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Effect of Cryosolvent on Transient Kinetics of the Glutamate Dehydrogenase Reaction†

Alan H. Colen, Robert E. Johnson, and Harvey F. Fisher*

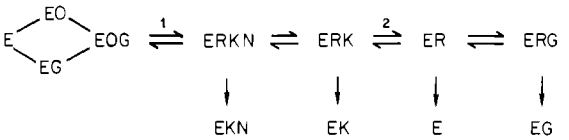
ABSTRACT: The transient and steady-state kinetics of the oxidative deamination of L-glutamate by glutamate dehydrogenase and NADP in both aqueous solution and 30% methanol are compared. Methanol causes an approximately 5-fold tightening of the enzyme–L-glutamate binary complex and an approximately 2-fold reduction of the interaction parameter for the ternary enzyme–NADP–L-glutamate complex. The most dramatic effect of methanol on the time course of the reaction is what appears to be a conversion of the enzyme at substoichiometric initial levels of reactant NADP to a form from which product α-ketoglutarate does not readily dissociate. This conversion appears only at NADP concentrations over one-third of the enzyme active site concentration.

Cryoenzymological studies of the glutamate dehydrogenase reaction (Johnson et al., 1981a,b) have revealed the existence of a stable less reactive form of the enzyme, identifiable by its distinctive spectral characteristics in the aromatic chromophore region. At normal temperatures in the absence of the cryosolvent (antifreeze) methanol, these spectral characteristics are observed only in the enzyme–NADPH–α-ketoglutarate ternary complex, but at cryogenic temperatures in solutions containing methanol the enzyme can be converted to a state for which the spectral characteristics are observed regardless of which enzyme–coenzyme complexes are formed. The purpose of the present experiments is to determine at which point in the reaction mechanism the existence of this new enzymatic form becomes mechanistically important and whether these effects are produced by the effects of temperature on free energy barriers, by methanol, or by both.

As it is currently understood from experiments in aqueous solution (diFranco, 1974; Colen et al., 1975, 1977, 1981; Brown et al., 1978; Fisher & Colen, 1978), the mechanism of the oxidative deamination of L-glutamate by glutamate dehydrogenase and NADP is given in Scheme I.

The catalytic hydride transfer step (step 1) converts oxidized coenzyme to complexes consisting of enzyme, reduced coenzyme, and α-ketoglutarate, which exhibit a blue-shifted reduced nicotinamide absorption peak, observed as a rapid transient production ("burst") of 336-nm absorbance. The release of α-ketoglutarate (step 2) is accompanied by a spectral shift of bound reduced nicotinamide absorbance to the red (346 nm) (Fisher et al., 1970; diFranco, 1971; diFranco & Iwatsubo, 1971, 1972). When free NADPH is produced (arrows pointing downward in Scheme I), its absorbance is, of course, unshifted (340 nm).

Scheme I[a]



[a] Abbreviations: E, enzyme, glutamate dehydrogenase; O, oxidized coenzyme, NADP; G, L-glutamate; N, ammonia; R, reduced coenzyme, NADPH; K, α-ketoglutarate.

Steps 1 and 2 may be studied directly by stopped-flow spectrophotometry and the production of free NADPH by studies of steady-state kinetics. Since step 2 is rate-limiting in steady-state studies under most experimental conditions, effects observed in transient-state studies will also be reflected in steady-state measurements (diFranco, 1974; Colen et al., 1975). In the work that follows, we compare the rapid initial burst of blue-shifted absorbance and the steady-state and transient modes of breakdown of these blue-shifted complexes at 5 °C both in aqueous solution and in 30% methanol.

Materials and Methods

Bovine liver glutamate dehydrogenase was obtained in ammonium sulfate suspension from Boehringer Mannheim. Before use, the enzyme was exhaustively dialyzed against 0.1 M potassium phosphate buffer at the appropriate pH and treated with activated charcoal to remove tightly bound organic material. We calculated enzyme concentrations using $A^{1\%}_{280}$ = 9.7 and M_r 56 100. The ratio A_{280}/A_{260} was always greater than 1.92, indicating the absence of significant amounts of nucleotide impurities.

