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262. Interaction of Ophidian L-Amino Acid Oxidase with its Substrates and Inhibitors: Role of Molecular Geometry and Electron Distribution[1])

Communication 6 on ophidian L-amino acid oxidases[2])

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Dedicated to Professor *Franz Leuthardt*, Ph. D., M.D.h.c., University of Zurich

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Summary. Molecular activities, determined polarographically for the degradation of phenylalanine and 21 meta- and para-substituted derivatives by crystalline L-amino acid oxidase, yielded a linear *Hammett* plot of positive slope. However, the presence of large moieties in the para-position led to negative deviations which were linearly correlated with *van der Waals'* radius of the substituent. This suggested that the large substituents cause the substrate molecules to be displaced from their normal (eutopic, productive) position at the active site, resulting in lower velocities. In analogous experiments with competitive inhibitors (ring-substituted benzoic and phenylacetic acids), the logarithm of K_I was found to be a linear function of *Hansch's* hydrophobic constant π. Lines of different slope characterized the meta- and para-derivatives, suggesting that the nature of the substituent affects the strength of hydrophobic binding, while the locus determines the orientation of the inhibitor on the active site. An analysis of the data reported here and in the literature for meta- and para-compounds supports the idea that the geometry of the ring-substituted substrate affects the orientation of the molecule on the active site, which may in turn determine which step of the reaction sequence becomes rate-limiting.

Whenever a substrate molecule is bound to the active site of an enzyme, a catalytic event is presumed to take place. During the Fifties, however, an increasing number of observations obtained with several enzymes suggested the existence of enzyme-substrate complexes which are incapable of breaking down to products and free enzyme. A series of papers on the specificity of chymotrypsin by *Niemann et al.* and reviewed by *Niemann* in 1964 [5], stand out as milestones in this development. During the same period of time, a considerable body of experimental data on oxidative deamination was collected in this laboratory. This information was interpretable

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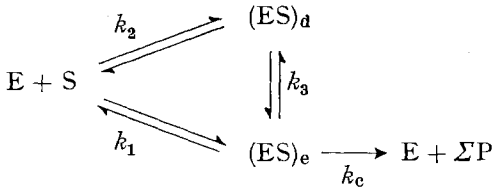
by the concept of multiple enzyme-substrate complexes [6] [7]. While in their most general form such enzyme reactions can be described by reaction sequence 1 [3],



(1)

the first step in formulation of the corresponding *Michaelis-Henri* equations was made in both laboratories by starting from a simpler reaction scheme which did not include the step characterized by k_3 in the vector sequence 1. (See Discussion, section on Displacement Effects.) The indices e and d refer to eutopic (productive) and dystopic (non-productive, wrong-way, dead-end, mis-oriented) complexes, respectively.

The L-amino acid oxidase of snake venoms (L-aminoacid: O_2 oxidoreductase [deaminating] EC 1.4.3.2.)[5]), first described by *Zeller & Maritz* [8–10], seemed to be a suitable system for providing additional information about the existence of multiple enzyme-substrate complexes. Previously, several para-substituted phenylalanines were shown to be much more slowly degraded by crude and purified L-AAO than the corresponding meta-isomers [4]. From these and other observations it was tentatively concluded that large para-residues force the substrate molecule into non-productive complexes. Hoping to find a broader basis for this model, we studied the rate of degradation of a large number of ring-substituted phenylalanines by crystalline L-AAO, using mainly a polarographic method. The data were organized according to the *Hammett* relationship (equ. 2), where V represents molecular activity (see 'Assay Conditions'), σ the

$$\log V_X - \log V_H = \sigma\varrho + i \tag{2}$$

electron density at the reaction center of the substrate, and ϱ and i the slope and intercept. The indices H and X refer to phenylalanine and the substituted substrates, respectively. The results revealed that the rate-limiting step of the reaction is affected not only by electron-withdrawing and electron-releasing residues [1], but also by the locus of the substituent. Similarly, the depressed rates observed for the degradation of certain para-derivatives permitted quantitation of the formation of dystopic (non-productive) complexes.

Since competitive inhibitors such as ring-substituted benzoic acids [8] [9] [11] and phenylacetic acids can be considered to be substrate substitutes, it was of interest to study their mode of action in a similar way. While no simple *Hammett* relationship between the inhibitor constant K_i and σ could be generated, we fared better when we expressed the logarithm of the inhibitor constant K_i as a function of *Hansch*'s constant $\pi = \log (P_X/P_H)$, where P_X is the octanol/water partition co-

[5]) L-AAO.

efficient of a derivative and P_H, that of the parent molecule [12]. Finally, the results are interpreted with respect to orientation and displacement of substrate molecules on the active site of L-AAO.

Experimental Part

Substrates and Inhibitors. The origin and synthesis of the substrates has previously been reported [4]. The substituted phenylacetic acids were obtained from *K & K* Laboratories, Inc., Plainview, N.Y., while the substituted benzoic acids, with the exception of the fluorocompounds (obtained from *Aldrich Chemical* Co., Inc., Milwaukee, Wis.), were purchased from *Eastman Organic Chemicals*, Rochester, N.Y.

Enzyme Purification. We isolated and crystallized L-AAO from the venom of *Crotalus adamanteus* (*Sigma Chemical* Co., St. Louis, Mo.) according to the method of *Wellner & Meister* [13]. The yields of crystalline enzyme varied from 25–37%. The crystals could be stored suspended in water or dissolved in 0.1M KCl for months at 5° without significant loss of activity. The enzyme concentration was determined both by the protein method of *Folin-Ciocalteau* [14] and by FAD absorption at 462 nm (molar extinction coefficient: 11,300). The specific activities of the crystalline preparations varied from 6000 to 6900[6]), where specific activity is defined as units per ml divided by the absorbance at 275 nm (1 cm pathlength), while a unit is designated as the quantity of enzyme required to catalyze the consumption of 1 μl of oxygen in 30 minutes under the specified assay conditions [16]. Unless stated otherwise, the crystalline enzyme at this stage of purification was used for all kinetic studies.

