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The University of Michigan, Ph.D., 1975
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STUDIES ON

1-ALKYL-*sn*-GLYCERO-3-PHOSPHATE:ACYL-CoA ACYLTRANSFERASE

IN RAT BRAIN

by

Patrick John Fleming

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Biological Chemistry)
in The University of Michigan
1975

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LIST OF ABBREVIATIONS

All abbreviations not listed here are standard abbreviations as defined by the Journal of Biological Chemistry.

BBS-3	Beckman Bio-Solv 3
dimethyl POPOP	1,4-bis[2-(4-methyl-5-phenyl-oxazolyl)]benzene
EDTA	ethylenediamine-tetraacetic acid
(n-3), (n-6)	Position of the double bond nearest the methyl end of unsaturated fatty acids; where n equals the number of carbon atoms in the chain.
PPO	2,5-diphenyloxazole
rac	racemo (1)
radyl	Substituent of glycerol of unspecified nature
sn	Stereospecific numbering (1)

INTRODUCTION

Phospholipids are ubiquitous components of living cells. The bulk of the cellular phospholipids are contained in the membranes surrounding and within the cell. It is believed that these membrane phospholipids play both a general structural role and a more specific role as effectors of membrane protein function. The most widely accepted model of the membrane depicts a fluid bilayer of phospholipids with proteins of various shapes interspersed either partially or wholly within the bilayer (2). The major structural function of the phospholipids is thought to be that of providing a matrix in and on which the proteins can carry out their metabolic functions. The nature of this matrix is determined by the conglomerate physical characteristics of the individual lipids. The relevant characteristics include fluidity (2), lateral compressibility (3), and surface charge (4).

As more knowledge is gained about the functions of phospholipids in membranes it will be important also to know how the cell produces and controls the various populations of phospholipids. This requires a familiarity with the composition of each population and the individual metabolic steps leading to that population. To that end this dissertation is an investigation of one metabolic step in the synthesis of one class of phospholipids - the ether containing phosphoglycerides.

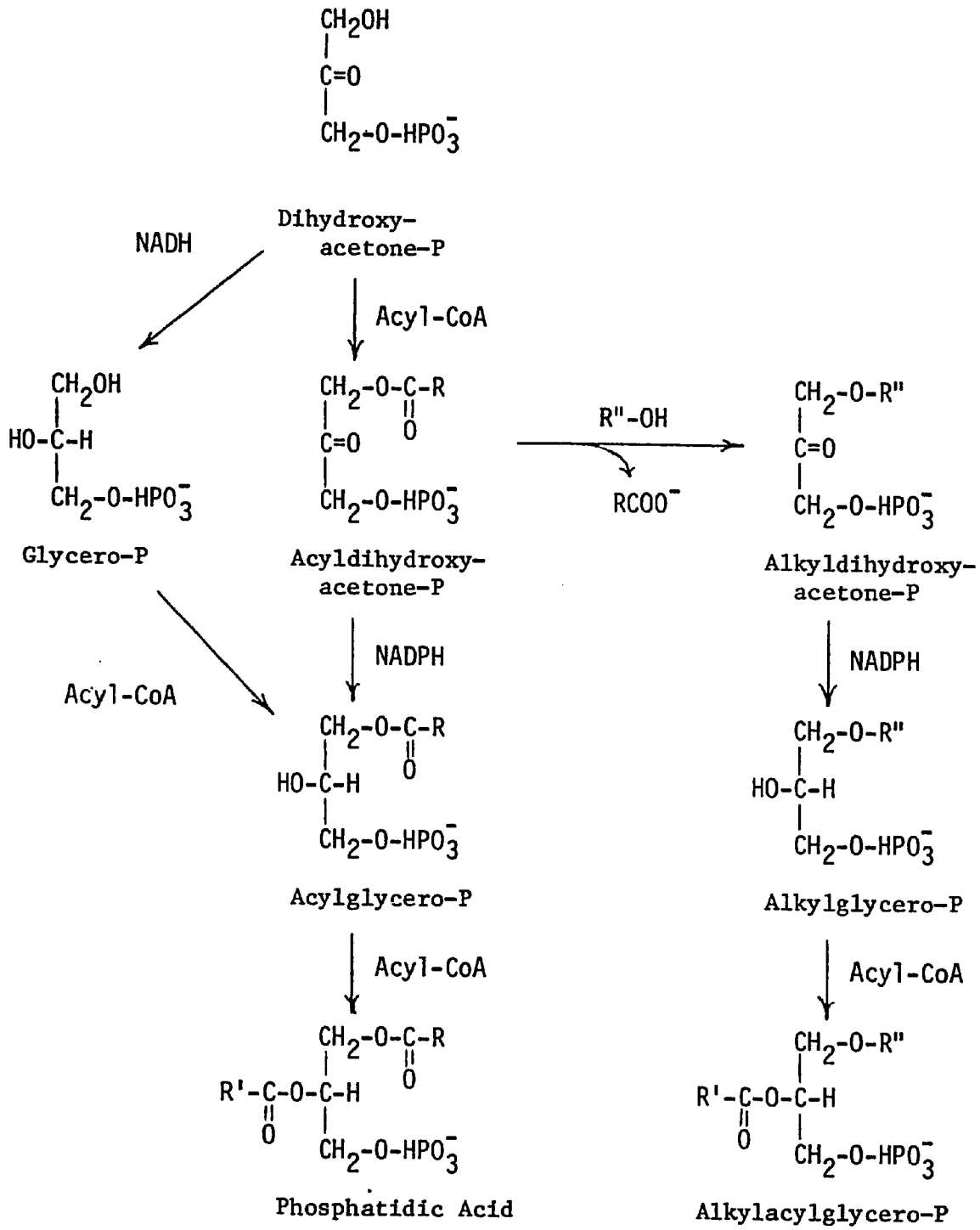
The dissertation research was a continuation of work by Hajra and coworkers on the ether containing phosphoglycerides which began with the discovery in 1969 that dihydroxyacetone phosphate was a precursor to the glycerol moiety of ether phosphoglycerides (5). Since that time

much work on the metabolism of the ether containing lipids has been done. Reports from this laboratory have described the first two enzymatic reactions involved in the biosynthesis of the ether containing phosphoglycerides, a) formation of the ether bond to produce alkyldihydroxyacetone phosphate (5,6) and b) reduction with NADPH to form 1-alkylglycero-3-phosphate (7,8). (See Scheme A on next page). But no detailed description of the enzyme which introduces the first fatty acyl group into the ether lipids had been reported. Therefore a detailed study of this enzyme, 1-alkyl-sn-glycero-3-phosphate:acyl-CoA acyltransferase, was undertaken.

Brain was chosen as the organ for study because the ether content of brain phosphoglycerides is high when compared to other organs (9-13). In addition, the mammalian brain exhibits a well defined increase in ether phosphoglyceride content (14), and the enzyme under study would be expected to play an important metabolic role during this time.

The goals of this research were 1) to describe the enzyme 1-alkyl-sn-glycero-3-phosphate:acyl-CoA acyltransferase in terms of its *in vitro* activity and substrate specificities, 2) investigate the *in vivo* distribution of the enzyme, and 3) address the question of whether this enzyme can control the final fatty acyl distribution of the ether containing phosphoglycerides in brain.

The following paragraphs of this introduction will review the basic biochemistry of lipids that is relevant to the understanding and interpretation of the dissertation work. Discussion of the phospholipids is limited for the most part to the simple phosphoglycerides and those containing choline and ethanolamine.


Dihydroxy-acetone-P
NADH
Acyl-CoA
Glycero-P
Acyldihydroxy-acetone-P
R"-OH
RCOO⁻
Alkyldihydroxy-acetone-P
NADPH
Acyl-CoA
Acylglycero-P
Alkylglycero-P
Acyl-CoA
Acyl-CoA
Phosphatidic Acid
Alkylacylglycero-P

Scheme A

A. Phosphoglyceride Metabolism Diacyl phosphoglycerides are now known to be biosynthesized from the precursor phosphatidic acid (1,2-diacyl-sn-glycero-3-phosphate). This lipid was first discovered in an extract of cabbage leaves in 1927 (15). Thirty-three years later it was isolated in chemical amounts from animal tissue by Hubscher (16). Even before this isolation, made difficult by the low metabolic steady state levels, the biosynthesis in animal tissues was being studied. Kornberg and Pricer published the primal paper on phosphoglyceride metabolism when they reported that phosphatidic acid was biosynthesized by transfer of the acyl group from two acyl-CoA donors to glycerophosphate (17). The original studies were done with particulate liver enzymes and the enzyme was later reported to exist in brain (18).

Lands and coworkers differentiated the enzyme responsible for the acyltransfer to the C-1 position of glycerophosphate from the C-2 acyltransferase by its sensitivity to sulfhydryl reagents (19). Subsequent studies have confirmed this differentiation and descriptions of the separate enzyme activities have been published. Glycerophosphate acyltransferase has been found to be active in the mitochondria and microsomes (20-23). Acylglycerophosphate acyltransferase activity is greatest in the endoplasmic reticulum as evidenced by the high specific activity in microsomes (24,25,26). However, it is clear that both acyltransferases are present in both mitochondrial and microsomal preparations (27-31). The substrate specificities and kinetic description of these acyltransferases will be discussed in detail below under "Acyltransferases".

The glycerophosphate acceptor for this biosynthetic pathway can

be produced de novo from dihydroxyacetone phosphate by enzymatic reduction with NADH (32). In addition, a mechanism for salvage of free glycerol exists through the action of a glycerol kinase (33). However, both of these enzymes exhibit low activity in some tissues, e.g. brain (34,35), which maintain a high metabolic rate of phospholipid biosynthesis. This paradox is eliminated by the existence of a second pathway for the biosynthesis of phosphatidic acid as postulated by Hajra and Agranoff in 1968 (36,39,41).

In this second pathway dihydroxyacetone phosphate is acylated directly as the initial step to form acyldihydroxyacetone phosphate (Scheme A). The acyltransferase catalyzing this reaction was first discovered in guinea pig liver mitochondria and microsomes (37) and has been shown to exist in other major organs of the rat (38). The acyldihydroxyacetone phosphate is enzymatically reduced by NADPH (39) and this oxidoreductase has been described in detail (8). Acylation of the resulting 1-acylglycero-3-phosphate may then proceed (40) to form phosphatidic acid. The complete series of reactions for this second pathway was demonstrated with in vitro incubation of guinea pig liver particulate fractions plus dihydroxyacetone phosphate, acyl-CoA, and NADPH (41).

Since the acyldihydroxyacetone phosphate pathway is active in tissues such as brain which may have a low capacity to form glycerophosphate, it was of interest to determine the relative importance of each pathway in different tissues. In the glycerophosphate pathway, dihydroxyacetone phosphate is reduced by NADH; whereas in the acyldihydroxyacetone phosphate pathway, NADPH is used. This difference was utilized (42) to investigate the relative role of each pathway in mouse

liver homogenates and Ehrlich ascites tumor cells. ^{3}H-Labeled NADH or NADPH was used in incubation mixtures with glucose or dihydroxyacetone phosphate, and the ^{3}H incorporated into the glycerol moiety of labeled lipid was determined. The results showed that the acyldihydroxyacetone phosphate pathway was significant in liver and dominant in the tumor cells (42). This correlated with the low level of glycerophosphate:NAD oxidoreductase in tumor cells.

From the results reported by Okuyama and Lands (43) one could question the importance of the acyldihydroxyacetone phosphate pathway in liver slices. They used a mixture of ^{14}C-labeled and ^{3}H-labeled glycerol with the ^{3}H in the C-2 position. After incubation with liver slices the resulting labeled lipids showed an increase in the $^{3}H/^{14}C$ ratio when compared to the starting glycerol mixture. This is the opposite result expected if the acyldihydroxyacetone phosphate pathway were being used at all, because ^{3}H would be lost in the formation of dihydroxyacetone-P. However, it was later shown that the [2-^{3}H]glycero-3-phosphate is protected by an isotope effect on the mitochondrial glycerophosphate dehydrogenase (EC 1.1.99.5). Thus the pool of labeled glycerol would gradually be enriched in ^{3}H. When this was taken into consideration in experiments by Manning and Brindley, the data showed that up to 60 percent of the lipid synthesis in the liver slices was by way of acyldihydroxyacetone phosphate (44). Their experimental design was to incubate rat liver slices with a mixture of sn-[2-^{3}H]glycerol and sn-[1-^{14}C]glycerol and then determine the $^{3}H/^{14}C$ ratio in the isolated glycero-3-phosphate and glycerolipid. This permitted calculation of the ^{3}H enrichment in the glycerophosphate pool. Their data showed a more rapid increase

in the $^{3}H/^{14}C$ ratio in glycerophosphate than in glycerolipid. It was from this difference that they calculated a major involvement of the second pathway in glycerolipid synthesis. Similar recent studies by Rognstadt et al (45) with isolated rat liver cells showed that the $^{3}H/^{14}C$ ratios of glycerophosphate and glycerolipid became different only after the labeled glycerol precursor was substantially depleted. These workers concluded that glycerolipid was being synthesized directly from glycerophosphate with little involvement of dihydroxyacetone phosphate. Their results pointed out the difficulties in controlling all variables in these metabolic studies, and made any conclusion based on this approach equivocal. A different approach to this problem, which overcomes some of the difficulties associated with varying metabolic flux of radioactive tracers, was reported by Curstedt and Sjovall (45a). They administered [1,1-$^{2}H_2$]ethanol to rats and determined the deuterium incorporation in bile phosphatidylcholine. Such incorporation was assumed to be either directly or indirectly from [^{2}H]-NADH, which was labeled during oxidation of [1,1-$^{2}H_2$]-ethanol. The amount of labeled ethanol administered to the rats was sufficient to substantially label the metabolic pools which had access to the reduced pyridine nucleotide. Their data showed that the C-2 glycerol position of phosphatidylcholine contained a greater amount of deuterium than the C-2 of total endogenous glycerophosphate. The authors concluded that a separate pool of glycerophosphate was being used for lipid synthesis. In addition, they stated that the labeling of the C-2 position could not be due to reduction of acyl-dihydroxyacetone phosphate because the intramitochondrial NADPH would not be significantly labeled and they evidently assumed that the

acyldihydroxyacetone phosphate reduction only occurred in mitochondria. This latter assumption is wrong and, in fact, their data connot be used to differentiate the relative utilization of either pathway or, for that matter, to differentiate between a compartmentalization of glycerophophate or NAD(P)H. Other studies with isolated rat hepatocytes show that dihydroxyacetone and glycerol are incorporated into phospholipid to the same extent but that dihydroxyacetone is a better precursor for triglycerides (46). If the cultured hepatocytes were incubated with [1,3-^{3}H]glycerol and [U-^{14}C]dihydroxyacetone plus fatty acids, the labels were equally incorporated into phosphatidylcholines and phosphatidylethanolamines. The incorporation of ^{14}C into triglycerides was approximately 50 percent greater than incorporation of ^{3}H. This indicates that there may be a functional separation of the pathways, but further studies will be necessary to determine the relative involvement of each pathway under normal in vivo circumstances.

Not all phosphatidic acid is synthesized de novo by acylation of glycerophosphate or dihydroxyacetone phosphate. Diglyceride may be phosphorylated by a kinase using ATP (47). A kinase which accepts monoglyceride has also been described (48), and the resulting monoacylglycerophosphate could then be acylated to form phosphatic acid as outlined above. The neutral mono- and diglycerides used in these reactions could derive from endogenous lipid catabolism or from newly ingested lipids by way of intestinal absorption.

The precursor phosphatidic acid is converted into the phosphoglycerides by two basic mechanisms. The first is dephosphorylation and condensation of the resulting diglyceride with an activated base. This series of reactions was first demonstrated by Kennedy and Weiss

with CDP-choline (48), and later with CDP-ethanolamine (50). The reactions have also been shown to occur in brain (51,52). In liver (53), but not in brain (54), phosphatidylethanolamine can be converted into phosphatidylcholine by stepwise methylation.

The phosphoglycerides such as the phosphatidylinositols and phosphatidylglycerols are formed by a different mechanism from phosphatidic acid. In these cases, phosphatidic acid is activated with a cytidine dinucleotide to form CDP-diglyceride and this is condensed with the inositol (55,56) or glycerol (57) alcohol.

B. Ether containing phosphoglycerides The phosphoglycerides containing an ether bond are comprised of two main classes. One type contains a glycerylalkyl ether where the alkyl chain is saturated between the C-1 and C-2 positions. This will be called an alkylphosphoglyceride. The second type is a glycerylalkyl ether with a double bond between C-1 and C-2 of the alkyl chain forming a vinyl ether linkage (1-alk-1'-enylglyceryl ether). This type is commonly called a plasmalogen but will be referred to as an alkenylphosphoglyceride. Both types may have double bonds in other positions of the alkyl chain, and in both the ether bond is with the C-1 of the glycerol moiety.

The alkylglycerides were first isolated from neutral fish oils in 1915 (60). After saponification of total lipid extract, an ether-soluble alcohol was found, although at that time its structure was unknown. Similarly, the unsaponifiable lipid from the liver oil of elasmobranch fishes was being studied by Japanese workers in the 1920's. Toyama, who together with Tsujimoto determined that the unsaponifiable alcohol contained two hydroxy groups, suggested that

these alcohols were condensation products of long chain alcohols and glycerol (61). Proof that these alcohols contained an ether bond came in 1928 when Heilbron and Owens (62) cleaved the ether with hydroiodic acid to obtain an alkyl iodide. Davies et al showed that the ether bond was on the C-1 (or C-3) of glycerol by cleaving the diol (alkylglyceryl ether) with lead tetraacetate to yield a glycoaldehyde and formaldehyde (63).

Early studies on the alkyl ether lipids were done with neutral fish oil, and it was shown that the natural form of the glyceryl ethers was a triglyceride (64,64a). In his comprehensive study on brain lipids, Brante discovered an alkali-stable phospholipid fraction and called it "cephalin B" (65). Soon thereafter, these lipids were found in other mammalian tissues and were eventually identified as ethanolamine derivatives of a glyceryl ether. The reader is referred to a recent monograph on the ether lipids for a detailed history of these compounds (58).

The alkenyl phosphoglycerides were discovered in 1924 by Robert Feulgen (58). The original histological observation was that an aldehyde-producing substance was present in the cytoplasm of many cells (see ref. 58). Hence the name "plasmalogen". It was later shown that brain contained large amounts of this aldehydogenic substance (59).

Elucidation of the structure of the alkenylethanolamine phosphoglyceride was essentially arrived at independently by Rapport (66) and Klenk and Debuch (59,67). But it required further clarification to establish that the ether bond was on the C-1 position of the glycerol moiety (68,69). At about the same time (1958), Carter published the structure of alkylethanolamine phosphoglyceride (70).

The majority of alkyl and alkenyl chains in the ether-containing lipids are 16 or 18 carbons in length. In many tissues, including brain, the 18 carbon chain has a double bond in the $\Delta 9$ position (71,71a,71b).

The alkenyl phosphoglycerides are found in most animal tissues (72). The highest levels ($>$ 20 percent of phospholipids) are in spermatozoa, brain, heart muscle, and skeletal muscle. Moderate levels (10-20 percent) exist in the adrenal glands, kidney, spleen, bone marrow, testes, and erythrocytes (71,73). When present, the amounts of alkyl phosphoglycerides (2-10 percent of phosphoglycerides) are much less than the alkenyls, but the proportional tissue distribution is similar. Many tumors have also been shown to contain relatively high levels of ether containing lipids (74).

The specific purpose of the ether containing phosphoglycerides in the cell is unknown. Comparisons of the physical properties of membranes made with diester or ether phosphoglycerides have given conflicting results (75,76). Enzyme hydrolysis rates with the ether phosphoglycerides (both alkyl- and alkenyl-) as substrates are usually lower than corresponding rates with diester phosphoglycerides (77,185), and this lessened reactivity to hydrolysis may be an important functional characteristic. Synthetic enzymic reaction rates with ether phosphoglycerides have, in at least one case, been shown to be similar to the acyl phosphoglycerides [CDP-choline:diglyceride cholinephosphotransferase, (121)], and in other cases to be slower [NADPH:alkyldihydroxyacetone phosphate oxidoreductase, (8) and alkyl- or alkenylglycerophosphorylcholine acyltransferase, (185,189)].

Studies with the dogfish (78,79) and the starfish (80) provided the first evidence that the alkyl chain precursor for the ether

lipids was long chain alcohol. The glycerol source was unknown, although the data suggested it was a triose phosphate (79). Ether phosphoglyceride formation was demonstrated in vitro in 1969. Snyder and coworkers reported that glyceraldehyde-3-phosphate plus long chain alcohol with ATP(ADP), and CoA were the necessary substrates and cofactors for ether bond formation (81). Hajra reported that dihydroxyacetone phosphate was the phosphorylated substrate and that long chain alcohol, ATP, and CoA were necessary (5). Both laboratories reported that alkyldihydroxyacetone phosphate was the product. It was subsequently confirmed that dihydroxyacetone phosphate was the direct precursor (6,82,83). In addition, Hajra showed that the requirement for ATP and CoA was to allow formation of acyldihydroxyacetone phosphate as an intermediate (6). The evidence for this was that acyldihydroxyacetone phosphate was a more efficient in vitro precursor than dihydroxyacetone phosphate, and that CoA was not necessary when acyldihydroxyacetone phosphate was used. Thus, the ether bond formation apparently involved displacement of the acyl group from acyldihydroxyacetone phosphate by the long chain alchol (Scheme A). Confirmatory evidence for this mechanism has been published (84,85) and it has been established that dihydroxyacetone phosphate stereospecifically exchanges the pro-R hydrogen on C-1 with water during the formation of alkyldihydroxyacetone phosphate (84,86,87,88). This was first demonstrated through the loss of tritium from [1,3-$^{3}H_{2}$]-dihydroxyacetone phosphate during ether bond formation (86). Subsequent studies have proven that this exchange occurs when the alcohol displaces the acyl group (84) and that the exchange is stereospecific (87).

After the metabolic role of long chain alcohols was discovered,

their content in various tissues was determined, and brain and heart were found to contain low but significant quantities of these alcohols (89,90). Evidence has now been published that interconversion of long chain alcohols, aldehydes, and carboxylic acids occurs in various tissues and that the pyridine nucleotides function as the electron donors and acceptors in these reactions (91,92,93).

Reduction of alkyldihydroxyacetone phosphate, the first ether containing phosphoglyceride in the biosynthetic pathway, is catalyzed by the same NADPH reductase acting on acyldihydroxyacetone phosphate (7,8) which was discussed above. This reduction produces 1-alkyl-sn-glycero-3-phosphate, the substrate for the enzyme with which this dissertation is concerned. The reductase has a wide tissue distribution (38) and is present in the subcellular fractions used during this work on alkylglycero-P acyltransferase. This fact permitted experiments in which the acyltransferase substrate, 1-alkyl-sn-glycero-3-phosphate was made *in situ* during the enzyme incubation.

In his original report on the *in vitro* formation of ether phosphoglycerides, Hajra presented evidence to suggest that 1-alkylglycero--3-phosphate could be acylated to form 1-alkyl-2-acylglycero-3-phosphate (5). Subsequent studies by Wykle *et al* have confirmed the *in vitro* formation of 1-alkyl-2-acylglycero-3-phosphate during incubation of ascites tumor cell microsomes with the proper cofactors (85,94). Recently this ether phosphoglyceride was shown to be formed during incubations with rabbit harderian gland microsomes (95). In all of these studies, free fatty acids, either added or present in the enzyme preparation, were assumed to be activated by CoA before transfer to the phosphoglyceride.

1-Alkyl-2-acylglycero-3-phosphate is the precursor to all the ether containing phosphoglycerides and plays a pivotal metabolic role anaologous to that of phosphatidic acid, as discussed above for the diester phosphoglycerides. A phosphatase forming 1-alkyl-2-acylglycerol has been described (94), as well as choline and ethanolamine phosphotransferases (96,97) which use this alcohol as acceptor. In addition, neutral ether-containing lipids have been shown to be biosynthesized by acylation of alkylacylglycerols (98,99,100).

Early investigations on the origin of the alkenyl glycerolipids in the slug and dogfish suggested that they were derived from the alkyl glycerolipids (101,102). This precursor-product relationship was strengthened by a number of ^{3}H and ^{14}C tracer studies *in vivo* (103-109) and in tissue culture (110). Similar confirmatory evidence was obtained using ^{18}O-labeled long chain alcohol for experiments *in vivo* and *in vitro* which also demonstrated that alkylglyceryl ethers are precursors of alkenylglyceryl ethers (111,112). The labeled oxygen is incorporated into alkyl ethers preceding incorporation into alkenyl ethers in a classical precursor-product relationship. The desaturation reaction was shown to be a *cis* elimination by injection of stereospecifically labeled [1-^{3}H]-[1-^{14}C]hexadecanol and [2-^{3}H]-[1-^{14}C]hexadecanol into rat brain and analysis of the alkenyl glycerolipids formed (113). The enzyme responsible for the desaturation was first studied with small intestine (114), and later with tumor and brain particulate fractions (115,116,117). 1-Alkyl-2-acylglycero-3-phosphorylethanolamine was found to be the substrate for the desaturase, and NADPH and O_2 were necessary for maximal activity. The enzyme is inhibited by KCN (114,117) and requires cytochrome

b_5 (118), but does not seem to be identical to the fatty acid desaturase in microsomes (119). The evidence for this non-identity is that the activity of the ether phosphoglyceride desaturase is not under the same dramatic dietary control as the fatty acid desaturase in liver (119). More definitive studies in other tissues are needed to confirm this point. A different alkylacylglycerophosphorylethanolamine desaturase activity has been described by Horrocks and Radominska-Pyrek (120). This enzyme, found in rat brain, does not require NADPH or O_2 and is stimulated by a heat-stable soluble factor.

