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UNIVERSITÉ D'OTTAWA
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LIPID ENZYMES OF THE RABBIT HEART: Some Properties
of Acyl-CoA:sn-Glycerol-3-Phosphate-O-Acyltransferase,
Palmitoyl-CoA Hydrolase, and Phosphatidate Phospho-
hydrolase in Cardiac Subcellular Fractions

by Maw-Shung Liu, D.D.S., M.S.

Thesis presented to the School of Graduate Studies
in partial fulfillment of the requirements for the
degree of Ph.D. in Physiology

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LIST OF ABBREVIATIONS

The following abbreviations are used throughout the text except in titles, subtitles, and the summary.

Substances

ADP	= Adenosine diphosphate
AMP	= Adenosine monophosphate
ATP	= Adenosine triphosphate
CoA	= Coenzyme A
CTP	= Cytidine triphosphate
DFP	= Diisopropylfluorophosphate
DG	= Diglyceride = Diacylglycerol
DHAP	= Dihydroxyacetone phosphate
Diacyl- <u>sn</u> -G3P	= Diacyl- <u>sn</u> -glycerol-3-phosphate = Phosphatidic acid*
DTT	= Dithiothreitol
EDTA	= Ethylenediaminetetraacetate
FA	= Fatty acid
FFA	= Free fatty acid
<u>sn</u> -G3P	= <u>sn</u> -glycerol-3-phosphate
GTP	= Guanosine triphosphate
MG	= Monoglyceride = Monoacylglycerol
Monoacyl- <u>sn</u> -G3P	= Monoacyl- <u>sn</u> -glycerol-3-phosphate = Lysophosphatidic acid

NADH	= Nicotinamide adenine dinucleotide, reduced form
NADP	= Nicotinamide adenine dinucleotide phosphate
NADPH	= Nicotinamide adenine dinucleotide phosphate, reduced form
Pi	= Inorganic phosphate
PL	= Phospholipid
PL FA	= Phospholipid fatty acid
PP	= Pyrophosphate
T3	= 3,3',5-triiodothyronine
TG	= Triglyceride = Triacylglycerol
TG FA	= Triglyceride fatty acid
TLC	= Thin-layer chromatography

Enzymes

ATP ase	= Adenosine triphosphatase
G3P acyltransferase	= Acyl-CoA: <u>sn</u> -glycerol-3-phosphate-0-acyltransferase (EC 2.3.1.15)
Monoacyl- <u>sn</u> -G3P acyltransferase	= Acyl-CoA:monoacyl- <u>sn</u> -glycerol-3-phosphate-0-acyltransferase (EC 2.3.1.?)
1-acyl- <u>sn</u> -G3P acyltransferase	= Acyl-CoA:1-acyl- <u>sn</u> -glycerol-3-phosphate-0-acyltransferase
2-acyl- <u>sn</u> -G3P acyltransferase	= Acyl-CoA:2-acyl- <u>sn</u> -glycerol-3-phosphate-0-acyltransferase
MG acyltransferase	= Acyl-CoA:monoglyceride-0-acyltransferase

MG kinase = Monoglyceride kinase

DG acyltransferase = Acyl-CoA:sn-1,2-diglyceride-0-
acyltransferase (EC 2.3.1.20)

DG kinase = Diglyceride kinase

Acyl-CoA synthetase = Acid:CoA ligase (AMP) (EC 6.2.1.3)

* Diacyl-sn-G3P and phosphatidic acid are both used
without discrimination because the latter terminology
has been widely used, e.g., phosphatidate phosphohy-
drolase

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SUMMARY

The enzymes involved in triglyceride biosynthesis in the subcellular fractions of rabbit heart were characterized in an attempt to elucidate the controlling mechanisms of myocardial fatty acid esterification. The enzymes acyl-CoA:sn-glycerol-3-phosphate-0-acyltransferase and acyl-CoA:monoacyl-sn-glycerol-3-phosphate-0-acyltransferase, which catalyze the acylation reactions of sn-glycerol-3-phosphate and monoacyl-sn-glycerol-3-phosphate, respectively, were initially studied. Subcellular fractions were prepared by differential centrifugation. The enzymes were assayed by incubating the subcellular preparation with sn-[U-¹⁴C]-G3P and FAs in the presence of various cofactors (CoA, ATP, MgCl₂, serum albumin, and Tris-phosphate buffer). The reaction products, monoacyl- and diacyl-sn-G3P, were extracted by butanol and separated by thin-layer chromatography.

Kinetic data on the sn-G3P and monoacyl-sn-G3P acylating enzymes in heart subcellular fractions revealed that the acylation rates in mitochondria were slower than, but constant for a longer time period (up to 20 min) than, those in the microsomal fraction. The range of palmitate,

oleate, and linoleate concentrations yielding optimal sn-glycerol-3-phosphate acylation was broader for mitochondria than for the microsomal fraction, the latter showing a preference for linoleate. The mitochondrial fraction synthesized a relatively large quantity of monoacyl-sn-glycerol-3-phosphate, reaching 135 % of the microsomal biosynthesis during an assay period of 15 min. In contrast, the microsomal fraction formed considerably more diacyl- than monoacyl-sn-glycerol-3-phosphate, except with linoleate as the acyl donor, in which case approximately equal quantities of the two products were produced. Additional evidence was provided for these findings by observations on the biosynthesis of monoacyl-sn-glycerol-3-phosphate in experiments in which hepatic subcellular fractions were used. Cardiac mitochondrial diacyl-sn-glycerol-3-phosphate formation was less than 17 % of the microsomal formation. However, evidence was presented to exclude the possibility that monoacyl-sn-glycerol-3-phosphate in the mitochondrial fraction is formed by deacylation of the contaminating microsomal diacyl-sn-glycerol-3-phosphate. The extent of participation of the dihydroxyacetone phosphate pathway in the

biosynthesis of mono- and diacyl-sn-glycerol-3-phosphate was found to be minimal in cardiac mitochondria. The addition of CTP and the fatty acid specificity of the reactions both provided results reinforcing the postulate that mitochondrial differs from microsomal acylation. Cardiac acyl-CoA:sn-glycerol-3-phosphate-0-acyltransferase was more sensitive to sulphhydryl-binding reagents and aging of the enzyme preparation than were the hepatic enzymes. All of these findings demonstrate that the characteristics of acyl-CoA:sn-glycerol-3-phosphate-0-acyltransferase and acyl-CoA:monoacyl-sn-glycerol-3-phosphate-0-acyltransferase in rabbit heart mitochondria are distinct from those of the cardiac microsomal enzymes and hepatic enzymes.

The responses of sn-glycerol-3-phosphate acylation reactions in the mitochondrial and microsomal fractions of heart to triiodothyronine administration are not uniform. The mitochondrial monoacyl-sn-glycerol-3-phosphate was augmented two-fold, whereas the microsomal biosynthesis of monoacyl-sn-glycerol-3-phosphate was unchanged, in the hearts of hyperthyroid rabbits. The formation of diacyl-sn-glycerol-3-phosphate in mitochondrial and microsomal

fractions was increased about four-fold. These findings indicate that myocardial acyl-CoA:sn-glycerol-3-phosphate-0-acyltransferase⁷ and acyl-CoA:monoacyl-sn-glycerol-3-phosphate-0-acyltransferase are under the influence of a thyroid hormone, namely, triiodothyronine.

The positional specificity of monoacyl-sn-glycerol-3-phosphate formation in heart mitochondria was analyzed by using a method utilizing lysosomal phosphatidate phosphohydrolase and borate-impregnated thin-layer chromatography. Contrary to the results of studies with liver particulates, approximately one-third of the palmitate was found at position 2 of the glycerol moiety with the remainder at position 1, whereas one-third of oleate was found at position 1. Only a slight asymmetric distribution (42.8-48.5 % and 51.5-57.2 % for positions 1 and 2, respectively) was observed with linoleate as the acyl donor. This indicates that preferential acylation of individual fatty acids during the formation of monoacyl-sn-glycerol-3-phosphate in the heart mitochondria is different from that in the liver mitochondria and microsomes, suggesting that acyl-CoA-sn-glycerol-3-phosphate-0-acyltransferase is organ-specific.

Appropriate assay conditions for palmitoyl-CoA hydrolase were established with respect to stability of substrate in the incubation medium, time course of reactions, amounts of enzyme source, and pH optima. The assays were carried out by incubating either subcellular fraction or homogenate with $[1-^{14}\text{C}]$ palmitoyl-CoA in the presence of bovine serum albumin and Tris-phosphate buffer. The hydrolytic product was extracted by 10 ml of isopropanol-heptane-1 N H_2SO_4 (20:5:1, v/v/v) and separated from its precursor by subsequent addition of heptane (6 ml) and H_2O (4 ml). The results showed that the sulfhydryl-protective agents DTT and cysteine accelerated spontaneous decomposition of palmitoyl-CoA, particularly at alkaline pH. The rate of hydrolysis of palmitoyl-CoA by rabbit heart homogenate was constant up to 2 hr of incubation. The optimal pH value for this enzyme was 8.5. (+)-Decanoylcarnitine (4-12 mM) potentiated palmitoyl-CoA hydrolase activity of rabbit heart homogenates, while 5 mM of diisopropylfluorophosphate inhibited its activity by half. The apparent activity of palmitoyl-CoA hydrolase was highest in the lysosomal and the microsomal fractions, followed by the mitochondrial and

soluble fractions.

The activity of phosphatidate phosphohydrolase was determined by measuring the rate of release of inorganic phosphate from aqueous dispersion of phosphatidate in the presence of an enzyme preparation. The activity of the enzyme in heart homogenate was proportional to increasing concentrations of enzyme protein up to 2.5 mg, and the rate of reaction was linear with incubation time for up to 3 hr. Magnesium ions at low concentration (1 mM) did not influence the phosphatidate phosphohydrolase activity in heart homogenate. The optimal pH value for this enzyme in dialyzed homogenate was found to be 7.0. The K_m of phosphatidate phosphohydrolase in the lysosomal-microsomal fraction was 0.33 mM. This enzyme showed its highest specific activity in the lysosomal and microsomal fractions. The activity of the phosphatidate phosphohydrolase in the soluble fraction was less than 1/15 of the microsomal enzyme activity when phosphatidate suspension was used as the substrate. The ability of this enzyme to react with membrane-bound phosphatidate in soluble fraction of heart was very low.

I. INTRODUCTION*

The mechanism regulating fatty infiltration of the heart has been the subject of study in various laboratories. Fatty infiltration appears in the heart not only under experimental conditions, but also in human subjects (Opie, 1968 and 1969). Experimentally, lipids accumulate in the heart under the following states: diphtheritic myocarditis, alloxan-diabetes, alcohol-infusion, norepinephrine-infusion, thyrotoxic cardiomegaly, and myocardial hypoxia. Clinically, cardiac TG is found to increase in human diphtheritic myocarditis, alcohol cardiomyopathy, and myocardial infarction.

There are three possible causative factors responsible for the increase in TG biosynthesis in the heart. These are:

- (1) increased availability of the substrates FA and/or sn-G3P,
- (2) increased uptake of TG or TG FA, and (3) enhanced activity of enzymes for TG biosynthesis.

*The experimental work presented in this thesis was done before January, 1974, with the major parts of the thesis being written around the time of March, 1974. The references cited, therefore, are dated prior to and including that year.

Increased substrate availability has been frequently noted as an etiology of increased myocardial TG formation. In alloxan-diabetes (Denton and Randle, 1967) and norepinephrine-infusion (Regan et al., 1966a), mobilization of FFA from adipose tissue is increased, resulting in an increased plasma concentration of FFA. This increased plasma concentration enhances uptake of FFA by the myocardium, and thus more intracellular FFA is made available for TG synthesis. An increase in intracellular FFA can also be associated with decreased FFA oxidation by the heart as seen in diphtheritic myocarditis (Bressler and Wittels, 1965) and alcohol-infusion (Kikuchi and Kako, 1970). In the ischemic heart, increased anaerobic glycolysis results in an increased availability of sn-G3P (Scheuer and Brachfeld, 1966). Depressed FFA oxidation during cardiac ischemia (Wood et al., 1972 and 1973) may lead to increased levels of intracellular FFA and fatty acyl-CoA. The increased level of sn-G3P, however, would be more important in determining the increased myocardial TG content, because the K_m value of sn-G3P is higher than that of acyl-CoA for the G3P acyltransferase reaction (Lamb and Fallon, 1970).

Increased myocardial TG formation could be a result of

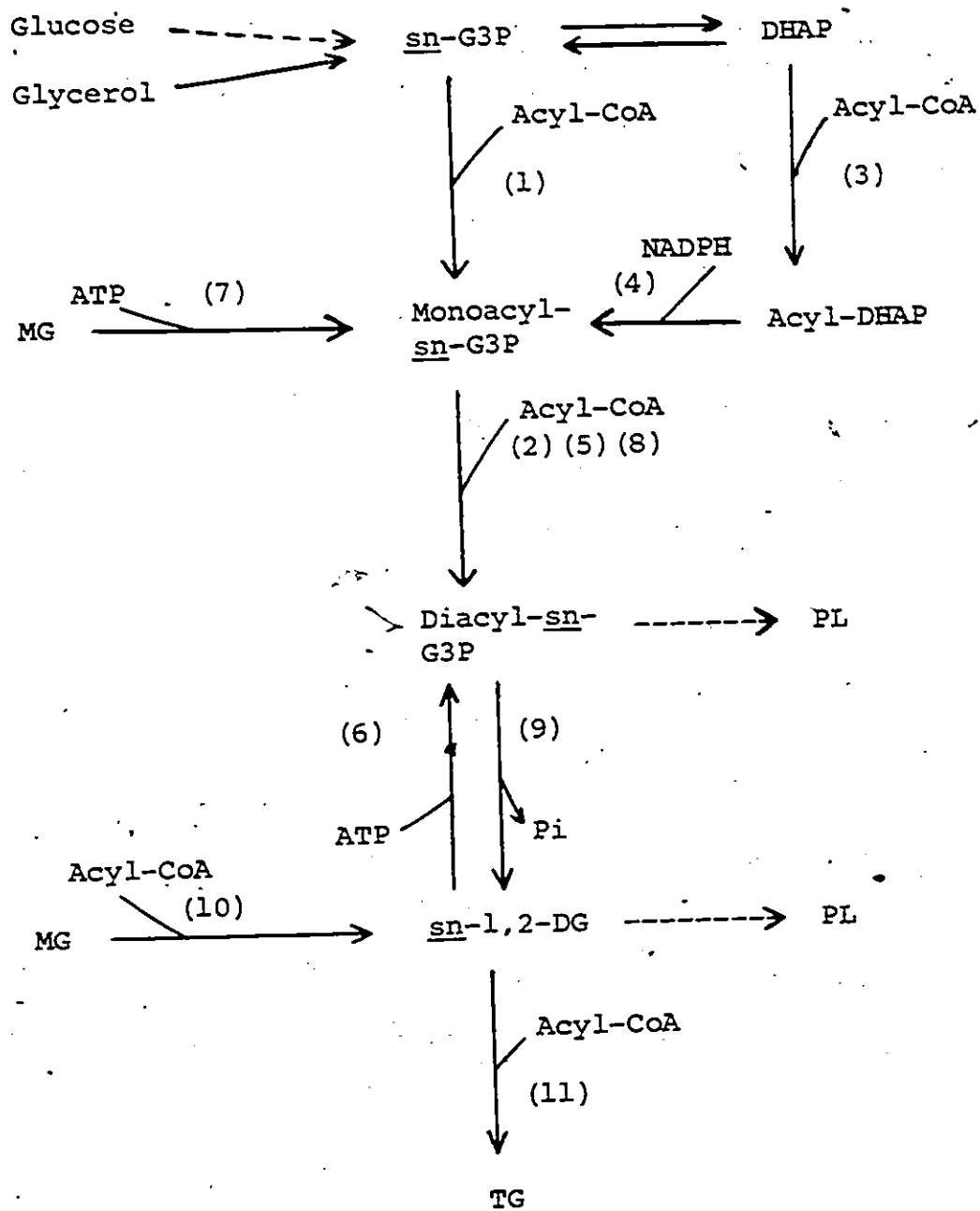
increased TG and/or TG FA penetration due to altered membrane permeability. This idea was proposed by Regan et al. (1966b and 1969) in ethanol cardiomyopathy. Opie (1968 and 1969) suggested the possibility that an increase in activity of myocardial lipoprotein lipase (clearing factor lipase) may increase TG FA uptake by the heart, thus resulting in an accumulation of TG. This possibility was demonstrated in hyperthyroidism (Alousi and Mallov, 1964), epinephrine-infusion (Alousi and Mallov, 1964; Mallov and Cerra, 1967), and ethanol intoxication (Mallov and Cerra, 1967). However, in the case of ethanol-induced fatty infiltration, the activity of myocardial lipoprotein lipase was reported to be unaltered (Kikuchi and Kako, 1970). Because of the existence of conflicting data, special attention must be given to the techniques for assay of lipoprotein lipase in order to insure that the lipase being studied is, in fact, clearing factor lipase and not one of a number of other lipases (Yamamoto and Drummond, 1967; Opie, 1968 and 1969). By doing so, it is possible to investigate the significance of changes in activity of this enzyme in relation to the TG FA uptake by the heart.

The possibility of TG accumulation in the myocardium as a result of enhanced activity of enzymes for TG biosynthesis has not been explored. Moreover, there is very little information available concerning the basic characteristics of and the subcellular localization of these enzymes in the normal heart. In an attempt to elucidate the controlling mechanism(s) of TG biosynthesis in diseased hearts, it is essential to obtain data concerning fundamental characteristics of enzymes synthesizing TG in the rabbit heart. An attempt is made in the following introductory sections to give a brief description concerning the metabolic pathways of TG biosynthesis. Intracellular distribution of enzymes for biosynthesis of and the FA and positional specificity of phospho- and glycerolipids in mammalian organs, primarily the liver, are also discussed.

I-1. The Metabolic Pathways of Triglyceride Synthesis

Present knowledge concerning the metabolic pathways of TG biosynthesis and related reactions is summarized in Fig. 1. The numbers appearing in Fig. 1 correspond to those stated with all the chemical reactions written below.

Fig. 1. Metabolic pathways of triglyceride biosynthesis
in mammalian tissues



I-1-1. Formation of diacyl-sn-glycerol-3-phosphate

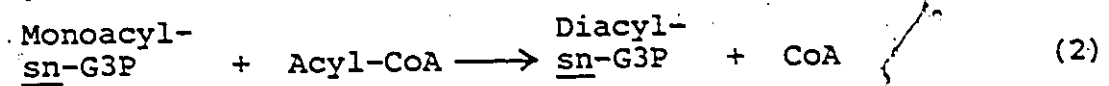
In 1961, Kennedy formulated a schematic diagram of the pathways involved in complex lipid biosynthesis. In this diagram, sn-G3P, diacyl-sn-G3P, and sn-1,2-DG are shown to be successive intermediates in the biosynthesis of TG. The diagram also indicates that diacyl-sn-G3P is needed for the formation of five major phospholipids, namely, phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, phosphatidylglycerol, and sphingomyelin. Since Kennedy suggested this pathway in 1961, diacyl-sn-G3P has been recognized as a key intermediate in and the common precursor for the biosynthesis of glycerides and phosphoglycerides. Kennedy's pathway leading to synthesis of diacyl-sn-G3P is shown in Fig. 1.

I-1-1-i. Stepwise acylation of sn-glycerol-3-phosphate by acyl-CoA

Kornberg and Pricer (1953b) discovered that the acylation of sn-G3P takes place in guinea pig liver microsomes and produces diacyl-sn-G3P. Following this discovery, Stein et al. (1957), Buell and Reiser (1959), Weiss et al. (1960), and many other investigators have confirmed that diacyl-sn-G3P

plays a key role as an intermediate in the biosynthesis of glycerides and phosphoglycerides in mammalian organs. It has been established that monoacyl-sn-G3P is an intermediate for diacyl-sn-G3P synthesis in E. Coli (Pieringer et al., 1967; Ray et al., 1970). In the mammalian system, it was thought that diacyl-sn-G3P was the sole product of acylation (Lands and Hart, 1964; Abou-Issa and Cleland, 1969; Sánchez de Jiménez and Cleland, 1969; Possmayer et al., 1969; Sarzala et al., 1970; Eibl et al., 1969; Davidson and Stanacev, 1972). It was only recently that investigators found reason to doubt this. Fallon and Lamb (1968) and Daae and Bremer (1970) were able to detect monoacyl-sn-G3P as one of the products in liver microsomes and mitochondria. Moreover, the studies by Barden and Cleland (1969), Lamb and Fallon (1970), and Okuyama et al. (1971) of liver microsomes established that a product-precursor relation existed between monoacyl- and diacyl-sn-G3P. Thus, the acylation of sn-G3P by acyl-CoA to form monoacyl-sn-G3P, followed by subsequent acylation to diacyl-sn-G3P was then established in mammalian tissues. The enzyme that catalyzed the initial acylation of sn-G3P was partially purified from liver mitochondria by Monroy et al.

(1973) and from microsomes by Yamashita and Numa (1972). The enzyme involved in the subsequent acylation reaction was also partially purified from rat liver microsomes by Yamashita *et al.* (1973). The reaction sequence is shown:

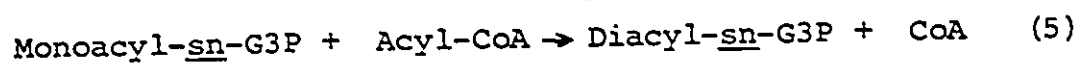
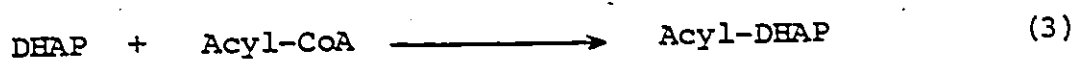


The enzymes G3P acyltransferase and monoacyl-sn-G3P acyltransferase catalyze equations (1) and (2), respectively, and are reported to possess distinct properties in terms of relative resistance to sulfhydryl-binding reagents (Lands and Hart, 1965) as well as in terms of substrate and positional specificity (Lamb and Fallon, 1970; Yamashita and Numa, 1972; Monroy *et al.*, 1973; Yamashita *et al.*, 1973). The roles of these two enzymes in regulating the positional arrangement of FAs in monoacyl- and diacyl-sn-G3P molecules will be discussed later.

Although the acylation reactions of sn-G3P and monoacyl-sn-G3P involve two different enzymes with distinct properties, the name G3P acyltransferase has been used by many investigators (Mårtensson and Kanfer, 1968; Daae and Bremer, 1970; Daae, 1972; Davidson and Stanacev, 1972; Mangiapane *et al.*,

1973) to indicate both reactions. In order to avoid ambiguity, the name G3P acyltransferase used in this thesis refers only to the enzyme which catalyzes the formation of monoacyl-sn-G3P from sn-G3P and acyl-CoA.

I-1-1-ii. Acylation of dihydroxyacetone phosphate followed by reduction and reacylation

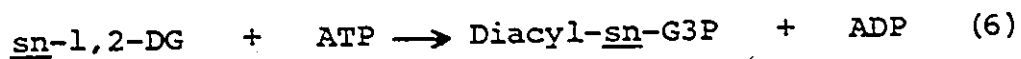


Hajra and Agranoff (1968a) and Hajra (1968a and 1968b) first described the operation of these reactions in microsomal and mitochondrial fractions of guinea pig liver. The existence of these reactions was subsequently confirmed by Puleo *et al.* (1970) in liver microsomes. Manning and Brindley (1972) compared the relative activity of the DHAP pathway to Kennedy's de novo pathway for the synthesis of glycerolipids from radioactive glycerol in rat liver slices and found that 50 to 60 % of the glycerolipids were produced via the DHAP pathway.

I-1-1-iii. Phosphorylation of sn-1,2-diglyceride in the presence of ATP

An ATP-dependent phosphorylation of the free primary .

hydroxyl group of sn-1,2-DG has been described in particulate fraction of the brain (Hokin and Hokin, 1959; Pieringer and Hokin, 1962a) and in erythrocyte membrane (Hokin and Hokin, 1961b; Hokin and Hokin, 1963). In microsomes prepared from guinea pig brain, synthesis of diacyl-sn-G3P was dependent upon the addition of sn-1,2-DG, an ATP generating system, and magnesium ions (Hokin and Hokin, 1959). The pathway has been described as follows:

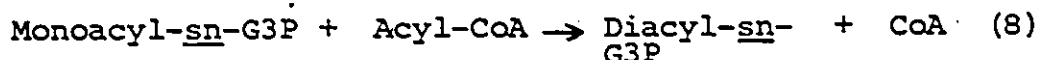


The enzyme which catalyzes the formation of diacyl-sn-G3P via this pathway has been termed DG kinase. The activity of the enzyme was very low in brain (Hokin and Hokin, 1959). However, the rate of formation of diacyl-sn-G3P was greatly increased by the treatment of the microsomal preparation with a suitable concentration of a surface-active agent, for example, deoxycholate. The enzyme exhibited a strong substrate specificity. DG prepared from cabbage phosphatidate was the most effective substrate, while DG prepared from brain lecithin was a less suitable substrate, and 1-palmitoyl,2-oleyl DG was the least effective (Hokin and Hokin, 1959).

The combined actions of DG kinase and phosphatidate phosphohydrolase form the phosphatidic acid cycle to synthesize and to break down phosphatidic acid. Hokin and Hokin (1961a) postulated that this cycle may be identical to the ATPase activity related to the sodium-potassium pump in erythrocyte membrane, since the combined activities of the two enzymes of the phosphatidic acid cycle require sodium, potassium, and magnesium, and the membrane ATPase activity is also dependent on the presence of these three ions. The physiological significance of the phosphatidic acid cycle in other organs has not been explored further, although the existence of DG kinase in other organs such as liver has been reported (Yoshida and Nukada, 1961). The sodium potassium pump hypothesis, based on the phosphatidic acid cycle proposed, has not been supported unanimously by all investigators.

I-1-1-iv. Phosphorylation of monoglyceride with ATP to monoacyl-sn-glycerol-3-phosphate followed by acylation with acyl-CoA to form diacyl-sn-glycerol-3-phosphate

The observation of Pieringer and Hokin (1962a and 1962b) showed that diacyl-sn-G3P can be synthesized via the following sequence of reactions:



MG kinase, which catalyzes the formation of monoacyl-sn-G3P (equation (7)), and monoacyl-sn-G3P acyltransferase, which catalyzes the acylation reaction shown in equation (8), have been found in guinea pig brain and liver (Hokin and Hokin, 1960a; Pieringer and Hokin, 1962a and 1962b).

In deoxycholate-treated extracts of either the cytoplasmic particulate or the microsomal fraction of guinea pig or calf brain (Pieringer and Hokin, 1962a), synthesis of monoacyl-sn-G3P required only ATP and MG as substrates. Both α - and β -MGs are equally active as substrates. Phosphorylation of 1-monopalmitoylglycerol, 1-monoooleylglycerol, and 1-monolinolylglycerol gave rates of 0.4, 0.5, and 0.8 μ mole of monoacyl-sn-G3P formed per mg protein per hour, respectively, indicating a slight specificity towards unsaturated FAs.

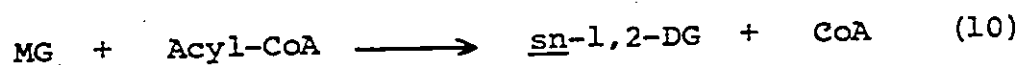
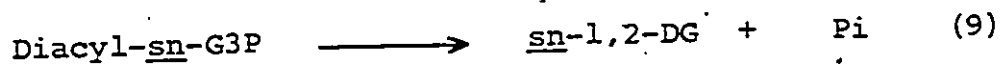
The rate of incorporation of ^{32}P -ATP into monoacyl-sn-G3P and the formation of diacyl-sn-G3P were both stimulated by the addition of free FAs (Pieringer and Hokin, 1962a). Monoacyl- and diacyl-sn-G3P labellings from ^{32}P -ATP (Pieringer and Hokin, 1962a; Hokin and Hokin, 1959) did not decrease

upon the addition of sn-G3P, indicating a direct phosphorylation of the glycerides without hydrolysis to glycerol and subsequent incorporation involving sn-G3P as an intermediate.

The biosynthesis of monoacyl-sn-G3P from ATP and 1-monooleylglycerol by intestinal mucosa of rat was reported to take place both in vivo (Paris and Clément, 1965) and in vitro (Paris and Clément, 1969).

I-1-2. Formation of sn-1,2-diglyceride

Sn-1,2-DG is synthesized by dephosphorylation of diacyl-sn-G3P or by acylation of MG as shown in reactions (9) and (10), respectively:



The evidence for the enzymatic dephosphorylation of diacyl-sn-G3P by animal tissues was first provided by Smith, Weiss, and Kennedy in 1957. These investigators showed that the enzyme catalyzing equation (9), phosphatidate phosphohydrolase (EC 3.1.3.4), is widely distributed in mammalian organs. In the rat, the enzymes located in the kidney, liver, and brain are the most active, while heart

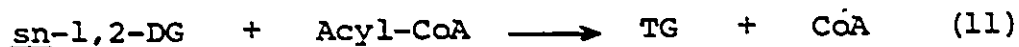
and skeletal muscle enzymes are less active.

Phosphatidate phosphohydrolase plays an important role in the regulation of hepatic neutral lipid biosynthesis. The in vitro activity of phosphatidate phosphohydrolase (Vavrečka et al., 1969; Mangiapane et al., 1973; Lamb and Fallon, 1974a) was significantly lower than the estimated in vitro rates of acyl-CoA synthetase (Lloyd-Davies and Brindley, 1973), G3P acyltransferase (Shepard and Hübscher, 1969; Lloyd-Davies and Brindley, 1973; Mangiapane et al., 1973; Lamb and Fallon, 1974a), 1-acyl-sn-G3P acyltransferase (Eibl et al., 1969; Lamb and Fallon, 1974a), and DG acyltransferase (Young and Lynen, 1969; Lamb and Fallon, 1974a). If the estimated in vitro rates of the reactions catalyzed by these enzymes accurately reflect their in vivo rates, the phosphatidate phosphohydrolase appears to be rate limiting for hepatic TG biosynthesis (Lamb and Fallon, 1974a and 1974b). This hypothesis was supported by the observations that subtotal hepatectomy (Mangiapane et al., 1973) and high carbohydrate diet (Waddell and Fallon, 1973; Lamb and Fallon, 1974b) increased the TG content of the liver and enhanced the activity of phosphatidate

phosphohydrolase.

In the intestine, an alternative pathway which employs MG for the formation of DG by the action of MG acyltransferase has been established by Clark and Hübscher (1960 and 1961), Senior and Isselbacher (1962), Johnston and Brown (1962), Ailhaud et al. (1963), and Gallo et al. (1968) and is illustrated in equation (10). Furthermore, Mattson and Volpenhein (1964) and Kern and Borgström (1965) have evaluated the extent of participation of the two pathways both in vitro and in vivo and have shown that the MG acyltransferase pathway accounts for the major portion of DG and TG synthesis in the intestinal mucosa. Other evidence suggests that the MG acyltransferase pathway occurs also in the kidney, pancreas (Hübscher, 1961), adipose tissue (Belfrage, 1964), wall of the aorta (Stein et al., 1963), and the mammary glands of guinea pigs (McBride and Korn, 1964) and goats (Pynadath and Kumar, 1964; Dimick et al., 1966). However, Dils and Clark (1962) and Senior and Isselbacher (1962) failed to demonstrate the existence of this pathway in both the mammary glands and the liver of the rat.

I-1-3. Formation of triglyceride



The hypothesis that sn-1,2-DG serves as an immediate precursor for TG synthesis was originated because of the work of Weiss and Kennedy (1956) with particulate subcellular preparations from chicken liver, and also due to the work of Tietz and Shapiro (1956) and Stein and Shapiro (1957) in liver homogenates and Stein et al. (1957) in liver mitochondria. The existence of the enzyme DG acyltransferase, which catalyzed reaction (11), was later confirmed in chicken liver (Weiss et al., 1960), adipose tissue (Steinberg et al., 1961; Goldman and Vagelos, 1961), rat and rabbit intestinal mucosa (Clark and Hübscher, 1961; Ailhaud et al., 1964), mammary glands of lactating pigs and goats (McBride and Korn, 1964; Pynadath and Kumar, 1964), and rat diaphragm (Neptune et al., 1962).

The structure and configuration of DG plays a role in its acylation reaction (Hübscher, 1970). 1,3-DGs are poor acyl acceptors, whereas 1,2-DGs are more suitable substrates. The D-isomer gave higher reaction rates than did the L-isomer or the DL-mixture. The FA composition of the 1,2-DGs also

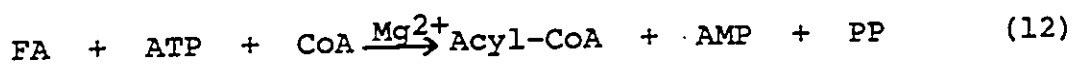
influences the rate of their acylation reaction. 1,2-dioleoyl-glycerol gave the highest reaction rate, whereas the rate was lowest with mixed DGs containing oleic and palmitic acid. 1,2-dipalmitoylglycerol was a poor substrate for the enzyme.

DG acyltransferase shows a specificity towards individual FAs. When 1,2-dipalmitoylglycerol was used as substrate, higher reaction rates were observed with palmitoyl-CoA than with caproyl-CoA. Oleyl-CoA produced only half the reaction rate of stearyl-CoA (Ailhaud et al., 1964). Magnesium ions exerted a stimulating effect, whereas fluoride produced an inhibitory effect on the DG acyltransferase reaction (Weiss et al., 1956).

The studies described above were carried out by using DGs dispersed in surface-active agents. The results, therefore, could have been modified by the state of substrates in an aqueous assay medium.

I-1-4. Other reactions related to the biosynthesis of triglyceride

I-1-4-i. Activation of long-chain fatty acids



In order to be esterified, FAs must first be activated

by CoA, ATP, and magnesium ions to form fatty acyl-CoAs. The reactions are of two types: ATP-dependent and GTP-dependent. The GTP-dependent activating enzyme plays only a minor role in glyceride biosynthesis. When GTP, at a range of concentrations similar to that of ATP, was used to provide the energy necessary for the formation of acyl-CoA derivatives in a microsomal fraction from cat intestine, no activation of palmitate was detected (Brindley and Hübscher, 1966). Furthermore, substitution of GTP for ATP in the study of the activation of long-chain FAs by rat liver microsomes failed to activate octanoate and oleate (Pande and Mead, 1968b). Therefore, only the enzyme involved in the ATP-dependent formation of acyl-CoA will be discussed in this study.

In 1953, Kornberg and Pricer discovered the activation of long-chain FAs up to C22:0 taking place in the liver of the guinea pig, rat, mouse, pigeon, and chicken. The enzyme acyl-CoA synthetase, which catalyzes equation (12), was found to exist in the mucosa of the small intestine of rabbits (Clark and Hübscher, 1960), rats (Senior and Isselbacher, 1960), hogs (Ailhaud et al., 1962), guinea pigs and cats

(Brindley and Hübscher, 1966), as well as in rat liver (De Jong and Hülsmann, 1970; Pande, 1972) and heart (De Jong and Hülsmann, 1970). Pande and Mead (1968a) have studied the distribution of this enzyme in various rat tissues. Its highest specific activity was found in liver and, in a decreasing order, epididymal fat pad, heart, kidney, diaphragm, skeletal muscle, brain, lungs, intestine, and testes. The activity in serum was not detectable. Bar-Tana and Shapiro (1964) and Bar-Tana et al. (1971 and 1973) were able to partially purify palmitoyl-CoA synthetase from rat liver microsomes and study its general properties.

The ATP-dependent long-chain acyl-CoA synthetase showed different substrate specificity in various subcellular preparations. The microsomal fractions from guinea pig liver (Borgström and Wheeldon, 1961) and those from the intestinal mucosa of the guinea pig (Brindley and Hübscher, 1966) exhibited the maximum reaction rate with $C_{10:0}$ and $C_{14:0}$, respectively. A microsomal preparation from the intestinal mucosa of cat (Brindley and Hübscher, 1966) and one from rat liver (Pande and Mead, 1968b) gave maximum reaction rates with $C_{16:0}$. The unsaturated FA, oleate, was reported to give the highest

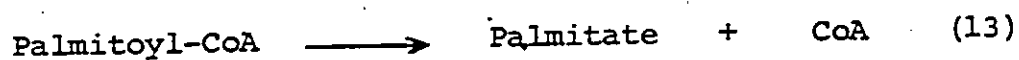
rate (111 nmoles/mg protein per min) of acyl-CoA formation by a microsomal preparation of rat liver when measured by the hydroxamate assay method (Pande, 1972).

Since fatty acyl-CoA is the only form in which FAs can be oxidized and esterified, it is of importance to know whether or not the acyl-CoA synthetase is rate limiting for FA oxidation and esterification. A comparison of the acyl-CoA synthetase activity of different tissues with their maximal rates of FA uptake and oxidation should be relevant (Pande, 1971). Rat liver was able to activate 856 μ moles of palmitate per hr per g of fresh tissue (Pande and Mead, 1968a), but the maximal rate of uptake of FA was only 10 to 50 μ moles per hr per g (Van Harken *et al.*, 1969), suggesting that any FAs taken up would be activated to form acyl-CoA. It was found that the rate of palmitate oxidation was 2.46 μ moles per min per g in the heart (Pande and Mead, 1968a). Working rat heart consumed about 9.5 μ moles of O_2 per min per g (Neely *et al.*, 1967), and if palmitate were the only substrate for respiration, this would correspond to a utilization of 0.41 μ mole of palmitate per min. This indicates that the ability of heart to activate palmitate

is much greater than its ability to oxidize this FA. Unlike the liver, which not only oxidizes FAs but also extensively esterifies them for secretion, heart and skeletal muscle use FAs mainly for oxidation, at least in the working state. Hence, it is unlikely that acyl-CoA synthetase activity limits FA oxidation and esterification.

I-1-4-ii. Palmitoyl-CoA hydrolase

Since acyl-CoA is the only form of acyl donor available for esterification, and since it is one of the two precursors for G3P and DG acyltransferases, the enzymatic deacylation of this compound could alter the formation of phosphatidate and the synthesis of TG. Observations on the characteristics of palmitoyl-CoA hydrolase appeared in a number of publications not directly dealing with this enzyme but with various FA esterification reactions. Generally, whenever an assay was carried out by measuring CoA ester disappearance or free CoA appearance, the action of palmitoyl-CoA hydrolase had to be accounted for. The reaction is shown in equation (13).



Acyl-CoA hydrolase of rat liver microsomes exhibits

some specificity for acyl thioesters of chain length greater than C₁₂, with palmitoyl and stearyl thioesters being the preferred substrates. Myristyl, oleyl, and lauryl thioesters are less active substrates (Barden and Cleland, 1969).

I-2. Intracellular Localization of Enzymes Related to Triglyceride Biosynthesis in Mammalian Tissues

The current status of knowledge regarding the intracellular localization of enzymes related to TG biosynthesis in mammalian tissues* is summarized in Table 1.

I-2-1. Distribution of acyl-CoA:sn-glycerol-3-phosphate-0-acyltransferase and acyl-CoA:monoacyl-sn-glycerol-3-phosphate-0-acyltransferase

As mentioned earlier, a number of investigators have used the term G3P acyltransferase for the acylation reactions of sn-G3P and monoacyl-sn-G3P, and the enzymes involved in these reactions have been studied simultaneously. Therefore,

* The animals referred to are mainly mammals. The exceptions are references to the chicken and albatross which were deemed too important to exclude.

the subcellular distribution of G3P and monoacyl-sn-G3P acyltransferases will be described together.

When this study was initiated in 1970, there was no unequivocal opinion concerning the subcellular distribution of G3P and monoacyl-sn-G3P acyltransferases in mammalian tissues because the available data differed widely. To study biosynthesis of diacyl-sn-G3P from sn-G3P or monoacyl-sn-G3P in cell free enzyme systems, earlier experiments were carried out with microsomal fractions of rat liver (Lands and Hart, 1964; Fallon and Lamb, 1968; Abou-Issa and Cleland, 1969; Possmayer et al., 1969; Barden and Cleland, 1969; Lamb and Fallon, 1970), guinea pig liver (Brandes et al., 1963), and rat brain (Sánchez de Jiménez and Cleland, 1969; Possmayer and Mudd, 1971). Mitochondrial fractions of rat liver and brain were used successfully as enzyme sources for acylation of sn-G3P by Smith and Hübscher (1966), Stoffel and Schiefer (1968), and Mårtensson and Kanfer (1968). Finally, Shephard and Hübscher (1969), Zborowski and Wojtczak (1969), and Sarzala et al. (1970) provided

evidence that mitochondrial fractions of rat liver were capable of synthesizing diacyl-sn-G3P, and that the enzymes responsible for diacyl-sn-G3P synthesis were found mainly in the outer membrane. By using palmitoylcarnitine, CoA, and carnitine palmitoyltransferase as the acyl donor system, Daae and Bremer (1970) and Daae (1972) demonstrated a bimodal intracellular distribution of G3P and monoacyl-sn-G3P acyltransferases, that is, their distribution in both mitochondrial and microsomal fractions of rat liver. The mitochondrial enzymes were found to be somewhat more active than the microsomal enzymes, per unit mass of protein.

Eibl et al. (1969) and Davidson and Stanacev (1972) raised a question about the intracellular localization of G3P and monoacyl-sn-G3P acyltransferases in mammalian tissues. Based upon the subcellular distribution of glucose-6-phosphatase, a microsomal marker enzyme, Eibl et al. (1969) concluded that the measured mitochondrial acyltransferase activity in rat liver represents an activity due to contamination by microsomal enzyme. This finding was later supported by Davidson and Stanacev (1972) in rat and guinea pig liver by the use of an additional microsomal marker enzyme,

NADPH:cytochrome C reductase. The technique which these workers utilized is similar to that used earlier in a study by McMurray and Dawson (1969), who demonstrated that the synthesis by rat liver mitochondria of either nitrogen- and inositol-containing phosphoglycerides from ^{32}P or phosphatidylcholine from CDP- ^{14}C -choline was dependent upon a small microsomal component that invariably contaminated the mitochondria preparation.

More recently, Parkes and Thompson (1973) studied the metabolic relationship between molecular classes of phosphatidylcholine in the mitochondria and endoplasmic reticulum of guinea pig liver using intraperitoneal injections of $[2\text{-}^3\text{H}]\text{glycerol}$, $[1\text{-}^{14}\text{C}]\text{palmitic acid}$, or $[1\text{-}^{14}\text{C}]\text{stearic acid}$. They found that the percentage distribution of radioactivity in mitochondrial classes of phosphatidylcholine was very similar to that in microsomes. From this, and because of the fact that the activity of microsomes was greater than that of mitochondria, the authors concluded that the profile of molecular classes of phosphatidylcholine in guinea pig liver mitochondria was regulated primarily through mechanisms involving the transfer of lipids from the endoplasmic reticulum. Although this work dealt with

the biosynthesis of phosphatidylcholine from glycerol and FAs in vivo, it indirectly supported the conclusions drawn by Eibl et al. (1969) and Davidson and Stanacev (1972); they state that the mitochondrial capacity to synthesize diacyl-sn-G3P represents the extent of cross contamination by microsomal enzymes.

In view of the uncertainties concerning the question of whether the mitochondria of rat liver possessed an acyltransferase activity, and whether the reported presence of this activity was a reflection of microsomal contamination as described above, Monroy et al. (1972) have studied the characteristics of acyltransferases in these organelles. They demonstrated that in rat liver, mitochondrial G3P acyltransferase possesses characteristics different from that in microsomes, namely, the relative sensitivity to sulfhydryl-binding reagents and response to magnesium ions of the two enzymes differed (Monroy et al., 1972). This indicates that the mitochondrial fraction was capable of acylating sn-G3P to monoacyl-sn-G3P independently of the microsomal enzymes. Furthermore, the acyltransferase has been purified partially (four-fold) from the mitochondrial fraction of rat liver (Monroy et al., 1972). Yamashita

and Numa (1972) have successfully purified G3P acyltransferase (six-fold) from rat liver microsomes. Although the extent of purification achieved by these authors was not very high, it provided convincing evidence supporting the fact that, in rat liver, G3P acyltransferase was distributed both in the mitochondrial and microsomal fractions. Independently, a study undertaken by our laboratory with rabbit hearts provides further evidence in support of the distinctly different characteristics of mitochondrial and microsomal G3P acyltransferases, and thus clarifies the question of the mitochondrial existence of this enzyme in the rabbit heart.

All of the above studies have dealt with either G3P acyltransferase alone, or both G3P and monoacyl-sn-G3P acyltransferases. It is of particular interest to note that monoacyl-sn-G3P acyltransferase, which has been shown to exist in the microsomal preparation of rat liver (Lands and Hart, 1964; Barden and Cleland, 1969; Okuyama et al., 1971) and guinea pig liver (Lands and Hart, 1964), was purified partially from rat liver microsomes by Yamashita et al. (1973).

I-2-2. Distribution of acyl-CoA: dihydroxyacetone phosphate acyltransferase

Hajra and Agranoff (1967), who first described the existence of the acyl-DHAP pathway in guinea pig liver, incubated mitochondrial preparation with γ - ^{32}P -ATP in the presence of MgCl_2 , and isolated a rapidly labelled phospholipid. When ^{32}P -orthophosphate was substituted for γ - ^{32}P -ATP, the added ^{32}P -orthophosphate was first converted to labelled ATP which subsequently phosphorylated the mitochondrial lipid. On the basis of comparing its chemical properties with synthetic palmitoyl-DHAP, the isolated product was identified as acyl-DHAP (Hajra and Agranoff, 1967). Furthermore, synthetic DHAP was converted to acyl-DHAP by mitochondria of guinea pig liver in the presence of FA, CoA, ATP, and Mg^{2+} . The requirement for FA, CoA, ATP, and Mg^{2+} could be replaced by acyl-CoA. The product, acyl-DHAP, was again verified by comparing its structure with infrared spectra of synthetic and biosynthetic palmitoyl-DHAP (Hajra, 1968a).

By using the technique described above, Hajra and Agranoff (1967 and 1968a) and Hajra (1968a and 1968b) claimed that the acyl-DHAP pathway was localized in the mitochondria and microsomes of guinea pig liver. It was later confirmed by La Belle and Hajra (1972) that the acyl-DHAP pathway was

distributed in the mitochondria and microsomes of the liver, brain, kidney, heart, and adipose tissue of the rat.

In contrast, Puleo et al. (1970) and Rao et al. (1971a and 1971b) provided evidence that the acyl-DHAP pathway in rat liver was present in microsomal fraction but absent in the mitochondria. Davidson and Stanacev (1972) observed that in guinea pig liver, the rates of sn-[U-¹⁴C] G3P and sn-[2-³H] G3P acylation by mitochondria and microsomes were similar. From these results they reached a conclusion that the acyl-DHAP pathway was absent in these subcellular organelles. By using a similar technique, Monroy et al. (1972) could not demonstrate the involvement of the DHAP pathway in the biosynthesis of monoacyl-sn-G3P in rat liver mitochondria.

In studying the DHAP pathway, Manning and Brindley (1972) employed a method which involves measurement of the 2-³H/1-¹⁴C ratio in both sn-G3P and glycerolipid. The measurements were carried out at various time intervals after incubation of rat liver slices in the presence of [2-³H]glycerol and [1-¹⁴C]glycerol. It was found that the 2-³H/1-¹⁴C ratio in the sn-G3P was considerably higher than the cumulative 2-³H/1-¹⁴C ratio in glycerolipid synthesized throughout the corresponding time-period. This indicated that the sn-G3P, labelled

with 2-³H and 1-¹⁴C, is not the only precursor of glycerolipid in rat liver slices. Another metabolite, which was labelled exclusively with ¹⁴C, was also incorporated. This was DHAP. Based on these findings, Manning and Brindley (1972) calculated that 50-60 % of the glycerol incorporated into lipids by rat liver slices proceeded via the DHAP pathway. In this study, however, no information regarding the subcellular distribution of the DHAP pathway was given.

Many other investigators have previously failed to show the presence of the DHAP pathway in subcellular organelles (Davidson and Stanacev, 1972; Monroy et al., 1972) or in intact liver slices (Okuyama and Lands, 1970). However, the technique which they utilized has been criticized for the following reason: [2-³H]-sn-G3P was oxidized 2.17 times more slowly than the unlabelled substrate by rat liver mitochondria in the absence of oxidative phosphorylation inhibitors and uncouplers (Carnicero et al., 1972). Accordingly, the use of [2-³H]-sn-G3P does not account for the isotope effect and hence produces erroneous data in assessing the DHAP pathway (Manning and Brindley, 1972). Consequently, more precise subcellular distribution of the enzymes of the

acyl-DHAP pathway in animal tissues is to be further investigated.

I-2-3. Distribution of diglyceride kinase

DG kinase was found to be present in microsomal fractions of guinea pig brain (Hokin and Hokin, 1959; Pieringer and Hokin, 1962a) and in microsomal, mitochondrial, and soluble fractions of albatross salt gland (Hokin and Hokin, 1960b). The existence of this enzyme in mitochondrial and microsomal preparations from rat brain was reported later by Strickland (1962) and De Pury and Collins (1963), respectively.

I-2-4. Distribution of monoglyceride kinase

Hokin and Hokin (1960a) and Pieringer and Hokin (1962a and 1962b) provided evidence that MG kinase was localized in microsomal fractions of guinea pig brain and liver and calf brain. Both mitochondrial and microsomal preparations from rat intestinal mucosa were reported to be capable of synthesizing monoacyl-sn-G3P from ATP and 1-monooleylglycerol (Paris and Clément, 1969).

I-2-5. Distribution of phosphatidate phosphohydrolase

Since the discovery of phosphatidate phosphohydrolase by Kates (1955) in plants, the enzyme has been described in numerous mammal tissues (Smith et al., 1957; Hokin and Hokin,

1959). In earlier studies, it was generally assumed that the enzyme was distributed in mitochondrial and microsomal fractions (Hokin and Hokin, 1960b; Coleman and Hübscher, 1962; Stoffel and Schiefer, 1968). However, the observations described by Wilgram and Kennedy (1963) cast some doubt on this postulate. It was pointed out that the phosphatidate phosphohydrolase of rat liver is found neither in mitochondrial nor microsomal fractions. It is, instead, principally localized in subcellular structures having intermediate sedimentation characteristics identical to lysosomal fractions. In addition, the soluble fraction was found to contain some activity.

In cat intestinal mucosa, a different distribution pattern, almost exclusively microsomal, was reported by Brindley and Hübscher (1965). In 1965, Sedgwick and Hübscher found that phosphatidate phosphohydrolase in rat liver is a true constituent of all three particulate subcellular fractions including mitochondrial, lysosomal, and microsomal fractions. Some activity also occurred in the soluble fraction (also referred to as the cell sap or the cytosomal fraction). Furthermore, it was shown that two types of mitochondrial phosphatidate phosphohydrolase exist; one is

readily soluble and could be released by freezing and thawing, while the other is insoluble and tightly bound to mitochondrial membrane (Sedgwick and Hübscher, 1965). In 1967, the soluble phosphatidate phosphohydrolase was partially purified from rat liver mitochondria (Sedgwick and Hübscher, 1967) and was claimed to be the major enzyme responsible for the hydrolysis of phosphatidic acid. However, the insoluble enzyme (membrane-bound) acted only slowly on the endogenous substrate (Hübscher et al., 1967).

Soluble fractions prepared from either rat liver or cat intestinal mucosa (Hübscher et al., 1967; Smith et al., 1967) stimulated glyceride biosynthesis via the acylation of sn-G3P. This stimulation appeared to be due mainly to the presence of phosphatidate phosphohydrolase in the preparation. However, the soluble fractions of Hübscher et al. (1967) and Smith et al. (1967) contained no detectable acyl-CoA synthetase, DG acyltransferase, and phosphatidate phosphohydrolase which is measured by using exogenous phosphatidic acid (Smith and Hübscher, 1966). From this, it is difficult to conclude that phosphatidate phosphohydrolase in the soluble fraction is a real constituent of cell sap. Perhaps it leaked out from the membrane of the mitochondria and/or

the endoplasmic reticulum during preparation.

Johnston et al. (1967) have independently examined a functionally similar soluble fraction from intestinal mucosa and have concluded that it stimulated glyceride synthesis by hydrolyzing the phosphatidic acid formed endogenously by microsomes. However, this soluble fraction did not act on exogenous phosphatidic acid suspension. Conversely, Johnston et al. (1967) found that microsomal phosphatidate phosphohydrolase did not act on endogenous microsomal phosphatidic acid, but did act on added phosphatidic acid.

Hübscher and his coworkers (Sedgwick and Hübscher, 1965; Smith et al., 1967; Brindley et al., 1967; Smith and Hübscher, 1966; Mitchell et al., 1971), Johnston et al. (1967), and Rao et al. (1971b) studied the properties of phosphatidate phosphohydrolase of the mitochondrial fraction of rat liver and of the microsomal and supernatant fractions of cat intestine by using both aqueously dispersed phosphatidate and membrane-bound phosphatidate as substrates. They have concluded that the supernatant fraction is the major source of phosphatidate phosphohydrolase and that mitochondrial and microsomal phosphatidate phosphohydrolase activity is not significant in neutral lipid biosynthesis. The low

activity observed by these authors in mitochondrial and microsomal fractions is probably due to the presence of F^- in the assay medium, since F^- has an inhibitory effect on phosphatidate phosphohydrolase (Smith *et al.*, 1967).

In studying the role of phosphatidate phosphohydrolase in glycerolipid biosynthesis from sn-G3P by rat liver sub-cellular fractions, Lamb and Fallon (1974a) found that with the addition of supernatant fraction or a 40 % $(NH_4)_2SO_4$ precipitate of the supernatant fraction to the microsomal preparation, the formation of neutral lipids was increased. The supernatant phosphatidate phosphohydrolase was more active with microsomal-bound phosphatidate. This result confirmed the previous finding that phosphatidate phosphohydrolase was localized in the soluble fraction (Smith and Hübscher, 1966; Hübscher *et al.*, 1967; Smith *et al.*, 1967; Johnston *et al.*, 1967). However, unlike the previous authors, Lamb and Fallon (1974a) found that microsomal phosphatidate phosphohydrolase activity was higher with an aqueous dispersion of phosphatidate, and that Mg^{2+} , at a concentration below 4 mM, activated microsomal but not supernatant phosphatidate phosphohydrolase.

In another study, Jamdar and Fallon (1973) demonstrated

that there are two types of phosphatidate phosphohydrolase existing in rat adipose tissue: Mg^{2+} -dependent and Mg^{2+} -independent. In the presence of Mg^{2+} , both the utilization of aqueous substrate and membrane-bound substrate was stimulated. Mitochondrial phosphatidate phosphohydrolase was not stimulated by Mg^{2+} and did not utilize membrane-bound substrate. In the absence of Mg^{2+} , the rates of utilization of aqueous substrate by the phosphatidate phosphohydrolase of mitochondrial, microsomal, and soluble fractions were detectable, but the membrane-bound substrate was not utilized under these conditions. From this data, Jamdar and Fallon (1973) concluded that in rat adipose tissue, Mg^{2+} -dependent phosphatidate phosphohydrolase was responsible for the utilization of membrane-bound substrate and was localized in the microsomal and soluble fractions, while the Mg^{2+} -independent enzyme existed chiefly in mitochondria with some activity in microsomes.

In a study to be described, the intracellular localization of phosphatidate phosphohydrolase in rabbit heart was examined. The highest specific activity of this enzyme was found to be in the microsomal and lysosomal fractions. Mitochondrial phosphatidate phosphohydrolase was approximately

1/3 to 1/2 as active as the microsomal enzyme. The activity found in the cytosomal (soluble) fraction was extremely low.

I-2-6. Distribution of acyl-CoA:monoglyceride-O-acyltransferase

Mitochondrial fractions of rabbit and rat intestinal mucosa were used as enzyme sources when Clark and Hübscher (1960 and 1961) first suggested the existence of MG acyltransferase in mammals. In 1962, Senior and Isselbacher established a dual intracellular localization of this enzyme, stating that the mitochondria and microsomes of rat intestinal mucosa contained the enzyme. Microsomal MG acyltransferase was later confirmed to exist in guinea pig mammary glands (McBride and Korn, 1964) and hamster and rat intestinal mucosa (Rao and Johnston, 1966; Ailhaud et al., 1964; Gallo et al., 1968). In addition, Hübscher (1961) reported that MG acyltransferase was present in soluble fractions of rat intestinal mucosa and liver, pig kidney, and rabbit pancreas. However, Senior and Isselbacher (1962) claimed that this enzyme was absent from the soluble fractions of rabbit intestinal mucosa and rat liver.

I-2-7. Distribution of acyl-CoA:sn-1,2-diglyceride-O-acyltransferase

DG acyltransferase was reported to be present in both the mitochondrial (Stein et al., 1957) and microsomal (Young and Lynen, 1969; Mangiapane et al., 1973; O'Doherty et al., 1972) fractions of rat liver. In addition, mitochondrial DG acyltransferase has been shown to exist in chicken liver (Weiss et al., 1960), rabbit intestine (Clark and Hübscher, 1961), goat mammary glands (Pynadath and Kumar, 1964), and rat diaphragm (Neptune et al., 1962). Microsomes of chicken liver (Weiss et al., 1960), rat intestine (Ailhaud et al., 1964), guinea pig and goat mammary glands (McBride and Korn, 1964; Pynadath and Kumar, 1964), as well as soluble fractions of goat mammary glands (Pynadath and Kumar, 1964) also contained DG acyltransferase.

I-2-8. Distribution of acyl-CoA synthetase

The microsomal fraction of guinea pig liver was used as the enzyme source by Kornberg and Pricer (1953a) in their original study on the activation of long-chain FAs. Since then, a predominantly microsomal localization of this enzyme has been established for rat liver (Korchak and Masoro, 1964; Farstad et al., 1967; Pande and Mead, 1968b; Pande, 1972; Marcel and Suzue, 1972) and for the mucosa of the small intestine of the rat (Senior and Isselbacher, 1960; Ailhaud

et al., 1963) and the cat (Hübscher et al., 1963). De Jong and Hülsmann (1970) performed a comparative study of palmitoyl-CoA synthetase activity in subcellular fractions of rat liver, heart, and gut. They found that palmitoyl-CoA synthetase was localized mainly in the sarcosomes of the heart and the microsomes of the intestine. Liver mitochondria and microsomes were about equally active (50:50 to 30:70 %) in catalyzing palmitoyl-CoA synthesis (De Jong and Hülsmann, 1970).

A somewhat different subcellular distribution of medium- and short-chain FA activating enzymes has been reported. Approximately 70-80 % of the total activity in the liver and kidney was found in the cytoplasm with the remainder of the activity being located in the mitochondrial fraction. In heart muscle, the distribution of total activity between these two fractions was approximately equal (Barth et al., 1971). Furthermore, Aas and Bremer (1968) provided evidence to show that acyl-CoA synthetases for acids of chain lengths C_2 , C_4 , and C_7 were exclusively localized in mitochondria, mainly in the matrix. The acyl-CoA synthetases for C_{12} and C_{16} , however, existed in the outer membrane of the mitochondria and in the microsomes.

Barth et al. (1971), Aas and Bremer (1968), Creasey

(1962), and Pande (1972), who all observed FA specificity of acyl-CoA synthetase, claimed that there is more than one synthetase, each having a markedly different substrate specificity. However, other investigators do not support this hypothesis. The kinetic studies by Marcel and Suzue (1972) suggest that there is one long-chain acyl-CoA synthetase which contains several active sites, i.e., a main site which binds the carboxyl group, thus common for all FAs, and other sites which bind different types of FA molecules with specific affinity for unsaturated FAs with a $\Delta 9$ double bond. Lippel and his coworkers studied the activation of naturally occurring cis- and trans-unsaturated FAs by rat liver microsomes and mitochondria (Lippel, 1973; Lippel et al., 1973a and 1973b). Their data agrees with that of Marcel and Suzue (1972) in that a single activating enzyme catalyzes positional isomers of long-chain FAs.

The GTP-dependent long-chain acyl-CoA synthetase is absent from the outer membrane of the rat liver mitochondria and is exclusively associated with the inner membrane-matrix fraction (Lippel and Beattie, 1970). Thus, it is unlikely that the activation of FA through this pathway contributes to the G3P acyltransferase reaction which takes place outside

of the inner membrane. Furthermore, the specific activity of GTP-dependent synthetase is only about 4 % of the ATP-dependent enzyme present in the outer membrane (Lippel and Beattie, 1970).

I-2-9. Distribution of palmitoyl-CoA hydrolase

Palmitoyl-CoA hydrolase was reported to be present in the microsomal fraction of rat liver (Lands and Merkl, 1963; Fallon and Lamb, 1968; Barden and Cleland, 1969) and also in dog lung and liver (Frosolono et al., 1971). However, according to Brandes et al. (1963), palmitoyl-CoA hydrolase is a constituent of both microsomal and mitochondrial fractions of rat and guinea pig liver.