In view of the observation that the crystalline enzyme can be separated into 3 fractions by electrophoresis [13], we isolated Fraction A (see next paragraph) from the purified enzyme by disc-gel electrophoresis using a *Canalco* preparative disc unit. The anode and cathode solutions were both buffered by 0.3M glycine/NaOH, pH 9.0. The sample, applied in a dense polyacrylamide solution, was allowed to migrate under a current density of 5 Ma for the first hour, followed by a 7 Ma for the duration of the run.

Physical Characterization of Purified Enzyme Preparations. The sedimentation velocity of the twice crystallized enzyme was measured in a *Spinco* Model E analytical ultracentrifuge equipped with schlieren optics. The run was made at a rotor speed of 59,780 rpm and a temperature of 22°, using a protein concentration of 0.28% in a solution of 0.05M Tris-buffer, pH 7.2, 0,1M with respect to KCl. The sedimentation coefficient ($s_{20,w}$) was 6,60 S. This compares favorably with earlier values of 6.63 and 6.54 [13] determined with 0.23 and 1.0% protein, respectively.

Fifty and 100 μg samples of the enzyme preparation were subjected to electrophoresis at a running pH of 9.5 on 7.5% crosslinked polyacrylamide gels. The tests, run at 4° with a current of 5 Ma per sample column, revealed 3 major bands.

Kinetic Measurements. The polarographic rate determinations were made with the Biological Oxygen Monitor (*Yellow Springs Instrument* Co., Yellow Springs, O.). The apparatus was equipped with a membrane-covered electrode of the *Clark* type [17] which was connected to a *Bausch & Lomb* VOM 5 recorder operating at 100 mv. The manometric procedure was described previously [4].

Assay Conditions. For the determination of pI_{50}-values, kinetic measurements were obtained manometrically with crude venom as the enzyme source and pure oxygen as the gas phase. All components of the reaction system with the exception of the venom, which was dissolved in 0.9% NaCl-solution, were taken up in 0.067M phosphate buffer, pH 7.2, yielding a final concentration of 0.060M phosphate buffer and 0.015M NaCl. Each vessel contained 0.1 to 0.5 mg venom and 40 μg crystalline catalase. The reactions were carried out at 38° in a total volume of 2 ml. Readings were taken at intervals of 2 to 3 min for 30 min.

All other rate measurements were carried out by the polarographic method. We ran the assay system generally for 2 to 5 min, usually before 3% of the substrate had been degraded. The reactions took place at 38° in air-saturated solutions, in a total volume of 3 ml. Five different substrate concentrations between 0.4 and 2 mM, and sometimes as low as 0.1 mM, were used for

[6]) These values are about 10% lower than those reported previously [13]. A detailed discussion for the possible reason for the difference is given in [15].

the conventional determination of maximal velocities by means of the *Lineweaver-Burk* diagram. Inhibitors were present in 1 to 10 mM concentrations. Enzyme concentrations ranged from 2 to 25 μg crystalline enzyme or 0.1 to 0.5 mg crude enzyme per vessel. The assays were performed in 0.067 M Tris-buffer, pH 7.6 (at 38°). The enzyme was dissolved in 0.1M KCl (final concentration: 0.067 M).

Since the amount of CO_2 produced as a result of the reaction of H_2O_2 with the keto acid [8] did not affect the polarographic measurements, in contrast to the manometric readings, catalase was omitted. In addition, the initial velocities were measured early enough so that the amount of H_2O_2 produced would be too small to inactivate the enzyme measurably. This was evidenced by the fact that the velocity in the absence of catalase was exactly twice that in its presence.

Maximal velocities obtained with the crude enzyme were expressed as microequivalents of O_2 uptake per h per mg of dry venom, while maximal velocities determined with the crystalline preparation were defined as the number of substrate molecules converted by one enzyme molecule (M.-W.: 130,000 [16]) per min ('molecular activity').

Although D,L-amino acids were generally used as substrates, concentration data refer to the L-form. The following facts serve as a basis for this procedure: It is well-known that L-AAO does not attack D-amino acids. Moreover, the presence of a tenfold surplus of D-leucine does not influence the rate of degradation of the L-form [8]. Similarly, quantitative oxidation of L-alanine was observed in a solution containing 1000 times more of the D-enantiomer [18]. To be sure that D-amino acids do not measurably affect the kinetics of the L-forms under the exact conditions described here, the oxidation rates of several L-amino acids were compared with those of the corresponsing D,L-forms, including phenylalanine. We found no differences beyond experimental variation.

Selection of van der Waals' Radii, Partition Coefficients, and Electron Densities. Van der Waals' radii data were either taken from the literature or computed on the basis of interatomic distances, *van der Waals'* radii for single atoms, and bond angles, from the classical tables of *Pauling* [19]. When reliable data were not found, we used measurements of the molecular models of *Corey, Pauling & Koltun* (CPK) as a last resort. The *Hansch* substituent constants used for the various benzoic and phenylacetic acids were found in [12].

Although more than 40 sets of σ constants have been proposed [20], none exactly represents the electron distribution of substituted phenylalanines. Assuming that σ° may come sufficiently close to describe the situation in our system, we calculated the σ°-values for the *m*- and *p*-substituted phenylalanines according to *Swain & Lupton* (equ. 3), whereby σ° refers to electron densities

$$\sigma^\circ = fF + rR \tag{3}$$

at a locus separated by one carbon atom from the benzene ring, F stands for field effects, including induction, and R for resonance components; f and r are weighing factors.

