid stable (by virtue of survival of initial acidification and persistance in the mildly acid column chromatography eluent).

-is soluble in aqueous solution (is soluble in 50 mM formic acid).

-has very low absorbance at 220nm or has a very high specific activity.

-possibly sensitive to oxygen (since substitution of liquid nitrogen for liquid air in the method restored the potential of insulin-treatment low molecular weight fractions to enhance GPAT activity relative to the formic acid control).

There appear to be similarities between this low molecular weight factor(s) and others reported in the literature. In recent years there have been an increasing number of reports suggesting that the binding of insulin to its receptor causes the release of a factor or factor(s) from plasma membranes which mediates the action of the hormone on insulin-sensitive enzymes within the cell. In 1971 and 1972 novel, but not identified, nucleotide mediators of insulin action were suggested (Hersko et al., 1971; Larner, 1972), strongly influenced by the cyclic AMP "second messenger" concept of Rall & Sutherland (1958). A series of other small molecules or events have also been proposed as second messengers for insulin, such as cyclic AMP, cyclic GMP, Ca^{2+}, hydrogen peroxide, or turnover of membrane phospholipids (Czech, 1977, 1981; Fain & Butcher, 1976; Jefferson et al, 1968; Little & de Haen, 1980; May & de Haen, 1979; Mukherjee et al., 1982; Walaas & Horn, 1982). However, investigations along these lines have been inconclusive. Numerous attempts have failed to demonstrate correlations between the effect of insulin on concentrations of cAMP or cGMP and its metabolic effects under the same conditions (Czech, 1977; Larner, 1972; Walaas

& Horn, 1982). Likewise, while the release of Ca^{2+} may be of importance to the effect of insulin on membrane transport (Walaas & Horn, 1982), there is no indication that Ca^{2+} release is a primary mediator of insulin action on intracellular systems (Czech, 1977; Walaas & Horn, 1982). The need for any second messenger was questioned by Goldfine (Goldfine, 1977; Goldfine et al., 1981) who argued instead for direct action by insulin once internalised.

In 1974 Larner reported the isolation of an inhibitor of cyclic AMP-dependent protein kinase from insulin-treated muscle which was not cGMP or other previously reported inhibitors (Larner et al., 1974, 1976). The inhibitor was produced after 3-15 min of insulin treatment, was heat (100 ^{o}C) and trichloroacetic acid extractable and relatively stable to both of these conditions (Larner et al., 1976). The inhibition of the enzyme was proportional to the amount of inhibitor added to the assay and could be overcome with added cAMP (Larner et al., 1974, 1975, 1976, 1978, 1979a,b,c; Walkenbach et al., 1978).

In 1978 Jarett and co-workers (Seals et al., 1978) reported that insulin was able to decrease the labeling of a rat adipocyte plasma membrane preparation by [γ-^{32}P]ATP. Subsequent electrophoretic analysis revealed that the direct effect of insulin on autophosphorylation was accounted for by the decreased phosphorylation of two phosphoproteins of 120 000 and 42 000 M_r (Seals et al., 1979a). The former was of plasma membrane origin while the latter originated from mitochondria contaminating the plasma membrane preparation (Seals et al., 1979a,b). The mitochondrial phosphoprotein was identified as the α-subunit of pyruvate dehydrogenase and for insulin to decrease its

phosphorylation plasma membranes had to be present (Jarett & Seals, 1979a; Seals et al., 1979b). This effect of insulin on the phosphorylation of the α-subunit was consistent with the proposed mechanism by which insulin activates the pyruvate dehydrogenase complex in mitochondria (Denton & Hughes, 1978; Denton et al., 1981; Mukherjee & Jungas, 1975). These studies suggested that a "second messenger of insulin", which acts on mitochondrial PDH, is generated when plasma membranes are present. Hence direct addition of insulin to this subcellular system was expected to cause stimulation of PDH. Addition of insulin did stimulate PDH activity, but only when plasma membranes were present (Jarett et al., 1981; Seals & Jarett, 1980).

In 1979 both Larner and Jarett, with their co-workers, demonstrated that the same low molecular weight fraction of muscle extracts from insulin-treated rats was able to inhibit muscle cAMP-dependent protein kinase, stimulate both muscle glycogen synthase phosphoprotein phosphatase (Larner et al., 1979d,e), and adipocyte mitochondrial PDH (Jarett & Seals, 1979a,b). This material acted just like insulin and so the terms "insulin mediator substance" (IMS) (Larner et al., 1979d), and "chemical mediator for insulin action" (Jarett and Seals, 1979b) were proposed. It is unclear, however, whether IMS is one or a group of many substances that mediate the action of insulin. Subsequent studies suggested both "mediators" (stimulatory) and "antimediators" (inhibitory) are present in IMS preparations (Cheng et al., 1980; Jarett et al., 1982; Larner et al., 1979e; 1981a,b; 1982a,b,c; Saltiel et al., 1982b,c, 1983).

