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MECHANISM OF ETHER BOND FORMATION BY ALKYLDIHYDROXYACETONE-P SYNTHASE

A Dissertation

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Degree

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Alex Jay Brown

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DEDICATION

This dissertation would not have been possible without the love and encouragement of my parents.

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ABSTRACT

Alkyldihydroxyacetone-P (alkyl-DHAP) synthase catalyzes a unique reaction in which the fatty acid ester of acyl-DHAP is cleaved and replaced by a fatty alcohol to form alkyl-DHAP, the first ether-linked intermediate in the biosynthesis of the alkyl- or alk-1-enyl-glycerolipids. In order to study this reaction mechanism in greater detail, this enzyme was solubilized for the first time from Ehrlich ascites cell microsomal membranes using Triton X-100 and acetone, and purified 1000-fold by chromatography on DEAE-cellulose, QAE-Sephadex, Matrex Red, and hydroxylapatite with the aid of a new rapid assay using DEAE paper disks.

The acyl exchange activity in which [^{14}C]palmitic acid is incorporated into acyl-DHAP, and an analogous alkyl exchange reaction, in which [^{14}C]hexadecanol is incorporated into alkyl-DHAP, copurified with the forward reaction strongly supporting a ping-pong mechanism for alkyl-DHAP synthase. Further evidence was obtained by showing that palmitic acid is a competitive inhibitor, with respect to hexadecanol, of the forward reaction.

Highly purified alkyl-DHAP synthase slowly exchanged ^{3}H from acyl-[1-R-^{3}H]DHAP in the absence of cosubstrate. Addition of hexadecanol increased the ^{3}H exchange rate by an amount equal to the rate of alkyl-DHAP formation. Similarly, the increase in rate of ^{3}H exchange by addition of palmitic acid matched the rate of the acyl exchange reaction.

We have proposed that upon binding of acyl-DHAP to alkyl-DHAP synthase, the pro-R hydrogen at carbon 1 is exchanged by an enolization of the ketone, followed by release of the acyl moiety to form an

activated enzyme-DHAP intermediate. Carbon-1 of the intermediate is believed to carry a positive charge which may be stabililized by an essential sulfhydryl group; the incoming alkoxide ion reacts at carbon-1 to form alkyl-DHAP.

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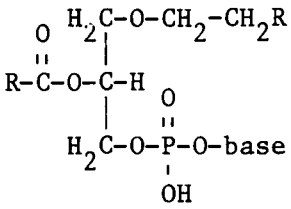
DHAP	Dihydroxyacetone-P
DNFB	Dinitrofluorobenzene
TNBS	Trinitrobenzenesulfonate
DTNB	Dithio-bis-(2-nitrobenzoate)
NEM	N-Ethylmaleimide
DFP	Diisopropylfluoro-P
QAE	Quaternary aminoethyl
DEAE	Diethylaminoethyl

CHAPTER I

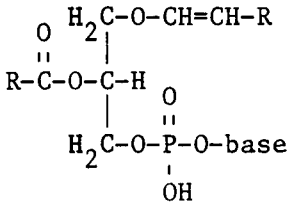
INTRODUCTION

Ether-Linked Lipids: Discovery, Distribution, Function

The compounds referred to collectively as ether lipids are analogs of the more common acylglycerolipids, i.e., triacylglycerols and diacylphospholipids, in which one of the fatty acids esterified to the glycerol backbone has been replaced by an ether-linked fatty alcohol. The common phospholipid analogs are the alk-1-enylacyl- and alkylacylphospholipids.



Alkylacyl-phospholipid



Alk-1-enylacyl-phospholipid

The alk-1-enylacyl-phospholipids are also called plasmalogens. This name was given by Robert Feulgen in 1924 to the precursor which, upon acid treatment, released into the plasma of cells an unknown aldehyde which he called plasmal (Feulgen and Voit, 1924). Because methods for isolating pure phospholipid fractions were not available, it was 30 years later that Klenk and Debuch (1954) proposed three structures, one of them correct, for ethanolamine plasmalogen. Rapport and co-workers (Rapport and Frazil, 1957; Rapport et al., 1957) presented evidence for the unsaturated ether linkage but predicted it to be at the sn-2 position with the fatty acid ester at the sn-1. The correct

structure for plasmalogens was finally confirmed by Marinetti et al. (1959) and Debuch (1959a,b).

The alkylacyl-phospholipids, on the other hand, were first detected nearly 15 years before Feulgen's discovery of plasmalogens. In 1909 Doree discovered a nonsterol alcohol, different from n-alkanols, in the unsaponifiable lipids of starfish (Doree, 1909). Six years later, Kossel and Edelbacher (1915) obtained a pure preparation of this alcohol from the unsaponifiable lipids from starfish testes and intestinal sacs, and although they obtained a melting point, molecular weight, and chemical formula consistent with that for octadecylglycerol, the structure was not determined. The connection with alkoxylipids was not made until Bergmann and Stansbury (1943) prepared this alcohol from starfish and found it to be identical to synthetic d-α-(n-octadecyl)glycerol. Similar compounds had been isolated from other marine sources such as shark and ray tissues by Tsujimoto and Toyama (1922), and Toyama (1924) proposed that a condensation reaction of a long chain alcohol with glycerol formed the ether bond. Heilbron and Owens (1928) confirmed the glyceryl ether structure by refluxing a pure preparation of octadecylglycerol from shark liver with hydriodic acid and obtaining octadecyliodide. Evidence that the alkylglycerol structure is found in phospholipids was presented by Carter et al. (1958). After mild alkali hydrolysis of egg yolk phospholipids, they obtained a phosphoethanolamine derivative of α-octadecylglycerol.

The alkyl linkage is also found in a neutral-type lipid, alkyldiacylglycerol. Andre and Bloch (1932) proposed that the two free hydroxyls of the alkylglycerols obtained by saponification may have been esterified with fatty acids. By a solvent partition technique, they

isolated a fraction from elasmobranch liver that was 74% alkyldiacylglycerol (Andre and Bloch, 1935). Twenty-five years later, Mangold and Malins (1960) prepared pure alkyldiacylglycerols by thin-layer chromatography for conclusive analysis of the applications.

The occurrence of ether lipids is widespread. Much of the early research investigated lipid extracts from marine animals because of their very high content of ether-linked glycerolipids. Karnovsky and Rapson (1946) used periodic acid oxidation to identify alkylglyceryl ethers in the nonsaponifiable lipid fraction from a series of marine and terrestrial animals and plants. They found ether lipids in 25 species of elasmobranch fishes, 20 species of Teleost fishes, molluscs, crustaceans, amphibians, reptiles, birds, and several mammals. These authors reported the presence of a small amount of O-alkylglycerols in tung nut oil, but oils from peanut, cottonseed, sesame seed, and castor seed contained none. Detailed reviews on occurrence of ether lipids in marine animals (Malins and Varanasi, 1972), molluscs (Thompson, 1972a), protozoa (Thompson, 1972b), bacteria (Goldfine and Hagen, 1972; Kates, 1972), plants (Mangold, 1972), and mammals and birds (Horrocks, 1972) have been published.

In mammals, ether-linked lipids have been quantitated in many tissues and although nearly all contained measurable amounts of alk-1-enyl-alkyl- glycerolipids, the content varied dramatically (for a review of the literature in this area, see Horrocks, 1972). In human brain, about 35% of the phospholipid is of the alk-1-enyl type, 1-2% is of the alkyl type, and the rest is of the diacyl type. Nearly all (>90%) of the ether-linked phospholipids in brain are ethanolamine plasmalogens. Heart also has a high plasmalogen content with about 30% of the

phospholipids as alk-1-enyl and 3-5% as alkyl. However, in heart, both choline and ethanolamine plasmalogens occur and constitute about 50% of each of these classes. Kidney phospholipids are about 10-15% alk-1-enyl type, most of which is ethanolamine plasmalogen. Other tissues such as spleen, bone marrow, and lung have levels similar to those of kidney. Liver, on the other hand, is very low in ether lipids; the alk-1-enyl and alkyl make up less than 5% of the total phospholipids.

The neutral fraction of mammalian lipids also contains significant amounts of alkyl and alk-1-enyl linkages. Eichberg et al. (1961) and Gilbertson and Karnovsky (1963) found glyceryl ether diesters in beef brain, beef bone marrow, calf heart, rat adipose fat, rat brown fat, polymorphonuclear leukocytes, human chylomicrons, and human β-lipoprotein. These authors found that the alk-1-enyldiacylglycerols comprised 29-82% of the glyceryl ether diesters in these tissues and that total neutral ether lipids accounted for about 0.1-2.9% of the total lipids. Other sources rich in glyceryl ether diesters are mouse preputial gland (Helmy and Hack, 1967; Snyder and Blank, 1969) and mouse L-M fibroblasts in suspension culture (Emmerie et al., 1952).

In the late 1960's, Snyder and co-workers discovered that neutral ether lipids occurred in a variety of transplantable animal tumors, Ehrlich ascites cells, and human neoplasms (Snyder et al., 1966; Wood and Snyder, 1967). The amount of glyceryl ether diesters in tumors was significantly higher than that found in the corresponding healthy tissues (Wood and Snyder, 1968).

The function of ether lipids is still not clear. The distribution of the ether-linked glycerol lipids is identical to that of their acyl counterparts. The glyceryl ether diesters are produced under conditions

that favor triacylglycerol synthesis (Scott et al., 1979a) and probably function as storage molecules. The ether-linked phospholipids appear to be made constitutively and are not affected by the metabolic state of the cell (Scott et al., 1979b). Although they presumably act as structural molecules in biological membranes, their difference in susceptibility to phospholipase cleavage (Wykle and Snyder, 1976) and different dipole moments must affect the membrane function to some degree. Model membranes containing ether-linked phospholipids have been shown to have altered fluidity compared to membranes of diacylphospholipids (Lee and Fitzgerald, 1980). Recently, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine has been shown to have potent platelet activating (Demopoulos et al., 1979) and hypotensive (Blank et al., 1979) properties. The mechanism by which the compound exerts its biological activities is currently under investigation by a number of laboratories.

Ether-Linked Lipids: Biosynthesis

Toyama (1924) suggested that the ether-linkage of O-alkyl lipids may be formed by the condensation of glycerol with a fatty alcohol. Over 40 years later, Toyama's proposal was partially confirmed when 1-^{3}H- and 1-^{14}C-labeled long-chain alcohols were incorporated intact into ether lipids in vivo (Friedberg and Greene, 1967; Ellingboe and Karnovsky, 1967; Snyder and Wood, 1968). Since the ^{3}H:^{14}C ratio did not change, oxidation of the fatty alcohol did not occur before incorporation. The source for the glycerol portion of ether-linked lipids was more difficult to ascertain.

Thompson and Hanahan (1963) showed that the labeled carbon-6 of glucose was ultimately found in carbon-3 of ether lipids. Friedberg and

Greene (1968) used [1,3-^{3}H]- and [^{14}C]glycerol and observed that the ^{3}H:^{14}C ratio decreased over 3-fold during incorporation into ether lipids suggesting that the glycerol was first oxidized to a triose-P.

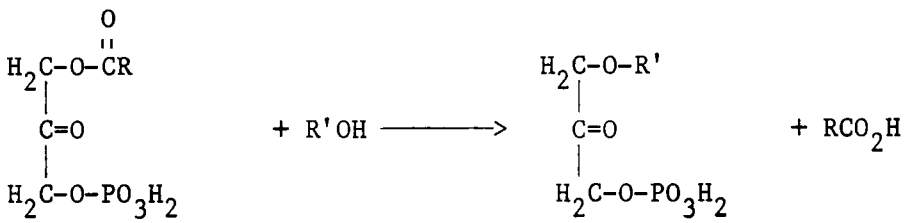
Development of a cell-free system by Snyder et al. (1969a) using microsomes and soluble fraction from mouse preputial gland tumors provided much greater detail on the synthesis of ether lipids. [^{14}C]Alkylglycerolipids could be produced in this system when ATP, CoA, Mg^{2+} and [^{14}C]hexadecanol were present. The soluble fraction could be replaced by glyceraldehyde-P but not glycerol-P (Snyder et al., 1969b). The requirement for fatty alcohols was confirmed in this cell-free system; fatty aldehydes and fatty acids were not incorporated into the alkyl linkage. It was subsequently found that the microsomal preparations contained high amounts of triose-P isomerase and that both DHAP and glyceraldehyde-P could act as precursors for alkylglycerolipids in the presence of this enzyme (Snyder et al., 1969c; Hajra, 1969; Wykle and Snyder, 1969; Snyder et al., 1970a). Hajra (1969) demonstrated that DHAP was the glycerol backbone source for ether lipids from time courses with ^{32}P-labeled triose-P, and from the effects of glyceraldehyde-P dehydrogenase and glycerol-P dehydrogenase on the system. Wykle and Snyder (1969) also showed this using an inhibitor of triose-P isomerase, 1-hydroxy-3-chloro-2-propanone-P. Hajra (1970) then explained the roles of the cofactors, ATP, Mg^{2+}, and CoA, by demonstrating that acyl-DHAP was an intermediate in glycerol ether synthesis. Acyl-DHAP was first isolated and identified by Hajra and Agranoff (1967) by incubating guinea pig liver mitochondria with [^{32}P]orthophosphate, and shown to be produced from DHAP when CoA, Mg^{2+}, and ATP were present (Hajra, 1968). The first ether-linked intermediate produced by preputial gland microsomes, brain

microsomes, or guinea pig mitochondria was 1-O-alkyl-DHAP (Hajra, 1969; Wykle and Snyder, 1969). The conversion of acyl-DHAP to alkyl-DHAP in the presence of fatty alcohol is catalyzed by alkyl-DHAP synthase. This enzyme is the subject of this dissertation and its properties will be discussed in detail in a later section.

Alkyl-DHAP was shown to be reduced in the presence of NADPH to alkylglycerol-P and subsequently acylated to alkylacylglycerol-P, the ether-linked analog of phosphatidic acid (Snyder and Wykle, 1969; Snyder et al., 1970a; Wykle and Snyder, 1970). The alkylacylglycerol-P can be metabolized by the enzymes of the triacylglycerol and phospholipid biosynthetic pathways to produce alkyldiacylglycerols and alkylacylphospholipids (Snyder et al., 1970b; Blank et al., 1974). The alk-1-enyl linkages were shown to be formed by desaturation of alkylacylphospholipids (Snyder et al., 1971; Blank et al., 1971, 1972; Wykle et al., 1972a; Paltauf, 1972; Paltauf and Holasek, 1973). The alk-1-enyl diacylglycerols, then, are presumeably synthesized from metabolites of the alk-1-enylacyl phospholipids.

Properties of Alkyl-DHAP Synthase

In the previous section, the identification of the intermediates of ether lipid biosynthesis was discussed. Since this dissertation concerns the mechanism of ether bond formation by alkyl-DHAP synthase, a more detailed review of the properties of this enzyme is necessary. As mentioned earlier, the substrates for alkyl-DHAP synthase are fatty alcohol and acyl-DHAP, which react with the enzyme to form alkyl-DHAP and fatty acid:



The fatty alcohols for ether lipid biosynthesis are derived from acyl-CoA by pyridine nucleotide-requiring reductases located in the soluble (Tabakoff and Erwin, 1970) and particulate (Snyder and Malone, 1970; Moore and Snyder, 1982a,b) fractions. In some tissues fatty alcohols are rapidly oxidized back to fatty acid by a NAD^+-requiring oxidoreductase (Snyder and Malone, 1970). In general, tissues that synthesize ether lipids have a high level of acyl-CoA reductase and low levels of the fatty alcohol oxidoreductase (Lee et al., 1980). Alkyl-DHAP synthase shows a very broad specificity for fatty alcohol. Of the n-alkanols, the 12-, 14-, and 16-carbon alcohols reacted similarly, whereas the 10- and 18-carbon alcohols were poorer substrates (Snyder et al., 1970a), and n-alkanol of less than 10 carbons or more than 22 did not react at all (Hajra, 1973). In all cases reported (Snyder et al., 1973; Hajra, 1973; Bandi et al., 1971), unsaturated species reacted as well as, if not better than, the parent saturated alcohol. The alkanediols, hexadecane-1,16-diol and hexadecane-1,2-diol, were not incorporated into ether lipids by preputial gland microsomes (Snyder et al., 1973), while hexadecane-1,2-diol and chimyl alcohol (α-hexadecylglycerol) were weakly incorporated into glyceryl ethers by Ehrlich ascites cell microsomes (Hajra, 1973). Two branched chain

alcohols, 14-methylpentadecanol and 12-methyltetradecanol, were excellent substrates, the latter reacting much better than hexadecanol. Naturally produced ether lipids, however, incorporate mostly hexadecanol, octadecanol, and octadecenol (49%, 21%, and 14%, respectively) into Ehrlich ascites cell alkyldiacylglycerols (Wood and Snyder, 1967). The composition of the alkyl chains, then, is probably determined by the limited specificity of the acyl-CoA reductase (Bishop and Hajra, 1978).

The specificity of alkyl-DHAP synthase for acyl-DHAPs with various acyl groups is not as well defined due to the difficulties in synthesizing these substrates. Davis and Hajra (1981) recently reported that 100 μM tetradecanoyl-, hexadecanoyl-, and octadecanoyl-DHAP gave activities of 0.42, 1.20, and 0.95 nmol/min/mg protein, respectively, using Ehrlich ascites cell microsomes. They also pointed out, as have others (Wykle et al., 1972b), that enzyme activity increased with hexadecanoyl-DHAP concentrations up to 100 μM and then decrease rapidly at higher concentrations. It was not shown whether 100 μM is also the optimal concentration for tetradecanoyl- and octadecanoyl-DHAP, and hence, the relative activities presented by Davis and Hajra may only reflect different optimal concentrations for the other substrates.

The ketone group of acyl-DHAP is essential. When NADPH was added to the microsomal systems, ether lipid synthesis was inhibited (Snyder et al., 1969b). The oxidoreductase that reduces alkyl-DHAP uses this cofactor to reduce acyl-DHAP to acyl-glycerol-P, which is inactive as a substrate for alkyl-DHAP synthase. 1-Acylpropanediol-3-P, an analog of acyl-DHAP with no ketone group, and the dimethylketol of acyl-DHAP also failed to serve as substrates (Wykle and Snyder, 1976). But unlike the interaction of the ketone group of DHAP with aldolase, there appears to

be no Schiff base linkage between acyl-DHAP and alkyl-DHAP synthase (Friedberg et al., 1980; Davis and Hajra, 1981; Brown and Snyder, 1980).

The reaction catalyzed by alkyl-DHAP synthase, the exchange of an ester-linked fatty acid for an ether-linked fatty alcohol, is unique in higher biological systems, and, therefore, its mechanism has received considerable attention. Using ^{18}O-labeled hexadecanol, Snyder and co-workers showed that the oxygen in the ether linkage of O-alkyl lipids is donated by fatty alcohols (Snyder et al., 1970c; Bell et al., 1971).

Friedberg and co-workers found that one of the hydrogens from the carbon of DHAP that forms the ether linkage is exchanged with water during ether bond formation by Tetrahymena pyriformis (Friedberg et al., 1971, 1972; Friedberg and Heifetz, 1973). The exchangeable hydrogen (1-pro-R) is the same one exchanged from DHAP by triose-P isomerase; the 1-pro-S hydrogen is not exchanged (Friedberg et al., 1972). [^3H]DHAP was used in these experiments and the endogenous acyltransferase activity formed acyl-[^3H]DHAP in situ; it was demonstrated the acylation of [^3H]DHAP did not involve an exchange of ^3H (Friedberg and Heifetz, 1973). The configuration of the carbon that exchanges this hydrogen is retained during the alkyl-DHAP synthase reaction (Friedberg and Alkek, 1977; Davis and Hajra, 1977a, 1979).

In addition to converting acyl-DHAP to alkyl-DHAP, alkyl-DHAP synthase has also been shown to catalyze several other reactions involving these compounds. Davis and Hajra (1977b) showed that when acyl-DHAP and [^{14}C]palmitic acid were incubated with Ehrlich ascites cell microsomes, [^{14}C]palmitoyl-DHAP was formed. Addition of 5 mM DTNB to inactivate endogenous CoA did not affect the rate of exchange, indicating that DHAP cleaved from acyl-DHAP was not acylated with [^{14}C]palmitic acid

by DHAP acyltransferase. Addition of fatty alcohol, on the other hand, dramatically inhibited this exchange and suggested that alkyl-DHAP synthase was responsible for this activity. An analogous reaction in which [^{14}C]hexadecanol and alkyl-DHAP form [^{14}C]hexadecyl-DHAP was documented by Brown and Snyder (1981), and the reverse reaction, in which [^{14}C]palmitic acid and alkyl-DHAP form [^{14}C]palmitoyl-DHAP, was found to be only about 1-2% the rate of the forward reaction (Brown and Snyder, 1980).

Most recently, Friedberg and co-workers have presented evidence that alkyl-DHAP synthase can cleave the fatty acid ester of acyl-DHAP in the absence of fatty alcohol (Friedberg et al., 1980; Friedberg and Gomillion, 1981). Using [1-R-^{3}H, 1,3-^{14}C]DHAP and relying on an endogenous acylation system, they found that incubation with Ehrlich ascites cell microsomes resulted in an exchange of the ^{3}H with the aqueous medium and that the rate of exchange was independent of the presence of fatty alcohol. Examination of the DHAP after the incubation revealed a decrease in the ^{3}H:^{14}C ratio. The amount of ^{3}H calculated from the decreased isotope ratio to be lost from DHAP during the reaction matched the amount recovered as [^{3}H]water. The rate of hydrolysis of acyl-DHAP, based on ^{3}H exchanged with the medium, was approximately 15 times faster than the synthesis of alkyl-DHAP, even in the presence of saturating fatty alcohol. Therefore, it appeared that alkyl-DHAP synthase reacted with acyl-DHAP and acted primarily as a hydrolase; fatty alcohol or fatty acid could react in place of water to give alkyl-DHAP and acyl-DHAP, respectively, but at slower rates.

Friedberg et al. (1980) have proposed a detailed molecular mechanism for alkyl-DHAP synthase based on the observations discussed

above (Figure 1).[1] It accounted for the stereospecific exchange of the pro-R hydrogen at carbon-1 with retention of configuration, the reaction with either alkoxide or hydroxide (R′=H) ions to form alkyl-DHAP or DHAP, respectively, the incorporation of the entire alkoxide moiety, including the oxygen, into alkyl-DHAP, and the lack of a Schiff base or intermediate during the reaction.

Hixson and Wolfenden (1981) recently synthesized monopalmitoyl-1,2,3-trihydroxyicosane-1-P, an analog of the central acyl-alkyl-intermediate in Friedberg's scheme, in which a methylene group replaced the ether oxygen. Although this compound was inhibitory at rather high levels (37% and 75% inhibition at 130 and 260 μM, respectively), it was not clear if it was due to the parent compound or to fatty acids hydrolyzed from it by microsomal acylhydrolases; the alkyl-DHAP synthase activity is very sensitive to fatty acid. Furthermore the assay system used DHAP, CoA, Mg^{2+}, ATP, and [^{14}C]hexadecanol and, thus, relied on the activities of acyl-CoA synthetase and DHAP acyltransferase activities as well. No data were presented to show that these enzymes were not inhibited by the analog.

Friedberg's mechanism, however, does not account for the observation by Davis and Hajra (1977b) that alkyl-DHAP synthase may be able to catalyze an exchange of the fatty acid esterified to acyl-DHAP, a characteristic of a ping-pong reaction mechanism, not a sequential mechanism like that proposed by Friedberg et al. (1980). In the ping-pong mechanism suggested by Davis and Hajra (1977b), the fatty acid is released to give an enzyme-DHAP intermediate that can then interact

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[1]All of the figures appear in the Appendix.

either with another fatty acid to regenerate acyl-DHAP or with fatty alcohol to give alkyl-DHAP. Thus, the order of addition of substrate and release of products is still very controversial.

CHAPTER II

STATEMENT OF THE PROBLEM

The mechanism of ether bond formation by alkyl-DHAP synthase has been under study for over a decade, and although many characteristics of this enzyme reaction have been elucidated, several very important properties remain uncertain. Friedberg et al. (1980) suggested that the complex mechanism they proposed may require more than one enzyme and may explain the inability of several laboratories to solubilize or purify alkyl-DHAP synthase. Also, due to the unusual nature of the reaction, it has been suggested that a coenzyme may be needed (Metzler, 1977). As discussed in the Introduction, controversy exists over the order of addition of substrates and release of products; Friedberg et al. (1980) proposed a sequential mechanism, while Davis and Hajra (1977b) had presented data to support a ping-pong mechanism. Proof for one mechanism over the other would allow further investigation of the reaction intermediates and suggest appropriate substrate analogs for active site mapping. Finally, the recent indirect evidence indicating that alkyl-DHAP synthase catalyzes the hydrolysis of acyl-DHAP with a concomitant hydrogen exchange needs to be more clearly documented.

The purpose of this dissertation is to resolve these questions by developing a purified enzyme system that is free of competing enzymes and endogenous lipid substrates and products. A significant purification of the alkyl-DHAP synthase activity would indicate that a single enzyme or very tightly associated enzymes are responsible for ether bond formation. It should also be possible to more clearly define any cofactor requirements. The copurification of the partial reactions, the acyl

exchange and alkyl exchange reactions, with the forward reaction of alkyl-DHAP synthase would be strong evidence for a ping-pong mechanism as opposed to the sequential mechanism proposed by Friedberg et al. (1980). The reaction kinetics of the purified enzyme, free of competing enzymes and lipids, may also be studied with much less ambiguity. Finally, removal of other acyl-DHAP acylhydrolase activities known to be present in whole microsomes would allow careful study of the proposed acylhydrolase activity of alkyl-DHAP synthase.

