Alkyldihydroxyacetone-P (alkyl-DHAP) synthase, the enzyme that forms the ether linkage of alkyl and alk-1-ethyl glycerolipids, has been solubilized from Ehrlich ascites cell microsomes using Triton X-100 and acetone precipitation. The solubilized enzyme, which is stabilized by glycerol or ethylene glycol, was then purified further by chromatography on DEAE-cellulose, QAE-Sephadex, Matrex Red, and hydroxyapatite with the aid of a new rapid assay method using DEAE-cellulose disks. Four enzymes involved in the metabolism of acyl-DHAP and alkyl-DHAP (acyl-DHAP/alkyl-DHAP oxidoreductase, DHAP acyltransferase, alkyl-DHAP phosphohydrolase, and a dinitrofluorobenzene-insensitive acyl-DHAP acylhydrolase) are removed under these conditions along with endogenous fatty acids and fatty alcohols. Two other activities copurify with the alkyl-DHAP synthase forward reaction: an acyl exchange reaction, in which [1-14C]palmitic acid is incorporated into palmitoyl-DHAP, and an alkyl exchange reaction, in which [1-14C]hexadecanol is incorporated into hexadecyl-DHAP. Exchange reactions of this type are characteristic properties of a ping-pong mechanism but not a sequential mechanism. This is confirmed by documentation that palmitic acid is a competitive inhibitor with respect to hexadecanol. In addition, low levels of palmitoyl-DHAP (<100 μM) show competitive inhibition with respect to hexadecanol, possibly due to palmitic acid formed from palmitoyl-DHAP by alkyl-DHAP synthase under these conditions. Based on the observations presented here and previously, a molecular mechanism for alkyl-DHAP synthase is proposed.

Alkyldihydroxyacetone-P 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-ethyl glycerolipids.

Previous studies of the alkyl-DHAP synthase reaction revealed that (a) the oxygen in the ether linkage is derived from the fatty alcohol substrate (3), (b) the pro-R hydrogen of the C-1 carbon of the DHAP portion of acyl-DHAP is exchanged with water during the reaction (4, 5) and that this hydrogen exchange occurs with retention of configuration of the C-1 carbon (6), and (c) there appears to be no Schiff's base formed between the ketone of acyl-DHAP and a functional group of the enzyme during the reaction (7-9). These observations led Friedberg and his co-workers (8, 10) 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 (11) proposed a ping-pong mechanism for alkyl-DHAP synthase in which acyl-DHAP is 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 [1-14C]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 [14C]acyl-DHAP produced was not a product of the acyl-CoA:DHAP acyltransferase, Davis and Hajra (11) 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%) (7) as well as the acyl exchange reaction. 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 (11), we have solubilized and partially purified alkyl-DHAP synthase from Ehrlich ascites cell microsomes.

**EXPERIMENTAL PROCEDURES AND RESULTS**

**DISCUSSION**

The improved solubilization procedure, stabilization by ethylation of the microsomes, and the use of a standard magnifying glass for full-size photocopies are available from the Journal of Biological Chemistry, 9560 Rockville Pike, Bethesda, MD 20814. Request Document No. 81M3151, cite authors, and include a check for $6.00 per set of photocopies. Full-size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

---

*Abbreviations used are: DHAP, dihydroxyacetone-P; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); DNB, dinitrofluorobenzene; QAE, quaternary aminoethyl.*
reaction. Our preliminary results indicate that an acylhydratase, it is possible that the competitive inhibition by palmitoyl-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 Fig. 4, 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 (4, 5); (b) configuration of the C-1 carbon is preserved (6); (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 (8, 11, 19); (e) a Schiff's base intermediate cannot be demonstrated (7-9); and (f) the oxygen in the ether linkage of alkyl-DHAP is donated by the fatty alcohol (3). 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 Fig. 4 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 [18O]palmitoyl-DHAP.

Acknowledgments—We thank Dr. Salil Nyogi for his suggestion of using DEAE-cellulose disks to assay alkyl-DHAP synthase, and Dr. Bruce Jacobson for critically reviewing this manuscript.