The presence of IMS has been inferred in many studies involving

fractionation of muscle extracts (Cech et al., 1980; Jarett & Seals, 1979a,b; Larner et al., 1974, 1976, 1978, 1979a,b,d,e, 1982b), adipocytes (Begum et al., 1982b; Jarett et al., 1980; Kikuchi et al., 1981; Kiechle & Jarett, 1981a,b; Kiechle et al., 1980a,b; Seals & Czech, 1982a), adipocyte plasma membranes (Jarett et al., 1981, 1982; Kiechle & Jarett, 1981b; Kiechle et al., 1981; McDonald et al., 1981; Seals & Czech, 1980,1981b;), rat liver particulate fraction (Amatruda & Chang, 1983; Begum et al., 1983; Saltiel et al., 1982a,c), rat liver plasma membranes (Larner et al., 1979a; Saltiel et al., 1981, 1983; Suzuki et al., 1984), human placenta (cited by Suzuki et al., 1984), H4-II-EC3'hepatoma cells (Parker et al., 1982a,b), and IM-9 lymphocytes (Jarett et al., 1980, 1982). It appears therefore that IMS may be widely distributed among various cell types. However, as the exact chemical nature of IMS has not been determined, this cannot be proven.

There have been various suggestions as to the possible nature of IMS. A peptide or oligopeptide nature has been proposed (Larner et al., 1979e, 1981a,b, 1982b,c; Seals & Czech, 1980, 1981a,b, 1982b; Suzuki et al., 1984; Zhang et al., 1983) and two provisional amino acid (plus carbohydrate) compositions have been suggested (Larner et al., 1982b,c), but these have not yet been generally accepted. Despite the suggested peptide nature, IMS does not react with ninhydrin, as once implied (Larner et al., 1979d), or fluorescamine (Larner, 1982). Reported destruction of the IMS by proteases supports the above contention (Seals & Czech, 1980, 1981a; Suzuki et al., 1984; Zhang et al., 1983), although negative results of others with proteases does not (Jarett et al., 1983; Larner, 1982). Experiments which demonstrated the destruction of IMS activity by neuraminadase and β-D-galactosidase

(Begum et al., 1983) added weight to suggestions that IMS has a carbohydrate nature (Larner, 1982). However, doubt has been cast on the association of sugars or amino-sugars with IMS activity as it was contended, though without supporting data, that IMS does not bind to lectins (Jarett et al., 1983). Phospholipid or proteolipid natures for IMS have also been proposed (Jarett et al., 1983; Kelly et al., 1984; Kiechle & Jarett, 1983; Kiechle et al., 1982; Macaulay et al., 1982, 1983a,b; Parker et al., 1982c). Reports that the mediator is phospholipase-C insensitive (Zhang et al., 1983), insoluble in absolute ethanol (Saltiel et al., 1983), and not very soluble in chloroform-methanol (Larner, 1982) conflict with this view. A nucleotide nature for IMS has been ruled out (Cheng et al., 1980; Larner, 1982; Larner et al., 1979a,d, 1982c) supported by the lack of absorption to charcoal (Kiechle et al., 1980a; Kikuchi et al., 1981; Larner, 1982; Larner et al., 1979d; Saltiel et al., 1981, 1982c, 1983). There are no correlations between the activity of the IMS and absorbance at 230nm, 254nm, or 260nm (Kiechle et al., 1980a; Larner, 1982; Seals & Czech, 1981a) although, after chromatography on several Waters HPLC columns, a single peak of absorbance measured at 210nm did correlate with IMS activity (Seals & Czech, 1981a). Therefore the exact chemical nature of IMS or IMSs is still unresolved. Hence the only reliable method of identifying IMS(s) is by biological assay (Kiechle et al., 1980a; Larner, 1982), i.e. through the ability of fractions, extracts, or supernatants from insulin/control preparations to alter the activity of insulin-sensitive enzymes.