CHAPTER III

SOLUBILIZATION OF ALKYL-DHAP SYNTHASE FROM EHRLICH ASCITES CELL MICROSOMAL MEMBRANES[2]

Acyl-DHAP, formed by the acylation of DHAP, is enzymatically converted to alkyl-DHAP by alkyl-DHAP synthase (Hajra, 1969, 1970; Snyder et al., 1969c; Wykle and Snyder, 1969). In this reaction, which is unique in higher biological systems, the ester-linked fatty acid of acyl-DHAP is replaced by a fatty alcohol to form the first detectable ether-linked intermediate in the biosynthetic pathway that forms O-alkyl glycerolipids and plasmalogens. The membrane-bound form of this enzyme has been well characterized in microsomal preparations from mouse Ehrlich ascites cells (Hajra, 1973; Rock et al., 1977; Wykle et al., 1972b), rabbit harderian glands (Rock, 1977; Rock et al., 1977) and Tetrahymena (Friedberg and Alkek, 1977; Friedberg and Heifetz, 1975), and in mitochondrial preparations from guinea pig liver (Hajra, 1970). Although the precise mechanism of catalysis is not yet known, isotope studies have shown that the hydroxyl oxygen of the fatty alcohol is incorporated into alkyl-DHAP (Snyder et al., 1970c). Also, one of the hydrogens attached to the carbon of acyl-DHAP that forms the ether linkage is exchanged with water (Friedberg and Alkek, 1977; Friedberg and Heifetz, 1975). Interpretation of kinetic data is limited due to the asymmetric localization of this enzyme on the lumenal surface of the microsomal vesicles (Rock et al., 1977). However, recent reports have suggested

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[2](Brown and Snyder, 1979)

that the enzyme is located in the peroxisomes of mouse brain and guinea pig liver (Jones and Hajra, 1977). Attempts to remove the enzyme from its membrane environment have been unsuccessful until now. In this communication we describe an effective procedure for solubilizing the enzyme to a low molecular weight form using Triton X-100 and phospholipase C. Under these conditions, the alkyl-DHAP synthase is fully active, is obtained in high yields, and has a molecular mass of less than 300,000 daltons as judged by Sepharose 4B chromatography.

Materials and Methods

Materials

[1-^{14}C]Hexadecanol was synthesized from [1-^{14}C]palmitate (New England Nuclear, spec. act. = 50.2 Ci/mol) by reduction with Vitride (Snyder et al., 1971). Palmitoyl-DHAP was obtained as described by Piantadosi et al. (1972a); Triton X-100 was purchased from Fisher Scientific Co.; Bio-Beads SM-2 from BioRad; phospholipase C of *Bacillus cereus* and phospholipase A_2 of *Naja Naja* from Sigma; and Sepharose 4B from Pharmacia.

Methods

Enzyme Source. Ehrlich ascites cells, transplanted in Swiss albino mice (HA/ICR) by intraperitoneal innoculation, were harvested after 7 days of growth. The cells were washed extensively with 0.1 M Tris-HCl (pH 7.4) containing 0.25 M sucrose and 0.1 M KCl, and the microsomal fraction was prepared by differential centrifugation as described previously (Wykle and Snyder, 1970).

Enzyme Assay. The assay system was a slight modification of one described recently (Rock et al., 1977). A substrate solution was prepared by mixing 100 nmol of [1-^{14}C]-hexadecanol and 300 nmol of acyl-DHAP in chloroform. After evaporating the solvent under nitrogen, 1 ml of assay buffer (0.1 M Tris-HCl at pH 8.2, 0.25 M sucrose, 0.1 M KCl) containing 0.5 M NaF was added and the lipid substrates were dispersed by sonication. The reaction was initiated by adding 0.05 ml of substrate solution to 0.45 ml of assay buffer that contains the enzyme. After incubation at 37°C for 60 min, the reaction was terminated by extraction of total lipids (Bligh and Dyer, 1959). The lipids were resolved on Silica Gel HRB layers developed in chloroform:methanol:acetic acid (90:10:10, v/v). Distribution of radioactivity was determined by zonal profile scanning or area scraping and utilizing a liquid scintillation spectrometer.

Solubilization. Ehrlich ascites cell microsomes (10 mg protein/ml) were solubilized in the assay buffer containing 0.5% Triton X-100. The mixture was homogenized with five strokes of a loose fitting Kontes glass homogenizer and immediately centrifuged at 100,000 x g for 60 min. The supernatant, which contained over 90% of the enzyme activity, was then incubated at 37°C for 10 min with either phospholipase C or phospholipase A_2 (0.2 units/mg protein). Lipid extraction (Bligh and Dyer, 1959) of the sample and resolution of the lipids on HRB plates developed in chloroform:methanol:acetic acid:water (50:25:8:3, v/v) followed by H_2SO_4 charring verified that the degradation of phosphoglycerides was nearly quantitative under these conditions. Protein was determined by the method of Lowry et al. (1951) or by absorbance at 280 nm.

Gel Filtration. A 1-ml sample of the enzyme preparation from the various steps of solubilization was applied to a Sepharose 4B column (1.5 x 24 cm) previously equilibrated with the assay buffer or with the buffer containing 0.5% Triton X-100. A flow rate of 2 ml/hr was maintained. Sephacryl S-200 and Sephadex G-200 columns of the same dimensions were used under identical conditions. When Triton X-100 was absent from the column buffer, protein eluting from the column was measured spectrophotometrically at 280 nm.

Removal of Triton X-100 with Bio-Beads SM-2. Bio-Beads SM-2, washed with methanol and water, were used in the batch procedure described by Holloway (1973). The washed Bio-Beads were pipetted carefully into a screw-top tube and the aqueous supernatant was removed and replaced with the protein solutions (0.3 g Bio-Beads/ml protein solution). After the suspension was mixed gently on a rotary wheel for 1 hr at 4°C, the protein solution was removed and filtered through glass wool. We determined the solution was free of Triton X-100 by extracting a portion of the sample (Bligh and Dyer, 1959), resolving the lipids on Silica Gel HRB layers developed in chloroform:methanol:acetic acid:water (50:25:8:3, v/v), and visualizing the lipids after H_2SO_4 charring. Triton X-100 had an Rf of 0.85 in this system.

Results and Discussion

Previous results from our laboratory (Rock et al., 1977) indicated that the alkyl-DHAP synthases of rabbit harderian gland and Ehrlich ascites cells were inhibited by high concentrations of deoxycholate, but were stimulated by concentrations of Triton X-100 from about 0.025 to

0.05%. This maintenance of activity in the presence of Triton X-100 served as a successful starting point for our attempts to solubilize the enzyme from the microsomal fraction of Ehrlich ascites cells. We found that higher levels of Triton X-100 (> 0.5%) released over 90% of the alkyl-DHAP synthase activity into the supernatant after centrifugation at 100,000 x g for 60 min (Figure 2). In order to establish an approximate molecular weight of this form of the enzyme, we chromatographed the material solubilized by Triton X-100 on Sephadex G-200 and Sepharose 4B (exclusion limits: 8×10^5 and 2×10^7 daltons, respectively). The enzyme activity eluted with the excluded components in the void volumes of both columns; this indicated the enzyme was still in a form with a molecular mass of several million daltons. Thus, even though the enzyme activity was not associated with the pellet after high speed centrifugation, it was not completely solubilized. This high molecular weight form was still active even when the Triton X-100 was removed with Bio-Beads SM-2 as described under Materials and Methods.

In an effort to obtain a smaller molecular weight component, several other methods, by themselves and in combination with Triton X-100, were tried. The list of unsuccessful treatments included sonication, trypsin, urea, high salt, low salt, lyophilization, delipidation, freeze-thawing, other nonionic detergents such as Tweens and Lubrol, and various cholate derivatives. Success was finally achieved by treatment of the Triton X-100 solubilized material with phospholipases. Both phospholipases A_2 and C yielded lower molecular weight forms of the enzyme. The form obtained with phospholipase A_2 eluted from Sepharose 4B with an approximate molucular mass of 500,000 daltons, whereas the form obtained by phospholipase C treatment was

estimated to be from 250,000 to 300,000 daltons. The lower value was obtained by the elution of the enzyme activity in the void volume of Sephacryl S-200 (exclusion limit: 2.5×10^5 daltons). The upper value was estimated by measuring the elution volume of the enzyme from Sepharose 4B. Figure 3A shows the elution profile of the enzyme from Sepharose 4B equilibrated with the assay buffer and Figure 3B shows a profile from an identical Sepharose 4B column equilibrated with assay buffer containing 0.5% Triton X-100. The elution volumes of the enzyme activity from the two columns were nearly identical. The small amount of activity eluting earlier from the column in Figure 3A was due to incomplete degradation of phospholipid by the phospholipase C treatment; in Figure 3B, complete degradation of the phosphoglycerides was achieved. The large A_{280} peak with an elution volume of 30 to 40 ml is due to Triton X-100, which absorbs strongly at this wavelength.

The data obtained at various stages of solubilization are shown in Table I.[3] The Triton X-100 solubilization consistently gave a 2-fold purification since approximately one half of the protein and nearly all of the alkyl-DHAP synthase activity were solubilized as evidenced by their appearance in the supernatant. The phospholipase treatments did not affect the enzyme activity while converting it to lower molecular weight forms. Analysis of the lipids in the sample showed that the phospholipase C treatment quantitatively degraded all of the phospholipids present, except sphingomyelin, to diacylglycerols. A small amount of monacylglycerols was also detected, indicating some additional hydrolysis by other lipases. The fatty acids produced by this latter

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[3]All of the tables appear in the Appendix.

hydrolysis, when appreciable, inhibited the enzyme slightly as observed previously (Davis and Hajra, 1977b). Removal of the Triton X-100 after phospholipase C treatment had little effect on the enzyme activity, but when this material was chromatographed on Sepharose 4B all of the activity eluted in the void fraction. It appears, then, that the enzyme must remain in detergent to prevent aggregation after hydrolysis of the lipids. The procedure developed by us for preparing the partially purified alkyl-DHAP synthase is an important prelude to its purification for subsequent studies of its mechanism of action.

CHAPTER IV

ALKYL-DHAP SYNTHASE: SOLUBILIZATION, PARTIAL PURIFICATION, NEW ASSAY METHOD, AND EVIDENCE FOR A PING-PONG MECHANISM[4]

Alkyl-DHAP synthase catalyzes a unique reaction in which the ester-linked fatty acid of acyl-DHAP is cleaved and replaced by a fatty alcohol in an ether linkage to form alkyl-DHAP. The latter compound serves as a precursor for the alkyl and alk-1-enyl glycerolipids (Hajra, 1970; Wykle et al., 1972b).

Previous studies of the alkyl-DHAP synthase reaction revealed that a) the oxygen in the ether linkage is derived from the fatty alcohol substrate (Snyder et al., 1970b), b) the pro-R hydrogen of the C-1 carbon of the DHAP portion of acyl-DHAP is exchanged with water during the reaction (Friedberg and Heifetz, 1975; Friedberg et al., 1971) and that this hydrogen exchange occurs with retention of configuration of the C-1 carbon (Davis and Hajra, 1979), and c) there appears to be no Schiff base formed between the ketone of acyl-DHAP and a functional group of the enzyme during the reaction (Brown and Snyder, 1980; Davis and Hajra, 1981; Friedberg et al., 1980). These observations led Friedberg and his co-workers (Friedberg and Gomillion, 1981; Friedberg et al., 1980) to propose a sequential mechanism for alkyl-DHAP synthase in which acyl-DHAP and then fatty alcohol bind to the enzyme to form a ternary complex, after which the fatty acid and then alkyl-DHAP are released.

On the other hand Davis and Hajra (Davis and Hajra, 1977b) proposed a ping-pong mechanism for alkyl-DHAP synthase in which acyl-DHAP is

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[4](Brown and Snyder, 1982)

bound, followed by release of the fatty acid to give an enzyme-DHAP complex that can then react with a fatty alcohol to produce alkyl-DHAP. This proposal was based on the ability of Ehrlich ascites cell microsomes to catalyze an exchange of the acyl group of acyl-DHAP with [1-^{14}C]palmitic acid. Inhibition of this acyl exchange by hexadecanol suggested that alkyl-DHAP synthase was responsible. The results, however, must be interpreted cautiously since intact microsomes contain endogenous lipid substrates and products, as well as competing enzymes. Furthermore, in order to eliminate the possibility that the [^{14}C]acyl-DHAP produced was not a product of the acyl-CoA:DHAP acyltransferase, Davis and Hajra (Davis and Hajra, 1977b) measured the acyl exchange in the presence of 1 mM DTNB to inactivate endogenous CoA. They found no inhibition of the acyl exchange reaction. In contrast, we subsequently showed that 1 mM DTNB inhibits the forward reaction (>90%) (Brown and Snyder, 1980) as well as the acyl exchange reaction (unpublished results). Therefore, because of the presence of the endogenous lipids and competing enzymes in the microsomal preparations used previously and the discrepancy in the DTNB sensitivities in our system compared to that of Davis and Hajra (Brown and Snyder, 1980), we have solubilized and partially purified alkyl-DHAP synthase from Ehrlich ascites cell microsomes.

Materials and Methods

Materials

[1-^{14}C]Palmitic acid (51 Ci/mol) was obtained from New England Nuclear. [1-^{14}C]Hexadecanol was prepared from [1-^{14}C]palmitic acid by Vitride reduction (Snyder et al., 1971). Palmitoyl-DHAP and

hexadecyl-DHAP were prepared as described previously (Piantadosi et al., 1971, 1972). Trizma base, KCl, QAE-Sephadex A-25, and dithiothreitol were purchased from Sigma Chemical Co., Triton X-100 from J. T. Baker Chemicals, DEAE-cellulose DE52 and DEAE-cellulose circles from Whatman, Inc., ethylene glycol from Aldrich Chemical Co., Matrex Red from Amicon Corp., hydroxylapatite (Bio-Gel HTP) from BioRad Laboratories, and DNFB from Pierce Chemical Co.

Methods

Solubilization. Ehrlich ascites cell microsomes were prepared by a previously described method (Wykle and Snyder, 1970). Solubilization was achieved by a two-step procedure. First, the Ehrlich ascites cell microsomes were diluted to 10 mg protein/ml in 0.25 M sucrose, 0.1 M KCl, and 0.05 M Tris-HCl (pH 7.4), and 20% Triton X-100 was added to a final concentration of 1%. The mixture was centrifuged at 100,000 x g for 90 min and the supernatant, which contained about 90% of the recovered enzyme activity, was then added dropwise with stirring to 10 volumes of ice-cold acetone. The gummy precipitate formed under these conditions allowed the acetone to be decanted. The precipitate was dried under a stream of nitrogen to remove residual acetone and taken up in buffer A (20% ethylene glycol, 0.2% Triton X-100, 50 mM Tris-HCl (pH 9.0) and 1 mM dithiothreitol). This solution (15 mg protein/ml) was centrifuged at 100,000 x g for 60 min and the resultant supernatant containing the solubilized alkyl-DHAP synthase was dialyzed overnight against buffer A. The enzyme was kept at 4°C during all subsequent purification steps and was stored at -20°C.

Purification. Solubilized alkyl-DHAP synthase was applied to a DEAE-cellulose column (DE 52; 2.5 x 30 cm) equilibrated in buffer A and, after washing the column with 2 bed volumes of buffer A, was eluted with a linear gradient of 0 to 0.2 M KCl in buffer A (5 bed volumes) at a flow rate of 25 ml/hr. The active fractions were pooled, dialyzed against buffer B (20% ethylene glycol, 0.2% Triton X-100, 50 mM Tris-HCl (pH 7.4) and 1 mM dithiothreitol) overnight and applied to a QAE-Sephadex A-25 column (2.5 x 30 cm) equilibrated in buffer B. After washing the column with 2 bed volumes of buffer B, a linear gradient of 0 to 0.4 M KCl in buffer B (5 bed volumes) was applied at a flow rate of 25 ml/hr.

Active fractions from the QAE-Sephadex column were pooled, diluted 2-fold with buffer B, and applied to a 0.9 x 10 cm column of Matrex Red equilibrated with buffer B. After washing with 5 bed volumes of buffer B, a linear gradient of 0 to 1 M KCl in buffer B (10 bed volumes) was applied at a flow rate of 5 ml/hr. Active fractions were pooled and dialyzed against buffer C (20% ethylene glycol, 0.2% Triton X-100, 10 mM potassium phosphate (pH 7.4) and 1 mM dithiothreitol).

The Matrex Red fraction in phosphate buffer was applied to an 8 ml hydroxylapatite column equilibrated in buffer C. After washing with 3 bed volumes of buffer C, a 40 ml linear gradient of 10 to 300 mM potassium phosphate (pH 7.4) containing 20% ethylene glycol, 0.2% Triton X-100, and 1 mM dithiothreitol was applied. Active fractions were pooled and used for subsequent kinetic studies.

Assays. Due to the presence of Triton X-100 in the enzyme preparations, protein was measured using the BioRad protein assay kit (Bradford, 1976) using bovine serum albumin as a standard.

The forward reaction catalyzed by alkyl-DHAP synthase was assayed in buffer D, which contained 0.25 M sucrose, 0.1% Triton X-100, 50 mM NaF, and 50 mM Tris-HCl (pH 8.0). Palmitoyl-DHAP and [1-^{14}C]hexadecanol were added as sonicated suspensions in buffer D; final concentrations were 120 μM and 40 μM, respectively, for routine assays, but were varied as described in the figure legends for the kinetic studies. In the inhibition experiment, palmitic acid was added as a sonicated suspension in buffer D to give the desired final concentration. The reaction was initiated by addition of enzyme to give a total volume of 0.25 ml. Incubations were done a 37°C for 10 min at which time an aliquot (usually 50 μl) was spotted onto a DEAE-cellulose disk. Disks from an entire experiment were washed three times in absolute ethanol for 15 min, blotted dry, and placed into vials for scintillation counting.

When comparing the forward, acyl exchange, and alkyl exchange reactions, all three activities were measured identically. The forward reaction contained 40 μM [1-^{14}C]hexadecanol and 120 μM palmitoyl-DHAP; the acyl exchange reaction contained 200 μM [1-^{14}C]palmitic acid and 200 μM palmitoyl-DHAP; and the alkyl exchange reaction contained 200 μM [1-^{14}C]hexadecanol and 200 μM hexadecyl-DHAP. Substrates were added as sonicated suspensions in buffer D (see above). Reactions were initiated by addition of enzyme to give a total volume of 0.25 ml. Incubations were done at 37°C for 10 min and then extracted by the method of Bligh and Dyer (1959), with 2% acetic acid in the methanol. The extracts were concentrated and separated on Silica Gel HR plates developed in chloroform:methanol:acetic acid (90:10:10, v/v/v). Bands corresponding to the product (palmitoyl-DHAP or hexadecyl-DHAP) were visualized by spraying with a 0.2% solution of p-nitrophenylhydrazine in 2N HCl and

scraped into vials for radioassay by liquid scintillation spectroscopy.

Acyl-DHAP/alkyl-DHAP oxidoreductase was measured using [^{3}H]NADPH and unlabeled acyl-DHAP as substrates. The [^{3}H]NADPH was formed in a reaction mixture containing 10 mM ATP, 10 mM $MgCl_2$, 100 µM NADP, 50 µM [1-^{3}H]glucose (20 Ci/mol), hexokinase (80 units/ml), and glucose-6-P dehydrogenase (80 units/ml) that was incubated at 37°C for 30 min. The oxidoreductase assay mixture contained 50 nmol palmitoyl-DHAP, 6 µmol EDTA, 50 µmol NaF, and enzyme in 0.9 ml of 0.25 M sucrose, 0.1 M KCl, and 0.05 M Tris-HCl (pH 7.4). Reactions were initiated by adding 100 µl of the [^{3}H]NADPH solution. After incubation at 37°C for 15 min, the samples were extracted by the method of Bligh and Dyer (1959) with 2% acetic acid in the methanol. Lipid extracts were applied to Silica Gel HR layers and resolved in chloroform: methanol:acetic acid (80:20:10, v/v/v). Bands corresponding to acylglycero-P were located with an external standard and scraped into vials for radioassay in a liquid scintillation spectrometer.

Alkyl-DHAP phosphohydrolase was measured by omitting NaF from the alkyl-DHAP synthase forward reaction assay. Samples were extracted as in the alkyl-DHAP/acyl-DHAP oxidoreductase assay and the extracts were applied to Silica Gel G layers and resolved in hexane:ether:methanol: acetic acid (70:30:5:1, v/v/v/v). The alkyl-DHA bands were located using an internal standard visualized with 0.4% dinitrophenylhydrazine in 0.2 N HCl. [^{14}C]Alkyl-DHA was quantitated by zonal scraping (2 mm) of the chromatoplate and subsequent liquid scintillation assay.

DHAP acyltransferase was assayed in a mixture containing 50 nmol [1-^{14}C]palmitoyl-CoA (51 Ci/mol), 1 µmol DHAP, 2 mg bovine serum albumin, 4 µmol $MgCl_2$, 50 µmol NaF, and enzyme in 1 ml of 0.25 M sucrose, 0.1 M KCl, and 0.05 M Tris-HCl (pH 7.4). Reactions were initiated by addition

of enzyme and then incubated for 30 min at 37°C. Extraction and quantitation of [^{14}C]acyl-DHAP were done as described for the alkyl-DHAP synthase forward reaction.

The acyl-DHAP acylhydrolase reactions contained 100 μM [^{14}C]acyl-DHAP and enzyme in 0.25 μl of 0.25 M sucrose and 0.05 M Tris-HCl (pH 8.0) in the presence and absence of 5 mM DNFB. After incubating for 30 min at 37°C, the reaction mixtures were extracted as described for the acyl-DHAP/alkyl-DHAP oxidoreductase assay; the extracts were applied to Silica Gel G layers and resolved in hexane: ether:acetic acid (60:40:1, v/v/v). Fatty acids were located with oleic acid as an internal standard visualized with iodine vapors. Fatty acid bands were scraped and the label quantitated by liquid scintillation assay.

Results

The solubilization procedure presented here differs from our previously published method (Brown and Snyder, 1981) in which the Triton X-100 supernatant was treated with phospholipase C to convert the large form of the enzyme (molecular mass > 4 x 10^6) recovered in the Triton X-100 supernatant to a smaller form (molecular mass = 300,000). This lower molecular weight form could be purified by various chromatographic steps, e.g., DEAE-cellulose, QAE-Sephadex, etc. However, attempts to perform large scale purification of alkyl-DHAP synthase using several consecutive column steps were unsuccessful due to contamination of the phospholipase C preparations with proteases. Therefore, we developed an alternate procedure, where the Triton X-100 supernatant is added dropwise to a 10-fold excess of cold acetone to produce the same lower molecular weight form of the enzyme. In addition, thin-layer chromatography

reveals that approximately 80-85% of the total phospholipid and nearly all (approximately 95%) of the neutral lipids, including fatty acids and fatty alcohols, are removed by this acetone treatment. Yields of activity are variable due to the denaturing effect of the acetone and the removal of inhibiting lipids (especially fatty acids). Therefore, the acetone precipitation is done as quickly as possible, usually in 10 to 15 min, to minimize loss of enzyme activity. Attempts to stimulate the activity of the solubilized enzyme by adding back the extracted lipids or various purified lipids to the assay mixture resulted in a slight inhibition of activity, which suggested the lack of a lipid requirement for alkyl-DHAP synthase.

Despite the greater stability of the acetone solubilized enzyme, extended storage in buffers containing 0.25 M sucrose resulted in a gradual loss of activity. Increasing the sucrose concentration to 20% (w/v) was more effective in stabilizing the enzyme activity, but adding ethylene glycol or glycerol (20% v/v) dramatically preserved the activity of the solubilized enzyme and made possible a multi-step purification (Figure 4).

Another obstacle to the purification was the time-consuming assay of the enzyme activity. The separation of labeled product and substrate was previously accomplished by extracting each sample by the method of Bligh and Dyer (1959) with 2% acetic acid in the methanol. The samples were extracted three times with chloroform to quantitatively partition the alkyl-DHAP into the organic phase. An aliquot was chromatographed on Silica Gel HR plates and the alkyl-DHAP band was scraped and counted. Because it was not feasible to assay the large number of samples generated during a purification by this method, a new procedure was

developed, taking advantage of the charge differences between hexadecanol and hexadecyl-DHAP. At the end of the incubation, an aliquot of the reaction mixture is spotted onto a DEAE-cellulose paper disk. After air drying, the disk is immersed in ethanol which elutes the hexadecanol but not the phosphorylated product. In practice the disks from many samples can be washed in the same ethanol bath. Three washes of 15 min each quantitatively remove the [1-^{14}C]hexadecanol (background <0.5%), but blank disks added to the ethanol washes do not become labeled, i.e., no transfer of [^{14}C]hexadecyl-DHAP occurs between the disks. Approximately 95% of the [^{14}C]hexadecyl-DHAP remains bound to the disks. Thus, as many as 100 samples can be analyzed in as little as 2 hours, allowing rapid screening of column fractions for enzyme activity peaks.

