Noncooperative vs. Cooperative Reactivation of D-β-Hydroxybutyrate Dehydrogenase: Multiple Equilibria for Lecithin Binding Are Determined by the Physical State (Soluble vs. Bilayer) and Composition of the Phospholipids†

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Received January 21, 1987; Revised Manuscript Received April 9, 1987

ABSTRACT: D-β-Hydroxybutyrate dehydrogenase (BDH) is a lecithin-requiring mitochondrial enzyme that catalyzes the interconversion of β-hydroxybutyrate and acetoacetate. The purified soluble enzyme devoid of lipid (i.e., the apodehydrogenase) can be reactivated with soluble lecithin or by insertion into phospholipid vesicles containing lecithin. Lipid activation curves have a sigmoidal shape, and two models have been proposed to explain them. We have previously reported that the kinetics of reactivation with short-chain lecithins in the soluble state is consistent with a model in which the enzyme contains two identical, noninteracting lecithin binding sites, both of which must be occupied to activate the enzyme [noncooperative mechanism; Cortese, J. D., Vidal, J. C., Churchill, P., McIntyre, J. O., & Fleischer, S. (1982) *Biochemistry 21*, 3899–3908]. More recently a kinetic model involving cooperative interactions between lecithin binding sites was proposed for the reactivation of the membrane-bound enzyme [Sandermann, H., Jr., McIntyre, J. O., & Fleischer, S. (1986) *J. Biol. Chem. 261*, 6201–6208]. This study reinvestigates the basis for the different conclusions in these two studies. The previous study with soluble lecithins was limited to about 34% of maximal activation compared with mitochondrial phospholipid, due to inactivation of the enzyme at the critical micellar concentration. We could now extend this study to 91% activation by increasing the ethanol concentration. This experimental evidence confirms that the soluble system follows a noncooperative equation. We provide a new kinetic approach to test the cooperative model. A velocity equation is derived for a Hill-type cooperative ligand binding system interacting with a mixture of ligands. This equation predicts a proportionality between an *overall weighted cooperative dissociation constant* [$K_{coop(w)}$] and a dissociation constant for a single lecithin (PC) species from interacting sites (K_{PC}), regulated by the PC molar fraction (X_{PC}): $1/K_{coop(w)} = X_{PC}/K_{PC}$. The equation was applied to the data of Sandermann et al. [Sandermann, H., Jr., McIntyre, J. O., & Fleischer, S. (1986) *J. Biol. Chem. 261*, 6201–6208] as well as to newly obtained data. The results obtained over a wide range of PC molar fractions and different mixtures of bilayer phospholipids fit this equation, confirming the cooperative behavior. We conclude that BDH has a different mode of reactivation depending on the nature of the lipid environment. With soluble lecithin, the activation is noncooperative, whereas in the bilayer, mixtures of phospholipids give cooperative behavior that fits a Hill equation. Both models conform to two lecithin binding sites per active enzyme unit, which appears to be a characteristic of the enzyme.

D-β-Hydroxybutyrate dehydrogenase (EC 1.1.1.30, D-(−)-3-hydroxybutyrate:NAD oxidoreductase; BDH)[1] is one of the best studied examples of a phospholipid-requiring enzyme (Fleischer et al., 1983, 1985; Latruffe et al., 1986). The enzyme is normally membrane bound, inserted into the matrix face of the mitochondrial inner membrane (McIntyre et al., 1978). The apoenzyme (apodehydrogenase, apoBDH), i.e., the enzyme devoid of phospholipid, has been purified to homogeneity from beef heart mitochondria (Bock & Fleischer, 1974, 1975) and rat liver mitochondria (Vidal et al., 1977a,b). The apodehydrogenase, as isolated, is soluble and catalytically inactive but can be reactivated by the addition of soluble PC or mixtures of phospholipid in the form of vesicles containing PC, with formation of an active BDH–PC complex (Gazzotti et al., 1975). For the activation with soluble lecithins, the enzyme–lecithin complex exists as a dimer in solution (McIntyre et al., 1978). The apoenzyme inserts unidirectionally into preformed phospholipid vesicles (McIntyre et al., 1979; Maurer et al., 1985; Deese et al., 1986; Berrez et al., 1985). As carried out for the activation by vesicles, practically all the enzyme and phospholipid are present within the bilayer, and there is no soluble BDH or phospholipid (McIntyre et al., 1979). The enzyme exists as a tetramer in the bilayer (McIntyre et al., 1983). The mixture of mitochondrial phospholipids (MPL) was found to give optimal reactivation of the enzyme (Isaacson et al., 1979; Gazzotti et al., 1975).

† This investigation was supported in part by National Institutes of Health Grants AM 21987 and AM 14632.
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[1] Abbreviations: apoBDH, D-(−)-3-hydroxybutyrate dehydrogenase apoenzyme; BDH, D-(−)-3-hydroxybutyrate dehydrogenase; BOH, β-hydroxybutyrate; BSA, bovine serum albumin; cmc, critical micellar concentration; DCP, dicetyl phosphate; DPG, diphosphatidylglycerol; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HA, hexadecylamine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; L, phospholipid as a ligand; MPL, mitochondrial phospholipid (a mixture of PC–PE–DPG in 1.0/0.8/0.2 phosphorus molar ratio, respectively) isolated from rat liver or beef heart mitochondria; PA, L-α-phosphatidic acid; PA(10:0), 1,2-didecanoyl-*sn*-glycerol 3-phosphate; PC, phosphatidylcholine; PC(8:0), 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine; PC(10:0), 1,2-didecanoyl-*sn*-glycero-3-phosphocholine; PE, phosphatidylethanolamine; PE(10:0), 1,2-didecanoyl-*sn*-glycero-3-phosphoethanolamine; PP, phosphatidylpropane-1,3-diol; SPM, sphingomyelin; TLC, thin-layer chromatography.

0006-2960/87/0426-5283$01.50/0

The activation by MPL can be mimicked by a mixture of three single molecular species of PC, PE, and an acidic phospholipid (Churchill et al., 1983).

Lipid activation curves for D-β-hydroxybutyrate dehydrogenase have a sigmoidal shape. A plausible model for the lipid reactivation was proposed on the basis of the analysis of the kinetic mechanism of reactivation of beef heart and rat liver soluble apoBDH with soluble short-chain lecithins (Cortese et al., 1982; Cortese & Vidal, 1983). The model suggested simultaneous occupation of two identical noninteracting lecithin binding sites through a rapid equilibrium mechanism with random addition of ligands to achieve the catalytically active conformation. This model displays sigmoidal reactivation curves albeit there is no interaction between sites (Cortese et al., 1982; Cortese & Vidal, 1983, 1984). Subsequently, Sandermann et al. (1986) applied a kinetic theory for lipid-dependent enzymes (Sandermann, 1982, 1984) and suggested that only a model involving cooperativity between the lecithin binding sites could explain the relative high degree of cooperativity reported for the activation of apoBDH by bilayer-forming phospholipids [data of Fleischer et al. (1979) and Churchill et al. (1983); Sandermann, 1984].

This study reinvestigates the basis for the different conclusions reached by using soluble vs. bilayer phospholipids.

Experimental Procedures

Reagents. Sucrose, tris(hydroxymethyl)aminomethane (Tris), dithiothreitol (DTT), β-NAD^+, sodium DL-3-hydroxybutyrate, bovine serum albumin (BSA, type F), sphingomyelin (SPM), dicetyl phosphate (DCP), diphosphatidylglycerol (DPG), hexadecylamine (HA), and L-α-phosphatidic acid (PA) were purchased from Sigma Chemical Co. (St. Louis, MO). 1,2-Dioctanoyl-*sn*-glycero-3-phosphocholine [PC(8:0)], 1,2-didecanoyl-*sn*-glycero-3-phosphocholine [PC(10:0)], 1,2-didecanoyl-*sn*-glycero-3-phosphoethanolamine [PE(10:0)], and 1,2-didecanoyl-*sn*-glycerol 3-phosphate [PA(10:0)] were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). All other reagents and solutions were of analytical grade, and solutions were prepared in double-distilled water unless otherwise stated.

Chromatography Materials. Sepharose 4B, Sepharose 4B-CL, DEAE-Sephadex A-50, and CM-Sephadex C-50 were purchased from Pharmacia, Ltd. (Uppsala, Sweden). Thin-layer chromatography (TLC) was performed on glass plates covered with a 250-μm layer of silica gel H (E. Merck, Darmstadt, Federal Republic of Germany), or alternatively, precoated silica gel G thin-layer sheets were used for current tests of lipid purity.

Analytical Methods. Protein concentration was measured according to the method of Lowry et al. (1951) using BSA as standard. When the samples contained DTT, the modification described by Ross and Schatz (1973) was used. Phospholipid concentrations were determined as inorganic phosphorus according to the method of Chen et al. (1966) modified by Rouser and Fleischer (1967), and TLC of the phospholipid was performed as described previously (Cortese et al., 1982).

Lipids. Mitochondrial phospholipids from rat liver mitochondria were extracted and purified as described by Gazzoti et al. (1975) for beef heart MPL. The purified phospholipids exhibit a single spot when analyzed by TLC using chloroform–methanol–water (65:25:4 by volume) as the developing system. Egg phospholipids were prepared by chromatography on silicic acid according to the method of Smith (1969). Small unilamellar vesicles were prepared by sonication and sized by chromatography on Sepharose 4B-CL or Sepharose 4B as described by Huang and Thompson (1974) or by differential ultracentrifugation according to Barenholz et al. (1977).