There have, however, been many observations made during these studies and, as a result, physical characteristics of IMS have been

deduced. Sephadex G-25 chromatography (in 50mM-formic acid) indicates that IMS has a molecular weight within the range of 1000-3000 daltons (Larner et al., 1979d,e; Kiechle et al, 1980b, 1981; Saltiel et al., 1981, 1982c; Zhang et al., 1983). A higher apparent molecular weight (2000-4000 daltons), when chromatographed at neutral pH (Parker et al., 1982a; Seals and Czech, 1981b), suggests it may aggregate or associate (Larner, 1982). Therefore, variation in the molecular weights reported for IMS may be a function of the pH and ionic strength of the buffer used for the chromatography (Parker et al., 1982a). An ionic character appears likely as IMS binds to ion exchange resins on which it has been fractionated (Larner, 1982; Larner et al., 1975, 1976, 1978, 1979a,b; Parker et al., 1982a; Seals & Czech, 1981b, 1982b). It has a negative charge at pH 7.4 (Larner et al., 1976; Seals & Czech, 1981b, 1982b) and an isoelectric pH of 4.5 (Seals and Czech, 1981b). IMS is soluble in aqueous solution (50mM-formic acid) (Larner et al., 1979d; Parker et al., 1982a; Seals & Czech, 1981b, 1982b), and in trichloroacetic acid (Larner et al., 1979a). Hence it appears to be hydrophilic (Larner, 1982; Seals & Czech, 1982b). It is stable to heating (Larner et al., 1974, 1976, 1979d; Parker et al., 1982a; Saltiel et al., 1981, 1982a; Seals & Czech, 1981b) and mildly acid conditions (Larner et al., 1974, 1976, 1979a,d; Kiechle et al, 1980b; Parker et al., 1982a; Saltiel et al., 1981; Seals & Czech, 1981b). It is alkaline labile (Larner, 1982; Larner et al., 1976, 1978, 1979a,b,c,e; Saltiel et al., 1982a, 1983; Seals & Czech, 1981b). There is general agreement that IMS is unstable although reports of the duration of stability differ (Larner, 1982; Larner et al., 1978, 1979a,b, 1982c; Saltiel et al., 1981; Seals & Czech, 1981b; Kiechle et al., 1981). It has been suggested that for maximum stability, acid pH, presence of

an -SH compound (e.g. cysteine or mercaptoethanol), presence of a metal chelator, such as EDTA, and the absence of oxygen, are necessary (Kiechle et al., 1981; Larner, 1982; Larner et al., 1982c).

IMS preparations are reported to stimulate: glycogen synthase phosphoprotein phosphatase (Cheng et al., 1980; Kikuchi et al., 1981; Larner et al., 1979d,e, 1982a); glycogen synthase (Kikuchi et al., 1981; Seals & Czech, 1981a, 1982b); pyruvate dehydrogenase (Amatruda & Chang, 1983; Begum et al., 1982b; Jarett & Kiechle, 1981; Jarett & Seals, 1979a,b; Jarett et al., 1980, 1981; Kiechle et al., 1981; Kikuchi et al., 1981; Saltiel et al., 1981, 1982a,b; Seals & Czech, 1981b, 1982a; Seals & Jarett, 1980); pyruvate dehydrogenase phosphatase (Jarett et al., 1981; Kiechle & Jarett, 1981a; Kiechle et al., 1980a,b; Popp et al., 1980; Seals et al., 1979b); high affinity (Ca^{2+} + Mg^{2+})ATPase (McDonald et al., 1981); low K_m cyclic AMP phosphodiesterase (Kiechle & Jarett, 1981a,b; Parker et al., 1982a,b); and acetyl-CoA carboxylase (Saltiel et al., 1983). IMS preparations inhibit: cyclic AMP-dependent protein kinase (Cheng et al., 1980; Jarett et al., 1982; Larner et al., 1974, 1976, 1978, 1979a,b,d,e, 1982a); adenylate cyclase (Larner, 1982; Saltiel et al., 1982c); and glucose 6-phosphatase (Suzuki et al., 1984). Although similar IMS preparations have been shown to stimulate/inhibit different insulin-sensitive enzymes, it is still unclear whether these preparations contain one or many IMS. Conceivably, similar IMS preparations could stimulate/inhibit different enzymes, but with the actual IMS acting on the enzymes being entirely different. However, until the chemical nature is elucidated, this cannot be resolved.

Given the available information on putative IMS, it appears possible that the factor responsible for the insulin-dependent stimulation of GPAT activity in the present study could be similar to the putative IMS. Similarities in molecular weight, apparent sensitivity to oxygen, heat and acid stability, and aqueous solubility would tend to support this suggestion. Therefore, the possibility exists that the GPAT stimulatory factor(s) may even be an IMS. However, the experiments conducted in the present study are unable to establish this. It would be possible in further experiments to ascertain whether the low molecular weight fractions that enhance GPAT activity in an insulin-dependent manner also enhance the activity of other enzymes known to be stimulated both by insulin in vivo and IMS preparations in vitro. It is possible, however, that fractions which stimulate GPAT activity may not enhance the activity of these other enzymes. This would not rule out the possibility that the GPAT stimulatory factor(s) is (are) an IMS(s). It is possible that there could be a unique substance, released in response to insulin, that is specific to microsomal GPAT regulation. Hence this would allow targeting of insulin action, to the required extent, on GPAT and glycerolipid metabolism. This is not unlikely as other, apparently insulin-dependent, low molecular weight substances have been found which are not entirely similar to the putative IMS. A low molecular weight (M_r approximately 1000 daltons) compound from rat liver perfused with insulin stimulates the synthesis of RNA in isolated nuclei (Horvat, 1980). An antagonist of cAMP, generated in the presence of either adrenaline or insulin, has also been reported. This substance mediates some of the actions of insulin including inhibition of cyclic AMP-dependent protein kinase and adenylate cyclase (Wasner, 1981).

