A Hydrocarbon–Water Model for the Formation of the Enzyme–Inhibitor Complex in the Case of α-Chymotrypsin*

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ABSTRACT: It is possible to predict the dependence of K_i, the association constant for enzyme–competitive inhibitor complex formation, upon the size of the inhibiting molecule. This can be accomplished provided that the dependence of the distribution coefficient between water and a liquid hydrocarbon phase is known for the same homologous series of inhibitors. The "normal" or most common slope obtained for the extraction process for a $\Delta F°$ *vs.* molecular surface area plot is approximately 0.12. The same slope is generally obtained when $\Delta F°$ of enzyme–inhibitor complex formation is plotted against inhibitor surface area. An exception is a series of substituted nitrobenzenes; the extraction plot has a slope of 0.07 and is matched almost perfectly by the enzyme plot. This matching of slopes for the extraction plots and the enzyme plots is observed in homologous series of low polarity hydrocarbons, nitrobenzenes, and carboxylate ions. This shows that EI formation for these simple substances may be completely analagous to an extraction process, with the enzyme site acting as a second nonaqueous phase.

On the other hand, substrates such as the hippuric acid and hydrocinnamic acid esters do not show this effect. The extraction plots are "normal," but the corresponding free energy *vs.* molecular surface area plots for the enzyme situation, while linear, have a much smaller slope than the extraction process. These substrate molecules consist of a central polar group or groups with hydrocarbon residues on either end, which would allow a two-point hydrophobic attachment to the enzyme. It is shown that the inhibition constants for a series of phenones, which have this same general structure, also yield a linear slope of similarly small value in the $\Delta F°$ *vs.* surface area plot. This is taken as evidence for a two-point hydrophobic attachment to the enzyme. It is suggested that complex formation may be treated either as a phase transfer (extraction) or as a transfer from the aqueous phase to the enzyme–water interface. In either case the enzyme may in some cases be treated as the second nonaqueous phase.

In previous publications from this laboratory (Miles and Canady, 1963; Miles *et al.*, 1963; Hymes *et al.*, 1965), it was suggested that an extraction mechanism is prominent in the formation of α-chymotrypsin–inhibitor complexes. It was considered that the active site of the enzyme contained an area of considerable hydrocarbon character, where the inhibitor or substrate molecule could rest at a much lower potential energy level. At a later time a similar suggestion was made by Belleau and Lacasse (1964) for acetylcholinesterase. We have treated complex formation in terms of extraction theory (Hymes *et al.*, 1965). In addition the solubilities of a series of aromatic hydrocarbons of low polarity were related to their ability to inhibit chymotrypsin. This treatment was applicable to a series of inhibitors which were sparingly soluble liquids of low polarity. In that series, the free energy of interaction per unit surface area between molecules of hydrocarbon in the liquid hydrocarbon phase could be considered to be approximately constant for the whole series. Such a solubility treatment is not directly applicable to polar-soluble substrates and inhibitors, nor to solid substances which are of interest. Hence it was deemed advisable to study the distribution of some substrates and inhibitors across a hydrocarbon–water interface. The purpose of this communication is to show that under certain conditions, it is possible to predict the dependence of K_i, the association constant for enzyme–inhibitor complex formation, upon the size of the inhibiting molecule, provided that the dependence of the distribution coefficient between the water and liquid hydrocarbon phase is known for the same series.

Experimental Section

The enzyme employed was a salt-free twice-crystallized sample of α-chymotrypsin supplied by the Sigma Chemical Co. The pH was 6.9 and the rates were determined by titration using a Radiometer pH-Stat. The substrate used for the inhibition work was methyl hippurate obtained from the Mann Chemical Co. All experiments included in this work were done at a concentration of 0.1 M in potassium chloride and

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3.3×10^{-3} M Tris buffer. The temperature was 25 ± 0.1°. Other details have been described in the first series of references in this present paper. Each point in Figures 1–6 is based upon approximately 24 rate measurements.

The methyl, ethyl, and propyl hydrocinnamates were prepared by refluxing 5 g of hydrocinnamic acid with 750 ml of the appropriate alcohol along with a few drops of concentrated sulfuric acid. The reaction mixture was refluxed for 2 days. The excess alcohol was evaporated under reduced pressure and the residue was extracted with ether. The ether layer was washed with ten 100-ml portions of 5% sodium bicarbonate. The ether was evaporated under reduced pressure and the ester was distilled under reduced pressure. The boiling points at 760 mm were: methyl, 236°; ethyl, 243°; and propyl, 250°.