Results. – *Kinetics of Substrate Degradation and* Hammett *Relationship.* The kinetic data and electron densities (σ°) for phenylalanine and its substituted derivatives are given in Table 1. When we plotted the logarithms of all *V*-values (set A) against σ° (Fig. 1), a regression line of positive slope (ϱ) and very low correlation coefficient resulted (Table 2). The line obtained for the meta-substituted substrates (set B), however, was characterized by a high correlation coefficient, in sharp contrast to the results obtained for the para-substituted compounds (set C). Since a number of para-substitutions (OH, NH_2, NO_2, F, SO_2CH_3, and SO_2NH_2) fell close to the line defined by phenylalanine and its meta-substituted derivatives, they were combined with the data from set B. The new group (set D) was characterized by a *Hammett* line which, based on regressional data (Table 2), was almost identical to that representing set B (Fig. 1). Whether or not this procedure is arbitrary may be judged by the relationships uncovered for the non-fitting *V*-values (see next two

paragraphs). The slope for all sets of *Hammett* relationships was positive (Table 2), indicating that electron withdrawal produced by the ring-substituents facilitates a rate-limiting step in the process of substrate degradation.

Table 1. *Maximal velocities,* Michaelis *constants, and* Hammett *constants for the degradation of ring-substituted phenylalanines by crystalline* L-*amino acid oxidase*

Substituent	V[a])	K_m(mM)	$\sigma°$[b])	Substituent	V[a])	K_m(mM)	$\sigma°$[b])
H	1780	0.08	0.000	*p*-Hydroxy	1970	0.07	−0.15[8]
m-Fluoro	2480	0.12	0.328	*p*-Amino	1420	0.16	−0.47[4]
p-Fluoro	2120	0.09	0.190	*m*-Nitro	3200	0.15	0.71[0]
m-Chloro	2470	0.09	0.367	*p*-Nitro	3400	0.25	0.77[4]
p-Chloro	1690	0.07	0.301	*p*-Sulfamyl	2900	2.20	0.53[8]
m-Bromo	2580	0.08	0.385	*p*-Methylsulfonyl	3030	1.60	0.692
p-Bromo	1240	0.06	0.313	*p*-Methylmercapto	550	0.02	0.069
m-Iodo	3000	0.12	0.346	3,5-diiodo, 4-hydroxy[c])	1380	0.15	0.534
p-Iodo	860	0.04	0.265				
m-Methyl	1760	0.10	−0.072	3,5-dinitro, 4-hydroxy[c])	1220	0.16	1.262
p-Methyl	1020	0.06	−0.130				
m-Hydroxy	2300	0.13	0.106				

[a]) Expressed as molecular activity, moles of substrate oxidized per minute per mole enzyme (M.W.: 130,000).

[b]) Electron density computed from field and resonance components according to *Swain & Lupton* [20].

[c]) Assayed with crude venom and computed with reference to rate of phenylalanine oxidation; $\sigma = \Sigma\sigma°$.

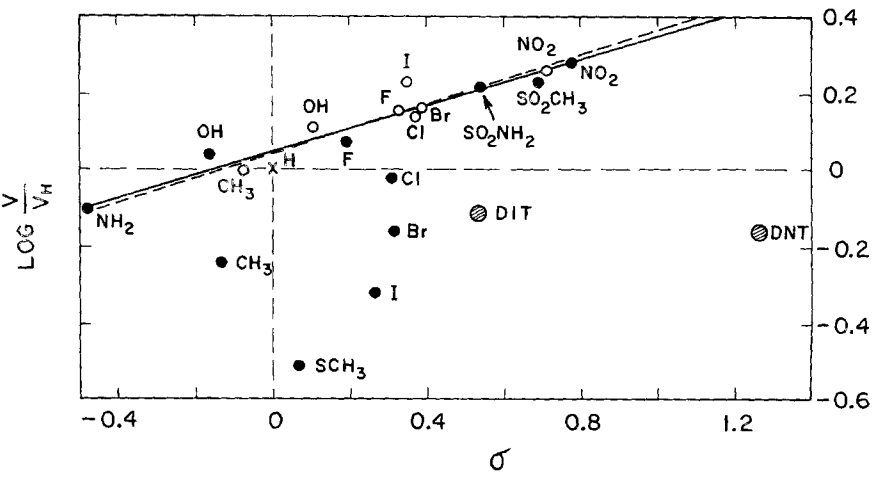


Fig. 1. Hammett *plot for the degradation of unsubstituted and substituted phenylalanines by crystalline* L-*AAO of* C. adamanteus (see Table 1). Cross: phenylalanine; open circles: meta-derivatives; black circles: para-derivatives; cross-hatched circles: multiple substituents; solid and dashed line: linear regression functions for sets B and D, respectively. Maximal velocity is expressed in terms of molecular activity, based on a M.W. of 130,000

The linear *Hammett* relationship for set D established a basis for the quantitative evaluation of the negatively-deviating maximal velocities within set A: Cl, Br, I, CH_3, and SCH_3, all substituted in the para-position. These will be referred to as set E.

Table 2. *Regression analysis of* Hammett *data for various sets of substrates*

Substrate sets	Preparation	Number of substrates	ρ[a]	i[b]	r[c]	p[d]
A: Phe, meta- and para-subst.	crystall.	21	0.17	0.04	0.31	<0.2
phenylalanines	crude	18	0.38	−0.01	0.67	<0.005
B: Meta-subst. phenylalanines	crystall.	7	0.31	0.05	0.91	0.0025
	crude	7	0.34	0.07	0.93	0.005
C: Para-subst. phenylalanines	crystall.	11	0.36	−0.12	0.56	<0.1
	crude	11	0.29	−0.06	0.64	<0.05
D: Phe, meta- and selected	crystall.	14	0.30	0.05	0.96	$<<0.001$
para-subst. phenylalanines	crude	14	0.36	0.06	0.97	$<<0.001$

[a]) Slope. [b]) Intercept. [c]) Correlation coefficient. [d]) Probability that curve is not linear.

