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Inhibition of triacylglycerol biosynthesis in the regenerating rat liver by 3,4-dihydroxybutyl-1-phosphonate

Stein, Theodore Anthony, Ph.D.

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INHIBITION OF TRIACYLGLYCEROL BIOSYNTHESIS IN THE REGENERATING RAT LIVER BY 3,4-DIHYDROXYBUTYL-1-PHOSPHONATE

by

Theodore Anthony Stein

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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ABSTRACT

INHIBITION OF TRIACYLGLYCEROL BIOSYNTHESIS IN THE REGENERATING RAT LIVER BY 3,4-DIHYDROXYBUTYL-1-PHOSPHONATE

BY

THEODORE ANTHONY STEIN

ADVISOR: PROFESSOR BURTON E. TROPP

Mitochondrial, microsomal and peroxisomal membranes were partially purified by differential sedimentation. sn-Glycerol 3-phosphate analogues, (RS)-3,4-dihydroxybutyl-1-phosphonate,(RS)-glyceraldehyde 3-phosphate,(RS)-3-hydroxy-4-oxobutyl-1-phosphonate, (1S,3S)-1,3,4-trihydroxybutyl-1-phosphonate, and (1R,3S)-1,3,4,-trihydroxybutyl-1-phosphonate, were competitive inhibitors of both mitochondrial and microsomal sn-glycerol 3-phosphate acyltransferase reactions. An isosteric analogue of dihydroxyacetone phosphate, 4-hydroxy-3-oxobutyl-1-phosphonate, was a competitive inhibitor of the microsomal enzyme. Phenethyl alcohol was a noncompetitive inhibitor of the microsomal enzyme. The Ki-values of the reactions, inhibited by (RS)-3,4-

dihydroxybutyl-1-phosphonate, were 3.18 and 1.76 mM with the mitochondrial and microsomal enzymes, respectively. The mitochondrial acyltransferase reaction with (RS)-3,4-dihydroxybutyl-1-phosphonate resulted almost exclusively in 4-palmitoyl-sn-3-hydroxybutyl-1-phosphonate. The microsomal acylation reaction generated both the monoacyl product and 3,4-dipalmitoyl-sn-butyl-1-phosphonate. The apparent Km for (S)-3,4-dihydroxybutyl-1-phosphonate was 2.50 and 1.38 mM for the mitochondrial and microsomal enzymes, respectively. Using whole cell preparations, (S)-3,4-dihydroxybutyl-1-phosphonate incorporated into the chloroform-soluble material of liver, adipose tissue, and enterocytes. The rates of uptake were linear over 3 h, and varied from 0.78 to 0.95 pmol/h/g tissue.

Following two-thirds partial hepatectomy the triacylglycerol content of the residual liver increased five-fold at 18 h, and six-fold at 24 h. The microsomal sn-glycerol 3-phosphate acyltransferase activity was increased at 18 and 24 h after partial hepatectomy. A significant correlation, $r = 0.608$, occurred between the triacylglycerol content and the microsomal enzyme activity. The mitochondrial acyltransferase activity was unchanged. Peroxisomal dihydroxyacetone phosphate acyltransferase was increased at 24 h, but the microsomal activity was not statistically different from control values.

Intraperitoneal injections of (RS)-3,4-dihydroxybutyl-1-phosphonate during liver regeneration caused a significant decrease in hepatic triacylglycerol and the mitotic index. Sodium chloride and sn-glycerol 3-phosphate decreased the triacylglycerol content and mitotic index to a much lesser degree. DNA biosynthesis was increased by (RS)-3,4-dihydroxybutyl-1-phosphonate, while the liver content of DNA, RNA, phospholipid and protein was similar to the levels obtained by sodium chloride and sn-glycerol 3-phosphate administration.

In cell culture, butanol-extractable 3,4-dihydroxybutyl-1-phosphonate acylation products were obtained from mouse 3T3 fibroblasts and human Hep-G2 hepatoma. A linear incorporation of the phosphonate occurred in the 3T3 cells over 5 h at a rate of 4.1 pmol/h/million cells. In the hepatoma cells the rate of phosphonate-incorporation gradually declined from 1.5 to 5 h, and was 3.2 and 1.8 pmol/h/million cells, respectively.

DEDICATION

I wish to dedicate this work to my wife, Jeanette, who gave support and encouragement over these many years even when she was ill and needed my support.

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INTRODUCTION

Glycerolipids are important components of hepatic cellular membranes. During rapid growth of the liver, which occurs after partial liver resection, the need of glycerolipids for membrane biosynthesis increases. The effect of administering an inhibitor of glycerophospholipid synthesis is studied now during the early period of liver regeneration.

The hepatocyte contains several different types of membranes. The endoplasmic reticulum comprises half of the total membrane mass. In contrast, the mitochondria, Golgi and plasma membrane account for about 30, 6 and 5 percent of the membrane mass, respectively (1). Glycerophospholipids make an important contribution to membrane structure and function. Among different membranes there can be two-fold variations in the phospholipid to protein ratio (1). Organelle-specific variations in the phospholipid composition also occur (1). The relative proportion of cholesterol to glycerophospholipid is greatest in plasma membranes and is five to six-fold less in the endoplasmic reticulum and mitochondria. The relative proportion of phosphatidylcholine to phosphatidylethanolamine is approximately 3:1 in the endoplasmic reticulum, and is less than 1.5:1 in mitochondria. Cardiolipin is found primarily in the inner membrane of mitochondria (2). The relative distribution of phospholipids in body

organs also varies (1). Although cellular membrane phospholipid heterogeneity must be related to the physiological function of healthy cells, and the pathophysiology of disease, little is known how the relative distribution of lipids in organelle membranes influences cellular function.

Intracellular triacylglycerol accumulation in the liver can be caused by a deficiency of lipotropic substances, chronic alcoholism, malabsorption, malnutrition, diabetes mellitus, and obesity. Although a fatty liver is commonly associated with disease, during liver regeneration the transient storage of triacylglycerol may be physiological. The biosynthesis of triacylglycerol and phospholipids occurs from a common percursor, phosphatidic acid.

Enzymes of Glycerolipid Biosynthesis

Fatty Acid Coenzyme A Ligase

A scheme of the biosynthesis of glycerophospholipids is shown in Figure 1. Fatty acyl-CoA thioesters may be synthesized by the activation of long-chain free fatty acids which are derived from the liver, adipose tissue or the diet depending on the nutritional state of the individual. Although the activation of fatty acids is necessary for the acylation reactions which occur during phospholipid biosynthesis, the reaction of fatty acid CoA ligase (EC 6.2.1.3) is probably not rate-limiting for phospholipid

biosynthesis (Figure 1,#1). In rat hepatoma microsomes, the low phospholipid content of these tumor cells is not caused by a restricted activity of the ligase, but is probably due to a limited availability of glycerol 3-phosphate (3).

Glycerol 3-Phosphate Acyltransferase and Dihydroxyacetone Phosphate Acyltransferase

Animal cells have two distinct pathways for the synthesis of phosphatidic acid. The primary pathway in the liver involves the sequential reactions of sn-glycerol 3-phosphate acyltransferase (EC 2.3.1.15) and lysophosphatidic acid acyltransferase (EC 2.3.1.-), while that in the rabbit harderian gland involves the sequential reactions of dihydroxyacetone phosphate acyltransferase, monoacyl dihydroxyacetone phosphate reductase, and lysophosphatidic acid acyltransferase (4,5).

sn-Glycerol 3-phosphate acyltransferase (Figure 1,#2) activity is distributed almost equally in mitochondrial and microsomal subcellular fractions of the liver (6). There is ample evidence in the literature to indicate that the mitochondrial and microsomal activities represent two isoenzymes (6,7,8,9,10,11,12,13). The active site of the isoenzyme in mitochondria is located in the outer membrane (7,12,14,15,16,17). N-Ethylmaleimide, iodoacetamide, 5,5-dithiobis (2-nitrobenzoate), acetone, divalent cations, and heat inhibit the microsomal

activity, but stimulate or have no effect on the mitochondrial isoenzyme (6,7,8,10,11,12,13,18). Saturated long-chain fatty acyl-CoA thioesters are more reactive with the mitochondrial enzyme than are unsaturated acyl-CoA thioesters; the microsomal enzyme shows no preference (6,7,18). Starvation for 48 hour and diabetes decreases mitochondrial but not microsomal glycerol 3-phosphate acyltransferase activity (12,13). Other investigators show that the mitochondrial isoenzyme is decreased by glucagon, and the microsomal isoenzyme is decreased by insulin and increased by glucagon (19).

Hepatic microsomal isoenzyme may have a dual catalytic function for sn-glycerol 3-phosphate acyltransferase activity and dihydroxyacetone phosphate acyltransferase (EC 2.3.1.42) activity, because many inhibitors and inactivators act on both enzyme activities to the same degree (18,20,21). Acetaldehyde also inhibits both acyltransferase activities (22). In rat brain these two microsomal acyltransferase activities, however, may represent different enzymes (23).

The biosynthesis of phosphatidic acid can also occur from the acylation of dihydroxyacetone phosphate (Figure 1,#3). Most of the acylation activity is found in the peroxisomal subcellular fraction and less is in the microsomes (24,25,26,27). N-Ethylmaleimide and 5

mM glycerol 3-phosphate inhibits the microsomal dihydroxyacetone phosphate acyltransferase activity, but not the peroxisomal activity (4,21,28).

Acyl(Alkyl) Dihydroxyacetone Phosphate Oxidoreductase and 1-Acyldihydroxyacetone Phosphate Synthase

The activity of acyl (alkyl) dihydroxyacetone phosphate oxidoreductase (EC 1.1.1.10) is also found in the microsomal and peroxisomal subcellular fractions and has a specific activity greater than that of dihydroxyacetone phosphate acyltransferase (26,29,30,31). This enzyme (Figure 1,#4) can either reduce 1-acyldihydroxyacetone phosphate to 1-acylglycerol 3-phosphate, or 1-alkyldihydroxyacetone phosphate to 1-alkylglycerol 3-phosphate which would be further metabolized to ether lipids (24,32,33). 1-Acyldihydroxyacetone phosphate synthase (EC 1.1.1.10) (Figure 1,#5) also occurs in peroxisomes (24).

1-Acylglycerol 3-Phosphate (Lysophosphatidic Acid) Acyltransferase

Phosphatidic acid is synthesized on the surface of the endoplasmic reticulum by the acylation of 1-acylglycerol 3-phosphate (Figure 1,#6) (34,35,36,37,38). The specific activity of 1-acylglycerol 3-phosphate acyltransferase (EC 2.3.1.-) is greater than that of sn-glycerol 3-phosphate acyl-transferase and does not seem to be rate-limiting in the liver (36,39,40). This enzyme has been partially purified and separated from sn-glycerol 3-phosphate

acyltransferase and other enzymes (41,42).

Phosphatidic Acid Phosphatase

Dephosphorylation of phosphatidic acid produces diacylglycerol (Figure 1,#7) which can be further metabolized to triacylglycerol, phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine (43,44,45,46,47). Phosphatidic acid phosphatase (EC 3.1.3.4) is primarily found in the cytosol and microsomal subcellular fractions which is unique for triacylglycerol forming enzymes (48,49,50). Magnesium and calcium stimulate cytosolic phosphatidic acid phosphatase activity, while chloropromazine, sodium fluoride and p-chloromercuribenzoic acid inhibit the activity (50,51,52,53,54,55,56). The microsomal enzyme appears to be only slightly stimulated by magnesium (55,56,57). When the fatty acid content increases in hepatocytes, the magnesium-dependent phosphatidic acid phosphatase activity in the cytosol translocates to the endoplasmic reticulum membranes (37,38,50,58). The activity of the cytosolic enzyme appears to be regulated by phosphorylation-dephosphorylation reactions, which may be stimulated by hormones (59). Glucocorticoids, glucagon, and growth hormone stimulate phosphatidic acid phosphatase activity, and insulin inhibits the activity (19,38,60,61). There is much evidence that phosphatidic acid phosphatase regulates triacylglycerol formation in the liver (62).

Diacylglycerol Kinase

The ATP-dependent phosphorylation of diacylglycerol generates phosphatidic acid (Figure 1,#8). Diacylglycerol kinase (EC 2.7.1.-) is also found in the microsomal and cytosolic subcellular fractions (63,64). The role of this enzyme in modulating triacylglycerol formation is unknown.

Diacylglycerol Acyltransferase

Diacylglycerol can be converted to triacylglycerol, phosphatidylethanolamine, and phosphatidylcholine. Diacylglycerol acyltransferase (EC 2.3.1.20) acylates sn-1,2-diacylglycerol (Figure 1,#9) to form triacylglycerol (48,65,66,67,68,69,70). This microsomal enzyme has a broad specificity for fatty acyl-CoA thioesters, and for several 1-saturated 2-unsaturated fatty acyl-glycerols when acylated with palmitoyl-CoA thioester (69,70,71,72,73,74,75,76). Glucagon and cyclic AMP can inhibit the activity of the enzyme, suggesting that triacylglycerol synthesis may be regulated independently of phosphatidylcholine and phosphatidylethanolamine biosynthesis (71). Increasing fatty acid concentrations increase diacylglycerol acyltransferase activity and triacylglycerol biosynthesis (72).

Diacylglycerol Ethanolaminephosphotransferase, Diacylglycerol Cholinephosphotransferase, and Choline-P Cytidyltransferase

In the rat liver the most abundant phosphoglyc-

erides in membranes are phosphatidylcholine and phosphatidylethanolamine (1). The synthesis of CDP-choline (Figure 1,#12) and CDP-ethanolamine is catalyzed by choline-P cytidyltransferase (EC 2.7.7.15) and ethanolamine-P cytidyltransferase (EC 2.7.7.16), respectively (73). The enzymes are found in both the microsomal and cytosolic subcellular fractions (74). Choline and ethanolamine are phosphorylated by cytosolic choline and ethanolamine kinases (EC 2.7.1.32), respectively (75,76,77,78). Phosphatidylethanolamine (Figure 1,#10) is generated from diacylglycerol and CDP-ethanolamine by the action of diacylglycerol ethanolaminephosphotransferase (EC 2.7.8.1.), and phosphatidylcholine (Figure 1,#11) is synthesized from diacylglycerol and CDP-choline by the action of diacylglycerol cholinephosphotransferase (EC 2.7.8.2) (48,79,80). The microsomal enzymes appear to be uniquely different enzymes (81,82,83,84,85).

Phosphatidylethanolamine N-Methyltransferase and Phosphatidylethanolamine Serinetransferase

Phosphatidylcholine can also be generated by the transfer of methyl groups from S-adenosyl-L-methionine to phosphatidylethanolamine. This reaction is catalyzed by phosphatidylethanolamine N-methyltransferase (EC 2.1.1.17), and occurs in the endoplasmic reticulum (79,86). Several different methyltransferases have been reported, and they may be

located on opposite sides of the microsomal membrane (87,88,89,90,91). Approximately 20 percent of phosphatidylcholine appears to be synthesized by this reaction (92). Phosphatidylserine is synthesized in the liver by a calcium-dependent exchange between serine and the ethanolamine moiety of phosphatidyl-ethanolamine (79,93,94). This microsomal reaction is considered the major route for phosphatidylserine formation (73).

Phosphatidic Acid Cytidyltransferase and Phosphatidylinositol Synthase

Phosphatidic acid can also be metabolized to cytidine diphosphodiacylglycerol (CDP-diacylglycerol) by a magnesium-dependent reaction with CTP (Figure 1,#15). Phosphatidic acid cytidyltransferase (EC 2.7.7.41) is found in the microsomal and mitochondrial subcellular fractions (79,80,95). Although the enzyme has a broad specificity for the fatty acid composition of phosphatidic acids, hepatic CDP-diacylglycerols are rich in stearic and arachidonic acids (96,97,98). Phosphatidylinositol synthase (EC 2.7.8.11) is only found in the microsomal subcellular fraction, and catalyzes (Figure 1,#16) the reaction between CDP-diacylglycerol and myo-inositol to yield phosphatidylinositol (96,99,100).

Monoacylglycerol Acyltransferase

Diacylglycerol can also be generated in the liver by the acylation (Figure 1, #17) of 2-

monoacylglycerol, but not 1-monoacylglycerol (101). The enzyme is found in the microsomal subcellular fraction, and is stereospecific for the sn-2-monoacyl isomers (102,103). 2-Monoacylglycerol acyltransferase (EC 2.3.1.22) activities decrease 700-fold from the suckling period to adulthood, and suggests that the monoacylglycerol pathway is only a minor route for diacylglycerol biosynthesis in the adult rat (101).

sn-Glycerol 3-Phosphate Dehydrogenase

In the rat liver, sn-glycerol 3-phosphate:NAD oxidoreductase (EC 1.1.1.8) catalyzes the reduction of dihydroxyacetone phosphate to sn-glycerol 3-phosphate with the oxidation of NADH or NADPH (Figure 1,#18). Since this cytosolic enzyme has an equilibrium constant favoring glycerol 3-phosphate formation at physiological pH, glycolysis and gluconeogenesis should increase glycerol 3-phosphate for storage as triacylglycerols (104).

Biosynthesis of Very Low Density Lipoprotein

The liver primarily secretes lipoproteins rich in triacylglycerol, called very low density lipoproteins. These lipoprotein particles contain approximately 54 percent triacylglycerol, and 18 percent phospholipid, 15 percent cholesterol, and 13 percent protein (105). The synthesis of very low density lipoproteins begins with the synthesis of triacylglycerol and apoproteins. The enzymes

associated with the synthesis of triacylglycerol, phospholipid and cholesterol are in the membranes of the smooth endoplasmic reticulum (106). Phosphatidylcholine, the primary phospholipid of rat liver lipoprotein, is also synthesized in the Golgi (107,108). The Golgi contain diacylglycerol cholinephosphotransferase, phosphatidylethanolamine N-methyltransferase, and choline-P cytidyltransferase activity. The methylation pathway appears to be more important than the cholinephosphotransferase pathway in the Golgi for the synthesis of phosphatidyl-choline, which is destine for secretion. The synthesis of apoprotein B, the major apoprotein of rat nascent very low density lipoprotein, occurs on the ribosomes of the rough endoplasmic reticulum (109). The lipids and protein are translocated into the endoplasmic cisternae. The assembly of the apoprotein and lipids into the very low density lipoprotein begins in the endoplasmic reticulum and continues in the Golgi before secretion (110). Fasting decreases the assembly of triacylglycerol with apoprotein and slows the formation of nascent very low density lipoprotein, but does not alter the transfer of triacylglycerol to cytoplasmic lipid droplets for storage (111).

Regulation of Hepatic Phosphoglyceride Biosynthesis

In the fed animal, food is digested and absorbed in the small bowel. Blood concentrations of carbohydrates, amino acids and lipids increase shortly

after a meal. Most of the dietary lipid is delivered by the small bowel to the interstitial fluid as a chylomicron, which is synthesized on the small bowel endoplasmic reticulum (112). The chylomicron enters the lymphatic system and the venous blood by way of the thoracic duct (113,114). The adipose tissue and muscle rapidly remove the triacylglycerol component from the chylomicron particle, whereas the cholesterol component is removed by the liver (114,115). Carbohydrates and amino acids are the primary fuels for hepatic metabolism in the fed state.

During starvation or stress, the stored triacylglycerol in adipose tissue is hydrolyzed to free fatty acids and glycerol by the action of hormone-sensitive triglyceride lipase (116,117). Glycerol and free fatty acids are released in the blood (118,119). Epinepherine, norepinepherine, glucagon, adreno-corticotrophic hormone (ACTH), glucocorticoids and growth hormone promote the fat mobilization from adipose tissue (120,121,122). Glycerol and free fatty acid are removed from the blood by the liver. During starvation the greater portion of the free fatty acid taken up by the liver is used for oxidation and a lesser part for acylation reactions with glycerol phosphates (123). In the rat the entire synthesis of hepatic triacylglycerol, which occurs 48 hours after fasting, is derived from serum non-esterified fatty

acid and serum triacylglycerol (124). Recycling of serum triacylglycerol appears to contribute significantly to the total hepatic triacylglycerol turnover in the starved rat, whereas the intestinal synthesis of triacylglycerol contributes little (125). Low concentrations of sn-glycerol 3-phosphate in hepatocytes has been considered a limiting factor in hepatic triacylglycerol biosynthesis, although the livers from both fed and fasted rats seem to be able to esterify the same amount of fatty acids (126). Following a 16 hour fast, triacylglycerol synthesis can be stimulated in rats with 2-tetradecylglycidic acid, and the low hepatic levels of sn-glycerol 3-phosphate which occur do not appear to limit the rate of triacylglycerol production (111).

Many enzymes of hepatic glycerolipid synthesis may require phosphorylation and dephosphorylation reactions for their activity (127). Reversible phosphorylation may regulate sn-glycerol 3-phosphate acyltransferase, phosphatidic acid phosphatase, diacylglycerol acyltransferase, phosphatidyl-ethanolamine methyl-transferase, and phosphocholine cytidyltransferase (71,128,129,130,131,132). Although glucagon increases the total phosphatidic acid phosphatase activity, it does not change the total sn-glycerol 3-phosphate acyltransferase activity (133). Glucagon increases the microsomal sn-glycerol 3-phosphate acyltransferase activity, but decreases the

mitochondrial isoenzyme activity. Glucocorticoids further increase the glucagon-induced stimulation of total phosphatidic acid phosphatase activity; the membrane bound activity increases with higher concentrations of fatty acids. Spermine inhibits the glucagon, dexamethasone and cyclic AMP stimulated activities of phosphatidic acid phosphatase (134). Glucagon can also inhibit triacylglycerol synthesis by limiting the acylation of glycerol 3-phosphate by stimulating mitochondrial fatty acid oxidation in isolated hepatocytes (135). Cortisol increases the cytosolic phosphatidic acid phosphatase activity, but has no effect on triacylglycerol synthesis (136). Cortisol has no effect on the microsomal sn-glycerol 3-phosphate acyltransferase activity (136). The rate of phosphoglyceride synthesis, however, is dependent upon the concentration of fatty acids (136). The stimulatory effects of fatty acids on triacylglycerol synthesis may be related to the enhanced activities of diacylglycerol acyltransferase (72).

Insulin decreases the microsomal sn-glycerol 3-phosphate acyltransferase activity, and the total phosphatidic acid phosphatase activity (133). In insulin-dependent diabetes mellitus, suboptimal insulin levels in patients cause an overproduction of very low density lipoproteins due to the increased utilization of free fatty acids and decrease in very low density

lipoprotein clearance (137). With severe insulin deficiency, the hypertriacylglyceridemia is caused by a decreased lipoprotein lipase activity and not by an increase in triacylglycerol synthesis (137). Insulin helps mediate the de novo synthesis of triacylglycerol in the liver, and secretion of nascent very low density lipoprotein (139). In rabbits insulin also regulates high density lipoprotein metabolism (139). Current evidence indicates that many enzymes involved with glycerolipid synthesis are regulated by hormonal control, that phosphatidic acid phosphatase is probably one of the rate-limiting enzymes of glycerolipid synthesis, and that the hepatocyte sn-glycerol 3-phosphate and fatty acid content may alter the rate of glycerolipid biosynthesis.

Liver Regeneration

Extensive loss of normal liver tissue is compatible with survival, because the remnant has the capacity to regenerated. During the period of rapid liver growth, glycerolipid synthesis must increase to meet the need for new membrane formation. The liver is in a dynamic metabolic state, and the synthesis of DNA, RNA, protein, lipid and carbohydrate increases (140). The rate of DNA synthesis depends upon the extent of liver resection, the age of the rats, and hepatotrophic factors such as hormones (141). When less than one-third of the liver is resected, there is only a small increase in DNA synthesis. Significant DNA synthesis

occurs with a 43 percent resection, and increases proportionally up to a loss of 68 percent of the liver. If liver loss is greater than two-thirds, DNA synthesis and mitosis is delayed. After a 90 percent subtotal hepatectomy, no DNA synthesis or mitosis occurs. Hepatic metabolic function, such as phenobarbital-induced synthesis of microsomal enzymes, is compromised even after a loss of two-thirds of the liver. Most studies of liver regeneration in the rat have been performed after two-thirds partial hepatectomy.

Following two-thirds partial there is a rapid increase in the size of the hepatocytes and the number of cells increase (142,143). Peak mitotic activity of parenchymal cells occurs 24 to 36 hours after partial hepatectomy, that of Kupffer cells occurs at 48 hours, and that of endothelial cells occurs at 96 hours (144,145,146,147). DNA synthesis in parenchymal cells precedes the increase in mitotic activity by 6 to 8 hours (148,149). Parenchymal cells constitute 90 to 95 percent of the total liver cell mass, while being only 60 to 65 percent of the total number of cells (150).

Within 30 minutes after partial hepatectomy, basophilic bodies are dispersed in the cytoplasm, and probably represent ribosomes which are lost from the endoplasmic reticulum (151). Some mitochondria become short and dumbbell in shape (152). Within a few hours hyalin protein droplets appear in the cytoplasma, and

at 6 hours lipid vacuoles appear (145,152). Glycogen granules disappear by 10 hours (155). By 12 hours an increase in the size of cells, nuclei and nucleoli occurs, and becomes maximal at 24 hours (145,153,154). The normal lobular pattern of the parenchyma is lost due to the enlarged cells. At 16 to 18 hours after partial hepatectomy, the endoplasmic reticulum begins to reorganize and is seen close in contact with mitochondria (155). The endoplasmic reticulum is completely restored at 36 to 48 hours (156). Mitosis occurs between 24-28 hours and exhibits diurnal periodicity (157). The rapid growth phase slows after the third day and regains its orginal mass by 10-15 days (145,153). Following 2/3 partial hepatectomy one-third of the liver mass remains, at 18 hours there is 45 percent, at 36 hours there is 53 percent, at 60 hours 71 percent, at 4 to 7 days 74 percent, at 7 to 14 days 93 percent, and at 14 to 21 days 102 percent (158).

Metabolism in the regenerating liver gradually changes from the time of the surgical removal of liver mass to its full restoration. Several reviews of the biochemical changes in the liver which are associated with partial hepatectomy have been published (141,144,159,160,161). In order to understand the effect of extensive liver loss on metabolism, the metabolic response to abdominal surgery without liver resection must also be considered. It has been

demonstrated in man that following surgery serum ACTH and growth hormone levels are elevated (162). When growth hormone is administered to patients after gastrointestinal surgery, fat oxidation is increased by 33 percent and protein oxidation is decreased by 24 percent (163). Abdominal surgery also causes increased secretion of vasopressin and cortisol (164,165). The increase in plasma catecholamine levels probably causes the decrease in testosterone and insulin secretion (166,167). In response to the increased energy demands after major surgery, thyroxine secretion increases (168). Hyperglucagonemia is probably caused by elevated plasma catecholamine levels (169,170). The postsurgical alterations of the secretion of hormones affects metabolism, the management of the patient and recovery.

Following partial hepatectomy in the rat similar hormonal changes occur. Plasma glucagon levels uniformly increase after partial hepatectomy; whereas plasma insulin levels have been reported to be either decreased, unchanged or elevated (171,172,173,174). Hyperglucagonemia and hypoinsulinemia follow major liver resection in well-fed rats, but hyperinsulinemia occurs in starved rats (174). Both insulin and glucagon are considered hepatotrophic factors for the regenerating liver, and act synergistically to promote growth (175). Although regeneration is not prevented

in adrenalectomized rats, the accumulation of triacylglycerol is interferred with, and liver growth is slowed (152,176,177). Pretreatment with glucocorticoids before partial hepatectomy delays DNA synthesis and mitosis (178,179). Cortisone, hydrocortisone and corticosterone promote lipid accumulation (153,180). Catecholamines also delay DNA synthesis (179). Thymidine kinase synthesis is inhibited by glucocorticoids and catecholamines (179). Pretreatment with growth hormone before partial hepatectomy accelerates DNA synthesis, as does minor surgery up to 3 days before hepatectomy (181). Serum estrogen increases while serum testosterone decreases from 1 to 3 days after partial hepatectomy (182). Total hepatic and nuclear estrogen receptors increase with a concomitant decline in cytosolic estrogen receptors, whereas the total and nuclear androgen receptors decrease (182,183). Other hormones which can influence hepatic regeneration are parathyroid hormone and calcitonin (159,184).

It is apparent that the metabolic response to extensive liver loss is very complex being influenced by the nutritional status of the patient, age, and the health of the liver (185,186,187). Insulin therapy promotes liver growth in fasted rats, but does not in fed rats (188). In spite of starvation for days or prolonged protein deprivation, DNA synthesis and mitosis is only delayed (185,186,187,190). Glycogen

depletion and triacylglycerol accumulation occurs (159). Following partial hepatectomy fat is mobilized from the adipose tissue, and serum levels of unesterified fatty acids and glycerol increase (191). The uptake of fatty acids and glycerol is increased, and is related to the increase in blood flow to the residual liver and to the elevated blood levels (192,193,194,195). Although fatty acid oxidation increases, a significant increase in fatty acid incorporation into triacylglycerol occurs during the first day (196,197).

Although many toxin-induced fatty livers can be attributed to a decrease in very low density lipoprotein secretion, the regenerating liver at 20 hours secretes lipids at a rate equal to the normal liver (198). In another study, triacylglycerol secretion at 3 and 8 hours after partial hepatectomy was higher than in laparotomized controls (199). These studies indicate that very low density lipoprotein synthesis and secretion are not decreased when the triacylglycerol content is highest during liver regeneration, and that the triacylglycerol accumulation results from increased synthesis. The activities of enzymes associated with the synthesis of glycerolipids can also change during liver regeneration, and may be partly responsible for triacylglycerol accumulation. Phosphatidic acid phosphatase activity is increased in

the regenerating rat liver at 6,10,16 and 24 hours after subtotal (82%) hepatectomy, and palmitoyl-CoA synthetase and sn-glycerol 3-phosphate acyltransferase activities are increased at 24 hours (200). The increased activities of these microsomal enzymes may be related to the increase in triacylglycerol synthesis, when the phospholipid content of the liver is unchanged (191).

Metabolic Inhibitors of Glycerol 3-Phosphate and Dihydroxyacetone Phosphate Acyltransferases

Since the biosynthesis of glycerolipids begins with the acylation of either sn-glycerol 3-phosphate or dihydroxyacetone phosphate, inhibitors of the reactions in hepatocytes may modulate the synthesis of glycerolipids and very low density lipoproteins. These metabolic inhibitors could be used to provide a better understanding of the early reactions that lead to triacylglycerol and phospholipid biosynthesis. In recent years several isosteric and nonisosteric analogues of sn-glycerol 3-phosphate and dihydroxyacetone phosphate have been developed. The effects of the analogues on phosphoglyceride metabolism have been primarily studied in bacteria. (S)-3,4-Dihydroxybutyl-1-phosphonate inhibits the growth of Escherichia coli and is a competitive inhibitor for phosphatidylglycerol phosphate synthetase and CDP-diacylglycerol:sn-glycerol 3-phosphate phosphatidyltransferase activities (201,202,203,204).