The following compounds were obtained from Eastman Kodak: propiophenone, butyrophenone, nitrobenzene, *p*-nitrotoluene, *t*-butylbenzoic acid, 2-naphthoic acid, 1-naphthoic acid, and hydrocinnamic acid. K and K Laboratories supplied *m*-toluic acid, *p*-toluic acid, *p*-nitroethylbenzene, β-nitronaphthalene, and γ-phenyl-*n*-butyric acid. Fisher Scientific supplied the acetophenone and benzoic acid. The acids, except for benzoic, were recrystallized from water before use. The nitrobenzenes were distilled before use. The remaining compounds were used without further treatment.

Distribution Determinations. The equilibrium concentrations of solute in each phase were determined by direct spectrophotometric measurement of solute concentration in the aqueous phase. The solute was dissolved in the aqueous phase and an aliquot was transferred to a conical flask along with a suitable volume of the extracting hydrocarbon phase. The total volume varied from 50 to 100 ml. The flasks were sealed and placed on a wrist action shaker for 1 hr, which was found to be sufficient time for all systems to reach equilibrium under the conditions used. The shaker was positioned so that the flasks were submerged in a constant temperature water bath maintained at 25 ± 0.01°. The amount of solute was varied from 1 to 0.2 g/l. The variation of volume of each phase was designed to show any adsorption at the liquid–liquid interface by causing this interface to vary in area. No such effect was observed, the distribution coefficient being independent of the above conditions.

After shaking had ceased, the flasks were allowed to stand for 10 min and an aliquot of the aqueous phase was removed for analysis. The solute concentration in the water phase was read after suitable dilution in a Beckman DUV spectrophotometer at 230 mμ (330 mμ for the nitrobenzenes). All of the solutes were found to follow Beer's law from 0 to 0.5 OD. The concentration of solute in the hydrocarbon phase was estimated by difference.

Results

The free energy change for the extraction situation was written as equal to $-RT \ln C_1/C_2$, where C_1 represents the concentration of solute in the hydrocarbon phase, and C_2 represents the concentration in the aqueous phase at equilibrium. The free energy change for complex formation was written in the usual way as equal to $-RT \ln K$, where K is either an association constant for enzyme–inhibitor complex formation (K_i) or an apparent association constant for enzyme–substrate complex formation ($\bar{K}$). The results may be seen in Tables I–V.

TABLE I: Standard Free Energy Changes for Extraction and Enzyme–Substrate Complex Formation for Hippuric Acid Esters.

Hippurate	$\Delta F_{EX}°$ (kcal/mole)[a]	$-\Delta F_{EX}°$ (kcal/mole)[b]	$-\Delta F_{ES}°$ (kcal/mole)
Methyl	1.80	0.040	3.49
Ethyl	1.15	0.795	3.57
Propyl	0.47	1.43	3.77
Isopropyl	0.52	1.51	3.62
Butyl	−0.16	2.07	Not done

[a] Pentane–water. [b] Xylene–water.

TABLE II: Standard Free Energy Changes for Extraction and Enzyme–Substrate Complex Formation for Hydrocinnamic Acid Esters at 25°.

Hydrocinnamate	$\Delta F_{EX}°$ (kcal/mole)[a]	$-\Delta F_{ES}°$ (kcal/mole)
Methyl	1.42	4.44
Ethyl	0.745	4.57
Propyl	0.025	4.73

[a] Pentane–water.

TABLE III: Standard Free Energy Changes for Extraction and Enzyme–Inhibitor Complex Formation for *p*-Nitrobenzenes at 25°.

p-Nitro-	$-\Delta F_{EX}°$ (kcal/mole)[a]	$-\Delta F_{EI}°$ (kcal/mole)
Benzene	2.01	4.14
Toluene	2.52	4.51
Ethylbenzene	2.77	Not done
2-Naphthalene	3.40	5.57

[a] Hexane–water.

TABLE IV: Standard Free Energy Changes for Extraction and Enzyme–Inhibitor Complex Formation for Some Phenones at 25°.

Phenone	$-\Delta F_{EX}°$ (kcal/mole)[a]	$-\Delta F_{EI}°$ (kcal/mole)
Aceto-	1.44	3.64
Propio-	2.23	3.88
Butyro-	2.85	4.06

[a] Heptane–water.

