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Solvation effects upon the thermodynamic substrate activity; correlation with the kinetics of enzyme catalyzed reactions. I. Effects of added reagents such as methanol upon alpha-chymotrypsin

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Abstract

Solvents, detergents, etc., have often been added to the medium to study the kinetics of enzyme action and for binding studies. They have been employed for diverse reasons such as solubilization of substrates or to stabilize an enzyme that was originally membrane bound. Thermodynamic considerations dictate that any added substance, such as methanol, which is present in significant quantity *must* affect the thermodynamic activities of the enzyme, enzyme–substrate complex, substrate and any other intermediates although cancellation effects may occur in this regard. The influence upon substrate activities is the only one that is easily experimentally accessible. These effects are shown, from the data of Bernard and Laidler, to be large in the case of the α-chymotrypsin catalyzed hydrolysis of methylhydrocinnamate. The variation of the Michaelis–Menten constant is quantitatively explainable in terms of the alteration of the thermodynamic activity of the substrate by methanol.

Keywords: Enzyme catalyzed reactions; α-Chymotrypsin; Modifiers; Enzyme kinetics; Enzyme thermodynamics

1. Introduction

The purpose of this communication is to inaugurate a start to this difficult problem by beginning with the effects of additives such as methanol, ethanol, etc., on the chemical potential or activity of the substrate by means of substrate solubility studies.

An added substance or solvent modifier ("mod") which has a net effect only upon the thermodynamic activity of the substrate may mimic classical competitive, noncompetitive or mixed inhibition. This explains the findings of some previous authors who suggested that methanol is a noncompetitive or mixed type inhibitor for α-chymotrypsin.

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The primary experimental contribution of this paper is to suggest a possible empirical method based on molecular size for determining the substrate activities in methanol–water solutions.

Many membrane bound enzymes have come under intensive study in the last ten years or so. In many cases, once such an enzyme is removed from the membrane, it is stabilized by significant amounts of detergent, glycerol, etc., in order to keep it in solution and/or to keep it from denaturing. Therefore, for example, many experiments on constitutive cytochrome P-450 enzymes have been performed in a 25% solution of glycerol in water as suggested by Ichikawa and Yamano [1]. Also to be shown in this communication using α-chymotrypsin as an example, roughly a fifty to seventy-five percent error in K_m, the Michaelis–Menten constant, will be introduced by the addition of methanol. This could have been avoided by using substrate activities rather than concentrations; it depends to a considerable extent on the nature of the substrate. If the substrate were very large and hydrophobic the error could be even larger. The term "mod" or modifier will be used for such an added substance.

Unstable or insoluble enzymes are not the only ones to which this discussion applies. For example, substances such as methanol and acetonitrile have been used routinely with α-chymotrypsin to increase the solubility of difficultly soluble substrates [2–5]. The same is true for many spectral studies with cytochrome P-450 [15]. The effects of the modifier, if any, on the enzyme and enzyme intermediates would be superimposed on this substrate effect. The first task is to separate these effects. The simplest first step for accomplishing this is to determine the effect of added modifier on the solubility of the substrate.

Applewhite et al. [6] following a treatment by Dimroth [7] exhibited remarkable foresight in first proposing the general approach which we are espousing here. It is unfortunate that they regarded that approach as a failure. We will show in the paper immediately following this one that they simply failed to take another important concept into consideration.

In order to cover the material to be presented, the following five topics must be discussed.

(1) The determination of substrate activities and the variation of those activities with modifier (mod) concentration; the relationship of the McMillan–Mayer second virial coefficient to the subject. The possibility of an empirical method based on molecular size is proposed for the determination of the McMillan–Mayer second virial coefficient in methanol–water solutions.
(2) The enzyme kinetic data of Barnard and Laidler [9] in which Michaelis–Menten constants and v_{max} values were determined in the presence of various concentrations of methanol. The enzyme was α-chymotrypsin and the substrate was methylhydrocinnamate.
(3) The striking parallel between the effects methanol on the activity of the substrate and the effects of methanol on the Michaelis–Menten constant. Maximum velocities are also affected.
(4) The simplest possible thermodynamic models which quantitatively explain the observed results upon K_m entirely in terms of thermodynamic substrate activities.
(5) The solution effects (effects on substrate activities) in points (1)–(4) above can mimic such classical types of behavior as noncompetitive, mixed or competitive inhibition depending on the particular circumstances. Thus some suggestions in the literature that methanol is a noncompetitive or mixed inhibitor for α-chymotrypsin is explainable in terms of substrate solvation.

We emphasize that we are not dealing with a case of modifier combining with substrate to form a discrete complex as exemplified by Segel's treatment of magnesium and ATPase [8].

2. Experimental procedures

The aqueous solutions were prepared with HPLC grade water from Baker. The toluene and methanol were of the highest HPLC grade from Baker. The other aromatic hydrocarbons were HPLC standards from Fluka. Their purity was confirmed as meeting or surpassing specifications by mass spectrometry. The hydrocinnamic acid and butyrophenone were supplied by Kodak. The hydrocinnamic acid was recrystallized from water before use. The butyrophenone was distilled *in*

vacuo. The benzophenone from Fisher was recrystallized from an ethanol–water mixture.

The McMillan–Mayer second virial coefficients in methanol–water solutions [10] were determined from solubility studies [11]. The method of Smith et al. [11] was used for toluene, ethylbenzene, propylbenzene, butylbenzene, butyrophenone and benzophenone. The high-speed stirring technique of Larese et al. [12] was used in the case of hydrocinnamic acid; it is essential that no significant esterification take place. The absorbances of the saturated solutions of toluene, ethylbenzene, propylbenzene and butylbenzene were determined at 260, 259, 268 and 268 nm, respectively. Butyrophenone was examined at 275 nm, while 257.5 nm was used for hydrocinnamic acid. Benzophenone was assayed at 361 nm. All measurements were made with a Cary 17D spectrophotometer. The values quoted are for 25 ± 0.05°C.