Although initial studies have indicated that (S)-3,4-dihydroxybutyl-1-phosphonate is not an inhibitor of sn-glycerol 3-phosphate acyltransferase activity in E. coli, recent evidence suggests that this analogue is a competitive inhibitor of a highly purified sn-glycerol 3-phosphate acyltransferase of E. coli with a Ki of 25 mM (205). Other studies with Bacillus subtilus indicate that (RS)-3,4-dihydroxybutyl-1-phosphonate inhibits the synthesis of phosphatidylglycerol and to a lesser extent phosphatidylethanolamine (206). Several analogues of sn-glycerol 3-phosphate, which inhibit the growth of E. coli, are transported across the cell membrane by the hexose phosphate transport system (207); this type of transport system has not been demonstrated in animal cells. 3,4-Dihydroxybutyl-1-phosphonate is also a substrate for rabbit muscle sn-glycerol 3-phosphate dehydrogenase (sn-glycerol 3-phosphate:NAD^+ oxidoreductase) (208,209). (1R,3S)-1,3,4-Trihydroxybutyl-1-phosphonate, a structural analogue of sn-glycerol 3-phosphate, is also an inhibitor of E. coli sn-glycerol 3-phosphate acyltransferase and is bacteriostatic (210).

(S)-Glyceraldehyde 3-phosphate is a bactericidal drug, and is a competitive inhibitor for sn-glycerol 3-phosphate acyltransferase and CDP-diglyceride:sn-glycerol 3-phosphate phosphatidyltransferase from E. coli (211). It has no effect on lysophosphatidate

acyltransferase reactions, but inhibits DNA, RNA, phospholipid and protein synthesis in bacteria (212). Recent studies from our laboratory indicate that E. coli can convert (S)-glyceraldehyde 3-phosphate into sn-glycerol 3-phosphate in a reaction catalyzed by a NADPH-dependent reductase. The phosphonic acid analogue of glyceraldehyde 3-phosphate, 3-hydroxy-4-oxobutyl-1-phosphonate is a competitive inhibitor of sn-glycerol 3-phosphate in sn-glycerol 3-phosphate acyltransferase reactions and in CDP-diglyceride:sn-glycerol 3-phosphate phosphatidyltransferase reactions in E. coli; this analogue is bacteriostatic (211,212).

The phosphonic acid analogue of dihydroxyacetone phosphate, 4-hydroxy-3-oxobutyl-1-phosphonate, is a substrate for sn-glycerol 3-phosphate dehydrogenase and aldolase from rabbit muscle, but is not a substrate for chicken muscle triose phosphate isomerase (213,214). Although phenethyl alcohol is not structurally related to glycerol 3-phosphate, it is a noncompetitive inhibitor of E. coli sn-glycerol 3-phosphate acyltransferase (215). The synthesis of phospholipids is inhibited more than the synthesis of DNA, RNA or protein. Since the carbon-phosphorus bond is not metabolized in rats, the phosphonate analogues of sn-glycerol 3-phosphate and dihydroxyacetone phosphate may be useful probes to perturb metabolic reactions using the natural substrate (216).

Aim of Investigations

The purpose of this study was to evaluate the effect of sn-glycerol 3-phosphate analogues on liver regeneration. In the first study various agents were evaluated as possible inhibitors of mitochondrial and microsomal sn-glycerol 3-phosphate acyltransferase reactions. Chemicals used were isosteric analogues of sn-glycerol 3-phosphate (3,4-dihydroxybutyl-1-phosphonate, 3-hydroxy-4-oxobutyl-1-phosphonate, and glyceraldehyde 3-phosphate), an isosteric analogue of dihydroxyacetone phosphate (4-hydroxy-3-oxobutyl-1-phosphonate), nonisosteric analogues of sn-glycerol 3-phosphate [(1S,3S)- and (1R,3S)-1,3,4-trihydroxybutyl-1-phosphonate], and phenethyl alcohol. 3,4-Dihydroxybutyl-1-phosphonate was also evaluated as a substrate for mitochondrial and microsomal acyltransferase reactions. In the next study the possible incorporation of a tritiated analogue of sn-glycerol 3-phosphate into the lipid fraction of whole cells was determined. Following partial hepatectomy the relationship of the degree of triacylglycerol accumulation to the activity of sn-glycerol 3-phosphate acyltransferase and dihydroxyacetone phosphate acyltransferase was determined. In the last study the effect of administering 3,4-dihydroxybutyl-1-phosphonate during liver regeneration was evaluated in the rat liver.

METHODS AND MATERIALS

Chemicals

The following chemicals were purchased from the Sigma Chemical Co., St. Louis,MO: glycine, free base, essentially ammonia-free; hydrazine hydrate, 100 % liquid; $MgCl_2$, anhydrous; NaF, crystalline; bovine serum albumin, essentially fatty acid-free and globulin-free; Eagle's minimum essential medium with Hank's salts and L-glutamine; fetal calf serum; streptomycin sulfate; N-ethylmaleimide, crystalline; ethylenediaminetetraacetic acid (EDTA), disodium salt, Sigma grade; sucrose, crystalline, Sigma grade I; tris(hydroxymethyl) aminomethane base, Sigma reagent grade; n-nonane; K-Na tartrate, crystalline; Na_2CO_3, anhydrous; $CuSO_4$, pentahydrate, Sigma grade I; phenol reagent, Folin and Ciocalteu's, 2 N; Dowex-50 (H^+), ion exchange resin, specially washed (50x4-200R); NaCl; LiOH; $FeCl_3$; 5-sulfosalicylic acid; (RS)-dithiothreitol, Sigma grade; indole, crystalline; deoxyribonucleic acid (DNA), sodium salt, from calf thymus; periodic acid; aminonaphthol-sulfonic acid; $NaSO_3H$, anhydrous, Sigma grade I; Na_2SO_3, anhydrous, Sigma grade I; o-dianisidine dihydrochloride; 2-(p-iodophenyl)-3-p-nitrophenyl 5-phenyltetrazolium chloride (p-iodonitrotetrazolium violet); K_2HPO_4, crystalline; KH_2PO_4, crystalline; ammonium acetate,

crystalline; ammonium formate; ammonium bicarbonate, crystalline; ammonium molybdate, tetrahydrate; triethanolamine HCl, crystalline; glycylglycine; Triton X-100 (Rohm & Haas, Co.); α-ketoglutarate; glucose 6-phosphate, monosodium salt, Sigma grade; sodium deoxycholate; sodium cholate, from ox or sheep bile; sodium cacodylate; trichloroacetic acid, Sigma ACS reagent grade; benzylamine HCl; N-phenyl-p-phenylene diamine; sodium arsenate; sodium urate; NaOH; cytochrome c, Sigma type VI, from horse heart; adenosine 5′-diphosphate (ADP), potassium salt, Sigma grade XVIII, from yeast; adenosine 5′-triphosphate (ATP), disodium salt, from equine muscle; dihydroxyacetone, crystalline; dihydroxyacetone phosphate, dimethyl ketal salt, monohydrate; (S)-glyceraldehyde 3-phosphate, diethyl acetal, di(cyclohexylammonium) salt; (RS)-glyceraldehyde 3-phosphate, diethyl acetal, monobarium salt; β-nicotinamide adenine dinucleotide, oxidized form (NAD^+), Sigma grade V, from yeast; β-nicotinamide adenine dinucleotide, reduced form (NADH), Sigma grade III, disodium salt; β-nicotinamide adenine dinucleotide phosphate, oxidized form ($NADP^+$), Sigma type I, sodium salt; β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), Sigma type I, tetrasodium salt; phenethyl alcohol; (RS)-glycerol 3-phosphate, disodium salt; and palmitoyl coenzyme A.

The following chemicals were purchased from the

Mallinckrodt Chemical Co., Inc., St. Louis, MO: perchloric acid, HCl, H_2SO_4, acetic acid, formic acid, hydrogen peroxide, and KCN. Diethyl ether was obtained from the Fisher Scientific Co., St. Louis, MO. A triolein standard (Bio Trol T) , and a kit (# 126012) to measure triacylglycerols were obtained from Boehringer Mannheim Biochemicals, Indianapolis,IN. A purified Dowex AG 1-X8 anion exchange resin, formate form, 200-400 mesh was purchased from Bio-Rad Laboratories, Richmond CA. Chloroform, methanol, and isopropanol were obtained from Burdick and Jackson Laboratories, Muskegon, MI. DEAE Cellulose DE-52, preswollen resin; silica gel thin layer chromatography plates (LK5DF), 250 um thick, 20 x 20 cm; and filter paper (#2) was obtained from Whatman Inc., Clifton, NJ). Absolute ethanol was produced by the Publicker Industries, Inc., Dayton, NJ. Whatman 3M chromatography paper, 20 cm width, was kindly provided by A. Rosenthal, Long Island Jewish Medical Center. Formaldehyde, 5 % in potassium phosphate buffer, pH 7.4, was generously provided by the Histology Department, Long Island Jewish Medical Center. Nitrogen gas, ultra pure, was obtained from a distributor for the Linde Division, Union Carbide Corp., New York, NY.

Radioactive Chemicals and Scintillation Fluids

sn-[^{14}C(U)]-Glycerol 3-phosphate, 118.5

Ci/mole; [γ-^{32}P]-adenosine 5'-triphosphate, 25 Ci/mole; and [^{3}H-methyl]-thymidine, 20 Ci/mmole, were purchased from ICN, Radiochemical Division, Irvine, CA. The lithium salt of (S)-3,4-dihydroxy-[3-^{3}H]-butyl-1-phosphonate, 175 Ci/mole was kindly provided by B. Tropp and R. Engel. Biofluor and Aquasol liquid scintillation fluids, and internal standards were obtained from the New England Nuclear Co., Boston, MA. Quenching was corrected by internal standardization with [^{3}H]-toluene or [^{14}C]-toluene for chloroform soluble material, and [^{3}H]-water for aqueous soluble material.

Synthesis of [32-P]-Dihydroxyacetone Phosphate

[32-P]-Dihydroxyacetone phosphate, 42.9 Ci/mol, was prepared by the enzymatic phosphorylation of dihydroxyacetone with [γ-^{32}P]-ATP using the reaction system described by Schlossman and Bell (20). The 0.5 ml reaction mixture contained 40 mM triethanolamine HCl, pH 7.4, 4 mM $MgCl_2$, 30 mM dihydroxyactone, 20.5 mM [γ-^{32}P]-ATP, 200 Ci/mol, and 40 µg of glycerol kinase, and was incubated at 23^{o} C for 2 hours. The reaction was terminated by adding 50 µl of 0.5 g/ml trichloroacetic acid. Following centrifugation at 1500 x g for 10 minutes, the supernatant was applied to a 1 x 5 cm column of 4.5 g of Dowex AG 1-X8 anion exchange resin in the formate form. The column was washed with 100 ml of distilled water, and the [^{32}P]-dihydroxyactone phosphate was eluted from the column

with 200 ml of 2 N formic acid. Fractions containing the greatest counts were pooled, lyophilized and dissolved in distilled water. With this method there was only a 2 to 8 percent contamination with inorganic ^{32}P. The amount of [^{32}P]-dihydroxyacetone phosphate which was generated was determined spectrophotometrically by the oxidation of NADH using the glycerol 3-phosphate dehydrogenase reaction (217). The 3 ml reaction mixture contained 200 mM triethanolamine HCl, pH 7.5, 17 μM NADH, sample and 50 units of enzyme. The change in absorbancy was measured at 340 nm using a pen recorder, and determining the linear rate over 45 seconds. A standard of dihydroxyacetone phosphate, which was prepared according to the procedure of the Sigma Chemical Co., St. Louis,MO, was used to validate the assay conditions.

Enzymes

Monoamine oxidase (EC 1.4.3.4) from bovine plasma, 40-60 units/g protein; L-glutamate dehydrogenase (EC 1.4.1.3), Type I, from bovine liver, 40 units/mg protein; glucose 6-phosphatase (EC 3.1.3.9), a microsomal preparation from rabbit liver, 100 units/g protein; urate oxidase (EC 1.4.2.2) from porcine liver, Sigma type V, 20 units/g protein; catalase (EC 1.11.1.6) from Aspergillus niger, 5,000 units/mg protein; esterase (EC 3.1.1.1) from porcine liver, 100 units/mg protein; and triacylglycerol

acylhydrolase (lipase, EC 3.1.1.3) from Rhizopus arrhizus, 400,000 units/mg protein were purchased from the Sigma Chemical Co., St. Louis, MO. Diaphorase (EC 1.6.4.3); glycerol kinase (EC 2.7.1.30) from Candida mycoderma, 170-320 units/ml; glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) from rabbit muscle, 340 units/ml; D-glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) from rabbit muscle, 80 units/mg protein; triosephosphate isomerase (EC 5.3.1.1) from rabbit muscle, 10,000 units/ml; and lactate dehydrogenase (EC 1.1.1.27), 1,100 units/mg protein were purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN.

Analogues

The lithium salt of (RS)-3,4-dihydroxybutyl-1-phosphonate was provided by B.E. Tropp. The diethyl acetal of (RS)-3-hydroxy-4-oxobutyl-1-phosphonate, and the monosodium salt of 4-hydroxy-3-oxobutyl-1-phosphonate were provided by R. Engel. (-)-(1S,3S)-1,3,4-Trihydroxy-butyl-1-phosphonate, and (+)-(1R,3S)-1,3,4-trihydroxy-butyl-1-phosphonate, were provided by S. Niess. 4-Palmitoyl-sn-3-hydroxybutyl-1-phosphonate, and 3,4-dipalmitoyl-sn-butyl-1-phosphonate were generously provided by P. Waters and R. Engel.

(RS)-Glyceraldehyde 3-phosphate, which was purchased from the Sigma Chemical Co. as the diethyl acetal salt required hydrolysis before use. The procedure for generating the acid form was the method as described by the Sigma Chemical Co. Dowex-50 (H^+)

resin, 1.5 g, was added to a tube containing 6 ml of distilled water, and was vortexed for 5 seconds. Then 100 mg of the diethyl acetal salt was added. The tube was placed in boiling water for 3 minutes, and was shaken intermittently. The tube was then placed in an ice bath for 10 minutes. After centrifugation at 1000 x g for 10 minutes, the supernatant was decanted. The resin was resuspended in 2 ml of water, vortexed for 1 minute, and centrifuged again. The supernatants were combined and the resin was washed two additional times to complete the extraction of the glyceraldehyde 3-phosphoric acid. The diethyl acetal salt of (RS)-3-hydroxy-4-oxobutyl-1-phosphonate was also hydrolyzed using the identical procedure to generate the free acid form. (R)-Glyceraldehyde 3-phosphate was purchased as the dimethyl ketal, di(cyclohexylammonium) salt from the Sigma Chemical Co., St. Louis, MO. Before enzymatic analysis 10 mg of the salt was hydrolyzed with 0.25 ml of 2 M sulfuric acid overnight at room temperature according to the method of the Sigma Chemical Co. Sodium hydroxide was added to adjust the pH to 6. Then the solution was passed through 1 g of Dowex-50 (H^+) resin, and the pH was adjusted as before. Dihydroxyacetone phosphate was purchased from the Sigma Chemical Co. as the dimethyl ketal salt, and required hydrolysis before use. The procedure of the Sigma Chemical Co. was used to hydrolyze the ketal. The

dihydroxyacetone phosphate-ketal was dissolved in 2 ml water, and 0.5 g of Dowex-50 (H^+) resin was added. The tube was vortexed for 1 minute. The solution and resin were poured onto a Whatman #2 filter paper, and the filtrate was collected. The resin was washed 3 times with 1 ml of distilled water. The combined filtrates were incubated for 4 hours at 40° C.

Tissue Culture Cells

Mouse 3T3 fibroblasts and human Hep-G2 hepatoma cells were kindly provided and cultured by C. S. Shopsis, Rockefeller University, New York, NY. All studies with these cell lines were performed at Rockefeller University.

Equipment

Chemicals were weighed with either a Sartorius model 2474 analytical balance, Brinkmann Instrument Co., Westbury, NY, or an Ohaus Dial-O-Gram balance, Ohaus Scale Corp., Port Washington, NY, depending on the amount of material weighed. Low speed centrifugation was performed using a refrigerated centrifuge, model PR-6 with a 276 rotor, International Equipment Co., Needham Heights, MA. High speed centrifugation was performed using a Beckman model L5-75 ultracentrifuge with a 50 Ti rotor, Beckman Instruments, Inc., Los Angles, CA. Tissue was disrupted with a Polytron homogenizer with a microtip, Brinkman Instrument Co., Westbury, NY. A Gilford model 250 spectrophotometer and recorder,model 6050, Gilford

Instrument Laboratories, Inc., Oberlin, OH, was used to determine marker enzyme activities; circulating water bath, Polyscience model 80, Warrington, PA, was used to maintain the reaction temperature. A Packard liquid scintillation spectrophotometer, model 3385, Downers Grove, IL, was used to measure radioactive decay.

Animals

Male Holtzman rats, weighing 150 to 200 g, were purchased from the Holtzman Co., Madison, WI, and were housed in wire mesh cages in a well ventilated room with a 12-hour light/dark cycle. Animals were kept in groups of 6 rats per cage. Before experimentation all animals were conditioned to their environment for at least one week. Rats were fed Rat Chow (Ralston Purina Co., St. Louis, MO) and water was available ad libitum. Two-thirds partial hepatectomies were performed as described by Higgins and Anderson, except that 50 to 75 mg/kg of ketamine HCl (Bristol Laboratories, Syracuse, NY) was injected intraperitoneally to induce anesthesia (158,218). The duration of surgical anesthesia was 15 to 25 minutes, and lacked cardiorespiratory depression (218). Ketamine HCl has little adverse effect on liver metabolism, in contrast to the effect of diethyl ether (219). Surgery was performed between 9:00 and 11:00 AM to minimize the effect of diurnal variations on regeneration. The median and left lateral lobes were

excised, weighed, and found to comprise 66 ± 2 percent of the total weight; the right lateral and caudate lobes comprised the remainder of the mass. Sham operated rats had midline laparotomies and the livers had an untied ligature temporarily placed about the lobes. Rats were fasted after surgery, but were allowed water. Animals were sacrificed by cervical dislocation, and the livers were excised rapidly, placed on ice, and weighed before being used in an experiment. In other studies, fed rats were terminated as above, and liver, kidneys, adipose tissue and small bowel were excised for intact cell experimentation.

Statistical Methods

Measurements on samples were performed at least in duplicate and the results were averaged. An analysis of variance (ANOVA) was used to test the statistical significance of the differences between the sham and partial hepatectomy groups (220). A product-moment correlation analysis of enzyme specific activity and the triacylglycerol content was performed (221). A significant difference between groups was inferred when $p < 0.05$.

Isolation and Purification of Mitochondrial, Peroxisomal and Microsomal Membranes

After one week of conditioning to the environment, 5 non-fasted rats were sacrificed by cervical dislocation; the remaining 5 rats were sacrificed one week later. Livers were excised, rinsed

in 0.25 M sucrose - 1 mM EDTA - 10 mM Tris HCl, pH 7.4 at 4°C, and blotted dry with a gauze sponge. Large vessels and connective tissue were trimmed off, and the livers were weighed. The livers were minced with a surgical scissors in a 50 ml stainless steel tube which was precooled to 4°C. Fractionation of subcellular membranes was obtained by a modification of the methods of Weinbach (222), Monroy et al. (223), and Jones et al. (224) to collect partially purified mitochondrial, peroxisomal and microsomal membranes. Liver homogenates, 25 % (w/v) in sucrose-EDTA-Tris HCl buffer, pH 7.4, were produced using a Polytron Homogenator (Brinkman Co., Great Neck, NY) at a low setting using 10 strokes, cooling for 30 seconds and then using 10 additional strokes, while the tube remained in an ice bath. One-tenth of the volume was removed and saved as the crude homogenate fraction; the remainder was centrifuged at 600 x g for 10 minutes. The supernatant was saved for the collection of the subcellular fractions,and the pellet was resuspended in nine-tenths volume of buffer. Following centrifugation at 500 x g for 10 minutes, the nuclear fraction (pellet) was discarded. Post-nuclear supernatants were combined and centrifuged at 2,400 x g for 20 minutes to pellet mitochondria. The layer of fat floating on the top of the fluid was removed carefully with cotton-tipped applicators. This supernatant was saved for the

collection of peroxisomes and microsomes. The pellet, containing mitochondria, was again resuspended in buffer and centrifuged at 2,400 x g for 20 minutes. Discarding the supernatant, the pellet was again resuspended in buffer and centrifuged at 2,400 x g for 20 minutes to sediment the mitochondria, but not peroxisomes and microsomes. The washing procedure was repeated three additional times to partially purify the mitochondrial fraction.

The peroxisomal-microsomal supernatant was centrifuged at 9,000 x g for 30 minutes and the pellet was saved for collection of peroxisomes. The supernatant was again centrifuged at 9,000 x g for 30 minutes, and the supernatant was saved for the collection of microsomes. The two pellets were resuspended in buffer, pooled and centrifuged at 5,000 x g for 20 minutes to pellet the peroxisomes; the supernatant was saved. The pellet was resuspended in buffer and centrifuged again at 5,000 x g for 20 minutes. The two post-5,000 x g supernatants, which contained peroxisomes and lysosomes, were pooled and were centrifuged at 5,000 x g for 20 minute. The pellets were resuspended in buffer and combined; this was the peroxisomal fraction. The supernatant was an intermediate fraction.

The 9,000 x g supernatant which was saved for the isolation of microsomes was then centrifuged at 100,000 x g for 90 minutes. The supernatant was

discarded, and the pellet was resuspended in the buffer and centrifuged again at 100,000 x g for 90 minutes. This was repeated and the pellet was the microsomal fraction. Pellets were resuspended in the sucrose-Tris HCl buffer , pH 7.4, at a concentration of 10 to 20 mg protein/ml, and were stored at -90 ^{o}C in small aliquots.

Membrane Marker Enzymes

The purity of the subcellular fractions were determined on the fresh preparations by the relative concentrations of organelle enzymes. The presence of marker enzymes (monoamine oxidase, EC 1.4.3.4, mitochondrial outer membrane; succinate cytochrome c reductase, EC 1.3.99.1, mitochondrial inner membrane; glutamate dehydrogenase, EC 1.4.1.3, mitochondrial matrix; glucose 6-phosphatase, EC 3.1.3.9, endoplasmic reticulum luminal surface; NADPH-dependent cytochrome c reductase, EC 1.6.2.4, endoplasmic reticulum cytoplasmic surface; urate oxidase, EC 1.4.2.2, peroxisomal cores; catalase, EC 1.11.1.6, peroxisomal matrix) was determined in all of the collected fractions. Monoamine oxidase activity was assayed by the method of Schnaitman et al. (225), measuring the production of benzaldehyde from 0.25 mM benzylamine at 37 ^{o}C in 0.05 M potassium phosphate buffer, pH 7.6, at 250 nm with a spectrophotometer. Succinate cytochrome c reductase activity was determined spectro-

photometrically by the method of Schnaitman and Greenawalt (226). The assay mixture contained 3 mM succinate, 0.3 mM KCN, 0.1 mM cytochrome c, 50 mM potassium phosphate buffer, pH 7.5, and the sample was diluted in 0.25 M sucrose to stablize the enzymatic activity. The reduction of cytochrome c was measured at 550 nm at 30 °C. Glutamate dehydrogenase activity was assayed by the method of Schmidt measuring spectrophotometrically the oxidation of NADH at 340 nm (227). At 25 °C, the reaction mixture contained in 1 ml: 0.2 mM NADH, 1 mM ADP, 0.1 M ammonium acetate, 50 mM triethanolamine HCl buffer at pH 8.0, 2.5 mM EDTA, 2 units of lactate dehydrogenase, 7 mM α-ketoglutarate, and the sample diluted in buffer containing 0.05 % Triton X-100. Glucose 6-phosphatase activity was measured colorimetrically by the method of Baginski et al. at 25 °C (228). The assay mixture contained 20 mM glucose 6-phosphate, 50 mM sucrose, 0.02 mM EDTA, 40 mM cacodylate buffer at pH 6.5, 5 g/l deoxycholate and sample. After incubation at 37 °C for 15 minutes, the reaction was stopped with 10 % (w/v) trichloroacetic acid. Liberated inorganic phosphate was measured by the reaction with 3 mM ammonium molybdate and 310 mg/l of N-phenyl-p-phenylenediamine at 700 nm. NADPH-dependent cytochrome c reductase activity was determined spectrophotometrically by the method of Phillips and Langdon measuring the reduction of cytochrome c at 550 nm (229). The assay mixture

contained in 3 ml: 0.1 M potassium phosphate buffer, pH 7.5, 1 mM KCN, 0.042 mM NADPH, 0.05 mM cytochrome c, and sample; and was incubated at 30° C. Urate oxidase activity was determined by the method of Leighton et al. (230). The reaction mixture was incubated at 37° C and contained in 3 ml: 0.042 mM sodium urate, 30 mM potassium phosphate, pH 7.4, 1 mM EDTA, 1 mg/ml of Triton X-100, and sample. The rate of urate oxidation was measured spectrophotometrically at 292 nm. The total catalase activity was measured after pretreating the sample with 10 mg Triton X-100 per ml for 2 minutes as described by Declercq et al. (4). Free catalase activity was determined in 0.25 mM sucrose in the absence of detergent. Both activities were assayed spectrophotometrically by the method of Luck at 25° C, measuring the decline in hydrogen peroxide concentration at 240 nm in the presence of enzyme (231). In 3 ml of reaction mixture there was 0.05 % hydrogen peroxide in 50 mM potassium phosphate buffer, pH 7.0, and the sample. Sedimentable catalase was calculated by subtracting free catalase activity from the total catalase activity (4).

The relative purity of the subcellular fractions was determined by the methods of Wattiaux et al. and Leighton et al. as modified by Declercq (4,230,232). The percentage of the activity of a marker enzyme in a subcellular fraction was determined by dividing the

amount of enzyme activity in a fraction by the total enzyme activity in the homogenate, and indicated the portion of the total enzyme recovered in the fraction. The relative specific activity of a marker enzyme was then calculated by dividing this percent of enzyme activity present in a fraction by the corresponding percent of the total protein present in the fraction. The percentage of protein in a fraction was determined by dividing the amount of protein in the fraction by the total protein present in the homogenate.

The contamination of the microsomal fraction by mitochondria was calculated as follows. The total activity of the marker enzymes of microsomes (i.e. NADPH-cytochrome c reductase) and mitochondria (i.e. monoamine oxidase), and the amount of protein was determined in the homogenate. The activity of the marker enzymes, and protein content in each fraction was then measured. The distribution or percentage of the activity of the enzymes was calculated for each fraction; i.e. 23 percent of the NADPH-cytochrome c reductase activity, and 1.6 percent of the monoamine oxidase activity were present in the microsomal fraction. The percentage of protein in each fraction was also calculated; 3.5 percent of protein was in the microsomal fraction. The relative specific activity of each marker enzyme was then determined by dividing the calculated percentage of enzyme activity by the calculated percentage of protein present in the

fraction. In the microsomal fraction, the relative specific activity of NADPH-cytochrome c reductase was 23/3.5 or 6.5, and the activity of monoamine oxidase was 1.6/3.5 or 0.45. Since NADPH-cytochrome c reductase was a marker for the endoplasmic reticulum, and monoamine oxidase was a marker for the mitochondria, the contamination of the microsomal fraction by mitochondria was calculated to be 6 percent or 0.45/(6.5 + 0.45). The relative purity of the microsomal fraction was then 94 percent or 6.5/(6.5 + 0.45). Calculations for the purity of the mitochondrial fraction from microsomal enzymes were also performed.

Protein Determination

The concentration of protein in each fraction was determined by the method of Lowry et al. (233) and was confirmed by the method of Hartree (234) for insoluble protein. Samples were hydrolyzed in 0.5 N NaOH containing 2 g/l of potassium-sodium tartrate and 100 g/l of sodium carbonate at 50° C for 10 minutes. After cooling to 20° C a solution of 20 g/l of potassium-sodium tartrate and 10 g/l of copper sulfate in 0.1 N NaOH was added, and the reaction mixture was incubated at 20° C for 10 minutes. Phenol reagent (1:15) was then added and the mixture was heated at 50° C for 10 minutes. After cooling to 20° C the chromagen was measured at 650 nm. Bovine serum albumin was used

as the standard.

Glycerol 3-Phosphate Acyltransferase Activity

Glycerol phosphate acyltransferase (acyl-CoA:sn-glycerol 3-phosphate O-acyltransferase, EC 2.3.1.15) activity was determined by the method of Coleman and Hayes (21). The reaction mixture contained 75 mM Tris-HCl, pH 7.4; 4 mM magnesium chloride; 2 mg/ml bovine serum albumin, essentially fat free; 8 mM NaF; 80 uM palmitoyl CoA; and 33-400 uM [^{14}C]-glycerol 3-phosphate, 15.6 Ci/mol. In addition, there was 1 mM KCN in mitochondrial reactions, and 1 mM dithiothreitol in microsomal reactions. Palmitoyl CoA was omitted from the blank assays. The reactions in a final volume of 0.2 ml were initiated with 15 to 30 ug of protein which produced a linear rate of ^{14}C incorporation into chloroform-soluble material for at least 20 minutes at 23^{o} C. Kinetic studies were performed by allowing the reaction to proceed for 10 minutes, and then the reaction was terminated by the modified method of Bligh and Dyer as described by Coleman and Hayes (20,21,235). The reaction of the 0.2 ml assay mixture was stopped by the addition of 3 ml of chloroform-methanol (1:2,v/v) and 0.6 ml of 1 % perchloric acid. After mixing well, a single solvent phase was present. Five minutes later 1 ml of chloroform and 1 ml of 1 % perchloric acid was added to break the single phase. After mixing well and centrifuging at 500 x g for 10 minutes at 4^{o} C, the upper aqueous layer was removed. The lower chloroform

layer was washed 4 times by adding 2 ml of 1 % perchloric acid, mixing, centrifuging and removing the aqueous layer. The chloroform layer was then transferred to a 7 ml glass scintillation vial and was evaporated under nitrogen. After the addition of 5 ml of Biofluor, Packard Instrument Co., and mixing well, the samples were counted for ^{14}C in a Packard Model 3385 liquid scintillation spectrophotometer.