TABLE V: Standard Free Energy Changes for Enzyme–Inhibitor Complex Formation for Some Carboxylic Acid Anions at 25°.

Acid	$-\Delta F_{EI}°$ (kcal/mole)
Benzoic	2.70
m-Toluic	3.29
p-Toluic	3.30
Hydrocinnamic	3.79
γ-Phenyl-*n*-butyric	4.38
2-Naphthoic	5.25
p-*t*-butylbenzoic	5.28

Discussion

One point of very great interest is whether or not $\bar{K}$, the apparent association constant for ES complex formation, represents an equilibrium constant or a steady-state constant. Bernhard *et al.* (1960) and Epand and Wilson (1963) have presented very good evidence that, under conditions very similar to those used here, the association constant for the chymotrypsin–methyl hippurate complex is an equilibrium constant.

To the knowledge of the authors, no similar evidence is available for the esters of hydrocinnamic acid. All of the association constants are considered to represent equilibrium constants. The reader should keep in mind that while this is not an unreasonable assumption, it may not necessarily be true. The results that follow, however, indicate that this may well be the case. Of course, no difficulty arises with the inhibitors, all of which were found to be of the competititve type.

The concept of extraction as an important mechanism in complex formation was originally suggested to us by the effects of added potassium chloride on the association constant using methyl hippurate as a substrate (Miles *et al.*, 1961, 1963). The pH was 7.05. It was shown that $\bar{K}$, the apparent association constant for the formation of the enzyme–methyl hippurate complex, could be written

$$\bar{K}_u = \bar{K}_w \frac{\gamma_1}{\gamma_2\gamma_2} \quad (1)$$

where $\bar{K}_u$ represents the association constant at a given ionic strength, $\bar{K}_w$, the association constant in pure water, and γ_1, γ_2, and γ_3 represent the activity coefficients of the ES complex, enzyme, and substrate, respectively. If S_w is the solubility of the substrate or inhibitor in pure water where its activity coefficient is γ_w, and S_u is the solubility in the presence of added electrolyte where the activity coefficient is γ_3, it was shown that

$$\gamma_3 = \gamma_{rel}$$

where

$$\gamma_{rel} = \frac{S_w}{S_u}$$

Solubility studies performed in this laboratory (Larese *et al.*, 1962) showed that log γ_{rel} (or log S) values for the methyl, ethyl, propyl, and isopropyl hippurates vary in a linear manner with ionic strength (potassium chloride). The logarithms of the association constants for the same series of hippuric acid esters with α-chymotrypsin were also found to be linear from a concentration of about 0.2–2 *m* potassium chloride (Miles *et al.*, 1961, 1963). The behavior of the enzyme at ionic strengths below 0.2 *m* appears to involve other factors and will be considered at a later time. In any case, as long as the ionic strength is at least moderately high, both the free energies of solution and enzyme complex formation vary in a linear fashion with salt concentration and with almost exactly the same slope. That is to say, the effect of ionic strength upon the association constant may be entirely explained on the basis of the effect of ionic strength on the chemical potential of the substrate in aqueous solution. Inspection of eq 1 shows that there are two possible ways in which this finding could be explained: the first is that γ_1 and γ_2 vary with ionic strength, but in the same direction and to the same extent, thus canceling out. The second possibility is that γ_1 and γ_2 are simply independent of ionic strength. It is possible that when the enzyme is not actually interacting with the substrate, the site is not visible to the surroundings and a change in conformation then takes place upon the approach of the substrate with the hidden active site becoming attainable from the outside.

Another possibility is that at least a portion of the active site might present a nonpolar "face" to the solvent. The simplest situation involving such a nonpolar area would be that the nonelectrolyte substrate or inhibitor is extracted from the aqueous medium. In such a case the solute molecule (substrate or inhibitor) would pass completely from the water phase to the enzyme phase as the complex is formed. Put in another way, this simple theory assumes that water surrounding the inhibitor (or substrate) molecule

