Glyceryl-Ether Monooxygenase [EC 1.14.16.5]. A Microsomal Enzyme of Ether Lipid Metabolism

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Abstract: The history, biological, and medical aspects of glyceryl ethers, as well as their chemical syntheses, biosynthesis, and their chemical and physical properties are briefly reviewed as background information for appreciating the importance of the enzyme glyceryl-ether monooxygenase, and for embarking on new studies of this enzyme. The occurrence, isolation and general properties of the microsomal, membrane-bound, glyceryl-ether monooxygenase from rat liver are described. Radiometric, nonradiometric, and coupled and direct spectrophotometric assays for this enzyme are detailed. The effects of detergents on the kinetics of this enzyme are described together with the stoichiometry and the effects of inhibitors. The structure-activity relationships of pterin cofactors and of ether lipid substrates, including their stereospecificities, have been summarized from enzyme kinetic data which are also tabulated. The mechanism of enzymic hydroxylation of glyceryl ethers and a model for the active site of glyceryl-ether monooxygenase are proposed from these apparent kinetic data. Notes on useful future studies of this monooxygenase have been made.
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Key words: glyceryl-ether monooxygenase; ether lipid substrates; tetrahydropterin cofactors; structure-activity relationships; stereospecificity; inhibition; active site model; medical aspects

1. INTRODUCTION

Glyceryl-ether monooxygenase is a mixed function oxidase which hydroxylates the α-carbon atom of the lipid carbon chain in glyceryl ethers. The enzyme inserts one oxygen atom of an oxygen molecule into the glyceryl ether substrate and the other oxygen atom ends up as a water molecule. The

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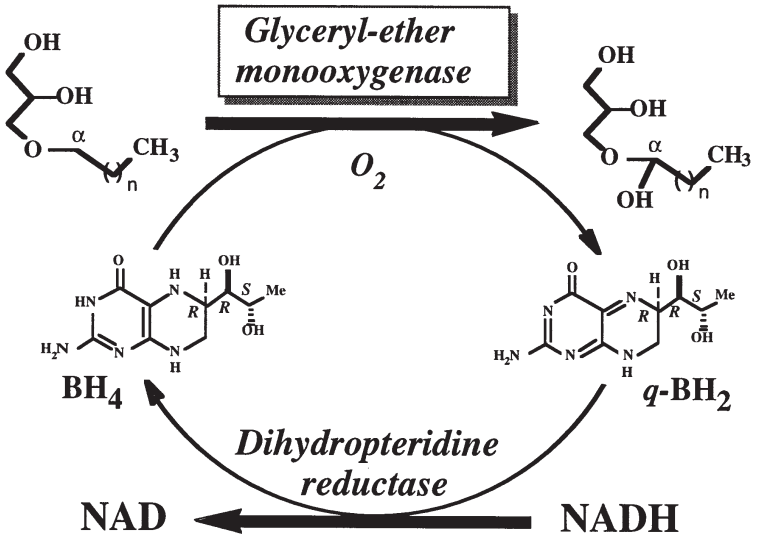
enzymic reaction was first studied by Tietz, Lindberg, and Kennedy[1] in 1964 who showed that it had many features in common with those of phenylalanine hydroxylase which was intensively investigated by Kaufman.[2–5] Glyceryl-ether monooxygenase, like phenylalanine hydroxylase [EC 1.14.16.2],[6] tyrosine hydroxylase [EC 1.14.16.3],[7] and tryptophan hydroxylase [EC 1.14.16.4],[8] has strict requirement for oxygen and a 5,6,7,8-tetrahydropterin as cofactor (Scheme 1). Its Enzyme Commission number is EC 1.14.16.5 and its official name is glyceryl-ether monooxygenase: 1-alkyl-*sn*-glycerol, tetrahydropteridine: oxygen oxidoreductase, and the reaction is given as 1-alkyl-*sn*-glycerol + tetrahydropteridine + O_2 = 1-hydroxyalkyl-*sn*-glycerol + dihydropteridine + H_2O.[9] Other names have been used for this enzyme including glyceryl etherase, glyceryl ether hydroxylase, glyceryl ether oxidase, and *O*-alkyl glycerol monooxygenase, but the name in the title will be used in this review because it is the name recommended by the Nomenclature Committee of the International Union of Biochemistry.[9]

Glyceryl-ether monooxygenase belongs to a class of enzymes in which the protein is membrane-bound and insoluble in aqueous medium like its substrates, the glyceryl ethers. In order to study thoroughly any enzyme, it is imperative that a good assay(s) for enzyme activity is available. For the present enzyme it is therefore important to devise means of "solubilizing" the enzyme and the substrate. An understanding of the physical and chemical properties of the ether substrates as well as their syntheses is necessary for getting further information about the monooxygenase. Consequently a substantial portion of the present review deals also with glyceryl and related ethers which should be useful to readers who are working, or proposing to work, with this enzyme.

A. Brief History of Glyceryl Ethers

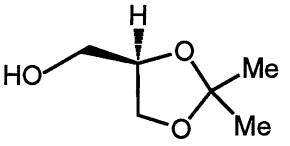
The isolation of a nonsteroidal unsaponifiable partially pure alcohol fraction from the starfish *Asterias rubens* by Dorée[10] in 1909 was the first indication of a naturally occurring glyceryl ether lipid of a long-chain fatty alcohol. A similar alcohol from another starfish *Astrospecten aurantiacus* was obtained in a pure state by Kossel and Edlbacher[11] in 1915. This alcohol was later shown by Bergmann and Stansbury[12] to be batyl alcohol (3-1′-octadecyloxypropane-1,2-diol, 1-*O*-stearyl



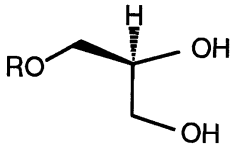
Scheme 1. The glyceryl-ether monooxygenase cycle.

glyceryl ether) by comparison with an authentic sample synthesized by Baer and Fischer[13] in 1941. Tsujimoto and Toyama,[14,15] found during the 1920s, large amounts of 1-glyceryl ethers in the unsaponifiable fractions of liver oils of elasmobranchs which they named after the respective fishes. Batyl alcohol, chimyl alcohol (3-1′-hexadecyloxypropane-1,2-diol, 1-*O*-palmityl glyceryl ether), and selachyl alcohol (3-1′-octadec-9′-enyloxypropane-1,2-diol, 1-*O*-oleyl glyceryl ether) were named after the respective Elasmobranch families of sea rays (Batoidei), ratfish (Chimera) and sharks (Selachoidei). These are the most plentiful ethers, but ethers from as low as C8 alcohols to as high as C24 alcohols have been found in trace amounts. The ethers are mostly from straight chain alcohols which are saturated or with only a low degree of unsaturation with even numbers of carbon atoms, although trace amounts of glyceryl ethers of odd-numbered carbon atoms, e.g., C17, have been found. These alcohols exist mainly as diacyl derivatives of long chain fatty acids.[15–18]

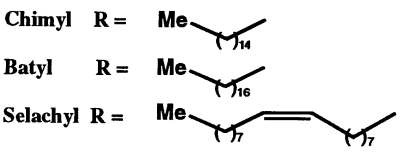
The elucidation of the structures of these alcohols took several years by separate research groups which involved oxidation procedures and finally unequivocal syntheses.[19–25] The experimental difficulties were due to the low melting and waxy nature, and close similarities of the physical properties of these alcohols. The natural alcohols were optically active, but their optical rotations were small and were concentration dependent (see below), which made it difficult for investigators to compare samples.[13,15,23,24] Batyl, chimyl and selachyl alcohols have one chiral center and Baer and Fischer,[13] and later with Rubin,[26] showed that the naturally occurring alcohols belonged to the "D" series by synthesizing them from L(−)-2,3-isopropylidene-*sn*-glycerol whose configuration was known.[27–29]



1 *R* (-)



2 *S* (+)



In modern terms L(−)-2,3-isopropylidene glycerol has the absolute configuration "*R*" at the chiral center, i.e., it is *R*(−)-2,2-dimethyl-1,3-dioxolane-4-methanol **1,** and upon acid hydrolysis of its *O*-alkyl ethers it yields the respective *S*-(+)-1-alkyl glyceryl ethers **2.** The absolute configurations of the above three naturally occurring alcohols **2** are also consistent with optical rotatory dispersion studies.[30,31] On the rational assumption that glyceryl ethers, with a large variety of alkyl chains, from marine animals,[32,33] mammals and birds,[34] plants,[35] protozoa,[36] and bacteria[37,38] are formed by common biosynthetic pathways it is likely that they all have the same stereochemical configurations at C2 of the glycerol moiety. Early accounts on the history of glyceryl ether lipids have been admirably reviewed.[39–42]

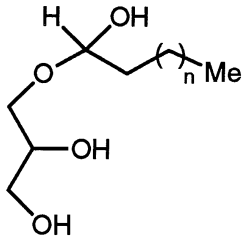
B. Early History of Glyceryl-Ether Monooxygenase

Studies of the metabolic fate of chimyl alcohol were carried out in the late 1950s using chimyl alcohol labeled with ^{14}C at the α carbon atom of the alkyl chain.[43–45] When the labeled alcohol was fed to rats and humans, it was rapidly absorbed and [1-^{14}C]-palmitic acid was excreted. The results

revealed that the glyceryl ether bond was cleaved. As alkyl ether bonds are relatively unreactive and require a strongly protonating medium for cleavage then there must be an enzymic system capable of splitting the ether bond in glyceryl ethers. This argument led to the discovery in 1964 by Tietz, Lindberg, and Kennedy[1] of an oxidase in rat liver which catalyzed the conversion of glyceryl ethers to glycerol and the respective aliphatic acid. This enzyme is glyceryl-ether monooxygenase. Two years later Snyder and Pfleger[46] used singly labeled [1′-^{14}C]-batyl alcohol and doubly labeled [2-^{3}H, 1′-^{14}C]-chimyl alcohol to demonstrate that >94% cleavage of the ether bond occurred within 6 h *in vivo* in rats (intravenous injection) and *in vitro* in rat liver slices. In these experiments the fatty acid products entered the fatty acid pool and were incorporated into triglycerides and phospholipids. It should be noted that the product from the monooxygenase reaction is a hemiacetal **3** (and Scheme 1) which has not yet been identified, but undergoes hydrolysis in aqueous buffer, e.g., during an enzymic assay. However in tissues the hydrolysis may well be enzyme catalyzed. Hydrolysis provides the respective aldehyde which has been identified and is converted to the corresponding acid and alcohol by oxidoreductases present in tissues or in crude homogenates (see Section III-C).

C. Biological Importance of Glyceryl Ethers and the Monooxygenase

Glyceryl ethers are ubiquitous in nature and are present in various animal tissues. They have been found in intestinal fat, cavity fat and liver oil of rat, in the domestic hen (particularly in the egg yolk),[16,47] bone marrow and milk of cattle and man,[48] spleen of pigs,[49] erythrocytes,[50] and in atherosclerotic arteries of man,[51] to name a few tissues. Glyceryl ethers are present in neonatal calf but their concentrations decrease rapidly after birth.[52] Their presence in bone marrow and appearance in mesenchymal tissue[52] suggested that they may play specific roles in haemopoiesis. The feeding of batyl alcohol to normal rats resulted in erythrocytosis because of the production of microcytes, together with reticulocytosis, thrombocytosis, leucocytosis, and hyperplasia of all myeloid tissue similar to the effect produced by the heat-stable erythropoietic factor from plasma.[53] The erythropoietic activity was further confirmed.[54]



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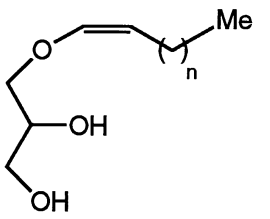

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In most of the above, glyceryl ethers refer to 3-alkoxypropane-1,2-diols, but there is another class of ethers which consist of alk-1-enyl glyceryl ethers (3-alk-1′-enyloxypropane-1,2-diols), and are commonly known as plasmalogens. They occur widely in nature as 2-acylated-phosphoethanolamine or phosphocholine derivatives of the diol **4.** Theoretically the alk-1-enyl ethers **4** can be produced by dehydration of the glycerol-hemiacetal **3,** the product from the glyceryl-ether monooxygenase reaction (see Formulae 3 and 4). At present there is no evidence that this dehydration reaction takes place in nature. However, plasmalogens **4** were known to be formed *in vivo* from 1-alkyl glyceryl ethers in a reaction that was mediated by a desaturase. This enzyme, which may also

be a mixed function oxidase, requires oxygen and NADH or NADPH, with no apparent difference in specificity for these pyridine nucleotides,[55–60] to form a *cis* double bond[61] most probably by removal of the 1′-pro*S* and 2′-pro*S* hydrogen atoms.[57] 1-Alkyl-glycero-3-phosphoethanolamine (2-*lyso*-alkyl-GPE) uses this (or similar) enzyme to produce 1-alk-1′-enyl-glycero-3-phosphoethanolamine; and a desaturase, probably the same enzyme, may also carry out the same reaction with the 2-acyl derivatives under certain circumstances.[60] The configuration of the double bonds of naturally occurring alk-1-enyl ethers isolated from pig heart lecithin were shown to be uniformly *cis*.[61] Hydrolysis of the vinyl ether link in plasmalogens is brought about in rat liver by a microsomal hydrolase (a plasmalogenase) which acts on, e.g., 1-alk-1′-enyl-2-acyl-(and 2-*lyso*) glycero-3-phospho-cholines and 1-alk-1′-enyl-2,3-diacylglycerols in the absence of cofactors to yield the respective fatty aldehydes and 2-acyl-(and 2-*lyso*) glycero-1-phosphocholines and 2,3-diacylglycerols, respectively.[39,62]

Alkyl glyceryl ethers are clearly important components of lipids but much is still to be revealed about their functional role in biology. Two main functions of natural glyceryl ethers appear to emerge however (see below for medical aspects). The first is as membrane components and the second as mediators of cell responses. Alkyl glycerophospholipids are mainly present on the plasma membranes of mammalian cells,[34,63] and as their related acyl analogues they tend to be localized in the inner faces of the membranes.[64] The polar nature and structure of the ether bonds compared with the ester bonds have an effect on the phase-transition temperatures[65] making the ether linked glycerophospholipids more loosely packed in membranes which increases their fluidity.[66] Ether lipid membranes appear to function as reservoirs for storing polyunsaturated fatty acids such as arachidonic acid and precursors of prostaglandins and thromboxanes by forming esters with them, e.g., at C2 of the glycerol moiety. The proximity of a 1-alkoxy group to a vicinal 2-fatty acid acyl group stabilizes the acyl group to hydrolysis, e.g., by phospholipase A_2, causing a large decrease in the rate of ester hydrolysis.[39,41,67]

The discovery of the soluble "fluid phase mediator" called platelet-activating factor (PAF) in the early 1970s by Henson, Sirganian, Osler, Benveniste, and Cochran had opened a new chapter on cell mediator responses.[68,69] Natural PAF is the general name for 1-alkoxy-2-acetylglycero-3-phosphocholines in which the alkoxy groups are mainly C16 and C18 aliphatic chains, i.e., they are derivatives of chimyl and batyl alcohols **2,** respectively. They were synthesized and identified by Hanahan, Benveniste, and Snyder and their respective coworkers during the 1970s, and by others in later years. The chemistry, biochemistry, and physiology of PAF have received considerable attention since the discovery and have been reviewed extensively.[70,71] Briefly, among the plethora of activities of PAF are: platelet aggregation, calcium uptake, protein phosphorylation, increased vascular permeability (it increases cell permeability several thousand orders of magnitude more than antihistamine), causes bronchoconstriction, intestinal necroses, systemic hypertension, hypersensitivity reactions in allergy and inflammation, chemo-taxis and kinesis, and at high concentrations it ultimately causes death.

Alkyl glyceryl ethers are mostly in the conjugated form as *O*-alkyl- monoacyl- or diacyl-glycerols and as monoacyl-glycero- phosphoethanolamines or phosphocholines. A brief survey of selected sources is in Table I.[72–85] The free and conjugated ethers are ultimately removed from circulation by glyceryl-ether monooxygenase, the enzyme that specifically cleaves the ether bond almost irreversibly. Since high levels of glyceryl ethers are toxic to some cells, a property which has been used to advantage in developing anticancer drugs (see below), the monooxygenase plays an important role in regulating the levels of these ethers. In tissues in which the ether levels are low, as in human liver,[16] the monooxygenase levels are high. Some neoplastic tissues were found to have low levels of the monooxygenase resulting in high levels of 1-alkyl-2,3-diacylglycerols.[86] The toxicity of some synthetic 1-alkylglycerophospholipids such as (±)-1-hexadecyl-2-*O*-methyl-glycero-3-phosphocholine towards tumor cells resulted in cell death because of their accumulation in these cells

Table I. Selected Sources of Naturally Occurring *O*-Alkyl Glyceryl Ethers

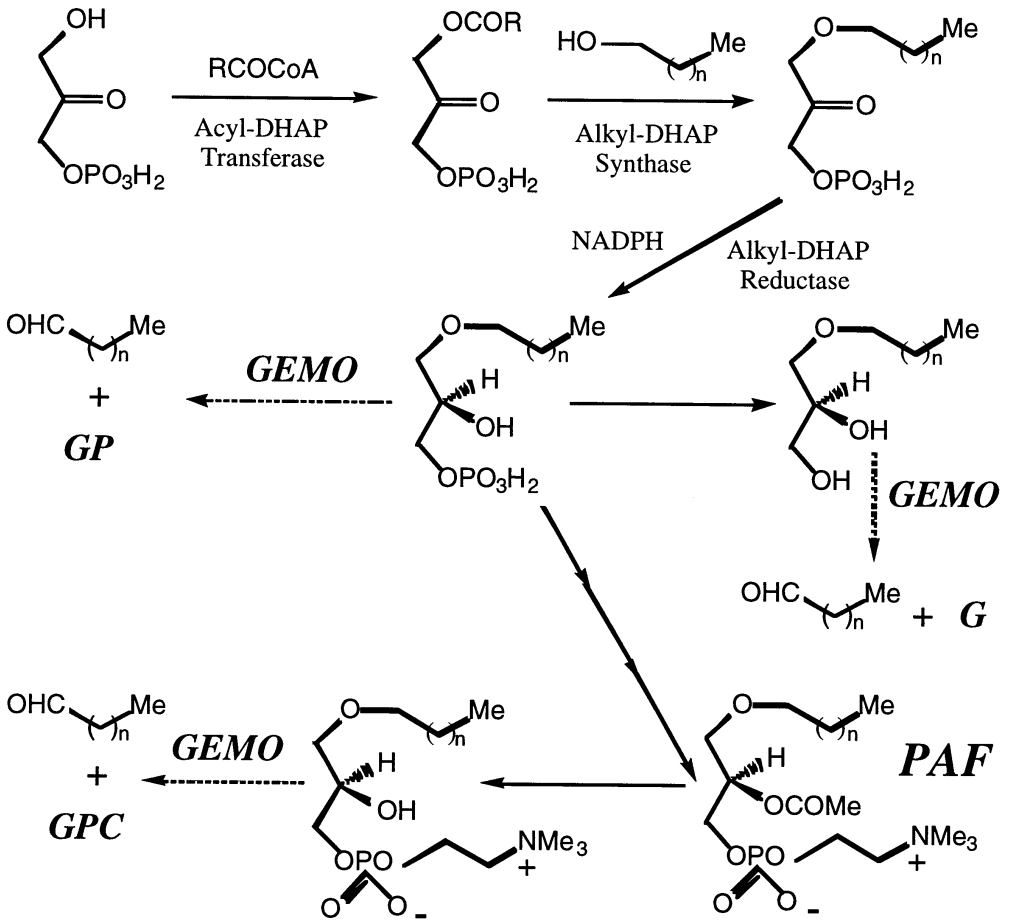
O-*Alkyl Glyceryl Ether*	*Source*	*References*
1-*O*-Alkyl ethers[a]	Human amniotic fluid	72
	Nonsaponifiable fractions from sources below	
1,2-Di-*O*-alkyl ethers and 3-phospho derivatives	Extremely halophilic bacteria	38
1-*O*-Alkyl-monoacyl ethers[a]	Mice preputial gland tumors	73
1-*O*-Alkyl-2,3-diacyl ethers[a]	Intestinal mucosa	74
	Bovine bone marrow	75
	Human adipose tissue	76
	Neoplastic tissue	77
	Marine species	32, 78
1-*O*-Alkyl-2-acyl-glycero-3-phosphoethanolamines	Rat brain	79
	Bovine spinal cord	80
	Bovine erythrocytes	81
	Egg yolk	82
1-*O*-Alkyl-2-acyl-glycero-3-phosphocholines	Ox brain	83
	Human heart	84
	Human serum	85

[a]Free 1-monoalkyl glyceryl ethers and 1-monoalkyl-2(or 3)-monoacyl glyceryl ethers were usually present in small or trace amounts in sources abundant in the 2,3-diacyl glyceryl ethers.

due to the low levels of monooxygenase.[87] The monooxygenase has a relatively wide range of specificity for glyceryl ether substrates (Sec. III-I) and can tolerate quite large changes in substrate structure. A glance at the biosynthesis of glyceryl ethers and PAF (Scheme 2) will show that the enzyme can act in three separate places to irreversibly degrade 1-alkyl-glycerols, 1-alkylglycero-3-phosphates,[88] 1-alkylglycero-3-phosphoethanolamines,[88] and 1-alkylglycero-3-phosphocholines (Sec. III-I).[89–91] This enzyme is thus capable of regulating the levels of a variety of glyceryl ethers either directly or indirectly.