Quenching by Acylglycerol 3-Phosphates

The efficiency of counting the chloroform-soluble ^{14}C-products of the sn-glycerol 3-phosphate acyltransferase reaction in liquid scintillation fluid was determined. The glycerolipids generated by the reaction were counted, and were compared to deacylated products which were obtained by the method of Kates (236). In the first experiment the identical acyltransferase reactions were performed in 2 tubes using 10 μCi [^{14}C]-glycerol 3-phosphate, and the [^{14}C]-glycerolipids were extracted into chloroform, as described previously (20). One extract was added immediately to a scintillation vial for evaporation under nitrogen. The other extract was evaporated in another tube, and 0.2 ml of chloroform, 0.3 ml of methanol and 0.5 ml of 0.2 N methanolic NaOH were added, mixed well and incubated at 20° C for 15 minutes. Then 0.2 ml of methanol, 0.8 ml of chloroform and 0.9 ml of water were added, mixed and centrifuged

at 600 x g for 10 minutes. The upper methanolic water phase was added to a tube,and was neutralized with Dowex 50 (H^+). After centrifugation as before the supernatant was transferred to a second vial. The chloroform phase was washed twice with 0.5 ml of methanol:water (10:9,v/v), and the mixture was centrifuged as before to separate the 2 phases. The methanol-water washings were mixed with the Dowex-50. After centrifugation, the washings were combined in the second scintillation vial, and were evaporated under nitrogen. Five ml of Biofluor was added to each vial and ^{14}C counts were determined. One vial contained [^{14}C]-glycerolipids and the other vial had the deacylated sample. When compared to a third vial containing 10 µCi [^{14}C]-glycerol 3-phosphate, the amount of [^{14}C]-deacylated sample was determined, and the counting efficiency of the acylated sample was calculated to be 90 %. Then 10 µCi of [^{14}C]-glycerol 3-phosphate was added to each vial, mixed, recounted and compared to the vial containing only [^{14}C]-glycerol 3-phosphate in Biofluor. The addition of [^{14}C]-glycerol 3-phosphate to the deacylated sample indicated that the standard was not quenched. The counts in the vial with the acylated sample plus [^{14}C]-glycerol 3-phosphate was quenched, and was 89 percent of the standard. In the second experiment 81 nmoles of glycerol 3-phosphate was added to 2 tubes and the acyltransferase reaction was again performed. After

extracting the glycerolipids with chloroform and evaporating under nitrogen, the sample of one vial was deacylated. Then 10 μCi of [^{14}C]-glycerol 3-phosphate was added to each vial and Biofluor was added. Counts of ^{14}C were determined. The counts of the acylated sample was 90 percent of the deacylated sample.

Determination of Substrate and Inhibitor Concentrations

The concentrations of sn-glycerol 3-phosphate, (S)-3,4-dihydroxybutyl-1-phosphonate, (1S,3S)- and (1R,3S)-1,3,4-trihydroxybutyl-1-phosphonate were confirmed by the reduction of NAD^+ with glycerol 3-phosphate dehydrogenase according to the method of Michal and Lang (237). The reaction ingredients in a final volume of 2.12 ml were: 0.19 M hydrazine, 0.47 M glycine, 2.7 mM EDTA, 2.31 mM NAD^+, and dilute sample. The reaction at pH 9.5 was initiated with 8 to 10 units of enzyme. The increase in absorbancy was recorded at 340 nm in a spectrophotometer at 25° C.

The concentrations of (R)-glyceraldehyde phosphate, and (R)-3-hydroxy-4-oxobutyl-1-phosphonate were confirmed by the method of Racker (238). The reaction mixture contained 25 mM glycylglycine buffer at pH 7.4, 0.6 μM NAD+, 5mM sodium arsenate, 4,000 units of (R)-glyceraldehyde 3-phosphate dehydrogenase, and dilute sample in a final volume of 1.08 ml. The production of NADH was recorded at 340 nm at 25° C.

The concentration of 4-hydroxy-3-oxobutyl-1-

phosphonate was verified by the method of Bucher and Hohorst for the assay of dihydroxyacetone phosphate (239). In a final volume of 1.03 ml, the assay mixture contained 0.4 M triethanolamine HCl buffer, pH 7.6, 40 mM EDTA, 50 nM NADH, 4 units of glycerol 3-phosphate dehydrogenase, and sample. The change in absorbancy was recorded at 340 nm at 25 °C.

Inhibition of Glycerol 3-Phosphate Acyltransferase Activity

In order to study the inhibition of the sn-glycerol 3-phosphate acyltransferase reaction, the assay was performed as previously described, but now in the presence of potential inhibitors. [^{14}C]-Glycerol 3-phosphate was diluted with water, and the final concentration was 0.033, 0.04, 0.05, 0.067, 0.10, 0.20 and 0.40 mM. Microsomal or mitochondrial fractions were used to initiate the reactions; dilution of the enzyme was with 0.25 M sucrose - 1 mM Tris HCl buffer, pH 7.4. In uninhibited reactions, no inhibitor was added, and the volume was replaced with 0.25 M sucrose - 1 mM Tris HCl buffer, pH 7.4. The concentrations of the inhibitors in the reaction mixture were: 2 mM (RS)-3,4-dihydroxybutyl-1-phosphonate, 2.5 mM (RS)-glyceraldehyde 3-phosphate, 2.5 mM (RS)-3-hydroxy-4-oxobutyl-1-phosphonate, 2.5 mM (1R,3S)-1,3,4-trihydroxybutyl-1-phosphonate, 2.5 mM (1S,3S)-1,3,4-trihydroxybutyl-1-phosphonate, 8 and 18.8 mM 4-hydroxy-3-oxobutyl-1-phosphonate for microsomal and

mitochondrial enzyme, respectively, and 4 mM phenethyl alcohol. After determining the quantity of [^{14}C]-glycerolipids which were produced, the data were plotted according to the method of Lineweaver and Burk (240) and subjected to least square analysis. The apparant Michaelis-Menten constants (Km) and the maximum reaction velocities (Vmax) were determined from the double reciprocal plot of glycerol 3-phosphate concentrations and the reaction rates. The apparent Km for the substrate is represented by the reciprocal of the absolute value of the x-axis intercept. The reciprocal of the x-axis intercept of the double reciprocal plot of competitive-inhibited reactions is equal to Km(1+[I]/Ki) and is the Kp (241). The inhibitor constant (Ki) was calculated from the inhibitor concentration ([I]), the Kp, and the apparent Km (competitive inhibition) or the apparent Vmax (noncompetitive).

N-Ethylmaleimide Inhibition

The selective inhibition of microsomal glycerol 3-phosphate acyltransferase activity by N-ethylmaleimide can be used to distinguish between mitochondrial and microsomal glycerol 3-phosphate acyltransferase activities (21). Mitochondrial and microsomal fractions were diluted with 0.25 M sucrose - 1 mM Tris HCl buffer, pH 7.4, to a concentration of 1.0 mg protein/ml, and incubated with 2 M N-ethylmaleimide

for 15 minutes at 4° C. In control tubes the diluted enzyme was incubated without N-ethylmaleimide. Samples (20 µl) were removed to initiate the acyltransferase reactions. The difference in the amount of chloroform-extractable ^{14}C-products after N-ethylmaleimide inhibition was determined for both mitochondrial and microsomal preparations. Thus, the percent of the acyltransferase activity which decreased in the mitochondrial fraction after N-ethylmaleimide treatment was taken as the percent of microsomal contamination. The percent of the acyltransferase activity remaining in the microsomal fraction after N-ethylmaleimide inhibition was considered the percent of mitochondrial contamination.

Acyltransferase Activity in Total Membranes

Livers were obtained from rats following either sham or partial hepatectomy at 18 and 24 hours. One to two grams of liver were minced and homogenized in cold 0.25 M sucrose - 1 mM EDTA, pH 7.4, with a Polytron homogenizer at a moderate speed using 10 strokes, cooling for 30 seconds, and then using 10 additional strokes. A total particulate suspension was obtained from the liver homogenate by centrifugation at 105,000 x g for 60 minutes and resuspending the pellet in 0.25 M sucrose/EDTA.

A unique property of mitochondrial and microsomal glycerol 3-phosphate acyltransferase is useful for distinguishing the two activities in the

total particulate suspension. N-Ethylmaleimide, a sulfhydryl reactive reagent, selectively inhibits the microsomal enzyme by more than 90 percent (6,18,21,242). Similarly, N-ethylmaleimide selectively inhibits the microsomal dihydroxyacetone phosphate acyltransferase activity and has been used to differentiate between peroxisomal and microsomal activities (20,21). Total glycerol 3-phosphate acyltransferase and dihydroxyacetone phosphate acyltransferase activities were measured. At the same time, the N-ethylmaleimide-resistant (nonmicrosomal) activities were determined by incubating 1 mM N-ethylmaleimide with 1 mg of the particulate protein for 15 minutes at 4^{o} C before assay. The difference between the total activity and N-ethylmaleimide-resistant activity represented the activity of the microsomal enzyme.

The activities of glycerol 3-phosphate acyltransferase and dihydroxyacetone phosphate acyltransferase were determined by the method of Coleman and Hayes (21). The reactions were initiated with 15 to 30 ug protein which produced a linear rate of [^{14}C]-glycerol 3-phosphate or [^{32}P]-dihydroxyacetone phosphate incorporation into chloroform-soluble material for at least 10 minutes. The 0.2 ml incubation mixture contained 75 mM Tris-HCl, pH 7.4; 4 mM $MgCl_2$; 2 mg/ml bovine serum albumin, essentially fat

free; 8 mM NaF; 80 μM palmitoyl CoA and either 0.3 mM [^{14}C]-glycerol 3-phosphate, 15.6 Ci/mol, or 1.5 mM [^{32}P]-dihydroxyacetone phosphate, 42.9 Ci/mol. The reaction time was 3.5 minutes. Palmitoyl CoA was omitted from blank assays. The modified Bligh and Dyer extraction was used to determine the incorporation of ^{14}C or ^{32}P into chloroform-soluble material (20).

Triacylglycerol Measurement

A 100 mg sample of liver was homogenized in 5 ml of nonane:isopropanol (2:3.5,v/v) using the Polytron homogenizer at a high setting for 10 strokes. One ml of 0.08 N sulfuric acid was added to the tube, and the mixture was agitated vigorously with a Vortex mixer for 1 minute. The extraction system efficiently extracts triacylglycerol into the nonane layer, while less than 1 percent of the available phospholipids are extracted (243). The nonane layer was removed, and evaporated to dryness under nitrogen at 40° C. The residue was resuspended in 0.25 to 1 ml of 50 mg/ml bovine serum albumin, fat free, which solubilized the triacylglycerol in the aqueous medium (244).

The nonane-extracted triacylglycerol samples, nonane-extracted bovine serum albumin (blank), and triolein standards (0.5 to 8 mg/ml) were assayed by the method of Bucolo and David as modified by Megraw et al. (245,246). The 2 ml reaction mixture contained 100 mM triethanolamine HCl, pH 7.5, 0.5 mg p-iodonitro-tetrazolium violet, 2 mg bovine serum albumin,

fat free, 1 mM ATP, 2 mM NAD^+, 3000 units of lipase, 0.1 unit of esterase, 3 units of glycerol kinase, 8 units of sn-glycerol 3-phosphate dehydrogenase, and 3 units of diaphorase. The reaction mixture was preincubated at 37 °C for 5 minutes. After the addition of 10 µl of sample, the reaction solution was mixed for 5 seconds using a Vortex mixer. The reaction tubes were incubated for 10 minutes at 37 °C, and was terminated with 1 ml of 100 mM HCl. The formation of the formazan was measured at 525 nm. The method followed Beer's law to at least 8 mg/ml of triacylglycerol. The reagent blank consisted of replacement of the sample by 10 µl of 50 mg/ml of the bovine serum albumin. The principle of the assay was that the hydrolysis of triacylglycerol proceeded quantitatively to glycerol and free fatty acids, the phosphorylation of glycerol occurred, and glycerol 3-phosphate was converted to dihydroxyacetone phosphate. The reduced nicotinamide adenine dinucleotide (NADH) produced in the reaction then reduced p-iodonitrotetrazolium violet to iodonitrotetrazolium formazan. The assay was validated by reproducing the results of 12 samples, using a method which was specific for triacylglycerol, and was available in a kit form from Boehringer Mannheim, Indianapolis, IN (247).

To verify the adequacy of the solubilization method, liver samples from 5 of the most fatty livers

were homogenized and extracted in nonane as before. After evaporization under nitrogen, the dried pellets, triolein standards, and nonane-blank were hydrolyzed in 1 ml of 0.5 N ethanolic KOH, at 70^{o} C for 30 minutes in stoppered tubes (248). After cooling, the contents of the tubes were neutralized to pH 7 with 2.5 N perchloric acid, and the potassium perchlorate precipitate was removed by centrifugation at 1700 x g for 10 minutes. The supernatant fluid was used for assay of glycerol by the same enzymatic method. The results indicated that triacylglycerol can be solubilized in dilute solutions of bovine serum albumin for triacylglycerol assay. The nonane extraction method combined with the enzymatic hydrolysis of triacylglycerol appears to be specific for triacylglycerol.

sn-Glycerol 3-Phosphate Dehydrogenase

The supernatant obtained during the preparation of the total particulate suspension was used to determine sn-glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) activity by a modification of the method of Lee and Craine (217). The reaction mixture contained 0.2 M glycine, 1 M hydrazine, 2 mM $MgCl_2$, 25 mM sn-glycerol 3-phosphate and 1 mM nicotinamide adenine dinucleotide (NAD^+) at pH 9.8 in 2.2 ml. The dihydroxyacetone phosphate produced by the reaction was trapped as the hydrazone, and the amount of NADH formed was determined at 366 nm.

Protein concentrations were measured by the method of Lowry et al. (233), using bovine serum albumin as the standard. Enzyme specific activities are expressed as either picomoles or nanomoles of product formed per minute per milligram protein.

Acylation of 3,4-Dihydroxybutyl-1-Phosphonate

In order to test the hypothesis that (S)-3,4-dihydroxybutyl-1-phosphonate is a substrate for acylation reactions involving mitochondrial and microsomal enzyme, the lithium salt of (RS)-3,4-dihydroxy-[3-^{3}H]-butyl-1-phosphonate was prepared. The diethyl ester of (RS)-3,4-dihydroxy-[3-^{3}H]-butyl-1-phosphonate, 220 Ci/mol, was transferred to a thick-walled vial, and was evaporated with a gentle stream of nitrogen. One ml of 1 N LiOH was added to the vial. The vial was capped and heated in an autoclave for 7 hours at 121^{o} C. The contents were allowed to cool to room temperature, and were cloudy with a white precipitate. The sample was slightly acidified by adding washed Dowex 50 (H^{+}), and the resin suspension was filtered on a Whatman #2 filter. The resin bed was washed twice with 2 ml water, and the filtrate was collected.

(RS)-3,4-Dihydroxy-[3-^{3}H]-butyl-1-phosphonate was purified by elution from a DEAE cellulose column. A column was prepared by suspending 7.2 g of DEAE cellulose in 100 ml of 0.5 mM ammonium bicarbonate at

pH 8.5. The suspension was shaken, the heavier particles were allowed to settle to the bottom of the cylinder and the lighter fine particles were decanted into a waste reservoir. Another 100 ml of buffer were added, and the process was repeated 10 times to remove most of the fines. A 1.0 x 15 cm column was poured and allowed to pack by gravity. The sample was applied to the surface of the DEAE cellulose column. Successive 25 ml volumes of 0.5, 2, 25, 50, 75, and 100 mM ammonium bicarconate, pH 8.5, were percolated through the column to elute the purified tritiated 3,4-dihydroxybutyl-1-phosphonate salt. Ten µl of each elutate were then counted in Biofluor. Nearly 93 % of the radioactivity was eluted in the 25 and 50 mM fractions. The two fractions were pooled, and Dowex-50 (H^+) was added to adjust the pH to 5.0. The contents of the tube were placed on a filter and the filtrate was collected in a tube. The resin was washed twice with water and the filtrates were combined. The tube was heated at 45 ^{o}C for one hour to remove carbon dioxide. The volume was reduced under a gentle stream of nitrogen at 40 ^{o}C, and the solution was neutralized with 1 N NaOH. Enough 80 mM (RS)-3,4-dihydroxybutyl-1-phosphonate, unlabeled, was added to the preparation to result in a 8 mM (RS)-3,4-dihydroxy-[3-^{3}H]-butyl-1-phosphonate solution, 29.7 Ci/mol.

The purity of the (RS)-3,4-dihydroxy-[3-^{3}H]-butyl-1-phosphonate solution was determined by paper

chromatography. Ten µl of the radioactive preparation and 10 µl of 80 mM of a nonradioactive 3,4-dihydroxybutyl-1-phosphonate solution were spotted on alternative areas on Whatman 3M chromatography paper, 8 x 8 inches. After allowing the spots to dry, the paper was placed in a glass chromatography tank which contained 200 ml of methanol:water:formic acid, 91 % (80:16:14, v/v/v). Care was taken that the solution was approximately 1 cm below the spots. In about 2 hours the solvent front was 2 to 3 cm from the top. The paper was removed and allowed to dry. The lanes were cut out, and the paper strips containing nonradioactive samples were stained to visualize the phosphonate (249). A solution containing 1 g/l of ferric chloride in 80 % ethanol was sprayed on the paper. After the paper dried, a solution containing 10 g/l of sulfosalicyclic acid in 80 % ethanol was sprayed to visualize the phosphonate. 3,4-Dihydroxybutyl-1-phosphonate migrated with an R_f of 0.75 and appeared as a white spot on a reddish background. The radioactive strips were marked at one end with a ^{14}C reference before the strips were counted in a Packard Radiochromatogram scanner. The strip contained only one area of tritium which matched the R_f of the 3,4-dihydroxybutyl-1-phosphonate standard.

The reaction kinetics of the acylation of 3,4-dihydroxybutyl-1-phosphonate were studied. The assay

ingredients in 0.2 ml were: 75 mM Tris-HCl, pH 7.5; 2 mM magnesium chloride; 4 mM NaF; 400 mg bovine serum albumin; 80 µM palmitoyl CoA; and (RS)-3,4-dihydroxy-[3-^{3}H]-butyl-1-phosphonate, 29.7 Ci/mol, at concentrations of 0.125, 0.25, 0.5, 1.0, 2.0, and 4.0 mM. In addition mitochondrial reaction mixtures contained 1 mM KCN, and microsomal assays contained 1 mM dithiothreitol. The reaction was initiated with 40 µg of either mitochondrial or microsomal protein which was diluted with 0.25 M sucrose - 1 mM Tris-HCl buffer, pH 7.4. The reaction proceeded for 4 minutes at 25^{o} C, while being constantly agitated, and was terminated by the previously described method of Coleman and Bell (20,21). Palmitoyl CoA was omitted in the blank tubes. The assay was linear for at least 10 minutes. The Vmax and the apparent Km were calculated as previously described for sn-glycerol 3-phosphate acyltransferase reactions.

The products of the acylation reaction with 3,4-dihydroxybutyl-1-phosphonate were determined. The incubation mixture contained 75 mM Tris-HCl, pH 7.4; 2 mM magnesium chloride; 4 mM NaF; 2 mg bovine serum albumin; 80 µM palmitoyl CoA; 8 mM (RS)-[3^{3}H]-3,4-dihydroxybutyl-1-phosphonate, 29.7 Ci/mol; and 200 ug of either mitochondrial or microsomal protein in a final volume of 1 ml. In addition, mitochondrial reaction mixtures contained 1 mM KCN and microsomal assays contained 1 mM dithiothreitol. At 5, 10, 15 and

20 minutes 0.2 ml of the reaction mixture was removed and pipetted to tubes containing 3 ml chloroform:methanol (1:2,v/v) and 0.6 ml 1 % perchloric acid. The acylation products were extracted as previously described for sn-glycerol 3-phosphate acyltransferase reactions. The chloroform layers were evaporated by using a gentle stream of nitrogen. Then 50 µl of chloroform:methanol (4:1,v/v) was added to solubilize the acylation products of 3,4-dihydroxybutyl-1-phosphonate. The standards, 4-palmitoyl-sn-3-hydroxybutyl-1-phosphonate and 3,4-dipalmitoyl-sn-butyl-1-phosphonate were also solubilized in the chloroform-methanol mixture at a concentration of 20 mg/ml. The extracts were applied to 20 x 20 cm channeled silica gel (8 nm) thin-layer plates (LK5DF, 250 um thick, Whatman Inc., Clifton, NJ), and were developed in chloroform:formic acid, 91 %:ethanol:water (180:16:20:1,v/v/v). When the solvent front was 3 cm from the top of the plate, the plate was removed and dried. The acylated derivatives of 3,4-dihydroxybutyl-1-phosphonate were located by exposure to iodine. In this solvent system the radioactivity separated into 2 spots, which were located at the position of the standards. The R_f of monoacyl and diacyl products were 0.29 and 0.69, respectively. The plate was scored in 1 cm segments from the origin to the solvent front. The silica gel was scraped into

liquid scintillation vials, and 0.4 ml of 10 % acetic acid in ethanol was added. Vials were capped and kept overnight. Then 6 ml of Aquasol was added and mixed well. Samples were counted for tritium at an effiency of 39 percent.

Incorporation of 3,4-Dihydroxybutyl-1-Phosphonate into Tissue

In another experiment the liver, kidneys, small bowel and abdominal adipose tissue were excised, and placed on a block of ice. The small bowel was slit longitudinally, the luminal contents were gently removed by washing in a bath of isotonic saline at 2 ^{o}C, and the mucosa was removed by scraping with a glass slide. The other tissues were finely minced, 4 - 8 mm^3, with a single edge razor blade on a cold glass plate which was on a block of ice. Tissue minces were rinsed in a cold isotonic saline bath to remove blood and then were blotted dry. To three tubes for each tissue, 100 mg of small bowel mucosa, or 250 mg of liver, kidney or fat were added to 3 ml of Eagle's Minimum Essential Media which contained 10 % fetal calf serum, 250 units of streptomycin, and 7.5 nmoles of (RS)-3,4-dihydroxy-[3- ^{3}H]-butyl-1-phosphonate, 401 Ci/mol. All tubes were then vortexed for 5 to 10 seconds. Tubes were incubated at 38 ^{o}C for either 0, 90, or 180 minutes. The uptake of the sn-glycerol 3-phosphate analogue was stopped by the addition of 3 ml of 2 % perchloric acid in 0.9 % saline. The tubes were

mixed and centrifuged at 1,800 x g for 10 min at 20° C. The supernatant was decanted. The pellet was gently resuspended in 3 ml 1 % perchloric acid in saline and was again centrifuged. The pellet was washed two additional times in the 1 % perchloric acid-saline solution before being homogenized in a mortar with a pestle using approximately 100 mg of 0.45 mm diameter glass beads as an abrasive surface. Five ml of methanol were added to the mortar and the contents were poured into a 50 ml glass centrifuge tube. The remaining particles were then washed into the tube with 5 ml water. The bowl of the mortar was washed twice with 5 ml of chloroform which was added to the tubes. The tubes were vortexed, corked and allowed to remain undisturbed overnight at room temperature. The tubes were centrifuged at 800 x g for 5 minutes, and the polar layer was removed. The chloroform layer was transferred to a 50 ml glass tube. The pellet was resuspended in 5 ml of chloroform, and centrifuged at 800 x g for 5 minutes. The chloroform was decanted and pooled with the previous chloroform layer. The combined chloroform layers were washed 3 times with 5 ml water. The tubes were vortexed and centrifuged as before. The water layer was aspirated and discarded. The chloroform layer was decanted into a 20 ml liquid scintillation vial, and evaporated under a gentle stream of nitrogen. Fifteen ml of Biofluor was added

to the vial, and the amount of tritium that was incorporated in the lipid fraction was determined.

In Vivo Effects of 3,4-Dihydroxybutyl-1-Phosphonate

The effect of 3,4-dihydroxybutyl-1-phosphonate administration on rats during liver regeneration was evaluated at 20 and 24 hours. The sodium salt of (RS)3,4-dihydroxybutyl-1-phosphonate was prepared for injection. Approximately 1.5 g Dowex 50 (H^+) was suspended in 60 ml 1 N NaOH and was swirled several times before pouring onto a Whatman #2 filter. The resin bed was washed with 60 ml water, then with 60 ml 1 N HCl, and finally with water until the pH is 6 to 7. A slurry of the washed resin was mixed with 150 mg of 3,4-dihydroxybutyl-1-phosphonate, lithium salt, and mixed for 5 minutes. The mixture was filtered, and resin was washed twice with water. The filtrate was adjusted to a pH of 6-7 with 1 N NaOH. The concentration of the sodium salt of 3,4-dihydroxybutyl-1-phosphonate was determined by the method of Michal and Lang (237).

In the first study, 2 µmoles/100 g body weight of either NaCl or 3,4-dihydroxybutyl-1-phosphonate were administered intraperitoneally to rats 2 hours before and 6 and 14 hours after partial hepatectomy. The rats were sacrificed at 20 hours. The livers were excised, rinsed in 9 g/l of saline at 0 °C, blotted dry, and weighed. The caudate lobe was placed in 5 % formaldehyde in 50 mM sodium phosphate, pH 7.0, to fix

the tissue for histological sectioning (10 to 15 µm thickness) and staining with iron hematoxylin, a basic stain, and eosin, an acid stain. The number of cells in mitosis (the mitotic index) was determined in 1000 cells, and the degree of fat was graded from 0, when no fat vacuoles were present, to 4+, when fat vacuoles filled the cytoplasm. The right lateral lobe of the liver was trimmed of fascia and large blood vessels before the parenchyma was minced. The protein content was measured in 50 mg samples by the method of Lowry et al. (233). Triacylglycerols were determined by the method of Megraw et al. using 150 mg of tissue (247).

In the second study, 2 µmoles/100 g body weight of either NaCl, sn-glycerol 3-phosphate, or 3,4-dihydroxybutyl-1-phosphonate were given by intraperitoneal injection to rats 2 hours before, and 6 and 14 hours after partial hepatectomy. One hour before sacrifice, 25 µCi/ 100 g body weight of tritiated thymidine was also administered by intraperitoneal injection to measure DNA synthesis. At 24 hours the rats were sacrificed, and the caudate lobe was used for histology. The remaining lobe was again minced. In this study, 250 mg of tissue was extracted to measure DNA, [^{3}H]-DNA, RNA, triacylglycerol, phospholipid and protein, using the method of Munro and Fleck (250). A 1:20 (w/v) homogenate was prepared in 5 ml ice-cold water by placing the suspension in a Polytron

homogenizer at a high setting for 10 seconds. A small aliquot was saved for protein assay by the method of Lowry et al. (233). The homogenate was then treated with 2.5 ml of ice-cold 0.6 N perchloric acid, mixed, allowed to stand undisturbed for 10 minutes at 0° C, and centrifuged at 1,500 x g for 10 minutes. The supernatant was decanted into a tube containing 5 ml chloroform:methanol (2:1,v/v) to extract free lipids (251). The pellet was extracted for phospholipids using successive treatments of cold absolute ethanol, ethanol:chloroform (3:1,v/v), ethanol:ether (3:1,v/v), and ether according to the method of Hutchison et al. (252). Extracts including the chloroform layer were combined and evaporated under a gentle stream of nitrogen at 40° C. The residue was solubilized in 2 ml of chloroform:methanol (2:1,v/v) for triacylglycerol and phospholipid assay. The pellet was then washed twice with 5 ml of cold 0.2 N perchloric acid. Then 4 ml of 0.3 N KOH was added to the precipitate, mixed and incubated at 37° C for 1 hour. After cooling the tubes in an ice bath, 2.5 ml of cold 1.2 N perchloric acid was added, and the mixture was allowed to stand undisturbed for 10 minutes to precipitate protein and DNA. Tubes were centrifuged at 3000 x g for 15 minutes, and the RNA supernatant fraction was decanted and saved. The pellet was washed twice with 5 ml of 0.2 N perchloric acid. The supernatants were combined with the RNA fraction, which was then diluted to 100 ml

with water and additional 0.6 N perchloric acid to result in a RNA solution in 0.1 N perchloric acid. The precipitate was solubilized in 5 ml of 0.3 N KOH by incubating at 37 ^{o}C for 5 minutes. The mixture was diluted to 50 ml with water and additional KOH to result in a DNA solution in 0.1 N KOH.

The DNA content was determined by the method of Ceriotti (253). The reaction mixture contained 2 ml of the DNA solution, 1 ml of 0.04 % indole and 1 ml of 3 N HCl. Tubes were incubated at 100 ^{o}C for 15 minutes. After cooling to room temperature, 4 ml of chloroform was added and mixed. The chromagen in the aqueous layer was measured at 490 nm. The amount of tritiated thymidine incorporated in DNA was determined by mixing 1 ml of the DNA solution with 6 ml of Aquasol in a liquid scintillation vial and counting for tritium decay. The effiency was 31 % using tritiated water as the internal standard.

RNA concentrations were determined in the RNA solution according to the method of Munro and Fleck (250). The ultraviolet absorption of RNA at 260 nm has an extinction of 1.000 for 32 µg/ml.

Triacylglycerols were extracted by the nonane-sulfuric acid method, and determined by the method of Megraw et al. (247). The phospholipid content was measured by the method of Rosenthal and Han (254). A sulfuric-periodic acid reagent was prepared daily by

mixing 10 N sulfuric acid:0.1 N periodic acid:95 % ethanol (3:1:6,v/v/v). A solution of 2.5 g/l aminonaphtholsulfonic acid, 75 g/l sodium bisulfite and 5 g/l sodium sulfite was prepared. A 2.5 g/dl ammonium molybdate solution was also prepared. To 0.25 ml of the chloroform-methanol extract 1 ml of the sulfuric-periodic acid reagent was added, and the mixture was incubated at 100 ^{o}C for 45 minutes. After 4.8 ml of a 5:3 mixture of the aminonaphtholsulfonic acid reagent and the ammonium molybdate reagent were added to the reaction tubes, the incubation proceeded for another 10 minutes. The tubes were cooled to room temperature, and 2 ml of chloroform were added to the tubes. The upper, blue aqueous layer was read at 660 nm. A potassium phosphate solution was used as the standard.

Uptake of 3,4-Dihydroxybutyl-1-Phosphonate by Tissue Culture Cells

Mouse 3T3 fibroblasts were transferred to new tissue culture dishes containing sterile Medium 199 and 10% fetal calf serum, and were incubated overnight at 38 ^{o}C. Human Hep-G2 hepatoma cells were also transferred to new tissue culture dishes containing sterile Medium 199 and 10% fetal calf serum, but were incubated 6 days at 38 ^{o}C. There were approximately 20,000 cells per dish. On the day of the study, the medium was removed, and 0.5 ml of sterile Medium 199 with 10% fetal calf serum and 160 nmol (RS)-3,4-dihydroxy-[^{3}H]-butyl-1-phosphonate, 50 Ci/mol, was

added to each dish. The cells were incubated for 0, 1.5, 3 and 5 hours at 38 °C. The incubation was terminated by aspirating the medium, and washing the cells 3 times with 0.5 ml Medium 199. In order to lysis the cells, 1 ml of n-butanol was added to the dish. After 10 minutes, the butanol was removed and added to a tube containing 1 ml of water. The tube was vortexed for 5 seconds and centrifuged at 500 x g for 10 minutes to separate the phases. The butanol layer, which contains the cellular lipids, was washed one additional time with water. The butanol was added to a liquid scintillation vial, containing 12 ml of liquid scintillation fluid (Liquidscint). Vials were counted for 10 minutes to determine the amount of tritium incorporation. A standard of 3,4-dihydroxy-[^{3}H]-butyl-1-phosphonate in butanol was used to determine the efficiency of tritium decay. The amount of the phosphonate incorporated into lipids over 5 hours was determined.