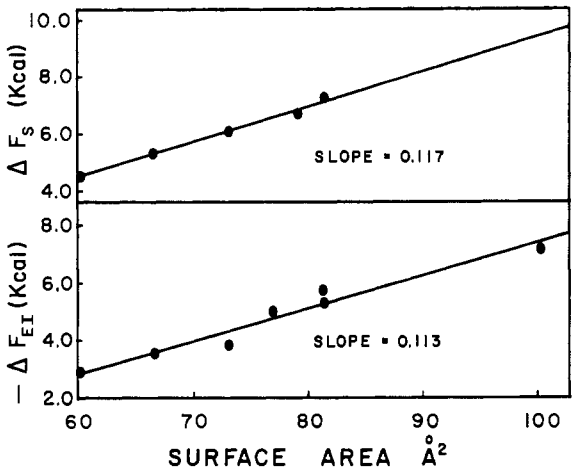

FIGURE 1: (Top) ΔF° of solution for some aromatic hydrocarbons *vs.* molecular surface area. The compounds are, in ascending order, benzene, toluene, ethylbenzene, the isomers *p*- and *m*-xylene, and naphthalene. (Bottom) ΔF° of enzyme–inhibitor complex formation for some aromatic hydrocarbons *vs.* molecular surface area. The compounds are, in ascending order, benzene, toluene, ethylbenzene, indene, the isomers naphthalene and azulene, and anthracene.

in the aqueous phase is completely excluded when complex formation takes place. Such a complete disappearance of the inhibitor or substrate from the water phase into the enzyme is not inconceivable, but as is pointed out later may be an oversimplification. It is also important to realize that the portion of the enzyme involved need not necessarily be pentanelike or benzenelike. Work by Hansch and his collaborators (Iwasa *et al.*, 1965) have shown that the dependence of distribution coefficients upon molecular size is very similar to our values when a higher alcohol rather than a hydrocarbon is used as the nonaqueous phase. Therefore, it would seem to follow that the enzyme site would only have to provide a *predominantly* hydrocarbon site rather than a more completely nonpolar one.

Simple extraction theory treats the solvent as a continuum. This is not uncommon and is, for example, the assumption made by Debye and Hückel in their treatment of ionic activities. The fact that the enzyme would not allow as much freedom as an ordinary nonaqueous phase might be expected to affect the entropy and/or the heat but not the *dependence* of the free energy of transfer upon molecular surface area. The great attractiveness of this theory is due to its simplicity. The purpose of the present communication is to point out the successes and failures of this simple theory in predicting the dependence of the affinity constants of various homologous series of compounds upon their molecular surface areas.

Successes of the Extraction Theory

The extraction theory is very successful in predicting the effects of added hydrocarbon groups on the free energy of complex formation for a group of inhibitors which are quite simple in structure. Figure 1 shows the free energy change for the solution process (ΔF°_s) and the free energy change for complex formation (ΔF°_{EI}) plotted against molecular surface area. The surface areas were calculated from the molar refractions. The molar refraction at infinitely long wavelength is related to the volume occupied by 1 mole of the substance under consideration. It should be noted that almost all of our work deals with various homologous series where we start with a parent compound and add carbon atoms (generally methylene groups). The fact that the molar refraction is indeed proportional to the molar volume in such series can easily be demonstrated by plotting the molar volume from molecular weight and density data against molar refraction; where suitable data are available very good linearity for many series of liquids was obtained. In all cases the slopes were constant at about 0.28. Hence, it follows that we are justified in using molar refraction to calculate molar volumes where there are no data available to estimate them by other means. The molar refraction is calculated from the additive values for the D line of sodium which are to be found in any handbook of chemistry or chemistry and physics. Exaltation accounts for a maximum error of only 6% in the various series of compounds considered in this paper. The problem disappears completely if exaltation makes a constant contribution in a homologous series such as the phenones. In such a case the exaltation would be about the same in each member of the series, hence the above-mentioned proportionality would not change. If we consider the molecule to be a sphere, knowledge of the molar volume allows the calculation of the molecular volumes. Then, treating the molecule as a sphere of known volume, the surface area is calculated. This approach is applicable to the many solid substances in which we are interested. The substances are, in the upper plot, benzene, toluene, ethylbenzene, *p*- and *m*-xylene, and naphthalene. The lower plot shows a similar presentation for the free energy of enzyme–inhibitor complex formation against molecular surface area. It should be kept in mind by the reader that the concepts of molecular volume and polarizability are not always easily separable; any parameter which is related to the molecular radius (and hence volume or surface area) is also related to the polarizability. Whether one should use molecular surface area or molecular volume in studies such as this one is a moot question. With all of the homologous series studied in this work, good linearity is obtained whether the surface area (treating the molecule as a sphere) or volume of the added group is used. This is true because these two parameters happen to be proportional to one another. This was demonstrated by plotting the surface area against volume of the added group for each series of compounds

investigated. Straight lines of very constant slope were obtained (plots not shown). This indicates that for our purposes, either total surface area or volume of added group may be used. Since our original work made use of surface areas, this parameter has been used here so that the results may be directly compared.