Recent work from this laboratory demonstrates the presence of palmitoyl-CoA hydrolase in all four subcellular preparations of rabbit heart (Liu and Kako, 1975). The highest specific activity was found in lysosomal and microsomal fractions. Mitochondrial and soluble fractions were less active.

LEGEND TO TABLE 1Intracellular localization of enzymes related to tri-
glyceride biosynthesis in mammalian tissues

Unless otherwise indicated, + signifies the use of that particular subcellular fraction by the author(s). A ++ indicates that the enzyme is reported to be localized exclusively in the fraction specified. A (+) indicates a partially purified enzyme, and - signifies the absence of an enzyme. The abbreviations MT, LY, MC, and SL stand for mitochondrial, lysosomal, microsomal, and soluble fractions, respectively.

TABLE 1

Intracellular localization of enzymes related to triglyceride biosynthesis in mammalian tissues

Enzyme and reaction catalyzed	Species and tissue	Localization				References
		MT	LY	MC	SL	
G3P and monoacyl- <u>sn</u> -G3P acyl-transferases (Eqs. 1 & 2)	Rat liver			+		Lands and Hart (1964)
	"			+		Fallon and Lamb (1968)
	"			+		Abou-Issa and Cleland (1969)
	"			+		Possmayer <u>et al.</u> (1969)
	"			+		Barden and Cleland (1969)
	"			++		Eibl <u>et al.</u> (1969)
	"			+		Lamb and Fallon (1970)
	"			++		Davidson and Stanacev (1972)
	"			+		Okuyama <u>et al.</u> (1971)
	"			+		Yamashita and Numa (1972)
	"			+		Mangiapane <u>et al.</u> (1973)
	"			(+)		Yamashita <u>et al.</u> (1973)
	"	+		+		Stein <u>et al.</u> (1957)
	"	+		+		Smith and Hübscher (1966)
	"	+		+		Stoffel and Schiefer (1968)

Table 1 (page 2)

Enzyme and reaction catalyzed	Species and tissue	Localization				References
		MT	LY	MC	SL	
	rat liver	+		+		Shephard and Hübscher (1989)
	"	+		+		Zborowski and Wojtczak (1969)
	"	+		+		Sarzala <u>et al.</u> (1970)
	"	+		+		Daae and Bremer (1970)
	"	+		+		Daae (1972)
	"	+		+		Monroy <u>et al.</u> (1972)
	"	(+)				Monroy <u>et al.</u> (1973)
	guinea pig liver			+		Kornberg and Pricer (1953b)
	"			+		Brandes <u>et al.</u> (1963)
	"			++		Davidson and Stanacev (1972)
	chicken liver	+		+		Weiss <u>et al.</u> (1960)
	guinea pig intestinal mucosa			+		Brindley (1973)
	rat brain			+		Sánchez de Jiménez and Cleland (1969)
	"			+		Possmayer and Mudd (1971)
	"	+				Mårtensson and Kanfer (1968)
	rabbit heart	+		+		Liu and Kako (1974)

Table 1 (page 3)

Enzyme and reaction catalyzed	Species and tissue	Localization				References
		MT	LY	MC	SL	
Acyl-CoA: DHAP acyl-transferase (Eqs. 4 & 5)	guinea pig liver	+				Hajra and Argranoff (1967)
	"	+		+		Hajra and Argranoff (1968a)
	"	+		+		Hajra (1968a and 1968b)
	"	-		-		Davidson and Stanacev (1972)
	rat liver	-		+		Puleo <u>et al.</u> (1970)
	"	-		+		Rao <u>et al.</u> (1971a and 1971b)
	"	+		+		La Belle and Hajra (1972)
	"	-				Monroy <u>et al.</u> (1972)
	rat brain, kidney, & adipose tissue	+		+		La Belle and Hajra (1972)
	rabbit heart	-				Liu and Kako (1974)
DG kinase (Eq. 6)	guinea pig brain			+		Hokin and Hokin (1959)
	"			+		Pieringer and Hokin (1962a)
	albatross salt gland	+		+	+	Hokin and Hokin (1960b)
	rat brain	+				Strickland (1962)

Table 1 (page 4)

Enzyme and reaction catalyzed	Species and tissue	Localization				References
		MT	LY	MC	SL	
	rat brain			+		De Pury and Collins (1963)
MG kinase & monoacyl-sn-G3P acyltransferase (Eqs. 7 & 8)	guinea pig brain, liver and calf brain			+		Hokin and Hokin (1960a)
	rat intestinal mucosa	+		+		Pieringer and Hokin (1962a and 1962b) Paris and Clément (1969)
Phosphatidate phosphohydrolase (Eq. 9)	albatross salt gland	+		+		Hokin and Hokin (1960a)
	pig kidney	+		+		Coleman and Hübscher (1962)
	rat liver			+		Wilgram and Kennedy (1963)
	"	+	+	+	+	Sedgwick and Hübscher (1965)
	"	(+)				Sedgwick and Hübscher (1967)
	"	+		+		Stoffel and Schiefer (1968)
	cat intestinal mucosa				++	Brindley and Hübscher (1965)

Table 1 (page 5)

Enzyme and reaction catalyzed	Species and tissue	Localization				References
		MT	LY	MC	SL	
	rat adipose tissue	+		+	+	Jamdar and Fallon (1973)
	rat liver			+	+	Lamb and Fallon (1974a)
	rabbit heart		+	+		Liu and Kako (1975)
MG acyl-transferase (Eq. 10)	rat intestinal mucosa	+				Clark and Hübscher (1960)
	"	+			+	Hübscher (1961)
	"	+		+		Senior and Isselbacher (1962)
	"			+		Ailhaud <u>et al.</u> (1964)
	"			+		Gallo <u>et al.</u> (1968)
	rabbit intestinal mucosa	+				Clark and Hübscher (1960)
	"	+				Clark and Hübscher (1961)
	"	+		+	-	Senior and Isselbacher (1962)
	hamster intestinal mucosa			+		Rao and Johnston (1966)
	rat liver	+			+	Hübscher (1961)
	"			-		Senior and Isselbacher (1962)

Table 1 (page 6)

Enzyme and reaction catalyzed	Species and tissue	Localization				References
		MT	LY	MC	SL	
	pig kidney				+	Hübscher (1961)
	guinea pig mammary gland			+		McBride and Korn (1964)
	rabbit pancreas				+	Hübscher (1961)
DG acyl-transferase (Eq. 11)	rat liver	+				Stein <u>et al.</u> (1957)
	"			+		Young and Lynen (1969)
	"			+		Mangiapanne <u>et al.</u> (1973)
	"			+		O'Doherty <u>et al.</u> (1972)
	chicken liver	+		+		Weiss <u>et al.</u> (1960)
	rabbit small intestine	+				Clark and Hübscher (1961)
	rat intestinal mucosa			+		Ailhaud <u>et al.</u> (1964)
	guinea pig mammary glands			+		McBride and Korn (1964)
	goat mammary glands	+		+	+	Pynadath and Kumar (1964)

Table 1 (page 7)

Enzyme and reaction catalyzed	Species and tissue	Localization				References
		MT	LM	MC	SL	
	rat diaphragm	+				Neptune <u>et al.</u> (1962)
Acyl-CoA Synthetase (Eq. 12) (long-chain)	guinea pig liver			+		Kornberg and Pricer (1953a)
	rat liver			+		Korchak and Masoro (1964)
	"			+		Farstad <u>et al.</u> (1967)
	"			+		Pande and Mead (1968b)
	"			+		Pande (1972)
	"	+		+		De Jong and Hülsmann (1970)
	"			+		Marcel and Suzue (1972)
	"	+		+		Lippel (1973) and Lippel <u>et al.</u> (1973a and 1973b)
	rat intestinal mucosa			+		Senior and Isselbacher (1960)
	"			+		Ailhaud <u>et al.</u> (1963)
	"			+		De Jong and Hülsmann (1970)
	cat intestinal mucosa			+		Hübscher <u>et al.</u> (1963)
	rat heart			+		De Jong and Hülsmann (1970)

Table 1 (page 8)

Enzyme and reaction catalyzed	Species and tissue	Localization				References
		MT	LY	MC	SL	
Acyl-CoA synthetase (Eq. 12) (medium- & short-chain) (C ₂ , C ₄ & C ₇) (C ₁₂ & C ₁₆)	rat liver	+			+	Barth <u>et al.</u> (1971)
	"	++				Aas and Bremer (1968)
	"	+			+	"
	rat kidney	+			+	Barth <u>et al.</u> (1971)
	rat heart	+			+	"
Palmitoyl-CoA hydro-lase (Eq. 13)	rat liver			+		Lands and Merkl (1963)
	"			+		Fallon and Lamb (1968)
	"			+		Barden and Cleland (1969)
	dog liver, lung			+		Frosolono <u>et al.</u> (1971)
	rat & guinea pig liver	+		+		Brandes <u>et al.</u> (1963)
	rabbit heart	+	+	+	+	Liu and Kako (1975)

I-3. Fatty Acid and Positional Specificity of Phospho- and Glycerolipid Biosynthesis

It has been shown that the FA composition of complex lipids differs widely among various organs and species (Van Deenen, 1965; Hill and Lands, 1970; Hübscher, 1970). Before the advancement of analytical techniques in lipid chemistry, however, analyses were not sufficiently precise to draw an exact map of the FA composition of lipids of various sources. Introduction of the gas-liquid chromatographic technique has dramatically changed the amount of knowledge regarding this problem. Since then, numerous analyses on various naturally occurring lipids have been carried out, and the results confirm earlier findings: there exists a non-uniform distribution of FAs with respect to chain length and degree of unsaturation in complex lipids.

This information focused attention to the diverse characteristics of individual FAs. For example, it is a well known fact that they have different melting points. Therefore, from a teleological view point, it may be postulated that living organisms regulate their FA composition when required to live in a cold or a hot environment. If the characteristics of individual FAs with different chain

lengths and degree of unsaturation are dissimilar, it is possible that the complex lipids derived from them may have different characteristics depending on their different molecular configurations. The chemical and physical properties of the hydrophobic fatty acyl side chains of complex lipids not only constitute the major permeability barrier of the cell membrane but also enable these complex lipids to serve as cofactors or coenzymes in many enzymatic reactions occurring within the cell membrane (Lennarz, 1970). From this, the importance of understanding the molecular configuration of complex lipids becomes apparent.

Analytical methods were devised in which pancreatic lipase and, later, more specific snake venom phospholipase A₁ were used to hydrolyze lipids stereospecifically. The chromatographic separation of the hydrolytic products was then carried out (Brockhoff et al., 1963; Brockhoff and Hoyle, 1963; Lands and Hart, 1964; Van Deenen, 1965; Kuksis, 1972). By these techniques, investigators were able to analyze not only the FA composition of complex lipids, but also their positional distribution (Brockhoff et al., 1963; Brockhoff and Hoyle, 1963; Lands and Hart, 1964 and 1965; Stoffel et al., 1967; Possmayer et al., 1969). In the earlier days of investigation, there

were two opposing schools of thought, these being a non-random distribution hypothesis (Hanahan et al., 1960; Tattrie, 1959; De Haas and Van Deenen, 1961; Husbands and Reiser, 1966) and a random distribution hypothesis (Lands and Hart, 1964; Stoffel et al., 1967).

There have been numerous studies to explain the non-random distribution of FAs in the glycerolipid molecule. One such explanation states that specific acylation could take place at various stages of Kennedy's de novo pathway of TG synthesis (Kennedy, 1961; see also Section I-1 of this thesis) because of the substrate and positional specificity of the enzymes involved. Another possibility is that the biosynthesis of phospholipids or neutral lipids may take place in a random manner with respect to FAs, but with rearrangement of fatty acyl moieties, such as

deacylation-reacylation process, acyl migration of complex lipids, or desaturation of FA molecules taking place after complex lipid formation (Van Deenen and De Haas, 1966; Goldfine, 1968).

Although wide varieties of phospholipids in many different organs and species have not yet been analyzed exhaustively, the general consensus with the liver

glycerolipids in several species is that saturated FAs, particularly 16:0 and 18:0, are preferentially esterified at position 1 of the glycerol moiety, while unsaturated FAs are esterified at position 2 (Van Deenen, 1965; Lands, 1965). It would seem logical that the first and second acylation steps of sn-G3P are specific to particular FAs since one of the products of these reactions, diacyl-sn-G3P, a key intermediate for complex lipid formation, provides the backbone structure which contains the FA asymmetry. Consequently, numerous studies have been published dealing with the specificity of the first and second acylation steps of sn-G3P, as described below.

p-3-1. Fatty acid and positional specificity of acyl-CoA:
sn-glycerol-3-phosphate-0-acyltransferase

The present knowledge concerning the substrate and positional specificity of acyltransferases leading to the formation of diacyl-sn-G3P in certain animals is summarized in Table 2. ©

Lands and Hart (1964) have investigated the acylation of sn-G3P in microsomal preparations of liver and concluded that the specificity of the acyltransferase reactions leading to diacyl-sn-G3P formation could not adequately

account for the asymmetric distribution of saturated and unsaturated FAs known to occur naturally in glycerolipids in this tissue. However, subsequent investigations with 1-acyl-sn-G3P acyltransferase from rat liver showed that unsaturated FAs were preferentially attached at position 2 of the synthesized diacyl-sn-G3P (Lands and Hart, 1965), indicating that some specificity exists in the second step of the sn-G3P acylation reactions. In earlier experiments, Lands and his coworker (Lands and Merkl, 1963; Merkl and Lands, 1963) found that in rat liver, acyltransferases involved in the acylation of monoacylphosphoglycerides exhibited a preferential esterification of saturated FAs at position 1 and unsaturated FAs at position 2 in both lecithin and phosphatidylethanolamine synthesis. Accordingly, they concluded that with a position-specific redistribution of FA constituents in tissue, phosphoglycerides may arise in part from a redistribution of fatty acyl moieties between positions 1 and 2 (deacylation-reacylation process) after the nitrogenous base has been attached to the molecules.

The question concerning the origin of the asymmetric distribution of FAs in naturally occurring lipids became an important issue in 1968. Hill et al. (1968) studied the

incorporation of FAs into diacyl-sn-G3P in rat liver slices and found that the acyltransferase reactions proceed in a nonrandom manner. Saturated FAs are preferentially incorporated into position 1 and unsaturated FAs into position 2 of the glycerol moiety. Van Golde et al. (1969) made a similar observation from experiments with liver slices and arrived at the same conclusion. Contrary to their findings with liver slices, Hill et al. (1968) found that in an in vitro study with a solubilized preparation from liver microsomes, the specificity with respect to fatty acyl-CoA esters did not exist during the acylation of sn-G3P. This finding was in accord with an earlier study of Lands and Hart (1964). Why the positional specificity of G3P acyltransferase is manifested in tissue slices but not in subcellular fractions presents a problem that is as yet unsolved.

Possmayer et al. (1969) studied the distribution of a number of radioactive FAs incorporated into diacyl-sn-G3P, phosphatidylcholine and phosphatidylethanolamine in rat liver microsomes. By analyzing the diene species produced from a mixture of radioactive palmitate and linoleate, they found that 96 % and 31 %, respectively, of the esterified radioactive FAs were at the 1 position, while 4 % of palmitate

and 69 % of linoleate were esterified at the 2 position. Accordingly, Possmayer et al. (1969) concluded that the majority of the saturated FAs were at position 1 while the polyunsaturated FAs were confined mostly to position 2. This finding rendered strong support for the idea that the FA asymmetry of phospholipids is introduced at the first and second phases of the overall de novo biosynthetic pathway, that is, the formation of diacyl-sn-G3P.

Hill and Lands (1970) recalculated these data using the total nmoles of each FA incorporated and the percentage of its distribution and reached a different conclusion. The total amount of palmitate and linoleate incorporated into phosphatidic acid was 0.15 and 0.42 nmoles per 15 min, respectively (Table 5 of Possmayer et al., 1969), while 96 % of the esterified palmitate and 31 % of the linoleate were found at position 1 (Table 6 of Possmayer et al., 1969). This would indicate that 0.14 nmoles of palmitate and 0.13 nmoles of linoleate were incorporated at the 1 position of the diene species during 15 min incubation. Thus, one may calculate that almost 50 % of the radioactive acids at the 1 position were unsaturated. Because of these calculations, Hill and Lands (1970) interpreted such data as indicating

that there was a lack of positional specificity. If similar calculations are made for the monoene produced from stearate and oleate or the diene from stearate and linoleate, one finds that a greater amount of radioactive unsaturated FA than saturated FA was esterified at both the 1 and 2 positions.

On the basis of the detailed kinetic studies of various fatty acyl-CoA esters in the acylation of sn-G3P to form diacyl-sn-G3P by microsomes of rat brain and liver, Cleland and his co-workers (Abou-Issa and Cleland, 1969; Sánchez de Jiménez and Cleland, 1969; Zahler and Cleland, 1969) found no selectivity of acyltransferase reactions using various acyl-CoA esters. However, in their study, the location of the acyl moiety in esterified products was not analyzed. The micellar nature of fatty acyl-CoA esters, the considerable amounts of endogenous FAs in enzyme sources (Abou-Issa and Cleland, 1969; Sánchez de Jiménez and Cleland, 1969; Zahler and Cleland, 1969), and the selectivity of acyl-CoA hydrolase (Barden and Cleland, 1969) could be the factors causing the diverse results reported by these investigators.

In 1970, monoacyl-sn-G3P was isolated (Lamb and Fallon, 1970) as an intermediate of sn-G3P acylation in rat liver microsomes, and it was claimed to be the immediate precursor

for diacyl-sn-G3P biosynthesis as described above. Consequently, the positional analysis and nature of acyl group in the monoacyl-sn-G3P molecules offered a more direct assessment of the specificity of the G3P acyltransferase reaction. Therefore, the kind of FA and its position in the monoacyl-sn-G3P molecule have been examined in order to clarify factors controlling the asymmetric FA distribution in naturally occurring lipids.

Lamb and Fallon (1970) isolated monoacyl-sn-G3P as the major product at pH 6.5 in their study of the acylation of sn-G3P by rat liver microsomes in the presence of acyl-CoA thioesters. Stereospecific analysis of monoacyl-sn-G3P molecules indicates that the 1-isomer is the primary product when palmitoyl-CoA is the substrate, and that the 2-isomer is the predominant product formed from oleoyl-CoA. More recently, Daae (1972) studied the mitochondrial acylation of sn-G3P in rat liver using fatty acid carnitine esters indirectly as the acyl donor and found that palmitoylcarnitine is the only substrate that serves as an effective acyl donor. It is esterified exclusively at position 1 of monoacyl-sn-G3P. Monroy et al. (1972) reported pronounced substrate and positional specificity during the acylation of sn-G3P by

rat liver mitochondria to form monoacyl-sn-G3P. It was found that palmitoyl-CoA was esterified exclusively in position 1 of the glycerol molecule, and that oleoyl-CoA and linoleyl-CoA failed to show any significant incorporation into monoacyl-sn-G3P. The results obtained by using partially purified mitochondrial G3P acyltransferase (Monroy *et al.*, 1973) confirmed their earlier findings in which sonicated or non-disrupted mitochondria were used (Monroy *et al.*, 1972).

Microsomal G3P acyltransferase has also been purified, and its properties studied by Yamashita and Numa (1972). These authors found that the purified enzyme catalyzes the formation of monoacyl-sn-G3P from sn-G3P and palmitoyl-CoA. The acyl moiety is esterified preferentially at position 1 of the glycerol molecule. Palmitoyl-CoA is utilized most efficiently, while unsaturated fatty acyl-CoA thioesters are poor substrates.

From these observations, one can conclude that both mitochondrial and microsomal enzymes in the rat liver possess similar FA and positional specificity. Stated another way, formation of monoacyl-sn-G3P, the first step of acylation of sn-G3P by fatty acyl-CoA in liver mitochondria and

microsomes, takes place at position 1 of the glycerol molecule and shows a strong affinity towards palmitate. A similar study with other organ preparations has not been published.

Baker and Thompson (1972) approached the problem from a different angle. They injected radioactive FAs into rats and analyzed the FA distribution in brain phosphatides at various time intervals. Their results were slightly different from those obtained in experiments using liver particulates, and the FA and positional specificity was less pronounced. Some palmitate was found in position 2 and some unsaturated FAs at position 1 of the diacyl-sn-G3P molecule. The composition of complex lipids was different from that of diacyl-sn-G3P, indicating that the acyl exchange reactions contribute to formation of the final molecular configuration of complex lipids.

I-3-2. Fatty acid and positional specificity of acyl-CoA: monoacyl-sn-glycerol-3-phosphate-0-acyltransferase

Barden and Cleland (1969) conducted a thorough investigation of 1-acyl-sn-G3P acyltransferase from rat liver, examining its substrate specificity as a function of the FA composition of both acyl donors (acyl-CoAs) and acyl acceptors (1-acyl-sn-G3Ps). They found that the nature of

fatty acyl-CoA esters influences the reaction rate to a great extent. The rate of reactions in a decreasing order follows this sequence: oleyl > palmitoleyl $\approx$ linoleyl $\approx$ palmityl > myristyl > stearyl > lauryl. The acyl moiety of the 1-acyl-sn-G3P was not decisive in this regard. These results indicate that unsaturated FAs are preferred substrates for the acylation at position 2 of 1-acyl-sn-G3P. These findings agree with the preliminary work of Hill and Lands (1968) discussed earlier in this thesis.

In 1971, Okuyama et al. prepared 2-acyl-sn-G3P and then examined its esterification to diacyl-sn-G3P. In the presence of microsomal preparations from rat liver, the rate of acylation of 2-acyl-sn-G3P by acyl-CoA was found to be one tenth of the rate observed when 1-acyl-sn-G3P served as the acyl acceptor. The acylation became even more selective when both 1-acyl-sn-G3P and 2-acyl-sn-G3P were present in the incubation mixture. Oleate was esterified more rapidly than palmitate or stearate under the experimental conditions. This work provided evidence that, as well as the nature of the acyl donor (Barden and Cleland, 1969), the molecular configuration of the acceptor, monoacyl-sn-G3P, plays an important role in diacyl-sn-G3P biosynthesis.

Because the rate of acylation of 1-acyl-sn-G3P was higher than that of 2-acyl-sn-G3P (Okuyama *et al.*, 1971), it is more likely that 1-acyl-sn-G3P serves as the intermediate for diacyl-sn-G3P synthesis. Because of the work of Barden and Cleland (1969) quoted above, it seems that a pronounced FA specificity must exist in the acylation of 1-acyl-sn-G3P. Okuyama and Lands (1972), however, found that the FA preference *in vitro* of microsomal 1-acyl-sn-G3P acyltransferase in rat liver could be influenced by the incubation conditions. Briefly stated, when the acylation takes place at very low concentrations of 1-acyl-sn-G3P, palmitate and arachidonate tend to be excluded from the 2 position of diacyl-sn-G3P, whereas relatively non-selective acylation occurs at high concentrations of 1-acyl-sn-G3P. Oleoyl-CoA and linoleyl-CoA are relatively readily acylated to position 2 at any concentration of 1-acyl-sn-G3P. From a kinetic analysis of enzyme reactions with various acyl-CoAs, these authors suggest that there are multiple acyltransferases and, possibly, multiple reactive sites of an enzyme in rat liver.

Recently, Yamashita *et al.* (1973) succeeded in partially purifying 1-acyl-sn-G3P and 2-acyl-sn-G3P acyltransferases

from rat liver microsomes and obtained results supporting the view that the asymmetric FA distribution found in naturally occurring glycerolipids results, to a large extent, from preferential acylation in the steps leading to diacyl-sn-G3P synthesis. It is thought that the final molecular configuration of phospholipid and, possibly, neutral lipid could be further modified by the deacylation-reacylation reaction after their de novo synthesis.

I-3-3. Fatty acid and positional specificity of acyltransferase involved in the dihydroxyacetone phosphate pathway

Original studies on the DHAP pathway by Hajra and Agranoff (1968a and 1968b) were done using ^{32}P -labelled ATP and guinea pig liver mitochondria. Hajra (1968a) then extended these studies in mitochondria of liver, brain, kidney, and heart of guinea pig by using ^{32}P -DHAP and FAs of various chain lengths and degrees of unsaturation. The formation of acyl-DHAP was highest with palmitate as the acyl donor, and the specificity became more noticeable at high FA concentrations. In other words, the activity of the DHAP pathway was dependent upon the nature and concentration of acyl donors. For example, 1 mM of palmitate, which was the

optimal concentration under these experimental conditions, produced 120 nmoles of acyl-DHAP, whereas the optimal concentration of oleate or linoleate was only 0.1 mM and produced 7 nmoles of acyl-DHAP. In contrast, the FA specificity of microsomal acylation was not as evident. Hajra (1968b) reported that phosphatidate formed via the DHAP pathway in mitochondria of guinea pig liver contained more palmitate in position 1 of the glycerol moiety than that formed via the Kornberg-Kennedy pathway. This finding suggests that the DHAP pathway participates in the asymmetric FA distribution observed in complex lipids. However, many other investigators (Okuyama and Lands, 1970; Davidson and Stanacev, 1972; Monroy et al., 1972; Liu and Kako, 1974) have not confirmed the existence of the DHAP pathway in guinea pig and rat liver or rabbit heart. Therefore, studies on subcellular distribution and substrate specificity of acyl-CoA:DHAP acyltransferase are to be investigated further.

I-3-4. Selectivity of acyltransferases leading to the formation of other phosphoglycerides

Lands et al. (1966) have studied the acylation of 1-acyl-lysolecithin and 2-acyl-lysolecithin by rat liver microsomes. By comparison of the distribution of a series

of cis- and trans-isomers of unsaturated FAs in vivo with their relative esterification rates in vitro, they found that the location and configuration of a double bond in an FA could control both its distribution in vivo between the 1 and 2 positions of lecithins and TGs and the rate of its acyltransferase-catalyzed esterification to form lecithin. Similar observations were reported by Privett et al. (1966). In 1967, Stoffel et al. provided evidence that the structure of an FA constituent in an acceptor molecule such as monoacyl-sn-G3P could influence the acyl transfer reaction in the presence of rat liver microsomes. The rate of acyl transfer to unsaturated 1-acyl-lysolecithin was higher than that observed with saturated 1-acyl-lysolecithin. In addition, a greater rate of acylation for polyunsaturated FAs was seen when saturated lysolecithin was used as an acceptor instead of unsaturated lysolecithin. The studies described above re-emphasize the importance of the enzymatic acylation of lysolecithin in directing the asymmetric distribution of saturated and unsaturated FAs in tissue lecithins.

A more physiological approach in studying the positional and metabolic asymmetry of the lecithin molecule was conducted

by Stein and Stein (1963) using isolated perfused rat heart. These investigators perfused isolated rat heart with a medium containing both [^3H]palmitate and [^{14}C]linoleate and found that the linoleate was the preferential substrate for incorporation into lecithin. In the lecithin molecule, labelled linoleate was found almost exclusively in the 2 position, while labelled palmitate was distributed evenly between the 1 and 2 positions. More recently, Åkesson and co-workers (Åkesson et al., 1970a and 1970b; Åkesson, 1970) injected either [^3H]glycerol, [$9,10\text{-}^3\text{H}$]palmitate, or [$1\text{-}^{14}\text{C}$]linoleate into rats and studied the incorporation of isotope into different liver glycerolipids. They found a non-random combination of FAs in glycerolipid synthesis. In phosphatidic acid formation, linoleic acid was the most active substrate, and it was esterified almost exclusively at position 2 of the phosphatidate molecule. In the DG molecule, 90 % of labelled palmitate was found at position 1.

Brockerhoff et al. (1963) and Brockerhoff and Hoyle (1963) analyzed the FA distribution in TG and lecithin of marine animals and found that polyunsaturated FAs of the linoleate-type were preferentially located at the 2 position. They postulated that the linkage of the unsaturated FAs to the 2 position

of glycerol may have been established originally in plants and plankton, being retained through the food chains and through most steps of the lipid metabolism of the individual organism until they were eventually oxidized. Support was lent to this hypothesis by the observations that the 2-MG structure was retained during intestinal absorption of TGs in rats (Savary et al., 1961); and that the MG acyltransferase pathway was an alternative route for DG, TG, and lecithin biosynthesis (Section I-1-2). The hypothesis of the metabolic stability of the 2-MG can not, however, completely explain the phenomenon of specific FA distribution. In addition, the question of the origin of a specific stable linkage of an unsaturated FA to position 2 of MG is open to further investigation.

A selective incorporation of FAs into phosphoglycerides of red cells has been recorded by Mulder et al., (1963) and Robertson and Lands (1964). The observed selectivity concerning the nature and location of the FA constituents was believed to be governed by the metabolic pathway involving the diacyl-monoacyl phosphoglyceride cycle (Robertson and Lands, 1964; Van Deenen, 1965).

The nature of the dietary FAs can also have an effect

on FA patterns in human erythrocyte PLs (Farquhar and Ahrens, 1963). On ad libitum diets, the linoleate content of erythrocytes was about 10 % of the total PL FA. It fell to 5 % or less on diets low in linoleate and rose to as much as 27 % on diets rich in linoleate. De Gier and Van Deenen (1964) observed that feeding rats on a diet of saturated fats free of essential FAs resulted in an increase of oleate and a decrease of palmitate and arachidonate in the FA pattern in PLs from erythrocyte membrane. These findings demonstrated the role of dietary habits in regulating the FA composition of PLs in red blood cells.

TABLE 2

Summary of the substrate and positional specificity of acyltransferases leading to the formation of diacyl-sn-glycerol-3-phosphate in certain animal tissues

Species, tissue, & subcellular fraction	Acyl Donor	Acyl Acceptor	Substrate Specificity	Positional specificity	References
Rat liver microsomes	linoleate & stearate	G3P	no preference	random (DAGP) position 1: both saturated and unsaturated FAs position 2: both saturated and unsaturated FAs	Lands and Hart (1964)
Guinea Pig liver microsomes	FA	MAGP	linoleic > oleic > palmitic > stearic		Lands and Hart (1964)
Pigeon liver microsomes	acyl CoA	G3P	no preference	random (DAGP) position 1: both saturated and unsaturated position 2: both saturated and unsaturated	Hill et al. (1968)

Table 2 (page 2)

Species, tissue, & subcellular fraction	Acyl donor	Acyl acceptor	Substrate specificity	Positional specificity	References
Rat liver slices	FA	glycerol		non-random (DAGP) position 1: saturated FAs position 2: unsaturated FAs	Hill et al. (1968) Van Golde et al. (1969)
Rat liver microsomes	FA	G3P	16:0 > 18:1 > 18:2 > 18:0	non-random (DAGP) position 1: saturated FAs position 2: unsaturated FAs	Possmayer et al. (1969)
Rat brain microsomes	acyl CoA	G3P	no preference	(not analyzed)	Sánchez de Jiménez and Cleland (1969)
Rat liver microsomes	acyl CoA	G3P	no preference		Zahler and Cleland (1969) Abou-Issa and Cleland (1969)

Table 2 (page 3)

Species, tissue, & subcellular fraction	Acyl donor	Acyl acceptor	Substrate specificity	Positional specificity	References
Rat liver microsomes	acyl CoA	G3P	palmitoyl CoA > linolenoyl CoA > palmitoleoyl CoA ≈ oleoyl CoA > stearoyl CoA	non-random (MAGP) position 1: saturated FAs position 2: unsaturated FAs	Lamb and Fallon (1970)
Rat liver mitochondria	acyl carnitine, CoA, & CPT	G3P	palmityl carnitine is the only active substrate	non-random 1-MAGP is the only product from palmityl carnitine	Daae (1972)
Rat liver microsomes	"	"	linoleyl-carnitine > linoleyl-carnitine > palmityl-carnitine ≈ oleylcarnitine >		Daae (1972)

Table 2 (page 4)

Species, tissue, & subcellular fraction	Acyl donor	Acyl acceptor	Substrate specificity	Positional specificity	References
			arachidonyl- carnitine > stearyl- carnitine		
Rat liver mitochondria	acyl CoA	G3P	palmitoyl CoA is the only active substrate	non-random 1-MAGP (only product from pal. CoA)	Monroy et al. (1972)
Rat liver mitochondria (partially purified)	acyl CoA	G3P	palmitoyl CoA > stearyl CoA ≈ myristoyl CoA > decanoyl CoA	1-MAGP (only product from pal. CoA)	Monroy et al. (1973)
Rat liver microsomes (partially purified)	acyl CoA	G3P	palmitoyl CoA (the only active substrate)	non-random 1-MAGP (the only esterified product)	Yamashita and Numa (1972)

Table 2 (page 5)

Species, tissue, & subcellular fraction	Acyl donor	Acyl acceptor	Substrate specificity	Positional specificity	References
Rat liver microsomes	acyl CoA	1-MAGP	oleyl > palmitoleyl ≈ linoleyl ≈ palmityl > myristyl > stearyl > lauryl		Barden and Cleland (1969)
Rat liver microsomes	acyl CoA	2-MAGP	(1) oleyl > palmityl > stearyl (2) <u>acceptor preference</u> 2-MAGP > 1-MAGP		Okuyama et al. (1971)
Rat liver microsomes	acyl CoA	1-MAGP (low conc.)	highly selective (unsaturated > saturated)		Okuyama and Lands (1972)

Table 2 (page 6)

Species, tissue, & subcellular fraction	Acyl donor	Acyl acceptor	Substrate specificity	Positional specificity	References
		1-MAGP (high conc.)	less selective (no incorporation for palmitate and arachidonate)		
Rat liver microsomes (partially purified)	acyl CoA	1-MAGP	oleyl CoA > linoleyl CoA ≈ palmitoleyl CoA > palmityl CoA ≈ myristyl CoA		Yamashita et al. (1973)
"	acyl CoA	2-MAGP	stearyl CoA > palmityl CoA		

Abbreviations: MAGP: monoacyl-sn-glycerol-3-phosphate
 DAGP: diacyl-sn-glycerol-3-phosphate

II. AIM OF RESEARCH

Fatty infiltration of the heart is not only of academic interest, but is also of considerable clinical importance because of its common occurrence in experimental animals and human subjects. An attempt is made in this study to elucidate the mechanisms controlling myocardial lipid deposition. Among the several possible causative mechanisms of fat deposition described in the "INTRODUCTION" (Section I), special attention is given to one, that is, possible changes in the activity of FA esterifying enzymes.

In order to search for the factors affecting FA esterification, as well as to locate the rate-limiting step(s) in the biosynthesis of neutral lipids, it is essential to delineate the characteristics of the enzymes involved in TG biosynthesis in the heart. Since no detailed information has been made available in this regard in the literature, the properties of the following enzymes were studied.

(1) Acyl-CoA:sn-glycerol-3-phosphate-0-acyltransferase

Studies on the characteristics of this enzyme in

rabbit heart form the major part of this thesis not only because it is the key enzyme involved in the biosynthesis of TG, but also because there has been a controversy regarding its intracellular localization in the liver, which has been widely studied. Since there was no data concerning its subcellular distribution in the heart at the time of this study, an effort was made to clarify whether or not this enzyme exists in cardiac mitochondrial fraction. Furthermore, the substrate and positional specificity of this enzyme were analyzed in an attempt to explain its role in regulating FA asymmetry in phospho- and glycerolipids. Finally, the activity of this enzyme in the heart was compared under normal and hyperthyroid states in order to locate the possible rate-governing step in TG biosynthesis under abnormal conditions.

(2) Palmitoyl-CoA hydrolase

Since acyl-CoA thioester is the only form of acyl donor for sn-G3P, monoacyl-sn-G3P, and DG acyltransferase reactions, the enzymatic deacylation of this compound, which is catalyzed by acyl-CoA hydrolase, could affect the biosynthesis of monoacyl-sn-G3P, diacyl-sn-G3P, and TG. Therefore, studies of the characteristics of

palmitoyl-CoA hydrolase are pertinent. The first attempt was made to establish satisfactory conditions for activity measurement in respect to stabilization of substrate in the incubation medium, duration of incubation time, amounts of enzyme source, and pH optima. Finally, with the appropriate assay procedure, the properties and subcellular distribution of this enzyme in the myocardium were investigated.

(3) Phosphatidate phosphohydrolase

It is generally believed that phosphatidate phosphohydrolase plays an important role in the regulation of hepatic neutral lipid synthesis because the in vitro activity of this enzyme is lower than that of acyl-CoA synthetase, G3P acyltransferase, monoacyl-sn-G3P acyltransferase, and DG acyltransferase. Furthermore, the activity of this enzyme in the liver increased after subtotal hepatectomy and high carbohydrate diet (Section I-1-2). Therefore, studies on the characteristics of phosphatidate phosphohydrolase in rabbit heart were initiated in order to clarify the role of this enzyme in the regulation of myocardial TG biosynthesis. The appropriate conditions for enzyme activity measurement were

established in relation to the amounts of enzyme protein, the duration of incubation time, various amounts of substrate, and different pH values. Then, the effect of magnesium ions on this enzyme activity was studied.

Finally, emphasis was placed on studies of the role of soluble fraction to convert membrane-bound phosphatidate into glycerides and of the subcellular distribution of this enzyme in the heart.

(4) Acyl-CoA:sn-1,2-diglyceride-0-acyltransferase

An attempt was made to develop a working assay method for measuring the activity of this enzyme in the heart.

III. MATERIALS AND METHODS

III-1. Materials and Methods for Studies on Acyl-CoA:

sn-Glycerol-3-Phosphate-0-Acyltransferase

III-1-1. Materials

Disodium ATP, CoA, palmitoyl-CoA, palmitic acid, oleic acid, linoleic acid, 1-palmitoyl-sn-glycerol, tripalmitoyl-glycerol, and T3 (sodium salt) were purchased from Sigma Chemical Co. (St. Louis). Potassium salts of FAs were prepared by titrating FAs with 1N KOH. L-carnitine chloride and sodium phosphatidate were obtained from Nutritional Biochemical Corp. (Cleveland) and Pierce Chemical Co. (Rockford, Illinois), respectively. The FA composition of the latter substance was 36 % palmitic acid, 37 % oleic acid, 15 % stearic acid, and 12 % linolenic acid. CTP, NADH, and NADPH were products of C.F. Boehringer Corp. (New York). Cyclohexylammonium salt of dimethylketal DHAP (C.F. Boehringer Corp.) was converted into the sodium salt of DHAP by treating it with Dowex 50 (H^+ form) (Ballou and Tomita, 1960). The monoacyl-sn-G3P was donated by Dr. M. Kates (Ottawa), the acyl-DHAP was obtained from Dr. A.K. Hajra (Ann Arbor, Michigan), and the L-palmitoylcarnitine was supplied by Otsuka Pharmaceutical Co., Osaka, Japan. Bovine serum

albumin (fraction V from the Sigma Chemical Co.) was treated with charcoal to remove FAs (Chen, 1967). In most of the experiments, sn-[U- ^{14}C]G3P (New England Nuclear Corp., Boston) was diluted with rac-G3P (Sigma Chemical Co.) to a specific activity of approximately 350,000 d.p.m./ μ mole sn-G3P. The radio-chemical purity of the ^{14}C -labelled sn-G3P was verified by paper chromatography with phenol-water (5:2, w/v) (Benns and Proulx, 1971) and was found to be free of cyclic G3P and other labelled impurities. [1- ^{14}C]palmitic acid was purchased from New England Nuclear Corp. and converted into a potassium salt. ^{14}C -labelled monoacyl- and diacyl-sn-G3P were synthesized enzymatically by incubating rabbit liver microsomal fractions with sn-[^{14}C]G3P and isolating the products by TLC. [^{14}C]phosphatidylethanolamine and [^{14}C]phosphatidylcholine were generously supplied by Dr. P.R. Proulx (Ottawa). Carnitine palmitoyltransferase (EC 2.3.1.23) was prepared by the method of Kopec and Fritz (1971). Thin-layer chromatographic plates coated with silica gel G (E. Merck, Darmstadt), prepared either by our laboratory or purchased from Brinkmann Co. (Toronto), were activated at 120°C for 1 hour and then stored in a dessicator. All other reagents were of analytical grade.

III-1-2. Preparation of subcellular fractions

Albino male rabbits (2.0 to 3.0 kg) were anaesthetized by an intraperitoneal injection of 2 % (w/v) α -chloralose (J.T. Baker Chemical Co., Phillipsburg, N.J.) in 20 % (w/v) urethane. The dosage was 2.5 ml per kg body weight. The hearts were excised, trimmed to remove fat, weighed, and chopped into small pieces. The tissue was then homogenized with four volumes of 0.25 M sucrose containing 0.02 M Tris-HCl, pH 7.4, in a Potter-type homogenizer with a Teflon pestle rotating at 1,000 rev/min. The homogenization was standardized by performing two strokes up and down with a loose-fitting pestle and then one stroke with a tight-fitting pestle (Kako, 1969). The homogenate was centrifuged at 800 x g for 15 min to remove nuclei and cell debris, and the supernatant was carefully decanted and centrifuged at 10,000 x g for 15 min. The resulting supernatant was removed and further centrifuged at 100,000 x g for 60 min. This fraction is contaminated by lysosomal particles. The pellet thus obtained was used as a microsomal fraction after being suspended in the homogenizing medium to give 2.0 to 5.0 mg protein/ml. The pellet from the 10,000 x g centrifugation was resuspended and centrifuged at 8,000

x g for 15 min, yielding a mitochondrial pellet which was suspended in the homogenizing medium to give 8.0 to 10.0 mg protein/ml. Although the yield of mitochondria could have been improved by the use of the proteinase Nagarse, the method adapted in this study did not use proteinase because acyl-CoA synthetase (acid: CoA ligase (AMP) EC 6.2.1.3) is easily destroyed by this procedure (De Jong and Hülsmann, 1970; Pande and Blanchaer, 1970). In some experiments, the lysosomal fraction was obtained by centrifuging the 10,000 x g supernatant at 24,000 x g for 15 min. The resulting pellet was used as the lysosomal fraction. Hepatic sub-cellular particles were isolated and compared with those of heart organelles. The liver microsomal fraction which was isolated also served as the source of the enzyme to synthesize labelled diacyl-sn-G3P, as stated in Section III-1-1.

III-1-3. Electron microscopic examination of subcellular preparations

In order to examine the cross contamination of sub-cellular fractions and the possible contamination by microorganisms during preparation procedures, rabbit heart mitochondrial and lysosomal fractions were examined under an electron microscope. Mitochondrial and lysosomal pellets

were prepared by the procedures described above, and they were then fixed in a solution of 1 % osmium tetroxide in phosphate buffer, pH 7.4 (Millonig, 1961), for one hour at 4°C. The dehydration was accomplished by passing the materials through a series of increasing concentrations of ice cold ethanol (30 %, 50 %, and 90 %, each for 5 min) and finishing with two changes of ice cold absolute ethanol. The specimens were then treated with propylene oxide (two changes, 10 min each). Araldite resin 502 was used as an embedding medium according to the method of Luft (1961). The blocks were polymerized by incubation at 35°, 45°, and 60°C (approximately 12 hours at each temperature).

Sections of approximately 70-80 mμ in thickness were cut with glass knives using an LKB Ultramicrotome. These were then stained with a saturated solution of uranyl acetate in 50 % ethanol for 40 min, followed by lead citrate for 5 min (Reynolds, 1963). A Philips Electron Microscope, Model 300, was used to examine the specimens. The electron micrographs of the isolated rabbit heart mitochondrial and lysosomal fractions are shown in Figs. 2 (mitochondrial fraction, 33,858 x magnification) and 3 (lysosomal fraction, 33,858 x magnification).

The isolated cardiac mitochondrial fraction, as shown in Fig. 2, displays three types of ultrastructures: orthodox, intermediate, and condensed. These structures closely resemble the rabbit heart mitochondria isolated by Deshpande et al. (1961) and the hepatic mitochondria described by Hackenbrock (1966). Although various conformations of mitochondria are associated with their metabolic steady states (Hackenbrock, 1966), the techniques applied for the preparation of isolated mitochondria most frequently resulted in an alteration of the morphological characteristics (Deshpande et al. 1961). Accordingly, vacuolation, loss of matrix, rearrangement of the cristae, a change to spherical forms, and, sometimes, swelling was commonly observed in electron microscopic studies.

Although the technical difficulties involved in preparation of the electron micrographs may discount the exact interpretation of the morphological characteristics of isolated rabbit heart subcellular fractions, the primary object of this study is to examine the extent of cross contamination among different subcellular particles. A direct comparison with the same magnification of isolated mitochondrial (Fig. 2) and lysosomal (Fig. 3) fractions clearly

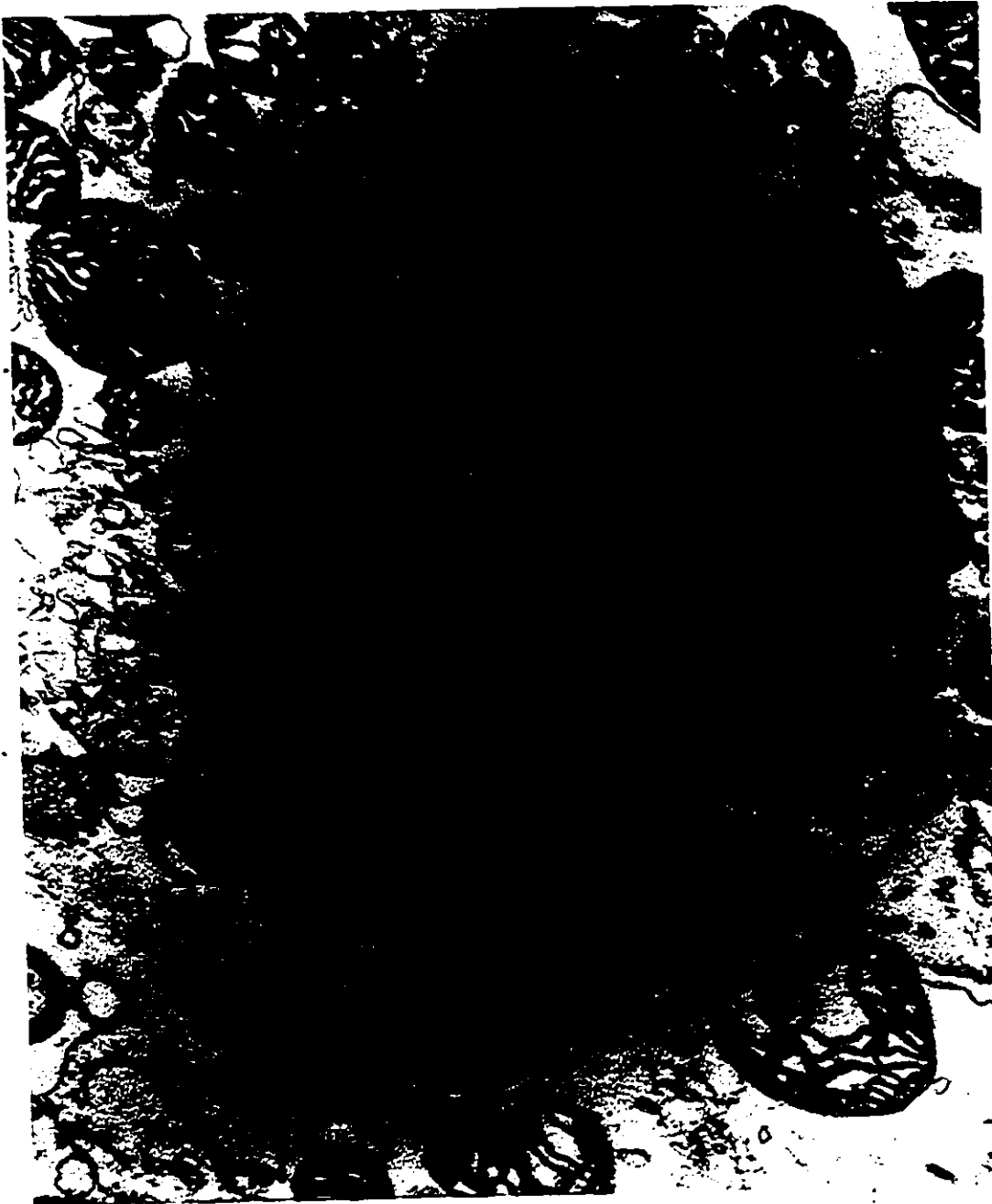


FIGURE 2

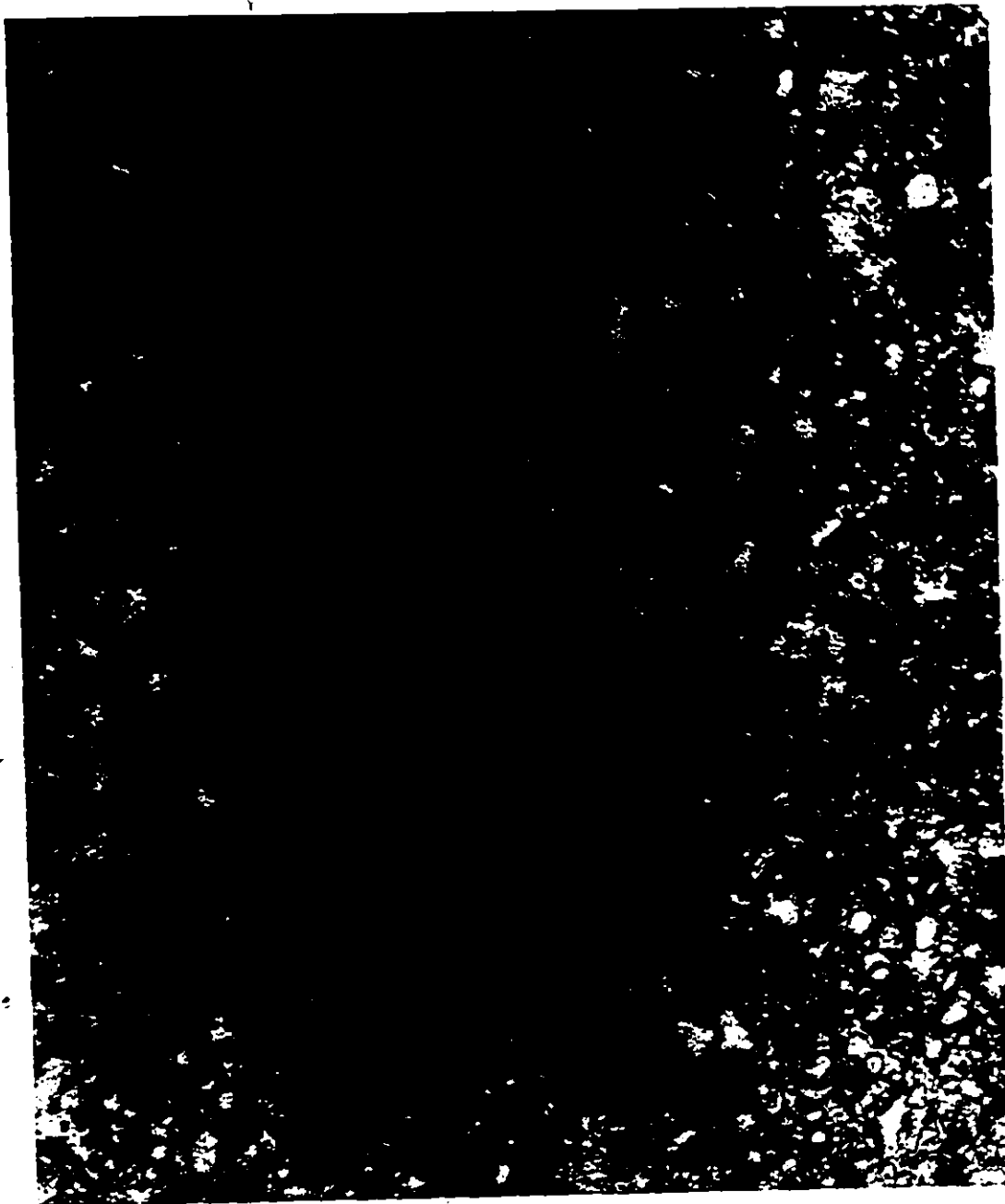


FIGURE 3

indicates that the mitochondrial preparations were very slightly contaminated by the lysosomal fractions. From this, it would seem that cross contamination of the mitochondrial fraction by microsomes should be minimal since the density of the microsomal fraction is even less than that of the lysosomal fraction. It must be mentioned, however, that lysosomal particles were not free of microsomal contamination. The electron micrographs showed no evidence of existence of microorganisms. Examination of the specimens was carried out randomly, and, in all cases, similar pictures were obtained.

III-1-4. Assay procedure

The reaction mixture contained, in a final volume of 2.0 ml, 100 μ moles Tris-phosphate buffer, pH 7.4, 2.0 μ moles potassium palmitate (also oleate or linoleate), 0.8 μ mole CoA, 12 μ moles ATP, 6.0 μ moles $MgCl_2$, 20 mg FA-poor bovine serum albumin, 6.0 μ moles sn-[U- ^{14}C]-G3P (0.8 μ Ci), and a fresh cardiac enzyme preparation containing approximately 2.5 mg mitochondrial protein or 0.8 mg microsomal protein. When liver subcellular fractions instead of cardiac fractions were used as the enzyme source, the amounts of mitochondrial and microsomal proteins were decreased to approximately 1.5

mg and 0.5 mg, respectively, because of their higher enzyme activity. In some experiments, 0.8 μ mole palmitoyl-CoA was used instead of CoA, ATP, palmitate, and $MgCl_2$. When L-palmitoylcarnitine served as the acyl donor (Daae & Bremer, 1970), CoA, ATP, palmitate, and $MgCl_2$ were replaced with 0.8 μ mole L-palmitoylcarnitine, 1.6 μ moles CoA, and purified carnitine palmitoyltransferase containing 10.3 μ g protein with a specific activity of 3.7 μ moles/min per mg protein at 25°C. These concentrations of acyl donors were chosen from the substrate-product relationships obtained from a preliminary experiment. Other conditions are specified in the legends to the figures and tables.

The reaction commenced with the addition of the enzyme preparations, and the incubation was carried out in a metabolic shaker at 37°C in air. In the experiments of Figs. 11 and 12, the incubation temperature was 30°C because, at this temperature, the reaction rate of purified palmitoylcarnitine acyltransferase is reduced so that palmitoyl-CoA can be generated continuously for the acyltransferase reaction (Fig. 11). In order to compare the results of the experiments in Figs. 11 and 12 directly, a 30°C incubation temperature was adapted for the experiment of Fig. 12. The

duration of incubation is stated for individual experiments. The reaction was stopped by the addition of 3.0 ml of water-saturated butanol followed by the addition of 4.0 ml of butanol-saturated water (Daas & Bremer, 1970). After separation of the two phases, the upper phase was washed with butanol-saturated water and the lower phase with water-saturated butanol. This modified procedure gave a very low blank value for zero-time incubation which improved the accuracy and reproducibility of the assay. A portion of the combined butanol extracts was suspended in 10 ml of Bray's solution (Bray, 1960) for radioactivity determination. The remainder was used for product analysis.

When [$1-^{14}\text{C}$]palmitate was used as a tracer, the specific radioactivity was adjusted to approximately 500,000 d.p.m./ μmole , and the assay was carried out as described, except that the unused labelled palmitate was removed first by TLC with a solvent system of petroleum ether (b.p. 30° to 60°C)-diethyl ether-acetic acid (40:10:1, v/v/v) (Possmayer & Mudd, 1971; Huang & Kako, 1970). The phospholipid and the neutral lipid fractions were eluted with chloroform-methanol (1:1, v/v); the eluates were evaporated to a small volume under nitrogen gas and re-chromatographed in

another system for further identification. Between 94 and 104 % of the radioactivity of the mono-, di-, and tri-glycerides was recovered after elution, evaporation, and the second chromatography, but the calculated amounts of the reaction products, monoacyl-sn-G3P and diacyl-sn-G3P, had to be corrected by their recovery values, which were approximately 70 %.

III-1-5. Remarks on enzymatic assay procedure

In the past two decades, there has been a great amount of research dealing with the acylation of sn-G3P in the liver, yet the results obtained by different investigators varied widely in respect to activities, subcellular localization, reaction products, and FA specificity of G3P acyltransferase. The various results obtained by the many different investigators can in part be attributed to the fact that different assay procedures were employed. These varieties can be best illustrated in table form (Table 3). In order to make the table concise, data was reduced and greatly simplified while still demonstrating marked disagreements. It is evident from this table that the use of a small amount of enzyme and a short incubation time and/or a low temperature are essential since G3P acyltransferase

TABLE 3

Summary of the experimental conditions and results of the acylation of sn-glycerol-3-phosphate

Source & amount (mg)	Activity nmole mg. min	Product	FA specificity	Incubation temp. time	Extraction	GP (mM)	Acyl donor (mM)	Buffer	Albumin	References
MT	2.9	LPA, PA	(+)	37° 5'	butanol	6.5 (H)	0.07 (CoA)	MES TES Glygl	0.2	(159)
MC (1)	2.8	LPA, PA		37° 2.5'	butanol	7.5	.4-.8 (CoA)	"	0.4	(158)
MT (.5-1)	1.5-3.0	LPA, PA	(+)	37° 2.5'	butanol	1.5	.4-.8 (CoA)	"	0.4	(158)
MC	0.6	PA	(-)	20° 5'	CMA	0.7	.028 (CoA)	Tris		(242)
MT (.75)	4.3-3.6	LPA, PA	(+)	30° 7'	butanol	5 (H)*	.25 (PC)	TES	0.5	(47) (48)
MC (.75)	1.6-10.9	PA, DG		30° 7'	butanol	5	.25 (PC)	TES	0.5	(47) (48)
MC* (.2-.4)	26.0	PA	(-)	37° 5'	B.D.	10 (H)	.2	Pi	0.5	(50)
MC	15.0	PA		37° 5'	B.D.	10 (H)	.2	Pi	0.5	(50)
MT	1.1	PA		37° 5'	B.D.	10 (H)	.2	Pi	0.5	(50)
MC ⁺ (1)	.9	PA		37.5° 10'	B.D.	(C)	.5	Tris, Pi		(182)

Table 3 (page 2)

Source & amount (mg)	Activity nmole/mg. min	Product	FA specificity	Incubation temp. time	Extraction	GP (mM)	Acyl donor (mM)	Buffer	Albumin	References
MC (10)	4.0	PA		37° 90'	B.D.	.125	.01 (C)	Tris		(181)
MC (.3)	.69	LPA	(+)	37° 15'	F	.18 (C)	.12 (CoA)	Tris	.74	(124)
MC	1.25	LPA	(+)	37° 15'	F	.18 (C)	(Palm)	Tris	.74	(124)
MC (3)	.02	PA		30'	B.D.	.05 (H)	.01 (C)	Tris		(199)
MT (3)	.02	PA		30'	B.D.	.05 (H)	.01 (C)	Tris		(199)
MC (1)	3.0-5.0	PA		37° 2'	B.D.	1.5 (C)	.03 (CoA)	Pi		(3)
MC (0.5)	.85	PA	(-)	20° 3'	CMA	.29 (C)	.017 (CoA)	Tris		(63)
MT	.19	PA		20° 3'	CMA	.29 (C)	.017 (CoA)	Tris		(63)
MC (6)	.04		(+)	23° 60'	F	.5 (P)	.33 (C)	Tris		(247)
MT (21)	.16		(+)	23° 60'	F	.5 (P)	.33 (C)	Tris		(247)
MC (2)	15.6			37° 20'	B.D.	20	.8 (C)	Pi		(207)

Table 3 (page 3)

Source & amount (mg)	Activity nmole/mg. min	Product	FA specificity	Incubation temp. time	Extraction	GP (mM)	Acyl donor (mM)	Buffer	Albumin	References
MT	4.0-6.0			37° 40'	B.D.	20	.8 (C)	Pi		(207)
MC	9.1	LPA	(-)	35° 15'	F	.91 (C)	.68 (CoA)	Tris	1.1	(66)
MT	3.1	LPA	(-)	35° 15'	F	.91 (C)	.68 (CoA)	Tris	1.1	(66)
MC (5-10)	.48	PA		30° 30'	F	1 (P)	.1 (CoA)	Pi		(219)
MT (5-10)	.13	PA		30° 30'	F	1 (P)	.1 (C)	Pi		(219)
MT ⁺	.1			37° 60'	F	.014 (C)	.1 (C)	Tris		(150)
MC	11.7	PA		37° 20'	B.D.	3.3	.8 (C)	Pi		(210)
MT	1.2	PA		37° 40'	B.D.	20	.8 (C)	Pi		(210)
MC	.08	PA	(-)	20° 90'	CMA	1.0	.04 (C)	Tris		(128) (130)
MC*	0.01			30° 10'	CoA	41.7	.4 (CoA)	Pi	1.7	(25)

Table 3 (page 4)

All experiments were carried out by using rat liver fractions (MC=microsomes, ~~MT~~^M=mitochondria), except those with * (guinea pig liver) and + (rat brain). Abbreviations used are: { CMA=chloroform-methanol-acid extraction, F=Folch et al.'s method (1957), and B.D.=Bligh and Dyer's method (1959). C, H and P indicate radioactive tracers used (¹⁴C, ³H, and ³²P, respectively), whereas CoA and PC, signify acyl-CoA and palmitoylcarnitine, respectively. GP, PA, LPA and DG stand for sn-glycerol-3-phosphate, diacyl-sn-glycerol-3-phosphate, monoacyl-sn-glycerol-3-phosphate, and diglyceride, respectively.

activity is fairly high and non-linear with respect to time. The addition of albumin and Mg^{2+} , the various extraction procedures, and the general experimental conditions have a profound influence on the enzymatic activity, the reaction products, and the FA specificity. It was found that this generalization is applicable to studies dealing with heart subcellular fractions, although there have been, as mentioned before, no systematic studies dealing with heart enzymes.