Purification of the solubilized enzyme was accomplished by chromatography on DEAE-cellulose at pH 9.0, QAE-Sephadex at pH 7.4, Matrex Red at pH 7.4, and hydroxylapatite. Elution profiles are shown in Figure 5. A summary of the purification is shown in Table II. The overall purification from microsomes is 97-fold with a specific activity of 20.8 nmol/min/mg. After completion of the purification, the activities of several other enzymes involved in the metabolism of acyl-DHAP and alkyl-DHAP were measured. Acyl-DHAP and alkyl-DHAP oxidoreductase, DHAP acyltransferase, alkyl-DHAP phosphohydrolase and DNFB-insensitive acyl-DHAP acylhydrolase activities were absent in all purification fractions except the whole microsomes. The oxidoreductase and phosphohydrolase seem to be unstable in the buffers used for the 2-week purification procedure even when stored at -20°C. In a previous purification (Brown and Snyder, 1981) the activities of these enzymes were measured immediately after each purification step. The

oxidoreductase, which had a specific activity of 3.9 nmol/min/mg in whole microsomes, was partially co-solubilized with a 30% recovery of activity in the Triton X-100 supernatant and a 9% recovery after acetone treatment. The remaining oxidoreductase activity was completely separated from alkyl-DHAP synthase after Matrex Red chromatography. The phosphohydrolase is also co-solubilized with alkyl-DHAP synthase and is stimulated 13-fold by the Triton X-100 and acetone treatments. Both DEAE-cellulose and finally Matrex Red chromatography remove this activity completely. The DHAP acyltransferase (0.38 nmol/min/mg in whole microsomes) is sensitive to the detergent and no attempt was made to remove the Triton X-100 to recover the activity. Friedberg and co-workers recently demonstrated that alkyl-DHAP synthase can also catalyze a net hydrolysis of acyl-DHAP when fatty alcohols are absent (Friedberg and Gomillion, 1981; Friedberg et al., 1980). DNFB (5 mM) totally inhibits alkyl-DHAP synthase (Brown and Snyder, 1980) and, therefore, the insensitive hydrolysis activity (about 50% of the total hydrolysis activity in microsomes) was measured. Only about 1% of the initial DNFB-insensitive acylhydrolase activity is found in the Triton X-100 supernatant and acetone treatment totally removes this activity. The DNFB-sensitive activity appears to copurify with alkyl-DHAP synthase and exceeds the synthase rate by about 20- to 30-fold. As mentioned above, the acetone extraction removed nearly all of the endogenous neutral lipids. Analysis of the purified alkyl-DHAP synthase preparation for residual fatty acids and fatty alcohols was not possible because of the small amounts of these lipids and the limited enzyme available for subsequent experiments. Controls in which acyl-DHAP was omitted gave

only background levels of alkyl-DHAP synthase activity indicating the total absence of this substrate in purified preparations.

The forward, acyl exchange, and alkyl exchange activities were measured during purification as described in Materials and Methods. Table III shows the relative activities expressed as percentage of the forward reaction. Although slight variations exist between the purified fractions, it is clear that the acyl exchange and alkyl exchange activities copurify with the alkyl-DHAP synthase forward reaction. Since the relative activities of the two exchange reactions do not decrease during purification, it appears that alkyl-DHAP synthase also catalyzes these reactions, a characteristic of an enzyme catalyzing a reaction by a ping-pong mechanism, but not by a sequential mechanism.

Inhibition patterns can also suggest the types of mechanism involved. The ping-pong mechanism for alkyl-DHAP synthase would predict an "enzyme-DHAP" intermediate that can react with fatty acids to produce acyl-DHAP or with fatty alcohols to produce alkyl-DHAP. Thus, fatty acids would compete with fatty alcohols for this intermediate and plots of 1/v vs. 1/[fatty alcohol] at various fatty acid concentrations should converge at the 1/v axis. Although we (unpublished results) and others (Davis and Hajra, 1981) have attempted such an analysis using intact microsomes, the data were inconclusive, whereas with the purified enzyme, plots showing competitive inhibition by palmitic acid were obtained (Figure 6). Each data point was done in triplicate; regression analysis showed that the data fit the lines with correlation coefficients greater than 0.95 and the 1/v intercepts were within the experimental error.

Finally, initial velocities were measured at various substrate levels in order to further confirm the ping-pong mechanism for alkyl-DHAP

synthase. Since palmitoyl-DHAP has been shown to inhibit the reaction at concentrations greater than 100 M even at saturating concentrations of hexadecanol (Wykle et al., 1972b; Davis and Hajra, 1981), we used levels of 100 μM and lower for these measurements. But as seen in Figure 7A, at low hexadecanol concentrations, palmitoyl-DHAP is inhibitory at levels less than 100 μM, whereas this inhibition is less at higher concentrations of hexadecanol and suggests a competitive type of inhibition by palmitoyl-DHAP. This is confirmed by the results in Figure 7B where 1/v is plotted against 1/[hexadecanol]. The slopes of the lines corresponding to 10, 25, 50, and 100 μM palmitoyl-DHAP increase linearly with palmitoyl-DHAP (Figure 7B, insert). These results are consistent with a ping-pong mechanism in which acyl-DHAP is a competitive substrate inhibitor with respect to fatty alcohol. The lower apparent V_{max} at 100 μM palmitoyl-DHAP in Figure 7B reflects the previously noted inhibition by palmitoyl-DHAP at saturating concentrations of hexadecanol.

Discussion

The improved solubilization procedure, stabilization by ethylene glycol, and new rapid assay method have facilitated the purification of alkyl-DHAP synthase from Ehrlich ascites cell microsomes. The partially purified enzyme is free of competing enzymes and lipid substrates and products present in intact microsomes that have prevented the kinetic analysis of the reaction mechanism of alkyl-DHAP synthase.

The acyl exchange reaction noted previously (Davis and Hajra, 1977b) and the alkyl exchange reaction copurify with the alkyl-DHAP synthase forward reaction. These exchange reactions are characteristic of enzymes that catalyze by a ping-pong mechanism but not by a sequential

mechanism. This was confirmed by demonstrating that palmitic acid is a competitive inhibitor of the forward reaction with respect to hexadecanol. Interpretation of the competitive inhibition by low levels of palmitoyl-DHAP is clouded somewhat by the recent reports by Friedberg and co-workers (Friedberg and Gomillion, 1981; Friedberg et al., 1980) that show alkyl-DHAP synthase can also catalyze a net hydrolysis of acyl-DHAP in the absence of fatty alcohol. They proposed that in the absence of fatty alcohol, the hydroxide ions from water could bind to an intermediate of the reaction in a manner analogous to the alkoxide ions from fatty alcohols. Thus, the release of fatty acid appeared to be independent of fatty alcohol concentration and alkyl-DHAP formation. An exact quantitation of the amount of fatty acid released by alkyl-DHAP synthase was not possible because of the presence of an acylhydrolase, but it was assumed (Friedberg and Gomillion, 1981; Friedberg et al., 1980) that the rate of exchange of the tritium-labeled pro-R hydrogen at C-1 of the DHAP portion of acyl-DHAP equalled the cleavage of the acyl group by alkyl-DHAP synthase. Since the moles of tritium exchanged exceeded the moles of alkyl-DHAP formed, it appeared that release of fatty acid exceeded formation of alkyl-DHAP even in the presence of saturating amounts of fatty alcohol. Therefore, it is possible that the competitive inhibition by palmitoyl-DHAP may be due to the palmitic acid released during the reaction. Our preliminary results indicate that an acylhydrolase activity, which is DNFB-sensitive, copurifies with alkyl-DHAP synthase and exceeds the rate of alkyl-DHAP formation by 20- to 30-fold. Thus, inhibition constants for palmitic acid and palmitoyl-DHAP could not be calculated. This net hydrolytic activity is currently being investigated using a purified enzyme preparation and

multilabeled substrates. Regardless of whether the apparent substrate inhibition is by palmitoyl-DHAP itself or by the palmitic acid cleaved from it, our results are consistent with a ping-pong mechanism.

A molecular mechanism for alkyl-DHAP synthase is presented in Figure 8, and is based on the following observations: a) the pro-R hydrogen at C-1 of the DHAP portion of acyl-DHAP is exchanged with water during the reaction (Friedberg and Heifetz, 1975; Friedberg et al., 1971); b) configuration of the C-1 carbon is preserved (Davis and Hajra, 1979); c) the fatty acid ester is cleaved before addition of fatty alcohol; d) either fatty acid, fatty alcohol, or water (R equals H) can bind the activated enzyme-DHAP intermediate to produce acyl-DHAP, alkyl-DHAP, or DHAP (Brown and Snyder, 1981; Davis and Hajra, 1977b; Friedberg et al., 1980); e) a Schiff base intermediate cannot be demonstrated (Brown and Snyder, 1980; Davis and Hajra, 1981; Friedberg et al., 1980); and f) the oxygen in the ether linkage of alkyl-DHAP is donated by the fatty alcohol (Snyder et al., 1970c). The X refers to a nucleophilic cofactor, possibly an amino acid functional group at the active site which covalently binds the DHAP portion of the substrate. Attempts to demonstrate an enzyme-DHAP intermediate have been hampered by the reactivity of this complex with water. We are currently investigating analogs of acyl-DHAP and alkyl-DHAP that can bind covalently to the enzyme but not be cleaved. The scheme in Figure 8 also proposes that the oxygen in the ester linkage of acyl-DHAP leaves with the fatty acid. The fate of this oxygen during the reaction is being studied using the purified enzyme and [^{18}O]palmitoyl-DHAP.

CHAPTER V

FORMATION OF [^{3}H]WATER FROM ACYL-[1-R-^{3}H]DHAP BY PURIFIED ALKYL-DHAP SYNTHASE FREE OF ACYLHYDROLASE ACTIVITY[5]

The first committed step in the biosynthesis of ether-linked glycerolipids is the formation of the ether linkage by alkyl-DHAP synthase. In this reaction, the ester-linked fatty acid of acyl-DHAP is replaced by a fatty alcohol in an ether linkage to form alkyl-DHAP (Hajra, 1970; Wykle et al., 1972b). Because of its position in the ether lipid synthetic pathway and its catalysis of this unique exchange reaction, alkyl-DHAP synthase has received considerable attention during the past 10 years. An important step in the reaction mechanism is the exchange of the pro-R hydrogen at C-1 of the DHAP portion of acyl-DHAP with the medium (Freidberg et al., 1971, 1972; Freidberg and Heifetz, 1973); this exchange occurs with retention of configuration at the C-1 position (Friedberg and Alkek, 1977; Davis and Hajra, 1977a, 1979). It is also known that the incoming fatty alcohol donates the oxygen to the ether bond (Snyder et al., 1970c). In addition, we have recently presented evidence that the reaction proceeds by a ping-pong mechanism in which acyl-DHAP binds to the enzyme with a subsequent release of the fatty acid to produce an activated enzyme-DHAP complex, which then reacts with a fatty alcohol to produce alkyl-DHAP (Brown and Snyder, 1981). This enzyme-DHAP complex may also react with fatty acid to regenerate the substrate, as demonstrated initially in whole microsomes by Davis and Hajra (1977b) and later confirmed by us with a solubilized and partially

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[5]Brown and Snyder, J. Biol. Chem. (submitted)

purified enzyme preparation (Brown and Snyder, 1981, 1982). The complex may also be formed from alkyl-DHAP as evidenced by the formation of [^{14}C]hexadecyl-DHAP from [^{14}C]hexadecanol and unlabeled alkyl-DHAP (Brown and Snyder, 1981, 1982).

Recently, it was proposed that alkyl-DHAP synthase can also cleave acyl-DHAP to fatty acid and DHAP with the concurrent exchange of the pro-R hydrogen (Friedberg et al., 1980, Friedberg and Gomillion, 1981). The evidence for this was obtained using whole microsomes and the endogenous acylation system for DHAP to produce acyl-[1-R-^{3}H; 1,3-^{14}C]DHAP in situ from the double-labeled DHAP. Exchange of the ^{3}H from DHAP was greatly stimulated by addition of the cofactors necessary for acylation suggesting that alkyl-DHAP synthase was involved. However, the amount of ^{3}H exchanged with water during the reaction exceeded the production of alkyl-DHAP under the conditions used. The excess ^{3}H was eventually accounted for by a slight decrease in the ^{3}H:^{14}C ratio of the [^{3}H, ^{14}C]DHAP. Direct proof of an acyl cleavage activity by alkyl-DHAP synthase was not presented, possibly due to the difficulty of producing large amounts of the required acyl-[^{3}H]DHAP, but more likely due to the presence of acylhydrolases for acyl-DHAP in microsomes.

In order to study this excess pro-R hydrogen exchange and acyl cleavage activity in greater detail with less ambiguity, we have solubilized and partially purified (1000-fold) alkyl-DHAP synthase from Ehrlich ascites cell microsomes. In addition, a procedure was developed to chemically acylate [1-R-^{3}H]DHAP in high yield, since the enzymatic acylation system is not present in the purified preparations (Brown and Snyder, 1982). Using [^{32}P]palmitoyl-DHAP to follow the fate of the DHAP portion of the molecule, however, we were unable to detect any hydrolysis

of acyl-DHAP in the purified preparations. Furthermore, exchange of the pro-R hydrogen at C-1 occurs at a rate equal to that of alkyl-DHAP formation. Finally, we found that exchange of the pro-R hydrogen also occurs during the acyl exchange reaction, supporting our recently published molecular mechanism (Brown and Snyder, 1982).

Materials and Methods

Materials

[1-^{14}C]Palmitic acid and carrier-free [^{32}P]H_3PO_4 were purchased from New England Nuclear, [^{3}H]KBH_4 from Amersham, ATP, NAD^+, NADH, pyruvate, palmitic anhydride, dimethylaminopyridine, glycerokinase (from Escherichia coli), glycero-P dehydrogenase (from rabbit muscle), lactic dehydrogenase (from rabbit muscle), aldolase (from rabbit muscle), triose-P isomerase (from rabbit muscle), and alkaline phosphatase (from chicken intestine) from Sigma and hydroxylapatite (Bio-Gel HTP) from BioRad Laboratories. Chloroacetol-P was a gift from Dr. Fred Hartman, Biology Division, Oak Ridge National Laboratory.

Methods

Enzyme Preparation. Microsomes were prepared from Ehrlich ascites cells as described previously (Wykle and Snyder, 1970). Solubilization and purification of alkyl-DHAP synthase were carried out as outlined (Brown and Snyder, 1982).

Synthesis of Labeled Substrates. [1-^{14}C]Hexadecanol was synthesized by Vitride reduction of [1-^{14}C]palmitic acid (Snyder et al.,

1971). Unlabeled palmitoyl-DHAP was prepared by the method of Piantadosi et al. (1972).

[^{32}P]Palmitoyl-DHAP was synthesized as described by Davis and Hajra (1979) using the intermediate, palmitoxydiazoacetone, prepared as described by Schlenk et al. (1952). The palmitoxydiazoacetone (20 µmol in 50 µl of dioxane) was added slowly during 20 min with stirring to [^{32}P]H_3PO_4 (15.8 mCi; 10 µmol) in 50 µl dioxane at 70°C. After 90 min the reaction was extracted by the method of Bligh and Dyer (1959) with 2% acetic acid in the methanol. The extract was concentrated under nitrogen and purified by preparative thin-layer chromatography. The yield was 15% based on ^{32}P and the radiopurity was approximately 98%.

[1-R-^{3}H]DHAP was synthesized by modification of previously described methods (Davis and Hajra, 1979; Friedberg et al., 1980). L-Glyceraldehyde (50 µmol) was dissolved in 2 ml 0.001 N NaOH and added to 100 mCi (10 Ci/mmol) [^{3}H]KBH_4. After 1 hr at 22°C, 8 mg (200 µmol) $NaBH_4$ were added. An hour later, 1 ml of 1 N acetic acid was added and the mixture was passed through a 5-ml column of Dowex 50x8-hydrogen form and dried by rotary evaporation. Five milliliters of methanol containing 100 µl of 1 N acetic acid were added to convert the boric acid to the volatile methyl borate and the mixture was dried by rotary evaporation. The methanol/acetic acid treatment was repeated twice and the [^{3}H]glycerol was taken up in methanol and passed through a 3 ml column of mixed bed exchange resin (Dowex MR-3). The [^{3}H]glycerol (60 mCi) was dried and a solution containing 100 µmol ATP, 100 µmol $MgCl_2$ and 500 µmol triethanolamine, adjusted to pH 9.0 with 1 N NaOH, was added. After addition of 40 units of glycerokinase, the mixture was incubated at 37°C for 1 hr. Then another 40 units of glycerokinase were added and after an

additional hour at 37°C, 200 µl glacial acetic acid were added and the precipitated protein was removed by centrifugation at 800x*g* for 10 min. The supernatant was mixed with 250 mg activated charcoal and centrifuged at 800x*g* for 10 min. The pellet was washed with 2 ml water and, after centrifuging again, the supernatants were combined, treated once more with 250 mg activated charcoal, and centrifuged, and the pellet was washed as before. The final supernatants were combined and filtered through a pasteur pipet plugged with glass wool, diluted to 100 ml with water, and applied to a 5-ml column of Dowex 1x8 equilibrated with 50 mM pyridinium acetate at pH 5.0. After washing the column with 20 ml of equilibration buffer, a linear gradient of 50 mM to 500 mM pyridinium acetate buffer (pH 5.0) was applied. Fractions containing [^{3}H]glycero-P, which were detected by scintillation counting of 1 µl of the 2-ml fractions, were pooled and lyophilized.

The purified [^{3}H]glycero-P (57 mCi) was taken up in 3 ml water and a 2-ml solution containing 100 µmol NAD^+, 100 µmol pyruvate and 500 µmol triethanolamine at pH 9.0 was added. Lactic dehydrogenase (100 units) and glycero-P dehydrogenase (100 units) were added and after 1 hr at 37°C, the reaction was stopped by the addition of 200 µl glacial acetic acid. The sample was centrifuged to remove precipitated protein and treated twice with charcoal as described above. After dilution to 100 ml with water, the sample was applied to a 5-ml Dowex 1x8 column equilibrated with 50 mM pyridinium acetate (pH 5.0) containing 5 mM boric acid. A 200-ml linear gradient of 50 mM to 500 mM pryidinium acetate (pH 5.0) containing 5 mM boric acid was applied and 2 ml fractions were collected and sampled for radioactivity (Figure 9). After lyophilization of the DHAP peak, boric acid was removed by converting it to methyl

borate by addition of 5 ml MeOH and 100 µl of 1 N acetic acid, and drying by rotary evaporation.

Purified [1-RS-^{3}H]DHAP (31 mCi) was taken up in 5 ml of 50 mM ammonium acetate (pH 7.0) and 6 units of fructose diphosphate aldolase were added to remove the ^{3}H from the pro-S position of C-1. After 15 min 43.4% of the ^{3}H was exchanged with water. Another 6 units of aldolase were added and after 15 more min, no further ^{3}H was exchanged. The reaction was acidified with 200 1 glacial acetic acid, diluted to 80 ml with H_2O, and purified by chromatography on a 5-ml Dowex 1x8 column in the presence of boric acid as described above for DHAP purification. Recovery of purified [1-R-^{3}H]DHAP was 13 mCi. An aliquot of the aldolase-treated DHAP was incubated for 30 min at 37°C with 10 units of triose-P isomerase, which exchanges the pro-R ^{3}H at C-1. Approximately 11% of the ^{3}H in the [1-R-^{3}H]DHAP was not exchangeable, indicating it was at carbon 3. All specific activities given for [^{3}H]DHAP and acyl-[^{3}H]-DHAP are for the 1-R-^{3}H only.

To produce the amounts of palmitoyl-[1-R-^{3}H]DHAP required for the experiments below, a method for chemically acylating DHAP was developed. [1-R-^{3}H]DHAP was diluted with unlabeled DHAP (lithium salt) to a specific activity of about 100 Ci/mol and the mixture was converted to the pyridinium salt by passing it through a 5-ml column of Dowex 50x8-pyridinium form. The DHAP (1 mCi; 10 µmol) was dried under nitrogen at 30°C and then dried overnight under vacuum over P_2O_5. Palmitic anhydride (30 µmol) and dimethylaminopyridine (30 µmol) were added in 1 ml dry, ethanol-free chloroform. The reaction was stirred for 3 days at 22°C. At this point, approximately 75 to 90% of the ^{3}H was in solution.

Thin-layer chromatography on a Silica HR plate developed in chloroform: methanol:acetic acid (80:20:10, v/v/v), however, revealed that most of the radioactivity migrated near the solvent front, with only about 10% migrating with the acyl-DHAP standard. The reaction mixture was applied directly to a 2-ml silicic acid (Unisil) column equilibrated in chloroform and washes of 60 ml chloroform, 24 ml chloroform:methanol (19:1), 24 ml chloroform:methanol (9:1), 24 ml chloroform:methanol (3:1), and 24 ml methanol were applied. Under these conditions, the bulk of the ^{3}H that migrated near the solvent front, probably a mixed anhydride of the phosphate of palmitoyl-DHAP and palmitic acid, was converted to palmitoyl-DHAP. Several batches of palmitoyl-[^{3}H]DHAP were produced this way and yields ranged from 50 to 70% of the starting [^{3}H]DHAP. The chloroform:methanol (3:1) and methanol washes contained more than 90% of the recovered palmitoyl-DHAP. These fractions were pooled, dried, and purified by preparative thin-layer chromatography to yield palmitoyl-[1-R-^{3}H]DHAP with a radiopurity of approximately 98%. Specific activity was determined by resolving an aliquot on Silica Gel HR plates developed in chloroform:methanol:acetic acid (90:10:10, v/v/v) and, after charring, the acyl-DHAP band was scraped into a test tube and oxidized in 0.9 ml 70% perchloric acid at 200°C for 20 min. The free phosphate released was then determined by the method of Rouser et al. (1966).

Product Analysis. Reduction of labeled acyl-DHAP and alkyl-DHAP to acylglycero-P and alkylglycero-P was accomplished by dissolving samples in 1 ml methanol containing 5 mg $NaBH_4$. After 12 hr at room temperature, excess $NaBH_4$ was decomposed by adding 1 ml of 1 N acetic acid. The samples were then extracted by the method of Bligh and Dyer (1959) and

analyzed on Silica Gel HR plates developed in chloroform:methanol:acetic acid (90:10:10, v/v/v) after spotting 25 µl of 1% phenylhydrazine in methanol over the sample. Under these conditions acylglycero-P and alkylglycero-P had R_f's of 0.2 while the phenylhydrazones of acyl-DHAP and alkyl-DHAP had R_f's of 0.5. Radioactive products were quantitated by zonal scraping and scintillation counting of the silica gel. $NaBH_4$ reductions went to completion and recoveries were approximately 80%.

Products formed after $NaBH_4$ reduction were saponified in 0.5 N ethanolic KOH at 95°C for 3 hr. After acidifying with HCl, the samples were extracted three times with chloroform. Under these conditions, all of the acylglycero-P but less than 2% of the alkylglycero-P was hydrolyzed. Fatty acids produced from acylglycero-P were analyzed by thin-layer chromatography on Silica Gel G plates developed in ether:hexane:acetic acid (40:60:1, v/v/v) with oleic acid as an internal standard and visualizing with I_2 vapors. Fatty acid bands and the nonsaponifiable starting material at the origin were scraped from the plate and the isotope measured by scintillation counting.

Labeled alkylglycero-P was dephosphorylated by sonicating the dried samples into 1 ml of 0.1 M Tris-HCl (pH 8.5), adding 20 units of alkaline phosphatase, and incubating for 1 hr at 37°C (Blank and Snyder, 1970). Samples were extracted by the method of Bligh and Dyer (1959), with 2% acetic acid in the methanol. Alkylglycerols were analyzed by thin-layer chromatography on Silica Gel G plates developed in ether:ammonium hydroxide (100:1, v/v) with selachyl alcohol (α-octadec-9-enylglycerol) as internal standard. After visualization with I_2 vapors, product bands were scraped and counted.

The structures of alkylglycerols were confirmed by several methods.

Isopropylidene derivatives were made by the method of Hanahan et al. (1963). Lipids were dissolved in 0.5 ml acetone containing 1 mg chimyl alcohol (α-hexadecylglycerol) and 2 µl 70% perchloric acid. After addition of 1 drop NH_4OH, 2.5 ml ether and then 2.5 ml water, the samples were extracted three times with ether. The ether extracts were pooled, washed once with 2.5 ml water and then dried over Na_2SO_4. The samples were then analyzed by thin-layer chromatography on Silica Gel G plates developed in hexane:ether (2:1, v/v). Products were located, using the isopropylidine of selachyl alcohol as an internal standard, by staining with I_2 vapors. Bands corresponding to the product were scraped into scintillation vials and counted.

Hydriodic acid cleavage of alkyglycerols was done by adding 1 ml HI to the dried lipids spiked with 1 mg chimyl alcohol, and incubating at 105°C for 3 hr (Hanahan, 1965). After addition of 1 ml water the samples were extracted three times with chloroform. Products were analyzed on Silica Gel G plates developed in hexane:ether:acetic acid (50:50:1, v/v/v) and detected with dichlorofluorescein using hexadecyliodide as a standard. Hexadecyliodide bands were scraped into scintillation vials and counted.

Periodiate cleavage was done as described by Baumann et al. (1969) after spiking samples with 1 mg of carrier chimyl alcohol. Cleavage products were separated on Silica Gel G plates developed in hexane:ether: acetic acid (50:50:1, v/v/v) and quantitated by zonal scraping (5 mm) and scintillation counting.

Periodate/permanganate oxidation was done as outlined by von Rudloff (1956) after addition of chimyl alcohol as carrier. Products were analyzed and quantitated as described for the periodate cleavage.

Results

Measurement of Acyl-DHAP Acylhydrolase Activity During Purification of Alkyl-DHAP Snythase

Purification of alkyl-DHAP synthase was accomplished as before (Brown and Snyder, 1982) except that a larger column of hydroxylapatite (8 ml) relative to protein (3 mg) and a larger gradient volume (60 ml) were used allowing resolution of the main activity peak of alkyl-DHAP synthase from the bulk of the protein (Figure 10). A summary of the purification is given in Table IV.

Pooled fractions of alkyl-DHAP synthase activity at each purification step were measured for their ability to cleave palmitoyl-DHAP to DHAP. In addition, when present, this activity was also measured in the presence of 10 mM DNFB. Reactions contained 5% ethylene glycol, 0.2% Triton X-100, 0.25 M sucrose, 50 mM NaF, 50 mM Tris (pH 8.0), and 1 mM chloroacetol-P with or without 10 mM DNFB in a total volume of 75 µl. After preincubation at 37°C for 10 min, 12.0 nmol of palmitoyl-[1-R-^{3}H, ^{32}P]DHAP (513,000 cpm ^{3}H, 502,000 cpm ^{32}P) was added as a sonicated suspension in 25 µl of assay buffer and the incubation was continued 30 more min at 37°C. Then 5 µl glacial acetic acid was added to stop the reaction and 25 µl of the sample was spotted onto a cellulose thin-layer plate (500 µ layer prepared from 20 µ Sigmacell, Sigma Chemical Co.) and resolved using a solvent system consisting of 80 ml ethyl acetate, 80 ml acetic acid, 40 ml water, and 2.5 g sodium bisulfite. Palmitoyl-DHAP migrates to the solvent front in this system, whereas free phosphate and DHAP have R_f's of 0.5 and 0.2, respectively. Zonal scrapings of 1 cm each were taken from the bottom half of the plate

and total ^{32}P and ^{3}H were measured by shaking scrapings vigorously for 30 sec in 3.3 ml water, which elutes greater than 95% of the radioactivity from the gel, and then adding 10 ml Insta-Gel (Packard Instrument Co. Inc.). Counting efficiencies were 21% for ^{3}H and 96% for ^{32}P with spillover of ^{32}P into ^{3}H of 1.9% and no spillover of ^{3}H into ^{32}P. For direct comparison to alkyl-DHAP synthase activity, another set of samples were run under identical conditions except that unlabeled palmitoyl-DHAP (12 nmol) and [1-^{14}C]hexadecanol (5 nmol) were added instead of palmitoyl-[^{3}H, ^{32}P]DHAP. These samples were also incubated at 37°C for 30 min and then extracted by the method of Bligh and Dyer (1959) with 2% glacial acetic acid in the methanol. Extracts were concentrated under nitrogen and applied to Silica Gel HR plates and resolved in chloroform: methanol:acetic acid (90:10:20, v/v/v). Zonal scrapings of 0.5 cm in the area of the external standard (visualized with dinitrophenylhydrazine spray) were counted in an organic solvent-based cocktail.