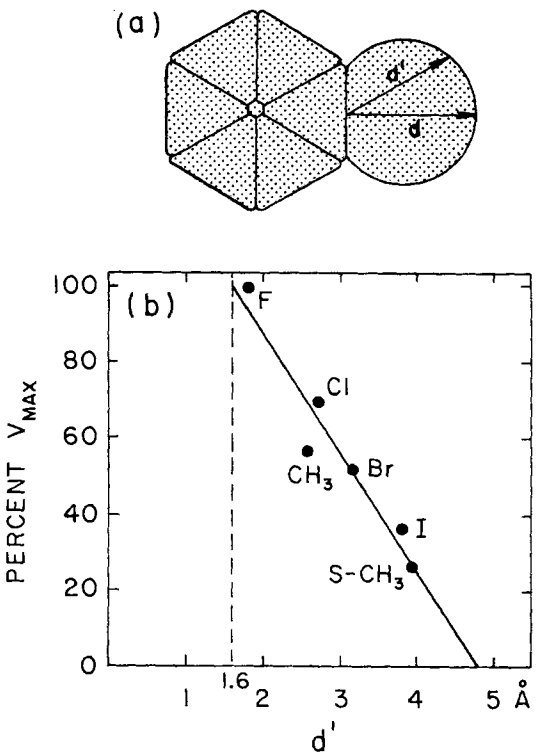


Fig. 2. *Correlation between size of large para-substituents (set E) and deviation of* V *from* Hammett *line* (see text). The *van der Waals'* parameters d and d' are defined in Fig. 2a. Fig. 2b: Plot of deviation as a function of d' (deviation expressed as per cent of *V* defined by the *Hammett* relationship)

At a given σ-value, the maximal velocity read from the *Hammett* line was assigned unity, and the experimentally determined rate for the deviating compound was expressed as a fraction thereof. These relative velocities were then correlated with the *van der Waals'* radii of the substituents. When we plotted the distance d as defined in Fig. 2 against the relative rates (in %), a relationship was obtained (Fig. 2b) which, in spite of the uncertainty regarding the *van der Waals* dimensions, exhibited a fair degree of linearity ($r = 0.82$, $P < 0.02$). However, the highest correlation coefficient ($r = 0.96$, $P < 0.01$) was noted when vector d', forming an angle of 30° with d, was drawn. No deviation from the *Hammett* line was observed when the vectors d or d' were below 1.7–1.8 Å.

To rule out possible complications arising from the fact that crystalline L-AAO consists of several components (see Experimental Section), we separated the three major constituents and compared the maximal velocities obtained for component A with those observed for the original mixture (A, B, C). Phenylalanine and 4 metasubstituted derivatives (CH_3, F, Br, and I), encompassing a considerable range of σ-values ($\Delta = 0{,}46$), were chosen as substrates. The correlation coefficients ($r_A = 0.97$, $r_{ABC} = 0.98$) for the *Hammett* regression line again indicated a considerable degree of linearity. The slope ($\varrho_A = 0.49$, $\varrho_{ABC} = 0.51$) and the intercept ($i_A = i_B = 0.02$) of the two lines were within experimental variation. Thus, on the basis of the kinetic data, no significant difference could be detected between the two enzyme preparations[7]). Apparently, the various isozymes respond in much the same way to changes in electron distribution within the substrate molecule. It must be emphasized, however, that fraction A can be further separated into 5 components by electrofocusing [22].

To investigate the possibility of a change in conformation and thus of the reactivity of the enzyme during its isolation, we compared the kinetic constants for the crude and crystalline preparations. The results of the regression analysis of log V/V_H vs. σ° for sets A to D (Table 2) indicate that no major irreversible change took place in the enzyme during the purification procedure, at least not within the range of our analytical data.

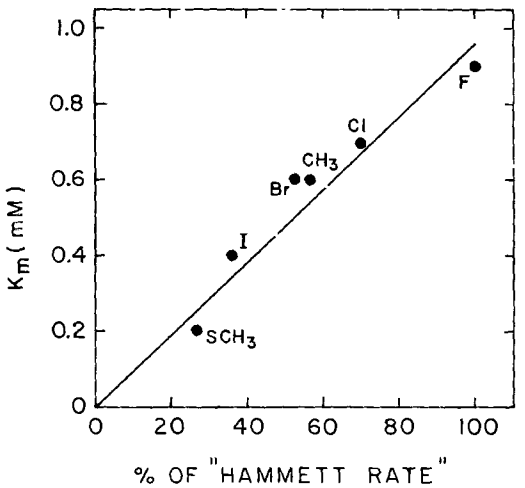


Fig. 3. *Slater plot for the degradation of phenylalanines possessing large para-substituents* (set E). V is defined as in Fig. 2

[7]) For further details, see [21].

When the logarithm of the *Michaelis* constant was plotted against σ or *Hansch*'s hydrophobic constant π (see next section), no obvious pattern appeared. However, when we selected the *Michaelis* constants of the para-substituted compounds of set E and plotted them against the rate of reaction according to *Slater* [23], as expressed in % of the *Hammett* values, a linear relationship having a correlation coefficient of 0.97 was obtained, with the line passing close to the zero point (Fig. 3). Thus, the *Slater* plot indicated that both the V and the K_m-terms of members of set E are multiplied by the same factor. No such correlation was found for the other substrates.

