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Glyceryl-ether Monooxygenase [EC 1.14.16.5] Part VII Effects of Alkyl Phosphocholines and Acyl L- α -lyso-Phosphatidylcholines Detergents on Enzyme Activity

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Summary

The effects of the ionic detergents dodecyl, tetradecyl, hexadecyl and octadecyl phosphocholines, and dodecanoyl, tetradecanoyl, hexadecanoyl and octadecanoyl L- α -lyso-phosphatidylcholines on the enzyme activity of glyceryl-ether monooxygenase are reported. All these detergents inhibit the monooxygenase at concentrations above a threshold value which is above the relative critical micelle concentrations implying that the micelles interact passively with the microsomal membrane below the threshold concentration. The kinetics of inhibition by the C14 and C16 detergents above the threshold concentrations have been selected for study and are found to be competitive with apparent K_i values in the range 316-365 μ M. The apparent K_m and V_{max} values for RS-batyl alcohol with 6-MePH₄ as cofactor in the presence of the choline detergents (at or below the threshold inhibiting concentrations) are in the range 24-44 μ M and 46-64 nmoles/min.mg of monooxygenase protein respectively. These data are compared and discussed with data reported for the monooxygenase in the presence of the non-ionic detergent Mega-10.

Keywords : Glyceryl-ether monooxygenase, Activity, K_m and V_{max} values, Inhibition, Alkyl phosphocholines, Acyl L- α -lyso-phosphatidylcholine, Critical micelle concentration,

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Enzymes : Bovine liver catalase [EC 1.11.1.6]. Dihydropteridine reductase (DHPR), NAD (P)H : *q*-7, 8-dihydropterin oxidoreductase [EC 1.6.99.7]. Glyceryl-ether monooxygenase, 1-alkyl-sn-glycerol, tetrahydropterin : oxygen oxidoreductase [EC 1.14.16.5]. Abbreviations : 6-MePH₄=RS-6-methyl-5, 6, 7, 8-tetrahydropterin; lyso-PAF=3-(1-hexadecyloxy)-2-hydroxypropane-1-phosphocholine; Mega-10=N-methyl-N-decanoylg-lucamide; 12PC=dodecyl phosphocholine; 14 PC=tetradecyl-phosphocholine; 16 PC=hexadecyl phosphocholine; 18 PC=octadecyl phosphocholine; 12 LPC=dodecanoyl (lauroyl) L- α -lyso-phosphatidylcholine; 14 LPC=tetradecanoyl (myristoyl) L- α -lyso-phosphatidylcholine; 16 LPC=hexadecanoyl (palmitoyl) L- α -lyso-phosphatidylcholine; 18 LPC=octadecanoyl (stearoyl) L- α -lyso-phosphatidylcholine; CMC=critical micelle concentration.

Introduction

The rat liver microsomal enzyme, glyceryl-ether monooxygenase, hydroxylates the α -methylene group of the alkyl sidechain (e.g. C16, C18) of 1-glyceryl-ethers (1) in the presence of oxygen and a tetrahydropterin (2) (Scheme 1)(1). A direct spectrophotometric assay for this enzyme has been developed and has been very useful for studying the kinetic parameters of a variety of substrates (2, 3) and was used to determine some important aspects of the stoichiometry (4). The major difficulty that had to be overcome for this assay was the general insolubility of the ether-lipid substrates in water or aqueous buffers. This has been

achieved by using detergents in water or aqueous buffers. Solubilisation of the ether-lipid stock solutions containing detergents was completed by gentle warming and/or sonication for short periods, and the clear solutions were stable for various periods of time (3). In all cases these solutions were clear before assaying. A most satisfactory detergent for this purpose was the non-ionic amide Mega-10 (5) which produced aqueous solutions of ether-lipids that were clear for extended periods.

The solutions did become turbid, however, after periods of time but could be made clear again by gentle warming, or sonication, without in any way affecting the enzyme activity with these substrates. Solutions of the substrate 3-1'-octadecylpropan-1, 2-diol (batyl alcohol, 1, $n=16$) in Mega-10 had maximum enzyme activities with concentrations of Mega-10 up to *ca* 6 mM (0.2%). However, the enzyme was inhibited at higher concentrations of detergent. Kinetic studies revealed that Mega-10 was inhibiting enzyme activity in a *non-competitive* manner, and because inhibition occurred at concentrations above the CMC of Mega-10 (7.15 mM), it was deduced that the inhibiting species were Mega-10 micelles. Since *RS*-

lyso-PAF was an active substrate well above its CMC value in the *absence of any detergent*, and batyl alcohol was active in Mega-10 solutions below the CMC of Mega-10, it was concluded that the active substrates were in micelle form for *lyso*-PAF and in mixed micelle form for batyl alcohol-Mega-10 solutions (6).