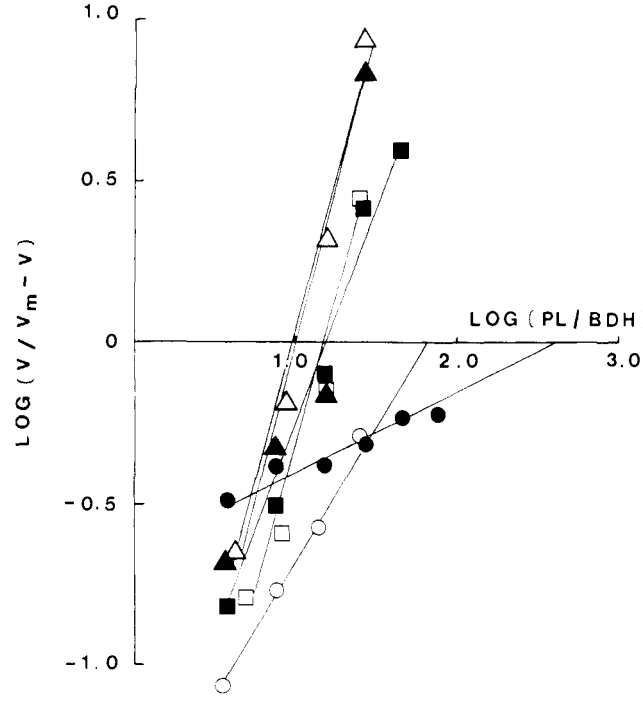

FIGURE 1: Hill plot of reactivation curves of bovine heart D-β-hydroxybutyrate apodehydrogenase (apoBDH). Data were taken from original experiments shown in Figure 1 of Sandermann et al. (1986). Initial velocities (v) are used as percent of maximum activation with mitochondrial phospholipid (MPL), and apparent maximal velocities (V_m) are estimated from double-reciprocal plots [v^{-1} as a function of the reciprocal of the ratio between total phospholipid (PL) and BDH monomers]. Different fixed molar fractions of PC (X_{PC}) were used in each reactivation assay: X_{PC} = 0.02 (●, the Hill plot exhibits a linear regression coefficient r = 0.990); X_{PC} = 0.05 (○, r = 0.995); X_{PC} = 0.1 (■, r = 0.967); X_{PC} = 0.2 (□, r = 0.978); X_{PC} = 0.35 (▲, r = 0.988); and X_{PC} = 0.5 (△, r = 0.985). PP (phosphatidylpropane-1,3-diol) was kept at a molar fraction of 0.1 and $X_{PC} + X_{PE}$ = 0.9 in all the experiments.

D-β-Hydroxybutyrate Dehydrogenase. Purified apoBDH from bovine heart was obtained (Bock & Fleischer, 1975) and reconstituted as described by Churchill et al. (1983). ApoBDH from rat liver was purified as described by Cortese and Vidal (1983), and its enzymic activity was measured according to the reconstitution procedure described previously (Vidal et al., 1977b). When soluble short-chain phospholipid mixtures were used [PA(10:0), PE(10:0), and PC(10:0)], the enzymic activity was measured according to the method of Cortese et al. (1982). The assay mixture contained 10 mM K_2HPO_4–KOH (pH 7.45 at 37 °C), 0.5 mM EDTA, 0.3 mM DTT, 0.04% BSA, 5% ethanol, and 5 mM NAD^+. Prewarmed β-hydroxybutyrate (BOH) to a final concentration of 20 mM was added to start the catalytic reaction. Mixtures containing PC(10:0) or PC(8:0) as activating species contained 5% absolute ethanol to increase the critical micellar concentration (cmc). PC(8:0) was assayed at 25 °C in optimized conditions as described by Cortese et al. (1982). Reaction mixtures contained 10 mM Hepes–KOH (pH 8.0 at 25 °C), 1 mM EDTA, 2 mM DTT, 100 mM NaCl, 5% ethanol, and 5 mM NAD^+. Twenty microliters of 1 M BOH was added to start the reaction, and optical changes in 340-nm absorbance were recorded on a Cary 219 spectrophotometer.

Results

Derivation of a Hill-Type Equation for a Multiple Equilibrium System Interacting with Mixtures of Ligands. Cortese

and Vidal (1983) developed an experimental treatment of kinetic data obtained from reactivation curves in the presence of mixtures of soluble lecithins. This derivation makes use of site competition between ligands to discriminate between kinetic models exhibiting sigmoidal-shaped reactivation curves first proposed by Henis and Levitzki (1979) but not previously applied as a test of cooperative systems. When the pertinent assumptions about multiple equilibria present in the situation indicated above are considered (Cortese & Vidal, 1983; Cortese, 1984), a general equation is obtained:

$$v/V_m = 1/[1 + 1/(\sum[L_i]/K_i)]^n \quad (1)$$

where $[L_i]$ is the ith ligand concentration and K_i is the intrinsic dissociation constant for the ith ligand. If a mixture with fixed molar fractions of each ligand is used, the equation obtained for the noncooperative system is

$$v/V_m = 1/(1 + K_w/[L_t])^n \quad (2a)$$

for $n = 2$, we have

$$v/V_m = 1/(1 + K_w/[L_t])^2 \quad (2b)$$

that is, a simple binding isotherm (Sandermann, 1982; Cortese & Vidal, 1983), with

$$K_w = (\sum X_i/K_i)^{-1} \quad (2c)$$

K_w is an *overall weighted noncooperative dissociation constant*[2] for a noncooperative mechanism involving multiple equilibria, weighted with respect to the PC molar fraction (X_i). Equations 2a,b allow manipulation of ligand mixtures as single ligand species (Cortese & Vidal, 1983).

We next derive the velocity equation for a protein interacting through a Hill-type (one-step) cooperative multiple equilibrium in the presence of mixtures of ligands following a similar derivation described previously for the noncooperative situation (Cortese & Vidal, 1983). For an enzyme that exhibits cooperativity, with high Gibbs free energy of interaction between its sites (Hill model; Hill, 1910), the multiple equilibria established are

$$E + nL_1 \rightleftharpoons EL_{1(n)} \qquad K_1 = [E][L_1]^n/[EL_{1(n)}] \quad (3a)$$

$$E + nL_2 \rightleftharpoons EL_{2(n)} \qquad K_2 = [E][L_2]^n/[EL_{2(n)}] \quad (3b)$$

$$E + nL_p \rightleftharpoons EL_{p(n)} \qquad K_p = [E][L_p]^n/[EL_{p(n)}] \quad (3c)$$

where L_1, L_2, ... L_p are different ligand species (i.e., lecithin or other effective lipids in apoBDH reactivation); $EL_{1(n)}$, $EL_{2(n)}$, ... $EL_{p(n)}$ are enzyme species of the highest degree of occupancy by the ligand (L_1, L_2, ... L_p, respectively). K_1, K_2, ... K_p are overall dissociation constants for the cooperative process of dissociation of each enzyme–ligand$_n$ complex $[EL_{i(n)}]$ into free enzyme ([E]) and ligand ($[L_i]$). The observed velocity (v) for this situation will be

$$v = \sum_{i=1}^{p} k_i[EL_{i(n)}] \quad (4)$$

where k_i is the catalytic constant of the species $EL_{i(n)}$ at its equilibrium concentration. Dividing both sides of eq 4 by the total enzyme concentration $[E_t]$, we obtain

$$v/[E_t] = \sum_{i=1}^{p} k_i X_{EL_{i(n)}} \quad (5)$$

or

$$v = \sum_{i=1}^{p} V_{m(i)} X_{EL_{i(n)}} \quad (6)$$

where $X_{EL_{i(n)}}$ is the molar fraction of the species $EL_{i(n)}$ and $V_{m(i)} = k_i[E_t]$ is the maximal velocity obtained with the species $EL_{i(n)}$.

Equation 6 can be written as

$$v = \frac{V_{m(1)}[EL_{1(n)}] + V_{m(2)}[EL_{2(n)}] + ... + V_{m(p)}[EL_{p(n)}]}{[E] + [EL_{1(n)}] + [EL_{2(n)}] + ... + [EL_{p(n)}]} \quad (7)$$

Equation 7 can be rewritten so as to eliminate the [E] terms:

$$v = \frac{V_{m(1)}\{[L_1]^n/K_1\} + V_{m(2)}\{[L_2]^n/K_2\} + ... + V_{m(p)}\{[L_p]^n/K_p\}}{1 + \{[L_1]^n/K_1\} + \{[L_2]^n/K_2\} + ... + \{[L_p]^n/K_p\}} \quad (8)$$

Equation 8 can be rearranged (assuming $V_{m(1)} = V_{m(2)} = ... = V_{m(p)} = V_m$; Cortese & Vidal, 1983) to give

$$v = \frac{V_m\{([L_1]^n/K_1) + ([L_2]^n/K_2) + ... + ([L_p]^n/K_p)\}}{1 + ([L_1]^n/K_1) + ([L_2]^n/K_2) + ... + ([L_p]^n/K_p)} \quad (9)$$

and, alternatively

$$v/V_m = 1/\{1 + [1/(\sum_{i=1}^{p}[L_i]^n/K_i)]\} \quad (10)$$

When each ligand (L_1, L_2, ... L_p) is present in a fixed molar fraction (X_1, X_2, ... X_p, respectively) eq 10 can be simplified. Then, we obtain

$$v/V_m = 1/(1 + K_{coop(w)}{}^n/[L_t]^n) \quad (11)$$

where, analogous to eq 2c

$$K_{coop(w)}{}^n = [\sum(X_i{}^n/K_i)]^{-1} \quad (12)$$

The *overall weighted cooperative dissociation constant* can be expressed as a function of X_i and K_i [$K_{coop(w)}{}^n = \phi(X_i,K_i)$]. $K_{coop(w)}{}^n$ can be calculated for three limiting conditions: (a) different types of sites (with or without distinct kinetic properties; we do not discuss this situation further here); (b) an