NADP was obtained from Sigma and used without further purification. We calculated NADP concentrations using ϵ_{259} = 17 800 (Siegel et al., 1959). L-Glutamate acid was obtained from Calbiochem.

Solutions in 30% methanol were prepared with 0.1 M pH 7.6 phosphate buffer. On the basis of the data of Douzou et al. (1976), the pH of the solutions is estimated to be 8.2. For the sake of comparison, the experiments in aqueous solution

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were carried out in 0.1 M phosphate buffer pH 8.2.

For prevention of the denaturation of the enzyme in 30% methanol, these solutions were prepared by adding aqueous stock enzyme solution at 0 °C to the remaining components of the mixture, also maintained at 0 °C. The enyzme used in these studies exhibited no loss of activity when assayed in aqueous solution after incubation in 30% methanol under the experimental conditions employed here.

Rapid-Scan Spectrophotometry. A Hewlett-Packard 8450A spectrophotometer, an instrument capable of capturing a complete spectrum at 1-nm resolution in a single second, was used to obtain sequential spectra of the reaction mixtures as a function of time at 5 °C. Resolution of each of these spectra into components representing free enzyme, free NADPH, the ERK complex, and the ERG complex was carried out with the hard-wired program of the HP 8450A microprocessor. The statistical approach used by this program involves a combination of the "ordinary least squares" and the "maximum likelihood" methods as described by Beck (1977). The individual component spectra for the enzyme complexes were obtained on the HP 8450A spectrophotometer by the methods described by Cross (1972).

Stopped-Flow Experiments. Studies of transient kinetics were performed on a Durrum-Gibson stopped-flow spectrophotometer thermostated at 5 °C, equipped with a xenon arc lamp, and interfaced either to a Varian 620L digital computer or to a Declab 11/MNC data acquisition system. The initial velocity of the burst was measured at two successive time resolutions, and that of the blue-red spectral shift was measured at three. Transient initial velocities were obtained as the tangent at time zero to an exponential fit of the initial data segment as described previously (Colen et al., 1972, 1977).

First-order rate constants and amplitudes for the blue-red shift were obtained from multiple exponential fits by a modified Marquardt procedure (Dell et al., 1973).

All transient-state experiments were initiated by mixing a solution containing enzyme and NADP with a solution containing enzyme and L-glutamate. This procedure avoids light-scatter changes arising from dissociation of the enzyme upon dilution (a problem only in aqueous solution, since the enzyme does not appear to aggregate significantly in 30% methanol at low temperature). The transient initial velocities of the burst were measured at 340 nm, a wavelength approximately isosbestic for both blue- and red-shifted product complexes with $\epsilon_{340} = 5000$ (Colen et al., 1972; Cross, 1972). The data for the blue to red spectral shift were obtained at 330 and 363 nm. The absorbance difference between these two wavelengths reflects the concentration of blue-shifted complexes plus some contribution from free coenzyme without any significant contribution from red-shifted complexes. It is this absorbance difference, corrected for any free coenzyme production, that is used to determine the first-order rate constant and amplitude for step 2 in Scheme I.

Steady-State Kinetics. Initial velocities obtained under steady-state conditions were measured at 340 nm on a Gilford spectrophotometer equipped with a thermal cuvette set for 5 °C. The reaction was initiated by adding to the reaction mixture incubated at 5 °C that amount of stock enzyme solution, incubated at 5 °C, required to obtain a readily measurable rate less than 0.04 absorbance unit/min. The initial velocities were calculated by hand with $\epsilon_{340} = 6200$ for NADPH.

