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Use of Transition-State Theory in the Development of Bioactive Molecules

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The application of transition state theory to enzyme catalysis is presented in terms of mechanistic and energetic approaches. These treatments emphasize the rationale of using transition state analogs as bioactive molecules. Included in this chapter are some criteria for determining if a compound is a transition state analog. A hypothesis is then presented that the initial binding of some insecticidal carbamates and organophosphates to acetylcholinesterase is dependent in part upon their mimicking the transition state configuration of acetylcholine. As an indication of how transition state theory could be applied to problems in agricultural chemistry, we present data from this laboratory that some trifluoromethylketone inhibitors of insect juvenile hormone esterase(s) are transition state mimics and extend this argument by applying a quantitative structure-activity relationship approach to these analogs.

There has been an obvious decline in the number of commercial agricultural and pharmaceutical chemicals during the last decade. There are two major reasons for this decline. First, there are increasingly tighter requirements for market development. To use agricultural chemicals as an example, we see an increasingly narrow margin of profit for farmers and increasing concern over environmental health effects. The cost of registration, production and marketing also continues to increase. Secondly, the cost of discovery has increased dramatically. There are numerous components to this observation as well, but simplistically one can see that to meet the tight requirements for market development, more complex and expensive syntheses and bioassays are required. Random screening with the aid of some serendipity served effectively in the past for the discovery of new biological activities, yet we have reached the

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point of diminishing returns with regard to its application to future problems. Therefore, a theme of several of the manuscripts from this meeting relates to the development of new paradigms for the discovery of biological activity.

Molecules can react with a biological matrix in many ways. At least some biological activity is common among chemically reactive species. For instance, Michael acceptors and acetylating agents are commonly irritating agents or general cytotoxins. Molecules may reversibly react with biological systems based upon only solubility or weak molecular interactions with biological depressants as prime examples. However, these molecules lack the specificity desired for field application and the potency needed for economic feasibility. However, if an alkylating agent has a high affinity for nucleic acids, if an acetylating agent binds well to an enzyme active site, or if an inert molecule can form several weak molecular interactions with a receptor site, these molecules may display very high biological activity indeed.

During the last several years we have become impressed with the utility of transition state theory in the optimization as well as the discovery of biologically active structures. Empirical approaches indicate that a mimic of an enzyme-substrate transition state could have a K_I below 10^{-15} M (1,2). Although one is not likely to precisely mimic a transition state, even a vague mimic could still be a very potent inhibitor. Therefore, we feel that transition state theory is likely to be one of several approaches useful in agricultural chemistry. Thus in the following pages we present a brief review of the application of transition state theory in enzyme catalysis in terms of both mechanistic and energetic treatments. These treatments introduce the rationale of using transition state analogs as bioactive molecules. Data from this laboratory were presented as an example of the use of transition state theory in the development of potent inhibitors of insect juvenile hormone esterase(s).

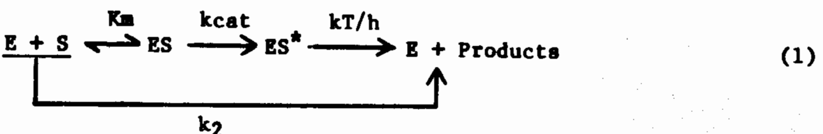
Transition State Theory

It is well known from structural and kinetic studies that enzymes have well-defined binding sites for their substrates (3), sometimes form covalent intermediates, and generally involve acidic, basic and nucleophilic groups. Many of the concepts in catalysis are based on transition state (TS) theory. The first quantitative formulation of that theory was extensively used in the work of H. Eyring (4,5). Noteworthy contributions to the basic theory were made by others (see (6) for review). As an elementary introduction, we will apply the fundamental assumptions of the TS theory in simple enzyme catalysis as follows.

(a) In every chemical reaction the reactants are in equilibrium with an unstable activated complex, the transition state complex, which decomposes to give products. In this complex chemical bonds are in the process of being formed or broken. Therefore, it occurs at the peak of the reaction coordinate diagram, i.e. at the saddle points of potential energy surfaces (7). In contrast, intermediates, whose bonds are fully established, occupy the troughs in the diagram (Figure 1). These intermediates can either be transient

or actual isolatable intermediates such as an acyl enzyme.

(b) It is postulated that the starting materials (in case of enzyme catalysis the enzyme (E) and its substrate (S)) are in equilibrium with all complexes which occur before the activated complex (ES^*) and also with the activated complex itself "Equation 1".



(1)

(c) The important postulate is made that in this theory all the activated complexes decompose to products at exactly the same rate for a given temperature. This means that the rate is proportional to the concentration of ES^* with a universal proportionality constant (kT/h) where k is the Boltzmann's constant, T is the absolute temperature and h is the Plank's constant. At 25° (kT/h) equals 6.212×10^{12} sec^{-1}.

$$\text{rate} = \frac{kT}{h}[ES^*] \quad (2)$$

However ES^* is in equilibrium with E and S and is governed by the equilibrium constant K_t^* as follows:

$$\frac{[ES^*]}{[E][S]} = K_t^* \quad (3)$$

Substitution for the value of $[ES^*]$ in "Equation 2" from "Equation 3" results in the following equation:

$$\text{rate} = [E][S]K_t^*\left(\frac{kT}{h}\right) \quad (4)$$

However, from "Equation 1" the rate also should be proportional to [E] and [S] and a second order rate constant (k_2):

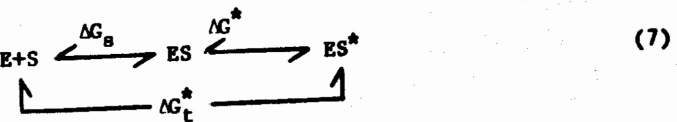
$$\text{rate} = k_2[E][S] \quad (5)$$

Comparing "Equation 5" with "Equation 4" indicates that:

$$k_2 = \frac{kT}{h}K_t^* \quad (6)$$

(d) A special property of the activated TS complex is that it has a unique, very loose internal mode of vibration, which is unstable with respect to dissociation into products. This "vibration" occurs along the reaction coordinate (Figure 1). Therefore we will consider the TS complex as the end point on the energy profile for simplicity.