[2] Kinetic definitions: v is the initial velocity. V_m is the maximal velocity extrapolated according to the various procedures described in the text. n is the number of ligands involved in multiple equilibria. $[L_t]$ is the total concentration of ligand; $[L_{10}]$, $[L_{25}]$, $[L_{50}]$, and $[L_{90}]$ are the respective ligand concentrations that give 10, 25, 50, and 90% of maximal activation; K_w, overall weighted noncooperative dissociation constant, represents an average dissociation constant for the multiple equilibria involving random addition of ligands (noncooperative model discussed here). We have previously used the term overall weighted intrinsic dissociation constant (Cortese & Vidal, 1983) and now are changing the nomenclature for clarity. Intrinsic dissociation constants for each ligand are weighed with respect to the molar fraction (X_i). This constant is extrapolated from x intercepts in $v^{-1/2}$ vs. $[L_t]^{-1}$ and is an $[L_{25}]$ value (see Figure 5). $K_{coop(w)}$, overall weighted cooperative dissociation constant, represents an average dissociation constant for the multiple equilibria involving interconversion between free enzyme (E) and maximal liganded enzyme (EL_n). It is weighed with respect to the individual molar fractions of ligand (X_i), and it is an intrinsic constant because it represents the addition of one of the n molecules involved in the equilibria. [$K_{coop(w)}{}^n$ represents the total addition of n molecules.] This constant is obtained from x intercepts in Hill plots as an $[L_{50}]$ value (see Figure 1). K_i, cooperative dissociation constant, represents the intrinsic dissociation constant for the interconversion between free enzyme (E) and a maximal liganded enzyme with one class of ligand $[EL_{i(n)}]$. It is called intrinsic because only one of the p classes of ligand species (L_i) is considered. K_{PC} is the cooperative equilibrium constant for single dissociation within the multiple Hill equilibria. It is equivalent to the geometric mean of the overall constant K_i ($K_i{}^{1/n}$). $K_{Hill(app)}$ is an apparent Hill-type dissociation constant obtained when noncooperative data are plotted in a Hill plot.

exclusive binding[3] [where $EL_{i(n)}$ is the only enzyme–ligand species with the highest degree of occupancy]; or (c) a nonexclusive binding behavior [where there are mixed ligand species of enzyme–ligand complex with the highest degree of occupancy, $EL_{i(1)}L_{j(n-1)}$, $EL_{i(2)}L_{j(n-2)}$, ... $EL_{i(n-2)}L_{j(2)}$, $EL_{i(n-1)}L_{j(1)}$ (Rubin & Changeaux, 1966)]. The latter two situations are considered in the Appendix and the main conclusion is that a simple scheme with one ligand species always has a solution [i.e., eq 13; see also Wyman (1967, 1968)].

For the activation of BDH by bilayer phospholipids containing different lecithin molecular species, we make the approximation that each of these lecithins interacts approximately the same. Therefore, $K_{coop(w)}$ is considered as an overall weighted intrinsic cooperative binding constant for PC binding to apoBDH. For only one ligand species, and weighted with respect to the PC molar fraction, eq 12 becomes

$$1/K_{coop(w)} = X_{PC}/K_{PC} \qquad (13)$$

where X_{PC} is the molar fraction of PC in lecithin-containing vesicles and K_{PC} is a redefined cooperativity constant ($K_{PC} = K_i^{1/n}$) for the dissociation of a single PC molecule from PC-binding sites on apoBDH (when these n sites are considered equivalent). With this definition, we use a linear plot that is the $K_{coop(w)}^{-1}$ value as a function of X_{PC}. Both limiting models considered in the Appendix (exclusive and nonexclusive binding) give the same linear equation when only one ligand species is present in the reaction mixture (eq A4 and A11 in the Appendix).

Application of the Site-Competition Model for the Reactivation of D-*β-Hydroxybutyrate Dehydrogenase.* Hill plots of experimental data for reactivation of apoBDH by PE–PC–PP vesicles are given in Figure 1. Each plot is a titration of enzymic activity as a function of the phospholipid concentration. The set of data were taken from Figure 1 of Sandermann et al. (1986). The different plots represent reactivation in the presence of distinct molar fractions of lecithin, constructed with estimated maximal velocities (V_m). Values of $K_{coop(w)}$ (overall weighed intrinsic cooperative binding constant; see eq 13) were interpolated from the x intercepts.

A plot of $K_{coop(w)}^{-1}$ as a function of X_{PC} was constructed for three sets of reactivation data (Figure 2). K_{PC} values, obtained from the slope (according to eq 13), were 5.23 mol of PL/mol of BDH monomer for PE–PC–SPM vesicles, 4.90 mol of PL/mol of BDH monomer for PE–PC vesicles, and 4.47 mol of PL/mol of BDH monomer for PE–PC–PP vesicles [cf. Sandermann et al. (1986)]. Unilamellar vesicles from egg PC vesicles ($X_{PC} = 1.0$) sized both by gel filtration and differential ultracentrifugation provided the maximal activation point on the line and gave K_{PC} values of 4.94 and 4.75 mol of PL/mol of BDH monomer, respectively.

Other mixtures of phospholipid bilayer vesicles were tested for reactivation of apoBDH. We have used PE–PC–L (lipid in assay) vesicles, with X_{PE} ranging from 0.34 to 0.42 and X_{PC} ranging from 0.42 to 0.53. Linear plots of $K_{coop(w)}^{-1}$ as a function of X_{PC} were obtained in the range studied. The K_{PC} values were 11.8 (2.9–20.8) mol of PL/mol of BDH monomer for PE–PC–HA (hexadecylamine) vesicles (values in parentheses are 95% confidence limits), 1.39 (0.32–4.56) mol of PL/mol of BDH monomer for PE–PC–CP (dicetyl phosphate) vesicles, and 0.75 (0.08–1.44) mol of PL/mol of BDH monomer for PE–PC–PA (phosphatidic acid) vesicles (J. O. McIntyre, unpublished results; plots not shown). Vesicles containing DPG (diphosphatidylglycerol) do not fit in our linearizing plot, probably due to the high negative charge density as pointed out previously (Churchill et al., 1983; Berrez et al., 1985).

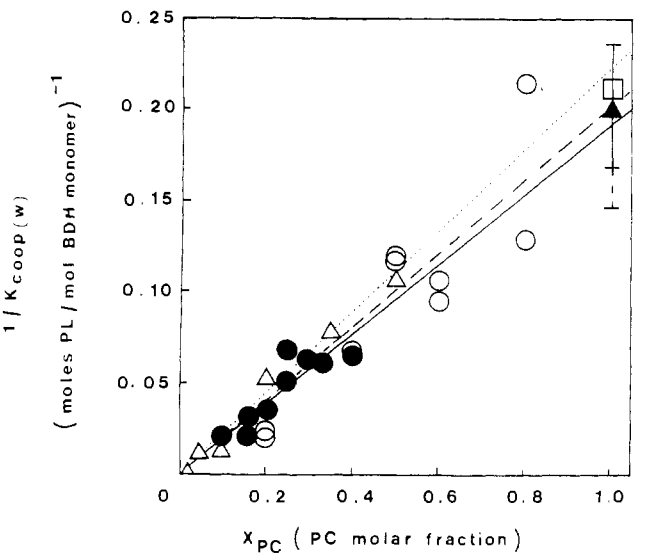

FIGURE 2: Plot of the reciprocal overall weighted intrinsic cooperative binding constant [$K_{coop(w)}$] as a function of the PC molar fraction (X_{PC}) according to eq 13 (see Results). A set of straight lines is obtained by linear regression analysis comparing results of several phospholipid reactivation mixtures of D-β-hydroxybutyrate apodehydrogenase with different lipid mixtures (three curves are shown). $K_{coop(w)}$ values obtained from Figure 1 (PE–PC–PP vesicles, Sandermann et al., 1986) are shown here [dotted line, open triangles; $K_{coop(w)}^{-1} = 0.2236X_{PC} - 8.53 \times 10^{-4}$; $r = 0.994$]. Other values of $K_{coop(w)}$ were estimated from x intercepts of Hill plots similar to those shown in Figure 1, constructed for activation curves of rat liver apoBDH by PE–PC–SPM vesicles [solid line, closed circles; $K_{coop(w)}^{-1} = 0.1911X_{PC} + 8.26 \times 10^{-4}$; $r = 0.960$] and PE–PC vesicles [J. D. Cortese and J. C. Vidal, unpublished results; dashed line, open circles; $K_{coop(w)}^{-1} = 0.2042X_{PC} - 2.4 \times 10^{-3}$; $r = 0.960$]. Additional values of $K_{coop(w)}^{-1}$ for single-bilayer egg lecithin vesicles prepared according to Huang and Thompson [1974; closed triangle and solid standard deviation bar (SD), mean ± SD = 0.202 ± 0.034, $n = 5$) and Barenholz et al. [1977; open square and dashed standard deviation bar (SD), mean ± SD = 0.211 ± 0.064, $n = 6$] are shown. Statistical variation of these values (calculated as the ratio of standard deviation to average slope values) were 14.3% for PE–PC–SPM vesicles, 31.0% for PE–PC vesicles, 17.1% for PE–PC–PP vesicles, 16.7% for chromatographically sized unilamellar vesicles (Huang & Thompson, 1974), and 30.3% for ultracentrifugation sized unilamellar vesicles (Barenholz et al., 1977).