RESULTS

Characterization of the Mitochondrial Subcellular Fraction

The mitochondrial and microsomal glycerol 3-phosphate acyltransferases were partially purified by differential sedimentation. In order to determine the degree of purification of the mitochondrial and microsomal fractions relative to other organelles, several enzymes unique to the mitochondrial membranes and matrix, the peroxisomal matrix and cores, and the endoplasmic reticulum cytoplasmic and luminal surfaces were determined. The percentages of the total homogenate activity and the relative specific activities of the mitochondrial fraction are shown in Table I. The relative specific activities of enzymes of mitochondria were significantly greater than the enzyme activities of either microsomes or peroxisomes. Mitochondrial membrane enzyme activities were similar to mitochondrial matrix enzyme activity. Total catalase activity was similar to urate oxidase activity. Glucose 6-phosphatase activity was slightly greater than NADPH-dependent cytochrome c reductase activity. Calculated from the relative distribution of the marker enzymes, the purity of the mitochondrial preparation was approximately 89 percent when the activities of succinate cytochrome c reductase, monoamine oxidase, or glutamate dehydrogenase were

compared to the microsomal marker, NADPH-dependent cytochrome c reductase. When the contamination of peroxisomal matrix enzyme was included, the purity was calculated to be about 81 percent.

Characterization of the Microsomal Subcellular Fraction

The percentages of the total homogenate activity and the relative specific activities of the microsomal fraction are shown in Table II. The relative specific activities of enzymes of the endoplasmic reticulum were significantly greater than the activities of peroxisomal or mitochondrial enzymes. Comparing the relative specific activities of the microsomal enzymes, the NADPH-dependent cytochrome c reductase activity was greater than the activity of glucose 6-phosphatase. The relative specific activity of sedimentable catalase was greater than the activity of urate oxidase. Monoamine oxidase activity was greater than the activity of succinate cytochrome c reductase.

Calculated from the distribution of the organelle markers, the microsomal preparation was approximately 94 percent pure compared to the mitochondrial marker enzymes. With the addition of the contamination by peroxisomal matrix enzyme, catalase, the purity was approximately 89 percent.

Characterization of the Peroxisomal Subcellular Fraction

The percentages of the total homogenate activity and the relative specific activities of the

peroxisomal fraction are shown in Table III. The relative specific activities of the marker enzymes of the peroxisomes were greater than the activities of the enzymes representative of the mitochondria and endoplasmic reticulum. The relative specific activities of urate oxidase and sedmentable catalase were similar. From the relative specific activities, this subcellular fraction appears to be more contaminated by microsomal enzymes than by mitochondrial enzymes. Calculated from the distribution of these organelle markers, the peroxisomal fraction was was approximately 99 percent pure when compared to the mitochondrial enzymes. The peroxisomal preparation was, however, only 88 percent pure when the contamination of the enzymes from the endoplasmic reticulum and mitochondria were included.

N-Ethylmaleimide Inhibition

Enzyme preparations were also preincubated with 1 mM N-ethylmaleimide to inhibit the microsomal reaction. Mitochondrial acyltransferase reactions were inhibited by only 9 percent, indicating that the mitochondrial preparation contained only a small amount of microsomal enzyme. These results agreed with the calculated purity of the fraction determined with marker enzymes. The acyltransferase activity in microsomal preparations was almost completely prevented by N-ethylmaleimide pretreatment, which decreased the activity by more than 99 percent. Since the remaining activity was less than

1 percent of the inhibitor-free activity, the microsomal preparation appeared to be nearly free of mitochondrial enzyme.

Reaction Kinetics of Mitochondrial sn-Glycerol 3-Phosphate Acyltransferase and Inhibition by Isosteric Analogues of sn-Glycerol 3-Phosphate

Mitochondrial glycerol 3-phosphate acyltransferase activity was inhibited by 2 mM (RS)-3,4-dihydroxybutyl-1-phosphonate, 2.5 mM (RS)-glyceraldehyde 3-phosphate, and 2.5 mM (RS)-3-hydroxy-4-oxobutyl-1-phosphonate. The reaction rates of mitochondrial sn-glycerol 3-phosphate acyltransferase at various concentrations, 33 to 400 uM, of sn-glycerol 3-phosphate were plotted by the method of Linweaver-Burk (Figure 2). The mean and standard deviation of the apparent Km was 0.59 $\pm$ 0.01 mM, and the Vmax was 4.30 $\pm$ 0.20 nmol/min/mg protein. The Kp for (RS)-3,4-dihydroxybutyl-1-phosphonate was 0.97 $\pm$ 0.02 mM, and the Vmax was 4.40 $\pm$ 0.29 nmol/min/mg protein. The kinetics of the (RS)-3,4-dihydroxybutyl-1-phosphonate inhibition resembled a competitive inhibition (Figure 2), and the Ki was 3.18 mM. The Kp for (RS)-glyceraldehyde 3-phosphate was 0.93 $\pm$ 0.02 mM, and the Vmax was 4.25 $\pm$ 0.27 nmol/min/mg protein (Figure 3). (RS)-Glyceraldehyde 3-phosphate inhibition of the enzyme was also competitive, and the Ki was 4.44 mM. (R)-Glyceraldehyde 3-phosphate at 10 mM showed no

inhibition. The inhibition of the acyltransferase reaction by (RS)-3-hydroxy-4-oxobutyl-1-phosphonate resembled a competitive inhibition, and had a Kp of 0.93 ± 0.01 mM and a Vmax of 4.45 ± 0.20 nmol/min/mg protein (Figure 4). The Ki was 4.42 mM, which was similar to the Ki value of (RS)-glyceraldehyde 3-phosphate.

Inhibition of Mitochondrial Acyltransferase by Nonisosteric Analogues of sn-Glycerol 3-Phosphate

The inhibition of the acyltransferase reaction by the non-isosteric analogues of sn-glycerol 3-phosphate also resembled the competitive type. With 2.5 mM (1S,3S)-1,3,4-trihydroxybutyl-1-phosphonate, the Kp was 0.87 ± 0.05 mM, and the Vmax was 4.46 ± 0.58 nmol/min/mg protein (Figure 5). The Ki was 5.27 mM. The reaction with 2.5 mM (1R,3S)-1,3,4-trihydroxybutyl-1-phosphonate had a Kp of 0.89 ± 0.02 mM, and a Vmax of 4.38 ± 0.38 nmol/min/mg protein (Figure 6). The Ki was 4.92 mM, and was similar to that of the diastereoisomer. The Ki values of these stereoisomers were greater than the Ki value of (RS)-3,4-dihydroxybutyl-1-phosphonate.

Inhibition of Mitochondrial Acyltransferase by an Isosteric Analogue of Dihydroxyacetone Phosphate and Phenethyl Alcohol

The isosteric analogue of dihydroxyacetone phosphate, 4-hydroxy-3-oxobutyl-1-phosphonate, had a Kp of 0.95 ± 0.01 mM and a Vmax of 4.26 ± 0.19 nmol/min/mg

protein at 18.8 mM (Figure 7). A competitive inhibition was suggested by the double reciprocal plot of the kinetic data, and Ki was 31.64 mM, which was greater than the Ki values of the sn-glycerol 3-phosphate analogues. Although the inhibition of the acyltransferase by 4 mM phenethyl alcohol appeared to be noncompetitive (Figure 8), additional studies are needed to confirm the type of inhibition. The Kp was 0.60 ± 0.1 mM and the Vmax was 2.97 ± 0.15 nmol/min/mg protein. The Ki was 8.93 mM.

Reaction Kinetics of Microsomal sn-Glycerol 3-Phosphate Acyltransferase and Inhibition by Isosteric Analogues of sn-Glycerol 3-Phosphate

The microsomal sn-glycerol 3-phosphate acyltransferase activity was inhibited by 2 mM (RS)-3,4-dihydroxybutyl-1-phosphonate, 2.5 mM (RS)-glyceraldehyde 3-phosphate, and 2.5 mM (RS)-3-hydroxy-4-oxobutyl-1-phosphonate. Microsomal glycerol 3-phosphate acyltransferase reactions were performed using 33 to 400 μM of sn-glycerol 3-phosphate. The double reciprocal plot of the data gave an apparent Km of 0.29 ± 0.02 mM and was less than that of the mitochondrial enzyme (Figure 9). The Vmax was 5.70 ± 0.65 nmol/min/mg protein. The Kp for (RS)-3,4-dihydroxybutyl-1-phosphonate was 0.62 ± 0.02 mM, and the Vmax was 6.03 ± 0.61 nmol/min/mg protein. The Ki was 1.76 mM, and the kinetics of the inhibition

suggested a competitive inhibition of the acyltransferase (Figure 9). The inhibition by (RS)-glyceraldehyde 3-phosphate also resembled a competitive inhibition (Figure 10). The Kp was 0.46 ± 0.03 mM, and the Vmax was 5.87 ± 0.66 nmol/min/mg protein; the Ki was 4.16 mM, and was similar to the Ki obtained with the mitochondrial preparation. (R)-Glyceraldehyde 3-phosphate, however, did not inhibit the reaction at 10 mM. The kinetics of the (RS)-3-hydroxy-4-oxobutyl-1-phosphonate inhibition suggested a competitive inhibition (Figure 11). The Kp was 0.44 ± 0.03 mM, and the Vmax was 6.24 ± 0.38 nmol/min/mg protein. The Ki was 4.88 mM. The Ki values for (RS)-glyceraldehyde 3-phosphate, and (RS)-3-hydroxy-4-oxobutyl 1-phosphonate were similar for microsomes and mitochondria.

Inhibition of Microsomal Acyltransferase by Nonisosteric Analogues of sn-Glycerol 3-Phosphate

Microsomal glycerol 3-phosphate acyltransferase activity was competitively inhibited by 2.5 mM (1S,3S)-1,3,4-trihydroxybutyl-1-phosphonate (Figure 12). The Kp was 0.89 ± 0.18 mM, and the Vmax was 5.80 ± 0.85 nmol/min/mg protein. The Ki was 1.21 mM which was less than the Ki value obtained with mitochondria. The inhibition of the acyltransferase activity by 2.5 mM (1R,3S)-1,3,4-trihydroxybutyl-1-phosphonate also suggested a competitive inhibition (Figure 13). The Kp was 0.91 ± 0.05 mM, and the Vmax was 5.96 ± 0.30 nmol/min/mg protein. The Ki was 1.17 mM, which was

similar to the Ki value of the diastereoisomer, and was also less than the Ki value for the mitochondrial inhibition.

Inhibition of Microsomal Acyltransferase by an Isosteric Analogue of Dihydroxyacetone Phosphate and Phenethyl Alcohol

The kinetics of the inhibition by 8 mM 4-hydroxy-3-oxobutyl-1-phosphonate, the dihydroxyacetone phosphate analogue, gave a competitive inhibition of the acyltransferase activity (Figure 14). The Kp was 0.70 $\pm$ 0.07 mM, and the Vmax was 5.77 $\pm$ 0.73 nmol/min/mg protein. The Ki was 5.50 mM, which was greater than the Ki values of the sn-glycerol 3-phosphate analogues, but less than the Ki value for the inhibitor obtained with mitochondria. When 4 mM phenethyl alcohol was used, a non-competitive inhibition was observed (Figure 15). The Kp was 0.24 $\pm$ 0.03 mM, and the Vmax was 3.56 $\pm$ 0.65 nmol/min/mg protein. The Ki was 7.35 mM, and was slightly less than the Ki value obtained with mitochondria.

Acylation Products of 3,4-Dihydroxybutyl-1-Phosphonate

The acylation derivatives of 3,4-dihydroxybutyl-1-phosphonate by the reaction of mitochondrial and microsomal acyltransferases with palmitoyl CoA were identified. The mitochondrial reaction from 5 to 20 minutes generated almost exclusively the monoacyl product, which was 97 percent

of the total 3,4-dihydroxybutyl-1-phosphonate incorporation (Figure 16). In contrast, the microsomal reaction resulted in a gradual change in the proportion of monoacyl to diacyl products over 20 minutes. The proportion of 3,4-dipalmitoyl-sn-butyl-1-phosphonate, compared to the total acylation product, increased from 3 percent at 5 minutes to 13 percent at 20 minutes.

Kinetics of the (S)-3,4-Dihydroxybutyl-1-Phosphonate Acylation Reaction

Mitochondrial and microsomal (S)-3,4-dihydroxybutyl-1-phosphonate acylation reactions were performed. The apparent Km and Vmax were determined from the Lineweaver-Burk plots of the data. The apparent Km for the mitochondrial enzyme is 2.50 $\pm$ 0.12 mM, and the Vmax was 2.21 nmol/min/mg protein (Figure 17). The microsomal enzyme had an apparent Km of 1.38 $\pm$ 0.27 mM, and a Vmax of 4.58 $\pm$ 0.21 nmol/min/mg protein (Figure 18).

Incorporation of 3,4-Dihydroxybutyl-1-Phosphonate into Tissue

When a tissue preparation of either liver, fat or small bowel was incubated with Eagle's minimum essential media in the presence of tritiated (RS)-3,4-dihydroxybutyl-1-phosphonate, increasing amounts of the radioactivity appeared in the chloroform-extractable fraction during the 3 hour incubation (Table IV). The rates of 3,4-dihydroxybutyl-1-phosphonate incorporation in the liver, 0.96 pmol/h/g tissue, and adipose tissue,

0.95 pmol/h/g tissue, were nearly linear over 3 hours. Enterocytes also had an increase in the amount of the phosphonate which incorporated over time, but the rate, 0.78 pmol/h/g tissue, was more variable. Compared to the zero time, the kidney preparation did not incorporate significant amounts of tritiated 3,4-dihydroxybutyl-1-phosphonate into chloroform soluble material.

Acyltransferase Contribution to Lipid Accumulation

Eighteen hours after partial hepatectomy the triacylglycerol content increased five-fold in the residual liver and at 24 hours there was a six-fold increase (Table V). There was no statistical difference between the absolute levels at 18 and 24 hours. sn-Glycerol 3-phosphate dehydrogenase activity was decreased by 24 percent at 18 hours after partial hepatectomy, and by 32 percent at 24 hours.

After partial hepatectomy, microsomal sn-glycerol 3-phosphate acyltransferase activity increased both at 18 and 24 hours (Table VI). The combined microsomal and mitochondrial (total) activity of this enzyme was increased only at 24 hours, but there was no change in the mitochondrial activity. In the sham groups microsomal and mitochondrial acyltransferase activities were similar in magnitude. Following partial hepatectomy the microsomal acyltransferase activity was almost double the mitochondrial activity at 24 hours.

There was a correlation between the tissue triacylglycerol content and the microsomal glycerol 3-phosphate acyltransferase activity; the correlation coefficient (r) was 0.608, and was statistically significant with $p < 0.05$ (Figure 19).

Peroxisomal dihydroxyacetone phosphate acyltransferase activity was increased at 24 hours after partial hepatectomy, as was the combined peroxisomal and microsomal (total) cellular activity (Table VII). Although there was no statistically significant change in the microsomal dihydroxyacetone phosphate acyltransferase activity, the mean value for the enzymatic activity was 18 percent greater at 24 hours after partial hepatectomy, compared to the shams. The peroxisomal activity was approximately 75 percent of the total.

Appearance of the Regenerating Rat Liver After Intraperitoneal Injection of 3,4-Dihydroxybutyl-1-Phosphonate

The effect of 3,4-dihydroxybutyl-1-phosphonate, glycerol 3-phosphate and sodium chloride treatment on the regenerating liver was studied at various times. At sacrifice the gross appearance of the livers after 3,4-dihydroxybutyl-1-phosphonate treatment was usually reddish brown, and occasionally mottled with red and yellow blotches. On the other hand, treatment with either sodium chloride or glycerol 3-phosphate resulted in homogeneous yellow livers. Livers from the sham-

operated rats were shiny and bright red, and were similar to those from unoperated rats. Untreated rats with partial hepatectomies had livers which appeared dull and were very yellow.

Histological Studies on the Regenerating Rat Liver

The effect of sodium chloride, glycerol 3-phosphate, or 3,4-dihydroxybutyl-1-phosphonate treatments on partial hepatectomy-induced accumulation of lipids and mitosis was studied in histologically stained liver sections, and the results were summarized in Table VIII. Hepatocytes from sham-operated rats had little cytoplasmic fat, and were arranged in cords of cells radiating from the central vein (Figure 20). The sinusoids were clearly visible and contained erythrocytes. Mitosis occurred in only 0.1 percent of hepatocytes. After 18 to 24 hours from partial hepatectomy, intracellular lipids coalesced into large vacuoles, which almost completely filled the cytoplasm of the hepatocytes (Figure 21). These cells were hypertrophic, and displaced the sinusoids. The number of cells which were in mitosis increased gradually from 0 to 24 hours, and occurred in approximately 1.4 percent of the hepatocytes at 20 and 24 hours (Table VIII). Sodium chloride administration decreased the cytoplasmic fat which occurred in the regenerating liver at 20 and 24 hours, compared to livers from rats which had received no injections (Table VIII). Mitosis

was slightly decreased, and occurred in about 1.2 percent of hepatocytes. The photomicrograph showed that the lipid was dispersed in small vacuoles within the cytoplasm, and that the hepatocytes were hypertrophic, displacing the sinusoids (Figure 22). Intraperitoneal injection of 3,4-dihydroxybutyl-1-phosphonate significantly reduced lipid vacuolation to levels approaching those of the sham-operated rats, and the number of hepatocytes in mitosis was 0.12 percent (Table VIII). The photomicrograph showed little cytoplasmic lipid, cells of normal size, and visible sinusoids (Figure 23). sn-Glycerol 3-phosphate administration also decreased the content of cytoplasmic fat, but only to the same degree as sodium chloride treatment (Table VIII). The number of hepatocytes in mitosis was 0.6 percent, which was less than that with sodium chloride treatment, but more than that in sham-operated animals. The cytoplasm was dispersed with small lipid vacuoles, hepatocytes were hypertrophic, and the sinusoids were displaced (Figure 24).

Effect of 3,4-Dihydroxybutyl-1-Phosphonate on the 20 Hour Regenerating Rat Liver

At 20 hours following partial hepatectomy, the hepatic content of triacylglycerol and protein was determined from homogenates (Table IX). Treatment with 3,4-dihydroxybutyl-1-phosphonate caused a significant decrease in the triacylglycerol level, when compared to

the group receiving sodium chloride. The amount of the lipid present was similar to the triacylglycerol content of sham-operated rats (Table V). Unexpectedly, the lipid level in the sodium chloride treated group was half of that found in untreated rats at 18 and 24 hours after partial hepatectomy.

Effect of 3,4-Dihydroxybutyl-1-Phosphonate on the 24 Hour Regenerating Rat Liver

The content of DNA, RNA, triacylglycerol, phospholipid and protein, and DNA synthesis was determined in the regenerating liver at 24 hours (Table X). Although there was no change in the DNA content in the groups, 3,4-dihydroxybutyl-1-phosphonate treatment caused a 42 percent increase in DNA synthesis, and a 44 percent decrease in triacylglycerol content. No change in RNA, phospholipid and protein content occurred with the treatments. The hepatic triacylglycerol content of 3,4-dihydroxybutyl-1-phosphonate injected rats gradually increased from 20 to 24 hours, whereas the levels were unchanged in saline-treated rats.

3,4-Dihydroxybutyl-1-Phosphonate Incorporation into Lipids of Tissue Culture Cells

A linear incorporation of tritiated (S)-3,4-dihydroxybutyl-1-phosphonate into the lipid fraction of mouse 3T3 fibroblasts occurred from 1.5 to 5 hours (Table XI). The rate of incorporation of the phosphonate was approximately 4.1 pmoles/hour/million

cells. Although the incorporation of the phosphonate into lipids of human Hep-G2 hepatoma cells occurred at a rate of 3.2 pmoles/hour/million cells during the initial 90 minutes of the incubation, the rate gradually decreased. At 5 hours, the rate of 3,4-dihydroxybutyl-1-phosphonate incorporation was 1.8 pmoles/hour/million cells.

DISCUSSION

Partial Purification of Subcellular Fractions

The relative specific activities of the marker enzymes in the mitochondrial fraction suggest that the mitochondria are nearly intact organelles. The relative specific activities of glutamate dehydrogenase, succinate cytochrome c reductase and monoamine oxidase are nearly equal. Contamination by microsomal membranes is about 11 percent when calculated from the relative activity of the marker enzymes. Since N-ethylmaleimide inhibits the sn-glycerol 3-phosphate acyltransferase activity in the mitochondrial fraction by only 9 percent, 91 percent of the activity is due to the mitochondrial enzyme.

The relative specific activity of NADPH cytochrome c reductase is greater than that of glucose-6-phosphatase in the microsomal fraction. It is possible that when the endoplasmic reticulum is disrupted, some membrane fragments containing glucose 6-phosphatase activity adhere to the membranes of other organelles, and are centrifuged in other subcellular fractions. A slightly larger portion of the glucose-6-phosphatase activity is in the mitochondrial fraction, compared to NADPH cytochrome c reductase. The purity of the microsomal fraction is calculated to be 94 percent based on the relative activity of the marker enzymes.

Inhibition by N-ethylmaleimide suggest that the fraction is 99 percent pure and contain few mitochondria. It has been suggested that sn-glycerol-3-phosphate acyltransferase activity may be a better microsomal marker than NADPH cytochrome c reductase (20).

The Km of the microsomal and mitochondrial preparations using sn-glycerol 3-phosphate for the substrate are compared in Table XIV. In this study the Km for microsomes was 0.29 mM, and was within the reported range of other investigators. Although fewer kinetic studies had been made on the mitochondrial acyltransferase, the Km obtained in this study, also compared favorably with previously reported values.

(S)-3,4-Dihydroxybutyl-1-Phosphonate

In this study, (RS)-3,4-dihydroxybutyl-1-phosphonate is a competitive inhibitor of mitochondrial and microsomal acyltransferases, and appears to be a substrate for the enzymes. Since (S)-3,4-dihydroxy-butyl- 1-phosphonate is likely to be the biologically active isomer in the rat liver as it is in E. coli (206), the Ki values of the (S)-isomer have been calculated (Tables XI and XII). The Ki values of (S)-glyceraldehyde 3-phosphate are also determined, because (R)-glyceraldehyde 3-phosphate is not an inhibitor of sn-glycerol 3-phosphate acyltransferase. Since the isosteric analogue of (S)-glyceraldehyde 3-phosphate is (S)-3-hydroxy-4-oxobutyl-1-phosphonate, the Ki values

of this analogue are given. Compared to the other inhibitors, the Ki values of (S)-3,4-dihydroxybutyl-1-phosphonate are the smallest for both membrane preparations, and are nearly twice the respective apparent Km values for sn-glycerol 3-phosphate acylation. Kinetic studies of the acylation of (S)-3,4-dihydroxy-[3-^{3}H]-butyl-1-phosphonate indicate that the apparent Km for the mitochondrial enzyme is 3.2 times greater than the apparent Km for sn-glycerol 3-phosphate acylation. The apparent Km for the acylation of (S)-3,4-dihydroxybutyl-1-phosphonate by the microsomal enzyme is 3.8 times the apparent Km for sn-glycerol 3-phosphate acylation. Thus (S)-3,4-dihydroxybutyl-1-phosphonate is not only an inhibitor of sn-glycerol 3-phosphate acyltransferase activity, but appears to be a substrate for the enzyme. It is also a substrate for rabbit muscle (S)-glycerol 3-phosphate dehydrogenase (143).

The acylation products of 3,4-dihydroxybutyl-1-phosphonate have been indentified. Essentially only the monoacyl product is present in the mitochrondrial reaction. The small amount of diacyl product is likely due to microsomal contamination with the subsequent acylation by 1-acylglycerol 3-phosphate acyltransferase. The microsomal reaction shows an initial predominance of the monoacyl product, and in time an increase in the diacyl product occurs. Since

the 1-acylglycerol 3-phosphate acyltransferase activity is primarily found in the microsomal preparation, the data suggest that this enzyme also can use the acylated analogue as a substrate (34). Further studies are indicated to confirm this hypothesis.

Other Isosteric Analogues of Glycerol 3-Phosphate

Since (S)-glyceraldehyde 3-phosphate can exist as a gem diol in solution, it is also an isosteric analogue of sn-glycerol-3-phosphate, and is able to function as a competitive inhibitor. The analogue of (S)-glyceraldehyde 3-phosphate, (S)-3-hydroxy-4-oxobutyl-1-phosphonate, also causes a competitive inhibition which is similar to that of (S)-glyceraldehyde 3-phosphate. The Ki values of both inhibitors are almost identical.

Inhibition by Nonisosteric Analogues of Glycerol 3-Phosphate

The Ki values of the two diastereoisomers of 1,3,4-trihydroxybutyl-1-phosphonate are greater for mitochondria than for microsomes. The addition of a hydroxyl group to carbon 1 of (S)-3,4-dihydroxybutyl-1-phosphonate moderates the inhibition of the microsomal enzyme. Since the steric position of the carbon 1 hydroxyl does not alter the degree of inhibition, the orientation of the hydroxyl group does not seem important in the reaction with the microsomal enzyme. The presence of the hydroxyl group on carbon 1 appears to interfere with the inhibition of the mitochondrial

enzyme, when compared to (S)-3,4-dihydroxybutyl-1-phosphonate.

Other Inhibitors

The largest Ki value is observed with 4-hydroxy-3-oxobutyl-1-phosphonate, an analogue of dihydroxyacetone phosphate. Both mitochondrial and microsomal enzymes are competitively inhibited, and the Ki values are 31.64 and 5.50 mM, respectively. These results suggest that the oxidation of the third carbon of 3,4-dihydroxybutyl-1-phosphonate especially interferes with the inhibition of the mitochondrial enzyme. Dihydroxyacetone phosphate is a competitive inhibitor of the rat liver microsomal enzyme, and has a Ki value of 1.4 mM (18). Phenethyl alcohol is a noncompetitive inhibitor of glycerol 3-phosphate acyltransferase activity in E. coli (149), and in this study it is a noncompetitive inhibitor of the microsomal enzyme and, possibly, the mitochondrial enzyme.

The results obtained in this study also support the hypothesis that there are two isoenzymes for palmitoyl-CoA:sn-glycerol 3-phosphate acyltransferase activity (12), as suggested by the action of the phosphonates with mitochrondria and microsomes. Although the enzyme has not been purified from either organelle, much evidence suggests two isoenzymes. The mitochondrial enzyme is more affected by diet and hormones (12,13), and is more active with long-chain saturated

acyl-CoA thioesters than with unsaturated acyl-CoA thiosters (7,8). The microsomal enzyme is sensitive to thiol group reagents, heat and proteolytic enzymes (10, 17), and shows less preference for the type of acyl-CoA thioesters (10,17,18). While the catalytic site for the acyltransferase activity of the endoplasmic reticulum is on the cytoplasmic surface, the site of the mitochondrial activity seems to be on the inner surface of the outer membrane (17). Some of the differences in the degree of inhibition between mitochondrial and microsomal enzymes by the phosphonates may be related to the location and accessibility of the catalytic site.

Glycerol 3-Phosphate Acyltransferase Activity in Total Membranes After Partial Hepatectomy

Following two-thirds partial hepatectomy triacylglycerol accumulates in the regenerating liver (176). Our study confirms the increase and accumulations of triacylglycerol 18 hours after resection, and to an even greater extent at 24 hours (Table VI). At this time microsomal glycerol 3-phosphate acyltransferase activity is increased as was the total activity (Table VII). Mangiapane and colleagues found an 89% increase in the microsomal enzyme at 24 hours after a subtotal (82%) hepatectomy, but no significant increase at 6, 10 and 16 hours (201). In our study, the activity of microsomal glycerol 3-phosphate acyltransferase correlates with the increase in

triacylglycerol content during liver regeneration at 18 and 24 hours. This suggests that the activity of microsomal glycerol 3-phosphate acyltransferase plays a role in promoting triacylglycerol synthesis at both 18 and 24 hours after partial hepatectomy.

Phosphatidate phosphohydrolase activity also increases during subtotal hepatectomy (201). The activity of this enzyme may also be important for the regulation of triacylglycerol synthesis during liver regeneration. Mitochondrial glycerol 3-phosphate acyltransferase activity is unchanged after partial hepatectomy in our study. The role of this enzyme is difficult to evaluate, because it is insulin-sensitive and decreases with fasting (13).

Dihydroxyacetone Phosphate Acyltransferase Activity

In unoperated rats dihydroxyacetone phosphate acyltransferase activity contributes only a minor role in glycerolipid synthesis (4). In our study the peroxisomal enzyme activity is increased only at 24 hours after partial hepatectomy (Table VII). Since there is no correlation between the enzyme activity and content of triacylglycerol, peroxisomal dihydroxyacetone phosphate acyltransferase activity probably plays a minor role, if any in the increased synthesis of triacylglycerols during liver regeneration. The microsomal dihydroxyacetone phosphate acyltransferase activity does not statistically increase in

our study. Since it has been suggested that a single microsomal enzyme may have a dual catalytic function for both glycerol 3-phosphate and dihydroxyacetone phosphate acyltransferase activities (18), it is surprising that both microsomal activities are not increased to the same degree. While the glycerol 3-phosphate acyltransferase activity increases from 35 percent at 18 hours to 62 percent at 24 hours compared to the sham values, the dihydroxyacetone phosphate acyltransferase activity increases from 8 per cent at 18 hours to 18 percent at 24 hours after partial hepatectomy. The inability to dectect a significant increase in the dihydroxyacetone phosphate acyltransferase activity can be ascribed to the method which is used to determine the microsomal activity. Using N-ethylmaleimide to inhibit the microsomal activity, the microsomal activity is calculated from the difference between the total activity and the peroxisomal (N-ethylmaleimide-insensitive) activity. When small changes in activity occur, the method is overly sensitive to changes in the peroxisomal activity which represents the greater portion of the total dihydroxyacetone phosphate activity. The method using N-ethylmaleimide to inhibit the microsomal glycerol 3-phosphate acyltransferase activity to calculate the mitochondrial and microsomal activity, however, is adequate, because the activity of the mitochondrial enzyme is unchanged, and the total glycerol 3-phosphate

acyltransferase activity found in the liver is nearly equally divided between these two organelles (6,8,15).

Glycerol 3-Phosphate Dehydrogenase Activity

The cytoplasmic NAD^+-dependent glycerol 3-phosphate dehydrogenase activity is decreased after partial hepatectomy. This enzyme activity is also decreased in rat hepatomas, and a correlation of low activity with fast growth has been suggested (105). This would favor the availability of sn-glycerol 3-phosphate, which comes from the phosphorylation of extrahepatic glycerol, for glycerolipid biosynthesis.

Uptake of 3,4-Dihydroxybutyl-1-Phosphonate by Tissue Slices

There is a linear rate for the incorporation of 3,4-dihydroxybutyl-1-phosphonate into chloroform extractable products in liver, adipose tissue and small bowel. Since there is no evidence for a plasma membrane receptor for the transport of phosphorylated compounds across the cell membrane in mammals, the mechanism by which the phosphonate enters the cell is unclear. In the liver it may be possible that the phosphonate is initially taken up by Kupffer cells by a pinocytic process, and then is transferred to the hepatocyte via gap junctions which connect the two cells. It is known that Kupffer cells, endothelial cells, and hepatocytes cooperate to clear the portal blood of substances. Hepatocytes, adipocytes, and

enterocytes of the small bowel could also incorporate the phosphonate directly by pinocytosis, but the mechanism is undefined.