Free Energy Change and TS Theory. We will now express the mechanism of enzyme catalysis "Equation 1" in terms of the change in the free energy as follows:



(7)

By applying the well-known relationship between the Gibbs-energy change and the equilibrium constant (8) to the above scheme, one finds:

$$\Delta G_t^* = -RT \ln K_t^* \quad (8)$$

where K_t^* is the equilibrium constant between the reactants and the TS. Analogous equations relate the equilibrium constant of any step and the corresponding free energy change. Upon rearrangement of "Equation 8" and introducing the value of K_t^* in terms of ΔG_t^* into "Equation 6", the following equation results:

$$k_2 = \frac{kT}{h} \cdot e^{-\Delta G_t^*/RT} \quad (9)$$

Using the familiar relationship between Gibbs free energy change (ΔG_t^*) and the change in the enthalpy (ΔH_t^*) and entropy (ΔS_t^*),

$$\Delta G_t^* = \Delta H_t^* - T\Delta S_t^* \quad (10)$$

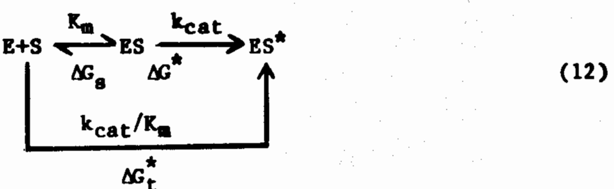
"Equation 9" can be expressed in terms of ΔH_t^* and ΔS_t^* as follows:

$$k_2 = \frac{kT}{h} \cdot e^{-\Delta H_t^*/RT} \; e^{\Delta S_t^*/R} \quad (11)$$

"Equation 11" indicates, from the theoretical point of view, that k_2 for a particular enzyme can be accelerated by either a decrease in the enthalpy and/or an increase in the entropy of the overall reaction E+S → ES^* so that ΔH_t^* will be negative and ΔS_t^* will be positive in the above equation. The role of entropy change in terms of different entropy vectors, translational, rotational and internal entropies in enzyme catalysis is not an easy task and the reader should refer to special textbooks in physical organic chemistry (see (8) and references therein). Theoretically forming a TS complex (ES^*) from an enzyme and substrate (2 → 1 reaction) would lead to a loss of the entropy of three degrees of translational freedom. However, a loose transition state with a high potential energy may, perhaps, be considered as two molecules in close juxtaposition but retaining considerable entropy freedom. Furthermore, enzymic reactions are different from pure organic chemical reactions since the former reaction takes place in the confines of the enzyme substrate complex. Therefore, the remarkably high catalytic activity of enzymes could be attributed to localization of S or S^* within the active site with susceptible bonds of the substrate or its TS configuration optimally oriented to the appropriate catalytic

moieties of the enzyme (3). Due to this localization and orientation, the substrate would be roughly considered as part of the same molecule as the catalytic group so there is no extensive loss in entropy as the reaction would be assumed to be intramolecular. If there is a great loss of entropy, it would be on forming the ES rather than ES^* complex which results in increasing K_m (8). The entropy change in terms of translational, rotational and internal rotational entropy has been discussed in more detail (9,10) in favor of contribution to the binding of E and S* rather than S. However, the Van der Waals attraction (hydrophobic bonding) in enzyme-substrate interactions might increase the entropy of either ES or ES^* as compared to that of the reactants by ejecting water molecules formerly bound to the catalytic site(s) (11). As illustrated in the above discussion, S* binds more tightly to the enzyme than S. Thus more molecules of solvating water would be released upon the formation of ES* resulting in an additional entropic advantage for its formation. Such hydrophobic bonding and entropy change in the reaction of enzyme with substrate or inhibitors will be fully explained in the next section.

Transition State and Binding Energy. There are at least two major factors which account for enzyme catalysis. The first one is a combination of entropic, acid base catalysis and electrostatic effects. The second one depends mostly on the enzyme substrate complementarity which results in a large amount of binding energy which may be used to distort the substrate to the structure of the products (12). The latter factor seems to be of high importance in lowering the activation energy of the overall reaction and subsequently increasing the magnitude of k_2 which is defined as k_{cat}/K_m in the simple Michaelis-Menten mechanism where $K_s = K_m$. Let us now put the simple scheme for enzyme catalysis in terms of both mechanistic and energetic entities.



(12)

The above scheme can be seen clearly from Figure 2 in which ΔG_s is algebraically negative, i.e. favorable reaction due to the realization of binding energy. However, ΔG^* is positive (unfavorable reaction) due to the activation energy of bond rearrangement in the activated TS complex. That is the activation energy ΔG_t^* for the whole process (E+S ⇄ ES^*) would be

$$\Delta G_t^* = \Delta G^* + \Delta G_s \quad (13)$$

Substitution for the value of ΔG_t^* in "Equation 9" by $\Delta G^* + \Delta G_s$ "Equation 13" results in the following equation:

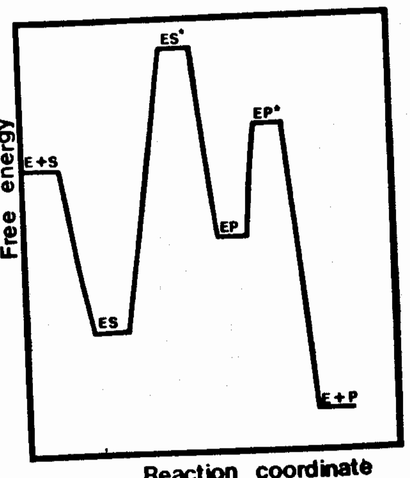


Figure 1. Schematic representation of the free energy changes in an enzyme-catalyzed reaction.

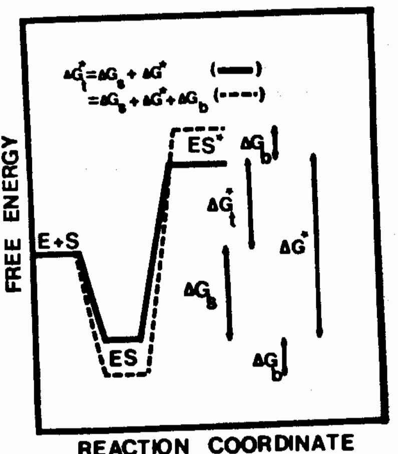


Figure 2. Schematic representation of the free energy changes in an enzyme-catalyzed reaction where the enzyme is complementary to either the substrate (broken lines) or to its transition state configuration (solid lines).

$$k_2 = k_{cat}/K_m = \frac{kT}{h}\, e^{-\Delta G^*/RT}\, e^{-\Delta G_s/RT} \qquad (14)$$

From the above equation it can be shown that the binding energy of ES complex and/or of ES* complex can play a crucial role in lowering ΔG_s or ΔG^* respectively and eventually increasing the catalytic activity (k_{cat}/K_m). However, since the structure of the substrate (based on TS theory) changes throughout the reaction, it is likely that the undistorted enzyme can have maximum complementariness to only one species (S or S*) of the substrate (8). At this point an important question arises regarding which form of substrate would have the highest complementarity and the highest binding energy in its reaction with the enzyme. The importance of the above question comes from the fact that its answer is considered to be the main rationale for the development of TS-analogues (TSA) as enzyme inhibitors. The qualitative answer to this question was introduced by Haldane (12) and Pauling (1,2) as the favored complementarity would be between the enzyme and the transition state portion (S*) of the substrate rather than between the enzyme and the substrate itself. In supporting the great insight of Haldane and Pauling, Fersht (8) proved collectively that the intrinsic binding energy in driving k_{cat}/K_m would be in favor of the transition state. In the following paragraphs we will slightly modify Fersht's approach to show that it is energetically expensive for enzyme catalyzed reactions to have maximum binding interactions with the substrate rather than its TS configuration.