The inhibitors in the lower plot seen in Figure 1 are benzene, toluene, ethylbenzene, indene, naphthalene, azulene, and anthracene. This figure is taken from a previous paper on this subject (Hymes *et al.*, 1965) and serves as a convenient starting point for the work in the present communication. This previous treatment assumed that the solution of a series of liquid hydrocarbons could be treated as a series of extractions. It can be seen that agreement is excellent between the two slopes and is about 3.5%. If the assumptions made about the solution process of liquid nonpolar hydrocarbons being analagous in every way to an extraction process are correct, then any mechanism involving extraction, whether it be a distribution between a water and hydrocarbon phase or between water and an enzyme phase, should always produce a slope of roughly 0.12 when $\Delta F°$ for the process in question is plotted against molecular surface area of the solutes involved. Such an assumption is not unreasonable; the solution process of a liquid hydrocarbon would be expected to be a special case of extraction where the standard partial molar free energy change for the process is that free energy change involved in the transfer of 1 mole of hydrocarbon from the hydrocarbon phase to the aqueous phase. It is a well known fact that solutions of nonpolar aromatic hydrocarbons in one another approach ideality. Thus a toluene molecule could be considered to be in approximately the same state whether it were dissolved in benzene or surrounded by other molecules of toluene. If the toluene were distributed between benzene and water, we would be dealing with a conventional extraction process; if the toluene molecules were distributed between toluene and water, we would be considering a solution process. It is important to note that this relationship between solubility and extraction for a homologous series of hydrocarbons such as that seen in Figure 1 will hold only if the individual hydrocarbons are capable of forming ideal solutions when one is dissolved in another. The free energy of solution of nitrobenzene in water would not be expected to fall on the above-mentioned plot, and indeed it does not. One other case in which the above relationship would hold would be for a series of liquid solutes, the solutions of which deviated from Raoult's law in the same direction and approximately to the same extent.

The results indicate that our surmises in this regard are correct, since when the various homologous series of solutes are distributed between water and an organic solvent the slopes of $\Delta F°$ *vs.* molecular surface area plots are indeed approximately 0.12. The nitrobenzenes are the exception, the slope being 0.07. The deviations from this value are generally found, as one might expect, in enzyme–substrate or enzyme–inhibitor

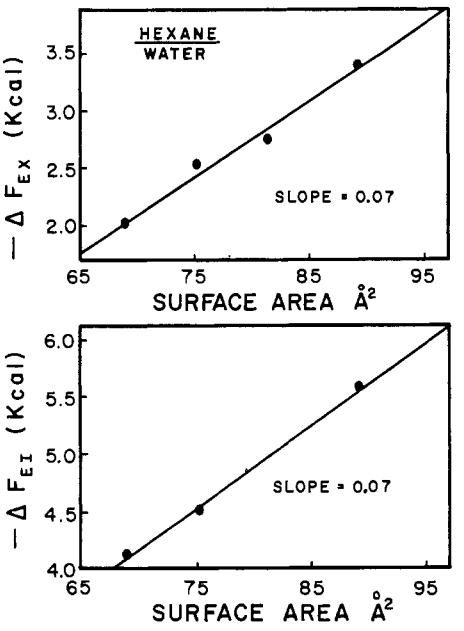

FIGURE 2: (Top) $\Delta F°$ of distribution for some nitrobenzene derivatives between hexane and water *vs.* molecular surface area. The compounds are, in ascending order, nitrobenzene, *p*-nitrotoluene, *p*-nitroethylbenzene, and β-nitronaphthalene. (Bottom) $\Delta F°$ of enzyme–inhibitor complex formation for some nitrobenzene derivatives *vs.* molecular surface area. The compounds are, in ascending order, nitrobenzene, *p*-nitrotoluene, and β-nitronaphthalene.