In the present work we describe studies with ionic detergents related to phosphocholines, namely alkyl phosphocholines (6-9) and acyl L- α -lyso-phosphatidylcholines (10-13) with C12, C14, C16 and C18 fatty chains. These cholines were investigated because they were similar in structure to membrane lipids without monooxygenase activity, and because tetradecyl phosphocholine (8) and 1-lauroylpropanediol-1, 3-phosphocholine were used to solubilise 1-alkyl glyceryl-ethers for the coupled (continuous spectrophotometric) assay for glyceryl-ether monooxygenase with dihydropteridine reductase in the presence of NADH developed by Eibl and coworkers (7, 8). When batyl alcohol was used as substrate and 6-MePH₄ as cofactor these detergents behaved like Mega-10 except that they inhibited the monooxygenase in a *competitive* manner with only minor differences in apparent *K_i* values between the two sets of detergents. Also, unlike with Mega-10, the concentration of choline detergent at which monooxygenase inhibition occurred was well above the CMC of the respective cholines.

Materials and Methods

RS-Batyl alcohol (*RS*-3-1'-octadecyloxypropan-1, 2-diol, referred to as batyl alcohol), Mega-10, lauroyl-, myristoyl-, palmitoyl- and stearoyl-L- α -lyso-phosphocholines, dodecan-1-ol, tetradecan-1-ol, hexadecan-1-ol and octadecan-1-ol, and bovine catalase (Sigma), ethanolamine (Fluka) and magnesium 8-anilino-1-naphthalene sulphonate (Kodak) were purchased and *RS*-6-methyl-5,6,7,8-tetrahydropterin (6-MePH₄) hydrochloride was prepared as before (9). The microsomal glyceryl-ether monooxygenase was prepared from male Wistar rat livers as before (3) and had consistent specific activities of *ca* 27 nmoles/min.mg protein in the standard assay (below). A double beam UV-VIS spectrometer (Cary 118) was used for kinetic measurements with the cuvette holders thermostated at 25°C, and fluorescence measurements were obtained on a Perkin Elmer LS5 luminescence spectrometer. Critical micelle concentrations were determined as before using magnesium 8-anilino-1-naphthalene sulphonate as fluorescent dye (6, 10). IR spectra were in KBr discs and measured on a Perkin Elmer 1600 Series FTIR spectrometer, and ¹H NMR spectra were measured on a Varian Gemini 300 spectrometer

at 300 MHz.