A plot of V_m and $n_{H(app)}$ vs. X_{PC} is shown in Figure 3. The apparent n_{Hill} was estimated from Hill plots of the maximal slope in the mid- (50%) saturation region as a function of PC molar fraction (X_{PC}) in PE–PC–PP vesicles (data taken from Figure 1). The $n_{H(app)}$ extrapolates to a maximal value of 1.85, obtained for this sigmoidal (nonhyperbolic) curve (see Discussion). The first two points of $n_{H(app)}$ at low X_{PC} appear to be complicated by the strong anionic charge ($X_{PP} > X_{PC}$; see also Discussion). If these two points are discounted, the $n_{H(app)}$ varies mainly from approximately 1 to somewhat less than 2.

Reactivation Studies Using Short-Chain Phospholipids. In the analysis of the noncooperative model proposed by Cortese et al. (1982), one limitation was that inactivation of BDH occurred when the soluble lecithin reached its cmc, so that experimental data for the reactivation in the presence of short-chain lecithins was available up to 34% of the maximal activation with MPL. In this study, ethanol was used to increase the cmc of dioctanoyllecithin, and conditions of temperature and ionic strength were optimized in the current kinetic assays (see Experimental Procedures). Under these conditions, it is possible to obtain activation curves up to 91%

[3] Exclusive vs. nonexclusive binding is used to refer to the presence of only one class vs. more than one class, respectively, of enzyme–ligand complex with the maximal degree of occupancy.

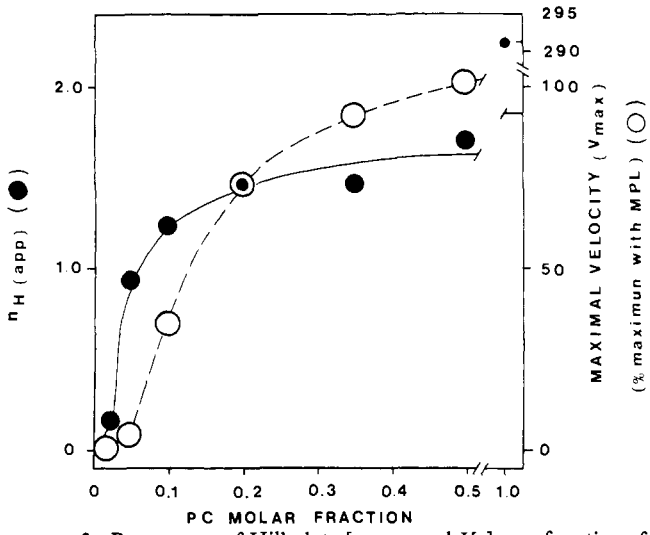

FIGURE 3: Parameters of Hill plots [$n_{H(app)}$ and V_m] as a function of PC molar fraction. Average data taken from experiments similar to those shown in Figure 1. Maximal velocities (V_m; open circles) are expressed as percent maximum activation with mitochondrial phospholipid (MPL). Reciprocal polynomial regression gives $V_m = [3.45 \times 10^{-3} + (4.18 \times 10^{-3})X_{PC}^{-1} - (6.84 \times 10^{-4})X_{PC}^{-2} + (5.19 \times 10^{-5})X_{PC}^{-3}]^{-1}$ with a regression coefficient $r = 0.993$. Apparent number of Hill [$n_{H(app)}$, closed circles] as defined in the text fits the equation $n_{H(app)} = [0.54 + 0.04X_{PC}^{-1} - (2.06 \times 10^{-3})X_{PC}^{-2} + (6.69 \times 10^{-5})X_{PC}^{-3}]^{-1}$ with $r = 0.999$. Maximal values of both quantities as extrapolated from the polynomials are indicated in the right ordinate. Values of $n_{H(app)}$ ± standard deviation were calculated by considering data dispersion on experiments presented in Figure 1: (a) for $X_{PC} = 0.02$, $n_{H(app)} = 0.35 \pm 0.27$; (b) for $X_{PC} = 0.05$, $n_{H(app)} = 0.94 \pm 0.20$; (c) for $X_{PC} = 0.10$, $n_{H(app)} = 1.24 \pm 0.23$; (d) for $X_{PC} = 0.20$, $n_{H(app)} = 1.40 \pm 0.17$; (e) for $X_{PC} = 0.35$, $n_{H(app)} = 1.44 \pm 0.04$; (f) for $X_{PC} = 0.50$, $n_{H(app)} = 1.69 \pm 0.12$. V_m are believed to be ±20%. An error analysis shows that when V_m was varied 4-fold, i.e., for the Hill plot at $X_{PC} = 0.02$, when V_m was varied from 1.21% to 5.00% of maximal activation with MPL, $n_{H(app)}$ changed 70%.

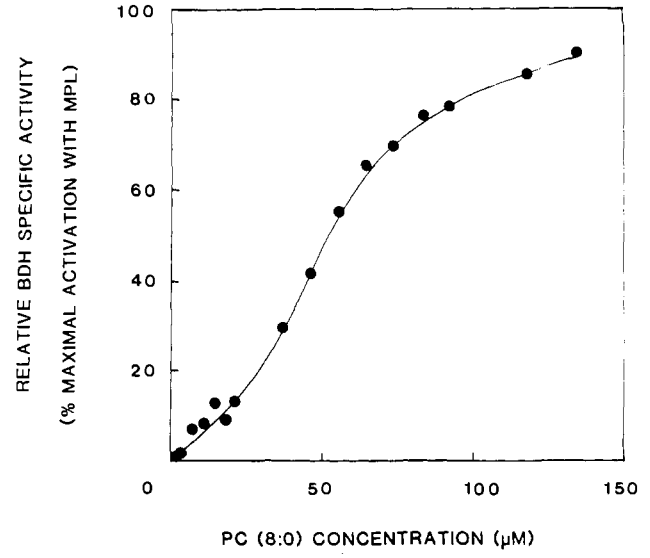

FIGURE 4: Reactivation of D-β-hydroxybutyrate dehydrogenase in the presence of PC(8:0). BDH specific activity measurements were performed at 25 °C according to Experimental Procedures, with 3 μg of protein per assay and variable amounts of dioctanoyllecithin. Absolute ethanol was added at 5% v/v concentration to ensure that all the experimental PC(8:0) concentrations were under its cmc value. BDH specific activity measurements of MPL were carried out under similar conditions and considered as 100% activation [mean ± standard deviation (SD) = 23.55 ± 1.47 μmol of NAD$^+$ reduced·min^{-1}·(mg of protein)$^{-1}$; $n = 4$].

of maximal activity with MPL (Figure 4). A maximal value of 90.9% was obtained at 135 μM PC(8:0) concentration. Higher concentrations showed inhibition by micelles (58.1 ± 4.0% of maximal with MPL at 186 μM PC(8:0), mean ± standard deviation; $n = 3$). When the data are represented according to the linear function described by Cortese et al. [1982; i.e., $v^{-1/2}$ as a function of $1/[PC(8:0)]$], a straight line is obtained over the full range (see Figure 5). Our experimental data also satisfy the requirements of the noncooperative model as indicated by Cortese et al. (1982), i.e., biphasic nonlinear v^{-1} vs. $1/[PC(8:0)]^2$ plots. The Hill plot obtained is linear ($r = 0.969$), with a slope of 1.765 and an apparent K_{Hill} of about 42 μM (not shown).[4]

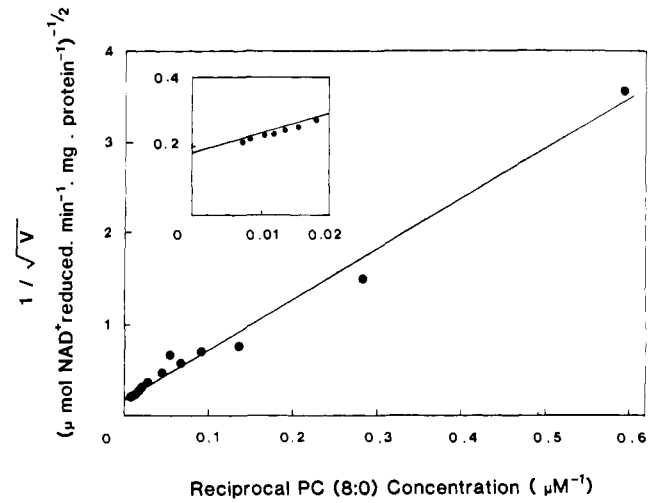

FIGURE 5: Double-reciprocal plot of the square root of initial velocity [(BDH specific activity)$^{-1/2}$] as a function of the reciprocal PC(8:0) concentration [[PC(8:0)]$^{-1}$]. The experimental data were taken from Figure 4. The linear regression straight line obtained for the full range of concentrations (see insert) was $v^{-1/2} = 5.464[PC(8:0)]^{-1} + 0.1894$, with a regression coefficient $r = 0.993$. Maximal velocity was obtained from the y intercept as $V_m = 27.9$ μmol of NAD$^+$ reduced·min^{-1}·(mg of protein)$^{-1}$. Maximal activities of MPL obtained were 23.6 ± 1.5 μmol of NAD$^+$ reduced·min^{-1}·(mg of protein)$^{-1}$ [mean ± standard deviation (SD), $n = 4$] under the same experimental conditions. $K_L = 28.84$ μM was extrapolated according to the method of Cortese et al. (1982).