Assume that the extra binding energy resulting from complementarity to the substrate or to its TS equals ΔG_b. If this extra binding energy is in the enzyme substrate complex, it will decrease the value of K_m and reduce its free energy change to become $\Delta G_s - \Delta G_b$. However, since the formation of TS complex will lead to a reduction in binding energy as the substrate geometry changes to give poorer fit and eventually this will increase ΔG^* for k_{cat} to be $\Delta G^* + 2\Delta G_b \cdot \Delta G_t^*$ for the overall reaction (k_{cat}/K_m) can be calculated (see Figure 2) to be the sum of ΔG values for the preceding steps as follows:

$$\Delta G_t^* = \Delta G^* + 2\Delta G_b + \Delta G_s - \Delta G_b = \Delta G^* + \Delta G_s + \Delta G_b \qquad (15)$$

Substitution for the components of ΔG_t^* ("Equation 15") into "Equation 9":

$$k_2 = k_{cat}/K_m = \frac{kT}{h} \cdot e^{-\Delta G^*/RT}\, e^{-\Delta G_s/RT}\, e^{-\Delta G_b/RT} \qquad (16)$$

Comparing "Equation 16" with "Equation 14" where in the latter the maximum intrinsic binding energy was assumed to be in the activated TS complex and taking into consideration ΔG_b to be originally

positive in "Equation 16" one finds that the favorable maximum fit is likely to be with the transition state configuration rather than the substrate itself. In fact the enzyme can have an extra catalytic activity by a factor of $e^{\Delta G_b/RT}$ (by dividing "Equation 14" over "Equation 16") just by using the same amount of binding energy in interaction with S^* rather than with S. It is not hard to realize the above arguments since k_{cat}/K_m is independent of the interactions in the initial enzyme-substrate complex (Figure 2).

Although the overall catalytic activity of an enzyme catalyzed reaction can be accounted for both by the affinity of the substrate to the enzyme (K_m or more accurately $1/K_m$) and the substrate reactivity (k_{cat}), the latter value seems to be more important in reflecting the extra binding energy in the ES^* complex. This binding energy, as mentioned before, will decrease the energy of activation for the reactivity process (k_{cat}).

This hypothesis has been supported with some serine proteases where increasing the length of the leaving group increased k_{cat} for chymotrypsin (13,14) or increasing the length of the polypeptide chain of the substrate increased k_{cat} for elastase (15). The catalytic rate constant (k_{cat}) for juvenile hormone esterase from the larval hemolymph of Trichoplusia ni using JH_I, JH_{III} (16) and JH_{II} (17) as substrates was kinetically measured to be respectively 37.1, 19.4 and 31.8 min^{-1}. Mumby and Hammock (18) reported the partition coefficient (log P values) for 3 series of geranyl derivatives and JH_I. The log P value for the latter compound was 3.71. Log P values for the 2,3-unsaturated-6,7-epoxides, as the closest series to the structure of JH homologs, were subjected to Hansch's approach (19,20) to measure the substituent hydrophobicity (π). The π value for CH_3 was calculated from different comparisons to be 0.47, 0.49, 0.54 with an average value of 0.5 in an excellent agreement with the reported values for CH_3 (0.49-0.56) calculated from the octanol/water partition coefficients of four different systems (21). Therefore it is reasonably accepted to calculate the expected log P values for JH_{II} and JH_{III} from that of JH_I using the π value for CH_3 group. Interestingly a good correlation between log P and k_{cat} for the three homologs was obtained (Figure 3). This correlation might indicate that the binding energy through hydrophobic interactions is likely to be involved in the TS complex (ES^*). Since values of k_{cat} were obtained from different hemolymph pools, the activity of the enzyme from one single hemolymph pool was measured towards the three homologs at a final molar concentration of $5x10^{-6}$ and the data were plotted against log P. The same relation was obtained as with k_{cat} which is expected since at the substrate concentration used (~two orders of magnitude greater than the values of K_m for the three homologs), the velocity would approximate V_{max} and the latter equals $k_{cat}[E_t]$. An excellent linear function relationship (r^2=0.999) between the velocity and k_{cat} (Figure 3, inset) was obtained and supports the above discussion. One of the advantages of the above approach is that one can estimate the molar equivalency of a specific enzyme in crude preparations. Since at enzyme-substrate saturation conditions $v = V_{max} = k_{cat}[E_t]$, the above relation (Figure 3, inset) enables the calculation of $[E_t]$ from the slope of the inset (1.22 nmoles/ml plasma) which is equivalent to $1.22x10^{-6}$M of juvenile hormone esterase in the hemolymph of T. ni. In fact this average number is in close agreement with that calculated from using each substrate separately using different hemolymph pools (16). The ability to determine the molarity of a catalytic site in situ is of tremendous benefit in the elucidation of physiological or pharmacokinetic parameters.

Transition State Analogs (TSA)

The qualitative description of enzyme catalysis in terms of the TS theory (Pauling, 1,2) that the enzyme is complementary to an unstable molecule with only transient existence; namely, the activated complex (ES^*) for which the power of attraction by the enzyme is much greater than that of the substrate itself has been discussed energetically (8) and mechanistically (10,22-25). Pauling's assertion has opened a new era in enzymology, and relevant to our discussion is the stabilization of the activated complex and TSA as powerful enzyme inhibitors. As the transition state is a mathematical construction (with a typical half-life of 10^{-10} msec., (22)), its structure cannot be defined in common chemical sign language. In fact difficulty in isolating and defining transition state complexes was the major reason behind the recreation of Pauling's concept to design stable analogs approaching the structure of the altered substrate in the transition state without undergoing catalytic conversion. Eventually these TSA are expected to be stabilized upon the reaction with the enzyme to such a degree that they approach ground state energy minima (Figure 4). This stabilization would in fact enable indirect observation of the structure of the enzyme-TS by using the available techniques. Furthermore, if a TSA takes advantage of the additional, favorable binding interactions that are inherent in ES^* interactions, it could be an extremely powerful inhibitor to the limit of stoichiometric reaction. Theoretically a perfect TSA would have the same affinity for the enzyme as the transition state of the substrate (Figure 4). The question at issue now is: what is the magnitude of enzyme-TS affinity as compared to enzyme substrate affinity? This question has been fully declared by several workers (10,22,23,25). In the following paragraphs we will summarize their mathematical approach in a simple way. Note that in this section all equilibria are defined as association constants.