complex formation. In addition to the aromatic hydrocarbons of low polarity seen in Figure 1, the extraction model is strikingly successful in the case of the substituted nitrobenzenes. The results of these experiments are seen in Figure 2. The compounds used were nitrobenzene, *p*-methylnitrobenzene, *p*-ethylnitrobenzene and β-nitronaphthalene. The upper part of Figure 2, showing the free energy change for the transfer of these competititve inhibitors from water to hexane, indicates that if the extraction model is correct, one would expect that a similar plot of free energy of complex formation would have an abnormal slope of about the same value of 0.07 (a "normal" slope would be approximately 0.12 as pointed out before). Examination of the lower part of Figure 2 shows that it is indeed the case. This must be considered as quite a striking success. It has been determined that the anions of a series of carboxylic acids also follow the "normal" law as well as the simple aromatic hydrocarbons. This is shown in Figure 3. The acids are: benzoic, *m*- and *p*-toluic, hydrocinnamic, γ-phenyl-*n*-butyric, α-naphthoic and *p*-t-butylbenzoic. Since these acids are almost completely ionized at the working pH, no distribution experiments were done. To summarize, the extraction model is successful in predicting the dependence of the inhibition constants upon molecular size in such homologous series as the

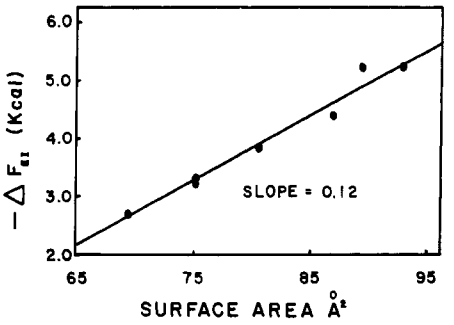

FIGURE 3: $\Delta F°$ of enzyme–inhibitor complex formation for some carboxylic acid anions *vs.* molecular surface area. The anions are, in ascending order, benzoate, the isomers *m*- and *p*-toluate, hydrocinnamate, γ-phenyl *n*-butyrate, 2-naphthoate, and *p-t*-butylbenzoate.

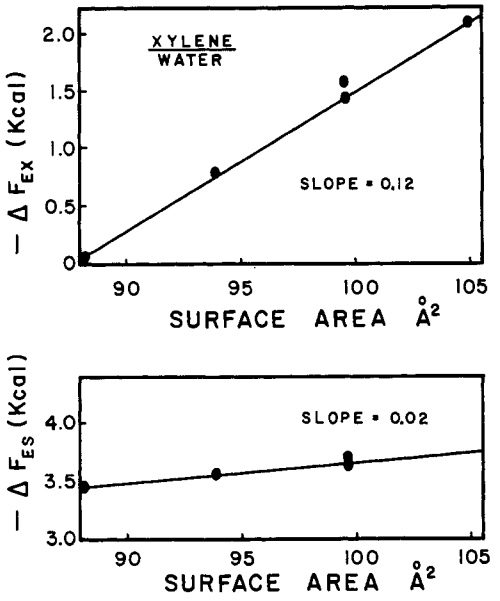

FIGURE 4: (Top) $\Delta F°$ of distribution for some esters of hippuric acid between xylene and water *vs.* molecular surface area. The compounds are, in ascending order, methyl, ethyl, the isomers propyl and isopropyl, and butyl hippurates. (Bottom) $\Delta F°$ of enzyme–substrate complex formation for all of the above compounds but butyl hippurate *vs.* molecular surface area.

simple aromatic hydrocarbons of low polarity, a series of highly polar nitrobenzene derivatives, and also can be applied to a series of carboxylic acid ions with discrete electrical charges.

Failures of the Extraction Theory

Inspection of Figure 4 shows that when such ester substrates as methyl, ethyl, propyl, isopropyl, and butyl hippurates are studied, the free energy of transfer from the water phase to the hydrocarbon phase pro-

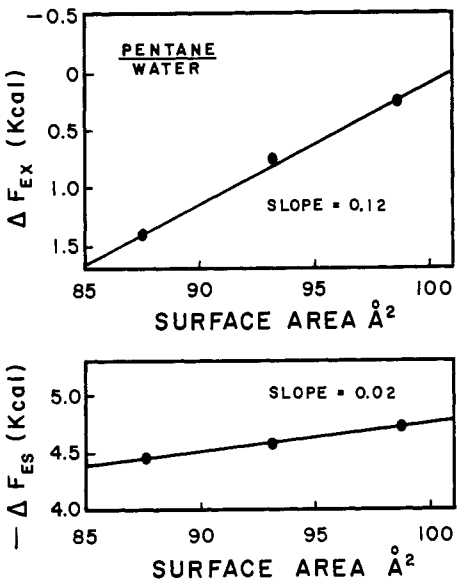

FIGURE 5: (Top) $\Delta F°$ of distribution for some esters of hydrocinnamic acid between pentane and water *vs.* molecular surface area. The compounds are, in ascending order, methyl, ethyl, and propyl hydrocinnamates. (Bottom) $\Delta F°$ of enzyme–substrate complex formation for the above compounds *vs.* molecular surface area.