The reproducibility of the solubility measurements for the alkylbenzenes ranged from ±3% for butylbenzene to ±0.07% for toluene; the others were intermediate. The precision of the measurements involving butyrophenone was ±2% and for benzophenone ±2.6%. The repeatability for hydrocinnamic acid was ±2.8%, and for naphthalene ±0.68%. All studies were done in duplicate or triplicate.

3. Results and discussion

3.1 The determination of substrate activities and the variation of the activity of a substrate with mod concentration

The substrate activities in various methanol–water solutions may be determined as suggested by Miles et al. [13]. It is important that the nonelectrolyte substrate be sparingly soluble in water.

Bennett and Canady [14] showed, from their own data and reinterpretation of the data of Ueda et al. [24], that the logarithms of the aqueous solubilities, and hence the activities of a number of aromatic hydrocarbon substrates for cytochrome P-450 vary in a linear manner with added methanol and ethanol modifiers. It was pointed out in eq. (6) of [14] that, in dilute solution, the above mentioned slope is approximately equal to

$$\text{slope} = \bar{P}_{\text{Smod}} = -\left(\frac{\partial \ln C_S}{\partial C_{\text{mod}}}\right)_{\mu_H, T, \mu_w} \quad (6)$$

where C_S is the concentration of hydrocarbon molecules per unit volume and w is the solvent (water). At low concentrations of mod it is approximately equal to the McMillan–Mayer second virial coefficient $\bar{P}_{\text{Smod}}$ [10]. The linearity of the slope was observed over a surprisingly wide range of modifier concentration. This term is a statistical thermodynamic parameter useful in applying statistical mechanics to the calculation of the properties of a model for the solution process. It can be represented by minus the slope of the plot of the logarithm of solubility vs. mod concentration [14].

3.1.1 The determination of $\bar{P}_{\text{Smod}}$ for methylhydrocinnamate in methanol–water solutions

Methylhydrocinnamate is an ester, and it was found that ordinary techniques for the determination of the solubility of such a compound are not satisfactory [12]. The rapid stirring method of Larese et al. [12] would be in order. The solubility can be determined in a few minutes by this method, thus avoiding the problem of constant increase in apparent solubility due to slow hydrolysis or decomposition of the solute. The utility of this ultra high speed stirring method (30,000 rpm) is, without a doubt, excellent for the study of solid solutes which are relatively unstable in aqueous solution. When the solute to be studied is a liquid or a heavy oil one sometimes obtains turbid suspensions that take a very long time to clear and which are difficult to sample. Such was the case with methylhydrocinnamate and water–methanol solutions in our hands. Therefore it was necessary to estimate the McMillan–Mayer second virial coefficient by other means. In some ways this was fortunate, since the method adopted for this purpose may help to give us some insight into what determines this coefficient, at least in methanol–water solutions. It also indicates, in a

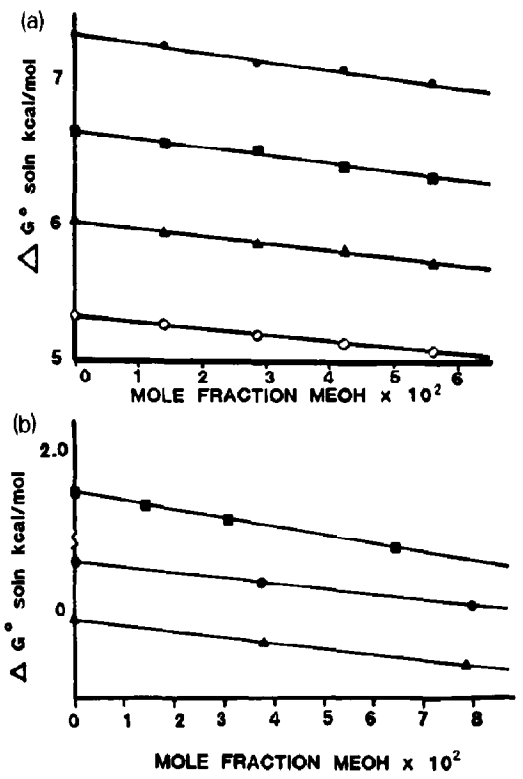

Fig. 1. (a) ΔG° of solution for a series of alkylated benzenes vs. concentration of methanol. They represent, in ascending order, (○) toluene, (▲) ethylbenzene, (■) propylbenzene and (●) butylbenzene. Concentrations are expressed in mole fraction. (b) ΔG° of solution (see text) vs. methanol concentration for, in ascending order, (▲) butyrophenone, (●) hydrocinnamic acid and (■) benzophenone.

preliminary manner, a possible method for estimating this second virial coefficient by empirical means.

Figures 1(a) and (b) show a series of plots of methanol concentration vs. ΔG° of solution for a series of aromatic hydrocarbons and other substances related to enzyme substrates. Concentrations are expressed in terms of mol fraction. The Gibbs free energy of solution was used in Fig. 1 rather than logarithms to emphasize an important point. Since the concentrations of methanol vary over a wide range, the higher concentrations not being dilute by any stretch of the imagination, it is clear that this is a linear free energy relationship which holds over a much wider concentration range than might have been imagined. Such plots illustrate a compensation law [16]. In many cases there is a compensation between the heat and entropy of solution producing a linear plot when the standard free energy of solution is plotted against alcohol concentration at constant temperature [11]. Leffler and Grunwald [20] have shown that this kind of compensation law is expected to hold when relatively small changes are introduced. Lumry and Rajender [16] have discussed many aspects of this phenomenon. Actually the heats of binding of various substances of the type studied here to α-chymotrypsin are not negligible and ΔG° values are quite large. However, a compensation between heat and entropy is still observed [25]. Discussion of possible reasons for this phenomenon is beyond the scope of the present communication.