The two reactions to be compared are:

$$E+S \underset{}{\overset{K_{ES}}{\rightleftharpoons}} ES \qquad (17)$$

where K_{ES} is the equilibrium association constant (= $1/K_m$) between the enzyme and substrate.

$$E+S^* \overset{K_{TX}}{\rightleftharpoons} ES^* \qquad (18)$$

However, there should be a hypothetical step that precedes "Equation 18" as follows:

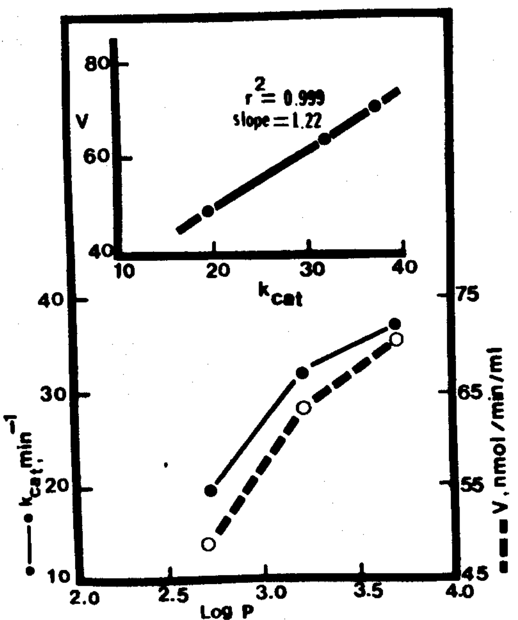


Figure 3. Plot of JH-hydrophobicity and JH-esterase hydrolytic activity of JH I, JH II and JH III.

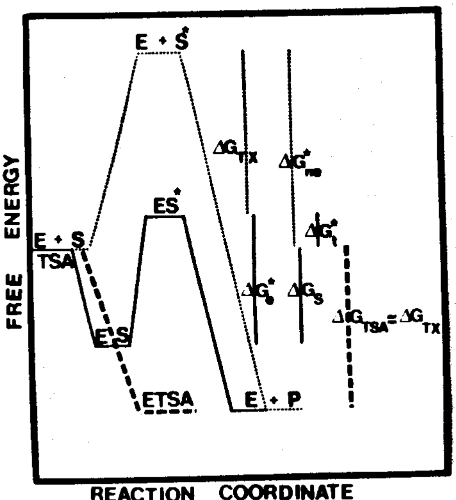
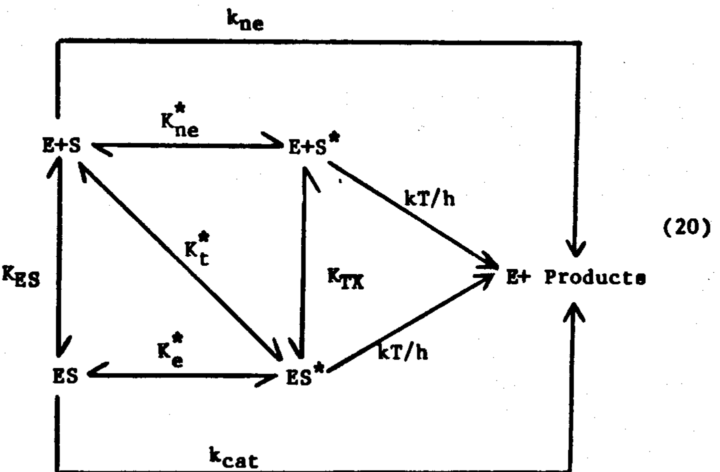


Figure 4. Schematic representation of the free energy changes in non-enzymatic and enzymatic reactions and in the reaction of a hypothetical transition state analog (TSA) with the enzyme.

$$E+S \underset{}{\overset{K^*_{ne}}{\rightleftharpoons}} E+S^* \tag{19}$$

In order to quantitate the relative affinity of the enzyme to S^* and S, a reasonable assumption is made that "Equation 19" stems from non-enzymatic reaction of the substrate, i.e. the mechanism of nonenzymatic reaction is the same as enzyme catalyzed reaction. It is useful to combine the above three reactions in a thermodynamic box as follows:



(20)

In the following table, a summary of the equilibrium constants (K) in relation to the changes of free energy of activation (-RT lnK) and the corresponding rate constants is displayed.

Table I. Equilibrium Constants and Free Energy Changes for Non-Enzymatic and Enzymatic Catalyzed Reactions

Equilibrium constant (K)	ΔG	Rate constant (k)
$K^*_{ne} = [S^*]/[S]$	ΔG^*_{ne}	$k_{ne}\ (t^{-1})$
$K_{ES} = [ES]/[E][S]$	ΔG_S	—
$K_{TX} = [ES^*]/[E][S^*]$	ΔG_{TX}	—
$K^*_e = [ES^*]/[ES]$	ΔG^*_e	$k_{cat}\ (t^{-1})$
$K^*_t = [ES^*]/[E][S]$	ΔG^*_t	$k_{cat} \cdot K_{ES} = k_{cat}/K_m$ $(M^{-1}\ t^{-1})$

From the above table an energy profile can be drawn for enzymatic vs. non-enzymatic reaction (Figure 4). From this figure, one easily can see how important it is that the enzyme should have un-

usual affinity for the altered substrate to make the transition state easier to reach, i.e. by decreasing the activation energy or the energy barrier of the overall reaction by a fraction equivalent to ΔG_{TX}. This amount of energy change must be due to the additional, favorable binding interactions that are inherited in the theoretical interaction between E and S^* ("Equation 18") and used to decrease the activation energy of the reaction as the collision complex or ground state complex (ES) approaches its transition state conformation (ES^*). Since K_{TX} is rather hypothetical, in order to make a prediction of its magnitude as compared with K_{ES}, the rate constants of the corresponding reactions (Table I) can be used according to the general relationship which is generated from the TS theory:

$$K^*_{ne} \cdot kT/h = k_{ne} \tag{21}$$

$$K^*_{e} \cdot kT/h = k_{cat} \tag{22}$$

$$K^*_{t} \cdot kT/h = k_{cat} \cdot K_{ES} \tag{23}$$

However, since ES^* is in equilibrium with the whole thermodynamic box, that is the overall K_{eq} of the reaction between E, S and ES^* must be the same regardless of the path, i.e.

$$K^*_{ne} \cdot K_{TX} = K_{ES} \cdot K^*_{e} = K^*_{t} \tag{24}$$

These relations can also be reached from the first column of Table I.

"Equation 24" is considered to be a cornerstone to the understanding of enzyme catalysis in terms of the magnitude of enzyme-substrate affinity in the transition state and how this affinity affects the energy of activation in favor of enzymatic catalysis rather than non-enzymatic reaction.