However, it appears to differ from IMS by being stable to neutral pH and having an apparent M_r of approximately 500 daltons. It may also contain prostaglandin E_1. Another recent report has described a potent inhibitor of insulin protease which is heat stable but with a M_r that is $\leq$ 3500 daltons (McKenzie & Burghen, 1984). A second messenger for another peptide hormone, prolactin, has also been suggested (Teyssot et al., 1981, 1982) although it now appears that results of earlier experiments cannot be repeated (Houdebine et al., 1985) and so the existance of this messenger now appears doubtful.

The results of experiments described in this thesis provide further evidence in favour of the hypothesis that hepatic microsomal GPAT activity can be modified by insulin and may contribute to the overall regulation of glycerolipid biosynthesis in liver. In future it will be important to determine whether the low molecular weight insulin-dependent material which stimulates hepatic microsomal GPAT is an IMS. Assay of this material with an enzyme such as pyruvate dehydrogenase would help establish this. It would also be important to establish, if possible, the identity of this low molecular weight material (by employing more powerful fractionation techniques such as ion-exchange or reversed-phase HPLC). Determination of the nature of, and extent to which, this material can be found in other tissues should provide valuable information concerning the general role of microsomal GPAT in the regulation of glycerolipid biosynthesis. It may also contribute to a greater understanding of the mechanism by which the action of insulin is mediated.

APPENDIX I

THE EFFECT OF FRUCTOSE 2,6-BISPHOSPHATE ON GPAT

THE EFFECT OF FRUCTOSE 2,6-BISPHOSPHATE ON GPAT

I.1 Introduction

Fructose 2,6-bisphosphate was discovered to be a very potent low molecular weight stimulator of phosphofructokinase (PFK) in 1980 (Van Schaftingen et al., 1980). PFK is a key regulatory enzyme of glycolysis. This naturally occurring sugar-diphosphate is present in micromolar amounts in liver cells, skeletal muscle, heart and brain of the rat (Hers & Van Schaftingen, 1982). It has also been found in lung, kidney, epididymal fat, pancreatic islets and hepatoma tumour cells (Hers et al., 1982) along with plants (Stitt et al., 1982), yeast (Hers & Van Schaftingen, 1982) and fungi (Hers et al., 1982). The concentration of fructose 2,6-bisphosphate depends upon nutritional state (Hers et al., 1982) and seems to be influenced by the animal's genotype, fructose 2,6-bisphosphate concentrations being elevated in the livers of genetically obese mice (Claus & Pilkis, 1982; Hue & Van de Werve, 1982). Fructose 2,6-bisphosphate concentrations respond to a number of hormonal treatments (Hers & Van Schaftingen, 1982). Insulin treatment of perfused rat hind limb muscle causes a 2-fold increase in concentration (Hue et al., 1982) and insulin treatment of human fibroblasts increases intracellular fructose 2,6-bisphosphate (Farnararo et al., 1984). Fructose 2,6-bisphosphate appears to be an intracellular signal which signifies that glucose is abundant (Hers & Van Schaftingen, 1982; Hers et al., 1982) and it seemed reasonable to suggest that this regulatory compound might enhance the glycolytic flux and the synthesis of glycerolipids simultaneously.

Low molecular weight fractions from heparinised rats treated with either insulin or saline were examined to determine whether carbohydrate-like material was present. Subsequently, the effect of authentic fructose 2,6-bisphosphate on GPAT activity was determined.