Figure 1 contains the above free energy plots for the solubilities of aromatic hydrocarbons toluene, ethylbenzene, propylbenzene and butylbenzene as determined by the method of Smith et al. [11]. Mole fraction concentrations were used, thus the number of particles involved do not make a contribution to the thermodynamic parameters. The free energy term was written as $-RT$ ln(solubility). In Fig. 1(a), ΔG° was written as $-RT$ ln(absorbance), the concentration units thus being arbitrary. The latter values are presented as preliminary ones, the not unreasonable assumption being made that the Beer–Lambert law is followed in each case. It makes very little difference to the linearity of the plots whether the mole fraction scale or molarity scale is used.

The data presented in Table 1 ($\bar{P}$ values) were calculated using the molarity scale and logarithms of the solubility. The slopes, ($\bar{P}$ values, determined by the method of least squares) from these lines were then plotted against the molecular weight of the solutes studied (Fig. 2). Carbonyl groups were not counted in calculating the molecular weights for the oxygen-containing compounds. The reasonably good linearity seen in Fig. 2 indicates that the McMillan–Mayer second virial coefficient for the types of substances investigated here seems to be determined primarily by the "hydrophobic" part of the solute molecule. The authors do not wish to infer that this is necessarily a general rule for all solutes and mod-

Table 1

$\bar{P}$-values for the interaction of a number of components with methanol in aqueous solution

Compound	$\frac{\partial \ln sol}{\partial c}(\bar{P})$
Benzene [a]	0.110
Toluene	0.124
Ethylbenzene	0.136
Hydrocinnamic acid	0.138
Propylbenzene	0.155
Naphthalene	0.170
Butyrophenone	0.139
Butylbenzene	0.190
Benzophenone	0.209

[a] The value for benzene is calculated from the data of Ueda et al. [24].

ifiers, but our preliminary results indicate that this is approximately true for methanol–water solutions and aromatic compounds of the molecular size range studied here. If one does include the carbonyl groups in the molecular weight calculations, the linearity of the above plot is reduced. Although there are good reasons to expect that molecular surface area would, in principle, be the best parameter, molecular weight is adequate for our purposes.

Our own preliminary results with certain chaotropes and various hydrocarbons indicate that the linearity observed above is not *necessarily* a universal type of law. Inspection of Fig. 2 and a least squares linear regression analysis indicate that, on the basis of its size, the numerical value for the McMillan–Mayer second virial coefficient for methylhydrocinnamate in methanol–water solution calculated from its molecular weight would be expected to be approximately 0.16. The variation of ln solubility with methanol for such nonelectrolytes and hence the activities are linear over a wide range of modifier (Fig. 1 and refs. [11,14]). Equations utilized by Martin et al. [27], Adjei et al. [28], Martin et al. [29,30], as well as Williams and Amidon [31–33], exemplify the approach to calculation of solubilities in mixed solvents (and hence activity coefficients and activities) from equations suggested by them and a minimal amount of experimental data. Such approaches may well prove worthwhile if the McMillan–Mayer second virial coefficient can be calculated with reasonable accuracy for standard enzyme substrates.

Since water is a highly structured liquid, the individual molecules being hydrogen bonded to each other, and since the tetrahedral symmetry of the oxygen orbitals and the tetrahedral structure of ice suggest a locally tetrahedral orientation of molecules in the liquid state as well, any solute dissolved in water might be expected to disrupt such structures [23]. Therefore virtually any added substance is a prospective "modifier". But as pointed out by Smith et al. [11], cancellation effects over a given temperature range are possible if an added cosolute were to re-form many of the hydrogen bonds initially broken by the first phases of the solution process. An example would be sucrose which we have observed to have a very small effect on the solubility of hydrocarbons of the type studied here at 25°C. Therefore one would expect that sucrose, in reasonable concentrations, would have little or no effect on substrate activities at 25°C; thus effects of the sort considered here would be minimal. Not all additives *necessarily* solubilize or desolubilize or alter the activities of substrates and related com-

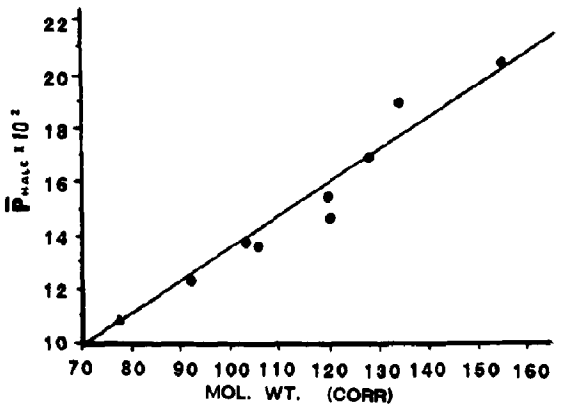

Fig. 2. Slopes ($\bar{P}_{Smod}$ values) of the plots shown in Fig. 1 as a function of corrected molecular weight. Carbonyl groups were omitted in calculating the molecular weight for the above plot. The solid triangular point on the line is for benzene and was calculated from the data of Ueda et al. [24]. In ascending order the compounds are benzene, toluene, hydrocinnamic acid, ethylbenzene, propylbenzene, butyrophenone, naphthalene, butylbenzene and benzophenone.

pounds, even when present in fairly high concentrations.

3.2 The enzyme kinetic data of Barnard and Laidler

Ordinarily, kinetic experiments are not done in a way well suited for the approach discussed here. A study by Barnard and Laidler [9] is one of the few which may be used as a preliminary test of the principles considered in this communication. They have determined $\bar{K}_{app}$ and k_{cat} (see eq. 5a) for the α-chymotrypsin catalyzed hydrolysis of methylhydrocinnamate at three methanol–water concentrations at 25°C and pH 7.8. The value of $\bar{K}$ in water was obtained from another study [17] from Laidler's laboratory (see Appendix). Obtaining the McMillan–Mayer second virial coefficient in methanol–water solutions for this oily substrate presented some technical difficulties which made it necessary to estimate it in the empirical way previously describe.