Effect of Intraperitoneal Injection of 3,4-Dihydroxybutyl-1-Phosphonate on the Regenerating Rat Liver

When 3,4-dihydroxybutyl-1-phosphonate is injected into rats 20 and 24 hours after partial hepatectomy, the appearance of intracellular fat in hepatocytes is drastically reduced to approach that found in livers of unoperated rats. The number of hepatocytes in mitosis is also similar in livers from unoperated rats and from 3,4-dihydroxybutyl-1-phosphonate treated rats after hepatectomy. The inhibition of triacylglycerol synthesis by this analogue of sn-glycerol 3-phosphate is confirmed by the significant decrease in the amount of hepatic triacylglycerol content at 20 and 24 hours after partial hepatectomy. Since both monoacyl and diacyl derivatives of 3,4-dihydroxybutyl-1-phosphonate occur in an *in vitro* system, it is likely that these products form in an *in vivo* system as well. The diacyl products presumably cannot be substrates for phosphatidic acid phosphatase, because the carbon-phosphorus bond is not likely to be cleaved.

Although the number of cells in mitosis is decreased by 3,4-dihydroxybutyl-1-phosphonate after partial hepatectomy, it does not inhibit DNA synthesis. On the contrary, there is a 42 percent increase in tritiated

thymidine incorporation into DNA. It is not likely that the phosphonate interacts directly with thymidine kinase to increase the utilization of thymidine. One effect of inhibiting triacylglycerol synthesis may be that the slowing of the acyltransferase reaction would lead to the accumulation of fatty acyl-CoA thioesters which could inhibit the synthesis of malonyl-CoA, and increase B-oxidation, increasing ATP synthesis and stimulate DNA synthesis. This study indicates that the microsomal sn-glycerol 3-phosphate acyltransferase activity is important in the triacylglycerol synthesis in the liver. Inhibition of glycerolipid biosynthesis by 3,4-dihydroxybutyl-1-phosphonate is related to the inhibition of sn-glycerol 3-phosphate acyltransferase and phosphatidic acid phosphatase. A decrease in the cellular content of triacylglycerol is related to a decrease in mitosis and an increase in DNA synthesis during the first day of liver regeneration.

Uptake of 3,4-Dihydroxybutyl-1-Phosphonate by Tissue Culture Cells

The linear rate of tritiated 3,4-dihydroxybutyl-1-phosphonate incorporation into the lipids of mouse 3T3 fibroblasts over a 5 hour period suggests that the acylation reactions are not inhibited by the accumulation of the products and the rate of the transfer of the phosphonate across the cell membrane is not limiting the acylation of 3,4-dihydroxybutyl-1-

phosphonate. In contrast, the hyperbolic rate of incorporation of the phosphonate into the lipids of human hepatoma cells suggests that either the acylation products or the rate of the movement of the phosphonate across the cell membrane may be limiting the rate of 3,4-dihydroxybutyl-1-phosphonate acylation.

The mechanism by which the phosphonate enters the cells is not clear. In these pure cell cultures, no phagocytic cells can help in the incorporation of the phosphonate. These cells, therefore, may also use a pinocytic mechanism.

Significance

The analogues of sn-glycerol 3-phosphate and dihydroxyacetone phosphate have been shown to be competitive inhibitors of sn-glycerol 3-phosphate acyltransferase reactions in the rat liver. The Ki of these chemicals suggest that they may be valuable probes for studying hepatic phosphoglyceride metabolism.

The molecular shape of (S)-3,4-dihydroxybutyl-1-phosphonate is most similar to the structure of sn-glycerol 3-phosphate. Whereas sn-glycerol 3-phosphate contains a 3 carbon atom chain and is a phosphate ester, 3,4-dihydroxybutyl-1-phosphonate contains a 4 carbon atom chain and is directly bounded to phosphorus. The Vmax of the mitrochondrial and microsomal sn-glycerol 3-phosphate acyltransferases for sn-glycerol 3-phosphate is similar to that for 3,4-

dihydroxybutyl-1-phosphonate. With the mitochondrial acyltransferase the Vmax for the natural substrate is 4.30 nmol/min/mg protein, and is 2.21 nmol/min/mg protein for the phosphonate. The microsomal enzyme has a Vmax of 5.70 nmol/min/mg protein for sn-glycerol 3-phosphate and a Vmax of 4.58 nmol/min/mg protein for the analogue. The apparent Km of the mitochondrial enzyme is 0.593 and 2.50 mM for sn-glycerol 3-phosphate and the analogue respectively; the apparent Km of the microsomal enzyme is 0.289 and 1.38 mM for the natural substrate and the phosphonate, respectively. The similarity of the respective kinetic constants suggest that, if 3,4-dihydroxybutyl-1-phosphonate could be delivered into a cell, the biosynthetic products of the analogue may be potent intracellular inhibitors of phosphoglyceride metabolism.

Since the in vitro studies suggest that the diacyl products of 3,4-dihydroxybutyl-1-phosphonate can be synthesized in vivo, and that the phosphonate carbon-phosphorus bond cannot be cleaved by phosphatidic acid phosphatase, the diacyl products should be further metabolized by cytidyltransferase to phosphonate analogues of phosphatidylinositol and cardiolipin (Figure 1). The synthesis of these products can be expected to alter cellular function.

Phosphatidylinositol can be phosphorylated to phosphatidylinositol 4-phosphate, and then again to phos-

phatidylinositol 4,5-bisphosphate (255). When a hormone or activating agent binds to certain plasma membrane receptors, a phosphatidylinositol-specific phospholipase C becomes activated and splits the phosphatidylinositol 4,5-bisphosphate to form inositol trisphosphate and diacylglycerol.

Inositol trisphosphate and diacylglycerol are important interacting second messengers, which regulate intracellular calcium levels and the action of protein kinase C, and control cellular secretion, contraction, metabolism and growth (256). Since the poly-phosphoinositol derivatives of 3,4-dihydroxybutyl-1-phosphonate cannot be cleaved by phospholipase C, the presence of these products may inhibit cellular function. It is possible that the inhibition of mitosis, which occurred during liver regeneration when 3,4-dihydroxybutyl-1-phosphonate was administered, is related to the interference of the polyphosphoinositol and second messenger system. The phosphonate analogues of sn-glycerol 3-phosphate may become an important new class of antimetabolites.

TABLE I

COMPOSITION OF THE MITOCHONDRIAL FRACTION

ENZYME	% TOTAL HOMOGENATE	RELATIVE SPECIFIC ACTIVITY
Succinate Cytochrome c Reductase	16.64 ± 0.29	3.06 ± 0.16
Monoamine Oxidase	15.22 ± 0.58	2.79 ± 0.01
Glutamate Dehydrogenase	14.16 ± 2.06	2.61 ± 0.34
Catalase	1.41 ± 0.36	0.26 ± 0.05
Sedimentable Catalase	3.53 ± 0.04	0.64 ± 0.01
Urate Oxidase	4.72 ± 0.12	0.86 ± 0.01
NADPH Cytochrome c Reductase	2.83 ± 0.98	0.51 ± 0.16
Glucose-6-Phosphatase	4.45 ± 1.07	0.81 ± 0.16
Protein	5.45 ± 0.19	

The percentages of the total homogenate activity and relative specific activities are expressed as the mean ± standard deviation of marker enzymes in the mitochondrial fractions of two different homogenates of a pool of five rat livers. The relative specific activity is the percentage of the total homogenate activity present in the mitochondrial fraction divided by the corresponding percentage of the total protein.

TABLE II

COMPOSITION OF THE MICROSOMAL FRACTION

ENZYME	% TOTAL HOMOGENATE	RELATIVE SPECIFIC ACTIVITY
NADPH Cytochrome c Reductase	22.96 ± 2.78	6.53 ± 1.55
Glucose-6-Phosphatase	13.44 ± 0.27	3.80 ± 0.37
Catalase	1.28 ± 0.10	0.35 ± 0.01
Sedimentable Catalase	1.57 ± 0.03	0.43 ± 0.03
Urate Oxidase	0.36 ± 0.01	0.10 ± 0
Succinate Cytochrome c Reductase	0.38 ± 0.13	0.11 ± 0.05
Monoamine Oxidase	1.60 ± 0.09	0.45 ± 0.03
Glutamate Dehydrogenase	1.04 ± 0.15	0.29 ± 0.06
Protein	3.53 ± 0.47	

The percentages of the total homogenate activity and relative specific activities are expressed as the mean ± standard deviation of marker enzymes in the microsomal fractions of two different homogenates of a pool of five rat livers. The relative specific activity is the percentage of the total homogenate activity present in the microsomal fraction divided by the corresponding percentage of total protein.

TABLE III

COMPOSITION OF THE PEROXISOMAL FRACTION

ENZYME	% OF TOTAL HOMOGENATE	RELATIVE SPECIFIC ACTIVITY
Urate Oxidase	12.93 ± 4.37	4.52 ± 1.25
Catalase	9.74 ± 2.36	3.42 ± 0.61
Sedimentable Catalase	15.27 ± 1.73	5.48 ± 1.95
Succinate Cytochrome c Reductase	0.19 ± 0.05	0.06 ± 0.01
Monoamine Oxidase	0.26 ± 0.01	0.09 ± 0.01
Glutamate Dehydogenase	0.49 ± 0.04	0.17 ± 0.01
NADPH Cytochrome c Reductase	2.01 ± 0.08	0.71 ± 0.03
Glucose-6-Phosphatase	4.41 ± 0.06	1.57 ± 0.09
Protein	2.66 ± 0.34	

The percentage of the total homogenate activity and relative specific activities are expressed as the mean ± standard deviation of marker enzymes in the peroxisomal fractions of two different homogenates of a pool of five rat livers. The relative specific activity is the percentage of the total homogenate activity presnt in the peroxisomal fraction divided by the corresponding percentage of total protein.

TABLE IV

CHLOROFORM-EXTRACTABLE 3,4-DIHYDROXYBUTYL-1-PHOSPHONATE PRODUCTS IN TISSUE

TIME (min)	LIVER (pmol/g)	FAT (pmol/g)	SMALL BOWEL (pmol/g)	KIDNEY (pmol/g)
0	1.05	0.86	0.17	1.50
90	2.55	2.55	1.16	1.43
180	3.75	3.57	2.88	1.53

The incorporation of (RS)-3,4-dihydroxy-[3-^{3}H]-butyl-1-phosphonate, 401.4 Ci/mole, in liver (250 mg) slices, adipose tissue (250 mg) slices, kidney (250 mg) slices, and small bowel mucosa (100 mg) scrapings was performed in the presence of Minimum Essential Media, containing 7.5 nmoles of 3,4-dihydroxybutyl-1-phosphonate, 10 % fetal calf serum, and streptomycin. Non-polar products were extracted from a homogenate of a thrice saline-washed pellet by the method of Folch et al. (251).

TABLE V

TRIACYLGLYCEROL CONTENT AND SN-GLYCEROL 3-PHOSPHATE DEHYDROGENASE ACTIVITY OF REGENERATING LIVER

GROUP	N	TRIACYLGLYCEROL (mg/g)	sn-GLYCEROL 3-PHOSPHATE DEHYDROGENASE (nmole NADH produced/min/mg)
SHAM, 18 HOURS	6	2.2 ± 0.4	81.3 ± 5.5
HEPATECTOMY, 18 HOURS	5	12.6 ± 1.1*	61.9 ± 7.2*
SHAM, 24 HOURS	7	1.9 ± 0.4	87.5 ± 6.5
HEPATECTOMY, 24 HOURS	6	13.9 ± 4.7*	59.6 ± 6.8*

* $p<0.05$, comparing hepatectomy with sham controls at each time period.

Data are given as mean ± standard error of the mean.

TABLE VI

SUBCELLULAR DISTRIBUTION OF SN-GLYCEROL 3-PHOSPHATE ACYLTRANSFERASE ACTIVITY IN REGENERATING LIVER

GROUP	N	MICROSOMAL	MITOCHONDRIAL	TOTAL
		(pmole glycerol 3-phosphate incorporated/min/mg)		
SHAM, 18 HOURS	6	708 ± 29	663 ± 49	1371 ± 74
HEPATECTOMY, 18 HOURS	5	956 ± 53*	565 ± 47	1521 ± 70
SHAM, 24 HOURS	7	742 ± 107	673 ± 47	1415 ± 107
HEPATECTOMY, 24 HOURS	6	1203 ± 144*	627 ± 63	1830 ± 131*

* $p<0.05$, comparing hepatectomy with sham controls at each time period.

Data are given as the mean ± standard error of the mean.

The total and N-ethylmaleimide-resistant activities were measured, and the difference between the total activity and N-ethylmaleimide-resistant activity was calculated as the microsomal activity.

TABLE VII

SUBCELLULAR DISTRIBUTION OF DIHYDROXYACETONE PHOSPHATE ACYLTRANSFERASE ACTIVITY IN REGENERATING LIVER

GROUP	N	MICROSOMAL (pmole dihydroxyacetone phosphate incorporated/min/mg)	PEROXISOMAL	TOTAL
SHAM, 18 HOURS	6	134 ± 33	420 ± 60	554 ± 33
HEPATECTOMY, 18 HOURS	5	145 ± 26	377 ± 36	522 ± 54
SHAM, 24 HOURS	7	117 ± 24	375 ± 24	492 ± 32
HEPATECTOMY, 24 HOURS	6	138 ± 43	523 ± 66*	661 ± 31*

* $p<0.05$, comparing hepatectomy with sham controls at each time period.

Data are given as mean ± standard error of the mean.

The total and N-ethylmaleimide-resistant activities were measured, and the differences between the total activity and N-ethylmaleimide-resistant activity was calculated as the microsomal activity.

TABLE VIII

EFFECT OF PARTIAL HEPATECTOMY ON INTRACELLULAR FAT AND MITOSIS

Treatment	N	Time (hours)	Fat (0-4 +)	Mitotic Index (#/1000 cells)
None	2	0	0.5 ± 0.5	1.0 ± 0
None	2	18	3.0 ± 0	9.0 ± 1.0
None	2	20	3.5 ± 0.5	13.5 ± 1.5
None	2	24	3.5 ± 0.5	14.0 ± 1.0
NaCl	4	20	2.2 ± 0.2	11.2 ± 1.5
NaCl	3	24	2.7 ± 0.3	12.3 ± 0.3
DBP	4	20	1.0 ± 0	1.2 ± 0.2
DBP	4	24	1.0 ± 0	1.2 ± 0.5
G3P	3	24	2.3 ± 0.3	6.0 ± 0.6

Abbreviations: DBP = 3,4-Dihydroxybutyl-1-Phosphonate; G3P = Glycerol 3-Phosphate

The degree of intracellular fat was graded from 0, when no fat vacuoles were present, to 4+, when fat vacuoles filled the cytoplasm.

The mitotic index was the number of cells in mitosis.

Animals had intraperitoneal injections of 2 μmoles/100 g body weight of either saline, DBP or G3P before partial hepatectomy, and at 6 and 14 hours after partial hepatectomy.

TABLE IX

EFFECT OF 3,4-DIHYDROXYBUTYL-1-PHOSPHONATE ON TRIACYLGLYCEROL AND PROTEIN CONTENT OF THE REGENERATING LIVER AT 20 HOURS

TREATMENT	N	TRIACYLGLYCEROL (mg/g)	PROTEIN (mg/g)
NaCl	10	6.12 ± 0.55	227 ± 18
DBP	9	1.85 ± 0.30*	242 ± 10

Abbreviation: DBP = 3,4-Dihydroxybutyl-1-Phosphonate

* $p<0.001$ with an ANOVA when DBP treated rats were compared to saline.

TABLE X

EFFECT OF SALINE, GLYCEROL 3-PHOSPHATE, AND 3,4-DIHYDROXYBUTYL-1-PHOSPHONATE ON DNA SYNTHESIS AND CONTENT OF DNA, RNA, TRIACYLGLYCEROL, PHOSPHOLIPID AND PROTEIN IN REGENERATING LIVER AT 24 HOURS

TREATMENT	N	DNA (mg/g)	(3H)-DNA (dpm/ug)	RNA (mg/g)	TRIACYLGLYCEROL (mg/g)	PHOSPHOLIPID (µmoles/g)	PROTEIN (mg/g)
NaCl	5	2.39 ± 0.19	125 ± 17	6.70 ± 0.34	7.21 ± 0.86	5.50 ± 0.84	238 ± 30
G3P	5	2.42 ± 0.07	116 ± 37	7.78 ± 1.14	7.28 ± 1.34	5.90 ± 1.50	248 ± 31
DBP	5	2.51 ± 0.13	177 ± 24*	7.80 ± 0.74	4.03 ± 0.66*	5.81 ± 0.97	265 ± 27

Abbreviations: G3P = Glycerol 3-Phosphate; DBP = 3,4-Dihydroxybutyl-1-Phosphonate

* $p<0.001$ with an ANOVA when DBP treated rats were compared to either saline or G3P treated rats.

Data are given as the mean ± standard error of the mean.

Animals received by intraperitoneal injection 2 µmoles/100 g body weight of either saline, glycerol 3-phosphate or 3,4-dihydroxybutyl-1-phosphonate at 2 hours before partial hepatectomy, and at 6 and 14 hours after partial hepatectomy. One hour before sacrifice all rats had 25 µCi/100 g body weight of tritiated thymidine injected intraperitoneally.

TABLE XI

BUTANOL-EXTRACTABLE 3,4-DIHYDROXYBUTYL-1-PHOSPHONATE ACYLATION PRODUCTS IN FIBROBLASTS AND HEPATOMA IN CELL CULTURE

Time (h)	3T3 (pmol/million cells)	HEP-G2 (pmol/million cells)
0	2.3	1.7
1.5	8.3	6.5
3.0	14.9	9.1
5.0	22.7	10.6

Values represent the mean of two experiments.

The incorporation of (S)-[^{3}H]-3,4-dihydroxybutyl-1-phosphonate, 50 Ci/mole, in mouse 3T3 fibroblasts and human Hep-G2 hepatoma cells was perfomed in 0.5 ml cell culture medium 199, containing 10% fetal calf serum and 160 nmol (RS)-3,4-dihydroxybutyl-1-phosphonate. Cells were incubated at 38 C. The incorporation of the radioactive tag was stopped by aspirating the media and washing three times with pure media 199. One ml of n-butanol was added to each dish and allowed to remain in contact with the cells for 15 minutes before pipeting the butanol into tubes containing 1 ml of water. The contents of the tubes were mixed vigorously with a vortex mixer. Tubes were centrifuged at 500 x g for 20 minutes. The upper butanol layer was placed in a liquid scintillation vial together with 12 ml of liquidscint, mixed and counted for tritium.

TABLE XII

MAXIMUM RATES AND KINETIC CONSTANTS OF MITOCHONDRIAL SN-GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE AND INHIBITORS

INHIBITOR *	APPARENT Km or Kp (mM)	Vmax (nmol/min/mg)	KI (mM)
NONE	0.59	4.30	-
(S)-DBP, 1 mM	0.97	4.40	1.59
(S)-GAP, 1.25 mM	0.93	4.25	2.22
(S)-GAPA, 1.25 mM	0.93	4.45	2.21
(-)TBP, 2.5 mM	0.87	4.46	5.27
(+)TBP, 2.5 mM	0.89	4.38	4.92
DHAPA, 18.8 mM	0.95	4.26	31.64
PEA, 4 mM	0.60	2.97	8.93

* Abbreviations used: (S)-DBP = (S)-3,4-Dihydroxybutyl-1-Phosphonate;
(S)-GAP = (S)-Glyeraldehyde 3-Phosphate;
(S)-GAPA = (S)-3-Hydroxy-4-Oxobutyl-1-Phosphonate;
(-)TBP = (-)-(1S,3S)-1,3,4-Trihydroxybutyl-1-Phosphonate;
(+)TBP = (+)-(1R,3S)-1,3,4-Trihydroxybutyl-1-Phosphonate;
DHAPA = 4-Hydroxy-3-Oxobutyl-1-Phosphonate;
PEA = Phenethyl Alcohol.

TABLE XIII

MAXIMUM REACTION RATES AND KINETIC CONSTANTS OF MICROSOMAL SN-GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE AND INHIBITORS

INHIBITOR *	APPARENT Km or Kp (mM)	Vmax (nmol/min/mg)	KI (mM)
NONE	0.29	5.70	
(S)-DBP, 1 mM	0.62	6.03	0.88
(S)-GAP, 1.25 mM	0.46	5.87	2.08
(S)-GAPA, 1.25 mM	0.44	6.24	2.44
(-)TBP, 2.5 mM	0.89	5.80	1.21
(+)TBP, 2.5 mM	0.91	5.96	1.17
DHAPA, 8 mM	0.71	5.77	5.50
PEA, 4 mM	0.28	3.56	7.35

* Abbreviations used: (S)-DBP = (S)-3,4-Dihydroxybutyl-1-Phosphonate;
(S)-GAP = (S)-Glyceraldehyde 3-Phosphate;
(S)-GAPA = (S)-3-Hydroxy-4-Oxobutyl-1-Phosphonate;
(-)TBP = (-)-(1S,3S)-1,3,4-Trihydroxybutyl-1-Phosphonate;
(+)TBP = (+)-(1R,3S)-1,3,4-Trihydroxybutyl-1-Phosphonate;
DHAPA = 4-Hydroxy-3-Oxobutyl-1-Phosphonate;
PEA = Phenethyl Alcohol.

TABLE XIV

LIST OF MICHAELIS-MENTEN CONSTANTS FOR HEPATIC SN-GLYCEROL 3-PHOSPHATE ACYLTRANSFERASE

Km (mM)	Reference
(MICROSOMES)	
0.14	18
0.16	21
0.29	*
0.33	12
0.67	255
2.00	256
(MITOCHONDRIA)	
0.18	12
0.59	*
1.00	7

* THIS STUDY

LEGEND TO FIGURE 1

Enzymes of glycerolipid biosynthesis. Permission to reproduce the Figure which appeared in the Journal of Lipid Research, Volume 22 was given by the Editor (106).

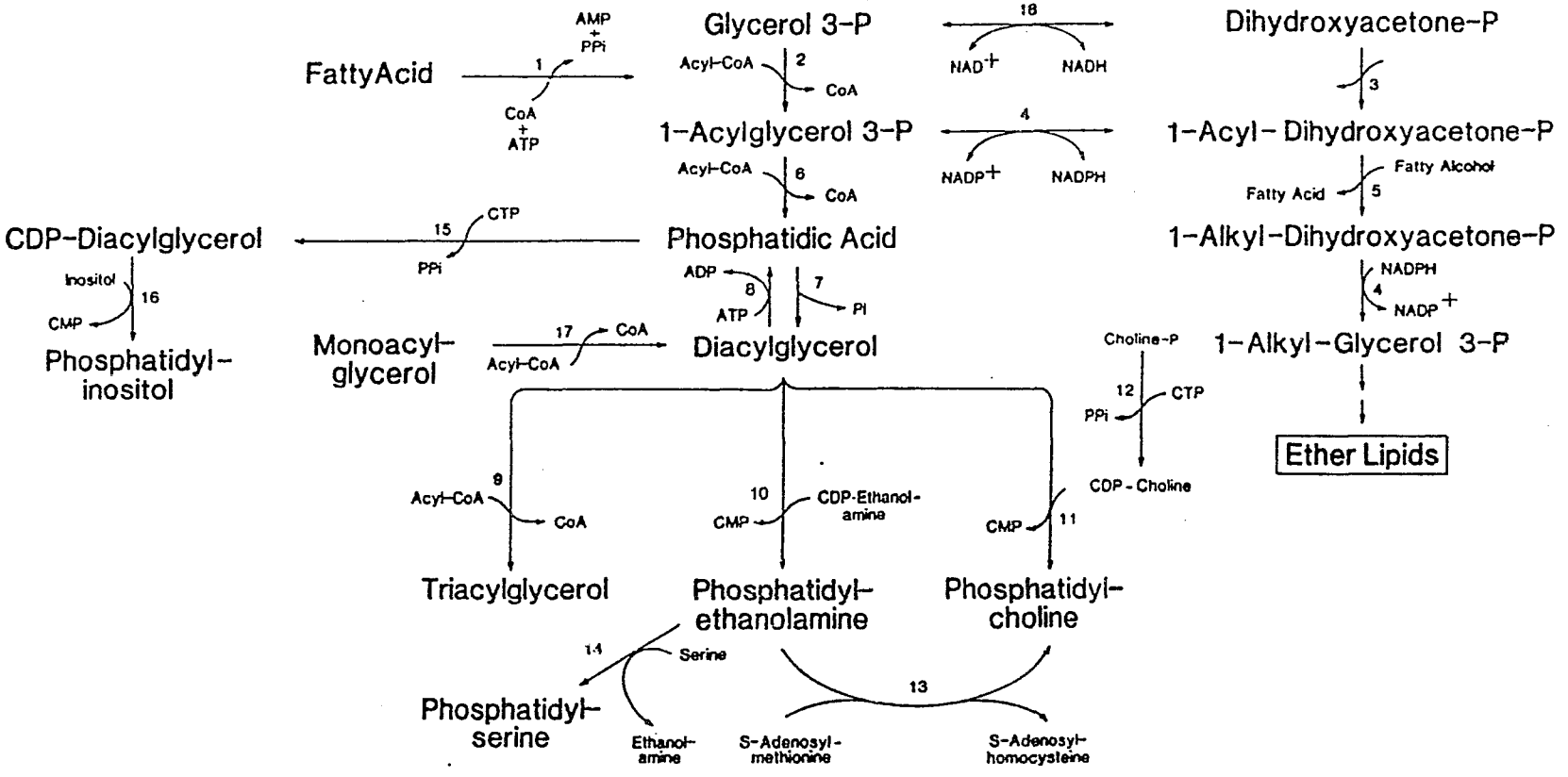
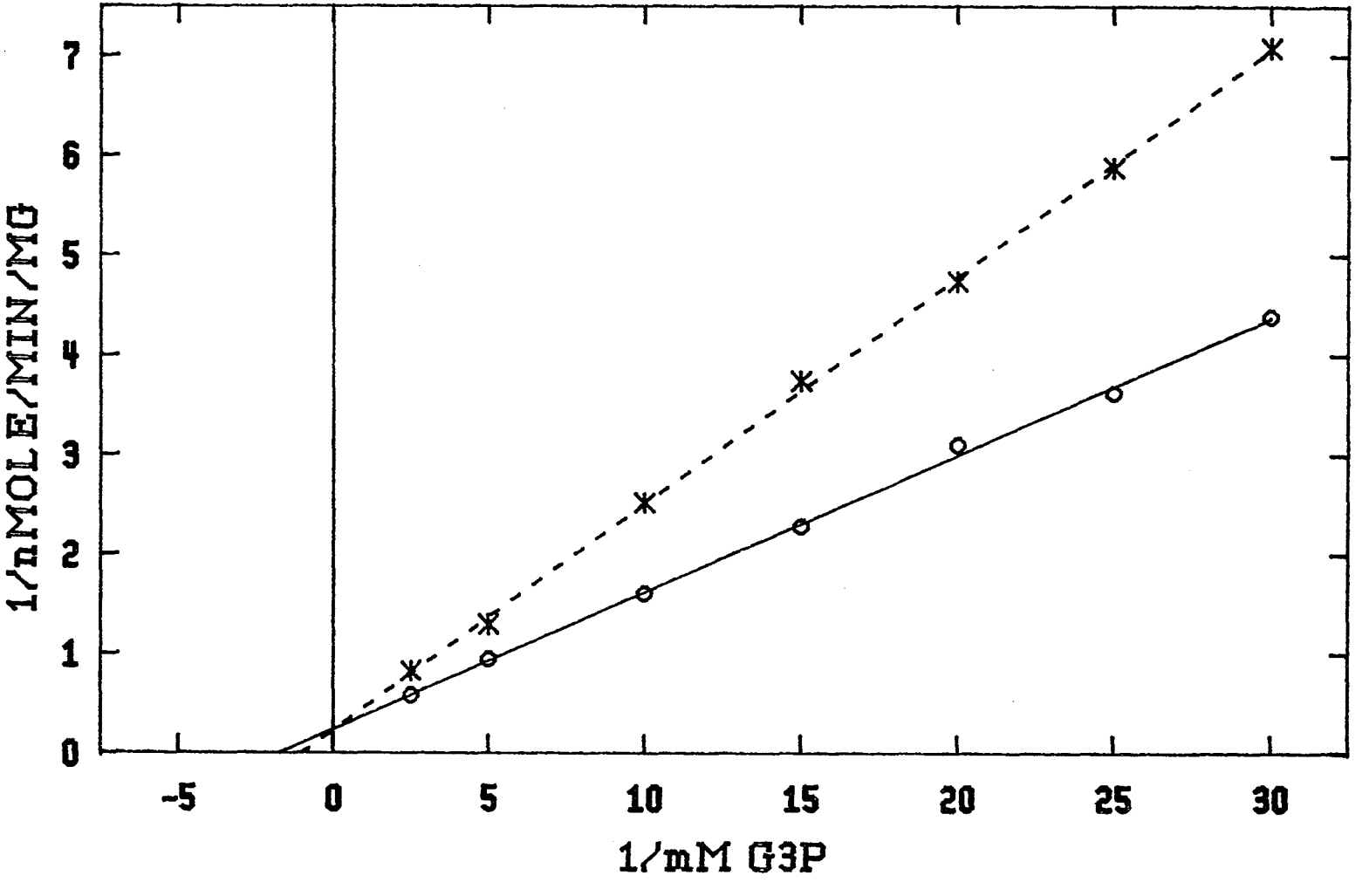


Fig. 1. Enzymes of glycerolipid biosynthesis. 1) Fatty acid CoA ligase (AMP) (EC 6.2.1.3); 2) *sn*-glycerol 3-P acyltransferase (EC 2.3.1.15); 3) dihydroxyacetone-P acyltransferase (EC 2.3.1.42); 4) acyl(alkyl)dihydroxyacetone-P oxidoreductase (EC 1.1.1.10); 5) alkyldihydroxyacetone-P synthase; 6) lysophosphatidic acid acyltransferase (EC 2.3.1.-); 7) phosphatidic acid phosphatase (EC 3.1.3.4); 8) diacylglycerol kinase; 9) diacylglycerol acyltransferase (EC 2.3.1.20); 10) diacylglycerol ethanolaminephosphotransferase (EC 2.7.8.1); 11) diacylglycerol cholinephosphotransferase (EC 2.7.8.2); 12) choline-P cytidyltransferase (EC 2.7.7.15); 13) phosphatidylethanolamine *N*-methyltransferase (EC 2.1.1.17); 14) phosphatidylethanolamine serinetransferase; 15) phosphatidic acid cytidyltransferase (CDP-diacylglycerol synthase) (EC 2.7.7.41); 16) phosphatidylinositol synthase; 17) monoacylglycerol acyltransferase; 18) glycerol-3-P dehydrogenase (EC 1.1.1.8).

LEGEND TO FIGURE 2

Lineweaver-Burk plot of kinetic data from mitochondrial sn-glycerol 3-phosphate acyltransferase reactions, and inhibition with 2 mM (RS)-3,4-dihydroxybutyl-1-phosphonate. Concentrations of sn-glycerol 3-phosphate (G3P) are from 33 to 400 μM. The plot of the uninhibited reaction (solid line) and the inhibited reaction (dashed line) suggests a competitive inhibition.