In the early phases of this study, the chlorform-methanol extraction procedures of Folch et al. (1957), Bligh and Dyer (1959), Hajra et al. (1968), and Eibl et al. (1969) were used and found to be unsatisfactory. These extraction procedures constantly produced a high control value in the presence of subcellular fractions prior to incubation. Radioactivity was high and variable even when the tissue fraction was omitted from the incubation mixture. This, in all probability, was due to the non-specific binding of albumin to the radioactive precursor (Barden and Cleland, 1969). Low and constant control values of the radioactivity are essential to a successful assay since the radioactivity found in the products is 0.1 to 0.01 % of that in the

precursor (0.7×10^6 to 17×10^6 d.p.m./tube) in most studies with liver particulate fractions (e.g. Abou-Issa and Cleland, 1969; Davidson and Stanacev, 1972; Monroy et al., 1972). Heart particulate fractions possess even lower activities than do the liver fractions. With the modified version of the butanol extraction procedure, more than 90 % of the radioactivity was recovered when ^{14}C -labelled diacyl-sn-G3P, monoacyl-sn-G3P, phosphatidylcholine, or phosphatidylethanolamine were tested individually. Thus, the application of the modified butanol extraction procedure with its low control values and quantitative extraction of the reaction products are the essential factors for the successful G3P acyltransferase assay in cardiac subcellular fractions. Bjerve et al. (1974) further showed that chloroform-methanol did not extract monoacyl-sn-G3P from animal tissue quantitatively, while butanol did.

III-1-6. Identification of reaction products

The butanol extract was evaporated to dryness under nitrogen gas. The residue was dissolved in chloroform-methanol (1:1, v/v) and then chromatographed on silica gel G plates. The quantities of phospholipids, neutral lipids, and FAs were determined by developing the plates with petro-

leum ether-ether-acetic acid (40:10:1, v/v/v) as the solvent system (Possmayer and Mudd, 1971). The quantities of monoacyl-sn-G3P and diacyl-sn-G3P were determined by developing the plates with chloroform-methanol-acetic acid-water (65:25:8:4, v/v/v/v) (Skipski et al., 1962). In general, the spots were identified by exposure to iodine vapour and their R_F values compared with authentic standards. The appropriate bands were scraped off and suspended in either Aquasol (New England Nuclear Corp.) or toluene containing 4 % Cab-O-Sil (Packard Instruments Co., Downers Grove, Ill.) for radioactivity determination. More than 90 % of the radioactivity was recovered after TLC. In order to verify that the products were monoacyl-sn-G3P and diacyl-sn-G3P, the developed chromatograms were scanned with an Actigraph III (Nuclear-Chicago Corp.) to localize the peaks of radioactivity which were then compared with those of authentic standards. In addition, they were stained with three reagents, namely, 0.3 % (w/v) ninhydrin in ethanol for amino groups (Jatzkewitz & Mehl, 1960), Schiff-periodate spray for vicinal hydroxyl groups (Waldi, 1965), and Dittmer & Lester's reagent to detect phosphorus (1964).

III-1-7. Radioactivity counting

A Nuclear-Chicago liquid scintillation spectrometer, Mark I, was used. The counting efficiency was calculated by the channels-ratio method and was found to be above 70 % for ^{14}C and 35 % for ^3H . The calibration graph for this method was constructed by using variously quenched standards in the presence or absence of 4 % Cab-O-Sil in toluene, or in Aquasol.

III-1-8. Determination of protein

The protein concentration of the subcellular fractions was measured either by the method of Lowry et al. (1951) after having dissolved the sample in 1N NaOH and diluting it, or by the biuret method of Lane & Mavrides (1969). Bovine serum albumin (Fraction V) was used as the standard.

III-1-9. Induction of the hyperthyroid state in rabbits

A group of albino male rabbits, whose body weights ranged from 1.9 to 2.5 kg, was made hyperthyroid by a daily intramuscular injection of 125 μg T3 per kg body weight for a period of six to ten days. This dosage induced the typical hyperthyroid state as seen in both the growth rate and the heart rate of the rabbits. T3 used for injection was dissolved in an aqueous solution with a final concentration

of 0.1 % by the addition of several drops of 1N NaOH. Some control animals were fed with laboratory chow for the same period as hyperthyroid rabbits, while others were starved for three to six days. The T3 treated animals lost 27.4 ± 2.0 % of their initial weight during the six to ten day period (Table 4). In contrast, the animals who received food regularly (the fed controls) gained 3.9 ± 1.2 % of their initial body weight during the same period. The starved controls lost 14.0 ± 0.9 % of their initial body weight during the three to six day period (Table 4).

The heart rates of the rabbits were recorded by a Hewlett-Packard Electrocardiograph, Model 1500B. They were recorded while the rabbits were under anesthesia via an intraperitoneal injection of 2 % (w/v) chloralose in 20 % (w/v) urethane (2.5 ml per kg body weight). The heart rate of both the fed and starved controls, as well as the T3 treated animals, before the start of injection was 256 ± 9 beats/min. The heart rate of both the fed and starved controls were identical throughout the experimental period. After T3 administration, however, the heart rate of the animals increased by 162 % to 417 ± 11 beats/min. (Table 4).

TABLE 4

Comparison of the euthyroid and hyperthyroid states in rabbits

Animal	Injection	Period (days)	Growth rate (%)	Heart rate (beats/min)	No. of experiments
Fed control	-	6-10	(+) 3.9 $\pm$ 1.2	256 $\pm$ 9	3
Starved control	-	3-6	(-) 14.0 $\pm$ 0.9	256 $\pm$ 9	6
T3	125 μ g T3 per kg body weight i.m., daily	6-10	(-) 27.4 $\pm$ 2.0	417 $\pm$ 11	8

T3 was dissolved in H₂O by the addition of several drops of 1N NaOH to give a final concentration of 0.1 %. Growth rate was expressed as % of gain (+) or loss (-) of their initial body weight. Heart rate was measured by a Hewlett-Packard Electrocardiograph (Model 1500B) under anesthesia (intraperitoneal injection of 2.5 ml of a mixture of 2 % chloralose in 20 % urethane, per kg body weight). i.m. stands for intramuscular injection.

III-2. Stereospecific Analysis of Monoacyl-sn-Glycerol-3-Phosphate Synthesized by the Rabbit Heart Mitochondrial Fraction In Vitro

III-2-1. Materials

1-monopalmitoyl-sn-glycerol, 2-monopalmitoyl-sn-glycerol, 1-monooleoyl-sn-glycerol, and 2-monooleoyl-sn-glycerol were obtained from Supelco Inc. and Applied Science Laboratories, Inc. Boric acid and disodium ethylenediaminetetraacetate (EDTA) were products of Matheson, Coleman, and Bell Co., and Fischer Scientific Co., respectively. Sodium phosphatidate prepared from chicken egg L- α -lecithin was supplied by Pierce Chemical Co. Borate-impregnated silica gel G plates were prepared either by spraying commercial plates (Brinkmann Co.) with 0.4 M boric acid in methanol, or by spreading silica gel G, 25 g in 50 ml 0.4 M boric acid (Thomas et al. 1965), on glass plates. For other chemicals and reagents, see Section III-1-1.

III-2-2. Préparation of heart mitochondrial fraction

The rabbit heart mitochondrial fraction was prepared as described in Section III-1-2.

III-2-3. Biosynthesis of monoacyl-sn-glycerol-3-phosphate

The biosynthesis of monoacyl-sn-G3P by the rabbit

heart mitochondrial fraction was measured by a method essentially identical to the enzyme assay described in Section III-1-4. The reaction mixture contained, in a final volume of 2.0 ml, 100 μ moles Tris-phosphate buffer, pH 7.4, 50 μ moles borate buffer, 2.0 μ moles potassium palmitate (also oleate or linoleate), 0.8 μ mole CoA, 12.0 μ moles ATP, 6.0 μ moles $MgCl_2$, 20 mg defatted bovine serum albumin, 6.0 μ moles sn-[U- ^{14}C]G3P (0.8 μ Ci), and a fresh mitochondrial preparation containing approximately 5.0 mg protein. The incubation was carried out in a metabolic shaker at 37° C for 10 min in air. The reaction was stopped by the addition of 3.0 ml 0.1 M boric acid-saturated butanol, followed by the addition of 4.0 ml butanol-saturated 0.1 M boric acid solution. After separation of the two phases, the upper phase was washed with butanol-saturated boric acid and the lower phase with boric acid-saturated butanol (Daae, 1972; Monroy et al., 1972). Butanol extracts from 20 to 40 incubations were combined and reduced to a small volume under nitrogen gas. The residue was dissolved in chloroform-methanol (1:1, v/v) for the isolation of monoacyl-sn-G3P.

III-2-4. Isolation of monoacyl-sn-glycerol-3-phosphate

The chloroform-methanol mixture, which contained radioactive products, was chromatographed on borate-impregnated silica gel G plates. The plates were developed with a solvent system containing chloroform-methanol-acetic acid-water (65:25:8:4, v/v/v/v) (Skipski *et al.*, 1962) in order to separate the monoacyl-sn-G3P from other products. Lipids were localized by exposure to iodine vapor and were compared to the authentic standards. The appropriate band of silica gel with the R_f value identical to that of the monoacyl-sn-G3P standard was scraped from the plate, and the removed silica gel was immersed in 2.0 ml of a solution containing 0.15 M potassium borate and 0.075 M EDTA, pH 6.8 (Monroy *et al.* 1972). After letting it stand for 5 to 10 min at room temperature, the slurry was extracted using the method of Bligh and Dyer (1959). The slurry was adjusted with chloroform-methanol- H_2O (1.25 : 2.5 : 1.0, v/v/v) to give a one phase system, shaken vigorously, and allowed to stand at room temperature for 5 to 10 min. The one phase system was then made to produce a two phase system by altering the chloroform-methanol- H_2O ratio to 2.5 : 2.5 : 2.25, v/v/v, and shaking gently. The silica gel was removed by centrifugation. The extraction procedure was repeated

three times to assure that at least 80 % of the radioactive monoacyl-sn-G3P was recovered in the chloroform phase.

III-2-5. Treatment of monoacyl-sn-glycerol-3-phosphate with phosphatidate phosphohydrolase

The chloroform phase, which contained monoacyl-sn-G3P, was allowed to dry under nitrogen gas and was then dispersed by sonication in 0.02 M Tris-HCl buffer, pH 7.4. The isolated monoacyl-sn-G3P was dispersed with a Biosonik (Bronwill Scientific Co., Model B10-II) which operated at half of the maximum intensity (20 K Hz, 150 W) for 15 sec at a temperature of 4° C. The total period of dispersion was 45 sec. The resulting suspension in 5-10 ml containing 16.5-35.8 nmoles monoacyl-sn-G3P was then treated with 45-60 mg protein of phosphatidate phosphohydrolase (EC 3.1.3.4) (prepared as described in Section III-2-7) in an incubation mixture containing 0.05 M borate buffer, pH 7.4 (the activity of purified phosphatidate phosphohydrolase was 3.1 μ moles/mg per hour). After 60 min of dephosphorylation at 37° C, the reaction was stopped by boric acid-saturated butanol as above, except that carriers 1- and 2-MG were added to the butanol extracts. Finally, isomeric

MGs were separated by borate-impregnated TLC plates using Thomas' solvent system E (Thomas et al., 1965) containing chloroform-acetone-methanol (71:25:4, v/v/v). Appropriate bands were removed and their radioactivities determined by a liquid scintillation counter (Section III-1-7).

III-2-6. Prevention of acyl migration

During the entire procedure, various precautions were taken to prevent acyl migration. These precautions included rapid handling of reaction products at low temperature until the final chromatograph was completed, the use of borate buffer in the incubation mixture (Monroy et al., 1972), performing of evaporation in a nitrogen atmosphere, the use of boric acid-saturated butanol and borate-impregnated TLC plates (Thomas et al., 1965), and the addition of carriers 1- and 2-MG to the final butanol extraction (Daae, 1972; Monroy et al., 1972).

III-2-7. Preparation of phosphatidate phosphohydrolase from rat liver

The phosphatidate phosphohydrolase was prepared according to the method of Wilgram and Kennedy (1963). Twenty male rats of Sprague-Dowley strain weighing approximately 250 g were killed by cervical dislocation. Their livers

were removed rapidly and transferred to beakers immersed in crushed ice. Portions of the chilled livers were minced with scissors and homogenized in ice cold 0.25 M sucrose (1:9, w/v) with a Potter-type homogenizer fitted with a Teflon pestle. The homogenization was performed for about 30 sec. The homogenate was centrifuged at 600 x g for 12 min. The supernatant fraction was centrifuged at 8,500 x g for 12 min; its supernatant was removed with a Pasteur pipet. A layer of loosely sedimented material was removed from the mitochondrial pellet without disturbing the bulk of the pellet by careful washing with a small amount of cold sucrose solution added from a capillary pipet. The mitochondrial pellet was then washed once with homogenizing medium and re-centrifuged. The fluffy lysosomal layer was collected, combined with the layer obtained from the first centrifugation, and these were suspended in 0.02 M Tris-HCl buffer, pH 7.4. The fraction was dialyzed overnight against 0.02 M Tris-HCl buffer, pH 7.4, containing 0.005 M EDTA, then stored at -20° C. The activity remained unchanged during storage for a period of 10 weeks. The activity of lysosomal phosphatidate phosphohydrolase was measured by the rate of release of inorganic phosphate from phosphatidic

acid (diacyl-sn-G3P) (Daniel and Rubinstein, 1968; Section III-4). It was found to be of 3.1 μ moles of inorganic phosphate released per hour per mg of protein. The method of Berenblum and Chain (1938), which is described in Section III-4-3, was employed to estimate the inorganic phosphate. As stated above, approximately 45-60 mg protein of purified lysosomal phosphatidate phosphohydrolase with an apparent activity mentioned above was used to dephosphorylate 16.5-35.8 nmoles of monoacyl-sn-G3P.

III-3. Studies on Palmitoyl-CoA Hydrolase

III-3-1. Materials

Palmitoyl-CoA and DFP were obtained from the Sigma Chemical Co. and K and K Laboratories, respectively. L-cysteine (free base) and DTT were purchased from Nutritional Biochemical Corp. $[1-^{14}\text{C}]$ palmitoyl-CoA and $[1-^{14}\text{C}]$ palmitic acid were supplied from New England Nuclear Corp. (+)-Decanoylcarnitine was prepared according to the method of Brendel and Bressler (1967) and crystallized twice by acidification in a hot aqueous solution. Its purity was checked by TLC with a solvent system consisting of chloroform-methanol-0.1 M sodium acetate (4:4:1, v/v/v) (Böhmer et al, 1966). The solvents were redistilled before being used.

III-3-2. Preparation of subcellular fractions

The method of preparation has been described in Section III-1-2, although the centrifugation procedure was somewhat modified. The homogenized tissue mixture was centrifuged at 800 x g for 15 min to remove nuclei and cell debris; the supernatant was used as a homogenate fraction. The latter was further centrifuged at 10,000 x g for 15 min. The precipitate from this centrifugation was resuspended and centrifuged at 8,000 x g for 15 min, yielding a mitochondrial fraction. The resulting supernatant from the 10,000 x g centrifugation, however, was then spun at 24,000 x g for 15 min, and its precipitate resuspended in the homogenizing solution as a lysosomal fraction. The 24,000 x g supernatant was further centrifuged at 100,000 x g for 60 min, and the resulting pellet was used as a microsomal fraction.

III-3-3. Enzymatic assay procedure

With slight modification, the method of Daniel and Rubinstein (1968) was utilized for the measurement of palmitoyl-CoA hydrolase activity. The enzyme preparation of either homogenates or subcellular fractions was incubated in 0.2 μ mole [1-¹⁴C]palmitoyl-CoA with a radioactivity of

approximately 35,000 c.p.m., 80 μ moles Tris-phosphate buffer, pH 7.4, and 20 mg bovine serum albumin. The final volume of the incubation mixture was adjusted with H_2O to 2.0 ml. In some experiments, the pH was adjusted by altering the amount of Tris-base and phosphoric acid. The incubation was carried out at 37° C in a metabolic shaker, either under nitrogen gas or in the presence of 4.0 μ moles (+)-decanoylcarnitine in the reaction mixture. This method was employed to prevent the possible oxidation of added substrate. The reaction was stopped by the addition of 10 ml isopropanol-heptane-1N H_2SO_4 (20:5:1, v/v/v) (Goss and Lein, 1967; Huang and Kako, 1970) after a period of incubation as stated in individual experiments. This was followed by the addition of 6.0 ml heptane and 4.0 ml H_2O . The mixture was then transferred to a separatory funnel, shaken vigorously, and allowed to stand for 5 to 10 min. After separating the two phases, the heptane phase was rewashed with heptane-saturated H_2O , and the aqueous phase with H_2O -saturated heptane (Huang and Kako, 1970). An aliquot from the combined heptane phases was then taken and dissolved in Bray's solution (Bray, 1960) for the determination of radioactivity. The combined heptane

extracts recovered 93 to 96 % of radioactivity when $[1-^{14}\text{C}]$ -palmitic acid was tested alone. In contrast, a negligible amount of radioactivity was found in heptane phases when $[1-^{14}\text{C}]$ palmitoyl-CoA was checked by this procedure. These results indicate that the extraction procedure employed in this assay gave a satisfactory separation of the radioactive product from its precursor.

For the determinations of radioactivity and protein, see the Sections III-1-7 and III-1-8.

III-4. Studies on Phosphatidate Phosphohydrolase

III-4-1. Materials

Sodium salt of phosphatidic acid was obtained from the Pierce Chemical Co. It consists of the following fatty acids: palmitic, 36 %; stearic, 15 %; oleic, 37 %; and linolenic, 12 %. For other materials, see Sections III-1-1, III-2-1, and III-3-1.

III-4-2. Enzyme preparation

The supernatant from the 800 x g centrifugation (Section III-1-2) was used as the homogenate fraction. Cardiac subcellular fractions were obtained as described in Sections III-1-2 and III-3-2.

III-4-3. Enzymatic assay procedure

The activity of phosphatidate phosphohydrolase was determined by measuring the rate of release of inorganic phosphate from sodium phosphatidate in the presence of an enzyme preparation. The incubation mixture was prepared immediately prior to each experiment and contained 3.0 μ moles sodium phosphatidate in an aqueous suspension, 160 μ moles Tris-acetate buffer, pH 7.0, and an enzyme preparation, all in a final volume of 2.0 ml. The reaction was allowed to proceed for a period of 60 to 90 min at 37° C in a metabolic shaker and was then stopped by the addition of 2.0 ml 12 % trichloroacetic acid. The specific activity of the enzyme was calculated by subtracting the value obtained in the control experiment. In the control tubes, the enzyme preparation was incubated in the absence of sodium phosphatidate for 1 hour, the substrate then added, and this followed immediately by trichloroacetic acid. After removing the precipitated protein by centrifugation, the inorganic phosphate in the reaction mixture was determined using the method of Berenblum and Chain (1938). The methods of Fiske and Subbarow (1925) and Lowry and Lopez (1946) were employed in the preliminary experiments for the determination of inorganic phosphate. The latter two

methods were found to be unsatisfactory due to the high control value obtained under the following conditions:

- (1) at zero time incubation with enzyme and substrate,
- (2) after incubation in the absence of enzyme preparation,
- or (3) in the presence of enzyme preparations and prolonged incubation. Fig. 4 illustrated the interference of tissue protein in the determination of inorganic phosphate by the method of Lowry and Lopez (1946). In contrast, the method of Berenblum and Chain (1938) gave good reproducibility of results and a negligible control value.

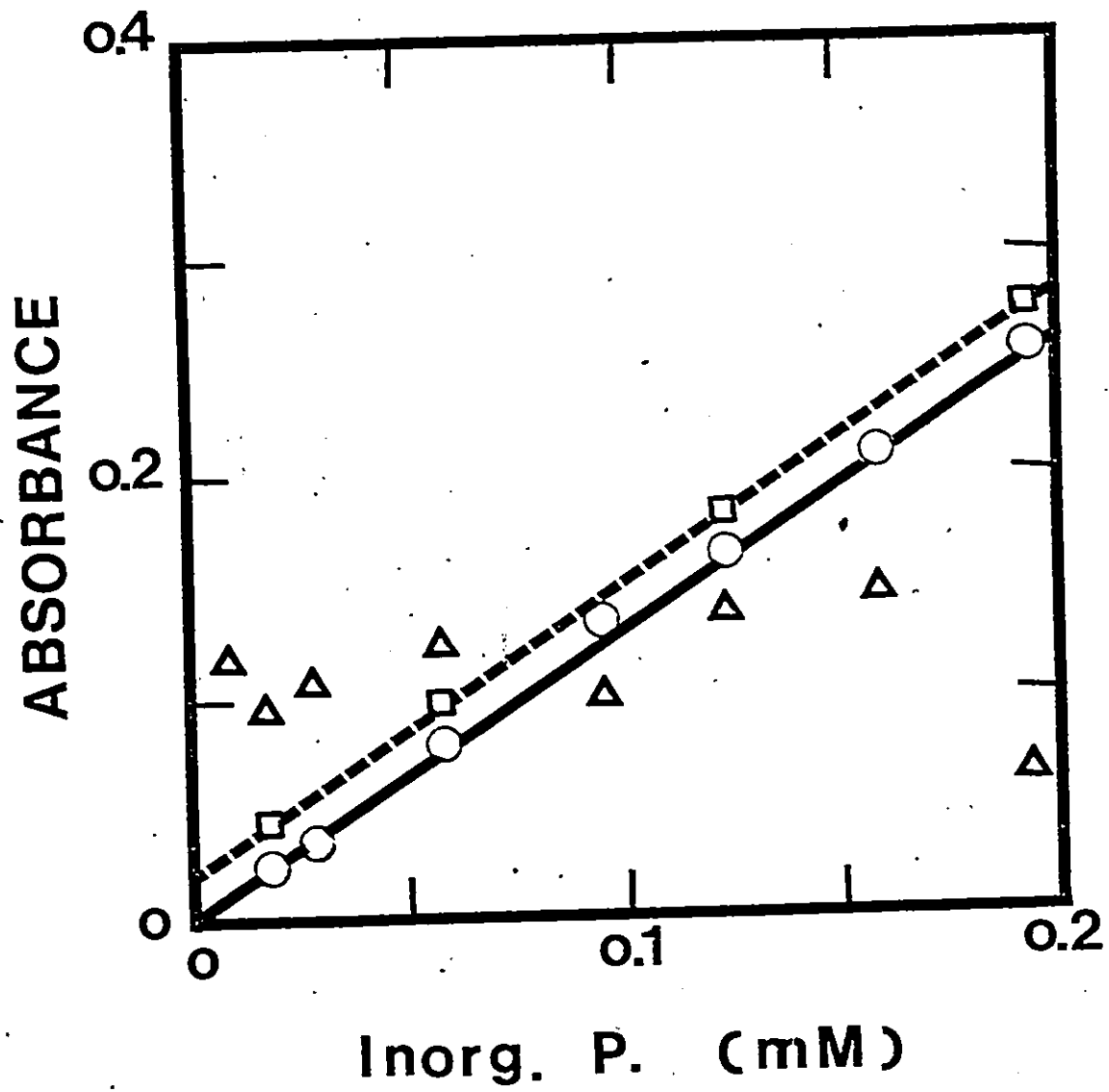
The effect of pH on the enzyme activity was examined by using the Tris-acetate buffer described above. To obtain a pH value of 11, this buffer was adjusted with 1N NaOH. Protein content of the enzyme preparations was measured as in Section III-1-8.

III-4-4. Preparation of particle-bound phosphatidic acid

The so-called particle-bound phosphatidic acid (Johnston et al., 1967; Smith et al., 1967; Mitchell et al., 1971) was prepared by incubating the cardiac microsomal preparation in the presence of sn-[U-¹⁴C]G3P for 5 min at 37° C. The incubation mixture was the same as that for, assaying the activity of G3P acyltransferase (Section III-1-4).

LEGEND TO FIGURE 4Interference in the inorganic phosphate determination
caused by the presence of homogenate in the assay mixture

Inorganic phosphate was determined by the method of Lowry and Lopez (1946). Incubation time was 60 min. The ordinate indicates the absorbance, and the abscissa indicates various concentrations of inorganic phosphate (Inorg. P.) in mM. Circles show the values obtained with various concentrations of inorganic phosphate standard. Triangles represent the results obtained by incubating the same amount of homogenate (approximately 2.0 mg) with various concentrations of inorganic phosphate, while squares show those values obtained when bovine serum albumin (2.0 mg) was used instead of homogenate. Three experiments were carried out.

FIGURE 4

After incubation, the contents of 10 tubes were combined and centrifuged twice at 100,000 x g for 60 min at 4° C. The resulting microsomal pellet was resuspended in a 0.25 M sucrose solution and used as a particle-bound phosphatidic acid. Under these experimental conditions, it was found that 98 % of the acylation products were monoacyl- and diacyl-sn-G3P.

III-4-5. Examination of the effect of the supernatant fraction on the conversion of particle-bound phosphatidate to glycerides

The freshly prepared particle-bound phosphatidic acid, 28 μ moles in 9.74 mg of microsomal protein containing a radioactivity of 46,000 d.p.m., was incubated with 160 μ moles Tris-acetate buffer, pH 7.0, in the presence or absence of 1.5 ml (10.8 mg protein) supernatant fraction. The final volume of the incubation mixture in either case was 3.0 ml. After 60 min of incubation, water-saturated butanol was added to the assay tubes, and lipids were extracted by the procedure previously described in Section III-1-4. The resulting products were analyzed by TLC in solvent systems of petroleum ether-ether-acetic acid (40:10:1, v/v/v)

(Huang and Kako, 1970; Possmayer and Mudd, 1971) and chloroform-methanol-acetic acid-water (65:25:8:4, v/v/v/v) (Skipski et al., 1962). After being analyzed, the radioactivity of these products was determined as described in Section III-1-7. From this data, the effect of the supernatant fraction on the conversion of particle-bound phosphatidate to glycerides was examined.

III-5. Studies on Acyl-CoA:sn-1,2-Diglyceride-0-Acyltransferase

III-5-1. Materials

1,2-dipalmitoyl-sn-glycerol, 1,3-dipalmitoyl-sn-glycerol, 1,2-dioleoyl-sn-glycerol, 1,3-dioleoyl-sn-glycerol, palmitoyl-CoA, Tween-20, and Triton X-100 were products of Sigma Chemical Co. Gum acacia was obtained from Matheson, Coleman, and Bell Manufacturing Chemists, Ltd.. [$1-^{14}\text{C}$]palmitoyl-CoA was purchased from New England Nuclear Corp. Other chemicals and reagents are described in Sections III-1-1 and III-2-1.

III-5-2. Preparation of subcellular fractions

The method of subcellular fractionation was described in Section III-1-2.

III-5-3. Assay procedure, comments, and preliminary results

The activity of DG acyltransferase was measured using the method of Daniel and Rubinstein (1968) with modifications. The enzyme preparation was incubated with 0.3 μ mole [$1-^{14}\text{C}$]palmitoyl-CoA with a specific activity of 800,000 d.p.m./ μ mole, 100 μ moles Tris-phosphate buffer, pH 7.4, 20 mg bovine serum albumin either in the presence or absence of 8.0 μ moles DG, and H_2O to make a final volume of 2.0 ml. The reaction was allowed to proceed for 30 min at 37°C , and was then stopped by the addition of 10 ml of a mixture of isopropanol-heptane-1N H_2SO_4 (20:5:1, v/v/v) (Goss and Lein, 1967). The separation of the heptane and aqueous phases was performed as described previously for the assay of palmitoyl-CoA hydrolase. The radioactivity in the combined heptane phases was counted, and the difference between the values found in the presence of DG and in its absence was taken as the DG acyltransferase activity. The radioactivity obtained in the absence of an acceptor represented the activity of palmitoyl-CoA hydrolase.

Since DGs are insoluble in H_2O , they were prepared as substrates for the enzyme by three different suspensions. Dioleoyl-sn-glycerol (50 % of 1,2-isomer and 50 % of 1,3-isomer) or dipalmitoyl-sn-glycerol (50 % of 1,2-isomer and

50 % of 1,3-isomer) was suspended by sonication in either 1 % Tween-20, 1 % Triton X-100, or 5 % gum acacia solution. The final concentration of either Tween-20, Triton X-100, or gum acacia in the reaction mixture was 0.2 %, 0.2 %, or 1 %, respectively. These different substrate preparations influenced the apparent activity of DG acyltransferase as shown in Fig. 5. The data in Fig. 5 was obtained by correcting for palmitoyl-CoA hydrolase activity, which is illustrated in Fig. 6. Palmitoyl-CoA hydrolase activity measured in the presence of gum acacia, Tween-20, or Triton X-100 (Fig. 6) was greatly modified as compared to the activity measured in the absence of gum acacia, Tween-20, Triton X-100, or DG (Section IV-2-3, Fig. 41). However, it was learned that the preparation of substrate (DG) in Tween-20 solution gave more reproducible results as compared to those obtained using Triton X-100 and gum acacia in the preliminary experiments. Accordingly, Tween-20 suspension was then used in the study of subcellular distribution of DG acyltransferase in rabbit heart. The activity of DG acyltransferase in the microsomal fraction obtained with Tween-20 was identical in 4 experiments (1.2 nmoles/mg min when dioleoyl-sn-glycerol served as substrate), while the activity obtained

with Triton X-100 and gum acacia varied from 0-0.8 nmole/mg min (dioleoyl-sn-glycerol was used as substrate). It should be noted that the different concentrations of surface active agents (Tween-20, Triton X-100, gum acacia) may produce different activities.

The radioactive counting procedure and the methods of measuring protein concentration in subcellular preparations were already described.

The technical difficulty in preparing a standardized DG suspension (Mangiapane et al., 1973) and the fact that different substrate preparations affect the apparent activities of DG acyltransferase (Fig. 5) probably hindered the attainment of consistent results among different experiments. An attempt to improve the accuracy and the reproducibility of the assay method was not too successful. Nevertheless, preliminary results of the subcellular distribution of DG acyltransferase in rabbit heart are shown in Fig. 7. The activities in lysosomal and microsomal fractions were highest (Fig. 7), while the measured activities in mitochondrial and soluble fractions (Fig. 7) could be attributed to contamination from microsomal or lysosomal fractions. The reported activity of DG acyltransferase in

hepatic microsomes was 0.75-1.8 nmoles/mg min (De Kruyff et al., 1970; O'Doherty et al., 1972), which was similar to that found in this study. The supernatant fraction of rat liver contained no DG acyltransferase activity, but it stimulated microsomal activity about 4-fold (Manley et al., 1974).

LEGEND TO FIGURE 5Acyl-CoA:sn-1,2-diglyceride-0-acyltransferase activity in rabbit heart mitochondrial and microsomal fractions

The incubation mixture, in a final volume of 2.0 ml, contained 0.3 μ mole [1-¹⁴C]palmitoyl-CoA with a specific activity of 800,000 d.p.m./ μ mole, 100 μ moles Tris-phosphate buffer, pH 7.4, 20 mg bovine serum albumin either in the presence or absence of 8.0 μ moles dioleoyl-sn-glycerol (Diole.) or dipalmitoyl-sn-glycerol (Dipalm.), and a freshly prepared subcellular fraction (1.02 mg mitochondrial or 1.3 mg microsomal protein). The incubation time was 30 min, and the temperature was 37° C. The values obtained in the presence of DG minus those obtained in its absence were taken as the DG acyltransferase activity (shown on ordinate in nmoles/mg min). The symbol $\square$ represents the mitochondrial activity, while $\blacksquare$ indicates microsomal activity. For further details of the assay, see Method Section III-5-3. The final concentrations of either Tween-20, Triton X-100, or gum acacia in the reaction mixture were 0.2 %, 0.2 %, or 1 %, respectively.

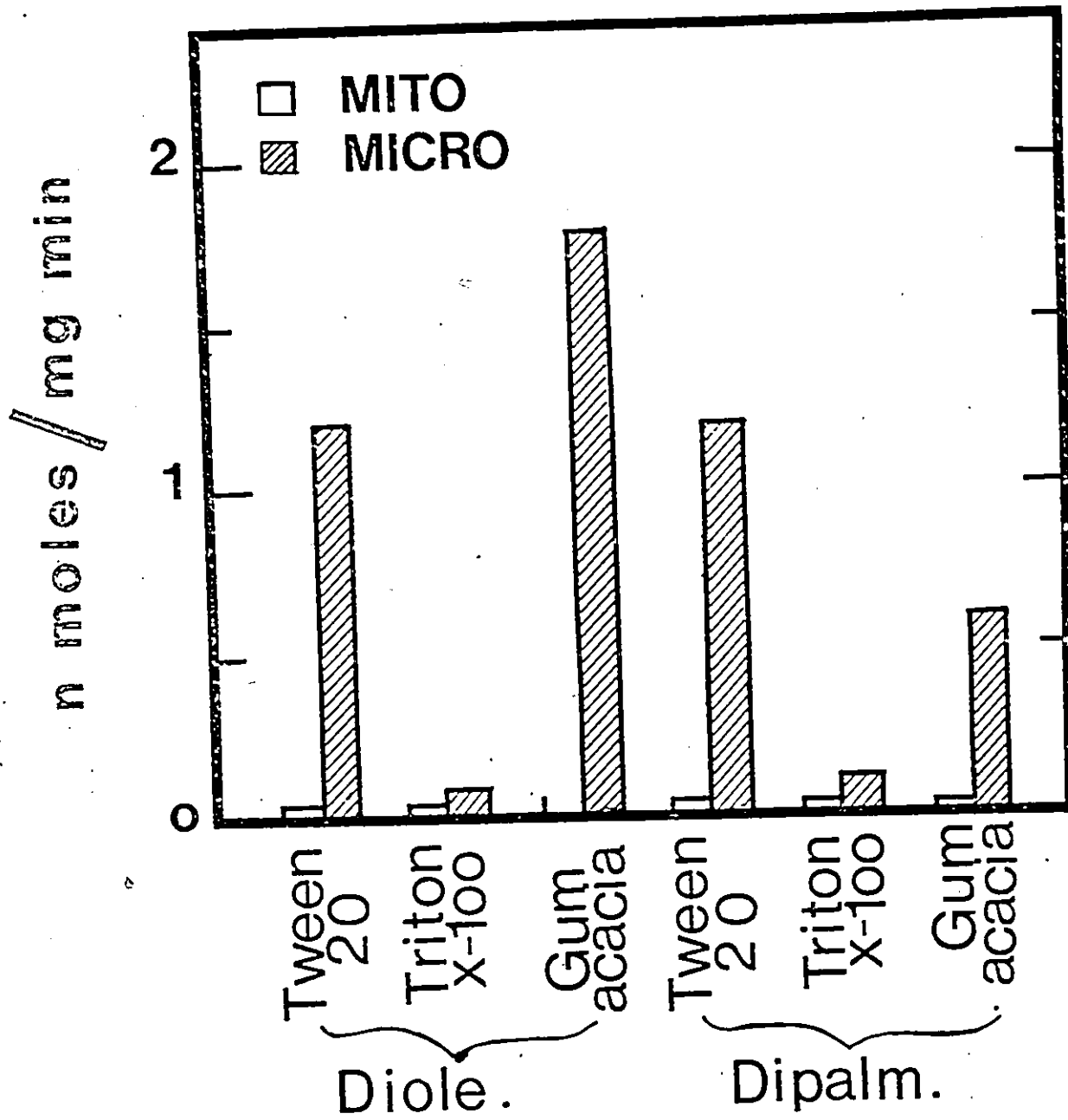
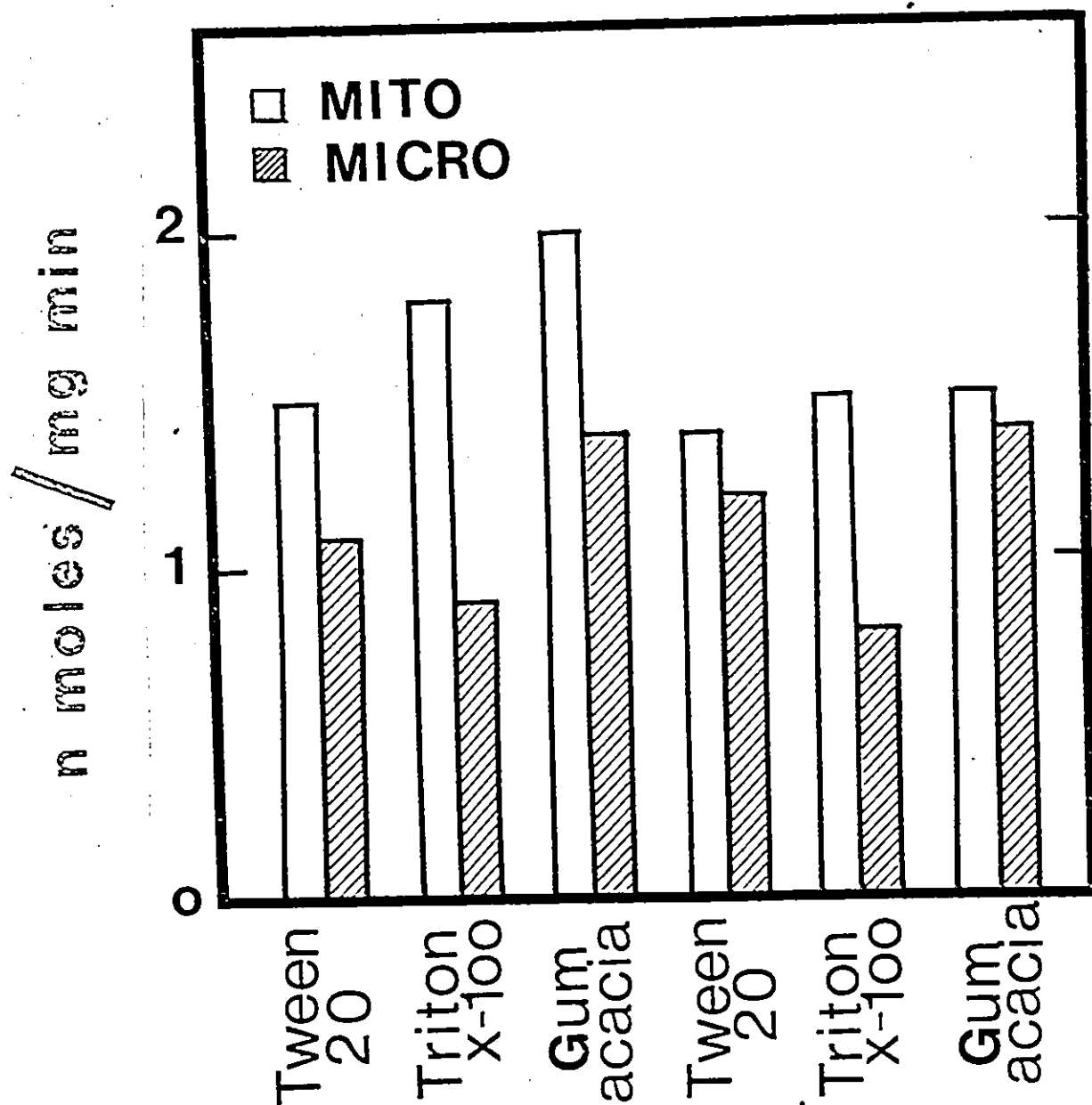


FIGURE 5

LEGEND TO FIGURE 6Palmitoyl-CoA hydrolase activity in rabbit heart subcellular fractions

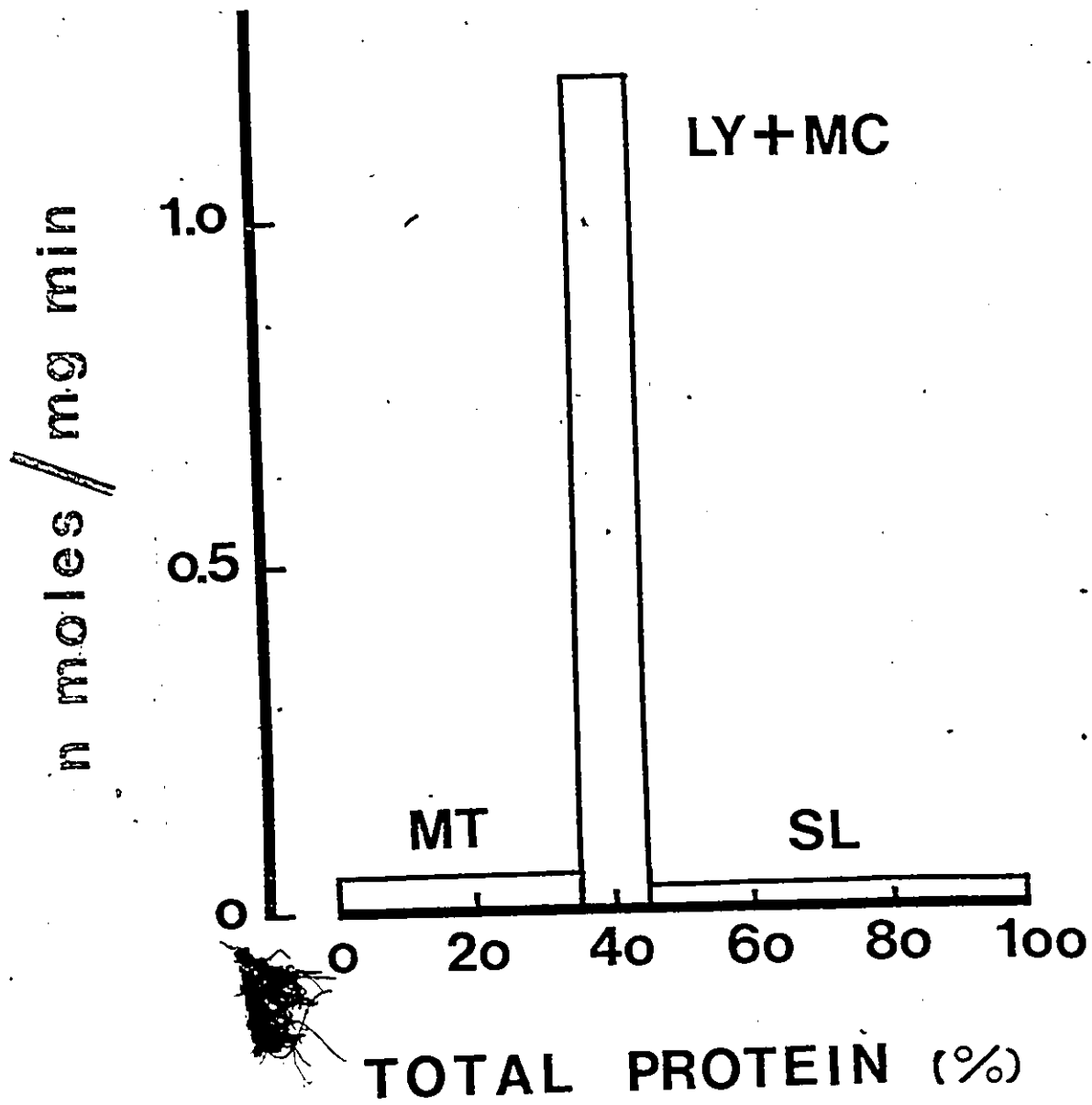
The incubation mixture and assay procedure were identical to those described in the legend to Fig. 5. Assays were carried out in the absence of dioleoyl-sn-glycerol and dipalmitoyl-sn-glycerol. The activity was expressed in nmoles/mg min on the ordinate. The symbol  represents the mitochondrial activity, while  indicates microsomal activity.

FIGURE 6

LEGEND TO FIGURE 7Subcellular distribution of acyl-CoA:sn-1,2-diglyceride-
O-acyltransferase in rabbit heart

The experimental conditions and assay procedure were described in the legend to Fig. 5 except that dioleoyl-sn-glycerol in Tween-20 suspension was used as the acyl donor. The ordinate shows the activity in nmoles/mg min. The abscissa represents total protein in %. MT, LY, MC, and SL stand for mitochondrial, lysosomal, microsomal, and soluble fractions, respectively.



FIGURE 7

IV. RESULTS AND COMMENTS

IV-1. Characteristics of Acyl-CoA:sn-Glycerol-3-Phosphate-0-Acyltransferase and Acyl-CoA:Monoacyl-sn-Glycerol-3-Phosphate-0-Acyltransferase in Mitochondrial and Microsomal Fractions of Rabbit Heart

IV-1-1. Effects of various cofactors on the activity of acyl-CoA:sn-glycerol-3-phosphate-0-acyltransferase in mitochondrial and microsomal fractions

The incorporation of sn-G3P into mitochondrial lipids was studied in the presence or absence of various cofactors (Table 5). The addition of CoA, ATP, and $MgCl_2$ was essential. The requirement for Mg^{2+} could be one of the characteristics of the sn-G3P acylating reaction (Yamashita and Numa, 1972; Monroy et al., 1973) and of acyl-CoA synthetase (Sánchez et al., 1973). It was previously found that when the sn-G3P concentration was 1.5 mM in the incubation mixture, Mg^{2+} stimulated the mitochondrial G3P acyltransferase reaction, while it had no effect on microsomal acylation activity. However, when the sn-G3P concentration was increased to 7.5 mM, Mg^{2+} had no effect on the mitochondrial enzyme, but it inhibited microsomal G3P acyltransferase activity (Monroy

et al., 1972). In both cases, palmitoyl-CoA, instead of palmitate, CoA, and ATP, was used as the acyl donor.

Doubling the ATP (6 mM) or CoA (0.4 mM) concentrations did not alter the reaction rate (not shown in Table 5). This is due to the fact that the optimal concentrations of ATP and CoA for acyl-CoA synthetase were reported to be 0.15 to 1.2 mM and 0.02 to 0.09 mM, respectively (Farstad et al., 1967; Possmayer and Mudd, 1971; Brindley, 1973). The apparent K_m values of acyl-CoA synthetase for ATP and CoA were 0.0015 to 0.02 mM and 0.003 mM, respectively (Pande, 1972; Bar-Tana et al., 1973). ATP not only provides energy for activation of FAs, but also stabilizes acyl-CoA synthetase activity (Pande, 1972). High concentrations of CoA have been reported to inhibit acyl-CoA synthetase activity (Farstad et al., 1967), but this must have been due to use of the carnitine palmitoyltransferase-coupled assay because no such effect was observed with the hydroxamate assay procedure (Pande, 1972).

Approximately 29 % of the G3P acyltransferase activity observed in the complete system (Table 5) was obtained in the absence of added palmitate, suggesting that there was a fairly large pool of endogenous FAs in rabbit heart

mitochondrial fraction. An even larger quantity of endogenous FAs appeared to exist in the microsomal fraction (Fig. 8 and Table 6). This result points to a possible error involved in assaying FA esterification using radioactive FA or fatty acyl-CoA. Unless the FA composition of the endogenous pool is determined and then taken into account for precursor specific activity, the results obtained with radioactive FA may be underestimated.

Although the omission of albumin neither stimulated nor inhibited mitochondrial G3P acyltransferase activity (Table 5), a sigmoid-shaped kinetic curve was produced with microsomal enzymes during an incubation period of 20 min as shown in Fig. 8 and Table 6. In liver microsomes, albumin at a concentration of 7.3 mg/ml stimulated G3P acyltransferase activity, but was inhibitory at higher levels (Fallon and Lamb, 1968). Lippel (1973) reported that albumin at concentrations below 4.9 mg/ml stimulated activation of mono- and di-unsaturated FAs in liver microsomes and mitochondria. Higher concentrations (19.5 mg/ml) of albumin were inhibitory. However, albumin has no effect on the activation of oleate and little stimulatory effect, or no effect, on palmitate activation (Lippel, 1973). In

our study, addition of albumin at a concentration of 10 mg/ml neither stimulated nor inhibited the G3P acyltransferase reaction (Table 5). The albumin has a protecting effect during the control stages of incubation (Fig. 8 and Table 6) presumably by binding with excess FAs in the incubation medium (The reaction was started by the addition of enzyme preparation). In the absence of albumin, excess FAs caused inhibition of acyl-CoA synthetase (Lippel, 1973) and activation of acyl-CoA hydrolase (Lamb and Fallon, 1970). Therefore, albumin was always added to the incubation mixture throughout the study. It is noteworthy that albumin possesses phospholipase-like activity (Elsbach and Pettis, 1973). However, the results obtained in Fig. 8 and Table 6 did not show the potentiation of phospholipase action.

Because a nitrogen atmosphere and NaF had no effect on G3P acyltransferase activity, incubation was carried out in air in the studies reported. However, it was found that NaF inhibited, among other enzymes, phosphatidate phosphohydrolase, which would remove the products of the G3P acyltransferase reaction (Smith et al., 1967). Therefore, the use of NaF in the incubation medium might cause

inaccuracy in the assay of G3P acyltransferase activity. CTP (5 mM) failed to show a regulatory effect on the biosynthesis of diacyl-sn-G3P, which disagrees with the inhibitory effect (Possmayer and Mudd, 1971) and stimulatory effect (Zborowski and Wojtczak, 1969) reported. The inhibition of G3P acyltransferase activity by CTP could be due to the indirect inhibition of phosphatidate phosphohydrolase in the presence of fluoride ion (Possmayer and Mudd, 1971), while the stimulatory effect was reported to be due to the formation of CDP-DG from phosphatidic acid (Zborowski and Wojtczak, 1969). L-carnitine depressed mitochondrial G3P acyltransferase activity (Table 5), presumably by enhancing the oxidation of activated FAs (Fritz, 1967). The effect of L-carnitine on the activity of microsomal G3P acyltransferase was not investigated in this study. Under our experimental conditions, reduced pyridine nucleotides did not affect G3P acyltransferase activity in the rabbit heart mitochondrial fraction (Table 5). This point will be discussed further in relation to the existence of the DHAP pathway.

The acyltransferase reaction was proportional to the amount of mitochondrial protein (up to 4 mg) added (Fig. 9

and Table 7). A similar linear relationship existed between sn-G3P acylation of the microsomal preparations (Fig. 10 and Table 8; Fig. 26) and the amount of enzyme protein (up to 1.5 mg).

IV-1-2. Nature of acyl donors

The extent of sn-G3P acylation when the optimal concentration (0.4 mM) of palmitoyl-CoA was used as the acyl donor was similar to the system using an optimum concentration (1 mM) of palmitate, CoA, ATP, and $MgCl_2$ (Fig. 9 and Table 7). This indicates that acyl-CoA synthetase does not limit sn-G3P acylation in heart mitochondrial fraction. The same conclusion was reached by Davidson and Stanacev (1972), Smith and Hübscher (1966), Shephard and Hübscher (1969), Abou-Issa and Cleland (1969), Brindley (1973), and Sánchez et al. (1973). The concentration of acyl-CoA used in our assay mixture was relatively high (0.4 mM). In rat liver microsomes, the optimal concentration of acyl-CoA for G3P acyltransferase was 0.03 mM (Abou-Issa and Cleland, 1969). This difference is in part due to the use of albumin in our assay system. Albumin binds palmitoyl-CoA and lowers the concentration of free monomeric palmitoyl-CoA in solution so that the incorporation proceeds at low palmitoyl-CoA

levels.

The use of (-)-palmitoylcarnitine together with purified carnitine palmitoyltransferase was proposed by Daae and Br mer (1970) who claimed that this donor system was the most suitable assay procedure for measuring G3P acyltransferase activity. On the other hand, a number of investigators have used acyl-CoAs in their assays (Lamb and Fallon, 1970; Yamashita and Numa, 1972; Monroy *et al.*, 1972; Brindley, 1973; S nchez *et al.*, 1973). Therefore, the microsomal G3P acyltransferase activity, measured by using (-)-palmitoylcarnitine (Fig. 11 and Table 9), was compared to that using palmitoyl-CoA (Fig. 12 and Table 10). It was found that the rate of sn-G3P acylation was similar when a concentration of 0.4 mM of either acyl donor was used (Fig. 11 and Table 9; Fig. 12 and Table 10). (-)-Palmitoylcarnitine exhibited a greater inhibition of the activity at a concentration above 0.4 mM, thus resulting in a narrow optimum range of concentrations (Fig. 11 and Table 9). This is presumably due to the stronger detergent action of (-)-palmitoylcarnitine as compared to that of palmitoyl-CoA.

The detergent property and complex physico-chemical

state in an aqueous solution of palmitoyl-CoA (Barden and Cleland, 1969) or palmitoylcarnitine produce an anomalous kinetic behavior of the enzyme reaction (Smith and Hübscher, 1966; Abou-Issa and Cleland, 1969; Zahler and Cleland, 1969; Lamb and Fallon, 1970). It is possible that owing to the action of palmitoyl-CoA hydrolase, a high concentration of acyl-CoA, which inhibits G3P acyltransferase activity (Shephard and Hübscher, 1969; Hübscher, 1970), could be required for the assay. The preparation of pure acyl-CoAs is not a simple procedure, and most acyl-CoAs are contaminated by thiol ester derivatives and other fluorescent and quenching impurities (Pullman, 1973). Furthermore, in mitochondria, G3P acyltransferase exists on the inner side of the outer membrane (Daae and Bremer, 1970; Monroy et al., 1972), whereas carnitine palmitoyltransferase II ("B") localizes inside the inner membrane (Hoppel and Tomec, 1972; Kopec and Fritz, 1973; Brosnan et al., 1973): This fact makes quite illogical the use of the carnitine palmitoyltransferase reaction as the means of producing an acyl donor. Although carnitine palmitoyltransferase I ("A") localizes at the outer surface of the inner membrane (Kopec and Fritz, 1971; Hoppel and

Tomec, 1972; Brosnan et al., 1973), the function of this enzyme is to produce palmitoylcarnitine from palmitoyl-CoA. These conditions did not justify the adoption of more elaborate assay methods using palmitoyl-CoA or palmitoylcarnitine, hence, the remaining experiments were performed with FAs as the acyl donors.

Microsomal acyl-CoA synthetase activity was reported to be 38 to 132 nmoles/min per mg protein (Abou-Issa and Cleland, 1969; De Jong and Hülsmann, 1970; Aas, 1971; Lippel, 1971 and 1973; Pande, 1972; Mangiapane et al., 1973), and the rate of sn-G3P acylation was found to be 0.6 to 11 nmoles/min per mg protein (Abou-Issa and Cleland, 1969; Possmayer et al., 1969; Daae and Bremer, 1970; Davidson and Stanacev, 1972; Monroy et al., 1972; Yamashita and Numa, 1972). Therefore, it is unlikely that acyl-CoA synthetase in microsomes becomes rate limiting for the acylation reaction when FAs are used as acyl donors. Furthermore, the substrate specificity of acyl-CoA synthetase towards individual long-chain FAs was not prominent (Hübscher, 1970). Accordingly, it is improbable that the reaction rate and substrate specificity of the G3P acyltransferase observed in our study reflect the characteristics of acyl-CoA synthetase.

IV-1-3. The acylation products

The major products of G3P acylation were localized by the radioactive scanning of the developed TLC plates. These spots were stained using three visualization techniques (Table 11). The radioactive scanning revealed two major peaks, the R_F values of which were 0.63 and 0.94. These values correspond to those of the authentic standards of monoacyl-sn-G3P and diacyl-sn-G3P (Table 11). The results indicate that monoacyl- and diacyl-sn-G3P are the major products of sn-G3P acylation.

To further characterize the molecular components of these reaction products, [^{14}C]palmitate and sn-[^{14}C]G3P were used individually as tracers in separate experiments under otherwise identical assay conditions (Table 12). The molar ratios of FA/sn-G3P in both monoacyl-sn-G3P and diacyl-sn-G3P were approximately one and two, which were close to the theoretical values. Additionally, it was found that a significantly greater quantity of neutral glycerides was produced when [^{14}C]palmitate served as the labelled precursor, whereas only a negligible amount of neutral lipids was formed from sn-[^{14}C]G3P. This indicates that the bulk of incorporation into glycerides was produced

from palmitate through pathways not involving G3P acyltransferase, but, perhaps, other acyltransferases (Elovson, 1965; McMurray and Magee, 1972). A relatively high rate of incorporation of FAs into glycerides has previously been reported (Scherphof and Van Deenen, 1966; Kikuchi and Kako, 1970; Monroy et al., 1972) and been postulated to be due to the acyl exchange reaction of the acyl moiety of glycerides.

IV-1-4. Distinction between mitochondrial and microsomal sn-glycerol-3-phosphate acylation

Under the standard assay conditions, the mitochondrial acylation yielded 32 to 42 % monoacyl-sn-G3P, 58 to 68 % diacyl-sn-G3P, and less than 2 % neutral lipids at the end of a 10 min incubation period (Fig. 13 and Table 13). However, the products of microsomal acylation were 70 to 89 % diacyl-sn-G3P, with the remainder being 11 to 20 % monoacyl-sn-G3P and 2 % neutral lipids after 5 min of incubation (Fig. 14 and Table 13). These values were obtained using palmitate as an acyl donor in the assay. When oleate was used as an acyl donor, mitochondrial enzymes produced approximately 40 % monoacyl-sn-G3P, 60 % diacyl-sn-G3P (calculated from Fig. 32 and Table 26), and a negligible amount of

neutral lipids (data not shown) during a 9 min incubation period. The products of microsomal acylation from oleate were not analyzed. The observed dissimilarities in the formation of mono- and diacyl-sn-G3P between the mitochondrial and microsomal preparations suggest that characteristics of the enzymes in the two subcellular fractions are different.

It was previously reported that MgSO_4 at a concentration of 11 mM stimulated monoacyl-sn-G3P acylation into diacyl-sn-G3P in microsomal preparation of guinea pig liver, but lower concentrations (1.1-5.5 mM) of MgSO_4 had no effect (Pieringer and Hokin, 1962b). In addition, Mg^{2+} (8 mM) depressed phosphatidate phosphohydrolase activity in rat brain homogenates (Strickland *et al.*, 1963). According to these observations (Pieringer and Hokin, 1962b; Strickland *et al.*, 1963), the stimulation of monoacyl-sn-G3P acyltransferase and the inhibition of phosphatidate phosphohydrolase by a high concentration of Mg^{2+} could cause a decrease in monoacyl-sn-G3P and an increase in diacyl-sn-G3P formation at the end of the G3P acyltransferase reaction. However, since the concentration of Mg^{2+} used in our study was only 3.0 mM, it is unlikely that the presence of Mg^{2+} in the

assay mixture alters the distribution of products of the sn-G3P acylation reaction.

A lysosomal preparation was found to inhibit the incorporation of linoleate into 1-acylglycerophosphorylethanolamine in outer membrane preparation of rat liver mitochondria (Waite et al., 1970). The effect of lysosomes on the mitochondrial G3P acyltransferase activity of rabbit heart was not investigated in this study.

The time-courses of the mitochondrial and microsomal sn-G3P acylation reactions were compared in Fig. 15 and Table 14. The rate of sn-G3P acylation in the mitochondrial fraction was constant during a 20 min incubation period, whereas the constant rate of reaction in the microsomal fraction was maintained for only 5 min. Since the activity of microsomal G3P acyltransferase was 6 times higher than that of the mitochondrial enzyme, and since the reaction rate of the microsomal enzyme was constant for only 5 min, one might suspect that the microsomal G3P acyltransferase reaction was limited by the availability of substrates in the assay mixture after 5 min of incubation. However, this is not the case. It was reported that the G3P acyltransferase activity in microsomes of rat liver and brain was 3.0 to 9.1

nmoles/min per mg protein, and the concentration of sn-G3P used was 0.125 to 1.5 mM (Fallon and Lamb, 1968; Abou-Issa and Cleland, 1969; Possmayer and Mudd, 1971; Monroy *et al.*, 1972). The concentration of sn-G3P used in our study, however, was 3.0 mM, and since the rate of the cardiac microsomal sn-G3P acylation reaction was found to be 3.0, nmoles/min per mg protein (Fig. 15 and Table 14), it is unlikely that the rate of the cardiac microsomal sn-G3P acyltransferase reaction observed in our study after 5 min of incubation (Fig. 15 and Table 14) is limited by the availability of sn-G3P in the incubation mixture. It is noteworthy that inhibition of the sn-G3P acylation reaction by a high concentration of sn-G3P has not been reported. Furthermore, the concentration of FA (1.0 mM) used in our study gave the maximal rate of the sn-G3P acylation reaction with both mitochondrial and microsomal fractions (Figs. 27 to 30; Tables 22 to 25). Therefore, the time-courses of the mitochondrial and microsomal sn-G3P acylation reactions shown in Fig. 15 and Table 14 illustrate the different kinetics of the acylating systems in the two fractions.