Direct conversion of alkyl to alkenyl ethanolamine phosphoglyceride may not be the only biosynthetic route to the alkenylphosphoglycerides. Schmid and Takahashi reported that, after intracerebral injection of labeled hexadecanol into rats the glycerolipid alkenyl chains showed increasing incorporation of 18:0 and 18:1 with time. These species were never found in the alkyl chains as would be expected if the alkylglycerolipid was a precursor to the alkenylglycerolipid (105). This difference suggests a separate source for the alkenyl chains.

Evidence has been found that choline and ethanolamine phosphotransferases exist which accept 1-alkenyl-2-acylglycerol (121,122), although the enzymatic formation of this neutral alkenyl ether lipid has not been studied. In fact, this would seem to be the only route for biosynthesis of the choline alkenylphosphoglycerides since 1-alkyl-2-acylglycerophosphorylcholine is not a substrate for the desaturase (123).

It was demonstrated in 1956 that alkyl glycerides were cleaved at the ether bond during intestinal absorption (124), and other in

vivo studies also showed cleavage by the liver (125). Tietz *et al* showed that alkylglycerol was oxidized to long chain aldehyde and glycerol in the presence of reduced pteridine, O_2, and NADH with rat liver enzyme (126). Snyder and coworkers showed that alkylglycerophosphorylethanolamine can also be cleaved in the liver by a similar pathway (127,128). A second type of ether cleavage enzyme has been described by Kapoulas *et al* (129). This enzyme is found in *Tetrahymena pyriformis* and requires O_2, NAD, and NADPH for maximal activity, but not pteridine. Only neutral glyceryl ethers are cleaved by this enzyme; it does not accept phosphorylated substrates.

An interesting distribution for the alkyl ether cleavage enzyme seems to exist in that the level of enzyme is lower in those tissues with relatively high ether content (130,131). This has led to the hypothesis that the level of ether lipids in various tissues is not controlled by the rate of synthesis, but rather by the rate of degradation (131,132). However, further quantitative data on the enzyme distribution will be needed to prove this hypothesis.

Enzymes capable of catalyzing metabolic turnover and reutilization of the alkylglycerides have been investigated. Pancreatic (133) and lysosomal (134) enzymes can deacylate alkylacylglycerides, and microsomal phosphohydrolases act on the alkylphosphoglycerides (135). The resulting alkyl- and alkylacylglycerols may be phosphorylated by kinases (95,135,136).

The first clear description of an alkenylphosphoglyceride ether cleavage enzyme was by Warner and Lands in 1961 (137). The enzyme from brain was later described (138,138a). Ethanolamine alkenylphosphoglyceride is the substrate, but the acylated form is more efficiently

cleaved by the brain enzyme (138) and the deacylated substrate by the liver enzyme (139).

The degree of metabolic turnover and re-utilization of the alkenylglycerol backbone is not well studied, except in the case of redistribution of acyl groups in the nitrogen-containing phosphoglycerides described below.

C. Acyltransferases The acyltransferases involved in the formation of phosphatidic acid were mentioned above under "Phosphoglyceride metabolism". These enzymes use a phosphoglyceride acceptor that does not contain a nitrogenous base or other substituent on the phosphate group. Another important class of acyltransferases is involved in acylation of monoradylphosphoglycerides containing choline, ethanolamine, inositol phosphate, and other substituents. The first indication that acyl groups may be introduced into these phosphoglycerides was reported by Lands in 1958 on the basis of different relative incorporations of fatty acid and glycerol (140). He later demonstrated the acylation of lysophosphatidylcholine in rat liver (141). The presence of this acyltransferase in brain was reported by Webster (142) who later showed the enzyme distribution in many rat tissues (143). The acylation of lysophosphatidylinositol has also been studied with pigeon pancreas microsomes (144).

The importance of the acyltransferases must be considered in relation to the acyl composition of the phosphoglycerides. Apparently, these enzymes use the fatty acids provided by a changing diet and variable metabolic state to form a lipid population which conforms to a limiting range of physical characteristics (145). This implies a

degree of specificity and control exhibited by the acyltransferases. In fact, it has long been recognized that the acyl distribution in phosphoglycerides is non-random. Those acyl groups esterified to the C-2 position of the glycerol moiety have been found to consist of a high percentage of unsaturated chains, and those on the C-1 of saturated chains (146,147,148). Analyses of the acyl distribution in the ether phosphoglycerides have generally exhibited this pattern. Studies on sarcoplasmic reticulum (149), intestinal mucosa (150), and ascites tumor cells (151) have shown that the acyl distribution on C-2 of the glycerol moiety in the ether phosphoglycerides consists of three major groups of acyl chains: a) the C-18 mono- and dienoic, b) the C-20 tetraenoic, and c) the C-22, penta- and hexaenoic species. The acyl groups attached to the C-2 glycerol position of the diacyl-phosphoglycerides in these tissues contain a major amount of group a), and few longer chain polyunsaturated acyl groups. In these tissues the ethanolamine and choline phosphoglycerides are similar in composition.

In brain the acyl compositions on the C-2 position of the ethanolamine and choline phosphoglycerides are different. The ethanolamine phosphoglycerides (both diacyl and ether containing) contain major amounts of mono-, tetra-, and hexaenoic species (71,152,153,154). The choline phosphoglycerides (again, both diacyl and ether containing) contain mostly 16:0 and 18:1 with very small amounts of polyunsaturated species (71,152,155). Therefore it is apparent that different tissues may have unique patterns of acyl ester distribution. Comparison of the C-2 acyl distribution in lecithin from various organs of four different animals has shown that the relative amounts

of mono-, di-, and polyenoic species do vary significantly (156). However, the majority of acyl chains in the C-2 position are unsaturated and those on the C-1 position are saturated. Lung is an exception to this pattern in that the major phosphatidyl choline is dipalmitoyl. Further heterogeneity is introduced by the fact that various subcellular fractions within a single organ (157,158) have different acyl chain compositions, although this may be controlled by factors such as specific intracellular transport and not the acyltransferases.

Acylation to the lysophosphoglycerides containing a nitrogenous base is selective when tested in vitro. Unsaturated acyl donors are preferred by 1-acylglycero-3-phosphorylcholine (31, 159, 160) and 1-acylglycero-3-phosphorylethanolamine acyltransferases (161). With 2-acylglycero-3-phosphorylcholine as the acceptor, saturated acyl donors are preferred (159).

Early studies by three laboratories on the in vitro acyltransfer to 1-acylglycero-3-phosphate showed almost no specificity for unsaturated fatty acyl groups (19,31,162,163,164). Therefore it seemed likely that phosphatidic acid was synthesized with a random distribution of acyl groups and that, after the nitrogenous base was added, turnover of the acyl groups would provide an opportunity for the specific acyltransferases to introduce asymmetry (165).

It was then discovered that in vivo the biosynthesis of phosphatidic acid, and hence diglycerides proceeds with the expected asymmetric incorporation of acyl chains (166,167,264). Studies with liver slices also showed a non-random fatty acid incorporation into phosphoglycerides (168,169). Later studies on 1-acylglycero-3-phosphate acyltrans-

ferase in which care was taken to obtain optimal, initial velocity measurements provided results which did show a selectivity for unsaturated acyl-CoA donors (24,25,26,170). One report by Hill and Lands (160) showed that, of twelve different acyl-CoA donors tested, 18:1, 18:2, and 18:3 were most efficiently transferred to 1-acylglycero-3-phosphate by liver microsomal acyltransferase. A high rate of transfer with 16:0 acyl-CoA was also noted, but longer chain unsaturated acyl groups were relatively poorly transferred. A comprehensive kinetic study by Barden and Cleland (24) obtained similar results on the acyl-CoA specificity for liver acyltransferase with 18:1 being the most efficient acyl donor, but 16:0 acyl-CoA also exhibited a high rate of transfer in this study.

In vitro acylation to the C-1 position of the glycerol moiety with either glycerophosphate (20,23,171) or dihydroxyacetone phosphate (172) as the acceptor has been shown to be selective for saturated acyl-CoA donors. A study involving incubations with pairs of labeled fatty acids, ATP, and CoA demonstrated that in vitro acylation of glycerophosphate can lead to the expected asymmetric acyl distribution on the glycerol moiety (170). Therefore it must be concluded that the acyltransferases are capable of synthesizing phosphatidic acid with an asymmetric fatty acyl distribution; predominantly saturated on the C-1 position and unsaturated on the C-2 position.

A partially purified preparation of liver acylglycero-P acyltransferase also exhibited maximal activity with 18:1 acyl-CoA (26) and the data obtained with this preparation can be interpreted to indicate that the greatest selectivity was shown when the enzyme was saturated with acceptor. The opposite conclusion, that the greatest

selectivity is obtained at low acceptor concentrations, was reached by Okuyama and Lands (25) from a kinetic evaluation of the data they obtained with liver microsomal acyltransferase. Using incubations with pairs of labeled acyl-CoA species, they demonstrated increased selectivity for 18:2 over 18:1, 16:0, and 20:4 acyl-CoA as the acceptor concentration was decreased. From these results it was postulated that there may be separate catalytic sites for each acyl-CoA with a different Km for the acceptor acylglycero-P. The data they reported indicate that the Km with 18:2 acyl-CoA was lower than that with 18:1, 16:0, or 20:4.

More recent studies with the 1-acylglycero-3-phosphorylcholine acyltransferase from rat liver show that 20:4 is the preferred unsaturated acyl donor (26), and that the preference is determined by a lower Km value for the acceptor with this acyl donor (173). As with the 1-acylglycero-3-phosphate acyltransferases, there is evidence that separate enzymes exist for each acyl-CoA. Reitz et al (174) found that the storage stability for acylglycerophosphorylcholine acyltransferase differed depending on the acyl-CoA donor. A difference in the Triton X-100-induced lability which was dependent on the acyl-CoA co-substrate, was observed with this acyltransferase activity (173). Dietary changes can also differentially affect the acyltransferase activities with 18:1 and 20:4 acyl-CoA (175). Since the acyl-CoA selectivities are different for the choline phosphoglyceride and ethanolamine phosphoglyceride acceptors (176), it seems likely that there are at least two types of acyltransferases.

It appears that glycero-3-phosphorylethanolamine and -choline are not suitable substrates for acyltransferases (141). These com-

pounds might be expected to be formed by the phospholipases known to occur (177), and in fact glycerophosphorylcholine and fatty acids have been shown to be produced by enzymatic hydrolysis of phosphatidylcholine (165).

The structural features of the acyl chains which are important to the acyltransferase specificities have been studied by Lands and coworkers. The natural unsaturated fatty acids contain cis double bonds, and the acyl donors with cis unsaturation transfer preferentially to the C-2 position of lysolecithin (178). Changing either the $\Delta 9$ or $\Delta 12$ double bond in linoleoyl-CoA to the trans configuration increases the incorporation into C-1 relative to C-2 and changing both double bonds to trans causes the incorporation in C-1 to be 4 to 5 times greater than in C-2 (178). The conclusion that the presence of the double bond per se was not as important as its position on the acyl chain was also reported (179,180). For example, in a series of dienoic C-18 acyl-CoA species the donors with 5,8; 9,12; 11,14; or 13,16 double bonds were transferred to the C-2 position much better than the donors with adjacent double bonds.

Analysis of the species of diester phosphoglycerides in liver by various laboratories confirmed that certain acyl chains frequently occur in pairs (145,181,182). For example, 16:0 is found with 18:1 and 18:0 with 20:4. An obvious answer as to the mechanism for this pairing would be a specificity of the acyltransferase for the acyl ester species on the lysophosphoglyceride acceptor. This was not found to be true with 1-acylglycero-3-phosphorylcholine acyltransferase in studies by two separate groups (183,184). Therefore it has been suggested that metabolic pooling of intracellular fatty

acids may occur (184). When [1,1-$^{2}H_{2}$]ethanol is injected into rats the deuterium is incorporated in newly synthesized palmitic acid (45a). From the incorporation of labeled palmitate in phosphoglyceride the half-life of unlabeled palmitic acid was determined by Curstedt and Sjovall (45a). They reported that, in fact, the data did indicate two pools of palmitic acid but that palmitic acid from the same pool could be paired with either oleic, linoleic, or arachidonic acid in phosphatidylcholine. Therefore, it is not possible to evaluate the contribution of separate fatty acid pools to the formation of specific molecular species.

The tissue distribution of acyltransferases for alkyl- and alkenyl-phosphoglycerides containing nitrogenous bases is variable and in fact the enzymes are lacking in some major organs. Tabulation of the available data (185-192) provides no apparent rationalization for the variable distribution, and therefore will not be discussed in detail. Of import to this discussion, however, is the fact that brain does not contain acyltransferases for the choline ether containing phosphoglycerides (186), but does have activity for the acylation of the ethanolamine ether phosphoglycerides (192). Where the ether acceptor acyltransferases have been studied in regard to substrate specificity, they have shown a preference for unsaturated acyl donors (186,189). Linoleoyl-CoA was found to be the most efficient acyl donor to alkenylglycerophosphorylcholine with sarcoplasmic reticulum (186,189) and Ehrlich ascites cell microsomes (190), the only preparations with which specificity studies have been done.

D. Acyl-CoA The enzymatic synthesis of the long chain acyl-CoA Thio-

ester was first demonstrated by Kornberg and Pricer (193). Free fatty acid, CoA, and ATP were the required substrates, and AMP plus pyrophosphate in addition to acyl-CoA were the products. The enzyme they described utilized a wide range of fatty acids (5:0 to 22:0 plus 18:1, 18:2, 18:3), but studies with a partially purified enzyme show a smaller range of acceptable chain lengths (12 to 18 carbons) (194). There are at least three different acyl-CoA synthetases and each has a more or less definitive range of carboxylic acids that are utilized (195).

The palmitoyl-CoA synthetase (defined as that accepting 12 to 18 carbon acids, (EC 6.2.1.3) has been demonstrated in the mitochondria and microsomes of liver (196) and brain (197). Liver mitochondria also contain an acyl-CoA synthetase using GTP instead of ATP (198,199).

The most detailed study on the specificity of palmitoyl-CoA synthetase toward different fatty acids suggested that both saturated and unsaturated acids were accepted equally well with the exception of Δ11 unsaturated acids, which were less well utilized (200). This discrimination was effected by an affinity of the enzyme towards acids with Δ11 double bonds which was 3 to 5 times less than towards other unsaturated or saturated fatty acids. The unsaturated fatty acid which contains a Δ11 double bond and which is relevant to the work in this dissertation is 20:4(n-6), arachidonic acid.

Long chain acyl-CoA is present in normal rat liver at levels from 15 to 38 nmoles/gm tissue (201,202). However, the level may be quite variable because, upon starvation, the concentration increases to 2 to 3 times those values (201,202). The monomer concentration of acyl-CoA in rat liver cytosol has been calculated to be less than

0.1 μM (24). This is well below the concentration at which long chain acyl-CoAs form micelles, i.e., 2-10 μM (24). The formation of such micelles is important to the in vitro assay of enzymes because a number of enzymes have been shown to be inhibited by acyl-CoA at approximately the critical micelle concentration (24).

E. Hydroxy Fatty Acids and Phosphoglycerides During this investigation on the acylation of alkylglycero-P with acyl-CoA an unknown lipid was discovered which migrated on thin layer chromatography in such a way as to suggest that it was similar to alkylacylglycero-P but more polar. Further investigation indicated that the long chain acyl group was responsible for the difference. It was finally proven that 3-hydroxy fatty acid was formed during enzymatic incubation and incorporated into alkylglycero-P to form 1-alkyl-2-(3-hydroxy)acylglycero-3-phosphate.

Previous studies have demonstrated that 3-hydroxy fatty acids can be formed from free fatty acids, mitochondria, and the proper cofactors (203). It has been suggested that significant amounts of 3-hydroxy fatty acids may occur in vivo either in the free form or activated with CoA (204). There have been no reports that these hydroxyacyl donors are transferred to phosphoglyceride acceptors. However, several authors have reported finding an unknown lipid formed in mitochondrial incubations similar to those used in this investigation (205,206,207). A comparison of the reported chromatographic behavior of this unknown lipid with the results reported here suggests that the lipid may have been a 3-hydroxy fatty acid containing phospholipid.

Oxidation of fatty acids to form hydroxy fatty acids may utilize either H_2O or O_2 as the oxygen donor. Of concern here is the hydroxylation of α,β unsaturated fatty acids to form the 3-hydroxy derivative. This reaction utilizes H_2O and the CoA activated form of the fatty acid. The α,β unsaturated substrate for the reaction is formed in the first step of the oxidative degradation of long chain fatty acids. The enzyme catalyzing this unsaturation was first described by Hauge et al (208) and was termed palmitoyl-CoA dehydrogenase (EC 1.3.99.3). It is found in the mitochondria, as are the other enzymes in this oxidative pathway, has a flavin adenine dinucleotide prosthetic group, and specifically forms the trans-α,β-unsaturated acyl-CoA. Hydration of this unsaturated acyl-CoA is catalyzed by a hydratase (EC 4.2.1.17) first described by Wakil and Mahler (209). The stereospecific product is S-3-hydroxyacyl-CoA. The third step in fatty acid oxidation is dehydrogenation of the hydroxyacyl-CoA to form 3-ketoacyl-CoA in an enzymatic (EC 1.1.1.35) reaction requiring NAD+ (210). Subsequent steps in fatty acid oxidation are the thiolytic cleavage of the keto compound liberating acetyl-CoA, and the recycling of the C(n-2) acyl chain through the oxidative process. Mitochondrial preparations used in this dissertation work contained these enzymes in addition to the acyltransferase under study. D-3-hydroxy acid derivatives are formed during fatty acid synthesis; however, such fatty acids remain attached to the acyl carrier protein of fatty acid synthetase in mammals (211).

MATERIALS AND METHODS

H_3-[^{32}P]PO_4, 1-[^{14}C]palmitic acid, and NaB-[^{3}H]H_4 were obtained from New England Nuclear (Cambridge, Mass.). Palmitoyl-CoA, stearoyl-CoA, oleoyl-CoA, linoleoyl-CoA, CoA, and 5,5'-dithiobis(2-nitrobenzoic acid) CoA were purchased from P-L Biochemicals (Milwaukee, Wis.). Palmitic acid, oleic acid, linoleic acid, arachidonic acid, docosatetraenoic(n-6) acid, and docosahexaenoic(n-3) acid were from Nu-Chek-Prep (Elysian, Minn.). Triton X-100, phosphatidic acid, ATP, D-glucose-6-phosphate, NADP+, NADPH, D-glucose-6-phosphate dehydrogenase, cytochrome c, and phospholipase C (B. cereus) were obtained from Sigma (St. Louis, Mo.). Phospholipase A_2 was from Boehringer (New York, N.Y.). The Pentex brand of bovine albumin (fatty acid free) from Miles Laboratories (Kankakee, Ill.) was used. N-methyl-N'-nitro-N-nitrosoguanidine was purchased from Aldrich (Milwaukee, Wis.). Palmitaldehyde sodium bisulfite was obtained from K & K Laboratories (Jamaica, N. Y.). 3-Hydroxypalmitic acid was from Applied Science (State College, Penna.). Standard thin layer chromatographic plates were the precoated silica gel type (0.25 mm thick) produced by EM Reagents (dist. by Brinkmann Inst., Westbury, N. Y.). Silicic acid used in preparation of $AgNO_3$ impregnated thin layer plates was Silica Gel G (Applied Science, State College, Penna.), and that for column chromatography was the Unisil brand of activated silicic acid (200 - 325 mesh) from Clarkson Chem. Co., (Williamsport, Penna.)

Sprague-Dawley rats and Hartley guinea pigs were purchased from a local supply house. Muscular dystrophic mice were obtained from the

Jackson Laboratory (Bar Harbor, Me.). Females of the 129/Re strain, both homozygous (dy/dy) and presumed heterozygous (?/+) for the dystrophic gene were used.

Acyldihydroxyacetone phosphate and alkyldihydroxyacetone phosphate were chemically synthesized by decomposing palmitoyldiazoacetone and hexadecyldiazoacetone with H_3PO_4 (6,36). Portions of the acyl- and alkyldihydroxyacetone phosphate were reduced with NaB-[^{3}H]H_4 to form 1-acyl- and 1-alkyl-rac-[2-^{3}H]glycero-3-phosphate, respectively. For this reaction 0.4 mg of the phospholipid plus 0.1 mg (1.0 mCi) of the ^{3}H-labeled $NaBH_4$ were reacted in 1.0 ml ethanol for 4 hours at 22°. Then 4 mg of unlabeled $NaBH_4$ was added and the reaction was run for 16 hours at 4°. The ethanol was evaporated under a stream of nitrogen and 1.0 ml of chloroform-methanol (2:1) plus 0.75 ml of HCl (1.0 N) was added. After mixing, the phases were separated by centrifugation and the lower layer was collected. The 1-alkyl-rac-[2-^{3}H]-glycero-3-phosphate formed by this procedure was purified by silicic acid column chromatography and was 98 percent pure as determined by thin layer chromatography with chloroform-methanol-acetic acid-water (25:10:3:1) as the developing solvent. Unlabeled alkyl- and acyl-glycerophosphate were made in a similar way by omission of the initial reaction with NaB-[^{3}H]H_4, and the reaction was done at room temperature. The unlabeled reduction products were greater than 95 percent pure as estimated from a thin layer chromatographic analysis similar to that described for the labeled compounds.

1-Hexadecyl-rac-glycero-3-[^{32}P]phosphate was prepared by using $H_3[^{32}P]PO_4$ to decompose hexadecyldiazoacetone and then reducing the product with $NaBH_4$. H_3PO_4 (0.1 mmole) and $H_3[^{32}P]PO_4$ (1 to 5 mCi,

carrier free) were dried under a stream of nitrogen at 45° with several additions of benzene, dissolved in 0.5 ml of dioxane, and heated to 70°. Then 1-alkyl-3-diazoacetone (25 μmoles in 0.5 ml dioxane) was added to the labeled H_3PO_4. The reaction mixture was stirred for 45 minutes at 70°. After cooling, 5 ml of diethyl ether and 3 ml of 2 M KCl (containing 0.02 M H_3PO_4) were added, mixed, and the two phases were separated by centrifugation. The lower aqueous layer was washed twice with ether and all ether extracts were combined and dried. The product was then purified by partition in a modified Bligh and Dyer (212) extraction mixture (chloroform-methanol-water, 12:12:10.8) with the upper phase adjusted to pH 8.5 with triethanolamine base and KOH. After mixing and separating the phases by centrifugation the upper phase was collected, acidified to pH 3.0, and extracted with chloroform. The chloroform extract was dried under nitrogen and the product was reduced with $NaBH_4$ in ethanol. Final purification of the product was accomplished by passage through a silicic acid column with methanol-chloroform mixtures (1, 20, and 50 percent methanol) as the eluting solvents. The yield was usually three percent of the radioactive phosphate and 15 percent of the diazoacetone derivative. Purity of the 1-alkyl-rac-glycero-3-[^{32}P]phosphate was 90 - 92 percent as determined by thin layer chromatography. The contaminating radioactivity remained on the origin with chloroform-methanol-acetic acid-water (25:10:3:1) as the developing solvent. A similar procedure was used to prepare 1-acyl-rac-glycero-3-[^{32}P]phosphate from 1-acyl-3-diazoacetone. Concentrations of stock solutions of phospholipid substrates were determined by inorganic phosphorus determination (213) after decomposition of the organic phosphate compounds with

$MgNO_3$ (214). Disposable tubes were used for phosphate determinations.

Linoleoyl-, arachidonoyl-, docosatetraenoyl(n-6)-, and docosahexaenoyl(n-3)-CoA were chemically synthesized from the free acids using the procedure of Seubert (215) as modified by Okuyama et al (179). All solutions used for the preparation and storage of acyl-CoA contained either butylated hydroxytoluene or Santaquin[1], and stock solutions of the acyl-CoA substrates were kept under nitrogen at -20^{o}. It was necessary to synthesize linoleoyl CoA because the commercial product (P-L Biochemicals) was not enzymatically active either in the acyltransferase assay described here or in the lysolecithin aclytransferase assay described by Lands and Hart (31)[2]. The linoleoyl-CoA chemically synthesized by the above procedure was active in both acyltransferase assays. Stock solutions of most acyl-CoA substrates were standardized by determination of the total CoA liberated in the lysolecithin acyltransferase spectrophotometric assay using guinea pig liver microsomes (31). However, docosatetraenoyl(n-6)-CoA reacted very slowly with lysolecithin. Therefore a similar enzymatic assay using the acyl-CoA hydrolase activity of rat brain microsomes was devised. For this assay, Tris-HCl buffer (0.85 ml, 0.05 M, pH 7.4), 5,5'-dithiobis(2-nitrobenzoic acid) (0.1 ml, 0.01 M), rat brain microsomes (approximately 0.1 mg protein) and acyl-CoA were added to a cuvette. The absorbance change at 413 nm was recorded until the reaction was completed. Duplicates were done

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[1] Monsanto Co., St. Louis, Mo.

[2] A recent batch of linoleoyl-CoA from P-L Biochemicals was approximately 25 percent enzymatically active on a weight basis.

with twice the amount of acyl-CoA, and the blank contained all additions minus acyl-CoA. The agreement between this assay and the lysolecithin assay for other acyl-CoA substrates was excellent. The rate of acyl hydrolase activity under these conditions is approximately 50 nmoles/min/mg protein. The concentration of each acyl-CoA as determined by the enzymatic assays was usually about 70 percent of the amount estimated by absorbance at 232/260 nm, and enzymatic reactivity decreased approximately 2 percent per month during storage at -20^{o}.