Results

Methanol Effects on Transient Initial Velocity. Figure 1 illustrates the effect of methanol on the time course of the oxidative deamination of L-glutamate by glutamate dehydrogenase and NADP at 5 °C. Curve A was obtained for aqueous solution at L-glutamate and NADP concentrations such that the initial ("time-zero") transient velocity is about 38% of the maximum transient initial velocity. Curve B represents an experiment in 30% methanol at approximately 30% of the maximum transient initial velocity. The concentrations used in these studies are below the range in which substrate inhibition of the transient-state rate is observed. The transient initial velocity, at comparable degrees of saturation, is very slightly higher in 30% methanol than it is in water. The velocity at the end of the transient phase (after about 0.6 s in Figure 1) is 7-fold higher in water than in 30% methanol. Thus, methanol has little effect on burst velocities but shows a substantial inhibitory effect on events after the initial transient period.

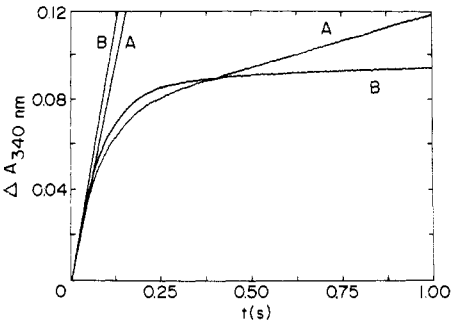
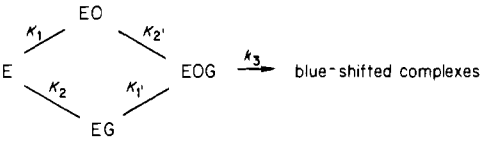


FIGURE 1: Stopped-flow progress curves at comparable degrees of saturation. The enzyme concentration was 1 mg/mL. (Curve A) Aqueous solution, 5 mM L-glutamate, 1 mM NADP; (curve B) 30% methanol, 2.5 mM L-glutamate, 0.5 mM NADP. The straight lines are initial velocity lines (computer fit): (line A) aqueous; (line B) 30% methanol.

Scheme II



The initial velocity of the rapid transient (burst) contains contributions only from the steps in the mechanism up to and including the catalytic hydrogen transfer step. Our previous research (Colen et al., 1977) indicates that the kinetic results may be summarized as shown in Scheme II where K_1, K_2, $K_{1'}$, and $K_{2'}$ are dissociation constants and k_3 is the first-order rate constant for EOG → ERKN.

We have measured the dependence of the transient initial velocity on substrate concentration at a number of fixed coenzyme concentrations at 5 °C both in aqueous solution and in 30% methanol. Figure 2 shows a double-reciprocal plot of the data obtained in aqueous solution. The solid lines represent fits to the equation:

$$\frac{[E_t]}{V_{transient}} = \frac{1}{k_3} + \frac{K_{1'}}{[NADP]} + \frac{K_{2'}}{[L\text{-glutamate}]} + \frac{K_1K_{2'}}{[NADP][L\text{-glutamate}]} \quad (1)$$

This equation is weighted on the basis that the experimental error in each measured velocity is proportional to the velocity itself. The constants for Scheme II, calculated in this manner both for the data obtained in aqueous solution (shown in Figure 2) and for those obtained from an identical series of experiments on solutions containing 30% methanol, are given in

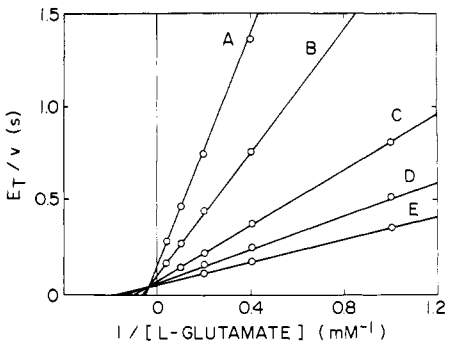


FIGURE 2: Double-reciprocal plot of transient initial velocities in aqueous solution. NADP concentration: (A) 50 μM; (B) 100 μM; (C) 250 μM; (D): 500 μM; (E) 1 mM.

