Ophidian L-Amino Acid Oxidase

THE NATURE OF THE ENZYME-SUBSTRATE COMPLEXES

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(Received 8 June 1964)

1. To investigate the kinetics of ophidian L-amino acid oxidase, V and K_m were determined for phenylalanines that were substituted in every ring position with groups of various size and reactivity, and for a few ring-substituted tryptophans and histidines. The venom of one representative from each of three major classes of poisonous snakes, *Naja melanoleuca*, *Vipera russelli* and *Crotalus adamanteus*, served as a source of the ophidian L-amino acid oxidase. Both crude and crystalline enzyme from the venom of *C. adamanteus* were tested. 2. The introduction of a benzene ring into glycine and alanine caused some increase of V and a very marked depression of K_m . 3. With the exception of fluorine, residues in the *ortho* position of phenylalanine led to a decrease of V . The rates induced by various substitutions follow the pattern: *meta* $\geq$ *para* $\geq$ *ortho*. Within the halogen series, the effects become more pronounced with increasing atomic number. 4. Ring substitution in heterocyclic amino acids also affected the V values markedly. For methyl-substituted tryptophans the pattern was: 5-methyl $\geq$ 6-methyl $\geq$ 4-methyl. In a few instances ring substitution accounts for a considerable elevation of V , as shown for β -quinol-4-ylalanine and its 6-methoxy derivative. 5. The kinetic constants appear to be unaffected by relatively high concentrations of the corresponding D-amino acids. 6. A general principle that permits a uniform interpretation of a vast body of information is suggested. It is based on the assumption that most substrates form not only eutopic but also dystopic complexes with the enzyme. The latter, in contrast with the former, do not permit the formation of reaction products. K values for eutopic and dystopic complexes are computed. Similar concepts have been presented to elucidate the action of α -chymotrypsin (Hein & Niemann, 1962) and of monoamine oxidase.

In studying the substrate pattern and kinetics of the monoamine oxidase reaction, we collected experimental results that could be uniformly interpreted by the assumption that two kinds of enzyme-substrate complexes occur. The eutopic complex formed between the active site and the substrate leads to chemical alterations of the latter, but the dystopic one does not (Zeller, 1963*a,b*). In the present paper experiments similar to those performed with monoamine oxidase are reported for the ophidian L-amino acid oxidase [L-amino acid-oxygen oxidoreductase (deaminating), EC 1.4.3.2]. [Our original suggestion (Zeller & Maritz, 1944, 1945) to characterize this enzyme by the Greek prefix 'ophio' ($\delta\phi\iota\varsigma$), pertaining to snakes, has been replaced by some authors by L-amino acid oxidase 'of snake venoms'. The latter designation is not only different in various languages,

but it is not strictly correct, because this oxidase is also found in tissues of snakes (Zeller, Iselin & Maritz, 1946). We propose to replace the prefix 'ophio' by the more correct term 'ophidian'.] Ophidian L-amino acid oxidase, discovered by Zeller & Maritz (1944, 1945) and found to be a flavoprotein (Zeller, 1948; Singer & Kearney, 1950), occurs in almost all snake venoms tested (Zeller, 1948). It was chosen for study because extensive, albeit incomplete, investigations of its substrate pattern (Zeller, 1948, 1951; Meister & Wellner, 1963) suggested the occurrence of dystopic complexes and because crystalline preparations were available.

Phenylalanines, substituted in every position of the benzene ring by groups of various sizes and reactivity, and a small number of derivatives of tryptophan and histidine, were tested as substrates.

The venoms of one representative of each of the three major classes of poisonous snakes, *Naja melanoleuca* (colubrid), *Crotalus adamanteus* (pit viper) and *Vipera russelli* (true viper), served as sources of the L-amino acid oxidase.

A preliminary account of part of this work has already been published (Zeller & Ramachander, 1964).