3.3 The striking parallel between the effects of methanol on the activity of the substrate and the effects of methanol on the Michaelis–Menten constant

Figure 3 consists of a plot of ln $\bar{K}_{app}$ for α-chymotrypsin and methylhydrocinnamate vs. concentration of methanol. Of course, concentrations rather than activities were used by Barnard and Laidler [9] to calculate the apparent $\bar{K}$-values ($\bar{K}_{app}$). It was necessary to use the $\bar{K}$-value of Canady and Laidler [17] for water since the other values quoted in the literature were determined in the presence of some methanol or at a nonstandard temperature or pH (2–4) (see Appendix). The slope of the line, determined by the method of least squares and shown in Fig. 3, is -0.17, certainly not far from the -0.16 which reflects only the effects of methanol on the activity of the substrate alone. Put in another way, if Barnard and Laidler had used substrate *activities* rather than concentrations in their calculations, they would have observed virtually no apparent change in $\bar{K}$, (or $1/K_m$) with methanol concentration. The simplest possible interpretation of

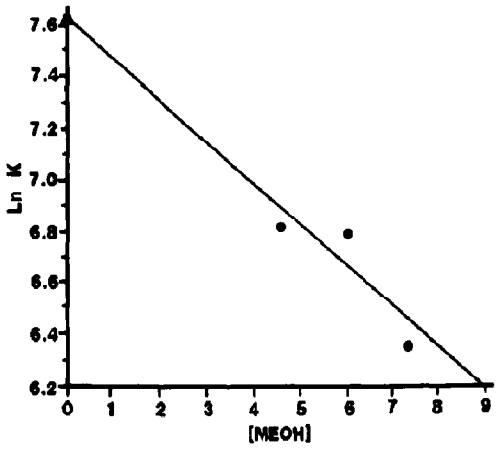

Fig. 3. The natural log of $\bar{K}$ for the α-chymotrypsin catalyzed hydrolysis vs. concentration of methanol in moles per liter. Data calculated from the work of Barnard and Laidler [9] (circles). The value represented by the closed triangle on the y-axis is from Canady and Laidler [17].

this finding is that $\bar{K}$ is possibly a pseudoequilibrium constant (see Appendix) and perhaps there are no significant contributions to enzyme–substrate complex formation from electrostatic effects. We must hasten to point out that room still certainly exists for cancellation and additive effects. For example, considerations to be presented tacitly assume that the working pH is reasonably removed from the pK_a's of important groups of the enzyme associated with enzyme activity, thus the small shifts in these values brought about by the change in dielectric constant caused by the addition of methanol or other mod molecule will not make a serious contribution to the observed kinetic effects. The finding by Kaplan and Laidler [26] that a medium consisting of 20% (w/v) isopropanol caused little change in the pH profile of K_m for α-chymotrypsin indicates that this kind of shift is probably not contributing in this case. We do feel, though, that such effects upon the substrate as discussed here are sufficiently important to merit serious thought and investigation.

A glance at Fig. 4 will confirm that for the α-chymotrypsin experiments v_{max} or k_{cat} is also affected by the presence of methanol. This figure consists of a plot of the logarithm of k_{cat} vs.

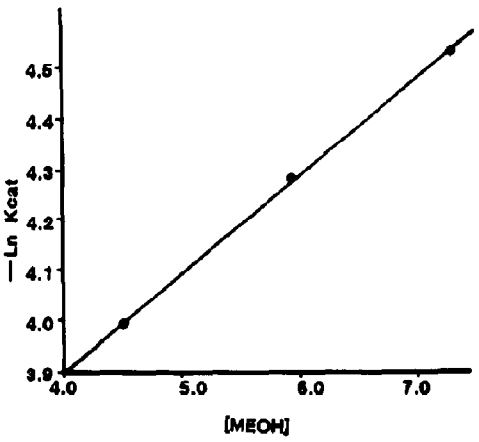

Fig. 4. Plot of the natural log of k_{cat} vs. methanol for the α-chymotrypsin hydrolysis of methylhydrocinnamate. Methanol concentration in moles per liter. Taken from the data of Barnard and Laidler [9].

methanol concentration also taken from the work of Barnard and Laidler [9]. Excellent linearity is obtained. Unfortunately, as opposed to $\bar{K}$, we do not have available a suitable value for k_{cat} at pH 7.8 and 25°C in water free of organic solvent. Apparently methanol was used by virtually all authors to help solubilize this rather difficult substrate. Thus only the three values of Barnard and Laidler, all in the presence of methanol, were suitable. These difficulties are discussed in more detail in the Appendix. The slope obtained from Fig. 4 is -0.22. This value is reasonably close to that for ln $\bar{K}$ vs. methanol concentration (-0.17, shown in Fig. 3). Whether the similarity of these two slopes is accidental or whether they are linked together by some as yet undiscovered principle remains to be seen; although such questions are good subjects for future research, they are beyond the scope of the present paper.

3.4 The simplest models which quantitatively explain the observed effects of methanol upon K_m entirely in terms of thermodynamic substrate activities

To attempt a steady state treatment would present too many complexities for the limited knowledge available, particularly experimental data, currently at our command; it will be assumed that $\bar{K}$ or its reciprocal K_m represents an approximate equilibrium constant or, alternatively, that the *variation* or $\bar{K}$ or K_m with methanol (mod) concentration reflects the *variation* of an equilibrium constant with methanol even though they themselves may be steady state constants. The reader is referred to the Appendix where the most commonly used steady state treatment for α-chymotrypsin is examined in some detail and the conditions under which at least one of the above assumptions is true are pointed out. Actually, equilibrium is not unlikely for this particular substrate (methylhydrocinnamate). The overall catalytic constant is one of the smallest values for k_{cat} to be found in the literature being orders of magnitude smaller than for "good" substrates [18].

Spectral binding constants determined for cytochrome P-450 enzymes are not subject to this uncertainity about whether the steady state or equilibrium applies; no product is formed under the experimental conditions employed. The reaction stops with the formation of enzyme–substrate complex [15]. Equilibrium being thus assured.