Syntheses of alkyl phosphocholines

The synthesis described by Eibl and Engel (11) for hexadecyl phosphocholine (16 PC) was adopted with slight modifications for the preparation of dodecyl (12 PC), tetradecyl (14 PC), hexadecyl (16 PC) and octadecyl (18 PC) phosphocholines, and details for 14 PC are given here. To POCl₃ (14.7 g, 96 mmoles) in dry THF (8 ml) was added a solution of Et₃N (8.9 g, 88 mmoles) and tetradecan-1-ol (17.1 g, 80 mmoles) in dry THF (80 ml) in an ice bath (<15°C) and stirred at room temperature for 1 h. A solution of ethanolamine (5.9 g, 97 mmoles) and Et₃N (19.6 g, 194 mmoles) was added to the above mixture keeping the temperature below 30°C, and then stirred at room temperature for 1 h followed by 40°C for 1 h. The mixture was filtered and the filtrate was evaporated *in vacuo*. The residue was dissolved in acetic acid (30 ml) and water (15 ml) was added. The mixture was heated at 70°C for 30 min and cooled. Acetone (600 ml) was added and the mixture was stirred at room temperature for 15 h. The supernatant was removed by decantation and the residue was stirred again with acetone (600 ml) for 3 h. The colourless solid was collected, washed with acetone and air-dried to give tetradecyl phospho-ethanolamine (22.1 g, 82%). This ethanolamine (6.74 g, 20 mmoles) was dissolved in CH₂Cl₂ (60 ml) and propan-2-ol (24 ml), and was warmed to 40°C. A solution of K₂CO₃ (2.76 g, 20 mmoles) in water (4 ml) was added to the mixture followed by Me₂SO₄ (2.3 g, 18 mmoles) in propan-2-ol (1 ml) at 40°C over a period of 1 h, and then stirred at 40°C for a further hour. The addition of K₂CO₃ and Me₂SO₄ was repeated twice more using the same quantities and then again once more using half the quantities but the same time period. After the mixture was cooled to room temperature without stirring, the upper layer was collected and evaporated *in vacuo*. The residue was extracted with EtOH and the extract evaporated *in vacuo*. The residue was extracted with warm propan-2-ol and again the extract was evaporated to dryness *in vacuo*. The residue was washed twice with acetone at room temperature with stirring, and the solid was collected, washed with acetone and air-dried to give pure 14 PC (6.1 g, 80.5%), m.p. >240° decomp. (Found : N, 3.10; P, 7.64. C₁₉H₄₂NO₄P. 4H₂O requires N, 3.33; P, 7.36%). Similarly prepared were 12 PC with m.p. 240° decomp. (Found : N, 3.18; P, 7.41. C₁₇H₃₈NO₄P. 6H₂O requires N, 3.27; P, 7.23%); 16 PC with m.p. 240° decomp. (Found : N, 2.52; P, 6.31. C₂₁H₄₆NO₄P. 11H₂O requires N, 2.79; P, 6.16%); and 18 PC with m.p. 240° decomp. (Found : N, 2.55; P, 5.93.

C₂₃H₅₀NO₄P. 10H₂O requires N, 2.77; P, 6.13%). The IR spectra all had ν : 970, 1065, 1087 (s, P-O-Calkyl), 1224 (P=O), 1472, 1464 (OH bend), 2852 and 2923 (CHstr) and 3446 (OH from H₂O) cm.⁻¹

All four alkyl phosphocholines were soluble in CDCl₃ and had 300 MHz ¹H NMR spectra which were almost identical to each other except for the sum of the integrals of the methylene groups of the long alkyl chain other than the 1- and 2-CH₂ groups; the δ values were 0.88 t / 6.8 Hz ω -CH₃ of the alkyl chain; 1.25 s 3 to ω -1 CH₂; 1.56 m 2-CH₂; 3.35 s N (CH₃)₃; 3.77 m 1-CH₂-O and 1'-CH₂O-, and 4.26 m 2'-CH₂-N ppm downfield from tetramethylsilane. Water in the samples appeared at 4.19, 4.33, 3.77 and 4.29 ppm for 12 PC, 14 PC, 16 PC and 18 PC respectively.

Enzyme activity-detergent concentration profile

The standard assay using 0.1 mM batyl alcohol as substrate and 0.5 mM 6-MePH₄ as cofactor (final concentration and almost saturating) was adopted (3, 6). Stock solutions (100 μ l, 10 $\times$ concentration) of the alcohol were made in water containing the smallest concentration of choline detergent to solubilise it were added to the reaction cuvette containing 670 μ l of Tris-catalase (100 μ l of M Tris-HCl pH 7.5, 100 μ l of 1 mg/ml catalase and 470 μ l of water), detergent solution in water (100 μ l, adjusted to the required concentration) and microsomal enzyme (30 μ l, of 54 or 12.8 mg/ml protein). The blank cuvette contained the same ingredients without batyl alcohol. The analytical wavelength was set at 354 nm and the maximum recorder deflection was set at 0.02 absorbance units. At this wavelength the reaction extinction coefficient was 3440 M⁻¹cm.⁻¹ i.e. one absorbance unit was 0.291 μ moles.ml⁻¹ for a 1 cm cell pathlength (3). When a steady baseline was obtained (2~5 min), the reaction was initiated by adding simultaneously 5 mM 6-MePH₄ (100 μ l) and the rate of change of absorbance was determined from the recorder trace. The mean of reproducible runs were then plotted against the detergent concentrations (Figure 1 and 2). Prior to each set of runs, enzyme activity was measured using the standard assay (6) with Mega-10 as detergent and if that had changed from the original value all the rates were normalised to this value. Enzyme activity did not usually vary from day to day and never from the first to the last measurement during a set of runs.