MATERIALS AND METHODS

Snake venoms. The venom of an African cobra, *N. melanoleuca*, was obtained from Dr Roulet and Dr Wansson (Zeller *et al.* 1946), that of an Indian viper, *V. russelli*, from the collection of the late C. H. Kellaway (Zeller, 1949), and the freeze-dried venom of the rattlesnake *C. adamanteus* from Sigma Chemical Co., St Louis, Mo., U.S.A. The last one was the starting material for the preparation of the crystalline enzyme by the method of Wellner & Meister (1960). It displayed the highest amino acid-oxidase activity of any venom observed in this Laboratory (see Table 4). Since the activity of the crystalline enzyme, suspended in water, dropped markedly within 10 days under the conditions of measurement adopted (see below), the rate determinations with this material were carried out within a few days of crystallization.

Amino acids. The three chlorophenylalanines were given by Dr H. Blaschko (University of Oxford) (Blaschko & Stiven, 1950), the *m*-methylphenylalanine and the sulphur-containing amino acids of Table 4 by Dr R. L. Colescott (Armour Pharmaceutical Co., Kankakee, Ill., U.S.A.) (Herr, Enkoji & Dailey, 1957; Colescott, Herr & Dailey, 1957), the amino acids listed in Table 3 by Sir Charles Harington (Medical Research Council, London, W.1) (Elliott, Fuller & Harington, 1948; Elliott & Harington, 1949), L-tryptophan, L-phenylalanine and L-histidine by Hoffmann-La Roche (Nutley, N.J., U.S.A.), and thienylalanine by Eli Lilly Research Laboratories (Indianapolis, Ind., U.S.A.). Bromo-, iodo-, and nitro-phenylalanines and *o*- and *p*-methylphenylalanine were synthesized in this Laboratory from the corresponding benzyl bromides, which were condensed with diethyl acetamidomalonate and isolated as hydrochlorides (Albertson & Archer, 1945; Snyder, Shekleton & Lewis, 1945; Burekhalter & Stephen, 1951). When the benzyl bromides were not available, they were synthesized from the analogous toluene derivatives by photochemical bromination (Brewster, 1918). The rest were commercial 'chromatographically pure' preparations. Unless otherwise specified, DL-amino acids were used.

Methods of measurement. For the manometric determina-

tion of oxygen consumption, all components were made up with 0.067 M-phosphate buffer, pH 7.2, except the venoms, which were dissolved in 0.2 ml. of 0.9% sodium chloride. The final phosphate buffer concentration in the total volume of 2 ml. was therefore 0.06 M. The assay system contained 40 µg. of crystalline catalase (Sigma Chemical Co.), added in 0.2 ml. of buffer solution. If not otherwise stated, the reaction was carried out in an atmosphere of oxygen at 38°. These conditions of measurement are referred to below as 'standard'. In some experiments, 0.05 M-tris or 0.05 M-collidine buffer was used. In most instances, ammonia was determined after 30 min. of incubation. The results, mostly omitted from the present paper, clearly indicated that the catalase sufficiently destroyed the hydrogen peroxide formed during the reaction (Zeller & Maritz, 1944, 1945), since the ratio equiv. of oxygen consumed/moles of ammonia formed (O/N ratio) was unity within experimental error.

For the determination of *V* and *K_m*, four to six concentrations of substrate were tested, ranging from 10 to 2 mM, unless low rates (Table 1) or low solubilities necessitated

Fig. 1. Degradation of phenylalanine and iodinated phenylalanines by *N. melanoleuca* venom. The rate of oxidation of L-phenylalanine (○) and its *ortho*- (●), *meta*- (▲) and *para*- (△) iodo derivatives were determined in the presence of 1 mg. of venom (standard conditions).

Table 1. Oxidative deamination of aliphatic amino acids and their phenyl derivatives by snake venoms

The reactions were carried out under standard conditions, with 2 mg. of venom (exception: 1 mg. for the phenylalanine experiment).