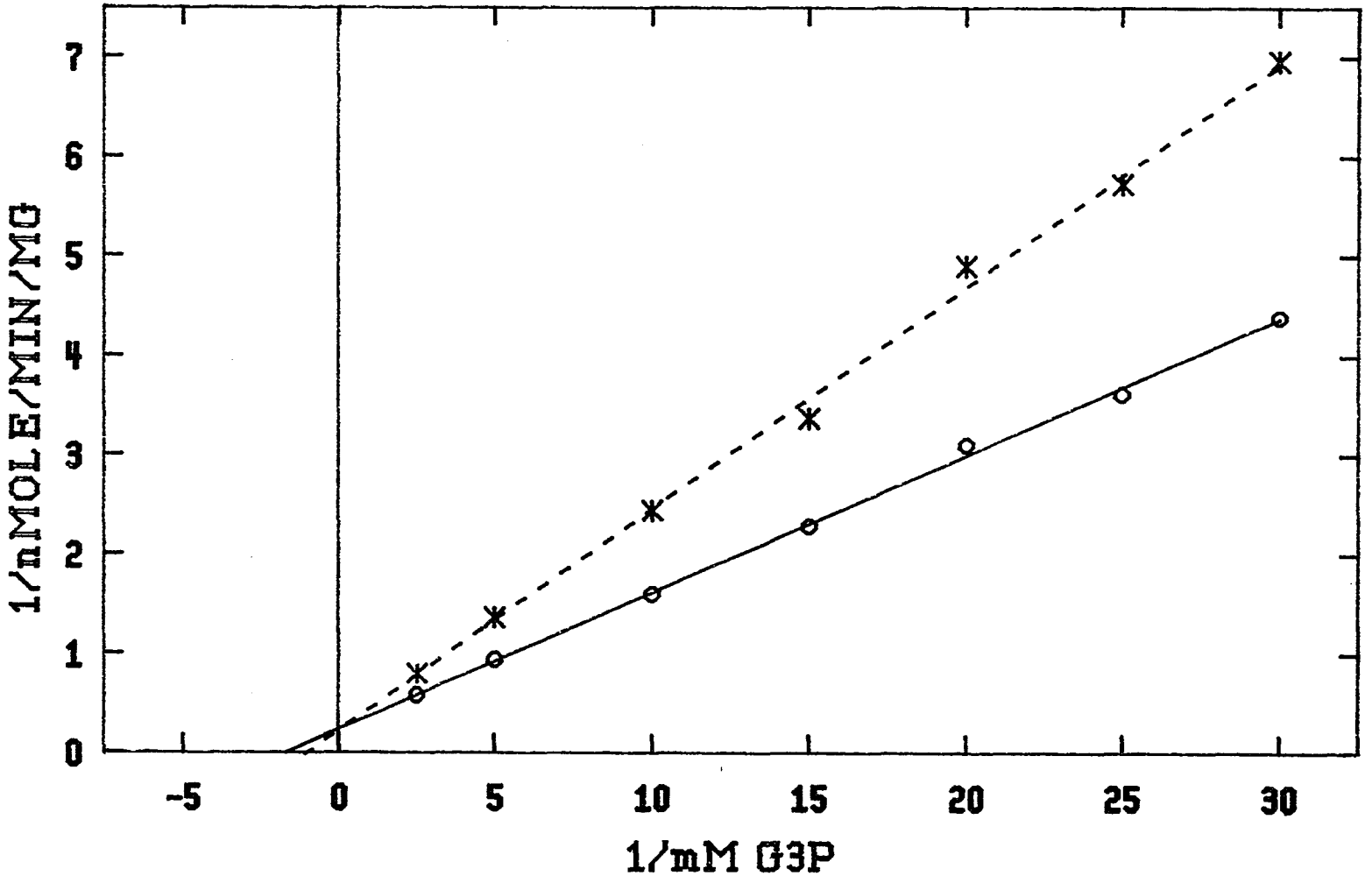
Figure 2



LEGEND TO FIGURE 3

Inhibition of mitochondrial sn-glycerol 3-phosphate acyltransferase activity by 2.5 mM (RS)-glyceraldehyde 3-phosphate. The Lineweaver-Burk plot shows the effect of the inhibitor (dashed line) on reaction rate compared to the uninhibited reaction (solid line), and suggests a competitive inhibition. Concentrations of sn-glycerol 3-phosphate (G3P) are from 33 to 400 μM.

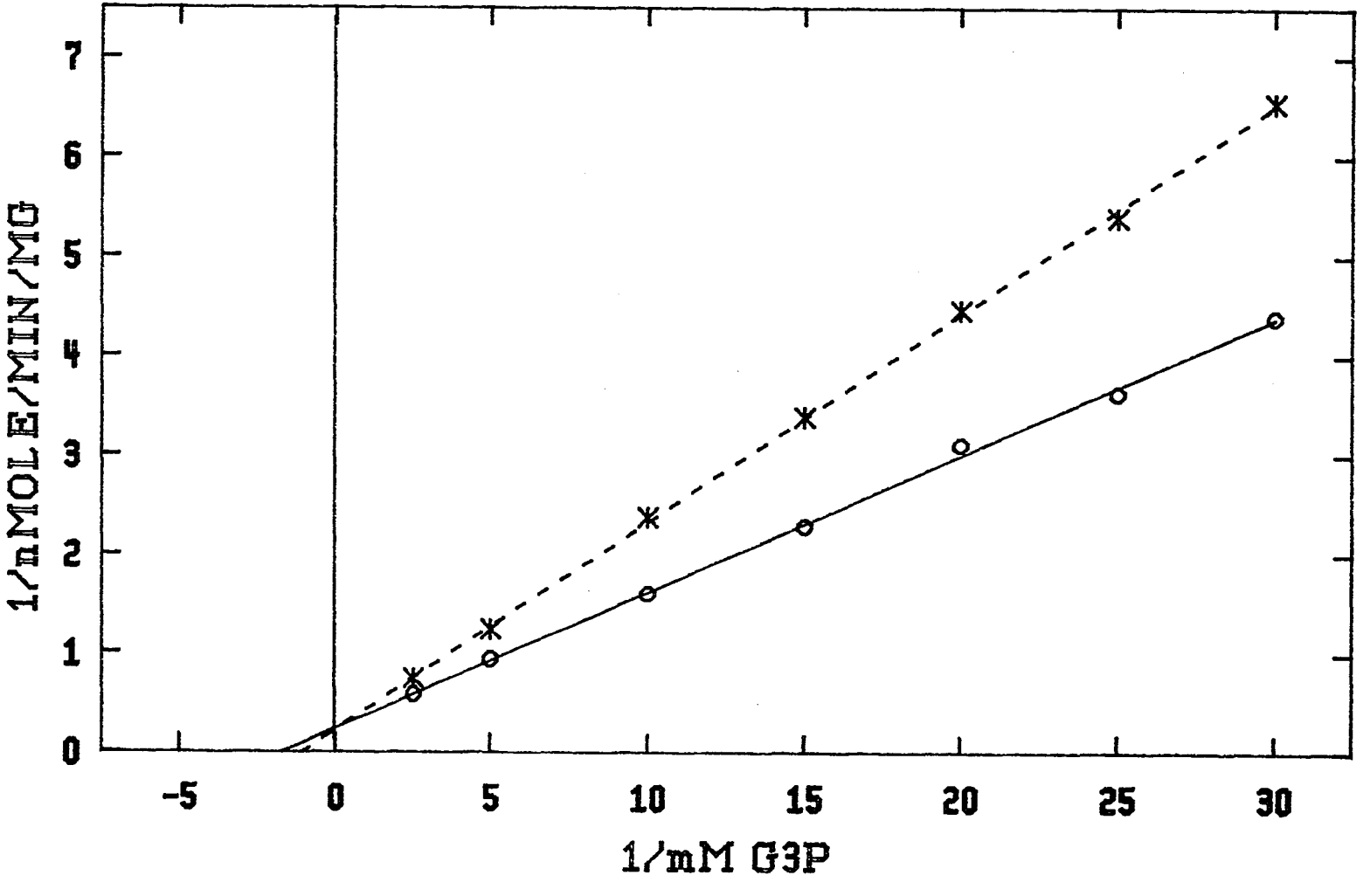
Figure 3



LEGEND TO FIGURE 4

Inhibition of mitochondrial sn-glycerol 3-phosphate acyltransferase activity by 2.5 mM (RS)-3-hydroxy-4-oxobutyl-1-phosphonate. The Lineweaver-Burk plot shows the effect of the inhibitor (dashed line) on reaction rates compared to the uninhibited reaction (solid line), and suggests a competitive inhibition. Concentrations of sn-glycerol 3-phosphate (G3P) are from 33 to 400 µM.

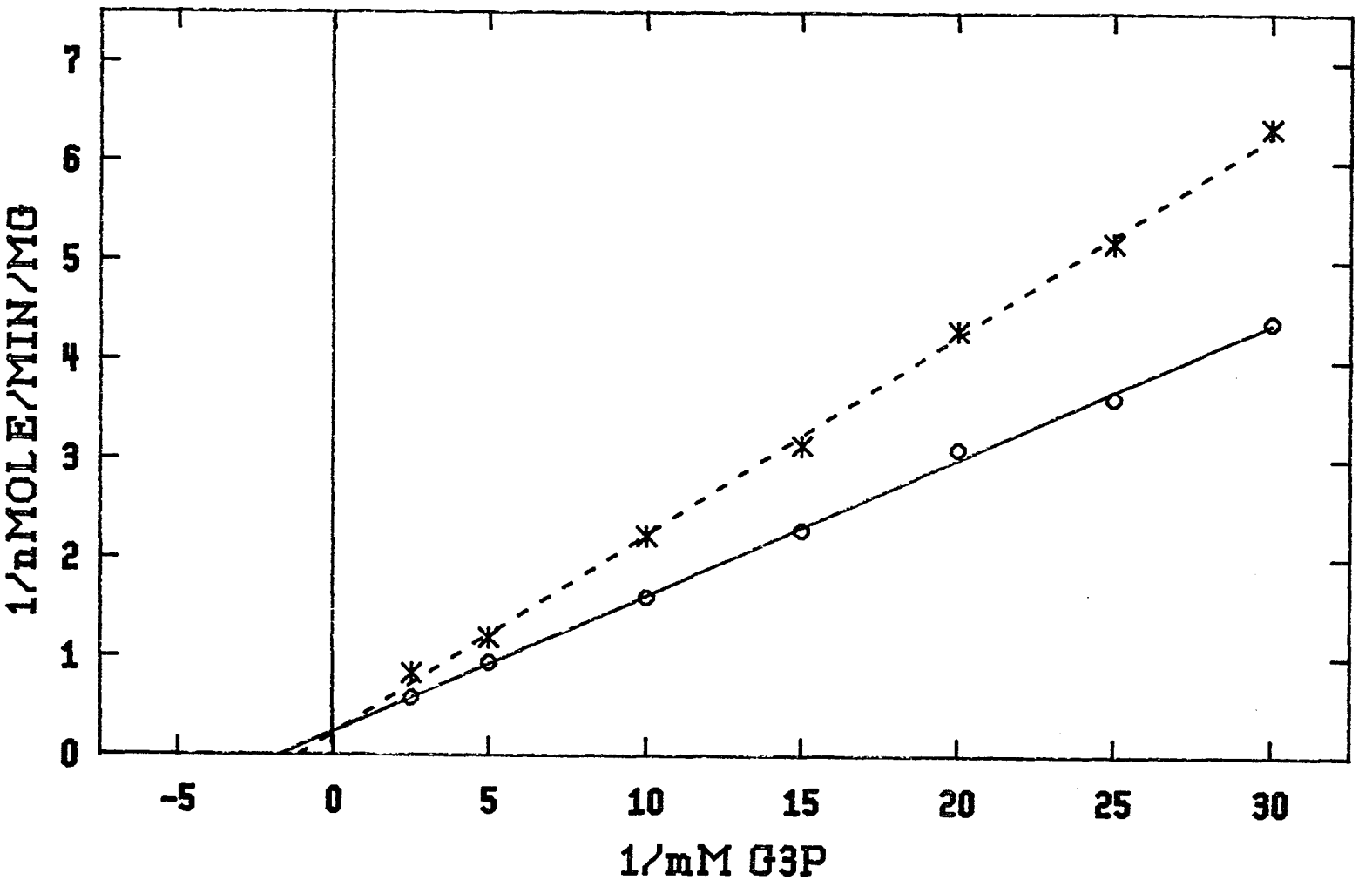
Figure 4



LEGEND TO FIGURE 5

Inhibition of mitochondrial sn-glycerol 3-phosphate acyltransferase activity by 2.5 mM (1S,3S)-1,3,4-trihydroxybutyl-1-phosphonate. The Lineweaver-Burk plot shows the effect of the inhibitor (dashed line) on reaction rates compared to the uninhibited reaction (solid line), and suggests a competitive inhibition. Concentrations of sn-glycerol 3-phosphate (G3P) are from 33 to 400 μM.

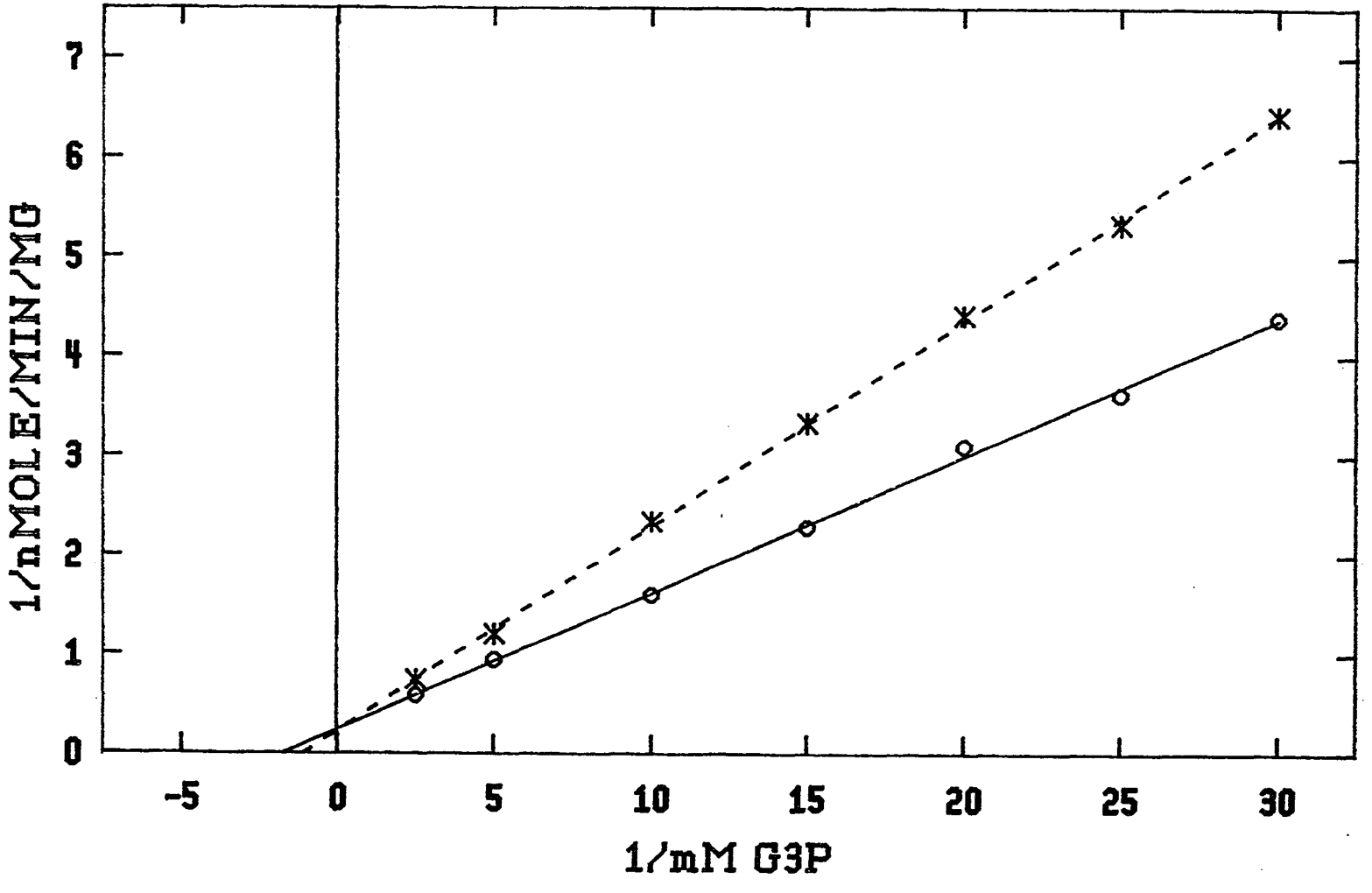
Figure 5



LEGEND TO FIGURE 6

Inhibition of mitochondrial sn-glycerol 3-phosphate acyltransferase activity by 2.5 mM (1R,3S)-1,3,4-trihydroxybutyl-1-phosphonate. The Lineweaver-Burk plot shows the effect of the inhibitor (dashed line) on reaction rates compared to the uninhibited reaction (solid line), and suggests a competitive inhibition. Concentrations of sn-glycerol 3-phosphate (G3P) are from 33 to 400 µM.

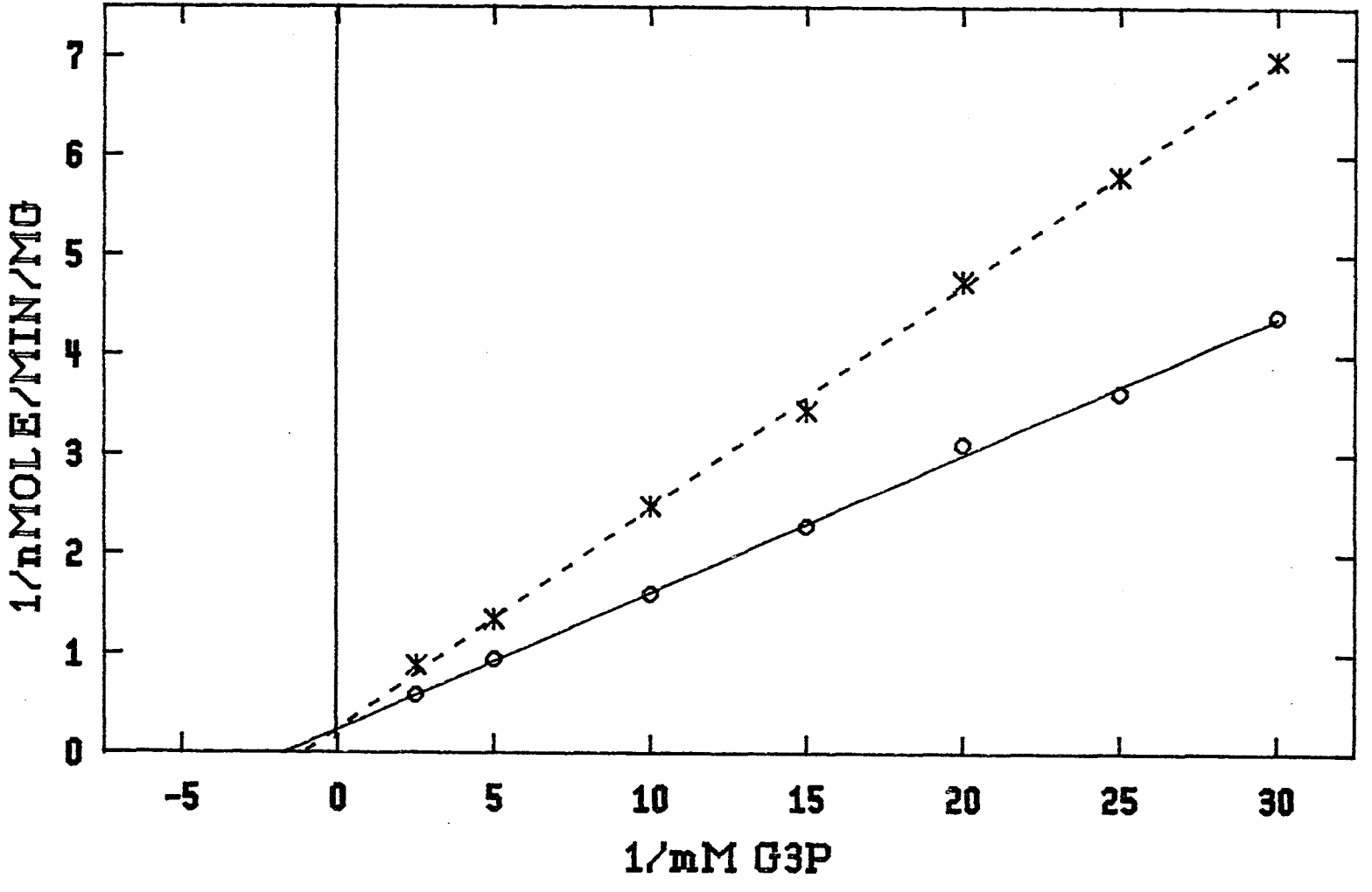
Figure 6



LEGEND TO FIGURE 7

Inhibition of mitochondrial sn-glycerol 3-phosphate acyltransferase activity by 18.8 mM 4-hydroxy-3-oxobutyl-1-phosphonate. The Lineweaver-Burk plot shows the effect of the inhibitor (dashed line) on the reaction rates compared to the uninhibited reaction (solid line), and suggests a competitive inhibition. Concentrations of sn-glycerol 3-phosphate (G3P) are from 33 to 400 μM.

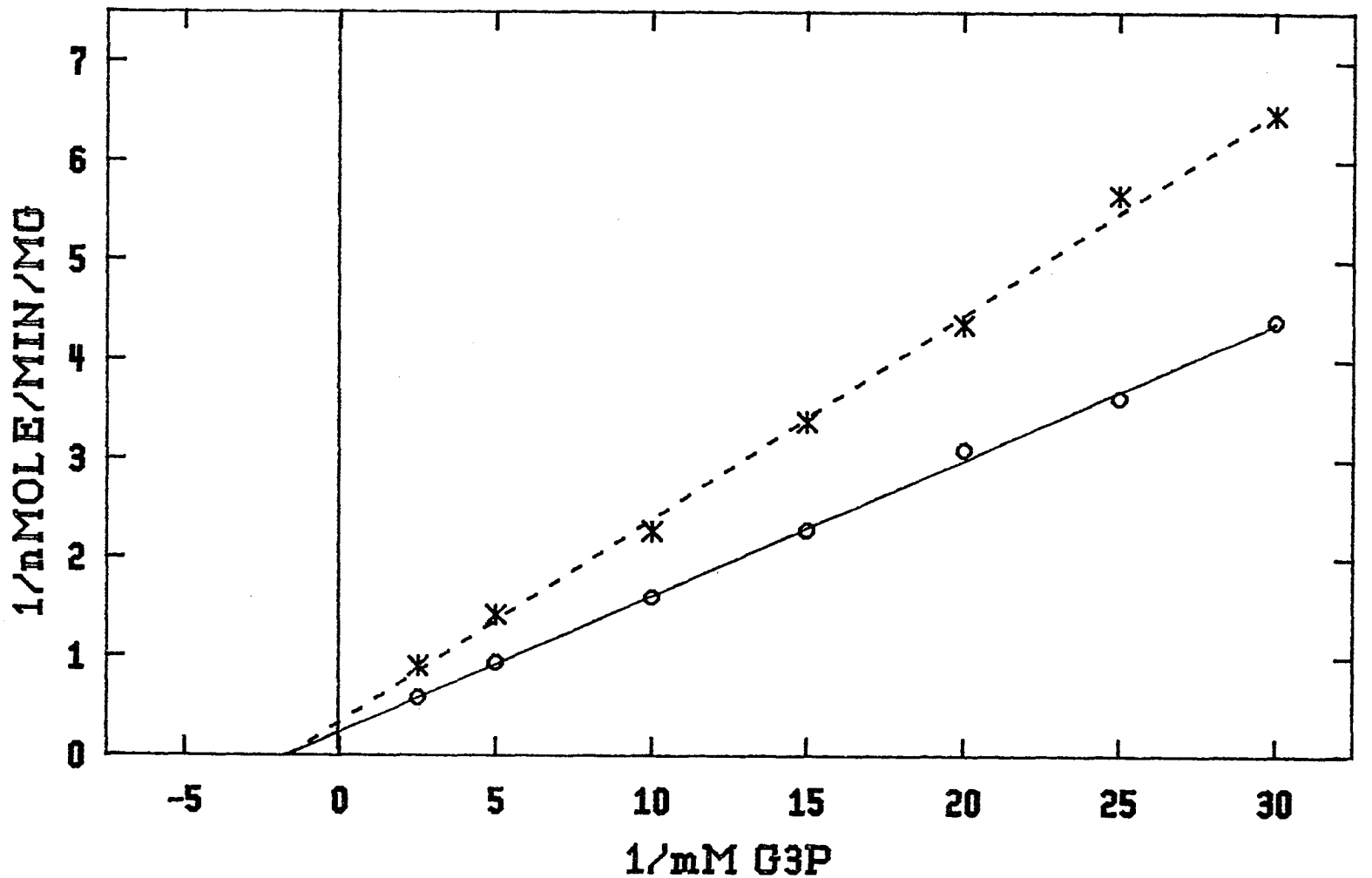
Figure 7



LEGEND TO FIGURE 8

Inhibition of mitochondrial sn-glycerol 3-phosphate acyltransferase activity by 4 mM phenethyl alcohol. The Lineweaver-Burk plot shows the effect of the inhibitor (dashed line) on the reaction rates compared to the uninhibited reaction (solid line), and suggests a noncompetitive inhibition. Concentrations of sn-glycerol 3-phosphate (G3P) are from 33 to 400 μM.

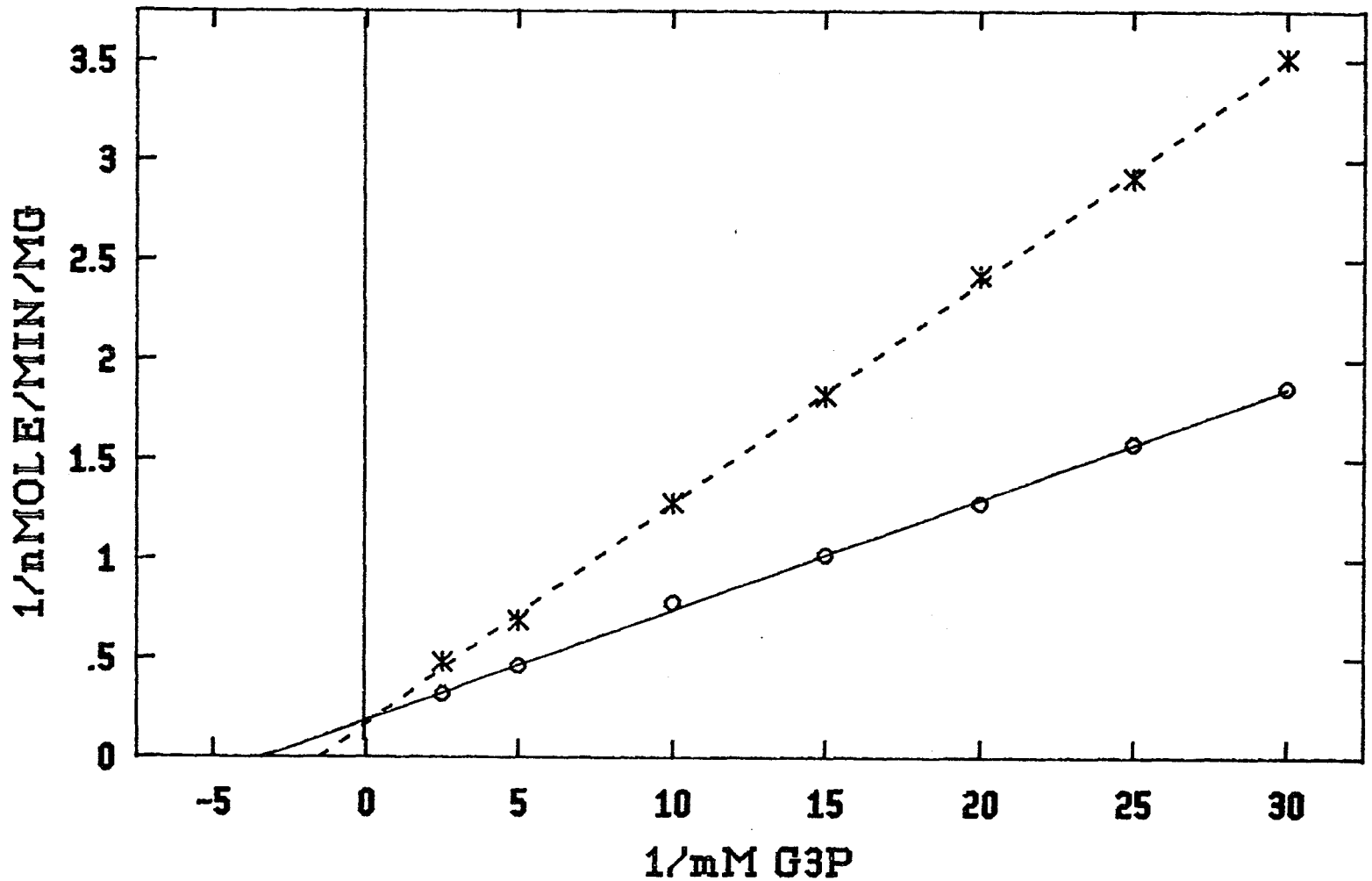
Figure 8



LEGEND TO FIGURE 9

Lineweaver-Burk plot of kinetic data from microsomal sn-glycerol 3-phosphate acyltransferase reactions, and inhibition with 2 mM (RS)-3,4-dihydroxybutyl-1-phosphonate. Concentrations of sn-glycerol 3-phosphate (G3P) were from 33 to 400 μM. The plot of the unihibited reaction (solid line) and the inhibited reaction (dashed line) suggests a competitive inhibition.