Therefore, consider this equilibrium where the symbols have their usual significances.

$$E + S \overset{\bar{K}}{\rightleftharpoons} ES$$

The *association* constant describing this equilibrium is given by

$$\bar{K} = \frac{a_1}{a_2 a_3} = \frac{1}{K_m} \tag{1}$$

where a_1, a_2 and a_3 represent the activities of ES, E and S respectively. The use of an association constant makes keeping track of the signs of the thermodynamic constants easier.

If one adds a given increment of mod which alters the activity of, among other things, S, thus altering $\bar{K}$ to $\bar{K}_{app}$.

$$\bar{K}_{app} = \frac{\gamma_1[ES]}{\gamma_2[E]\gamma_3[S]} \tag{2}$$

Where γ_1, γ_2 and γ_3 are activity coefficients for enzyme-substrate complex, enzyme and substrate

respectively in the mod–water medium and $\bar{K}_{app}$ is the observed or apparent association constant for the formation of enzyme–substrate complex in the presence of a given amount of mod. These relative activity coefficients relate activities of the various species to those in pure water.

To evaluate γ_3 for a substrate independently of any enzyme kinetic experiment: consider a solid or liquid substrate for the enzyme, the crystalline or liquid phase of which is at equilibrium with the saturated aqueous phase. If S_w is the solubility of the substrate in water where its activity coefficient is γ_w and S_m is the solubility in the presence of an added quantity of mod where the activity coefficient is γ_3, the activities of these two substrate solutions will be the same since the standard states are the same [13]; hence

$$\gamma_w S_w = \gamma_3 S_m \tag{3}$$

It is a simple matter to determine the ratio of γ_3 to γ_w

$$\frac{\gamma_3}{\gamma_w} = \frac{S_w}{S_A} = \gamma_R \tag{4}$$

where γ_R is a relative activity coefficient determined from solubility measurement and which refers γ_3 to γ_w, the conventional activity coefficient of the substrate in a saturated solution of pure water. If the nonelectrolyte substrate is only sparingly soluble in water, the saturated aqueous solution therefore being quite dilute, γ_w will have a value close to unity in water; so

$$\gamma_3 = \gamma_R$$

Therefore to determine the activity or activity coefficient for the substrate, one determines the solubility of the substrate in pure water and in mod–water solutions at various desired concentrations of mod. The ratios of the solubility in water/solubility in mod–water mixture at each mod concentration yield the desired activity coefficients. The addition of mod (alcohols, ketones, urea, and urea derivatives, etc.) generally increases the solubility of the nonelectrolyte substrate (decreases the activity or free energy of the substrate dissolved in the solution).

Substituting γ_R for γ_3 in the equilibrium expression for $\bar{K}_{app}$.

$$\bar{K}_{app} = \frac{\gamma_1[ES]}{\gamma_2[E]\gamma_R[S]} = \frac{1}{K_{m\,app}} \tag{5a}$$

and the appropriate Michaelis–Menten equation

$$v = \frac{k_{cat}\bar{K}_{app}[E]_0[S]}{1 + \bar{K}[S]} \tag{5b}$$

where v is the initial velocity of the enzyme catalyzed reaction, and the other terms have their usual significances. Therefore, it is clear that, of the three activity coefficients involved, one of them can be determined quite easily from solubility measurements. If all of them could be determined from non-enzyme kinetic experiments, one could predict in advance the dependence of the apparent binding constant on added mod by means of the Michaelis–Menten equation, eq. (5b). We show in the case being discussed that sometimes it is possible to anticipate that dependence when only the activity coefficients for the substrate are known.

In this first communication attention will be limited almost entirely to the initial step of enzyme–substrate complex formation from enzyme and substrate.

It is useful to review the conditions under which the slopes of ln (solubility) and ln $\bar{K}$ vs. mod concentration are numerically identical. In other words $\bar{K}_{app}$ in eq. (5) is determined *entirely* by the variation of γ_3 (or γ_R) since, as we have just pointed out from the data of Barnard and Laidler [9] this indeed appears to be the case with α-chymotrypsin and the substrate methylhydrocinnamate. Two possibilities spring immediately to mind.

(A) It is possible that the values of γ_1 and γ_2 (for ES complex and free enzyme, respectively) do not vary to any extent with methanol concentration. This does not seem very likely, although an example from our own work mentioned earlier in the literature [11] indicated that fairly high concentrations of sucrose did not greatly alter the activity coefficient for a hydrocarbon cosolute. Not all added substances *necessarily* significantly alter the activity of a cosolute at a given tempera-

ture. It was suggested that perhaps this occurred because of cancellation effects involving such factors as rupturing of old hydrogen bonds and formation of new ones, etc. Therefore, similar cancellations leaving the activity coefficients γ_1 and γ_2 essentially unchanged by the presence of methanol can not be ruled out with absolute certitude.

(B) In our view, a more likely explanation of the experimental data is that γ_1 and γ_2 may indeed vary with methanol or mod concentration, but to about the same extent. Therefore the *difference* in chemical potential or free energy between these two molecules remains about the same whether methanol is present or not. This may not be too unreasonable when one considers that, insofar as overall structure is concerned, E and ES are very similar molecules, differing from one another to only a small extent. Data presented in Part II of this series which follows immediately further supports the concept that either (A) or (B) is approximately true for α-chymotrypsin.

3.5 The solvation effects discussed here may mimic classical types of enzyme inhibition

Previous reports such as the one by Bender and Glasson [5] that methanol is a noncompetitive inhibitor for α-chymotrypsin might seem to be confusing in light of our approach to the subject. A similar result was reported from our own laboratory [19] that methanol was essentially a noncompetitive inhibitor with a very small competitive component (see Fig. 5 of that paper). The question now to be resolved is whether or not such apparent classical noncompetitive behavior can be explained in terms of solvation effects of methanol or other modifiers on the substrate.