Measurement of the kinetic parameters

Measurements were carried out as above except that the batyl alcohol concentrations were varied and

Table 1. Critical Micelle concentrations of alkyl phosphocholines and acyl L- α -lyso-phosphatidylcholines in the presence and absence of 0.1 mM batyl alcohol in 0.1 M Tris-HCl pH 7.5 and 22°C~25°C.

Phosphocholines	CMC (μ M)		L- α -lyso-phosphatidylcholines	CMC (μ M)	
	—	0.1 mM Batyl alcohol		—	0.1 mM Batyl alcohol
Dodecyl (12 PC)	622 (0.020%)	482 (0.016%)	Lauroyl (12 LPC)	470 (0.019%)	395 (0.016%)
Tetradecyl (14 PC)	53 (0.0018%)	58 (0.0020%)	Myristoyl (14 LPC)	59 (0.0026%)	77 (0.0034%)
Hexadecyl (16 PC)	7.2 (0.0003%)	22 (0.0008%)	Palmitoyl (16 LPC)	2.8 (0.00013%)	8 (0.00037%)
Octadecyl (18 PC)	3.5 (0.00014%)	3.0 (0.00012%)	Stearoyl (18 LPC)	0.2 (0.00001%)	— ^a
Mega-10 ^b	7150 (0.25%)	5720 (0.20%)			

^a0.1 mM Batyl alcohol could not be dissolved in the small amount of 18 LPC required for the initial solution^bFrom ref. 3

the concentrations of choline detergents were set at or near the values which gave maximum rates (see Figure 1 and 2) for each detergent. A computer program was used to evaluate the apparent K_m and V_{max} values (12).

Inhibition measurements

These were carried out as before (6) (see also above) and the data were evaluated using a computer program (12) to fit competitive, noncompetitive and mixed inhibition equations. The equations which gave apparent K_i values with the smallest errors were taken as the correct type of inhibition and agreed very well with Lineweaver-Burk plots (e.g. Figure 3).

Discussion

Critical micelle concentration of choline detergents

The alkyl phosphocholines and acyl L- α -lyso-phosphatidylcholines have ionic detergent properties because they have long fatty unbranched hydrocarbon chains with polar groups at one end. They are capable of forming micelles and we have measured the critical micelle concentrations (CMC) for the two sets of choline detergents in 0.1 M Tris-HCl pH 7.5 as well as in the presence of 0.1 mM batyl alcohol (Table 1). The conditions chosen were appropriate for other studies discussed here. In each set of detergents the CMC values decreased rapidly with increase of hydrocarbon chain length from C12 to C18. In 0.1 M Tris-HCl the decrease in CMC values for the alkyl phosphocholines was about two orders of magnitude,

and for the phosphatidylcholines it was greater than three orders of magnitude. It should be noted that the CMC values are not sharp concentrations but are concentration ranges that can be as high as $\pm 10\%$ of the extrapolated value. With high CMC values as with 12 PC this range was $\pm 80 \mu$ M, whereas with 18 PC the range was smaller than *ca* 0.5 μ M. It was generally found that as the CMC value decreased for a particular set of detergents the change in fluorescence was sharper as the CMC value was reached, e.g. the range was of the order of $<5\%$ of the CMC value for C18 fatty chains. In the presence of 0.1 mM batyl alcohol the CMC values were decreased for 12 PC and 12 LPC but increased progressively as the hydrocarbon chains increased. We had shown⁶ that the CMC of Mega-10 in Tris-HCl (pH 7.5) also decreased from 7.15 mM to 5.72 mM when the detergent contained 0.1 mM batyl alcohol and that below 5.72 mM the latter solution contained mixed batyl alcohol/Mega-10 micelles. It can be inferred that solutions of batyl alcohol and the choline detergents with concentrations below the CMC, batyl alcohol formed mixed micelles with the detergents. This is further supported by the almost insolubility of batyl alcohol in aqueous buffer; sonicated suspensions of batyl alcohol in water or aqueous buffer without detergents elicited no monooxygenase activity (3).

Monooxygenase activity vs detergent concentration profiles

The profiles for the four alkyl phosphocholines (6-9) and the four phosphatidyl cholines (10-13) were determined using 0.1 mM batyl alcohol as substrate

Figure 1. Monooxygenase activity *vs* alkyl phosphocholine (PC) concentration profiles (see Materials and Methods).