Chemical synthesis of 3-hydroxyfatty acid was from palmitaldehyde and ethyl bromoacetate by the Reformatsky reaction according to Stoffel et al (216). Palmitaldehyde was liberated from its bisulfite addition complex by reacting 0.5 gm of palmitaldehyde sodium bisulfite with 0.25 gm $NaCO_3$ in 5 ml boiled water. After adding 5 ml diethyl ether and mixing, the phases were allowed to separate. The ether phase was collected, washed once with water, dried over Na_2SO_4, and evaporated to dryness under a stream of nitrogen. A mixture of palmitaldehyde (1 gm) and ethylbromoacetate (95 mg) in 5 ml benzene was added dropwise to a stirred suspension of zinc dust in the same solvent. The reaction mixture was refluxed for 2 hours at 80^{o}. After addition of 3 ml ice cold 6 N HCl, the mixture was extracted with diethyl ether. This was dried and the 3-hydroxystearate ethyl ether present was hydrolyzed by a 1 hour reflux in ethanolic KOH. The unreacted aldehyde was extracted with petroleum ether and, after acidification the 3-hydroxystearic acid was extracted with chloroform. A small portion of the product (100 mg) was purified by column chromatography on silicic acid. After loading, the column was eluted with hexane-chloroform (25:75) and then methanol-chloroform (2:98). The lipid in the

second eluent was run on a thin layer chromatogram using hexane-diethyl ether-acetic acid (30:70:1) as the developing solvent, and purity of the 3-hydroxystearic acid was judged to be 95 percent. Acetylation of 3-hydroxyfatty acid methyl esters was done with acetic anhydride in pyridine (217).

Esterification of fatty acids for thin layer and gas liquid chromatography was with acidic methanol. In some cases, a rapid method of reacting 0.2 ml methanol, 0.5 ml 2,2-dimethoxypropane, and 0.02 ml HCl with the free fatty acid for 1 hour was used (218). This mixture was evaporated to dryness with a stream of nitrogen after several additions of benzene during the drying procedure. In other cases, or when transesterification of phosphoglyceride acyl esters was done, the apparatus described by Kishimoto and Radin was used to react the free or esterified fatty acids with methanol-HCl for 16 hours at 76^{o} (219). The fatty acid methyl esters were extracted with hexane, and loaded on a small silicic acid column. The column was eluted with hexane first, and then hexane-benzene (1:1). The fatty acid methyl esters elute in the latter solvent.

Diazomethane was prepared from N-methyl-N'-nitro-N-nitrosoguanidine in the apparatus described by Fales et al (220), and obtained commercially (Kontes Co., Vineland, N. J.). The guanidine derivative (133 mg) was reacted with 0.6 ml of 5 N NaOH for 60 minutes and the diazomethane was collected in 3 ml diethyl ether. The whole apparatus was kept in ice. Successive preparations were made and stored for use the same day. An aliquot of diazomethane in ether was reacted with 0.2 N benzoic acid, also in ether. After 30 minutes the excess benzoic acid was titrated with 0.1 N NaOH, and the original concentration of

diazomethane was found to be 0.18 mmole/ml ether.

Alkaline methanolysis of phosphoglycerides to cleave the ester linkages was accomplished by reacting the lipid in 1 ml chloroform and 1 ml of 0.5 N NaOH in methanol for 30 minutes. Then 0.9 ml of 1.0 N HCl was added and, after mixing and phase separation, the lower chloroform layer containing the lipids was collected.

Enzymatic hydrolysis of phosphoglyceride esters was done using phospholipase A_2 as described by Wu and Tinker (221). The enzyme (5 mg) was dissolved in 1 ml Tris-HCl (pH 7.5) containing 4 mM $CaCl_2$. The lipid was dissolved in 5 ml diethyl ether and 0.05 ml of enzyme solution was added. The reaction was stirred vigorously for 16 - 24 hours at 22^{o}. The solvent was then evaporated with a stream of nitrogen and 0.1 ml of citric acid solution (7.6 mg/ml) was added. The lipid hydrolysates were extracted into chloroform-methanol (2:1) for further analysis.

NADPH:cytochrome c reductase in subcellular fractions was assayed by the spectrophotometric method of Williams and Kamin (222). The sample cuvette contained cytochrome c (0.4 mg), NADPH (74 μg), and subcellular fraction (0.05 to 0.5 mg protein) in K_2HPO_4 buffer (50 mM, pH 7.7) containing KCN (1.0 mM) in a total volume of 1.1 ml. The reaction was started by addition of NADPH and followed at 550 nm against a blank containing everything but NADPH. A unit of enzyme activity was assigned the value of 1.0 absorbance unit/min/cm.

In all cases where silicic acid column chromatography was used, not more than 20 mg of lipid were loaded on the column for each gm of silicic acid. The volume of each eluent fraction was 20 ml of solvent for each gm of silicic acid.

Silica gel thin layer plates impregnated with $AgNO_3$ were prepared according to Renkonen (223). Clean, dry glass plates were wiped with toluene and placed side-by-side in a holder. $AgNO_3$ (5 gm) was dissolved in 50 ml water and Silica Gel G (25 gm) was added. The suspension was shaken vigorously for 30 seconds and then spread on the plates in a layer 0.25 mm thick. The plates were air dried and stored. Before use each plate was activated at 110^{o} for 40 minutes.

Determination of pH was usually done using a Corning Model 10 pH meter with a Combination-Triple Purpose electrode. The meter was standardized before each use with the respective Beckman Buffer Solution.

Radioactivity determinations on labeled lipids were done in a Beckman LS133 liquid scintillation counter. Aliquots of radioactive lipids were dried in vials and a solvent containing toluene-ethanol-PPO and dimethyl POPOP (950 ml:50 ml:5 gm:0.2 gm) was added for counting. For radioactive lipids in aqueous suspension, the scintillation solvent contained toluene-BBS-3-PPO-dimethyl POPOP (1 liter: 200 ml:4 gm:0.25 gm). Ten ml of solvent was added to 0.5 ml of lipid suspension.

Location of labeled compounds on thin layer chromatograms was usually accomplished using autoradiography. After the plates were developed and air-dried, a few drops of ink containing [1-^{14}C]palmitic acid were placed in each corner of the plate. In the darkroom a standard 8 x 10 inch X-ray film (Kodak No-Screen NS54T) was placed over the thin layer plate and both were stored in the dark overnight or long enough to allow for approximately 10^{6} disintegrations per spot on the chromatogram. The film was developed and fixed using the

standard solutions. When dry, the film was placed on an overhead projector and the thin layer plate was positioned on the film by aligning the ink spots with the respective spots on the film. The outline of radioactive compounds could then be visualized and marked on the chromatogram.

Subcellular fractionation of whole brain from 22 day-old male rats was accomplished using discontinuous sucrose gradient centrifugation as described by Eichberg et al (224). Twelve rats were decapitated at a time, and the brains were removed and immediately washed in ice cold sucrose (0.32 M, pH 7.0). They were then homogenized in 9 volumes (1 gm: 9 ml) of 0.32 M sucrose containing EDTA (0.1 mM). All homogenizations were done in a glass homogenizer with Teflon pestle. The preparations were kept at 0^{o} to 4^{o} throughout the procedure. The cellular debris and nuclei were sedimented by centrifugation at 1000 x g for 10 minutes and discarded. The supernatant was centrifuged at 18,000 x g for 30 minutes to sediment mitochondria, myelin, and nerve endings. This second supernatant was centrifuged at 100,000 x g for 60 minutes to sediment the microsomes. The microsomal pellet was resuspended once in sucrose-EDTA, resedimented at 100,000 x g , and then suspended in a minimal volume of 0.32 M sucrose (no EDTA). The pellet containing mitochondria, myelin, and nerve endings was suspended in 0.32 M sucrose-EDTA and layered on a discontinuous sucrose-EDTA gradient consisting of an upper 0.8 M and lower 1.2 M sucrose layer, both containing 0.1 mM EDTA. This gradient was centrifuged at 70,000 x g for 110 minutes in a swinging bucket rotor. The myelin fraction was found at the 0.32-0.8 M sucrose interface, the nerve endings at the 0.8-1.2 M sucrose interface, and the mitochondria as a pellet at the bottom.

Each of these fractions was collected, and the myelin and nerve endings were washed once with 0.32 M sucrose - 0.1 mM EDTA. Finally, all fractions were resuspended in a minimal volume of 0.32 M sucrose (no EDTA) and frozen. Microsomal contamination of the brain subcellular fractions was estimated by the activity of NADPH:cytochrome c reductase using the assay described by Williams and Kamin (222). Guinea pig liver mitochondria and microsomal fractions were prepared by standard centrifugation techniques which have been previously described (37). The subcellular particles were stored in sucrose at -20° in small batches. In the tissue distribution study described under "Results", the particulate fractions from various rat tissues were prepared as follows: Hearts, spleens, brains, kidneys, livers, and lungs were dissected from three rats (male, 22 day old) and homogenized in nine volumes of 0.25 M sucrose containing 0.1 mM EDTA (pH 7.0). The homogenate was centrifuged at 1000 x g for 10 min. The supernatant was decanted and centrifuged at 100,000 x g for 60 min. The pellet was resuspended in sucrose-EDTA and resedimented at 100,000 x g for 60 min. The washed pellet was suspended in a small volume of 0.25 M sucrose and stored at -20°. Protein concentrations were determined by the method of Lowry et al (225).

Two assay systems were devised to measure alkylglycero-P acyltransferase activity. The first was based on the differential partition of substrate (alkylglycero-P) and product (alkylacylglycero-P) in a chloroform-methanol-water two phase system. The second involved separation of substrate and product on thin layer chromatograms. For both assays, incubations of labeled phospholipid substrates and cofactors with enzyme preparations were done at 37° with shaking. The

incubation mixture was prepared by sonicating the ^{3}H- or ^{32}P-labeled phospholipid substrates, Triton X-100 (0.4 mg), NaF (21 mM), $MgCl_2$ (1.7 mM), and acyl-CoA (20 nmoles) in buffer (Tris-PO_4, 75 mM, pH 8.0). Then albumin (1.0 mg), water, and enzyme were added to a total volume of 1.2 ml. These are the standard conditions referred to in the remainder of this report. The reaction was started by addition of enzyme (< 0.1 mg protein) after 2 min temperature equilibration. The reaction was stopped by addition of 4.5 ml chloroform-methanol (1:2). When using the partition assay, this one phase mixture was centrifuged to precipitate the protein, and the supernatant was transferred to another tube. Then 1.5 ml methanol and 1.5 ml water were added, and the mixture was vortexed and centrifuged to separate the phases. The upper aqueous phase was removed and the lower chloroform-rich phase was washed with 2 ml of methanol-water (1:1). An aliquot of the lower phase containing the product was dried and the radioactivity was determined in a liquid scintillation counter. Throughout this extraction the aqueous phase was maintained at pH 8 by the Tris buffer in the original incubation mixture. Fig. 1 shows the partition of alkylglycero-P and alkylacylglycero-P in a chloroform-methanol-water two phase system at various pH values. The labeled lipids used for the experiment shown in Fig. 1 were purified on thin layer chromatograms and, when they were eluted from those chromatograms (as described below), a scintillation quenching agent was also collected. Removal of this quenching agent during the experimental partition caused an apparent recovery of greater than 100 percent for the alkylacylglycero-P. Control experiments with pure ^{32}P-labeled product showed an actual recovery of 95 to 96 percent in the lower

Fig. 1 Partition of alkylglycero-P and alkylacylglycero-P in a chloroform-methanol-water two phase system at various pH values. 1-Alkyl-[2-^{3}H]glycero-3-P (●—●) was prepared as described under "Materials and Methods". 1-Alkyl-2-[1-^{14}C]palmitoylglycero-3-P (○—○) was prepared by incubating alkylglycero-P and [1-^{14}C]palmitic acid with guinea pig liver microsomes and cofactors. Both labeled lipids were purified by thin layer chromatography. Each partition was done as follows: 1-Alkyl-[2-^{3}H]glycero-3-P (50,000 cpm, 5 μg) or 1-alkyl-2-[1-^{14}C]palmitoylglycero-3-P (50,000 cpm, 10 μg) was added to a tube with chloroform (3.0 ml), methanol (3.0 ml), and buffer (2.7 ml, phosphate (74 mM) or citrate-phosphate (37 mM:74 mM)). After mixing the phases were separated by centrifugation. An aliquot of the lower chloroform-rich phase was collected for counting.

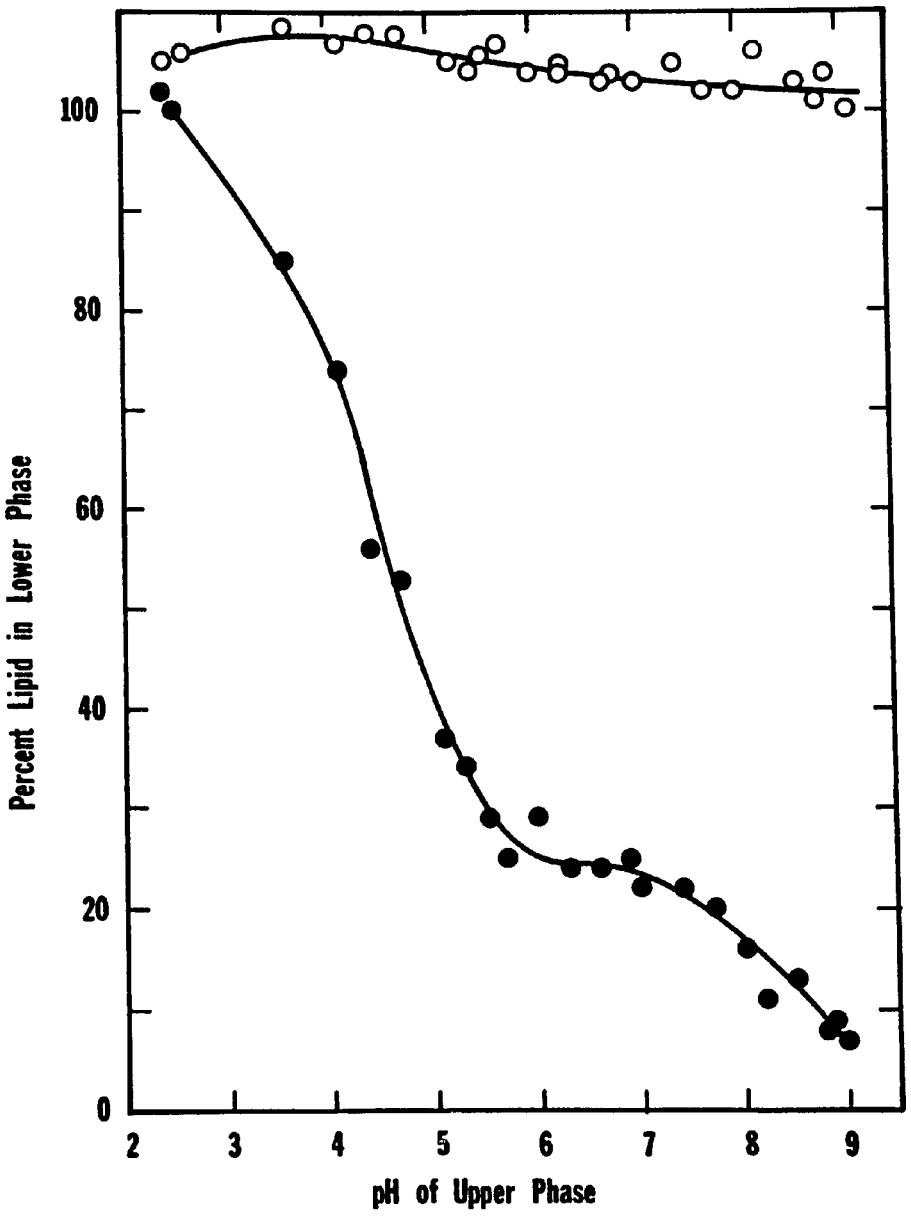

Percent Lipid in Lower Phase
100
80
60
40
20
0
2
3
4
5
6
7
8
9
pH of Upper Phase

Figure 1

phase. The partition of the substrate (alkylglycero-P) shows a dramatic change with varying pH values. Below pH 3.0, more than 90 percent of this lipid partitions into the lower phase, but at pH 8.0 only 15 percent is in the lower phase. Since two extraction-partitions are done at pH 8.0 during the assay procedure, only 2.25 percent of the substrate should remain in the lower phase with the product. In control experiments with cofactors and boiled enzyme added to the partition, the average proportion of substrate remaining in the lower layer was 5.5 percent. This was subtracted as a blank value from the experimental determinations. For the assay invclving separation of substrate and product by thin layer chromatography, the reaction was stopped by addition of 4.5 ml chloroform-methanol (1:2). Then 1.5 ml chloroform and 1.5 ml of 2 M KCl containing H_3PO_4 (0.2 M) were added (226). Centrifugation resulted in two clear phases being formed. The lower chloroform rich phase which contained the labeled lipids was collected and evaporated under a stream of nitrogen. [1-^{14}C]palmitic acid (0.1 μg, 40,000 cpm) was added as an internal standard. This labeled standard was necessary to correct for less than quantitative transfer during the next step. The labeled lipids were redissolved in chloroform-methanol (2:1) and an aliquot was transferred to a thin layer plate for development in chloroform-methanol-acetic acid-water (25:10:3:1). Radioactive lipids were located by autoradiography. The Rf values were as follows: alkylglycerophosphate, 0.3; alkylacylglycerophosphate, 0.6; palmitic acid, 0.9. The respective spots on the plate were scraped into counting vials, the powder was dispersed in 0.5 ml water by sonic oscillation, and after adding 10 ml of scintillation solvent containing BBS-3 the

radioactivity was determined. The amount of substrate acylated was calculated after correcting for the aliquot spotted on the chromatogram by using the internal standard, [1-^{14}C]palmitic acid. Blank values obtained with incubations without acyl-CoA were subtracted from each experimental value.

In some experiments the radioactive lipids were eluted from the chromatograms by collecting the appropriate area of silica gel and extracting twice with 2 ml chloroform-methanol-HCl (10:10:0.01). Chloroform (2 ml) and water (1.5 ml) were added to the combined extract, mixed, and centrifuged to separate the phases. The lower chloroform rich phase containing the extracted phospholipids was then collected.

To determine acyltransferase selectivity using endogenous fatty acid or a combination of acyl-CoA species, the extraction and isolation of labeled lipid product was different from the standard procedure. The incubation was stopped with 4.5 ml chloroform-methanol (1:2). Then 1.5 ml of chloroform, 1.0 ml of water, and 0.5 ml of Tris-PO4 (pH 8.0) were added. After mixing, the phases were separated by centrifugation and the upper aqueous phase was removed. The lower phase was washed once with basic methanol-water (1:1, pH 8.0) and then with acidic methanol-water (1:1, pH 3.0). This extraction procedure removes the unreacted alkylglycero-[^{32}P]phosphate and converts the acylated product to the acid form for the next step. After drying the extracted lipid product under nitrogen, it was dissolved in a small volume of ether and reacted with a large excess of diazomethane (>1 ml) prepared for immediate use as described above. The methylated product, 1-alkyl-2-acylglycero-3-[^{32}P]phosphoryldimethyl

ester, was separated according to the degree of unsaturation in the acyl chain on silver nitrate impregnated thin layer chromatograms using essentially the method of Renkonen (223). To obtain separation of all lipid species for quantitation it was necessary to divide the product sample and separate the saturated and monounsaturated species on one plate and the polyunsaturated species on a separate plate. The relative amounts of saturated and monounsaturated products were determined using 99:1 chloroform-methanol as the developing solvent (plate A, fig. 2). To separate the polyunsaturated product species 90:10 chloroform-methanol was used (plate B, Fig. 2). No single solvent system was found to adequately separate all species on one plate.

The distribution of free fatty acids in the rat brain microsomes was determined using the method described by Lunt and Lowe (233). An aliquot (3 mg) of total lipid extract from the microsomes (extracted by the method of Folch et al (227)) was suspended in ether and applied to a pre-washed thin layer chromatogram in a band. The plate was developed in petroleum ether (bp 30 - 60^{o}):diethyl ether:acetic acid (80:20:1) (228) and then sprayed with dichlorofluoroscein (0.1 percent in ethanol). The area corresponding to free fatty acid was located by comparison with a standard (palmitic acid) on the same plate. The respective area of silica gel was collected and the acids were eluted five times with ether. An odd chain fatty acid (23:0) was added as an internal standard. Esters of the fatty acids were made and then analysed on a Hewlett-Packard 5710A gas chromatograph with a flame ionization detector. Details of the chromatographic conditions are given in the legend to Fig. 3.

The acyl chain distribution of the ether containing ethanolamine

Fig. 2 Diagram representing a typical separation of individual species of 1-alkyl-2-acylglycero-3-phosphoryldimethyl esters on silver nitrate-impregnated thin layer chromatograms. The developing solvent was a mixture of chloroform-methanol, 99:1 for part A and 90:10 for part B. The drawings represent observed spots on the respective autoradiograms. The labeled lipids applied to each lane were obtained from incubations of microsomes and cofactors with the following combinations of substrates: lanes 1,2,5, and 6 contained 1-alkylglycero-3-[^{32}P]phosphate plus palmitoyl-CoA (16:0), oleoyl-CoA (18:1), arachidonoyl-CoA (20:4), or docosahexaenoyl-CoA (22:6) respectively. Lanes 3 and 7 contained alkyldihydroxyacetone-[^{32}P]phosphate and NADPH plus a mixture of equal proportions of six acyl-CoA species (16:0, 18:0, 18:1, 20:4, 22:4, 22:6). Lanes 4 and 8 contained 1-alkylglycero-3-[^{32}P]phosphate plus the mixture of acyl-CoA species. The dotted or heavy lines signify light or heavy density of grains on the autoradiogram, respectively. Details of the incubations are given in Table IV and details of the isolation and chromatographic separation are in the text under "Materials and Methods".

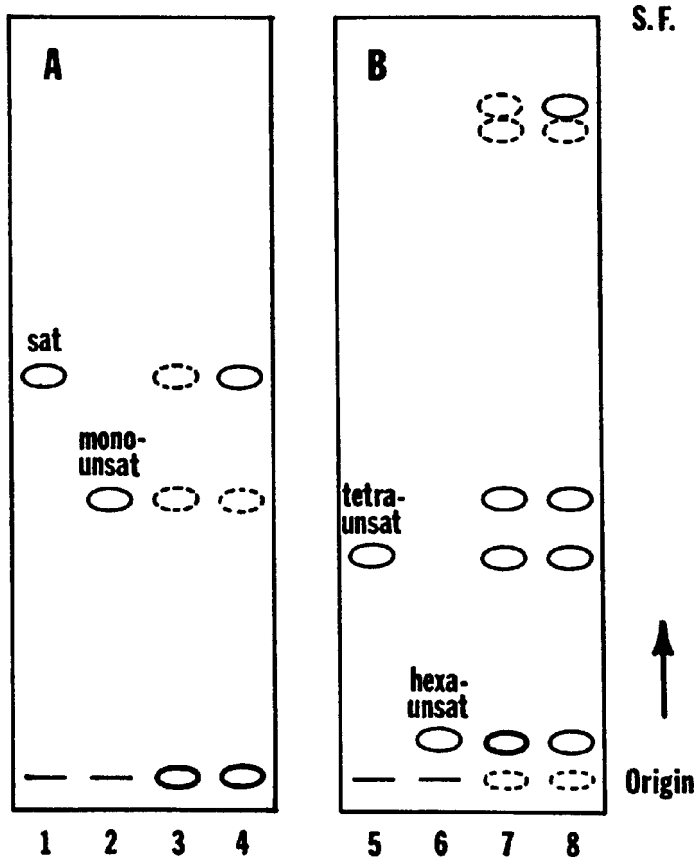


Figure 2

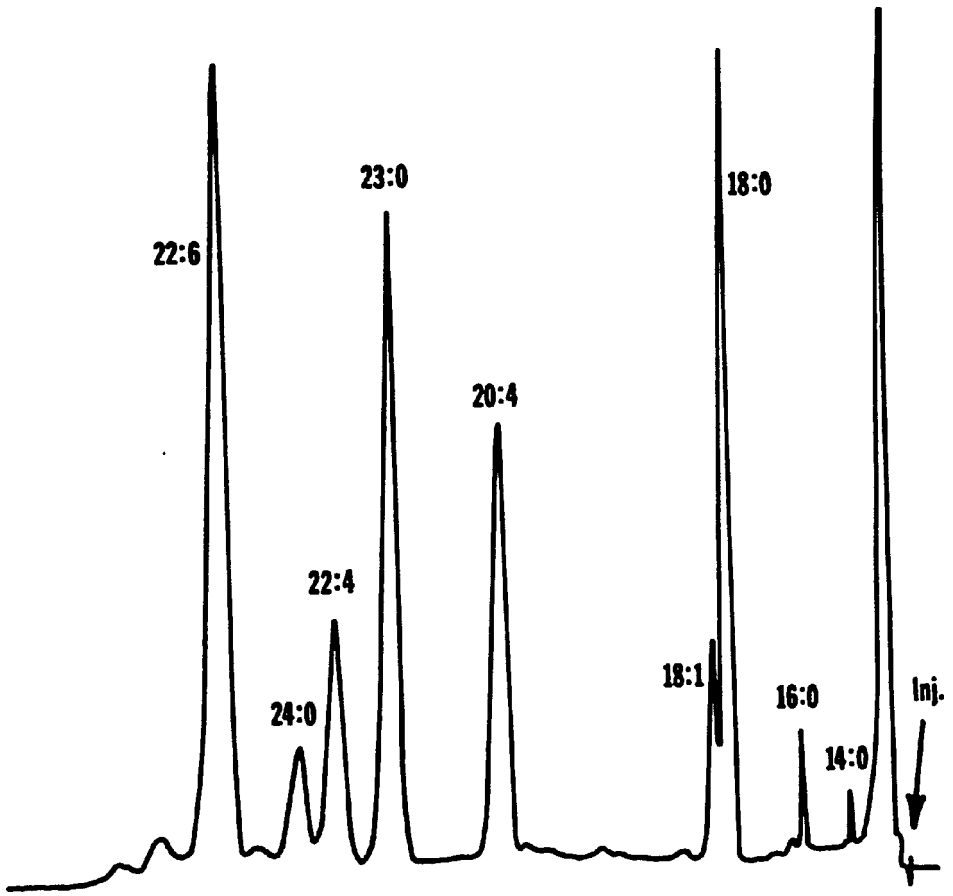


Fig. 3 Separation of fatty acid methyl esters by gas liquid chromatography. The example shown is the mixture of acyl groups isolated from the 1-alkyl-2-acylglycerophosphorylethanolamine fraction in rat brain microsomes. The isolation procedure and preparation of methyl esters is described in the text. An odd chain fatty acid (23:0) was added as an internal standard. The area under each peak was quantitated in early studies by cutting out and weighing each peak and later by automatic integration. The 6 ft x 1/8 inch column contained 5 percent diethylene glycol succinate on Chromasorb G-AW, 80/100 mesh. Nitrogen was the carrier gas and a flame ionization detector was used. Temperature of the column was increased from 170° to 220° at 2° per minute starting at the time of injection.

and choline phosphoglycerides in rat brain microsomes was determined essentially by the procedures described by Waku et al (]49). The lipids in brain microsomes (100 mg protein) were extracted by the method of Folch et al (227) and separated into lipid classes by silicic acid column chromatography (229) using methanol-chloroform mixtures (1, 10, 30, and 50 percent methanol) for elution. The eluent was collected in small fractions and assayed for lipids by thin layer chromatography (Silica gel G; chloroform-methanol-water, 100:36:7). A molybdate-sulfate spray (230) was used to detect phospholipids. After the phospholipids were identified the plate was charred to visualize any non-phospho compounds. Those fractions containing ethanolamine phosphoglycerides were eluted with 10 percent methanol-chloroform and were combined. The fractions containing choline phosphoglycerides were eluted with 50 percent methanol-chloroform and were also combined. The ethanolamine phosphoglycerides were contaminated with approximately 5 percent sulfatides as estimated by thin layer chromatography. The plasmalogens in the two phospholipid fractions were cleaved by treatment with $HgCl_2$ (1.0 percent) in acetic acid, and the resulting 2-acylphosphoglycerides were isolated from the other phospholipids by silicic acid chromatography as described by Waku et al (149). The acyl chains of these 2-acylphosphoglycerides were transesterified with acidic methanol for analysis by gas-liquid chromatography as above. The ethanolamine phosphoglyceride fraction contained 80 percent plasmalogen and the choline fraction 17 percent. The phosphoglycerides not cleaved with acetic acid-$HgCl_2$ were hydrolyzed using phospholipase C from B. cereus. After acetylation of the resulting alkylacyl- and diacyl-glycerides, the ether containing lipids

were separated by thin layer chromatography using hexane-diethyl ether (4:1) to develop the plate twice. Dichlorofluoroscein was used to detect the neutral lipids. No alkylglycerolipid was detected in the diglycerides obtained from choline phosphoglycerides. The alkylglycerolipid from ethanolamine phosphoglycerides was eluted from the chromatogram and the acyl chain distribution determined by gas-liquid chromatography as above. An example of the chromatographic separation of fatty acid methyl esters obtained from the alkenylethanolamine phosphoglycerides is shown in Fig. 3. A peak representing the internal standard (23:0) can be seen. This fatty acid methyl ester was added to each fraction just before transesterification.