The data for the acylhydrolase and alkyl-DHAP synthase activities are shown in Table V. The acylhydrolase activity is present in the microsomal and solubilized fractions and a small amount is still present after DEAE and QAE chromatography. Subsequent fractions contain no acylhydrolase activity. On the other hand, alkyl-DHAP synthase is still very active in each fraction under the conditions of the assay.

A portion of the DHAP produced from palmitoyl-[^{3}H, ^{32}P]DHAP by the microsomal and solubilized fractions is detritiated as evidenced by the decrease in ^{3}H:^{32}P in the DHAP compared to that of the substrate mixture. Several possibilities exist for this phenomenon: 1) Trace amounts of triose-P isomerase activity may still be present despite the inclusion of chloroacetol-P. Spectrophotometric assay indicated no detectable

isomerase activity under these conditions, but since the uninhibited activity is approximately 3000-4000 times that of alkyl-DHAP synthase in the microsomal and solubilized preparations, a trace of remaining isomerase activity could produce a small amount of detritiation of [1-R-^{3}H]DHAP. 2) More than one acylhydrolase may be present, one of which causes exchange of the C-1 pro-R hydrogen during its reaction. 3) Alkyl-DHAP synthase may catalyze the exchange of some ^{3}H from palmitoyl-[1-R-^{3}H]DHAP through its acyl exchange reaction (see below) and this detritiated acyl-DHAP may then be cleaved by an acylhydrolase.

It should be noted that the acylhydrolase activity in the crude fractions is sensitive to DNFB. A DNFB-insentitive acylhydrolase for acyl-DHAP that is also present in fresh microsomes is not stable under the storage conditions used for alkyl-DHAP synthase (Brown and Snyder, 1982). The DNFB-sensitive acylhydrolase was found to copurify with alkyl-DHAP synthase in an earlier report (Brown and Snyder, 1982), but improved resolution in the present purification has allowed us to separate this acylhydrolase activity from alkyl-DHAP synthase.

To eliminate the possibility that the ^{3}H- or ^{32}P-labeled palmitoyl-DHAP used in the acylhydrolase assay may contain a contaminant that specifically inhibited alkyl-DHAP synthase, the ability of the double-labeled substrate to support the formation of alkyl-DHAP was compared to a parallel set of samples containing unlabeled palmitoyl-DHAP. Reactions were carried out exactly as described in the previous experiment, but scaled up 5-fold with [1-^{14}C]hexadecanol (50 μM) present and using the 1000-fold purified enzyme. The same concentration and proportions of the ^{3}H- and ^{32}P-labeled palmitoyl-DHAP's were used. After 30 min at 37°C, the samples were extracted by the method of Bligh

and Dyer (1959) with 2% glacial acetic acid in the methanol. After $NaBH_4$ reduction, saponifcation, and alkaline phosphatase treatment, recoveries of [^{14}C]hexadecylglycerol were 6.00 $\pm$ 0.48 nmol for the samples incubated with unlabeled palmitoyl-DHAP and 6.40 $\pm$ 0.80 nmol for the samples incubated with the double-labeled palmitoyl-DHAP. This indicated that the double-labeled palmitoyl-DHAP was as good a substrate as the unlabeled palmitoyl-DHAP and did not contain any inhibiting components.

Since the [^{3}H]palmitoyl-DHAP made up approximately 95% of the total palmitoyl-DHAP in these incubations, the ^{32}P label was followed during the product analysis. The ^{14}C to ^{32}P molar ratio in the hexadecylglycero-P recovered from the double-labeled sample after saponification was 0.99 $\pm$ 0.03 and the ratio of [^{14}C]hexadecylglycerol to [^{32}P]phosphate recovered after alkaline phosphatase was 1.03 $\pm$ 0.03, confirming the identity and purity of the [^{32}P]palmitoyl-DHAP.

The ^{14}C-labeled products recovered after alkaline phosphatase treatment were confirmed to be hexadecylglycerol by the following criteria. Treatment with acetone and perchloric acid yielded ^{14}C-labeled derivatives which comigrated with standard isopropylidenes of alkylglycerol on thin-layer plates. Yields for the unlabeled and double-labeled samples were 96.2 and 95.4%, respectively. Cleavage with hydriodic acid gave hexadecyliodides in yields of 96.7 and 97.8% for the unlabeled and double-labeled samples, respectively. In addition, the molar amount of [^{3}H]glycerol in the aqueous phase was about 10 times less than the molar amount of [^{14}C]hexadecyliodide produced, consistent with the previously determined 11% ^{3}H at the sn-3 position of acyl-[1-R-^{3}H]-DHAP (see Methods). Periodate cleavage gave hexadecyloxyacetaldehydes in yields of 70.1 and 69.4%, respectively, for the unlabeled and double-

labeled samples. Finally, periodate/permanganate oxidation produced hexadecyloxyacetates in yields of 79.1 and 80.2% for the unlabeled and doubled-labeled samples, respectively.

Measurement of ^{3}H-Exchange from Acyl-[1-R-^{3}H]DHAP

To investigate the stoichiometry of exchange of the pro-R hydrogen at C-1 versus either formation of alkyl-DHAP or acyl-DHAP labeling via the acyl exchange reaction, incubations were done with palmitoyl-[1-R-^{3}H]DHAP with and without [1-^{14}C]hexadecanol or [1-^{14}C]palmitic acid. The reactions contained 0.25 M sucrose, 0.4% Triton X-100, 10% ethylene glycol, 50 mM NaF, 50 mM Tris-HCl (pH 8.0), 120 μM palmitoyl-[1-R-^{3}H]DHAP (75 Ci/mol) and 1000-fold purified enzyme to a total volume of 150 μl. When [1-^{14}C]hexadecanol (50 μM) or [1-^{14}C]palmitic acid (100 μM) were added, they were sonicated together with the palmitoyl-DHAP. The reactions were initiated by addition of the substrates. After incubation at 37°C for 30 min, 1.85 ml of 0.01 N acetic acid was added and 1 ml of each sample was passed through a 1-ml Dowex 1x8-chloride column which was then washed with 5 ml of water. The column bound all palmitoyl-[1-R-^{3}H]DHAP, [1-^{14}C]palmitic acid, and [1-^{14}C]hexadecanol; the latter was retained presumably by hydrophobic interaction. A portion of the eluate was counted directly while another portion was lyophilized; less than 5% of the radioactivity (^{3}H) remained in the residue indicating that the unbound material was nearly all [^{3}H]water. All [^{3}H]water values were adjusted for this slight amount of radioactivity in the residue. The remaining 1 ml of the reaction mixture from samples containing [1-^{14}C]hexadecanol or [1-^{14}C]palmitic acid was extracted by the method of Bligh and Dyer (1959) with 2% glacial acetic

acid in the methanol. The extracted lipids were applied to Silica Gel HR plates and resolved in chloroform:methanol:acetic acid (90:10:10, v/v/v). [^{14}C]Hexadecyl-DHAP and [^{14}C]palmitoyl-DHAP bands were quantitiated by zonal scraping and counted under conditions to eliminate ^{3}H spillover from palmitoyl-[1-R-^{3}H]DHAP into the ^{14}C channel; under these conditions the ^{14}C efficiency was 56%. These data, based on triplicate determinations, are shown in Table VI.

The high amounts of [^{3}H]water produced in the absence of enzyme in these experiments was seen in other systems (Friedberg et al., 1980; Friedberg and Gomillion, 1981); it was consistent as evidenced by the small standard deviations and was not altered significantly by the presence of hexadecanol or palmitic acid. Therefore, this background was subtracted from the [^{3}H]water measured in the presence of enzyme to give the enzymatically produced [^{3}H]water. In the absence of cosubstrate, a small but significant amount of [^{3}H]water, 0.305 nmol, was formed enzymatically. When 50 μM [^{14}C]hexadecanol was included, the formation of [^{3}H]water increased 7-fold to 2.146 nmol, a net increase of 1.841 nmol due to the addition of the hexadecanol. In comparison, the amount of [^{14}C]hexadecyl-DHAP formed in the reaction was 1.879 nmol, indicating that for each molecule of alkyl-DHAP produced, a single pro-R hydrogen is exchanged. These data disagree with earlier reports that showed the rate of exchange of the pro-R hydrogen exceeded the rate of alkyl-DHAP formation (Friedberg et al., 1980; Friedberg and Gomillion, 1981). Furthermore, these earlier reports (Friedberg et al., 1980; Friedberg and Gomillion, 1981) presented evidence that the enzymatic exchange of the pro-R hydrogen was independent of the presence of fatty alcohol. The present data are also in disagreement with these earlier findings. A

possible explanation for the excess exchange seen by others, especially in the absence of fatty alcohol, is that the fatty acids present in whole microsomes stimulated the acyl exchange reaction and that this partial reaction also involved an exchange of the pro-R hydrogen. That this phenomenon does occur is demonstrated in Table VI. The addition of fatty acid also increased the enzymatic exchange of ^{3}H from the pro-R position from 0.305 nmol to 0.761 nmol, a net increase of 0.456 nmol, which agrees closely with the 0.458 nmol of [^{14}C]palmitoyl-DHAP formed in the reactions.

Discussion

Previous reports by Friedberg and co-workers (Friedberg and Heifetz, 1973; Friedberg et al., 1980; Friedberg and Gomillion, 1982) presented evidence that alkyl-DHAP synthase catalyzed the exchange of much more ^{3}H from acyl-[1-R-^{3}H]DHAP than could be accounted for by alkyl-DHAP formation and that this exchange was independent of the presence of the cosubstrate, fatty alcohol. The excess exchange was hypothesized to result from the alkyl-DHAP synthase-catalyzed cleavage of acyl-DHAP to DHAP during which an exchange of the pro-R hydrogen occurred. The production of detritiated DHAP in their experiments corroborated this proposal (Friedberg and Gomillion, 1981). The data presented here, using intact palmitoyl-[1-R-^{3}H]DHAP and alkyl-DHAP synthase that had been purified 1000-fold from Ehrlich ascites cell microsomes, indicate that although a small, but significant, pro-R hydrogen exchange occurs in the absence of a cosubstrate, addition of either fatty alcohol or fatty acid, increases this exchange. The amount of this increase in exchange is stoichiometric with product formation.

Furthermore, no cleavage of palmitoyl-DHAP to DHAP was observed and, therefore, our recently proposed molecular mechanism for the alkyl-DHAP synthase reaction (Brown and Snyder, 1982), which included this possibility, must be amended slightly (Figure 8), i.e., where R′ can only be alkyl and not H as originally suggested. However, the rest of the data presented here support our original scheme.

The observation that pro-R hydrogen exchange is stimulated by fatty acid via the acyl exchange reaction (Davis and Hajra, 1977b; Brown and Snyder, 1982) as well as by fatty alcohol may explain, in part, the excess exchange of ^{3}H observed by Friedberg and co-workers (Friedberg and Heifetz, 1973; Friedberg et al., 1980; Friedberg and Gomillion, 1981). The use of whole microsomes, containing fatty acids and at least two distinct acylhydrolases (based on DNFB sensitivity) for acyl-DHAP, may cause a two-step production of detritiated DHAP, first by ^{3}H loss from the pro-R position during the acyl exchange reaction, and then by hydrolysis of the detritiated acyl-DHAP. It is unlikely that this is the total explanation since the rate of the acyl exchange reaction is slower than alkyl-DHAP formation, whereas the rate of ^{3}H exchange observed by Friedberg et al. (1980) was much faster. Another possibility, that the [1-R-^{3}H]DHAP was detritiated by traces of triose-P isomerase, seems unlikely based on the lack of ^{3}H exchange from [^{3}H]DHAP observed by Friedberg et al. (1980) in the absence of the cofactors required for acylation. However, the existence of an acylhydrolase for acyl-DHAP that causes an exchange of the pro-R hydrogen cannot yet be ruled out.

Finally, our reaction mechanism predicts that the pro-R hydrogen is exchanged before the fatty acid is released (Figure 8). The possibility exists that under certain conditions, the reaction may be reversible

after the enolization step, but before the fatty acid release, resulting in a larger amount of ^{3}H release from palmitoyl-[1-R-^{3}H]DHAP than formation of products, i.e., [^{14}C]palmitoyl-DHAP or [^{14}C]acyl-DHAP. This would provide an alternate explanation for the small amount of ^{3}H exchange that we observed in the absence of a cosubstrate and the slight excess of ^{3}H exchange compared to ^{14}C-product formation in the presence of [^{14}C]hexadecanol or [^{14}C]palmitic acid. It may also possibly explain the results of Friedberg and co-workers (Friedberg et al., 1980; Friedberg and Gomillion, 1981) whose experimental conditions may have promoted this phenomenon to a greater extent. To test this possibility, we are currently preparing acyl-DHAP with both an ^{18}O in the ester linkage and a label in the fatty acid chain to carefully compare the fatty acid release to the pro-R hydrogen exchange under various conditions in the presence of highly purified alkyl-DHAP synthase.

CHAPTER VI

UNPUBLISHED EXPERIMENTAL DATA

The purpose of this chapter is to present experimental results that have not been included in the Chapters III, IV, and V but that point out several important characteristics of alkyl-DHAP synthase. These experiments are classified into two categories: 1) those pertaining to the purification of alkyl-DHAP synthase, and 2) those that illustrate various properties of the enzyme reaction. It is hoped that the first category will be useful for future investigators who wish to achieve a higher purification of the enzyme without repeating past unsuccessful procedures. Some of the experiments in the second category, especially those dealing with active site modifers and substrate analogs, may be extended to learn more about the alkyl-DHAP synthase reaction mechanism.

Materials and Methods

Materials. Carboxymethylcellulose was purchased from Whatman, Inc. Sulfopropyl-Sephadex, cellulose-P, sodium borohydride, iodoacetate, NEM, and DFP were from Sigma Chemical Co. DNFB and TNBS were from Pierce Chemical Co. Triton X-100 was from Fisher Scientific Co. Sephacryl S-300, Sephacryl S-200, Octyl-Sepharose, Phenyl-Sepharose, and the chromatofocusing system were from Pharmacia Fine Chemicals. Matrex Red and Matrex Blue were from Amicon Corporation. Acrylamide, N,N´-methylene-bis-acrylamide, N,N,N´,N´-tetramethylethylenediamine, riboflavin-P, ammonium persulfate, and bromophenol blue were from BioRad.

Purification. Carboxymethyl-cellulose was equilibrated in 0.5%

Triton X-100, 0.25 M sucrose, 0.05 M acetate at pH 4, 5, or 6. Four milliliters of gel at each pH was packed in a 5-ml plastic pipet. Solubilized enzyme samples (2 ml, 11 mg) prepared by Triton X-100 and phospholipase C treatments (Brown and Snyder, 1979) were dialyzed to the above buffers and centrifuged to remove precipitated protein. One ml of each sample was applied to the column of the same pH followed by elution with equilibration buffer. Fractions of 1 ml were collected, dialyzed overnight against 0.25 M sucrose, 0.5% Triton X-100, and 50 mM Tris at pH 7.4, and assayed as described by Brown and Snyder (1979). Recoveries of the fractions were based on comparisons to nonchromatographed samples at each pH that had been redialyzed to pH 7.4.

Sulfopropyl-Sephadex and cellulose-P were equilibrated in 0.5% Triton X-100, 20% ethylene glycol and 20 mM sodium phosphate at pH 6, 6.5, or 7.0, and 2 ml of each were transferred to 8 ml screw-top culture tubes. Solubilized enzyme (0.5 ml), prepared by Triton X-100 and acetone treatments (Brown and Snyder, 1982) were diluted 10-fold with equilibration buffer at each pH and added to the tubes containing the gel at the same pH. The samples were mixed gently on a rotary wheel for 60 min and allowed to settle. The supernatants were removed, filtered through glass wool and dialyzed to pH 7.4. Elution of the activity from carboxymethyl-cellulose was attempted by adding the same buffer containing 1 M KCl at each pH, mixing gently on the rotary wheel for 60 min, and filtering and dialyzing the supernatants. Samples were assayed by the disk method as described by Brown and Snyder (1982).

Chromatofocusing gel (1 ml) was equilibrated in 20% ethylene glycol, 0.5% Triton X-100 and 25 mM imidazole at pH 7.4, and packed into a pasteur pipet. Solubilized enzyme (7.3 mg), prepared by Triton X-100

and acetone treatments (Brown and Snyder, 1982), was dialyzed to the equilibration buffer and applied to the column. After washing with 5 ml of equilibration buffer, 32 ml of Polybuffer 74 (diluted 8-fold with water and adjusted to pH 4.5) was passed through the column and 1 ml fractions were collected. Protein was measured by the method of Bradford (1976) and alkyl-DHAP synthase activity was measured by the disk assay method (Brown and Snyder, 1982). The pH of each fraction was measured at room temperature.

Octyl-Sepharose (8 ml) was equilibrated to 0.25 M sucrose, 0.05% Triton X-100 and 0.1 M Tris pH 8.2. Solubilized enzyme, prepared by Triton X-100 and phospholipase C treatments (Brown and Snyder, 1979), was dialyzed to the equilibration buffer and, after washing with 16 ml of this buffer, a linear gradient of 0.05% to 2% Triton X-100 in the same buffer was applied. Fractions of 1 ml were collected and an aliquot of each was assayed as described by Brown and Snyder (1979).

Phenyl-Sepharose columns (5 ml) were equilibrated in (a) 0.1 M KCl, 0.5% Triton X-100, and 50 mM Tris at pH 7.4, (b) 0.1 M KCl, 0.1% Triton X-100, and 50 mM Tris pH 7.4, (c) 2M KCl, 0.5% Triton X-100, and 50 mM Tris at pH 7.4, or (d) 2 M KCl, 0.1% Triton X-100 and 50 mM Tris at pH 7.4. Solubilized enzyme samples (3.5 mg), prepared by Triton X-100 and phospholipase C treatments, were diluted 10-fold in each of the equilibration buffers and applied to the respective columns. Each column was washed with 8 ml of equilibration buffer and 2 ml fractions were collected. Columns (a) and (b) were washed with the same buffers at pH 9.0 and 2 ml fractions were collected. Column (b) was then washed with the same buffer made 20% in ethylene glycol and 2 ml fractions were collected. All fractions were assayed as described by Brown and Snyder

(1979). Another Phenyl-Sepharose column (5 ml) was equilibrated in 0.25 M sucrose, 0.1 M KCl, 0.5% Triton X-100 and 50 mM Tris at pH 7.4, and enzyme (3.5 mg) solubilized by Triton X-100 and phospholipase C treatments, was applied. The column was then washed with a solution containing 8 ml of ethylene glycol and 2 ml of buffer (0.5 Triton X-100, 0.1 M KCl, and 50 mM Tris at pH 9.0). Two milliliter fractions were collected and assayed as described by Brown and Snyder (1979).

Matrex Blue and Matrex Red columns (1 ml) were equilibrated in 0.5% Triton X-100, 0.25 M sucrose, 0.1 M KCl, and 50 mM Tris at pH 7.4. Solubilized enzyme (Triton X-100 and phospholipase C, 1 mg) was applied, and, after washing the column with 2 ml equilibration buffer, was eluted with 4 ml buffer containing 1 M KCl. Fractions were assayed as described by Brown and Snyder (1979).

Electrophoresis was performed by the Furlong modification (Furlong et al., 1973) of Ornstein (1964) and Davis (1964). The 5% resolving gel was prepared by mixing equal amounts of the following solutions: (a) 19% acrylamide, 1% N-N′-methylene-bis-acrylamide, (b) water, (c) 1.5 M Tris-HCl pH 8.8, 2.% Triton X-100, 1.0 M sucrose (or 40% ethylene glycol), and (d) 0.05% ammonium persulfate, 0.002% riboflavin-P. After degassing for 5 min, N,N,N′,N′-tetramethylethylenediamine (4 µl per ml resolving gel solution) was added. Electrophoresis tubes (5.5 mm i.d. x 7 mm o.d. x 125 mm) were filled to a level of 7.5 cm with the resolving gel solution. Water (100 µl) was layered on the top and gels were polymerized under fluorescent light (15 watts at 3 inches) for 2 hr. The water was shaken out and 2.5 cm of degassed stacking gel solution was added. This gel solution was prepared by mixing together (a) 1 ml of 16% acrylamide, 4% N,N′-methylene-bis-acylacrylamide, (b) 4 ml of water, (c)

2.5 ml of 0.128 M phosphoric acid, 0.238 M Tris, 1.0 M sucrose (or 40% ethylene glycol) and 2.0% Triton X-100 at pH 7.2, and (d) 2.5 ml of 0.05% ammonium persulfate and 0.002% riboflavin-P. After layering 100 µl of water over each, stacking gels were polymerized as for the resolving gel. Gels were then placed in the electrophoresis cell (Model 150 A from BioRad). The bottom chamber contained 0.1 M Tris HCl (pH 8.1) 0.25 M sucrose (or 10% ethylene glycol) and 0.5% Triton X-100. The upper chamber contained 0.052 M Tris, 0.055 M glycine (pH 8.8), 0.25 M sucrose (or 10% ethylene glycol) and 0.5% Triton X-100. Samples were made 20% in glycerol and each contained 10 µl of 0.25% bromophenol blue tracking dye. Gels were run at a constant amperage of 2.5 mA/tube. For activity determinations, gels were removed from the tubes, sliced into 5 mm sections, soaked in 1 ml assay buffer (0.25 M sucrose, 0.05 M Tris, 0.2% Triton X-100, pH 8.0) overnight and assayed by the disk method of Brown and Snyder (1982). Alternatively, gels were stained by soaking overnight in 45% methanol, 9% acetic acid, and 0.25% coomassie blue. After rinsing with water, background staining was removed by soaking in 7.5% acetic acid and 5% methanol in water.

Characterization. The possibility of a Schiff base-linked intermediate between alkyl-DHAP synthase and DHAP was tested using enzyme (solubilized by Triton X-100 and phospholipase C) at a protein concentration of 250 µg/ml. One aliquot was retained as a control, another was treated with $NaBH_4$, and a third was first saturated with acyl-DHAP (225 µM) and then treated with $NaBH_4$. The $NaBH_4$ treatment was done as follows. A 40-mg/ml solution of $NaBH_4$ in methanol was prepared and three 25-µl aliquots were added to 1 ml samples of the enzyme (+

acyl-DHAP) on ice at 15-min intervals. After the final addition, the samples were kept on ice for 4 hr to allow decay of the $NaBH_4$ and then assayed as described by Brown and Snyder (1979).

Results

Purification. In addition to the column procedures presented in the previous chapters, i.e., Sepharose 4B, DEAE-cellulose, QAE-Sephadex, Matrex Red, and hydroxylapatite, several other chromatography media were tested for their efficacy in purifying alkyl-DHAP synthase. Most of these procedures were unsuccessful but since the conditions used were somewhat arbitrary, they are outlined in detail in the methods section so that future investigators, using modified or different conditions, may possibly achieve efficient purifications with these media. Procedures that gave limited success will be discussed in greater detail.

In general, cation exchangers were unsuccessful. It was observed that dialyzing the enzyme to pH below 6.0 resulted in irreversible inactivation. No binding to carboxymethyl-cellulose was observed at pH 6.0 in 50 mM acetate buffer. Similarly, sulfopropyl-Sephadex did not bind any activity at pH 6.0, 6.5, or 7.0 in 20 mM sodium phosphate. In addition, neither carboxymethyl-cellulose nor sulfopropyl-Sephadex retained a significant amount of the protein (<20%) to make these feasible steps for removing nonspecific proteins. Cellulose-P, on the other hand, bound 90%, 80%, and 70% of the applied activity at pH's 6.0, 6.5, and 7.0 (20 mM sodium-P), respectively, but attempts to elute the protein at higher ionic strength with KCl (up to 1 M) were unsuccessful suggesting inactivation or very tight binding of alkyl-DHAP synthase.

The inability of the carboxymethyl-cellulose and sulfopropyl-

Sephadex columns to bind alkyl-DHAP synthase was explained later when the isoelectric point (pI) of the enzyme was determined using the chromatofocusing system. The solubilized enzyme, dialyzed to 25 mM imidazole buffer at pH 7.4 containing 20% ethylene glycol and 0.2% Triton X-100, was applied to a small chromatofocusing column and eluted with Polybuffer 74 (adjusted to pH 4.5) which generates a linear pH gradient when passed through the column. The negatively charged proteins are bound to the column and elute when the pH of the column is at their isoelectric point. The profiles of protein and alkyl-DHAP synthase activity are shown in Figure 11. The activity eluting in the void was probably due to overloading of the column, since reducing the amount of applied solubilized protein to about 4 mg/ml of exchanger resulted in a single activity peak eluting in the gradient. The pH of the peak fraction of alkyl-DHAP synthase activity, its pI, varied between pH 5.2 and pH 5.5. Therefore, alkyl-DHAP synthase had a net negative charge at the pH's used for the cation exchange procedures. Interestingly, the enzyme activity recovery was nearly quantitative in all three runs on chromatofocusing. Thus, the enzyme elutes from this column in active form whereas dialysis to this pH results in precipitation and inactivation. The lifetime of alkyl-DHAP synthase at this pH, however, is very short; approximately 50% of the activity is lost in 24 hr at 4 C.