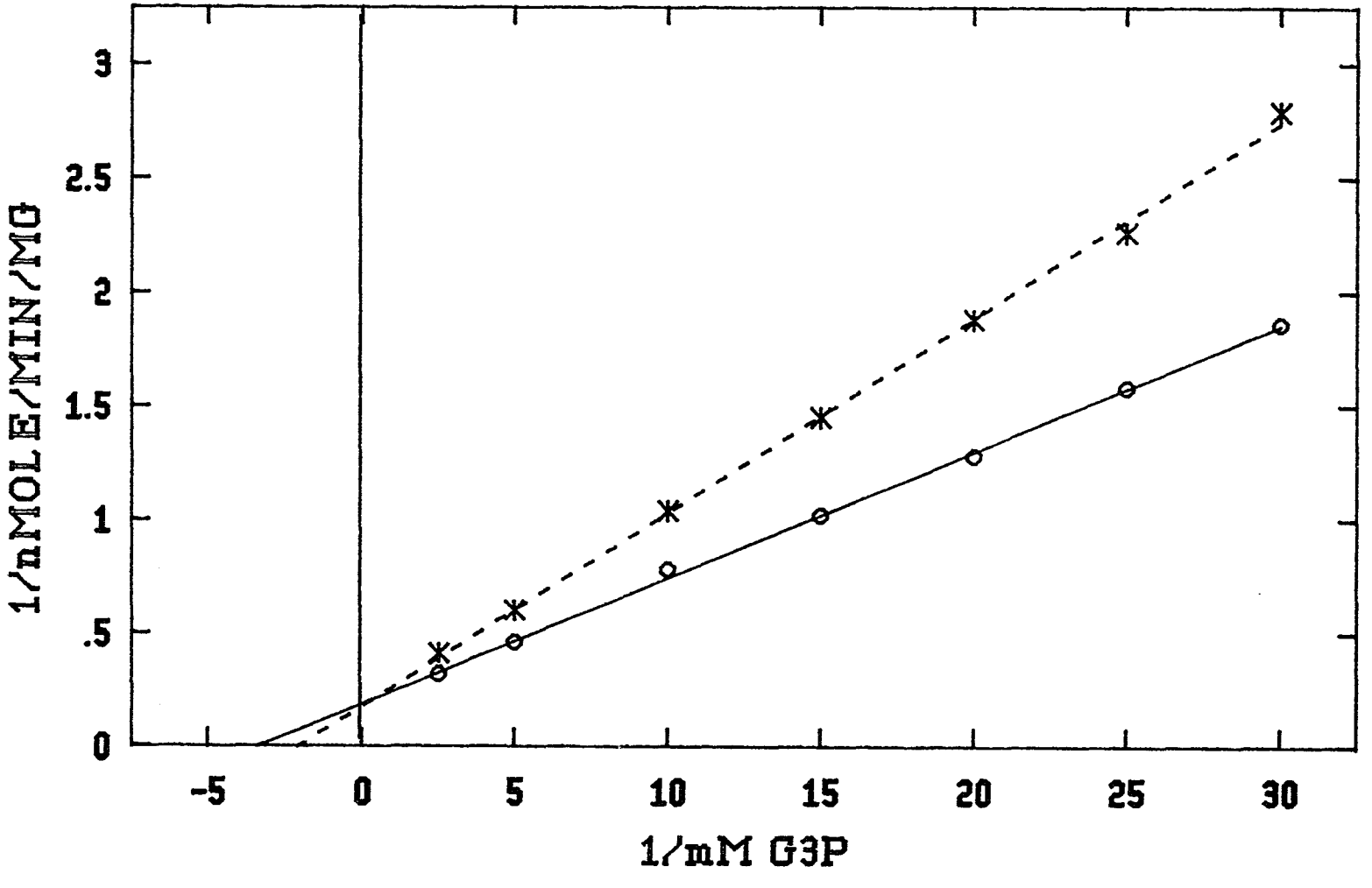
Figure 9



LEGEND TO FIGURE 10

Inhibition of microsomal sn-glycerol 3-phosphate acyltransferase activity by 2.5 mM (RS)-glyceraldehyde 3-phosphate. The Lineweaver-Burk plot shows the effect of the inhibitor (dashed line) on the reaction rates compared to the uninhibited reaction (solid line), and suggests a competitive inhibition. Concentrations of sn-glycerol 3-phosphate (G3P) are from 33 to 400 µM.

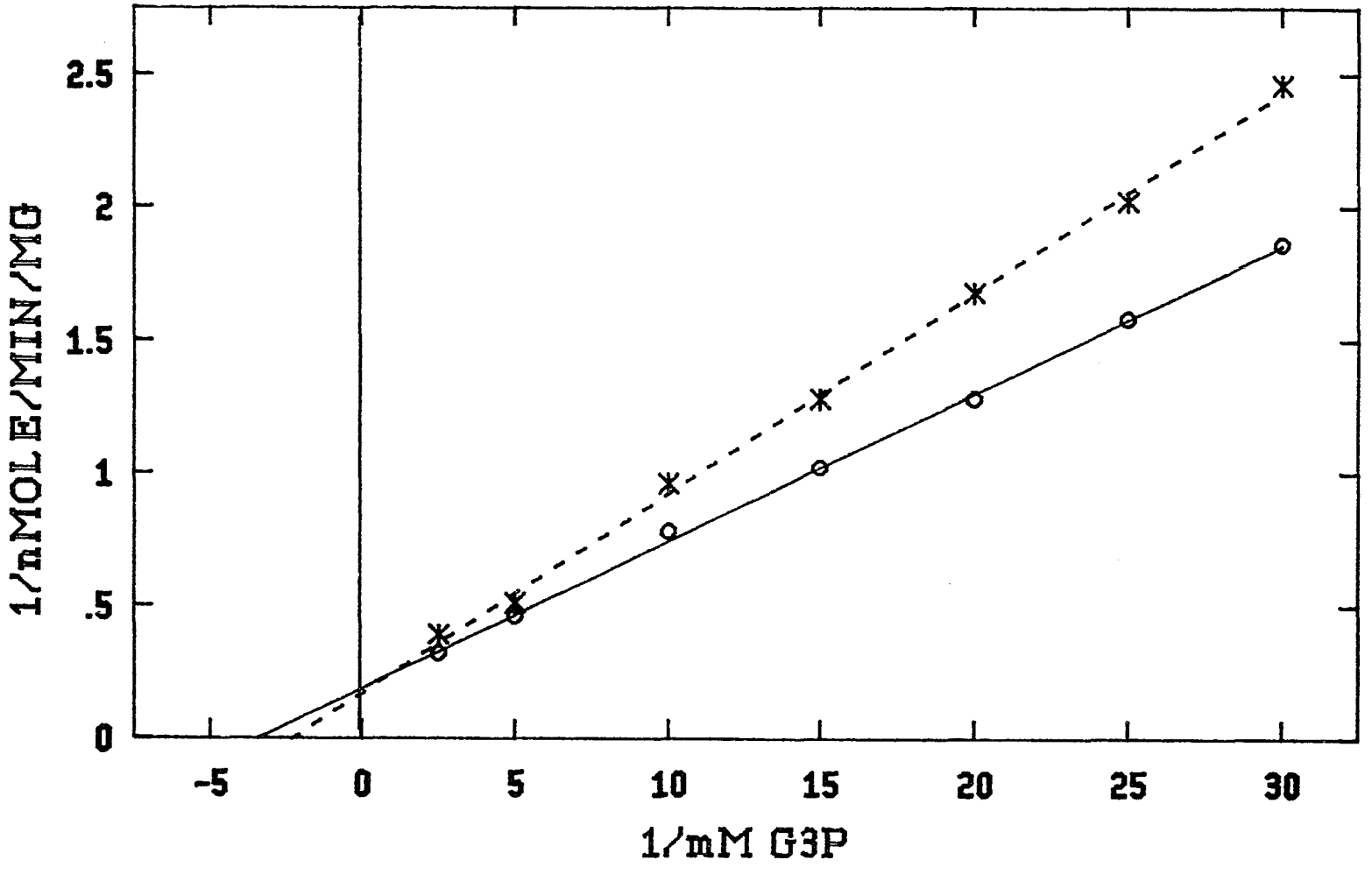
Figure 10



LEGEND TO FIGURE 11

Inhibition of microsomal sn-glycerol 3-phosphate acyltransferase activity by 2.5 mM (RS)-3-hydroxy-4-oxobutyl-1-phosphonate. The Lineweaver-Burk plot shows the effect of the inhibitor (dashed line) on the reaction rates compared to the uninhibited reaction (solid line), and suggests a competitive inhibition. Concentrations of sn-glycerol 3-phosphate (G3P) are from 33 to 400 µM.

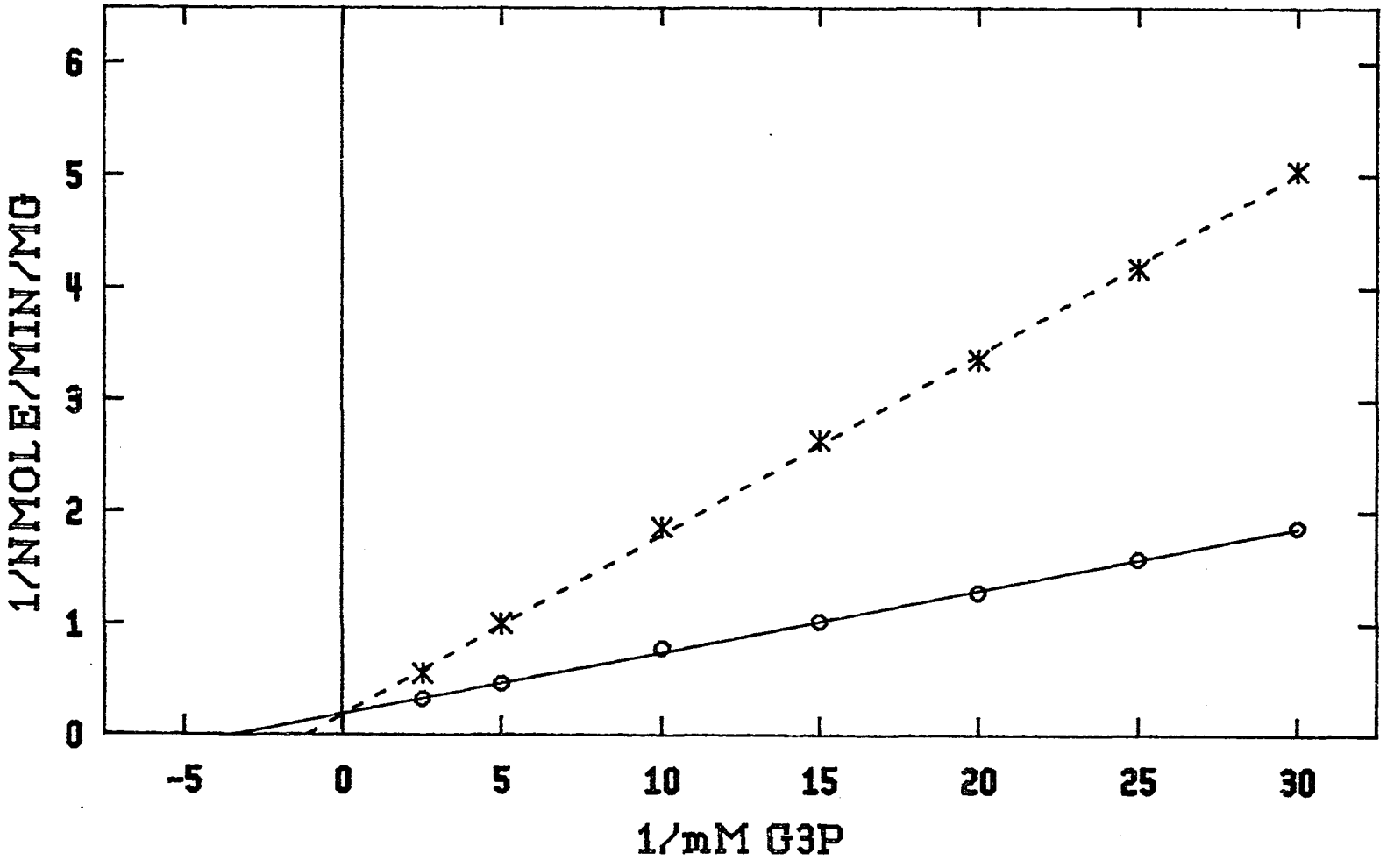
Figure 11



LEGEND TO FIGURE 12

Inhibition of microsomal sn-glycerol 3-phosphate acyltransferase activity by 2.5 mM (1S,3S)-1,3,4-trihydroxybutyl 1-phosphonate. The Lineweaver-Burk plot shows the effect of the inhibitor (dashed line) on the reaction rates compared to the uninhibited reaction (solid line), and suggests a competitive inhibition. Concentrations of sn-glycerol 3-phosphate (G3P) are from 33 to 400 μM.

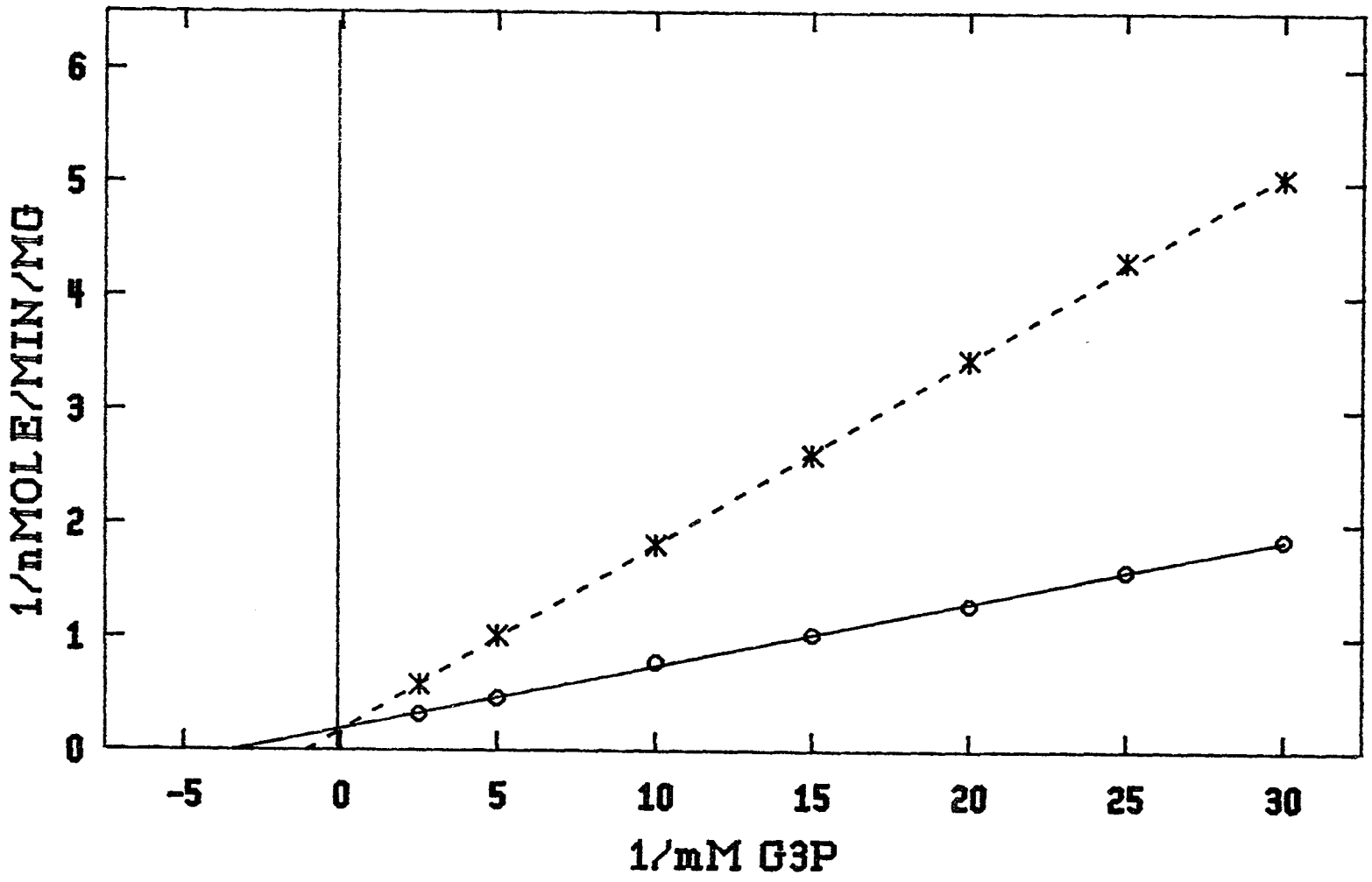
Figure 12



LEGEND TO FIGURE 13

Inhibition of microsomal sn-glycerol 3-phosphate acyltransferase activity by 2.5 mM (1R,3S)-1,3,4-trihydroxybutyl-1-phosphonate. The Lineweaver-Burk plot shows the effect of the inhibitor (dashed line) on the reaction rates compared to the uninhibited reaction (solid line), and suggests a competitive inhibition. Concentrations of sn-glycerol 3-phosphate (G3P) are from 33 to 400 μM.

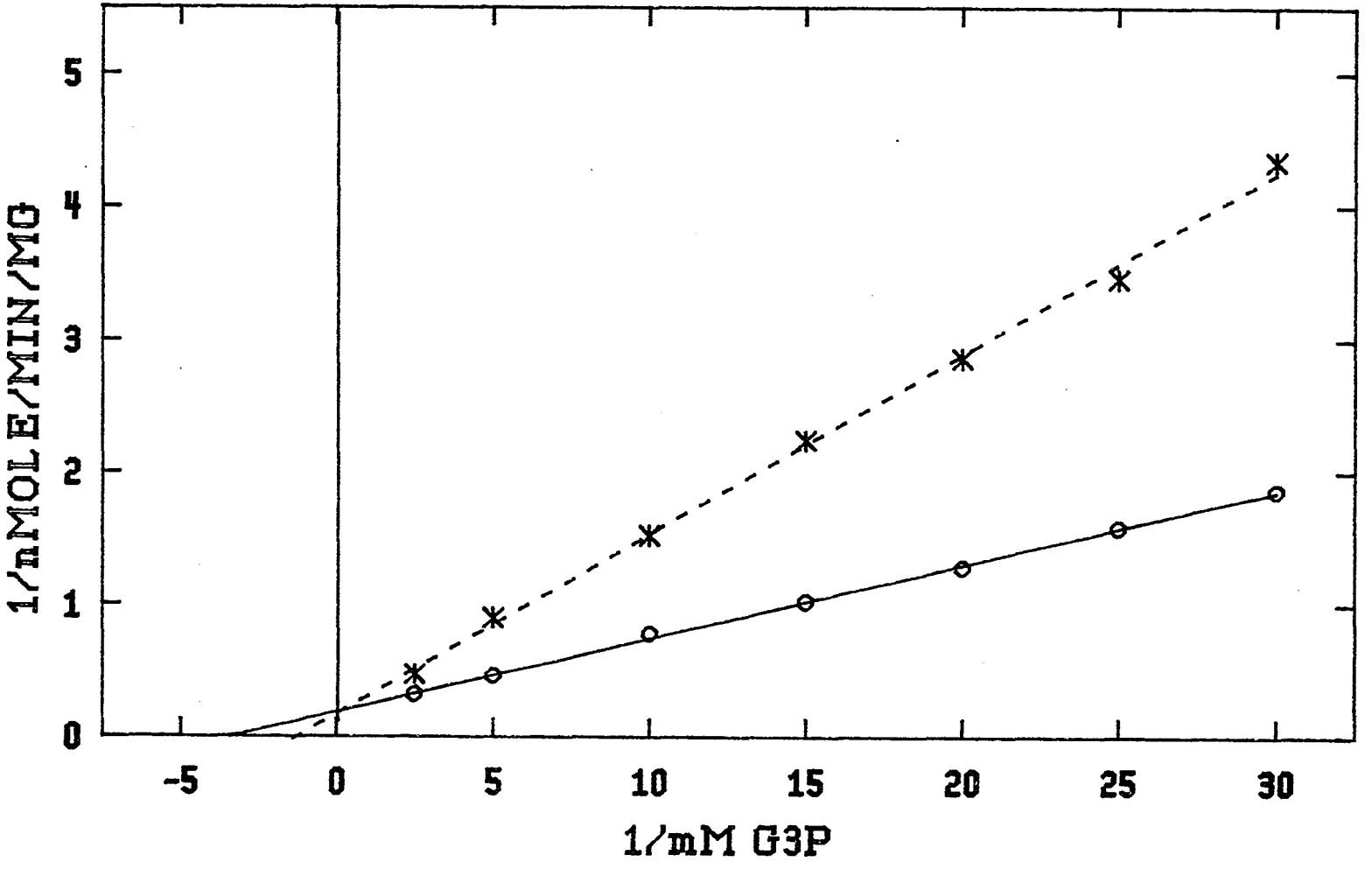
Figure 13



LEGEND TO FIGURE 14

Inhibition of microsomal sn-glycerol 3-phosphate acyltransferase activity by 8 mM 4-hydroxy-3-oxobutyl-1-phosphonate. The Lineweaver-Burk plot shows the effect of the inhibitor (dashed line) on the reaction rates compared to the uninhibited reaction (solid line), and suggests a competitive inhibition. Concentrations of sn-glycerol 3-phosphate (G3P) are from 33 to 400 μM.

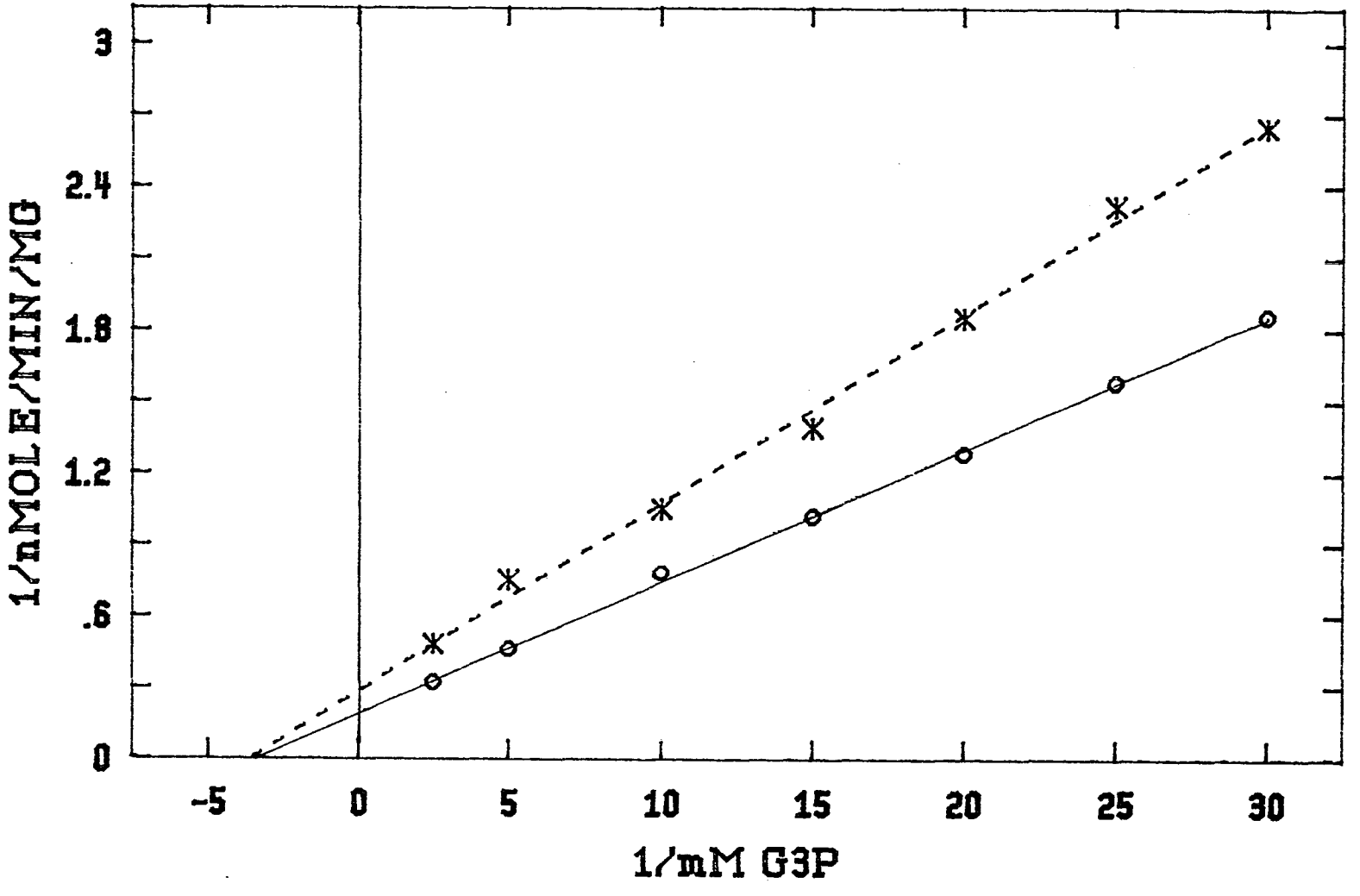
Figure 14



LEGEND TO FIGURE 15

Inhibition of microsomal sn-glycerol 3-phosphate acyltransferase activity by 4 mM phenethyl alcohol. The Lineweaver-Burk plot shows the effect of the inhibitor (dashed line) on the reaction rates compared to the uninhibited reaction (solid line), and suggests a noncompetitive inhibition. Concentrations of sn-glycerol 3-phosphate (G3P) are from 33 to 400 μM.

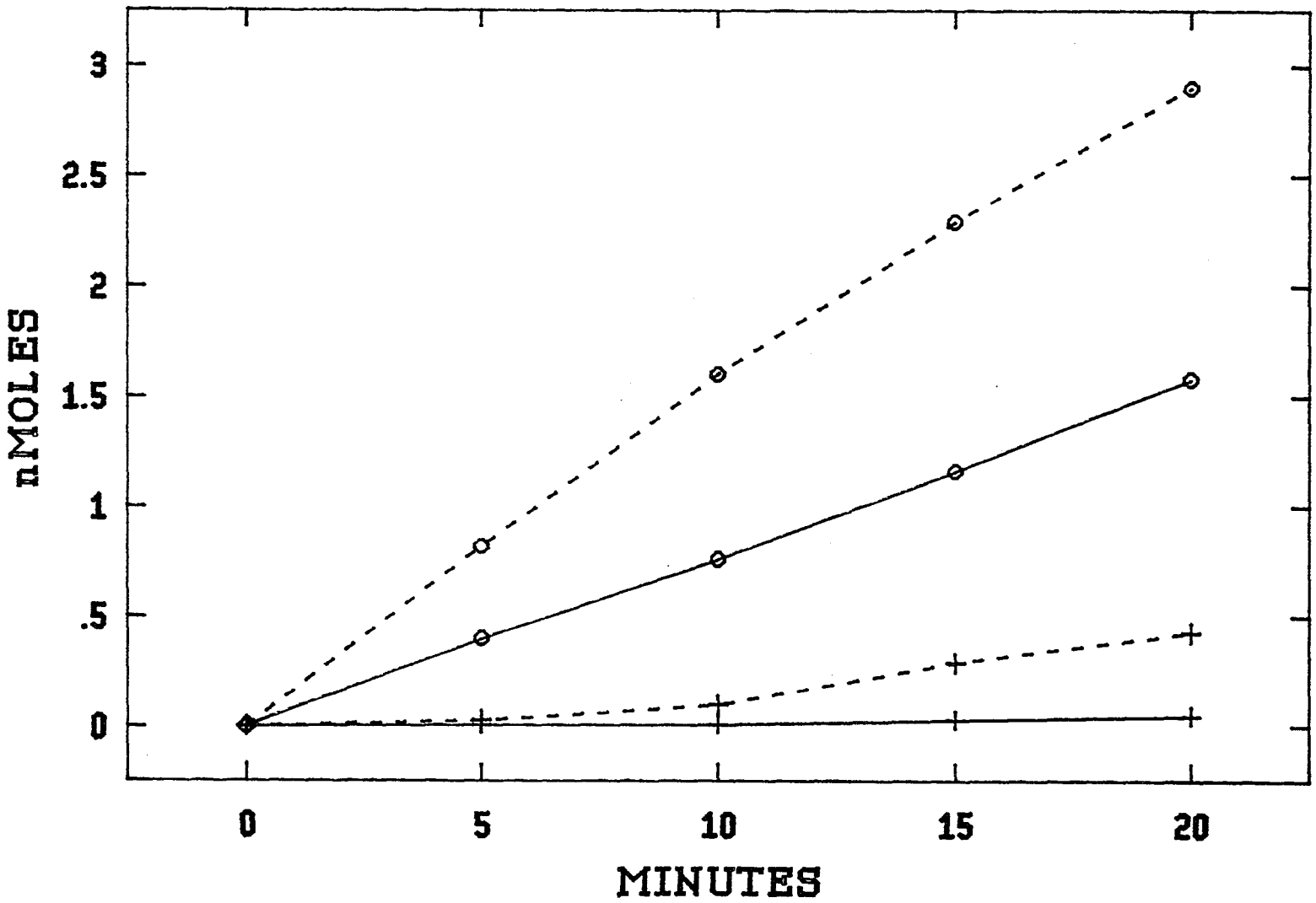
Figure 15



LEGEND TO FIGURE 16

Identification of acylation products of 3,4-dihydroxybutyl-1-phosphonate by mitochondrial and microsomal acyltransferase. The incubation mixtures contained 75 mM Tris-HCl, pH 7.4; 2 mM MgCl; 4 mM NaF; 2 mg bovine serum albumin; 80 µM palmitoyl CoA; 8 mM (RS)-3,4-dihydroxy-[3-^{3}H]-butyl-1-phosphonate, 29.6 Ci/mole; and 200 mg of mitochondrial or microsomal protein in a final volume of 1 ml. In addition mitochondrial reaction mixtures contained 1 mM KCN, and microsomal assays contained 1 mM dithiothreitol. At the indicated times 0.2 ml of the reaction mixture was removed and extracted as indicated in the Methods. The chloroform soluble acylation products were separated by thin layer chromatography as described in the Methods. The palmitoyl 1-monoacyl and diacyl derivative of DBP were identified. The amount of 4-palmitoyl-sn-3-hydroxybutyl-1-phosphonate (0) and 3,4-Dipalmitoyl-sn-butyl-1-phosphonate (*) which was synthesized in mitochrondrial (solid line) and microsomal (dashed line) reactions are plotted against time.