D. Medical Aspects of Glyceryl Ethers and the Monooxygenase

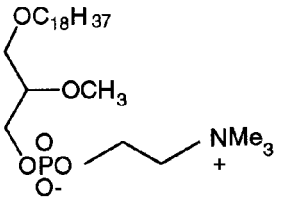
Early indications that alkyl glyceryl ethers had a very important part to play in cell metabolism was revealed by Sandler[92] in 1949 when he showed that batyl alcohol stimulated erythropoiesis in yellow bone marrow extracts of normal and benzene-treated rats. Stimulation of various cells in hematopoiesis was also confirmed and extended by Linman *et al.*[53,93] Brohult[94] investigated the effects of glyceryl ethers on such cells in cancer patients subjected to x-radiation. She demonstrated that these ethers could prevent or even cure leukocytopenia and thrombocytopenia in x-ray treated rats and humans. The alkyl glycerols were administered intraperitoneally. Selachyl alcohol appeared to be the most effective, reaching 61% of the normal unexposed rats (untreated radiated rats had 19% of the counts of normal rats). The effects were best at low doses, e.g., increasing the dose of selachyl alcohol from 0.12 to 0.18 mg per rat/day decreased the megakaryocyte count from 61 to 17% (ca. 50% decrease). When 30 children with leukopenias were treated with alkyl glycerols, the results were good but not clinically and hematologically uniformly successful. The good responses were with children where leuko- and thrombo-cytopenias had already occurred due to previous x-ray treatment. As noted above the dosages had to be regulated for optimum activity because the activity decreased



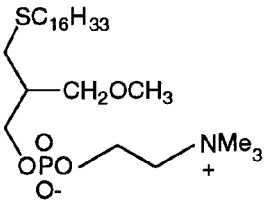
Scheme 2. Biosynthesis of α-alkyl glyceryl ethers and PAF. n = 14, 16. GEMO = glyceryl-ether monooxygenase, G = glycerol, GP = glycerol-1-phosphate, GPC = glycerol-1-phosphocholine, R = fatty alkyl chain (e.g., C16, C18).

at higher doses, and selachyl alcohol appeared to be very active. Bodman and Maisin[52] noted that alkyl glyceryl ethers originated in mesenchymal-type cells (see previous section) and showed that they can be used to initiate the healing of wounds when healing was pathologically inhibited as in nonhealing wounds (e.g., in septic wounds of elderly patients or from radiation injuries; see above). Here again minimal doses were more effective, although large less effective doses given orally to humans showed no long-term toxic effects.[52]

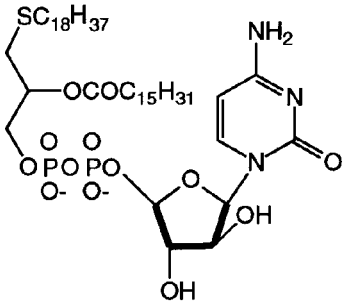
Further development came in the 60s when Fischer[95] found that complement-fixing immune reactions resulted in overproduction of lysolecithin, and with Munder and their collaborators showed that addition of lysolecithin stimulated strongly the phagocytic activity of peritoneal macrophages both *in vitro* and *in vivo*.[96] Munder, Westphal, and their coworkers, using a large variety of synthetic analogues of lysolecithins, demonstrated that several of these lipid compounds selectively destroyed tumor cells.[97–100] It turned out that the more active compounds were derivatives of 1-alkyl glyceryl ethers in which the alkyl group was a nonbranched carbon chain particularly of C16 or C18 length.[101] It is important to note that replacement of the 1-alkyl group by an acyl group resulted in diminished or loss of antineoplastic activity. A whole number of the journal "Lipids" was devoted to



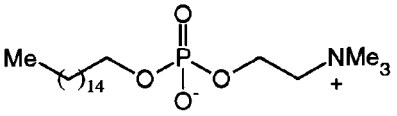
5, Edelfosine,
ET-18-OCH$_3$



6, Ilmofosine,
BM 14.440



7, Cytoros,
ara-CDP-*dl*-PTBA



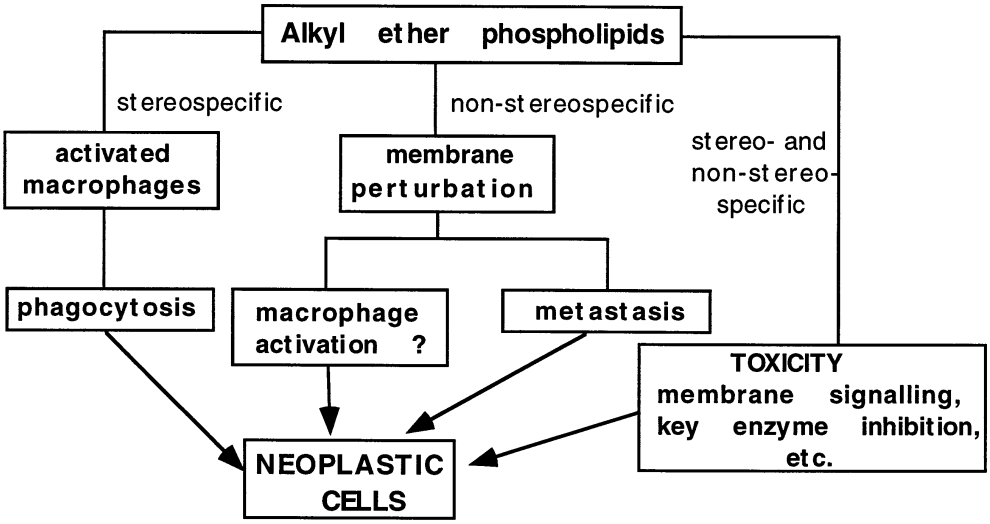
8, Miltefosine

35 articles delivered at the First International Symposium on "Ether Lipids in Oncology" and was coordinated by O. Westphal in 1987.[102]

Further stimulus into this work came from the discovery of platelet activating factor (PAF), and the variety of physiological effects that it produces on cells (see previous section),[69,70,103] which lead to the synthesis of a large number of alkyl glyceryl ether derivatives for oncology evaluation. The antineoplastic activities of several of these compounds tended to be cancer tissue specific, sometimes being more active for one tissue than for another. Some compounds were more attractive because they could be administered orally.[100] Among the more potentially useful compounds which were subjected to clinical trials were *Edelfosine* (**5,** *rac*-1-octadecyloxy-2-methoxypropyl-3-phosphocholine, ET-18-OCH$_3$) and *Ilmofosine* (**6,** *rac*-1-hexadecylthio-2-methoxymethylpropyl-3-phosphocholine, BM 41.440), and there is still more active work on these compounds as shown by their citations in Medline during 1992 to early 1997 (47 citations for compound **5,** and 20 for compound **6**) although these compounds were discovered much earlier. Conjugated thioether derivatives such as *Cytoros* (**7**) have antileukemic activity and inhibit metastasis.[104] The phosphocholine substituent is not always necessary for certain tumors, as for example 3-hexadecyloxy-2-methoxypropan-1-o1 which inhibited tumor growth of Melanoma B16, MCA-sarcoma MCG101, Lewis Lung Tumor, and lymphomas LAA and P1524, as well as inhibiting the spontaneous metastasis of the first two tumors named.[105]

The phospholipid nature of these molecules allows them to dissolve into lipid membranes of animal and tumor cells. Blitterswijk, Hilkmann, and Storme[106] found that ET-18-OCH$_3$ (**5**) was incorporated into various tumor cell membranes to the extent that it made up to 17% of the purified phospholipids of the membranes. This had the effect of altering the fluidity of the membranes and inhibited invasiveness (metastasis) of the cells. However, the mechanism of the antineoplastic effect is complex, and in addition to the cytotoxic nature of some of these compounds (*via* inhibition of some key enzymes), increased permeability, macrophage activation and their effect on membrane fluidity, they may also act by inhibiting transmembrane signaling *via* inactivating protein kinase C.[107] Vogler *et al.*[108] have demonstrated with these alkyl *lyso*-phospholipids that it was possible to selectively destroy leukemic cells from remission marrows for autologous bone marrow transplant, a very important application for cancer therapy.

In the advances made by F. Snyder and his coworkers on the antineoplastic nature of alkyl glyc-



Scheme 3. General outline for the mode of action of antineoplastic alkyl ether phospholipids.

erol phospholipids and the glyceryl-ether monooxygenase, they have observed that glyceryl ether diesters and alkyl acyl glycerophosphocholine lipids accumulated in the cells, e.g., as in the relatively fast-growing tumor hepatoma 7777 in which also unsaturated C18 and C20 fatty acids were poorly metabolized.[109] They demonstrated that the levels of the ether cleaving monooxygenase in their neoplastic cells were very low or almost absent.[86,87] One of the possible explanations that they gave for the selective antineoplastic action was the inability of tumor cells to metabolize the ether linkage due to the low activity of the monooxygenase.[110] This was supported by the fact that tumor cells with the lowest monooxygenase activity were more sensitive to the toxic properties of 1-alkoxy-2-methoxypropane-3-phosphocholines than cells with higher monooxygenase activity.[111] Thus toxic ethers are not metabolized extensively and can produce the desired toxic effects. Eibl and coworkers[112] studied the structure-activity relationships of these anticancer lipids and came up with a new class of anticancer phospholipids, the long unbranched-chain alkylphosphocholines of which hexadecyl-phosphocholine (**8,** *Miltefosine*) was the most promising.[113] It is still under active investigation and testing (77 citations in Medline during 1992–early 1997). The mode of action of these antineoplastic ether lipid substances has been depicted in generalized form by Kudo *et al.*[114] and is shown diagrammatically in Scheme 3.

Interest in alkylthio glyceryl ethers as possible physiologically active agents was proposed decades[115] before the discovery of the thioglycerol ether anticancer lipids related to *Ilmofosine* (**6**). Further interest in the syntheses of thioglycerol ethers was not surprising, as it is experimentally generally easier to make a thioether than an oxygen ether bond.[116] For comparing the metabolism of S-ethers with those of the naturally occurring O-ethers, Snyder *et al.*,[117] (who were interested in the nature of the biocleavage of the ether bond) fed rats with [1′-^{14}C]-3-hexadecylthiopropane-1,2-diol (thiochimyl alcohol) and [1′-^{14}C]-3-octadecylthiopropane-1,2-diol (thiobatyl alcohol), and found that, unlike the O-ether analogues, only very small amounts of ^{14}C label were found in the various tissues. Most of the radioactive thioethers appeared in the urine, feces, and intestinal contents, and were chemically unchanged. However, because a small amount was metabolized, Ferrell and Radloff[118] looked for their presence in nature. Indeed, they found that an extract from bovine heart contained a mixture of alkylthio-glyceryl ethers. They separated the components of the mixture by thin layer and gas chromatography and identified, by mass spectrometry, the following S-glyceryl

ethers (relative percentages are in brackets): C13 (1.4%), C14 (3.4%), unknown (4.0%), C15 (1.7%), C16 (49.2%), C16 with one double bond (4.5%), C18 (27.8%) and C18 with one double bond (8.1%).

The metabolism of glyceryl ethers involving the monooxygenase is affected (modulated) in three inborn errors of metabolism in humans. These are the autosomal recessive diseases of two variants of phenylketonuria (PKU) and of the cerebro-hepato-renal syndrome of Zellweger.

In classical PKU, children are born with a deficiency in phenylalanine hydroxylase which results in neurological disorders and mental retardation.[119] The enzyme phenylalanine hydroxylase converts phenylalanine to tyrosine in the presence of oxygen and the cofactor 6-*R*-5,6,7,8-tetrahydro-L-*erythro*-biopterin (BH_4). In this process the latter is oxidized to *quinonoid*-6-*R*-7,8-dihydro(6*H*)-L-*erythro*-biopterin (*q*-BH_2). The oxidized cofactor is then reduced by the enzyme dihydropteridine reductase (DHPR), in the presence of NADH, to tetrahydrobiopterin (BH_4) and thus recycling the cofactor. This cycle should be compared with that in Scheme 1, in which the monooxygenase is replaced by phenylalanine hydroxylase and the glyceryl ether substrate by phenylalanine. The two variant diseases of PKU mentioned above (some people prefer to classify them as separate diseases, albeit their close relationship) are dihydropteridine reductase deficiency,[120] and deficiencies in the biosynthesis of the cofactor BH_4[121] (which is biosynthesized in microorganisms and in humans) where both these deficiencies can be lethal in severe cases. Glyceryl-ether monooxygenase uses the same cofactor (Sec. III-H) and the same recycling enzyme DHPR as in PKU. Consequently children with these two variants of the disease have seriously impaired glyceryl-ether monooxygenase function and should in fact have high levels of glyceryl ethers in their livers. The general glyceryl ether levels in these children should vary depending on the severity of the lesions.

The lethal human familial syndrome of Zellweger was shown by Goldfischer *et al.*[122] to be characterized by the absence of peroxisomes (microbodies) in the cells.[123] Peroxisomes are ubiquitous cell organelles which contain catalase and also a variety of enzymes of lipid metabolism as well as the enzymes for the biosynthesis of glyceryl ethers (see Scheme 2). Hajra *et al.*[124] showed that the levels of dihydroxyacetone phosphate (DHAP) acyltransferase, the first step in Scheme 2, are reduced at least 20-fold in skin fibroblasts from Zellweger patients compared with normal fibroblasts. By adding [1-^{14}C]-hexadecanol and [9′,10′-$^{3}H_2$]-3-octadecyloxypropan-1,2-diol (labeled batyl alcohol) in the medium for growing skin fibroblasts, van den Bosch *et al.*[125] showed that ^{14}C label was not incorporated into plasmalogens (alk-1′-enyl glyceryl ethers) or alkyl phospholipids. The labeled hexadecanol was oxidized to fatty acid(s) and the incorporation of label into phospholipids was the same in Zellweger and control fibroblasts. The labeled batyl alcohol was incorporated into plasmalogens equally efficiently in Zellweger fibroblasts as in controls. This confirmed the earlier report[124] that only the reaction(s) involving the formation of the ether bond is deficient in the syndrome. The essential role of peroxisomes in glyceryl ether biosynthesis was demonstrated in CHO mutant cells which were defective in peroxisome biosynthesis and where the deficiency was corrected by supplementing the growth medium with alkyl glycerols. This also demonstrated that glycerol ether biosynthesis does not take place to any appreciable extent elsewhere in cells other than in peroxisomes.[126] Clearly, glyceryl-ether monooxygenase is not defective in the Zellweger syndrome, but as it affects at least three substrates in glyceryl ether phospholipid metabolism (Scheme 2), it may well have some modulating effect on the levels of the lipids involved.

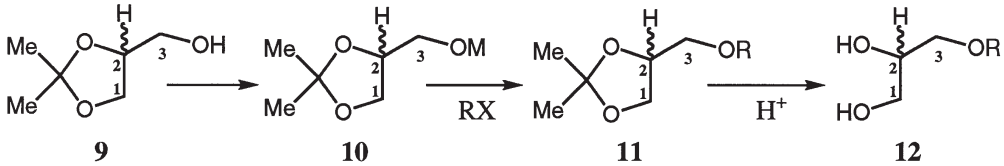
2. GLYCERYL ETHERS

Racemic chimyl and batyl alcohols are available commercially but their chiral enantiomers and their isotopically labeled (radio- and nonradioactive) analogues are not readily available and need to be synthesized. Other related alkyl ethers for structure activity, inhibition, and kinetic evaluation would have to be prepared specifically for a particular purpose. Consequently a knowledge of the general

syntheses, as well as the chemical and physical properties should be very helpful for those intending to work with glyceryl-ether monooxygenase. These are briefly described below only in so far as they could be useful for studies of the monooxygenase.

A. Syntheses

α-Alkyl glycerols, 1-*O*-alkyl glycerols, 3-alkoxypropane-1,2-diols with long unbranched alkyl chains (from *ca.* ~C12 to C20, Sec. III-I0 are viable substrates for glyceryl-ether monooxygenase. The first synthesis of alkyl glyceryl ethers was reported nearly a century and a half ago by Berthelot[127] and a variety of procedures and starting materials were used for them since then,[128,129] and for related lipids.[116,129–131] 3-Alk-1′-enyl glygerols, the plasmalogens, are not substrates for the monooxygenase and their syntheses[41,132] will not be reported here. The most useful and versatile synthesis of α-alkyl glycerols took advantage of the free hydroxyl group of 1,2-isopropylidene glycerol and was first adopted by E. Fischer *et al.*[133] for the preparation of α-monoglycerides (1-acylglycerols). A Williamson ether synthesis has generally been used involving the sodium or potassium salt of isopropylidene glycerol (**9**) with a reactive alkyl derivative in an inert solvent (Scheme 4). Today racemic isopropylidene glycerol, as well as its pure chiral enantiomers, are readily available commercially and have increased the popularity of this method considerably. Its metal salts (**10**) were prepared by reaction with sodium naphthalene,[13] dispersed sodium metal,[134] potassium metal,[135–137] and more recently sodium hydride (from a 60% dispersion in mineral oil)[31,138] in anhydrous inert solvents such as benzene, xylene, tetrahydrofuran or dimethyl formamide under dry nitrogen. After complete conversion to the salt (**10**), the reactive alkyl derivative such as the bromide,[138] iodide,[13,26,134,136] tosylate[13,26,31,135] or mesylate,[137] in an inert solvent as above was added, and the mixture refluxed for several hours (4–48 h). It is imperative that the reactive metal in the first step is in stoichiometric amounts otherwise extensive hydrolysis of the active alkyl derivative can occur with formation of the respective alkanol. For best results the isopropylidene ether (**11**) should be purified by chromatography (e.g., through an alumina column and elution with hexane) and the fractions monitored by TLC or HPLC to ensure the removal of small amounts of any alkanol formed. The major pitfall in this synthesis is the formation of large amounts of the latter fatty alcohols as their properties can be very similar to those of the respective ethers (**11**) and (**12**). It is therefore better to have slight excess of the salt (**10**) because its hydrolysis products (**9**) or glycerol can be readily removed from the respective desired ether. The isopropylidene ethers (**11**) were hydrolyzed to the required products (**12**) with hydrochloric acid in ethanol (*ca.* 4%) by boiling for one hour or by setting aside overnight.[31] Enantiomerically pure glyceryl ethers (**12**, chiral at C2) were prepared from the corresponding chiral 1,2-isopropylidene glycerols,[13,26,31,139] and by using radiolabeled (^{14}C and/or ^{3}H) reactive alkyl derivatives and/or radioactive isopropylidene glycerol appropriately labeled, radiolabeled α-alkyl glycerols have been prepared.[136] No inversion of configuration occurs at the prochiral carbon atom C3 in (**10**) or (**11**) in this reaction, but inversion at the α-carbon atom of the reactive alkyl derivative does take place. We have shown this by condensing the sodium salt (**9,** 2*RS*) with *R*-[1-^{2}H]-hexadecyl tosylate which gave (1′*S*)-[1′-^{2}H]-3-hexadecyloxy-(2*RS*)-propane-1,2-diol, whereas condensation of (2*RS*)-isopropylidene tosylate with the sodi-