Although the solubilized protein was purified 5- to 10-fold by chromatofocusing, efforts to incorporate this step into the purification scheme were hindered by an unexplained loss of activity when using an enzyme preparation partially purified by DEAE-cellulose chromatography as described by Brown and Snyder (1982). Reversing the order of the columns, i.e., purifying the solubilized protein by chromatofocusing

first followed by chromatography on DEAE-cellulose, resulted in yields expected for combining these two columns (50%). The same results were obtained when these experiments were repeated. Therefore, partially purified enzyme is inactivated by chromatofocusing or irreversibly bound to the column. However, because of the limited capacity of the chromatofocusing gel for the enzyme, it is not feasible to use this column as an initial purification step.

Hydrophobic interaction chromatography on Octyl-Sepharose and Phenyl-Sepharose was also tried with little success. The enzyme bound both columns very tightly under all conditions used. The only promising results with Octyl-Sepharose were obtained by applying the enzyme in 0.25 M sucrose and 0.1 M Tris at pH 8.2 with 0.05% Triton X-100, and increasing the Triton X-100 concentration in a linear gradient to 2%. A single activity peak eluted at around 0.6% Triton but it contained only 7% of the applied activity. Since it appeared that the enzyme bound too tightly to Octyl-Sepharose, the less hydrophobic Phenyl-Sepharose was tried. Because elution is favored by high pH, low ionic strength, detergents, and low solvent polarity, samples were applied in 50 mM Tris buffer at pH 7.4 containing either 0.1 or 0.5% Triton X-100 and either 0.1 or 1 M KCl (see Methods); binding occurred under all conditions. Raising the pH to 9.0 had no effect even at low ionic strength. Adding 20% ethylene glycol with 0.5% Triton X-100 and no KCl at pH 9.0, however, eluted about 10% of the applied activity. In another attempt, elution was tried with 50 mM Tris pH at 9.0, 0.5% Triton X-100 and 80% ethylene glycol. Even under these conditions, slightly less than 20% of the applied activity was eluted. It may be possible to achieve a better elution by increasing the Triton X-100 concentration also, since later

experiments showed that the enzyme remains active in solutions containing as much as 10% Triton X-100.

Although Matrex Red gel (Procion Red dye bound to 5% cross-linked agarose) was used successfully, the related gel, Matrex Blue (Cibacron Blue dye bound to agarose), efficiently bound alkyl-DHAP synthase at pH 7.4 but released only 25% of the applied activity when washed with 1 M KCl. Under identical conditions, most of the activity was eluted from Matrex Red by 1 M KCl. No further attempts were made to optimize Matrex-Blue chromatography.

Although gel filtration of alkyl-DHAP synthase on Sepharose 6B and 4B yielded good recoveries of activity, use of another gel filtration matrix, Sephacryl S-300, resulted in total loss of enzyme activity. Sephacryl S-300, composed of allyl-dextran cross-linked with acrylamide, has a fractionation range similar to that of Sepharose 6B. Columns were run using buffer (0.25 M sucrose and 50 mM Tris at pH 8.2) containing 0, 0.5%, and 10% Triton X-100, 0, 0.1 M, and 1 M KCl, and even 1% acrylamide without success. Pooling of fractions did not regenerate the activity and mixing of solubilized enzyme with each fraction did not indicate the presence of an inhibitor or reactivate the enzyme. Sephacryl S-300 did not inhibit when added to the assay mixture itself. In another experiment, samples were applied to 10 ml columns of Sephacryl and allowed to penetrate the gel to various degrees. Then, portions of the gel were removed and soaked in buffer overnight, and the eluant was assayed. No activity was observed. Thus, inactivation of the enzyme occurs very rapidly after application to the gel; the reason, however, is not clear. In addition, the large form of the enzyme obtained after Triton X-100 treatment elutes in the void of the Sephacryl S-300 with a

reasonable recovery; only the small molecular weight form is affected.

Native gel electrophoresis of alkyl-DHAP synthase was performed as outlined in the Methods section. Although the high molecular weight standards, ferritin and catalase, migrated in sharp bands, solubilized and even partially purified alkyl-DHAP synthase samples showed only a few discrete bands with a very high background of staining. When the gels were sliced into 5-mm sections and assayed, enzyme activity was detected only at the top of the resolving gel (Figure 12A). Despite the inclusion of 0.5% Triton X-100 in the gels and buffers, it is possible that during the concentration of proteins at the interface between the stacking and resolving gels the protein to detergent ratio increased to a point where aggregation occurred, preventing permeation of alkyl-DHAP synthase into the gel. When the stacking gel was omitted, enzyme activity was detected in a single, broad band migrating well into the resolving gel (Figure 12B). But as seen in the presence of the stacking gel, protein staining showed only a couple of sharp bands and a high background. Whether this background is due to the high number of different proteins present in the samples or to a random aggregating of the proteins is not clear. However, the diffuse staining in the partially purified preparations would suggest that the latter is probably the reason. This aggregation and disaggregation during electrophoresis may also explain the broad band of enzyme activity.

Characterization. The reaction mechanism originally proposed by Davis and Hajra (1977b) and later documented by Brown and Snyder (1982) using a purified enzyme preparation includes the formation of an enzyme-DHAP complex. Such complexes have been noted for aldolases and

triose-P isomerase. Grazi et al. (1962) demonstrated that DHAP formed a Schiff base with a lysine residue of aldolase that could be stabilized by reduction by $NaBH_4$. Reduction of the enzyme-DHAP complex inactivated the enzyme. To test whether the proposed complex between DHAP and alkyl-DHAP synthase involved a Schiff base linkage, solubilized enzyme (Triton X-100 and phospholipase C) was saturated with substrate (225 μM) and then treated with $NaBH_4$ (see Methods). After allowing the excess $NaBH_4$ to decay on ice for 4 hr, the activity was measured and compared to controls treated with $NaBH_4$ in the absence of acyl-DHAP and controls not treated with $NaBH_4$. The results are shown in Table VII (from Brown and Snyder, 1980). No inhibition was observed under these conditions. These results, later confirmed by Friedberg et al. (1980) and by Davis and Hajra (1981), indicated that a Schiff base linkage between alkyl-DHAP synthase and DHAP is unlikely.

Further attempts to define the nature of the active site of alkyl-DHAP synthase involved treatment of the enzyme with amino acid functional group modifiers. Table VIII (from Brown and Snyder, 1980) shows the effect of DNFB, TNBS, iodoacetate, NEM, DTNB, and DFP, when added at the given concentrations to the assay mixture. Assay conditions were those used in Chapter III (Brown and Snyder, 1979). The solubilized and microsomal forms of alkyl-DHAP synthase were affected identically by these reagents. DTNB, a sulfhydryl modifier, totally inhibited the enzyme at concentrations of 1 mM and higher. Iodoacetate and NEM, which also act on sulfhydryls, were less effective. However, due to the unknown age and known instability of these latter reagents, it is possible that they may have been partially inactive. DFP has been shown to covalently block active site serine groups. Its inhibition of

alkyl-DHAP synthase is incomplete even at 10 mM; therefore the effects of DFP are unclear. DNFB and TNBS are relatively nonspecific reagents which react readily with amino groups and occasionally with suflhydryls. Thus, it is not clear if the inhibition seen in Table VIII is due to modification of amino or sulfhydryl groupings.

To confirm that these agents are modifying alkyl-DHAP synthase and not altering the reaction in some other way, such as reacting with the substrates, solubilized enzyme (Triton X-100 and phospholipase C) at a concentration of 5 mg/ml was treated with either 1 mM DTNB or 1 mM DNFB for 10 min at 37°C and 1 ml of each was applied to a 10-ml Sephadex G-25 column equilibrated in 0.25 M sucrose, 0.5% Triton X-100, and 50 mM Tris-HCl at pH 7.4. Fractions of 1 ml were collected and the total activity recovered was compared to that for a similarly chromatographed, untreated enzyme sample. Direct assay of the treated enzyme before chromatography showed only 80% inhibition by DTNB and 98% inhibition by DNFB. After chromatography, a slight reversal of inhibition was seen for both reagents: 67% and 89% inhibition by DTNB and DNFB, respectively. The spontaneous hydrolysis of DTNB adducts has been documented by several laboratories (see Means and Feeney, 1971). It should be noted that although Tris buffers were used in these experiments, reaction of DNFB with Tris occurred relatively slowly and did not alter the pH significantly during the assay.

A number of analogs of acyl-DHAP, alkyl-DHAP, and proposed reaction intermediates were investigated for their effect on alkyl-DHAP synthase activity. The purpose was 2-fold: 1) to shed light on the molecular mechanism of the reaction, and 2) to search for suitable ligands for affinity chromatography.

Due to the cleavage of acyl-DHAP or alkyl-DHAP by the enzyme with subsequent release of the fatty acid or fatty alcohol, attachment to a support must be made via the DHAP portion of the molecule in order for the ligand to be effective. A convenient point of modification is at the phosphate moiety but, as seen with most affinity ligands, a spacer arm between the support and the ligand would probably be required. Thus, the effect of derivatizing the phosphate group was studied. Ethanolamine derivatives of acyl-DHAP and alkyl-DHAP had been previously synthesized (Piantadosi et al. 1972a,b) and were readily available. Acyl-DHAP-ethanolamine (Table IX, A) was a very poor substrate for alkyl-DHAP synthase supporting less than 10% of the activity of a comparable amount of acyl-DHAP. Similarly, alkyl-DHAP-ethanolamine (Table IX, B) was only mildly effective as an inhibitor, reducing the activity by 30% when used at 300 μM in a reaction containing a concentration of acyl-DHAP equal to its K_m (30 μM). As will be shown below, this amount of inhibition can be produced by other phospholipids under similar conditions. Therefore, it appeared that alkyl-DHAP attached to a support via the phosphate group was not feasible. On the other hand, these experiments pointed out the requirement for a free phosphate group on the substrate.

An alternative to anchoring acyl-DHAP or alkyl-DHAP to a support via the DHAP portion of the molecule was to synthesize a noncleavable ligand which could then be linked via the aliphatic chain. Such a compound, 2-ketohenicosanol-P, is shown in Table IX (C). This compound was synthesized by reacting icosanoyl-Cl with diazomethane, and then with phosphoric acid as in the synthesis of the ^{32}P-labeled acyl-DHAP. The final compound lacks an ester or ether linkage but retains the terminal phosphate and α-keto group. This compound was tested for its ability to

inhibit alkyl-DHAP synthase in the presence of 30 μM (K_m) or 120 μM acyl-DHAP. As shown in Table X, significant inhibition was observed. This inhibition was reduced by increasing the acyl-DHAP concentration but was never over 40%. Since addition of phospholipids to alkyl-DHAP synthase preparations always slightly inhibited the enzyme activity (unpublished results), phosphatidic acid was chosen as a general phospholipid control because of its similar free phosphate group. As seen in Table X, phosphatidic acid caused a similar reduction of activity, indicating that the effects of the 2-ketohenicosanol-P were probably not specific. Surprisingly, alkylglycerol-P at the same concentrations was much more inhibitory.

Discussion

While most of the experiments presented in this chapter gave seemingly negative results, several important properties of alkyl-DHAP synthase were discovered:

(1) Chromatofocusing indicated that the isoelectric point of the enzyme is between 5.2 and 5.5. These results explained the inability of cation exchangers to bind alkyl-DHAP synthase at the pHs tried. Although initial attempts to incorporate this very effective purification step (5- to 10-fold puriication with nearly quantitative recovery) into the overall scheme were disappointing, combination with other column steps or increasing the protein to column volume ratio may help preserve the activity.

(2) The enzyme appears to be very hydrophobic as evidenced by its aggregation in the absence of detergent (Brown and Snyder, 1979) and its very tight binding to Octyl- and Phenyl-Sepharose. The most recent

discovery that alkyl-DHAP synthase is stable in Triton X-100 concentrations up to 10% suggests that higher detergent concentrations could be combined with the promising results of elution with 80% ethylene glycol to allow eventual use of Phenyl-Sepharose as a purification step.

(3) The mysterious loss of activity during Sephacryl S-300 chromatography may be the result of an important property of the enzyme. Until conditions are found to elute or regenerate the activity, however, the phenomenon will remain an oddity.

(4) Native gel electrophoresis of alkyl-DHAP synthase can be accomplished with retention of sufficient activity to locate the enzyme in the gel. The diffuse staining pattern obtained with coomassie blue seems to be a property of the rather hydrophobic proteins in the samples and not the system itself, since ferritin and catalase gave sharp bands with no visible background staining. The rather broad band of activity suggests that alkyl-DHAP synthase may be reversibly aggregating during migration through the gel. If this is the case with the other proteins present, this phenomenon would explain the diffuse staining. Denaturing gels have not yet been tried but with the high degree of purification achieved recently (Chapter 5), it may be possible to identify bands corresponding to the enzyme.

(5) The inactivation of alkyl-DHAP synthase with DTNB suggests a possible sulfhydryl group in the active site. This moiety could be responsible for stabilizing the proposed activated enzyme-DHAP complex proposed by Brown and Snyder (1982).

(6) Results obtained with the ethanolamine derivative of acyl-DHAP indicates the requirement for a free phosphate moiety on the substrate.

(7) The necessity of either an ester or ether linkage for

interaction with alkyl-DHAP synthase is illustrated by the lack of effect of the 2-ketohenicosanol-P analog on enzyme activity.

CHAPTER VII

SUMMARY

Alkyl-DHAP synthase has received considerable attention since its discovery over a decade ago. The reaction mechanism is rather complex and not well understood. Early investigators determined that the oxygen in the ether linkage is donated by fatty alcohol (Snyder et al., 1970c) and that the pro-R hydrogen at C-1 of the DHAP portion of acyl-DHAP is exchanged with water during the reaction (Friedberg et al., 1971, 1972) with retention of configuration at the C-1 (Davis and Hajra, 1977a, 1979; Friedberg and Alkek, 1977). Further progress toward understanding the enzyme mechanism has been hampered by the use of whole microsomes as a source of alkyl-DHAP synthase. Thus, it was not clear if the overall reaction was catalyzed by a single enzyme or by two or more separate enzymes as suggested by Friedberg et al. (1980). The presence of endogenous substrates and competing enzymes prevented the detailed kinetic analysis required to determine the order of addition of substrate and release of products (Davis and Hajra, 1981). While Davis and Hajra (1977b) favored a ping-pong mechanism for the reaction, Friedberg et al. (1980) proposed a sequential mechanism. Competing enzymes in the microsomal preparations of Friedberg and co-workers (Friedberg et al., 1980; Friedberg and Gomillion, 1981) necessitated an extensive statistical treatment of their data before concluding that alkyl-DHAP synthase can cleave acyl-DHAP to fatty acid and DHAP. Therefore, in order to obtain a clearer understanding of the reaction mechanism we decided to purify alkyl-DHAP synthase from Ehrlich ascites cell microsomes.

Solubilization of the membrane-bound enzyme was achieved by a two-step procedure. Treatment of the microsomes with 1% Triton X-100 followed by high speed centrifugation released nearly all of the alkyl-DHAP synthase into the supernatant. This form of the enzyme had a very large molecular mass and could not be readily purified. Therefore, a second step was introduced to convert the enzyme to a smaller, purifiable form. Treatment of the Triton X-100 supernatant with phospholipase A_2 or phospholipase C yielded lower molecular weight form (Mr=300,000) that could be purified by several chromatographic methods. However, the enzyme was not stable, probably due to protease contamination of the phospholipase preparations. An alternate procedure, acetone precipitation of the Triton X-100 supernatant, produced the same low molecular weight form of alkyl-DHAP synthase without the problem of enzyme loss by proteolysis. Addition of either ethylene glycol or glycerol at 20% (v/v) further stabilized the enzyme.

Chromatography of the solubilized enzyme on DEAE-cellulose, QAE-Sephadex, Matrex Red, and hydroxylapatite columns yielded a highly purified preparation (up to 1000-fold) of alkyl-DHAP synthase. Use of cation exchange columns, however, was not possible due to the instability of the enzyme below pH 6.0. Chromatofocusing indicated that the pI of alkyl-DHAP synthase is around 5.3. Competing enzymes, including all acylhydrolases for acyl-DHAP, were removed during purification and the resulting preparations could be used to examine the reaction mechanism.

Purified alkyl-DHAP synthase retained the acyl exchange and alkyl exchange activities. The relative rates of these reactions to that of the forward reaction were constant during purification providing strong evidence for a ping-pong mechanism. Kinetic analysis confirmed the

ping-pong mechanism by showing that fatty acid is a competitive inhibitor of the forward reaction with respect to fatty alcohol. Thus acyl-DHAP binds to the enzyme with subsequent release of the fatty acid to yield an activated enzyme-DHAP complex. This complex in turn can react with fatty alcohol to produce alkyl-DHAP or with fatty acid to regenerate acyl-DHAP. In addition this complex can be formed by binding alkyl-DHAP and releasing the fatty alcohol.

The ability of alkyl-DHAP synthase to cleave acyl-DHAP to fatty acid and DHAP as proposed by Friedberg et al. (1980) was investigated in the purified preparations. Using ^{32}P-labeled acyl-DHAP as a substrate considerable acylhydrolase activity was detected in whole microsomes and solubilized preparations. This activity, relative to alkyl-DHAP synthase, was reduced after DEAE-cellulose and QAE-Sephadex chromatography and was totally absent in Matrex Red and hydroxyapatite fractions. Thus, alkyl-DHAP synthase does not produce DHAP from acyl-DHAP.

The stoichiometry of exchange of the pro-R hydrogen at C-1 of the DHAP portion of acyl-DHAP was also measured using purified alkyl-DHAP synthase. In the absence of cosubstrate, a small, but significant amount of ^{3}H was exchanged from acyl-[1-R-^{3}H]DHAP. Inclusion of saturating [^{14}C]hexadecanol stimulated the amount of ^{3}H exchanged by 7-fold. This increase in ^{3}H exchanged was identical to [^{14}C]alkyl-DHAP formation. Similarly, when [^{14}C]palmitic acid was added, the increase in ^{3}H exchanged matched the amount of [^{14}C]acyl-DHAP formed via the acyl exchange reaction. Therefore, the results of Friedberg and co-workers (Friedberg et al., 1980; Friedberg and Gomillion, 1981) indicating that the pro-R hydrogen exchange was independent of the presence of

cosubstrate and exceeded alkyl-DHAP formation by an order of magnitude were probably due to competing enzymes in the microsomes. In our experiments, the DHAP hydrolyzed from acyl-[1-R-^{3}H, ^{32}P]DHAP by microsomal preparations was partially detritiated, and the amount of detritiated DHAP produced exceeded the alkyl-DHAP formed by 13-fold. At least four possibilities may explain the production of detritiated DHAP by the whole microsomes. First, trace amounts of triose-P isomerase may be present despite the inclusion of the potent suicide inhibiitor, chloroacetol-P. This possibility is not likely since Friedberg et al. (1980) presented controls that demonstrated the lack of isomerase activity under these conditions. Second, the acyl exchange reaction is very active due to the presence of fatty acid in whole microsomes. We have shown that this reaction involves exchange of the pro-R hydrogen. Thus, the acyl exchange reaction can produce detritiated acyl-DHAP that can then be deacylated by acylhydrolases in the microsomes. However, this acyl exchange is much slower than alkyl-DHAP formation, whereas the ^{3}H exchange observed in whole microsomes (Friedberg et al., 1980) and by us is much faster. Therefore, this possibility would contribute only a small fraction of the total detritiated DHAP observed. Third, at least two acylhydrolases are present in the microsomal preparations based on DNFB sensitivity and it is possible that one of these involves the concurrent exchange of the pro-R hydrogen. This possibility has not yet been ruled out. Fourth, since our molecular scheme for the alkyl-DHAP synthase reaction (Brown and Snyder, 1982) proposes that the exchange at the pro-R hydrogen occurs before the release of the fatty acid, it is possible that under certain conditions, the reaction may be reversible after the pro-R exchange, but before the fatty acid release. This would

result in an excess of pro-R hydrogen exchange compared to alkyl-DHAP formation. It is possible that under the conditions of our experiments, this excess exchange was minimal, whereas the conditions employed by Friedberg and co-workers (Friedberg et al., 1980; Friedberg and Gomillion, 1981) may have promoted the excess exchange to a much greater extent.

The preparation of highly purified alkyl-DHAP synthase has permitted a much clearer investigation of the reaction mechanism. When freed of competing enzymes and endogenous lipid substrates and products, alkyl-DHAP synthase can be shown to catalyze the synthesis of alkyl-DHAP by a ping-pong mechanism and to exchange one pro-R hydrogen for each alkyl-DHAP formed. The proposed molecular mechanism for the reaction is based on these new findings as well as previous observations, and provides a working model of the alkyl-DHAP synthase reaction for future investigations.

LIST OF REFERENCES

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Andre, E. and Bloch, A. (1932) The etho-esters of glycerol or etho-glycerides of the oil from the livers of Scymnorhinas lichia Bonnaterre. C. R. Hebd. Seances Acad. Sci. 195, 627-629.

Andre, E. and Bloch, A. (1935) Oils of murine animals. A new group of lipides. The ether esters of glycerol. (Glyceryl alkoxy diglycerides.) Bull. Soc. Chem. Fr. [5] 2, 789-802.

Bandi, Z. L., Aaes-Jorgensen, E., and Mangold, H. K. (1971) Metabolism of unusual lipids in the rat. I. Formation of unsaturated alkyl and alk-1-enyl chains from orally administered alcohols. Biochim. Biophys. Acta 239, 357-367.

Baumann, W. J., Schmid, H. H. O., and Mangold, H. K. (1969) Oxidative cleavage of lipids with sodium metaperiodate in pyridine. J. Lipid Res. 10, 132-133.

Bell, O. E., Jr., Blank, M. L., and Snyder, F. (1971) The incorporation of ^{18}O and ^{14}C from long chain alcohols into the alkyl and alk-1-enyl ethers of phospholipids of developing rat brain. Biochim. Biophys. Acta 231, 579-583.

Bergmann, W. and Stansbury, H. A. (1943) Contributions to the studies of marine products. XIV. Astrol. J. Org. Chem. 8, 283-284.

Bishop, J. E. and Hajra, A. K. (1978) Specificity of reduction of fatty acids to long chain alcohols by rat brain microsomes. J. Neurochem. 30, 643-647.

Blank, M. L. and Snyder, F. (1970) Specificities of alkaline and acid phosphatases in the dephosphorylation of phospholipids. Biochemistry 9, 5034-5036.

Blank, M. L., Snyder, F., Byers, L. W., Brooks, B., and Muirhead, E. E. (1979) Antihypertensive activity of an alkyl ether analog of phosphatidylcholine. Biochem. Biophys. Res. Commun. 90, 1194-1200.

Blank, M. L., Wykle, R. L., Alper, S., and Snyder, F. (1974) Microsomal synthesis of the ether analogs of triacylglycerols. Biochim. Biophys. Acta 348, 397-403.

Blank, M. L., Wykle, R. L., and Snyder, F. (1971) Enzymic synthesis of ethanolamine plasmalogens from an O-alkyl glycerol. FEBS Lett. 18, 92-94.

Blank, M. L., Wykle, R. L., and Snyder, F. (1972) The biosynthesis of ethanolamine plasmalogens by a postmitochondrial fraction from rat brain. Biochem. Biophys. Res. Commun. 47, 1203-1208.

Bligh, E. G. and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911-917.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Brown, A. J. and Snyder, F. (1979) Solubilization of alkyldihydroxyacetone-P synthase from Ehrlich ascites cell microsomal membranes. Biochem. Biophys. Res. Commun. 90, 278-284.

Brown, A. J. and Snyder, F. (1980) Properties of membrane-bound and solubilized alkyldihydroxyacetone-P synthase. Fed. Proc. 39, 1993, (Abstr. 2032).

Brown, A. J. and Snyder, F (1981) Partial purification of alkyl synthase. Fed. Proc. 40, 1806 (Abstr. 1535).

Brown, A. J. and Snyder, F. (1982) Alkyldihydroxyacetone-P synthase: solubilization, partial purification, new assay method, and evidence for a ping-pong mechanism. J. Biol. Chem. 257, 8835-8839, 1982

Carter, H. E., Smith, D. B., and Jones, D. N. (1958) A new ethanolamine-containing lipide from egg yolk. J. Biol. Chem. 232, 681-694.

Davis, P. A. and Hajra, A. K. (1977a) Stereospecific exchange of hydrogen from 1-acyl dihydroxyacetone-3-P with water during the formation of ether lipid. Fed. Proc. 36, 850 (Abstr. 3050).

Davis, P. A. and Hajra, A. K. (1977b) The enzymatic exchange of the acyl group of acyl dihydroxyacetone phosphate with free fatty acid. Biochem. Biophys. Res. Commun. 74, 100-105.

Davis, P. A. and Hajra, A. K. (1979) Stereochemical specificity of the biosynthesis of the alkyl ether bond in alkyl ether lipids. J. Biol. Chem. 254, 4760-4763.

Davis, P. A. and Hajra, A. K. (1981) Assay and properties of the enzyme catalyzing the biosynthesis of 1-O-alkyl dihydroxyacetone 3-phosphate. Arch. Biochem. Biophys. 211, 20-29.

Debuch, H. (1959a) The position of the aldehyde in colamine plasmalogen from brain. Hoppe-Seyler's Z. Physiol. Chem. 314, 49-50.

Debuch, H. (1959b) The position of the aldehyde in cobalamin plasmalogen of brain. Hoppe-Seyler's Z. Physiol. Chem. 317, 182-189.

Demopoulos, C. A., Pinckard, R. N., and Hanahan, D. J. (1979) Platelet-activating factor. Evidence for 1-O-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine as the active component (a new class of lipid chemical mediators). J. Biol. Chem. 254, 9355-9358.

Doree, C. (1909) The occurrence and distribution of cholesterol and allied compounds in the animal kingdom. Biochem. J. 4, 72-106.