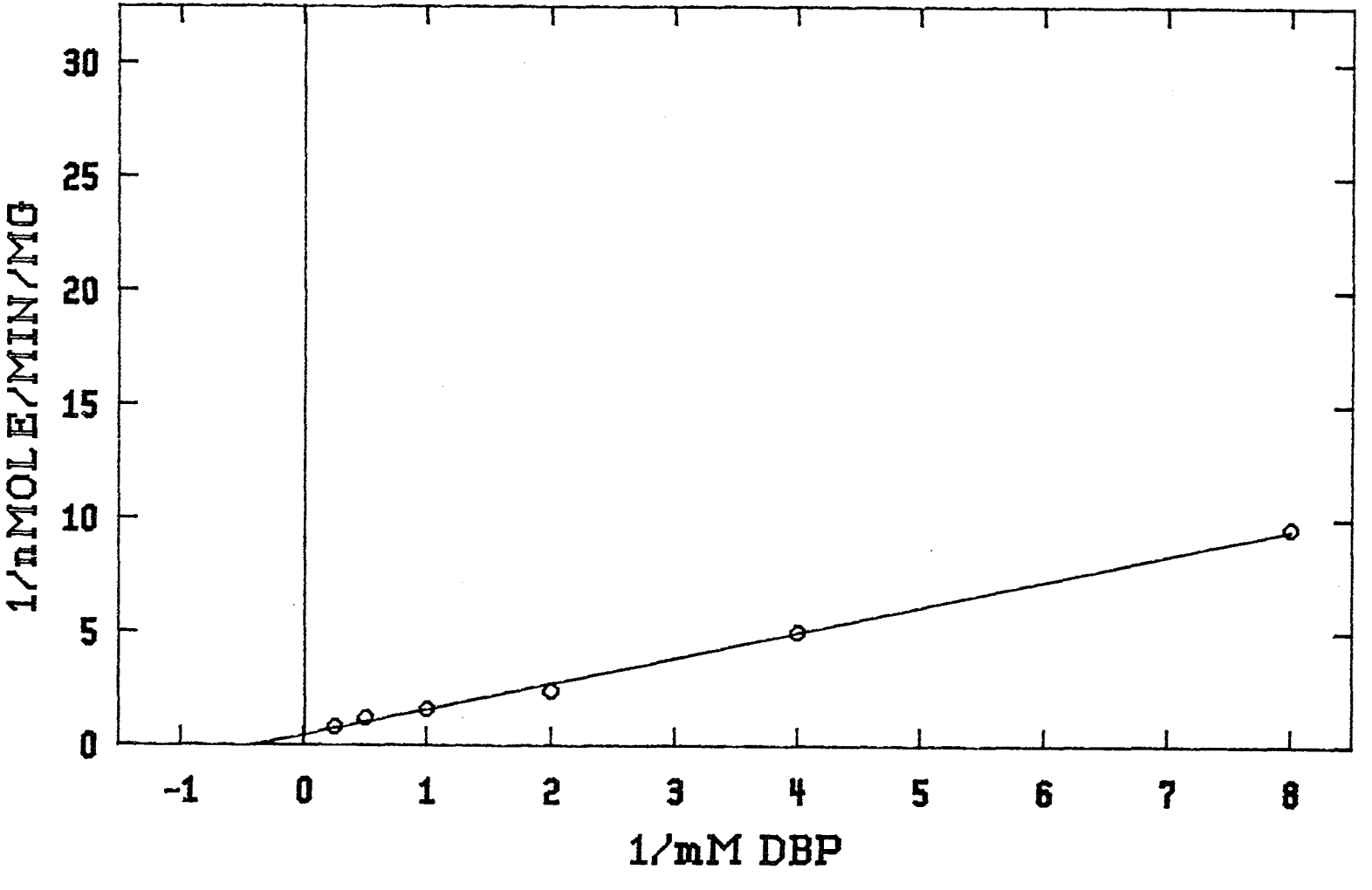
Figure 16



LEGEND TO FIGURE 17

Lineweaver-Burk plot of kinetic data from mitochondrial acylation reactions with (S)-3,4-dihydroxybutyl-1-phosphonate. The concentrations of (S)-3,4-dihydroxybutyl-1-phosphonate (DBP) are from 0.125 to 4.0 mM.

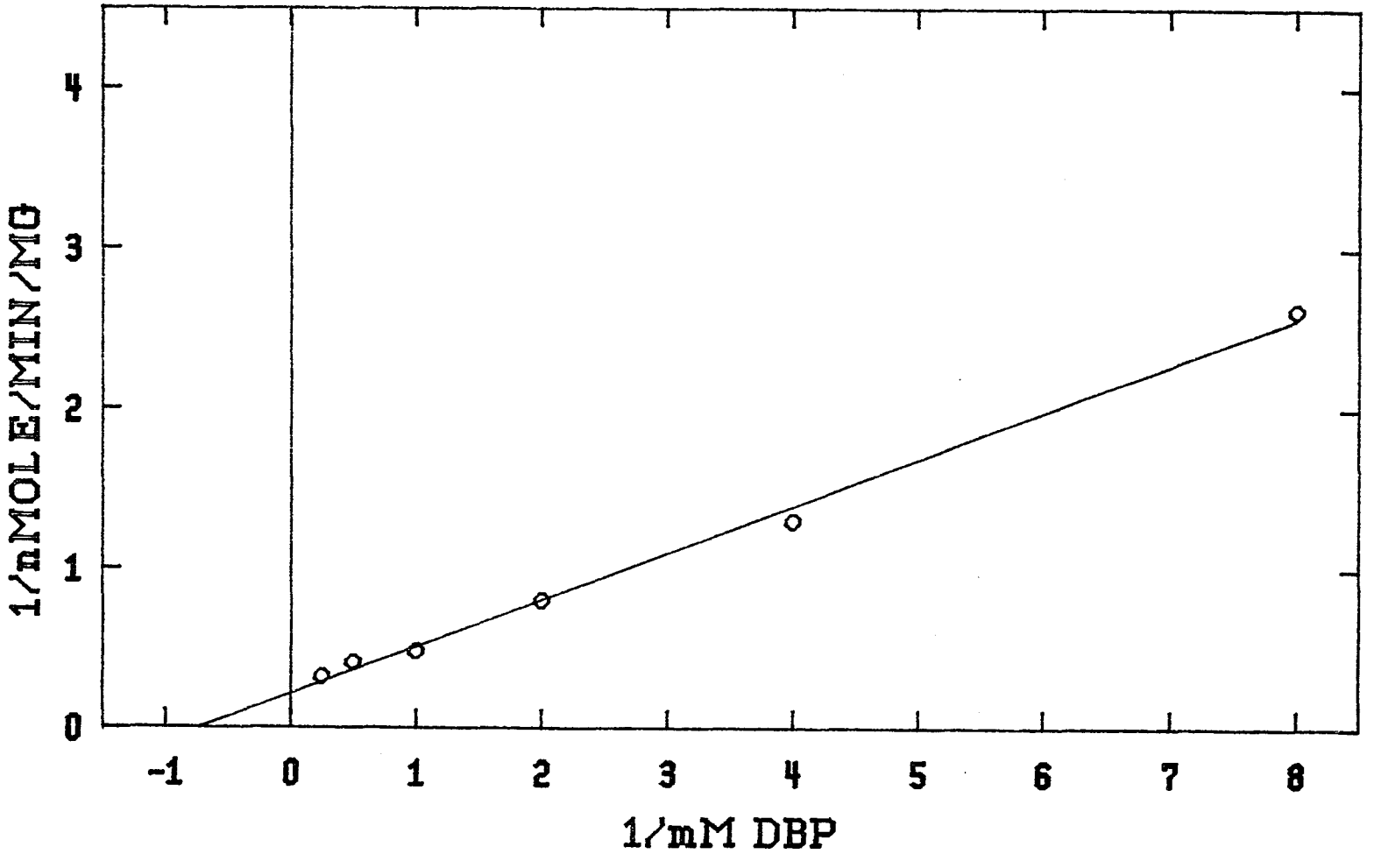
Figure 17



LEGEND TO FIGURE 18

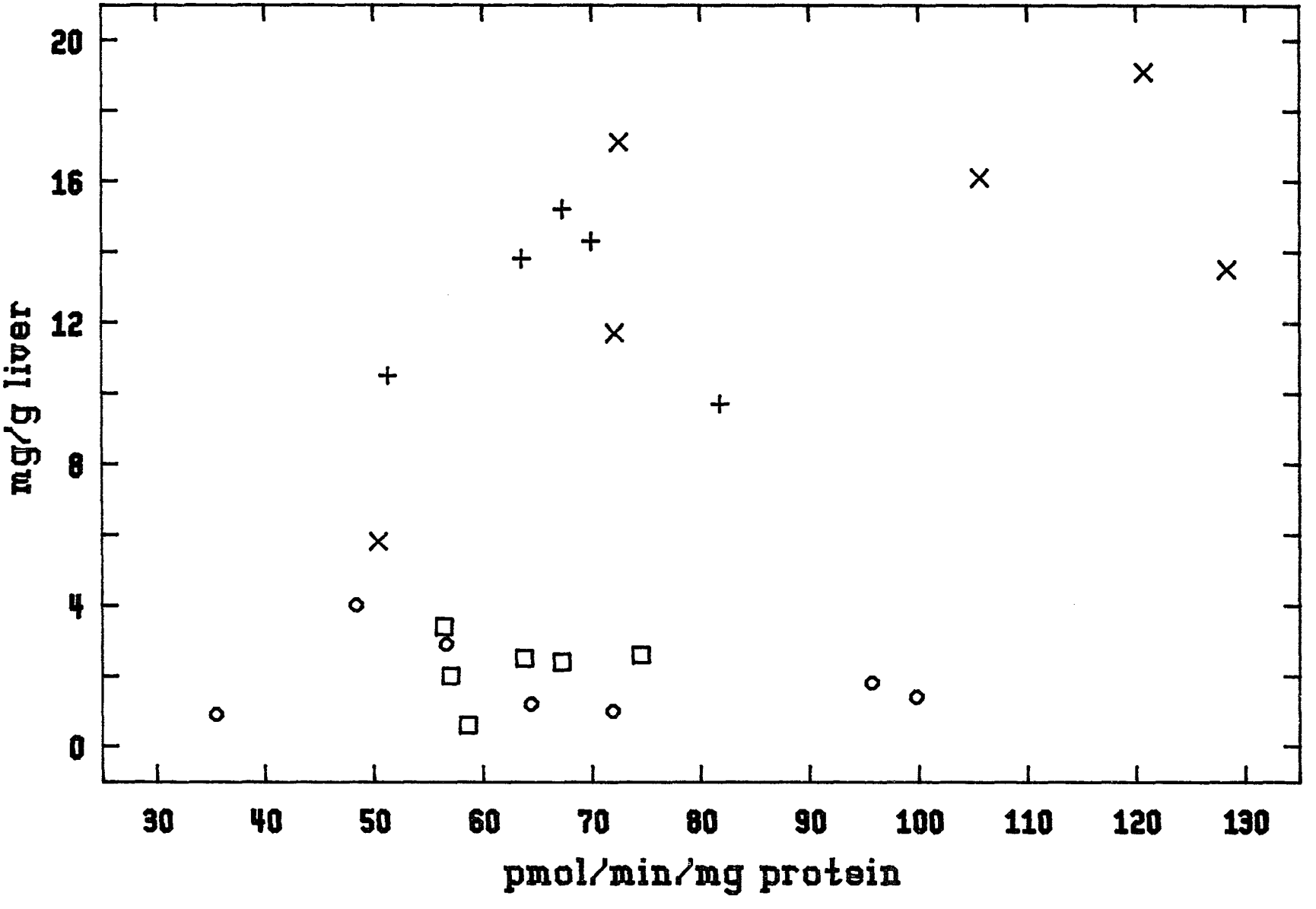
Lineweaver-Burk plot of kinetic data from microsomal acylation reactions with (S)-3,4-dihydroxybutyl-1-phosphonate. Concentrations of (S)-3,4-dihydroxybutyl-1-phosphonate (DBP) are from 0.125 to 4.0 mM.

Figure 18



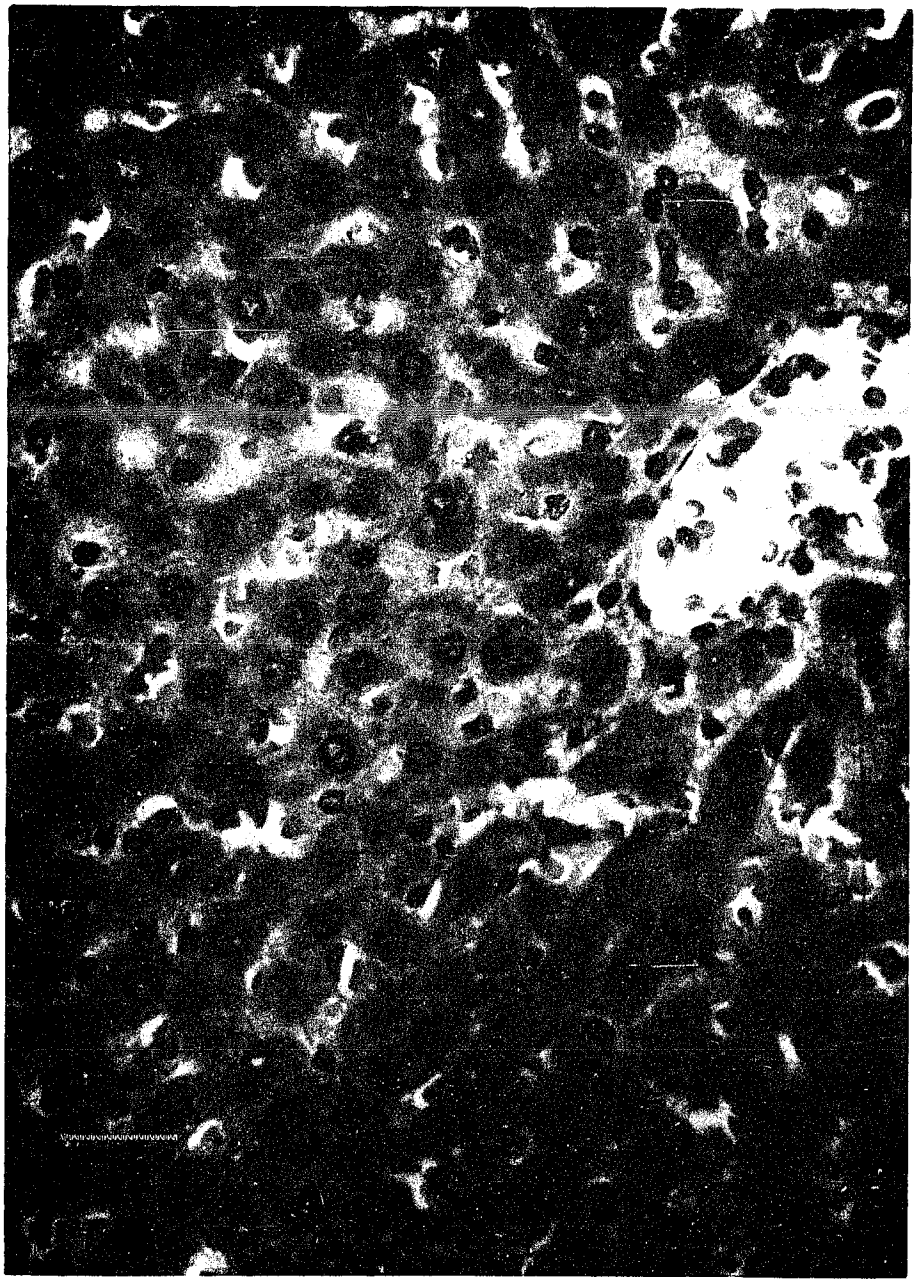
LEGEND TO FIGURE 19

Association between tissue triacylglycerol content and microsomal sn-glycerol 3-phosphate acyltransferase. Symbols used: □ , for 18 hour shams; o, for 24 hours shams; +, for 18 hour partial hepatectomies; and x, for 24 hour partial hepatectomy. The correlation coefficient (r) was 0.608, and was statistically significant, $p < 0.05$.


20
16
12
8
4
0
mg/g liver
30
40
50
60
70
80
90
100
110
120
130
pmol/min/mg protein

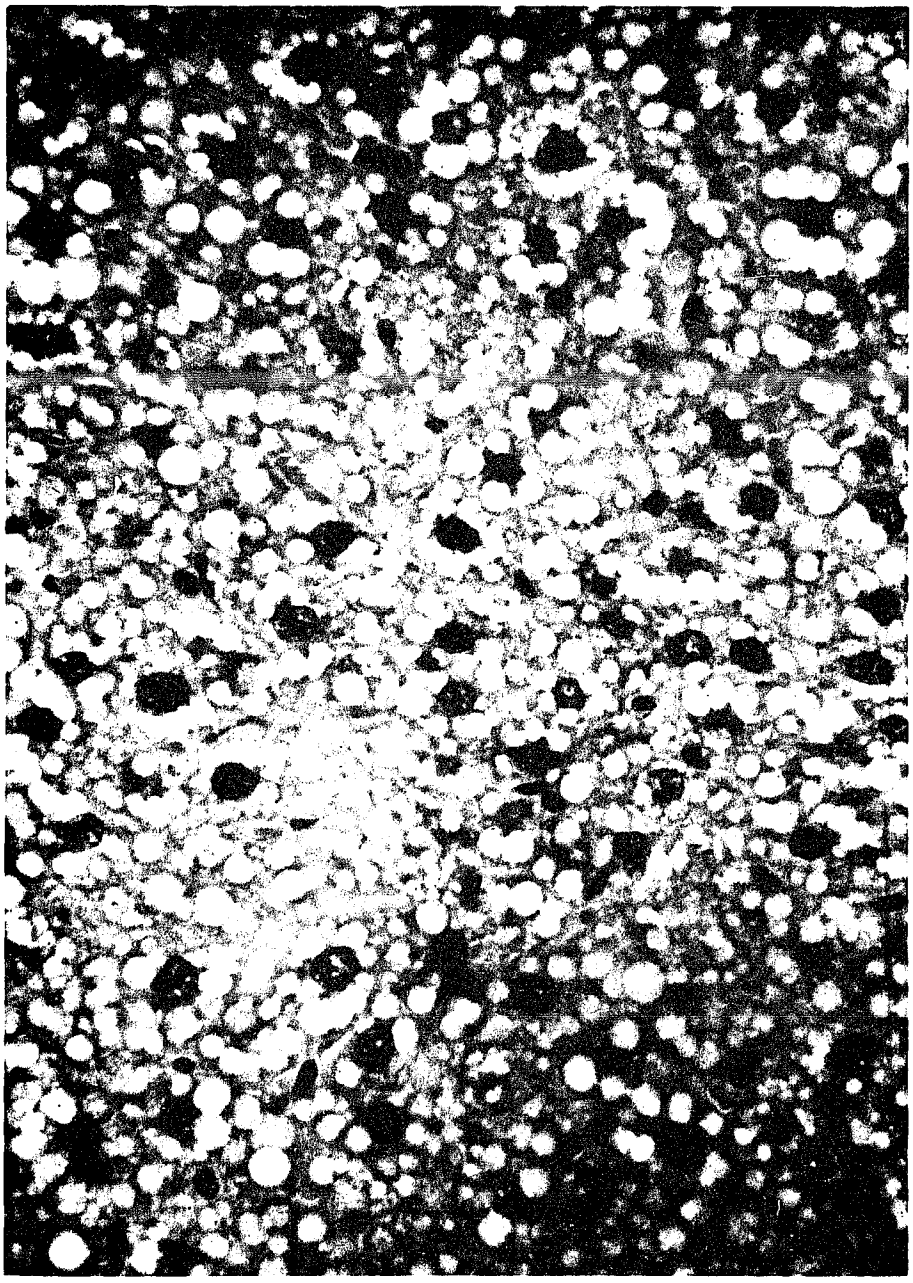
LEGEND TO FIGURE 20

Photomicrograph of the liver at 24 hours after sham operation. The magnification 450X, and the tissue stained with hematoxylin and eosin. Hepatocytes contain little cytoplasmic lipid and are arranged in radiating cords of cells. Sinusoids separate the cords of hepatocytes and contain erythrocytes.



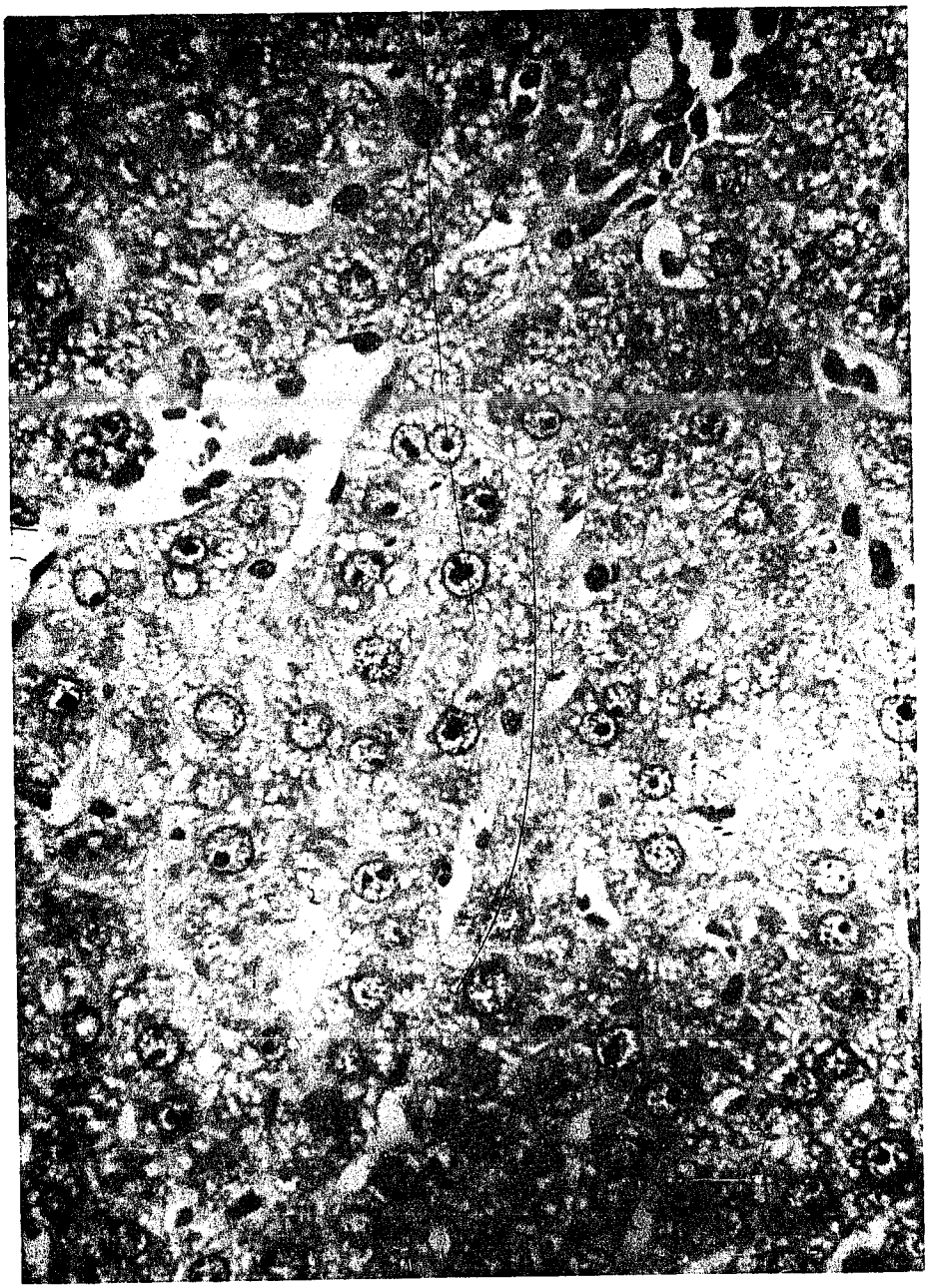
LEGEND TO FIGURE 21

Photomicrograph of the liver at 24 hours after partial hepatectomy. The magnification is 450X, and the tissue stained with hematoxylin and eosin. The cytoplasm of the hepatocytes is almost filled with large lipid vacuoles. The hepatocytes are hypertrophic, and the sinusoids are displaced.



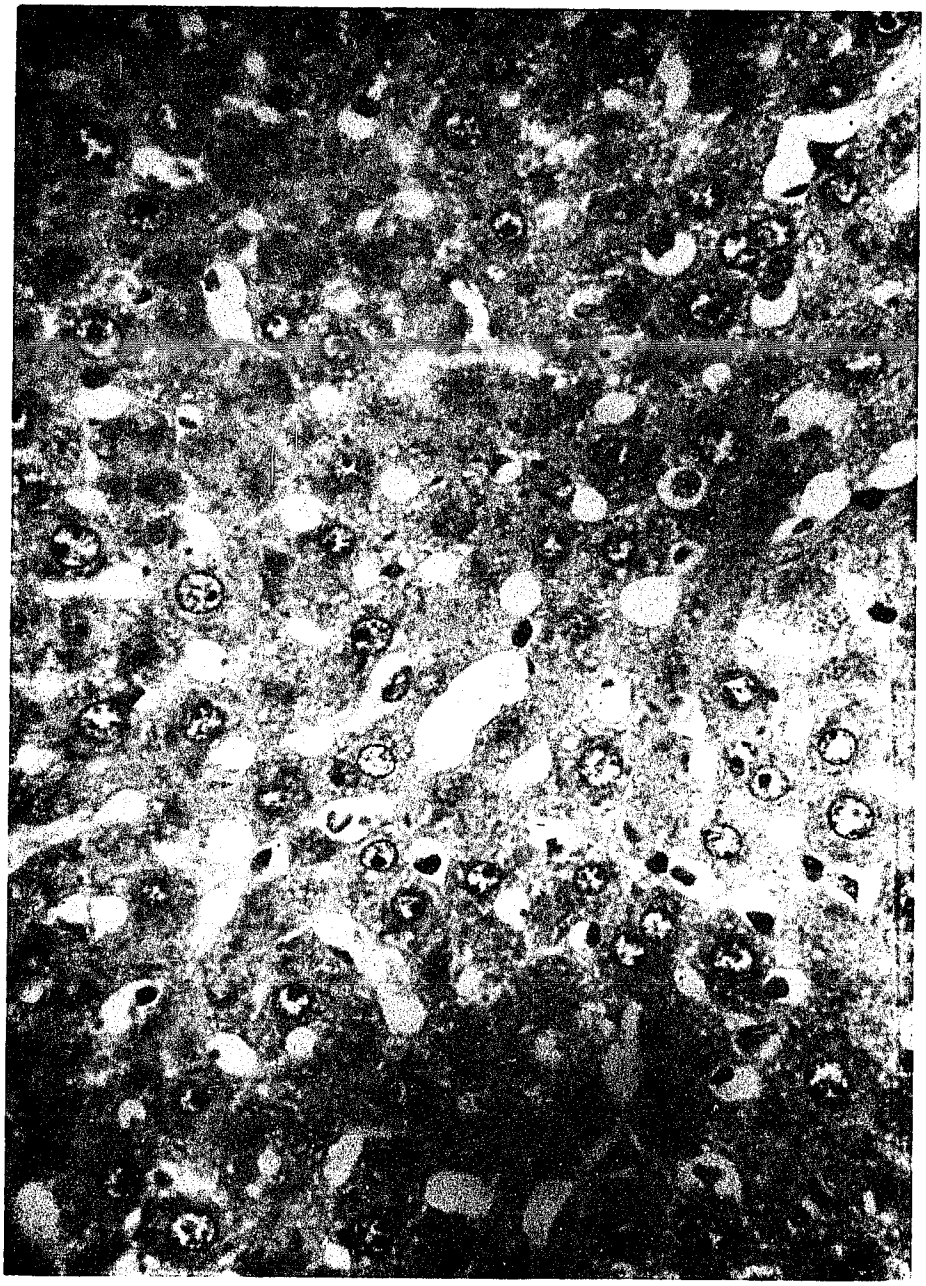
LEGEND TO FIGURE 22

Photomicrograph of the liver at 24 hours after partial hepatectomy and sodium chloride treatment. The magnification is 450X, and the tissue is stained with hematoxylin and eosin. The lipid vacuoles are smaller and diffusely dispersed in the cytoplasm. The hepatocytes are hypertrophic, and displace the sinusoids.



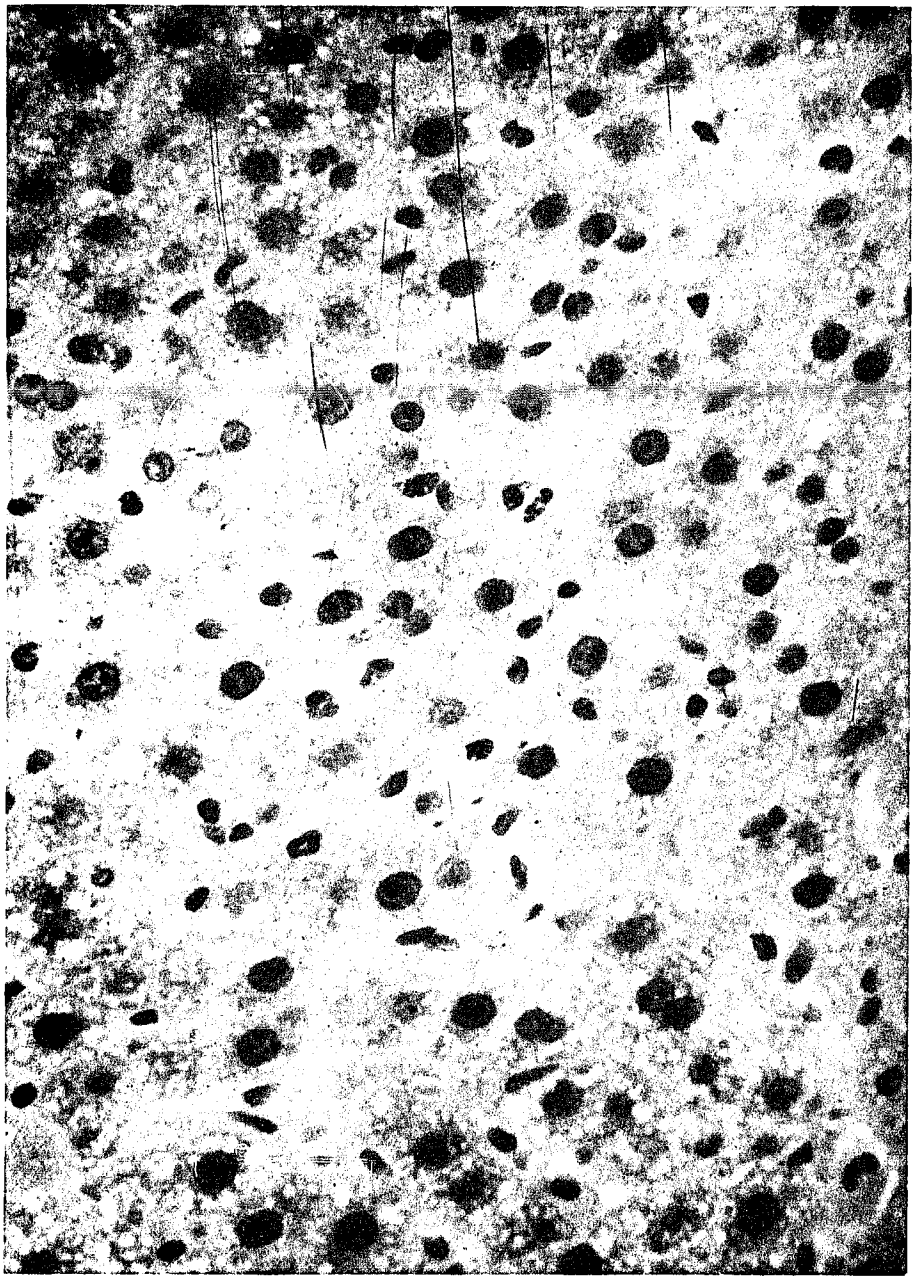
LEGEND TO FIGURE 23

Photomicrograph of the liver at 24 hours after partial hepatectomy and (RS)-3,4-dihydroxybutyl-1-phosphonate treatment. The magnification is 450X, and the tissue is stained with hematoxylin and eosin. The hepatocytes appear to be normal in size, and contain little lipid vacuoles. Sinusoids are visible, but the organization is well structured.



LEGEND TO FIGURE 24

Photomicrograph of the liver at 24 hours after partial hepatectomy and sn-glycerol 3-phosphate treatment. The magnification is 450X, and the tissue is stained with hematoxylin and eosin. The hepatocytes are hypertrophic and displace the sinusoids. The cytoplasm contains small, diffusely dispersed lipid vacuoles.



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