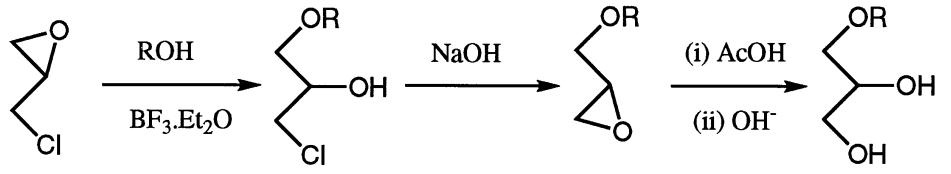


Scheme 4. General synthesis of 3-alkoxypropane-1,2-diols. M = Na, K; X = Br, I, tosyl, mesyl, R = alkyl.

um salt of *R*-[1-^{2}H]-hexadecanol provided the enantiomeric (1′*R*)-[1′-^{2}H]-3-hexadecyloxy-(2*RS*)-propane-1,2-diol.[140]

A modification of the above synthesis involved boiling a protected glycerol possessing at least one free OH group with the alkyl bromide in an inert solvent (e.g., benzene) over potassium hydroxide powder with azeotropic removal of water from the medium.[141–143] This procedure was useful for preparing dialkenyl and mixed alkyl–alkenyl glycerol ethers and was satisfactory in some instances for racemic ethers, but we have found it unsatisfactory for preparing monoalkyl glyceryl ethers directly without avoiding racemization. α-Alkyl thioglyceryl ethers, on the other hand, were obtained in very high yields by boiling unprotected thioglycerol with alkyl bromides or iodides in the presence of potassium hydroxide in 95% ethanol (or methanol with a crystal of iodine),[138] or with the alkyl tosylate in the presence of triethylamine in ethanol.[31] In a modification of this procedure ^{35}S and/or ^{14}C labeled 3-hexadecylthio and 3-octadecylthio propane-1,2-diols were prepared from ^{35}S-thioglycerol or thioglycerol with the respective alkyl bromide or [1-^{14}C]-alkyl bromide dissolved in hexane in the presence of N-alcoholic potassium or sodium hydroxides under nitrogen.[145]

A useful synthesis of chiral 1-*O*-alkyl-2-*O*-benzyl glycerols (which can be debenzylated by hydrogenolysis) starting from D- or L-1,2-isopropylidene-3-*O*-benzylthreitol was developed by Ohno *et al.*[146] for the preparation of the enantiomers of PAF and related lipids. It makes use of D- and L-tartaric acids which are commercially available cheap starting materials. Procedures which provide only racemic α-alkyl glyceryl ethers have been elaborated from earlier syntheses. These include the preparation of alkyl vinyl ethers, for example by condensation of the sodium salt of allyl alcohol with hexadecyl or octadecyl chlorides to form the respective alkyl allyl ethers followed by dihydroxylation of the double bond with hydrogen peroxide in acetic acid[23] or peracids;[147,148] a route which was used to prepare [1′-^{14}C]-3-hexadecyloxypropane-1,2-diol[43] (see ^{14}C-chimyl alcohol Sec. I-B). This procedure, however, was unsatisfactory for making ethers in which the alkyl chain was unsaturated, e.g., selachyl alcohol (see **2**), as it would hydroxylate the double bond of the fatty chain as well. A procedure which did not suffer from this drawback (Scheme 5) started with the reaction of the fatty alcohol with epichlorohydrin (**13**) in the presence of boron trifluoride etherate at 55°C,[149] or stannic chloride,[150] which produced the intermediate 3-alkoxy-2-hydroxypropyl chloride (**14**). This was dehydrohalogenated with concentrated aqueous sodium hydroxide to the glycidic ether (**15,** 3-alkoxy-1,2-epoxypropane) which can be isolated in a pure state. The epoxy group was then converted to the corresponding diol by boiling with acetic acid and basifying, followed by heating with strong ethanolic sodium hydroxide. A number of α-alkyl glyceryl ethers (**12**) with short and long fatty straight or branched chains, as well as with aryl groups, were prepared by this synthesis[149] which was also used when the alkyl chain was unsaturated, e.g., racemic selachyl alcohol from oleyl alcohol.[138] In a useful modification of this synthesis epichlorohydrin was converted into 3-benzyloxy-1,2-epoxypropane (**15,** R = $PhCH_2$-) by this procedure,[149] or by direct reaction with sodium benzylate.[150] The benzyloxyepoxide was then transformed to 3-alkoxy-1-benzyloxypropan-2-ol by reaction with a fatty alcohol in the presence of boron trifluoride etherate. Further alkylation of the 2-OH group (NaH/RX) provided 2,3-dialkoxy-1-benzyloxypropane from which 2,3-dialk-oxypropan-1-ol can be obtained by hydrogenolysis of the benzyl group. This propanol can also be further alkylated to furnish the racemic 1,2,3-trialkoxypropane,[31] and thus a variety of alkoxy groups can be introduced. If a variety of chiral dialkoxypropan-1-ols and trialkoxypropanes are required the methods developed by Eibl and coworkers[139] are very useful. These are best prepared from chiral 3-*O*-benzyl-1,2-isopropylidene glycerol (**11,** R = $PhCH_2$-) by acid hydrolysis to 3-benzyloxypropane-1,2-diol (**12,** R = $PhCH_2$-) followed by judicious use of protecting groups (trityl for primary hydroxy groups, allyl for secondary groups and rearrangement to propenyl with potassium *t*-butylate), selective removal of protecting groups and alkylation with mesylates of fatty alcohols. Potassium



13 **14** **15** **12**

Scheme 5. General synthesis of 3-alkoxypropane-1,2-diols from epichlorohydrin.

t-butylate in boiling *t*-butanol was found most effective with these protected glycerols for making the potassium salt(s) of the free hydroxy group(s) prior to addition of the activated alkyl derivatives.[139]

β-alkyl glyceryl ethers, 2-alkoxypropane-1,3-diols, with long fatty chains are active substrates for glyceryl-ether monooxygenase (Sec. III-I). They have been prepared by alkylation of 1,3-benzylidene glycerol, 2-benzyl-5-hydroxy-1,3-dioxane,[143,151] (alkyl bromide/KOH/benzene,[138,143] or alkyl iodide/18M aqueous potassium hydroxide[152]) followed by hydrolysis with dilute acid in alcohol. These molecules are nonchiral because they possess symmetry. Another nonchiral compound that was a substrate for the monooxygenase was 3-hexadecyloxypropan-1-ol which was best obtained directly in 67% yield by reacting the commercially available trimethylene oxide, oxetane, with hexadecanol in the presence of boron trifluoride etherate.[138] The alternative procedure from trityloxypropan-1-ol and equivalent amounts of octadecyl or *cis*-octadec-9-enyl mesylate provided, after acid hydrolysis, 3-octadecyl or *cis*-3-octadec-9′-enyl propan-1-ols, respectively, in overall yields below 50%.[153]

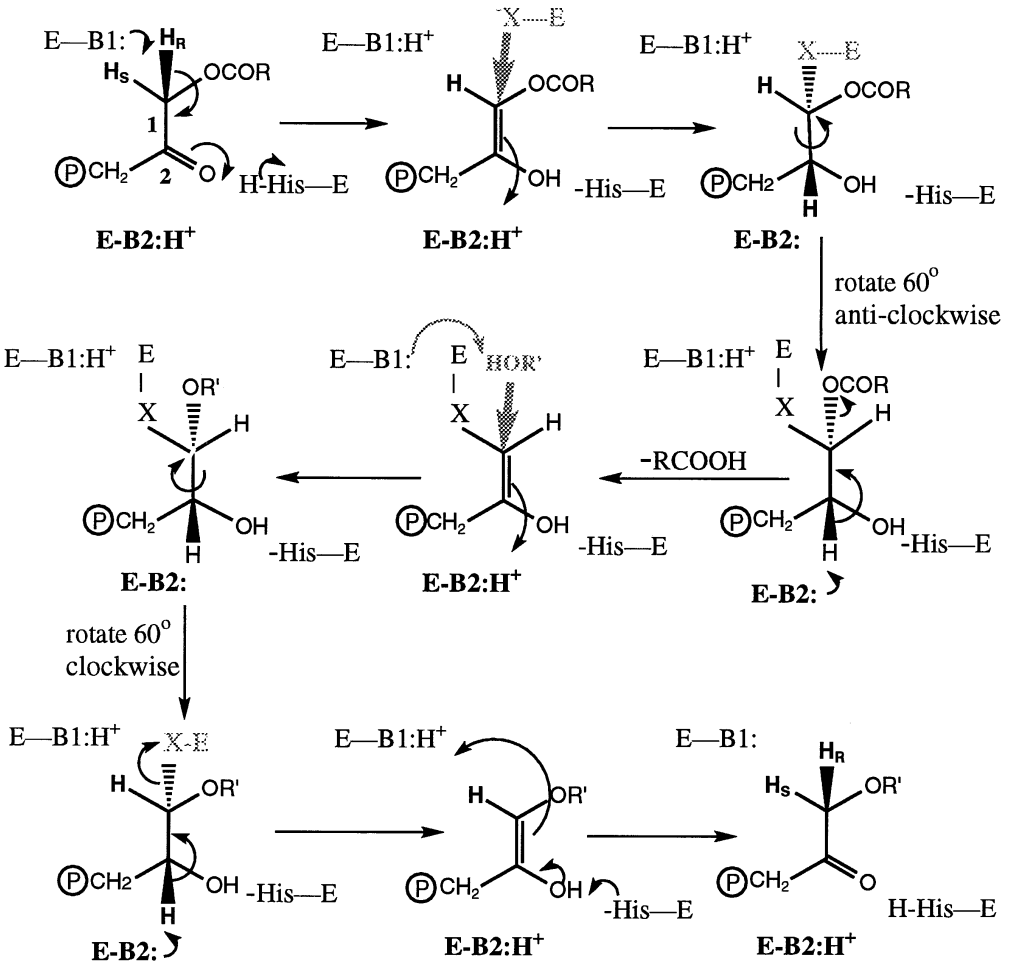
Biosynthesis

The early stages in the biosynthesis of α-alkyl glycerol lipids are depicted in the biosynthesis of PAF in Scheme 2. Much has been written on the biosynthetic steps, and of the enzymes and reaction mechanisms involved, by several authors,[34,41,126,154–156] but notably by F. Snyder[39,157–165] who has made substantial contributions to our knowledge of the biosynthesis of ether lipids and of glyceryl ether phospholipids in general. Only a few salient points need to be repeated briefly here.

The key enzyme in the biosynthesis of α-alkyl glyceryl ethers is alkyl-dihydroxyacetone phosphate synthase (alkyl-DHAP synthase, alkylglycerone-phosphate synthase, EC 2.5.1.26) which mediates the formation of the ether bond by displacing the fatty acid group from acyl dihydroxyacetone phosphate (Scheme 2). This reaction is unique and complex because it catalyzes several steps. It was studied extensively by Snyder,[159–165] Friedberg[156,166–170] and Hajra,[171–175] and their coworkers. The important features of the reaction were reported many times previously[165] and briefly are: (i) the acyloxy group in acyl-DHAP with its two oxygen atoms is completely displaced by the alkoxy group of the incoming fatty alcohol as shown by retention of the labeled oxygen atom when [^{18}O]-hexadecanol was used,[160] (ii) the 1-pro-*R* hydrogen atom of acyl-DHAP is exchanged with water of the solvent[162,166,167] with retention of configuration at the carbon atom bearing the acyloxy group (at C-1),[168,172] (iii) the enzyme binds covalently to acyl-DHAP.[162,164,165] Friedberg *et al.*[169] suggested an intermediate with both acyloxy and alkoxy groups attached to DHAP whereas Davis and Hajra[171] preferred a Ping-Pong Mechanism where an activated enzyme-DHAP complex is formed with release of the fatty acid which is then attacked by the alcohol followed by detachment from the enzyme. The latter mechanism was supported by Brown and Snyder from initial

rate studies and because the fatty acid was a competitive inhibitor with respect to the fatty alcohol.[160] The forward reaction was also inhibited by the sulfhydryl reagent 5,5′-dithio-bis-(2-nitrobenzoic acid).[161,164]

The reaction scheme, repeatedly drawn by Snyder,[157,159,165] described the mechanism by depicting several carbanion intermediates without showing the role of the synthase. A clearer, more detailed, picture of a mechanism which is consistent with that of Snyder and Hajra's was proposed by W. W. Cleland (Enzyme Institute, University of Wisconsin, Madison, personal communication) and invoked three catalytic groups at the enzyme active site (Scheme 6): a neutral histidine residue to hydrogen-bond the carbonyl group of the substrate and two basic residues, with one near C-1(B1) and the other near C-2(B2). In the first step, the substrate is enolized by proton removal by B1 to give an enediolate. The H-bond to the histidine now becomes a low barrier one and provides the energy for the enolization. B2 starts out in the protonated form. In the next step, a nucleophilic group in the enzyme (sulfhydryl or carboxyl, perhaps starting out as an ion-pair with B2) adds to C-1, and B2 protonates C-2 so that both C-1 and C-2 are tetrahedral. The proton removal and the attack by

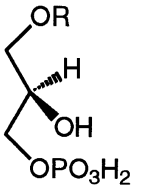


Scheme 6. Cleland's mechanism of alkyl-DHAP synthase consistent with data from Brown and Snyder *Methods Enzymol.*, **209,** 377 (1992).Ⓟ = phosphate, X = S- or COO-.

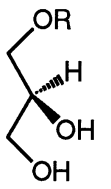
enzyme-sulfhydryl or enzyme-carboxylate(E—X) at C-1 occurs in the *out-of-plane* position. The substituents at C-1 now *must* rotate 60° so that the carboxyl group of the substrate at C-1 is *out-of-plane* and can be released. It then leaves (with protonation by B1) and the proton is removed from C-2 by B2. This gives the enediolate again with the enzyme-X replacing the original carboxyl group at C-1. The alcohol now attacks C-1, with general base assistance by B1, and with B2 putting the proton back on C-2. This makes C-1 and C-2 tetrahedral again. Now the group at C-1 rotates back to the position that puts the enzyme group in an *out-of-plane* position and it is eliminated by reversal of the steps by which it added. The final enediolate returns to the keto form to complete the reaction. The low barrier hydrogen bond between the enediolate and histidine stabilizes this structure, while the hydrogen bond will be weaker in the tetrahedral intermediates because the pK of the alcohol at C-2 will be higher than that of the enediol and the histidine. The final proof of the mechanism will have to come from an x-ray structure of the synthase.

Studies of fatty alcohol specificity in the alkyl-DHAP synthase reaction and displacing labeled palmitic acid from palmitoyl-[^{32}P]-DHAP was as follows: *n*-alcohol (chain length:unsaturation) % reactivity: octanol (8:0) 2, decanol (10:0) 22, dodecanol (12:0) 66, tetradecanol (14:0) 86, pentadecanol (15:0) 84, hexadecanol (16:0) 100, heptadecanol (17:0) 82, octadecanol (18:0) 80, eicosanol (20:0) 30, docosanol (22:0) 16; *cis*-octadec-9-en-1-ol (18:1) 116, *cis*-*cis*-octadec-9,12-dien-1-ol (18:2) 139, and *cis*-*cis*-*cis*-octadec-9,12,15-trien-1-ol (107). These reactivities were normalized with respect to hexadecanol (100%) which had an activity of 0.25 nmoles/min mg of microsomal alkyl-DHAP synthase from Ehrlich ascites cells.[174] The reactivities of acyl-DHAP in this reaction with [1-^{14}C]-hexadecanol were 0.42, 1.20, and 0.95 nmoles/min. mg protein for tetradecanoyl- (14:0), palmitoyl- (16:0), and stearoyl- (18:0) DHAP, respectively.[174] It is not surprising that alkyl groups found naturally in α-alkyl glyceryl ethers have chain lengths as above and that their relative proportions are somewhat in the same order as the above reactivities (see references in Sec. I-A,-B, and -C).[17,32,34,48] The most common and abundant lipids have C16:0 (chimyl alcohol), C18:0 (batyl alcohol), and C18:1 (selachyl alcohol) chain lengths.[34] Very small amounts of branched chain alkyl glyceryl ethers have been found in nature but these could be formed by enzymic C-alkylation (e.g., from unsaturated ethers).[14,17,32,34] Rare ethers, e.g., 2,3-phytanyl glyceryl ethers, were found in some bacteria but their biosynthesis is apparently different.[38,41]

Acyl-DHAP transferase,[175] alkyl-DHAP synthase, and alkyl/acyl-DHAP reductase are localized in peroxisome bodies in animal cells.[126,173] The reductase has been purified and shown to be more effective with NADPH than NADH and is stereospecific in providing the *R*-enantiomer (**16**) of *lyso*-glycero phosphates which on dephosphorylation provide *S*-α-alkyl glyceryl ethers (**17**). It should be



16 *R*- absolute configuration (natural)



17 *S*- absolute configuration (natural)

noted that no change in configuration at C-2 of the glycerol moiety takes place and they all belong to the "D series" (Secs. I-A, and II-B). The change from *R* to *S* is due to the different order of the atomic numbers of the substituents according to the sequence rules.[176–178]

C. Properties

Two isomeric alkyl glyceryl ethers are possible, the α- and the β- isomers, e.g., 3-alkoxypropane-1,2-diols and 2-alkoxypropane-1,3-diols. The latter are symmetrical with respect to the glycerol moiety.

The *chemical properties* of these ethers are typical of their structures,[179,180] and only brief mention is warranted here. The ether bond is relatively unreactive and stable to alkaline hydrolysis, e.g., compared to acyl glycerols. Strong acids, e.g., concentrated hydriodic acid, are required to cleave the ether bond. However, they are subject to photolytic oxidation, as are many lipids, where a hydroperoxy group displaces a hydrogen atom on the carbon atom α to the ether bond. α-Alkyl glyceryl ethers have two vicinal hydroxy groups, consequently they can be acylated, can form cyclic five-membered ring acetals, e.g., with acetone or benzaldehydes, and they can be oxidized with periodate or lead tetra-acetate to yield formaldehyde and α-alkoxy acetaldehyde. The latter reaction has been used for the quantitative estimation of these ethers and to distinguish them from the β-ethers or α-ethers in which one or two of the hydroxy groups are protected.[181,182] The β-ethers can be similarly acylated and form cyclic six-membered acetals but are stable to the above oxidation under the same conditions. Unsaturation in the alkyl chain provides a further center of reactivity making them subject to the usual reaction of alkenes.[183] The number of double bonds can be determined quantitatively,[184] and their positions can be determined by oxidation and identification of the fragments formed. Their stereochemistry (*cis* or *trans*), and in some cases their positions, can be identified by physical methods (see NMR below). Advantage of the greater reactivity of the primary hydroxy group over the secondary hydroxy group in α-ethers has been taken in order to prepare alkyl glycerophosphocholines or ethanolamines (e.g., cephalins). Thus batyl alcohol was converted to 1-octadecyloxy-3-tosyloxypropan-2-ol, then to 1-octadecyloxy-2-hydroxypropyl-1-iodide, 1-octadecyloxy-2-octadecanoylpropyl-1-iodide, and finally to 1-octadecyloxy-2-octadecanoyl-propane-1-phosphoethanolamine (stearoyl-batyl cephalin).[134]

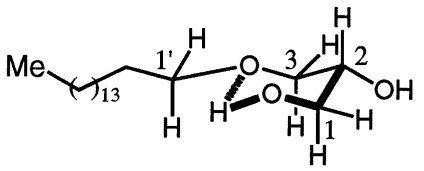
A variety of *physical properties* of alkyl glyceryl ethers have been used successfully to separate, identify, and characterize them. These include analytical and preparative liquid[1,17,62,136,139,145] gas-liquid[1,46,48,61,73,109,118,137,141,143,185,186,188] thin layer chromatography,[46,59,60,86,88,89,109,111,117,136–138,140–145,153,186–189] and in the early days paper chromatography,[7,72] which were also used in conjunction with radioactively labeled (^{3}H, ^{14}C, and ^{35}S) compounds for the identification of substrates and reaction products,[46,73,136,145,186,187] either directly or as acetates or trimethylsilyl ethers.[188] The ultimate identification was by direct comparison with authentic compounds prepared by unambiguous synthesis.