The mole percent of total phospholipid phosphate in each phosphoglyceride class was as follows: alkenylethanolamine, 24.0; alkylethanolamine, 1.7; diacylethanolamine, 4.6; alkenylcholine, 8.3; diacylcholine, 41.0.

All solutions used in the procedures for determination of acyl chain distributions contained 0.001 percent butylated hydroxytoluene and were kept under nitrogen when possible.

An assay for the determination of tissue phosphoglycerides containing a 3-hydroxy acyl group was devised. Fig. 4 is an outline of the assay which consisted of the following steps: The total lipid fraction was extracted from fresh tissue by the method of Folch et al (227). The total lipid extract was separated into lipid classes on a silicic acid column using methanol-chloroform mixtures as described above. The ethanolamine and choline containing phosphoglycerides were collected separately; other fractions were discarded. Alkaline methanolysis (described above) was used to liberate the

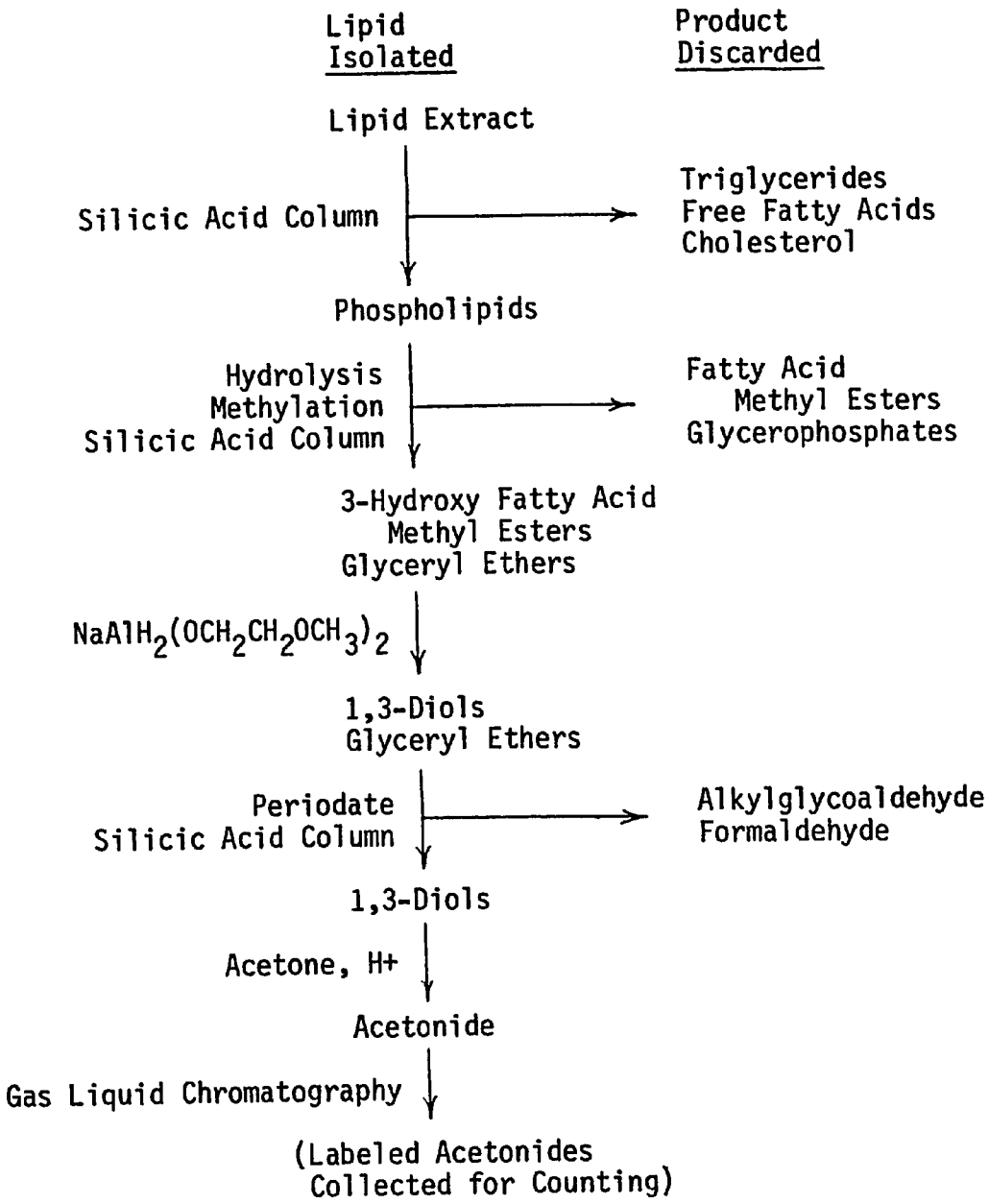


Fig. 4 Outline of assay for determination of phosphoglycerides containing a 3-hydroxyacyl group.

fatty acids from the phosphoglycerides, and complete methylation of the fatty acids was achieved with acidic methanol in 2,2-dimethoxypropane. The fatty acid methyl esters were loaded on a silicic acid column and the column was eluted with benzene. This eluent contained all normal fatty acid methyl esters. The second eluent was chloroform and this fraction contained any 3-hydroxy fatty acid methyl ester present plus glyceryl ethers. This latter fraction was evaporated under reduced pressure and the fatty acid methyl esters were reduced to the alcohol with sodium bis(2-methoxyethoxy)aluminum hydride; both Vitride (Eastman) and Red-al (Aldrich) were used. For this procedure the lipid was refluxed with excess reducing agent for 30 minutes in ether. The mixture was carefully acidified with HCl, mixed, and after phase separation, the ether layer was collected. The aqueous phase was washed twice with ether and the ether extracts were combined. The 3-hydroxyfatty acid methyl esters would be converted to 1,3-diols by this procedure. The diol fraction was then treated with periodate in pyridine (231) to cleave any vicinal diols present. For this reaction the dried lipid was dissolved in 0.5 ml pyridine. Sodium metaperiodate (10 mg) was added and the reaction was stirred at 22^{o} for 4 hours. The solvent was evaporated under a stream of nitrogen and 3 ml diethyl ether was added. The ether was washed twice with water and evaporated. The glyceryl ethers present in the original phosphoglyceride would be converted to the aldehydic compound and the diols derived from 3-hydroxyfatty acid would be unchanged. Long chain diols were separated from the aldehydes and other oxidation products by silicic acid column chromatography. After loading, the column was eluted with benzene first and then methanol-chloroform (1:4). The diols

were collected in the second fraction. Acetonides of the long chain diols were prepared by reaction with 5 μl of 70 percent $HClO_4$ in 1 ml acetone (231a). After 30 minutes at 22°, 4 ml of diethyl ether and 4 ml of water plus 2 drops of 17 N NH_4OH were added. The mixture was vortexed and centrifuged to separate the phases. The ether layer was collected and the aqueous phase was washed twice with ether. Gas liquid chromatography of the acetonides was done in a 5 ft x 5/32 inch OV-101 (5 percent on Chromasorb 9) column at 225°. The gas chromatograph (Varian-Aerograph, Model 920) was fitted with a thermal conductivity detector. The retention of long chain acetonides in the chromatographic column is shown in Fig. 5. Each acetonide was detected as a cluster of four compounds. Such heterogeneity was not due to impure 3-hydroxy fatty acid methyl esters from which the acetonides were derived because the former were homogeneous as determined by gas liquid chromatography. The acetonides of chimyl alcohol and 1,2-hexadecyldiol did not show evidence of heterogeneity when run through the same chromatographic column. Since a complete separation of species was possible (see Fig. 5), the origin of acetonide heterogeneity was not investigated further. Control experiments in which 3-hydroxy stearic acid was added to the original tissue homogenate showed that detection of 3-hydroxyfatty acid in concentrations of 0.1 percent of total lipid extract (w/w) were possible when using approximately 40 grams of wet tissue. In one experiment 3-hydroxy-[1-^{14}C]palmitic acid (100,000 cpm, 0.2 μg) was added to a tissue homogenate and the homogenate was run through the assay procedure. The fraction corresponding to hexadecylglyceryl ether acetonide was collected as it eluted from the gas chromatograph in a glass tube attached to a Millipore filter

Fig. 5 Retention of long chain acetonides in a gas liquid chromatographic column. The 1,3-diols were made by reduction of 3-hydroxy myristate, palmitate, or stearate methyl esters. Then the acetonides were made by reacting the diols with acetone and $HClO_4$. Details of preparation and purification of the acetonides are in the text. The 5 ft x 5/32 inch column contained 1.5 percent OV-101 on Chromasorb 9, 100/120 mesh and was maintained at 225°. Helium (75 ml/min) was the carrier gas and a thermal conductivity detector was employed. The carbon numbers in the figure refer to the number of carbons in the long chain hydrocarbon moiety.

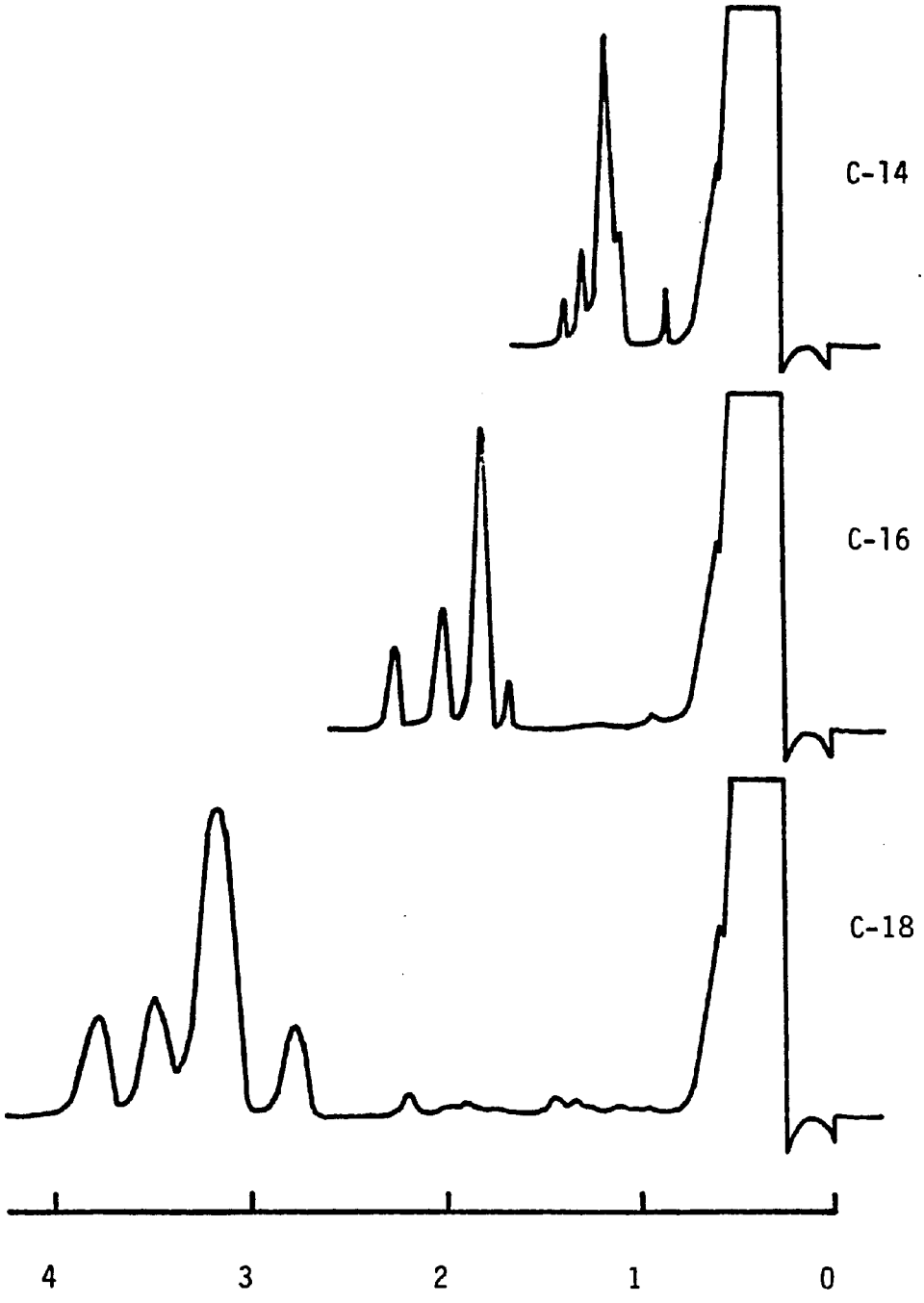


Figure 5

as described by Hajra and Radin (232). The filter with attached tube was twice flushed with scintillent solution directly into the counting vial using a syringe. The total recovery of radioactivity was 40 percent. Some of this loss of radioactivity could have been during gas liquid chromatography. The recovery of radioactivity injected into the chromatograph was tested with a sample of [1-^{14}C]palmitate methyl ester. Using the same collection procedure, 83 percent of the injected cpm was collected during the elution of carrier palmitate methyl ester and another 10 percent was collected at longer retention times. Fig. 6 shows the retention of the labeled palmitate methyl ester. The effluent collection periods can be identified from the blips in the baseline. These interruptions mark the insertion of a new collection device.

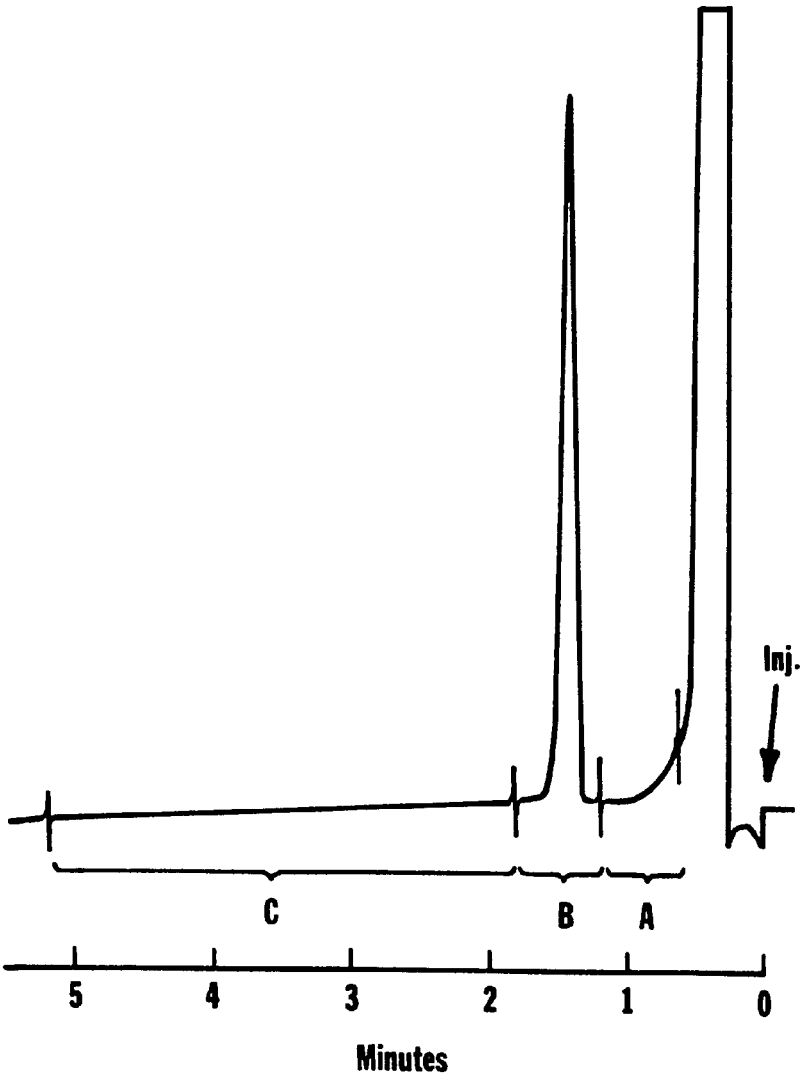


Fig. 6 Retention of [1-^{14}C]palmitate methyl ester on a gas chromatographic column. The methyl ester of [1-^{14}C]palmitic acid was made using acidic methanol and dimethoxypropane as described under "Materials and Methods". An aliquot (13,460 cpm, 10 μg) was injected into the chromatographic column and the effluent was collected as described in the text. The radioactivity recovered in each collection period was as follows: A) 145 cpm, 1.1 percent; B) 11,128 cpm, 83 percent; C) 1409 cpm, 10 percent. The chromatographic column and temperature were the same as described in the legend for Fig. 5.

RESULTS

A. Assay of 1-Alkylglycero-3-phosphate:Acyl-CoA Acyltransferase Activity Preliminary work on the measurement of alkylglycero-P acyltransferase activity used thin layer chromatography and autoradiography to isolate the ^{32}P-labeled product of the reaction, alkylacylglycero-[^{32}P]phosphate. As a routine assay this method has the disadvantage of being tedious. A second assay involving a simple partition to isolate the product was devised and, since autoradiography was not employed in this system, the radioisotope tritium which has a much longer half-life, could be used. Fig. 7 shows a comparison of the two assays with guinea pig liver microsomes as the enzyme source. This tissue was used in the preliminary studies when working up the assays. The agreement was excellent, although the results with the partition assay were more variable. When studies were started with the rat brain microsomes the partition assay was monitored by checking an aliquot of the final product solution on thin layer chromatography. Determination of the tritium in multiple one cm bands of these chromatograms showed that most of the radioactivity was not associated with either the substrate or product. The contaminating radioactivity co-migrated with chimyl alcohol, the dephosphorylation product of alkylglycero-P, in two different solvent systems (see below). This problem was apparently solved by increasing the NaF concentration in the incubation mixture to 80 mM in order to inhibit the very active phosphatase present in brain microsomes. At this concentration of NaF, approximately 5 percent of the substrate was dephosphorylated under the conditions of this test assay (< 5 nmoles

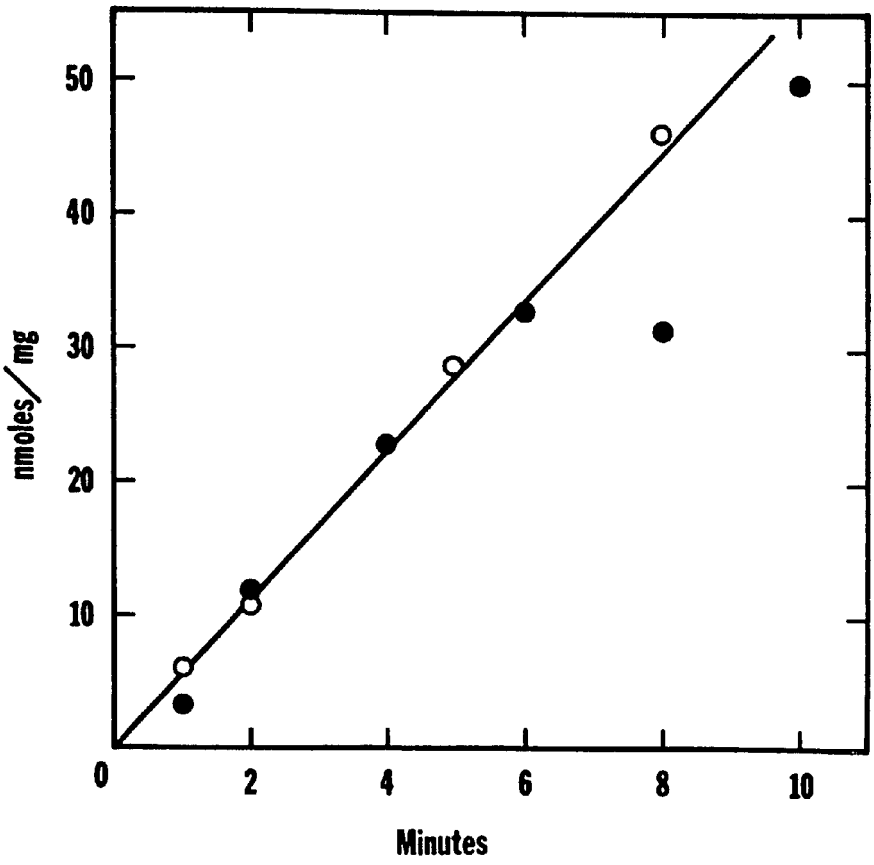


Fig. 7 Comparison of alkylglycerophosphate acyltransferase assay systems using guinea pig liver microsomes as the acyltransferase source. The data with ^{32}P-labeled substrate was obtained after isolation of the labeled product by thin layer chromatography (○—○). With ^{3}H-labeled substrate the product was separated from the substrate by differential partition in chloroform-methanol-buffer with the aqueous phase at pH 8 (●—●). The incubation conditions were as follows: For (○—○), 1-alkylglycero-3-[^{32}P]phosphate (43 nmoles, 11,000 cpm), oleoyl-CoA (30 nmoles), NaF (21 mM), $MgCl_2$ (4 mM), albumin (1.0 mg), and enzyme (0.15 mg protein) in a total volume of 1.2 ml. For (●—●), the conditions were essentially the same except that 1-alkyl-[2-^{3}H]glycero-3-phosphate (43 nmoles, 60,000 cpm) was the substrate and NaF was 84 mM and enzyme was 0.13 mg protein. Details of the assays are described under "Materials and Methods".

1-alkyl-[2-^{3}H]glycero-P, 0.2 mg protein).

After kinetic studies on the acyltransferase activity were underway, another chromatographic check on the partition assay showed that the proportion of ^{3}H-labeled chimyl alcohol to labeled alkylacylglycero-P varied considerably with the amount of added enzyme and concentration of substrate. In fact, at high substrate concentrations chimyl alcohol was the predominant product and the data represented the combined activities of two enzymes: acyltransferase and phosphatase. This problem could be circumvented by using ^{32}P-labeled substrate because the hydrolysis product remaining in the chloroform phase with alkylacylglycero-[^{32}P]phosphate would not be radioactive. However, an extensive re-evaluation of the partition assay at this stage showed that it produced results which were too variable for intended studies. The blank values due to contaminating substrate varied significantly between identical incubation tubes. All subsequent experiments reported herein were done using the assay involving thin layer chromatography. The reproducibility of this assay was better than $\pm$ 5 percent.

B. Requirements for Acyltransferase Activity Cofactor requirements for rat brain microsomal acyltransferase activity using 1-alkylglycero-[^{32}P]phosphate or alkyldihydroxyacetone-[^{32}P]phosphate plus NADPH as acceptor are listed in Table I. Either of these labeled lipids could serve as a substrate because NADPH:alkyldihydroxyacetone phosphate oxidoreductase is present in the microsomes used for the enzyme source (7). Thus, when alkyldihydroxyacetone phosphate plus NADPH is used, the direct acceptor, alkylglycerophosphate is produced in situ.

Table I

Cofactor requirements for acylation of alkylglycerophosphate by rat brain microsomes

The whole system contained Tris-HCl buffer (75 mM, pH 8.0), NaF (84 mM), $MgCl_2$ (42 mM), Triton X-100 (50 μg), either 1-hexadecyl-rac-glycero-3-[^{32}P]phosphate (52 nmoles, 22500 cpm) or hexadecyldihydroxyacetone-[^{32}P]phosphate (51 nmoles, 24000 cpm), oleoyl-CoA (30 μM), albumin (1.0 mg), and microsomes (0.18 mg protein) in a total volume of 1.2 ml. When hexadecyldihydroxyacetone phosphate was the substrate the whole system also included NADP+ (10 μg), D-glucose-6-P (5 mM), and D-glucose-6-P dehydrogenase (1.0 μg). The mixture was incubated at 37° for 15 minutes and the amount of lipid acylated was determined as described under "Materials and Methods".