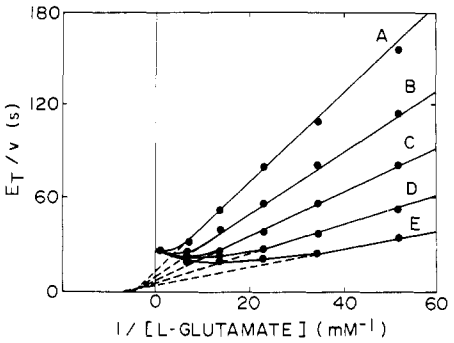


FIGURE 3: Double-reciprocal plot of steady-state initial velocities in 30% methanol. NADP concentrations: (A) 20 μM; (B) 30 μM; (C) 45 μM; (D) 75 μM; (E) 150 μM.

Table I: Comparison of Kinetic and Thermodynamic Constants for Transient Initial Velocity in the Presence and Absence of 30% Methanol

	30% methanol	aqueous
K_1 (mM)	0.8 ± 0.1	1.0 ± 0.1
K_2 (mM)	6.1 ± 0.6	30 ± 4
$K_{1'}$ (mM)	0.22 ± 0.05	0.12 ± 0.02
$K_{2'}$ (mM)	1.8 ± 0.5	3.6 ± 0.5
k_3 (s^{-1})	32 ± 5	24 ± 2
K_I	3.5 ± 0.7	8 ± 1

Table I. The only significant effect of 30% methanol is an approximately 5-fold tightening of the binary EG complex (as reflected in the values of K_2). Also shown in Table I is an interaction parameter K_1 (Colen et al., 1972; Fisher et al., 1980), a measurement of the heterotropic binding cooperativity in the formation of the EOG complex:

$$K_1 = K_1/K_{1'} = K_2/K_{2'} \quad (2)$$

The presence of 30% methanol reduces the size of this parameter by a factor of 2 relative to its value in aqueous solution.

Reexamination of Methanol Effects on Steady-State Initial Velocities. Whereas the effects of methanol on the transient velocities are reflected principally in the lowering of the EG complex binary dissociation constant, the 7-fold lowering of velocity after the completion of the initial transient suggests that methanol has a major kinetic effect on the steady-state velocity. We have measured the dependency of the steady-state velocity on L-glutamate concentration at a number of fixed enzyme concentrations both in aqueous solution and in 30% methanol. Figure 3 shows a double-reciprocal plot of the data contained in methanol. Under the present experimental conditions, we observe no inhibitory effects of substrate and coenzyme on the transient initial velocities and on steady-state velocities in aqueous solution. In 30% methanol, however, we do observe inhibitory effects in the steady state. In methanol, we must employ concentrations 5 times lower than those usable in aqueous solution to avoid the effects of substrate inhibition. Analysis of the data in the noninhibitory concentration range according to the phenomonological equation (3) (Dalziel,

$$\frac{V}{[E_t]} = \left(\phi_0 + \frac{\phi_1}{[\text{NADP}]} + \frac{\phi_2}{[\text{L-glutamate}]} + \frac{\phi_{12}}{[\text{NADP}][\text{L-glutamate}]} \right)^{-1} \quad (3)$$

1957) (values of the constants in Table II) shows only effects that parallel those observed in the transient-state experiments: no significant alteration of V_{max} (Table II), but alteration of the limiting Michaelis constants and especially of ϕ_{12} and ϕ_2 again reflecting a significant tightening of the binary EG complex.