REFERENCES
Alkyl-DHAP Synthase: Partial Purification and Mechanism

Alkyl-DHAP Synthase: Partial Purification and Mechanism

EXPERIMENTAL PROCEDURES

ALKYLD-4-ENOLPHTHALATE SYNTHASE: PARTICIPATION IN PARTIAL PURIFICATION, METHYL METHYL-

The alkyl-DHAP synthase reactions contained 150 mm 4-ENOL-PHTHALATE and enzyme in 0.25 ml of 0.25 M succinate and 0.03 M Tris-HCl (pH 8.2) in the presence and absence of 5 mm NADP. After incubation for 30 min at 37°C, the reaction mixtures were extracted as described for the alkyl-DHAP synthase assay (above). The reaction mixtures were then diluted 1:10 in 2% (w/v) NaOH and examined in neutralized chromotograph paper with indium vector, p-nitroaniline, and the labeled material was eluted. Neutralized chromotograph paper with indium vector, p-nitroaniline, and the labeled material was eluted. Neutralized chromotograph paper with indium vector, p-nitroaniline, and the labeled material was eluted.
Alkyl-DHAP Synthase: Partial Purification and Mechanism

The forward, acyl, and exchange activities were measured during purification as described in Experimental Procedures. Table II 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 and exchange activities overlap with the acyl-DHAP synthase forward reaction. Since the relative activities of the two exchange reactions do not decrease during purification, it appears that acyl-DHAP synthase catalyzes these reactions, 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 mechanisms involved. The ping-pong mechanism for acyl-DHAP synthase would predict an "inhibitor" that could react with fatty acids to produce acyl-DHAP or with fatty alcohols to produce aldehydes. Thus, fatty acids would compete with fatty alcohols for this intermediate and plots of V vs. 1/[S] would be linear. While the purified enzyme, plots showing competitive inhibition by palmitate and were obtained (Fig. 2B). Each data point was done in triplicate; regression analysis showed that the data fit the line with correlation coefficients greater than 0.95 and the 1/V intercepts were within the experimental error.

<table>
<thead>
<tr>
<th>Table II: Partial Purification of Acyl- and Exchange Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction</td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>Microsome</td>
</tr>
<tr>
<td>Triton X-100, exp.</td>
</tr>
<tr>
<td>Ammonium gels, exp.</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
</tr>
<tr>
<td>Uroplaxid</td>
</tr>
<tr>
<td>Avins</td>
</tr>
<tr>
<td>Histone-lysozyme</td>
</tr>
</tbody>
</table>

Finally, initial velocities were measured at various substrate levels in order to further confirm the ping-pong mechanism for acyl-DHAP synthase. Some palmitoyl-DHAP has been shown to inhibit the reaction at concentrations greater than 100 μM even at saturating concentrations of hexadecanol (3, 9). We used levels of 100 μM and lower for these measurements. As seen in Fig. 3A, at low hexadecanol concentrations, palmitoyl-DHAP in inhibitory at levels less than 100 μM, whereas this inhibition is least at higher concentrations. Hexadecanol modulates a competitive type of inhibition by palmitoyl-DHAP. This is confirmed by the results in Fig. 3B, where Vmax is plotted against 1/(S-1)-hexadecanol. The slope of the lines corresponds to 10, 50, and 100 μM palmitoyl-DHAP concentration linearly with palmitoyl-DHAP (Fig. 3B, inset). 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 Vmax at 100 μM palmitoyl-DHAP in Fig. 3B reflects the previously noted inhibition by palmitoyl-DHAP at saturating concentrations of hexadecanol.

Fig. 3. Initial velocity measurements of acyl-DHAP synthase reaction. Assays were done as described in Experimental Procedures section. All data points were measured at saturating concentrations of hexadecanol (3, 9). Any data points are averages of triplicate determinations and regression analysis indicated that the data fit the line with correlation coefficients greater than 0.95.