Eichberg, J., Gilbertson, J. R., and Karnovsky, M. L. (1961) Neutral plasmalogens analogous to the neutral triglycerides. J. Biol. Chem. 236, 15-16.

Ellingboe, J, and Karnovsky, M. L. (1967) Origin of glycerol ethers: biosynthesis from labeled acetate, stearic acid, stearaldehyde and stearyl alcohol. J. Biol. Chem. 242, 5693-5699.

Emmerie, A., Engel, C., and Klip, W. (1952) The tuberculostatic action in vitro of the unsaponifiable fraction of cod-liver oil. J. Sci. Food Agric. 3, 264-268.

Feulgen, R. and Voit, K. (1924) Aldehyde of wide distribution; its origin, its microchemical and microscopical detection. Pfluegers Archiv. Gesamte Physiol. Menchen Tiere 206, 389-410.

Friedberg, S. J. and Alkek, R. D. (1977) Absolute configuration of tritiated O-alkylglycerol synthesized enzymatically from [1,3-$^{3}H_2$, 1,3,-$^{14}C_2$]dihydroxyacetone phosphate. Biochemistry 16, 5291-5294.

Friedberg, S. J. and Gomillion, M. (1981) Hydrogen exchange in the formation of dihydroxyacetone phosphate from acyl dihydroxyacetone phosphate in O-alkyl lipid synthesis in Ehrlich ascites tumor cell microsomes. J. Biol. CHem. 256, 291-295.

Friedberg, S. J., Gomillion, D. M., and Stotter, P. L. (1980) The mechanism of ether bond formation in O-alkyl lipid synthesis. J. Biol. Chem. 255, 1074-1079.

Friedberg, S. J. and Greene, R. C. (1967) Glyceryl ether synthesis from long chain alcohols in Elasmobranch stomach. J. Biol. Chem. 242, 5709-5714.

Friedberg, S. J. and Greene, R. C. (1968) The synthesis of glyceryl ether glycerol in Tetrahymena pyriformis. Biochim. Biophys. Acta 164, 602-603.

Friedberg, S. J. and Heifetz, A. (1973) Hydrogen exchange in the synthesis of glyceryl ether and in the formation of dihydroxyacetone in Tetrahymena pyriformis. Biochemistry 12, 1100-1106.

Friedberg, S. J. and Heifetz, A. (1975) The formation of tritiated O-alkyl lipid from acyldihyroxyacetone phosphate in the presence of tritiated water. Biochemistry 14, 570-574.

Friedberg, S. J., Heifetz, A., and Greene, R. C. (1971) Loss of hydrogen from dihydroxyacetone phosphate during glyceryl ether synthesis. J. Biol. Chem. 246, 5822-5827.

Friedberg, S. J., Heifetz, A., and Greene, R. C. (1972) Studies on the mechanism of O-alkyl lipid synthesis. Biochemistry 11, 297-301.

Furlong, C., Cirakoglu, C., Willis, R. C., and Sastry, P. A. (1973) A simple preparative polyacrylamide disc gel electrophoresis apparatus: Purification of three branched-chain amino acid binding proteins from E. coli. Anal. Biochem. 51, 297-311.

Gilbertson, J. R. and Karnovsky, M. L. (1963) Nonphosphatide fatty acyl esters of alkenyl and alkyl ethers of glycerol. J. Biol. Chem. 238, 893-897.

Goldfine, H. and Hagen, P-O. (1972) Bacterial plasmalogens. in Ether Lipids: Chemistry and Biology (Snyder, F., ed.), pp. 329-350, Academic Press, New York.

Grazi, E., Rowley, P. T., C heng, T., Tcholon, O., and Horecker, B. L. (1962) The mechanism of action of aldolase III: Schiff base formation with lysine. Biochem. Biophys. Res. Commun. 9, 38-43.

Hajra, A. K. (1968) Biosynthesis of acyl dihydroxyacetone phosphate in guinea pig liver mitochondria. J. Biol. Chem. 243, 3458-3465.

Hajra, A. K. (1969) Biosynthesis of alkyl-ether containing lipid from dihydroxyacetone phosphate. Biochem. Biophys. Res. Commun. 37, 486-492.

Hajra, A. K. (1970) Acyl dihydroxyacetone phosphate: precursor of alkyl ethers. Biochem. Biophys. Res. Commun. 39, 1037-1044.

Hajra, A. K. (1973) The role of acyl dihydroxyacetone phosphate in tumor lipid metabolism. in Tumor Lipids: Biochemistry and Metabolism (Wood, R., ed.), pp. 183-199. The American Oil Chemists' Society, Champaign, Illinois.

Hajra, A. K. and Agranoff, B. W. (1967) Acyl dihydroxyacetone phosphate: a rapidly labeled lipid in guinea pig liver mitochondria. J. Biol. Chem. 242, 1074-1078.

Hanahan, D. L. (1965) Mode of attack of hydriodic acid on unsaturated glyceryl ethers. J. Lipid Res. 6, 350-355.

Hanahan, D. L., Ekholm, J., and Jackson, C. M. (1963) Studies on the structure of glyceryl ethers and the glyceryl ether phospholipids of bovine erythrocytes. Biochemistry 2, 630-641.

Hanes, C. S. and Isherwood, F. A. (1949) Separation of the phosphoric esters on the filter paper chromatogram. Nature 164, 1107-1112.

Heilbron, I. M. and Owens, W. M. (1928) Unsaponifiable matter from the oils of elasmobranch fish. IV. Establishment of the structure of selachyl and batyl alcohols as monoglyceryl ethers. J. Chem. Soc. London, 942-947.

Helmy, F. M. and Hack, M. H. (1967) Comparative lipid biochemistry. V. The neutral lipid plasmalogens and other lipids of some specialized sebaceous glands. Comp. Biochem. Physiol. 23, 329-334.

Hixson, S. and Wolfenden, R. (1981) Inhibitors of ether lipid synthesis. Biochem. Biophys. Res. Commun. 101, 1064-1070.

Holloway, P. W. (1973) A simple procedure for removal of Triton X-100 from protein samples. Anal. Biochem. 53, 304-308.

Horrocks, L. A. (1972) Content, composition, and metabolism of mammalian and avian lipids that contain ether groups. in Ether Lipids: Chemistry and Biology (Snyder, F., ed.), pp. 177-272, Academic Press, New York.

Jones, C. L. and Hajra, A. K. (1977) The subcellular distribution of acyl CoA: dihydroxyacetone phosphate acyl transferase in guinea pig liver. Biochem. Biophys. Res. Commun. 76, 1138-1143.

Karnovsky, M. L. and Rapson, W. S. (1946) South African fish products. XXIV. The occurrence of α-glyceryl ethers in the unsaponifiable fractions of natural fats. J. Soc. Chem. Ind., London 65, 425-428.

Kates, M. (1972) Ether-linked lipids in extremely halophilic bacteria. in Ether Lipids: Chemistry and Biology (Snyder, F., ed.), pp. 351-398, Academic Press, New York.

Klenk, E. and Debuch, H. (1954) Zur kenntnis der acetalphosphatide. Hoppe-Seyler's Z. Physiol. Chem. 296, 179-188.

Kossel, A. and Edelbacher, S. (1915) Chemistry of the echinodermata. Hoppe-Seyler's Z. Physiol. Chem. 94, 264-283.

Lee, T-c. and Fitzgerald, V. (1980) Phase transitions of alkyl ether analogs of phosphatidylcholine. Biochim. Biophys. Acta 598, 189-192.

Lee, T-c., Fitzgerald, V., Stephens, N., and Snyder, F. (1980) Activities involved in the metabolism of ether-linked lipids in normal and neoplastic tissues of rat. Biochim. Biophys. Acta 619, 420-423.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265-275.

Malins, D. C. and Varanasi, U. (1972) The ether bond in marine lipids. in Ether Lipids: Chemistry and Biology (F. Snyder, ed.), pp. 297-312, Academic Press, New York.

Mangold, H. K. (1972) The search for alkoxylipids in plants. in Ether Lipids: Chemistry and Biology (F. Snyder, ed.), pp. 399-405, Academic Press, New York.

Mangold, H. K. and Malins, D. D. (1960) Fractionation of fats, oils, and waxes on thin layers of silicic acid. J. Am. Oil Chem. Soc. 37, 383-385.

Marinetti, G. V., Erbland, J., and Stotz, E. (1959) The structure of beef heart plasmalogens. J. Am. Chem. Soc. 81, 861-864.

Mears, G. E. and Feeney, R. E. (1971) Chemical modification of proteins, Holden-Day, Inc., San Francisco.

Metzler, D. (1977) Biochemistry: The Chemical Reactions of Living Cells, p. 711, Academic Press, New York.

Moore, C. and Snyder, F. (1982a) Properties of microsomal acyl coenzyme A reductase in mouse preputial glands. Arch. Biochem. Biophys. 214, 489-499.

Moore, C. and Snyder, F. (1982b) Regulation of acyl coenzyme A reductase by a heat-stable cytosolic protein. Arch. Biochem. Biophys. 214, 500-504.

Paltauf, F. (1972) Plasmalogen biosynthesis in a cell-free system. Enzymic desaturation of 1-O-alkyl (2-acyl) glycerophosphoryl ethanolamine. FEBS Lett. 20, 79-82.

Paltauf, F. and Holasek, A. (1973) Enzymatic synthesis of plasmalogens. Characterization of the 1-O-alkyl-2-acyl-sn-glycero-3-phosphoryl-ethanolamine desaturase from mucosa of hamster small intestine. J. Biol. Chem. 248, 1609-1615.

Piantadosi, C., Chae, K., Ishaq, K. S., and Snyder, F. (1972a) Synthesis of acyl dihydroxyacetone phosphates and related derivatives. J. Pharm. Sci., 61, 971-974.

Piantadosi, C., Ishaq, K. S., Blank, M. L., and Snyder, S. (1972b) Synthesis of 1-O-alkyl-3-O-phosphorylethanolamine-2-propanone and related derivatives. J. Pharm. Sci. 61, 607-610.

Piantadosi, C., Ishaq, K. S., Wykle, R. L., and Snyder, F. (1971) Synthesis and characterization of 1-O-alkyldihydroxyacetone phosphates and derivatives. Biochemistry 10, 1417-1421.

Rapport, M. M. and Frazil, R. E. (1957) The structure of plasmalogens. I. Hydrolysis of phosphatidal choline by lecithinase A. J. Biol. Chem. 225, 851-857.

Rapport, M. M., Lerner, B., Alonzo, N., and Frazil, R. E. (1957) The structure of plasmalogens. II. Crystalline lysophosphatidal ethanolamine. J. Biol. Chem. 225, 859-867.

Rock, C. O. (1977) Harderian gland. in Lipid Metabolism in Mammals (F. Snyder, ed.) Vol. 2, pp. 311-321, Plenum Press, New York.

Rock, C. O., Fitzgerald, V., and Snyder, F. (1977) Activation of alkyldihydroxyacetone phosphate synthase by detergents. Arch. Biochem. Biophys. 181, 172-177.

Rouser, G., Siakotos, A. N. and Fleischer, S. (1966) Quantitative analysis of phospholipids by thin-layer chromatography and phosphorus analysis of spots. Lipids 1, 85-86.

Schlenk, H., Lamp, B. G., and DeHaas, B. W. (1952) Synthesis of derivatives of dihydroxyacetone and glycerides. J. Am. Chem. Soc. 74, 2550-2552.

Scott, C. C., Heckman, C. A., Nettesheim, P., and Snyder, F. (1979a) Metabolism of ether-linked glycerolipids in cultures of normal and neoplastic rat respiratory tract epithelium. Cancer Res. 39, 207-214.

Scott, C. C., Heckman, C. A., and Snyder, F. (1979b) Regulation of ether lipids and their precursors in relation to glycolysis in cultured neoplastic cells. Biochim. Biophys. Acta 575, 215-224.

Snyder, F. and Blank, M. L. (1969) Relationships of chain lengths and double bond locations in O-alkyl, O-alk-1-enyl, acyl, and fatty alcohol moieties in preputial glands of mice. Arch. Biochem. Biophys. 130, 101-110.

Snyder, F., Blank, M. L., and Malone, B. (1970b) Requirement of cytidine derivatives in the biosynthesis of O-alkyl phospholipids. J. Biol. Chem. 245, 4016-4018.

Snyder, F., Blank, M. L., and Wykle, R. L. (1971) The enzymic synthesis of ethanolamine plasmalogens. J. Biol. Chem. 246, 3639-3645.

Snyder, F., Clark, M., and Piantadosi, C. (1973) Biosynthesis of alkyl lipids: Displacement of the acyl moiety of acyldihydroxyacetone phosphate with fatty alcohol analogs. Biochem. Biophys. Res. Commun. 53, 350-356.

Snyder, F., Cress, E. A., and Stephens, N. (1966) An unidentified lipid prevalent in tumors. Lipids 1, 381-386.

Snyder, F. and Malone, B. (1970) Enzymic interconversion of fatty alcohols and fatty acids. Biochem. Biophys. Res. Commun. 41, 1382-1387.

Snyder, F., Malone, B., and Blank, M. L. (1969c) The biosynthesis of alkyl glyceryl ethers by microsomal enzymes of digestive glands and gonads of the starfish, Asterias forbesi. Biochim. Biophys. Acta 187, 302-306.

Snyder, F., Malone, B., and Blank, M. L. (1970a) Enzymic syntheis of O-alkyl bonds in glycerolipids. J. Biol. Chem. 245, 1790-1799.

Snyder, F., Malone, B., and Wykle, R. L. (1969a) The biosynthesis of alkyl ether bonds in lipids by a cell-free system. Biochem. Biophys. Res. Commun. 34, 40-47.

Snyder, F., Rainey, W. T., Jr., Blank, M. L., and Christie, W. H. (1970c) The source of oxygen in the ether bond of glycerolipids: ^{18}O studies. J. Biol. Chem. 245, 5853-5856.

Snyder, F. and Wood, R. (1968) The occurrence and metabolism of alkyl and alk-1-enyl ethers of glycerol in transplantable rat and mouse tumors. Cancer Res. 28, 972-978.

Snyder, F., Wykle, R. L., and Malone, B. (1969b) A new metabolic pathway: Biosynthesis of alkyl ether bonds from glyceraldehyde-3-phosphate and fatty alcohols by microsomal enzymes. Biochem. Biophys. Res. Commun. 34, 315-321.

Tabakoff, B. and Erwin, V. G. (1970) Purification and characterization of a reduced nicotinamide adenine dinucleotide phosphate-linked aldehyde reductase from brain. J. Biol. Chem. 245, 3263-3268.

Thompson, G. A., Jr. (1972a) Ether-linked lipids in molluscs. in Ether Lipids: Chemistry and Biology (F. Snyder, ed.), pp. 313-320, Academic Press, New York.

Thompson, G. A., Jr. (1972b) Ether-linked lipids in protozoa. in Ether Lipids: Chemistry and Biology (F. Snyder, ed.), pp. 321-337, Academic Press, New York.

Thompson, G. A., Jr. and Hanahan, D. J. (1963) The nature and formation of -glyceryl ether lipids in bovine bone marrow. Biochemistry 2, 641-646.

Toyama, Y. (1924) The unsaponifiable constituents (higher alcohols) of shark- and torpedo-liver oils. Chem. Umsch. Geb. Fette, Ocle, Wachse Harze 31, 61-67.

Tsujimoto, M. and Toyama, Y. (1922) The unsapanifiable constituents (higher alcohols) of the liver oils of sharks and rays. Chem. Umsch. Geb. Fette, Oele, Wachse Haraze 29, 27-29, 43-45.

Von Rudloff, E. (1956) Periodate-permanganate oxidations. V. Oxidation of lipides in media containing organic solvents. Can. J. Chem. 34, 1412-1418.

Wood, R. and Snyder, F. (1967) Characterization and identification of glyceryl ether diesters present in tumor cells. J. Lipid Res. 8, 494-500.

Wood, R. and Snyder, F. (1968) Quantitative determination of alk-1-enyl- and alkyl-glyceryl ethers in neutral lipids and phospholipids. Lipids 3, 129-135.

Wykle, R. L., Blank, M. L., Malone, B., and Snyder, F. (1972a) Evidence for a mixed-function oxidase in the biosynthesis of ethanolamine plasmalogens from 1-alkyl-2-acyl-sn-glycero-3-phosphoryl-ethanolamine. J. Biol. Chem. 247, 5442-5447.

Wykle, R. L., Piantadosi, C., and Snyder, F. (1972b) The role of acyldihydroxyacetone phosphate, NADH, and NADPH in the biosynthesis of O-alkyl glycerolipids by microsomal enzymes of Ehrlich ascites tumor. J. Biol. Chem. 247, 2944-2948.

Wykle, R. L. and Snyder, F. (1969) The glycerol source for the biosynthesis of alkyl glyceryl ethers. Biochem. Biophys. Res. Commun. 37, 658-662.

Wykle, R. L. and Snyder, F. (1970) Biosynthesis of an O-alkyl analogue of phosphatidic acid and O-alkylglycerols via O-alkyl ketone intermediates by microsomal enzymes of Ehrlich ascites tumor. J. Biol. Chem. 245, 3047-3058.

Wykle, R. L. and Snyder, F. (1976) Microsomal enzymes involved in the metabolism of ether-linked glycerolipids and their precursors in mammals. in The Enzymes of Biological Membranes, (Martonosi, A., ed.) Vol. 2. pp. 87-117, Plenum Press, New York.

APPENDIX

TABLE I

SUMMARY OF PROTEIN AND ENZYME ACTIVITY DURING DIFFERENT STEPS OF SOLUBILIZATION OF ALKYL-DHAP SYNTHASE FROM EHRLICH ASCITES CELLS

Step	Total Protein (mg)	Total Activity (nmol/min)	Specific Activity (nmol/min mg)	% Yield Protein	% Yield Activity
Microsomal fraction	15.6	0.827	0.052	100	100
Triton X-100 treatment	9.3	0.818	0.088	60	99
Phospholipase C treatment	9.0	0.747	0.083	58	90

TABLE II

SUMMARY OF PURIFICATION OF ALKYL-DHAP SYNTHASE FROM EHRLICH ASCITES CELL MICROSOMES[a]

	Protein		Activity		Specific activity	
Fraction	mg	%	nmol/min	%	nmol/min/mg	Purification
Microsomes	1635	100	351	100	0.22	1
Triton X-100 sup.	803	49	272	77	0.34	1.6
Acetone ppt. sup.	393	24	205	58	0.52	2.4
DEAE-cellulose	79	4.8	125	36	1.30	9.6
QAE-Sephadex	15.6	0.95	121	35	7.8	36
Matrex Red	4.7	0.29	72	21	15.5	72
Hydroxylapatite	2.3	0.14	47	13	20.8	97

[a]Assays were done as described in Materials and Methods section using the DEAE-cellulose disk method. Remaining reaction mixtures were extracted by the method of Bligh and Dyer. Products were verified by (1) comigration with hexadecyl-DHAP on Silica Gel HR layers developed in chloroform: methanol:acetic acid (80:20:10, v/v/v), (2) comigration of phenylhydrazones with hexadecyl-DHAP phenylhydrazone on Silica Gel HR layers developed in chloroform:methanol:acetic acid (80:20:10, v/v/v), and (3) comigration of label after acid methanolysis with hexadecanol on Silica Gel G layers developed in hexane:diethyl ether:acetic acid (60:40:1, v/v/v).

TABLE III

COPURIFICATION OF ACYL AND ALKYL EXCHANGE REACTION ACTIVITIES WITH ALKYL-DHAP SYNTHASE FORWARD REACTION[a]

	% Forward rate	
Purification step	Acyl exchange	Alkyl exchange
Solubilized	20	14
DEAE-cellulose	16	11
QAE-Sephadex	24	9
Matrex Red	17	12
Hydroxylapatite	19	13

[a]Assays were done as described in Materials and Methods. Products were verified as described in Table I except that palmitoyl-DHAP, the phenylhydrazone of palmitoyl-DHAP, and methyl palmitate were used as standards in methods 1), 2), and 3), respectively, when analyzing the acyl-exchange reaction products.

TABLE IV

SUMMARY OF PURIFICATION OF ALKYL-DHAP SYNTHASE[a]

	Protein		Activity		Specific Activity	
Fraction	mg	%	nmol/min	%	nmol/min/mg	Purification
Microsomes	1556	100	104.3	100	.067	1
Solubilized	227	14.6	100.1	96	.441	6.6
DEAE-Cellulose	41.2	2.6	58.4	56	1.42	21
QAE-Sephadex	9.97	0.64	27.5	26.4	2.94	44
Matrex Red	3.08	0.19	11.92	11.4	2.87	58
Hydroxylapatite	0.064	0.004	4.32	4.1	67.1	1001

[a]Protein was assayed by the dye-binding method of Bradford (1976). Alkyl-DHAP synthase assays contained 0.25 M sucrose, 5% ethylene glycol, 0.2% Triton X-100, 50 mM NaF, 50 mM Tris-HCl pH 8.0, 120 μM palmitoyl-DHAP, 50 μM [1-^{14}C]hexadecanol and enzyme in a total volume of 250 μl. After incubation at 37°C for 10 min, 75 μl of the reaction was applied to a DEAE-cellulose paper disk. The disks were washed three times for 15 min in 95% ethanol, dried and counted in organic-based scintillation cocktail.

TABLE V

ACYL-DHAP ACYLHYDROLASE AND ALKYL-DHAP SYNTHASE ACTIVITIES IN PURIFICATION FRACTIONS[a]

		Acylhydrolase		Alkyl-DHAP-Synthase
Fraction	DNFB	[^{32}P]DHAP	[^{3}H]DHAP	[^{14}C]Alkyl-DHAP
		(pmol)	(pmol)	(pmol)
No Enzyme	-	265 ± 8	276 ± 4	15 ± 10
	+	247 ± 1	269 ± 6	13 ± 5
Microsomes	-	1506 ± 95	558 ± 42	72 ± 3
	+	276 ± 13	233 ± 6	12 ± 1
Solubilized	-	1074 ± 1	472 ± 4	319 ± 1
	+	247 ± 9	264 ± 3	8 ± <1
DEAE-Cellulose	-	578 ± 7	587 ± 11	437 ± 7
QAE-Sephadex	-	359 ± 7	300 ± 16	573 ± 2
Matrex Red	-	239 ± 1	272 ± 1	333 ± 1
Hydroxylapatite	-	184 ± 1	188 ± 4	538 ± 66

[a]Details for assay procedures are given in the text. All values for acylhydrolase and alkyl-DHAP synthase activities are expressed as mean ± standard deviation and are based on triplicate determinations.

TABLE VI

FORMATION OF [^{3}H]WATER FROM ACYL-[1-R-^{3}H]DHAP BY ALKYL-DHAP SYNTHASE[a]

Cosubstrate	Enzyme	Total [^{3}H]Water	Enzymatic [^{3}H]Water	Total ^{14}C-Product	Enzymatic ^{14}C-Product
		(nmol)	(nmol)	(nmol)	(nmol)
None	-	1.385 ± 0.074			
	+	1.690 ± 0.090	0.305		
[1-^{14}C]Hexadecanol	-	1.344 ± 0.039		0.005 ± 0.001	
	+	3.490 ± 0.101	2.146	1.884 ± 0.080	1.879
[1-^{14}C]Palmitic acid	-	1.283 ± 0.027		0.003 ± 0.001	
	+	2.044 ± 0.135	0.761	0.461 ± 0.024	0.458

[a]Details of the incubation conditions are presented in the text. All values are based on triplicate determinations and are expressed as mean ± standard deviation.

TABLE VII

ATTEMPT TO TRAP A SCHIFF BASE LINKAGE BETWEEN ALKYL-DHAP SYNTHASE AND ACYL-DHAP BY $NaBH_4$ REDUCTION[a]

Treatment	Activity (nmol/hr)	% Inhibition
None	0.186	—
$NaBH_4$	0.180	0
Acyl-DHAP and $NABH_4$	0.189	0

[a]A solubilized preparation of alkyl-DHAP synthase was diluted to 250 µg/ml. One aliquot was retained as a control; another was treated with $NaBH_4$; and a third aliquot was first saturated with acyl-DHAP (225 nmol/ml) and then treated with $NaBH_4$. A methanolic solution of $NaBH_4$ (40 mg/ml) was prepared and three 25-µl aliquots were added to 1-ml samples of the enzyme (± acyl-DHAP) at 15-min intervals. After the final addition, the samples were kept on ice for 4 hr to allow decay of the $NaBH_4$. All samples were then assayed by the standard procedure.

TABLE VIII

EFFECT OF VARIOUS AMINO ACID MODIFIERS

ON ALKYL-DHAP SYNTHASE ACTIVITY[a]

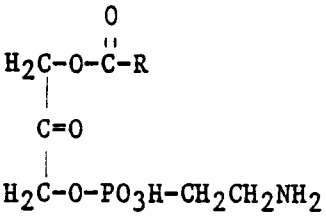
		% Inhibition	
Inhibititor	[I]	Microsomal	Solubilized
DNFB	10 mM	100	100
	1	100	100
	0.1	89	80
TNBS	10 mM	100	100
	1	95	94
	0.1	77	78
Iodoacetate	10 mM	100	100
	1	10	6
	0.1	29	13
NEM	10 mM	100	98
	1	70	58
	0.1	16	44
DTNB	10 mM	100	100
	1	100	98
	0.1	89	91
DFP	10 mM	82	79
	1	49	48
	0.1	25	34

[a]Various amino acid modifying agents were preincubated with either 250 μg of microsomal protein or 250 μg of solubilized protein at 37°C for 10 min. The two substrates were then added and the incubation was continued for 60 min.