The relative affinity of E to S^* and to S can be calculated from "Equation 24" to be

$$K_{TX}/K_{ES} = K^*_{e}/K^*_{ne} \tag{25}$$

Substituting for K^*_e and K^*_{ne} by their corresponding rate constants ("Equations 21 and 22") results in the following equation:

$$K_{TX}/K_{ES} = k_{cat}/k_{ne} \tag{26}$$

Thus "Equation 26" shows that the tighter binding of S^* than S to the enzyme as expressed by K_{TX}/K_{ES} must be equivalent to the rate constant ratio (k_{cat}/k_{ne}). Since the latter ratio is typically 10^{10} or more (10, 23,26), the binding of the enzyme to the transition state configuration of the substrate should be at least 10 orders of magnitude tighter than the binding of the enzyme to the substrate itself. On the other hand by applying the free energy relationship ($\Delta G = -RT \ln K$) to "Equation 24", the changes in the free energy for the thermodynamic box, "Equation 20", would be calculated as follows:

$$\Delta G^*_{ne} + \Delta G_{TX} = \Delta G_S + \Delta G^*_e = \Delta G^*_t \tag{27}$$

where ΔG^*_{ne}, ΔG^*_e and ΔG^*_t are algebraically positive and ΔG_{TX} and ΔG_S are negative (Figure 4). From the above equation one can see that the activation energy for the reaction is less in the presence of enzyme (ΔG^*_t) than in its absence (ΔG^*_{ne}) by an amount of energy equivalent of ΔG_{TX} due to the greatest power of attraction between the enzyme and the TS configuration of the substrate. Accordingly the enzyme would speed the reaction. This picture explains the main concept of TSA as extraordinarily powerful enzyme inhibitors. The rationale for this approach is to design molecules which are structured in such a way that they resemble the transition state configuration of the substrate to the extent that they can take advantage of the binding interactions similar to ΔG_{TX} without undergoing catalytic conversion, and therefore would exhibit tighter binding to the enzyme than the substrate itself (Figure 4). However, it would be unrealistic to suppose than an ideal TSA, perfectly resembling the substrate in its transition state can ever be synthesized so that K_I/K_M would be in the same order as k_{ne}/k_{cat} (10^{-10} or lower). This is an important point from the practical point of view since it raises the question of which criteria one can use to differentiate between substrate analogs and TSA as inhibitors of a particular enzyme. In general TSA should exhibit abnormally low dissociation constants with the enzymes when compared with their K_m values (3). Exploitation of this approach has been reviewed (10,22-25,27). The simple numerical comparison of K_I with K_M fails to distinguish between either a TSA and a substrate analog for at least two reasons. First, no inhibitor will be a perfect mimic of either the substrate or its TS configuration (28). Second, most enzymes show overlapping specificity to different substrates and one enzyme can have a range of K_m values over several orders of magnitude. For example K_m values for the hydrolysis of N-acyl-L-amino acid esters by α-chymotrypsin (29) varied from 0.018 mM for benzoyl tyrosyl methyl ester to 862 mM for acetyl glycyl methyl ester indicating a difference of about five orders of magnitude. Fortunately, enzymes with overlapping substrate specificity can be of great help in distinguishing TSA from substrate analogs if a series of related inhibitors can be compared with the corresponding members of a series of related substrates. By taking the log of both sides of "Equation 26" and rearranging the log values,

$$-\log K_{TX} = -\log K_{ES} - \log k_{cat} + \log k_{ne} \tag{28}$$

However, $-\log K_{ES} = \log K_M$ and $-\log K_{TX}$ can be substituted by $\log K_I$ in the case of TSA, where K_I is the enzyme-inhibitor dissociation constant.

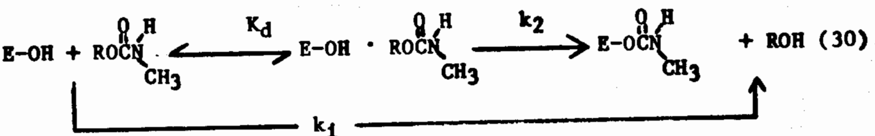
$$\log K_I = \log(K_M/k_{cat}) + \log k_{ne} \tag{29}$$

If one assumes that the rate of the nonenzymatic reaction does not vary among the substrates used, application of "Equation 29" can be a rigorous criterion for justifying the TSA since a linear function relationship would be obtained between log K ...

and $\log(K_M/k_{cat})$ for the corresponding parallel substrates. In case of using the same enzyme preparation the substrates' $\log(K_m/V_{max})$ can be used in the above relationship. Correlations of this kind have been reported for TSA of different enzymes (28,30-33). On the other hand, if a better correlation between $\log K_I$ and $\log K_M$ were obtained, the inhibitors would likely be acting as substrate analogues.

Application of TS Theory to Organophosphate and Carbamate Insecticides

It is well known that organophosphates, carbamates and sulfonates are acid-transfer-inhibitors of serine hydrolases because they transfer the acid moiety of the inhibitor to the serine hydroxyl of the enzyme active site (34). Extensive evidence indicates that the reaction of these inhibitors with acetylcholinesterases (AChE) appears to involve the same reaction pathway as that for the esters of carboxylic acids, i.e. acetylcholine (see (35) for review), and in fact these inhibitors are considered to be poor substrates of AChE (36), especially the carbamic acid esters ("Equation 30").



Therefore, it is not surprising that the basic kinetics ("Equation 31") for the inhibition by these compounds (37,38) has precisely the same form as the Michaelis-Menten rectangular

$$\rho = k_2 I/(K_d + I) \tag{31}$$

hyperbolic equation $(v = \frac{V_{max}S}{K_m + S})$ by replacing ρ, the first order inhibition rate constant, with v, k_2 with V_{max}, K_d with K_m and I with S (39). In this respect it came to us that these compounds might in fact resemble the transition state configuration of the substrate and accordingly part of their effect might be due to acting as TSA somewhere along the reaction coordinate of the catalytic reaction. Recently it has been found that the N-hydroxy (40), and N-methoxy (40,41) in contrast to N-methyl, and N,N-dimethylcarbamates inhibit AChE reversibly in a complete competitive manner. These findings stimulated our hypothesis that N-methyl and N,N-dimethylcarbamates, as carbamylating agents of AChE (36), can be considered poor substrates and the corresponding N-hydroxy N-methyl or N-methoxy N-methylcarbamates may be truly TSA. Supportive to our hypothesis is that m-trimethylammonium phenyl N-methyl N-methoxycarbamate, the most active reversible competitive inhibitor among the whole series (41) has a dissociation constant (K_I) of about three orders of magnitudes lower than the K_m values of the three tested enzymes. To test the above hypothesis "Equation 29" was applied to the inhibition of housefly head AChE by substituted phenyl N-methyl-carbamates using the I_{50} values reported by Metcalf (42) and the K_I values of the corresponding N-methyl-N-methoxycarbamates measured by Wustner et al. (41). The equivalent values of substrate (K_m/k_{cat}) would be the reciprocal of the bimolecular reaction constant $(1/k_i)$ since k_i equals k_2/K_d (37). k_i values were calculated from the I_{50} values and 15 minute preincubation time (t) from the following relationship (43):

$$k_i = \frac{0.693}{I_{50}t} \tag{32}$$

A plot of $\log K_I$ against $\log 1/k_i$ (Figure 5) clearly reveals an excellent linear relationship (r=0.97) indicating that these N-methoxy N-methylcarbamates may in fact act as TSA, and the corresponding N-methylcarbamates are poor substrates and part of their inhibitory potency is likely to be due to their resemblance to the TS complex in the reaction of the enzyme with its carboxy ester substrate. Additional supportive evidence is that when Wustner et al. (41) tried to prove that the ring substituents in both series of compounds interact similarly with the bovine erythrocyte AChE active site they found a disappointing correlation between K_I and K_d and a better correlation between K_I and k_i respectively for N-methoxy N-methyl and N-methylcarbamates.