Infrared spectroscopy was used for comparison as the ethers possess characteristic absorption frequencies.[73,142,179,186,187] In particular the bands in the region 1040 to 1070 cm^{-1} arising from the C—O—C antisymmetric stretching vibrations and bands at 1108 to 1120 cm^{-1} arising from the C—O vibration of the ether linkage as well as characteristic bands at ca. 1455 to 1470 cm^{-1} attributed to C—H bending (deformation) skeletal vibrations can be identified. Free OH groups, if present, provide strong bands at 2830 to 2926 cm^{-1}.[136,138,185]

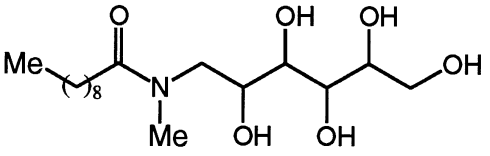
Alkyl glyceryl ethers are transparent in the *ultraviolet spectrum* except for increasing tail absorption below 215 nm. However, by conversion into their respective ester or urethane derivatives of aromatic acids or amines characteristic UV spectra could be obtained. *Mass spectrometry* of glyceryl ethers has not been used extensively. The spectra, after methylating the free hydroxy groups to prevent excessive fragmentation, have been used to identify structures after gas chromatographic separations.[17] The spectra of the unsubstituted chimyl alcohol and its 1′*R* and 1′*S* 1′-monodeuterated derivatives were used to determine the deuterium content of these compounds which was an indication of their optical purity. Although the molecular ion peaks of the free alcohols were weak, the M - $CH_3O\cdot^{+}$ peaks after loss of the terminal CH_2OH, were intense and could be compared with the related peak (with one mass unit less) of the unlabeled fragment which appeared in the same spectrum.[140]

α-Alkyl glyceryl ethers but not the β-ethers have *optical rotatory* properties because of the chiral center at C2 (see **2**) (Sec. I-A).[13,26,27,30] In the early periods of study of these ethers a lot of confusion about the optical rotations of the natural chimyl, batyl, and selachyl alcohols arose, and the identities of these ethers from various sources.[24,40,129] This was partly because of the small rotations using the sodium D line (λ 589 nm), and the solvent and concentration dependence of the magnitude of the rotations. Toyama and Ishikawa[189] had shown that the optical rotation of selachyl alcohol (see **2**) in chloroform decreased with increase in concentration reaching 0° at ca. 10% concentration and became negative below this concentration. Baer *et al.*[13,26] later showed that the di-*p*-nitrobenzoates or diphenylurethane derivatives had higher rotations, did not suffer seriously from these solvent problems and were better for identifying these ethers because of higher and sharper melting points. We have measured the rotatory dispersions of 2*R*-3-tridecyloxypropane-1,2-diol (non-natural configuration) in chloroform (and $CDCl_3$) between 260 and 600 nm at concentrations varying from 0.76 to 25% (Fig. 1).[31] It should be noted that at a concentration of ca. 10% the rotation was ca. 0° up to ca. 350 nm, below which a plane positive curve was obtained. At lower concentrations the curves exhibit weak anomalous dispersion with negative maxima between 275 and 300 nm. This behavior could be due to micelle formation (see below) or aggregation of molecules which change with concentration.

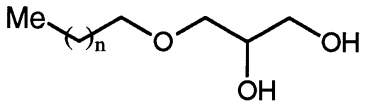
^{1}H NMR Spectroscopy at low magnetic fields (60 MHz) gave a general picture of alkyl glyceryl ethers and was used to distinguish α- from β-ethers, and revealed the presence of double bonds in the alkyl chain.[143,185] The spectra at higher fields (>350 MHz), on the other hand, were much more informative and in $CDCl_3$ displayed all the protons in the glycerol moiety as well as those on the α-carbon atom of the fatty chain.[31,138] The spectra showed that in dilute $CDCl_3$ solution(ca. 0.1% w/v) the 3-carbon glycerol backbone was in a rigid 6-atoms (but not 5-atoms) chair conformation which was stabilized by hydrogen bonding as in structure **18** for chimyl alcohol. All the hydrogen atoms



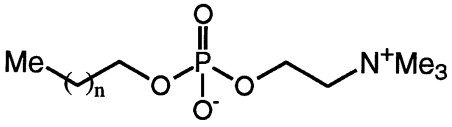
18



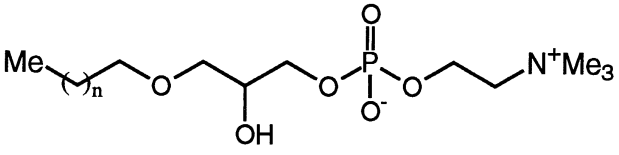
20 Mega-10



19 Chimyl: n = 14
Batyl: n = 16



21 n = 10,12,14 or 16



22 n = 10,12,14 or 16

denoted by H in the structure were clearly observed with the chemical shifts, multiplicities, and coupling constants dictated by the rigid chair structure with the 2-OH group in the equatorial conformation. We obtained almost identical spectra for the ethers with longer (up to C22) and shorter alkyl carbon chains (down to C1) which we examined.[31,138] 3-Octadecylthiopropane-1,2-diol gave simi-

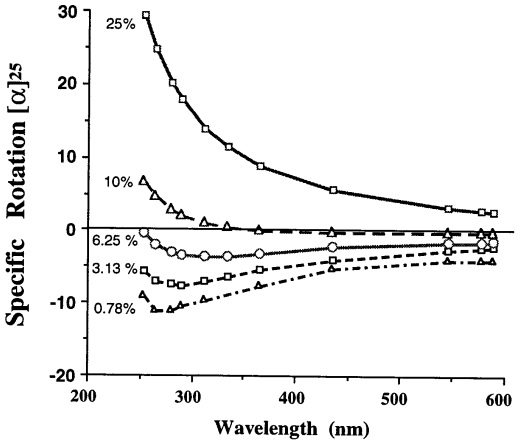


Figure 1. ORD of 3-1′-tridecyloxypropane-1,2-diol in $CDCl_3$.

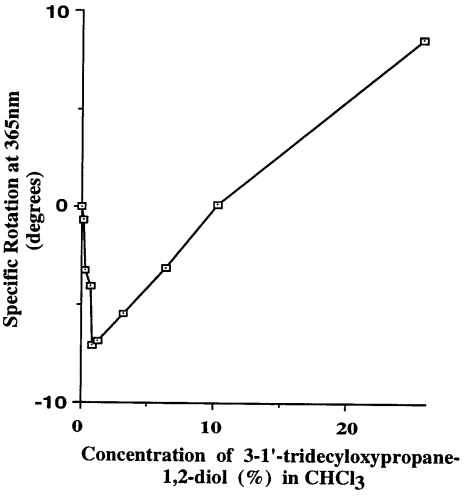


Figure 2. Effect of concentration on Specific Rotation.

lar spectra.[138] It was interesting that as the concentration of the ether was increased to 25% all the peaks broadened markedly and the 16 peaks for the two H1′ and the two H3 protons coalesced to three broad peaks implying some changes in the physical state of the molecules in solution. It should be pointed out that the optical rotation of *R*-3-tridecyloxypropane-1,2-diol at 365 nm also changed in magnitude at a concentration of ~0.78% and increased sharply upon further dilution (Fig. 2). The spectrum of 3-hexadecyloxypropan-1-ol, on the other hand, was characteristic of a molecule with a linear conformation. However, the spectra of β-alkyl glyceryl ethers, e.g. 2-hexadecyloxypropane-1,3-diol, possessed numerous peaks from which peaks for structures such as **18** could be identified, but other conformations were present in solution including a linear conformation.[138]

X-ray examinations of alkyl glycerol derivatives, e.g., cephalin derivatives of chimyl and batyl alcohols,[134] were used for comparing the products of various preparations in the days when other physical techniques (above) were not at the advanced stage that they are today.

A close look at the structures of α-alkyl glyceryl ethers with long aliphatic chains such as chimyl alcohol (**19,** n = 14) and batyl alcohol (**19,** n = 16) will reveal that they are similar to those of nonionic *detergent* molecules. They possess a long fatty chain with a polar (di-hydroxy) head. These have very poor solubility in water or aqueous buffer and have the ability to foam. They are readily solubilized in the presence of nonionic detergents such as Mega-10 (**20,** N-methyl-N-decanoylglucamide), as well as ionic detergents such as the alkylphosphocholines (**21**) and acyl-*lyso*-phosphatidylcholines (**22**), by shaking, warming or by sonication to give clear solutions that are stable for many hours at room temperature.[191] Ether lipids which are generally very insoluble in aqueous buffers had to be solubilized when used as substrates, or inhibitors, for the determination of glyceryl-ether monooxygenase activity by spectrophotometric methods (Secs. III-C and -D). This was possible with the detergents **20** to **22.** For example up to 2 mM (or more) stock solutions of batyl or chimyl alcohols in 0.8% (23 mM) of Mega-10 in water or 0.1 M tris-HCl buffer (pH 7.5) at 25°C were solubilized by sonication, and the solutions were clear for several hours at 25°C.[192] The CMC (critical micelle concentration)[193] of Mega-10 was found to be 7.44 mM and 7.15 mM in water (pH 7.4) and in 0.1 M Tris-HCl buffer (pH 7.5), respectively,[194,195] and decreased to 5.72 mM (0.1 M Tris-HCl, pH 7.5) when 0.1 mM batyl alcohol was present in solution. With the ionic detergents **21** and **22,** 0.1 mM batyl alcohol decreased or increased the CMC values depending on the chain length

n.[191] From the evidence presented it was concluded that glyceryl ethers were solubilized in aqueous medium by forming mixed micelles with the soluble detergents and that the solutions contained a mixture of micelles of the detergent alone and mixed micelles of detergent and glyceryl ether, and that the concentration of free glyceryl ether in solution was usually negligible.[194] It was demonstrated for example that batyl alcohol suspensions which were sonicated (or heated) for several hours displayed no monooxygenase activity at 25°C.[138,191] α- And β- alkyl glyceryl ethers exhibit different surface monolayer film properties such as film compression, surface area and temperature of half expansion at 1.4 dynes/cm.[25] These properties were used to show that batyl alcohol and synthetic 3-octadecyloxypropane-1,2-diol were identical.[22,25,196]

3. GLYCERYL-ETHER MONOOXYGENASE

A. Occurrence and Isolation

Glyceryl-ether monooxygenase activity was first described by Tietz, Lindberg, and Kennedy,[1] who found it in the microsomal fraction of rat liver homogenates. They measured the enzymic activity by the amount of radiolabeled stearic acid, or aldehyde formed, when 9′,10′-ditritiobatyl alcohol (obtained by catalytic hydrogenation of selachyl alcohol **2** with platinum oxide and tritium gas) was used as substrate. With this assay (see Sec. III-C) they determined the specific requirements of the monooxygenase with regard to the cofactor, substrates, pH, etc. The activity was mainly in the microsomal fraction, after removal of cell debris, mitochondria, and nuclei. A more detailed study by F. Snyder and his coworkers,[197,198] using the same assay in principle, confirmed the subcellular localization (microsomal) of this activity and with [1′-^{14}C]-chimyl alcohol showed that the levels of this activity (by the relative release of [1-^{14}C]-palmitic acid) in perirenal fat, brain, intestine, and liver of rat were in the order: 0.039, 0.059, 0.163, and 1.00, respectively. The relative levels of monooxygenase activity in the livers of rabbit, hamster, mouse, gerbil (sand rat), guinea pig, dog, female rat, and male rat (and whole slugs) were 0.129, 0.159, 0.246, 0.358, 0.454, 0.781, 1.000, and 1.681 (and 0.042), respectively, using the same assay procedure. The lower activity in the female rat compared with the male rat livers was confirmed by Kosar-Hashemi and Armarego[138] who found them to be consistently in the ratio of 1:3.3 (female:male) with Wistar rats and using a spectrophotometric assay (see Sec. III-C).

The isolation procedures by various authors were generally the same. The livers from overnight starved rats that were decapitated, or killed in a CO_2 atmosphere, were homogenized in 0.25*M* sucrose in 0.125*M* Tris-HCl buffer (pH 7.5) in the absence,[1,89,138,192,197] or presence of 1 mM EDTA,[91,198,199] in 0.125*M* sucrose alone,[200] or in buffer containing 8 mM $CaCl_2$.[201] We did not find it necessary to add EDTA to obtain optimal activity. Homogenization was usually done by several passages in a Potter-Elvehjem homogenizer (all at 4°C), or in a Waring blender for large quantities.[197] The cell debris was removed at low centrifugation speeds (3000 × g, 15 min), larger particles such as mitochondria and nuclei at 35,000 × g (30 min) and the microsomal fraction containing most of the activity was collected as a pellet by centrifugation of the clear supernatant at 105,000 × g (1 to 1.5 h) or higher speeds. The pellet was resuspended (Potter-Elvehjem) in one of the aqueous solutions or in 50 mM Tris-HCl (pH 8.8) containing 20% glycerol (w/v),[89] but preferably in 0.25 M sucrose in 0.125 M Tris-HCl buffer (pH 7.5) and adjusted to a concentration of 15 to 20 mg protein/ml. These were stored at −20°C, or at −70°C where they were stable for many months. Usually, thawing provided samples with the original activity, but if the activity had decreased it was recovered by resuspending in the same homogenizer at 4°C. Such preparations were used for most of the work described in later sections (III-B to III-J) without further purification because the information required was for the monooxygenase bound to its natural lipid membrane.

At high magnification (Fig. 3) the microsomes are seen to be of varying sizes. Extensive washing of the microsomal pellets with buffer caused some loss of enzyme activity,[198] probably due to solubilization, or removal of some of the smaller vesicles or of the protein from the lipid membranes. Ishibashi and Imai[200] had solubilized the microsomal fraction in 2% of Triton X-100 and partially purified the protein on a 6-aminohexyl-Sepharose column, followed by a DEAE-cellulose column and sucrose density gradient centrifugation, whereby they isolated the membrane-free protein in 36% yield with a *ca.* 83-fold purification. The protein was enzymically active (using the radioactive assay) in the presence of Triton X-100 and other phospholipids (see Sec. III-B). We have repeated this purification but were unable to get active fractions using our spectrophotometric assay due to considerable loss of activity. Ishibashi and Imai[202] further purified their protein to apparent homogeneity using an affinity column of 3-*O*-hexadecyl-1,2-(4-aminobenzylidene)-glycerol bound to 6-carboxyhexyl-Sepharose 4B and elution by increasing the ionic strength of the medium.

A preparation of rat liver microsomes using differential centrifugation as above or $CaCl_2$ precipitation[203] was shown to cleave 2-hexadecyloxyethanol and evidence was presented that it may well be the same monooxygenase that cleaves monoalkyl glyceryl ethers.[204]

In studies of glyceryl ether metabolism by the protozoan *Tetrahymena pyriformis*, the 100,000 or 250,000 × g pellet was found to actively cleave α-alkyl glyceryl ethers such as chimyl alcohol.[205] Two glyceryl ether pools were found in this organism with different sensitivities to ether cleavage. Ether cleavage increases when the cells were at a stationary phase or when excess of external glyceryl ether was provided to the cell medium.[206] The enzyme was apparently different from the monooxygenase in this review as it did not require a pterin cofactor, but required an oxygen and NADPH regenerating system.[36,207] Similarly the 100,000 × g microsomal fraction from the parasite *Leishmania donovani* has a NADPH-dependent alkyl glyceryl ether cleavage enzyme which does not require a pterin cofactor.[208] This may well have desaturase activity (see Sec. I-C) and perhaps also α-alk-1′-enyl glyceryl ether hydrolase activity (plasmalogenase) to carry out the necessary cleavage (see Sec. I-C) if alk-1′-enyl glyceryl ethers were the products of the reaction.

B. General Properties

The monooxygenase activity of the microsomal fractions from male rats varied slightly with the preparation but were in the range of 23 to 28 nmoles/min.mg protein with the standard direct spectrophotometric assay (Sec. III-C below) using 0.1 mM *RS*-batyl alcohol as substrate and 0.1 mM *RS*-6-methyl-5,6,7,8-tetrahydropterin (6-MeTHP) as cofactor in 0.1M Tris-HCl buffer (pH 7.5) containing catalase (0.1 mg/ml) and 0.08% Mega-10 at 25°C.[138140,192] The activities of the heavy microsomal fractions (35000 × g, 20 min) contained some cell debris (electron micrograph not shown) and were *ca* 65% of the activities of the light microsomal fractions (105000 × g, 60 min, Fig. 3).[192] Three washings of the latter fraction with Tris-sucrose buffer results in up to a *ca.* 30% increase in activity whereas further excessive washing causes loss of activity (Sec. III-A above). The apparently lower activities of earlier preparations[90,197,198,200] were more difficult to assess because of the different assay procedures used (see following section), except for the first preparation[1] which appeared to have the same order of activity as in the standard direct spectrophotometric assay above. This was possibly coincidental. The pure preparation of monooxygenase protein by Ishibashi and Imai[202] had a specific activity of 34 nmoles/min.mg, when using the radioactive assay with chimyl alcohol, 6,7-dimethyl-5,6,7,8-tetrahydropterin and asolectin.[200]

The monooxygenase activity of the microsomes was unaltered after 3 days at 4°C, and was slightly affected by freezing and thawing, losing 10% of the activity after 6 thaw–freeze cycles during 3 days.[138] At 25°C the activity decreased with a half life of 27 h,[209] at 60°C it dropped to 10%,[91] and was completely lost on boiling.[200,209] The protein was finally purified by affinity chromatography and gave a single band on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with an M_r val-

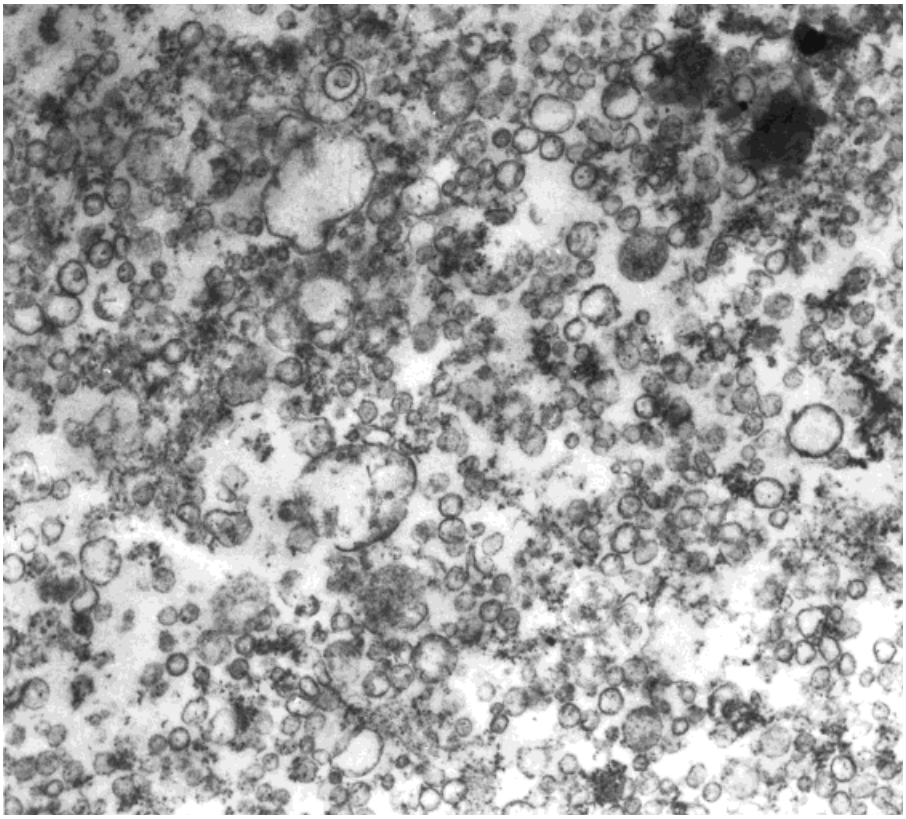


Figure 3. Electron micrograph of rat liver microsomes containing glyceryl-ether monooxygenase activity.

ue of 45K,[202] whereas the molecular weight of the native protein as measured by Sepharose 6B column chromatography in 20 mM Tris-HCl (pH 7.5) containing 0.1% Triton X-100 was *ca.* 400K Dal.[200] Unpurified microsomal protein was heterogeneous consisting of at least 16 bands with the most intense bands (silver staining) between 45 and 50K on SDS-PAGE.

The requirement of oxygen for monooxygenase activity was demonstrated by a marked decrease in activity when the reaction mixture was incubated under a nitrogen atmosphere,[1] and complete loss of activity in an oxygen depleted assay mixture.[200] A tetrahydropterin was required as cofactor for the oxidation of glyceryl ethers (see Secs. C and H below). Monooxygenase activity was linear with increase in microsomal protein at least up to a concentration of ca. 1 to 2 mg/ml with a slope of 45°, i.e., the activity doubled when the protein concentration was doubled, at pH 7.5 and 8.7.[138,198] Many cations have an inhibiting effect (Sec. III-F), and although above 4.8 mM NH_4^+ ions, as sulphate or chloride, were said to double the activity in the radioactive assays,[198,204] this was not confirmed in a similar assay,[200] and we have found no effect of this ion on the enzyme activity in the direct spectrophotometric assay.[138] The discrepancy could well be due to the borate-KCl buffer used by the first authors compared with Tris-HCl buffer used by the later authors. For the inhibition by metal chelating agents see Sec. III-F.