Incubation conditions	Lipid acylated	
	Hexadecyl-glycero-P	Hexadecyl-dihydroxyacetone-P + NADPH
	nmoles	
Whole system	4.5	3.8
" - Triton	3.9	3.4
" - Oleoyl-CoA	0.1	0.4
" - Oleoyl-CoA + oleic acid (40 nmoles) + ATP (83 μM) + CoA (50 μg)	4.1	3.2
" - NaF	5.8	1.3
" - Albumin	3.0	2.4
" + EDTA	5.9	-
" - NADPH generating system	-	0.6
" with boiled enzyme*	0.3	0.3

*The enzyme was heated at 100° for 10 minutes before use.

Either acyl-CoA or fatty acid plus ATP and CoA can be used to provide the acyl group for transfer to the phosphoglyceride acceptor, although acyl-CoA gives a slightly higher rate. NaF (84 mM) inhibits the reaction slightly when alkylglycero-[^{32}P]phosphate is the substrate, but it is included in the standard incubation mixture to prevent hydrolysis of the phospholipid substrate (see below). The amount of phosphoglyceride acylated was increased when EDTA was added to the incubation, but with EDTA present, 50 percent of the substrate was dephosphorylated. This was confirmed in experiments with the ^{3}H-labeled substrate in standard incubations. In this case the dephosphorylated substrate, 1-alkyl-2-[^{3}H]glycerol, was isolated on thin layer chromatographic plates and quantitated by scintillation counting. Two different solvent systems were used to identify the dephosphorylated compound: chloroform-methanol-acetic acid-water (25:10:3:1) and hexane-ether-acetic acid (25:25:1). The labeled compound co-migrated with authentic chimyl alcohol in both solvents and no other dephosphorylated compound was detected in the lipids isolated from the incubation mixtures. Therefore only the substrate, alkylglycerophosphate and not the product, alkylacylglycerophosphate was dephosphorylated under the standard incubation conditions. When alkyldihydroxyacetone-[^{32}P]phosphate is used as the phospholipid substrate, the incorporation into 1-alkyl-2-acylglycero-3-[^{32}P]phosphate is stimulated by NaF. Preliminary experiments[1] showed that both alkylglycero-[^{32}P]-phosphate and alkyldihydroxyacetone-[^{32}P]phosphate were dephosphorylated at the same rate, so the difference in the effect of NaF on

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[1] Berent, S. and Hajra, A. K., Unpublished experiments.

alkylglycero-P and alkyldihydroxyacetone-P as substrates cannot be due to inhibition of different initial rates of dephosphorylation. Rather, the fact that when alkylglycero-P is made in situ from alkyldihydroxyacetone-P, the concentration of alkylglycero-P would be very low and depletion by a phosphatase would be an important rate limiting factor. Therfore, inhibition of the phosphatase by NaF in this case would have a more noticable effect.

C. Characterization of Product The labeled phospholipid product from the enzymatic reaction of 1-alkyl-rac-glycero-3-[^{32}P]phosphate and acyl-CoA was shown to be 1-alkyl-2-acyl-sn-glycero-3-[^{32}P]phosphate by chromatographic behavior and chemical and enzymatic degradation. The ^{32}P-labeled product co-migrated with phosphatidic acid on thin layer chromatograms in two different solvent systems: chloroform-methanol-acetic acid-water (25:10:3:1), Rf = 0.77; chloroform-methanol-30 percent methyl amine (65:25:8), Rf = 0.17. Double labeled product was formed during incubation of 1-alkylglycero-3-[^{32}P]phosphate and 1-[^{14}C]palmitic acid with enzyme and cofactors. Alkaline methanolysis of this purified, doubly labeled product resulted in two homogeneous compounds with chromatographic properties identical to the two radioactive substrates, i.e. palmitic acid and 1-alkylglycero-3-phosphate (see below). The purified lipid product was also shown to be hydrolyzed by phospholipase A_2. The procedure described by Wu and Tinker (221) was used to incubate ^{32}P-labeled product with phospholipase A_2 for 24 hours. After incubation the lipids were separated by thin layer chromatography. The radioactivity in the areas on the chromatogram corresponding to alkylacylglycero-P and alkylglycero-P were determined. The relative

amounts of radioactivity showed that 73 percent of the 1-alkyl-2-acyl-glycero-3-[^{32}P]phosphate was hydrolyzed by phospholipase A_2.

D. Discovery of a Second Acyltransferase Product Heterogeneity of the product was observed when guinea pig mitochondria were used as the enzyme source. The labeled lipids formed from either 1-alkylglycero-3-[^{32}P]phosphate or from [1-^{14}C]palmitic acid by guinea pig liver mitochondria and microsomes in the presence of different cofactors are illustrated by the autoradiogram in Fig. 8. It can be seen that two labeled products are formed (spots I and II) by the mitochondria. When microsomes were used as the enzyme source, only spot I was observed. The lipids corresponding to spots I and II in Fig. 8 were separately eluted and rechromatographed in the same solvent system. Each compound retained its respective Rf value and was homogeneous, showing that I and II are different compounds and not artifacts of the chromatography system. Both lipids I and II were formed when myristic, oleic, and linoleic acids were incubated with alkylglycero-[^{32}P]phosphate and proper cofactors. Mitochondrial fractions from rat liver were also capable of forming lipids I and II when used in similar incubation mixtures.

The main product (spot I, Fig. 8) formed from either labeled substrate was identified as 1-alkyl-2-acyl-sn-glycero-3-phosphate as described above. Hydrolysis of the lipid corresponding to spot II also gave 1-alkyl-sn-glycero-3-phosphate. However, the fatty acids liberated from II by hydrolysis migrated differently than normal long chain fatty acids on thin layer chromatograms. Spots I and II in lane 4 (Fig. 8) were formed using ^{14}C-labeled fatty acid. After alkaline

Fig. 8 Autoradiogram of lipids extracted from incubation mixtures after separation by thin layer chromatography. All incubations contained the folowing: Tris-HCl (75 mM, pH 8.5), Triton X-100 (50 μg), NaF (21 mM), ATP (8.4 mM), CoA (40 μM), and $MgCl_2$ (4.2 mM) in a final volume of 1.2 ml. In addition, respective incubations contained: (1) alkylglycero-[^{32}P]phosphate (3×10^4 cpm, 90 nmoles), palmitic acid (40 nmoles), mitochondria (0.81 mg protein). (2) as in 1, except microsomes (0.15 mg protein) in place of mitochondria were used. (3) alkylglycero-P (90 nmoles), [1-^{14}C]palmitic acid (40 nmoles, 10^6 cpm), mitochondria (0.81 mg protein). (4) as in 3, except microsomes (0.15 mg protein) in place of mitochondria were used. (5) standard phosphatidic acid. (6) alkylglycero-P. The mitochondrial incubations were done at 37° for 15 minutes and the microsomal for 5 minutes. Lipids were extracted and developed on commercial silica gel plates in chloroform-methanol-acetic acid-water (25:10:3:1). Spots I and II, in the case of the mitochondrial incubations, each represent approximately 4 nmoles of labeled lipid. Spot I, in the case of the microsomal incubations, represents 7 nmoles of labeled lipid.

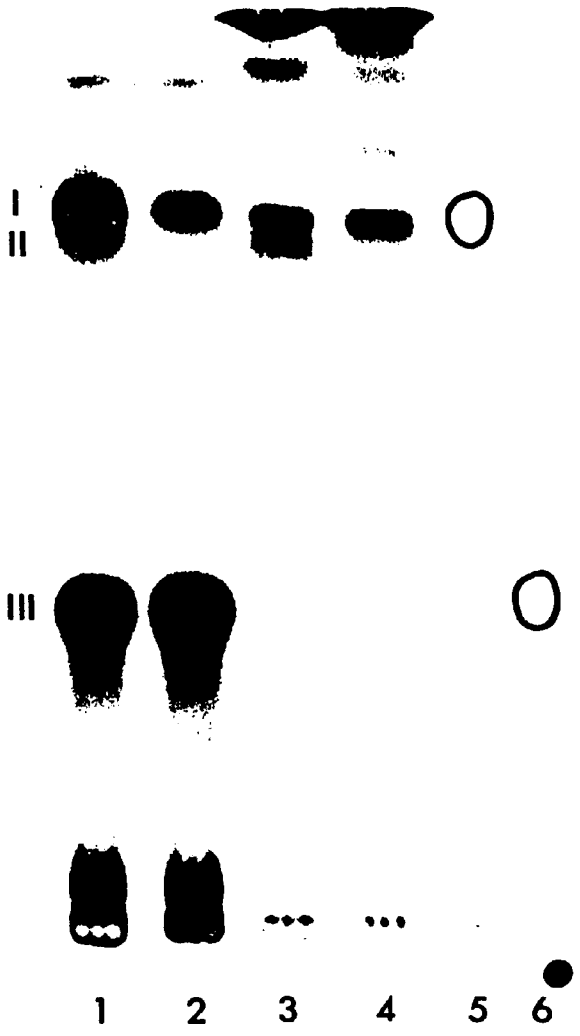


Figure 8

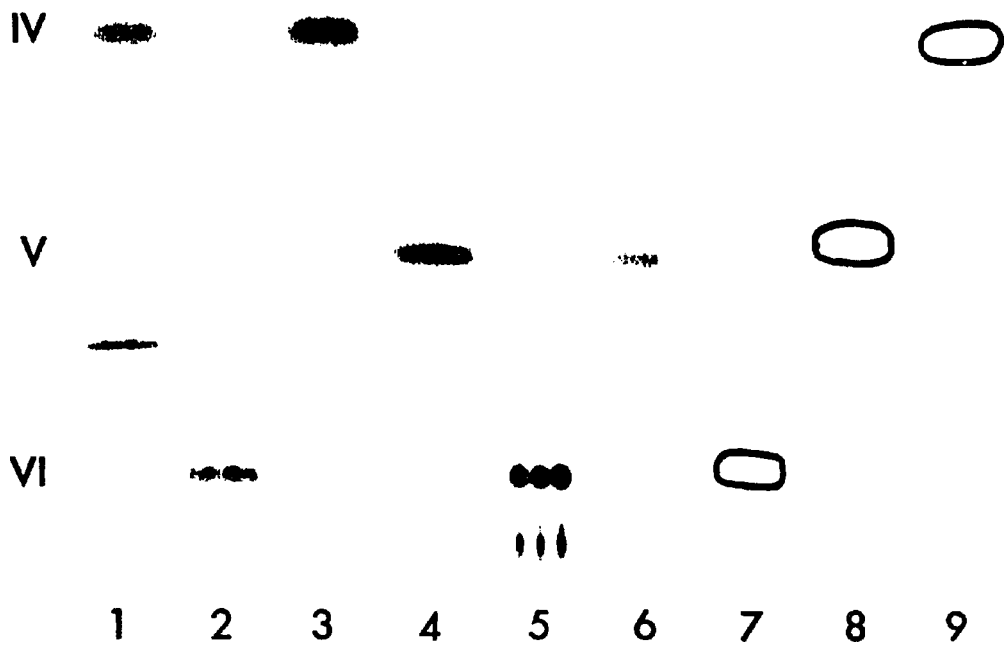


Fig. 9 Autoradiogram of radioactive fatty acids and their derivatives obtained on hydrolysis of 1-alkyl-2-acylglycero-3-phosphate formed during incubation with guinea pig liver mitochondria. Lane (1) represents the fatty acids liberated by alkaline methanolysis from the phosphoglyceride corresponding to spot I, Fig. 8. The fatty acids in lane (2) are from lipid II, Fig. 8. Lanes (3) and (4) show the respective products obtained when the labeled lipids in lanes (1) and (2) were treated with methanol, HCl, and dimethoxypropane. The polar fatty acid extracted from the mitochondrial incubation with [1-^{14}C]-palmitic acid is in lane (5), and the methyl ester of this labeled fatty acid is in lane (6). Lanes (7) - (9) represent the following respective standards: (7) 3-hydroxy fatty acid, (8) methyl ester of 3-hydroxy fatty acid, (9) methyl ester of normal fatty acid. The silica gel plate was developed in hexane-diethyl ether-acetic acid (30:70:1).

methanolysis, the labeled fatty acid from spot I migrated on thin layer chromatography with the same Rf as the methyl ester of normal fatty acid (see lanes 1 and 9, Fig. 9). The labeled fatty acid from spot II migrated as two compounds with Rf values identical to 3-hydroxy fatty acid and its methyl ester (see lanes 2,7 and 8,Fig. 9). Treatment of these labeled fatty acids with acidic methanol converted all species to the methyl esters (see lanes 3 and 4, Fig. 9). Thus the slower migration of spot II in Fig. 8 was attributed to an extra hydroxyl group which was present as an hydroxy fatty acid ester.

It was also found that, when [1-^{14}C]palmitic acid was incubated with guinea pig liver mitochondria under the same conditions as described in Fig. 8, but without alkylglycero-P, a polar fatty acid was formed. This polar fatty acid was extracted with the standard H_3PO_4-KCl-methanol-chloroform extraction and purified by silicic acid column chromatography. The column was eluted with two solvents: I) hexane-chloroform (25:75) and II) methanol-chloroform (2:98). The lipid in fraction II was methylated with acidic methanol and dimethoxypropane. From the chromatographic properties of the polar fatty acid and its methyl ester (lanes 5 and 6, Fig. 9), and also from its chemical properties (see below), this polar fatty acid was shown to be 3-hydroxy fatty acid.

The fatty acid released from spot II (Fig. 8) was identified as 3-hydroxy fatty acid by the following methods as outlined in Fig. 10. On treatment with dimethoxypropane and methanol, the radioactive compound which migrated with 3-hydroxy fatty acid disappeared, and only one major product was found which migrated with the methyl ester of 3-hydroxy fatty acid (Fig. 9, lane 4, spot V). Under the same

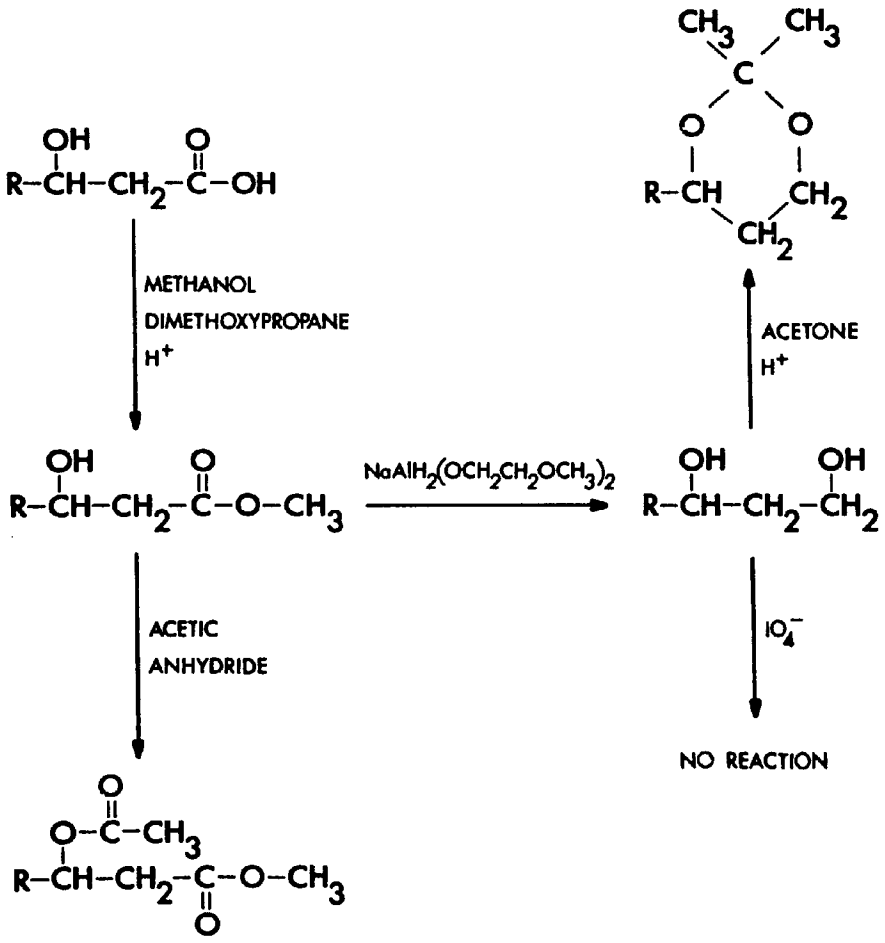


Fig. 10 Chemical characterization of 3-hydroxy fatty acid. (See text for details).

conditions, standard 3-hydroxy fatty acids are methylated to their methyl esters. The methyl ester of the fatty acid from lipid II upon treatment with acetic anhydride in pyridine formed a compound which, on thin layer chromatography on silica gel (hexane:diethyl ether:acetic acid, 30:70:1), co-migrated with the acetylated derivative of standard methyl 3-hydroxy fatty acid (Rf = 0.64). On reduction with Vitride the fatty acid from lipid II formed a product which had the same migration rate as long chain 1,3-diol on thin layer chromatography using hexane-diethyl ether (212) as the solvent system. The reduced compound was unchanged with respect to its migration in the same thin layer chromatographic system after treatment with periodate. Under the same conditions periodate completely oxidized 1,2-diols. On treatment with acetone and perchloric acid the reduced compound (diol) was completely converted to a less polar product which had the same migration rate as the acetonide of long chain 1,3-diols (Rf = 0.66 compared to diol, Rf = 0.11). Finally, the acetylated derivative of the methyl ester of the radioactive polar fatty acid formed from [1-^{14}C]palmitic acid was injected with the same derivatives of unlabeled carrier 3-hydroxy fatty acids into the gas chromatographic column, and different fractions eluting from the column were collected. The chromatographic conditions for this analysis are given in the legend of Fig. 11, which shows the separation of 3-acetoxy fatty acid methyl esters. The effluent collection periods can be identified from the blips in the baseline in Fig 11. When the radioactivity of these fractions was determined, the major radioactive peak had the same retention time (Rt = 3.8 min) as the methyl ester of 3-acetoxypalmitate. Radioactivity determinations in cpm were as fol-

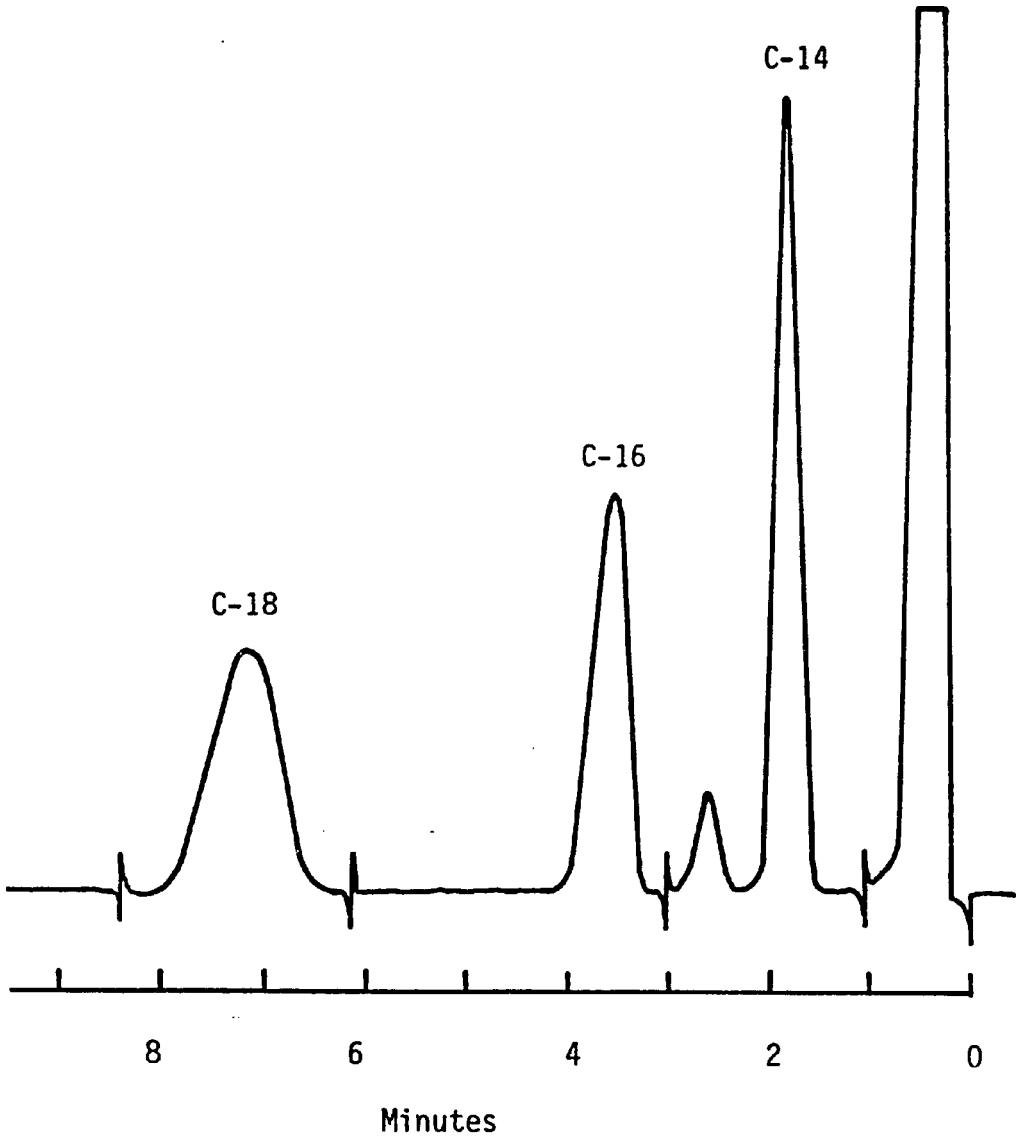


Fig. 11 Separation of 3-acetoxy fatty acid methyl esters in a gas liquid chromatographic column. 3-Hydroxy myristate, palmitate and stearate methyl esters and 3-hydroxy-[1-^{14}C]palmitate methyl ester were acetylated with acetic anhydride as described in the text. The 3 ft x 5/32 inch column contained 5 percent OV-101 on Gas-chrom A, 100/120 mesh and was maintained at 200°. Helium (75 ml/min) was the carrier gas and a thermal conductivity detector was employed. The carbon numbers in the figure refer to the long chain hydrocarbon moiety. The peak at 2.6 minutes Rt was unidentified. The blips in the base line at 1,3,6, and 8 minutes are the points where the collection apparatus was changed.

lows: 3-hydroxy myristate, 420; 3-hydroxy palmitate, 2019; 3-hydroxy stearate, 218. This showed that the [1-^{14}C]palmitic acid was converted to the 3-hydroxy derivative in the mitochondria without significant degradation or chain elongation during the incubation.

The polar fatty acid formed from [1-^{14}C]palmitic acid behaved chemically exactly as the fatty acid from spot II (see Fig. 10). When this labeled 3-hydroxy fatty acid was substituted for [1-^{14}C]-palmitic acid in the incubation mixture with alkylglycero-P, only spot II and no spot I was observed on the thin layer chromatogram. Guinea pig liver microsomes would also form spot II when provided with 3-hydroxy fatty acid in the incubation mixture. Addition of NAD+ (2 mg) to the incubation mixture prevented the formation of spot II.

The formation of a phosphoglyceride *in vitro* which contained 3-hydroxy fatty acid raised the possibility of a similar lipid being formed *in vivo*. Using the assay described under "Materials and Methods", a search was made for 3-hydroxy fatty acid esterified to ethanolamine and choline phosphoglycerides in various tissues. Since the hydroxy lipid had been found in incubations with guinea pig liver mitochondria, the search was started with guinea pig liver. The ethanolamine and choline phosphoglycerides were purified from a total lipid extract as described under "Materials and Methods". No 3-hydroxy fatty acids were found in either phosphoglyceride fraction using the assay procedure described above. This result meant that, if such a lipid existed in the liver, it was present in quantities of less than 0.1 percent of the total lipid, which was the detection level of the assay being used. Although lower amounts could have

been detected by starting with more tissue or using a chromatograph with a more sensitive detector, it was decided that for this initial survey it was important to determine if the 3-hydroxy lipid was present in significant amounts, i.e. greater than 0.1 percent of the total lipid. The next tissues assayed were rat brain and liver. No 3-hydroxy fatty acid was found esterified to the phosphoglycerides of these tissues either. At that point in time it was thought that, if normal tissue did not contain the hydroxy lipid, a tissue undergoing pathological degeneration might form this lipid. The reasoning behind this hypothesis was that 3-hydroxy fatty acids were presumably made inside the mitochondria where the enzymes for fatty acid oxidation are known to be located. Only in the case of damaged or "leaky" mitochondria would these acids be available to the phosphoglyceride acyltransferases located outside this organelle. Gross structural changes in the mitochondria have been shown to occur in the pathological condition of muscular dystrophy (233). It is also possible to obtain tissue samples from mice which have an inherited form of this disease. The muscular dystrophy appeared as a spontaneous mutation in these mice and they have been developed by the Jackson Laboratory, Bar Harbor, Maine as an animal model for similar diseases in man. The first external symptom exhibited by these mice is an impairment in the use of the hind legs. As the condition progresses the hind legs, become extended and the animal uses only the forelimbs for locomotion. When the disability was clearly evident in both hind legs a group of mice was killed and the muscle around the hind leg was dissected away and homogenized in a Virtis blender-type homogenizer with 6 volumes methanol (1 gm: 6 ml). Chloroform was then added and

a standard Folch extract of the total lipids was done. The ethanolamine and choline phosphoglycerides were assayed for 3-hydroxy fatty acid esters and the results were negative. Tissue samples from both homozygotes and heterozygotes for the dystrophic gene were assayed by this procedure and neither contained 3-hydroxy fatty acid esters.