The time course of sn-G3P incorporation into monoacyl-sn-G3P by mitochondrial and microsomal fractions (Fig. 16

and Table 15) resembled that of the total sn-G3P acylation (Fig. 15 and Table 14), i.e., microsomal acylation was linear only for a short period of time when compared to mitochondrial acylation. The rate of formation of diacyl-sn-G3P from the labelled precursor by the mitochondrial fraction was much slower than that by the microsomal enzyme (Fig. 17 and Table 15). With the mitochondrial fraction as an enzyme source, monoacyl- and diacyl-sn-G3P formation increased linearly with time (Fig. 18). This could mean that the supply of monoacyl-sn-G3P for the monoacyl-sn-G3P acyltransferase reaction was probably sufficient. On the other hand, microsomal monoacyl-sn-G3P formation seems to control the rate of diacyl-sn-G3P formation (Fig. 19). This might be part of the reason that a high diacyl-sn-G3P/monoacyl-sn-G3P ratio is maintained with the microsomal system. The ratio of mitochondrial to microsomal monoacyl-sn-G3P increased linearly to 135 % during 15 min of incubation, whereas the ratio of mitochondrial to microsomal diacyl-sn-G3P formation remained at 11-17 % during the same time period (Fig. 20 and Table 15). These findings demonstrate that mitochondria are capable of synthesizing monoacyl-sn-G3P from sn-G3P and FAs, and also that the activity of

the enzyme catalyzing this reaction differs from that existing in the microsomal fraction.

The results obtained from experiments with mitochondrial and microsomal preparations of rabbit liver (Figs. 21, 22, and 23; Table 16) supported those obtained with heart subcellular fractions, namely, the relationship between the incubation time and the rate of monoacyl- and diacyl-sn-G3P formation was similar. The formation of monoacyl-sn-G3P in the mitochondrial fraction of rabbit liver was constant for 15 min, while microsomal monoacyl-sn-G3P formation was maintained constantly for only 5 min of incubation (Fig. 21 and Table 16). The rate of microsomal diacyl-sn-G3P formation was faster than that of the mitochondrial fraction and remained constant for a short period of time (Fig. 22 and Table 16). The ratio of mitochondrial to microsomal monoacyl-sn-G3P formation increased linearly with incubation time and reached 205 % at the end of 15 min incubation, while the ratio of mitochondrial to microsomal diacyl-sn-G3P formation was constant for only 5 min and then increased slowly to 31.4 % at the end of 15 min incubation (Fig. 23 and Table 16). Liver mitochondria incorporated sn-¹⁴C]G3P into monoacyl-sn-G3P to an even greater extent (0.48 nmole/

min per mg of protein) than did the heart mitochondria (0.16 nmole/min per mg of protein). In agreement with the results obtained by previous workers (Daae and Bremer, 1970; Eibl et al., 1969; Davidson and Stanacev, 1972; Monroy et al., 1972; Sánchez et al., 1973), the activities of hepatic enzymes were extremely high in comparison with those of heart enzymes.

As described in the INTRODUCTION, the production of monoacyl-sn-G3P as an intermediate of phospho- and neutral glyceride biosynthesis in the liver has only been recently established (Fallon and Lamb, 1968; Daae and Bremer, 1970; Barden and Cleland, 1969; Lamb and Fallon, 1970; Okuyama et al., 1971; Yamashita and Numa, 1972; Yamashita et al., 1973; Monroy et al., 1972 and 1973). It was found, however, that the chloroform-methanol extraction method for tissue lipids did not effectively extract monoacyl-sn-G3P (Bjerve et al., 1974). Some investigators attributed the loss of lipids, notably monoacyl-sn-G3P, to this particular extraction procedure (Daae and Bremer, 1970; Sánchez et al., 1973), whereas others did not (Pieringer et al., 1967; Yamashita and Numa, 1972; De La Roche et al., 1973). An assay procedure of short duration is essential for measuring the acylation

of sn-G3P because of the non-linear kinetics with incubation time when microsomal preparation is used as enzyme source (Figs. 15 to 17; Table 15) and because of further synthesis of neutral glycerides when incubation time is prolonged (Figs. 13 and 14; Table 13). As described in Section III-1-5, we have examined the chloroform-methanol extraction procedures of Folch et al. (1957), Bligh and Dyer (1959), Hajra et al. (1968), and Eibl et al. (1969) in the early phase of this study and have found that these extraction procedures produced high blank values at zero time incubation with poor reproducibility. Therefore, use of the chloroform-methanol extraction procedure is a factor responsible for different results obtained among investigators (Table 3). For this reason, the validity of our results is not at issue as far as the extraction procedure is concerned.

There are some other possibilities that could account for the observed differences in the acylation products in the mitochondrial and microsomal fractions. Monoacyl-sn-G3P acyltransferase might possibly be inhibited by lysosomes (Waite et al., 1970) which could be contaminating the mitochondrial preparations (Eibl et al., 1969). The inhibition

of monoacyl-sn-G3P acyltransferase could result in an increase in monoacyl-sn-G3P formation and a decrease in diacyl-sn-G3P. On the other hand, microsomal diacyl-sn-G3P formation could be explained by the high activity of monoacyl-sn-G3P acyltransferase (Barden and Cleland, 1969). Barden and Cleland (1969) reported that monoacyl-sn-G3P acyltransferase activity in rat liver microsomes was 24 to 97 nmoles/min per mg protein, while G3P acyltransferase activity was 0.6 to 15.6 nmoles/min per mg protein (Yamashita and Numa, 1972; Monroy et al., 1972 and 1973; Lamb and Fallon, 1970; (and many others)). The high monoacyl-sn-G3P acyltransferase activity could lead to an increased formation of diacyl-sn-G3P and a decrease in monoacyl-sn-G3P, as seen in heart microsomes (Fig. 14; Tables 13 and 15). It is unlikely that the results obtained on the differences of the acylation products in the mitochondrial and microsomal fractions are due to the effect of phosphatidate phosphohydrolase. The reason for this is because the activity of this enzyme in heart is high in the microsomal fraction as compared to that in the mitochondrial preparation (Section IV-3-3; Fig. 47). A high activity of phosphatidate phosphohydrolase in the microsomal fraction would convert diacyl-

sn-G3P to form neutral lipids, resulting in a decrease in diacyl-sn-G3P.

The slow biosynthesis of diacyl-sn-G3P by the mitochondrial fraction and the relatively constant ratio of mitochondrial to microsomal diacyl-sn-G3P formation can be taken to indicate, at least in part, an effect of microsomal contamination of the mitochondrial preparation as has been inferred by some investigators (Davidson and Stanacev, 1972). However, no attempt was made to investigate this postulate further by measuring the activities of microsomal marker enzymes since marker enzymes are not necessarily exclusively localized in one subcellular compartment. The most commonly used microsomal marker enzymes are rotenone-insensitive NADPH-cytochrome C reductase and glucose-6-phosphatase. The rotenone-insensitive NADPH-cytochrome C reductase, which has been suggested to be present solely in the microsomal fraction of rat liver (Green et al., 1968), was found to be a true constituent of the outer mitochondrial membrane (Sottocasa et al., 1967; Brunner and Bygrave, 1969; Shephard and Hübscher, 1969). The glucose-6-phosphatase was also found to localize in rat mitochondria (Sarjala et al., 1970; Brunner and Bygrave, 1969). In addition, glucose-6-phos-

phatase activity was not measurable in microsomes of rat heart (Hülsmans, 1961). Furthermore, it is obvious that if the apparent distribution of marker enzymes were similar to that of the enzymes producing mitochondrial diacyl-sn-G3P, it would not be possible to support or disclaim the contention that the latter enzymes do exist. It is my belief that the evidence presented in this study to support the existence of separate mitochondrial enzymes provided more definite information regarding this problem than that which can be obtained with marker enzymes.

IV-1-5. The non-uniform response to triiodothyronine of sn-glycerol-3-phosphate acylation by mitochondrial and microsomal fractions

The view that the characteristics of mitochondrial and microsomal acylation are dissimilar is reinforced further by the experiments with hyperthyroid rabbits. Since it has been known that thyroid hormone increases the TG content of the heart (Bressler and Wittels, 1966), it was thought possible that the activity of enzymes acylating sn-G3P in the myocardium increases, thereby causing the accumulation of TG in the hyperthyroid heart. Experiments were therefore designed to see if the administration of

T3 affects mitochondrial and microsomal sn-G3P acylating systems to different degrees.

The results obtained from these experiments are shown in Figs. 24 and 25. and Tables 17 and 18. The formation of diacyl-sn-G3P by mitochondrial and microsomal fractions was increased about four-fold in the hearts of hyperthyroid rabbits as compared to the starved controls. In addition, mitochondrial monoacyl-sn-G3P formation was augmented two-fold (Fig. 24 and Table 17), whereas the microsomal biosynthesis of monoacyl-sn-G3P was unchanged (Fig. 25 and Table 18). The results indicate that the activity of G3P and monoacyl-sn-G3P acyltransferases is under hormonal control, and that this increase in activity is the possible cause of the accumulation of TG in the hyperthyroid heart.

Since the plasma FFA is known to increase in the hyperthyroid state (Opie, 1968 and 1969) and could possibly be the cause of the observed change in enzyme activity, we examined the formation of monoacyl- and diacyl-sn-G3P in starved and fed euthyroid rabbits. The acylation reaction in starved rabbits, in which plasma FFA concentration is higher than in fed animals (Opie, 1968 and 1969), was similar, but not identical, to the reaction observed in

fed rabbits (Tables 17 and 18). Interestingly enough, fasting has been shown to decrease G3P acyltransferase activity in rat liver homogenates (Vavrečka et al., 1969). In starved rats, plasma FFA concentration increased, resulting in an elevation in hepatic acyl-CoA levels, however, the decreased G3P acyltransferase activity minimized the effect of increased FFA. The formation of phospho- and glyceroglycerides was not altered (Vavrečka et al., 1969). The incorporation of palmitate into hepatic phospho- and glyceroglycerides in starved rats was decreased to 68 to 75 % of that observed with fed animals (Vavrečka et al., 1969). During an investigation on the control of glyceride biosynthesis in rat adipose tissue, Denton and Halperin (1968) found that the increased rate of TG biosynthesis could not be correlated with the increased concentration of either sn-G3P or long-chain fatty acyl-CoA. From these observations, plus our findings with the different magnitudes of increase in monoacyl- and diacyl-sn-G3P formation between the fed and starved animals (Tables 17 and 18) and between normal and hyperthyroid states (Figs. 24 and 25; Tables 17 and 18), it is unlikely that the plasma FFA level plays a role in regulating the formation of monoacyl- and diacyl-

sn-G3P in hyperthyroid hearts.

Our findings, which show that there are different magnitudes of increase in monoacyl- and diacyl-sn-G3P synthesis in the mitochondrial and microsomal fractions, implicates three underlying mechanisms: (1) The first and second acylations are catalyzed by two different enzymes in the heart, as was found in the liver (Eibl *et al.*, 1969; Barden and Cleland, 1969; Hübscher, 1970; Yamashita and Numa, 1972; Monroy *et al.*, 1973); (2) The mitochondrial enzyme possesses characteristics different from those of the microsomal enzyme, confirming the view obtained by the experiments with euthyroid rabbits; (3) The thyroid hormone does not act on these enzymes indiscriminately, i.e., the microsomal monoacyl-sn-G3P acyltransferase activity was stimulated to a greater extent than was the G3P acyltransferase activity.

The magnitude of increase in monoacyl- and diacyl-sn-G3P formation by the mitochondrial fraction differed from that by the microsomal fraction, suggesting the possibility that mitochondrial G3P and monoacyl-sn-G3P acyltransferases were potentiated by T3 equally, whereas microsomal G3P acyltransferase was stimulated to a lesser extent than was

monoacyl-sn-G3P acyltransferase (Figs. 24 and 25; Tables 17 and 18). It is also possible that microsomal phosphatidate phosphohydrolase is inhibited, hence diacyl-sn-G3P accumulates greatly. Further reactions leading to the formation of glycerol- and phospholipids could be curtailed, resulting in a decrease in TG biosynthesis. However, the activity of phosphatidate phosphohydrolase was reported to be increased, rather than decreased or inhibited, during starvation (Vavrečka et al., 1969; Mangiapane et al., 1973) and after subtotal hepatectomy (Mangiapane et al., 1973). In both cases TG accumulation was observed in the liver. In the above work, phosphatidate phosphohydrolase activity was measured using a particle-free supernatant fraction of the liver with membrane-bound phosphatidate serving as substrate. It is not clear whether phosphatidate phosphohydrolase, when assayed with aqueous dispersions of phosphatidate, would show the same effect because it was previously reported that the highest specific activity of phosphatidate phosphohydrolase was localized in lysosomal fraction when assayed with aqueous dispersion of phosphatidate (Wilgram and Kennedy, 1963; Sedgwick and Hübscher, 1965). The problem related to the supernatant and particle-

bound phosphatidate phosphohydrolases will be discussed in a later chapter. Whether or not phosphatidate phosphohydrolase, rather than monoacyl-sn-G3P acyltransferase, could be the rate limiting step of not only hepatic, but also cardiac, TG synthesis is to be investigated further.

IV-1-6. Possible formation of monoacyl-sn-glycerol-3-phosphate by deacylation of diacyl-sn-glycerol-3-phosphate in mitochondrial fraction

During the study on the biosynthesis of diacyl-sn-G3P in mitochondria and microsomes of guinea pig liver via the acylation of sn-G3P, Davidson and Stanacev (1972) measured the acylation activity of sn-G3P by mitochondrial fraction containing increasing amounts of added microsomes. They found that values obtained with each addition of microsomes were as expected for the additive synthesis of mitochondria and microsomes. The mitochondrial fraction contained 2.3 % and 2.1 % microsomes as determined by glucose-6-phosphatase and NADPH:cytochrome C reductase, respectively. A plot of the acyltransferase activity versus the content of microsomal protein resulted in a straight line which extrapolated through zero, indicating that the mitochondrial fraction possessed no acyltransferase activity in the absence of

added microsomes. By using this technique, plus the assay for the activities of microsomal marker enzymes, Davidson and Stanacev (1972) concluded that G3P acyltransferase activity in the mitochondria of guinea pig brain, heart, and kidney and rat liver was due exclusively to contaminating microsomal enzymes. Accordingly, we attempted to test the effect of microsomal contamination on mitochondrial G3P acyltransferase in rabbit heart. The mitochondrial fraction (4.16 mg protein) was incubated with various amounts of microsomal preparation (0 to 2.3 mg protein as shown in Fig. 26). If the mitochondrial fraction possesses its own G3P acyltransferase, then a figure such as Fig. 26, which illustrates the relationship between the total enzyme activity and the sum of mitochondrial and microsomal contents, would indicate an additive effect of the two enzyme actions. If there was no mitochondrial activity, the line should intercept point zero activity at zero mitochondrial concentration. However, the activity obtained with mitochondrial fraction in the absence of added microsomes did not intercept at zero activity (Fig. 26), suggesting that the mitochondrial fraction possessed its own enzyme activity. The microsomal acylation was suppressed

by the addition of mitochondrial fraction (Fig. 26). Since the formation of neutral lipids was found to be unchanged, it was unlikely that this effect was related to phosphatidate phosphohydrolase.

Careful inspection of Figure 6A (mitochondrial fraction incubated with increasing amounts, 0 to 1.4 mg protein, of added microsomes) and Figure 7 (microsomal fraction incubated with increasing amounts, 0 to 4.0 mg protein, of the mitochondrial fraction) in the paper by Davidson and Stanacev (1972) and Figure 4 (liver mitochondrial preparation equivalent to 1.0 gm tissue incubated with various amounts of added microsomes, 0 to 1.0 gm tissue) in the publication by McMurray and Dawson (1969) reveals similar suppressive, rather than additive, effects of the two subcellular fractions. These investigators did not comment on this puzzling question.

One of the possibilities was whether or not phospholipase of the mitochondrial fraction or albumin (Elsbach and Pettis, 1973) plays a role in deacylating the diacyl-sn-G3P formed by the microsomal fraction. Elsbach and Pettis (1973) reported that human and bovine serum albumin preparations contained phospholipase A which hydrolyzed

the acyl moiety at the 2 position of E. Coli phospholipids such as phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin. In an attempt to clarify the possible deacylation of diacyl-sn-G3P by the action of phospholipase, the mitochondrial fraction was incubated under standard conditions, but in the presence of ^{14}C -labelled diacyl-sn-G3P instead of sn- ^{14}C -G3P. The results (Table 19, Experiment 2) demonstrated that only one tenth of the monoacyl-sn-G3P was formed by the action of phospholipases when either palmitate or oleate served as an acyl donor, thus excluding the possibility that the formation of monoacyl-sn-G3P by the mitochondrial fraction was a result of diacyl-sn-G3P deacylation. It is noteworthy that the rate of incorporation of oleate into mitochondrial monoacyl-sn-G3P was greater than that of palmitate (Table 19, Experiment 1). This indicates the FA specificity of the sn-G3P acylating enzymes which will be discussed in a later section (Section IV-1-9-i).

If the monoacyl-sn-G3P formed was the deacylated product of diacyl-sn-G3P, an addition of exogenous diacyl-sn-G3P to the assay mixture would not only decrease the specific activity of the newly synthesized diacyl-sn-G3P,

but would also proportionally decrease the labelling of monoacyl-sn-G3P. This experiment was not carried out in this investigation because the composition of endogenously synthesized diacyl-sn-G3P may not be identical to exogenous diacyl-sn-G3P, and thus the addition of exogenous diacyl-sn-G3P to the incubation mixture would not achieve the isotope diluting effect on the endogenously synthesized diacyl-sn-G3P.

A technique employed by Monroy et al. (1972) to establish the precursor-product relationship of monoacyl- and diacyl-sn-G3P in isolated liver mitochondria is useful. However, the relatively low activity of the acylating enzyme in the heart did not permit us to follow the time course of labelling with desirable accuracy during the very early phase of incubation. Therefore, results thus far obtained have not provided an answer to the question concerning the apparent inhibition of acylation which took place when the two subcellular fractions were added simultaneously (Fig. 26).

IV-1-7. Effect of sulphhydryl-binding reagents and aging of the enzyme preparation on the acylation of sn-glycerol-3-phosphate

In order to compare the characteristics of cardiac versus hepatic G3P acyltransferase, the effect of thiol inhibitors and storage on cardiac enzymes was examined. As shown in Table 20, both mitochondrial and microsomal fractions of rabbit heart were extremely sensitive to N-ethylmaleimide and P-chloromercuribenzoate. When palmitate was used as the acyl donor, a complete inhibition of the enzyme activity for both mitochondrial and microsomal fractions was observed at the lowest concentration (1 mM) of both inhibitors tested: When palmitoyl-CoA served as the acyl donor, mitochondrial enzyme activity was completely inhibited by both inhibitors at the lowest concentration (1 mM) tested. Microsomal enzyme activity was inhibited by N-ethylmaleimide to 89 % and 92 % at concentrations of 1 and 2 mM, respectively. 4 mM N-ethylmaleimide and 1 mM P-chloromercuribenzoate completely inhibited microsomal enzyme activity (Table 20). In contrast, hepatic microsomal G3P acyltransferase was less sensitive to these inhibitors. (Lands and Hart, 1965), i.e., N-ethylmaleimide and P-chloromercuribenzoate gave 71-84 % of inhibition at a concentration of 2 mM with palmitoyl-CoA as acyl donor.

Cardiac mitochondrial G3P acyltransferase lost 56 %

of its activity after three weeks of storage at -20°C (data not shown), while hepatic mitochondrial enzymes remained unchanged after one year of storage at -60°C (Monroy et al., 1972). Whether or not the difference in results obtained by us and by Monroy et al. (1972) was due to the difference in storage temperatures was not investigated.

These findings with thiol inhibitors and aging illustrate the different characteristics of cardiac G3P acyltransferase as opposed to the hepatic enzyme.

IV-1-8. The dihydroxyacetone phosphate pathway

Hajra and Agranoff (1968a) and Hajra (1968a and 1968b) discovered an alternative pathway of FA esterification (Section I-1-1-ii). This pathway is operative in hepatic particular preparations, therefore, it is possible that the DHAP pathway may be operative in rabbit heart mitochondria. Since acylated DHAP is reduced to monoacyl-sn-G3P in the presence of NADPH, some monoacyl-sn-G3P may be formed via this pathway (LaBelle and Hajra, 1972). To test this possibility, the effect of NADH or NADPH on the acylation of sn-G3P in the presence or the absence of DHAP and NaCN was investigated (Tables 5 and 21).

Under normal incubation conditions, some of the labelled G3P used in our experiments was oxidized by mitochondrial glycerophosphate oxidase to produce labelled DHAP. Thus, if the DHAP pathway would have been operative to a measurable extent, labelled acyl-DHAP or monoacyl-sn-G3P should have been produced. However, we could not detect the formation of labelled acyl-DHAP using TLC. It was previously found that 3 mM of NaCN (the concentration used in the experiments of Tables 5 and 21) inhibited the oxidation of exogenous and endogenous reduced pyridine nucleotides (Colli *et al.*, 1969); this might slow down the reaction rate of acyl-DHAP reduction. Furthermore, cyanide had an inhibitory effect on the DHAP pathway (Hajra *et al.*, 1968). Accordingly, in the presence of cyanide, sn-G3P can not be oxidized to form DHAP, and hence the production of labelled acyl-DHAP and monoacyl-sn-G3P from labelled G3P via the DHAP pathway would be decreased. The results shown in Table 5, however, demonstrated that the acylation of sn-G3P into mitochondrial lipids (32-42 % of the total acylation products was monoacyl-sn-G3P as shown in Fig. 13 and Table 13) was not decreased by the addition of NaCN, indicating that the DHAP pathway did not operate to a

measurable extent.

Under the experimental conditions of Table 21, in which DHAP (non-labelled), NADPH (or NADH), and NaCN are present in the assay mixture for the acylation of sn-G3P to mono- and diacyl-sn-G3P, and assuming that the DHAP pathway is of a detectable magnitude, the following possibilities exist: (1) Cyanide at a concentration of 0.5 mM was reported to give 96 % of inhibition on the DHAP pathway in guinea pig liver mitochondria (Hajra *et al.*, 1968; Hajra and Agranoff, 1968a). If cyanide at the concentration given in the experiment of Table 21 (3.0 mM) blocks the DHAP pathway completely and the mitochondrial glycerophosphate oxidase is reversible, the added excess NADH and DHAP would force the glycerophosphate oxidase reaction to glycerophosphate formation, thereby diluting labelled sn-G3P and resulting in a decreased formation of labelled mono and diacyl-sn-G3P. If, however, cyanide blocks the DHAP pathway completely, but the mitochondrial oxidase is irreversible, the results would be no DHAP and no acyl-DHAP formed from sn-G3P, and thus a decreased labelling of monoacyl-sn-G3P. (2) If cyanide at the concentration of 3 mM does not block the DHAP pathway completely, some

non-labelled monoacyl-sn-G3P could be formed via this pathway in the presence of NADPH (Table 21), thus decreasing labelled diacyl-sn-G3P formation by the dilution of its precursor, labelled monoacyl-sn-G3P. Hajra (1968b) reported that when ^{14}C -palmitoyl-CoA (0.05 mM) served as the only acyl donor, the yields of diacyl-sn-G3P from DHAP and sn-G3P were similar in guinea pig liver. When linoleoyl-CoA (non-labelled, 0.05 mM) was present in the assay mixture, more ^{14}C -palmitoyl-CoA was incorporated into diacyl-sn-G3P from DHAP than from sn-G3P. If the competition between the two pathways for available acyl-CoAs, as described by Hajra (1968b), does exist in heart mitochondria, and if the rate of the sn-G3P reaction is influenced by the accumulation of its products (labelled and unlabelled monoacyl-sn-G3P), the labelled monoacyl-sn-G3P production may decrease. However, two points should be mentioned: i. The K_m value of acyl-CoA for G3P acyltransferase is low (Abou-Issa and Cleland, 1969; Zahler and Cleland, 1969), and the FA used to generate acyl-CoA in the experiment of Table 21 is in excess. ii. The monoacyl-sn-G3P acyltransferase reaction was reported to be more active than was the G3P acyltransferase reaction (Barden and Cleland, 1969), so that monoacyl-

sn-G3P may be used as soon as it is formed for the synthesis of diacyl-sn-G3P. The results shown in Table 21 demonstrated that the incorporation of sn-G3P into mitochondrial, monoacyl- and diacyl-sn-G3P was unaltered in the presence of DHAP (non-labelled), NADPH (or NADH), and NaCN (3.0 mM), indicating that the activity of the DHAP pathway in rabbit heart mitochondria under the selected experimental conditions is of an undetectable magnitude.

A direct measurement of the formation of acyl-DHAP from ^{32}P -DHAP by rat heart mitochondria was reported to be 5.28 nmoles/hr per mg of protein (LaBelle and Hajra, 1972). Since DHAP is extremely labile (Hajra *et al.*, 1968; Benne and Proulx, 1971) and hence is easily destroyed during mild alkaline hydrolysis, a direct measurement of the formation of acyl-DHAP was not used as a method for confirming the non-existence of acyl-DHAP before hydrolysis or during the G3P acyltransferase assay period. An elegant experimental approach, utilizing $[2\text{-}^3\text{H}]$ - and $[1\text{-}^{14}\text{C}]$ -labelled glycerol with liver slices, was recently proposed (Manning and Brindley, 1972). This new approach may produce a different result regarding the DHAP pathway in heart tissue, although a direct application of this method is difficult

because the glycerophosphate shuttle pathway, glycerophosphate oxidase, and glycerol kinase in the normal cardiac mitochondria are all not very active (Safer et al., 1971).

IV-1-9. Fatty acid and positional specificity of acyl-transferase reactions

IV-1-9-i. Fatty acid specificity of monoacyl- and diacyl-sn-glycerol-3-phosphate formation by mitochondrial and microsomal fractions

FA analysis of glycerolipids in mammalian organs has shown unequivocally that the distribution of FAs is stereospecific (Van Deenen, 1965; Lands, 1965; Hill and Lands, 1970), that is, the saturated FAs such as palmitate and stearate are preferentially esterified at position 1, while unsaturated FAs such as oleate and linoleate are esterified at position 2 of the glycerol molecule. The G3P acyltransferase reaction of heart mitochondrial and microsomal fractions indeed demonstrates some substrate specificity. The amounts of the two products, monoacyl- and diacyl-sn-G3P, formed from various concentrations of palmitate, oleate, and linoleate, are shown in Figs. 27 to 30 and Tables 22 to 25. The incorporation of sn-G3P into monoacyl-sn-G3P (Fig. 27 and Table 22) and diacyl-sn-G3P (Fig. 29 and Table 24)

by the mitochondrial enzyme was highest between 0.5 and 1.0 mM oleate, exhibiting a broad optimum. In contrast, the optimal concentration of oleate for the production of monoacyl-sn-G3P (Fig. 28 and Table 23) and diacyl-sn-G3P (Fig. 30 and Table 25) in microsomes was 1.0 mM. With exogenous palmitate as acyl donor, the precursor-product relationship in the mitochondrial fraction showed a slightly different pattern from that with oleate, i.e., the rate of acylation was lower with palmitate (Fig. 27 and Table 22; Fig. 29 and Table 24) and was almost identical between the concentrations of 0.5 and 2.0 mM (Fig. 27 and Table 22).

The patterns of mitochondrial and microsomal monoacyl- and diacyl-sn-G3P formation from linoleate were quite different from those with other FAs (Figs. 27 to 30; Tables 22 to 25). When linoleate served as an acyl donor for the mitochondrial fraction, it exhibited the lowest rate of acylation among the three FAs tested (Fig. 27 and Table 22; Fig. 29 and Table 24). In contrast, the rates of incorporation of linoleate into microsomal phosphatides were highest (Fig. 28 and Table 23; Fig. 30 and Table 25). This is true for the formation of both monoacyl- and diacyl-

sn-G3P.

In the formation of microsomal monoacyl-sn-G3P, a strong affinity was shown for linoleate (Fig. 28 and Table 23), whereas mitochondrial monoacyl-sn-G3P had a preference for oleate (Fig. 27 and Table 22). In addition, the specificity for linoleate was more marked in microsomal diacyl-sn-G3P biosynthesis than in mitochondrial diacyl-sn-G3P formation (Fig. 29 and Table 24; Fig. 30 and Table 25). The peak observed was not due to the effect of different molar ratios of FA to albumin at various FA concentrations, although the concentration of albumin was constant in these experiments. Similar patterns of the activities versus various substrate concentration curves were recorded when the molar ratio of FA to albumin was kept constant at each different FA concentration (data not shown). In another series of experiments, as has already been explained (Experiment 1 of Table 19; Section IV-1-9-i), it was found that the biosynthesis of monoacyl-sn-G3P by heart mitochondrial fraction was greater when oleate, rather than palmitate, was used. In summary, the shapes of the activity-substrate concentration curves of the mitochondrial enzymes are not identical to those of the microsomal enzymes. (Figs.

27 to 30). The results thus support the previous conclusion that the mitochondrial acylating system differs from the microsomal system.

Some investigators working with liver mitochondria observed a more prominent substrate preference in monoacyl-sn-G3P production (Monroy et al., 1972; Daae, 1972), that is, an almost exclusive acylation of palmitate at position 1. According to Monroy et al. (1972), the effectiveness of oleyl- or linoleyl-CoA as an acyl donor in the formation of monoacyl-sn-G3P in liver mitochondria was never more than 15 % of that observed with palmitoyl-CoA. Daae (1972) used acylcarnitine esters plus other cofactors as an acyl donor system and observed that the acylation activity of unsaturated acylcarnitine esters such as oleyl-, linolenyl-, ~~linoleyl~~-, stearoyl-, and arachidonylcarnitine was not more than 27 % of palmitoylcarnitine acylation activity during the first acylation reaction of sn-G3P. The G3P acyltransferase reaction was found to be more selective than the monoacyl-sn-G3P acyltransferase reaction in rat liver microsomes (Lands and Hart, 1964; Barden and Cleland, 1969; Lamb and Fallon, 1970; Okuyama et al., 1971; Okuyama and Lands, 1972; Yamashita and Numa, 1972; Yamashita et al., 1973), that

is, microsomal monoacyl-sn-G3P formation showed a strong preference toward saturated rather than unsaturated FAs, while the FA specificity for diacyl-sn-G3P formation was less pronounced.

The biosynthesis of monoacyl-sn-G3P and diacyl-sn-G3P by cardiac mitochondrial preparation, with either palmitate or oleate as the substrate, was examined at various time-intervals in other experiments (Figs. 31 and 32; Table 26). These results demonstrated that oleate was incorporated into monoacyl- and diacyl-sn-G3P in heart mitochondrial preparation as effectively as was palmitate, thus confirming the data shown in Tables 19, 22, and 24 and Figs. 27 and 29 that oleate was acylated into mitochondrial monoacyl- and diacyl-sn-G3P to a similar or even greater extent than was palmitate. Therefore, these findings (Figs. 27 to 32; Tables 19 and 22 to 25) demonstrated that the substrate specificity of cardiac particulate enzymes during the sn-G3P acylation reaction is not as pronounced as that of the hepatic particulates. Hepatic mitochondria acylated palmitoyl-CoA 50 times faster than oleyl-CoA to form monoacyl-sn-G3P (Monroy *et al.*, 1972). The rate of acylation of palmitoyl-CoA was 2 to 4 times greater than the

acylation of oleyl-CoA into liver microsomal monoacyl-sn-G3P (Lamb and Fallon, 1970; Yamashita and Numa, 1972).

Cardiac mitochondria, in contrast, utilized unsaturated FAs extremely well in comparison to hepatic mitochondrial and microsomal fractions. Therefore, preference for individual FAs in heart mitochondrial and microsomal monoacyl-sn-G3P and diacyl-sn-G3P formation is quite different from that reported for liver particulate fractions.

IV-1-9-ii. The positional specificity of monoacyl-sn-glycerol-3-phosphate biosynthesis by cardiac mitochondrial fraction

It was previously reported that palmitate was the most active substrate for the first step of the sn-G3P acylation process in liver mitochondria (Monroy et al., 1972), and that it was almost exclusively esterified at position 1 of the monoacyl-sn-G3P molecule (Monroy et al., 1972; Daae, 1972). Similarly, liver microsomes acylated palmitoyl-CoA most actively, with the only esterified product being 1-monoacyl-sn-G3P (Yamashita and Numa, 1972). Our results described above demonstrated that oleic and linoleic acids were as effective as palmitate as substrates for the formation of monoacyl-sn-G3P by heart mitochondria, indicating

that the substrate specificity for G3P acyltransferase in heart differed from that in the liver.

There are three possible situations which may occur during the first step of the sn-G3P acylation reaction in heart mitochondria: (1) Only 1-monoacyl-sn-G3P is formed as the product of the sn-G3P acylation reaction regardless of the nature of the acyl donor, (2) both 1- and 2-monoacyl-sn-G3P are formed as products of sn-G3P acylation, the position of the acyl moiety in the monoacyl-sn-G3P molecule depending upon the nature of the acyl donor, or (3) both 1- and 2-monoacyl-sn-G3P are formed from the same acyl donor, the acyl moiety being interchangeable between the 1 and 2 positions of the glycerol molecule. Since the substrate specificity for G3P acyltransferase of heart mitochondria is not marked, and since it is well known that most saturated FAs are localized at position 1 of the glycerol molecule, situation (2) stated above would most probably occur during the 1st step of the sn-G3P acylation reaction in cardiac mitochondrial fraction. In order to test this contention, the positional specificity of the first acylation reaction of sn-G3P in heart mitochondrial preparation was analyzed.

There are two methods of studying this problem. In the first method, monoacyl-sn-G3P synthesized by heart mitochondrial enzyme is exposed to the hydrolytic reaction of phosphatidate phosphohydrolase. The resultant 1- and 2-MGs are separated and quantified using TLC. In the second method, snake venom phospholipase A2 is used to deacylate monoacyl-sn-G3P. The resultant sn-G3Ps, FAs, and monoacyl-sn-G3Ps are isolated and quantified. In this study, the method with phosphatidate phosphohydrolase was applied as described in the "MATERIALS AND METHODS" (Section III-2).

Monoacyl-sn-G3P synthesized by heart mitochondrial preparation was hydrolyzed with phosphatidate phosphohydrolase isolated from rat liver lysosomes. The hydrolytic products, 1- and 2-MGs, were separated by TLC. The radioactive scanning of the developed TLC plates revealed three peaks (Fig. 33). The first peak, which appeared at the origin, was unreacted monoacyl-sn-G3P. The R_F values of the other two peaks corresponded to those of authentic (Applied Science Laboratory) 1-MG (0.54) and 2-MG (0.63). No radioactivity was detectable in the area corresponding to DG, indicating that the isolated monoacyl-sn-G3P was free of diacyl-sn-G3P contamination.

The relative proportion of 1- and 2-MGs derived from 1- and 2-monoacyl-sn-G3P synthesized by the heart mitochondrial preparation when three different FAs and one acyl-CoA were used individually as acyl donors for monoacyl-sn-G3P synthesis is shown in Table 27 and Fig. 34. Palmitic acid, as well as palmitoyl-CoA, was preferentially, but not exclusively, acylated at position 1 of the glycerol molecule. That is, approximately 2/3 of the monoacyl-sn-G3P contained palmitate at position 1, and 1/3 contained it at position 2. In contrast, 2/3 of the monoacyl-sn-G3P containing oleic acid was 2-oleoyl-sn-G3P, and the remaining 1/3 was 1-oleoyl-sn-G3P. Linoleic acid showed an almost equal distribution at positions 1 and 2. Approximately 64 to 78 % of the radioactive monoacyl-sn-G3P was hydrolyzed by the phosphohydrolase, indicating that the results of this analysis can be taken to represent the original composition of monoacyl-sn-G3P.

To add greater support to this assumption, a second hydrolysis by phosphatidate phosphohydrolase was performed on the remaining, unhydrolyzed monoacyl-sn-G3P containing 22 to 36 % of the starting material. The repetition of hydrolysis by means of phosphatidate phosphohydrolase produced results which resemble those of the first hydrolysis

(Table 28). Since the extent of hydrolysis by these enzyme preparations is approximately 70 %, and the recovery of monoacyl-sn-G3P is about 80 %, a third hydrolysis would probably produce a larger technical error without resulting in a complete hydrolysis of the original monoacyl-sn-G3P. As shown in Table 28, the overall hydrolysis was 85 to 86 %, which is higher than the value obtained by Lamb and Fallon (1970), Daae (1972), Yamashita and Numa (1972), similar to that obtained by Monroy et al. (1972), and slightly lower than that reported by the most recent work of Monroy et al. (1973).

These results are in direct contrast to those obtained with liver particulates in which palmitate was almost exclusively esterified to the 1-position (Possamayer et al., 1969; Daae, 1972; Monroy et al., 1972). Furthermore, a very small quantity of palmitate was acylated to position 2 during the first step of the sn-G3P acylation process in liver (Monroy et al., 1972). Recent studies with partially purified hepatic enzymes confirmed the results of those with hepatic sub-cellular fractions (Yamashita and Numa, 1972; Monroy et al., 1973).

The results under Section IV-1-9-i showed that the

de novo synthesis of monoacyl-sn-G3P by rabbit heart mitochondrial fraction occurred to a similar extent with palmitic, oleic, and linoleic acid as acyl donors. It was postulated that, contrary to hepatic enzymes, the heart enzyme is capable of acylating both positions 1 and 2 of the sn-G3P moiety with little discrimination. The results in this section (IV-1-9-ii) confirmed this postulate and support the view that both the substrate and positional specificity of cardiac G3P acyltransferase clearly differ from those of the liver enzyme, and that the enzyme appears to be organ-specific. In other words, the enzyme of the heart mitochondrial fraction can acylate both 1 and 2 positions of the glycerol moiety of sn-G3P, whereas the enzyme of the liver mitochondria appears to acylate only the 1-position of sn-G3P. The cardiac enzyme can acylate linoleate, whereas the hepatic enzyme can not. Positional specificity of diacyl-sn-G3P formation in the brain (Baker and Thompson, 1972) appears to be similar to that found in this study which dealt with monoacyl-sn-G3P biosynthesis by heart mitochondria and supports the organ-specificity hypothesis. The second acylation, that is, the reaction catalyzed by monoacyl-sn-G3P acyltransferase, is also known to be selective with respect to individual FAs

in the liver (Hill and Lands, 1970), but no data has been available on the heart enzyme.

Although acyl-CoA synthetase has been reported to have some substrate specificity in liver microsomes as described by Marcel and Suzue (1972), the reported V_{max} for this enzyme is extremely high (151.5-306.6 nmoles/min per mg protein). Furthermore, acyl-CoA synthetase activity is five times greater than the G3P acyltransferase activity in liver mitochondria, i.e., 80.3 nmoles/min per mg protein for acyl-CoA synthetase versus 16.0 nmoles/min per mg protein for G3P acyltransferase (Sánchez *et al.*, 1973). In a separate experiment, the activity of acyl-CoA synthetase in heart mitochondria is reported to be higher than that in liver mitochondria (71 versus 47 nmoles/min per mg of mitochondrial protein) (Pande, 1971). Therefore, it is unlikely that the results described in this study regarding the substrate and positional specificity of G3P acyltransferase in heart mitochondria are influenced by the specificity of acyl-CoA synthetase. The results shown in Table 27 demonstrated that localization of the acyl moiety at the 1 and 2 positions of the monoacyl-sn-G3P molecule was identical when either palmitate (plus necessary cofactors) or palmitoyl-

CoA served as an acyl donor. This suggests that the observed positional specificity was not modified by the specificity of acyl-CoA synthetase.

It would be desirable to analyze the FA distribution in TG in the heart in order to clarify our hypothesis that both positions 1 and 2 of sn-G3P can be acylated equally by both saturated and unsaturated FAs in cardiac mitochondria. To my knowledge, very little work has been done in this area. Christie and Moore (1970) examined pig organs in detail and found a striking difference in FA distribution between hepatic and cardiac TG. Liver TG contained 41.6 % palmitate and 28.1 % oleate and linoleate at position 1, with 16.6 % palmitate and 65.2 % oleate and linoleate at position 2. Heart TG had 12.7 % palmitate and 55.8 % oleate and linoleate at position 1, with 64.8 % palmitate and 22.5 % oleate and linoleate at position 2. In other words, cardiac TG contained considerably less palmitic acid, but more oleic and linoleic acids, at position 1 than did the liver TG (Christie and Moore, 1970). In addition, it was reported earlier that the distribution of labelled palmitic acid in positions 1 and 2 of lecithin in the isolated perfused heart was dissimilar to that in the liver following the

injection of labelled palmitic acid (Stein and Stein, 1963). Palmitic acid was distributed evenly between the 1 and 2 positions of heart lecithin (46-50 % versus 50-54 % for the 1 and 2 positions, respectively), while it was almost completely confined to the 1 position of the liver lecithin molecule (85-88 % versus 12-15 % for the 1 and 2 positions, respectively). Thus, these results dealing with heart tissue are comparable to those obtained in our study.

Hill and Lands (1970) reported that rearrangement and desaturation of FA moieties in liver PLs takes place after diacyl-sn-G3P has been formed. A similar rearrangement and desaturation probably takes place after the formation of diacyl-sn-G3P, DG, and TG in heart tissue. This is implicated by some of our results (Table 12) which show that [^{14}C]palmitate is incorporated into diacyl-sn-G3P to a greater extent than is sn-[^{14}C] G3P. Furthermore, G3P and monoacyl-sn-G3P acyltransferases of heart subcellular fractions that were found in my work showed a rather indistinct substrate specificity (Figs. 27-32; Tables 22-26), suggesting that the initial acylation reaction of sn-G3P takes place in both positions 1 and 2 of the glycerol moiety. Thus, the low degree of non-random FA distribution found in the tissue lipids (Tables

27 and 28; Fig. 34) must be explained by assuming the existence of active exchange of acyl moieties in complex lipids. It should be noted at this point that in experiments utilizing non-cell-free systems, such as perfused heart or heart slices, very little diacyl-sn-G3P accumulates, and the amount of labelling of diacyl-sn-G3P is relatively small (Stein and Stein, 1963; unpublished observation). The conditions for FA acylation existing in such non-cell-free systems, therefore, seems to be quite different from those adopted in our experiments with mitochondrial and microsomal fractions.

LEGEND TO TABLE 5

The effects of substances omitted from or added to the incubation mixture on the activity of acyl-CoA:sn-glycerol-3-phosphate-O-acyltransferase from heart mitochondria

Heart mitochondrial fractions were isolated by differential centrifugation. The assay mixture of the control experiments contained 100 μ moles Tris base adjusted to pH 7.4 with phosphoric acid, 2.0 μ moles potassium palmitate, 0.8 μ mole CoA, 12 μ moles ATP, 6.0 μ moles $MgCl_2$, 20 mg fatty acid-poor bovine serum albumin, 6.0 μ moles sn-[U- ^{14}C]glycerol-3-phosphate (12 μ moles rac-glycerol-3-phosphate) containing 350,000 d.p.m./ μ mole, and water in a final volume of 2.0 ml. After 10 min incubation at 37° C, the reaction was stopped, and lipids were extracted by butanol as described in Sections III-1-4 and III-1-6. A portion of the butanol phase was used to determine the radioactivity. The rate of acylation is expressed as nmoles sn-glycerol-3-phosphate incorporated per mg min. Each value represents the average of three assays, in which mitochondrial preparations contained 2.42 mg, 2.50 mg, and 2.61 mg protein, respectively. All experiments were carried out in air except for the one indicated as N₂ in which the incubation flask was flushed with nitrogen gas throughout the incubation period.

TABLE 5

The effects of substances omitted from or added to the incubation mixture on the activity of acyl-CoA:sn-glycerol-3-phosphate-0-acyltransferase from heart mitochondria

Condition	<u>sn-glycerol-3-phosphate</u> incorporated
	nmols/mg min
Experiment 1 (omission)	
None (Complete system) ----	0.52
CoA -----	0.07
ATP -----	0
MgCl ₂ -----	0.33
Albumin -----	0.50
Palmitate -----	0.15
Experiment 2 (addition)	
None (complete system) ----	0.53
None + N ₂ -----	0.52
NaF (3mM) -----	0.52
CTP (5mM) -----	0.52
L-carnitine chloride (2 mM)	0.40
NADPH (5 mM) + NaCN (3 mM)	0.54
NADH (3 mM) -----	0.48

LEGEND TO FIGURE 8

A comparison of the rates of acylation of *sn*-glycerol-3-phosphate by rabbit heart microsomal fraction using endogenous fatty acids, palmitate, or albumin-bound palmitate as the acyl donor

The incubation mixture and assay procedure are described in the legend to Table 5. When endogenous fatty acids were used as the acyl donor, palmitate was omitted from the assay mixture. Albumin was present only in the experiments in which albumin-bound palmitate served as the acyl donor. The molar ratio of palmitate to albumin was 2. The amount of microsomal protein was 1.14 mg and 0.92 mg, respectively, for two experiments. The ordinate represents the incorporation of *sn*-glycerol-3-phosphate into lipids in nmoles/mg. The abscissa indicates the incubation time in min.

- — ○ : indicates the results obtained when endogenous fatty acids were used as acyl donors,
- — □ : indicates the results obtained when albumin-bound palmitate served as the acyl donor,
- ▲ — ▲ : indicates the values obtained in the absence of albumin, but presence of palmitate.

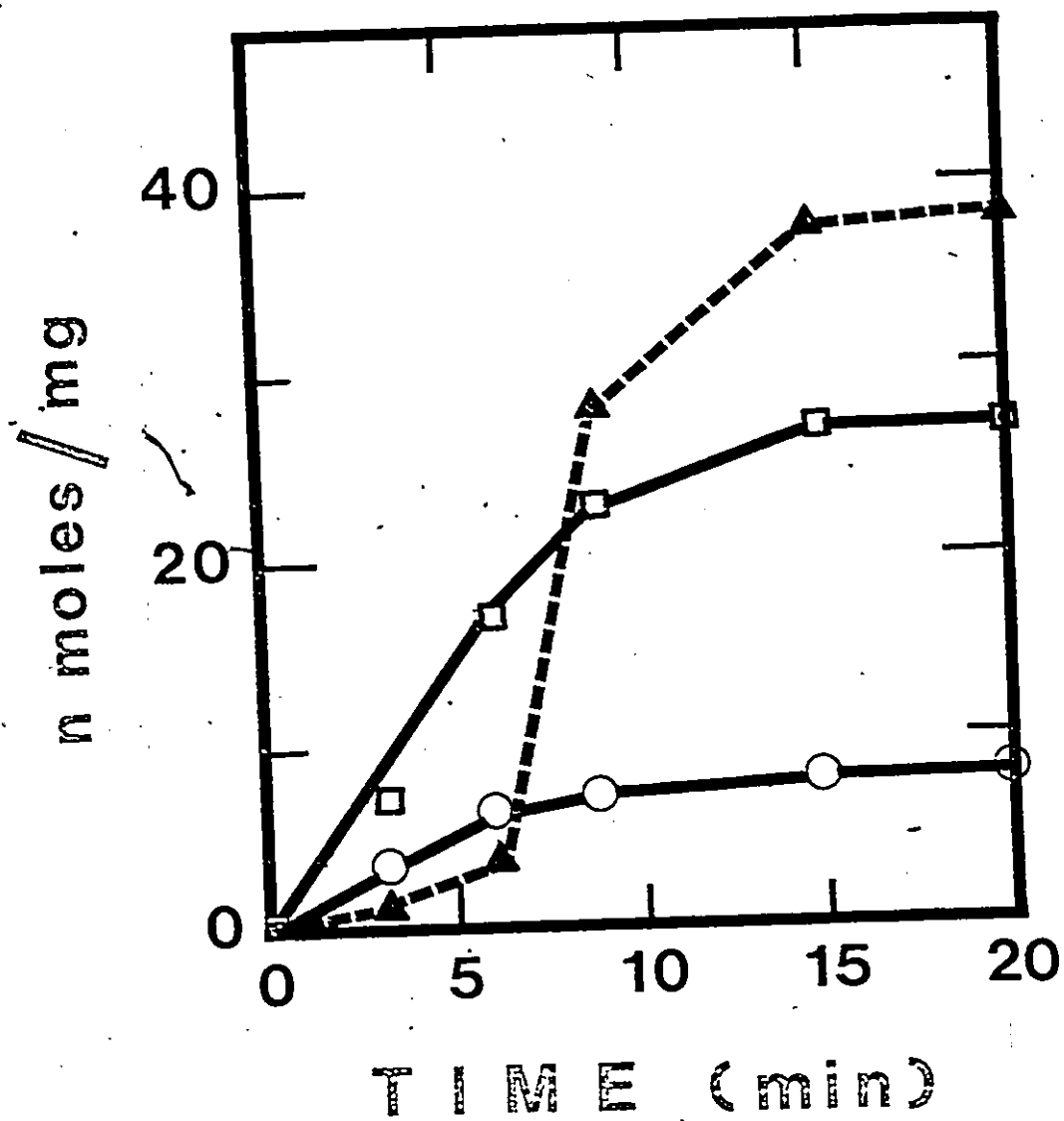
FIGURE 8

TABLE 6

A comparison of the rates of acylation of sn-glycerol-3-phosphate by rabbit heart microsomal fraction using endogenous fatty acids, palmitate, or albumin-bound palmitate as the acyl donor

Acyl donor	Incubation time				
	3 min	6 min	9 min	15 min	20 min
	nmoles/mg				
Endogenous	3.0	6.0	7.5	8.0	8.2
Palmitate	1.7	4.0	28.0	37.5	38.0
Albumin-bound palmitate	7.6	17.0	23.2	25.5	25.4

For the experimental details, see the legend to the previous figure.

LEGEND TO FIGURE 9

The relationship between different amounts of mitochondrial proteins and the incorporation of sn-[U-¹⁴C]glycerol-3-phosphate into lipids with two different acyl donor systems

The composition of the incubation mixture and the assay procedure are described in the legend to Table 5 and in Sections III-1-4 and III-1-6. In this experiment, two different acyl donor systems were used, namely, either potassium palmitate (2.0 μ moles), CoA (0.8 μ mole), and ATP (12 μ moles) were used as an acyl donor system ($\blacktriangle \longrightarrow \blacktriangle$), or palmitoyl-CoA (0.8 μ mole) was used ($\triangle \longrightarrow \triangle$). The incubation time was 8 min. The ordinate indicates the rate of acyltransferase reaction in nmoles/min, and the abscissa indicates the amounts of mitochondrial protein in mg.

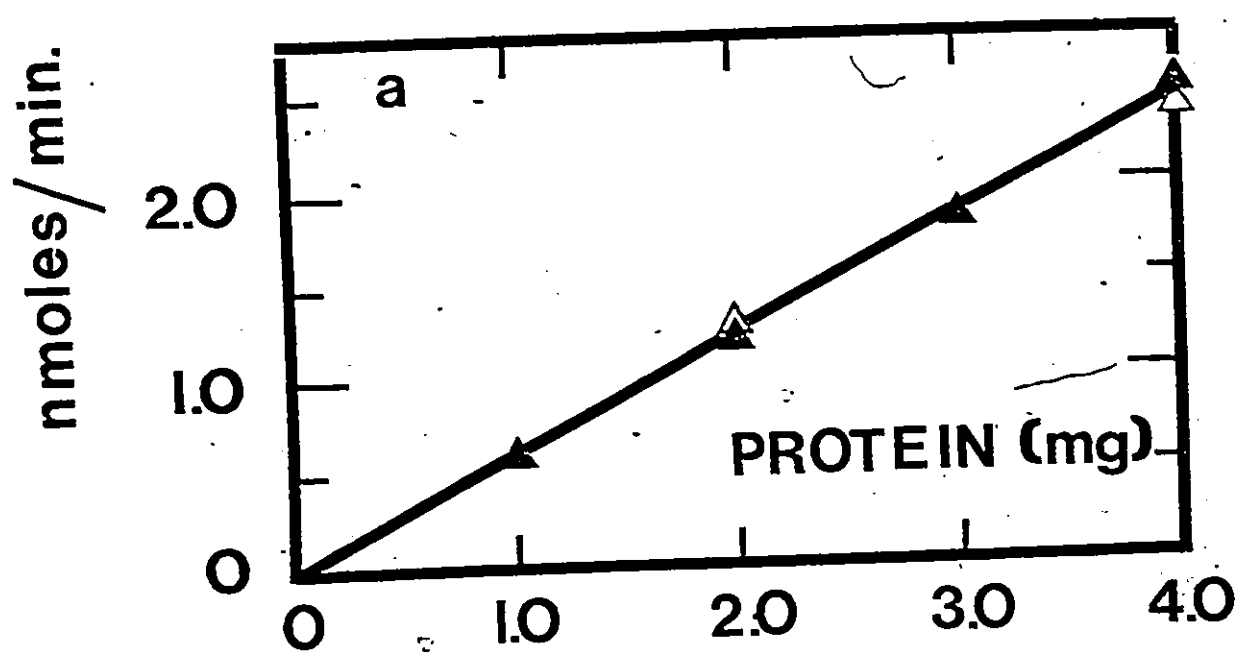


FIGURE 9

TABLE 7

The relationship between different amounts of mitochondrial proteins and the incorporation of sn-[U-¹⁴C]glycerol-3-phosphate into lipids with two different acyl donor systems

Acyl donor system	Amount of mitochondrial fraction	sn-glycerol-3-phosphate incorporated
	mg protein ^m	nmoles/min
Palmitate, CoA, and ATP	0	0
	1.0	0.6
	2.0	1.1
	3.0	1.8
	4.0	2.5
Palmitoyl-CoA	0	0
	2.0	1.2
	4.0	2.4

For the experimental procedure, see the legend to

Fig. 9.

LEGEND TO FIGURE 10

The relationship between various amounts of microsomal protein and the acylation of sn-glycerol-3-phosphate

The experimental conditions and the assay procedure were described in the legend to Table 5 and in Sections III-1-4 and III-1-6. The incubation time was 5 min. The ordinate indicates the rate of incorporation of sn-[U-¹⁴C]-glycerol-3-phosphate into lipids in nmoles/min. The abscissa represents the amounts of microsomal protein in mg.

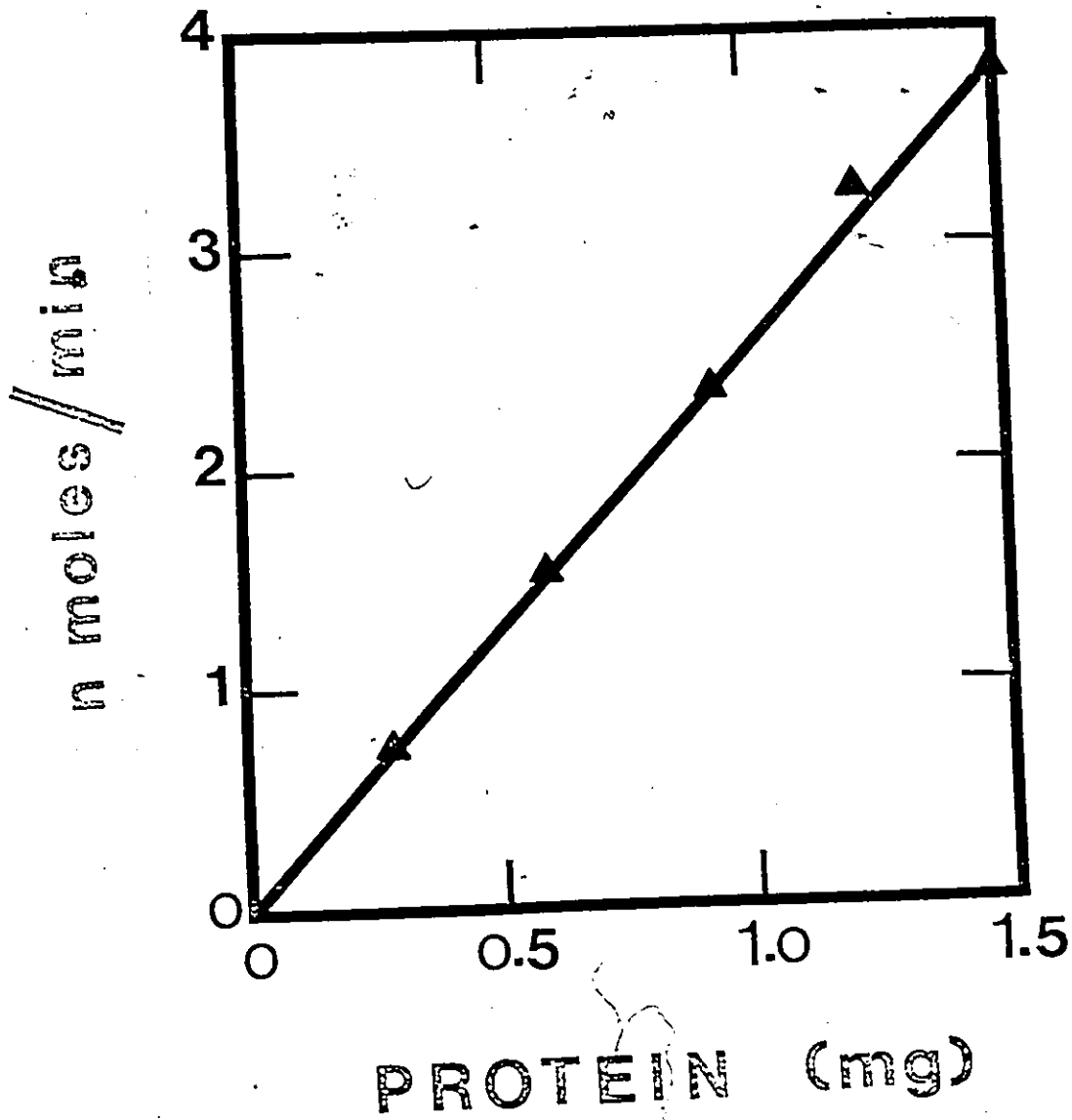
FIGURE 10

TABLE 8

The relationship between various amounts of microsomal protein and the acylation of *sn*-glycerol-3-phosphate

Amount of microsomal protein	Incorporation of <i>sn</i> -glycerol-3-phosphate
mg	nmoles/min
0	0
0.3	0.8
0.6	1.6
0.9	2.3
1.2	3.3
1.5	3.8

For the experimental details, see the legend to Fig. 10.

LEGEND TO FIGURE 11

The effect of various concentrations of L-palmitoyl carnitine on the acylation of *sn*-glycerol-3-phosphate in the microsomal fraction of rabbit heart

The experiments were carried out in the same manner as described in the legend to Table 5, except that (-)-palmitoyl carnitine was used as the acyl donor. The incubation time was 6 min, the temperature was 30° C, and the gas phase was air. The amounts of microsomal enzyme were 0.34, 0.5, and 0.6 mg, respectively, for three experiments. The ordinate indicates the rate of the acylation reaction in nmoles/mg min, and the abscissa represents the various concentrations of (-)-palmitoyl carnitine.

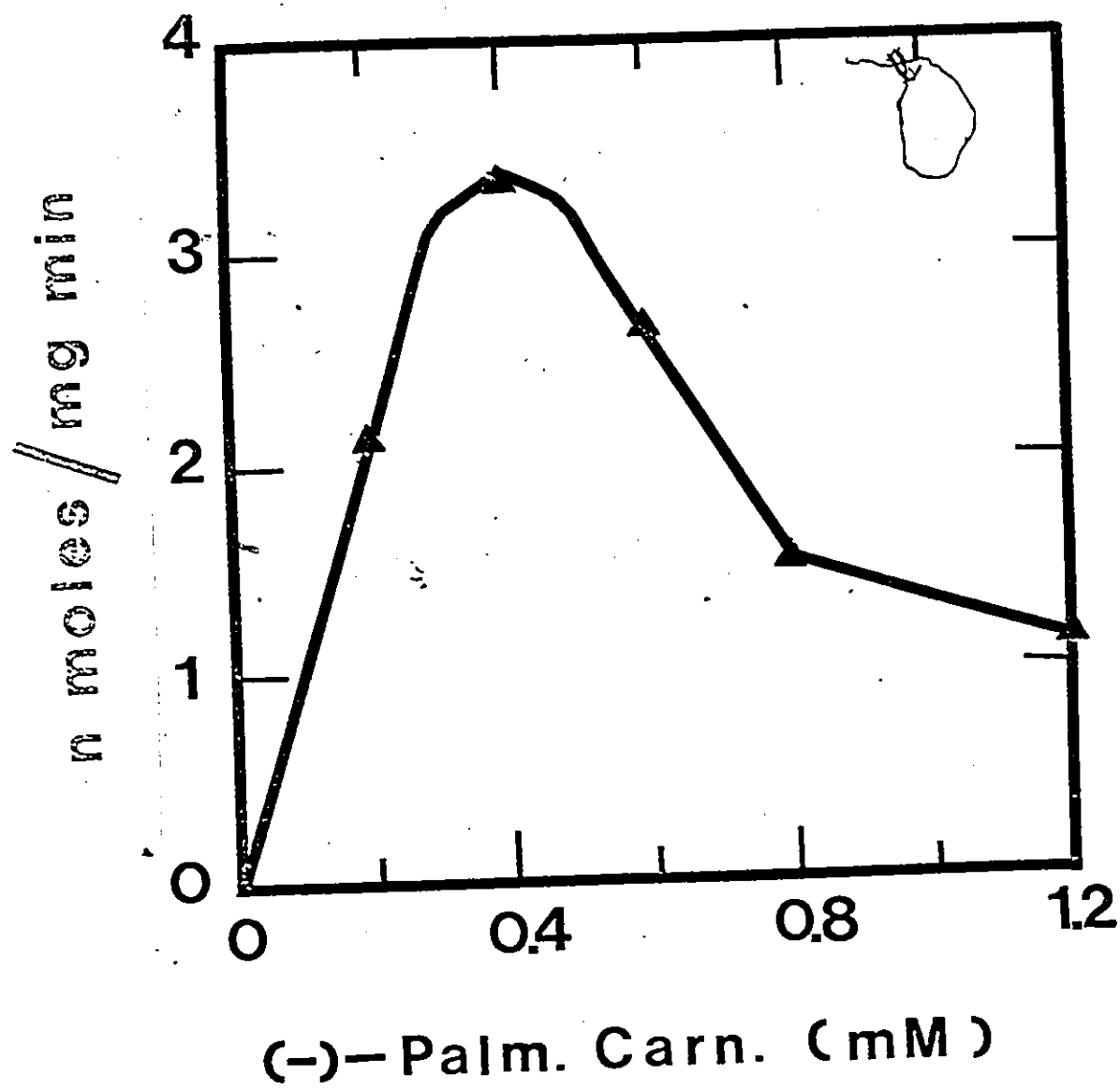


FIGURE 11

TABLE 9

The effect of various concentrations of (-)-palmitoyl
carnitine on the acylation of sn-glycerol-3-phosphate
in the microsomal fraction of rabbit heart

Concentration of palmitoyl carnitine	Incorporation of <u>sn</u> -glycerol- 3-phosphate into lipids
mM	nmoles/mg min.
0	0
0.2	2.2
0.4	3.3
0.6	2.6
0.8	1.5
1.2	1.1

The experimental details were described in the legend
to Fig. 11.