Figure 2. Monooxygenase activity *vs* acyl L- α -lyso-phosphatidylcholines (LPC) concentration profiles (see Materials and Methods).

and 0.5 mM 6MePH₄ as cofactor, and are illustrated in Figure 1 and 2 respectively. The reason for decreased enzyme activity at very low detergent concentrations was due to the lower solubility of batyl alcohol in the very low concentrations of detergent as demonstrated by the turbidity of the solutions. The profiles show that 12 PC and 18 LPC in each set stand out, but all the maximum enzyme velocities (35–45 nmole/min.mg protein) were somewhat higher than the maximum activity of 27.6 nmole/min.mg protein for the maximum initial rate in 2.3 mM Mega-10 under the same conditions (0.1 mM batyl alcohol, 0.5 mM 6-MePH₄ and 0.1 M Tris-HCl at pH 7.5). This suggests that the ether-lipid substrate in the mixed micelles was more accessible to the monooxygenase, or the detergent may have modified the nature of the enzyme such that the activity increased.

Inhibition of glyceryl ether monooxygenase by detergents

All the phosphocholines and phosphatidylcholines inhibit monooxygenase activity at higher detergent concentrations, as was observed with other detergents, e.g. Mega-10, TritonX-100, sodium deoxycholate (3). However, unlike Mega-10 which started to inhibit the monooxygenase at a concentration threshold immediately above the CMC, all the cholines had maximum enzyme activities well above their respective CMC values. The threshold concentrations for 12 PC, 14 PC, 16 PC and 18 PC are 1.63 mM (0.07%), 0.832 mM (0.035%), 0.348 mM (0.02%) and 0.600 mM (0.035%) respectively (see Figure 1 and 2). The threshold concentrations for 12 LPC, 14 LPC, 16 LPC and 18 LPC are 1.22 mM (0.05%), 0.660 mM (0.29%), 0.540 mM (0.025%) and 1.02 mM (0.05%) respectively. This implies that below their threshold concentrations the detergent micelles are passively associated with the microsomal membrane and do not seriously affect enzyme activity. However, it is possible that the inhibiting species above the threshold concentrations of these detergents are detergent micelles with higher aggregation numbers because *competitive* inhibition was observed (see below). Alternatively, inhibition of the enzyme above the threshold concentrations may be due to disruption, by the detergent, of the microsomal lipid associated with the monooxygenase. Tetradecyl phosphocholine (14 PC) had been used as a detergent to solubilise ether-lipid substrates, including batyl alcohol, by dissolving them in this detergent at a concentration of 7 mg/ml, i.e. 0.7%, *ca* 20 mM (7, 8). The solubilised substrates were used in the coupled (continuous spectrophotometric) assay with DHPR and NADH. The authors had not stated the final concentration of 14 PC in their assay solutions, but if the dilution was tenfold, i.e. 0.07%, then considerable inhibition of monooxygenase activity must have resulted (see Figure 1). This was reflected in the low monooxygenase activity that had been observed (7.4 nmol/min.mg protein for batyl alcohol) (8).

The kinetics of inhibition of glyceryl-ether monooxygenase with batyl alcohol as substrate and 6-MePH₄ as cofactor by 14PC, 14LPC, 16PC and 16LPC were investigated. The data were evaluated as described for Mega-10 (6), and the threshold concentrations, i.e. the concentrations at which enzyme inhibition begins, were taken as zero detergent concentration. Only by setting these values as zero all the data could be evaluated satisfactorily by computer analysis (12). The data fitted best for *competitive* inhibition and corresponded with the respective Lineweaver-Burk plots (see Figure 3 for 14PC). The ap-

Figure 3. Lineweaver-Burk plots for the inhibition of glyceryl-ether monooxygenase by 14 LPC using *RS*-batyl alcohol as substrate and 0.5 mM 6-MePH₄ as cofactor in 0.1 M Tris-HCl pH 7.5.

Table 2. Inhibition of glyceryl-ether monooxygenase by C14 and C16 phosphocholines and L- α -lyso-cholines using batyl alcohol as substrate and 6-MePH₄ (0.5 mM) as cofactor in 0.1 M Tris-HCl pH 7.5 and 25°C.