The behavior of a soluble short-chain lecithin [PC(10:0)] in mixture with other nonactivating short-chain phospholipids [PE (10:0) and PA(10:0)] was also tested (Figure 6). Mixtures of PC(10:0) and PE(10:0) were prepared at different molar fractions of PC(10:0), and BDH enzymic activity was measured (Figure 6A). Mixtures of PC(10:0), PE(10:0), and PA(10:0) were also prepared at a fixed PA molar fraction of 0.1 and assayed (Figure 6B). Values of MPL activation are given in the same figure for comparison (Figure 6B, right ordinate).

Apparent dissociation constants obtained by Hill plots of data presented in Figure 6 are plotted as a function of molar fraction of PC(10:0) in Figure 7 [$1/K_{Hill(app)}$ vs. $X_{PC(10:0)}$]. Mixtures of PC(10:0) and PE(10:0) gave nonlinear plots with a minimum binding at $X_{PC(10:0)} \sim 0.5$; for $X_{PC(10:0)} > 0.5$, the binding of PC was enhanced (Figure 7A). Ternary mixtures of PC(10:0), PE(10:0), and PA(10:0) gave nonlinear plots with a maximum at $X_{PC(10:0)} \sim 0.5$ (Figure 7B).

In Figure 7B, a theoretical plot of $K_{Hill(app)}^{-1}$ vs. X_{PC} is shown (dashed line) for the noncooperative expression (eq 2b). The Hill equation for a two site noncooperative model was derived

[4] However, the $K_{Hill(app)}$ (extrapolated from 50% of maximal activity) and K_L (the intrinsic dissociation constant extrapolated from the $v^{-1/2}$ vs. $[PC]^{-1}$ plot) are not equal. It may be noted that when $v/V_m = 0.5$, $[L_{50}] = K_{Hill(app)}$, and the actual ligand concentration is higher than K_L (from eq 2b); i.e., $[L_{50}]/[L_{25}] = 2.41$. A value near this ratio is obtained in the PC(8:0) experiments ($K_{Hill(app)}/K_L = 2.14$).

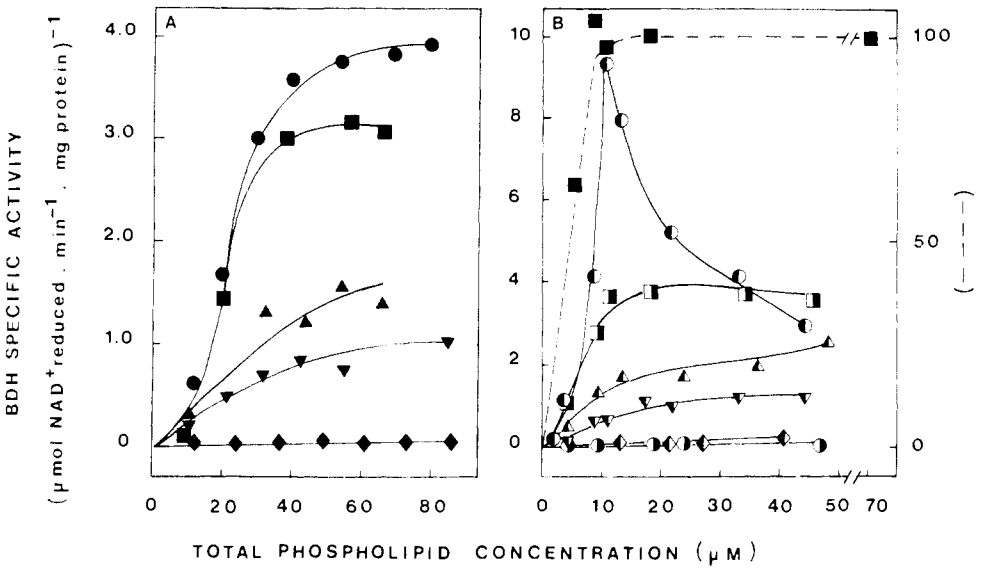

FIGURE 6: Reactivation curves of D-β-hydroxybutyrate dehydrogenase in the presence of mixtures of short-chain phospholipids. BDH specific activity measurements were performed according to Experimental Procedures, using 3 μg of protein per assay and variable amounts of preformed mixtures of PC(10:0), PE(10:0), and/or PA(10:0). Absolute ethanol (5%) was added to ensure that all the experimental concentrations were below cmc values. (A) Mixtures of PC(10:0) and PE(10:0) at molar fractions of PC(10:0) = 0.2 (◆), 0.5 (▼), 0.7 (▲), 0.9 (■), and 1.0 (●). (B) Mixtures of PC(10:0), PE(10:0), and PA(10:0). PA(10:0) molar fraction was kept at 0.1, and PC(10:0) molar fraction in the mixture varied to 0.25 (◑), 0.3 (◈), 0.5 (▼), 0.6 (▲), 0.75 (◨), and 0.9 (◐). The activation with MPL is provided for comparison. BDH specific activity measurements of MPL are shown in the same range of total phospholipid concentrations (dashed curve, right ordinate).

previously for a single ligand (Cortese et al., 1982). When the treatment of site competition of more than one ligand to a single site is applied, we obtain

$$\log (v/V_m - v) = -n \log K_{Hill(app)} + n \log [L_t] - \log \{[2[L_t]/K_{Hill(app)}] + 1\} \quad (14)$$

using definitions of v, V_m, n, and $[L_t]$ given in footnote 2.

For the noncooperative model (eq 2c), $K_{Hill(app)}$ is obtained from the x intercepts of Hill plots in the same way as $K_{coop(w)}$ is obtained for the cooperative model (eq 13). Rearrangement of eq 14 gives x intercepts $[K_{Hill(app)}]$ as a function of X_{PC}:

$$1/K_{Hill(app)} = (2^{1/n} - 1)(X_{PC}/K_{PC}) \quad (15)$$

Equation 15 is a function of n [i.e., $n_{H(app)}$] as obtained from experimental data (i.e., those from Figure 6). Then, it is possible to calculate $K_{Hill(app)}^{-1}$ vs. $X_{PC(10:0)}$ from $n_{H(app)}$ and average K_{PC} values [obtained from $X_{PC(10:0)} = 1.0$; not shown]. By contrast, the cooperative model is linear over the entire range of X_{PC} as indicated and goes through the origin (see Figure 2). This alternative noncooperative model (eq 2c) is the closest to a highly cooperative model with respect to a double-reciprocal graphical representations ($1/v^{1/2}$ vs. $1/[L]$) of the kinetic data. This plot is linear for the noncooperative model and deviates upward only slightly for the noncooperative model (Cortese et al., 1982). Other previously studied noncooperative models [which include association between active subunits and inhibition by excess of substrate; see also Cortese et al. (1982)] do not fit the plot of the reciprocal average cooperative constant as a function of X_{PC} (calculations not shown).

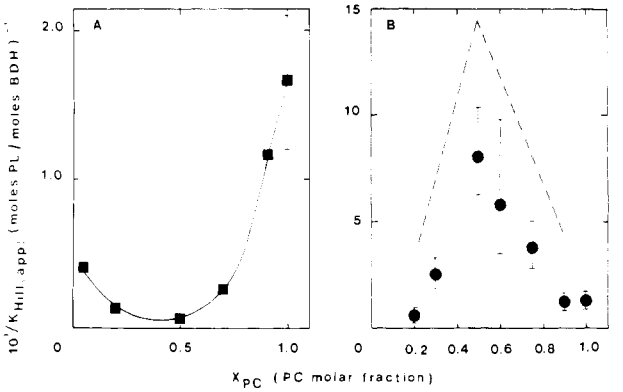

FIGURE 7: Apparent cooperative binding constants $[1/K_{Hill(app)}]$ as a function of PC(10:0) molar fraction for soluble short-chain phospholipid mixtures. The experimental data used to construct Hill plots are those shown in parts A and B of Figure 6, respectively. Maximal velocities are extrapolated from double-reciprocal plots of the square root of initial velocity $v^{-1/2}$ as a function of the reciprocal total phospholipid concentration ($[PL]^{-1}$) as described by Cortese et al. (1982). $K_{Hill(app)}$ was extrapolated from x intercepts of a Hill plot as in Figure 1. (A) Mixtures of PC(10:0) and PE(10:0); (B) mixtures of PC(10:0), PE(10:0), and PA(10:0) (solid curve; standard deviations for three experiments are indicated with solid bars). A theoretical curve of $K_{coop(w)}^{-1}$ vs. X_{PC} as expected for a noncooperative two-site model presented previously (Cortese & Vidal, 1983) is also plotted (dashed curve). We use $1/K_{Hill(app)} = (2^{1/n} - 1)\, X_{PC(10:0)}/K_{PC(10:0)}$ as an approximate equation (see Results; eq 15), with n as the apparent maximal slope in Hill plots [or $n_{H(app)}$]. $n_{H(app)}$ values obtained for a given $X_{PC(10:0)}$ [indicated as mean ± standard deviation (SD), number of experiments (n)] are (a) for $X_{PC(10:0)} = 1.0$, 1.65 ± 0.25 ($n = 6$); (b) for $X_{PC(10:0)} = 0.9$, 1.34 ± 0.01 ($n = 2$); (c) for $X_{PC(10:0)} = 0.7$, 0.77 ± 0.28 ($n = 2$); (d) for $X_{PC(10:0)} = 0.5$, 0.33 ± 0.28 ($n = 2$); (e) for $X_{PC(10:0)} = 0.2$, 1.01 ± 0.39 ($n = 2$); (f) for $X_{PC(10:0)} = 0.05$, 1.51 ± 0.06 ($n = 2$). We used an average reciprocal K_{PC} value [obtained from K_{Hill}^{-1} at $X_{PC(10:0)} = 1.0$] of 315.8 (mol of PL/mol of BDH)$^{-1}$.