I.2 Section 1 - Examination of muscle extract fractions from heparinised rats treated with insulin or saline for the presence of phenol-sulphuric reactive carbohydrate

I.2.1 Methods

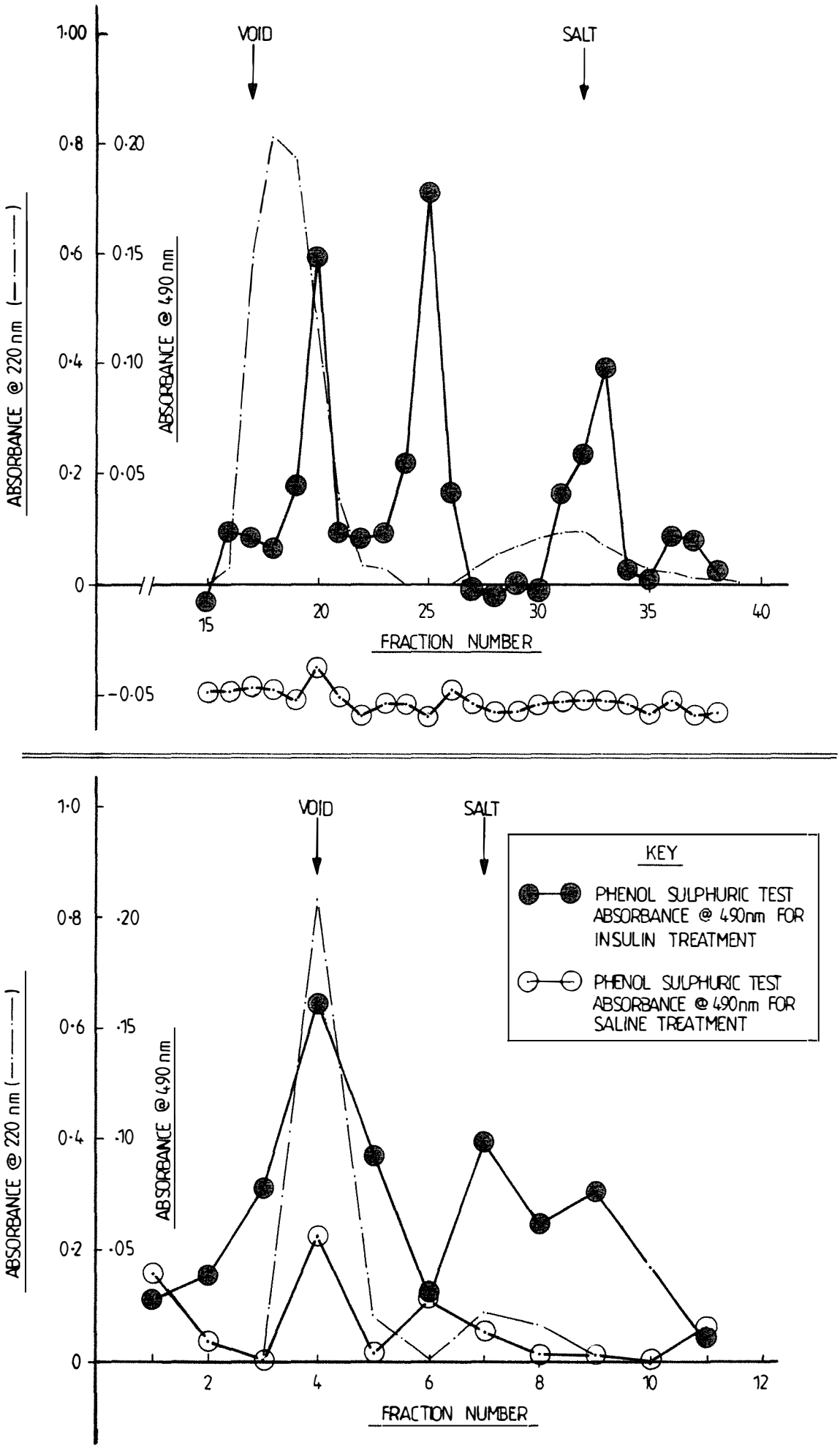
Extracts were prepared and fractionated as described in Chapter 4. Extracts C, C_s, D and F were used. Fractions of Extract C_s (saline treatment) were not assayed with GPAT, but fractions from the others were as reported in Section 2, Chapter 4. The presence or absence of carbohydrate in the respective fractions was determined through reacting an aliquot of each fraction with phenol and sulphuric acid according to the following method (Pers. Comm., Dr I. G. Andrew, Dept of Chemistry and Biochemistry, Massey University):

1. Reagents required for this determination were 5% (w/v) phenol in distilled, deionised water and 18 M sulphuric acid (Analar grade).
2. A 0.5 ml aliquot of the Sephadex G-25 fraction to be tested was pipetted into a clean pyrex test tube. 0.5 ml of 50 mM formic acid served as the blank.
3. 0.5 ml of 5% (w/v) phenol was added to the tube followed by the rapid addition of 3.2 ml of 18 M sulphuric acid from an automatic dispenser. The solution was immediately vortexed.
4. After cooling to about room temperature, the absorbances were read at 490nm against the blank.