duces a slope of "normal" value (0.12) but the slope for the enzyme situation is very much smaller than one would expect on the basis of the simple extraction theory. Figure 5 shows similar results for methyl, ethyl, and propyl hydrocinnamate. In each case the slope for the enzyme data is only about one-sixth of the expected value calculated from the hydrocarbon–water distribution coefficients. Therefore, up until this time only inhibitors appeared to follow the extraction law while substrates produced a linear plot but of much smaller slope. One possible explanation for such a result might be that K does not represent a pseudo-equilibrium constant but rather a complex steady-state constant which may be only remotely related to a true equilibrium constant. If it were possible to find a series of inhibitors which would behave in a similar way to the substrates, our fears in this regard would be somewhat allayed.

Consideration of the structure of the substrates used in this work (the hippurates and hydrocinnamates) indicates that these molecules may be considered to consist of polar groups to which are attached two hydrocarbon moieties, a benzene ring on one end and an aliphatic group on the ester portion. Thus the substrates may be bound to the enzyme by a two-point hydrophobic interaction while the polar group remains exposed to the aqueous phase. Under such conditions, a certain amount of enzyme–water interface would be destroyed, since the substrate could not pass completely into the enzyme during complex formation. Previous work from this laboratory (Hymes *et al.*, 1965) indicated that when the solute molecule reached

a potential energy minimum between the two phases (this would be a type of adsorption) accompanied by such an interface destruction, a plot of ΔF° *vs.* molecular surface area would be expected to be linear but of less negative slope than for the extraction process. This is true when values of the free surface energy for the enzyme–water interface are used which correspond to those of water–hydrocarbon or water–organic solvent interfaces generally encountered.

From the above considerations, it is clear that if a series of inhibitors with the same general structural characteristics as the substrates (hydrocarbon–polar group–hydrocarbon) were studied, it might be expected that a similar "abnormal" slope of approximately 0.02 would be obtained for the enzymatic process. That this is indeed the case can be ascertained by inspection of Figure 6. This figure shows the distribution and inhibition data for a series of phenones: acetophenone, propiophenone, and butyrophenone. The slope for the extraction process is 0.12 as would be expected. The slope for the enzymatic process is only 0.03, which is quite close to the values obtained for the substrates. Hartley (1964) has suggested a "hydrophobic slot" on the basis of the kinetic constants for a number of substrates. The authors of this present paper feel that the evidence presented concerning the molecular surface area dependence of the association constants for the substrates and the phenones is the strongest yet produced for such a two-point (or perhaps more in some cases) hydrophobic attachment to the enzyme. It is not possible at this state of our knowledge to decide whether a slot or some other configuration may be involved. A future communication will deal with this problem in more detail.

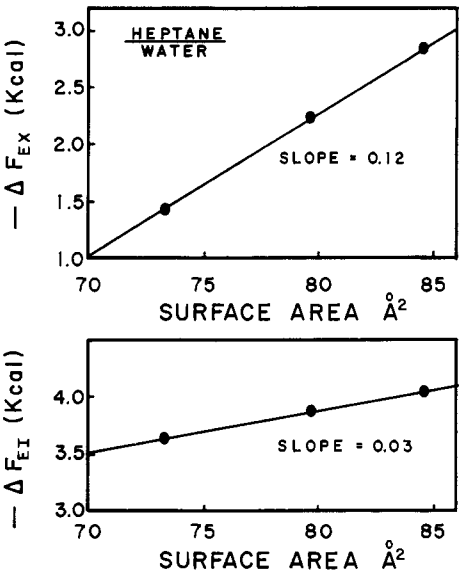

FIGURE 6: (Top) ΔF° of distribution for some phenones between heptane and water *vs.* molecular surface area. The compounds are, in ascending order, acetophenone, propiophenone and butyrophenone. (Botton) ΔF° of enzyme–inhibitor complex formation for the above compounds *vs.* molecular surface area.