Effect of Ring-substitution on the Strength of Competitive Inhibitors. Our initial studies involved the determination of the pI_{50}-values of certain substrate-substitutes, viz. 3 sets of benzoic acids having substituents (F, Cl, Br, I, OH, CH_3, and NO_2) in the ortho-, meta-, or para-positions. The rates were measured manometrically in the presence of 5 mM L-leucine with the crude enzyme as catalyst. While the nature of the substituent had some effect on the degree of inhibition, its locus exerted an overriding power, as shown by the average pI_{50}-values ($\pm$ s.d.) computed for each group of 8 inhibitors:

ortho-substitution	2.25 ± 0.14
meta-substitution	2.61 ± 0.29
para-substitution	2.25 ± 0.17

The data for the meta-substituted acids differ significantly from those of the two other sets ($P < 0.001$), indicating a free energy of binding approximately 0.5 kcal higher for these compounds.

With the aid of the polarographic method, a detailed analysis of the inhibitory effect of a series of meta- and para-substituted benzoic and phenylacetic acids was then undertaken with the crystalline enzyme. Since this was the first time to our knowledge that the phenylacetic acids were tested as inhibitors, we set out to determine whether the inhibition was competitive. In order to avoid bias in the drawing of the *Lineweaver-Burk* diagrams, we treated our kinetic data by regression analysis. The linearity of the double reciprocal plots was substantiated by high correlation coefficients (average $r \simeq 0.99$). In each case the intercept computed for the inhibitor-free system differed from the intercept of the inhibitor system by less than 3%. Thus, the inhibition can be considered to be competitive, suggesting that the inhibitor acids interact with the active site of L-AAO. The K_i-values are presented in Table 3. Another set of benzoic acids has been investigated with the manometric procedure [11]; the K_i-values are similar to ours for the few compounds tested in both laboratories.

The question arose as to whether the action of these 'substrate-substitutes' is also governed by electron densities and thus by the *Hammett* relationship. In plotting log K_i against σ for 5 pairs of meta- and para-derivatives of benzoic acid, *de Kok* & *Veeger* [11] concluded that a non-linear relationship exists between the two parameters. We found a low degree of linearity for their data (slope $= -0.40$, $r = -0.43$). Our compounds (benzoic acid plus 7 pairs of meta- and para-derivatives) yielded

Table 3. *Effect of ring-substituted benzoic and phenylacetic acids on the degradation of* L-*leucine as Expressed by* K_i

Substituents	K_i			
	Benzoic acids[a])		Phenylacetic acids[b])	
	Meta-subst.	Para-subst.	Meta-subst.	Para-subst.
F	0.53×10^{-3}	1.10×10^{-3}	0.76×10^{-3}	1.10×10^{-3}
Cl	0.27	0.88	0.50	0.83
Br	0.25	0.83	0.47	0.75
I	0.20	0.76	0.36	0.54
CH_3	0.63	1.40		
OH	1.20	1.50		
OCH_3	0.47	1.60		

[a]) K_i benzoic acid (unsubstituted) $= 1.3 \times 10^{-3}$.
[b]) K_i phenylacetic acid (unsubstituted) $= 1.2 \times 10^{-3}$.

similar results (slope $= -0.36$, r $= -0.65$). Apparently, a lower electron density results in a decreased K_i, as indicated by the negative slope; however, a relationship more complex than the classical *Hammett* equation appears to relate electron density to the free energy of binding of the inhibitors to the enzyme. Since hydrophobic bonding is dependent on many factors, including electron density, we plotted log K_i^X – log K_i^H against π (Fig. 4). Two separate lines emerged for the meta- and

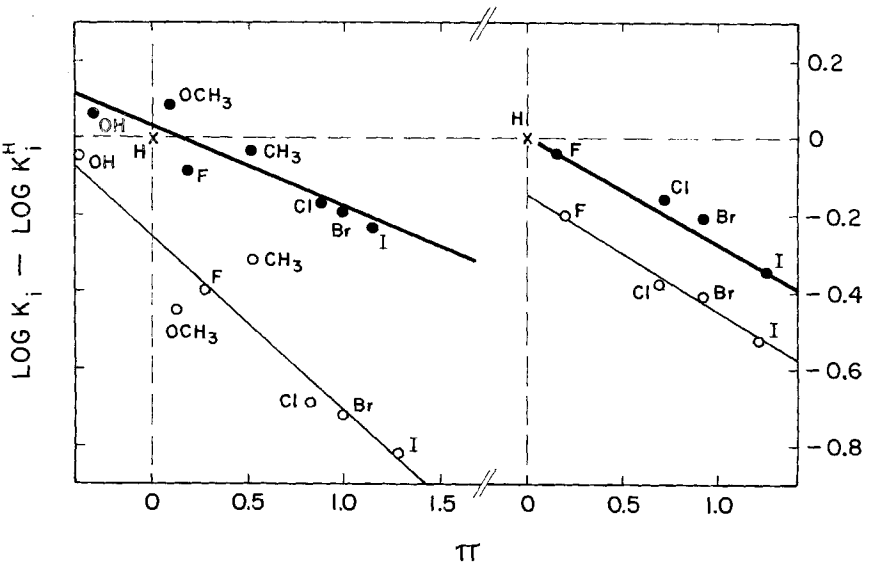


Fig. 4. Hansch *plot for the competitive inhibition of* L-*AAO* (C. adamanteus) *with* L-*phenylalanine as substrate* (see Table 3). Conditions of experiment are given in Experimental Section. Left diagramm: benzoic acid (×) and its meta-substituted (open circles) and para-substituted (black circles) derivatives. Right diagram: phenylacetic acid (×) and its ring-substituents

para-compounds. Regression analysis showed this approach, including the separate analysis for the meta- and para-series, to be a vast improvement over the *Hammett* treatment (Table 4).