I.2.2 Results

The results presented in Fig I.1 show that when fractions from muscle extracts were assayed in the phenol-sulphuric acid test for carbohydrate, fractions from the insulin treatments appeared to have carbohydrate present. The amount of carbohydrate present in the saline treatment fractions appeared to be substantially less than in the corresponding insulin treatment fractions, although the absolute amounts were not determined.

Fig I.1: Elution of carbohydrate, as shown by Phenol-sulphuric reactivity, in Sephadex G-25 fractions of muscle extracts from heparinised rats treated with either insulin or saline. 3.2 mg of the respective extract (see section I.2.1) was chromatographed on a 2.54 x 27 cm Sephadex G-25 column in 50 mM formic acid. The presence of carbohydrate was estimated by reactivity of the fractions with the Phenol Sulphuric acid reagent and measurement of the resultant absorbance at 490nm (I.2.1). In the experiment shown in the upper panel, 3.8 ml fractions were collected while in the experiment shown in the lower panel 16 ml fractions were collected.


VOID
SALT
1·00
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ABSORBANCE @ 220 nm (—·—·—)
ABSORBANCE @ 490 nm
15
20
25
30
35
40
FRACTION NUMBER
-0·05
1·0
·20
·15
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2
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12
KEY
PHENOL SULPHURIC TEST ABSORBANCE @ 490nm FOR INSULIN TREATMENT
PHENOL SULPHURIC TEST ABSORBANCE @ 490nm FOR SALINE TREATMENT

I.3 Section 2 - Examination of effect of fructose 2,6-bisphosphate on microsomal GPAT

I.3.1 Methods

Authentic fructose 2,6-bisphosphate was obtained and resuspended in distilled deionised H_2O to give a 3.5 mM stock solution. This was diluted appropriately to deliver the required amount of fructose 2,6-bisphosphate to the GPAT reaction mixture. The effect of the fructose-2,6-bisphosphate on liver microsomal GPAT (microsomes, see Fig 3.1)) was determined. GPAT was assayed according to the method described in section 3.8 except that fructose-2,6-bisphosphate was included in the assay. The volume of the reaction was kept constant by the addition of distilled water. The method adopted for the assay of GPAT also differed in that the reactions were thermally equilibrated for at least 4 min after which the microsomal enzyme protein was added to the tubes at 2 min intervals. After 20 min the reaction was started by the addition of palmitoyl-CoA as described (3.8) and treated according to the standard method (3.8). Assays were conducted in triplicate.

I.3.2 Results

It was found that fructose 2,6-bisphosphate did not enhance the activity of hepatic microsomal GPAT (see Table I.1). However, it appeared that fructose 2,6-bisphosphate could cause slight inhibition of GPAT activity.

Table I.1: The effect of fructose 2,6-bisphosphate on microsomal GPAT.

Fru 2,6-P_2 conc^n (μM)	GPAT activity nmol/min/mg	% of control
0	2.97 ± 0.04[a]	100.0
1.0	3.05 ± 0.06	102.7
2.5	2.90 ± 0.07	97.6
5.0	2.70 ± 0.01[a]	90.9
10.0	2.92 ± 0.10	98.3
25.0	2.91 ± 0.04	98.0
50.0	2.87 ± 0.11	96.6
100.0	2.86 ± 0.11	96.1

Values are the means of duplicate assays ± SEM. Values with superscript differ significantly, $P<0.05$

I.5 Discussion

Experiments described in Chapter 4 provided evidence that fractions of muscle extracts from rats treated with insulin stimulated GPAT activity relative to the effect of fractions from saline treatment muscle extracts. These fractions would have contained material with an apparent molecular weight of 1000-4000 daltons as judged by Sephadex G-25 chromatography. Similarly, prior to the identification of the stimulator of phosphofructokinase (PFK) as fructose 2,6-bisphosphate, it had been reported that the activator of PFK had an apparent molecular weight of 3000 to 4000 daltons as judged by gel filtration (Furuya & Uyeda, 1980). Subsequent to this, however, it was reported that this activator of PFK was actually fructose 2,6-bisphosphate and that the discrepancy in molecular weight was due to an apparent anomalous behaviour of highly charged molecules on Sephadex chromatography (Uyeda et al., 1981). It seemed possible therefore, in the absence of knowledge of the actual chemical identity of the low molecular weight material causing the stimulation of GPAT activity, that it might have some relationship to fructose 2,6-bisphosphate. Also the concentration of fructose 2,6-bisphosphate would be increased at the time that the synthesis of triglyceride would need to be increased. Hence the possibility that this molecule stimulated both the glycolytic flux and synthesis of triglyceride in a concerted manner seemed attractive.