Competitive inhibitor	Apparent K _i (μ M)
Tetradecyl phosphocholine [14 PC]	320 ($\pm$ 72)
Myristoyl L- α -lyso-phosphatidylcholine [14 LPC]	326 ($\pm$ 36)
Hexadecyl phosphocholine [16 PC]	365 ($\pm$ 63)
Palmitoyl L- α -lyso-phosphatidylcholine [16 LPC]	316 ($\pm$ 55)

parent K_i values using batyl alcohol as substrate were very similar, within experimental error, and in the range 316-365 μ M (Table 2). By analogy the K_i values for 12 PC, 12 LPC, 18 PC and 18 LPC can be taken as being similar to the C14 and C16 analogues and

that inhibition would be competitive. These inhibitions were different from the value for Mega-10 (using batyl alcohol and 0.5 mM 6-MePH₄ in 0.1 M Tris-HCl at pH 7.5) in magnitude (apparent K_i 1.74 $\pm$ 0.37 mM) and in type (*noncompetitive*). Clearly the various choline micelles were interacting with the monooxygenase most probably at the active site and competing with mixed micelles containing the ether-lipid substrate.

Kinetic parameters

The apparent K_m and V_{max} values were determined for batyl alcohol with 0.5 mM 6-MePH₄ (at or near saturating) in 0.1 M Tris-HCl buffer at pH 7.5 in the presence of all the PC and LPC detergents at concentrations near or below the respective threshold concentrations. The parameters together with the respective detergent concentrations (all above the CMC values) are in Table 3. The apparent K_m values were in the range 24-44 μ M and the apparent V_{max} values were in the range 46-64 nmoles/min.mg protein. The similarities of these kinetic parameters suggest that the mechanism by which the ether-lipid substrate was directed to the active site of the monooxygenase was the same for these detergent solutions. The K_m values were of the same magnitude as that for batyl alcohol in Mega-10 (at 2.3 mM, below the CMC), i.e. 25 μ M, whereas the apparent V_{max} values were consistently higher by a factor of about 2 (see Table 3 and Figure 1 and 2). If the K_m values were taken as a rough indication of the binding of batyl alcohol at the active site of the monooxygenase, then the various detergents, ionic and non-ionic, do not appear to affect this interaction seriously. The turnover of batyl alcohol (as deduced from the apparent V_{max} values) was more efficient with the choline detergents than with Mega-10. This can be

Table 3. Kinetic parameters for glyceryl-ether monooxygenase with batyl alcohol as substrate and 6-MePH₄ (0.5 mM) as cofactor in 0.1 M Tris-HCl (pH 7.5) at 25°C.^a

	12 PC	14 PC	16 PC	18 PC
Concn of detergent, μ M	815 (0.035%)	382 (0.016%)	348 (0.02%)	600 (0.035%)
K _m , μ M	27.7 ($\pm$ 2.0)	27.0 ($\pm$ 2.6)	30.1 ($\pm$ 2.8)	36.3 ($\pm$ 2.4)
V _{max} nmoles/min.mg protein	48.7 ($\pm$ 1.1)	46.1 ($\pm$ 1.9)	64.2 ($\pm$ 2.0)	60.6 ($\pm$ 1.5)
	12 LPC	14 LPC	16 LPC	18 LPC
Concn of detergent, μ M	979 (0.04%)	332 (0.0145%)	538 (0.025%)	1170 (0.05%)
K _m , μ M	33.8 ($\pm$ 3.8)	27.4 ($\pm$ 2.7)	24.0 (2.7%)	43.7 ($\pm$ 4.3%)
V _{max} nmoles/min.mg protein	54.4 ($\pm$ 2.2)	48.5 ($\pm$ 0.8)	50.0 ($\pm$ 2.2)	61.0 ($\pm$ 2.5)

^aWith Mega-10 [2.3 mM, 0.08%] as detergent the K_m and V_{max} values were 25.1 ($\pm$ 2.9) μ M and 27.6 nmoles/min.mg protein. All K_m and V_{max} values are apparent.

attributed to greater interaction of the detergents with the monooxygenase and/or the microsomal membrane. Studies with the microsomal-free monooxygenase are in progress, as well as analytical ultracentrifugation studies of the micelle composition of these detergents.

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