Four cases are proposed:

3.5.1 Case I

The added substance mod interacts *only* with the *substrate* altering its activity and exerting no other net effect. Since a plot of log [S] vs. [mod] would be expected to be linear over a considerable range of mod [14], we may write

$$\log[S] = \log[S_0] + \alpha[\text{mod}] \tag{7}$$

Where $[S_0]$ is the activity of S in pure water (approximately equal to the concentration of S) and [S] is the corresponding activity of S in the presence of a given quantity of mod. The slope of this line is α, and since the addition of mod lowers the activity of S, α is negative. Alpha is numerically equal to $\bar{P}_{\text{Smod}}/2.303$; $\bar{K}_0$ is the true association constant in the absence of mod.

In the absence of mod the Michaelis–Menten equation is expressed as

$$v_0 = \frac{k_{\text{cat}}[E]_0\bar{K}_0[S_0]}{1+\bar{K}_0[S_0]} \tag{8}$$

In the presence of mod

$$v = \frac{k_{\text{cat}}[E]_0\bar{K}_0[S]}{1+\bar{K}_0[S]}$$

where [S] represents the activity of the substrate in that concentration of mod; $\bar{K}_0$ appears in both equations since the association constant as well as $v_{\max}$ are assumed to be unaffected by mod, only the change in effective concentration of S is contributing to the change in rate. An approximate example is discussed in the following paper in this series.

The log of the ratio v_0/v is

$$\log\frac{v_0}{v} = \log[S_0] - \log[S] + \log\left(1+\bar{K}_0[S]\right) - \log\left(1+\bar{K}_0[S_0]\right) \tag{9}$$

Keeping in mind eq. (7) and rearranging

$$\log\frac{v_0}{v} = -\alpha[\text{mod}] + \log\frac{1+\bar{K}_0[S]}{1+\bar{K}_0[S_0]} \tag{10}$$

For values of v_0/v between 1 and 1.5 the following relationship holds with a maximum deviation within the usual experimental precision for the determination of this ratio [1] ($\pm 3.5\%$).

$$\log\frac{v_0}{v} = 0.39\left(\frac{v_0}{v}-1\right) \tag{11}$$

[1] This is not meant to be considered as a truly mathematically satisfactory relationship. However, one can easily demonstrate for oneself that, given errors of up to three percent or so in v_0/v, it becomes impossible to distinguish in a significant way between v_0/v calculated from this relationship and the experimental value actually obtained.

hence

$$\frac{v_0}{v} = -2.56\alpha[\text{mod}] + 2.56 \log\frac{1+\bar{K}_0[S]}{1+\bar{K}_0[S_0]} + 1 \quad (12)$$

This is the general equation which is a reasonable approximation for inhibition up to about 50%. At relatively low values of [S], and [S_0], the second term on the right will tend to disappear and

$$\frac{v_0}{v} \cong -2.56\alpha[\text{mod}] + 1 \quad (13)$$

Remembering that α is negative, compare this with

$$\frac{v_0}{v} = K_i[I] + 1 \quad (14)$$

Which is a way of writing this ratio for simple classical noncompetitive inhibition, K_i being the noncompetitive association constant and [I] the concentration of noncompetitive inhibitor [18]. Virtually all of the ester substrates that have been used with α-chymotrypsin are sparingly soluble in water, therefore the concentrations of substrate employed experimentally ranged from low to quite moderate. It is easy to see how methanol might be mistaken for a noncompetitive inhibitor.

Therefore at moderate or low substrate concentrations, mod would be expected to mimic a classical noncompetitive inhibitor. For that matter, at very low substrate concentrations, competitive inhibition would be imitated as well, because the corresponding equation for classical competitive inhibition is [18]

$$\frac{v_0}{v} = \frac{K_i[I]}{1+\bar{K}[S]} + 1 \quad (15)$$

which at very low values of [S] reduces to eq. (14) since $\bar{K}[S]$ in the denominator of eq. (15) becomes small compared to unity; K_i in this case represents the affinity constant of the competitive inhibitor for the enzyme.

If [S] is very high, eq. (15) for competitive inhibition becomes $v_0/v = 1$. That is to say sufficiently high substrate concentrations will completely reverse the action of a competitive inhibitor.

Now let us examine eq. (12) at very high substrate concentrations. If [S] and [S_0] are very large, keeping eq. (7) in mind it is easy to show that

$$2.56 \log\frac{1+\bar{K}_0[S]}{1+\bar{K}_0[S_0]} = 2.56 \log\frac{\bar{K}_0[S]}{\bar{K}_0[S_0]} = 2.56\alpha[\text{mod}] \quad (16)$$

and eq. (12) reduces to $v_0/v = 1$. Therefore, at very high substrate concentrations, mod would be expected to mimic a classical competitive inhibitor, confirming our intuitive prediction that despite the fact that mod decreases the effective concentration of the substrate, it should, in principle, be possible to raise substrate concentrations high enough to eventually achieve saturation at some concentration of mod.

To summarize: Even though we allowed mod to exert an effect *only* by altering the activity or effective concentration of the substrate calculated from the effect on solubility, conventional analysis will indicate noncompetitive, mixed or competitive inhibition depending upon the specific conditions.

The plots will be approximately linear over considerable substrate ranges (low and high) and a tendency to be nonlinear over intermediate ranges. This curvature may be masked by scatter of the experimental data.

3.5.2 Case II

The maximum velocity or k_{cat} is also affected by the addition of mod. It might be unrealistic to expect that we would be able to find a large number of cases where an aqueous solvent modifier would affect only the chemical potential of the substrate and exert no net effect or a very small effect upon v_{max} or k_{cat} $[E]_0$. As mentioned earlier, this has been shown to be the case for the activation by potassium chloride (increase in binding of substrate) with the α-chymotrypsin hydrolysis of methylhippurate [13] and, in a very preliminary manner, the binding of a hydrocarbon to cytochrome P-450 [15]. Other solvent mod-

ifiers might be expected to alter v_{max} considerably.