	Concn. of substrate	Venom	<i>V</i>	<i>K_m</i> (mm)	O/N ratio
Glycine	0.1–1.0 M	<i>C. adamanteus</i>	0.7	500	1.09
Phenylglycine	4–8 mm	<i>C. adamanteus</i>	1.4	2.2	0.84
Alanine	20–400 mm	<i>V. russelli</i>	2.7	83	0.91
L-Phenylalanine	5–20 mm	<i>V. russelli</i>	30	1.7	0.93

other concentrations. Q values, calculated from the initial portion of the curve of oxygen consumption, are defined as $\mu\text{equiv. of oxygen consumed/hr./mg. of dry venom}$. Fairly straight Lineweaver-Burk graphs were obtained (Fig. 1), except for those amino acids (L-leucine, β -pyrid-4-ylalanine) whose oxidation rate dropped at the highest concentrations (Zeller & Maritz, 1944; 1945; Marcus & Feeley, 1961). Since in these cases the Lineweaver-Burk relationship was linear at concentrations below 5 mM, this part of the curve was used for determination of K_m and V ; the latter is expressed in the same way as Q ($\mu\text{equiv. of oxygen/hr./mg. of dry venom}$).

RESULTS

Effect of the benzene ring on the oxidative deamination of amino acids. Introduction of a benzene ring

into the glycine and alanine molecules increased V and sharply decreased K_m (Table 1). Glycine is barely, if at all, attacked by the venoms of *Bitis gabonica* and *N. melanoleuca* (Zeller *et al.* 1946), but, by using large amounts of *C. adamanteus* venom and of substrate, oxidative deamination could be demonstrated (Table 1). Phenylglycine (6 mM) was also oxidized in the presence of 2 mg. of the venoms of *Bothrops jararac* (Q 0.8), *N. melanoleuca* (Q 0.04) and *Crotalus horridus* (Q 0.3). The claim by Meister & Wellner (1963) that phenylglycine 'is rapidly oxidized by L-amino acid oxidase' is hardly justified, because 25 mg. of *C. adamanteus* venom was required to ensure rapid oxidation of this substance.

Table 2. *Oxidation of amino acids in the presence of Vipera russelli venom*

The coefficient δ gives the ratio of maximal velocities (as determined under standard conditions and with 1 mg. of venom/vessel) for a given amino acid and L-leucine; the definition and calculation of the constants K_s and K_d are given in Discussion section. $V_{\text{Leu}} 74$ indicates the maximal velocity for L-leucine in $\mu\text{equiv. of oxygen/hr./mg. of dry venom}$.

Amino acid	100 δ	K_m (mM)	K_s (mM)	K_d (mM)
L-Leucine	100	10	10	
L-Phenylalanine	42	1.8	4.3	3.1
<i>o</i> -Fluorophenylalanine	53	3.7	7.0	7.9
<i>m</i> -Fluorophenylalanine	47	2.7	5.7	5.1
<i>p</i> -Fluorophenylalanine	38	2.0	5.3	3.2
<i>o</i> -Chlorophenylalanine	17	2.9	17	3.5
<i>m</i> -Chlorophenylalanine	28	1.3	4.6	1.8
<i>p</i> -Chlorophenylalanine	19	1.2	6.3	1.5
<i>o</i> -Bromophenylalanine	12	1.9	16	2.2
<i>m</i> -Bromophenylalanine	35	2.5	7.2	3.8
<i>p</i> -Bromophenylalanine	14	0.8	5.7	0.9
<i>o</i> -Iodophenylalanine	11	1.1	10	1.2
<i>m</i> -Iodophenylalanine	30	1.9	6.3	2.7
<i>p</i> -Iodophenylalanine	12	1.2	10	1.4
<i>o</i> -Nitrophenylalanine	9.5	0.4	4.0	0.4
<i>m</i> -Nitrophenylalanine	36	1.7	4.7	2.7
<i>p</i> -Nitrophenylalanine	38	1.7	4.5	2.8
<i>o</i> -Hydroxyphenylalanine	11	6.7	61	7.5
<i>m</i> -Hydroxyphenylalanine	31	1.6	5.2	2.3
<i>p</i> -Hydroxyphenylalanine	24	1.5	6.3	2.0
<i>o</i> -Methylphenylalanine	18	1.5	8.3	1.8
<i>m</i> -Methylphenylalanine	35	3.0	8.6	4.6
<i>p</i> -Methylphenylalanine	20	1.7	8.5	2.1
3,5-Dibromotyrosine	38	3.9	10.0	6.3
3,5-Di-iodotyrosine	33	2.4	7.3	3.6
<i>p</i> -Methylmercaptophenylalanine	15	1.6	11	1.9
<i>p</i> -Ethylmercaptophenylalanine	12	2.6	22	3.0
<i>p</i> -Methylsulphinylphenylalanine	19	2.2	12	2.7
<i>p</i> -Methylsulphonylphenylalanine	34	3.6	11	5.5
<i>p</i> -Methylsulphamylphenylalanine	20	2.6	13	3.2
L-Tryptophan	41	2.2	5.4	3.7
4-Methyltryptophan	7	1.9	27	2.0
5-Methyltryptophan	19	3.7	19	4.6
6-Methyltryptophan	18	0.50	2.8	0.6
L-Histidine	34	1.0	2.9	1.5
1-Methyl-L-histidine	19	0.55	2.9	0.7
3-Methyl-L-histidine	8	0.25	3.0	0.3