One property of fructose 2,6-bisphosphate not consistent with the proposed relationship between GPAT stimulatory substances and fructose 2,6-bisphosphate was the acid sensitivity of the sugar diphosphate,

i.e. it is destroyed completely upon incubation with 0.01 M HCl for 10 min at 20°C (Van Schaftingen et al., 1980). GPAT stimulatory factor in low molecular weight fractions of muscle extracts appeared stable to acid conditions as judged by the ability of this material to survive a preparative protocol that involved acidification and manipulation in 50 mM formic acid.

There was carbohydrate present in the Sephadex G-25 fractions of the rat muscle extracts (see Fig I.1). Therefore, it seemed possible that there might be a link between the presence of this carbohydrate, in insulin treatment fractions which enhanced GPAT activity relative to the effect of the saline treatment fractions, and the PFK activator fructose 2,6-bisphosphate. The effect of fructose 2,6-bisphosphate on microsomal GPAT was determined over the range at which it is reported to have its effect of phosphofructokinase (Hers & Van Schaftingen, 1982). However, this compound had very little effect on GPAT activity, despite use of a wide range of concentrations covering that usually found with the cell (Hers & Van Schaftingen, 1982). It did, however, appear to cause slight inhibition of GPAT activity (see Table I.1). In contrast, phosphofructokinase is stimulated several hundred-fold by fructose 2,6-bisphosphate at micromolar or submicromolar concentrations (Hers & Van Schaftingen, 1982; Van Schaftingen et al., 1980). Therefore, it seemed that fructose 2,6-bisphosphate, while a potent activator of phosphofructokinase (Hers & Van Schaftingen, 1982), was not the insulin-dependent low molecular weight factor, present in fractions of muscle extracts, reponsible for the activation of liver microsomal GPAT activity observed in earlier experiments (Chapter 4).

APPENDIX II

THE EFFECT OF ADENINE NUCLEOTIDES ON GPAT

THE EFFECT OF ADENINE NUCLEOTIDES ON GPAT

II.1 Introduction

There have been reports of nucleotides having variable and inconsistent effects on GPAT from various sources (see 1.3.4.4). The effect of three adenine nucleotides on liver microsomal GPAT was examined.

II.2 Methods

6.25 mM solutions of ATP, ADP, and AMP, in 50 mM Tris HCl buffer (pH 7.4), were prepared. Varying volumes were included in the microsomal GPAT assay to give a final range in concentration of 0 - 2.0 mM which is near the physiological concentration in rat liver (Woods & Krebs, 1973).

Microsomal GPAT (microsomes; see Fig 3.1, shortened) was assayed according to the standard method (3.8) except that GPAT and the nucleotide were incubated together for 10 min at 30^{o}C prior to starting the reaction.

II.3 Results and Discussion

ATP was found to enhance GPAT activity at 0.2 mM and 0.5 mM ($P<0.01$ for both; Table III.1). ADP did not enhance GPAT activity significantly at any of the concentrations examined. The effect of AMP on GPAT activity was also non-significant. There have been no reports that these adenine nucleotides can alter the activity of microsomal GPAT. Bacterial GPAT is stimulated by ATP (Rock et al., 1981). Therefore, it appears that microsomal GPAT can be stimulated by low concentrations of ATP. However, this may have little physiological meaning as the concentration of ATP in rat liver is considerably higher than this at about 2.22 μmol/g wet weight (Woods & Krebs, 1973). At 2.0 mM ATP there was no significant effect on GPAT activity.

Table II.1: The effect of ATP, ADP, and AMP on hepatic microsomal GPAT from fed rats.

	Concentration of nucleotide (mM)				
	0	0.2	0.5	1.0	2.0
ATP					
GPAT rate[a]	1.84 ± 0.05	2.16[b] ± 0.07	2.29[b] ± 0.07	2.14 ± 0.07	2.06 ± 0.07
ADP					
GPAT rate[a]	2.33 ± 0.09	2.44 ± 0.12	2.59 ± 0.12	2.56 ± 0.12	2.39 ± 0.16
AMP					
GPAT rate[a]	1.85 ± 0.05	1.95 ± 0.08	1.86 ± 0.08	1.87 ± 0.08	1.97 ± 0.08

[a]Values given table are Least Square Means (nmol/min/mg ± SEM) derived from a two way analysis of variance using the "REG" statistical package on the Massey University Computer Unit's Prime 750. Three experiments per nucleotide were conducted. Control (0 mM nucleotide) assays were conducted in quadruplicate for each experiment and assays with nucleotide present were conducted in duplicate. Values = mean ± SEM. Means with superscripts differ from control, [b]$P<0.01$.