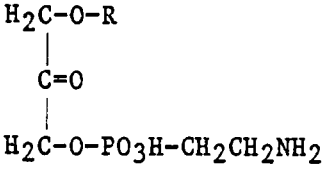
TABLE IX

STRUCTURES OF ANALOGS OF

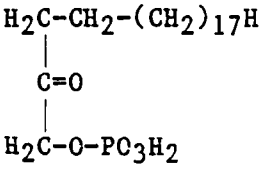
ACYL-DHAP AND ALKYL-DHAP



A. Acyl-DHAP-ethanolamine



B. Alkyl-DHAP-ethanolamine



C. 2-Ketohenicosanol-P

TABLE X

INHIBITION OF ALKYL-DHAP SYNTHASE BY PHOSPHOLIPIDS[a]

Phospholipid	[Phospholipid] (μM)	[Acyl-DHAP] (μM)	% Inhibition
2-Ketohenicosanol-P	30	30	37
	30	120	19
	120	30	37
	120	120	29
	250	30	18
	250	120	23
Phosphatidic acid	120	30	28
	120	120	30
	250	30	45
	250	120	35
Alkylglycero-P	120	30	58
	120	120	45
	250	30	87
	250	120	77

[a]Assays contained 150 μg of Ehrlich ascites cell microsomes, 30 or 120 μM acyl-DHAP, 40 M [^{14}C]hexadecanol and the phospholipid at the specified concentrations in 0.25 M sucrose, 50 mM NaF, and 50 mM Tris-HCl at pH 8.0. Incubations were initiated by addition of enzyme and carried out at 37°C for 15 min. Products were quantitated by the disk assay method (Brown and Snyder, 1982) and % inhibition was based on activities obtained with either 30 or 120 μM acyl-DHAP in the absence of phospholipid.

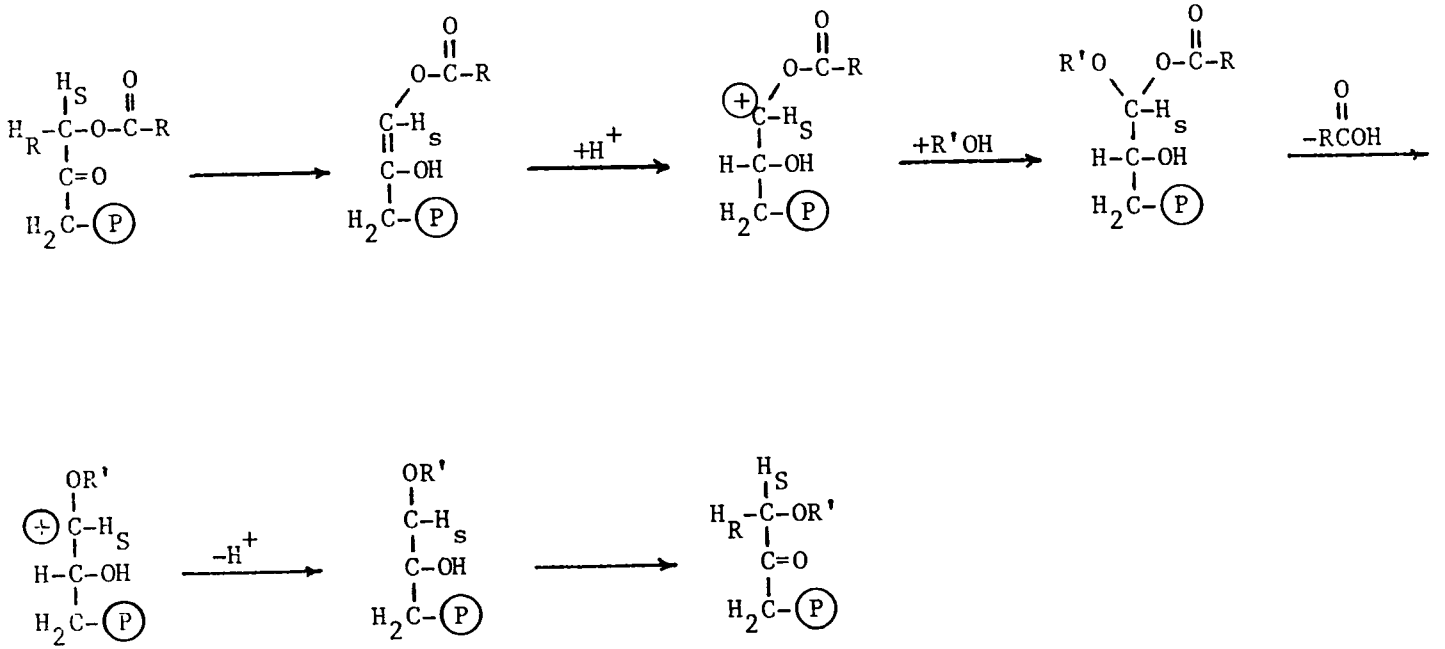


Figure 1. Sequential mechanism for alkyl-DHAP synthase.

When R′=alkyl, product is alkyl-DHAP. When R′=H, product is DHAP (Friedberg et al., 1980).

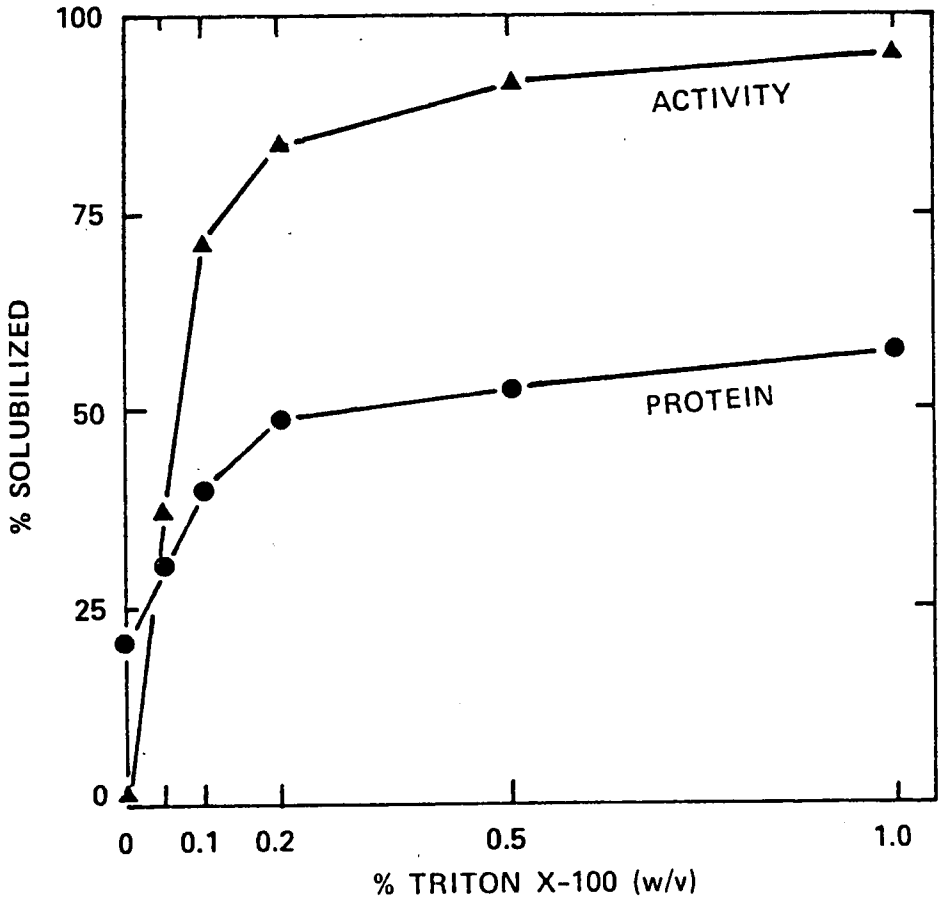


Figure 2. Release of total protein and alkyl-DHAP synthase activity into the supernatant by Triton X-100 treatment.

Triton X-100 treatment of the microsomal fraction of Ehrlich ascites cells was performed as described under Materials and Methods. Symbols represent total protein (●) and alkyl-DHAP synthase activity in the supernatant (▲).

Figure 3. Sepharose 4B chromatography of the Triton X-100/phospholipase C-treated microsomal preparation.

Panel A shows the protein (•——•) and enzyme activity (•- - -•) profiles on Sepharose 4B (1.5 x 24 cm) equilibrated in 0.25 M sucrose, 0.1 M KCl, 0.1 M Tris-HCl at pH 8.2. Panel B shows the enzyme activity profile on the same Sepharose 4B column equilibrated with the above buffer containing 0.5% Triton X-100.

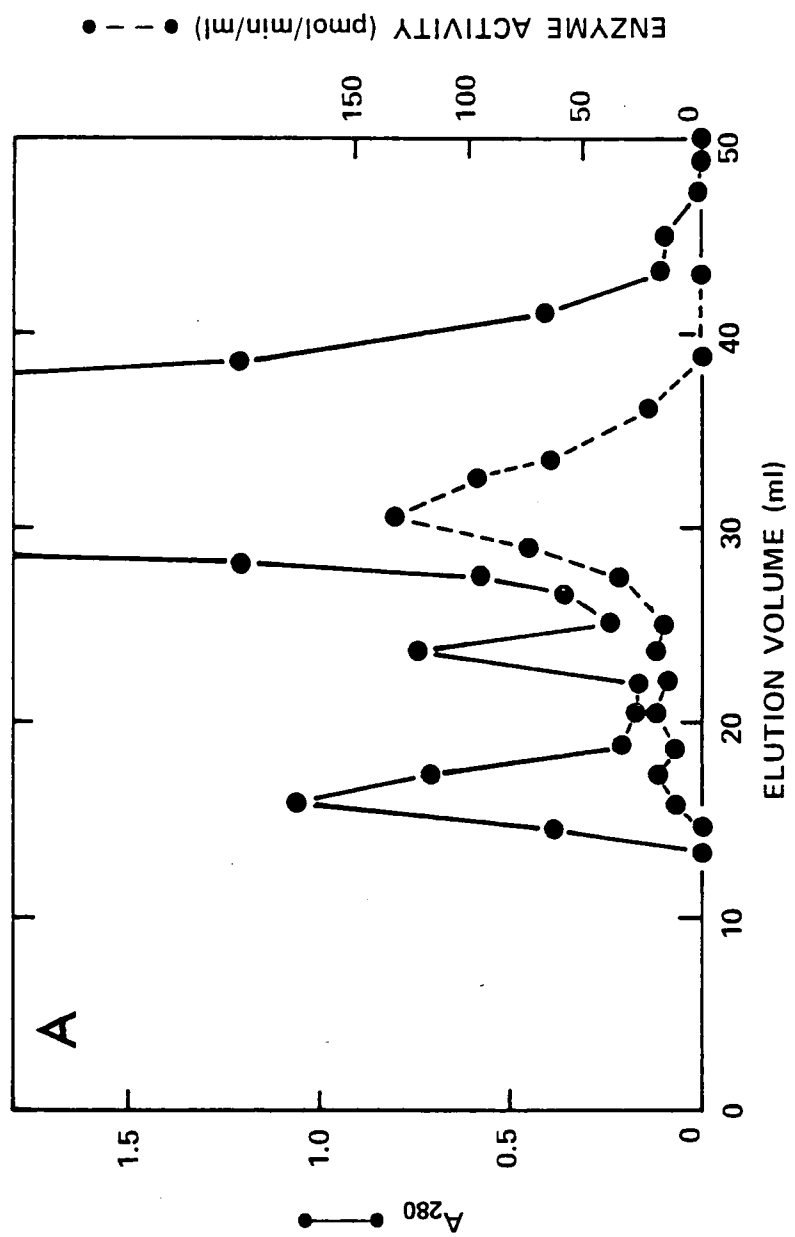

A
A_{280}
ENZYME ACTIVITY (pmol/min/ml)
ELUTION VOLUME (ml)
0
0.5
1.0
1.5
10
20
30
40
50
100
150

Figure 3.

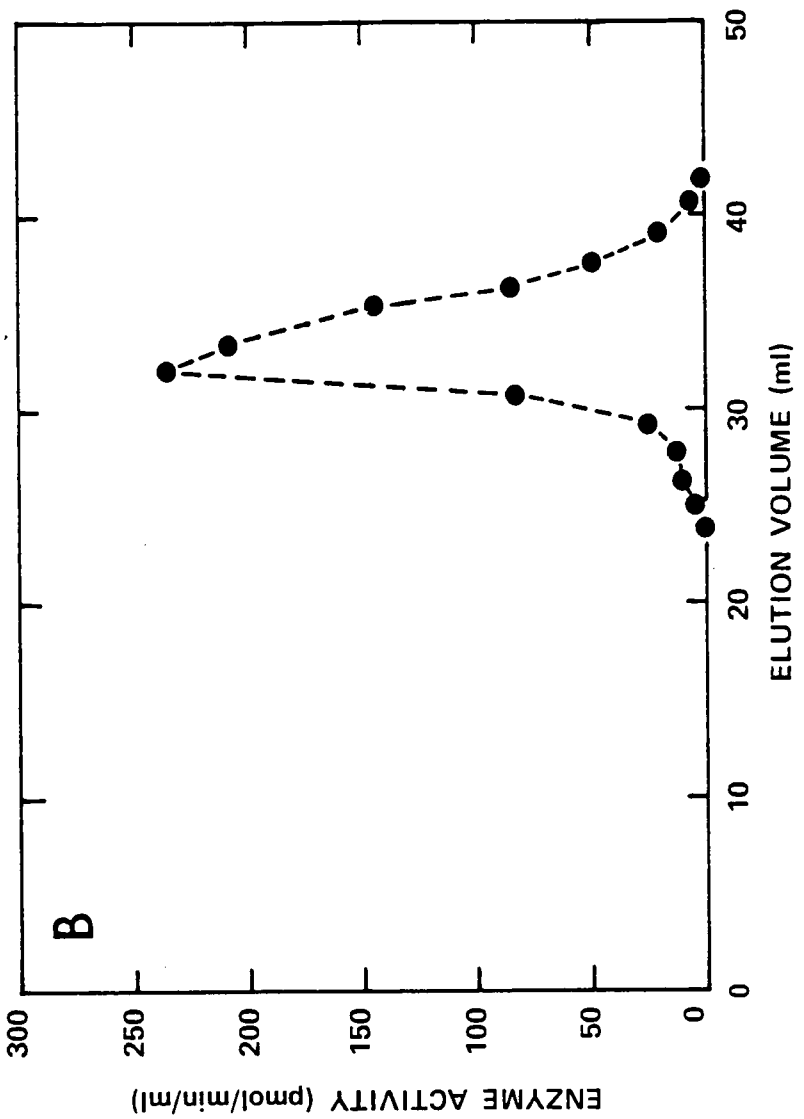


Figure 3 (continued).

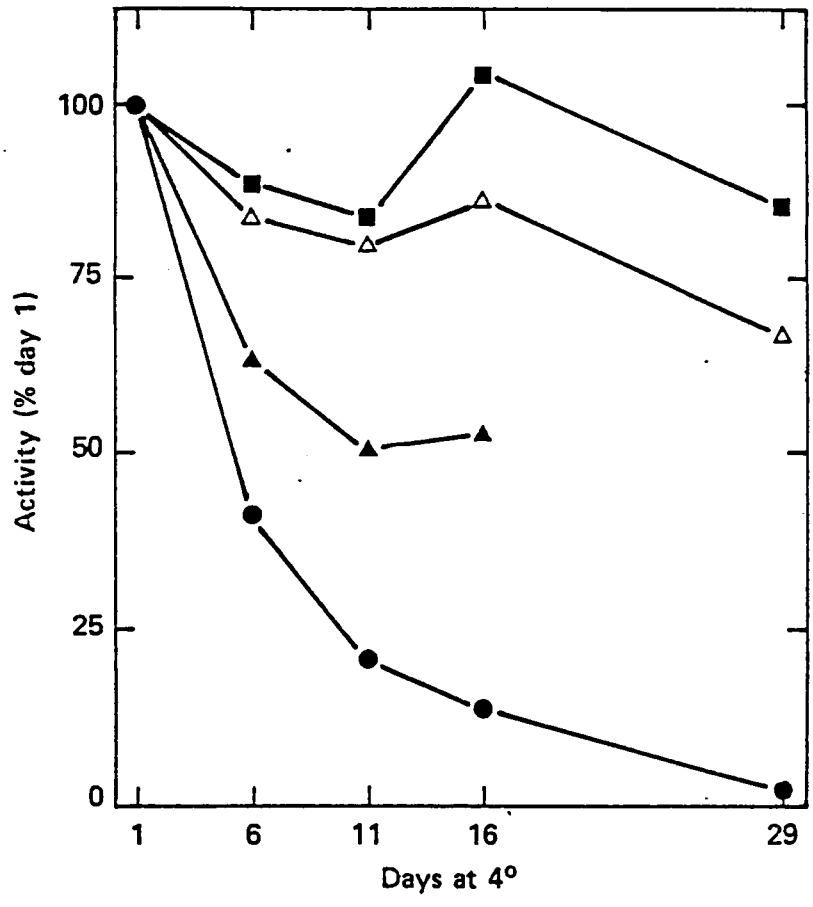


Figure 4. Stabilization of alkyl-DHAP synthase by sucrose, glycerol, and ethylene glycol.

Solubilized enzyme (acetone ppt-sup.) was stored at 4°C at a concentration of 2 mg/ml in 0.1 M KCl, 0.05 Tris-HCl (pH 7.4), 0.5% Triton X-100, and 2 mM dithiothreitol containing either 0.25 M sucrose (●), 20% (w/v) sucrose (▲), 20% (v/v) ethylene glycol (Δ), or 20% v/v glycerol (■). After the indicated number of days, aliquots of 80 μg enzyme were incubated with 0.05 M Tris-HCl (pH 7.4), 50 mM NaF, 120 M palmitoyl-DHAP, and 40 μM [1-^{14}C]hexadecanol (25.5 Ci/mol) in 0.25 ml total volume. Incubations were done at 37°C for 15 min and products were analyzed by the DEAE-cellulose disk method.

Figure 5. Column profiles of protein and alkyl-DHAP synthase activity.

(A) DEAE-cellulose DE 52, (B) QAE-Sephadex A-25, (C) Matrex Red, (D) Hydroxylapatite. Protein (———) was measured using the BioRad protein assay kit. Alkyl-DHAP synthase activity (-----) was measured as described in the Materials and Method section using the DEAE-cellulose disk assay.

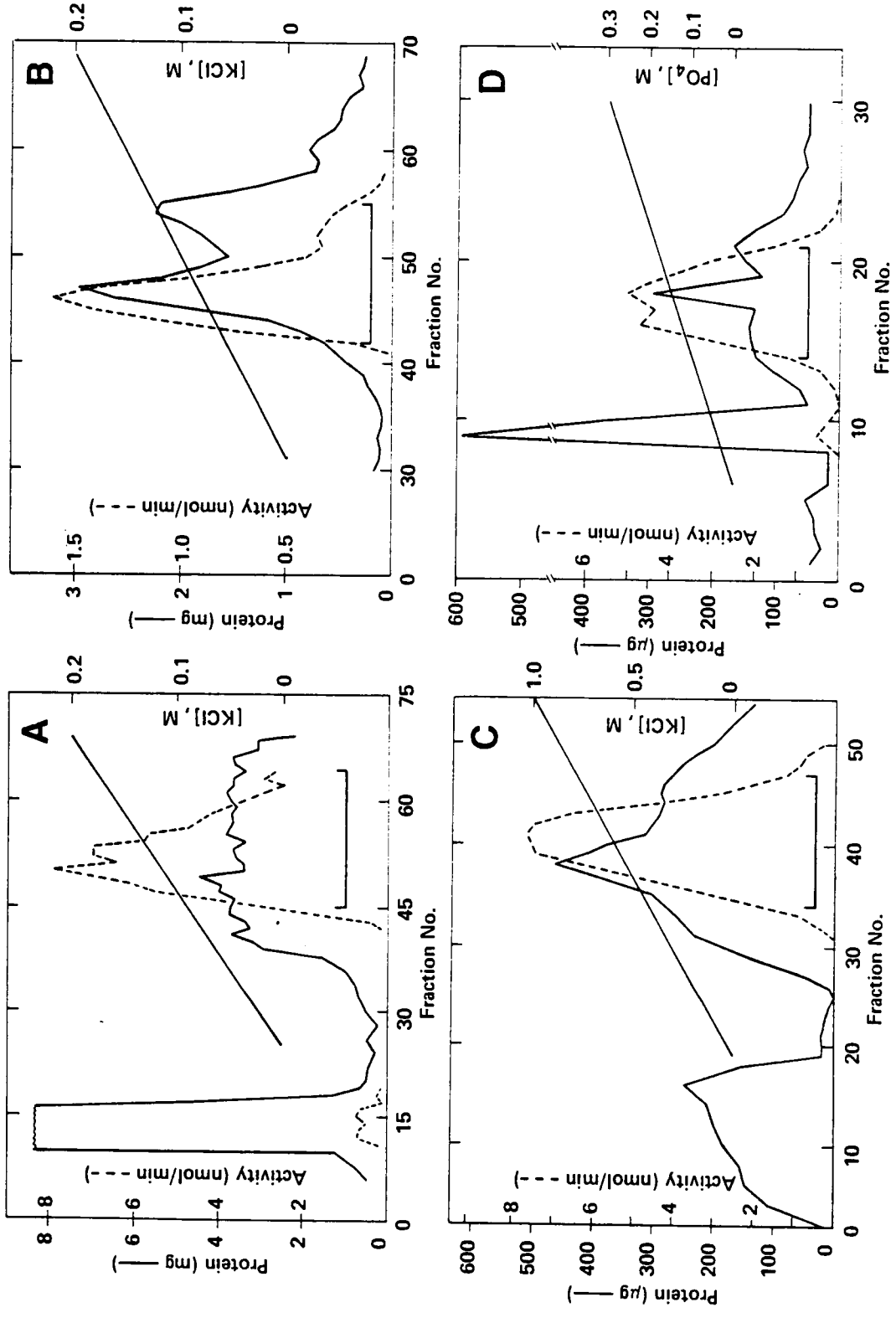

A
B
C
D
Protein (mg ——)
Activity (nmol/min - - -)
[KCl], M
Protein (μg ——)
[PO4], M
Fraction No.

Figure 5

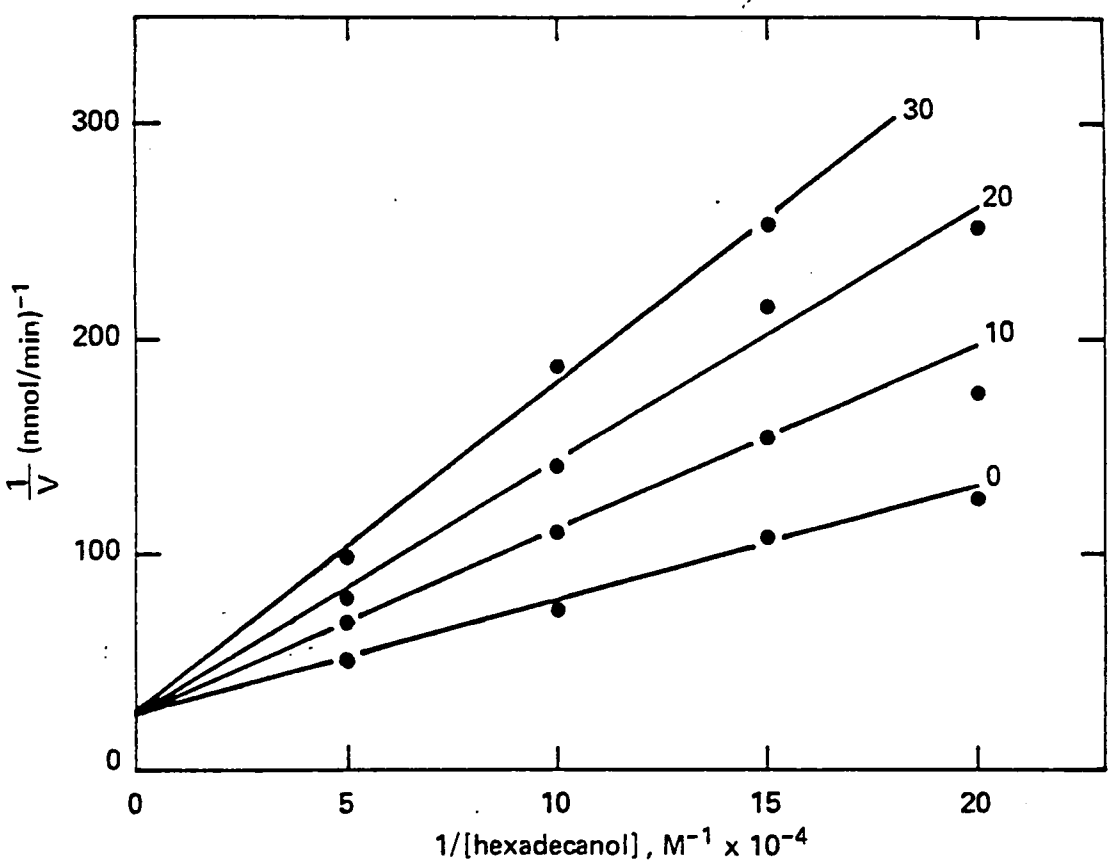


Figure 6. Inhibition of the forward reaction of alkyl-DHAP synthase by fatty acid.

Assays were carried out as described in Materials and Methods section using 120 μM palmitoyl-DHAP. Numbers to the right of each line refer to μM palmitic acid. All data points are averages of triplicate determinations and regression analysis indicated that the data fit the lines shown with correlation coefficients of greater than 0.95.

Figure 7. Initial velocity measurements of alkyl-DHAP synthase reaction.

Assays were done as described in the Materials and Methods section. (A) 1/v vs. 1/[palmitoyl-DHAP]. Numbers to right of line refer to μM hexadecanol. (B) 1/v vs. 1/[hexadecanol]. Numbers to right of line refer to μM palmitoyl-DHAP. All data points are averages of triplicate determinations. Regression analysis of data fitting lines corresponding to 10, 25, 50, and 100 μM palmitoyl-DHAP gave correlation coefficients of 0.82, 0.95, 0.99, and 0.98, respectively.