Table II: Comparison of Kinetic Constants for Steady-State Initial Velocities in the Presence and Absence of 30% Methanol

	30% methanol	aqueous
ϕ_0 (s)	2.8 ± 1.7	2.3 ± 0.1
ϕ_1 (mM·s)	0.24 ± 0.05	0.08 ± 0.01
ϕ_2 (mM·s)	0.19 ± 0.05	0.26 ± 0.04
ϕ_{12} (mM^2·s)	0.05 ± 0.016	0.22 ± 0.01
$K_{m,NADP} = \phi_1/\phi_0$ (mM)	0.08 ± 0.07	0.03 ± 0.004
$K_{m,Glu} = \phi_2/\phi_0$ (mM)	0.07 ± 0.06	0.11 ± 0.02
$V_{max}[E_t]$ (s^{-1})	0.4 ± 0.2	0.44 ± 0.02

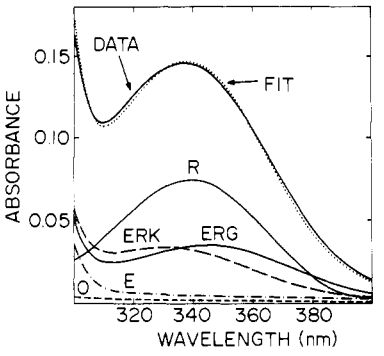


FIGURE 4: Spectral resolution after 86 s of a reaction mixture containing 30% methanol, 1 mg/mL glutamate dehydrogenase, 38 μM NADP, and 45 mM L-glutamate, measured against a reference cuvette containing buffer alone.

The 7-fold rate decrease observed in steady-state studies in 30% methanol then is not a result of the alteration of V_{max} by methanol as had been previously suggested (Bradley et al., 1979) but rather the result of enhanced substrate and coenzyme inhibition in methanol.

Methanol Effects on Breakdown of Intermediates That Have Blue-Shifted Reduced Nicotinamide Spectra. Inhibitory L-glutamate concentrations and high enzyme concentrations provide conditions under which one may most readily measure the rate of conversion of complexes with blue-shifted reduced nicotinamide absorption maxima to ones with red-shifted spectra (Colen et al., 1975). Figure 4 shows a spectral resolution of a reaction mixture in 30% methanol after the reaction has proceeded for 86 s. Under these conditions only half of the bound NADPH is converted to the red-shifted form, whereas in aqueous solution the conversion at this point in time is virtually complete.

Stopped-flow studies in aqueous solution show that the blue-red conversion equilibrates in less than 12 s, becoming progressively more rapid with increasing NADP concentration (Figure 5) but remaining independent of L-glutamate and enzyme concentration. The first-order rate constants for this

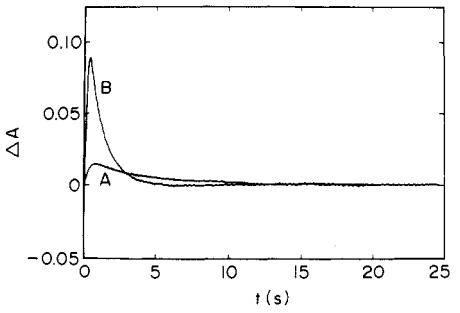


FIGURE 5: Progress curves in aqueous solution for the blue to red spectral shift. The curves were obtained by subtracting data taken at 363 nm from data at 330 nm. Both experiments contained 2 mg/mL glutamate dehydrogenase and 100 mM L-glutamate. NADP concentrations were (A) 4.06 μM and (B) 87.1 μM. Curve B is corrected for the production of free NADPH.

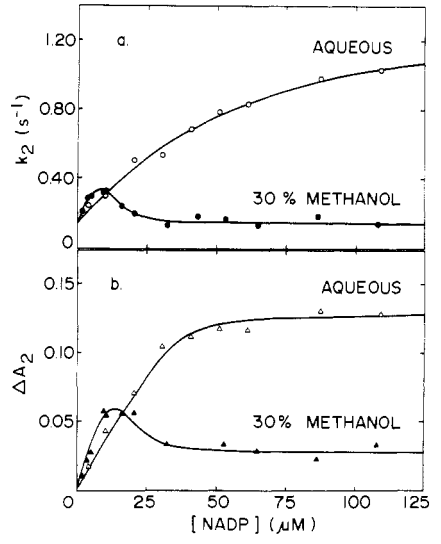


FIGURE 6: Plot of NADP concentration dependence for the blue to red shift. The enzyme concentration was 2 mg/mL, and the L-glutamate concentration was 100 mM. (a) First-order rate constants; (b) amplitudes.

process are plotted against NADP concentration in Figure 6a. The data indicate that the presence of NADP causes the first-order rate constant to increase from 0.17 ± 0.02 s^{-1} to 1.27 ± 0.15 s^{-1} with a Michaelis constant for the NADP effect of 37 ± 12 μM.