LEGEND TO FIGURE 12

The influence of different concentrations of palmitoyl-CoA on the acylation of *sn*-glycerol-3-phosphate in rabbit heart microsomal fraction

The incubation mixture, in a final volume of 2.0 ml, contained 100 μ moles Tris-phosphoric acid buffer, pH 7.4, 10 mg bovine serum albumin, 6.0 μ moles *sn*-[U- 14 C]glycerol-3-phosphate, and various concentrations of palmitoyl-CoA (mM) as indicated on the abscissa. The amounts of microsomal protein were 0.32 mg and 1.2 mg, respectively, for two experiments. The incubation time was 6 min, the temperature was 30 $^{\circ}$ C, and the gas phase was air. The reaction products were isolated and quantified as described in Section III-1-6. The activity of *sn*-glycerol-3-phosphate acylation was expressed as nmoles/mg min (ordinate).

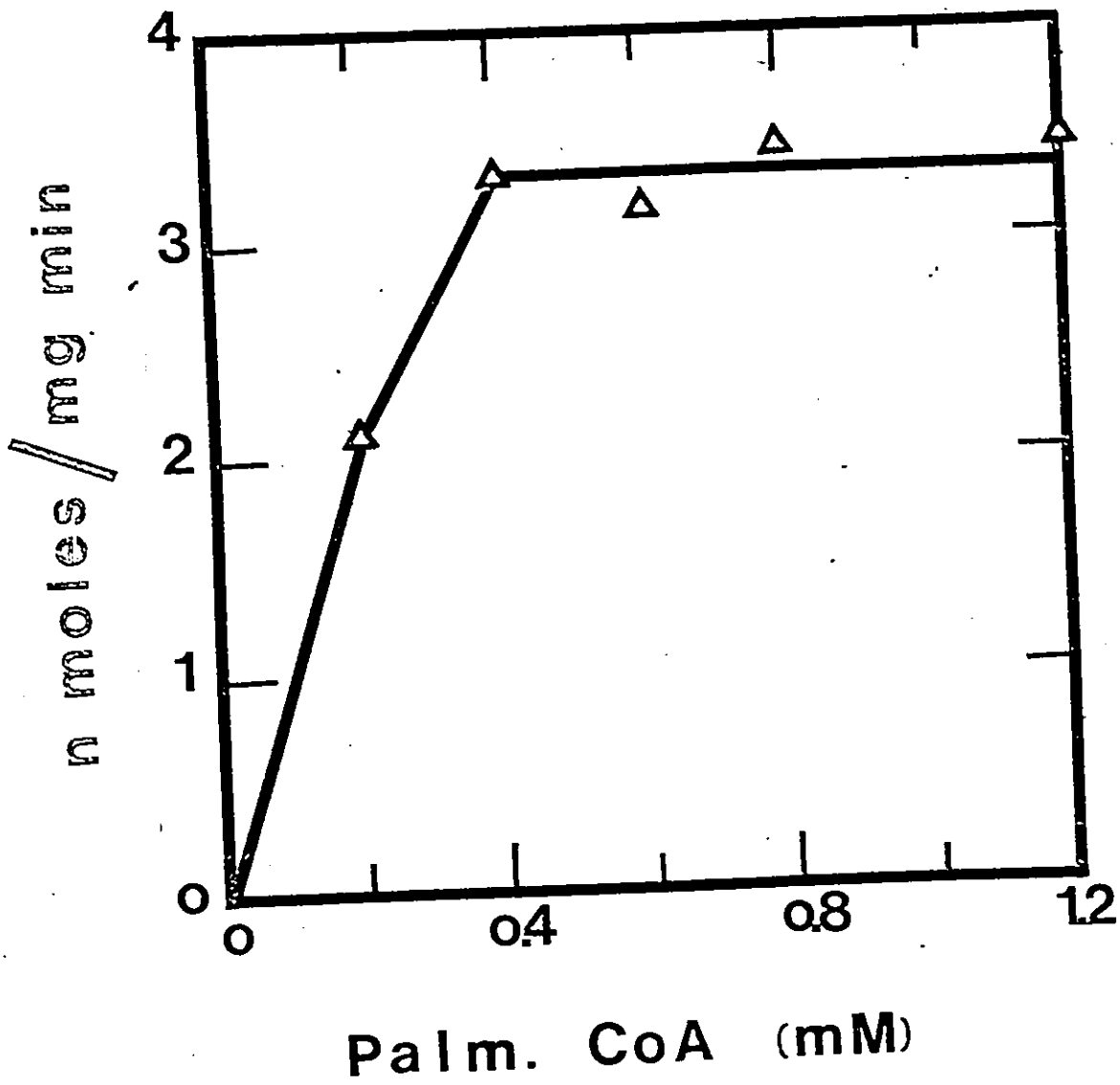
FIGURE 12

TABLE 10

The influence of different concentrations of palmitoyl-CoA on the acylation of sn-glycerol-3-phosphate in rabbit heart microsomal fraction

Concentration of palmitoyl-CoA	Incorporation of <u>sn</u> -glycerol-3- phosphate into lipids
mM	nmoles/mg min
0	0
0.2	2.2
0.4	3.3
0.6	3.2
0.8	3.4
1.2	3.4

The experimental procedure was described in the legend to the previous figure.

TABLE 11

The identification of acylation products from sn-glycerol-3-phosphate in heart mitochondrial fraction

Products	Reaction with specific stains			R _F
	Ninhydrin	Schiff-periodate	Phosphorus	
Monoacyl- <u>sn</u> -glycerol-3-phosphate	-	-	+	0.63
Diacyl- <u>sn</u> -glycerol-3-phosphate	-	-	+	0.94

The assays were carried out as described in the legend to Table 5. Monoacyl-sn-glycerol-3-phosphate and diacyl-sn-glycerol-3-phosphate were separated by TLC with chloroform-methanol-acetic acid-water (65:25:8:4, v/v/v/v). The developed plates were scanned to localize the radioactive peaks, the R_F values of which were verified by the authentic standards.

LEGEND TO TABLE 12Analysis of the products synthesized from two different
labelled precursors

In experiments with [$1-^{14}\text{C}$] palmitate, approximately 500,000 d.p.m. of [$1-^{14}\text{C}$] palmitate/ μmole replaced non-radioactive palmitate while maintaining a final concentration of 2.0 $\mu\text{moles}/2\text{ml}$. The assay procedure was described in the legend to Table 5 and in Section III-1-4. The incubation was for 10 min and contained 2.67 mg of mitochondrial protein. The unreacted, labelled palmitate was separated by TLC, and lipids were eluted with chloroform-methanol (1:1, v/v). The neutral lipid fraction was further separated into mono-, di-, and tri-glycerides with a solvent system of light petroleum (b.p. 30° - 60° C)-acetone-formic acid (38:12:1, v/v/v). The phospholipid fraction was separated into monoacyl-sn-glycerol-3-phosphate and diacyl-sn-glycerol-3-phosphate by developing the plate with chloroform-methanol-acetic acid-water (85:25:8:4, v/v/v/v). The assay with sn-[$U-^{14}\text{C}$] glycerol-3-phosphate (350,000 d.p.m./ μmole) was carried out in the same manner as the experiments described above,

(Legend to Table 12, page 2)

except that part of the butanol extract was used directly for the isolation of monoacyl-sn-glycerol-3-phosphate and diacyl-sn-glycerol-3-phosphate. The remainder was used for the separation of mono-, di-, and tri-glycerides. The recovery of phosphatides in these procedures was estimated, and the values obtained were taken into account in the calculation of the products formed.

TABLE 12

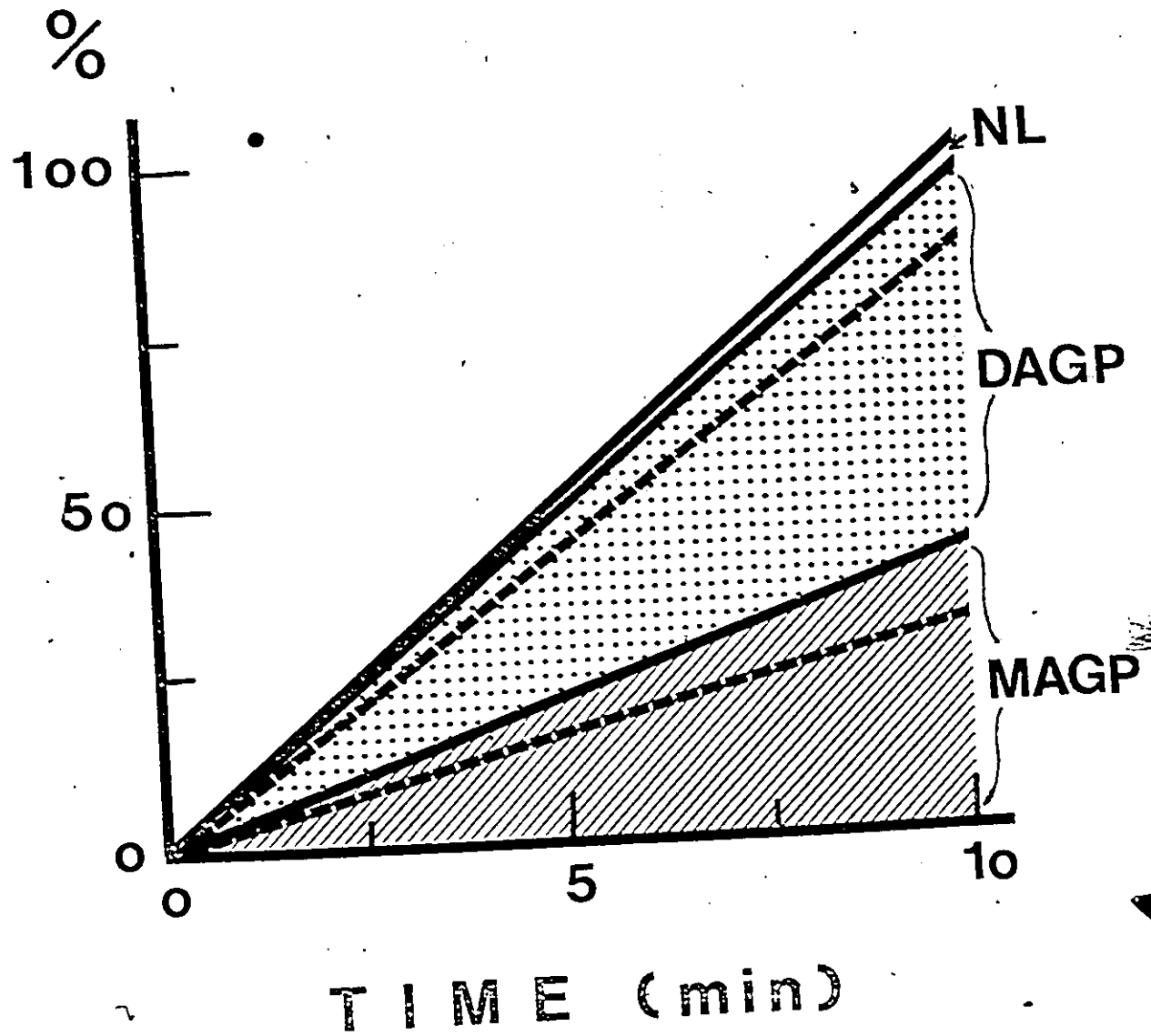
Analysis of the products synthesized from two different labelled precursors

Radioactive precursor Product	[1- ¹⁴ C] palmitate incorporated	sn-[U- ¹⁴ C]-glycerol-3-phosphate incorporated	Ratio of [¹⁴ C]-palmitate to [¹⁴ C]glycerol-3-phosphate
	nmoles/mg min		
Monoacyl-sn-glycerol-3-phosphate	0.16	0.15	1.06
Diacyl-sn-glycerol-3-phosphate	0.70	0.33	2.12
Monoglyceride	0.05	0.01	
Diglyceride	0.06		
Triglyceride	0.04		

LEGEND TO FIGURE 13

The products of *sn*-glycerol-3-phosphate acylation by the mitochondrial fraction

The standard assay conditions and procedure were described in Section III-1-4. The products were identified and separated as stated in Section III-1-6. The ordinate indicates the total acylation products in %, the abscissa the incubation time in min. MAGP, DAGP, and NL stand for monoacyl-*sn*-glycerol-3-phosphate, diacyl-*sn*-glycerol-3-phosphate, and neutral lipids, respectively. Solid lines represent the highest values, while dotted lines indicate the lowest values, obtained in 3 experiments.

FIGURE 13

LEGEND TO FIGURE 14

The products of *sn*-glycerol-3-phosphate acylation by the
microsomal fraction

The experimental conditions were described in Sections III-1-4 and III-1-6. The ordinate indicates the total acylation products in %, the abscissa the incubation time in min. MAGP, DAGP, and NL stand for monoacyl-*sn*-glycerol-3-phosphate, diacyl-*sn*-glycerol-3-phosphate, and neutral lipids, respectively. Solid lines represent the highest values, while dotted lines indicate the lowest values, obtained in 3 experiments.

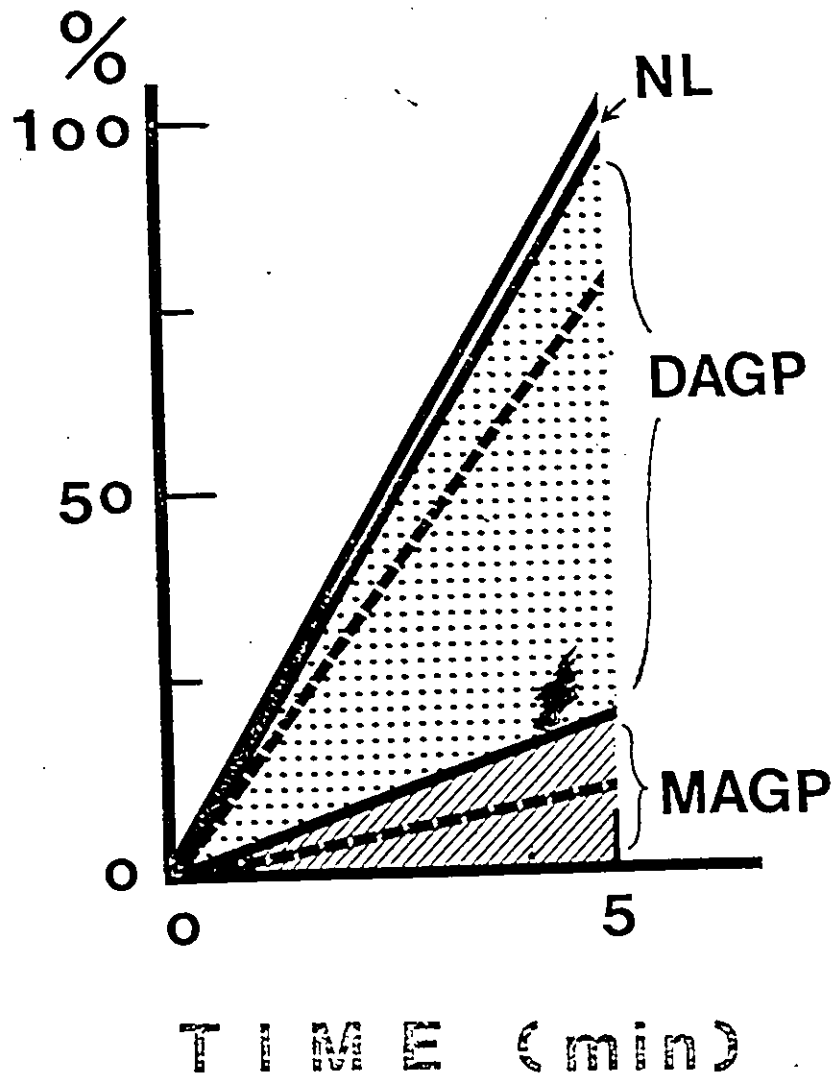


FIGURE 14

TABLE 13

The products of sn-glycerol-3-phosphate acylation by
subcellular fractions of rabbit heart

Subcellular fraction	Time	Products		
		Monoacyl- <u>sn</u> -glycerol-3-phosphate	Diacyl- <u>sn</u> -glycerol-3-phosphate	Neutral lipids
	min	% of total products		
Mitochondrial	5	16-22	23-28	1
	10	32-42	58-68	2
Microsomal	5	11-20	70-89	2
	10	-	-	-

The experimental conditions and assay procedure were described in the legends to Figs. 13 and 14.

LEGEND TO FIGURE 15

The time course of the acylation of *sn*-glycerol-3-phosphate by heart subcellular fractions

The assay mixture, in a final volume of 2.0 ml contained 100 μ moles Tris-phosphate buffer, pH 7.4, 2.0 μ moles potassium palmitate, 0.8 μ mole CoA, 12 μ moles ATP, 6.0 μ moles $MgCl_2$, 20 mg fatty acid-poor bovine serum albumin, and 6.0 μ moles *sn*-[U- ^{14}C]glycerol-3-phosphate (12 μ moles *rac*-glycerol-3-phosphate) containing 350,000 d.p.m./ μ mole. The reaction was started by the addition of enzyme preparation, and the incubation was carried out at 37° C for various time intervals as indicated on the abscissa. The reaction was stopped, and lipids were extracted by butanol (Sections III-1-4 and III-1-6). A portion of the butanol phase was used to determine the radioactivity. Each point represents the average of two assays. Amounts of enzyme used were 0.8 mg and 1.0 mg for the microsomal fraction and 2.7 mg and 2.8 mg for the mitochondrial fraction. The symbol  indicates mitochondrial acylation and  microsomal acylation. The ordinate represents the extent of *sn*-glycerol-3-phosphate incorporation into lipids in nmoles/mg and the abscissa the duration of incubation time in min.

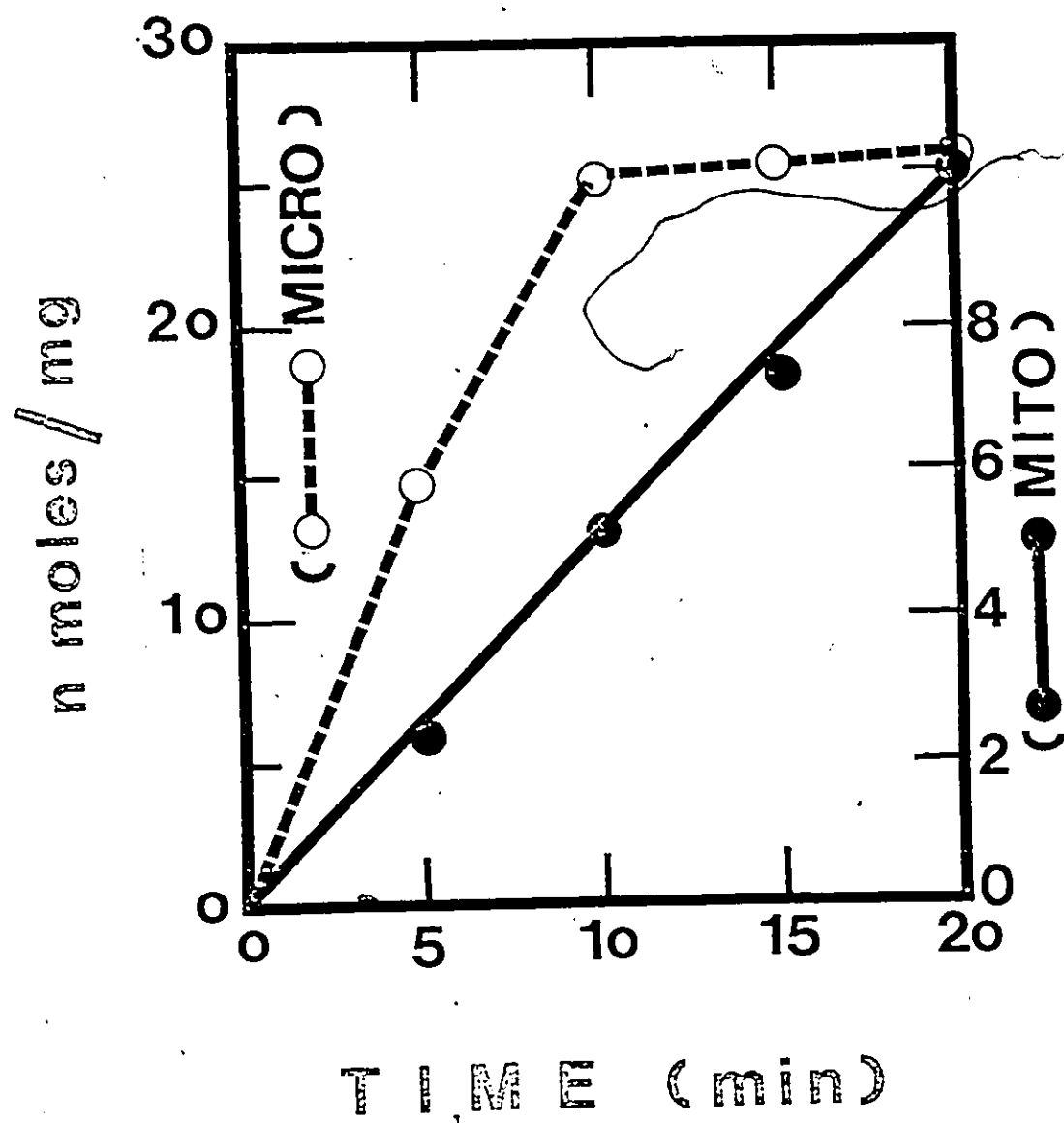


FIGURE 15

TABLE 14

The time course of the acylation of *sn*-glycerol-3-phosphate
catalyzed by heart subcellular fractions

Incubation time	Incorporation of <i>sn</i> -glycerol-3-phosphate	
	Mitochondrial fraction	Microsomal fraction
min	nmoles/mg	nmoles/mg
0	0	0
5	2.4	14.8
10	5.1	25.2
15	7.4	25.3
20	10.1	25.4

For experimental details, see the legend to the previous figure.

LEGEND TO FIGURE 16

The time course of sn-[U-¹⁴C]glycerol-3-phosphate incorporation into monoacyl-sn-glycerol-3-phosphate in mitochondrial and microsomal fractions of rabbit heart

The experiments were carried out as described in the legend to Fig. 15. The ordinate indicates the formation of monoacyl-sn-glycerol-3-phosphate in nmoles/mg protein. The abscissa represents the incubation time in min. Black circles represent mitochondrial monoacyl-sn-glycerol-3-phosphate formation. The squares show microsomal monoacyl-sn-glycerol-3-phosphate formation.

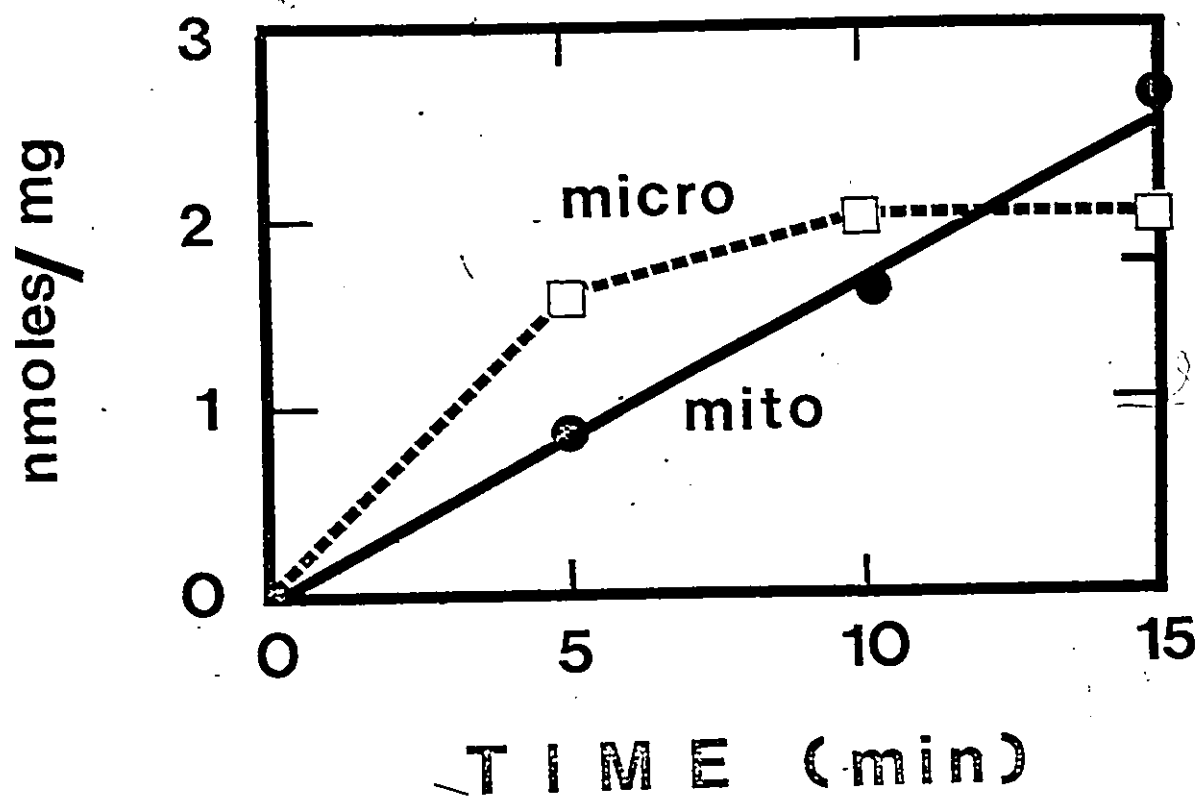


FIGURE 16

LEGEND TO FIGURE 17

The time course of $\text{sn-[U-}^{14}\text{C]}$ glycerol-3-phosphate incorporation into diacyl- sn -glycerol-3-phosphate in mitochondrial and microsomal fractions of rabbit heart

For the experimental details, see the legend to Fig.

15. The ordinate indicates the formation of diacyl- sn -glycerol-3-phosphate in nmoles/mg. The abscissa represents the incubation time in min. Black squares represent mitochondrial diacyl- sn -glycerol-3-phosphate formation, and circles represent microsomal diacyl- sn -glycerol-3-phosphate formation.

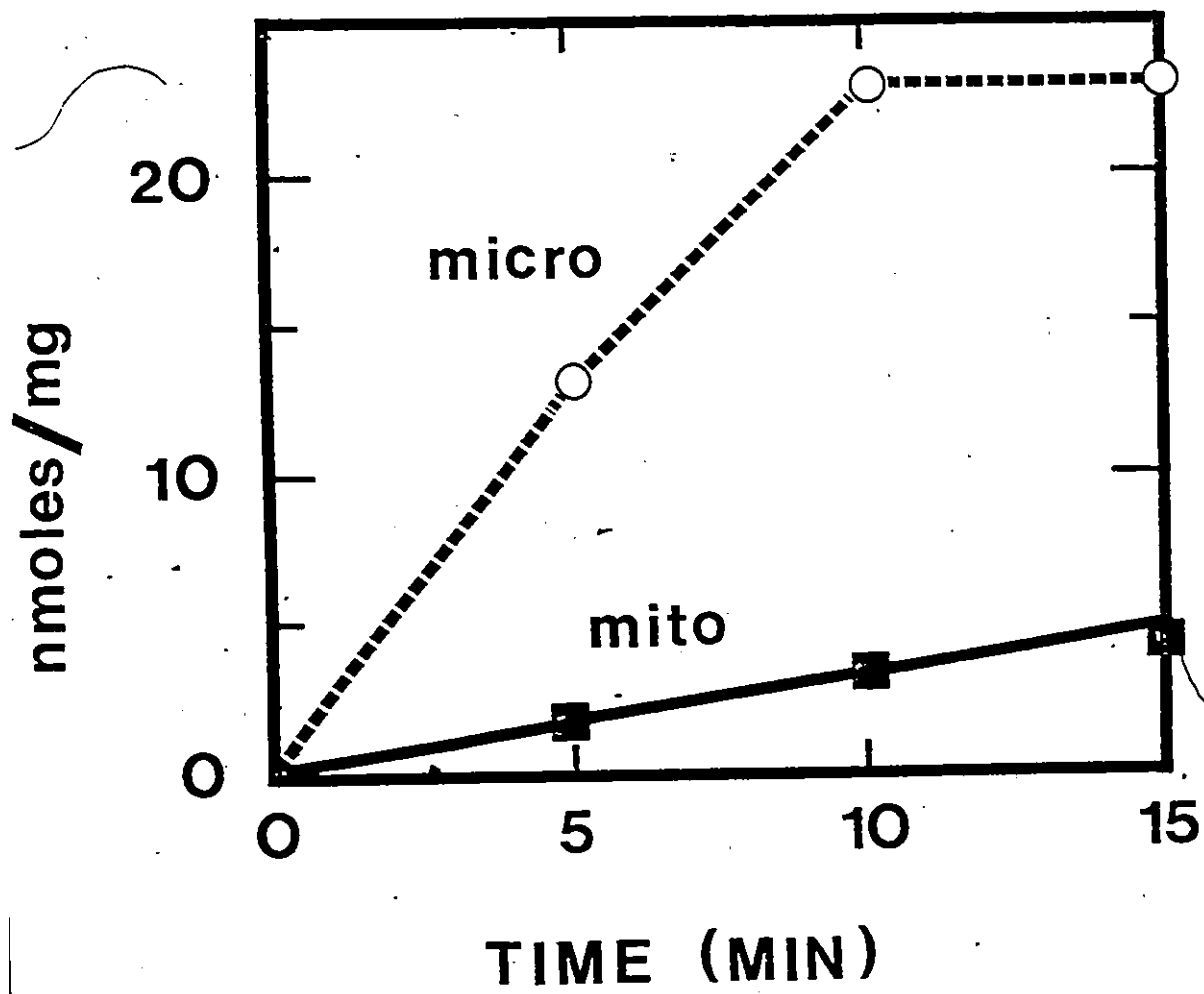


FIGURE 17.

LEGEND TO FIGURE 18

The time course of $\text{sn-}[\text{U-}^{14}\text{C}]\text{glycerol-3-phosphate}$ incorporation into monoacyl- and diacyl- sn-G3P in mitochondrial fraction of rabbit heart

This figure was replotted from the data of Figs. 16 and 17. The abscissa represents the incubation time in min. The ordinate indicates the incorporation of $\text{sn-}[\text{U-}^{14}\text{C}]\text{glycerol-3-phosphate}$ into monoacyl- sn-G3P (MAGP; $\blacktriangle$ — $\blacktriangle$) and diacyl- sn-G3P (DAGP; $\square$ — $\square$) in nmoles/mg of mitochondrial protein.

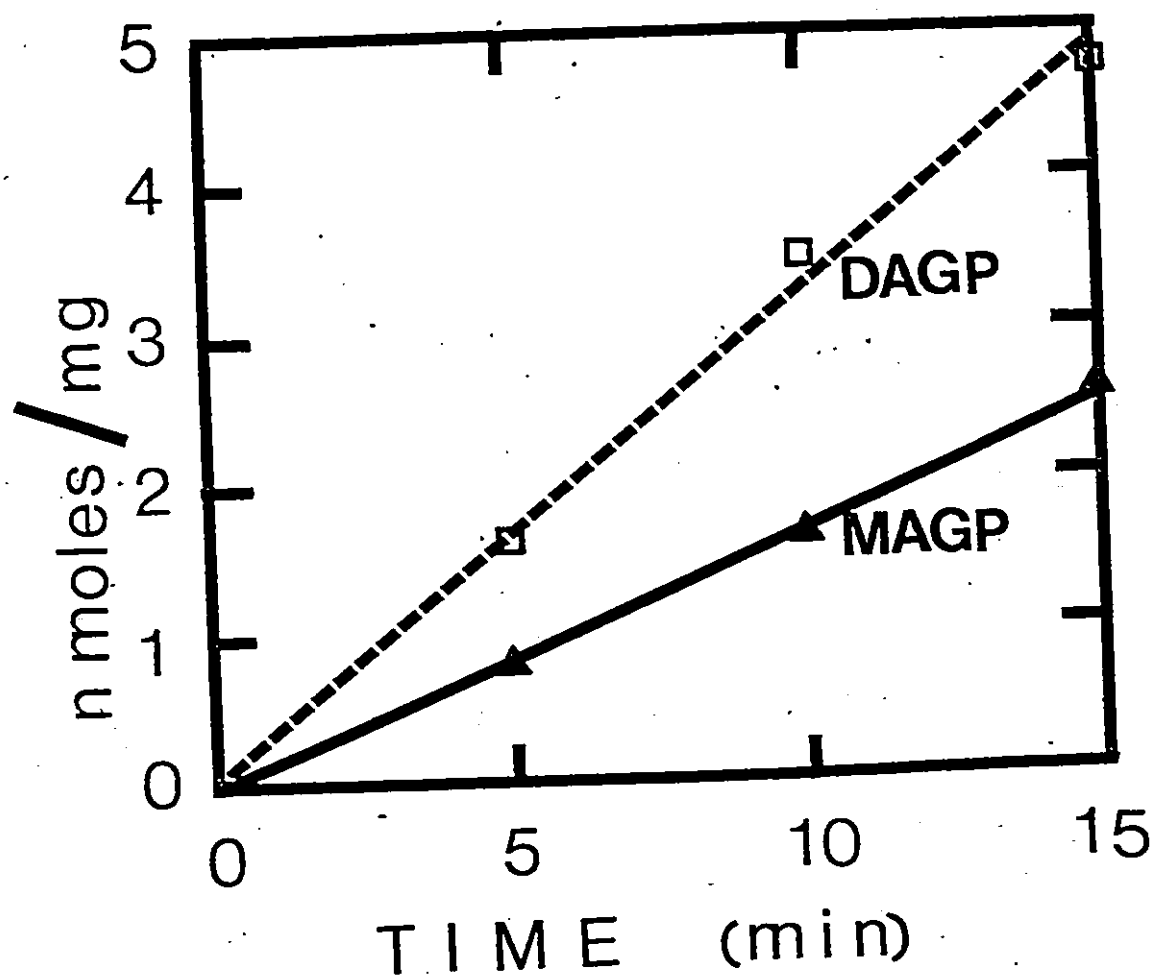
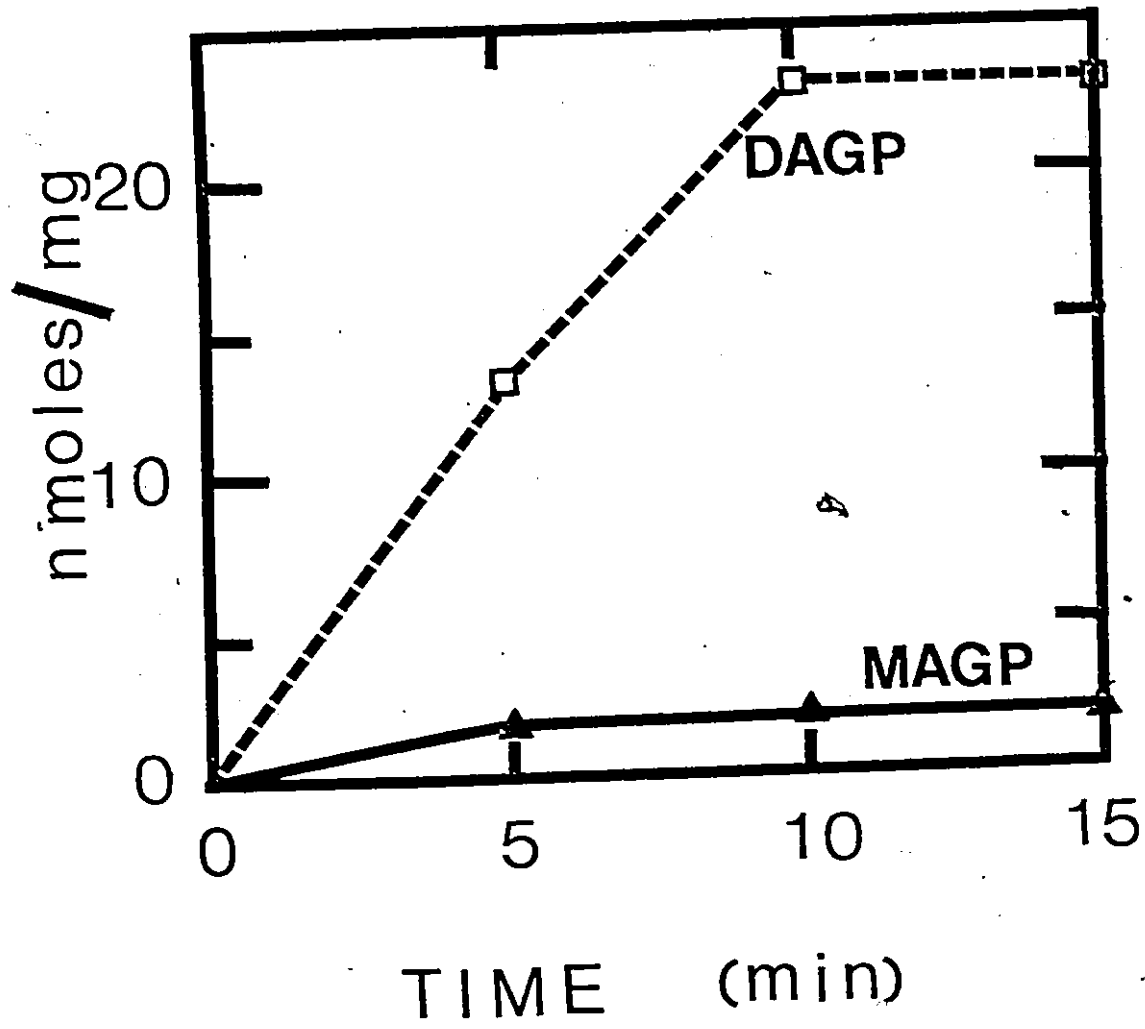


FIGURE 18

LEGEND TO FIGURE 19

The time course of $\text{sn-[U-}^{14}\text{C]glycerol-3-phosphate}$ incorporation into monoacyl- and diacyl- sn-G3P in microsomal fraction of rabbit heart

This figure was replotted using the data on Figs. 16 and 17. The abscissa represents the incubation time in min. The ordinate indicates the incorporation of $\text{sn-[U-}^{14}\text{C]glycerol-3-phosphate}$ into monoacyl- sn-G3P (MAGP; $\blacktriangle \longrightarrow \blacktriangle$) and diacyl- sn-G3P (DAGP; $\square \text{---} \square$) in nmoles/mg of microsomal protein.

FIGURE 19

LEGEND TO FIGURE 20.

The ratio of mitochondrial to microsomal monoacyl-sn-glycerol-3-phosphate and diacyl-sn-glycerol-3-phosphate in rabbit heart

The assays were carried out as in Fig. 15 with palmitate as the acyl donor. The ordinate indicates the ratio of mitochondrial to microsomal acylation products in percentages. The abscissa represents incubation time in min. Black triangles represent the ratio of monoacyl-sn-glycerol-3-phosphate synthesized by the mitochondrial fraction to that synthesized by the microsomal fraction. Open triangles illustrate the ratio of mitochondrial to microsomal diacyl-sn-glycérol-3-phosphate. Each value was the mean of three experiments.

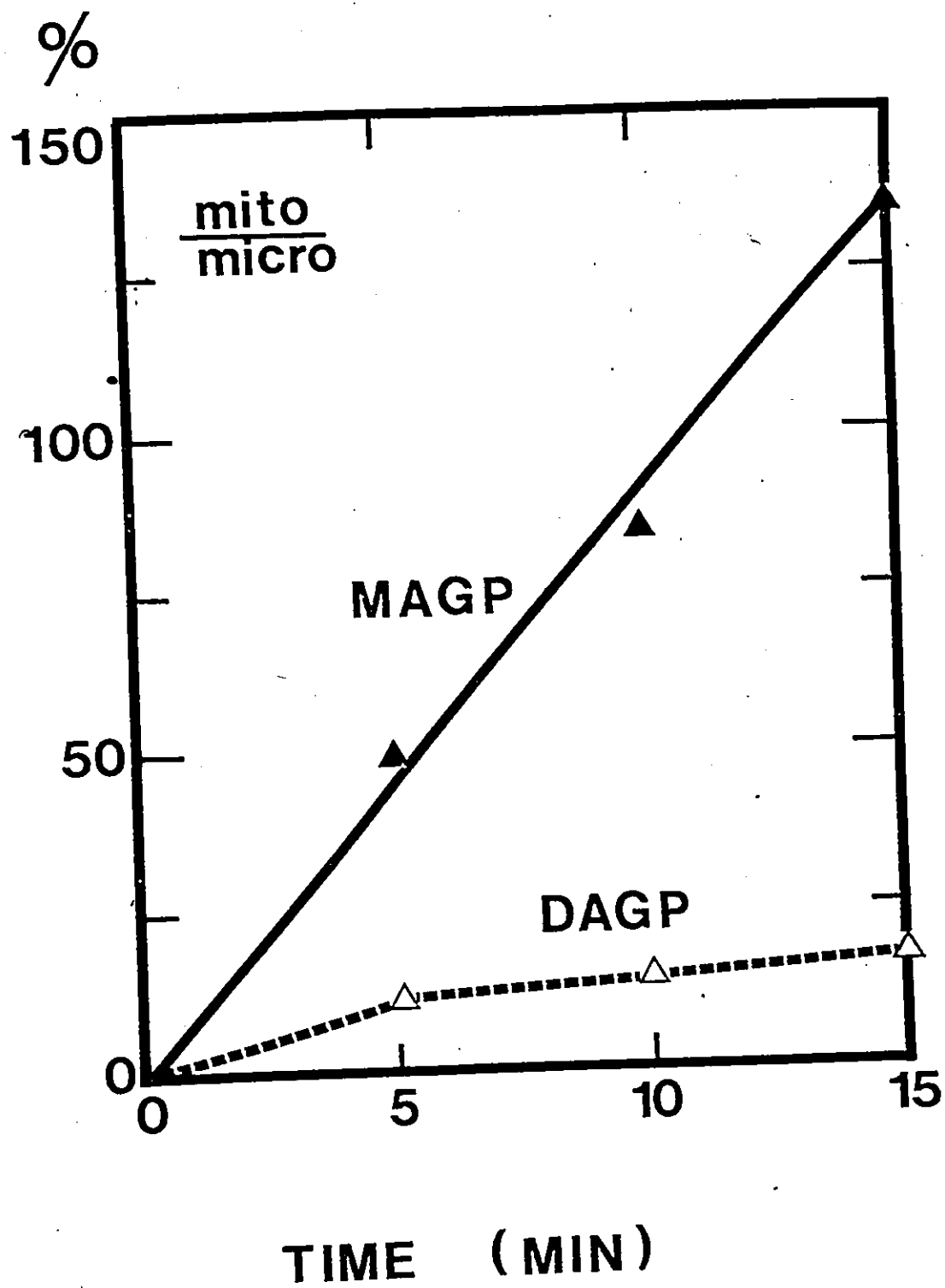


FIGURE 20

LEGEND TO TABLE 15

The time course of sn-[U-¹⁴C]glycerol-3-phosphate incorporation into monoacyl- and diacyl-sn-glycerol-3-phosphate by rabbit heart subcellular fractions

The assay procedure and product analysis were described in the legend to Fig. 15. In these experiments, 2.6 mg and 2.9 mg of mitochondrial protein and 0.9 mg and 1.0 mg of microsomal protein were used. Each value was obtained by the average of the two experiments. G3P is the abbreviation of glycerol-3-phosphate.

TABLE 15

The time course of sn-[U-¹⁴C]glycerol-3-phosphate incorporation into monoacyl- and diacyl-sn-glycerol-3-phosphate by rabbit heart subcellular fractions

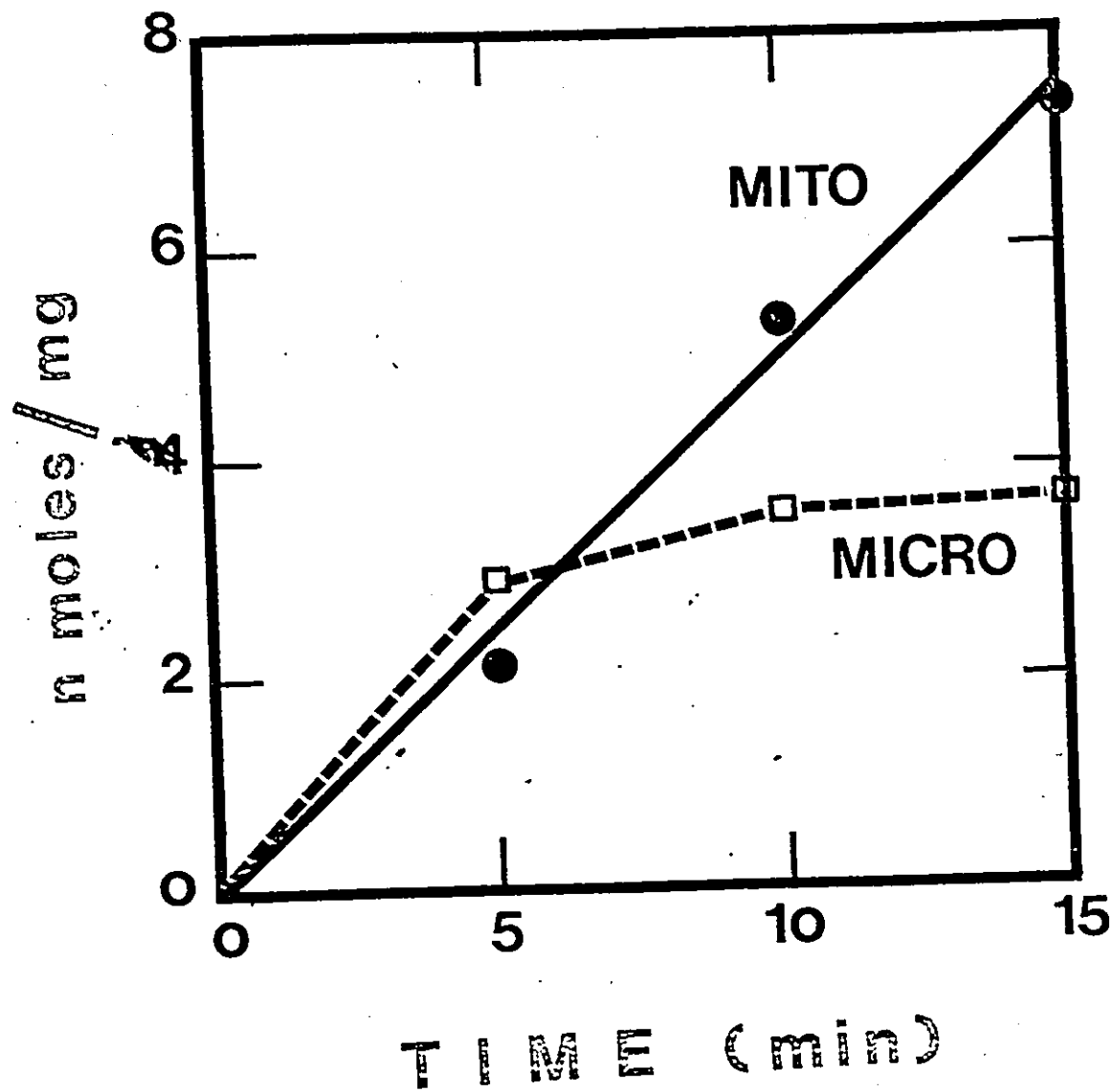
Subcellular fraction	Products	Incubation time		
		5 min	10 min	15 min
		nmoles/mg		
Mitochondrial fraction	Monoacyl- <u>sn</u> -G3P	0.8	1.6	2.6
	Diacyl- <u>sn</u> -G3P	1.6	3.5	4.7
Microsomal fraction	Monoacyl- <u>sn</u> -G3P	1.6	2.0	2.0
	Diacyl- <u>sn</u> -G3P	13.2	23.0	23.0
Ratio of mitochondrial to microsomal	Monoacyl- <u>sn</u> -G3P	50.0 %	80.0 %	130.0 %
	Diacyl- <u>sn</u> -G3P	12.1 %	15.2 %	20.4 %

For experimental details, see the legends to Figs. 16, 17, and 20.

LEGEND TO FIGURE 21

The time course of $\text{sn-[U-}^{14}\text{C]glycerol-3-phosphate}$ incorporation into monoacyl- $\text{sn-glycerol-3-phosphate}$ in mitochondrial and microsomal fractions of rabbit liver

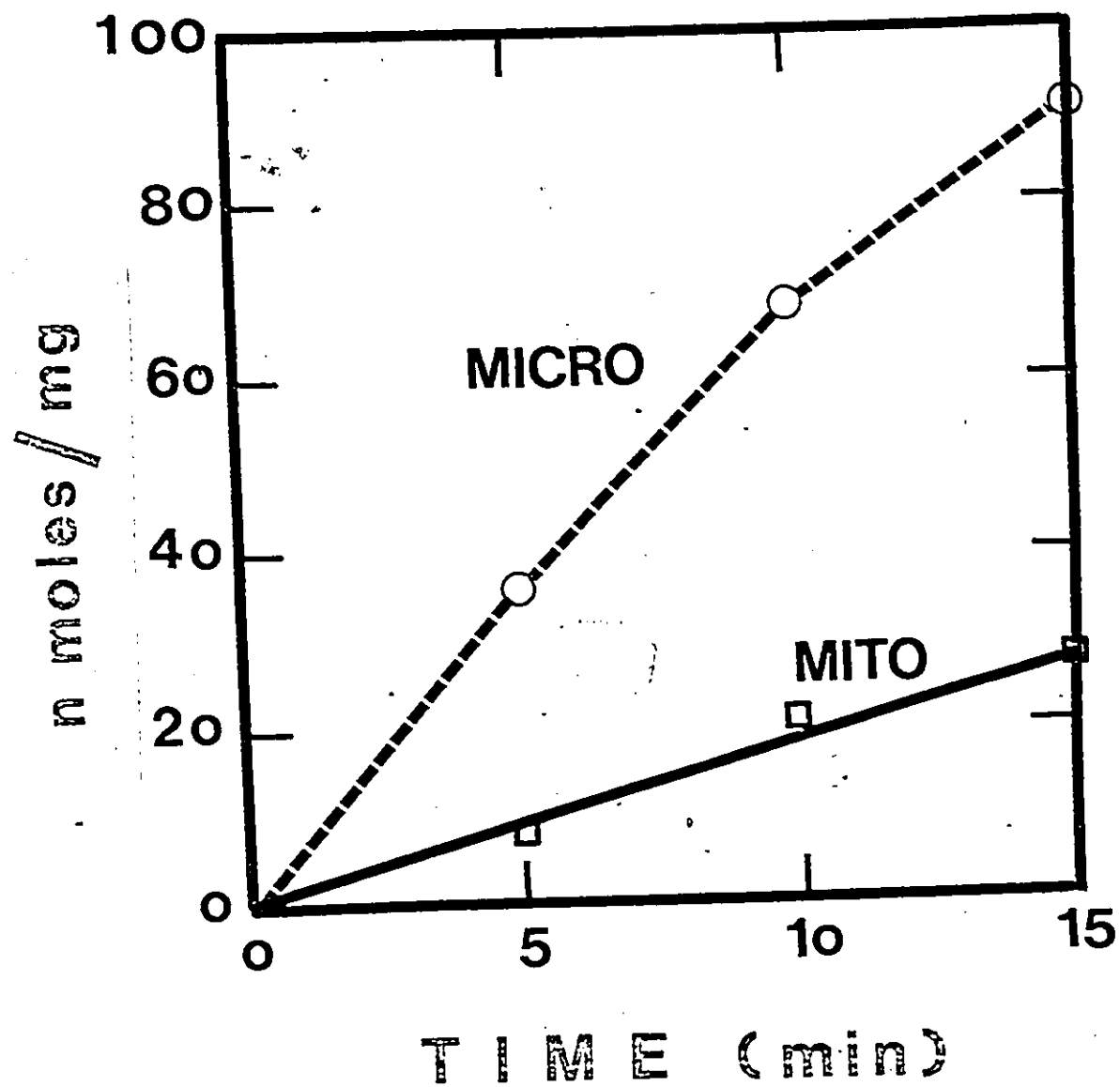
Rabbit liver subcellular fractions were prepared in the same manner as were heart fractions by differential centrifugation. Mitochondrial (1.5 mg protein) and microsomal (0.5 mg) fractions were incubated separately in the presence of potassium palmitate, and the enzyme activity was assayed by the same method as was used for heart particulate fractions (see Fig. 15). The reaction was stopped, lipids were extracted by butanol, and the products were analyzed as described in Section III-1-6. The ordinate indicates the formation of monoacyl- $\text{sn-glycerol-3-phosphate}$ in nmoles/mg protein. The abscissa represents the incubation time in min. Black circles signify mitochondrial monoacyl- $\text{sn-glycerol-3-phosphate}$ formation. The squares represent microsomal monoacyl- $\text{sn-glycerol-3-phosphate}$ formation.

FIGURE 21

LEGEND TO FIGURE 22

The time course of sn -[U- ^{14}C]glycerol-3-phosphate incorporation into diacyl- sn -glycerol-3-phosphate in mitochondrial and microsomal fractions of rabbit liver

For the experimental details, see the legend to Fig. 21. The ordinate indicates the formation of diacyl- sn -glycerol-3-phosphate in nmoles/mg. The abscissa represents the incubation time in min. The squares represent mitochondrial diacyl- sn -glycerol-3-phosphate formation, and the circles represent microsomal diacyl- sn -glycerol-3-phosphate formation.

FIGURE 22

LEGEND TO FIGURE 23

The ratio of mitochondrial to microsomal monoacyl-sn-glycerol-3-phosphate and diacyl-sn-glycerol-3-phosphate in rabbit liver.

The assays were carried out as in Fig. 21 with palmitate as the acyl donor. The ordinate indicates the ratio of mitochondrial to microsomal acylation products in percentages. The abscissa represents the incubation time in min. Black triangles represent the ratio of monoacyl-sn-glycerol-3-phosphate synthesized by the mitochondrial fraction to that synthesized by the microsomal fraction. Open triangles signify the ratio of the mitochondrial to microsomal diacyl-sn-glycerol-3-phosphate.

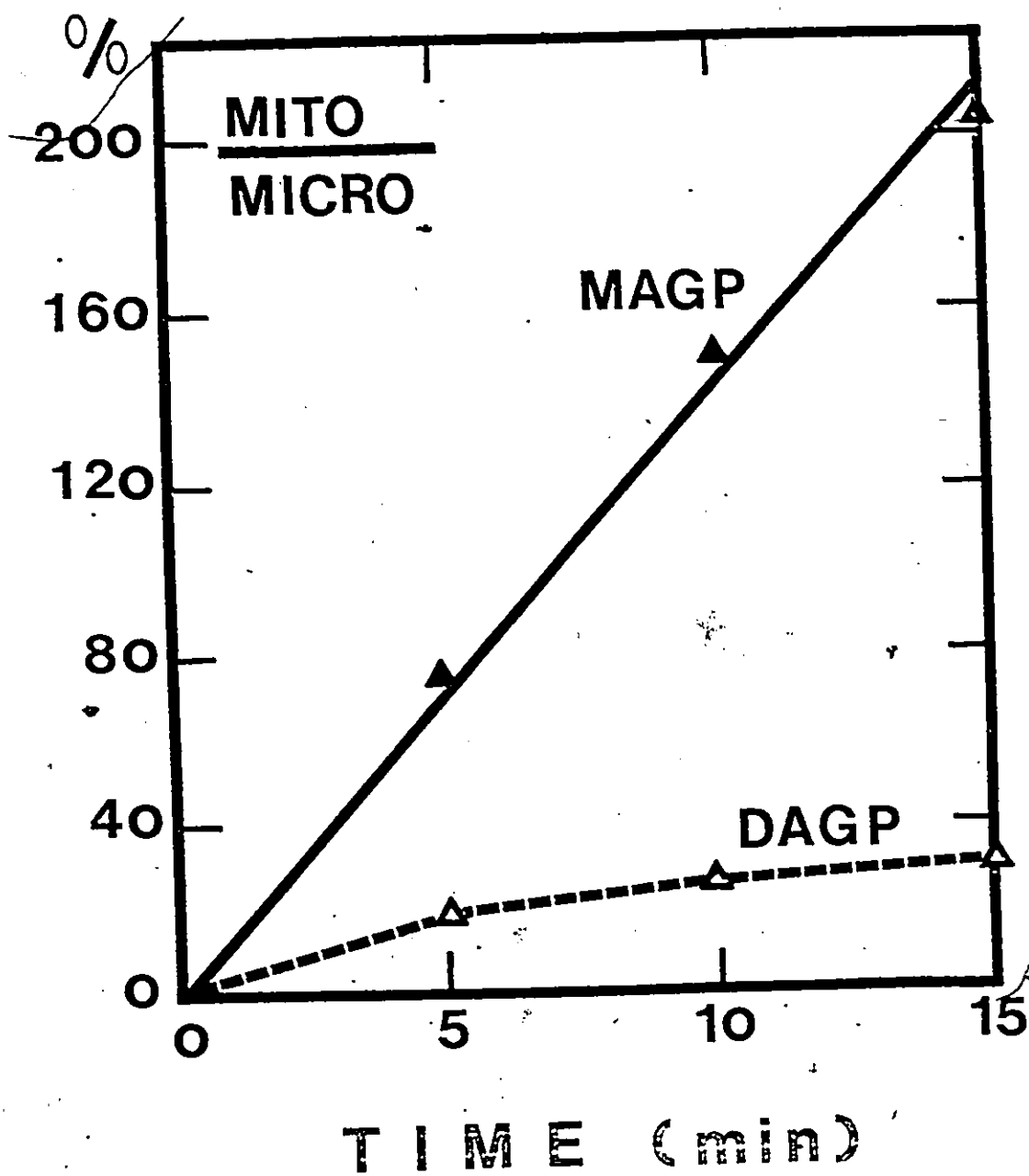


FIGURE 23

TABLE 16

The acylation of $\text{sn-[U-}^{14}\text{C]glycerol-3-phosphate}$ by rabbit
liver subcellular fractions

Subcellular fraction	Products	Incubation time		
		5 min	10 min	15 min
Mitochondrial	Monoacyl- <u>sn</u> -glycerol- 3-phosphate	2.2	5.2	7.4
	Diacyl- <u>sn</u> - glycerol-3- phosphate	8.5	20.0	28.6
Microsomal	Monoacyl- <u>sn</u> -glycerol- 3-phosphate	2.9	3.5	3.6
	Diacyl- <u>sn</u> - glycerol-3- phosphate	36.2	68.5	91.2
Ratio of mitochondrial to microsomal	Monoacyl- <u>sn</u> -glycerol- 3-phosphate	75.8 %	148.6 %	205.5 %
	Diacyl- <u>sn</u> - glycerol- 3-phosphate	23.5 %	29.2 %	31.4 %

For the experimental details, see the legends to Figs.