DISCUSSION

In this study, we provide a new theoretical framework for analyzing kinetic data in terms of cooperative interactions in enzyme systems. Within this context, the activation of BDH inserted into phospholipid vesicles containing lecithin was found to conform to a cooperative activation as proposed by Sandermann et al. (1986). In this study, two interacting sites are involved. Nonetheless, we also confirm that the activation of apoBDH with soluble lecithins follows a two-site noncooperative mechanism as proposed by Cortese et al. (1982) and extensively tested in this study.

Methodology is thereby provided to distinguish kinetically between noncooperative behavior and cooperative behavior.

The distinction between cooperative and noncooperative models for BDH–lecithin interaction could represent the basis of assigning a plausible role for the lipid as an effector of enzymic activity. Our finding that the cooperative behavior is determined by the physical state and the composition of the bilayer phospholipids represents a new insight into the role of phospholipids for this lipid-requiring enzyme.

Sandermann (1982) analyzed the cooperativity of a number of different lipid-requiring enzymes. The approach involved evaluating the sigmoidicity of the activation curve by two comparable criteria: (1) $[L_{90}]/[L_{10}]$ activation ratios and (2) Hill plot analysis. By these criteria, a number of lipid-requiring enzymes were found to be activated by phospholipid in a cooperative fashion. The activation of BDH was found to be one of the most cooperative in behavior (with one of the lowest $[L_{90}]/[L_{10}]$ ratios; Sandermann et al., 1986).

We provide a new approach to evaluate cooperativity. A generalized treatment is presented (see Results and the Appendix), and an expression has been derived relating the reciprocal value of an overall weighted cooperative dissociation constant $[K_{coop(w)}]$ to the molar fraction of activating phospholipid (X_{PC}; see eq 13). The activation of BDH by lecithin in a variety of different mixtures of bilayer phospholipid conforms to this relationship over the entire range of X_{PC} (0–100%) as predicted for a highly cooperative system (Figure 2). In this context, highly cooperative is used to express a Hill-type mechanism (Hill, 1910), in which practically only two enzyme forms exist, inactive (E) and active (EL_n).

A similar kinetic treatment for noncooperative interaction was previously applied to evaluate the mechanism of activation of BDH by soluble mixtures of short-chain lecithins (Cortese & Vidal, 1983). A noncooperative activation of apoBDH in a soluble phase was indicated with this approach. Both, the noncooperative activation by soluble lecithins and the cooperative activation by mixtures of bilayer phospholipid, give the same minimal number of sites (i.e., two). Thus, it would appear that the composition and physical state of the phospholipid determine the noncooperative vs. cooperative activation of BDH by lecithins.

The striking linearity for a Hill-type mechanism of the $K_{coop(w)}^{-1}$ vs. X_{PC} plots shown in Figure 2 indicates that a single dissociation constant is obtained with a diversity of phospholipid mixtures in the form of bilayer vesicles. This suggests that the nature of the interaction of lecithin with BDH is similar to that of the other nonactivating phospholipids in the bilayer (Churchill et al., 1983). Thus, there is a statistical independence between binding of lecithin and binding of nonactivating lipids (Wyman, 1965; Weber, 1972; Cortese, 1984). Other studies also indicate that the lecithin–BDH complex in the bilayer is short-lived on a time scale of NMR (10^{-5} s) and in a continuous state of association and dissociation (Deese et al., 1986).

The cooperative and noncooperative models discussed here are similar in that only EL_n, the maximally liganded species, is active, whereas any intermediate forms are inactive for the noncooperative model or vanishingly small in concentration for the cooperative model. The intermediate forms are thereby silent in both cases. These models are similar but can readily be distinguished by fitting eq 2b and 12 (see plots in Figure 2 and Figure 7). The main difference is that the cooperative model is linear in this representation (Figure 2); the straight line goes through the origin and always has a positive slope. In the noncooperative model discussed here, the slope of $K_{Hill(app)}^{-1}$ as a function of $X_{PC(10:0)}$ depends of the Hill coefficient, $n_{H(app)}$, (i.e., the Hill slope in these Hill plots; Figure 7 and eq 15). Other models, in which intermediate states of liganding and free enzyme are active, differ to an even greater extent (calculations not shown; Cortese et al., 1982).

There are two assumptions implicit in deriving the equations for both the cooperative and noncooperative models: (1) there is rapid equilibrium for ligand binding and (2) the different lecithin species used for the soluble system or the bilayer system give similar maximal velocity. The application of the tests for cooperative vs. noncooperative behavior should be carried out over a full range of X_{PC}. The discrimination is most sensitive in the middle range of X_{PC} (see Figures 2 and 7B).

Hill theory analyzes kinetic data in terms of cooperativity. A Hill coefficient is obtained that is used as an indication of the nature of site–site interactions (positive or negative cooperativity). This coefficient thus implies the number of sites that can be interacting and its magnitude whether the cooperativity is positive or negative (Neet, 1980). In addition, an affinity constant for the ligand can be obtained. It should be noted, however, that the inference of apparent cooperativity has more meaning when (1) a model of cooperativity is satisfied (Saroff & Minton, 1972; Saroff & Yap, 1972; Saroff, 1973) and (2) independent physical data is obtained to show intra- or intermolecular changes in conformation (Wyman & Phillipson, 1974; Neet, 1980).

The Hill plot is usually made over a limited range of ligand concentration, and the steepest part of the curve is generally used to obtain a Hill coefficient $[n_{H(app)}]$. Frequently, no effort is made and/or it is difficult to obtain the limiting values of $n_H = 1$ at low and high ligand concentration (Neet, 1980; Endrenyi et al., 1975). Thermodynamic and kinetic analyses are limited without such a complete plot (Cornish-Bowden & Koshland, 1975).

The number of ligands involved in enzyme catalysis or modulation is generally small (generally $n < 4$). Therefore, the Hill coefficient would also have to be a small number.[5] In the tabulation of lipid-activated enzymes presented by Sandermann (1982), n_H was found to be 3.17 or less (calculated from the general equation $([L_{90}]/[L_{10}])^{n_H} = 81$; see Segel, 1975).

It might be reasonable to suppose that in a membrane the phospholipids surrounding an intrinsic membrane protein each interact with the enzyme and that n could be a high value equivalent to the lipids at the boundary of the proteins in the membrane (Sandermann, 1982, 1983; Benga & Holmes, 1984). In actuality, the results referred to above for BDH indicate that n is a small number, i.e., 2, for both the models of noninteracting and interacting sites. This means that two lecithins are sufficient for activation of the active unit of enzyme (Cortese et al., 1982; Cortese & Vidal, 1983). In the case of the activation with soluble lecithin, the active unit is the dimer (McIntye et al., 1978). Thus, one lecithin per BDH polypeptide is sufficient for complete activation (Cortese et al., 1982).

In our studies with bilayer phospholipids, the maximal $n_{H(app)}$ obtained was somewhat less than 2 (1.85; Figure 3), whereas those obtained earlier were in excess of 2 (2.35; Sandermann et al., 1986). This difference is important in terms of implied mechanism. The lower value suggests a dimeric association for BDH and the higher value perhaps a tetramer. The results

[5] An exception is the cooperative binding of calcium ions by the calcium binding protein from terminal cisternae of sarcoplasmic reticulum. This protein has a high capacity for binding calcium (approximately 50 Ca^{2+} ions/molecule). The binding of calcium ions is a cooperative process and is associated with the crystallization of the protein; a Hill coefficient of 14 is obtained (Tanaka et al., 1986).

in this study appear to be more reliable since a V_m and a $K_{coop(w)}$ was obtained for each titration, in contrast with the previous study. A membrane-bound BDH tetramer with two interacting subunits could satisfy our experimental results. For a cooperative mechanism, a single n_H should be obtained when free energy of interaction approaches infinity. For this ideal case, the n_H would be constant and equal to the number of sites for any value of ligand concentration or X_{PC} (Heck, 1971). Weber (1975) calculated the n_H for two-site interaction using a Gibbs free energy of −2.8 kcal/mol; an n_H value of 1.81 was obtained, close to that we find here (cf. Figure 3). It is worth noting that Sandermann (1984) found a value of −2.8 kcal/mol for the free energy involved in cooperative behavior of membrane-bound BDH, using a two-site Adair equation (Adair, 1925).

In our studies, $n_{H(app)}$ values of less than 1.0 were obtained at X_{PC} less than 0.1. We suspect that for low X_{PC} (where $X_{PP} > X_{PC}$), the high negative charge density represents an additional complication. Discounting these two experimental points, the $n_{H(app)}$ varies from 1.0 to somewhat less than 2.0. Another possibility to explain $n_{H(app)}$ less than 1.0 would be a transition between symmetric and asymmetric tetramers of BDH [i.e., between tetramers with subunits in the same or in different conformations, respectively; see Viratelle and Seydoux (1974)], if a two-state Monod–Wyman–Changeaux model applies for membrane-bound apoBDH (Monod et al., 1965).