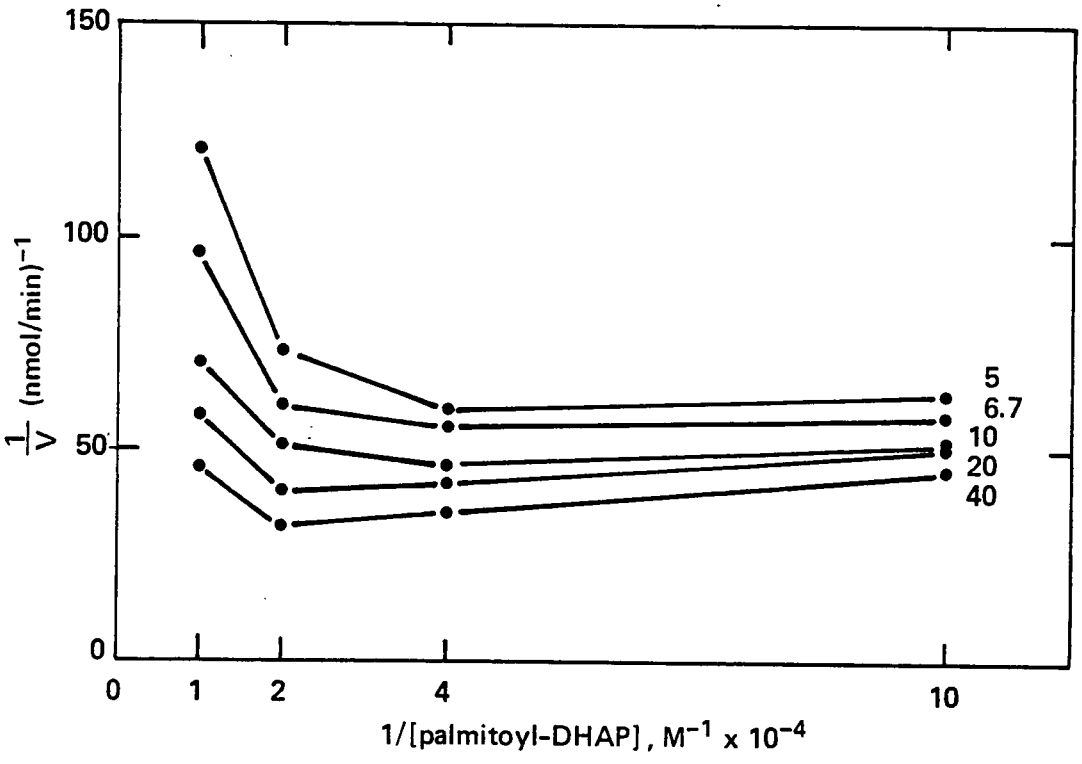

150
100
50
0
$\frac{1}{V}$ (nmol/min)$^{-1}$
0
1
2
4
10
1/[palmitoyl-DHAP], M^{-1} x 10^{-4}
5
6.7
10
20
40

Figure 7.

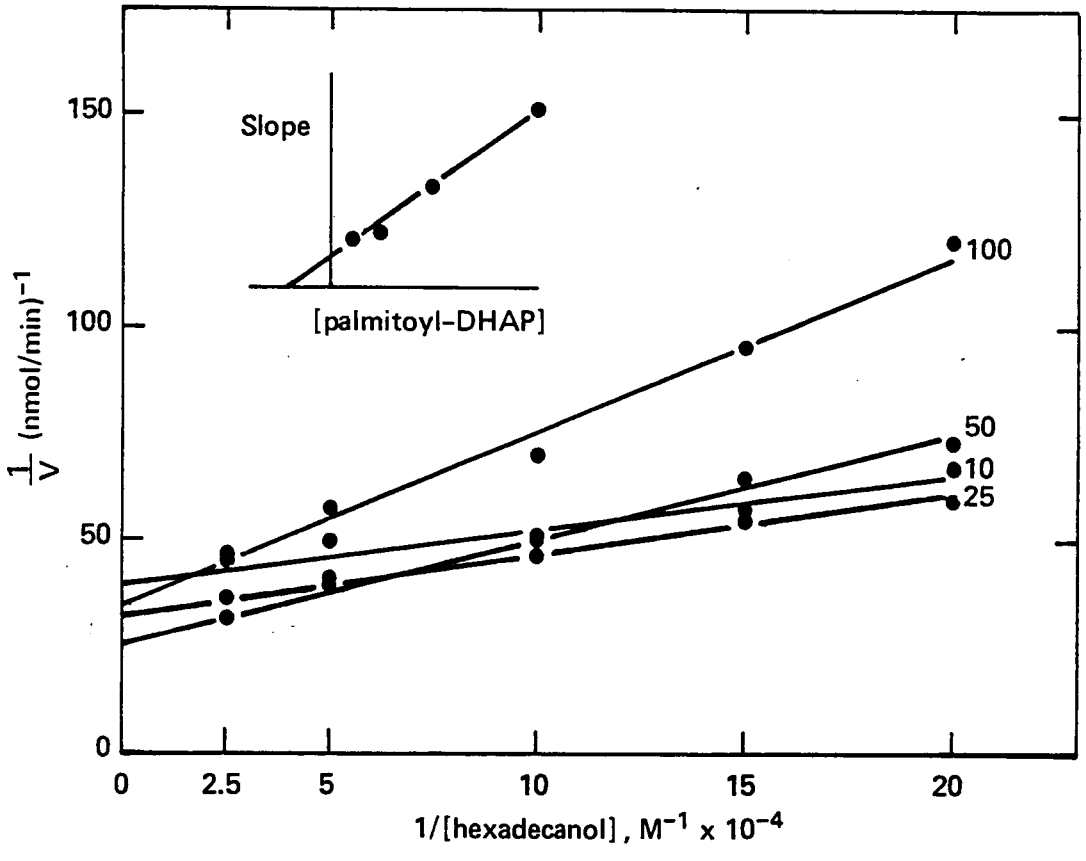


Figure 7 (continued).

Figure 8. Molecular mechanism for alkyl-DHAP synthase reaction.

When R′ is alkyl, the product is alkyl-DHAP. When R′ is H, the product is DHAP. X is a nucleophilic cofactor, possibly an amino acid functional group at the active site (see Discussion). [This scheme has been updated to exclude the possibility of R′=H (see p. 53).]

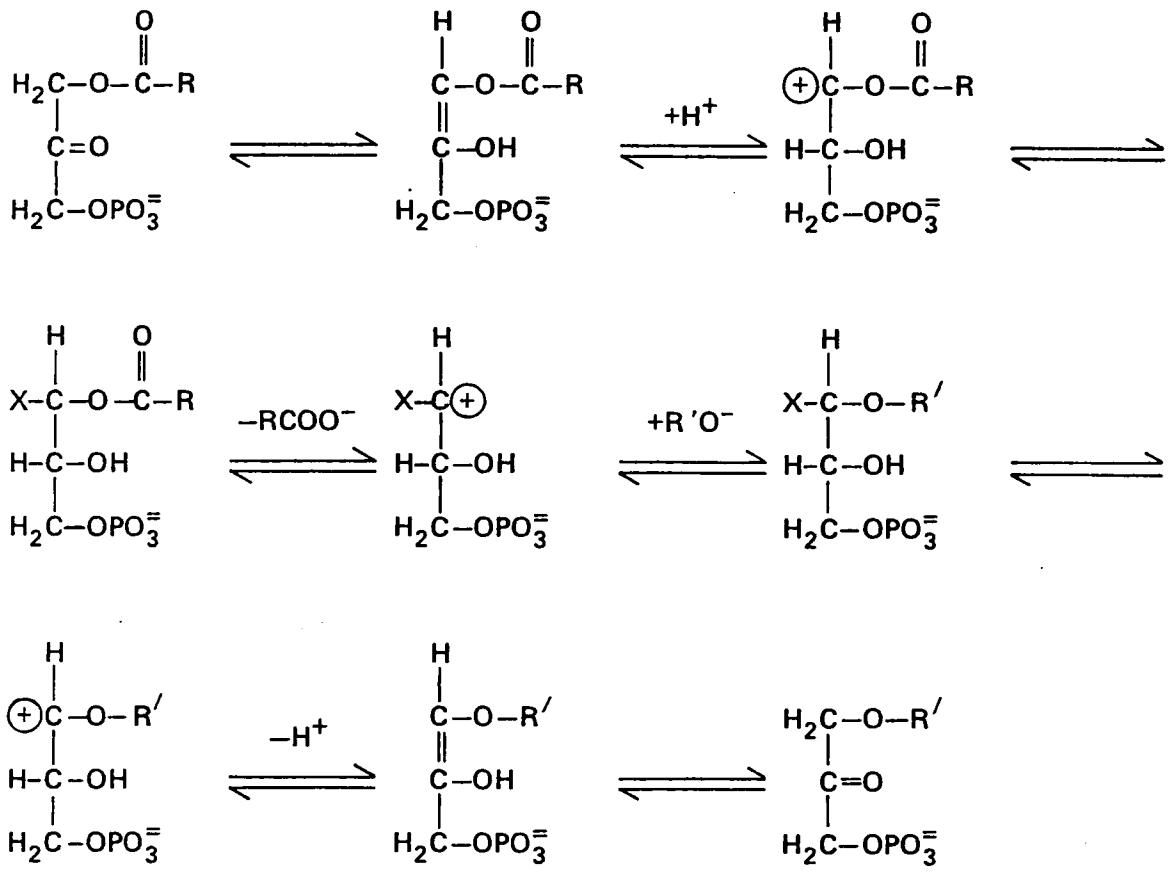


Figure 8.

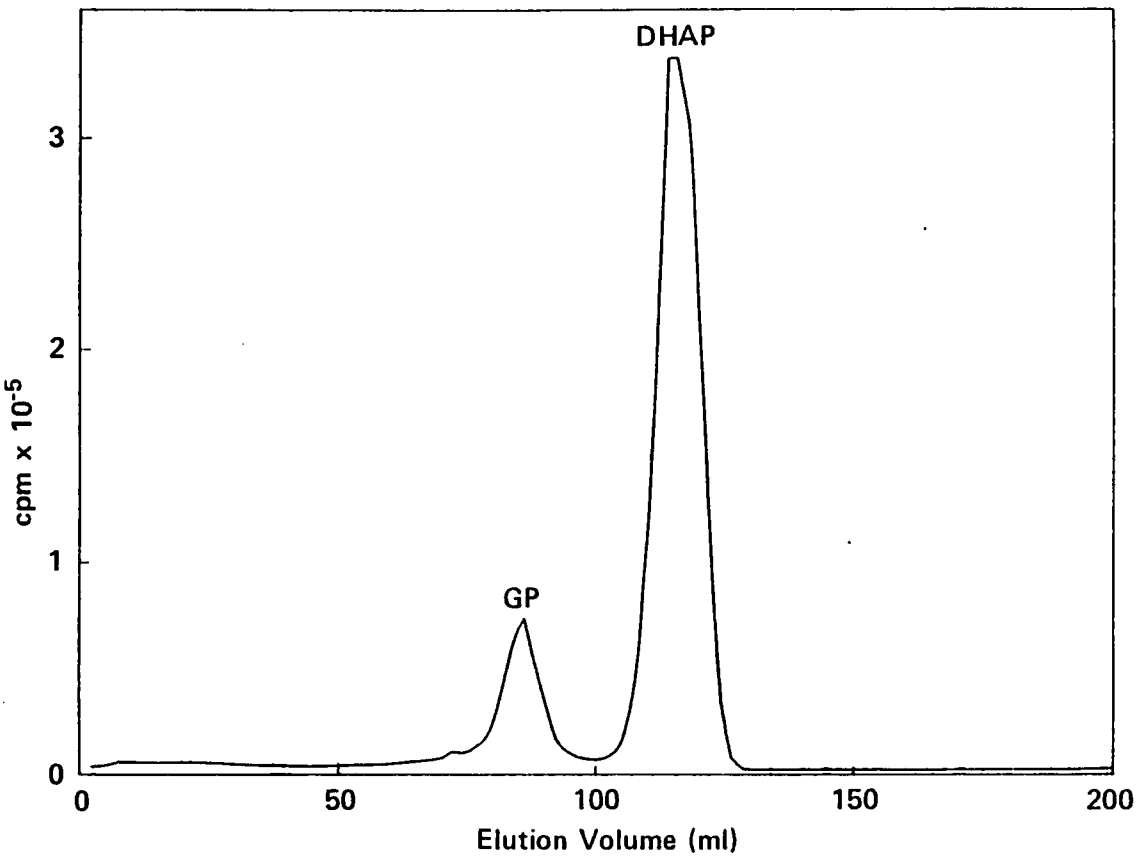


Figure 9. Radioactivity profile for the separation of [^{3}H]glycero-P and [^{3}H]DHAP by Dowex 1 chromatography.

The sample, prepared and applied to a 5-ml Dowex 1 column as described in the text, was eluted with a 200-ml linear gradient of 50 to 500 mM pyridinium acetate (pH 5.0) containing 5 mM boric acid at a rate of 20 ml/hr. One microliter of each 2-ml fraction was counted for ^{3}H. Peaks were were identified by comigration on cellulose thin-layer plates (500 μ layers prepared from 20 μ Sigmacell, Sigma Chemical Co.) developed in a solvent system containing 120 ml t-butanol, 75 ml water, 12 ml glacial acetic acid, and 3 g sodium bisulfite. External standards for glycero-P (GP) and DHAP were visualized using the Hanes and Isherwood (1949) molybdenum blue spray. Radioactivity was detected by zonal scraping (5 mm) and scintillation counting of the gel.

Figure 10. Hydroxylapatite chromatography of alkyl-DHAP synthase.

The active fractions from the Matrex Red column were pooled, dialyzed to 20% ethylene glycol, 0.2% Triton X-100, 10 mM potassium phosphate (pH 7.4) and 1 mM dithiothreitol as described previously (Brown and Snyder, 1982) and then applied to an 8-ml column of hydroxylapatite (BioRad) equilibrated in the above buffer. After washing with 5 bed volumes of the equilibration buffer, a 60-ml gradient of increasing phosphate buffer, 10 mM to 300 mM, containing 20% ethylene glycol, 0.2% Triton X-100, and 1 mM dithiothreitol was applied. One milliliter fractions were collected at a rate of 5 ml/hr and 50 µl of each was assayed as described in Table IV. Protein was determined by the method of Bradford (1976). Fractions of highest specific activity, 36-42, were pooled.

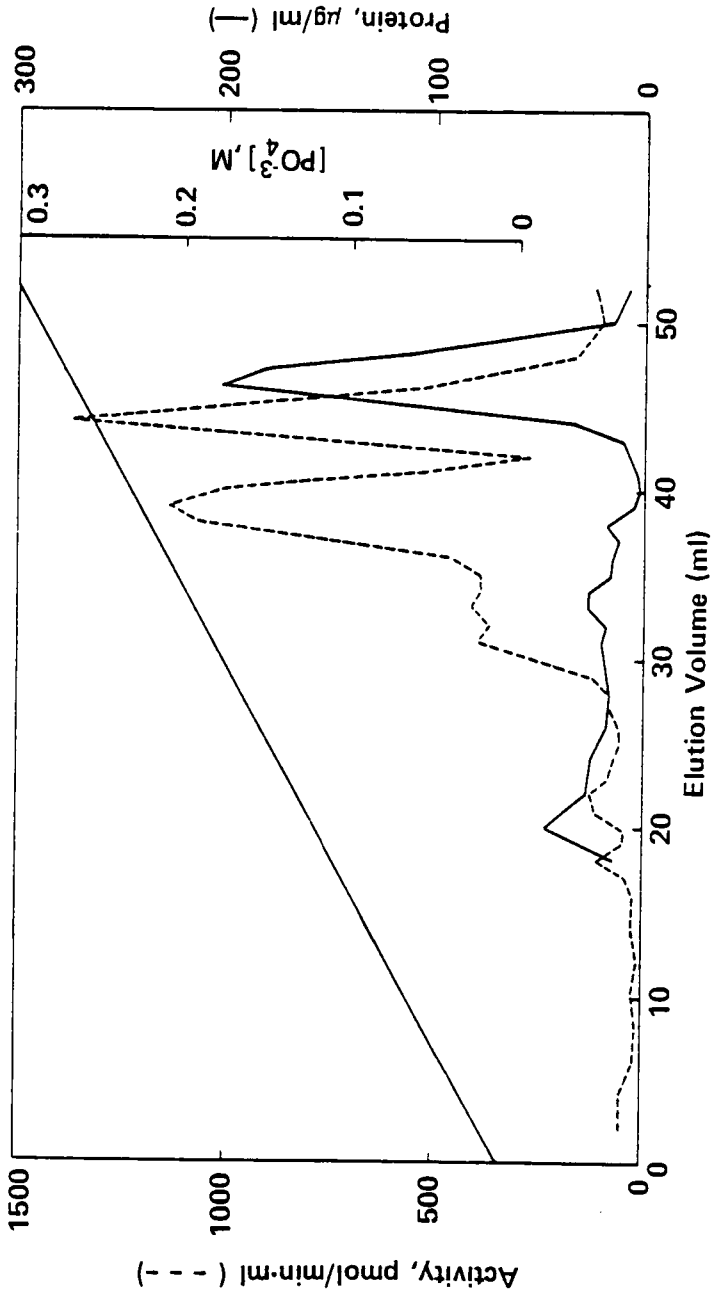

Activity, pmol/min·ml (- - -)
1500
1000
500
0
0
10
20
30
40
50
Elution Volume (ml)
[PO4-3], M
0.3
0.2
0.1
0
Protein, µg/ml (—)
300
200
100
0

Figure 10.

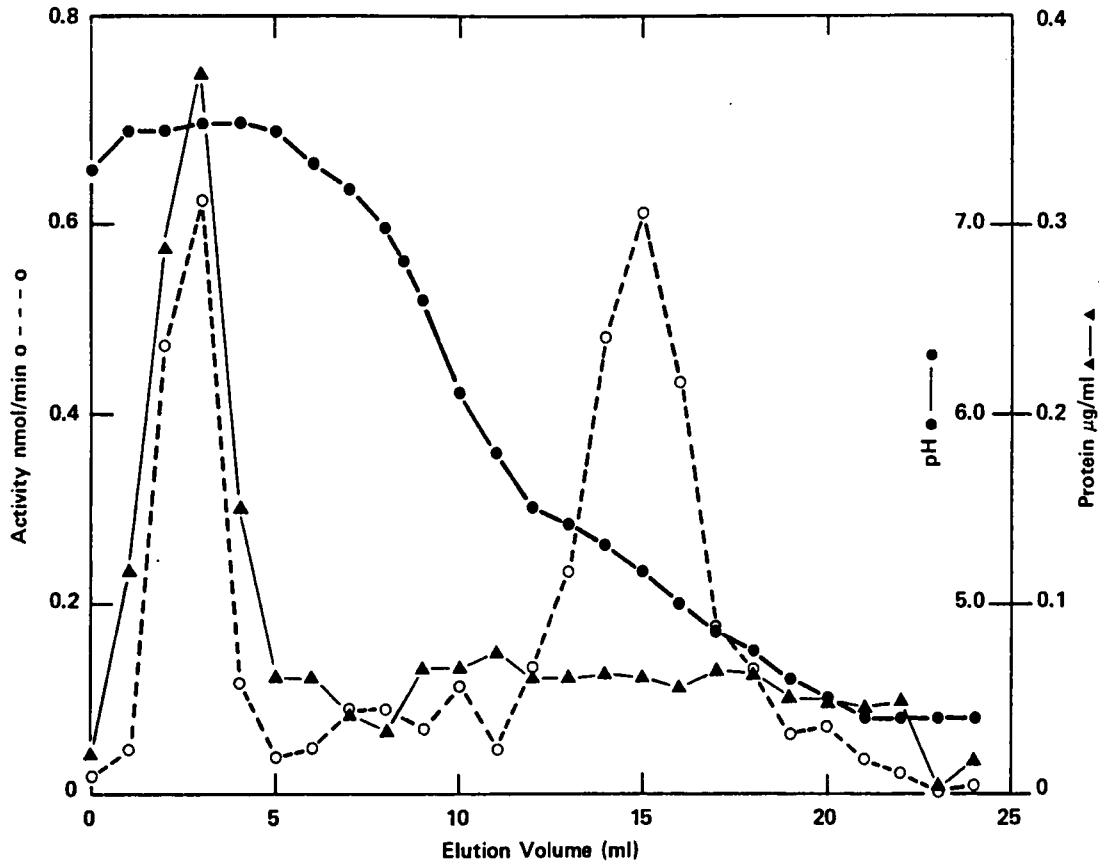


Figure 11. Chromatofocusing of solubilized alkyl-DHAP synthase.

Enzyme (7.3 mg) solubilized by Triton X-100 treatment and acetone precipitation was dialyzed against 20% ethylene glycol, 0.5% Triton X-100, and 25 mM imidazole at pH 7.4 and applied to a 1-ml PBE 94 chromatofocusing column equilibrated in the same buffer. After washing the column with 5 ml of this buffer, alkyl-DHAP synthase was eluted with 32 ml of Polybuffer 74 that had been diluted 8-fold with water and adjusted to pH 4.5. Protein (▲—▲) was measured by the method of Bradford (1976) and enzyme activity (o - - -o) was measured by the disk assay method (Brown and Snyder, 1982). The pH (●—●) of each fraction was measured at room temperature.

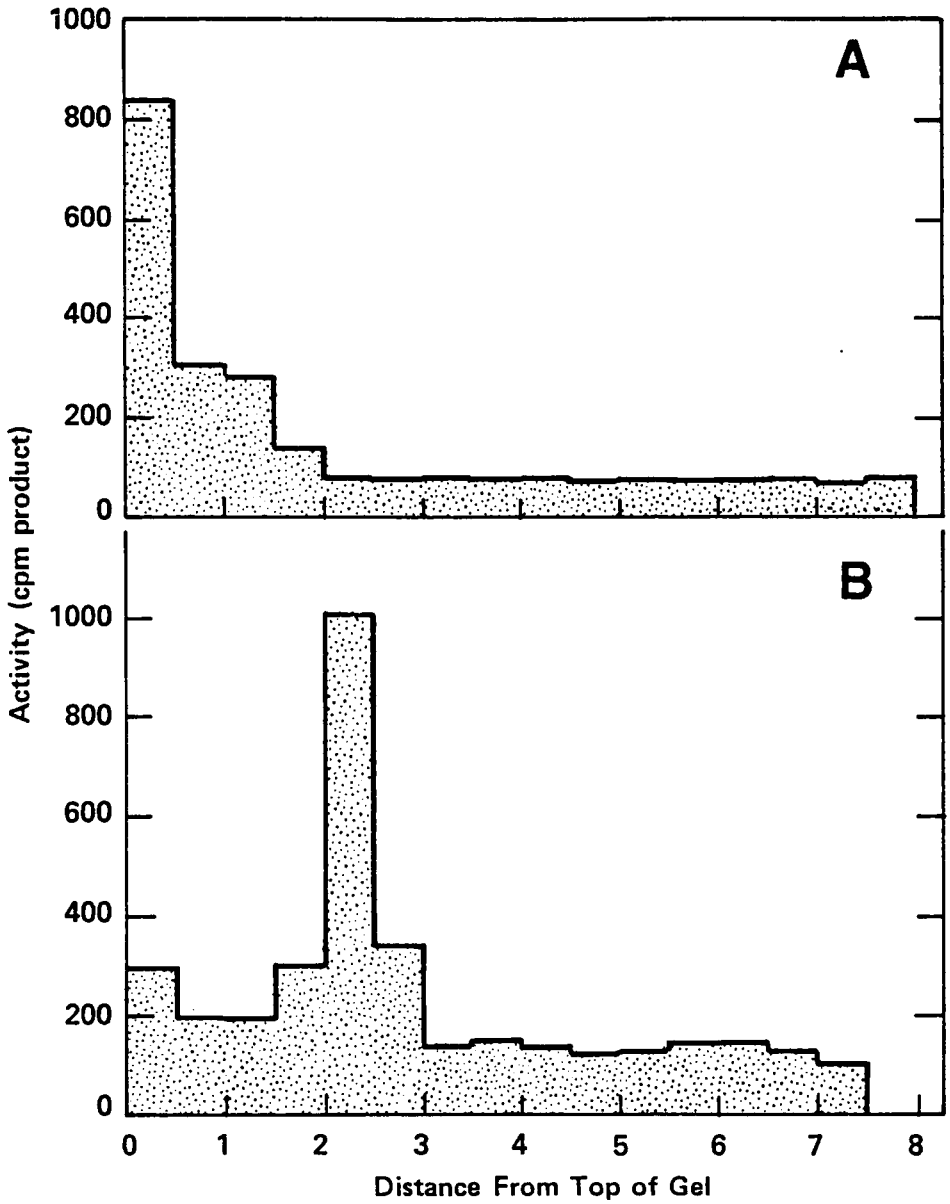


Figure 12. Native gel electrophoresis of solubilized alkyl-DHAP synthase.

Conditions for electrophoresis are described in the text. Alkyl-DHAP synthase activity was measured in 1-cm gel slices by the disk method (Brown and Snyder, 1982) after the slices had been soaked overnight in 1 ml of 0.25 M sucrose, 0.2% Triton X-100, and 50 mM Tris-HCl at pH 8.0.

VITA

Alex Jay Brown, the son of Mr. and Mrs. Edward J. Brown, was born in Kent, Washington on February 8, 1954. He graduated from Kent-Meridian High School in June, 1972; and obtained a Bachelor of Science degree in biochemistry from Washington State University in June, 1976. While at Washington State University, he worked for Dr. Pappachan E. Kolattakudy and completed an additional year of postgraduate study as he finished his research on the cutin-hydrolyzing activity of pancreatic lipase.

In Fall of 1977, he began graduate studies at the University of Tennessee Oak Ridge Graduate School of Biomedical Sciences and from June, 1978, to September, 1982, he worked with Dr. Fred Snyder at the Medical and Health Division, Oak Ridge Associated Universities, on the mechanism of ether bond formation by alkyl-DHAP synthase. During his studies in Oak Ridge, he was supported by the National Cancer Institute Training Grant (CA-09104). The Office of Energy Research, US Department of Energy (Contract DE-AC05-76OR00033) and the National Cancer Institute (CA-11949) provided facilities and other financial support for this project.