E. Optimal Conditions for Acyltransferase Activity The detergent Triton X-100 increased the amount of phosphoglyceride acylated during in vitro incubation with enzyme (Table I). Experiments were done to determine the optimal concentration of Triton X-100 for enzyme activity. The results with palmitoyl-CoA indicated a broad optimum from 0.2 - 0.4 mg/ml with inhibition at higher concentrations (30 percent at 0.7 mg/ml). And therefore 0.33 mg/ml was chosen for the standard condition in the incubation mixture.

The data in Table I indicate that NaF and divalent ions cause a slight inhibition of acyltransferase activity. However, it was necessary to add both fluoride and magnesium ions to incubation mixtures to prevent dephosphorylation of the substrate, alkylglycero-[^{32}P]phosphate. These two ions provide a synergistic inhibition of this dephosphorylation, and the optimal concentrations were determined as shown in Fig. 12. Approximately 30 percent of the phospholipid substrate was hydrolyzed when either NaF or $MgCl_2$ was omitted from the incubation mixture. But hydrolysis could be almost completely inhibited when both were added. The standard conditions (NaF, 21 mM; $MgCl_2$, 1.7 mM) were chosen as a compromise between inhibition of acyltransferase activity and inhibition of hydrolysis of substrate (Fig. 12).

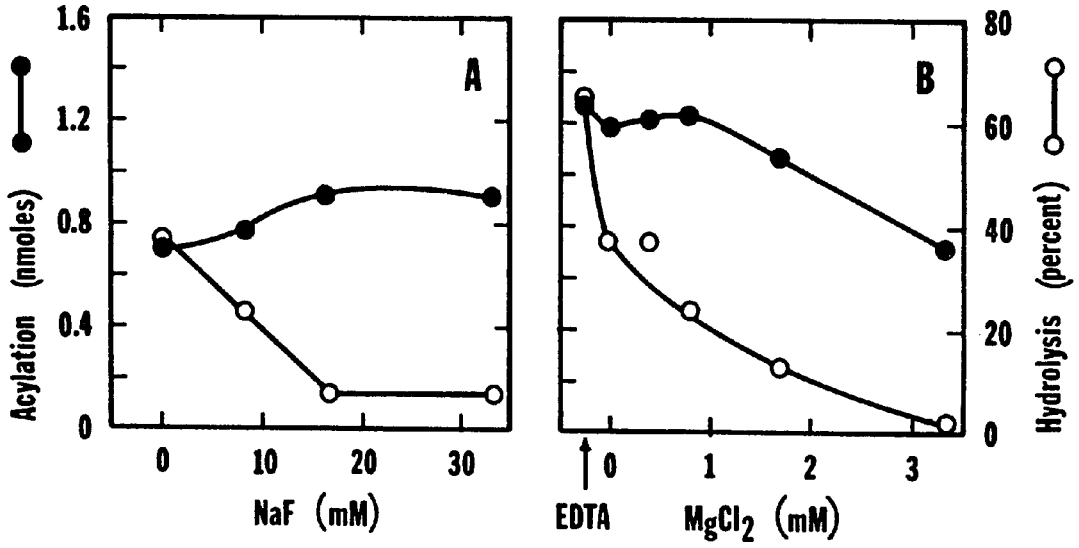


Fig. 12 Effect of increasing concentrations of NaF or $MgCl_2$ on the acylation (●—●) or hydrolysis (○—○) of alkylglycero-[^{32}P]phosphate. Alkylglycero-[^{32}P]phosphate (25 nmoles) was incubated under standard conditions except that the concentration of NaF (A) or of $MgCl_2$ (B) was varied, and in one case EDTA (8.3 μM) was added in place of $MgCl_2$. Radioactive lipids were extracted, isolated, and the amounts of acylation and hydrolysis of alkylglycero-[^{32}P]phosphate were determined as described under "Materials and Methods".

The increase in acylation of alkylglycero-[^{32}P]phosphate was linear with both increasing protein and time within limits (Fig. 13). The drop in rate of acylation at longer times was due to the fact that the acyl-CoA was approaching depletion. Acyl-CoA acylhydrolase was also present in the brain microsomal fractions. This enzyme was assayed in a standard incubation mixture (minus phospholipid acceptor) at 22^{o} by adding 1.0 mole 5,5'-dithiobis-(2-nitrobenzoic acid) and measuring the change in absorbance at 413 nm (234). Approximately 25 percent of the acyl-CoA (5 nmoles) was hydrolyzed in 5 minutes. Since this amount of hydrolysis would not diminish the acyl-CoA concentration below that permitting maximal rates of acylation (see Fig. 15), it was not considered limiting under the standard conditions of 5 minutes and less than 0.1 mg enzyme protein. However, if $MgCl_2$ and NaF were omitted from the incubation mixture, the acyl-CoA acylhydrolase activity increased dramatically to rates of approximately 50 nmoles/min/mg with rat brain microsomes. Triton X-100 (0.33 mg/ml) did not affect the rate of the acylhydrolase.

Acylation of acylglycero-[^{32}P]phosphate could also be assayed under the same conditions employed with alkylglycero-[^{32}P]phosphate, and when using the acyl phosphoglyceride substrate, the assay was linear at lower protein concentration and shorter incubation times (Fig. 13).

The effect of increasing concentrations of alkylglycero-[^{32}P]-phosphate on the rate of acyltransferase activity with varying amounts of albumin in the incubation mixture is shown in Fig. 14. When no albumin is present the reaction is inhibited at high phospholipid concentrations. The family of curves (Fig. 14) obtained with increasing amounts of albumin shows that albumin reduces the inhibition at

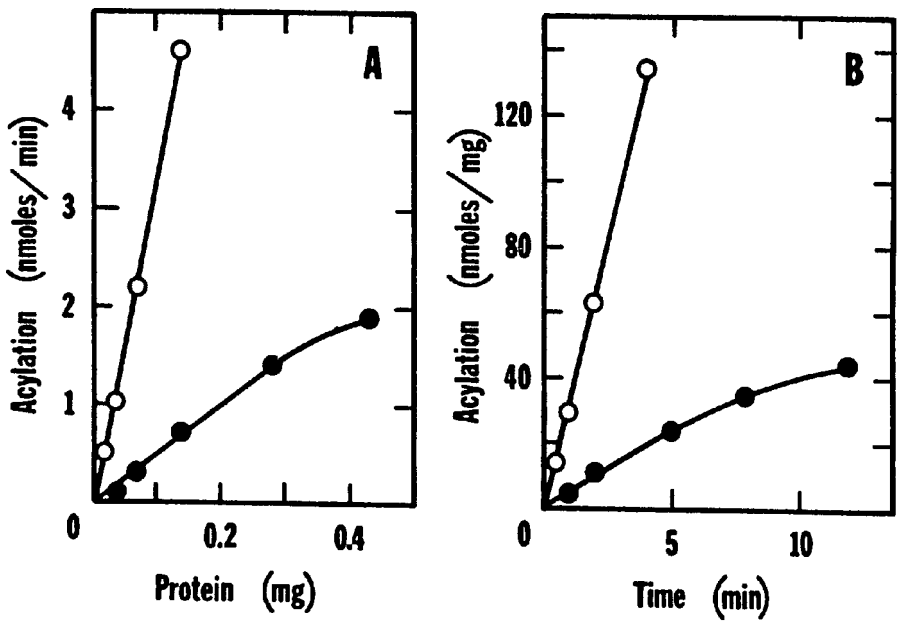


Fig. 13 A, Effect of increasing enzyme and B, time course of the acylation of alkyl- (●—●, 90 μM) or acyl- (○—○, 80 μM) glycero--[^{32}P]phosphate with palmitoyl-CoA (17 μM). Incubation times for part A were 5 minutes for alkyl-and 1 minute for acylglycero-[^{32}P]-phosphate . Amounts of protein in the incubation mixtures for part B were 0.14 mg for alkyl- and 0.04 mg for acyl-glycero-[^{32}P]phosphate. The rates of acyltransferase activity were determined as described under "Materials and Methods".

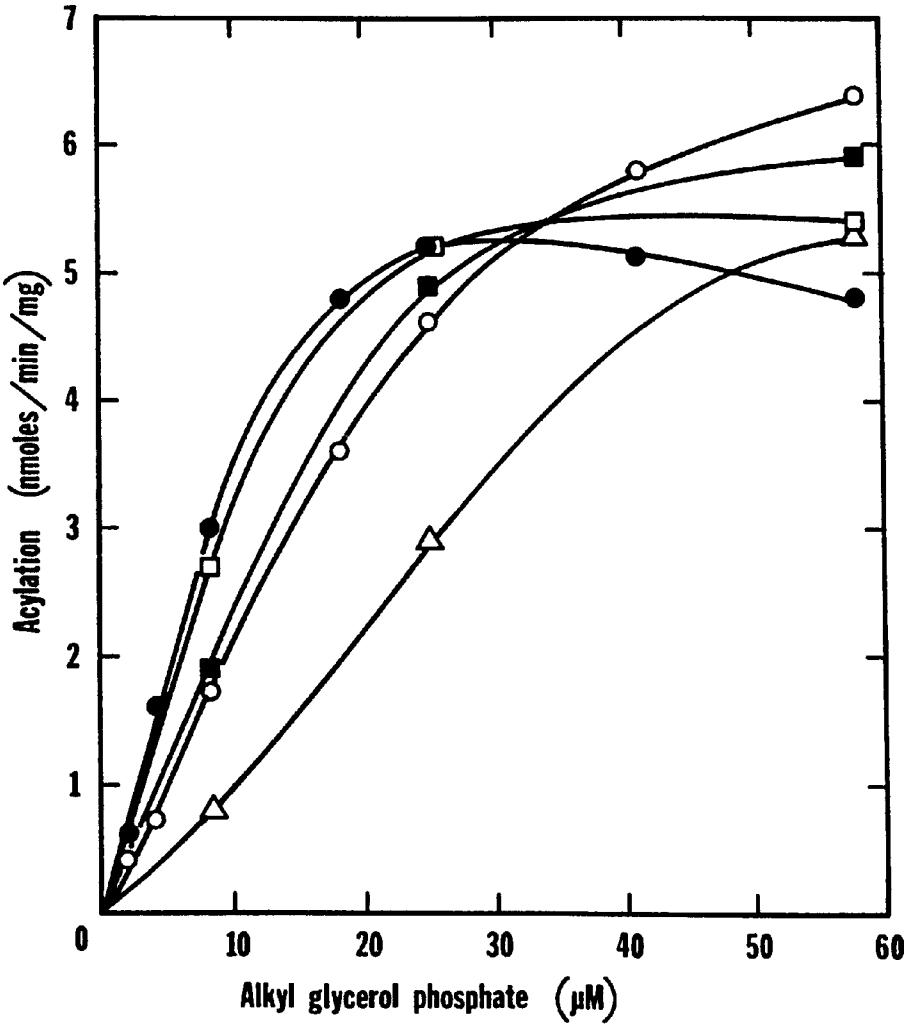


Fig. 14 Effect of albumin on the acylation of alkylglycero-[^{32}P]-phosphate. The rate of acyltransferase activity with increasing concentrations of alkylglycero-[^{32}P]phosphate was determined under standard conditions except that albumin was present in the following amounts: none, (●—●); 0.2 mg, (□—□); 0.6 mg, (■—■); 1.0 mg, (○—○); 2.0 mg, (△—△).

high substrate concentrations (>40 μM). However, the reaction is inhibited at low substrate concentrations (<25 μM) when increasing amounts of albumin are added. With the standard addition of 1.0 mg of albumin, the velocity versus substrate concentration curve is hyperbolic at substrate concentrations up to 30 μM, and can be described by Michaelis-Menten kinetics.

F. Tissue Distribution of Alkylglycero-P and Acylglycero-P Acyltransferase Activity The data in Table II show the rate of acyltransferase activity in the particulate fractions from six rat tissues. The enzymatic activity was assayed with both alkyl- and acylglycero-[^{32}P]phosphate. Acyltransferase activity toward the two acceptors did not vary proportionately in the different tissues. Heart and brain contained the highest rates of activity towards alkylglycero-P, and liver contained the highest activity with acylglycero-P. The ratios of alkyl- to acylglycero-P acyltransferase activities are also shown in Table II. From the data in this column it can be seen that heart, spleen, and brain particulate fractions contained the highest relative acyltransferase activities with alkylglycero-[^{32}P]phosphate as the acceptor.

G. Brain Subcellular Distribution of Alkylglycero-P Acyltransferase Activity All rat brain subcellular fractions exhibited acyltransferase activity with alkylglycero-[^{32}P]phosphate as the acceptor (Table III). However, much of the activity in the separate fractions was due to microsomal contamination. Table III also lists the percent microsomal contamination as determined by NADPH:cytochrome c reduct-

Table II

Tissue distribution of alkylglycerophosphate acyltransferase activity

The particulate fraction sedimenting between 1000 x g and 100,000 x g from various rat tissues was incubated with either alkyl- (28 μM) or acyl- (29 μM) glycero-[^{32}P]phosphate under standard conditions using 0.1 mg enzyme protein and arachidonoyl-CoA. The rate of acylation was determined as described under "Materials and Methods".

Source of Enzyme	Acyltransferase activity		Ratio of activities alkyl/acyl
	with alkyl-glycerophosphate	with acyl-glycerophosphate	
	nmoles/min/mg		
Heart	2.8	1.1	2.5
Spleen	1.3	1.3	1.0
Brain	2.6	4.1	0.6
Kidney	1.6	4.3	0.4
Liver	2.0	8.5	0.2
Lung	0.9	4.9	0.2

Table III

Brain subcellular distribution of alkylglycerophosphate acyltransferase activity

Various rat brain subcellular fractions prepared as described in the text were incubated with alkylglycero-[^{32}P]phosphate (110 μM) under standard conditions with oleoyl-CoA. The rate of acylation was determined by extracting and isolating the radioactive lipids on thin layer chromatography. The percent of microsomal contamination in each fraction was estimated by the activity of NADPH:cytochrome c reductase as described under "Materials and Methods".

Subcellular Fraction	NADPH:Cyto. c Reductase	Microsomal Contamination	Acyltransferase activity	
			uncorrected	corrected for percent microsomes
	units*	%	nmoles/min/mg	
Microsomal	.210	100	3.9	3.9
Mitochondrial	.020	9.5	1.2	0.8
Synaptosomal	.060	28.5	1.5	0.4
Myelin	.070	35.0	1.0	-0.3
Soluble	.016	7.5	0.7	0.4

*A unit of activity is defined as Δ1.0 absorbance unit/min/cm.

ase activity. Both myelin and synaptosomal fractions contained large proportions of microsomal membrane (approximately 30 percent) and when this was corrected for, these fractions contained essentially no acyltransferase activity. The microsomal fraction had the highest specific activity, but the mitochondrial fraction showed significant activity even when corrected for microsomal contamination.

H. Acyltransferase Specificity Towards Various Acyl-CoA Substrates

The effects of increasing concentrations of four different acyl-CoA substrates on the rate of acylation are shown in Fig. 15. The results obtained with both alkylglycero-P and acylglycero-P are included. Time course experiments with varying amounts of acyl-CoA demonstrated that the lower rate of acyltransferase activity shown at low acyl-CoA concentrations in Fig. 15 is due to limiting amounts of acyl-CoA. In an attempt to determine the apparent Km for acyl-CoA, the incubation time for the assay with alkylglycero-[^{32}P]phosphate as acceptor was shortened to one minute in separate experiments with arachidonoyl- and oleoyl-CoA. The acyl-CoA substrates were added in concentrations of 1.7 to 5.0 μM. Under these conditions the amount of acylation is small, and only 20 to 40 percent above the blank value. However, it appeared that maximal activity was obtained with acyl-CoA concentrations as low as 1.7 μM. Therefore no apparent Km could be determined. The data in Fig. 15 show that the acyltransferase reaction is inhibited by substrates at high concentrations (33 μM) of acyl-CoA. Specificity toward different acyl-CoA substrates was exhibited, and this specificity was different for alkylglycero-P acyltransferase when compared to acylglycero-P acyltransferase activity. With the ether-

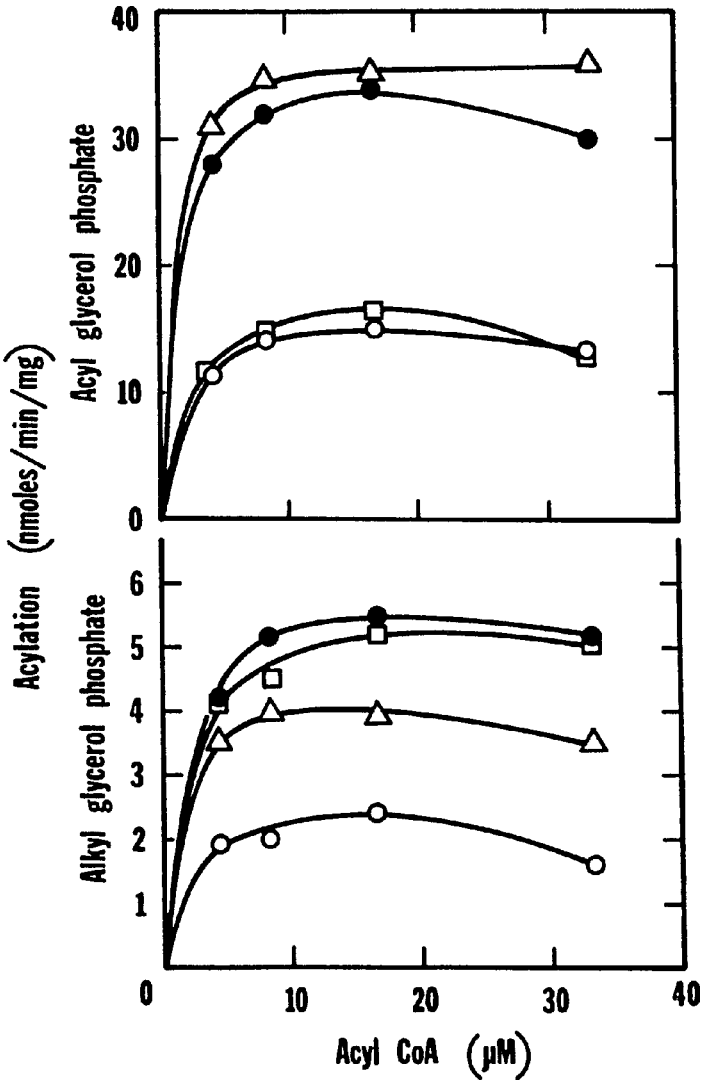


Fig. 15 Effect of increasing acyl-CoA concentrations on the rate of acyltransferase activity. Top, acylglycero-[^{32}P]phosphate (80 μM) was incubated for 1 minute or, Bottom, alkylglycero-[^{32}P]phosphate (90 μM) was incubated for 5 minutes under standard conditions with increasing concentrations of various acyl-CoA co-substrates and the rate of acyltransferase activity was determined as described under "Materials and Methods". Symbols for each acyl-CoA are: palmitoyl (●—●), oleoyl (○—○), linoleoyl (△—△), arachidonoyl (□—□).

containing substrate, palmitoyl- and arachidonoyl-CoA were the most efficient acyl donors, whereas with the ester containing substrate, linoleoyl- and palmitoyl-CoA were the better donors.

One explanation for the acyl-CoA selectivity could be the existence of different acyltransferases for different acyl-CoA substrates. If this were the case, addition of saturating amounts of two different acyl-CoA species together to the incubation should produce an additive increase in the maximum velocity. Therefore the rate of acylation was also assayed with combinations of two acyl-CoA substrates, with alkylglycero-[^{32}P]phosphate (28 μM) as the acceptor. The rates of acylation with saturating[1] amounts of one or two acyl-CoA species are shown in Fig. 16. Incubation with palmitoyl-CoA (10 μM) and arachidonoyl-CoA (10 μM) combined produced a rate of acylation 150 percent of the maximum obtainable with saturating concentrations of either acyl-CoA alone. However, combining oleoyl-CoA (10 μM) with either palmitoyl-CoA (10 μM) or with arachidonoyl-CoA (10 μM) did not produce an increase in the maximum rate, nor did it decrease the rate obtained with either palmitoyl- or arachidonoyl-CoA alone. Thus the results suggest that at least two acyltransferases are present; one that will accept palmitoyl-CoA and a separate enzyme that will accept arachidonoyl-CoA.

To further investigate the acyl-CoA selectivity of acyltransferase in brain microsomes the apparent Km for phospholipid acceptor

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[1]The term "saturating" is used in an empirical sense. The true concentration of acyl-CoA available to the enzyme as monomer is a function of the critical micelle concentration and may not be saturating the enzyme. But this is the concentration which gives maximal velocity in the in vitro incubation.

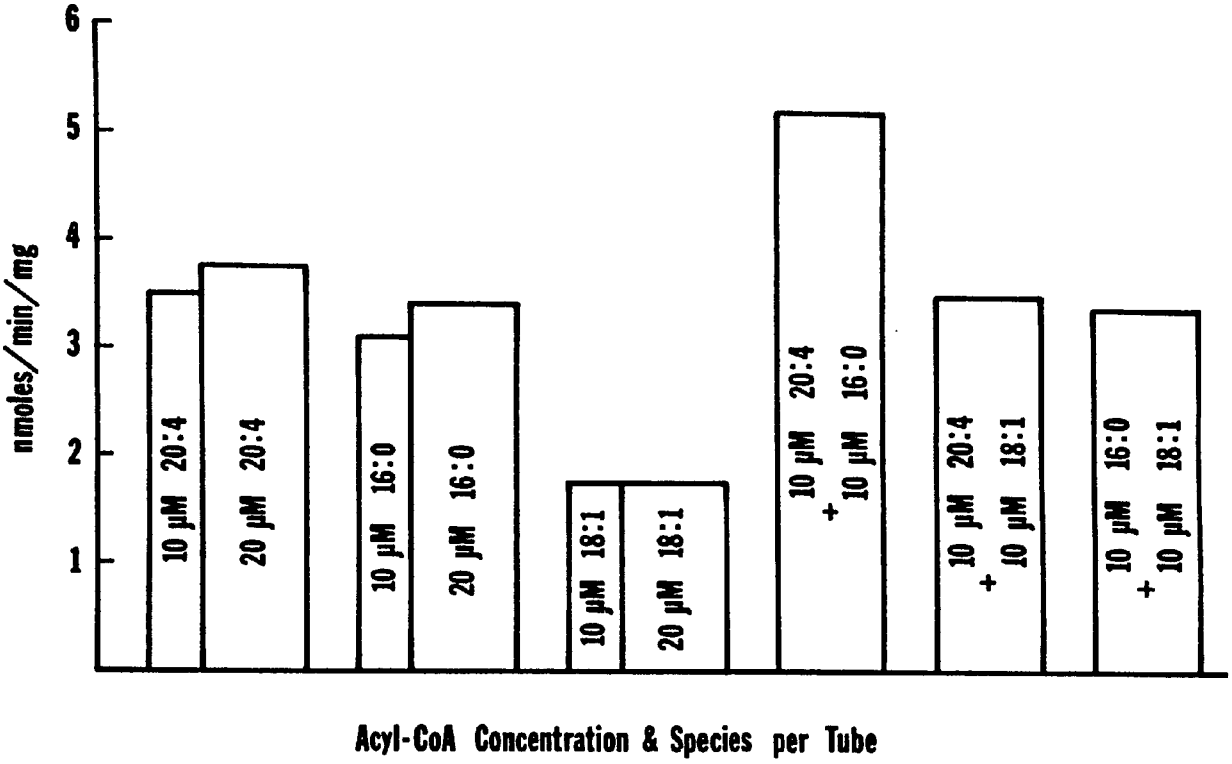


Fig. 16 Effect of acyl-CoA combinations on acyltransferase maximum velocity. The standard incubation conditions described under "Materials and Methods" were used with 34 μM alkylglycero-[^{32}P]phosphate and the concentrations of acyl-CoA shown in the figure. Velocity of acylation was determined with the standard assay involving thin layer chromatography.

and maximum velocity with each acyl-CoA was determined. The data obtained with alkylglycero-[^{32}P]phosphate as the acceptor are shown in Fig. 17. Most of the points shown are the mean values of two separate determinations. The absolute values for the apparent kinetic parameters varied by as much as 40 percent between enzyme preparations and changed somewhat with repeated thawing and freezing of the microsomes, but the relative values obtained with different acyl-CoA substrates were always of the same order of increasing values and approximately proportional. The data in Figs. 17 and 18 were obtained with the same preparation of microsomes. The data obtained with acylglycero-[^{32}P]phosphate as the acceptor are shown in Fig. 18. Each point represents one determination.

The apparent Km values for alkylglycero-[^{32}P]phosphate with different acyl donors increase in the order: arachidonoyl, 5 μM; oleoyl 7 μM; linoleoyl, 12 μM; palmitoyl, 100 μM (Fig. 17). The maximum velocities vary widely, but do not follow the same order. In contrast, the apparent Km values for acylglycero-[^{32}P]phosphate increase as follows: palmitoyl 25 μM; oleoyl, 100 μM; arachidonoyl, 125 μM; linoleoyl, 1 mM, and the maximum velocities again vary greatly and are much higher than the maximum velocities obtained with alkylglycero-[^{32}P]phosphate (Fig. 18). These kinetic parameters are also reflected in the data shown in Fig. 15 which were obtained at high phospholipid concentrations (80 - 90 μM).