Table 4. *Regression data for relationships given in Fig. 4*

Inhibitor sets	ϱ	i	r
Halogenated meta-BzA[a])	−0.43	−0.29	−0.99
All meta-BzA	−0.45	−0.25	−0.94
Halogenated para-BzA	−0.16	−0.04	−0.99
All para-BzA	−0.22	+0.04	−0.88
Benzoic acid, meta- and para-BzA	−0.36	−0.08	−0.65
meta-PhA[b])	−0.31	−0.15	−0.99
para-PhA	−0.27	−0.02	−0.98
Phenylacetic acid, meta- and para-PhA	−0.32	−0.04	−0.83

[a]) meta-Substituted benzoic acids; [b]) meta-Substituted phenylacetic acids.

From the data presented in Fig. 4 it is evident that the lines defined by the para-derivatives pass very close to the mark (x) designating the unsubstituted compounds, while the lines representing the meta-compounds are characterized by finite negative intercepts at the vertical axis. The intercept at $\pi = 0$ (constant b in equation 4) represents the effect of meta- or para-substitution *per se* on the free energy of binding as induced by the orientation of the inhibitor molecules on the active site, without taking into account the influence of the substituent on hydrophobic bonding. We attribute the downward shift for the meta-derivatives to steric factors which force the inhibitor molecule into a position of higher binding energy. Multiplying the intercepts (Table 4) of the meta-series by RT/2.30 yields differences between meta- and para-substitution of 0.35 kcal (benzoic acids) and 0.21 kcal (phenylacetic acids) for the free energy of hydrophobic bonding. Assuming that the free energy of hydrophobic bonding between two benzene rings is 2.0 kcal per mol [24], then the greater binding of the meta-compounds, compared to the para-analogues, could indicate that meta-substitution leads to a greater area of contact between the aromatic ring of the inhibitor and the corresponding enzyme moiety, viz. 17% more for the benzoic acids and 10% for the phenylacetic acids. If indeed the aromatic ring of the meta-derivatives shares a larger 'contact area' with the hydrophobic residue of the binding site than that of the para-compounds, the modulation of the hydrophobic bonding by ring-substitution should accordingly be larger, the greater the 'contact area'. This conclusion is supported by the fact that the ratio (0.60) of the intercepts of the lines representing the meta-halogenated phenylacetic and benzoic acids (Table 4) is similar to the ratio (0.69) of the corresponding slopes (constant a, equation 4).

Thus, the linear free energy relationship describing the hydrophobic binding (equation 4) can be written in a manner analogous to the *Hammett* equation, where the slope a is related to the size of the 'contact area' and the intercept b to the change in binding energy as induced by the geometry of the substituted ring. For

$$\log K_i^X - \log K_i^H = a\pi + b \tag{4}$$

the para-substituted compounds $b \simeq 0$, while for the meta-derivatives, $b < 0$.

Discussion. – This study has presented a number of new enzymic phenomena which we attribute to the effects of ring-substitution on the substrate's electron distribution, geometry, and ability to participate in hydrophobic bonding. Linear free energy relationships have been useful in separating the contributions of these parameters. It now remains for us to discuss the implication of these findings for an understanding of the interaction between L-AAO and its substrates.

1. *Binding of substrates and substrate substitutes to the active site.* The binding of substrate molecules to the active site of L-AAO is a rapid process. This important observation by *Massey & Curti* [25] was recently confirmed by *Page & van Etten* [26], who found the rate of the anaerobic reaction to be $1.2 \times 10^{-6}\,\text{M}^{-1}\,\text{min}^{-1}$. The readings were based on the formation of *Beinert*'s compound, which displays a high absorbance at 540 nm [27]. The high reaction velocity suggests a structural set-up which permits a rapid and proper adaption of the substrate to the active site. To facilitate this process and to overcome the considerable entropy loss accompanying it, the following forces appear to be operating: a) ionic bonding between the carboxylate ion of the substrate or competitive inhibitor and a positively charged moiety in the enzyme; and b) hydrophobic bonding between the aromatic ring of the substrate or inhibitor and appropriate groups in L-AAO. The first proposal is consistent with the fact that a wide variety of anionic molecules, *e.g.* aromatic carboxylic and sulfonic acids [8] [9] [11] and phenylacetic acids [15], function as competitive inhibitors. The effect of ring-substitution on the ionization of the carboxyl group would not appear to account for the binding of the competitive inhibitors because presumably the dissociation of the carboxylic acids in our measuring system was practically complete. The fact that the phosphonic acid analogue of phenylalanine is fairly well oxidized in the presence of this enzyme[8]) again stresses the importance of an anionic residue for the binding.

The existence of hydrophobic forces in the formation of the enzyme-substrate complex has been previously postulated [4]. This conclusion is reinforced by the results presented here which reveal linear correlations between *Hansch*'s hydrophobic constant π and the K_i-values obtained for four sets of competitive inhibitors.