The early rate measurements were at pH 7.4 and 8.0,[1,197] and the pH-rate profiles in phosphate or borate-KCl buffers showed activities which rose slowly from pH 3.0 to a maximum of 9.0 and dropped sharply to almost zero at pH~9.5.[198] In Tris-HCl buffers the curve was somewhat bell

shaped and had optimum pH near 8.5.[200] When the direct spectrophotometric assay was used in the absence of catalase the pH-rate profile in 0.1*M* Tris-HCl or phosphate buffers were also bell shaped with pH maxima at ~7.5; with *ca.* 20% lower activities in the phosphate buffer. In Tris-HCl buffer containing catalase the maximum rate at pH 7.5 was marginally lower but was almost flat between 7.0 and 9.5.[138]

C. Enzyme Assays

The development of methods for assaying glyceryl-ether monooxygenase activity has been hampered by the high insolubility of the ether substrates and to some extent by the suspended nature of the microsomes. Four main assays were developed namely the radiometric assay using radiolabeled ether substrates, a similar assay using nonlabeled substrates specifically for glyceryl ether phosphates, phosphoethanolamines, and phosphocholines in which phosphorus in the phospholipid products was determined, a coupled spectrophotometric assay where the monooxygenase reaction was coupled with a dihydropteridine reductase reaction and the rate of oxidation of NADH was measured (Scheme 1), and a direct spectrophotometric assay in which the oxidation of the cofactor tetrahydropterin (PH_4) to *quinonoid* 7,8-dihydro(6*H*)pterin (*q*-PH_2) was measured (top reaction in Scheme 1).

The *radiometric assay* was first developed by Kennedy and coworkers[1] and was used to determine the intracellular localization of the enzyme and its requirements for optimal activity. They used tritium labeled batyl alcohol. The assay mixture was complex and contained: 50 mM Tris-HCl buffer pH 7.5, 1 mM NADP, 1 mM NADPH, 5 mM glucose-6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase, 5 mM labeled batyl alcohol (in Cutscum 1% w/v), 50–750 μM PH_4 and the microsomal fraction (1–3 mg protein) and shaken in tubes at 37°C for 1 hr. Aliquots were taken and the reaction was terminated, e.g., by addition of ethanol, radioactive stearaldehyde and stearic acid were estimated by gas-liquid chromatography, or the aldehyde was converted to its *p*-nitrophenylhydrazone derivative and counted. Similar procedures with some modifications were used by Snyder *et al.*,[86,88,197] for example the total mixture contained: 40 mM borate-KCl buffer pH 9.0, 24 mM $(NH_4)_2SO_4$, 10 mM reduced glutathione, 750 μM PH_4, 350 μM [1′-^{14}C]-3-hexadecyloxypropane-1,2-diol (in 20 μl propylene glycol, sp. act. 0.14 μCi/μmole) and 1.15 mg of microsomal protein in a total volume of 1 ml;[198] the reaction was incubated at 37°C and the reactions in aliquots were terminated at noted intervals of time, the lipids were extracted as before,[210] separated by TLC and the identified radioactive bands were isolated and counted.[198] In the nonradioactive version of this procedure (see below) the *p*-nitrophenylhydrazone of the fatty aldehyde was prepared and estimated.[200,211] Ether substrates with the label, e.g., ^{3}H, in the glycerol moiety were also used to trace the glycerol or starting material.[46] In a later variation $(NH_4)_2SO_4$ was omitted but asolectin was added to solubilize the substrate and perhaps the enzyme.[200] The major advantage of this assay was that very low levels of monooxygenase activity could be detected when highly radioactive ether substrate was used. However the assay had many drawbacks. A regenerating system was required to keep the concentration of PH_4 constant or high. The aerobic autoxidation of the pterin cofactor affects this assay (see below), but has been considered in a more recent adaptation in which catalase was added to minimize this.[204] Radiolabeled fatty aldehyde, acid, and alcohol were formed and their ratio varied with time. The procedure was unsatisfactory for measuring the kinetic parameters of the enzyme because of the intensive labor involved, e.g., one incubation with the isolated aliquots which had to be analyzed at intervals of time provided only one plot for one concentration of substrate or cofactor, and several of these were required to obtain initial rates for Km and Vmax determinations. Accurate initial rates were difficult to obtain by this method.

The *nonradiometric* versions of the above assay where the products were estimated by e.g., gas–liquid chromatography, or the aldehydes formed were estimated as derivatives had some merit.

A radioactive substrate was not necessary—in some instances it was not easy, or it was time consuming, to synthesize it chemically. Eibl and coworkers[212] had used this procedure [0.1*M* borate buffer pH 8.8, 5 mM reduced glutathione, 5 mM $(NH_4)_2SO_4$, 1 mM PH_4, 500 nmol ether-phospholipid substrate (sonicated in water), 0.5 mg microsomal protein in 1 ml of final volume] for alkyl glycerol phosphates, phosphoethanolamines, and phosphocholines. After a time interval at 37°C the unreacted phospholipids were isolated and the phosphorus was determined by known analytical procedures. The sensitivity of this method was dictated by the phosphorus estimation.[213]

The *coupled spectrophotometric* assay was developed by Eibl and coworkers[89] which adopted the reactions in Scheme 1. This method was rapid because initial rates (oxidation of NADH to NAD) could be measured spectroscopically in a kinetic run by the change in optical densities at 340 nm with time which required only a few minutes. They have found it very useful for comparing the rates of reaction of a large variety of ether lipids and their phosphate derivatives. Their ether substrates were solubilized with 1-lauroyl(dodecanoyl)-propane-3-phosphocholine or tetradecylphosphocholine at 7mg/ml.[90] They had compared this method with the radiometric procedure and showed that it was satisfactory only when 6-$MePH_4$ [but not 6,7-dimethyl-5,6,7,8-tetrahydropterin (6,7-Me_2PH_4)], was used as cofactor. This was because with 6,7-Me_2PH_4 the system was not completely coupled. The acid-base catalyzed rearrangement[214] of *quinonoid* 6-methyl-7,8-dihydro(6*H*)pterin (*q*-6-$MePH_2$, oxidized cofactor) to 6-methyl-7,8-dihydropterin (6-$MePH_2$, nonreducible to 6-$MePH_4$ in this system) was considerably slower ($t_{0.5}$ 23 min at pH 7.2, 30°C)[215] than the rearrangement of *quinonoid* 6,7-dimethyl-7,8-dihydro(6*H*)pterin (*q*-6,7-Me_2PH_2) to 6,7-dimethyl-7,8-dihydropterin (6,7-Me_2PH_2, $t_{0.5}$ 11.8 min at pH 7.6, 25°C)[214] and produced lower concentrations of recyclable cofactor in the 6,7-dimethylpterin. Also tetrahydropterins readily undergo autoxidation in aqueous buffer[214,216] (the solubility of oxygen under 1 atmosphere of air in aqueous buffer is 0.25 mM)[217] with the formation of *quinonoid* species. Since catalase, which is known to inhibit the autoxidation of tetrahydropterins[217–219] probably by specific removal of H_2O_2 (product of the oxidation),[219] was not included in this assay then the rates of NADH oxidation were not exact measures of the monooxygenase reaction (although they were probably close to them, as initial rates were measured). Also the monooxygenase was sensitive to H_2O_2 and its removal by catalase was necessary.[199] However, for substrate activity comparison purposes this assay was quite satisfactory as long as the limitations were understood. In a recent use of this coupled assay it was shown that addition of catalase (1000 U/ml) was very necessary for optimal rates of reaction.[204] Dihydropteridine reductase had to be added for the recycling of cofactor to ensure that the rate of recycling was not rate-limiting, even though there was always residual dihydropteridine reductase in the microsomal preparations. Our preparations had dihydropteridine reductase activity of *ca.* 0.22 μmol of NADH oxidized/min.mg microsomal protein at pH 7.5 and 25°C with 6-$MePH_4$ (100 μM) and NADH (100 μM),[138] and accounted for the statement made previously that monooxygenase activity was not affected when dihydropteridine reductase was not added. It must be pointed out that coupled reactions are not good reactions for accurate measurements of kinetic parameters mainly because it may not be possible to keep the required reaction *always* the rate-limiting reaction when higher concentrations of substrate had to be used for obtaining the data, i.e., very large amounts of the second enzyme have to be added in order to keep the rates of the desired reaction rate-limiting at *all* concentrations of substrates. A typical assay had the following components (final concentrations in italics) in the cuvettes of a double beam spectrophotometer: M Tris-HCl buffer (100 μl, *0.1M*), 10 mM NADH (20 μl, *200* μM), 2.5 μg dihydropteridine reductase (5 μl), 50-500 nmol akyl glycerol, 7 mg tetradecylphosphocholine (or 7 mg 1-lauroylpropane-3-phosphocholine) and water to make a total volume of 1 ml. The reaction was initiated by addition of 20 mM 6-$MePH_4$ (10μl, *200* μM) to the reaction cuvette and the rate of change of optical density at 340 nm was measured.[89,90] For maximal rates catalase (1000 U) should be added to each cuvette.[204] See Sec. III-E for the stoichiometry of the coupled reaction.

We had developed a *direct spectrophotometric assay* in an endeavor to simplify the procedure and to overcome the deficiencies in the above assays. It involved only the reaction of the monooxygenase (top part of Scheme 1) and was an adaptation of the procedure of Ayling and coworkers[217] for assaying phenylalanine hydroxylase. It made use of the UV spectral changes that occurred when tetrahydropterin cofactors were oxidized to the respective *quinonoid* dihydropterins. Because *quinonoid* species rearrange to the 7,8-dihydropterins at different velocities with consequent spectral changes, an isosbestic analytical wavelength was chosen at a specific pH so that rearrangement did not interfere with the rate values. The analytical wavelengths varied from one pterin to another and had to be determined *a priori.* For example the isosbestic analytical wavelengths for 6-$MePH_4$, 6,7-Me_2PH_4, and natural 6*R*-BH_4 (see Scheme 1) in Tris-HCl buffer pH ca. 7.5 were 345, 335, and 340 nm with reaction extinction coefficients of 3440, 4000, and 3770 $M^{-1}.cm^{-1}$, respectively.[138,192,209] At these wavelengths the ether lipid substrates were transparent and although the microsomes had some absorbance, it remained constant during initial rates runs and was compensated in the reference cuvettes when a double beam spectrometer was used. The ether lipids had to be solubilized and several detergents were investigated for this purpose (see Sec. III-D). We found that the nonionic detergent Mega-10 was the most useful because it could be readily prepared,[190] was also available commercially, was transparent in the desired UV range and was very soluble in water. When it was used to solubilize batyl alcohol it was found that the monooxygenase activity did not alter much at concentrations up to 0.2% (w/v) of detergent (see Figs 4 and 5), and all the measurements were standardized at 0.08% Mega-10 in the final solutions. A typical assay consisted of the following ingredients in the cuvettes of a double beam spectrometer (final concentrations in italics): M Tris-HCl pH ca. 7.5 (100μl, *0.1M*), 0.04 to 2.0 mM ether lipid substrate (100 μl, stock prepared by sonicating a suspension in 0.8% w/v Mega-10 in water at 20°C, *4–200* μM), 0.1 g catalase (100μl, 1 mg/ml, 5000 U), 0.5 mg microsomal fraction (25 μl, 20 mg/ml protein), and water to a volume of 950 μl. The reference cuvette had the same components except that the ether lipid solution was replaced by 0.8% Mega-10 (100 μl). The cuvettes were shaken and placed in thermostated cell holders. When the optical density was steady 4 to 10 mM of tetrahydropterin hydrochloride (50 μl, in 4 mM HCl, *20–500* μM) was added to each of the two cuvettes simultaneously, shaken and the rate of change of absorbance was traced directly on a chart or recorded on a computer. Best results were obtained with a double-beam spectrometer where the cuvettes were close enough in distance for a gadget with two small platinum buckets to hold the pterin solution to be used for adding the pterin solutions simultaneously. If a single-beam spectrometer was used then it was important that the time laps between mixing the assay components and adding the pterin was kept constant (1–2 min) to avoid small changes in optical density due sedimentation between runs. With this assay it was possible to measure kinetic parameters with the usual accuracy (±5–10%).[31,138,140,191,194,220]

D. Effects of Detergents

The effects of detergents are quite complex and if some validity is to be attached to data for comparison purposes then one should standardize on the detergent and assay to be used (Secs. II-C, III-C, and -D). Enzyme activity varies at different concentrations of detergents as well as varying from one detergent to another.[89,138,191,200,201] The activity peaked sharply with some detergents, e.g., sodium deoxycholate, octylthioglucoside,[138] alkyl phosphocholines (**21**),[191] whereas with others, e.g., Mega-10 (**20**), CHAPS,[138] acyl *lyso*-phosphatidylcholines (**22**),[191] there was a relatively wide range of concentrations at which the activity varied slightly (Fig. 4). However in most cases the activity was low at low concentrations of detergents (even when the substrate could be solubilized), reached a peak or plateau of activity, then decreased (sometimes sharply) as the concentration of detergents increased further. High concentration of detergent therefore seriously

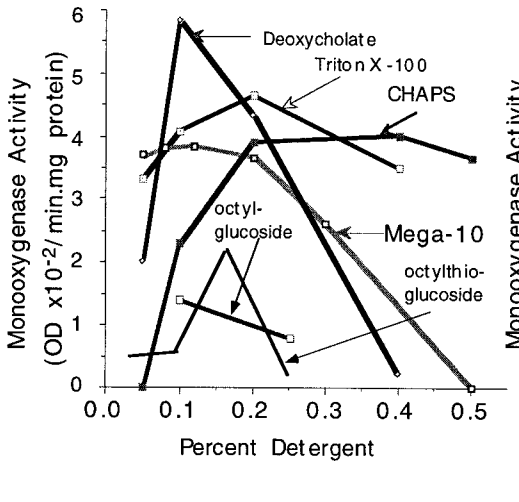


Figure 4. Enzyme activity in Tris buffer pH 7.5, with 0.1 mM batyl alcohol and 0.5 mM 6-MePH$_4$.

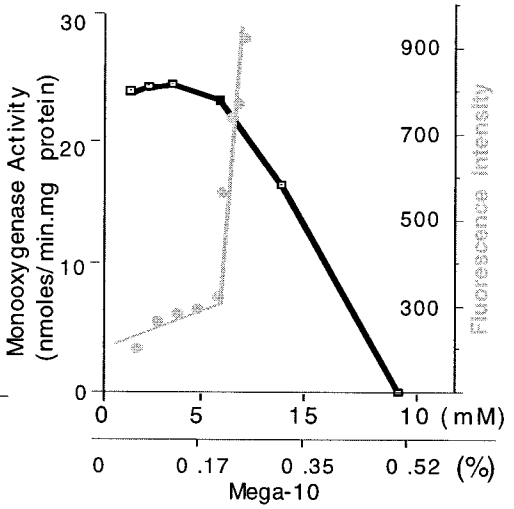


Figure 5. Enzyme activity in Mega-10 as in Figure 4, Fluorescence of Mega-10 + 0.1 mM batyl alcohol.

inhibits enzyme activity. With Mega-10, inhibition of the monooxygenase occurred at concentrations above ca. 0.18% (ca. 5.7 mM) in Tris-HCl buffer at pH 7.5, a concentration which was close to the CMC of Mega-10 (Fig. 5) (see Secs. II-C and III-F for effect of buffer, substrate, and octadecanol). This suggested that Mega-10 micelles were the inhibiting species and that mixed batyl alcohol—Mega-10 micelles had to be present in solution below the CMC of Mega-10 as that was how batyl alcohol could get into aqueous solutions (see Sec. IV for mechanism of action).[194] The conditions used in the standard direct spectrophotometric assay (Sec. III-C) were devised with this in mind. However not all detergents exhibited a direct correlation of CMC and monooxygenase inhibition. With some alkyl phosphocholines (**21**) and acyl *lyso*-phosphatidylcholines (**22**), e.g., with $n = 16$ and 18, maximum enzyme activity was obtained at concentrations more than an order of magnitude greater than the CMC. This was because their CMC values were very small. These detergents do, however, inhibit monooxygenase activity at high concentrations, e.g., activity was almost zero above ca. 0.2% of these phosphocholines (compare with Fig. 4).[191]

Triton X-100 at 0.05% (see Fig. 4) was found to stabilize the microsomal preparation without effect on initial rates. After separating the monooxygenase protein from its natural phospholipid membrane with DEAE-cellulose (see Sec. III-A) in Tris-HCl pH 7.5 buffer containing 0.1% of Triton X-100, the protein had lost 70% of its monooxygenase activity. However, addition of the natural phospholipids (which had been separated on the column) restored some activity, but addition of dipalmitoyl phosphatidylcholine or asolectin (see Sec. III-C) instead, enhanced the activity by ca. 50 and ca. 100%, respectively.[200] Dephosphorylation of the microsomal preparation with phospholipase A_2 resulted in a ca. 60% decrease in monooxygenase activity which was completely restored by addition of asolectin. The activity in lipid phosphate depleted microsomes using gel filtration in 1% deoxycholate, and removal of the latter by Sephadex G-25, was recovered to various extents by addition of the natural microsomal lipids, phosphatidyl-, and diphosphatidyl-glycerol but not phosphatidyl- (and *lyso*) choline, ethanolamine, serine, and inositol or sphingomyelin. The most effective reconstituting lipid was asolectin in this case,[201] as was found in other studies.[221] Asolectin is a soya bean mixture of phospholipids containing lecithins (acyl phosphatidylcholines, ca. 30%),

cephalins (acyl phosphatidylethanolamines, ca. 30%), acyl phosphatidylinositol and small amounts of other phosphates and polar lipids.

E. Stoichiometry

The monooxygenase reaction should produce an intermediate hemiacetal (Scheme 1), but this has not been isolated yet (see below). However, its presence was indicated by its rapid hydrolysis at the pH of the enzymic reaction (7 to 9) either by acid-base catalysis or by an unknown membrane bound hydrolase. The products were large amounts of fatty aldehyde and small amounts of the respective acid and alcohol in the early stages of the reaction. As the reaction proceeded, more of the latter two substances were formed indicating the presence of oxidoreductases in the membrane. It was shown that added NAD[1] or NADH[198] caused the disappearance of the respective aldehyde formed with increased production of fatty acid and alcohol. In the radiometric assay with 100 to 200 nmoles of 6-$MePH_4$ the amount of fatty aldehyde formed corresponded to 40 to 50% of pterin on a molar basis, and the authors[1] stated that perhaps one enantiomer of the racemic 6-$MePH_4$ used was enzymically active. This is not the case, however (see Sec. III-J). By using glycerokinase in the assay to estimate the amount of glycerol formed it was shown also that the amount of glycerol released was somewhat greater than the amount of aldehyde formed, at least in the first 5 min of reaction.[1]

Some aspects of the stoichiometry were studied using the coupled spectrophotometric assay together with the radiometric assay and using 6-$MePH_4$ and [11′,12′-$^3H_2$]-3-hexadecyloxypropane-1,2-diol (labeled chimyl alcohol).[89] It was shown that in the radiometric assay labeled hexadecanal was mainly formed within the first 0.5 min, and that the amounts of NADH consumed in the coupled assay were equal to the sum of labeled hexadecanal, hexadecanoic acid, and hexadecanol formed.[89] We had also studied the coupled assay using 6-$MePH_4$ and batyl alcohol (0.1 mM) with human dihydropteridine reductase and NADH at 83 μM. We measured the rate of NADH oxidation and we measured concurrently the amount of 6-$MePH_4$ oxidized to *q*-6$MePH_2$ by the direct spectrophotometric assay (monooxygenase activity alone, Sec. III-C), and determined the ratio of NADH oxidation to the amount of *q*-6-$MePH_2$ formed as a measure of the extent of coupling. We found that at concentration of 6-$MePH_4$ of 100 μM and below, the reaction was completely coupled, i.e., [NADH]/[*q*-6$MePH_2$], was 1.[187,220] At higher concentration of 6-$MePH_4$ (e.g., $>$ 480 μM) this ratio dropped (e.g., to $<$ 0.83). We attributed this to substrate inhibition of dihydropteridine reductase, as was well known (at *q*-6$MePH_2$ $>$ 200 μM), and to product (6-$MePH_4$) inhibition, and together they accounted for the uncoupling observed at high concentration of 6-$MePH_4$.[187,220]

The stoichiometry of 6-$MePH_4$ oxidation to batyl alcohol consumed was examined by the direct spectrophotometric assay and the radiometric assay (Sec. III-C) with [1′-^{14}C]-3-octadecyloxypropane-1,2-diol. The results are shown in Figure 6. Aliquots from the radiometric assay were sampled at time intervals and the labeled batyl alcohol, octadecanal, octadecanoic acid, and octadecanol were separated by TLC and counted. The sum of the radioactivity of the last three compounds together with a radioactive unidentified substance near the origin of the plate was about equal to the radioactivity of the batyl alcohol that reacted. The band near the origin may well have contained an intermediate (the hemiacetal which reacted with the TLC plate?). The amount of 6-$MePH_4$ oxidized in the direct assay at time intervals is shown in Figure 6. It was clear that at least in the first 2 min of reaction the amount of batyl alcohol consumed was equal to the amount of 6-$MePH_4$ oxidized and that the stoichiometry was 1:1.[187,222] Since all the kinetic parameters determined by the direct spectrophotometric assay involved initial rates measurements, i.e., within the first 2 mins, all the data obtained by this assay were valid.[31,138,140,191,192,194] It should be noted that in the radiometric assay the amount of octadecanol and octadecanoic acid formed were about the same, and as no pyridine nucleotide was added then the small amount of it bound to membrane oxidoreductases must have recycled to produce these products from octadecanal. The increased amounts of 6-$MePH_4$ consumed

after *ca.* 2 min (Fig. 6), compared with the hydroxylation of batyl alcohol, could be due to autoxidation which was not suppressed enough by the catalase after the first 2 min of reaction.