One of the simplest treatments for this case would be possible if log v_{max} were linear in mod concentration. As a matter of fact, if v_{max} were linear in mod concentration and did not change too much (40–50%), then plotting log v_{max} vs. mod concentration would be close to linear. As we have seen from Fig. 4, it is not unreasonable to postulate log v_{max} to be approximately linear in mod concentration. In other words

$$\log k_{cat}[E]_0 = \log k_{cat0}[E]_0 + \alpha'[mod] \quad (18)$$

As before:

In the absence of mod

$$v_0 = \frac{k_{cat0}[E]_0 \bar{K}_0[S_0]}{1 + \bar{K}_0[S_0]}$$

In the presence of mod

$$v = \frac{k_{cat}[E]_0 \bar{K}_0[S]}{1 + \bar{K}_0[S]}$$

By an argument similar to that used in Case I

$$\log \frac{v_0}{v} \cong -(\alpha' + \alpha)[mod] + \log \frac{1 + \bar{K}_0[S]}{1 + \bar{K}_0[S_0]} \quad (19)$$

Let $\alpha' = Z\alpha$

since

$$\log \frac{v_0}{v} \cong 0.39\left(\frac{v_0}{v} - 1\right)$$

$$\frac{v_0}{v} = -2.56(Z\alpha + \alpha)[mod] + 2.56\log\left(\frac{1 + \bar{K}_0[S]}{1 + \bar{K}_0[S_0]}\right) + 1 \quad (20)$$

At low values of [S] eq. (20) becomes

$$\frac{v_0}{v} = -2.56(Z\alpha + \alpha)[mod] + 1 \quad (21)$$

Thus at very low [S], the inhibition observed is apparently classical simple noncompetitive.

At very high substrate concentrations

$$\frac{v_0}{v} = -2.56\alpha Z[mod] + 1 \quad (22)$$

Thus very high substrate concentration leads to apparent simple noncompetitive inhibition with a somewhat smaller slope than that obtained at very low substrate concentrations. At intermediate substrate concentrations a competitive component will appear to exist with some tendency to deviate from linearity, quite possibly masked by scatter of the data.

This formulation correlates well with the data of Barnard and Laidler [9], Glasson and Bender [5] and results of Miles et al. [19]. The latter observed that the α-chymotrypsin catalyzed hydrolysis of methylhippurate appeared to be inhibited by methanol by means of a simple noncompetitive mechanism at relatively low substrate concentrations; when higher substrate concentrations were used, a slight tendency toward competitive inhibition was observed. Unfortunately, sufficiently high substrate levels to distinguish between Cases I and II could not be attained. Such results are compatible with eqs. (20) and (21).

3.5.3 Case IIA

In this case the dependences of log $\bar{K}$ (or log K_m) and log v_{max} upon mod concentration are about the same. In the special case specifically for α-chymotrypsin and methylhydrocinnamate considered $Z \cong 1$

$\alpha' \cong \alpha$

At low [S]

$$\frac{v_0}{v} = -5.12\alpha[mod] + 1 \quad (24)$$

and at high [S]

$$\frac{v_0}{v} = -2.56\alpha[mod] + 1 \quad (25)$$

Hence, in this case, apparent simple noncompetitive inhibition would be obtained at both very high and very low substrate concentrations with the slope at low substrate levels being approximately twice that obtained at high substrate concentrations. There is an apparent competitive

component at intermediate substrate concentrations.

3.5.4 Case III

We would place in this category cases in which the approximations assumed in Cases I and II regarding cancellation of modifier effects upon enzyme and enzyme-substrate complex activities apply, and the modifier also acts as a true competitive or noncompetitive inhibitor as well as exerting solvation effects upon the substrate. Examples are discussed in Part II of this series immediately following.

3.5.5 Case IV

Situations in which none of the approximations assumed in Cases I, II and III regarding cancellation of modifier effects on enzyme and enzyme–substrate complex activities apply. While we can make no predictions insofar as the variation of $\bar{K}_{app}$ or $K_{m\,app}$ with modifier concentration under these conditions, we can determine the effect of modifier on the substrate activity before doing the kinetic or binding experiments and thus be assured that the effects that are observed are indeed due to effects of the modifier on the enzyme and/or enzyme–substrate complex(es). For example, in studying the effect of dielectric constant on an association constant, we can, and have, in the case of α-chymotrypsin, taken into account the hydrophobic interaction between substrate and the solvent modifier (methanol) used to alter the dielectric constant. This still leaves the interactions with enzyme and enzyme–substrate complex to consider.

One might comment that the high concentrations of methanol that we have been considering here would not ordinarily be encountered in most practical situations. To this we would reply that our preliminary experiments indicate that, for example, the chaotrope tetramethylurea is capable of measurably altering the activity of naphthalene (a good substrate for cytochrome P-450 enzymes) at a concentration of 5×10^{-4} mol fraction. Therefore a number of substances that have previously been considered to interact only with enzyme and/or some enzyme intermediate, might be exerting an effect on the substrate activity as well. The present work indicates that such a possibility should be checked carefully in every case. One should be particularly suspicious of "weak inhibitors".

What would be required to more conclusively demonstrate the tentative conclusions reached here regarding α-chymotrypsin, methylhydrocinnamate and methanol? Aside from furnishing more evidence along the same lines, or variations thereon, the most immediately useful thing that might be done to confirm the tentative conclusions regarding our interpretation of the work of Barnard and Laidler [9] would be to investigate the temperature dependence of the McMillan–Mayer second virial coefficient with methanol for a given substrate such as methylhydrocinnamate or perhaps methylhippurate. There is a hydrophobic interaction between methanol and typical ligands for chymotrypsin; this means that the McMillan–Mayer second virial coefficient is quite negative [14]. In turn, it follows that the ability of methanol, or many other types of modifiers, to alter the activity of a typical substrate is thus quite temperature dependent. Lowering the temperature tends to abrogate the effects of modifier on the activity of such substances as those discussed here. Therefore, it would be expected that, if our interpretation were correct, the slope of the plot of ln $\bar{K}$ vs. mod concentration would dramatically *decrease* at low temperatures and conversely *increase* at temperatures higher than 25°C (the temperature used by Barnard and Laidler). Indeed, if the apparent agreement we observe at 25°C were *not* a coincidence, then knowing the McMillan–Mayer second virial coefficient for substrate and methanol at any temperature would allow the prediction of the methanol dependence of $\bar{K}$ (or K_m) at that temperature.