LEGEND TO FIGURE 24

The effect of triiodothyronine (T3) administration on the acylation of *sn*-glycerol-3-phosphate by the rabbit heart mitochondrial fraction

The assay procedure is described in the legends to Table 5 and Fig. 15. The amounts of enzyme source used in the assay were 3.7 to 4.3 mg mitochondrial fraction per incubation flask. T3 (125 µg/kg body weight) was injected intramuscularly for 6 to 10 days until the heart rate increased to approximately 160 % and the body weight decreased to approximately 27 % of the control values (see Section III-1-9 and Table 4). The rate of acylation is expressed on the ordinate, and the abscissa indicates the products, monoacyl-*sn*-glycerol-3-phosphate (MAGP) and diacyl-*sn*-glycerol-3-phosphate (DAGP). The mean, S.E.M., and number of experiments are shown on the graph. Each experiment represents the average of triplicate determinations. The values obtained with starved control animals are illustrated as "control".

MITOCHONDRIA

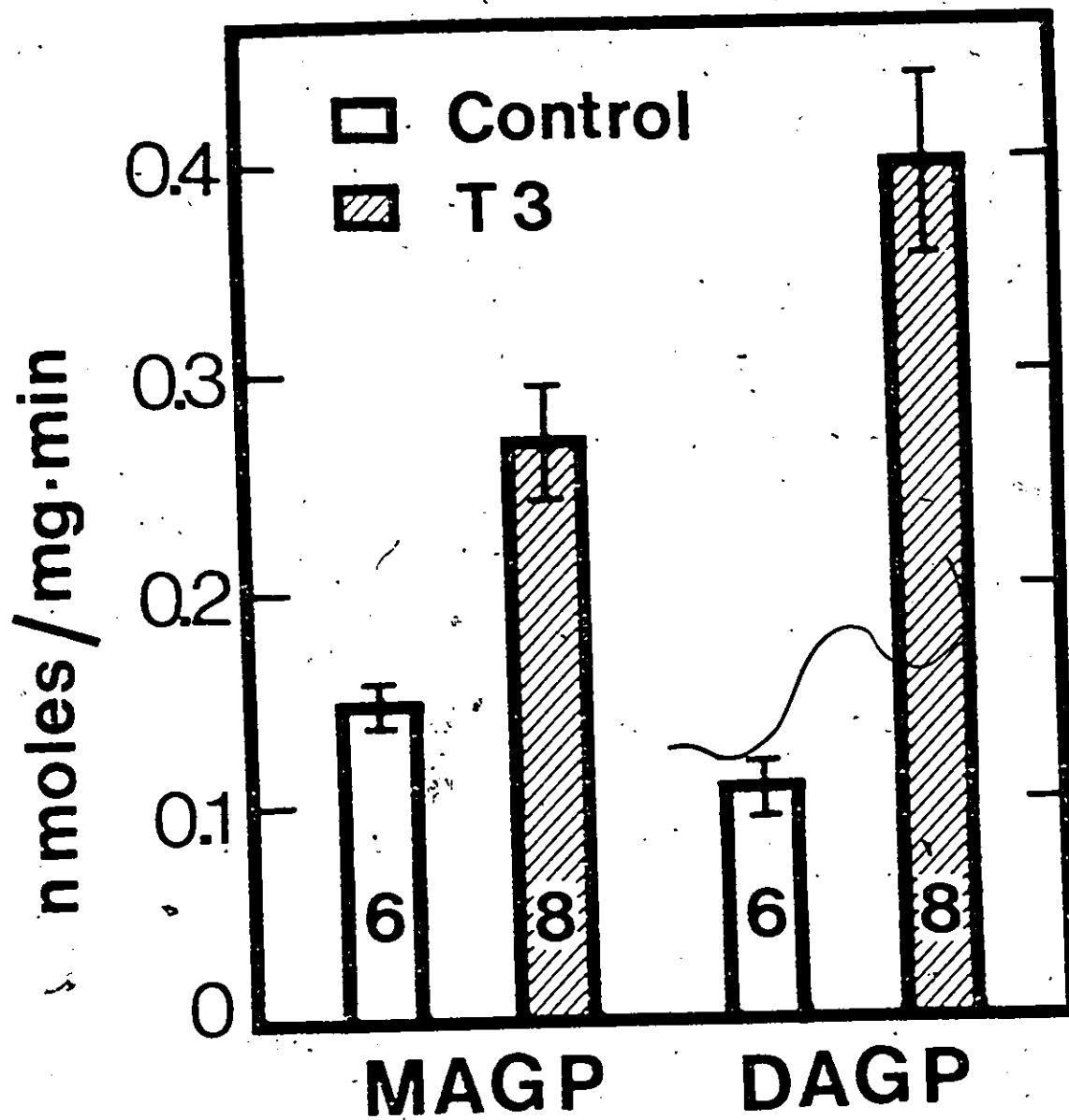


FIGURE 24

TABLE 17

The effect of triiodothyronine (T3) administration on the acylation of *sn*-glycerol-3-phosphate by the rabbit heart mitochondrial fraction

Experimental condition	Incorporation of <i>sn</i> -[U- ¹⁴ C]glycerol-3-phosphate into:		No. of experiments
	Monoacyl- <i>sn</i> -glycerol-3-phosphate	Diacyl- <i>sn</i> -glycerol-3-phosphate	
	nmoles/mg min		
Fed control	0.15±0.0173	0.12±0.0218	3
Starved control	0.147±0.0095	0.105±0.008	6
T3 administration	0.27±0.0293	0.40±0.0426	8
<u>P values between</u>			
T3 and Fed control	P < 0.025	P < 0.005	
T3 and Starved control	P < 0.005	P < 0.0005	

For the experimental details, see the legend to Fig. 24. Values shown are Mean ± S.E.M.

LEGEND TO FIGURE 25

The effect of triiodothyronine (T3) administration on the acylation of sn-glycerol-3-phosphate by the rabbit heart microsomal fraction

The experimental details were identical to those in Fig. 24, except that 2.0 to 2.5 mg of microsomal protein was used as the enzyme source. The ordinate indicates the rate of acylation in nmoles/mg min. The abscissa represents the reaction products, monoacyl-sn-glycerol-3-phosphate (MAGP) and diacyl-sn-glycerol-3-phosphate (DAGP). Each experiment represents the average of triplicate determinations. The values obtained with starved control animals are illustrated as "control".

MICROSOMES

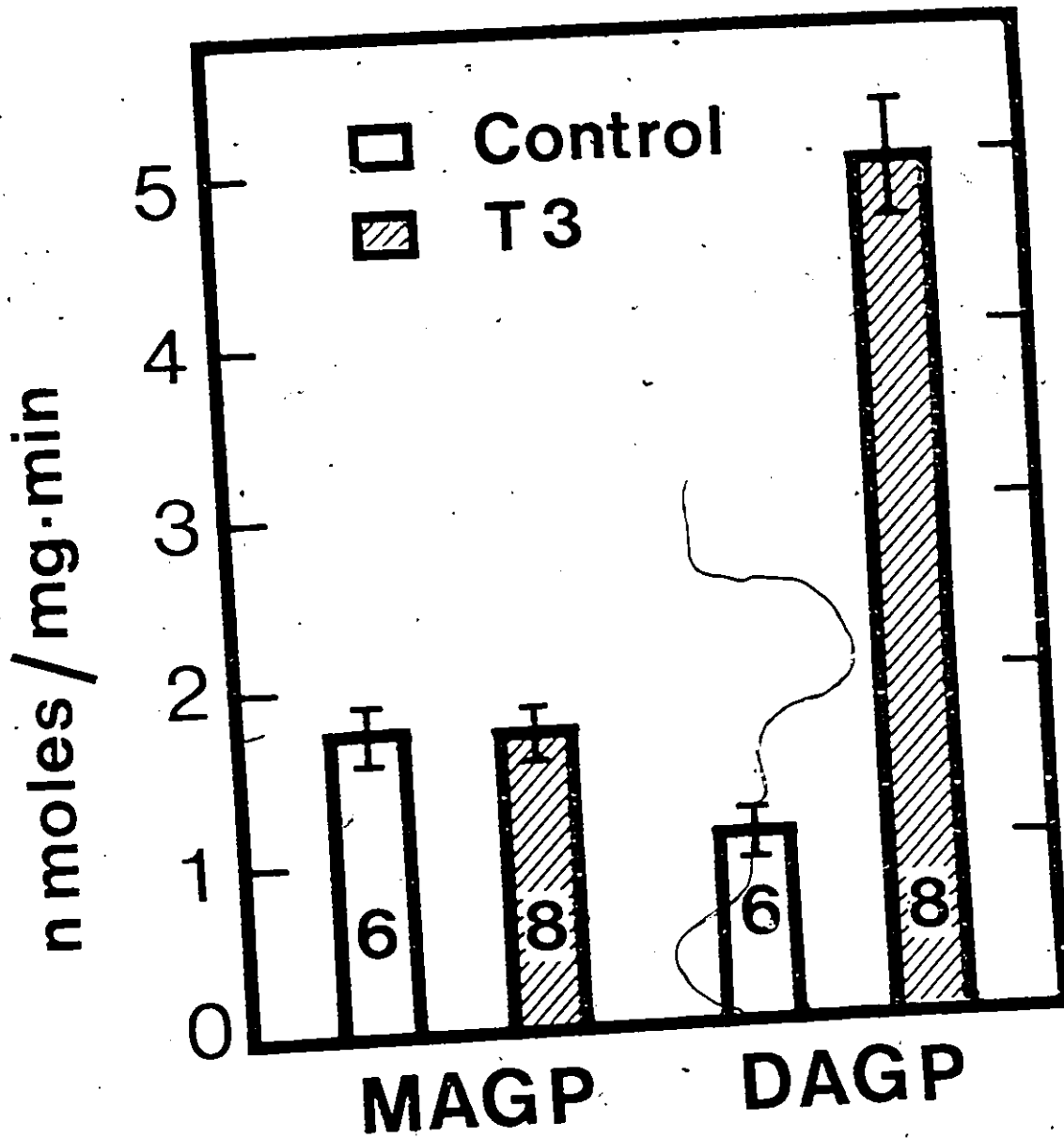


FIGURE 25

TABLE 18

The effect of triiodothyronine (T3) administration on the acylation of *sn*-glycerol-3-phosphate by the rabbit heart microsomal fraction

Experimental condition	Incorporation of <i>sn</i> -[U- ¹⁴ C] glycerol-3-phosphate into		No. of experiments
	Monoacyl- <i>sn</i> -glycerol-3-phosphate	Diacyl- <i>sn</i> -glycerol-3-phosphate	
	nmoles/mg min		
Fed control	1.43 ± 0.199	0.75 ± 0.113	3
Starved control	1.795 ± 0.133	1.10 ± 0.075	6
T3 administration	1.79 ± 0.118	5.02 ± 0.333	8
<u>P values between</u>			
T3 and Fed control	P < 0.1	P < 0.0005	
T3 and Starved control	P > 0.4	P < 0.0005	

The experimental conditions were described in the legend to Fig. 25. The values shown are Mean ± S.E.M.

LEGEND TO FIGURE 26

The relationship between the amounts of microsomal protein and the acylation of *sn*-glycerol-3-phosphate in the absence and presence of mitochondrial fraction

The experiment was carried out as described in the legend to Fig. 15. Black triangles indicate the activities obtained with various amounts of microsomal fraction as shown on the abscissa (mg protein) in the absence of the mitochondrial fraction, while open squares represent the values obtained in the presence of 4.1 mg mitochondrial protein. The rate of acylation of *sn*-glycerol-3-phosphate is expressed on the ordinate in nmoles/min.

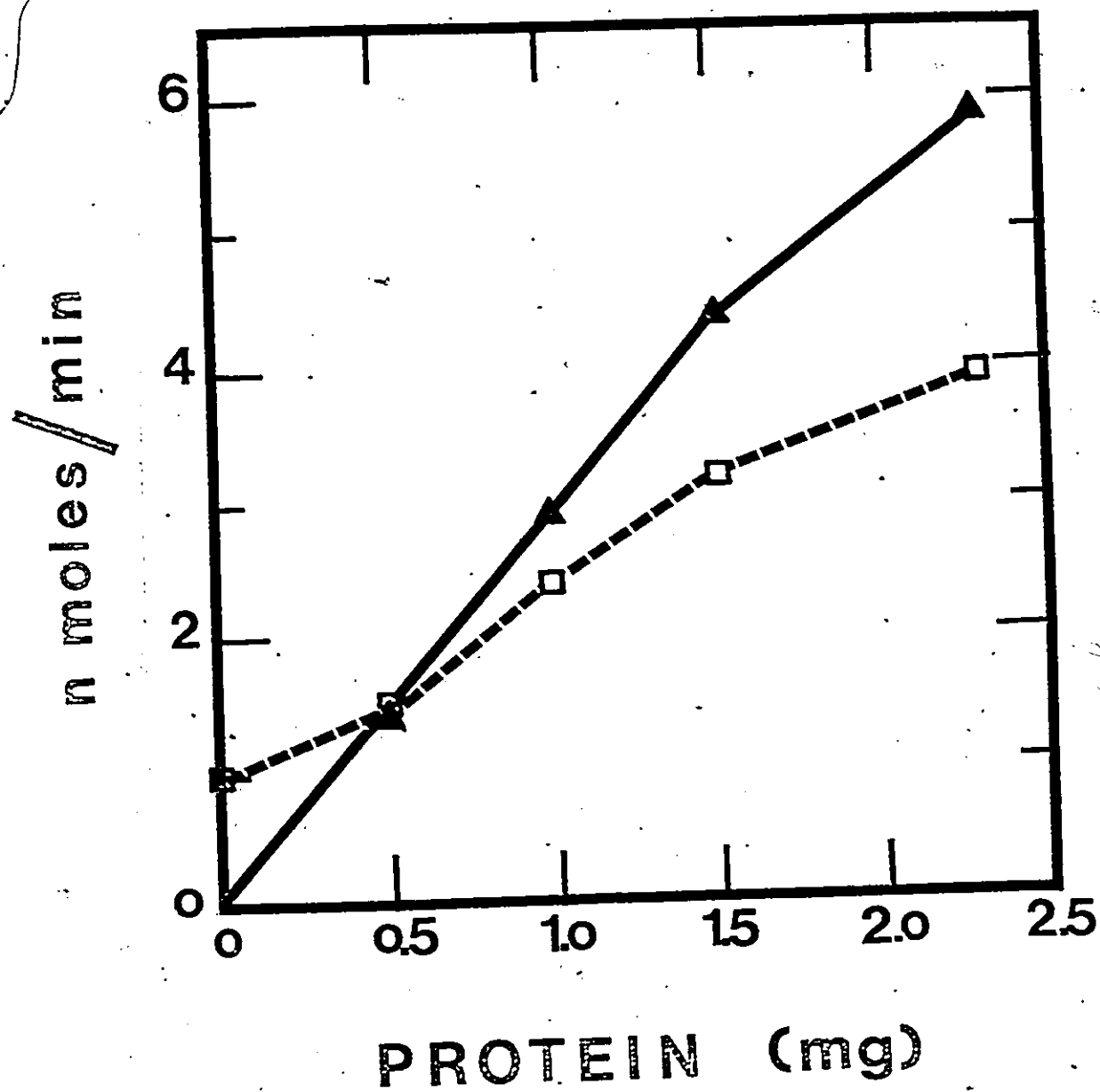


FIGURE 26

LEGEND TO TABLE 19

The rates of sn -[U- ^{14}C]glycerol-3-phosphate acylation and ^{14}C -labelled diacyl- sn -glycerol-3-phosphate deacylation in the rabbit heart mitochondrial fraction

The assay of acyl-CoA: sn -glycerol-3-phosphate-0-acyltransferase activity (Experiment 1, a and b) is described in the legends to Tables 5 and 11 and Fig. 15. The concentration of palmitate and oleate was 1.0 mM, and mitochondrial protein content was 2.4 to 2.9 mg. Experiment 2 was carried out under experimental conditions similar to those of Experiment 1, except that unlabelled glycerol-3-phosphate was used together with ^{14}C -labelled diacyl- sn -glycerol-3-phosphate. The latter substrate was isolated from the products of liver microsomal acylation when palmitate (Experiment 2 a) or oleate (Experiment 2 b) served as an acyl donor in the presence of [^{14}C]glycerol-3-phosphate. Each sample in all the experiments was incubated for 10 min at 37°C. The results of Experiment 1 are averages of six experiments, and those of Experiment 2 are averages of three.

TABLE 19

The rates of sn -[U- ^{14}C]glycerol-3-phosphate acylation and ^{14}C -labelled diacyl- sn -glycerol-3-phosphate deacylation in the rabbit heart mitochondrial fraction

Experiment	Labelled precursor	Acyl donor	Monoacyl- sn -glycerol-3-phosphate		Diacyl- sn -glycerol-3-phosphate	
			nmoles per mg min	d.p.m.	nmoles per mg min	d.p.m.
1, a	[^{14}C]glycerol-3-phosphate	Palmitate	0.16	1490	0.34	3167
		Oleate	0.25	2340	0.35	3241
2, a	[^{14}C]diacyl- sn -glycerol-3-phosphate	Palmitate		164		3316
		Oleate		230		3080

TABLE 20

The effect of thiol inhibitors on the activity of acyl-CoA:sn-glycerol-3-phosphate-0-acyltransferase in rabbit heart mitochondrial and microsomal fractions

Subcellular fraction	Acyl donor	Inhibitors	
		N-ethylmaleimide (1 mM) (2 mM) (4 mM)	P-chloromercuribenzoate (1 mM) (2 mM) (4 mM)
Mitochondrial	Palmitate	+	+
	Palmitoyl-CoA	+	+
Microsomal	Palmitate	+	+
	Palmitoyl-CoA	89 % 92 % +	+

The composition of the incubation mixture and the assay procedure were described in the legend to Fig. 15. N-ethylmaleimide was dissolved in H₂O, while P-chloromercuribenzoate was dissolved in a 0.03 N NaOH solution. The symbol + indicates 100 % inhibition.

LEGEND TO TABLE 21

The effect of dihydroxyacetone phosphate addition on the acylation of sn-[U-¹⁴C]glycerol-3-phosphate in the rabbit heart mitochondrial fraction

The sonicated mitochondrial fraction (2.55 mg protein) was used for all the experiments, except Experiment 1 (2.58 mg protein). For the disruption of isolated mitochondria, a Biosonik (Bronwill Scientific, Model B 10-11), operating at half of the maximum intensity (20 KHz, 150 W) for 15 sec at 4°C, was used for a total period of 45 sec. All assays were performed as stated in the legends to Tables 5 and 11.

TABLE 21

The effect of dihydroxyacetone phosphate addition on the acylation of $\text{sn-[U-}^{14}\text{C]}$ glycerol-3-phosphate in the rabbit heart mitochondrial fraction

Experiment	Addition	Incorporation of $\text{sn-[U-}^{14}\text{C]}$ glycerol-3-phosphate into:	
		Monoacyl- sn -glycerol-3-phosphate	Diacyl- sn -glycerol-3-phosphate
		nmoles/mg min	
1.	None	0.21	0.30
2	None	0.17	0.25
3	DHAP (4 mM) NADPH (5 mM) NaCN (3 mM)	0.16	0.27
4	DHAP (8 mM) NADPH (5 mM) NaCN (3 mM)	0.15	0.24
5	DHAP (4 mM) NADH (5 mM) NaCN (3 mM)	0.15	0.26
6	DHAP (8 mM) NADH (5 mM) NaCN (3 mM)	0.15	0.27

LEGEND TO FIGURE 27

The relationship between the concentrations of exogenous fatty acids and the incorporation of $\text{sn-[U-}^{14}\text{C]}$ glycerol-3-phosphate into monoacyl- sn -glycerol-3-phosphate by the rabbit heart mitochondrial fraction

The assays were carried out as described in the legends to Tables 5 and 11 and Fig. 15, but the amounts of fatty acids were varied as indicated in the abscissa (mM). The ordinate shows the amounts of monoacyl- sn -glycerol-3-phosphate formed. The incubation time was 10 min at 37°C. Each point represents the average of two experiments. Triangles signify the values obtained with palmitate, circles, those with oleate, and dots, linoleate.

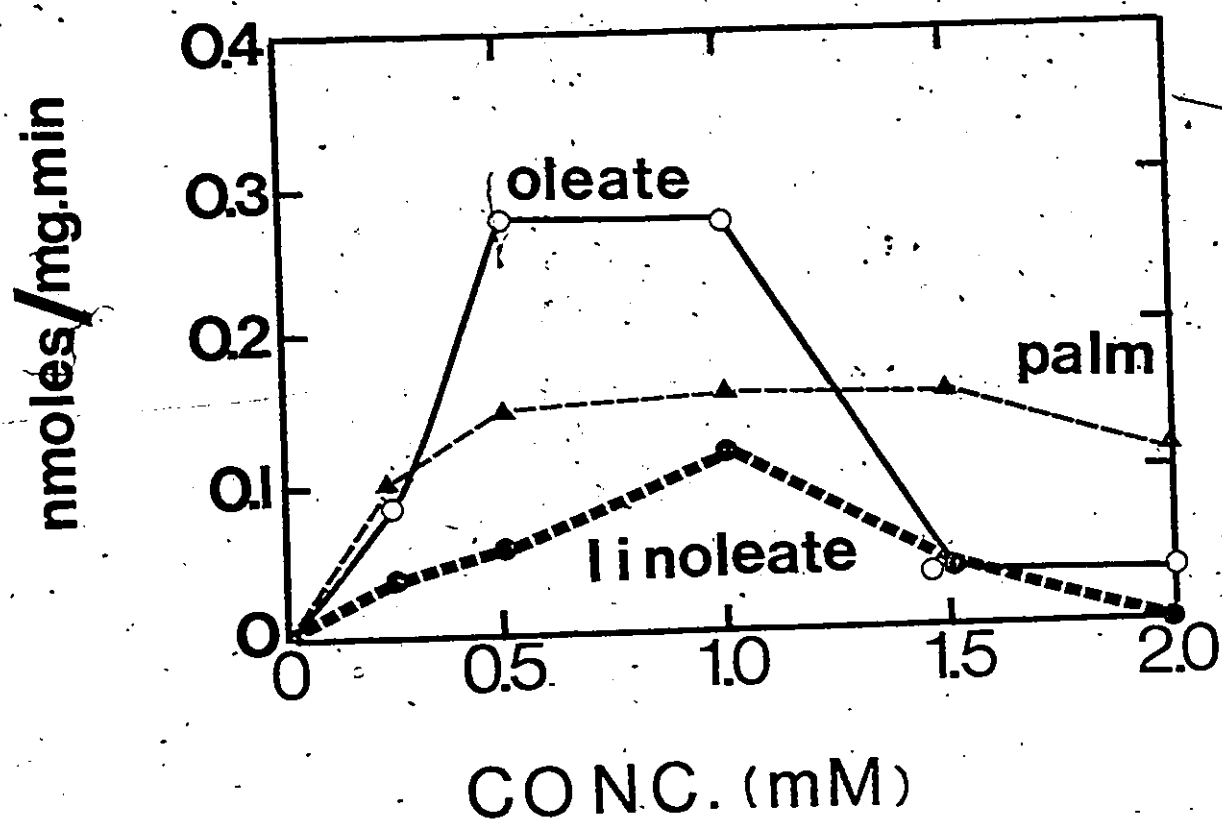


FIGURE 27

TABLE 22

The effect of different concentrations of exogenous fatty acids on the incorporation of $\text{sn-[U-}^{14}\text{C]glycerol-3-phosphate}$ into monoacyl- $\text{sn-glycerol-3-phosphate}$ by the rabbit heart mitochondrial fraction

Substrate	Enzyme activity Substrate concentration	Incorporation of $\text{sn-[U-}^{14}\text{C]glycerol-3-phosphate}$ into monoacyl- $\text{sn-glycerol-3-phosphate}$				
		0.25 mM	0.5 mM	1.0 mM	1.5 mM	2.0 mM
		nmoles/mg min				
Palmitate		0.11	0.15	0.16	0.16	0.12
Oleate		0.09	0.28	0.28	0.04	0.04
Linoleate		0.04	0.06	0.12	0.05 ^w	0

The composition of the incubation mixture and the assay procedure were stated in the legend to Fig. 27.

LEGEND TO FIGURE 28

The relationship between the amounts of acyl donors and the incorporation of $\text{sn-[U-}^{14}\text{C]}$ glycerol-3-phosphate into monoacyl- sn -glycerol-3-phosphate by the rabbit heart microsomal fraction

The assays were performed as described in the legends to Tables 5, and 11 and Fig. 15. The amounts of acyl donors are shown on the abscissa in mM, and the rate of monoacyl- sn -glycerol-3-phosphate formation is indicated on the ordinate. The incubation time was 5 min. Each point represents the average values of two experiments. Triangles signify the results of incorporation obtained with palmitate, circles, those with oleate, and dots, linoleate.

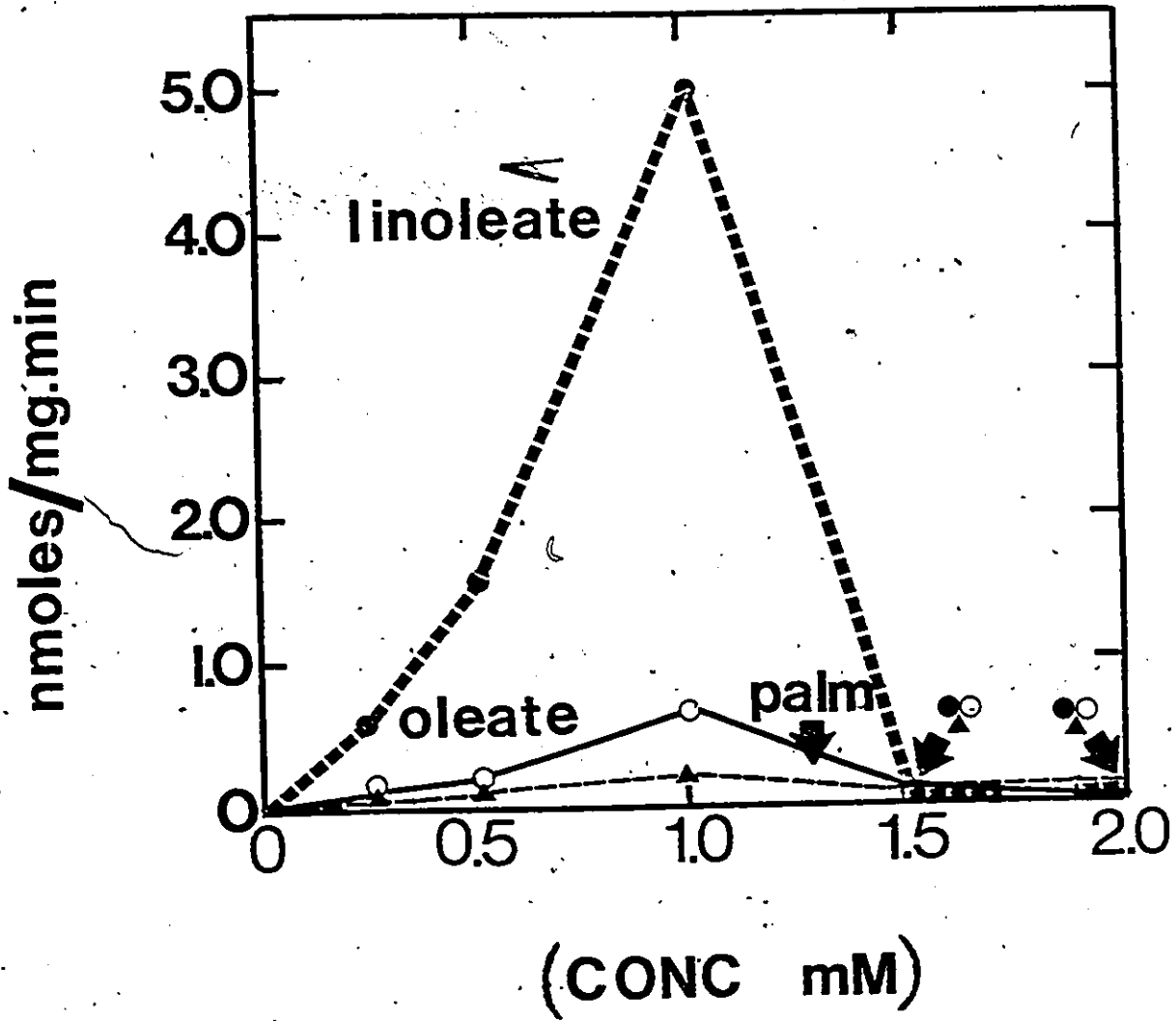
FIGURE 28

TABLE 23

The effect of various amounts of acyl donors on the incorporation of $\text{sn-[U-}^{14}\text{C]-}$ glycerol-3-phosphate into monoacyl-sn-glycerol-3-phosphate by the rabbit heart

microsomal fraction

Enzyme activity		Incorporation of <u>sn</u> -[<u>u-14C</u>]glycerol-3-phosphate into monoacyl- <u>sn</u> -glycerol-3-phosphate				
Substrate	Substrate concentration	0.25 mM	0.5 mM	1.0 mM	1.5 mM	2.0 mM
		nmoles/mg min				
Palmitate		0.09	0.16	0.20	0.15	0.12
Oleate		0.12	0.17	0.70	0.10	0.10
Linoleate		0.60	1.59	5.06	0.10	0.10

For the experimental details, see the legend to Fig. 28.

LEGEND TO FIGURE 29

Incorporation of sn -[U- ^{14}C]glycerol-3-phosphate into diacyl- sn -glycerol-3-phosphate by the rabbit heart mitochondrial fraction in the presence of different acyl donors

The assay conditions were identical to those for Fig. 15. The ordinate shows the rate of diacyl- sn -glycerol-3-phosphate formation in nmoles/mg min and the abscissa various amounts of acyl donors. Incubation time was 10 min, and the number of experiments was two. Triangles represent the results obtained with palmitate, circles; those with oleate, and dots, linoleate.

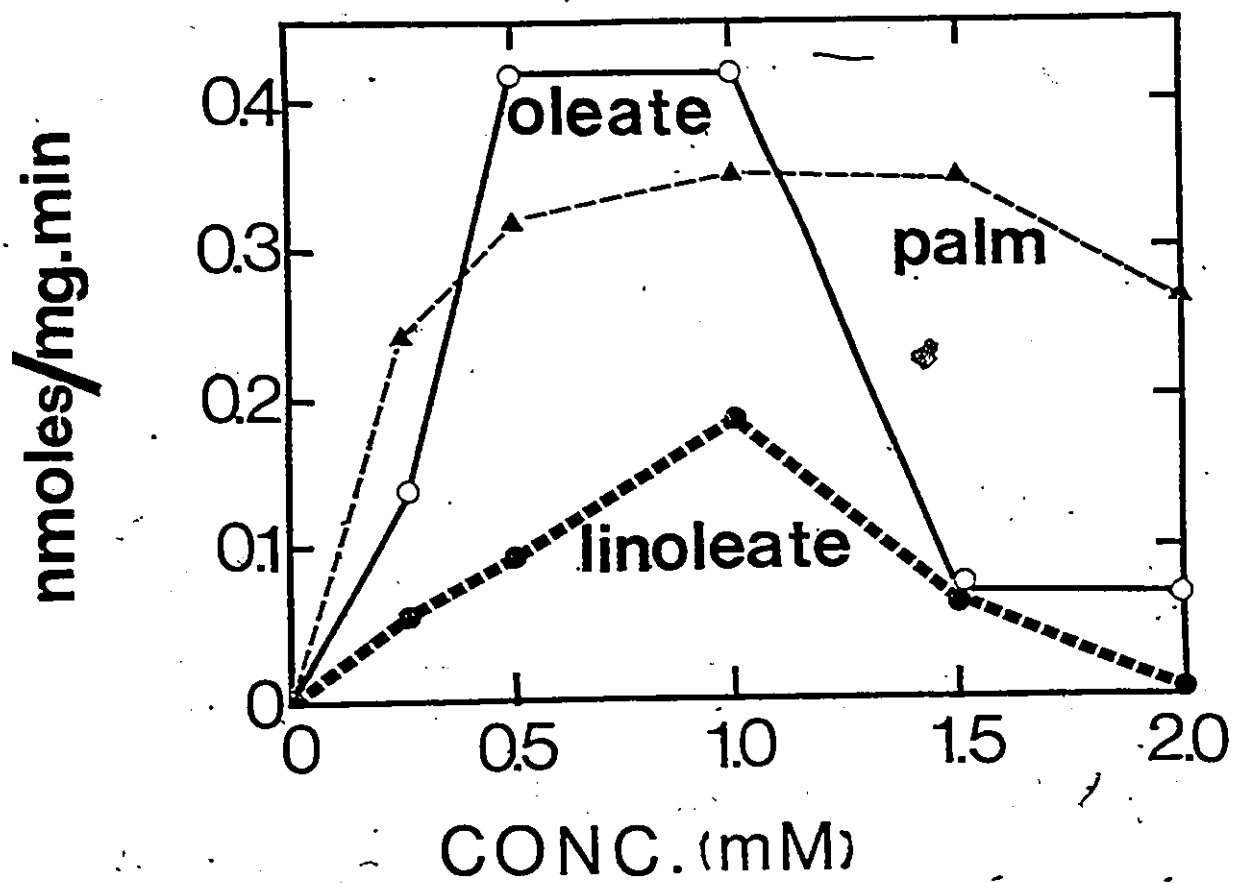
FIGURE 29

TABLE 24

A comparison of relative incorporation of sn-[$U-^{14}C$] glycerol-3-phosphate into diacyl-sn-glycerol-3-phosphate by the rabbit heart mitochondrial fraction using

different acyl donors

Enzyme activity Substrate concentration	Incorporation of <u>sn</u> -[U- ¹⁴ C]glycerol-3-phosphate into diacyl- <u>sn</u> -glycerol-3-phosphate				
	0.25 mM	0.5 mM	1.0 mM	1.5 mM	2.0 mM
Substrate	nmoles/mg min				
Palmitate	0.24	0.32	0.35	0.35	0.27
Oleate	0.14	0.42	0.42	0.07	0.07
Linoleate	0.06	0.10	0.19	0.07	0

The experimental procedure is stated in the legend to Fig. 29.

LEGEND TO FIGURE 30

Formation of diacyl-*sn*-glycerol-3-phosphate from *sn*-
[U-¹⁴C]glycerol-3-phosphate and different acyl donors
in the rabbit heart microsomal fraction

The experimental procedures were identical to those of Fig. 28. The formation of diacyl-*sn*-glycerol-3-phosphate in nmoles/mg min is indicated on the ordinate, and the various amounts of fatty acids in mM are shown on the abscissa. Incubation time was 5 min, and the number of experiments was two. Triangles represent the values obtained with palmitate, circles, those with oleate, and dots, linoleate.

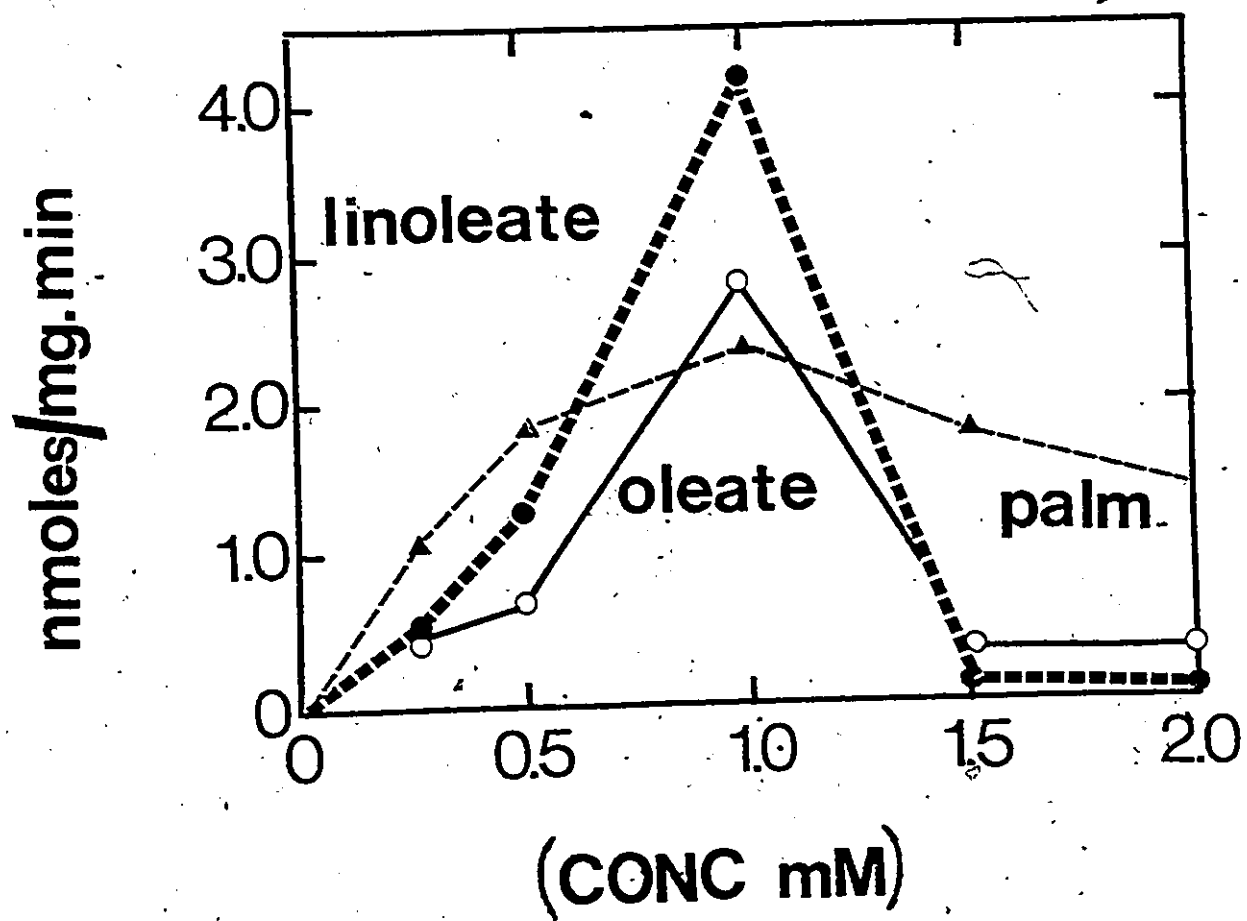


FIGURE 30

TABLE 25

A comparison of relative formation of diacyl-sn-glycerol-3-phosphate from sn-[U-¹⁴C]glycerol-3-phosphate in the rabbit heart microsomal fraction when different acids were used as acyl donors

Enzyme activity		Incorporation of <u>sn</u> -[U- ¹⁴ C]glycerol-3-phosphate into diacyl- <u>sn</u> -glycerol-3-phosphate				
Substrate concentration		0.25 mM	0.5 mM	1.0 mM	1.5 mM	2.0 mM
Substrate		nmol/mg min				
Palmitate		1.08	1.81	2.37	1.78	1.41
Oleate		0.45	0.66	2.80	0.35	0.35
Linoleate		0.49	1.30	4.16	0.08	0.04

The experimental conditions and assay procedure were identical to those in

Fig. 30.

LEGEND TO FIGURE 31

The incorporation of $\text{sn-[U-}^{14}\text{C]}$ glycerol-3-phosphate into lipids by the cardiac mitochondrial fraction in rabbits when palmitate served as acyl donor

The final concentration of palmitate was 1.0 mM, and the enzyme source was 2.1 mg mitochondrial protein. For further details of the assay procedure, see the legends to Tables 5 and 11. The ordinate represents the incorporation of $\text{sn-[U-}^{14}\text{C]}$ glycerol-3-phosphate into lipids in nmoles/mg. The abscissa indicates incubation time in min. MAGP and DAGP stand for monoacyl- and diacyl-sn-glycerol-3-phosphate, respectively.

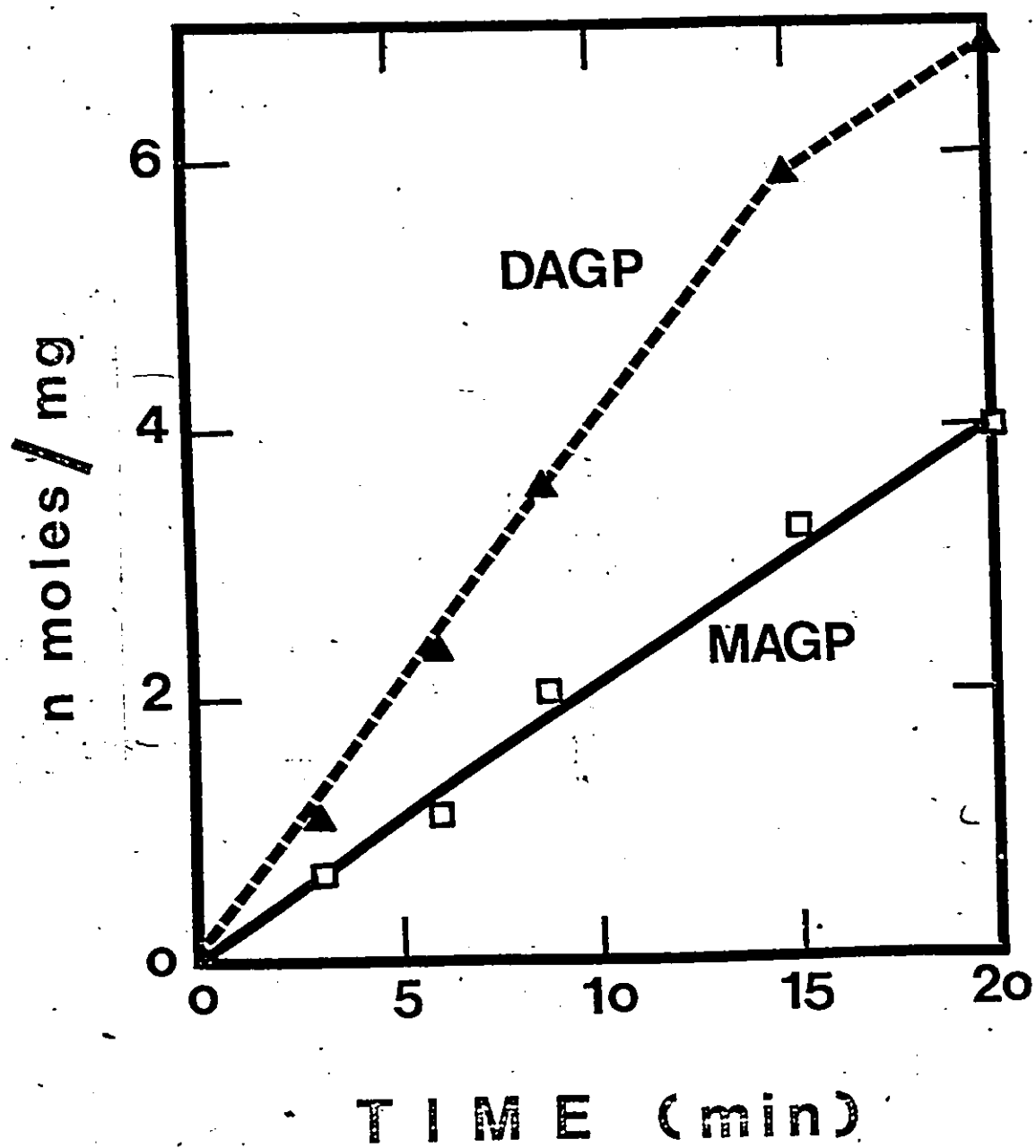


FIGURE 31

LEGEND TO FIGURE 32

The incorporation of $\text{sn-[U-}^{14}\text{C]}$ glycerol-3-phosphate into lipids by the cardiac mitochondrial fraction in rabbits when oleate served as the acyl donor

The experimental conditions and assay procedure were described in the legend to Tables 5 and 11, except that, 1.0 mM of oleate was used as acyl donor. Mitochondrial protein contents were 2.1 mg. The ordinate indicates the incorporation of $\text{sn-[U-}^{14}\text{C]}$ glycerol-3-phosphate into lipids in nmoles/mg. The abscissa represents the incubation time in min. MAGP and DAGP stand for monoacyl- and diacyl-sn-glycerol-3-phosphate, respectively.

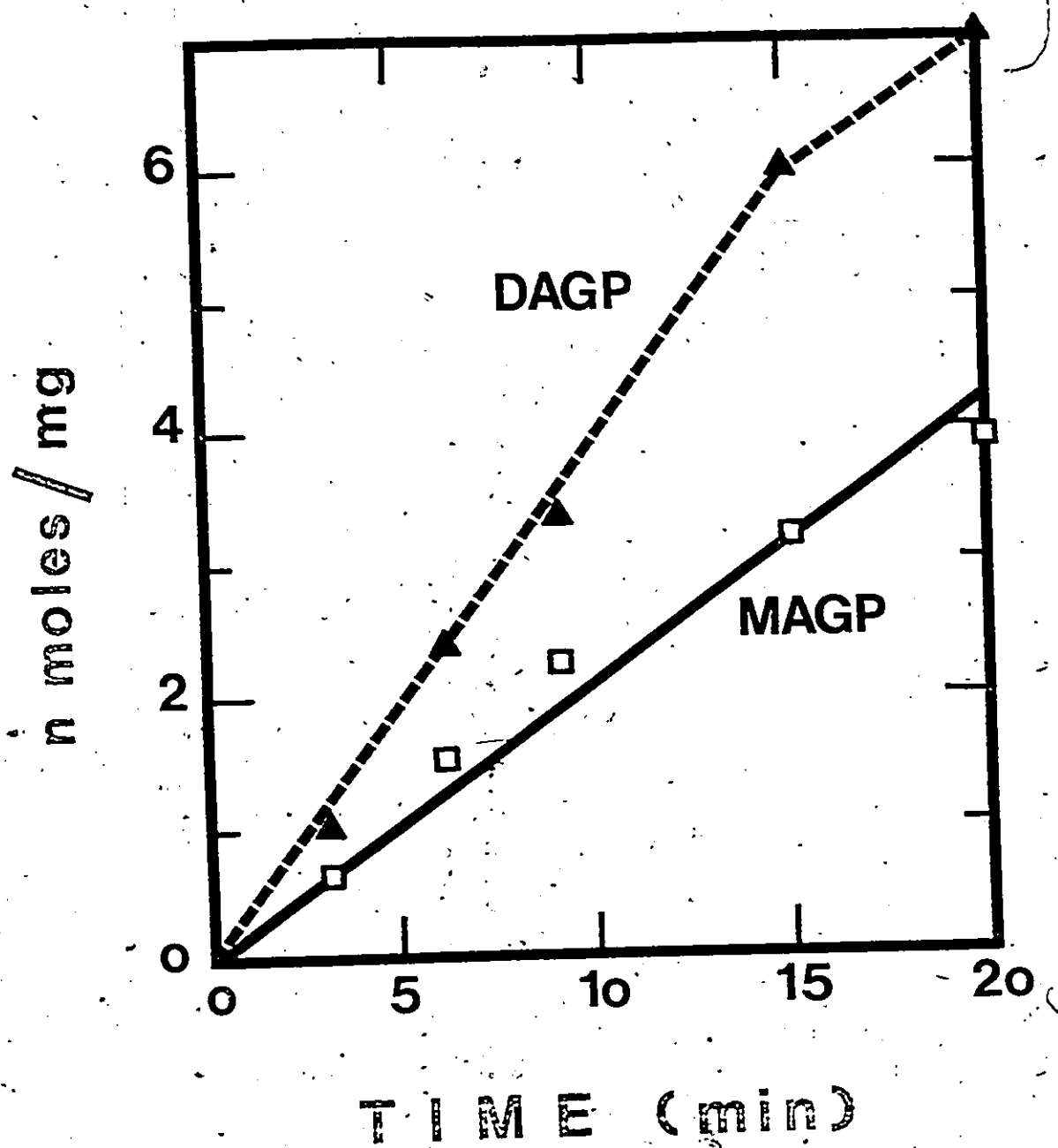


FIGURE 32

TABLE 26

A comparison of palmitate and oleate as an acyl donor for acyl-CoA:sn-glycerol-3-phosphate-0-acyltransferase in cardiac mitochondria in rabbits

Time min	Incorporation of palmitate into:		Incorporation of oleate into:	
	Monoacyl-sn- glycerol-3- phosphate	Diacyl-sn- glycerol-3- phosphate	Monoacyl-sn- glycerol-3- phosphate	Diacyl-sn- glycerol-3- phosphate
	nmoles/mg		nmoles/mg	
3	0.6	1.1	0.7	1.0
6	1.1	2.3	1.5	2.4
9	2.0	3.5	2.2	3.4
15	3.2	5.8	3.2	6.0
20	4.0	6.8	3.9	9.0

The experimental procedures were stated in the legends to Figs. 31 and 32.

LEGEND TO FIGURE 33Thin layer chromatography of the dephosphorylation reaction product from monoacyl-sn-glycerol-3-phosphate

Monoacyl-sn-glycerol-3-phosphate which was synthesized by the rabbit heart mitochondrial fraction with palmitate as the acyl donor was isolated by thin layer chromatography, pooled (29.8 nmoles), and then dephosphorylated by incubation with lysosomal phosphatidate phosphohydrolase (4 mg protein/ml) prepared from rat liver. The products were extracted and chromatographed with a solvent system containing chloroform-acetone-methanol (v/v/v) (Thomas et al., 1965) as described in Section III-2-5. The radioactivity of the developed plate was scanned by an Actigraph (Nuclear Chicago Corp.). The full scale of the latter equipment was set at 1000 cpm and a scanning speed of 60 cm/hr was used. In order to maintain a high resolution, the slit width of the scanner was narrowed to 3 mm, and the response time was adjusted to 20 sec to suppress the noise level. The positions of 1- and 2-monoglycerides (MG) were identified with authentic standards. MAGP stands for monoacyl-sn-glycerol-3-phosphate. The points represent the direct cpm reading from scanning.

RADIOACTIVE SCANNING

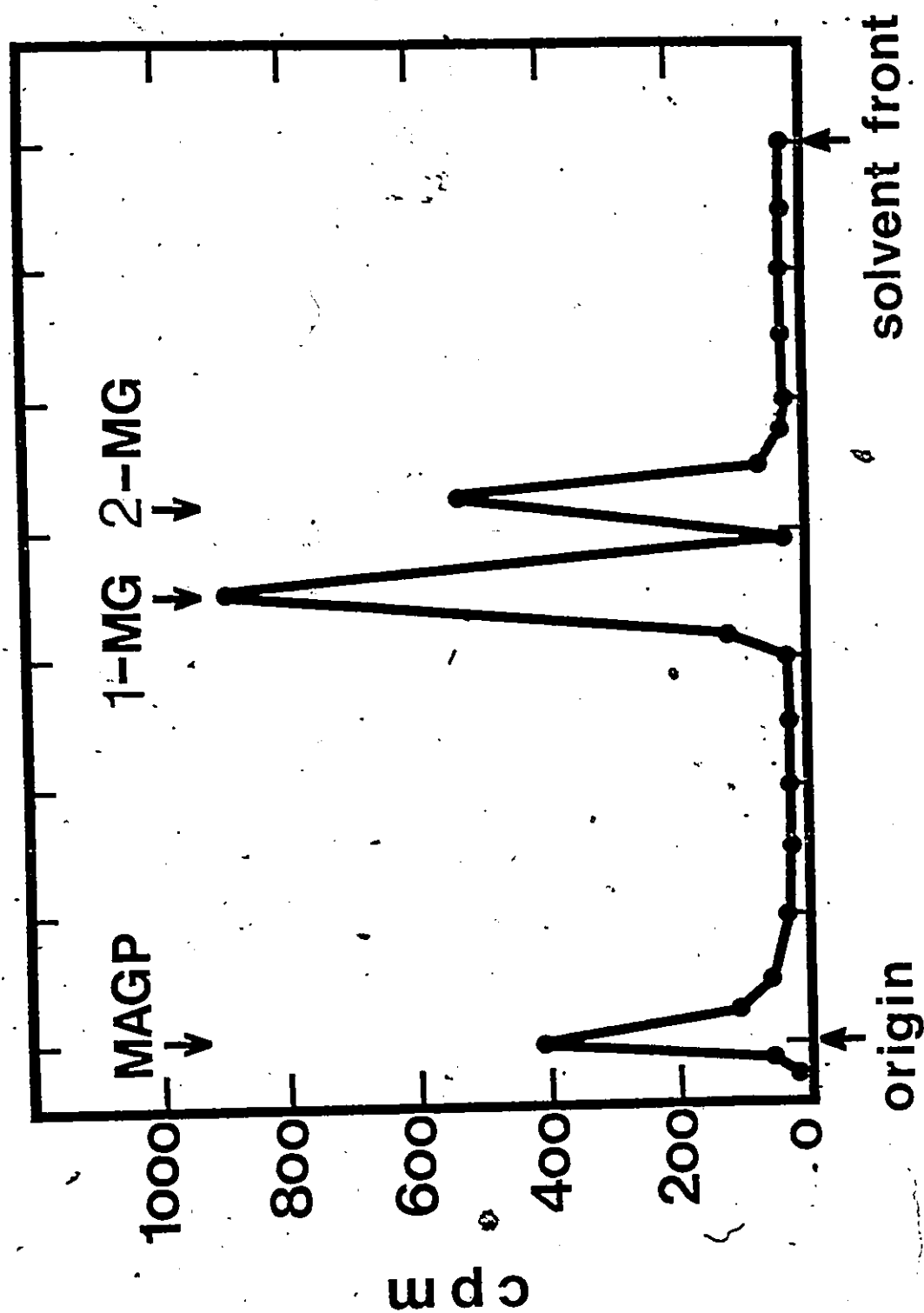


FIGURE 33

TABLE 27

The positional specificity of monoacyl-sn-glycerol-3-phosphate biosynthesis
by the rabbit heart mitochondrial fraction

Acyl donor	Monoacyl-sn-glycerol-3-phosphate used nmoles	Amount dephosphorylated nmoles (%)	Relative amount of radioactivity found in	
			1-monoglyceride %	2-monoglyceride %
Palmitate	29.8	20.7 (70.)	63.8	36.2
Palmitate	35.8	27.6 (78.)	60.7	39.3
Oleate	20.7	13.2 (64.)	35.5	64.5
Oleate	25.0	18.0 (72.)	32.8	67.2
Linoleate	16.5	11.6 (71.)	42.8	57.2
Linoleate	18.0	13.5 (75.)	48.5	51.5
Palmitoyl-CoA	19.8	15.5 (78.)	69.9	30.1

For the experimental details, see Section III-2.

LEGEND TO FIGURE 34

The positional specificity of monoacyl-sn-glycerol-3-phosphate biosynthesis by the rabbit heart mitochondrial fraction

The experimental details are identical to the legend in Table 28 (Section III-2). The value shown for palmitate (16:0), oleate (18:1), and linoleate (18:2) is the average of two experiments as indicated in Table 27. The number of experiments for palmitoyl-CoA is one. Open bars represent position 1, while shaded bars indicate position 2.

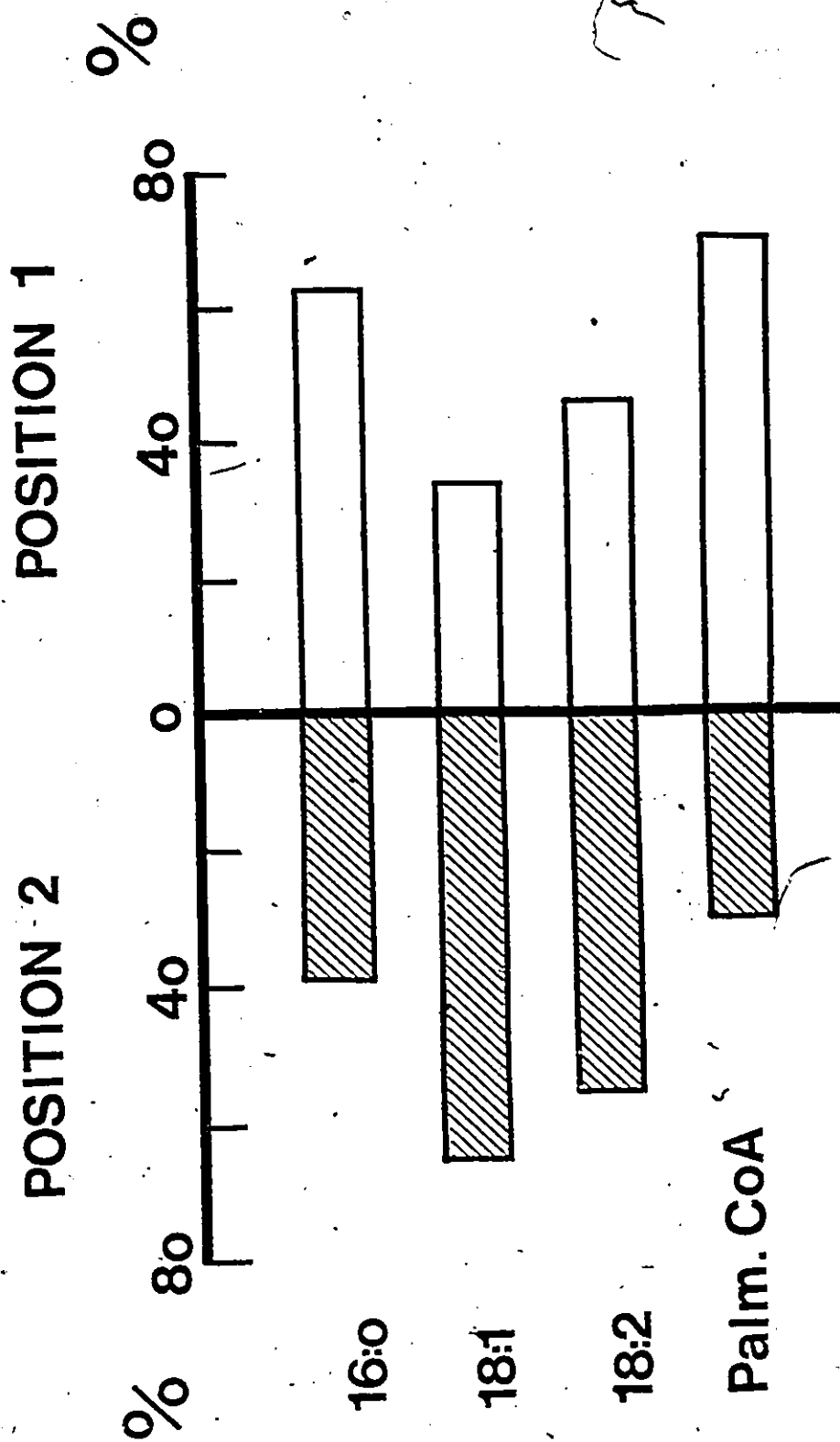


FIGURE 34

TABLE 28

Additional analyses of the positional specificity of monoacyl-sn-glycerol-3-phosphate (MAGP).

Acyl donor	Monoacyl- <u>sn</u> -glycerol-3-phosphate used nmoles	Amount dephosphorylated nmoles (%)	Relative amount of radioactivity found in	
			1-monoglyceride	2-monoglyceride
			%	%
1. After the first treatment of MAGP with phosphatidate phosphohydrolase.				
Palmitate	30.0	21.5 (72)	65.2	34.8
Oleate	26.7	18.7 (70)	36.0	64.0
2. After a second treatment of non-hydrolyzed MAGP with phosphatidate phosphohydrolase.				
Palmitate	6.8*	4.0 (59)	71.0	29.0
Oleate	6.9*	4.2 (61)	31.6	68.4
3. Combined results of the two treatments.				
Palmitate	30.0	25.5 (85)	66.0	34.0
Oleate	26.7	22.9 (86)	35.2	64.8

* 6.8 out of a theoretical value of 8.4, and 6.9 out of 8.0 (all in nmoles)

were recovered from the first experiment.

IV-2. Properties and Subcellular Distribution of
Palmitoyl-CoA Hydrolase in Rabbit Heart

IV-2-1. The effects of sulfhydryl protective agents on
the spontaneous decomposition of palmitoyl-CoA

The sulfhydryl protective agents DTT and cysteine, which were used previously for the activity measurements of palmitoyl-CoA hydrolase (Daniel and Rubinstein, 1968; Fallon and Lamb, 1968), were found to accelerate spontaneous decomposition of palmitoyl-CoA, particularly at alkaline pH (Fig. 35). The decomposition of palmitoyl-CoA was accelerated from 0.2 to 1.3 nmoles/30 min at pH 7.4 and from 0.4 to 2.6 nmoles/30 min at pH 9.5 in the presence of 5 mM DTT and 25 mM cysteine (Fig. 35). A similar observation was reported by Daniel and Rubinstein (1968) working with rat adipose tissue homogenates, in which they found that the spontaneous hydrolysis of palmitoyl-CoA was increased approximately twelve-fold at pH 10 as compared to that at pH 7.2 in the presence of 25 mM cysteine (240 versus 20 nmoles/30 min at pH 10 and 7.2, respectively). It is obvious that increased pH values in an incubation medium further potentiate the effect of DTT and cysteine on the hydrolysis of palmitoyl-CoA, i.e., the rate of spontaneous

hydrolysis was 0.4 nmoles/30 min at pH 5.0 and increased to 1.3 and 2.6 nmoles/30 min at pH 7.4 and 9.5, respectively (Fig. 35). Kurooka et al. (1972) studied the properties of palmitoyl-CoA hydrolase in various organs of rats and found that Tris-HCl buffer at pH values above 9.0 caused spontaneous hydrolysis of the substrate even when DTT and cysteine were not present in the incubation mixture. Our observations (Fig. 35) thus suggest that the presence of sulfhydryl protective agents in an incubation medium, particularly at alkaline pH, alter the stability of palmitoyl-CoA, which must be taken into account for successful assays of palmitoyl-CoA hydrolase activity.