The detergent does not appear to affect the kinetic parameters of the substrates very seriously as can be seen from Table II where the Km and Vmax values of batyl alcohol are reported in the presence of various detergents. The concentrations of the detergents in Table II were at values where the rates were not greatly affected by detergent concentrations.[191]

F. Inhibition

Inhibition of the monooxygenase by detergents was found to exhibit normal enzyme kinetic behavior when the rate data were subjected to kinetic analysis. The kinetics of inhibition were evaluated using computer programs devised by Cleland whereby the type of inhibition was the one in which all the data fitted the equation best.[223] The type of inhibition from the computed data agreed well with graphic plots of the data. Table III lists the inhibition constants Ki values of Mega-10 (**20**), two alkylphosphocholines (**21**), and two acyl L-α-*lyso*-phosphatidyl cholines (**22**). The Ki values showed that they were weak inhibitors of the noncompetitive and competitive type, i.e., Mega-10 acted generally on the protein or lipid bilayer such that it perturbed the active site, and the cholines competed with the substrate for the active site. Octadecanol, which is theoretically a detergent-like molecule, was also a weak competitive inhibitor suggesting that it may have occupied the active site of the enzyme which binds the octadecyl chain of batyl alcohol (3-octadecyloxypropane-1,2-diol).

The inhibition with *lyso*-PAF as substrate and Mega-10 as inhibitor was a particularly interesting case as it gave some insight into the nature of detergents in aqueous solution. Initial rates data fitted the *noncompetitive* type of inhibition with a Ki value of 185 μM.[194] The inhibition was of the same type as the inhibition of batyl alcohol by Mega-10 except that it was about one order of magnitude stronger (Table III). With *lyso*-PAF as substrate it appeared that Mega-10 micelles were not the inhibiting species since the concentrations of Mega-10 at which the measurements were made,

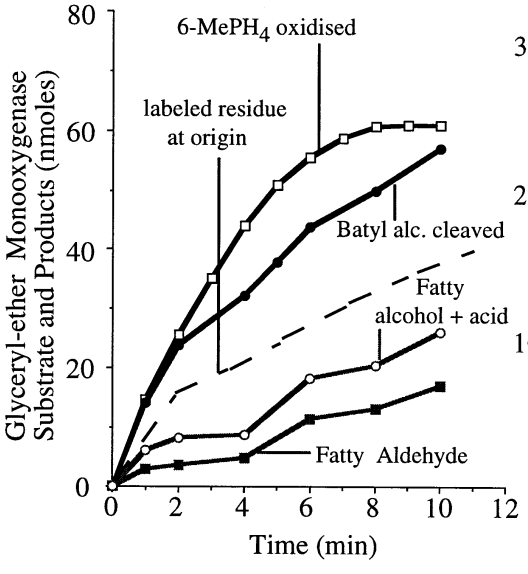


Figure 6. Batyl alcohol cleaved and 6-$MePH_4$ oxidized during the monooxygenase reaction (pH 7.5).

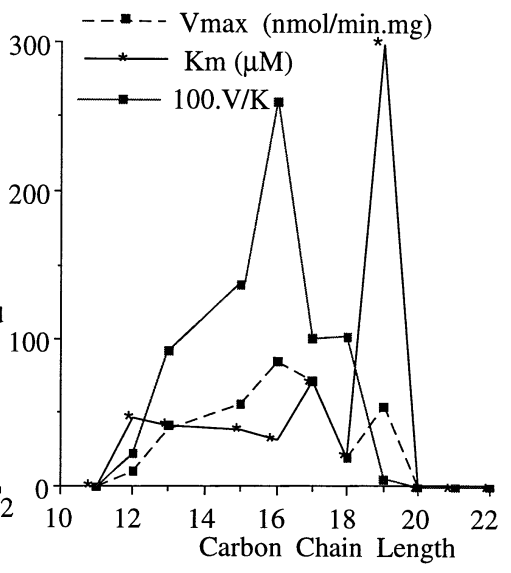


Figure 7. Monooxygenase activity of alkyl glyceryl ethers **19,** *n* = **9, 10, 11, 13, 14, 15, 16, 17, 18, and 20,** with 0.1 mM 6-$MePH_4$ in Tris (pH 7.5).

Table II. Apparent Km and Vmax of Glyceryl-Ether Monooxygenase with Batyl Alcohol[a]

Detergent[b] *(Concn, μM, %)*	*Km (μM)*	*Vmax nmoles/min.mg*
12-PC **(21, n = 10)**		
815, 0.035	27.7	48.7
14-PC **(21, n = 12)**		
382, 0.02	27.0	46.1
16-PC **(21, n = 14)**		
348, 0.02	30.1	64.2
18-PC **(21, n = 16)**		
600, 0.035	36.3	60.6
12-LPC **(22, n = 10)**		
979, 0.04	33.8	54.4
14-LPC **(22, n = 12)**		
332, 0.015	27.4	48.5
16-LPC **(22, n = 14)**		
538, 0.03	24.0	50.0
18-LPC **(22, n = 16)**		
1170, 0.05	43.7	61.0
Mega-10 **(20)**		
2300, 0.08	25.1	27.6

[a] 0.5 mM 6-$MePH_4$, in Tris-HCl, pH ca 7.5, 25°C.

[b] PC = alkyl phosphocholine, LPC = Acyl L-α-*lyso*-phosphatidylcholine (from Ref. 191).

were well below the CMC of Mega-10 (CMC ca. 7 mM). On the other hand the CMC of *lyso*-PAF was found to be 3.3 μM (in water at pH ca. 7.4) and decreased in the presence of Mega-10, i.e., the substrate was almost completely in the form of micelles during the measurements.[194] Inhibition was possibly caused by Mega-10 in mixed micelles being transported to the protein, because free Mega-10 molecules or aggregates were not inhibitory (see Fig. 5).

Many of the compounds examined for inhibition were not strong inhibitors (Table IV). We are yet to find out how compounds such as N-ethylmaleimide and *p*-chloromercuribenzoate interact with the monooxygenase. *o*-Phenanthroline, EDTA, and 8-hydroxyquinoline are strong chelating agents which could extract a metal from the enzyme as in the case of phenylalanine hydroxylase. However, *o*-phenanthroline was a relatively weak inhibitor of glyceryl-ether monooxygenase when compared with its inhibition of phenylalanine hydroxylase (100% inhibition at 50 μM).[224]

Most of the inorganic salts examined were very weak inhibitors requiring 40 mM concentrations in order to produce a noticeable effect. Zn^{2+} deserves further study as well as the inhibition by KCN and NaN_3 (Table IV). The stimulating effect reported for amethopterin (a potent dihydrofolate reductase inhibitor) was due to its effects on the supporting NADPH-linked enzymic reduction of oxidized pterin cofactor.[1]

The inhibition of monooxygenase activity by glyceryl ethers has been used successfully to identify that the activity observed, e.g., in specific tissues, was from the known glyceryl-ether monooxygenase. The enzyme which cleaved alkyl *lyso*-glycerophosphocholines was shown to be glyceryl-ether monooxygenase by the inhibition of cleavage of labeled chimyl alcohol (13.2 nmol, as substrate) by 20 and 30% upon addition of 2.9 and 5.7 nmols, respectively, of alkyl *lyso*-glycerophosphocholine (as inhibitor). Conversely, the release of total labeled hexadecanal, hexadecanol, and hexadecanoic acid in the enzymic reaction with [1′-^{14}C]-hexadecyl-2-*lyso*-glycerophosphocholine (14.5 nmol, as substrate) was found to be inhibited by 90 and 95% upon addition of 7.3 and

Table III. Inhibition of Glyceryl-Ether Monooxygenase (Tris-HCl pH ca. 7.5, 25°C)[a]

Inhibitor	*Apparent Ki (mM)*	*Type*	*Ref.*
Mega-10 **(20)**	1.74	Noncompetitive	194, 222
Octadecan-1-ol	0.77	Competitive	194, 222
Tetradecylphosphocholine **(21, n = 12)**	0.32	Competitive	191
Tetradecanolyl-L-α-*lyso*-phosphatidylcholine **(22, n = 12)**	0.33	Competitive	191
Hexadecylphosphocholine **(21, n = 14)**	0.37	Competitive	191
Hexadecanoyl-L-α-*lyso*-phosphatidylcholine **(22, n = 14)**	0.32	Competitive	191
RS-lyso-PAF as substrate and Mega-10 as inhibitor	0.185	Noncompetitive	194

[a]6-$MePH_4$ at 0.5 mM, various amounts of inhibitor and batyl alcohol in 0.08% Mega-10.

14.5 nmols, respectively, of alkyl glycerol. The identity of the monooxygenase as glyceryl-ether monooxygenase was further confirmed by the stimulating effect of catalase and by comparative heat stability studies of the enzyme.[91]

In a recent example of this approach to inhibition, it was shown that a monooxygenase cleaved the alkyl glycol ether 1-*O*-hexadecyl-[2-^{3}H]-ethylene glycol (2-hexadecyloxy-[1-^{3}H]-ethan-1-ol) and released [^{3}H]-ethylene glycol efficiently. Addition of 0.03, 0.06, and 0.12 mM chimyl alcohol (inhibitor) to the assay mixture caused the release of labeled ethylene glycol from 2-hexadecyloxy-[1-^{3}H]-ethan-1-ol (substrate) to the extent of 41, 31, and 19% of the chimyl alcohol-free assay reaction. This inhibition of alkyl ethylene glycol monooxygenase by chimyl alcohol exhibited competitive inhibition with a Ki value of 8 μM which was very close in value to the Km (12 μM) of chimyl alcohol in the same assay system. It implied that the monnoxygenase which catalyzed the oxidation of alkyl glycol ethers was the same as the one which catalyzed the oxidation of alkyl glycerol ethers.[204] 2,4,5-Triaminopyrimidin-6-one, which was previously shown to behave as a cofactor like tetrahydropterins in the dihydropteridine reductase[225] and phenylalanine hydroxylase reactions[226,227] was found to inhibit this "alkyl glycol = alkyl glycerol" monooxygenase when 2-hexadecyloxy-[1-^{3}H]-ethan-1-ol was the substrate and 6-$MePH_4$ was the cofactor. The inhibition was competitive with a Ki value of 240 μM for the triaminopyrimidinone.[204]

G. Structure-Activity Relationships

In the first article on glyceryl-ether monooxygenase, Tietz, Lindberg, and Kennedy[1] were aware of the necessity of studying the effects of the structure of the pterin cofactors and ether lipid substrates on this enzymic reaction. The radiometric assay that they used did not lend itself easily to initial rates measurements, and because nothing was known at the time about the requirements of the enzyme the data were only rough estimates of relative activities. As new spectrophotometric methods for measuring initial rates were developed, better and more rapid and convenient means of comparing the activities of cofactors and ether lipid substrates became possible. The measurements of kinetic parameters, Km, Vmax, and V/K, are the ultimate for comparison purposes as they take all the kinetic data into account. Vmax values alone, e.g., of lipid substrates at assumed saturating values of cofactor (assuming no inhibition at high concentrations) have been very helpful in informing roughly whether a substance is an excellent, medium, weak or inactive "substrate." The best parameters for comparison are the V/K values, the first-order rate constants at low substrate concentrations and are the best values when substrates have apparently closely similar activities.

Table IV. Inhibitors of Glyceryl-Ether Monooxygenase

Inhibitor	*Concn [mM]*	*% Activity*	*Ref.*
no inhibitor[a]	—	100	138
Digitonin	1.5%	52	138[b]
	1.5%	98	89[c]
Hexadecan-1,2-diol	0.1	84	138[b]
N-Ethylmaleimide	2.0	41	200[d]
	4.0	35	138[b]
	8.0	9	138[b]
p-Chloromercuribenzoate	2.0	11	200[d]
o-Phenanthroline	0.25	80	138[b]
	0.5	59	138[b]
	1.0	32	138[b]
EDTA	5.0	84	138[b]
8-Hydroxyquinoline	1.0	85	138[b]
NaN_3	15	13	138[b]
Na_2SO_4	24	84	198[e]
KCl	40	75	198[e]
KCN	4	7	200[d]
NaCl	40	64	198[e]
CsCl	40	59	198[e]
$MgSO_4$	40	35	198[e]
$CaCl_2$	40	19	198[e]
$(NH_4)_2\ Mo_7O_{24}$	40	11	198[e]
$ZnCl_2$	40	0	198[e]

[a]Standard spectrophotometric assay: 0.1*M* Tris-HCl pH ca. 7.5 at 25°C wth 6-$MePH_4$ (0.5 mM) and batyl alcohol (0.1 mM).

[b]Standard spectrophotometric assay and preincubating for 20 min at 25°C.

[c]Coupled assay in 0.05*M* Tris-HCl pH ca. 8.8 at 37°C and preincubating at 0°C for 15 min.

[d]Radiometric assay in 0.1*M* Tris-HCl pH ca. 8.8 for 30 min at 37°C, and spectrophotometric assay at pH ca. 7.5 (Ref. 138).

[e]Radiometric assay in 0.04*M* phosphate pH ca. 9.0 for 10 min. at 37°C.

The Km and Vmax values alone do not provide a complete assessment of the viability of substrates. It also gives information about which parameter, the Km or Vmax, accounts for the higher or lower V/K value. For a true comparison of a set of cofactor and lipid substrates initial rates at varying concentrations of cofactor and lipid substrate should be measured in the same experiment using the same amount of enzyme, and computed to provide the two Km values (one for cofactor and one for lipid substrate) and one Vmax value in one calculation.[223] A good example of such kinetic studies was the comparison of substrate and cofactor viabilities in mutated dihydropteridine reductases prepared by site directed mutagenesis of human cDNA, and expression of the proteins in *E.coli*.[228] However, if it was known that the cofactor does not inhibit enzyme activity at or near saturating concentrations then comparisons of lipid substrates activities could be made at a set high concentration of cofactor. This makes the assumption that the Km values of the cofactor were closely the same for all lipid substrates. The kinetic parameters were most conveniently determined by the direct spectrophotometric assay (Sec. III-C) and stated as [D] in the tables in the following sections.

The data in the tables were taken from the literature and provided a good indication of the structure-activity relationships, particularly of lipid ether substrates. All the data were "apparent" because

relatively crude microsomal preparations were used and the maximum velocities were given in nmol/min.mg of *total* protein in the microsomal aliquots used. All the data were obtained with the microsomal monooxygenase isolated from rat liver and was not specified in the tables. Also the sex and breed of the animals were not stated. Km, Vmax, and V/K values determined by the direct spectrophotometric assay [D] in the tables were evaluated from initial rate data using computer programs.[223]

H. Pterin Cofactors

Kennedy and coworkers[1] used the radiometric method in an NADPH regenerating system to show that at 2 mM batyl alcohol and 10 μM pterin in Tris-HCl buffer pH ca. 8 (37°C) the activities of the pterin cofactors were in the order: 5,6,7,8-tetrahydro-L-neopterin (10, freshly prepared) > 6-$MePH_4$ (5) ≈ 5,6,7,8-tetrahydrosepiapterin (5, old sample) > 6,7-Me_2PH_4 (1.7) ⩾ 5,6,7,8-tetrahydrofolic acid (0.1). The activity of the tetrahydrofolic acid may well be due to the presence of 6-$MePH_4$ which was a contaminant in all the preparations of tetrahydrofolic acid at the time of the experiments.[229] The data imply that conjugated pterins were not effective cofactors for the monooxygenase as in the case of the aromatic aminoacid hydroxylases.[6–8] "Apparent" kinetic data were collated and are in Table V. From the V/K values it was clear that *RS*-6-$MePH_4$ was a much better cofactor than *RS-cis*-6,7-Me_2PH_4, and that natural 6*R*-5,6,7,8-tetrahydro-*erythro*-biopterin, which is most probably the true natural cofactor for the monooxygenase, was the most active. The activity being "weighted" by the small Km value of this cofactor. The data were consistent with the relative activities in the first reported experiments.[1] These cofactors do not appear to cause substrate inhibition at the high concentrations of *ca* 0.5 mM.

I. Lipid Substrates

A considerable number of substrates were evaluated for monooxygenase activity and some of the early "rough estimate" comparisons were shown to be approximately consistent with data from later more accurate spectrophotometric assay procedures. Later procedures had provided more precise values of activities, which were still "apparent" values but were a very good indication of the *latitude* that the monooxygenase has for lipid substrates. Most of the available kinetic data have been collated in Tables VI to X with the respective cofactors and literature references. The pterin cofactors 6-$MePH_4$ and 6,7-Me_2PH_4 were racemic and the latter had the *cis* configuration of the 6,7-dimethyl groups in all the entries.