All of the above experiments were done using the standard incubation conditions with Triton X-100 present. Recently, Okuyama *et al* (173) reported that acylglycerophosphorylcholine:oleoyl-CoA acyltransferase from liver was inactivated by Triton X-100 at con-

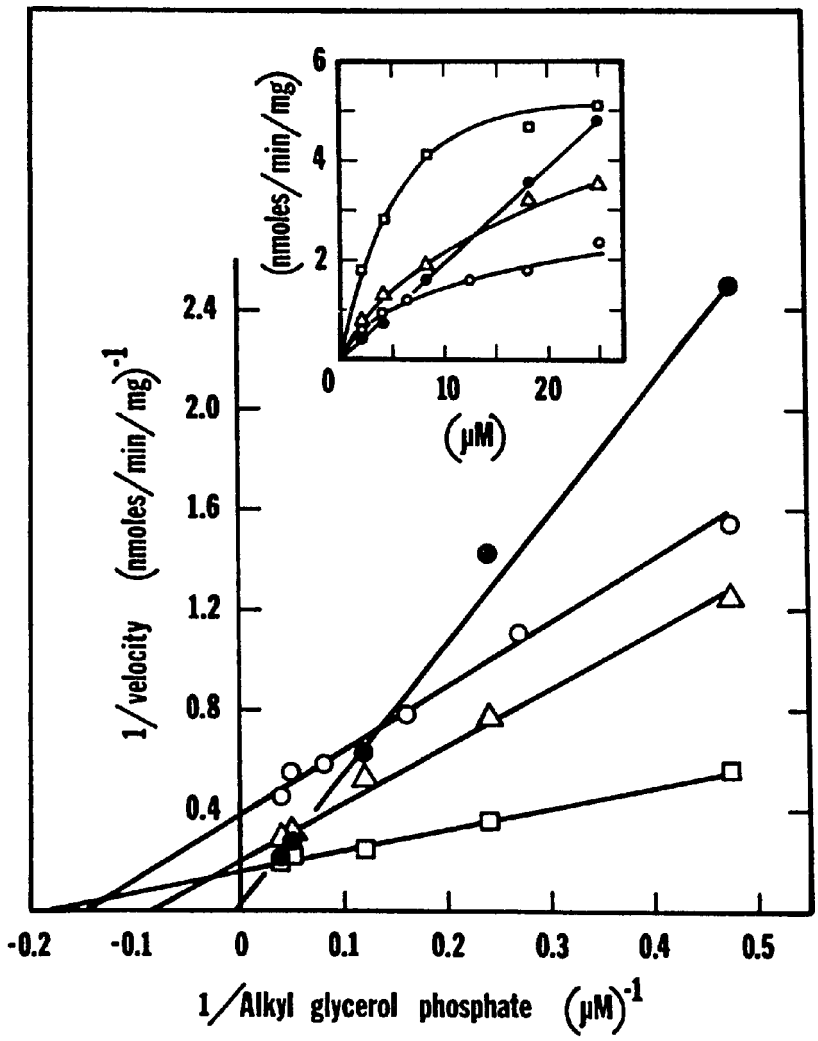


Fig. 17 Effect of increasing alkylglycero-[^{32}P]phosphate concentrations on the rate of acyltransferase activity with different acyl-CoA co-substrates. Alkylglycero-[^{32}P]phosphate was incubated for 5 minutes under standard conditions with either palmitoyl- (●—●), oleoyl- (○—○), linoleoyl-(△—△), or arachidonoyl- (□—□) CoA and the rate of acyltransferase activity was determined as described under "Materials and Methods".

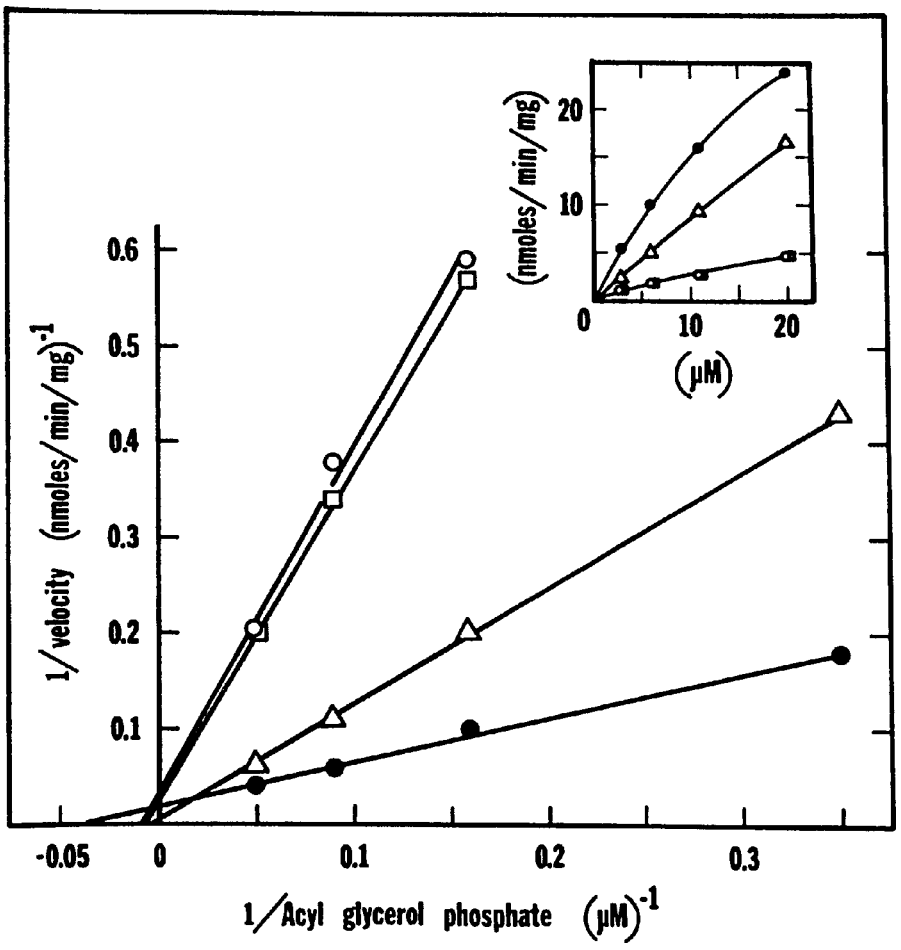


Fig. 18 Effect of increasing acylglycero-[^{32}P]phosphate concentrations on the rate of acyltransferase activity with different acyl-CoA co-substrates. Acylglycero-[^{32}P]phosphate was incubated for 1 minute under standard conditions with either palmitoyl- (●—●), oleoyl- (○—○), linoleoyl-(△—△), or arachidonoyl- (□—□) CoA and the rate of acyltransferase activity was determined as described under "Materials and Methods".

centrations that had minimal effect on the arachidonyl-CoA acyltransferase. The effects of increasing Triton concentration on the acylation of alkylglycero-[^{32}P]phosphate with oleoyl-CoA and arachidonoyl-CoA were examined in addition to the initial studies with palmitoyl-CoA. With arachidonoyl-CoA the effects of increasing Triton were similar to those described above for palmitoyl-CoA. With oleoyl-CoA the stimulation with low amounts of Triton (<0.1 mg/ml) was observed, but at 0.33 mg/ml the rate of acyltransfer was 75 percent inhibited.

This result indicated that the acyl-CoA selectivities observed above might have been due to differential effects of Triton X-100. Therefore, the effects of increasing acyl-CoA or increasing alkylglycero-P on rate of acylation were retested without Triton in the incubation mixture. The data are shown in Fig. 19. The rates of acylation in these experiments are lower than those shown in Figs. 17 and 18. This is due to both the lack of Triton and to a different preparation of enzyme. The relative rate with oleoyl-CoA is greater without Triton, confirming that this activity was inhibited in the previous experiments. However, the relative order of rates, i.e. palmitoyl $\cong$ arachidonyl > oleoyl is the same as described above with Triton. The data obtained with docosahexaenoic(n-3)-CoA are also shown in Fig. 19. This acyltransferase activity shows a dramatic decrease in the apparent Km for phospholipid acceptor, and concommitant increase in the inhibition caused by high concentrations of the acceptor, alkylglycero-P, when compared to the other acyl-CoA acyltransferase activities. The increase in rate with increasing alkylglycero-P is not hyperbolic when Triton is omitted; therefore the reciprocal plots for the curves in part B (Fig. 19) are nonlinear.

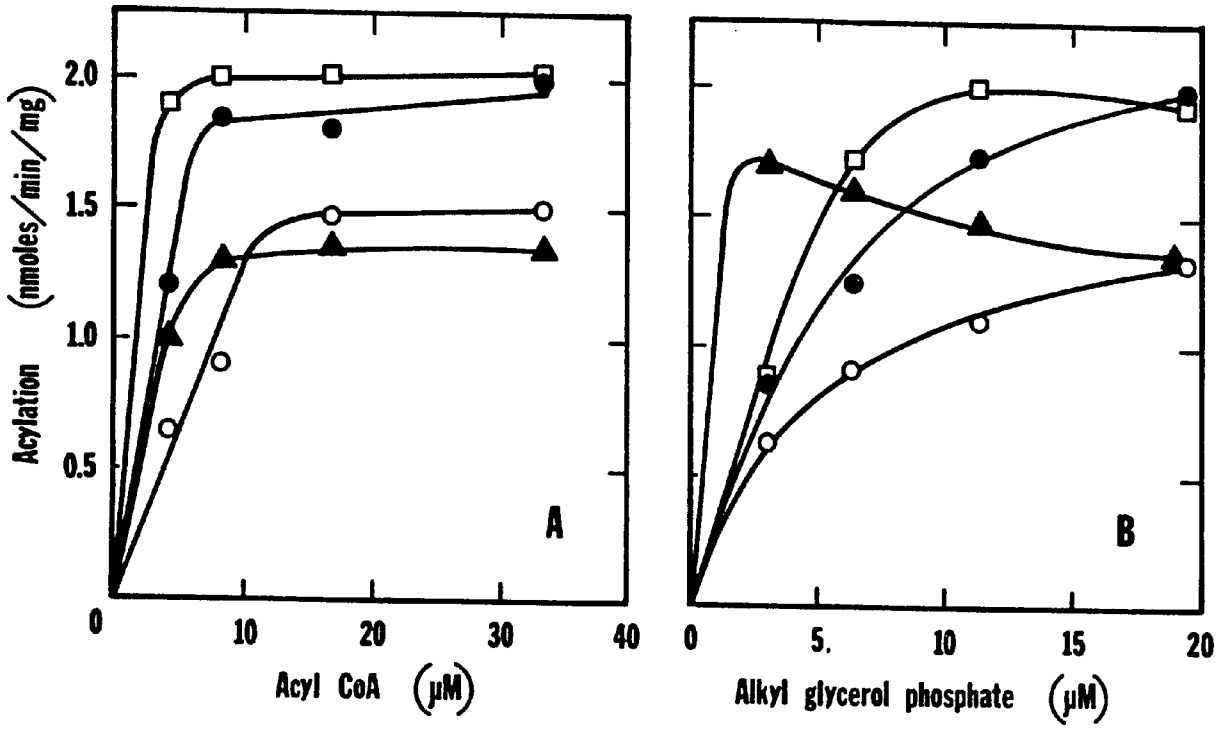


Fig. 19 A, Effect of increasing acyl-CoA and B, effect of increasing alkylglycero-[^{32}P]phosphate on the rate of acyltransferase activity without Triton X-100. Incubations were for 5 minutes under standard conditions (minus Triton). The concentration of alkylglycero-[^{32}P]-phosphate in (A) was 23 μM. The concentration of individual acyl-CoA substrates in (B) was 17 μM. Acyltransferase activity was determined as described under "Materials and Methods". Symbols for each acyl-CoA are: palmitoyl (●—●), oleoyl (○—○), arachidonoyl (□—□), docosahexaenoyl (▲—▲).

Qualitatively the curves in part B (Fig. 19) are similar to those in Fig. 17 for palmitoyl-, arachidonoyl-, and oleoyl-CoA donors.

I. Acyltransferase Selectivity Towards Combined Acyl-CoA Substrates

The results obtained both with and without Triton X-100 indicate that at low concentrations of alkylglycero-P, the polyunsaturated acyl species are incorporated more rapidly than the saturated species. To investigate further the conclusion that the alkylglycero-P acyltransferase was selective for acyl-CoA species, and that the concentration of the phosphoglyceride acceptor was important to that selectivity incubations were done with a mixture of different acyl-CoA species, and the alkylglycero-P concentrations were kept very low. To maintain low acceptor concentrations and still obtain significant acylation, the alkylglycero-P was made in the incubation mixture by enzymatic reduction of alkyldihydroxyacetone-P. As discussed above, the data in Table I show that alkyldihydroxyacetone-P plus NADPH can be added to the incubation mixture as precursors to alkylglycero-P. The NADPH: alkyldihydroxyacetone phosphate oxidoreductase present in the brain microsomes produces alkylglycerophosphate at approximately 4 - 5 nmoles/min/mg in a similar incubation mixture (7). Thus, as the alkylglycero-P was being formed in situ, it was simultaneously being acylated. The incubation mixture is described in Table IV and the data listed there show the acyltransferase selectivity with both low and high (29 μM) alkylglycero-P concentrations. When presented with equal concentrations of six different acyl-CoA species, the acyltransferase selectivity is dependent on the concentration of acceptor.

TABLE IV

Acyltransferase selectivity of combined acyl-CoA substrates

Rat brain microsomes (0.073 mg protein) were incubated with 7.5 nmoles of each of the following acyl-CoA species (16:0, 18:0, 18:1, 20:4, 22:4, 22:6) plus the standard amounts of Tris-PO_4 buffer (pH 8.0), NaF, $MgCl_2$, and albumin. The phospholipid substrate was either alkylglycero-[^{32}P]phosphate (35 nmoles) or alkyldihydroxyacetone-[^{32}P]phosphate (32 nmoles) plus NADPH (200 μM). With the latter substrate the incubation time was extended from 5 to 10 minutes. No Triton was added. The acylated radioactive lipid was extracted from the incubation mixture and the unreacted phospholipid substrate was separated from the acylated product by partition in a chloroform-methanol-buffer system. The product was treated with diazomethane and separated according to the degree of unsaturation in the acyl chain by silver nitrate impregnated silica gel plates. The relative proportions of individual lipid species were determined by counting the radioactivity in each respective area on the chromatogram. Details of the procedures are described under "Materials and Methods".

Acyl species	Acyl species incorporation	
	with low* alkylglycero-P concentration	with high (29 μM) alkylglycero-P concentration
	percent**	
16:0 + 18:0	9	29
18:1	9	10
20:4	21	25
22:4	15	18
22:6	45	19

*Low concentration produced in situ by enzymatic reduction of alkyldihydroxyacetone-[^{32}P]phosphate with NADPH.

**Data expressed as percent of total acylated product.

With low alkylglycero-P present, 45 percent of the acylated product contains hexaenoic acyl chains. This specificity for 22:6 acyl-CoA is lost to a large extent with a high concentration of acceptor. A diagrammatic representation of the different populations of labeled products obtained with either high or low acceptor concentrations can be seen in Fig. 2, which was included in "Materials and Methods". Plate A shows the good separation of saturated and monounsaturated products on $AgNO_3$ thin layer plates after the products were converted to the dimethyl esters with diazomethane. To obtain separation of the polyunsaturated products (4 or 6 double bonds), a second plate was run using a more polar solvent system (plate B). In lanes 7 and 8 the spots migrating slightly more than 1-alkyl-2-arachidonoylglycero-3-phosphate were presumed to be 1-alkyl-2-docosatetraenoylglycero-3-phosphate. Not shown in Fig. 2 are the faint spots migrating below each major spot. This minor spot in each case was presumed to be the monomethyl ester resulting from incomplete methylation with diazomethane. Radioactivity of the minor spot for each standard was consistently 20 percent of the major spot.

J. Acyltransferase Selectivity of Endogenous Fatty Acids Since the results above suggested that the acyltransferase could selectively use exogenously added acyl-CoA substrates for incorporation into the ether phosphoglyceride acceptor, an experiment was designed to determine if the acyltransferase could also selectively use acyl-CoA substrates derived from the endogenous supply of fatty acids. If ATP and CoA were added to the incubation mixture in place of acyl-CoA, the endogenous fatty acids from the microsomes were utilized for acylation. Rat

brain microsomes (0.73 mg protein) were incubated with ATP (8.3 mM), CoA (60 μM), alkylglycero-[^{32}P]phosphate (3.5 nmoles), and the standard amount of NaF, $MgCl_2$, and Tris-PO_4 buffer. No Triton or albumin was added. The distribution of acyl chains in the 1-alkyl-2-acylglycero-[^{32}P]phosphate formed from endogenous fatty acids was determined by separating the species of product according to degree of unsaturation in the acyl chains on $AgNO_3$ thin layer chromatograms. Identification of all the species was not possible, but approximately 70 percent of the product contained polyunsaturated ($\geq$4 double bonds) fatty acids. In order to evaluate this apparent selectivity of endogenous fatty acids it was necessary to determine the concentrations of individual fatty acids available in the microsomes. The composition of free fatty acids extracted from brain microsomes is shown in Fig. 20. Saturated fatty acids comprise 64 percent of the extracted acids. The unidentified polyunsaturated fatty acid fraction (12 percent) was composed of three separate species in approximately equal proportions. The distribution of species is similar to that reported for rat brain microsomes by Lunt and Lowe (235). When this distribution of fatty acids is considered, the production of a product with 70 percent polyunsaturated species as described above is a dramatic example of selectivity.

K. Acyltransferase Specificity Towards Phosphoglyceride Substrates

Since the naturally occuring phosphoglycerides are derivatives of the stereoisomer sn-glycero-3-phosphate, it was of interest to ascertain whether the acyltransferase would acylate other stereoisomers. With

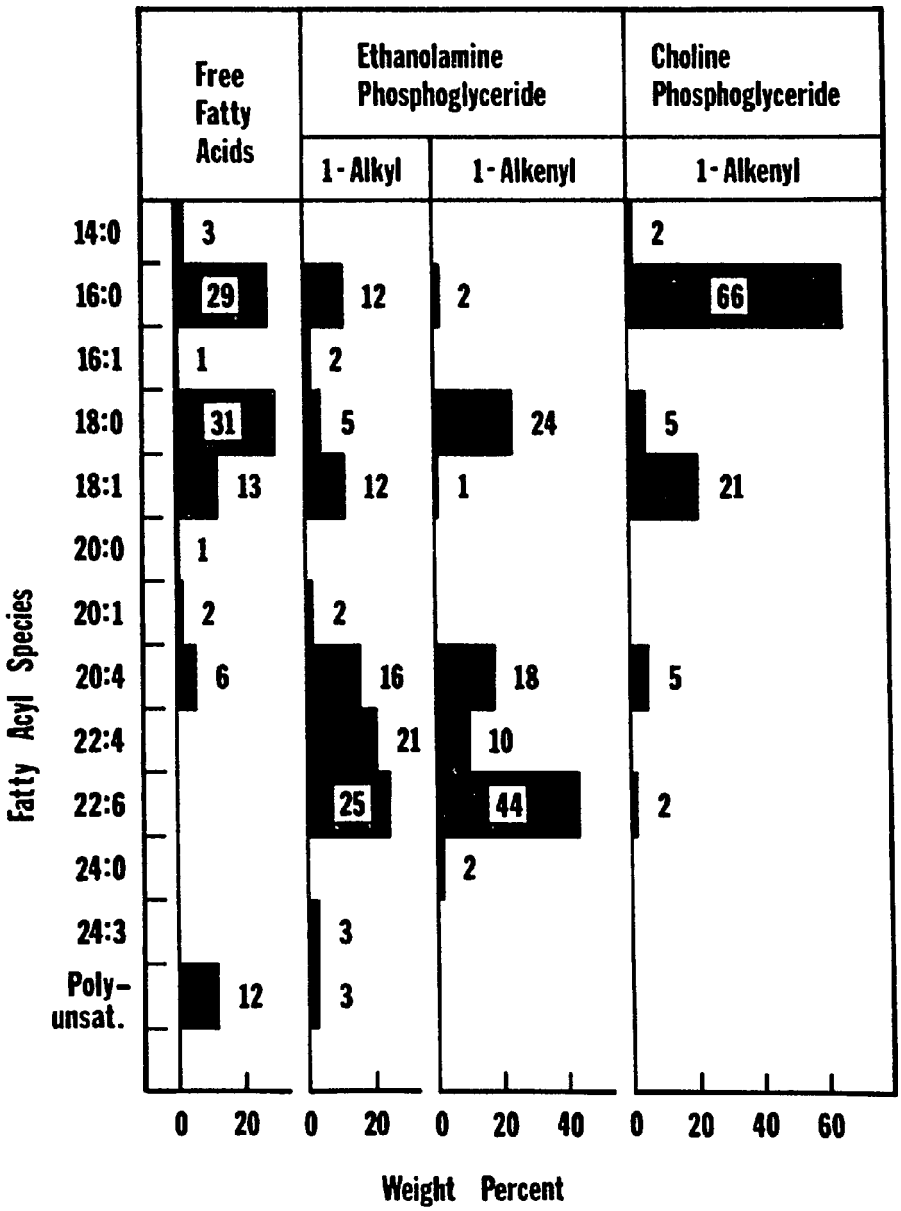


Fig. 20 Composition of fatty acids extracted from microsomes or hydrolyzed from the 2-position of microsomal ether containing phosphoglycerides. Details of extraction and fatty acid species determination are given under "Materials and Methods". The numbers corresponding to the horizontal bars are the exact values for the relative amount of each fatty acid in units of weight percent of total fatty acids in the respective lipid class. The data are mean values from two determinations.

the racemic substrate, the maximum amount acylated was never more than 50 percent, indicating that only one isomer was being enzymatically acylated. The activity of brain acyltransferase with 1-alkyl-sn-glycero-3-[^{32}P]phosphate and 3-alkyl-sn-glycero-1-[^{32}P]phosphate was assayed to determine the stereochemical specificity of the enzyme for the phospholipid substrate. To prepare the unreactive isomer, a racemic sample of labeled alkylglycero-P was exhaustively re-incubated with enzyme until no more product was formed in an incubation with enzyme and cofactors. The unacylated alkylglycero-[^{32}P]phosphate remaining after this procedure was isolated by extraction and separation on thin layer chromatography. The reactive isomer was prepared by alkaline methanolysis of enzymatically produced alkylacylglycero-[^{32}P]-phosphate. Both isomers were further purified by thin layer chromatography. Re-incubation of the purified reactive isomer for 30 minutes with excess acyl-CoA resulted in 78 percent acylation. However, none of the unreactive isomer was acylated. The above data are evidence that the brain acyltransferase specifically uses only one stereochemical enantiomer of alkylglycero-P as acyl acceptor, and data already presented indicate the active compound is 1-alkyl-sn-glycero-3-P. As reported above, 73 percent of the enzymatically formed product could be hydrolyzed by phospholipase A_2. This enzyme has been shown to be stereospecific for 1,2-diacyl-sn-glycero-3-phosphate (236). Therefore the phospholipase hydrolysis of the product from the acyltransferase reaction indicates that the latter enzyme accepts only the 1-alkyl-sn-glycero-3-P enantiomer.

L. Acyl Composition of Ether Containing Phosphoglycerides in Brain Microsomes The composition of fatty acyl chains at the 2-position of glycerol in the ether containing phosphoglycerides from rat brain microsomes is shown in Fig. 20. The 1-alkylethanolamine phosphoglycerides contain a large proportion (65 percent) of acyl chains with four or more double bonds. This distribution is reflected in the 1-alkenylethanolamine phosphoglyceride acyl composition in which 72 percent of the chains have four or more double bonds. However, there are significant differences in the 1-alkenylethanolamine phosphoglycerides when compared to the respective alkyl lipids. The former contain larger proportions of 18:0 (24 versus 5 percent) and also 22:6 (44 versus 25 percent). This redistribution is accompanied by smaller proportions of 16:0 and 18:1 in the 1-alkenylethanolamine phosphoglycerides. It is interesting to note that the whole brain 1-alkenylethanolamine phosphoglyceride acyl composition reported by Etzrodt and Debuch (154) is very similar to the acyl composition of the 1-alkylethanolamine phosphoglycerides in microsomes.

The 1-alkenylcholine phosphoglycerides contain 16:0 as the predominant acyl chain (66 percent). This was surprising in view of the reported acyl composition of the 1-alkenylcholine phosphoglycerides from ox brain where the predominant acyl chain is 18:1 (155). This lipid class contains a small proportion of acyl chains with four or more double bonds (7 percent).

DISCUSSION

Specific activities of 1-alkylglycero-3-phosphate:acyl-CoA acyltransferase in animal tissues are too low to be determined accurately with the convenient spectrophotometric assay described by Lands and Hart (31). Therefore assays involving radioactively labeled substrates were used for the study of this enzyme. An assay which separated the substrate (alkylglycero-P) and product (alkylacylglycero-P) by differential partition in a two phase system proved to be not reproducible enough for the kinetic studies reported above. The phenomenon that was the basis of this assay is important to the study of acidic phospholipids. As shown in Fig. 1, the partition of alkylglycero-P between a chloroform phase and an aqueous-methanol phase is dependent on the pH of the aqueous phase. A similar dependency was shown for other acidic phospholipids by Hajra (237). The chloroform-methanol-water solvent extraction has been a standard procedure used in lipid research for many years (212). The above results emphasize the importance of pH in quantitative extraction of phospholipids when using this method. The assay involving thin layer chromatographic isolation of the product was tedious, but produced reliable and reproducible data. In addition, the use of autoradiography to localize the labeled substrate and product on the chromatogram provided a daily visual check on any unusual side products or substrate impurities.

Enzyme kinetic studies on lipid substrates are difficult because of the physical nature of the substrate. Acyl-CoA is known to form micelles (24), and alkylglycero-P would be expected to form micellar

structures similar to acylglycero-P (24). The aim in these studies was to define an assay system which would enable meaningful comparison of kinetic data with different lipid substrates. To this end, the standard incubation mixture was optimized so that lipid substrates and their concentrations could be varied with minimal effect on the physical nature of the substrate complex. The assay used in this study enabled determination of acyltransferase activities of 0.4 nmole/min/mg protein with a reproducibility error of less than 5 percent.