Whether more extensive work shows our specific suggestions concerning α-chymotrypsin to be correct or incorrect is really not so important at this stage of development; the primary purpose of this paper is to convince, irritate, or inspire the reader to give further thought to this problem which has been virtually ignored since the beginning of research on the mechanism of enzyme action. The effects of modifiers on substrate activities is a logical place to start.

The second paper of this series demonstrates that certain mod compounds act both to alter the activity of the substrate and at the same time function as classical competitive inhibitors.

Appendix

Conditions under which one might expect to obtain a linear plot of ln K vs. methanol (or any other solvent modifier) the slope of which is approximately numerically equal to the McMillan–Mayer second virial coefficient

(Case I or II applies.) At least two conditions must be satisfied for this to happen; firstly, the free energy of E and ES are not affected at all by the addition of solvent modifier (thermodynamically impossible) or are each affected to about the same extent. Secondly, the *variation* of the logarithm of the apparent $\bar{K}$ with solvent modifier concentration must actually represent the approximate *variation* of the true equilibrium constant $\bar{K}$ with the concentration of modifier when dealing with Case II.

It is generally agreed that α-chymotrypsin and a number of other hydrolytic enzymes proceed through an acyl enzyme intermediate [21].

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{P_1}{\overset{k_2}{\rightleftharpoons}} ES^1 \overset{k_3}{\rightarrow} E + P_2$$

where P_1 and P_2 are the first and second products, respectively.

It can be shown that in the steady state

$$\bar{K}_{app} = \frac{1}{K_{m\,app}} = \frac{k_1(1 + k_2/k_3)}{k_{-1} + k_2}$$

and

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} = \frac{1}{1/k_2 + 1/k_3}$$

There are three major possibilities of particular interest to us, two of which are limiting, where conceptually clear cut results of the kind we are considering might be seen. Neither of the substrates specifically mentioned (methylhydrocinnamate and methylhippurate) are "good" substrates; k_{cat} values are extremely small. The assumption that k_2 and/or k_3 are very small compared to k_{-1} would be quite reasonable.

(A) *First possibility:* Suppose that $k_2 \ll k_3$. Thus, if acylation is the rate governing step, $\bar{K}_{app} = k_1/k_{-1} = \bar{K}_{true}$ (equilibrium constant); k_{cat} represents the first order rate constant for the acylation step.

(B) *Second possibility:* Suppose that $k_2 = k_3 = k$, then

$$\bar{K}_{app} = 2k_1/k_{-1} = 2\bar{K}_{true},$$

and

$$k_{cat} = \tfrac{1}{2}k$$

Of course, the slope of a plot of $\log 2\bar{K}_{true}$ vs. solvent modifier concentration would represent the variation of $\bar{K}_{true}$ with solvent modifier. It has been suggested that this is the situation with the substrate methylhippurate and α-chymotrypsin [21]. On the other hand, the work of Bernhard's laboratory with hydroxylamine [22] would indicate that probably possibility (A) might apply. It was found in our own laboratory that the effect of added potassium chloride between 0.5 and 2.0 molal KCl upon $\bar{K}$ for this enzyme–substrate combination could be accounted for simply in terms of the effect of potassium chloride on the activity of the methylhippurate substrate [13]. The catalytic constant or v_{max} was almost independent of KCl ($\pm 5\%$ from the mean). Hence it would appear that k_2 and k_3 varied to about the same extent or did not vary much. If possibility (A) applies for this particular example, the inference is that the rate of acylation is quite independent of ionic strength over this range.

(C) *Third possibility:* Suppose that $k_2 \gg k_3$, then

$$\bar{K}_{app} = \frac{k_1}{k_{-1}}\frac{k_2}{k_3} = \bar{K}\frac{k_2}{k_3}$$

and

$$k_{cat} = k_3$$

A plot of ln $\bar{K}$ vs. the concentration of a modifier such as methanol might be expected to

produce similar results to those already discussed provided that k_2 and k_3 are unaffected by modifier concentration, or vary to about the same extent with modifier concentration. In this case variation in k_{cat} or v_{max} will reflect the effect upon deacylation. In cases A, B and C, the predicted linear ln $\bar{K}$ (or ln K_m) vs. mod concentration plot would be expected.

In regard to obtaining values for $\bar{K}$ and k_{cat} in pure water: unfortunately, almost all of the experiments to which we referred earlier with α-chymotrypsin and methylhydrocinnamate involved at least moderate amounts of methanol and/or were not done at 25°C and pH 7.8. Laidler and Barnard, in another work [4] showed that $\bar{K}$ for methylhydrocinnamate (but not k_{cat}) was essentially independent of pH up to pH 8.0. A significant amount of organic solvent was present at all pH values investigated. On the other hand, Kaplan and Laidler [21] showed that $\bar{K}$ was also independent of pH in the same pH range for a number of other typical substrates for α-chymotrypsin in solutions containing no solvents other than water.

Therefore we have made the not unreasonable assumption that the value for $\bar{K}$ of Canady and Laidler [17] measured in water at neutral pH and 25°C could be used for the point representing zero methanol concentration. It is of interest that omitting this point and using only the values of Barnard and Laidler [9] at the three concentrations of methanol cited by them produces about the same slope (within 10%). Using all points the maximum deviation in $\bar{K}$ from the line shown is 11%, the average deviation is about 7.5%. This is the kind of precision that one would expect under the circumstances. The fact remains that omission of the point measured in the absence of methanol makes little difference when subjected to a least squares analysis.

On the other hand, we have only the three values of Barnard and Laidler [9] for k_{cat}; no approximations for k_{cat} in water may be made from the data that is available.

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