The following data were not included in the tables: The relative amounts of the respective fatty acids (nmol/mg protein in brackets) liberated in 120 min at 37°C using 6,7-Me_2PH_4 (0.125 mM) in Tris-HCl pH 7.5 and using the radiometric assay with a regenerating system (method [R] in tables) and ^{14}C labeled ether lipid substrate (79 μM) by rat liver microsomes were: 3-octadecyloxypropane-1,2-diol (2.35, batyl alcohol), 2-octadecyloxypropane-1,3-diol (2.60, 2-batyl alcohol), 3-hexadecyloxypropane-1,2-diol (4.44, chimyl alcohol), and 2-hexadecyloxypropane-1,3-diol (4.76, 2-chimyl alcohol).[197] These data should be compared with the data in Table VI. The "nonglycerol" ether 3-hexadecyloxy-propan-1-ol with 6-$MePH_4$ (0.5 mM, at pH 7.5 [25°C] as cofactor, assay [D] in Table VI, had Km 100 μM, Vmax 23.3 nmol/min.mg and V/K 0.23 and was a relatively good substrate.[138]

Discussions of the individual substrates would be prohibitive due to space, and a better approach has been adopted in which the general effects of structure on activity are listed. The lipid substrate structures required for monooxygenase activity using a viable cofactor, i.e., 6-$MePH_4$ or 6,7-Me_2PH_4 are:

1. The 1-monoalkyl glycerol ether (3-alkoxypropane-1,2-diol) with maximum activity had a normal C16 chain (chimyl alcohol) and was followed by ethers with normal C15, C18, and C19 chains, but dropped sharply in activity almost to zero at chain lengths of $<$ C11 and $>$ C20 (see Fig. 7, and Table VI). Isopropylidene derivatives, ethers with branched chain alkyl groups, e.g., 3-cyclohexylmethoxypropane-1,2-diol (Table VI), and aromatic groups, e.g., 3-benzyloxypropane-1,2-diol[1] were inactive.
2. 2-Monoalkyl glyceryl ethers (2-alkoxypropane-1,3-diols) were very active substrates when they had a normal C16 chain (2-chimyl alcohol) and C18 chain (2-batyl alcohol), and perhaps better substrates than the respective 1-alkyl isomers chimyl and batyl alcohols (see second paragraph of this section above and last entry in Table VI).
3. A *cis*-double bond adjacent to the ether oxygen atom, i.e., a 1′-double bond as in plas-

Table V. "Apparent" Kinetic Data of Glyceryl-Ether Monooxygenase with Pterin Cofactors

Cofactor	*Lipid Substrate mM [assay]*[a]	*Km μM*	*Vmax nmol/min.mg*	*V/K*	*pH (°C)*	*Ref*[c]
5,6,7,8-Tetrahydropterin PH_4	2-Hexadecyloxy-[^{3}H]-ethan-1-ol 0.4 [R]	60[b]	6.0[b]	0.10	8.8 (37)	204
RS-6-MePH_4	Batyl alcohol, 0.1 [D]	86.6	21.9	0.25	8.7 (25)	138, 220
	0.1 [D]	138[b]	29.3[b]	0.21[b]	7.5 (25)	138, 220
	0.1 [D]	136	32.5	0.24	7.5 (25)	138, 220
	2-Octadecyloxyethan-1-ol 0.14 [D]	171[b]	25.9[b]	0.15[b]	7.5 (25)	220
	2-Hexadecyloxy-[^{3}H]-ethan-1-ol 0.4 [R]	41	19.6	0.48	8.8 (37)	204
	3-*O*-Hexadecyl-G-l-PC 0.5 [C]	—	4.9	—	8.8 (37)	89
	[^{3}H]-Hexadecyl glycerol 0.05 [C]	—	13.5	—	8.8 (37)	89
	0.05 [R]	—	12.9	—	8.8 (37)	89
RS-6,7-Me_2PH_4	Batyl alcohol 0.1 [D]	176	21.9	0.12	7.5 (25)	138, 192, 220
	0.1 [D]	273	29.7	0.11	8.7 (25)	138, 192, 220
	3-Hexadecyloxy-G-1-PC 0.5 [C]	—	1.3	—	8.8 (37)	89
	[^{3}H]-Hexadecyl glycerol 0.05 [C]	—	3.1	—	8.8 (37)	89
	[^{3}H]-Hexadecyl glycerol 0.05 [R]	—	12.9	—	8.8 (37)	89
	2-Hexadecyloxy-[^{3}H]-ethan-1-ol 0.4 [R]	150[b]	20[b]	0.13[b]	8.8 (37)	204
6*R*-Tetrahydro-biopterin (BH_4)	2-Hexadecyloxy-[^{3}H]-ethan-1-ol 0.4 [R]	42[b]	18.3[b]	0.44[b]	8.8 (37)	204
	Batyl alcohol 0.1 [D]	24.6	30.6	1.24	8.7 (25)	138, 192, 220
5,6,7,8-Tetrahydrofolic acid	2-Hexadecyloxy-[^{3}H]-ethan-1-ol 0.4 [R]	600[b]	5.8[b]	0.01[b]	8.8 (37)	204

[a][D] = Direct spectrophotometric assay in Tris-HCl buffer with 0.08% Mega-10, [C] = coupled spectrophotometric assay, [R] = radiometric assay.

[b] With catalase.

[c] Apparent Vmax values in Ref. 192 have been normalized.

Table VI. "Apparent" Kinetic Data of Glyceryl-Ether Monooxygenase with Monoalkyl Glyceryl Ethers

Lipid Substrate Propane-1,2-Diol	*Cofactor mM [assay]*[a]	*Km μM*	*Vmax*[b] *nmol/min.mg*	*V/K*	*pH (°C)*	*Ref.*
RS-3-Propyloxy-	6-MePH_4, 0.5 [C]		Inactive at 90 mM		8.8 (37)	90
RS-3-Pentyloxy-	6-MePH_4, 0.5 [C]		Inactive at 90 mM		8.8 (37)	90
RS-3-Hexyloxy	6-MePH_4, 0.5 [C]	—	6.2 at 50 mM	—	8.8 (37)	90
RS-3-Heptyloxy-	6-MePH_4, 0.5 [C]	—	3.6 at 15 mM	—	8.8 (37)	90
RS-3-Heptyloxy-	6-MePH_4, 0.5 [D]	51300	86.9	0.002	7.5 (25)	31
RS-Cyclohexylmethoxy-	6-MePH_4, 0.5 [D]		Inactive at 10 mM		7.5 (25)	138
RS-3-Octyloxy-	6-MePH_4, 0.5 [C]	—	3.6 at 10 mM	—	8.8 (37)	90
RS-3-Nonyloxy-	6-MePH_4, 0.5 [C]	—	0.4 at 1 mM	—	8.8 (37)	90
R-3-Undecyloxy-	6-MePH_4, 0.5 [D]		Inactive at 0.2 mM		7.5 (25)	31
RS-3-Dodecyloxy-[c]	6-MePH_4, 0.5 [C]	—	3.5 at 2 mM	—	8.8 (37)	90
R-3-Dodecyloxy-	6-MePH_4, 0.5 [D]	47.4	10.7	0.23	7.5 (25)	31
R-3-Tridecyloxy-	6-MePH_4, 0.5 [D]	42.7	39.6	0.93	7.5 (25)	31
RS-3-Tetradecyloxy-[c]	6-MePH4, 0.5 [C]	—	5.1 at 1 mM	—	8.8 (37)	90
R-3-Pentadecyloxy-	6-MePH_4, 0.5 [D]	39.7	57.2	1.44	7.5 (25)	31
R-3-Hexadecyloxy-[d] (Non-natural chimyl alc.)	6-MePH_4, 0.5 [D]	32.9	79.4	2.41	7.5 (25)	31
RS-3-Hexadecyloxy- (Chimyl alcohol)	6-MePH_4, 0.5 [D]	13.8	18.0	1.30	8.7 (25)	192
	6-MePH_4, 0.5 [D]	11.1	39.9	3.5	7.5 (25)	138, 220
RS-3-Hexadecyloxy-[e]	6-MePH_4, 0.5 [C]	—	12.7 at 0.5 mM	—	8.8 (37)	90
RS-3-Hexadecyloxy-[2-^{3}H]-	6-MePH_4, 0.4 [R]	12	11	0.92	8.8 (37)	204
	6,7-Me_2PH_4, 1.0 [N]	660	17[f]	0.03[f]	8.8 (37)	200
S-3-Hexadecyloxy-[d] (Natural chimyl alcohol)	6-MePH_4, 0.5 [D]	37.5	92.2	2.46	7.5 (25)	31
R-3-Heptadecyloxy-	6-MePH_4, 0.5 [D]	71.8	72.6	1.01	7.5 (25)	31
R-3-Octadecyloxy-[d] (Non-natural batyl alc.)	6-MePH_4, 0.5 [D]	41.5	41.5	1.00	7.5 (25)	31, 138, 220
RS-3-Octadecyloxy- (Batyl alcohol)	6-MePH_4, 0.5 [D]	24.7	34.3	1.39	7.5 (25)	31, 138 220
	6,7-Me_2PH_4, ? [R]	72	41	0.57	8.8 (37)	87
RS-3-Octadecyloxy-[e]	6-MePH_4, 0.5 [C]	—	7.4 at 0.5 mM	—	8.8 (37)	90
S-3-Octadecyloxy-[d] (Natural batyl alcohol)	6-MePH_4, 0.5 [D]	20.4	30.5	1.5	7.5 (25)	31, 138, 220
R-3-Nonadecyloxy-	6-MePH_4, 0.5 [D]	937	55.9	0.06	7.5 (25)	31
R-3-Eicosanyloxy-	6-MePH_4, 0.5 [D]		Inactive		7.5 (25)	31
R-3-Heneicosanyloxy-	6-MePH_4, 0.5 [D]		Inactive		7.5 (25)	31
RS-3-Docosanyloxy-	6-MePH_4, 0.5 [D]		Inactive		7.5 (25)	31
2-Hexadecyloxypropane-1,3-diol[g]	6-MePH_4, 0.5 [D]	18.3	38.4	2.1	7.5 (25)	138, 220

[a][D] = Direct spectrophotometric assay in Tris-HCl buffer with 0.08% Mega-10, [C] = coupled spectrophotometric assay in Tris-HCl with microsomes solubilized in 1.5% w/v of digitonin, [R] = radiometric assay, [N] = nonradiometric assay by isolation of hexadecanal *p*-nitrophenylhydrazone in Tris-HCl containing 1.25 mg/ml of asolectin.

[b]Maximum velocity or rate at stated concentration of lipid substrate.

[c]Substrate solubilized in 1-lauroylpropane-3-phosphocholine.

[d]R and S notations are incorrectly reversed in literature references.

[e]Substrate solubilized in tetradecylphosphocholine.

[f] Vmax was estimated from the literature figure.

[g]2-Isomer of chimyl alcohol.

malogens (e.g., 3-hexadec-1′-enyloxypropane-1,2-diol) abolished activity, whereas a double bond in the 2′,3′-position or 9′,10′-position (e.g., selachyl alcohol) of the alkyl chain almost restored the activity to that of the saturated ether, i.e., batyl alcohol (Table VII).

4. The 2-OH group in 1-alkyl glycerols was not strictly essential for activity as 3-hexa-decyloxypropan-1-ol was a good substrate, albeit with diminished activity (see above, second paragraph of this section).
5. The presence of an OH (and not more than an OMe group) in the C3 glycerol chain (or C2 ethylene glycol chain, Table VIII) was a strict requirement because octadecyl propyl ether and octadecyl ethyl ether were inactive.[138]
6. An ether group was essential as shown by hexadecan-1,2-diol which was inactive,[138] and cannot be replaced by an ester group, e.g., 1-stearoyl glycerol.
7. An hydroxyethyl group can replace a 1,2-dihydroxypropyl group, as 2-hexadecyloxy-and 2-octadecyloxy-ethan-1-ols were good substrates (Table VIII). Again the C16 ethers were better substrates than C18 ethers. 1,2-Bis-ethers with long chains were inactive (see 8 below).
8. A second substituent on the oxygen atom adjacent to the ether oxygen atom decreased the activity somewhat as long as it had a much shorter carbon chain, e.g., 3-hexadecyloxy-2-methoxypropan-1-ol, and the activity decreased further as the length of the second chain increased, even if the positions were altered, e.g., 3-hexadecyloxy-1-pentyloxypropan-2-ol, and decreased much further if an acetoxy group replaced a methoxy group as in 3-hexadecyloxy-2-acetoxypropan-1-ol (Table VIII). Activity was abolished if an acyl chain was long and the ether substituent short as in 1-palmitoyl-3-prop-2′-enyloxypropan-2-ol.[90]
9. At least one OH group (or OMe and sometimes OAc) was necessary for activity in addition to the long fatty alkyl ether group which was cleaved (see 6 above). However, activity was abolished when two long fatty ether groups were present as in 2,3-(or 1,3)-bis-hexadecyloxypropan-1(or 2)-ol (see 7 above and Table VIII).
10. 1,2,3-Tris-hexadecyloxypropane and 1-hexadecyloxy-2-methoxyethane (see 7 above) as well as 1-acetylthio-2-hexadecyloxyethane (Table IX) were inactive, whereas 1-octadecyloxy-D-mannitol was very weakly active (Table VIII).
11. Sulphur analogues, e.g., thiochimyl alcohol (3-hexadecylthiopropane-1,2-diol), thiobatyl alcohol, and 2-octadecylthioethan-1-ol were active substrates but with 10 to 29% of the activity of their oxygen analogues. However, a free SH group as in 2-hexadecyloxyethane-1-thiol abolished activity (Table IX).
12. Ether phospholipids were active substrates (with less than half the activity, e.g., of batyl alcohol) as long as the glycerol moiety had a free OH group as in *lyso*-PAF (1-*O*-hexadecyl-*sn*-glycerophosphocholine), or no OH as in 1-hexadecyloxypropane-3-phosphocholine (with much reduced activity); but replacement by an OMe group, as in 1-*O*-octadecyloxy-2-methoxypropane-3-phosphocholine or OAc as in PAF considerably reduced or abolished activity (Table X). Replacement of a phosphocholine by a phosphoethanolamine group (and an N,N,N-trimethylphosphohexanolamine group) in an active ether lipid substrate slightly increases activity, but replacement of a phosphoethanolamine by a phosphoglycol group decreases the activity (Table X). The respective *lyso*-phosphatidic acids are devoid of activity.[212]

J. Stereospecificity

The statement made by Kennedy and coworkers[1] that because the amount of fatty aldehyde in the monooxygenase reaction was 50% (on a molar basis) of the amount of 6-MePH_4 added, then it was possible that only one enantiomer of the pterin cofactor had reacted, was incorrect. Although the experimental details were correct the explanation was not. The low production of aldehyde was prob-

Table VII. "Apparent" Kinetic Data of Glyceryl-Ether Monooxygenase with Alkenyl Glyceryl Ethers

Lipid Substrate Propane-1,2-diol	*Cofactor mM [assay]*[a]	*Km μM*	*Vmax*[b] *nmol/min.mg*	*V/K*	*pH (°C)*	*Ref.*
Cis-RS-3-Hexadec-1′-enyl-oxy- (Plasmalogen)	6-$MePH_4$, 0.5 [C]	—	Inactive at 200 mM	—	8.8 (37)	90
Cis-RS-3-Octadec-2′-enyl-oxy-[c]	6-$MePH_4$, 0.5 [C]	—	8.2 at 0.5 mM	—	8.8 (37)	90
Cis-RS-3-Octadec-9′-enyl-oxy- (Selachyl alcohol)[d]	6-$MePH_4$, 0.5 [D]	39.7	47.2	1.2	7.5 (25)	138 220
	6-$MePH_4$, 0.5 [C]	—	7.7 at 1mM	—	8.8 (37)	90
Cis-RS-3-Octadec-9′-enyl-oxy-2-methoxy-[d]	6-$MePH_4$, 0.5 [C]	—	6.6 at 1 mM	—	8.8 (37)	90
Cis-RS-3-Octadec-9′-enyl-oxy-2-acetyl-[d]	6-$MePH_4$, 0.5 [C]	—	8.8 at 1 mM	—	8.8 (37)	90
RS-3-Prop-2′-enyloxy-1-hexadecanoyl-[d]	6-$MePH_4$, 0.5 [C]	—	Inactive at 10 mM	—	8.8 (37)	90

[a][D] = Direct spectrophotometric assay in Tris-HCl buffer with 0.08% Mega-10, [C] = coupled spectrophotometric assay in Tris-HCl with microsomes solubilized in 1.5% w/v of digitonin.

[b]Maximum velocity or rate at stated concentration of lipid substrate.

[c]Substrate solubilized in 1-lauroylpropane-3-phosphocholine.

[d]Substrate solubilized in tetradecylphosphocholine.

ably due to excessive oxidation of the pterin and probably the formation of large amounts of fatty alcohol and acid in the particular experiment (see Fig. 6), and not to the stereospecificity of one enantiomer of 6-$MePH_4$. We have confirmed this by measuring the initial rates of oxidation of 6*R*-(+)-, 6*S*-(−),[230,231] and *RS*-6- $MePH_4$ (0.44 mM) by glyceryl-ether monooxygenase with batyl alcohol (0.1 mM) in 0.1M Tris-HCl buffer pH ca. 7.5 (25°C) which were 19.28, 21.46, and 21.32 nmol/min.mg protein, respectively.[187] The small difference in apparent specificity between the enantiomers was marginal and may have been due to the slightly lower optical and chemical purity of the 6-*R*-enantiomer. It should be pointed out that the cofactor recycling enzyme dihydropteridine reductase (lower reaction in Scheme 1) also uses both enantiomers of 6-$MePH_4$ with almost equal efficiency.[230]

The stereospecificity of the ether lipid substrate was checked by Kennedy and coworkers,[1] who found that there was no detectable difference in the rates of oxidation of DL-batyl alcohol and D-batyl alcohol (natural, *S*-enantiomer). This was confirmed more recently with 2*R*-, 2*S*-, and 2*RS*-chimyl[31] and batyl[138] alcohols. When the kinetic parameters (Km, Vmax, and V/K) were determined using the direct spectrophotometric assay, the V/K values showed that the natural *S*-enantiomers were slightly better substrates than the non-natural *R*-enantiomers but it should be noted that the *R*-enantiomers were still very good substrates (Table VI). Also very little stereospecificity was observed between natural *R-lyso*-PAF (V/K = 2.5) and *RS-lyso*-PAF (V/K = 2.0) in which the natural *R*-enantiomer was marginally better than the racemic mixture (Table X).[138] It must be pointed out that in the continuous assay of the PAF compounds, where Vmax values only were compared, the velocity of the non-natural enantiomer was marginally faster than that of the natural enantiomer (Table X).[90] This was also the case with the results by the direct spectrophotometric assay (compare Vmax values only of natural *R-lyso*-PAF with *RS-lyso*-PAF in Table X). *RS-lyso*-PAF had an overall activity slightly lower than the natural isomer (from V/K values) because the Km values were taken into account. This shows that for the comparison of substrates with closely similar activities all the kinetic parameters need to be measured.

Table VIII. "Apparent" Kinetic Data of Glyceryl-Ether Monooxygenase with Alkoxy-Ethanols and Dialkoxy-Propanols

Lipid Substrate	*Cofactor mM [Assay]*[a]	*Km μM*	*Vmax*[b] *nmol/min.mg*	*V/K*	*pH (°C)*	*Ref.*
Ethan-1-ol						
2-Hexadecyloxy-[c]	6-MePH$_4$, 0.5 [D]	62.4	73.6	1.18	7.5 (25)	31
	6-MePH$_4$, 0.4 [R]	60.0	22.0	0.37	8.8 (37)	204
2-Octadecyloxy-	6-MePH$_4$, 0.5 [D]	31.3	22.7	0.73	7.5 (25)	31, 138
2-Octadecyloxy-1-hydroxy (acetaldehyde hydrate)	6-MePH$_4$, 0.5 [D]	—	29.4 at 0.2 mM	—	7.5 (25)	138
Propan-1 (or 2)-ol						
3-Hexadecyloxy-2-methoxy-	6-MePH$_4$, 0.5 [D]	253	16.8	0.07	7.5 (25)	31
	6-MePH$_4$, 0.5 [D]	225[d]	24.1[d]	0.11[d]	7.5 (25)	31
3-Hexadecyloxy-2-acetoxy-	6-MePH$_4$, 0.5 [C]	—	5 at 1 mM[e]	—	8.8 (37)	90
3-Hexadecyloxy-1-methoxy-	6-MePH$_4$, 0.5 [D]	26.5	26.5	1.0	7.5 (25)	31
	6-MePH$_4$, 0.5 [C]	—	15 at 0.5 mM[e]	—	8.8 (37)	90
3-Hexadecyloxy-1-prop-2′-enyloxy-	6-MePH$_4$, 0.5 [C]	—	14.1 at 0.5 mM[d]	—	8.8 (37)	90
3-Hexadecyloxy-1-pentyloxy-	6-MePH$_4$, 0.5 [C]	—	7.3 at 0.5 mM[d]	—	8.8 (37)	90
3-Hexadecyloxy-1-octyloxy-	6-MePH$_4$, 0.5 [C]	—	1.1 at 0.5 mM[d]	—	8.8 (37)	90
3,2-*Bis*-hexadecyloxy-	6-MePH$_4$, 0.5 [D]	—	Inactive at 0.2 mM[d]	—	7.5 (25)	31
3,1-*Bis*-hexadecyloxy-	6-MePH$_4$, 0.5 [D]	—	Inactive at 0.2 mM[d]	—	7.5 (25)	31
2-Octadecyloxy-3-prop-2′enyloxy-	6-MePH$_4$, 0.5 [C]	—	6 at 1 mM[e]	—	8.8 (37)	90
2-Octadecyloxy-3-tetradecyloxy-	6-MePH$_4$, 0.5 [C]	—	Inactive at 10 mM[e]	—	8.8 (37)	90
3-Octadecyloxy-2-methoxy-	6-MePH$_4$, 0.5 [C]	—	6.6 at 1 mM[e]	—	8.8 (37)	90
3-Octadecyloxy-2-acetoxy-1-methoxypropane	6-MePH$_4$, 0.5 [D]	—	17.8 at 0.16 mM	—	7.5 (25)	138
1,2,3-*Tris*-hexadecyloxy-propane	6-MePH$_4$, 0.5 [D]	—	Inactive at 0.2 mM	—	7.5 (25)	31
1-Octadecyloxy-D-mannitol	6-MePH$_4$, 0.5 [C]	—	0.3 at 10 mM	—	8.8 (37)	90

[a]Assays as in Table VI

[b]Maximum velocity or rate at stated concentration of lipid substrate.