Triton X-100 was used in the earlier studies to provide a large molar excess of detergent to minimize the detergent effects of the lipid substrates. In addition, albumin was added which allowed assay of enzyme activity with phospholipid substrate concentrations up to 30 μM with no apparent inhibition by the substrate. However, at high concentrations of alkylglycero-P ($>$ 30 μM) or acyl-CoA ($>$ 20 μM) substrate inhibition was observed in the assay described here. By maintaining the substrate concentrations below these limits, Michaelis kinetics were observed for increasing alkylglycero-P concentrations when Triton was present. Inhibition of acyltransferase activity by high concentrations of acyl-CoA has been previously reported (31, 164), and Barden and Cleland (24) described the substrate inhibition of acyltransferase by high concentrations of the acceptor 1-acylglycero-3-phosphate. One explanation for the effects of albumin shown in Fig. 14 is that albumin binds the substrate alkylglycero-P, effectively taking it out of solution.

The brain microsomes used for the source of acyltransferase also contained enzymes which degraded the substrates used in the study.

Alkylglycero-P phosphohydrolase was especially active in the microsomal preparation, but it could be partially inhibited by addition of NaF and $MgCl_2$. The standard assay mixture contained both of these salts, and less than 10 percent of the phosphoglyceride substrate was hydrolyzed during the incubation time. Acyl-CoA thiohydrolase was also present in the microsomes and would hydrolyze approximately 25 percent of the acyl-CoA in the standard assay mixture. This did not cause the acyl-CoA concentration to fall below saturating amounts, however, so was not considered limiting.

The results above show that 1-alkylglycero-3-phosphate:acyl-CoA acyltransferase activity is present in the particulate fractions of at least six rat tissues. When compared to the distribution of 1-acylglycero-3-phosphate acyltransferase activity, the highest relative rates of activity with the ether phosphoglyceride acceptor were found in heart, spleen, and brain (Table II). These tissues are known to have relatively high contents of ether lipids (71). The ratios of acyltransferase activities with the diester and ether phosphoglyceride acceptors are approximately proportional to the ratios of incorporation of injected ^{32}P into diester and ether phosphoglycerides in liver (238). Thus the activities of specific acyltransferases correlate with both the lipid composition and the metabolic activity of various tissues.

Although the microsomal fraction of rat brain contained the highest specific activity of alkylglycero-P acyltransferase, mitochondria also contained a significant activity (Table III). Therefore it seems that brain mitochondria are able to catalyze each step in the biosynthesis of 1-alkyl-2-acylglycero-3-phosphate from

dihydroxyacetone phosphate, since both formation and reduction of alkyldihydroxyacetone-P can occur in brain mitochondria (5,6,7). Mitochondria in brain and liver are also able to synthesize phosphatidic acid (239,240). This raises an interesting question, because mitochondria are not able to convert this phosphatidic acid into ethanolamine or choline phosphoglycerides. One use the mitochondria can make of this precursor is to form cardiolipin (phosphatidylglycerophosphatidate). Biosynthesis of this compound does not involve activated bases, but uses CDP-diglyceride as an activated intermediate instead. The mitochondria are able to make CDP-diglyceride. Another metabolic fate of mitochondrial phosphatidic acid could be intracellular transport to other organelles (239). It is not known if alkylacylglycero-P, the ether containing analog of phosphatidic acid, participates in similar transformations. As with phosphatidic acid however, microsomes must be considered the major site of 1-alkyl-2-acylglycero-3-phosphate biosynthesis.

Mitochondria are capable of forming 3-hydroxy fatty acid in the course of fatty acid oxidation, and these hydroxy acids have been found in mitochondrial incubations <u>in vitro</u> (203,204). Since fatty acid oxidation of this type proceeds with the CoA activated fatty acids, one would expect the existence of 3-hydroxy fatty acyl-CoA inside the mitochondria. However, these acyl donors would not be expected to be available to the acyltransferases, which seem to be either on the outer mitochondrial membrane or on the endoplasmic reticulum. Therefore it was surprising to discover 1-alkyl-2-(3-hydroxy)acylglycero-3-P being formed during incubations with mito-

chondrial fractions. The formation of this lipid indicated that 3-hydroxy fatty acyl-CoA was available to the acyltransferase. A rigorous structural determination was carried out to identify positively the newly obtained lipid as 1-alkyl-2-(3-hydroxy)glycero-3-phosphate.

Fig. 10 describes the chemical methods employed to establish the structure of the polar fatty acid present in the phosphoglyceride fraction. The formation of an acetyl derivative and the reduction to a diol showed the presence of a hydroxyl group in the fatty acid. The cyclic ketalization of the reduced compound with acetone indicated that it was either 1,2-or 1,3-diol. However, the resistance of the diol to the periodate oxidation showed that vicinal hydroxyl groups were not present. The only alternative explanation was that the reduced compound was a 1,3-diol, and therefore the polar fatty acid was a 3-hydroxy fatty acid. Gas liquid chromatography proved that the 3-hydroxy fatty acid had the same carbon chain length as the parent fatty acid used in the incubation mixture.

A number of workers have reported the formation of some unknown phospholipid during the acylation of sn-glycero-3-phosphate (205, 206,207). It is possible that some of these unknown lipids are similar to the diacyl analog of 3-hydroxy fatty acid-containing phospholipid described here, i.e., 1-acyl-2-(3-hydroxy)acyl-sn-glycero-3-phosphate. Anderson and Sansone (207) actually indicated that some phosphorylated derivative of a 3-hydroxy fatty acid was formed in the incubation mixture when mitochondria were used as the enzyme source. The "phosphorylated derivative" may well have been a phosphatidic acid containing the esterified 3-hydroxy acid.

Since no naturally occurring phosphoglyceride with a 3-hydroxy

fatty acyl ester moiety was found in normal tissues, it was thought that the in vitro result was due to damaged mitochondria which permitted release of 3-hydroxy fatty acid, and hence availability to the acyltransferase. The logical next step was to look for this novel lipid formation in tissues which inherently contained "damaged" mitochondria. Skeletal muscle of mice with muscular dystrophy is one such tissue that contains mitochondria which show structural abnormalities when viewed in the electron microscope (233), and exhibit other biochemical abnormalities (241). Of importance to this work is the fact that muscle mitochondria from the dystrophic strain of mice have a decreased capacity to oxidize fatty acids and an accumulation of fat (242). If a defect in fatty acid oxidation were present in a step after the formation of S-3-hydroxyacyl-CoA, one would expect this lipid to increase in concentration. And since the mitocchondria are distended (243) and loosely coupled (242) in this tissue, it seemed likely that the mitochondria might be "leaky" enough to allow such a lipid to escape if it were present. If any of these events are taking place they do not lead to the formation of a 3-hydroxy fatty acyl phosphoglyceride, because no such lipid was found when the dystrophic tissue was assayed. The mice tested were up to 49 days old and showed serious progression of the disease in the hind legs.

The fact that no 3-hydroxy fatty acid was found in the above phosphoglyceride fractions points to the conclusion that such a lipid product is an artifact of the in vitro incubation. A recent report on the β-oxidation of fatty acids in cellular cytoplasm (244), where 3-hydroxy fatty acyl-CoA is presumably formed, begs the question of

how this intermediate is segregated from the acyltransferases. Either the cytoplasmic compartmentalization is very efficient or the hydroxy intermediate remains bound to the enzymes involved in its transformations.

The product of the acyltransferase reaction with normal acyl-CoA was shown to be 1-alkyl-2-acyl-sn-glycero-3-phosphate by its chromatographic behavior and chemical and enzymatic degradation. In addition, the nonreactivity of the optical enantiomer of the substrate demonstrated that the acyltransferase was stereospecific for the naturally occurring configuration of the glycerol moiety. This has been found to be true of 1-acylglycero-3-phosphorylcholine acyltransferase (245).

An important question to be addressed is whether the acyltransferase activity described herein is identical with the acylglycero-P acyltransferase previously described in brain (48). The specific activities shown in Table II for alkylglycero-P acyltransferase are relatively high in those tissues where the specific activities of acylglycero-P acyltransferase are low and vice versa. This finding indicates the presence of a separate enzyme activity for the acylation of the ether-containing phospholipid acceptor. In addition, the apparent kinetic parameters for the acyltransferase acting on acylglycero-P were different than those for the alkylglycero-P enzyme. These kinetic differences (Figs. 17 and 18) may be due to differential effects of Triton X-100 if, for example, Triton was partially denaturing the enzyme, but even taking this into consideration, the data suggest two different enzyme activities. Preliminary data have been obtained from experiments which determined the effects of acylglycero-P on the acylation of alkylglycero-P (and vice versa). Each acceptor

inhibits the acylation of the other and the inhibition appears to be competitive. However, the K_I value for each lipid, as an inhibitor, is much less than its respective Km value. This result also is evidence for separate enzyme activities. These experiments have not been repreated at the time of writing and the data are somewhat scattered, so they were not included as a part of the "Results". In view of the above results it was concluded that the enzyme activity described in this dissertation is a separate activity with preference for the ether containing phosphoglyceride acceptor. In contrast, Labelle and Hajra concluded that the preceding enzyme in the ether lipid biosynthetic pathway, NADPH:alkyldihydroxyacetone phosphate oxidoreductase, was not differentiated from the enzyme accepting the ester phospholipid substrate, acyldihydroxyacetone phosphate (7). The final solution to this question would be purification of the enzyme(s).

Solubilization of the acyltransferase activity from rat brain microsomes was attempted using various detergents and chaotropic agents. Triton X-100, Tween 80, and sodium cholate alone or in combination with NaCl, NaSCN, and/or dithiothreitol were tried, and Triton X-100 alone (0.1 percent) proved to be somewhat promising. The measure of solubilization was that activity remaining in the supernatant after centrifugation at 100,000 x g for 1 hour. By this definition, Triton X-100 did "solubilize" a small amount of activity, but with no enhancement in specific activity. It is likely that disintegration of the microsomal membrane to smaller mesophasic fragments is all that was accomplished. Column chromatography on Sephadex G-200 caused a complete loss of activity of this Triton solubilized acyltransferase.

Ammonium sulfate in various concentrations (20, 40, and 80 percent) would precipitate portions of protein from crude or Triton-solubilized microsomes, but there was no apparent increase in specific activity.

Acetone precipitation at -20^{o} of the Triton-solubilized enzyme caused a loss of acitvity. Addition of glycerol (15 percent) during the acetone precipitation did not protect the enzymatic activity.

Purification of the acyltransferase by differential partition in dextran-ficoll-polyethylene glycol phase systems, as described by Albertsson (246), was also tried. This was not successful because the polymers interfered with partition and thin layer assay of the lipid product. Separation of the polymers from protein was not tried.

Attempts were made to break up the membrane structure of the microsomes by lyophilization and then solubilization in pure Triton or glycerol before adding water. This procedure caused a complete loss of activity. No further work was done on the purification of the acyltransferase.

It has long been recognized that the acyl distribution in phosphoglycerides is non-random (146,147,148). Those acyl groups esterified to the 2-position of the glycerol moiety have been found to consist of a high percentage of unsaturated chains. Analyses of the acyl distribution in the ether phosphoglycerides have generally exhibited this pattern (149-154), with the exception of the alkylacylglycerophosphorylcholines in brain which contain a relatively high proportion of 16:0 ester together with 18:1 (155). The acyl composition of ether phosphoglycerides in rat brain microsomes reported here (Fig. 20) is similar to published data obtained with

whole brain (71,152-155), with the exception of alkenylacylglycerophosphorylcholine which, in microsomes contained 66 percent 16:0 ester and 21 percent 18:1 ester. Renkonen (155) found the reciprocal ratio for 16:0 and 18:1 in the alkenylcholine phosphoglycerides in ox brain. It may be that such a large difference is due to the difference in species.

The data reported here on the selectivity of alkylglycero-P acyltransferase suggest that this enzyme activity is able to exert selective control over the acyl distribution of 1-alkyl-2-acylglycero-3-P, the precursor of the ether containing phosphoglycerides. The data in Figs. 15 and 19(A) demonstrate that the maximal rates of acylation vary with the acyl-CoA substrate. It was not possible to determine apparent Km values for each acyl-CoA, so it is not known to what extent differences in acyl-CoA concentration have an effect on overall incorporation into alkylacylglycero-P. However, this may be an important factor in vivo. The acyl-CoA Km values for acylglycerophosphate acyltransferase have been determined to be in the same range as their intracellular concentrations in liver (24).

A qualifying point should be made on the subject of Km values. In the case of acyl-CoA, and as discussed below with alkylglycero-P, the substrate is a lipid which may exist in aggregates or associations more complex than dispersed monomeric solutions. For both the in vitro and in vivo situation, this means that the total amount of substrate per unit volume may not be the effective concentration available to the enzyme. Glycero-P acyltransferase is believed to accept the monomeric species of acyl-CoA (247). This conclusion was reached from kinetic studies on the rat liver enzyme and is a statement de-

scribing the *in vitro* action of the enzyme. There are two other possibilities which may describe the intracellular situation. Acyl-CoA may in fact reach the enzyme as a part of the membrane matrix. One can visualize the CoA portion, which may extend into the cytosol, binding to its area of the enzyme active site and aligning the acyl chain into its site, which could be on that part of the enzyme buried in the membrane. A second possibility is that the acyl-CoA may be presented to the acyltransferase by a carrier protein. Cytoplasmic proteins with very high affinity for acyl-CoA have recently been discovered (248). If, as a result of protein-protein interaction between a soluble binding protein and the membrane bound acyltransferase, the acyl-CoA were directly transferred to the active site the effective Km value for acyl-CoA would require more than a molar concentration for a meaningful definition. In spite of these reservations on the extrapolation of Km values to the *in vivo* situation, they do provide a useful descriptive parameter of an enzyme.

The apparent Km values for alkylglycero-P with four different acyl-CoA co-substrates varied by a factor of at least 30. This finding could not be a result of physical changes in the substrate complex caused by the different acyl-CoA substrates because the apparent Km values obtained for acylglycero-P with the same acyl-CoA co-substrates increased in a different order than those obtained with alkylglycero-P (compare Figs. 17 and 18). The maximum velocities obtained also varied with each acyl-CoA and were in different relative orders of value for the alkyl- and acylglycero-P acceptors.

When it was discovered that Triton X-100 had a differential effect on the acyltransferase activity which was dependent on the

acyl donor species, as suggested in a report by Okuyama et al, additional experiments without Triton were done. The results obtained from these experiments confirmed the conclusions reached with the earlier studies, but the kinetic parameters had different values. As discussed above, the values reported here may not be applicable to the in vivo environment. But when these values, obtained by comparison under identical physical conditions for different substrates, are considered in relative terms, information could be obtained as to substrate selectivities. The kinetic data lead to the conclusion that alkylglycero-P acyltransferase would select for polyunsaturated acyl-CoAs when the acceptor concentration was low, and would incorporate relatively more saturated acyl-CoA species at high acceptor concentrations. These results are in contrast to the results obtained for acylglycero-P acyltransferase with the brain microsomes. With this latter enzyme activity saturated acyl-CoA would be selected at low acylglycero-P concentrations.

To substantiate further the conclusion that the acyltransferase activity can control the distribution of acyl groups in alkylacylglycero-P experiments were done to test the selectivity of acyltransferase when provided with the endogenous microsomal mixture of fatty acids. The pool of free fatty acids in rat brain increases rapidly on decapitation (249). Possibly this release of free fatty acids is due to phospholipid acylhydrolases, since the lysolecithin concentration also increases with time in rat brain slices (250). To insure that the free fatty acid composition assayed was the same as that presented to the acyltransferase during in vitro incubations, the same batch of microsomes was used after one freezing. As reported above, the acyl distri-

bution in alkylacylglycero-P formed *in vitro* was comprised of approximately 70 percent polyunsaturated acyl species. The differential incorporation of acyl species was not due to the relative abundance of free fatty acids in the microsomes. Fig. 20 shows that free fatty acids with 4 or more double bonds were present in much smaller quantities than saturated acids, and yet these unsaturated acids were incorporated to a greater degree than the saturated acids. Such a result might also be due to a selectivity on the part of the acyl-CoA synthetase, but this does not seem to be the case in the present study. Marcel and Suzue concluded from studies with rat liver microsomes that activation of long chain acids with CoA was catalyzed by one enzyme with similar activities toward various long chain fatty acids (200). The slight preference exhibited by the synthetase in that study was for fatty acids with a $\Delta 9$ double bond and against those with a $\Delta 11$ double bond. This preference would favor 18:1 and exclude 20:4 and would lead to results opposite to those reported here.

A second way of testing acyltransferase selectivity, in this case that which would be due to differences in alkylglycero-P Km values, is to maintain a low concentration of alkylglycero-P in the incubation mixture. To accomplish this the alkylglycero-P was made *in situ* by enzymatic reduction of alkyldihydroxyacetone-P in the incubation mixture. When the acyltransferase was presented with such a low but continuous supply of acceptor and equal concentrations of a mixture of acyl-CoA species, the expected selectivity for polyunsaturated acyl donors, especially hexaenoic, was observed (Table IV).

The results clearly demonstrate that the acyltransferase can utilize the difference in Km for alkylglycero-P to specify the acyl

incorporation. The question arises as to the mechanism by which the acyltransferase can have differing apparent Km values for alkylglycero-P depending on the acyl donor involved in the reaction. The idea of separate catalytic sites for individual acyl-CoA species was postulated for the acylglycerophosphate acyltransferase by Okuyama and Lands (25) as mentioned in the "Introduction". This hypothesis was based on a kinetic evaluation which indicated separate Km values for the acceptor with individual acyl donors, and on previous work by Lands and coworkers on the stability of individual acyltransferases (174). Since that time the differing apparent Km values toward acylglycerophosphorylcholine have been confirmed (173) and, in addition, a differential sensitivity of the acyltransferase to Triton X-100, which is dependent on the acyl donor, has been reported (173).

A second mechanism for altering the apparent Km for the acceptor would be a single enzyme which was conformationally affected by the respective acyl-CoA donor. This model of a single allosterically-controlled acyltransferase is not supported by the data reported for acylglycerophosphorylcholine acyltransferase activity (174). The Δ12-octadecenoyl-CoA acyltransferase activity was less stable to storage than the Δ9-octadecenoyl-CoA activity with acylglycerophosphorylcholine as the acceptor.

A third mechanism[1] for the difference in apparent kinetic parameters with different acyl donors may be considered if the rate limiting step in the enzymic reaction were the release of the reaction product from the enzyme. If the rate constant for the desorption of product

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[1]Suggested by Dr. J.A. Shafer.

is similar in magnitude to the other rate constants of the reaction, the apparent Km and Vm will be influenced by the value of the product desorption rate constant (see Appendix I).

The results reported here are evidence that there are at least three active sites for alkylglycero-P acyltransferase activity. When saturating concentrations of both palmitoyl- and arachidonoyl-CoA are added to an incubation mixture, the rate of acyl transfer to alkylglycero-P is greater than the maximum velocity with either acyl-CoA alone. Without invoking the concept of synergistic allosteric effectors, the only reasonable conclusion is that more than one type of active site was engaged in catalytic activity. However, this result was obtained only with the pair of acyl-CoA species mentioned; combinations of either one with oleoyl-CoA did not obtain additive velocities. Since these studies were done with a large molar excess of Triton, it is unlikely that changes in the physical nature of the substrate complex could be responsible for the additive effect that was observed with both arachidonoyl- and palmitoyl-CoA. This result must be considered suggestive evidence for the existence of two catalytic sites, one for palmitoyl-CoA, and one for arachidonoyl-CoA. The presence of a third enzyme for oleoyl-CoA is suggested by the effect of Triton X-100 on this acyltransferase activity. At o.33 mg/ml of Triton, which was near the optimal concentration for stimulation of both palmitoyl-CoA and arachidonoyl-CoA acyltransfer, the activity of oleoyl-CoA acyltransfer was $\sim$75 percent inhibited.

In view of the above discussion a tentative conclusion may be drawn that there exists a separate series of acyltransferases for each acceptor, alkylglycero-P and acylglycero-P. Whether each series

contains a unique enzyme for each acyl donor or only several enzymes with one for each family of acyl donors is an open question. By extention it is logical to suggest that there may be a separate series of acyltransferases for each class of phospholipid acceptors.

In order to address the question of whether or not the alkylglycerophosphate acyltransferases can determine the acyl distribution in the ether containing phosphoglycerides the data in Fig. 20 were pooled to obtain the overall acyl distribution found in the total brain microsomal ether lipids. The respective acyl compositions were: (16:0, 18%), (18:0, 19%), (18:1, 6%), (20:4, 15%), (22:4, 8%), (22:6, 33%). Inspection of Table IV shows that neither the relative incorporation at low nor high acceptor concentrations can account for the observed acyl composition in the total microsomal ether phosphoglycerides. Such a correlation might be expected if alkylglycero-P acyltransferase were the only controlling factor in the distribution of acyl esters. Therefore, other means must be utilized by the cell to obtain the final acyl distribution in the ether containing phosphoglycerides. It was proposed by Robertson and Lands (165) that deacylation-reacylation reactions on the phosphatidyl ethanolamines and cholines could explain the acyl distribution. Evidence for deacylation-reacylation of these phosphoglycerides has been obtained in many studies (176,251-259). A consistent finding is that 20:4 is especially characteristic of an acyl species being introduced by these secondary acylations (255,257,258,259). The data presented here, however, indicate that the rate of incorporation of 20:4 into alkylacylglycero-P is greater than its proportional content in the total ether containing phosphoglycerides. It is not

known what the in vivo availability of arachidonoyl-CoA to the enzyme in brain is though. Considering the large differences reported here for alkyl- and acylphosphoglyceride acyltransferase specificities, this apparent difference in 20:4 incorporation is not surprising. In fact, in sarcoplasmic reticulum, the only tissue for which the appropriate data is available, the percent of 20:4 in alkenylacyl-glycerophosphorylcholine is more that would be expected from the relative arachidonoyl-CoA:alkenylglycerophosphorylcholine acyltransferase rate (186). In this tissue, a significant amount of 20:4 may be introduced in the alkylglycero-P acyltransferase step.

Incorporation of 20:4 and other polyunsaturated acyl groups into the ether-containing phosphoglycerides at the precursor stage of alkylacylglycerophosphate is an important point because it is in contrast to the current knowledge about the ester phosphoglycerides. An emerging consensus seems to be that phosphatidate synthesizing acyltransferases introduce a basic asymmetry utilizing mostly 16:0. 16:1, 18:0 and 18:1 acyl groups, and that the secondary acyltransferases introduce more subtle changes, utilizing longer, more unsaturated acyl groups (160,256,257,258,260). An exception to this theory is the incorporation of 22:6 into phosphatidic acid in liver (255). This latter fact, coupled with the knowledge that ethanolamine phosphoglyceride in liver is relatively rich in hexaenoic acyl species, led to the suggestion that the CDP-ethanolamine:diglyceride ethanolamine phosphotransferase was selective for hexenoic diglyceride (255). This selectivity has been shown by liver microsomal enzyme (261) and there is also evidence that the selectivity is expressed in isolated rat hepatocytes (46). No other specificity for ethanol-

amine or choline phosphotransferase has been observed (262,263). A selectivity for saturated 1-alkenyl-2-acylglycerol by brain CDP-choline phosphotransferase could explain the high percent of 16:0 ester found in the alkenyl choline phosphoglyceride fraction (Fig. 20). As mentioned above, no acyltransferase activity has been found which acylates 1-alkenylglycerophosphorylcholine. Both of these points require further study before a definite conclusion can be reached.

Other possibilites for selective control of acyl distribution include selective dephosphorylation of phosphatidic acid or selective removal by re-phosphorylation of diglyceride from the phosphoethanolamine and choline acceptor pool. Neither the phosphohydrolase (264) nor kinase (265) involved in these reactions is specific for esterified acyl species, however.

It seems likely that no single step, such as the alkylglycero-P acyltransferase reaction in the biosynthesis of the ether-containing phosphoglycerides, is solely responsible for the acyl distribution. Rather, a hierarchy of three levels may exert control over the acyl distribution in phosphoglycerides. The first point of control would be in the formation of the precursor phosphatidic acid, the second in the condensation of diglyceride with a phosphoethanolamine or phosphocholine, and the third an enzymatic retailoring of the "finished" phosphoglyceride. Indeed, if the cell must synthesize a lipid milieu which conforms to certain physical limitations, and must do this with a varying population of acyl donor species, it would require a control mechanism more complex than a single selective step. A hierarchy of controlling steps would be more suited to the ability to dynamically attenuate the lipid environment.

APPENDIX

The simplest mechanism describing an enzyme-catalyzed reaction in which both the product and substrate form reversible complexes with the enzyme is given by:

$$S + E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EP \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E + P$$

The following rate equation can be derived to describe this mechanism:

$$v = \frac{(k_1k_3k_5[S] - k_2k_4k_6[P])E_o}{k_2k_5 + k_2k_4 + k_3k_5 + k_1(k_3 + k_4 + k_5)[S] + k_6(k_2 + k_3 + k_4)[P]}$$

During initial velocity studies, when [P] is very low, the rate equation reduces to:

$$v = \frac{(k_1k_3k_5[S])E_o}{k_2k_5 + k_2k_4 + k_3k_5 + k_1(k_3 + k_4 + k_5)[S]}$$

This is identical to the Michaelis-Menten equation,

$$v = \frac{Vm\ [S]}{Km + [S]}$$

when,

$$Vm = \frac{(k_1k_3k_5)E_o}{k_1(k_3 + k_4 + k_5)} \quad \text{and} \quad Km = \frac{k_2k_5 + k_2k_4 + k_3k_5}{k_1k_3 + k_1k_4 + k_1k_5}$$

If k_5 is very large relative to other rate constants the equalities reduce to:

$$Vm = k_3 E_o \qquad \text{and} \qquad Km = \frac{k_2 + k_3}{k_1}$$

However, when k_5 is similar in value to the other rate constants the former equalities describe the kinetic constants and in this case both Km and Vm are influenced by k_5.

With enzymic reactions involving two substrates, such as alkylglycero-P acyltransferase, the reaction mechanisms would contain additional steps which may also influence the apparent kinetic parameters.

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