2. Hammett & Hansch *relationships as possible indicators for the orientation of substrates and substrate substitutes on the active site of* L-*AAO.* The rate of oxidation of phenylalanine by the L-AAO from *C. adamanteus* is accelerated by the withdrawal of electrons from the benzene ring of the substrate, indicating that the rate-limiting step is favored by low electron density at the reactive center of the substrate molecule. As we have shown, the meta-derivatives alone (set B) or together with selected para-derivatives (set D) yield a linear *Hammett* relationship of positive slope. On the other hand, *Radda* [28], in a stimulating publication, reported negative *Hammett* slopes for the oxidation of ring-substituted D, L-phenylglycines by the L-AAO of *Agkistrodon rhodastoma* and *C. adamanteus* venoms. The *Hammett* plot, based on rate measurements at a single substrate concentration, consisted of a biphasic line, the first portion having a positive slope ($\varrho = 1.8$), and the second exhibiting a negative slope ($\varrho \simeq -4.0$). This abrupt change in slope from positive to negative would indicate that the rate-limiting step shifted from one which is favored by low electron density

[8]) Unpublished data.

to one which is enhanced by high electron density. We then took the data of *Radda* and determined the regression lines separately for the meta- and para-derivatives. The analysis produced a straight line characterized by a high correlation coefficient ($\varrho = 1.7$, i = 0.03, r = 0.95) for the para-substituted phenylglycines (plus phenylglycine itself) and a line of negative slope for the meta-derivatives ($\varrho = -1.13$, i = -0.52, r = -0.96).

Similarly, *Hellerman et al.* [29] made use of a biphasic *Hammett* plot in their analysis of the degradation of D, L-phenylglycines by crystalline D-amino acid oxidase (D-AAO). Again applying our procedure, we found that the para-derivatives of the phenylglycines yielded a *Hammett* line of steep slope ($\varrho = 3.9$, r = 0.96). The corresponding data for the meta-substituted compounds were $\varrho = 0.2$ and r = 0.40.

A reasonable interpretation of these observations must take into account the fact that the interaction of L-AAO with its two substrates, amino acid and molecular oxygen, requires many individual steps [25] [26], involving the binding of substrates, electron transfer from amino acid to flavin to oxygen, and the release of products. Let us consider the set of reaction steps in which the substrate molecule participates directly, and let A and B be the steps which display the lowest rates (k_A, k_B) under the given conditions. We further assume that k_A is enhanced and k_B is depressed by low electron density. If a meta-substituent forces the substrate molecule into a geometric position within the enzyme-substrate complex such that a favorable situation is created for A and/or an unfavorable one for B, then $k_A > k_B$ and k_B will be rate-limiting. The slope of the *Hammett* relationship, therefore, will become negative. Para-substituents, on the other hand, may cause $k_A < k_B$ and produce a positive slope. Thus, with a minimum of assumptions, we have gained not only a simple interpretation for the data reported for L-AAO and substituted phenylglycines but also a new application of free energy relationships to the analysis of enzyme systems: they seem to permit us to detect shifts of rate-limiting steps and to project an internal enzymic event to the outside. Thus, it would appear that the procedure of separate analysis for the meta- and para-derivatives leads to a clearer picture of enzyme-substrate interaction than the somewhat arbitrary drawing of sharp-edged biphasic curves.

The existence of two *Hansch* plots, one for the meta- and one for the para-substituted competitive inhibitors, suggests an analogous interpretation of the binding of substrate substitutes to the active site. Again, molecular orientation as determined by the substituent seems to be the simplest explanation. Even fluorine (*van der Waals'* radius 1.35 Å) in the meta-position produces a shift relative to the unsubstituted and the para-substituted compounds. Unfortunately, substituents between the size of hydrogen (1.0 Å) and fluorine are not available to assess the minimum effective *van der Waals'* radius ($1.0 < r < 1.35$).

3. *Influence of Electron Distribution of Substrates on the Reactivity of L-AAO.* High electron density at the reaction center will either enhance or decrease the rates of certain steps of the enzyme-substrate interplay. The catalytic reaction for the flavoproteins L-AAO, D-amino acid oxidase, and lactate oxidase appears to be initiated by abstraction of a proton from the α-carbon atom [30]. According to the pioneering work of *Hemmerich* and his coworkers, covalent bonds are then formed between the C4a=N5 double bond and the substrate [31]. If and while the amino

acid is bound to the flavin nucleus, ring-substitution at the substrate molecule must affect the electron distribution in the flavin moiety and thus its reactivity. This hypothetical influence of ring-substitution should be more marked with ring-substituted phenylglycines than with phenylalanines, because the compound formed between amino acid oxidases and phenylglycines would contain only a single carbon atom between the benzene ring of the substrate and the 4a–5 area of the flavin moiety.

4. *Hybrid ϱ-Values.* A ϱ-value of 1.80 (*vide supra*) was computed for the degradation of para-substituted phenylglycines by L-AAO. Since the attenuation factor for the insertion of a methylene residue between the ring and the reaction center of the substrate is approximately 0.5 [32], the ϱ-value for the phenylalanine series should approximate 0.9. However, only one-third this value was found for the L-AAO of *C. adamanteus*. Even smaller slopes have been observed for the corresponding enzyme from *Vipera russelli* ($\varrho = 0.05$) and *Naia melanoleuca* ($\varrho = -0.24$), the latter values having been calculated from previously reported measurements [4]. The apparent discrepancy indicates that the ϱ-values obtained for the phenylalanines may be the resultant of several factors.

With the exception of the substituents of set E, both meta- and para-compounds can be represented by a single line of relatively small slope. Consequently, for these substrates, the topology of the enzyme-substrate complex could be such that more than one step becomes rate-limiting, with varying degrees of preponderance of one or the other, depending on the species from which the venom was collected. In this case, ϱ would appear not to be a direct indicator of the effect of ring-substitution on a single step, but rather, a hybrid with contributions from two or more steps, each characterized by a different slope. This analysis may serve as an example for the reaction described by equ. 7 [33].

$$\varrho\sigma = (\varrho_1 + \varrho_2 + \cdots)\,\sigma \tag{7}$$

5. *Displacement Effects.* A substituent effect different from that interpreted in terms of substrate orientation seems to influence the interaction between L-AAO and those phenylalanines which carry large residues in the para-position (set E). While substituent locus would appear to govern the orientation of the molecule, both locus and size are important for members of set E. Evidently, short distance forces prevent the establishment of proper enzyme-substrate complexes when the moiety in the para-position is larger than 1.8 Å (Fig. 2). Above this figure the deviation becomes a linear function of substituent size.