APPENDIX III

THE EFFECT OF OESTROGEN ON CHICKEN LIVER MICROSOMAL GPAT

THE EFFECT OF OESTROGEN ON CHICKEN LIVER MICROSOMAL GPAT

III.1 Introduction

It has been reported that oestrogen (diethylstilbestrol; DES) treatment of chickens leads to an increase in the total amount of hepatic microsomal GPAT without altering the specific activity of the GPAT (Coleman et al., 1977). The opportunity arose to consider the effect of oestrogen (as oestradiol 17β-dipropionate) on the microsomal GPAT of livers from young cockerals.

III.2 Methods

10 mg of 17-β-estradiol dipropionate was dissolved in 1 ml of peanut oil obtained from the local supermarket and 0.1 ml containing 1 mg of oestradiol 17β-dipropionate was injected into the breast muscle of the chicken. The controls received 0.1 ml of oil. The chickens, kept as described (2.4.2), were randomly assigned to the control and 2 treatment groups i.e. (1) Controls; (2) Treatment 1 = 1 mg oestradiol 17β-dipropionate 24 h before death; (3) Treatment 2 = 1 mg oestradiol 17β-dipropionate 40 h before death.

The chickens were 3 weeks old at death and microsomes were prepared from livers according to the method described (microsomes; see Fig 3.1, shortened). Three batches of microsomes per treatment group were each assayed in triplicate to determine GPAT activity (3.8). The protein content of the microsomal fractions was determined (3.4).

III.3 Results and discussion

24 h after the chickens were treated with oestradiol 17β-dipropionate the specific activity of GPAT (nmol/min/mg protein) was 70% of the activity in control liver microsomes ($P<0.01$). This contrasts with the results of Colman et al. (1977) who found no change in GPAT specific activity after injecting chickens with DES. However, 40 h after administration of oestradiol 17β-dipropionate the specific activity was similar to the controls (106%; non-significant; see Table III.1). In contrast to the marked decline in specific activity by 24 h after oestradiol 17β-dipropionate injection, microsomal GPAT activity per gram fresh liver did not differ significantly from that of the non-oestrogenised chickens (i.e. 97% of control activity). An increased amount of microsomal protein per gram of fresh liver (141%; $P<0.01$) at 24 h after treatment appeared to have effectively offset the lowered specific activity. It appears that, in the short term, protein other than GPAT protein must be induced (see Table III.1). Although the specific activity of GPAT from oestrogenised chickens at 40 h was not significantly different from controls, GPAT activity per gram liver was increased to 142% of controls ($P<0.01$). A slight increase in the specific activity of GPAT (106% of control) coupled with a significant increase in microsomal protein per gram liver (135%; $P<0.01$) observed after 40 h appears to have been responsible for this (Table III.1).

The mean weight of the livers from the 24 h and 40 h oestrogenised chickens was 135% ($P<0.10$) and 147% ($P<0.05$) that of the controls, respectively, (Table III.1). However, the mean liver weights of the two oestradiol 17β-dipropionate-treated groups did not differ

significantly. As a result of the increase in liver weight and increases in the amount of microsomal protein per gram liver in oestrogenised birds, the total GPAT activity per liver was increased significantly ($P<0.01$), i.e. to 131% and 208% of controls for the two treatments (Table III.1). These results compare with those of Coleman et al. (1977) who observed that total GPAT activity per liver, after 5 days DES treatment, was about double that of the controls.

Table III.1: The effect of oestradiol 17β-dipropionate on chicken liver microsomal GPAT activity.

GPAT activity	Control	Treatment 1	Treatment 2
nmol/min/mg	1.82 ± 0.09^{c}	1.27 ± 0.04^{d}	1.92 ± 0.20^{ce}
nmol/min/gram	18.61 ± 1.23^{a}	18.10 ± 0.39^{ac}	26.43 ± 2.70^{bd}
nmol/min/liver	99.01 ± 6.53^{c}	129.74 ± 2.81^{d}	206.15 ± 21.09^{e}
liver (g)	5.32 ± 0.58^{a}	7.17 ± 0.27	7.80 ± 0.10^{b}
mg microsomal protein/g liver	10.17 ± 0.45^{c}	14.34 ± 0.54^{d}	13.78 ± 0.18^{d}

Values are means of results for 3 batches of microsomes, each of which was assayed in triplicate (± SEM). Control = no oestradiol 17β-dipropionate; Treatment 1 = oestradiol 17β-dipropionate injected 24 h before death; Treatment 2 = oestradiol 17β-dipropionate injected 40 h before death. Means within rows with different superscripts differ; for superscripts a and b, $P<0.05$; for superscripts c, d and e, $P<0.01$.

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