[c]1-Hexadecyloxy-2-methoxyethane was inactive at 0.2 mM.

[d]Substrate solubilized in tetradecylphosphocholine.

[e]Substrate solubilized in 1-lauroylpropane-3-phosphocholine.

The absence of strong specificity by the monooxygenase for the configuration at C2 of the glycerol moiety was *not* reflected at C1′ of the alkyl ether chain—the carbon atom that is directly affected in the oxygen transfer during the oxidation. By measuring tritium release from (2*RS*, 1′*R*)-3-[1′-^{3}H]- and (2*RS*, 1′*S*)-3-[1′-^{3}H]- hexadecyloxypropane-1,2-diols (chimyl alcohols) in the monooxygenase reaction and measuring the tritiated water produced, we had found that the 1′-pro-*S* tritium atom was released considerably faster than the 1′-pro-*R* tritium. The small amount of tri-

Table IX. "Apparent" Kinetic Data of Glyceryl-Ether Monooxygenase with Sulphur Compounds

Lipid Substrate	*Cofactor mM [Assay]*[a]	*Km μM*	*Vmax*[b] *nmol/min.mg*	*V/K*	*pH (°C)*	*Ref.*
2-Hexadecylthioethan-ol-[c]	6-MePH_4, 0.5 [D]	228	49.9	0.22	7.5 (25)	31
2-Octadecylthioethan-1-ol-[c]	6-MePH_4, 0.5 [D]	521	16	0.03	7.5 (25)	31
2-Hexadecyloxyethane-1-thiol	6-MePH_4, 0.5 [D]	—	Inactive at 0.2 mM	—	7.5 (25)	31
1-Acetylthio-2-hexadecyloxy-ethane	6-MePH_4, 0.5 [D]	—	Inactive at 0.4 mM	—	7.5 (25)	31
RS-Hexadecylthiopropane-1,2-diol (Thiochimyl alcohol).[c]	6-MePH_4, 0.5 [D]	121	43.9	0.36	7.5 (25)	31
RS-Octadecylthiopropane-1,2-diol (Thiobatyl alcohol).[c]	6-MePH_4, 0.5 (D)	428	24.6	0.06	7.5 (25)	31
	6-MePH_4, 0.5 [C]	—	5.5 at 1 mM	—	8.8 (37)	90

[a]Assay as in Table VI.

[b]Maximum velocity or rate at stated concentration of lipid substrate.

[c]Substrate solubilized in tetradecylphosphocholine.

Table X. "Apparent" Kinetic Data of Glyceryl-Ether Monooxygenase with Glycerol Phosphates

Lipid Substrate[a]	*Cofactor mM [Assay]*[b]	*Km μM*	*Vmax*[c] *nmol/min.mg*	*V/K*	*pH (°C)*	*Ref.*
1-*O*-Hexadecyl-*sn*-glycero-3-PC	6-MePH_4, 0.5 [D]	137	33.7	0.25	7.5 (25)	138[d]
(Natural *R-lyso*-PAF)	6-MePH_4, 0.5 [C]	—	5.5 at 0.5mM	—	8.8 (37)	90
	6-MePH_4, 0.5 [C]	—	4.1 at 0.5 mM	—	8.8 (37)	89
	6,7-Me_2PH_4, 1.0 [R]	—	6.9 at 20 μM	—	8.8 (37)	91
	6-MePH_4, 1.0 [R]	—	6.3 at 0.5 mM	—	8.8 (37)	212
RS-Lyso-PAF	6-MePH_4, 0.5 [D]	173	34.1	0.20	7.5 (25)	138
S-Lyso-PAF (Non-natural)	6-MePH_4, 0.5 [C]	—	6.6 at 0.5 mM	—	8.8 (37)	90
	6-MePH_4, 0.5 [C]	—	4.9 at 0.5 mM	—	8.8 (37)	89
	6-MePH_4, 1.0 [P]	—	6.6 at 0.5 mM	—	8.8 (37)	212
1-Hexadecyloxypropane-3-PC	6-MePH_4, 0.5 [C]	—	5.7 at 0.5mM	—	8.8 (37)	90
1-Hexadecyloxypropane-3-PA	6-MePH_4, 1.0 [P]	—	6.4 at 0.5 mM	—	8.8 (37)	212
1-Hexadecyloxypropane-2-PC	6-MePH_4, 0.5 [C]	—	3.6 at 0.5 mM	—	8.8 (37)	90
1-*O*-Hexadecyl-*sn*-glycero-3-PH	6-MePH_4, 0.5 [C]	—	3.7 at 0.5 mM	—	8.8 (37)	90
1-Hexadecyloxypropane-3-PG	6-MePH_4, 1.0 [P]	—	2.5 at 0.5 mM	—	8.8 (37)	212
1-*O*-Hexadecyl-2-*O*-acetyl-*sn*-glycero-3-PC (PAF)	6-MePH_4, 0.5 [C]	—	1.5 at 1.0 mM	—	8.8 (37)	90
	6,7-Me_2PH_4, 1.0 [R]	—	0.59 at 20 μM	—	8.8 (37)	91
1-*O*-Methyl-2-*O*-octadecyl-*rac*-glycero-3-PC	6-MePH_4, 1.0 [P]	—	Inactive at 0.5 mM	—	8.8 (37)	212
1-*O*-[^{3}H]-Octadecyl-glycero-3-PC	6-MePH_4, 0.5 [C]	—	3.2 at 0.5 mM	—	8.8 (37)	89
1-*O*-[^{3}H]-Octadecyl-*sn*-glycero-3-PC	6-MePH_4, 1.0 [P]	—	4.1 at 0.5 mM	—	8.8 (37)	212
R-1-*O*-Octadecyl-2-*O*-methyl-glycero-3-PC	6-MePH_4, 1.0 [P]	—	Inactive at 0.5 mM	—	8.8 (37)	212
S-1-*O*-Octadecyl-2-*O*-methyl-glycero-3-PC	6-MePH_4, 1.0 [P]	—	Inactive at 0.5 mM	—	8.8 (37)	212
RS-1-*O*-Octadecyl-2-*O*-methyl-glycero-3-PC	6-MePH_4, 0.5 [C]		Inactive at	—	8.8 (37)	90
	6-MePH_4, 1.0 [P]		2 and 0.5 mM	—	8.8 (37)	212
2-Tetradecyl-*rac*-glycero-3-PA	6-MePH_4, 1.0 [P]	—	5.8 at 0.5 mM	—	8.8 (37)	212

[a]PC = phosphocholine, PA = phosphoethanolamine, PG = phosphoglycol, PH = phospho-N,N,N-trimethyl-hexanolamine.

[b]Assays as in Table VI and [P] refers to phosphate estimation in phospholipids (Ref. 212).

[c]Maximum velocity or rate at stated concentration of lipid substrate.

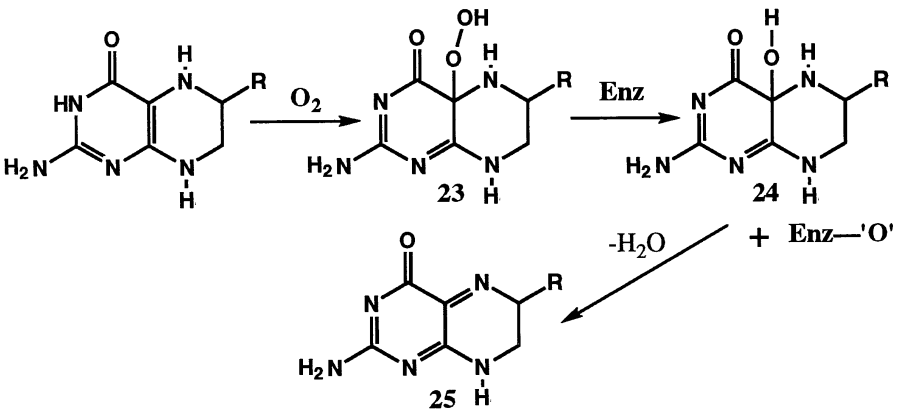
[d]Incorrectly stated as S in the reference.

tium released in the latter may well be due to incomplete stereospecificity during the syntheses of the tritiated substrates. Thus it was the pro-*S* hydrogen atom in the 1′-methylene group (adjacent to the ether oxygen atom) that was released, in preference to the pro-*R* hydrogen atom, during the oxidation. This was confirmed by measuring the kinetic parameters of unlabeled-(2*RS*)-3-, (2*RS*, 1′*R*)-3-[1′-^{2}H]-, (2*RS*, 1′*S*)-3-[1′-^{2}H]- and (2*RS*, -3-[1′,1′-$^2H_2$]- hexadecyloxypropane-1,2-diols. The apparent Km values (49.4, 53.7, 49.3, and 54.0 μM, respectively) were about the same, but the apparent maximum velocities (nmol/min.mg protein) of the first two substrates (37.5 and 37.5) were similar but faster than those of the latter two substrates (22.5 and 23.6), respectively. This was consistent with the relative difficulty in breaking the C—D bond compared with a C—H bond and the replacement of the pro-*S* hydrogen atom by the hydroxy group. The primary deuterium isotope effect was ca. 1.6.[140,232]

The formation of the carbon–oxygen bond can occur with retention or inversion of configuration at C1′, but this cannot be deduced from these experiments and should await the isolation of the intermediate hemiacetal (see Scheme 1). However, by analogy with the known stereospecificities of hydroxylations by mixed function monooxygenases, all of which occurred with retention of configuration,[233–237] the hydroxylation by glyceryl-ether monooxygenase can tentatively be said to occur with retention of configuration at C1′ of the glyceryl ethers.

4. MECHANISM OF THE GLYCERYL-ETHER MONOOXYGENASE REACTION

There is no evidence as yet that there are isoenzymes of glyceryl-ether monooxygenase. The monooxygenase from rat liver which hydroxylates the carbon atom on the alkyl chain attached to the ether oxygen atom of monoalkyl ethylene glycols,[204,238] and alkoxy *lyso*-glycerophosphocholines and ethanolamines[90,91,212] were shown to be apparently the same enzyme which hydroxylates alkyl glyceryl ethers (Secs III-F and -I). However, the data does not exclude the possibility that these isoenzymes have closely similar physical and mechanistic properties. Evidence was presented that the monooxygenase did not have properties similar to cytochrome P450,[204] and by analogy with the aromatic aminoacid hydroxylases which use tetrahydropterin cofactors,[6–8] the monooxygenase has most likely a nonhaem transition metal at the active site to mediate oxygen transfer and catalysis. Metal chelating agents do not strongly inhibit the monooxygenase, perhaps by removing the metal,



Scheme 7. Fate of the oxygen molecule in the mixed function oxidase glyceryl-ether monooxygenase.

to the same extent as they do with phenylalanine hydroxylase (Sec. III-F). The weaker inhibition may reflect the difficulty for the chelating agent to remove the metal from the monooxygenase since (unlike phenylalanine hydroxylase) the protein is embedded in the microsomal phospholipid membrane. The loss of activity when the natural lipid membrane was removed by detergents could be partly due to loss of the metal.

The tetrahydropterin cofactor is known to react with oxygen[239] to form an intermediate 4a-hydroperoxy-tetrahydropterin **23** (never actually isolated)[240] which could lose hydrogen peroxide, or bind at the active site of the monooxygenase and transfer one oxygen atom to the metal in the enzyme and is then released as 4a-hydroxy-tetrahydropterin **24.** This derivative then loses a water molecule to form the *quinonoid* 7,8-dihydro(6*H*)-pterin **25** (Scheme 7) which is then reduced by dihydropteridine reductase (Scheme 1). There is strong evidence from studies of phenylalanine hydroxylase[241] for the intermediate 4a-hydroxypterin **24,**[241–244] and a 4a-hydroxy-dehydratase which catalyses the dehydration **24** to **25** *in vivo*. The dehydration reaction can also occur rapidly by nonenzymic acid-base catalysis.[243] We have looked for the intermediate **24** in the monooxygenase reaction but have not yet seen it spectroscopically.[187] However, these reactions were not rate limiting in the acquisition of the data in Tables V to X as they did not appear to interfere with the kinetic measurements.

Preliminary structure-activity relationship data for the ether lipid substrates had lent support to four binding pockets at the active site of the monooxygenase,[220] *viz:* a strong large hydrophobic pocket to bind the long alkyl ether chain, an ether oxygen (sulphur) binding site most probably involving a metal (as no reaction occurred in the absence of the ether oxygen atom [or thioether sulphur atom]), a site to bind an OH group (or at least an OMe group) possibly also to the metal, and a site to which a second OH group could bind or a phosphocholine or ethanolamine group, but not a strongly acidic group as a phosphate in phosphatidic acids.[212]

The present more extensive structure-activity relationship data (Tables VI to X) give a still better picture in which the hydrophobic pocket is formed between the protein and the phospholipid layer because the optimal length C15 to C18 (Fig. 7) of the fatty ether chain which is similar to the length of the fatty chains in phospholipid membranes. Removal of the membrane results in loss of activity unless detergents or lipids with long carbon chains were used to reconstitute the enzyme. A diagrammatic picture of the active site of glyceryl-ether monooxygenase which accommodates most of the data in Tables VI to X is displayed in Figure 8.

The studies of detergents on monooxygenase activity have shed some light on the nature of the lipid substrates in aqueous solution.[138,191,194] The substrate is in micellar form in solution, either in mixed detergent micelles as in batyl alcohol—Mega-10 or substrate micelles as with the water soluble *lyso*-PAF (3-hexadecyloxy-2-hydroxypropyl-1-phosphocholine) which exists almost entirely as micelles in water at concentrations above the CMC (3.3 μM).[194] The detergents behave like water molecules normally do with soluble substrates which have to release the water molecules before they can bind at the active site.

The mechanism of the monooxygenase reaction can be postulated as follows:

a) The tetrahydropterin cofactor reacts with oxygen dissolved in the aqueous buffer to form a 4a-hydroperoxy-pterin which binds to the enzyme. It transfers one oxygen atom to the metal in the enzyme and is then released to the aqueous medium as a 4a-hydroxypterin. This is rapidly dehydrated by acid-base catalysis or by a dehydratase (see above and Scheme 7).[240,244]
b) The lipid substrate in micelle or mixed micelle form approaches the "oxidized" active site whereby the fatty ether chain of one molecule of the substrate binds to the hydrophobic pocket, while being released from the micelle, followed by the binding of other parts of the substrate to appropriate pockets.
c) The substrate is now in place for the stereospecific pro-H_S attack by the oxygen atom on the

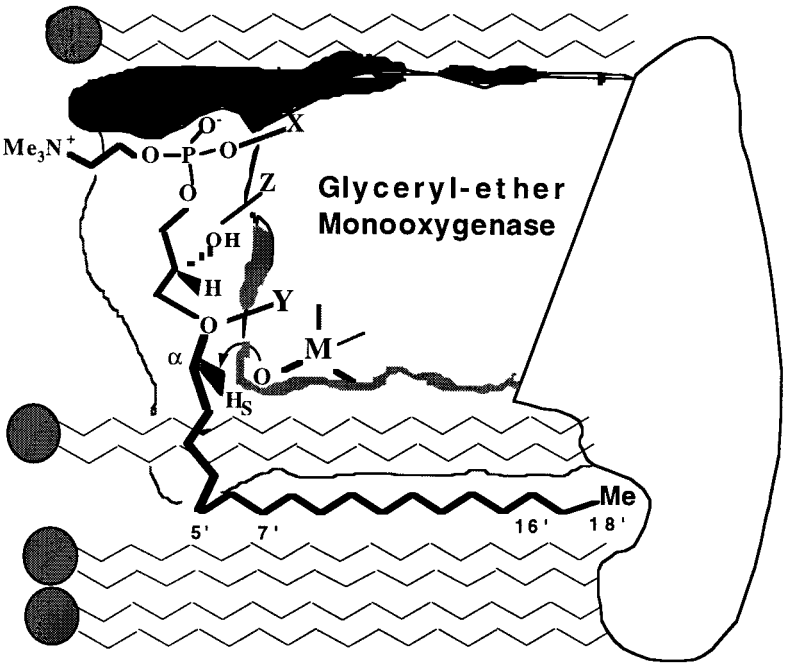


Figure 8. Proposed model of the active site of glyceryl-ether monooxygenase.

metal to C1′ of the alkyl chain, possibly with retention of configuration, and the released pro-*S* hydrogen atom or some other labile hydrogen atom in the protein, which protonates the oxygen atom to form the OH group.

d) The hydroxylated substrate is then released and undergoes rapid acid-base or enzyme catalyzed hydrolysis to the fatty aldehyde and glycerol.

The true mechanism will have to wait for an x-ray structure of crystals of the monooxygenase in its natural phospholipid membrane.

5. FUTURE STUDIES

A background on glyceryl ethers, their importance in ether lipid metabolism, as mediators of cell responses, in neoplasms and cancer therapy has been presented in order for the readers to appreciate the importance of glyceryl-ether monooxygenase. This monooxygenase has not been studied to the same extent as equally important enzymes have been investigated. However, the basic knowledge for it has been laid out, and very good assays are now available for its detection and kinetic investigations. Much more is yet to be revealed about this enzyme particularly because so much is already known about related monooxygenases with very similar reactions, the aromatic aminoacid hydroxylases. Studies which were successful for these hydroxylases should be applied to glyceryl-ether monooxygenase.

The pure protein needs to be studied further and better ways of purifying it have to be devised. Its physical properties and enzymology in the absence and presence of its natural phospholipid mem-

brane, as well as with other lipids and detergents, can then be studied in great detail. The nature of the metal has to be determined and the apo-protein needs to be reconstituted with other metals and the activities should be evaluated. Structure-activity relationships of the cofactors and lipid substrates will have to be re-examined with reconstituted pure enzyme, and the activities and stereospecificities can then be compared with the present data. The formation of a 4a-hydroxypterin intermediate and the possible action of a dehydratase enzyme should be looked at in the light of current data from work with phenylalanine hydroxylase.[242–244]

The aminoacid sequence of the pure protein will have to be determined, either partially or completely, and DNA probes corresponding to short aminoacid sequences should be synthesized in order to screen a cDNA library to isolate the monooxygenase cDNA. This cDNA will have to be sequenced, and cloned into an appropriate vector and the protein expressed in quantity in some appropriate microorganism, e.g., as was done in the case of human dihydropteridine reductase.[245] The monooxygenase protein can then be assessed enzymically in the absence and presence of lipids and detergents, and compared with the work proposed above.

The cDNA can be used to identify its respective messenger RNA and used to determine the levels of expression of the monooxygenase in various tissues. It is already known that tissues which have low levels of monooxygenase, e.g., neoplastic tissue, have high levels of glyceryl ethers.[86,91] This has been used to advantage for developing glyceryl ether analogues as anti-cancer agents (Sec. I-D). The cDNA probe can be used to show how the monooxygenase is regulated in cells and how its function affects the biochemical (e.g., in peroxisomes) and physiological processes. The cDNA or synthetic oligonucleotide sequences of it can be used to isolate glyceryl-ether monooxygenase genomic DNA which will have to be sequenced to determine the number and sequences of the introns. Segments of the DNA can be used to identify the chromosome on which the enzyme appears, as well as its position on the chromosome. Much of this work would have to be done on human DNA because it would be very useful for identifying any genetic disorders which would be associated with mutations in the monooxygenase, i.e., linkage studies.

With appropriate experimentation it should be possible to express high levels of protein and produce it in large amounts for crystallization, with and without the lipid membrane, and the three dimensional structure determined by x-ray studies. A strong inhibitor, very similar in structure to the ether lipid substrates, should be found which could be infused into the crystals to provide the crystal structure of the binary complex. The structure should then reveal the finer details of the active site and the reaction mechanism.

The effect of ether lipid metabolism in peroxisomes (see above) and in genetic disorders requires attention, e.g., as in Zellweger's syndrome (Sec. I-D). Also its importance in the synthesis and formation of myelin should be investigated as this is associated with classical phenylketonuria and other disorders of myelin degeneration, e.g., multiple sclerosis. The effects of dihydropetridine reductase and/or tetrahydrobiopterin and/or pterin dehydratase deficiencies on ether lipid metabolism in affected children will need to be evaluated in this light, as these enzymes and the cofactor are involved in the action of glyceryl-ether monooxygenase.

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