This K_m is not equivalent to the dissociation constant for the binding of a second mole of NADP, however, since almost all of the NADP in the reaction mixture has already been converted during the initial catalytic transient phase into NADPH with blue-shifted reduced nicotinamide absorbance. Instead, it must arise from effects produced by product NADPH and α-ketoglutarate. The concentration dependence of the amplitude (or extent) of the conversion step is shown in Figure 6b. In aqueous solution, none of the blue-shifted complexes formed during the initial transient remain after the conversion equilibrates.

Figure 6 also shows the dependence on NADP concentration of the first-order rate constant and amplitude in 30% methanol. Below one-third of the enzyme concentration, both the rate and amplitude of the equilibration process for blue- and red-shifted complexes increase just as in aqueous solution, but at higher NADP concentrations there is an abrupt drop to a constant value. It is as if the relative stabilization of blue- and red-shifted complexes changed drastically immediately above one-third saturation of enzyme with NADP. Above 25 μM NADP, the first-order rate constant is invariant and approximately equal to its extrapolated value at zero NADP. Thus, the presence of methanol, at NADP concentrations above one-third the enzyme stoichiometry, leads to the production of stable blue-shifted enzyme–NADPH–α-ketoglutarate complexes from which α-ketoglutarate does not dissociate as readily to form red-shifted complexes as it does in aqueous solution under the same conditions. This effect is an effect of methanol alone since in aqueous solution at the same temperature α-ketoglutarate dissociates quite readily.

Discussion

Recent cryoenzymological studies of the glutamate dehydrogenase reaction at −42 °C in 50% methanol (Johnson et al., 1981b) have shown that substoichiometric amounts of α-ketoglutarate in the presence of NADPH (both products of the rapid transient phase of the reaction studied here) cause the conversion of the enzyme to a long-lived less reactive form in which blue-shifted complexes are not as readily converted to red-shifted forms as we hitherto might have expected. It is probable that we are observing the very same phenomenon here at 5 °C in 30% methanol since, as the NADP concentration is increased, the concentrations of product α-ketoglutarate complexes increase. The NADP concentration threshold observed here for methanol effects may in fact simply correspond to an α-ketoglutarate concentration threshold under the experimental conditions reported here. Thus, the major effect of methanol (and perhaps the only mechanistically significant one) is to provide an environment in which the enzyme can be converted to a form in which the blue-shifted ternary complex of enzyme, NADPH, and α-ketoglutarate has enhanced stability, causing substrate and coenzyme inhibition at much lower concentrations than would otherwise be observed.

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Substrate Specificity of Bacterial Glycerophospholipid:Cholesterol Acyltransferase†

J. Thomas Buckley

ABSTRACT: The substrate specificity of a bacterial analogue of the plasma enzyme lecithin:cholesterol acyltransferase (LCAT) has been examined with small unilamellar liposomes and Triton mixed micelles. In contrast to LCAT, the microbial enzyme is capable of using all of the naturally occurring phospholipids as acyl donors. In general reaction rate depends more on the length or degree of unsaturation of the acyl chains than on the nature of the phospholipid head group. Among a series of disaturated phosphatidylcholines in liposomes, dilauroylphosphatidylcholine is the preferred acyl donor. Like LCAT, the enzyme will catalyze acyl transfer by using other alcohols in addition to cholesterol. Of saturated straight chain primary alcohols 1-decanol is the preferred acyl acceptor. Cholesterol, however, is a far better acceptor than any nonsterol alcohol tested. Other steroids with equatorial hydroxyls at position C-3 and trans-fused A:B rings will also act as acceptors whereas those steroids with axial hydroxyls at C-3 or cis-fused rings are inhibitors of acyl transfer. The ability of steroids to act as acyl acceptors may be due to the nature of their interaction with the phospholipid acyl donor.