A similar but intuitive argument can be made that the binding of organophosphorous compounds to the acetylcholinesterase may involve some aspects of TS theory. In the development of organophosphorous toxins, we classically assume that one is attempting to synthesize a molecule which mimics the substrate acetylcholine. With VX gas as an example one can assume that back bonding between oxygen and phosphorous leads to an electrophilic center mimicking the carbonyl carbon and that the tertiary amine mimics the quaternary ammonium of acetylcholine. However, one could argue that the tetrahedral phosphorous mimics the tetrahedral intermediate formed during hydrolysis of acetylcholine. It should be pointed out that such tetrahedral "transition states" often mentioned in the hydrolysis of esters are really transient intermediates occupying a slight dip near the peak of the reaction coordinates. The true transition states occur during the formation and breaking of the bonds of the tetrahedral intermediate. Thus, it is not surprising that some tetrahedral organophosphorous compounds can display an apparent affinity for an esterase which is even higher than that shown by the trigonal substrate. Perhaps an appreciation of TS theory may lead to better optimization of such organophosphorous and carbamate structures.

TSA as Inhibitors of Juvenile Hormone Esterase(s)

Background. The juvenile hormones (JHs) regulate a myriad of developmental and reproductive events in insects, and metamorphosis certainly is among the most striking of these events (44). The reduction in JH titer to initiate metamorphosis in Lepidoptera examined appears to be caused by degradative metabolism as well as reduction in the rate of biosynthesis (44,45). Ester cleavage of JHs is apparently the major route of metabolism. In the cabbage looper, Trichoplusia ni (T. ni) hydrolysis of JH is due largely to a

single enzyme (JH esterase) mainly present in the hemolymph and fat body (46-49). Although the specification of the precise binding mode through crystallographic or other means is eagerly awaited for JHEs, inhibition kinetics indicate indirectly that JHEs from T. ni (50) and Manduca sexta (51) are likely to be serine hydrolases. Typically, drug metabolizing carboxylesterases are serine hydrolases (EC 3.1.1.1.) and as such associate with the electrophilic moieties of esters or amides, organophosphates (52) and halomethyl ketones (53). Based on these indirect lines of evidence hydrolysis of the JHs by JHEs is thought to proceed with a change in the bond order of the substrate (54) to form tetrahedral adduct with the enzyme from the trigonal structure of the ester. The mode of operation of serine proteases is now fairly well understood in terms of their three dimensional geometries down to the level of individual atomic position (55). There appears to be a general agreement on the role of the charge relay system (proton relay system) in the catalytic activity of serine proteases with some little confusion in the detailed mechanism or the relative importance of each step in proton transfer (see (55) for review). Since JHs are considered to be less polarized at the carbonyl of the methyl esters due to the lack of electron withdrawing properties of the JH acids, one expects with caution a similar proton relay system is likely to be evolved in increasing the nucleophilicity of the serine oxygen to be reactive toward the less polarized carbonyl of the JH ester. This, of course, does not exclude the importance of the bulk of the long chain acid moiety and the methyl group of the alcohol moiety in the binding sites for the stabilization of the tetrahedral adduct. Based on the above discussion it was thought that the trifluoromethyl ketones would be more polarized and thus create a great electrophilicity on the carbonyl carbon which facilitates -OH attack by the serine residue. Yet there is no carbon-oxygen bond to be cleaved in the ketone moiety, and therefore the enzyme-trifluoromethyl ketone transition state complex does not undergo catalytic conversion. The above rationale seems reasonable as trifluoromethyl ketones were found to be extraordinary selective and potent inhibitors of cholinesterases (56) of JHE from T. ni (57) and of meperidine carboxylesterases from mouse and human livers (58). Since JH homologs are alpha-beta unsaturated esters, a sulfide bond was placed beta to the carbonyl in hopes that it would mimic the 2,3-olefin of JHs and yield more powerful inhibitors (54). This empirical approach was extremely successful since it resulted in compounds that were extremely potent inhibitors of JHEs from different species (51,54,59).

Quantitative Structure-Activity Relationships (QSAR) of Trifluoroketones as JHE Inhibitors. The great structural variety of trifluoromethyl ketones, and the reported biological response against JHE from T. ni (54,57) makes these results particularly well suited for a QSAR investigation. Except for the values of molar refractivity (MR), all the physicochemical parameters used in the present work were from the recent compilation of Hansch and Leo (21). To the best of our knowledge this will be the second QSAR study for the inhibition of JHE. The first one was done by Magee (60) in analyzing the data of Hammock et al. (61) for the inhibition of JHEs from cockroach (Blaberus giganteus) by some phosphoramidothioates.

In the first approach the activity of 3-alkyl and 3-alkylthio 1,1,1-trifluoro-propan-2-ones was considered for their structure-activity relationship (SAR) with MR. MR was used in the present study to model the enzyme-inhibitor attraction forces since MR is related to London dispersion forces (21,62,63) and has been also proposed to be really a corrected form of the molar volume (21). Figure 6 shows a clear parabolic relation between the molar I_{50} value and MR values for R groups in the general structure $RC(=O)CF_3$. It is worth noting that both series show an optimum MR value for strong inhibitory activity and that activity decreases when MR values are either larger or smaller than this value. This parabolic relation indicates that the compounds with an MR value smaller than the optimum value might have a positive coefficient for significant MR term which would indicate productive binding for JHE inhibition. However the rest of the compounds appeared to have a negative coefficient which might be due to steric inhibition of binding to the enzyme active site. Although the above discussion seems reasonable since a considerable collinearity between MR and Hancock's steric parameter (64) was found by Dunn (63), the attraction by dispersion forces for another region beyond the catalytic site of the enzyme could be involved. In general, it can be concluded that the affinity of these compounds for the enzyme is a linearly decreasing function of the sum of non-overlappable volumes of the inhibitor molecule and a receptor cavity on the enzyme active site. Since MR is an additive and constitutive property of the molecules (21), it was calculated from the fragment values of the atoms (C = 2.418, H = 1.1; ethereal oxygen = 1.643 and an increment for C = C double bond of 1.733) for both series except that the experimental MR value for C_4H_9S-(31.5) as quoted by Balaban et al. (65) was used as a basic group for the sulfide series. In an attempt to relate the MR value for these inhibitors with that of the corresponding straight chain of JHs, values of 47.1 and 52, respectively, for JH III and JH I were calculated. Considering the optical exaltation due to the conjugated double bond to be 2.85 (ΔMR between the enol and keto forms of ethyl acetoacetate) (66), the calculated value for the above JH homologs would be 50 and 54.9 respectively. When the above values scaled by 0.1 as in Figure 6, the optimum MR value for the most active compound in each series is identical with that of JH I and slightly larger than that of JH III. If one assumes that the octyl sulfide compound, as the most active compound in both series, is practically and roughly a perfect TSA it would be expected to have most of the available binding energy to the enzyme and eventually would behave as a tight binding inhibitor and can be used to titrate the active site of JHE. In fact this compound was found kinetically to act as a slow tight binding (16) and was used to evaluate the molar equivalency of JHE from T. ni to be 1.6×10^{-6}M in the hemolymph. Since in our laboratory a 1:500 diluted hemolymph is always used and if the above molarity is correct, this means that a molar equivalency of 3.2×10^{-9} is used in the inhibition assay. The molar I_{50} value of this compound was found to be consistent in at least five measurements (2.3×10^{-9}M) and almost identical with half the enzyme concentration, a behavi...