IV-2-2. Properties of palmitoyl-CoA hydrolase in rabbit heart homogenates

The rate of hydrolysis of palmitoyl-CoA by rabbit heart homogenates was constant up to 120 min under the experimental conditions selected as is shown in Fig. 36. The activity of palmitoyl-CoA hydrolase in heart homogenates was strongly pH dependent (Fig. 37). The optimal pH for the hydrolysis of palmitoyl-CoA by heart homogenates was approximately 8.5, and there was no measurable activity at a pH value of 9.7. The activity observed at pH 5.5-7.5 was only one-twelfth of

that at the optimal pH (Fig. 37). Similar observations were reported by Kurooka et al. (1972), Jansen and Hülsmann (1973), and Frosolono et al. (1971). Working with rat serum (Jansen and Hülsmann, 1973) and liver (Kurooka et al., 1972), these authors found that the activity of palmitoyl-CoA hydrolase was almost optimal at pH 8.0-8.5, and non-enzymatic palmitoyl-CoA hydrolysis became significant at pH values higher than 9. Similarly, Frosolono et al. (1971) reported that the hydrolysis of palmitoyl-CoA by dog lung microsomes was lowest at pH 6.0-7.5, however, the activity increased at both acid and alkaline pH. In contrast, Daniel and Rubinstein (1968) reported a completely different pH-activity curve for palmitoyl-CoA hydrolase in rat adipose tissue homogenates. These authors found that the optimal pH was 7.0 with the activity decreasing markedly at both acid and alkaline pH.

Williamson et al. (1968) reported that (+)-decanoyl-carnitine was a competitive inhibitor for carnitine palmitoyltransferase. From this observation, it was thought that the addition of this compound to the incubation mixture could prevent the oxidation of added substrate, palmitoyl-CoA, thus making it available in a larger quantity than

otherwise for the palmitoyl-CoA hydrolase reaction. Therefore, the effect of various concentrations of (+)-decanoyl-carnitine on the activity of palmitoyl-CoA hydrolase was examined.

For this purpose, the assays were carried out in an atmosphere of room air instead of N_2 gas. The results are illustrated in Fig. 38. The activity of palmitoyl-CoA hydrolase in heart homogenates was increased as the concentration of (+)-decanoylcarnitine increased up to 7.5 mM; it stayed constant as the concentration was further increased to 12 mM. However, it is unlikely that this increase in activity is due entirely to the inhibition of palmitoyl-CoA oxidation by (+)-decanoylcarnitine since the activities measured in air and in nitrogen atmospheres both in the absence (0.45 vs. 0.51 nmole/mg min) and in the presence (3.10 vs. 2.76 nmoles/mg min) of (+)-decanoylcarnitine were almost identical. The non-linear potentiation of the palmitoyl-CoA hydrolase activity by (+)-decanoylcarnitine shown in Fig. 38 suggests that besides the inhibition of carnitine palmitoyltransferase, (+)-decanoylcarnitine may have an effect in altering the physio-chemical states of the substrate or may modify the physical configuration of the enzyme-substrate complex, thus resulting in

non-linear kinetics.

Previous studies on acyltransferase reactions, Lands and Hart (1965) used spectrophotometric assay techniques which measured the rate of release of CoA from acyl-CoA, and in which both acyltransferase and acyl-CoA hydrolase activities were measured simultaneously. In order to minimize the influence of acyl-CoA hydrolase on the measurement of acyltransferase reactions, Lands and Hart (1965) attempted to selectively inhibit the hydrolase activity in their enzyme preparation. They found that among several agents tested, DFP was fairly effective in inhibiting hydrolase activity, and that the acyltransferase reaction was relatively unaffected by the DFP. The hydrolase activity in guinea pig liver microsomes was inhibited 78-88 % by preincubation of the enzyme with 3 mM DFP for 20-30 min. at 0° C. The DFP was most effective when the enzyme was preincubated for at least 1 hr at room temperature with 3 mM reagent (Lands and Hart, 1965). Accordingly, we have tested the effect of various concentrations of DFP on palmitoyl-CoA hydrolase activity in rabbit heart homogenates.

By preincubation of the enzyme preparation with DFP for 1 hr at 0° C, palmitoyl-CoA hydrolase activity in heart

homogenates was inhibited 38 % and 50 % at 3 mM and 5 mM DFP, respectively, and complete inhibition was achieved at a much higher concentration (30 mM) (Fig. 39). Frosolono et al. (1971) reported that in dog lung microsomes, the hydrolase activity was completely removed by treatment with 3 mM DFP in the same way as described by Lands and Hart (1965). Jezyk and Hughes (1971) observed that pig liver microsomes were relatively insensitive to DFP, while rat liver microsomes were strongly sensitive to DFP. By preincubation of enzymes with 1 mM DFP at 0° C for 60 min, the hydrolase activity in pig and rat liver microsomes was inhibited by 40 % and 70 %, respectively (Jezyk and Hughes, 1971). In contrast, palmitoyl-CoA hydrolase activity in various tissues (liver, kidney, heart, spleen, and brain) of the rat was not inhibited significantly by preincubation of enzyme with 4.4 mM DFP in 10 % 2-propanol or ethanol in 0.1 M Tris-HCl buffer (pH 8.0) at 38° C for 60 min (Kurooka et al., 1972). In comparison with hepatic palmitoyl-CoA hydrolase (Lands and Hart, 1965; Jezyk and Hughes, 1971), the cardiac enzyme was relatively insensitive to DFP (40-88 % of inhibition at 1-3 mM DFP for the hepatic enzyme vs. 50 % inhibition at 5 mM DFP for the cardiac enzyme). It

is possible that the DFP does not completely block the hydrolase activity, but alters the configuration of the enzyme sufficiently to produce an increased susceptibility to denaturation by detergent action of the acyl-CoA.

The relationship between rates of hydrolysis of palmitoyl-CoA and amounts of microsomal or soluble fractions was next examined (Fig. 40). The rate of hydrolysis of palmitoyl-CoA was proportional to the amount of the enzyme preparation up to 1.2 mg protein for the microsomal fraction and 3.0 mg protein for the soluble fraction. The activity was similar when either nitrogen gas or room air was used as the atmosphere during incubation (data not given).

IV-2-3. The distribution of palmitoyl-CoA hydrolase in rabbit heart subcellular fractions

The distribution pattern of the hydrolase in subcellular fractions of rabbit heart is shown in Fig. 41 and Table 29. The mitochondrial, lysosomal, microsomal, and soluble fractions all possess activities, namely, 0.106 nmole/min mg for mitochondrial, 0.169 and 0.166 nmole/min mg for lysosomal and microsomal, respectively, and 0.076 nmole/min mg for soluble fraction. The highest specific activity of palmitoyl-

7. 12

CoA hydrolase among various subcellular fractions was found to be in lysosomal and microsomal fractions, while the lowest specific activity was present in soluble fraction. However, the total activity for each fraction showed a different pattern of distribution (Table 29), that is, mitochondrial and soluble fractions contained 38.0 and 37.1 %, respectively, of the total palmitoyl-CoA hydrolase activity, with the remaining being in lysosomal (8.4 %) and microsomal (16.5 %) fractions. The results obtained with subcellular distribution in this study must be interpreted with some reservation because the distribution of marker enzymes for each particular fraction was not determined. The distribution pattern for protein content in each subcellular fraction in rabbit heart (Table 29) was not entirely identical to that in rat heart, as described by Hülsman^s (1961). The distribution of protein content in rabbit heart was 49 % for soluble, 36 % for mitochondrial, and 15 % for the combined microsomal and lysosomal fractions (Table 29); rat heart had a distribution of 26 % for soluble, 44 % for mitochondrial, and 30 % for the microsomal fraction (including lysosomal fraction) (Hülsmans, 1961). In both cases, the protein contents of the nuclei, unbroken cells,

cell debris, and myofibrils consisted of approximately 50 % of the total protein content of the whole homogenates.

The specific activity and the distribution of palmitoyl-CoA hydrolase, as well as the distribution of protein content, in subcellular fractions of rat liver (Kurooka et al., 1972) were entirely different from those of rabbit heart (Fig. 41 and Table 29). In rat liver, Kurooka et al. (1972) found that the specific activity of palmitoyl-CoA hydrolase was highest for soluble fraction (189.94 nmoles/min mg), moderate for microsomal fraction (31.25 nmoles/min mg), and lowest for mitochondrial fraction (23.15 nmoles/min mg). The protein content for rat liver subcellular fraction was highest in soluble (65.7 % of total protein), moderate in microsomal (contained lysosomes) (26.5 % of total protein), and lowest in mitochondrial (7.8 % of total protein) fraction (Kurooka et al., 1972). In rabbit heart (Fig. 41 and Table 29), however, the highest specific activity was found in the lysosomal and microsomal fractions which contained the least amount of protein (15.0 % of the total protein), while the lowest specific activity was observed in the soluble fraction which possessed the largest quantity of protein (49 % of total protein). Mitochondrial fraction

had a specific activity between the microsomal (plus lysosomes) and soluble fractions, and contained 36 % of the total protein. Considering these two factors, namely, the specific activity and the protein content, the calculated distribution of palmitoyl-CoA hydrolase in rat liver was 92 % in soluble, 6 % in microsomal (including lysosomes), and 2 % in mitochondrial fraction (Kurooka et al., 1972), while in rabbit heart, the distribution was 37.1 % in soluble, 38.0 % in mitochondrial, and 24.9 % in the combined fraction of microsomes and lysosomes (Table 29).

The specific activity of palmitoyl-CoA hydrolase reported by Kurooka et al. (1972) in rat liver was extremely high as compared to that in rabbit heart. It is not clear whether the discrepancy is due to the difference in species, in organs, or to the different technique employed in their study to measure enzyme activity, i.e., use of sonicated enzyme preparations and the DTNB colorimetric assay procedure.

LEGEND TO FIGURE 35

The effect of dithiothreitol and cysteine on the spontaneous decomposition of palmitoyl-CoA at various pH.

The reaction mixture, in a final volume of 2.0 ml, contained 0.15 μ mole [$1-^{14}\text{C}$]palmitoyl-CoA, 20 mg bovine serum albumin, 80 μ moles Tris-phosphate buffer at different pH, and the presence or absence of 5 mM dithiothreitol and 25 mM cysteine. The incubation temperature was 37° C, and the gas phase was nitrogen. The ordinate shows the amount of palmitoyl-CoA decomposed in nmoles, and the abscissa indicates the incubation time in min. The symbols are:

- | | | |
|-------|----------|--|
| --△-- | : pH 5.0 | } in the absence of dithiothreitol and cysteine |
| --○-- | : pH 7.4 | |
| --□-- | : pH 9.5 | |
| —△— | : pH 5.0 | } in the presence of dithiothreitol and cysteine |
| —○— | : pH 7.4 | |
| —□— | : pH 9.5 | |

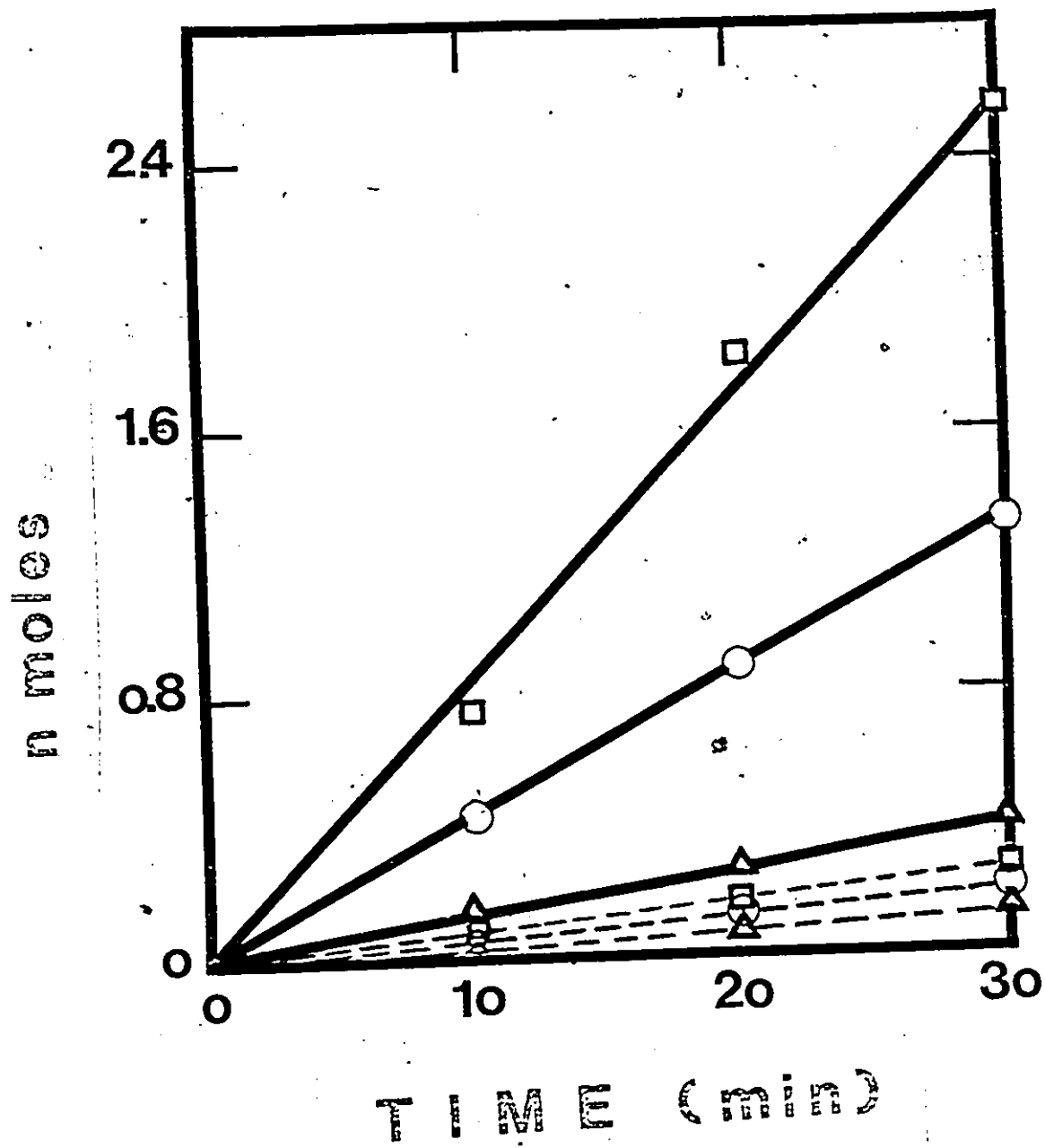
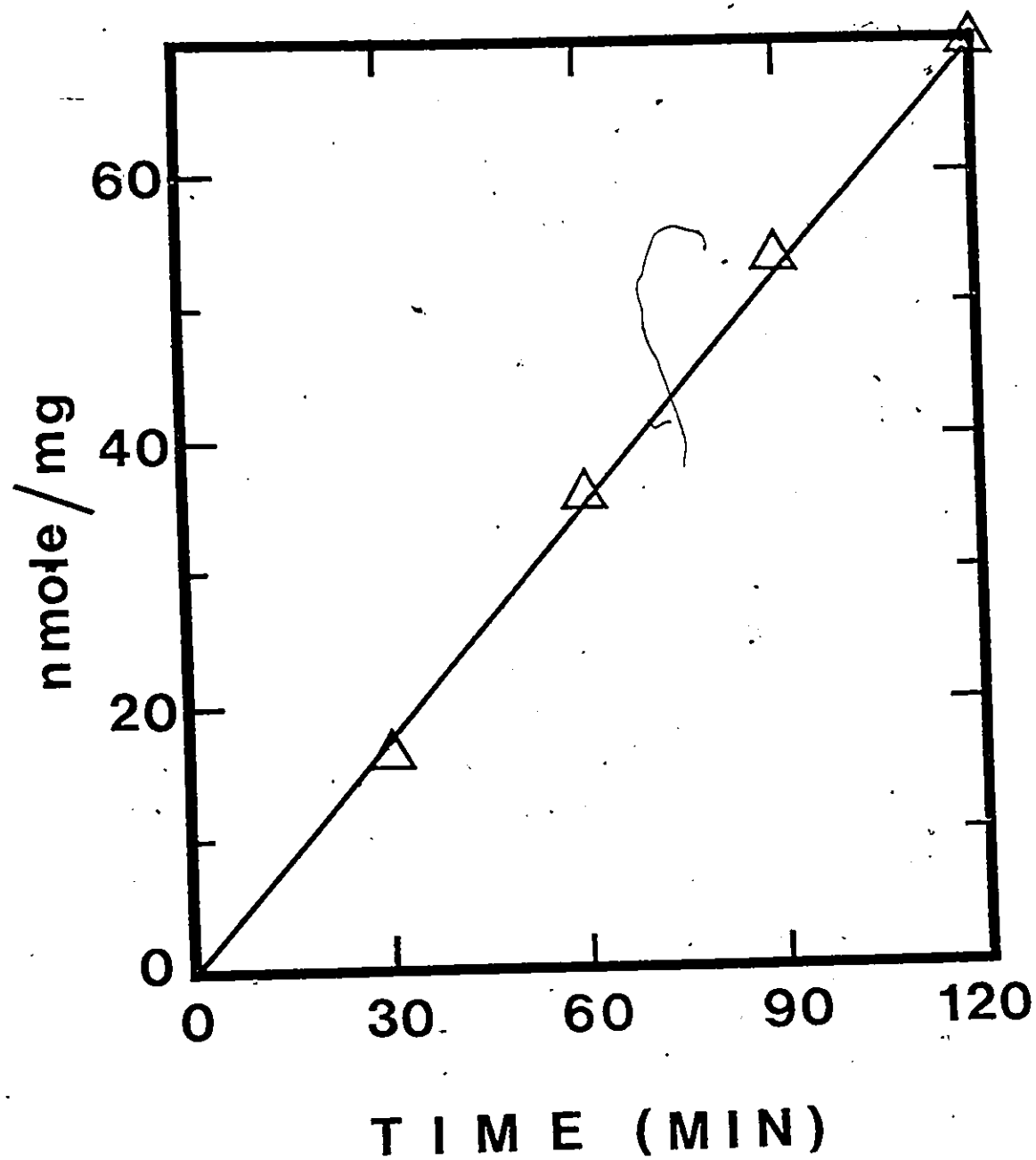


FIGURE 35

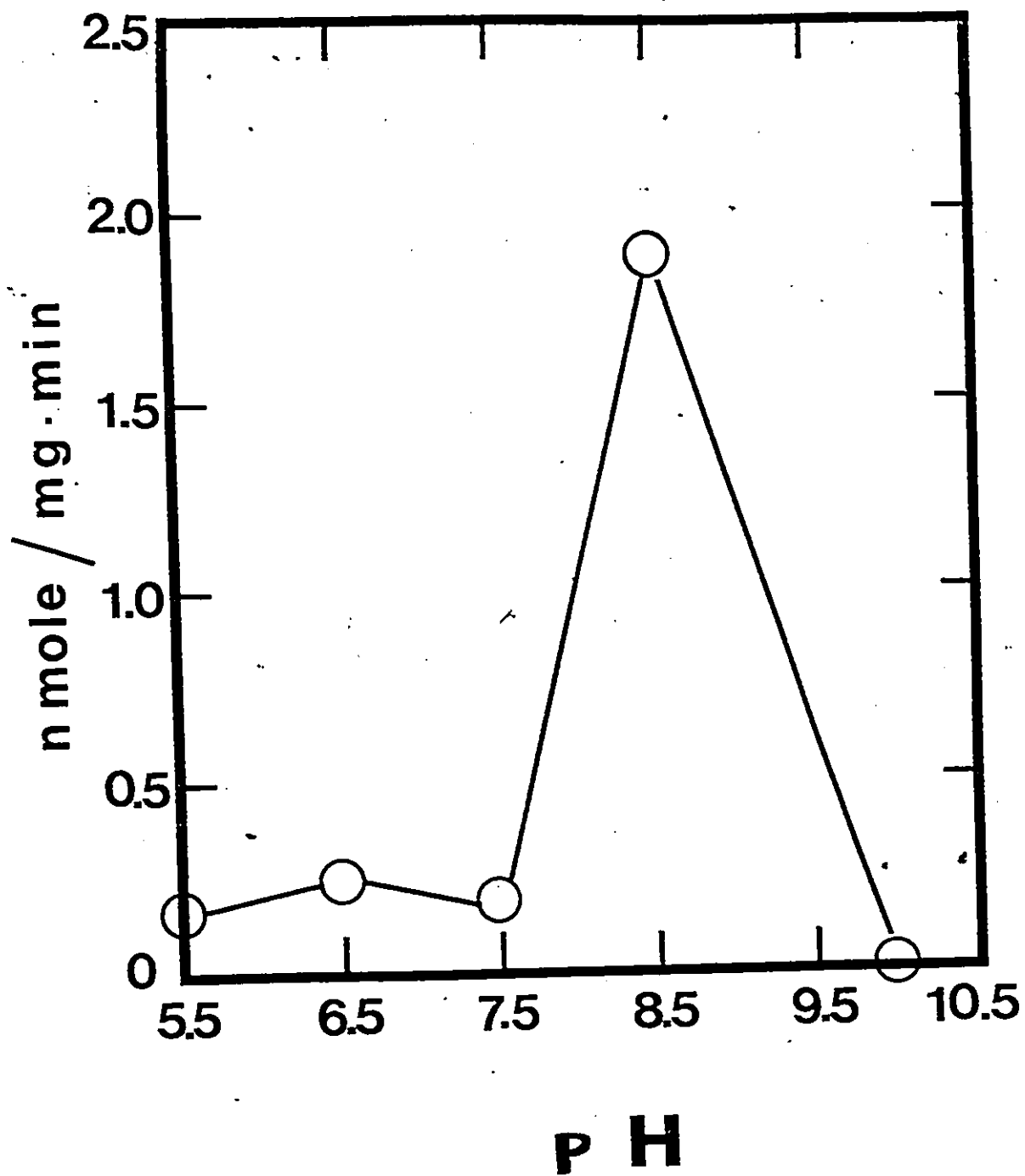
LEGEND TO FIGURE 36The time course of hydrolysis of palmitoyl-CoA by rabbit heart homogenate

The incubation mixture, in a final volume of 2.0 ml, contained 0.2 μ mole [$1-^{14}$ C]palmitoyl-CoA (226,000 d.p.m./ μ mole), 80 μ moles Tris-phosphate buffer, pH 7.4, 20 mg bovine serum albumin, 4.0 μ moles (+)-decanoylcarnitine, and 0.4 mg of freshly prepared homogenate. The temperature of incubation was 37° C, and the gas phase was room air. Activities in nmole/mg protein are expressed on the ordinate, while the duration of incubation in min is shown on the abscissa.

FIGURE 36

LEGEND TO FIGURE 37The effect of different pH values on the activity of
palmitoyl-CoA hydrolase in heart homogenates

The assay conditions are described in the legend to Fig. 36, except that the pH of the assay medium was varied as shown (abscissa), and (+)-decanoylcarnitine was omitted. The amount of heart homogenate was 0.3 mg, the incubation time was 60 min, the temperature was 37° C, and the gas phase was nitrogen. Each value was corrected by the blank value which was obtained in the absence of homogenate after the same period of incubation at the corresponding pH. The rate of hydrolysis is shown on the ordinate in nmole/mg protein min. The values obtained at pH 9.7 in the presence and absence of homogenate were similar.

FIGURE 37

LEGEND TO FIGURE 38

The effect of various concentrations of (+)-decanoylcarnitine on the activities of palmitoyl-CoA hydrolase in heart homogenate

The assay conditions are described in the legend to Fig. 36, except that the amount of (+)-decanoylcarnitine was varied as indicated on the abscissa (mM). The protein content of the homogenate was 0.39 and 0.41 mg protein in these experiments (n=2). Incubation time was 60 min, the temperature was 37° C, and the gas phase was room air. The ordinate indicates the rate of hydrolysis in nmole/mg min.

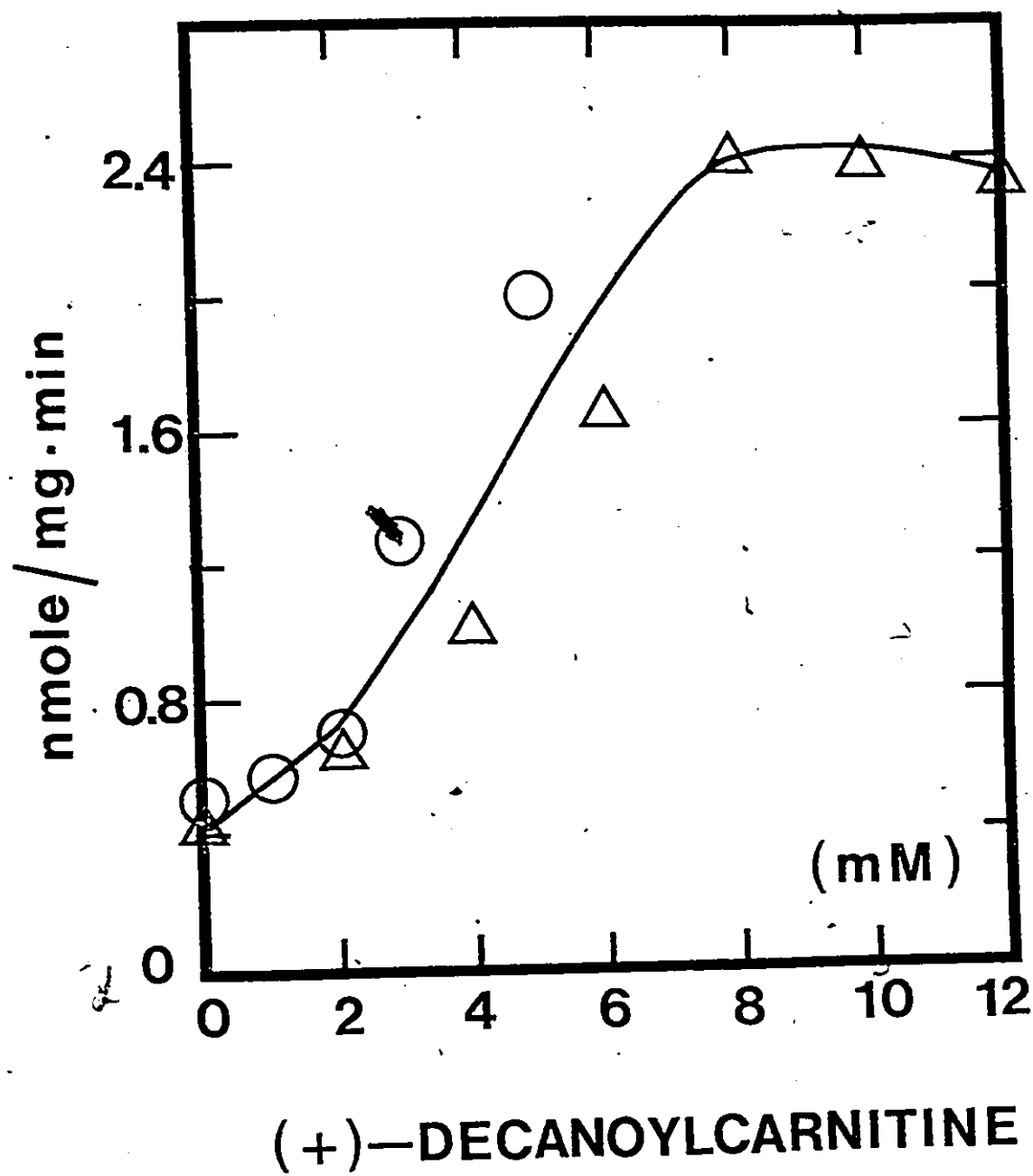


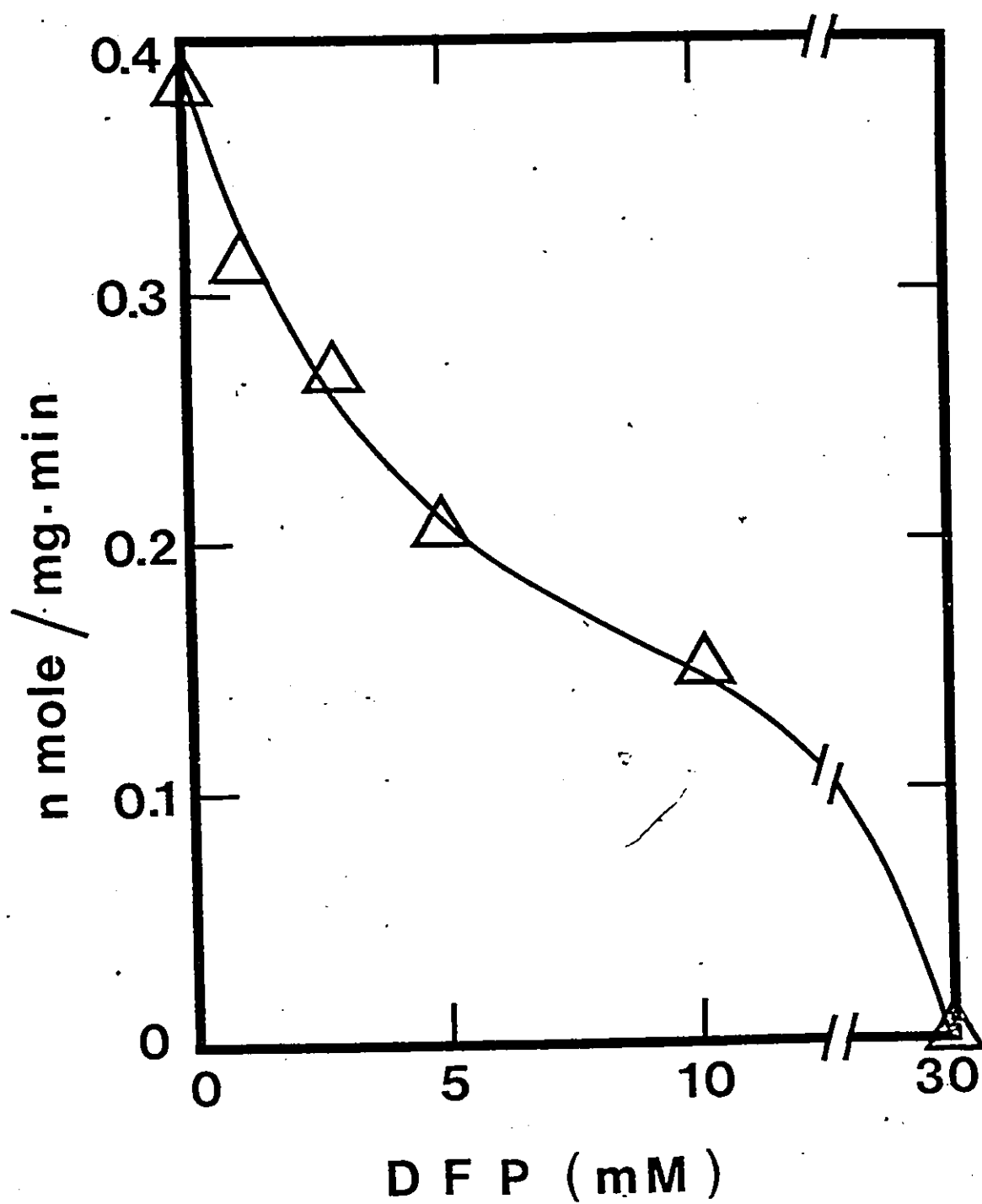
FIGURE 38

LEGEND TO FIGURE 39

The effect of preincubation of heart homogenate with
diisopropylfluorophosphate on the activity of palmitoyl-
CoA hydrolase

✓ The homogenate, containing approximately 4.9 mg protein in 2.0 ml, was preincubated in the presence of various concentrations (mM, abscissa) of diisopropylfluorophosphate (DFP) for 60 min at 0° C in this experiment (n=2). Palmitoyl-CoA hydrolase activity was subsequently assayed by taking 0.1 ml aliquots from the preincubation mixture under conditions similar to those described in the legend to Fig. 36. The incubation was performed under nitrogen gas for 60 min. The activity is indicated on the ordinate in nmole/mg min.



FIGURE 39

LEGEND TO FIGURE 40

The relationship between different amounts of microsomal
or soluble fractions and rates of hydrolysis of palmitoyl-

CoA

For the assay conditions, see the legend to Fig. 36, except that a nitrogen atmosphere was introduced during a 40 min incubation period, and (+)-decanoylcarnitine was omitted. The ordinate indicates the rate of hydrolysis of palmitoyl-CoA in nmole/min, while the abscissa indicates the amounts of subcellular preparations in mg protein. Dots signify the results obtained with a 100,000 x g precipitate containing microsomes and lysosomes, and circles signify results obtained with the soluble fraction.

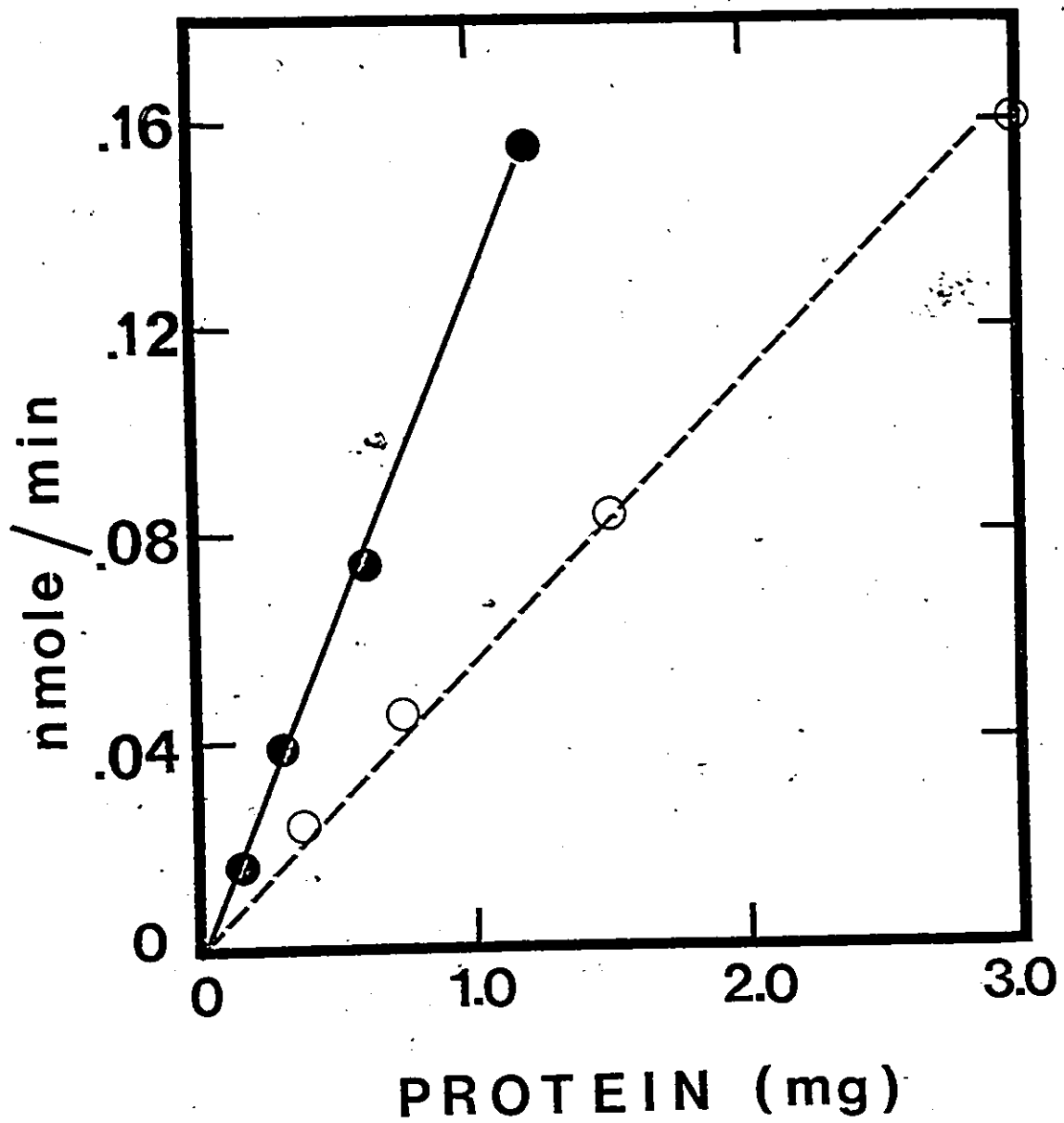


FIGURE 40

LEGEND TO FIGURE 41The distribution of palmitoyl-CoA hydrolase in rabbit heart subcellular fractions

The ordinate indicates the activity in nmole palmitoyl-CoA hydrolyzed per mg protein per min and the abscissa shows % distribution of protein in subcellular fractions. The symbols are: mitochondrial fraction = Mt, lysosomal fraction = Ly, microsomal fraction = Mc, and soluble fraction = S.

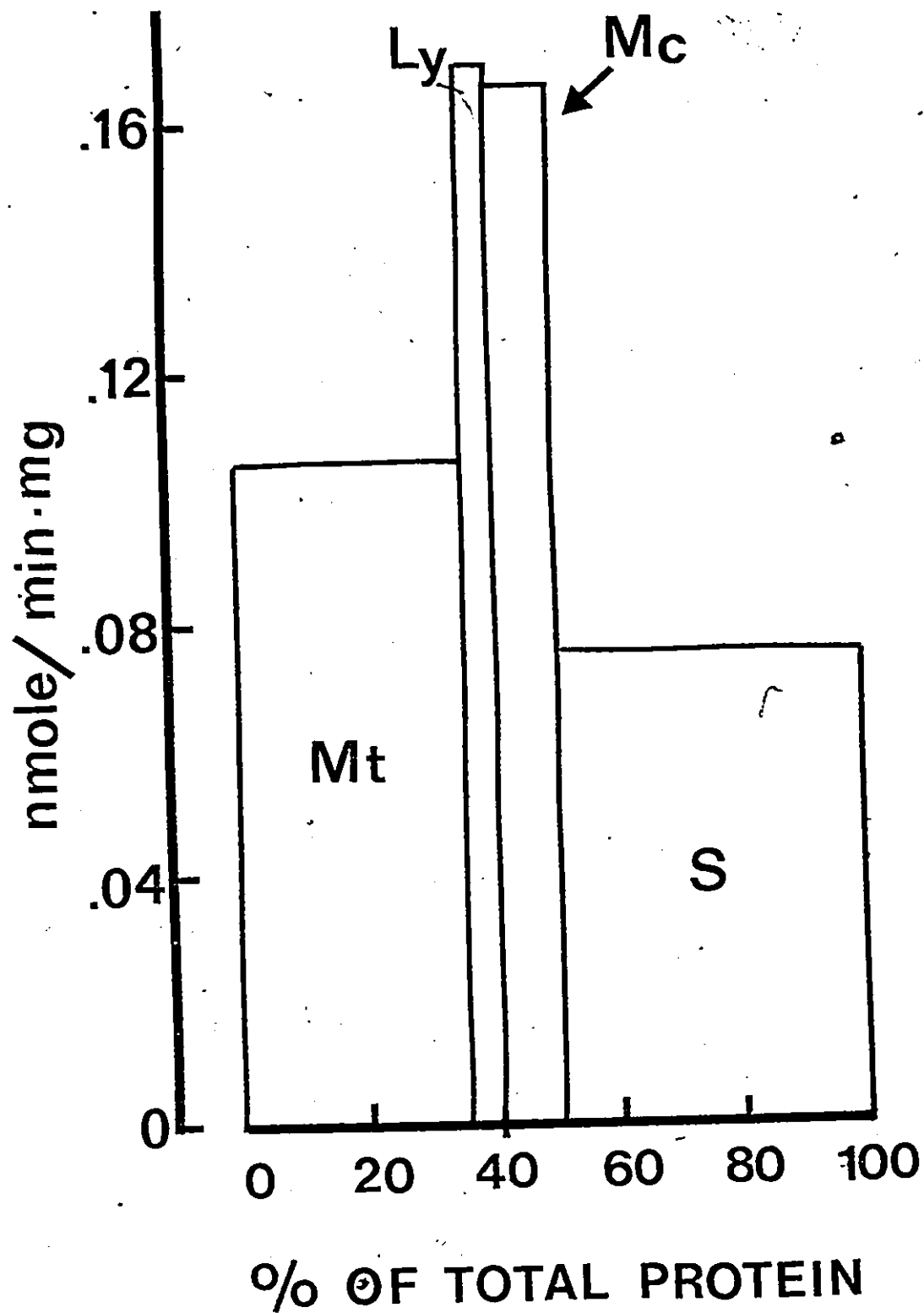


FIGURE 41

TABLE 29

Distribution of palmitoyl-CoA hydrolase in rabbit heart
subcellular fractions

Subcellular fraction	Percentage of total protein*	Specific activity	Distribution of total activity	
			nmole/min per g wet weight of heart	(%)
Mitochondrial	36.0	0.106	1.516	(38.0)
Lysosomal	5.0	0.169	0.338	(8.4)
Microsomal	10.0	0.166	0.664	(16.5)
Soluble	49.0	0.076	1.482	(37.1)

For experimental details, see the legend to Fig. 41.

*Values of total protein were obtained from the enzyme preparation after removal of nuclei, unbroken cells, cell debris, and myofibrils, which consist of approximately 50 % of the whole homogenates.

IV-3. Properties and Subcellular Distribution of
Phosphatidate Phosphohydrolase in Rabbit Heart

IV-3-1. Properties of phosphatidate phosphohydrolase

The relationship between the rate of hydrolysis of phosphatidic acid and the different amounts of heart homogenate from 0 to 2.5 mg protein was linear at the end of various incubation times (Fig. 42). Furthermore, the reaction rate was constant for nearly 3 hours with our assay system (Fig. 43). Since the activity of phosphatidate phosphohydrolase in heart homogenate measured with aqueous phosphatidate was dependent upon the concentration of enzyme protein (Fig. 42), and the rate of reaction was linear with incubation time (Fig. 43), this indicates that aqueous substrate is a suitable preparation for the activity measurement with the homogenate system. This is in agreement with the results obtained by other investigators with kidney (Coleman and Hübscher, 1962), adipose tissue (Jamdar and Fallon, 1973), and liver (Lamb and Fallon, 1974a and 1974b). With pig kidney microsomes, a time course study indicated that zero order conditions lasted for 40 min of incubation when 25-30 % of the aqueous substrate had been hydrolyzed (Coleman and Hübscher, 1962). With rat adipose tissue, the

activities of phosphatidate phosphohydrolase measured using aqueous suspension of substrate in mitochondrial, microsomal, and soluble fractions were linear for 60 min (Jamdar and Fallon, 1973). A similar linear relationship between the activity and incubation time for up to 50 min in rat liver microsomes was observed by Lamb and Fallon (1974a).

Magnesium ions have been reported to stimulate or inhibit the activity of phosphatidate phosphohydrolase from various sources (Smith et al., 1967; Sedgwick and Hübscher, 1967; Mitchell et al., 1971; Jamdar and Fallon, 1973; Lamb and Fallon, 1974a and 1974b). The rate of hydrolysis of phosphatidate (aqueous dispersion) by an ammonium sulphate precipitate from the particle-free supernatant of both cat intestinal mucosa and rat liver was stimulated by 53 to 83 % at 7.5 mM $MgCl_2$ (Smith et al., 1967). At this concentration of Mg^{2+} ions, these investigators showed that microsomal phosphatidate phosphohydrolase prepared from cat intestinal mucosa was strongly inhibited but, even then, the microsomal activity was more than twice that of the supernatant preparation. In the same study, Smith et al. (1967) reported that Mg^{2+} ions caused significant stimulation of the supernatant

enzyme from rat liver with the phosphatidate dispersion as substrate and 37 % inhibition with membrane-bound phosphatidate as substrate. From this, the authors concluded that the observed contradictory results were either due to the differences of the physical states in or to the fatty acid composition of the two substrates.

In another report, the hydrolysis of aqueous phosphatidate by a soluble fraction prepared from rat liver mitochondria was stimulated by 50 % at 3 mM $MgCl_2$ and was inhibited by 20 % at 20 mM (Sedgwick and Hübscher, 1967). In contrast, Jamdar and Fallon (1973) reported that with aqueous substrate, Mg^{2+} at a low concentration (2.5 mM) stimulated the phosphatidate phosphohydrolase from soluble and microsomal fractions of rat adipose tissue, but had no effect on the mitochondrial phosphohydrolase. At a higher concentration (5 mM), Mg^{2+} was inhibitory to the three fractions. When aqueous substrate was substituted for by membrane-bound substrate, the phosphatidate phosphohydrolase from soluble and microsomal fractions was stimulated by a low concentration of magnesium ions. No effect was observed when mitochondrial fraction was used as the enzyme source with membrane-bound substrate (Jamdar and Fallon, 1973).

More recently, Lamb and Fallon (1974) reported that with aqueous substrate, Mg^{2+} at a concentration below 5 mM promoted microsomal phosphatidate phosphohydrolase activity, but was inhibitory at higher concentrations in rat liver microsomes.

Following the reports cited above, we have attempted to clarify the effects of Mg^{2+} on phosphatidate phosphohydrolase activity in homogenates prepared from rabbit heart. It was found that the addition of 1 mM $MgCl_2$ to the incubation mixture did not influence the reaction rate of the enzyme in rabbit heart homogenate, that is, 0.74 nmole/mg min versus 0.81 nmole/mg min in the absence and presence of $MgCl_2$, respectively. The failure of Mg^{2+} to affect phosphatidate phosphohydrolase activity in heart homogenate as observed in our study is presumably due to the presence of a large endogenous pool of these ions in the homogenate preparation.

Figure 44 shows the effect of various pH values on the activity of phosphatidate phosphohydrolase in rabbit heart homogenate examined by using a dialyzed enzyme preparation. Dialysis was performed because it decreased the blank values which were obtained in the absence of substrate for

the same period of incubation time at the corresponding pH values. The activity of the dialyzed enzymes was increased about two-fold. The pH optimum of heart phosphatidate phosphohydrolase activity measured with aqueous substrate appeared to be 7.0 with a marked decrease in apparent activity at acidic pH. The activity at pH 4.0 was only one-sixth of that at optimal pH, while one-third of the activity at optimal pH was found at pH 11 (Fig. 44). The observed activity-pH curve in rabbit heart homogenate was similar to previous findings in rat liver (Sedgwick and Hübscher, 1965; Smith *et al.*, 1967), rat adipose tissue (Daniel and Rubinstein, 1968; Jamdar and Fallon, 1973), and pig kidney (Coleman and Hübscher, 1962). The optimal pH for the mitochondrial and microsomal enzymes of rat adipose tissue appeared to be 6.8 (Jamdar and Fallon, 1973), and that for the microsomal preparation from pig kidney was 6.0 (Coleman and Hübscher, 1962), when assayed with aqueous substrate. With aqueous phosphatidate dispersion, the supernatant enzyme of rat liver had a broad pH optimum between 6.0 and 7.5, while with membrane-bound phosphatidate as substrate, the optimum was sharp at pH 6.8 (Smith *et al.*, 1967). The discrepancy in pH optimum obtained among

investigators might, in part, be due to the different physical states of the substrates.

Next, the relationship between substrate concentrations and reaction rates was examined using the microsomal-lysosomal fraction (Fig. 45). The optimal phosphatidate concentration for heart enzyme was 1.0-1.5 mM which was in accord with that for rat liver microsomes (Lamb and Fallon, 1974). The kinetic data gave a Michaelis-Menten constant (K_m) of 0.33 mM for phosphatidate, a value similar to previous reports with other organs (Coleman and Hübscher, 1962; Sedgwick and Hübscher, 1967; Jamdar and Fallon, 1973). With pig kidney microsomes, Coleman and Hübscher (1962) observed a K_m value of 0.22 mM for aqueous substrate. Sedgwick and Hübscher (1967) reported a K_m value of 0.5 mM using aqueous phosphatidate as substrate for partially purified enzyme from rat liver mitochondria. Due to the stimulatory effect of Mg^{2+} , the K_m value for mitochondrial and microsomal enzymes from rat adipose tissue decreased from 0.55 mM in the absence of Mg^{2+} to 0.24 mM in the presence of 1 mM Mg^{2+} . The slight difference in K_m values reported among investigators could be due to the various degrees of emulsification in the phosphatidate preparations.

The rate of hydrolysis of phosphatidic acid was also proportional to the amount of subcellular fractions up to 1.0 and 3.2 mg protein per flask for microsomal-lysosomal and soluble fractions, respectively (Fig. 46). The linear relationship with mitochondrial fraction was sustained using up to 1.3 mg protein (Fig. 46). Lamb and Fallon (1974) reported that the release of inorganic phosphate from aqueous phosphatidate increased linearly with increasing amounts of microsomal protein prepared from rat liver up to 1.0 mg. In rat adipose tissue, the reaction velocity was proportional to the concentration of protein over a relatively low range, that is, up to 0.3 mg, 0.2 mg, and 0.72 mg for the mitochondrial, microsomal, and soluble fractions, respectively (Jamdar and Fallon, 1973).

IV-3-2. The effect of the supernatant fraction on the conversion of particle-bound phosphatidate to glyceride

In studying the biosynthesis of glycerides via the Kornberg-Kennedy de novo pathway, Smith and Hübscher (1966) found that the formation of neutral lipids from sn-G3P and palmitate by mitochondria of rat liver was stimulated markedly by the addition of the supernatant fraction of the

same organ. In order to evaluate the nature of the stimulation by the soluble fraction, various factors such as CoA, ATP, magnesium ions, bovine serum albumin, and CDP-glycerol, which are known to influence the biosynthesis of glycerides, were investigated. The results showed that none of these factors present in the supernatant fraction could account for the observed stimulatory effect (Smith and Hübscher, 1966). Furthermore, the supernatant fraction contained only negligible amounts of acyl-CoA synthetase, DG acyltransferase, and phosphatidate phosphohydrolase measured with aqueous substrate. From this finding, it was concluded that the stimulatory effect of the supernatant fraction could not be due to a leakage of acyl-CoA synthetase, DG acyltransferase, or phosphatidate phosphohydrolase from the mitochondria into the supernatant during the enzyme preparations (Smith and Hübscher, 1966).

Further investigations of the nature and mechanism of the stimulatory effect of the supernatant fraction on TG biosynthesis in mitochondrial fraction of rat liver (Smith et al., 1967) or in microsomal fraction of hamster and cat intestinal mucosa (Johnston et al., 1967; Smith et al., 1967) showed that the stimulation was due to the phosphatidate

phosphohydrolase present in the soluble fraction. It was reported that this enzyme preferentially acted on membrane-bound phosphatidate, while the mitochondrial and microsomal phosphatidate phosphohydrolase favored the aqueous dispersion of phosphatidate as substrate (Sedgwick and Hübscher, 1967; Johnston et al., 1967).

The hypothesis that the phosphatidate phosphohydrolase existing in the soluble fraction acts preferentially on membrane-bound substrate (Smith et al., 1967; Johnston et al., 1967; Sedgwick et al., 1967) was tested using cardiac subcellular fraction. The results are illustrated in Table 30. The microsomal fraction (9.74 mg protein), which was used for synthesizing the endogenous membrane-bound phosphatidate, contained 28 nmoles phosphatidate and 12 nmoles neutral lipids after one hour incubation in the absence of soluble fraction. However, in the presence of soluble fraction (10.8 mg protein) under identical experimental conditions, phosphatidate was diminished to 25.2 nmoles while neutral lipids were increased to 14.8 nmoles. Thus, the rate of conversion of labelled phosphatidate into neutral lipids by the action of phosphatidate phosphohydrolase in soluble fraction was only 0.0043 nmole/mg min

(Table 29). This finding demonstrates that the activity of the soluble enzyme in rabbit heart was very low with particle-bound phosphatidate in comparison to that with aqueous phosphatidate as the substrate, that is, 0.0043 nmole/mg min for particle-bound and 0.2 nmole/mg min (Table 30) for aqueous substrate. These results are in contrast to those reported with liver or intestine in which the phosphatidate phosphohydrolase of the supernatant fraction has a low activity with aqueous phosphatidate dispersion and a high activity with membrane-bound phosphatidate (Smith et al., 1967; Johnston et al., 1967).

The results presented in this thesis concerning the effect of the supernatant fraction on the conversion of membrane-bound phosphatidate to glyceride in rabbit heart are of a preliminary nature. In our study, the membrane-bound phosphatidate was prepared in the presence of magnesium ions (3 mM). These ions may have been absorbed by the microsomes and, therefore, could have influenced the measurement of phosphatidate phosphohydrolase activity. Magnesium ions at a low concentration (1-5 mM) had a stimulatory effect on rat liver mitochondrial enzyme (Sedgwick and Hübscher, 1965), but at a high concentration, they had an

inhibitory effect on erythrocyte phosphatidate phosphohydrolase (Hokin et al., 1963). It is also possible that the intrinsic G3P acyltransferase and phosphatidate phosphohydrolase present in the microsomes which were used to synthesize membrane-bound substrate could affect the activity measurement of phosphatidate phosphohydrolase in the soluble fraction. Although the assays of phosphatidate phosphohydrolase were carried out under conditions similar to those adopted by previous investigators (Smith et al., 1967; Jamdar and Fallon, 1973), more detailed investigation regarding the influence of incubation conditions on the activity measurement must be carried out. The effect of incubation time, enzyme concentration, intrinsic G3P acyltransferase, etc. on the phosphatidate phosphohydrolase assays must be studied. Therefore, further experimental work is required to establish the role of soluble phosphatidate phosphohydrolase in glyceride biosynthesis in the heart.

IV-3-3. Subcellular distribution of phosphatidate phosphohydrolase in rabbit heart homogenate

The subcellular distribution of phosphatidate phosphohydrolase assayed using aqueous phosphatidate dispersion

as substrate in the absence of Mg^{2+} is shown in Fig. 47 and Table 30. The highest specific activity of phosphatidate phosphohydrolase was found in the microsomal and lysosomal fractions. The activity of this enzyme in mitochondrial fraction was approximately four-tenths of that found in the microsomal fraction. The apparent activity of soluble phosphatidate phosphohydrolase was only 7 % of that found in the microsomal and 16 % of that found in the mitochondrial fraction (Fig. 47 and Table 30). These results are in close agreement with those found using adipose tissue (Jamdar and Fallon, 1973). In adipose tissue, microsomal phosphatidate phosphohydrolase was most active (20 nmoles/mg min) when measured with aqueous substrate in the absence of magnesium ions, while the mitochondrial enzyme was about 1/2 as active as the microsomal phosphatidate phosphohydrolase. The soluble enzyme contained only 6 % of the activity found in the microsomal fraction (Jamdar and Fallon, 1973).

By taking into account the distribution of the protein content of each subcellular fraction, the distribution of total phosphatidate phosphohydrolase in cardiac subcellular fraction was calculated. The results indicated that the

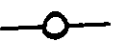
mitochondrial fraction contained the bulk of the enzyme, namely, 43.3 % of the total activity (Table 30). Microsomal fraction possessed 31.6 % of the enzyme, with 15 % of the activity being in the lysosomal and 10 % in the soluble fraction (Table 30). The results obtained in this study were not in agreement with those observed in liver (Sedgwick and Hübscher, 1965), kidney (Coleman and Hübscher, 1962), and adipose tissue (Jamdar and Fallon, 1973), in which the enzyme was assayed with aqueous substrate in the absence of magnesium ions. In rat liver, 37.8 % of the total activity was found in microsomal, 25 % in lysosomal, 18.5 % in mitochondrial, and 12 % in the soluble fraction (Sedgwick and Hübscher, 1965). The discrepancy in results obtained by us and by Sedgwick and Hübscher (1965) was due to the different distribution of protein content for each subcellular fraction, that is, the cardiac mitochondrial fraction contained a greater proportion of total protein than did the hepatic mitochondria (36 % versus 18.5 %), while cardiac microsomes contained a considerably smaller proportion of total protein as compared to that found in hepatic microsomes (10 % versus 37.5 %). In pig kidney, the microsomes contained the highest specific

activity (0.585 μ moles/mg hr) which consisted of 63 % of the total activity, with the remaining activity, 21.2 % and 14.6 %, being distributed in the mitochondrial and soluble fractions, respectively (specific activity, 0.197 nmole/mg hr for the mitochondrial fraction and 0.104 nmole/mg hr for the soluble fraction) (Coleman and Hübscher, 1962). In adipose tissue, 42 % of the phosphatidate phosphohydrolase activity was found in microsomes which contained only 8 % of total protein, while 27 % of the activity was found in mitochondria containing 9 % of total protein and 20 % in the soluble fraction possessing 75 % of total protein (Jamdar and Fallon, 1973).

Since the phosphatidate phosphohydrolase activity in soluble fraction was extremely low, it is difficult to deduce from the present data whether an independent enzyme exists in this fraction or whether the apparent activity observed is due to microsomal contamination. It is also possible that the observed phosphatidate phosphohydrolase activity in the soluble fraction is due to leakage of the mitochondrial enzyme during the preparation procedure since it has been shown that two types of mitochondrial phosphatidate phosphohydrolase exist, one being readily soluble

and the other being tightly bound to mitochondrial membrane (Sedgwick and Hübscher, 1965; see also Section I-2-5 of this thesis). Jamdar and Fallon (1973) proposed that there are two types of phosphatidate phosphohydrolase differing in their response to magnesium ions in rat adipose tissue, that is, Mg^{2+} -dependent and Mg^{2+} -independent enzymes (Section I-2-5). The Mg^{2+} -independent enzyme localized mainly in the mitochondrial fraction, with some activity existing in microsomes (Jamdar and Fallon, 1973). It is possible that there are two types of cardiac enzymes also, and that the low phosphatidate phosphohydrolase activity found in the soluble fraction in this study reflects the Mg^{2+} -dependent property.

LEGEND TO FIGURE 42The effect of various amounts of heart homogenate on the activity of phosphatidate phosphohydrolase

The incubation mixture, in a final volume of 2.0 ml, contained 3.0 μ moles sodium phosphatidate, 160 μ moles Tris-acetate buffer, pH 7.0, and various amounts of homogenate as indicated on the abscissa (mg protein). Incubation was carried out in air at 37° C. The ordinate indicates the activity of phosphatidate phosphohydrolase in nmoles inorganic phosphate released. The symbols  ,  , and  represent the results obtained at the end of 30, 60, and 90 min incubation, respectively.



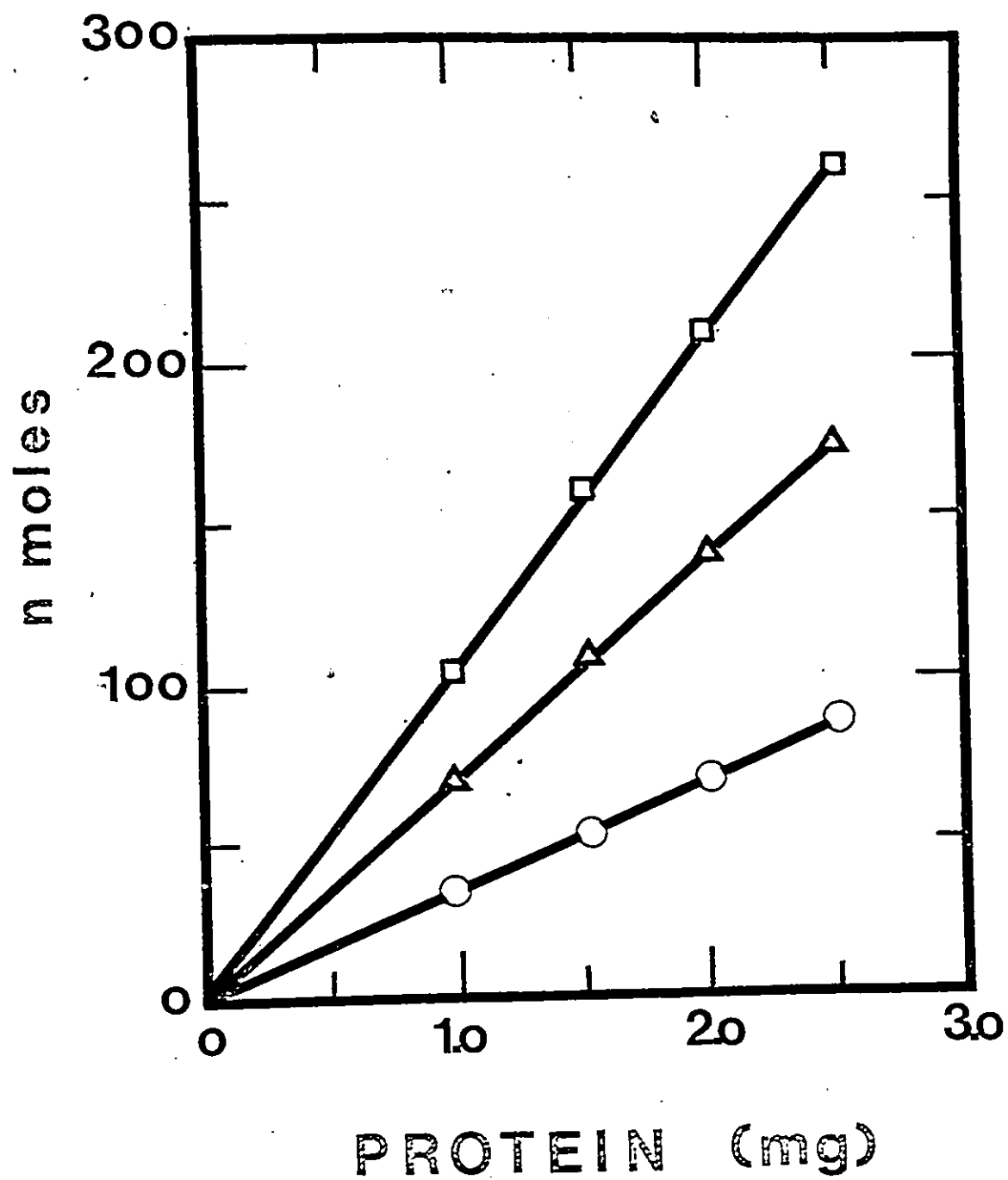


FIGURE 42

LEGEND TO FIGURE 43

The time course of the hydrolysis of phosphatidic acid
by rabbit heart homogenate

The assay mixture contained, in a final volume of 2.0 ml, 3.0 μ moles of phosphatidate, 160 μ moles Tris-acetate buffer, pH 7.0, and 2.55 or 3.10 mg protein of homogenate. Incubation was carried out in air at 37° C. The ordinate indicates the activity of phosphatidate phosphohydrolase in nmole inorganic phosphate released per mg protein, whereas the abscissa indicates the incubation time in min. Dots and circles represent two separate experiments.