In non-enzymic systems, the short distance forces exhibited by ortho-substituents create situations similar to those occurring during the degradation of meta- and para-derivatives of phenylalanine. The 'bulkiness' of ortho-substituents can markedly affect the equilibria and reaction rates and thus induce notable deviations from the *Hammett* relationship [32].

Within set E, the K_m-values change proportionally to the maximal velocity (Fig. 3). *Slater*-plots of this type appear essentially in two situations: a) in enzyme reactions characterized by $k_{-1} \ll k_c$, and b) in systems affected by the putative occurrence of dystopic (non-productive) complexes. In view of the high rate in establishing the equilibrium between L-AAO and the enzyme-substrate complex as compared with the overall reaction rate (see Section 1 of Discussion), hypothesis b is favored over a. Thus, we conclude that large moieties in the para-position seem to force the phenylalanine molecule into a mode of binding which reduces the probability of an efficient electron transfer to oxygen.

The role of the postulated dystopic (non-productive) complexes remains to be discussed in terms of reaction sequence (1):

$$\frac{d[S]}{dt} = -\frac{k_c[E][S]}{[K_e - k_{-3}(K_e + k_{+3}/k_{+2})]/k_{+1}(K_d + k_{-3}/k_{+1}) + f[S]} \quad (8)$$

$$K_e = (k_{-1} + k_{+3} + k_c)/k_{+1}; \quad K_d = (k_{-2} + k_{-3})/k_{+2};$$

$$f = 1 + (K_e + k_{+3}/k_{+2})/(K_d + k_{-3}/k_{+1}).$$

If equ. 8 in its general form (case 1) were applicable to our data, no constancy of K_m/V for set E would be expected because terms relating to multiple complexes appear in K_m as well as in factor f. However, if we assume k_{+2}, $k_{-2} \to 0$ (case 2) or k_{+3}, $k_{-3} \to 0$ (case 3), the 'apparent' K_m reduces to $(k_{-1} + k_c)/k_{+1}$ and does not contain 'dystopic' terms, while f becomes either $1 + k_{-3}/k_{+3}$ (case 2) or $1 + K_e/K_d$ (case 3). The result is that both K_m and V are multiplied by factor f. At the present time, we cannot differentiate experimentally between these two possibilities, both of which are in agreement with our empirical data.

The data for set E reflect a relationship between *van der Waals'* radii and reaction rates which can be quantitatively interpreted in terms of the concept of substrate displacement. Moreover, it seems within reach of the methodology of the near future to test this concept by measuring k_{-3} and k_{+3} (equ. (8), case 2). Thus, our observations add another example to the list of established cases of quantitative relationships between the non-reactive part of a molecule and its overall reactivity or, more generally expressed, quantitative relationships involving structure and reactivity [32].

6. *Application of* Hammett *relationships to the analysis of other enzyme systems.* The phenomena described here are not unique to L-AAO. Similar differences in the reactivity between meta- and para-substituted substrates have been observed for the pyridoxal phosphate enzymes diamine oxidase (EC 1.4.3.6.) [34] and spermine oxidase (EC 1.5.3.3.) [35], and for the flavoprotein monoamine oxidase (EC 1.4.3.4.) [35]. At the present time, we are engaged in characterizing the reaction of various monoamine oxidases through the use of linear free energy relationships. Astonishingly large differences occur between closely related monoamine oxidases, and new and interesting substrates for the determination of this enzyme have been found[8]).

Our studies show that the range of application of linear free energy relationships to the analysis of the enzyme-substrate interaction is still expanding and that new aspects of the nature of a given enzyme can be revealed by this approach.

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263. Synthesis of Benzo-Homotriasterenedione[1])[2])

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(10. X. 74)

Zusammenfassung. Kondensation von 6,8-dimethoxycarbonyl-benzocycloheptan-7-on (4,5-Benzo-tropon-2,7-dicarbonsäure-dimethylester) (**4**)[4]) mit Aceton-dicarbonsäuredimethylester ergab 68% 1,5-Hydroxy, methoxy-2,4,6,8-tetramethoxycarbonyl-10,10a-benzo-9-oxa-10a-homo-adamant-10-en (**6**) und 17% des bekannten 3,7-Dihydroxy-4,8,2β,6β-tetramethoxycarbonyl-9,10-benzo-bicyclo[3.3.2]dec-3,7,9-triens (**7**). Hydrolyse und Decarboxylierung überführte den Tetraester **6** (wie schon für **7** beschrieben) in 9,10-Benzo-bicyclo[3.3.2]dec-9-en-3,7-dion (**9**). Aus den durch Spinsimulierung bestimmten ^{1}H-NMR.-Parametern wurde für **9** die Bevorzugung des Sessel-Sessel-Konformers **9cc** abgeleitet.

Bromierung des Diketons **9** mit vier Äquivalenten Brom lieferte 74% 1,5-Dihydroxy-2β,4β-6β,8β-tetrabrom-10,10a-benzo-9-oxa-10a-homoadamant-10-en (**10**). Die homoadamantoide Struktur von **10** war – wie schon früher bei ähnlichen Systemen – durch hydratisierende Cyclisierung des primär gebildeten Tetrabrom-diketons entstanden; sie offenbarte sich in der Abwesenheit

[1]) Some of these results have been communicated in a preliminary note [2], where a different numbering system and correspondingly different names have been used.