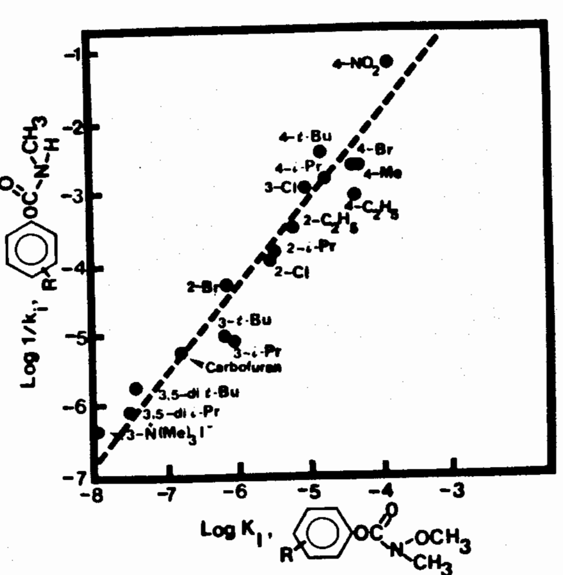


Figure 5. Relationship between the bimolecular inhibition reaction constant (k_i) of substituted phenyl N-methylcarbamates and the inhibition dissociation constant (K_I) of the corresponding N-methyl N-methoxycarbamates in their reaction with house fly acetylcholinesterase. k_i Values were recalculated from (42) and K_I values were from (41).

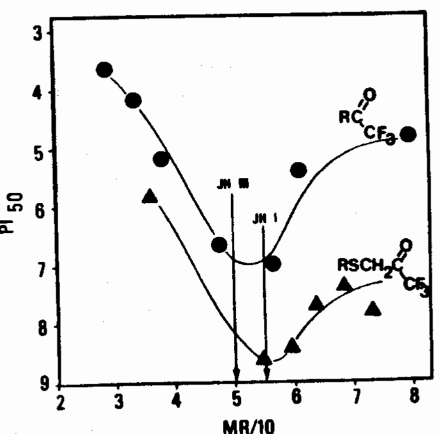
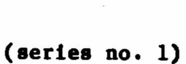


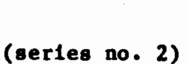
Figure 6. Relationship between the inhibitory activity of trifluoromethylketones against JH-esterase from Trichoplusia ni and the molar equivalents of these inhibitors.

of titrimetric and stoichiometric enzyme inhibitor. Additional supportive evidence for considering these compounds or more accurately the most active ones of the series to be TSA is that JH I which has the same MR as the octyl sulfide compound showed faster turnover number than JH III, with smaller MR than optimum, in consistence with the discussion mentioned before for the binding energy of the TS complex. The above relation is not consistent with K_m values for both homologs which excludes the possibility that these compounds act as substrate analogs. Therefore the unusually high activity of these compounds can be explained by forming hemiketal structures which mimic a transition state near the tetrahedral intermediate complex in ester hydrolysis (Figure 7). In moving from SAR to QSAR, log MR was used instead of 0.1 MR where the latter was used to generate Figure 6. This was done for at least two reasons. First, better correlation was obtained when log MR was used. Second, almost all the physicochemical parameters used for QSAR are originally from log terms in order to be linear free energy parameters. The alkyl and alkyl sulfide derivatives were analyzed separately at first and the following equations were obtained respectively for the following structures:



$RC(O)CX_3$ (series no. 1)

where R = alkyl, X = H or F



and $RSCH_2C(O)CF_3$ (series no. 2)

$$PI_{50} = -140.5 + 167.7 \log MR + 2.5 \Sigma F_{x3} - 48.9 (\log MR)^2 \quad (33)$$

n	r	s
9	0.95	0.6

$$PI_{50} = -226.6 + 269.9 \log MR - 77.5 (\log MR)^2 \quad (34)$$

n	r	s
6	0.94	0.44

where n represents the number of data points, r is the correlation coefficient and s is the standard deviation from the regression. It is rather interesting that the F term (polar effect) has a positive coefficient in "Equation 33" in agreement with our belief that the activity of these compounds might be due to the polarized ketone induced by the fluorine atoms on the α-carbon which facilitates the electrophilicity towards lone pair electrons on the oxygen atom of serine hydroxyl in the enzyme catalytic site. On the other hand, using the continuous variables MR, F in the above equations leaves a relatively large amount of variance in the data unaccounted for which might indicate that the basic group in the two series (trifluoromethyl) has the key structural requirement for their potency. At this point, structural modification of this group using different congeners might yield insights into whether the fluorine atoms act mainly through electronic transmission and/or steric

interaction with the catalytic site. In comparing members of the sulfide series with those lacking sulfide of the same MR, the former seems to have an average bestowed potency of 67.5. Therefore an indicator variable (X) was used to analyze both series in one regression, "Equation 35". X = 1.83 for the sulfide series and = 0 for the series lacking the sulfide bond is simply a device for merging the above two equations. To this point it is not certain how the sulfur increases the inhibitory potency of these compounds. The crystallographic study of a pure enzyme might offer clear opportunity to know whether the sulfur mimics the olefin structure of JH homologs or it contributes to the binding forces for example through hydrogen bonding since the extra bestowed activity is equivalent of about 2.54 Kcal/mole.

$$PI_{50} = -143.4 + 170.0 \log MR + 2.39\ \Sigma F_{x3} - 49.1 (\log MR)^2 + 0.89X \qquad (35)$$

n	r	s
15	.962	0.61

If the above equations represent a real model of quantitation to the interactions of these compounds with JHE, which seems to be the case since "Equation 35" holds for two series from two separate studies, one can assume that MR which dominates the above equations can explain more than 90% of the variation in the activity of members of both series. Interestingly, the hydrophobicity (π term) did not give better correlation when used instead of log MR in the above equations. Since the first use of MR for correlation of substituent effects (62), greater effort has been made to discuss the theory behind the use of this parameter in correlating ligand interactions (67) and to discuss the nature of interaction in which either MR and/or π model such interaction (68,69). Generally, MR reflects apolar as well as polar interactions where desolvation is not the main driving force. However, when π and not MR models the interaction, desolvation appears to play the dominant role (69). In relating the regression equations with the above working hypothesis, desolvation in the interaction of the above compounds with JHE appears not to be the driving force in the interaction process. Whether the interaction is apolar or polar needs further study in which π and MR should be orthogonal so that one can see exactly the role of each parameter in the interaction without being so collinear. Figure 8 shows the relation between the observed PI_{50} values vs. the expected values according to "Equation 35".