The Possible Physical Significance of the Experimental Findings

As has been pointed out above, the variation of the association constants with molecular size for the simple compounds which might be bound by a one-point attachment indicates that they behave *as though* complex formation were an extraction process; that is to say they behave as though the inhibitor disappears *completely* from the aqueous phase into the enzyme. Such a situation could be explained in a number of ways. In the following arguments, it is assumed that the enzyme behaves as a second phase and that complex formation can be treated in the same way as a distribution of solute molecules between two phases. (1) The inhibitor may truly disappear from solution. Such a situation might reasonably be the case for the hydrocarbons of low polarity such as benzene, toluene, etc. (2) More polar substances such as the nitrobenzenes or molecules with discreet electrical charges such as the carboxylic acid anions might be expected to orient themselves across the enzyme–water interface with the charged portions oriented toward the aqueous phase. Such a situation would be expected to give the *same results* as an extraction process as far as the slopes obtained are concerned, provided that, *for the whole series*, approximately the same amount of enzyme–water interface was destroyed in every case. The net result would be that the difference between each member of a homologous series and the next higher one would be due to the difference of free energy of transfer of the additional alkyl group from the aqueous phase entirely to the enzyme phase. On the other hand, charge neutralization might take place with the carboxylate ions. In the aforementioned case, the free energy of destruction of the enzyme–water interface brought about by the adsorption of the inhibitor would be a constant factor for the series. This surface free energy could not be recovered until the interface is re-formed, either by the inhibitor passing all the way into the enzyme, or by dissociating itself completely from the surface. Thus it can be seen that for any series of inhibitors in which the amount of interface destroyed is *not constant* the dependence of the free energy of complex formation upon the size of alkyl substituents present will be *different* than for an extraction process. A reasonable explanation for the striking similarity between the slopes for the phenones and those of the substrates might be that the more polar central portion of each type of compound prevents the two hydrocarbon groups on either end of the molecule from sinking completely into the hydrophobic area associated with the active site. Taking this polar group as a fixed point suspended in the aqueous phase only a short distance from the enzyme–water interface, it follows that as the size of either of the hydrocarbon groups is increased more and more enzyme–water interface would

be destroyed. When complex formation takes place, such a happening would also lead to a much less negative slope than that obtained for an extraction process.

While the evidence presented cannot be taken as absolute proof that nonpolar molecules such as benzene are extracted from the aqueous medium by the enzyme, it can definitely be stated that the phenones and substrates are *not* extracted, but are adsorbed perhaps by means of a two-point hydrophobic attachment. This can be seen in a rough qualitative way by considering the significance of the slopes of such plots first for the extraction case and then for the simple adsorption process.

For the extraction situation (Hymes *et al.*, 1965) the slope obtained from a plot of ΔF° *vs.* molecular surface area represents the term

$$\frac{R(\Lambda_1 - \Lambda_2)}{k}$$

where R is the gas constant, k is Boltzman's constant, and Λ_1 and Λ_2 are the interfacial free energies per unit area between the solute particles in the enzyme phase and aqueous phase, respectively. The corresponding expression for adsorption, where the particle lodges only part way between the two phases is

$$\frac{R(\Lambda_1 - \Lambda_2 - \Lambda_3)^2}{4k\Lambda_3}$$

where the additional term Λ_3 represents the interfacial free energy for the enzyme–water interface. Since Λ_1 will generally be small, it follows that in the first case the slope will tend to be negative, and reference to Figures 1, 2, 4, and 5 indicates that this is true for the extraction process. On the other hand, for the second case it can be seen that since Λ_1 will still be small, and since there is a squared term involved, this slope will tend to be positive since stable surfaces must have positive Λ values. The above equation for adsorption is based on a simple spherical model. A treatment using a more detailed and hence more realistic model is currently in preparation and will be presented at a later date. However, preliminary results with this more detailed model which takes the hydrocarbon–polar–hydrocarbon structure of the ketones into account indicate that either small or large negative slopes would be expected for adsorption, depending to a great extent on the value of Λ associated with the enzyme–water interface.

It should be emphasized that our proposed approach is not the same as that exemplified by Némethy and Scheraga (1962) although many of the same forces would be involved. We are suggesting that there may be much to be gained by treating enzyme–substrate or enzyme–inhibitor complex formation in terms of a simple phase-transfer (extraction) or a surface phenomenon (adsorption). This infers that the enzyme may be considered to supply a second phase where the substrate either passes into the interior or comes to rest at the interface. Thus the considerable body of knowledge of the thermodynamics of transfer and thermodynamics of surfaces may be brought to bear upon the problem. This way of thinking of complex formation is not meant to replace other methods, but should be considered as an alternative approach where much experimental data are available for the calculations involved. The first applications have been quite successful.

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