The glycerophospholipid:cholesterol acyltransferase (GCAT)[1] released by members of the *Vibrio* family (MacIntyre & Buckley, 1978; MacIntyre et al., 1979) and purified in this laboratory (Buckley et al., 1982) has a number of interesting features. In reaction mechanism it appears similar to the mammalian enzyme lecithin:cholesterol acyltransferase (LCAT). Like LCAT, the enzyme can act as a 2 position specific phospholipase or as an acyltransferase, depending upon the presence or absence of cholesterol. Furthermore, as with LCAT, the enzyme has no divalent cation requirement and its activity is stimulated by apolipoprotein A-1 (Buckley et al., 1982). Since GCAT is far more stable than LCAT and since it is available in large quantity, it should prove valuable in obtaining information in vitro to aid in further studies of the mechanism of LCAT action. In addition, because the enzyme (unlike LCAT) can carry out hydrolysis or acyl transfer using most if not all glycerophospholipids, because it has no divalent cation requirement, and because it apparently acts asymmetrically on plasma membranes (Buckley et al., 1982), it may be used to corroborate and expand information on phospholipid interactions in plasma membranes obtained in studies using eucaryotic phospholipases.

The lack of a divalent cation requirement and the 2 position specificity as well as the ability to acylate cholesterol distinguish the *Vibrio* enzyme from all other microbial phospholipases. It seems quite possible if not likely, however, that the transfer of a fatty acid from a phospholipid such as phosphatidylcholine to cholesterol is not the reaction carried out by the organism in vivo. Thus although it is conceivable that the bacteria use the enzyme to degrade eucaryotic cells, it may normally be used with quite different substrates, for example, in cell wall metabolism.

In this paper the specificity of the bacterial enzyme toward both acyl donors and acyl acceptors is examined in order to extend the comparison with LCAT and to shed more light on the probable function of the enzyme in vivo.

Materials and Methods

Materials. GCAT was purified from cell-free culture supernatants of *Aeromonas salmonicida* as previously described (Buckley et al., 1982). Bovine serum albumin (essentially fatty acid free), cholesterol, and aliphatic primary alcohols were purchased from Sigma Chemical Co. [4-^{14}C]Cholesterol (specific activity 58 mCi/mmol) and [1-^{14}C]oleic acid (57 mCi/mmol) were from Amersham Corp. 2-[1-^{14}C]Oleoylphosphatidylcholine was prepared according to the procedure of Pugh & Kates (1975). Individual phospholipids, and phosphatidylcholine molecular species, were obtained from Sigma, Serdary Research, or Supelco Inc. Steroids were supplied by Sigma, Supelco, or Research Plus Steroids. Individual lipids were examined by thin-layer chromatography prior to use and discarded if less than approximately 99% pure.

Preparation of Substrates. Small unilamellar vesicles of the compositions described in the text were prepared by sonication followed by ultracentrifugation at 110000*g* for 1 h to remove undispersed lipid (Barenholtz et al., 1977). The actual amounts of individual lipids in each preparation were quantitatively determined, and the liposomes were used immediately. Unless otherwise specified the chemical compositions of the liposomes were within 10% of the predicted amounts. Mixed micelles containing Triton X-100, phospholipids, and steroids or primary alcohols were prepared as described by Dennis (1973a,b). The ratio of detergent to total

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[1] Abbreviations: CE, cholesteryl ester; chol, cholesterol; PC, phosphatidylcholine; LCAT, lecithin:cholesterol acyltransferase; GCAT, glycerophospholipid:cholesterol acyltransferase; Tris, tris(hydroxymethyl)aminomethane.

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