The activity of 1,1,1-trifluoro 3-mercapto substituted phenyl propan-2-ones was not simply correlated with MR alone, therefore, a combination of Hammett σ constant, Taft steric parameter (E_s) and Hansch hydrophobic constant (π) was included in the regression analysis. "Equation 36" was found to be of the best fit for compounds substituted in the meta and para positions.

$$PI_{50} = 5.15 + 1.07\ \Sigma\pi + 1.23\ \Sigma \log MR - 1.08\ \Sigma\sigma + 0.59 (\Sigma\sigma)^2 + 0.35\ \Sigma E_s \qquad (36)$$

n	r	s
13	0.951	0.39

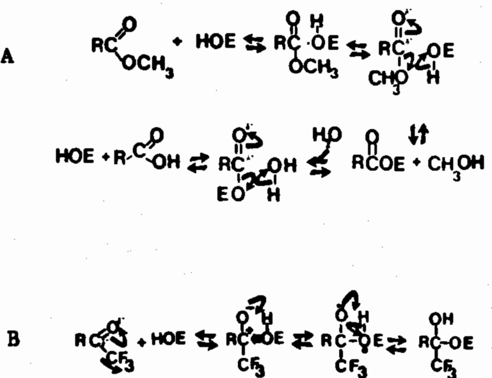


Figure 7. Proposed scheme for the reactions of JH-esterase with JH homologs (A) and with trifluoromethylketones (B).

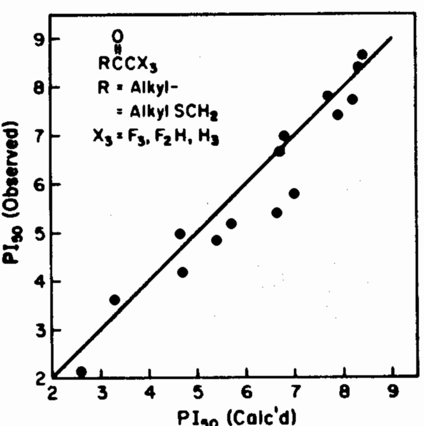


Figure 8. Relationship between the observed PI_{50} values and the PI_{50} values calculated from "Equation 35".

From the slope of the physicochemical parameters in "Equation 36" it appeared that π, MR and σ have about the same weight in modeling the activity of the 13 meta and para substituted compounds. The importance of E_s and σ^2 seems to be less although high covariance between E_s and both π and MR is a serious obstacle preventing the true delineation of E_s effects. Meanwhile the equation reveals that hydrophobic substituents (positive π term) with large MR and electron donating properties (negative σ term) would enhance the inhibitory potency of the trifluoromercaptophenylacetone as a parent compound. In fact this was the rationale behind synthesizing the para t-butyl analog, and it came out to be the most active congener among the tested compounds. Since covariance between MR and π is low ($r^2 = 0.46$), they are considered to be orthogonal and have different substituent effects. As discussed earlier for the alkyl compounds, these substituent effects might establish different type of interactions, i.e. polar and apolar interactions. Since these compounds are substituted in the same positions, it is not clear whether those types of interactions are in two different types of enzymic spaces or one space which is open to surrounding solution. In any case this leads to further research in which crystallographic structure of JHE might shed some light on the binding space(s) and the role of desolvation in their interaction with these novel inhibitors. At this moment, the coefficient of near 1 with π in "Equation 36" should not be ignored and as has been discussed before (70) for papain ligand interactions, brings out the close parallel between binding to JHE and partitioning into octanol. This indicates that there might be a true hydrophobic pocket on the enzyme active surface where interaction is driven by desolvation and the interaction is likely to be entropic in nature. This conclusion should not be oversimplified since extensive thermodynamic studies certainly are required to shed some light on the role of both entropy and enthalpy changes for the inhibition process by these compounds.

The selective inhibition of JHE and α-naphthyl acetate esterase(s) by ortho, meta and para substituted compounds showed that meta and para substitution offered selectivity towards JHE, however, the ortho substituted compounds favored inhibition of α-naphthyl acetate esterases (54). It was thought that substitution in the ortho position might be detrimental for inhibition of JHE. Therefore we decided to add E_s value for the ortho substituents in the regression analysis to merge the ortho compounds with their meta and para analogs in the same regression, "Equation 37".

$$PI_{50} = 5.05 + 0.74\ \Sigma\pi + 1.14\ \Sigma \log MR - 0.61\ \Sigma\sigma - 0.11\ (\Sigma\sigma)^2 + 0.61\ E_s\ (ortho)$$

n	r	s	
18	0.92	0.45	(37)

By comparing "Equations 36 and 37" one can see that the coefficients of π and MR are more stable to the addition of the ortho compounds to the regression analysis. However, the coefficients of σ terms became small upon the addition of E_s ortho. The stability of the

coefficients of π and MR in addition to the fact that the intercepts in both equations are so similar to the observed PI_{50} value (5.08) for the parent unsubstituted compound might suggest the validity of these equations and support the above discussion in modeling the interaction of these compounds with JHE in terms of their hydrophobicity and molar refractivity. Figure 9 shows the relation between the observed PI_{50} values for the members of the aromatic series and the PI_{50} values calculated from "Equation 37".

In conclusion, although our QSAR for the inhibition of JHE with trifluoromethyl ketones does not offer an excellent and sharp fit of the data to the regression ("Equations 33-37"), it might provide the chemist with a model and rough base line for testing new compounds. In general the interactions of these compounds with JHE are likely to be hydrophobic and nonhydrophobic. Whether the latter type is separable from the former or in fact it is hydrophobic without being dependent on desolvation is not clear. Nevertheless, as these compounds are considered to be stable TSA, they offer a valuable tool in studying the x-ray crystallography of JHE and its TS complex from which valuable information can be coupled with the QSAR study and would greatly increase our understanding of JH-JHE interaction. It is worth reporting that one of these analogs was attached to insoluble support and proved to be an excellent ligand for the purification of JHE by affinity chromatography (51). This is the first step in approaching the three dimensional structure of JHE and JHE-inhibitor complex.

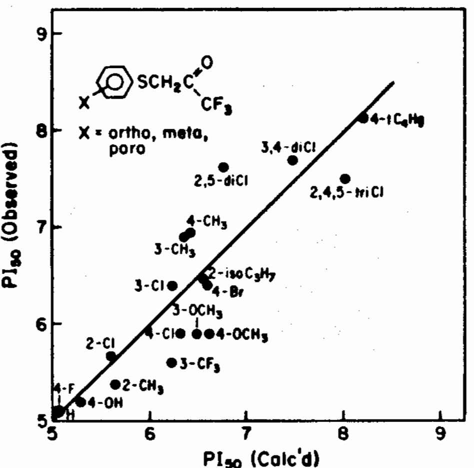


Figure 9. Relationship between the observed PI_{50} values and the PI_{50} values calculated from "Equation 37".

Acknowledgments

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