Studies presented previously (Cortese et al., 1982) showed that Hill plots obtained with short-chain lecithins are linear in all the range available for kinetic measurements (0.7–34% of maximal activation with MPL). At the higher concentrations for short-chain lecithins, BDH activity measurements were limited by inactivation when the cmc of the lecithin is reached (Cortese et al., 1982). For the soluble lecithins in this study (Figures 4 and 5), the Hill plot is linear over a much more extended range (approximately 1–91% of maximal activity). Further, reciprocal plots of the apparent cooperative dissociation constant [$K_{Hill(app)}$] as a function of $X_{PC(10:0)}$ are nonlinear (Figure 7), indicating that the data obtained with soluble lecithins do not satisfy a cooperative mechanism. Mixtures containing three different short-chain phospholipids, PC(10:0), PE(10:0), and PA(10:0), mimic some properties of bilayer phospholipids; i.e., the maximal activity is reduced when the molar fraction of PC(10:0) is lowered, and the maximal affinity or maximal reciprocal apparent dissociation constant is obtained at a composition of PC(10:0), PE(10:0), and PA(10:0) in the range similar to that in MPL [Figure 7B; also see Churchill et al. (1983)]. In Figure 7B the theoretical curve calculated according to the noncooperative model discussed here (eq 15) approximates the shape of our experimental curve. It is important to note that the mixtures of PC(10:0) and PE(10:0) do not conform to the noncooperative mechanism (Figure 7A). When the ternary mixture of phospholipids (PC–PE–acidic phospholipid) mimicking that of mitochondrial phospholipid is compared, the physical state (soluble vs. bilayer phospholipid) determines whether the behavior is noncooperative or cooperative.

Our finding that bilayer phospholipid determines cooperative behavior could be a reflection of (a) an allosteric regulation of the enzyme or (b) that the enzyme exists as a tetramer in the bilayer. In the case of hemoglobin it is only the tetramer that behaves cooperatively (Edelstein, 1975).

ApoBDH has an absolute and specific requirement for enzymic activity. It can be activated by lecithin in either the soluble or bilayer state (Nielsen & Fleischer, 1973). This study points out important differences in the activation of BDH by bilayer phospholipids vs. soluble lecithin. The following observations are relevant:

(a) The experimental results for bilayer phospholipids fit a cooperative model (i.e., a linear plot of a cooperative dissociation constant [$K_{coop(w)}$] is obtained as a function of PC molar fraction; Figure 2). Activation with soluble lecithins does not conform to this equation.

(b) We find here that the activation by soluble lecithin conforms with predictions for a noncooperative model (according to eq 1 and 2) over the measured range of 1–91% of maximal activity obtained with MPL (Figures 4 and 5).

(c) For noncooperative activation with short-chain lecithins, different plots are obtained depending on the type of lipid mixture. For the ternary mixture of PC(10:0), PE(10:0), and PA(10:0) the theoretical shape of the plot approximated the experimental data (see eq 15; Figure 7). The plot is not linear as a function of $X_{PC(10:0)}$ and therefore not cooperative.

We conclude that the state of the phospholipid determines the nature of activation of BDH. For soluble lecithins, it is noncooperative whereas for bilayer lecithins it is cooperative. Our studies do not address the mechanistic basis for these differences.[6] For both the stoichiometry is the same; i.e., two PC molecules are necessary for activation of the functional unit. This stoichiometry appears as a basic characteristic of the enzyme.

ACKNOWLEDGMENTS

We thank Professor J. Oliver McIntyre for providing valuable experimental data and Professor Juan C. Vidal for several helpful discussions regarding cooperativity. We are pleased to acknowledge the helpful comments of Drs. J. Oliver McIntyre and Thomas M. Duncan on the manuscript.

APPENDIX

Further development of the site-competition theory described previously (Cortese & Vidal, 1983) will be considered in this section for a Hill-type mechanism (see Results), for both exclusive binding and nonexclusive binding.[3] We show that convergent solutions (eq 13 under Results) are obtained in both limiting models that account for cooperativity in the simple situation with only one activating species in a mixture of nonactivating ligands (see Discussion).

We suggest in this section a general method to solve linear equations resulting for exclusive and nonexclusive binding models. This method is based on varying the molar fractions for each activating ligand in the mixture of activating and nonactivating species. Intrinsic dissociation constants can be obtained for each individual ligand equilibria. Experimental confirmation is necessary, and combined situations can be actually beyond the accuracy of kinetic methods. However, this approximation may have interest (when applicable to real systems) as a test of cooperative binding behavior of mixtures of ligands. To obtain the solutions for $K_{coop(w)}^n$ (overall weighted cooperative binding constant as defined in the text) by use of multiple linear equations, it is desirable to use iterative least-square statistical methods (Frazer & Suzuki, 1973). First, we have a situation with *exclusive binding*, when the binding of ligand species (L_1, L_2, ... L_p) with different affinity for binding sites is exclusive, given molecular species

[6] Our current studies indicate that an allosteric model can be used to describe the general kinetic behavior of BDH with soluble and bilayer phospholipids. In this regard, the composition of the phospholipid also modifies the allosteric behavior. Noncooperativity and cooperativity appear to be limiting cases of the general allosteric model (J. D. Cortese, J. O. McIntyre, and S. Fleischer, unpublished experiments).

with only one kind of ligand in their binding sites [there are no mixed species as $EL_{1(j)}L_{2(k)} \ldots L_{p(r)}$]. The multiple equilibria established are

$$E + nL_1 \overset{K_1}{\rightleftharpoons} EL_{1(n)} \qquad K_1 = [E][L_1]^n/[EL_{1(n)}] \tag{A1a}$$

$$E + nL_2 \overset{K_2}{\rightleftharpoons} EL_{2(n)} \qquad K_2 = [E][L_2]^n/[EL_{2(n)}] \tag{A1b}$$

$$E + nL_p \overset{K_p}{\rightleftharpoons} EL_{p(n)} \qquad K_p = [E][L_p]^n/[EL_{p(n)}] \tag{A1c}$$

where L_1, L_2, ... L_p are different ligand binding species (i.e., with distinct affinity for binding sites in E); $EL_{1(n)}$, $EL_{2(n)}$, ... $EL_{p(n)}$ are the catalytically active species of the enzyme. K_1, K_2, ... K_p are overall dissociation constants for the dissociation of each enzyme–ligand$_n$ complex into free enzyme ([E]) and ligand ([L_i]). This situation (that supposes p different ligand species as a generalization) was developed under Discussion (eq 4–11), giving

$$v/V_m = 1/[1 + 1/(\sum_{i=1}^{p}[L_i]^n/K_i)] \tag{A2}$$

Equation A2 gives an overall weighted cooperative binding constant (for n binding sites and p ligand species), that is

$$K_{\text{coop(w)}p}^{n} = (\sum_{i=1}^{p}X_i^n/K_i)^{-1} \tag{A3}$$

where X_i is the ligand molar fraction in the mixture of ligands and K_i is a dissociation constant that corresponds to the dissociation of p molecules of the ligand L_i from binding sites. To simplify eq A3 we can write K_i as a product of n individual constants (k_i). Then, $K_i = \prod_{i=1}^{n}k_i = k_i^n$. Taking this into account, we have

$$K_{\text{coop(w)}p}^{n} = [\sum_{i=1}^{p}(X_i/k_i)^n]^{-1} \tag{A4}$$

or, when only one ligand species is present

$$K_{\text{coop(w)}} = (X_i/K_i)^{-1} \tag{A5}$$

where $K_{\text{coop(w)}}$ is an overall weighted intrinsic cooperative binding constant. We use this solution for apoBDH–lecithin interaction (eq 12 in the text). The general situation can be solved as a simultaneous linear equations system. If we have p ligand species, and we perform p experiments with p different molar fractions for each ligand, we have

$$1/[K_{\text{coop(w)}}]^n = X_1^n(1/k_1^n) + X_2^n(1/k_2^n) + \ldots + X_p^n(1/k_p^n) \tag{A6a}$$

$$1/[K_{\text{coop(w)}}']^n = (X_1')^n(1/k_1^n) + (X_2')^n(1/k_2^n) + \ldots + (X_p')^n(1/k_p^n) \tag{A6b}$$

$$1/[K_{\text{coop(w)}}'']^n = (X_1'')^n(1/k_1^n) + (X_2'')^n(1/k_2^n) + \ldots + (X_p'')^n(1/k_p^n) \tag{A6c}$$

$$1/[K_{\text{coop(w)}}^{''\cdots p}]^n = (X_1^{''\cdots p})^n(1/k_1^n) + (X_2^{''\cdots p})^n(1/k_2^n) + \ldots + (X_p^{''\cdots p})^n(1/k_p^n) \tag{A6d}$$

We use sample programs with a $p \times p$ matrix and previously assigned values for all the k_i's to obtain solutions for these systems (calculations not shown). Actual experimental data can require some iterative statistical refinements to find the constants and their standard deviation (Frazer & Suzuki, 1973). Simple systems (as apoBDH interacting with lecithin) always have a graphical solution.