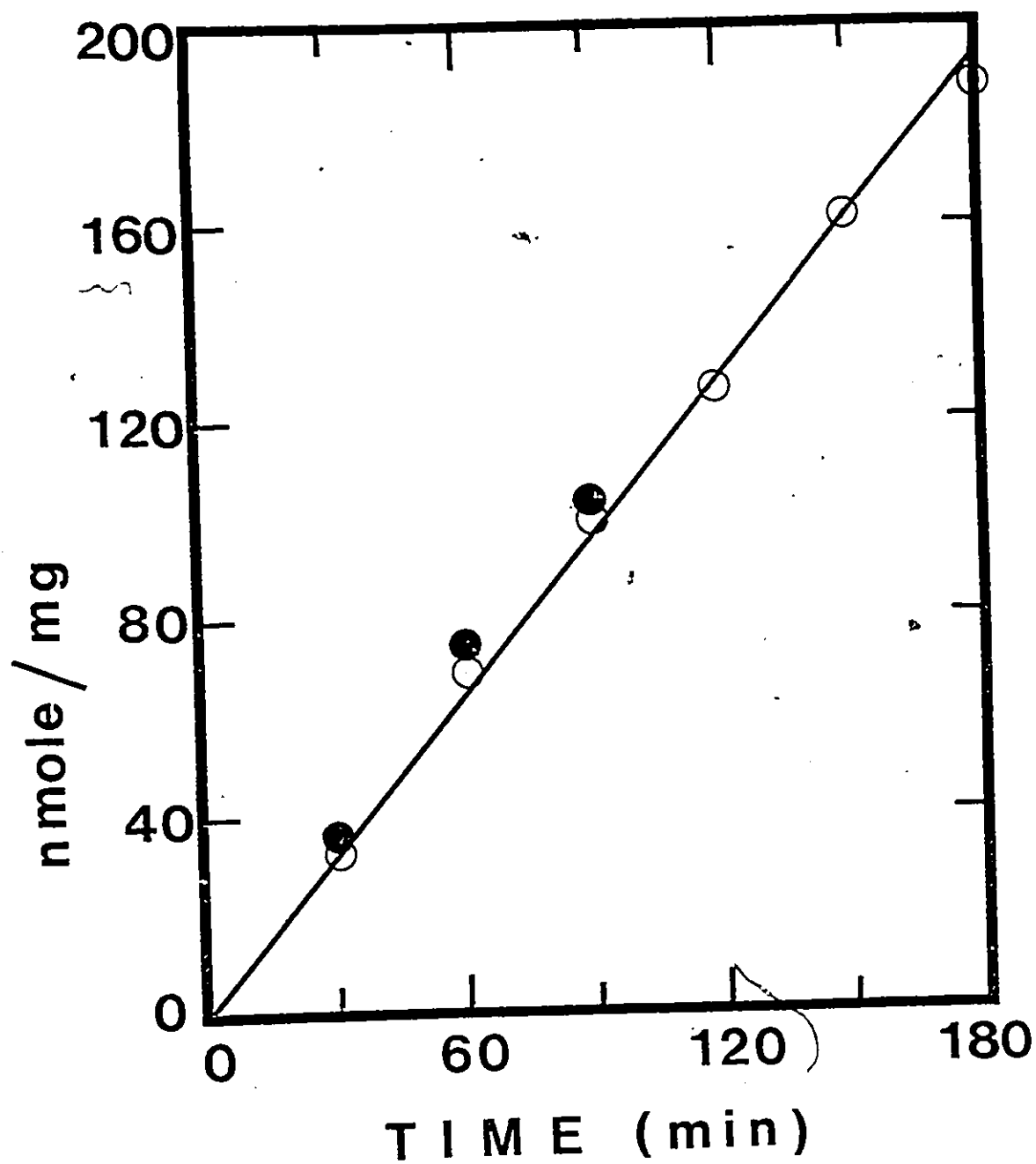
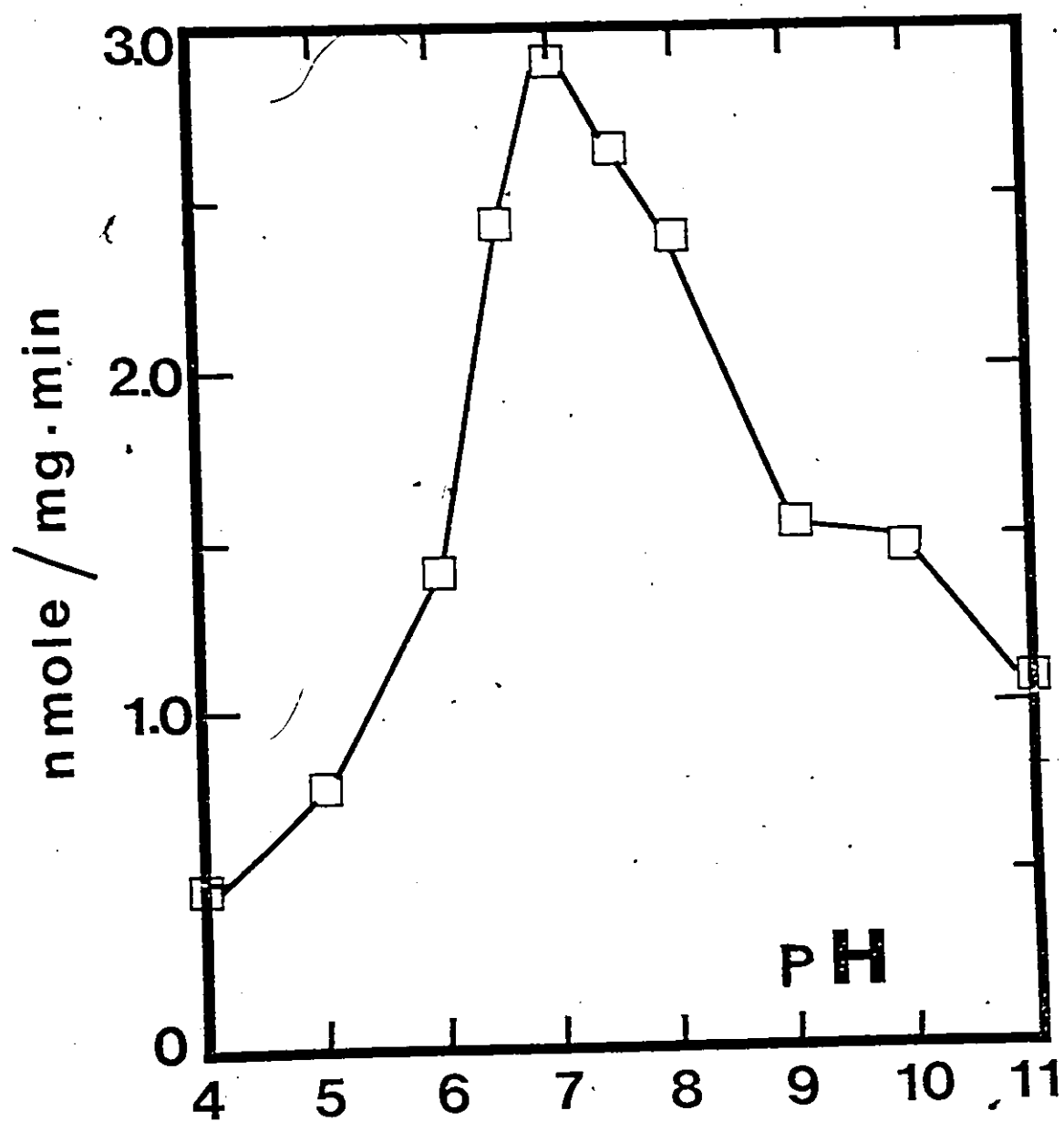


FIGURE 43

LEGEND TO FIGURE 44The pH-activity relationship of phosphatidate phospho-
hydrolase in rabbit heart homogenates

The assay conditions are identical to those described for Fig. 43. The heart homogenate in this experiment was dialyzed against a medium consisting of 0.25 M sucrose, 0.02 M Tris-HCl, and 0.001 M EDTA for 16 hours at 4° C. The amount of homogenate was 1.9 mg, and the incubation lasted for 45 min. Each value was corrected for a small blank value obtained at the same pH in the absence of phosphatidate. The activity is expressed as nmole/mg protein min (ordinate), and the pH values are plotted on the abscissa. Acetic acid and 1N NaOH were used to vary the pH.

FIGURE 44

LEGEND TO FIGURE 45

The effect of various concentrations of phosphatidate
on the activity of phosphatidate phosphohydrolase

The assay conditions are described in the legend to Fig. 43. The incubation was continued for 60 min, and the lysosomal-microsomal fraction (1.56 mg protein) was used as the enzyme source in this experiment. The activity is plotted on the ordinate (nmole/mg min), and the substrate concentration is on the abscissa (mM).

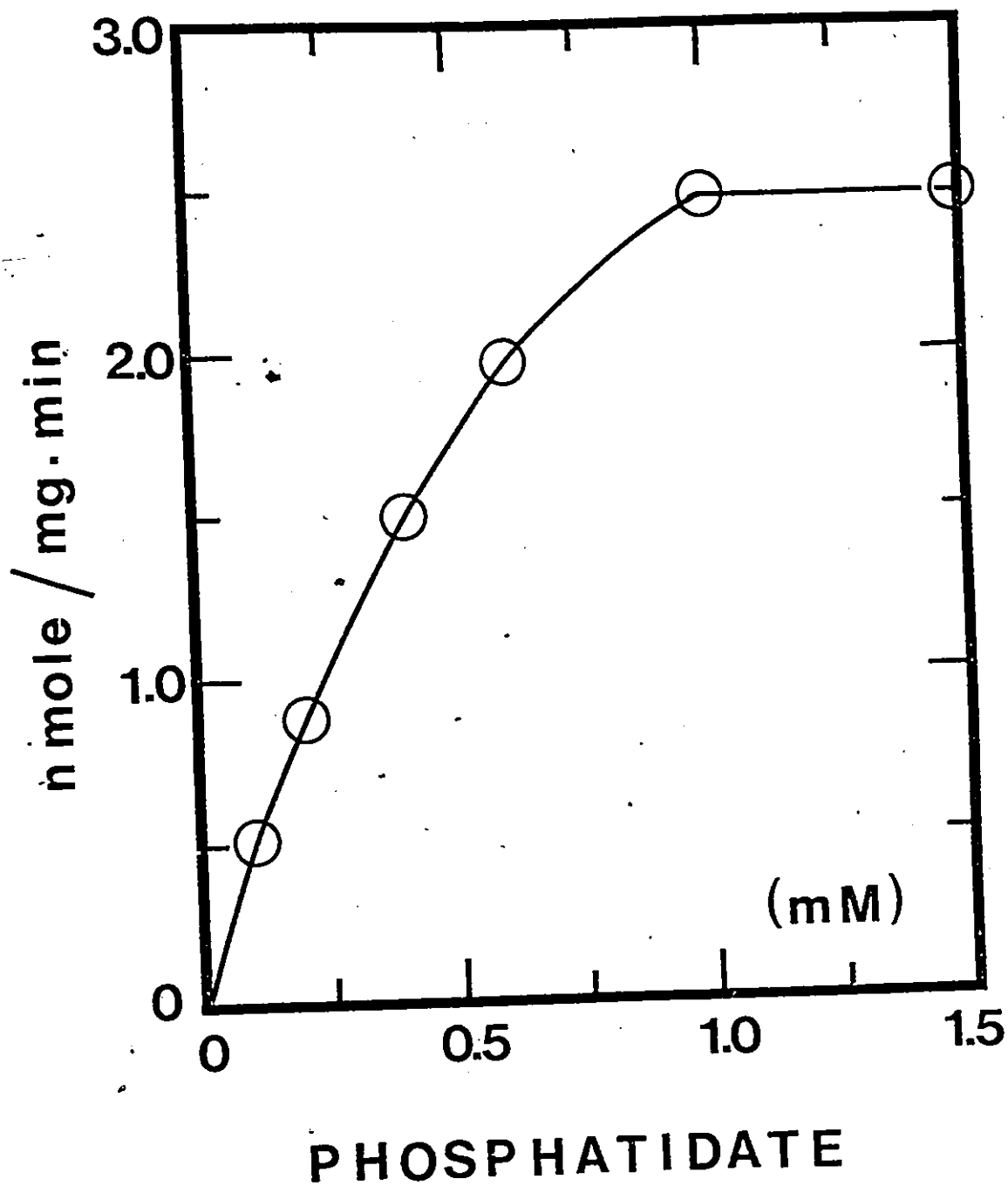


FIGURE 45

LEGEND TO FIGURE 46The relationship between amounts of different subcellular fractions and phosphatidate phosphohydrolase activity

The assay mixture contained, in a final volume of 2.0 ml, 3.0 μ moles of phosphatidate, 160 μ moles Tris-acetate buffer, pH 7.0, and various amounts of subcellular fractions (abscissa, mg protein). The assay was carried out for 45 min at 37° C in air. The activity is expressed as nmoles of inorganic phosphate released per min (ordinate). Squares represent activities of the microsomal-lysosomal preparations, dots represent those of the mitochondrial preparations, and circles represent those of the soluble fractions.

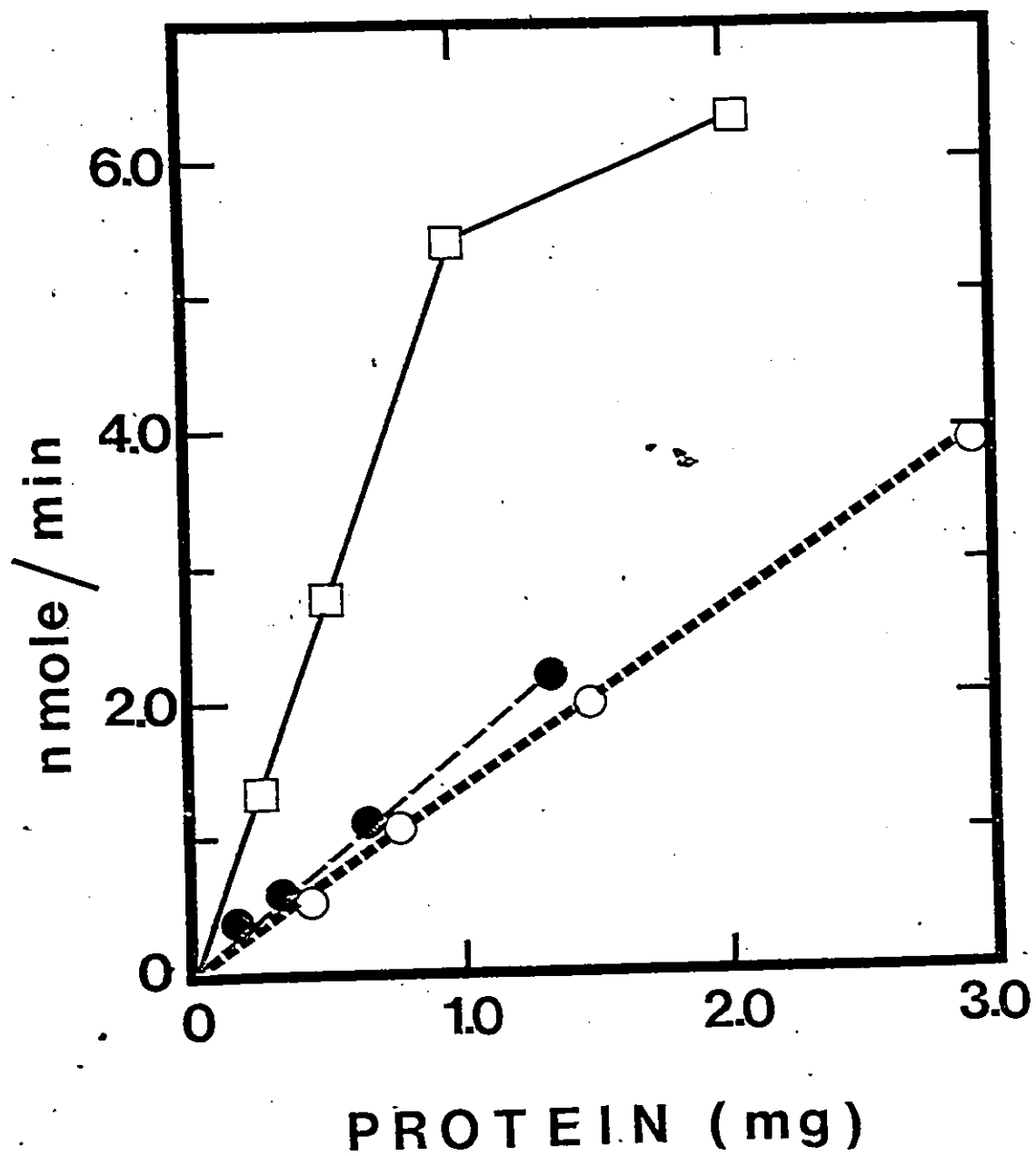


FIGURE 46

LEGEND TO TABLE 30The effect of the supernatant fraction on the conversion of particle-bound phosphatidate to glyceride

Membrane-bound phosphatidate was prepared by incubating the microsomal fractions with sn-[U-¹⁴C]G3P and palmitate in the presence of various cofactors as described in Materials and Methods (Sections III-1-4 and III-4-4). After 5 min incubation at 37° C, 10 tubes of reaction mixture were combined and centrifuged twice at 100,000 x g for 60 min at 4° C and the resultant pellets suspended in 0.25 M sucrose solution. The sucrose suspension was divided equally into three parts, each containing 9.74 mg protein. One of them was immediately extracted by butanol, and the radioactive compounds were analyzed by TLC with a solvent system of petroleum ether-ether-acetic acid (40:10:1, v/v/v) (Section III-4-5). The other two parts were incubated in the absence and presence of the soluble fraction (10.8 mg protein). After incubation for one hour at 37° C, the reaction was stopped by addition of butanol (Sections III-1-4 and III-4-5), and the lipid products were analyzed by TLC in a solvent system of petroleum ether-ether-acetic acid (40:10:1, v/v/v) (Section III-4-5).

TABLE 30

The effect of the supernatant fraction on the conversion
of particle-bound phosphatidate to glyceride

<u>Incubation for 1 hour</u>	<u>Products</u>			
	<u>Phosphatidic acid</u>		<u>neutral lipids</u>	
	(nmoles)	(d.p.m.)	(nmoles)	(d.p.m.)
Soluble fraction absent	28.0	46,000	12.0	19,500
Soluble fraction present	25.2	41,400	14.8	24,000

The rate of neutral lipids formed by the addition of
soluble fraction = 0.0043 nmole/mg min.

LEGEND TO FIGURE 47Subcellular distribution of phosphatidate phosphohydrolase
in rabbit heart homogenate

The ordinate indicates the activity in nmole/mg min,
and the abscissa indicates % distribution of protein in
subcellular fractions. The symbols are: Mt = mitochondrial
fraction, Ly = lysosomal fraction, Mc = microsomal fraction,
and S = soluble fraction.

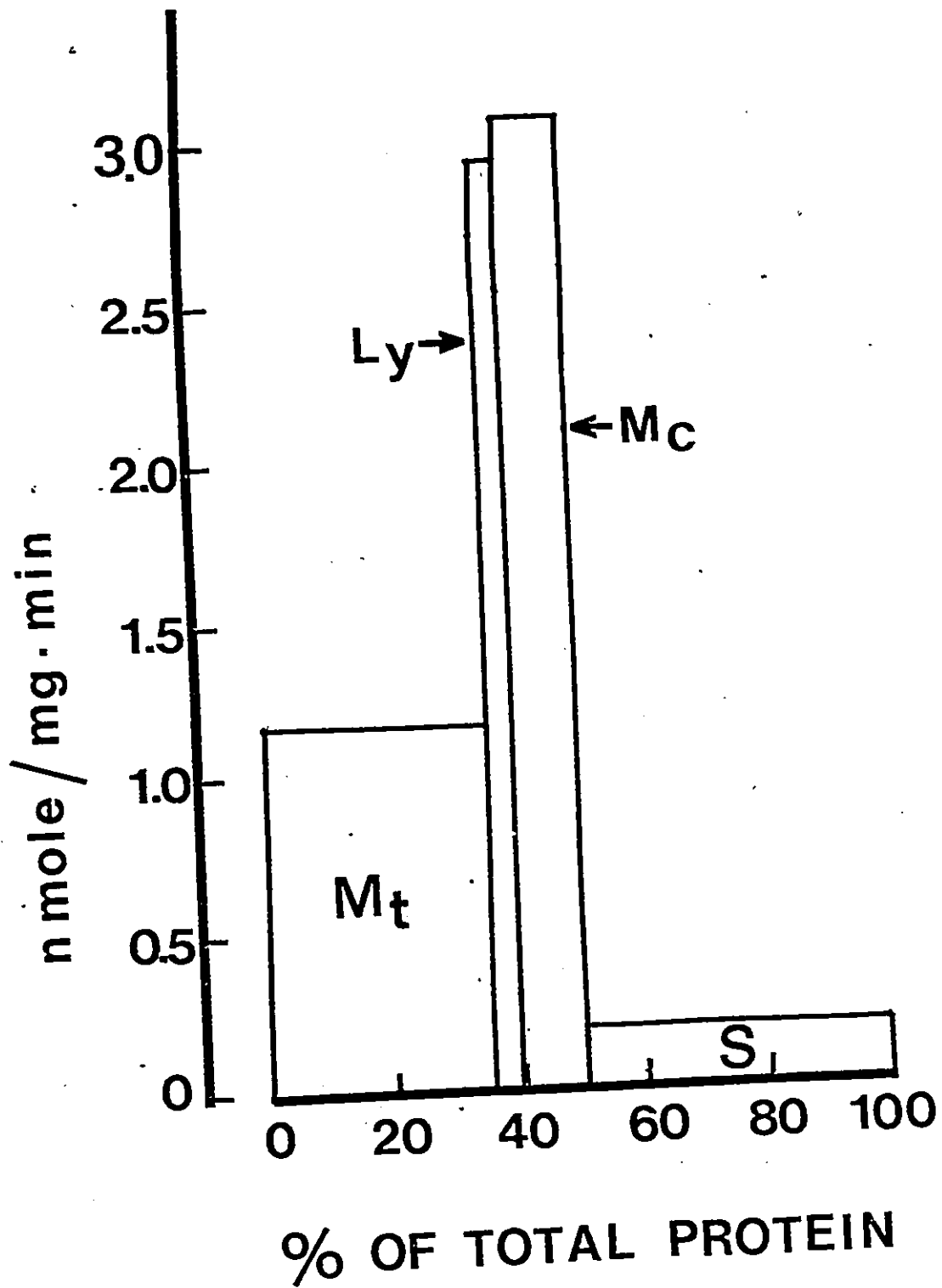


FIGURE 47

TABLE 31

Subcellular distribution of phosphatidate phosphohydrolase
in rabbit heart homogenate

Subcellular fraction	Percentage of total protein*	Specific activity	Distribution of total activity	
	%	nmole per min mg	nmole/min per g wet weight of heart	(%)
Mitochondrial	36.0	1.18	16.87	(43.3)
Lysosomal	5.0	2.96	5.92	(15.1)
Microsomal	10.0	3.10	12.40	(31.6)
Soluble	49.0	0.20	3.90	(10.0)

For experimental details, see the legend to Fig. 47.

* Values of total protein were obtained as described in Table 29.

V. DISCUSSION

This study provides evidence in support of the following conclusions: (1) Rabbit heart mitochondria are capable of acylating exogenous sn-G3P, and, furthermore, the acylating enzyme existing in the mitochondrial fraction is not a microsomal contamination; (2) The properties of cardiac sn-G3P acylating enzymes are dissimilar to those of hepatic enzymes, suggesting that G3P acyltransferase is organ specific; (3) The participation of the DHAP pathway in the biosynthesis of diacyl-sn-G3P in cardiac mitochondria under the conditions used in these studies is not demonstrable, (4) Administration of T3 enhances the activity of G3P acylating enzymes of the rabbit heart mitochondrial and microsomal fractions to different degrees. In addition, this study delineates the properties and subcellular distribution of palmitoyl-CoA hydrolase and phosphatidate phosphohydrolase in heart tissue. Observations made in this thesis should contribute to the understanding of the controlling mechanism of FA esterification in myocardium. Although the findings have been discussed in the appropriate sections of the RESULTS AND COMMENTS (Section IV), the points of particular

interest are selected for further discussion.

V-1. Acylation of sn-Glycerol-3-Phosphate by Rabbit Heart Subcellular Fractions

Acylation of sn-glycerol-3-phosphate by the mitochondrial fraction of rabbit heart: Differentiation from microsomal enzymes

With the observations of other workers, microsomal-acylation of sn-G3P was linear with respect to time for only a short duration, whereas the mitochondrial reaction was linear for up to 20 min incubation (Fig. 15 and Table 14). During the acylation process, the rate of monoacyl-sn-G3P accumulation by mitochondria was greater than that by microsomes, whereas the rate of diacyl-sn-G3P biosynthesis by mitochondrial enzyme was much slower than that by the microsomal enzyme (Figs. 13, 14, 16, 17; Tables 13, 15). Thus, the ratio of mitochondrial to microsomal monoacyl-sn-G3P formation increased linearly to more than two-fold during a period of 15 min, while the ratio of mitochondrial to microsomal diacyl-sn-G3P biosynthesis remained at a level of less than one-fifth (Fig. 20; Table 15). Although a quantitative difference existed, hepatic subcellular organelles exhibited a similar pattern of biosynthesis, that is, the mitochondrial fraction

synthesized relatively more monoacyl-sn-G3P than did the microsomes. However, the rate of diacyl-sn-G3P biosynthesis by the mitochondrial fraction was slower than that by the microsomal enzyme (Figs. 21-23; Table 16).

There is no convincing means of quantifying cross contamination of microsomes to the mitochondrial fraction, particularly in cardiac tissue. The technique most commonly used involves measuring the activity of microsomal marker enzymes (rotenone-insensitive NADPH-cytochrome C reductase and glucose-6-phosphatase) in the mitochondrial fraction. However, there is some criticism regarding the use of this method as a means of quantifying cross contamination, since these two enzymes have been shown to exist in both microsomal and mitochondrial fractions (Sottocasa et al., 1967; Brunner and Bygrave, 1969; Shephard and Hübscher, 1969; Sarzala et al., 1970). In addition, glucose-6-phosphatase activity is non-measurable in cardiac microsomes (Hülsmans, 1961). Thus, using the marker enzyme technique, it is not possible to decide whether or not a relatively low rate of diacyl-sn-G3P synthesis by mitochondria is a result of contaminating microsomal enzyme activity. It seems, however, that even if the mitochondrial



preparation was contaminated by microsomal fraction to an extent of 20 %, the mitochondrial enzyme would still mainly produce monoacyl-sn-G3P while the microsomal enzyme would synthesize both monoacyl- and diacyl-sn-G3P. This view is supported by the fact that monoacyl-sn-G3P acyltransferase activity is higher than the G3P acyltransferase reaction in the microsomes of liver (Lands and Hart, 1964; Eibl et al., 1969; Daae and Bremer, 1970). Additional experiments on the enzyme kinetics show that the characteristics of the distribution of acylating products, the time-courses of acylation, and the responses to T3 administration of acyltransferase reactions in the mitochondrial and microsomal fractions are all dissimilar. Thus, this study demonstrates definitively that cardiac mitochondria are capable of synthesizing monoacyl-sn-G3P from sn-G3P, and that the acylating enzyme existing in the mitochondrial fraction is not a microsomal contaminant.

It was observed that the mitochondrial enzyme possesses specificity towards various species of long-chain FAs different from the specificity of microsomal G3P acyltransferase. Among three FAs tested, the mitochondrial biosynthesis of monoacyl-sn-G3P from sn-G3P showed a substrate specificity

with an order of oleate > palmitate > linoleate (Fig. 27; Table 22), whereas the microsomal enzyme revealed an order of preference of linoleate $\gg$ oleate > palmitate (Fig. 28; Table 23). A similar pattern of substrate specificity was observed in the incorporation of sn-G3P into diacyl-sn-G3P in these suborganelles (Fig. 29 and Table 24; Fig. 30 and Table 25). The formation of diacyl-sn-G3P could be influenced by the specificity of G3P acyltransferase because the availability of monoacyl-sn-G3P may be one of the limiting factors in the monoacyl-sn-G3P acyltransferase reaction. The precursor-product relationship obtained by using palmitate, oleate, and linoleate thus supports the conclusion that different acylating enzyme systems exist in cardiac mitochondrial and microsomal fractions. It is not likely that the FA specificity of G3P acyltransferase in heart suborganelles is influenced by the specificity of acyl-CoA synthetase, since acyl-CoA synthetase does not appear to limit the G3P and monoacyl-sn-G3P acyltransferase reactions as has been described in the INTRODUCTION (Section I-1-4-i) and RESULTS AND COMMENTS (Sections IV-1-2 and IV-1-9-ii).

Cardiac versus hepatic enzymes: Organ specificity

The kinetics of monoacyl- and diacyl-sn-G3P formation.

in cardiac mitochondria differed greatly from those reported by Monroy *et al.* (1972) with hepatic mitochondria. Cardiac mitochondria synthesized monoacyl-sn-G3P linearly for up to 20 min incubation time (Figs. 16 and 18; Table 15), thereby continuously increasing the monoacyl-sn-G3P/diacyl-sn-G3P ratio (Fig. 20; Table 15). Hepatic mitochondria synthesized monoacyl-sn-G3P more rapidly than the diacyl derivative during the 1st min of incubation. The formation of the monoacyl compound ceased after 1 min while the appearance of diacyl-sn-G3P continued at a linear rate for 5 min, resulting in a dramatic reduction of the monoacyl-sn-G3P/diacyl-sn-G3P ratio during the first 5 min period (Fig. 5 of Monroy *et al.*, 1972). Factors such as pH or sonication treatment of the mitochondria may influence the formation of sn-G3P acylation products. At pH 6.0 and 6.5, the major product of the sn-G3P acylation reaction by liver microsomes was monoacyl-sn-G3P, while at higher pH values, diacyl-sn-G3P was rapidly deacylated to form DG (Lamb and Fallon, 1970). Sonicated hepatic mitochondria at pH 9.2 produced a product which had not been identified during the early time period (Monroy *et al.*, 1972). However, the aforementioned factors could not account for the observed

difference in the formation of monoacyl and diacyl derivatives between the cardiac and hepatic mitochondria, since the experimental conditions for heart (Figs. 16, 18, 20; Table 15) and those for liver (Fig. 5 of Monroy et al., 1972) are identical except for the nature of the acyl donor.

In this thesis, acylation of sn-G3P by cardiac subcellular fractions was carried out using palmitate, CoA, ATP, and $MgCl_2$ as an acyl donor system. Thus, the question of whether or not acyl-CoA synthetase limits acyltransferase reactions becomes a pertinent one. This question has been discussed in detail in the INTRODUCTION (Section I-1-4-i) and in RESULTS AND COMMENTS (Sections IV-1-2 and IV-1-9-ii). The subcellular localization of FA activation and esterification in different tissues is principally the same. The activation of long-chain FAs takes place both in the mitochondrial outer membrane and in the microsomal fraction (Aas, 1971). Esterification is found to occur also both in the outer membrane of mitochondria and in the microsomes (Daas and Bremer, 1970). In liver mitochondria, acyl-CoA synthetase activity is five times greater than the G3P acyltransferase activity (Sánchez et al., 1973). The activation of palmitate by heart mitochondria is not only higher

than that by liver mitochondria (Pande, 1971), but also is much greater than the ability of the heart mitochondria to oxidize this FA (Pande and Mead, 1968a and 1968b; Pande, 1971). These findings indicate that acyl-CoA synthetase does not limit the rate of G3P acyltransferase reactions in heart mitochondria. The conclusion is supported by the experimental data shown in Fig. 9 and Table 7 in which the extent of sn-G3P acylation by cardiac mitochondria was similar when palmitate, CoA, ATP, and $MgCl_2$ or palmitoyl-CoA served as the acyl donor system. The same conclusion can be drawn about hepatic microsomes since their rate of FA activation is 3-60 times greater than the rate of sn-G3P acylation (Section IV-1-2). The reported low degree of substrate specificity for acyl-CoA synthetase (Hubscher, 1970; Marcel and Suzue, 1972) is not likely to influence the specificity of the G3P acyltransferase reaction, since the V_{max} for this enzyme is 4-7 times greater than that of acyltransferase in rat liver microsomes (151.5-306.6 versus 41.2 nmoles/min per mg protein; Marcel and Suzue, 1972; Mangiapane *et al.*, 1973).

Addition of CTP to the reaction mixture during the acylation of sn-G3P by cardiac enzyme neither enhanced nor

depressed the rate of diacyl-sn-G3P biosynthesis in heart (Table 5). This disagreed with the stimulatory effect in the liver (Zborowski and Wojtczak, 1969) and inhibitory effect in the brain (Possmayer and Mudd, 1971). Zborowski and Wojtczak (1969) found that CTP (1.7 mM) strongly stimulated the incorporation of sn-glycerol-3- [32 P]phosphate into diacyl-sn-G3P in rat liver mitochondria, while Possmayer and Mudd (1971) observed that the presence of CTP (0.2 mM) markedly decreased the incorporation of sn- [14 C]glycerol-3-phosphate into diacyl-sn-G3P in rat brain homogenate. In addition, the enzymes in cardiac subcellular preparations were extremely sensitive to the thio inhibitors N-ethylmaleimide and P-chloromercuribenzoate (Table 20), whereas hepatic enzymes were less susceptible (Lands and Hart, 1965). Finally, cardiac mitochondrial fraction lost approximately half of its activity during 3 weeks of storage (Section IV-1-7), while hepatic mitochondrial enzyme activity remained unaltered for a longer period of time (Monroy *et al.*, 1972). These observations clearly reflect the different characteristics of cardiac and hepatic enzymes.

Studies on substrate specificity of the sn-G3P acylation reaction in cardiac subcellular fractions revealed a low

degree of selectivity for saturated and unsaturated FAs (Figs. 27-32; Tables 22-26). In the rat liver, some investigators have previously observed the preferential acylation of saturated FAs at position 1 of the glycerol moiety both by the mitochondrial and microsomal preparations (Possmayer et al., 1969; Lamb and Fallon, 1970; Monroy et al., 1972). In contrast, other workers have presented evidence against substrate specificity of the G3P acyltransferase reaction in microsomes of rat and guinea pig liver (Lands and Hart, 1965; Eibl et al., 1969). The problem of substrate specificity is a complex one, e.g., the study by Yamashita and Numa (1972) of liver microsomal enzyme demonstrated two contradicting characteristics: (1) The intact microsomal enzyme formed diacyl-sn-G3P from acyl-CoAs without preference, and (2) monoacyl-sn-G3P, which was synthesized by a partially purified enzyme of the same source, showed substrate and positional specificity. It is not known which form of enzyme preparation more closely resembles the physiological state. Possibly, the purification procedure might have removed some factors or modified the characteristics of the enzyme, hence producing different degrees of substrate specificity.

The studies on positional specificity with partially purified hepatic mitochondria (Monroy *et al.*, 1973) and microsomes (Yamashita and Numa, 1972) conclusively demonstrated that palmitate was the only active substrate for sn-G3P acylation, and it was exclusively esterified at position 1 of the glycerol molecule. The results obtained with cardiac mitochondria and presented in this thesis demonstrated that both saturated and unsaturated FAs can be acylated almost equally at positions 1 and 2 of the monoacyl-sn-G3P molecule (Tables 27 and 28; Fig. 34). These findings provide additional evidence in support of the proposal that G3P acyltransferase is organ-specific.

The substrate and positional specificity of monoacyl-sn-G3P acyltransferase in the heart has not yet been directly analyzed. However, in this study, we have found that the acylation of sn-G3P to diacyl-sn-G3P by cardiac mitochondrial and microsomal fraction proceeds without marked FA specificity, suggesting that monoacyl-sn-G3P acyltransferase of heart does not possess strong substrate and positional specificity. In microsomes of rat liver, the selectivity of monoacyl-sn-G3P acyltransferase depends upon the availability of the two precursors in the assay mixture. At

lower 1-acyl-sn-G3P concentrations, the acylation reaction is highly selective (palmitate and arachidonate are excluded from reaction), whereas relatively nonselective acylation occurs at high 1-acyl-sn-G3P concentrations (Okuyama and Lands, 1972).

Analysis of FA composition of glycerides isolated from various organs of the same species showed the existence of organ-specific characteristic (Hübscher, 1970). In the rat, the weight ratios of the FAs from the heart were different from liver and kidney lipids, i.e., the ratio $C_{18:1}/C_{18:2}$ of heart muscle was significantly higher than that of the liver (2.35 vs. 1.25), while the ratios $C_{16:0}/C_{18:0}$ and $C_{16:0}/C_{18:1}$ of heart were lower than those of the kidney (1.00 versus 2.39 for $C_{16:0}/C_{18:0}$; 0.41 versus 0.82 for $C_{16:0}/C_{18:1}$). There seems to have been little work done dealing with the positional analysis of heart glycerides (McMurray and Magee, 1972). However, analyses of the glyceride composition in pig organs revealed a striking difference in FA distribution between hepatic and cardiac TG, i.e., the latter contained considerably less palmitic acid at position 1 and more oleic and linoleic acids at positions 1 and 3 than did the liver TG.

(Christie and Moore, 1970). In addition, it was reported earlier that the distribution of palmitic acid in positions 1 and 2 of phosphatidylcholine in the heart was dissimilar to that in the liver (Stein and Stein, 1963). In the heart, palmitic acid was distributed equally between the 1 and 2 positions of phosphatidylcholine, while in the liver, this FA was almost completely esterified to the 1 position of the phosphatidylcholine molecule (Stein and Stein, 1963). These results are compatible to those obtained in our study, hence supporting the hypothesis of organ specificity of lipid enzymes.

The DHAP pathway

We have examined the extent of participation of the DHAP pathway in the biosynthesis of diacyl-sn-G3P in cardiac mitochondrial fraction. In our study, the addition of NADH or NADPH did not alter the incorporation of sn-[U-¹⁴C]G3P into glycerolipids (Table 5). In contrast, Rao et al. (1971), using mitochondrial-free supernatant of rat liver as the enzyme source, found that NADH or NADPH enhanced glyceride synthesis two- to three-fold in the presence of sn-G3P. In their experiments, 3.25 mM KF was included in the incubation mixture, which would most likely inhibit

the conversion of sn-G3P to DHAP. The net increase of glyceride synthesis, therefore, was due to the acylation of ~~endogenous DHAP~~ to form acyl-DHAP, followed by the reduction of acyl-DHAP to form glycerolipids in the presence of added reduced pyridine nucleotide.

In our experiment, the presence of 3.0 mM NaCN most probably blocked the oxidation of sn-G3P. It is possible that the mitochondrial preparation contained some endogenous DHAP, and that this contributed partly as a precursor for acyl-DHAP biosynthesis, with subsequent reduction of its product to form monoacyl-sn-G3P in the presence of added reduced pyridine nucleotide. As a consequence of this, the formation of labelled mono- and diacyl-sn-G3P from sn-[U-¹⁴C]G3P would have been diminished due to an isotope dilution effect. In other words, the labelled mono- and diacyl-sn-G3P were diluted by unlabelled compounds derived from the unlabelled endogenous DHAP. However, the results in Table 5 showed that the incorporation of sn-[U-¹⁴C]G3P into mitochondrial glycerophosphatides was not diminished in the presence of NaCN and reduced pyridine nucleotide. Moreover, the experiment in Table 21 demonstrated that the addition of exogenous unlabelled DHAP (4 or 8 mM) to the

incubation mixture did not decrease the formation of either labelled mono- or diacyl-sn-G3P from labelled sn-G3P by cardiac mitochondria in the presence of NaCN and reduced pyridine nucleotide. These observations indicate that cardiac mitochondria do not significantly convert either endogenous or exogenous DHAP to acyl-DHAP, with subsequent reduction and acylation to mono- and diacyl-sn-G3P, in the presence of reduced pyridine nucleotide. Therefore, it seems well justified to conclude that the DHAP pathway does not operate to an appreciable extent in the biosynthesis of phosphoglycerides in rabbit heart mitochondria. Other possibilities regarding the non-existence of the DHAP pathway in cardiac mitochondria have been discussed in Section IV-1-8. It should be noted that the mitochondrial preparation used in this study was sonicated to disrupt the membrane barrier for NADPH and NADH. Therefore, the acyl-DHAP reductase activity was not limited by lack of availability of reduced pyridine nucleotides under the experimental conditions selected.

In earlier studies, Puleo et al. (1970) and Rao et al. (1971a), using similar approaches described above, i.e., comparing the incorporation of labelled palmitate into

phospho- and glycerolipids in the absence and presence of NADH or NADPH, demonstrated that in the microsomes of rat liver and hamster intestinal mucosa, phospho- and glycerolipids can be synthesized from FAs and either sn-G3P or DHAP. In addition, with the aid of an irreversible inhibitor of triose phosphate isomerase, 1-hydroxyl-3-chloro-2-propanone phosphate, they showed that triose phosphate was the precursor for glyceride biosynthesis via the DHAP pathway. However, their work provided no information regarding whether DHAP was involved as an intermediate for the acylation of sn-G3P into glycerolipids.

Davidson and Stanacev (1972) and Monroy *et al.* (1972) studied the DHAP pathway by comparing the rate of incorporation of sn-[2-³H]G3P and sn-[U-¹⁴C]G3P into phospholipids in the liver. If sn-G3P was partially converted to DHAP, and then to acyl-DHAP, prior to the formation of phosphatidate under their experimental conditions, one would expect that the rate of incorporation of sn-[2-³H]G3P into lipids be lower than that of sn-[U-¹⁴C]G3P, since the tritium label at the 2 position of sn-G3P would be "leached out" when it was converted to DHAP. They found that both labels had similar rates of incorporation and concluded that the

DHAP pathway was not involved in the biosynthesis of phosphatides in the liver. However, Manning and Brindley (1972) pointed out that the rate of oxidation of sn-[2-³H]G3P was 2.17 times lower than that of cold sn-G3P in the absence of inhibitors and uncouplers of oxidative phosphorylation, confirming the report by Carnicero *et al.* (1972). Accordingly, the rate of incorporation of sn-[2-³H]G3P into lipids must be at least 2 times faster than that of sn-[U-¹⁴C]G3P in order to obtain equal rates of incorporation of tritium and ¹⁴C-labelled sn-G3P into lipids, as was aimed at by Davidson and Stanacev (1972) and Monroy *et al.* (1972). By taking this effect into account, the results obtained by Davidson and Stanacev (1972) and Monroy *et al.* (1972) can be interpreted to indicate that not only does the DHAP pathway contribute to the biosynthesis of phosphatides from sn-G3P, but it is even more active than the Kornberg-Kennedy *de novo* pathway in rat and guinea pig liver.

In order to avoid the factor of the unequal rate of oxidation of sn-[2-³H]G3P and unlabelled sn-G3P as described above, Manning and Brindley (1972) developed a technique involving the use of sn-[2-³H]G3P in deciding the existence or non-existence of the DHAP pathway. They incubated liver

slices with a mixture of $[2-^3\text{H}]\text{glycerol}$ and $[1-^{14}\text{C}]\text{glycerol}$ to produce endogenous double labelled sn-G3P which served as the precursor of glycerolipids. In examining the $2-^3\text{H} / 1-^{14}\text{C}$ ratios of sn-G3P and total glycerolipids at various time intervals, they found that the ratio of $2-^3\text{H} / 1-^{14}\text{C}$ in the sn-G3P was considerably higher than the cumulative $2-^3\text{H} / 1-^{14}\text{C}$ ratio in glycerolipid synthesized throughout the time period studied. Manning and Brindley (1972) concluded that the higher $2-^3\text{H} / 1-^{14}\text{C}$ ratio obtained in sn-G3P was due to the slower rate of oxidation of ^3H -labelled sn-G3P as compared to unlabelled sn-G3P, and that the lower $2-^3\text{H} / 1-^{14}\text{C}$ ratio obtained in glycerolipids was due to the loss of tritium as the sn- $[2-^3\text{H}]\text{G3P}$ passed through the DHAP pathway. By applying this calculation, it was shown that 50 to 60 % of the glycerol incorporated into lipids in the rat liver slice was produced via the DHAP pathway. Manning and Brindley (1972) claimed that their method as described above can be used to determine the relative importance of the sn-G3P and DHAP pathways with subcellular fractions, tissue slices, isolated cells, whole organs, or in vivo. However, this technique is not applicable for a study of.

the DHAP pathway in the heart, since cardiac glycerol kinase activity is not as active as in the liver (Safer *et al.*, 1971). The biosynthesis of cardiac glycerolipids would probably be limited by glycerol kinase if radioactive glycerol was used as the precursor.

From the references cited above, it is evident that the results obtained by different investigators using different experimental procedures were not in general agreement. The possibility still exists that the DHAP pathway may be operative in rabbit heart mitochondria, and that some monoacyl-sn-G3P may be formed via this pathway. However, we could not detect the formation of acyl-DHAP by TLC technique (Section IV-1-8). The results of the isotope dilution experiment (Table 21) indicated that the monoacyl- and diacyl-sn-G3P labels were not changed. Thus, it is not likely that cardiac phosphoglycerides were synthesized through the DHAP pathway to an appreciable quantity. The observed lack of distinct FA specificity (Figs. 27-32; Tables 22-26) does not support a view that the DHAP pathway is active under our experimental conditions, because Hajra showed that this pathway is characterized by the preferential acylation of individual FAs (Hajra, 1968).

The control of cardiac sn -glycerol-3-phosphate acylation
by thyroid hormone

The biosynthesis of diacyl- sn -G3P by the mitochondrial and microsomal fractions was increased about four-fold in the hearts of hyperthyroid rabbits (Figs. 24 and 25; Tables 17 and 18). Likewise, mitochondrial monoacyl- sn -G3P formation was stimulated two-fold (Fig. 24; Table 17), whereas microsomal biosynthesis of this intermediate was not altered (Fig. 25 and Table 18). The similar magnitudes of change were observed regardless of whether the results from the fed or the fasted controls were used in comparison with the results obtained from hyperthyroid animals (Tables 17 and 18). The data suggests that the activities of G3P and monoacyl- sn -G3P acyltransferase are under hormonal control. It has been found previously that myocardial TG content increases upon administration of thyroid hormone (Bressler and Wittels, 1966). The increase in the activities of G3P and monoacyl- sn -G3P acyltransferases observed in this study could be the cause responsible for the accumulation of TG in the hyperthyroid heart, since G3P acyltransferase has been postulated to be a rate-determining step in the biosynthesis of glycerolipids (Hübscher, 1970).

Although thyroid hormone has been known to cause an increase in protein synthesis and in some enzyme activities (Tata, 1964; Paterson, 1971; Kadenbach, 1966), there has been no report dealing with the FA esterifying system. It is not known at this point whether the observed change in enzyme activity is due to the new synthesis of enzyme proteins or the activation of existing enzymes.

Our study rules out the possibility that the biosynthesis of mono- and diacyl-sn-G3P in hyperthyroid heart is increased due to an increased plasma FFA concentration and an accompanying increase in FA uptake by the heart. The study showed that the acylation reactions in starved rabbits, in which plasma FFA concentration is higher than that in fed animals (Opie, 1968 and 1969), are similar to the rates of reaction observed in fed rabbits (Tables 17 and 18). In contrast, G3P acyltransferase activity was reported to decrease in fasted rat liver (Vavrečka *et al.*, 1969). The different type of response to fasting by cardiac enzyme on one hand, and the hepatic enzyme on the other, is additional evidence pointing to the organ-specific characteristics of these enzymes.

Although G3P acyltransferase and monoacyl-sn-G3P

acyltransferase are probably the rate-controlling enzymes of TG biosynthesis in the thyrotoxic heart, phosphatidate phosphohydrolase could be an additional factor controlling TG biosynthesis in the hyperthyroid state, since this enzyme has been postulated to be involved in the rate-governing step of TG synthesis in hepatectomized rats (Mangiapane *et al.*, 1973).

V-2. Properties and Subcellular Distribution of Palmitoyl-CoA Hydrolase

The assay of palmitoyl-CoA hydrolase activity was adopted from the method which was devised for activity measurements in adipose tissue (Daniel and Rubinstein, 1968), but it was equally satisfactory with cardiac subcellular fractions. Under our experimental conditions, hydrolysis was constant for up to two hours of incubation, and the rate of reaction was proportional to the amount of enzyme sources. The pH-activity relationship of this enzyme determined by using heart homogenates resembled that obtained by using dog lung microsomes (Frosolono *et al.*, 1971) and rat serum (Jansen and Hülsmann, 1973), but it differed from that found in the adipose tissue homogenates (Daniel and Rubinstein, 1968).

An apparent activation of palmitoyl-CoA hydrolase by a relatively high concentration of (+)-decanoylcarnitine has not been reported. It is unknown whether this enzyme is potentiated by the detergent property of (+)-decanoylcarnitine or whether the latter compound inhibits palmitoyl-CoA oxidation, thus providing a relatively greater amount of substrate for the enzyme. However, the high concentration of palmitoyl-CoA used in our assay system (100 μ M) and comparison of data obtained in the presence and absence of oxygen (the enzyme activity was identical in both cases) make the latter possibility less likely. It was reported earlier that palmitoyl-CoA hydrolase preferred the substrate in a micellar form (Barden and Cleland, 1969). The critical micellar concentrations of palmitoyl-CoA were 3-6 μ M. The enzyme was relatively inactive at low palmitoyl-CoA levels, but its activity increased proportionally to the increased concentration of palmitoyl-CoA up to 45 μ M and then reached a plateau (Barden and Cleland, 1969). It is possible, therefore, that the presence of (+)-decanoylcarnitine in our assay medium alters the physico-chemical state of the substrate and hence causes the potentiation of the enzyme.

An inhibition of enzyme activity by the detergent

property of the palmitoyl-CoA is improbable in view of a study with hepatic microsomes in which CoA esters of saturated FAs did not inhibit the hydrolase activity, i.e., the saturated acyl-CoA derivatives (12:0, 14:0, 16:0 and 18:0) produced typical substrate saturation curves without inhibition at higher levels (60 μ M) (Jezyk and Hughes, 1971).

There has been a lack of information in the literature regarding the subcellular distribution of palmitoyl-CoA hydrolase in heart tissue; much of the work has been done using hepatic tissue. In liver, microsomes have been used most frequently as an enzyme source (Lands and Merkl, 1963; Brandes et al., 1963; Lands and Hart, 1965; Fallon and Lamb, 1968; Barden and Cleland, 1969; Frosolono et al., 1971; Jezyk and Hughes, 1971); occasionally, mitochondrial fraction was also used (Brandes et al., 1963). In 1972, Kurooka et al. did a thorough study on the distribution of hydrolase in rat liver and found that a large proportion of the total activity was localized in the soluble fraction (92 %), with the remaining activity being in the microsomal and mitochondrial fractions (Kurooka et al., 1972). In rabbit heart, however, we found that the distribution

pattern is different from that in the liver, namely, 37.1 and 38.0 % of the total activity was localized in the soluble and mitochondrial fractions, respectively, with the remaining activity existing in the lysosomal and microsomal fractions. The specific activity of palmitoyl-CoA in the liver was also highest in the soluble and lowest in the mitochondrial fraction (Kurooka et al., 1972). In contrast, the highest specific activity of hydrolase in rabbit heart was found in the combined fraction of microsomes and lysosomes and the lowest specific activity found in the soluble fraction.

Acyl-CoA hydrolase was shown to exhibit some substrate specificity. Barden and Cleland (1969) reported that rat liver microsomes hydrolyzed palmitoyl-CoA two times faster than stearyl-CoA, and the rate of stearyl-CoA hydrolysis was twice that of oleyl-CoA. The lowest rates of hydrolysis were obtained with oleyl-, myristyl-, and lauryl-CoA. Jezyk and Hughes (1971) observed that acyl-CoA hydrolase from pig liver microsomes had a substrate specificity for different chain-length and degree of saturation of acyl-CoA thioesters in the following order: $16:0 > 14:0 > 12:0 > 18:1 > 18:2 > 18:3 > 18:0 > 20:4$. Kurooka et al. (1972) showed that C_{14}

and C₁₆ fatty acyl-CoAs were the best substrates for the partially purified hydrolase from various organs of rat, and other thioesters with chain lengths of less than C₈ were not hydrolyzed to an appreciable extent. The substrate specificity of acyl-CoA hydrolase from rabbit heart was not investigated in this study. Whether or not the specificity, if any, affects the activity and subcellular distribution of palmitoyl-CoA hydrolase in the heart, as reported in this study, is not known.

V-3. Properties and Subcellular Distribution of Phosphatidate Phosphohydrolase in Rabbit Heart

The importance of phosphatidate phosphohydrolase as the rate-governing step for biosynthesis of neutral glycerides was stressed in a recent paper describing a change in enzyme activity following subtotal hepatectomy (Mangiapanè *et al.*, 1973). Under their experimental conditions, the activity of phosphatidate phosphohydrolase was found to increase simultaneously with increased TG content in the liver. Treatment with actinomycin D suppressed the increased enzyme activity, but hepatic TG accumulation was uninfluenced (Mangiapanè *et al.*, 1973), suggesting that the control factors may be complex.

Our determinations of phosphatidate phosphohydrolase activity in heart muscle showed a pH optimum similar to that found in rat liver (Coleman and Hübscher, 1962; Sedgwick and Hübscher, 1965; Smith et al., 1967) and adipose tissue (Daniel and Rubinstein, 1968; Jamdar and Fallon, 1973). The subcellular distribution pattern described in this study, however, is not in agreement with the data obtained by some previous workers (Coleman and Hübscher, 1962; Sedgwick and Hübscher, 1965; Jamdar and Fallon, 1973) when the enzyme activity was assayed with aqueous dispersion of phosphatidate in the absence of magnesium ions. In rabbit heart, the largest proportion (43.3 %) of phosphatidate phosphohydrolase activity was localized in the mitochondrial fraction, with the least amount (10 %) being in the soluble fraction (our results). In rat liver, pig kidney, and rat adipose tissue, the microsomal fraction contained the largest proportion of the enzyme (37.5 % for liver, 63 % for kidney, and 42 % for adipose tissue), while the supernatant fraction had the smallest proportion of phosphatidate phosphohydrolase (12 % for liver, 14.6 % for kidney, and 20 % for adipose tissue) (Sedgwick and Hübscher, 1965; Coleman and Hübscher,

1962; Jamdar and Fallon, 1973).

Since phosphatidate phosphohydrolase in the soluble fraction has been reported to act preferentially on membrane-bound phosphatidate (Smith et al., 1967; Johnston et al., 1967; Sedgwick and Hübscher, 1967; see also Section IV-3-2), the subcellular distribution of this enzyme in soluble fraction varied depending upon the nature of the substrate. The apparent activity of soluble phosphatidate phosphohydrolase measured with membrane-bound phosphatidate was 16 to 18 times greater than that measured with aqueous substrate, that is, 5.5 versus 0.3 nmoles/min per mg protein in rat liver (Smith et al., 1967) and 65 versus 4 nmoles/min per g weight in hamster intestinal mucosa (Johnston et al., 1967). Furthermore, due to the stimulatory effect of low concentrations of magnesium ions (Smith et al., 1967; Sedgwick and Hübscher, 1967; Jamdar and Fallon, 1973; Lamb and Fallon, 1974), the subcellular distribution pattern of phosphatidate phosphohydrolase is likely to be modified by the presence of magnesium ions in the assay mixture. Magnesium ions at low concentrations (2.5-7.5 mM) stimulated phosphatidate phosphohydrolase activity by 50 % to seven-fold (Smith et al., 1967; Sedgwick and Hübscher, 1967;

Jamdar and Fallon, 1973) in soluble fraction and two-fold in microsomal fraction (Jamdar and Fallon, 1973; Lamb and Fallon, 1974) when measured with aqueous substrate. At this concentration, the soluble enzyme activity was also enhanced by Mg^{2+} with membrane-bound substrate (Jamdar and Fallon, 1973).

In our study, the phosphatidate phosphohydrolase activity in rabbit heart soluble fraction measured with membrane-bound substrate was only one-fiftieth of that with aqueous substrate. When the soluble enzyme activity obtained with membrane-bound substrate was compared to the microsomal enzyme activity with unbound substrate, the latter was still more active (0.0043 against 3.1 nmoles/min mg). These results are in support of those found in adipose tissue in which, in the presence of Mg^{2+} , the soluble enzyme is less active than the microsomal enzyme (8.8 vs. 19 nmoles/min mg; Jamdar and Fallon, 1973). Thus, in terms of apparent activity, the most active fractions in heart homogenate are the microsomal and lysosomal fractions, with the least activity found in the soluble fraction, regardless of the nature of the substrate.

Phosphatidate phosphohydrolase prepared from the

soluble and mitochondrial fractions of rat liver shows some FA specificity using membrane-bound phosphatidates of various FA compositions as substrates (Mitchell *et al.*, 1971). The soluble enzyme had the greatest activity with dimyristoyl- and palmitoyl-oleoyl phosphatidates, while dilauroyl and distearoyl phosphatidates were poor substrates. The mitochondrial enzyme showed the highest reaction rates with distearoyl and dioleoyl phosphatidates (Mitchell *et al.*, 1971). It should be pointed out that *sn*-G3P acyltransferase of rabbit heart mitochondrial fraction exhibited a low degree of substrate specificity in that both saturated and unsaturated FAs can be esterified almost equally at positions 1 and 2 of the *sn*-G3P molecule (Tables 27 and 28; Fig. 34). Whether or not cardiac phosphatidate phosphohydrolase shows some substrate specificity is as yet to be investigated.

VI. CONCLUSIONS

The following conclusions were drawn from the results obtained in this study:

- (1) Both mitochondria and microsomes of rabbit hearts are capable of acylating exogenous sn-G3P, however, the properties of the two acyltransferases are distinctly different. It is proposed that sn-G3P acyltransferase is a true constituent of mitochondria.
- (2) The properties of sn-G3P acyltransferase in the heart differ from those in the liver, i.e., the enzyme may be organ-specific.
- (3) The DHAP pathway does not play a significant role in the biosynthesis of diacyl-sn-G3P from sn-G3P in cardiac mitochondria.
- (4) Myocardial G3P and monoacyl-sn-G3P acyltransferases are under the influence of a thyroid hormone (T₃).
- (5) The G3P acyltransferase of rabbit heart mitochondria possesses a low degree of substrate and positional specificity.
- (6) Palmitoyl-CoA hydrolase in rabbit heart is distributed in all of the subcellular fractions. The

mitochondrial and soluble fractions each contained approximately one-third of the enzyme activity, with the remaining activity being localized in the microsomal and lysosomal fractions.

(7) Aqueous dispersed phosphatidate is the preferred substrate for myocardial phosphatidate phosphohydrolase. This enzyme exists in all of the subcellular fractions, with approximately four-tenths of its total activity in the mitochondrial fraction and three-tenths in the microsomal fraction. The lysosomal and soluble fractions contain only 15 % and 10 %, respectively, of the total enzyme activity.

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