A second situation that needs to be considered in our analysis is the *nonexclusive binding* of the ligands composing the mixture. When a protein binds several specific ligands with significant but unequal affinity, its binding process is nonexclusive [there are mixed species $EL_{1(j)}L_{2(k)} \ldots L_{p(r)}$ with intermediate degrees of occupancy by various or all the possible ligand species; Rubin & Changeaux, 1966]. From the whole range of situations that can occur in a given ligand binding system, we selected as a general example the appearance of all the intermediate forms. Thus, when the following multiple equilibria are established

$$E + nL_1 \overset{K_1}{\rightleftharpoons} EL_{1(n)} \tag{A7a}$$

$$K_1 = k_1^n = [E][L_1]^n/[EL_{1(n)}]$$

$$E + (n-1)L_1 + L_2 \overset{K_{1(n-1),2}}{\rightleftharpoons} EL_{1(n-1)}L_2 \tag{A7b}$$

$$K_{1(n-1),2} = k_1^{n-1}k_2 = [E][L_1]^{n-1}[L_2]/[EL_{1(n-1)}L_2]$$

$$E + (n-2)L_1 + 2L_2 \overset{K_{1(n-2),2(2)}}{\rightleftharpoons} EL_{1(n-2)}L_{2(2)} \tag{A7c}$$

$$K_{1(n-2),2(2)} = k_1^{n-2}k_2^2 = [E][L_1]^{n-2}[L_2]^n/[EL_{1(n-2)}L_{2(2)}]$$

$$E + L_1 + (n-1)L_2 \overset{K_{1,2(n-1)}}{\rightleftharpoons} EL_1L_{2(n-1)} \tag{A7d}$$

$$K_{1,2(n-1)} = k_1k_2^{n-1} = [E][L_1][L_2]^{n-1}/[EL_1L_{2(n-1)}]$$

$$E + nL_2 \overset{K_{2n}}{\rightleftharpoons} EL_{2(n)} \tag{A7e}$$

$$K_2 = k_2^n = [E][L_2]^n/[EL_{2(n)}]$$

we have a nonexclusive binding situation for two different ligand species (L_1 and L_2) that compete by n ligand binding sites in a protein molecule. If we express hypothetically the reduced specific velocity (v/V_m) with the same considerations done before with respect to the multiple equilibria present, we obtain

$$v/V_m = \{[EL_{1(n)}] + [EL_{1(n-1)}L_2] + \ldots + [EL_1L_{2(n-1)}] + [EL_{2(n)}]\}/[E_t] \tag{A8}$$

or, if we express v/V_m as a function of the free enzyme ([E)], it gives

$$v/V_m = \{[[L_1]^n/k_1^n + [L_1]^{n-1}[L_2]/k_1^{n-1}k_2 + \ldots + [L_1][L_2]^{n-1}]/[k_1k_2^{n-1} + [L_2]^n/k_2^n]\}/\{[1 + [L_1]^n/k_1^n + [L_1]^{n-1}[L_2]/k_1^{n-1}k_2 + \ldots + [L_1][L_2]^{n-1}]/[k_1k_2^{n-1} + [L_2]^n/k_2^n]\} \tag{A9}$$

In the presence of a fixed proportion of each ligand (constant molar fractions of L_1 and L_2), eq A9 can be explicitly written as

$$v/V_m = \{[L_t]^nX_1^n/k_1^n + [L_t]^nX_1^{n-1}X_2/k_1^{n-1}k_2 + \ldots + [L_t]^nX_1X_2^{n-1}/k_1k_2^{n-1} + [L_t]^nX_2^n/k_2^n\}/\{1 + [L_t]^nX_1^n/k_1^n + [L_t]^nX_1^{n-1}X_2/k_1^{n-1}k_2 + \ldots + [L_t]^nX_1X_2^{n-1}/k_1k_2^{n-1} + [L_t]^nX_2^n/k_2^n\} \tag{A10}$$

or, rearranging terms

$$v/V_m = [[L_t]^n(X_1^n/k_1^n + X_1^{n-1}X_2/k_1^{n-1}k_2 + \ldots + X_1X_2^{n-1}/k_1k_2^{n-1} + X_2^n/k_2^n)]/[1 + [L_t]^n(X_1^n/k_1^n + X_1^{n-1}X_2/k_1^{n-1}k_2 + \ldots + X_1X_2^{n-1}/k_1k_2^{n-1} + X_2^n/k_2^n)] \tag{A11}$$

Then, we define again an overall constant for the nonexclusive situation, that is

$$[K_{coop(w)}]_{(nonexclusive)_n}^{2} = [\sum_{j=0}^{n}(X_1/k_1)^{n-j}(X_2/k_2)^{j}]^{-1} \quad (A12)$$

and, when eq A12 is substituted into eq A11, we have

$$v/V_m = [L_t]^n/\{[K_{coop(w)ne}]_n^2 + [L_t]^n\} \quad (A13)$$

$K_{coop(w)}^{n}$ for the nonexclusive model considered here can be solved in a way similar to that for the exclusive case. If we rearrange eq A12, we have

$$1/[K_{coop(w)}]_n^2 = \sum_{j=0}^{n}[(X_1^j X_2^{n-j})(k_1^{-j}k_2^{j-n})] \quad (A14)$$

and we can calculate the $k_1^{-j}k_2^{j-n}$ products by a set of simultaneous linear equations obtained in experiments made with several different values of the X terms. The situation with more than two binding species (L_1, L_2, ... L_n, in general) becomes difficult to solve, except for computational methods of analysis. In such cases there is an exceptionally high number of terms to calculate and the statistical confidence is a factor hard to evaluate. Also, the contribution of linked potential function (Wyman, 1964, 1967) or partition functions (Hill, 1985) to the binding can affect the final results in many real systems that exhibit this kind of interactive behavior.

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Hydration of Carbon Dioxide by Carbonic Anhydrase: Internal Proton Transfer of Zn^{2+}-Bound HCO_3^- †

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ABSTRACT: Proton transfer within HCO_3^- has been examined under various conditions through molecular orbital methods: partial retention of diatomic differential overlap and 4-31G self-consistent field programs. These conditions include the absence or presence of Zn^{2+}, $Zn^{2+}(NH_3)_3$, or a water ligand on Zn^{2+}. In addition, 4-31G+ and some MP2/4-31G** results are obtained. The use of Be^{2+} to simulate Zn^{2+} reproduces reaction pathways and energy barriers, except for marginal cases. The barrier of 35.6 kcal/mol for direct internal proton transfer is reduced to 3.5 kcal/mol when one water molecule, not bound to Zn^{2+}, is included for proton relay and to 1.4 kcal/mol when two such water molecules are included. In the enzyme, either Thr-199 or solvent molecules could perform this relay function. Our results favor this facilitated proton transfer over a mechanism in which Zn^{2+}-bound OH^- attacks CO_2, a bidentate intermediate forms, and the OH moiety of the resulting HCO_3^- dissociates from Zn^{2+}, thus leaving one of the oxygens of the original CO_2 as a ligand to Zn^{2+}.

Carbonic anhydrase is a zinc metalloenzyme that catalyzes the reversible hydration of CO_2 to bicarbonate ion and a proton. In human carbonic anhydrase II (HCA II) the maximal turnover rate is 10^6 s^{-1} at 25 °C. It is now widely accepted that initial nucleophilic attack occurs by a Zn^{2+}-bound hydroxide ion and that subsequent proton transfer is catalyzed by a non Zn^{2+}-liganded histidine and by buffer in HCA II (Lindskog, 1983; Lindskog et al., 1984; Pocker & Sarkanen, 1978; Prince, 1979; Coleman et al., 1980; Lipscomb, 1983). A plausible sequence (Figure 1) for the hydration reaction is the following: (1) binding of CO_2 near Zn^{2+}; (2) conversion of CO_2 to HCO_3^- by nucleophilic attack of Zn^{2+}-bound OH^- on C of CO_2; (3) internal proton transfer of Zn^{2+}-bound HCO_3^-; (4) binding of H_2O to Zn^{2+} and ionization of this Zn^{2+}-bound H_2O to facilitate release of HCO_3^-; and (5) the coordinated transfer of H^+ from Zn^{2+}-bound H_2O to a proton-transfer group (His-64 in HCA II), then to buffer, and then to solvent. Not least among the ambiguities are five coordinated Zn^{2+} species, which can be formulated as intermediates in Figure 1. Examples are the transient binding of one oxygen of CO_2 to Zn^{2+} (step 2), the binding of both an OH and a terminal O of HCO_3^- to Zn^{2+} (step 3), and the binding of both H_2O and HCO_3^- (step 4).

In this paper we examine the proton transfer of step 3. The need for this transfer can be seen by comparing the forward reaction, in which the attack of Zn^{2+}-bound OH^- on CO_2 leaves the proton on a Zn^{2+}-bound oxygen, with the reverse reaction in which HCO_3^- may be expected to bind with an unprotonated oxygen to Zn^{2+} (Figure 1). Microscopic reversibility (Fersht, 1977) thus requires the proton transfer of step 3. In alkyl carbonate anions (RCO_3^-), which are known to bind as well as do alkyl carboxylates (RCO_2^-) to the enzyme, no substrate activity occurs (Pocker & Deits, 1983). Here, the proton is replaced by an alkyl group. If steric and pK_a factors are not problems, these results (Pocker & Deits, 1983) may support the proton transfer, which we now examine.

Methods

(*1*) *Basis Sets.* Partial retention of diatomic differential overlap (PRDDO) (Halgren & Lipscomb, 1973; Marynick & Lipscomb, 1982) uses an orthogonalized basis. It is a close approximation to the self-consistent field (SCF) MO calculations at the minimum basis set level. One-, two-, and three-center integrals of the form $(\chi_{ia}\chi_{jb}|\chi_{kc}^2)$ are retained, where χ_{ia} is a symmetrically orthogonalized AO mainly centered on the atom a. Also retained are one- and two-center exchange integrals of the form $(\chi_{ia}\chi_{ja}|\chi_{ia}\chi_{ja})$ and $(\chi_{ia}\chi_{ja}|\chi_{ia}\chi_{jb})$. Problems of rotational invariance are avoided by choice of local axes that are unique in anisotropic environments. Retention of $\sim N^3$ integrals in PRDDO is shown to simulate efficiently the STO-3G SCF results (Halgren et al., 1978; Scheiner et al., 1976). We also employ the Gaussian 82 program using the 4-31G basis of Pople (Ditchfield et al., 1971), and the 4-31G+ basis (Clark et al., 1983) obtained by adding sp type diffuse orbitals (with exponents 0.04 for C and 0.068 for O) on the heavy atoms in 4-31G. A limited number of 4-31G**

† This research was supported by the National Science Foundation (CHE-85-15347 and PCM-77-11398) and the National Institutes of Health (GM06920).

0006-2960/87/0426-5293$01.50/0