Degradation of phenylalanine and substituted phenylalanines. The values V 31 ± 2.3 and K_m 2.2 ± 0.4 (mm) were obtained for L-phenylalanine with *V. russelli* venom from ten determinations. Substitutions on the benzene ring led to marked changes in the constants (Fig. 1 and Tables 2-6). Relative reaction rates with *ortho*, *meta* and *para* isomers differed with amino acid oxidases of various venoms. In all but one case the magnitude

of the velocities was in the order: *meta* $\geq$ *para* $\geq$ *ortho*; the only exception was fluorophenylalanines. As the atomic number of the halogen residue was increased, the differences between the V values of the three isomers became larger.

It has been shown that a second aliphatic amino group (e.g. in ornithine) prevents oxidation of an otherwise suitable amino acid by snake venoms; acylation of this amino residue (e.g. as in

Table 3. *Oxidation of amino acids in collidine buffer and in the presence of Vipera russelli venom*

The rate measurements were carried out in 50 mm-collidine buffer, pH 7.2, with 0.2 mg. of venom/vessel, and in the absence of catalase. For DL-leucine, V was $56 \mu\text{equiv. of oxygen/hr./mg. of dry venom}$. See also the legend to Table 2.

Amino acid	100 δ	K_m (mm)	K_e (mm)	K_d (mm)
Leucine	100	5.0	5.0	
L-Phenylalanine	32	1.5	4.7	2.2
L-Phenylalanine*	34	1.3		
<i>p</i> -Nitrophenylalanine	41	1.7	4.2	2.9
<i>p</i> -Aminophenylalanine	38	2.9	7.6	4.7
<i>p</i> -Dimethylaminophenylalanine	25	1.4	5.6	1.9
<i>p</i> -Guanidinophenylalanine	36	14	39	22
<i>p</i> -Aminomethylphenylalanine	9	13	144	14
<i>p</i> -Guanidinomethylphenylalanine	20	4.0	20	5.0
β -Pyrid-4-ylalanine	72	16	22	57
β -Quinol-4-ylalanine	4	17	425	18
β -6-Methoxyquinol-4-ylalanine	30	8	27	11
β -Thien-2-ylalanine	38	0.8	2.1	1.3
β -Thianaphthen-3-ylalanine	72	2.5	3.5	0.9

* Phosphate buffer.

Table 4. *Oxidation of amino acids in the presence of Crotalus adamanteus venom*

See the legend to Table 2.

Amino acid	Sample	V_{Leu}	100 δ	K_m (mm)	K_e (mm)	K_d (mm)
L-Leucine	I	570	100	14	14	
L-Phenylalanine			19	1.7	9	2.1
<i>o</i> -Fluorophenylalanine			25	3.2	13	4.3
<i>m</i> -Fluorophenylalanine			17	1.7	10	2.1
<i>p</i> -Fluorophenylalanine			12	1.1	9	1.3
L-Leucine	II	400	100	13	13	
L-Phenylalanine			20	2.0	10	2.5
<i>o</i> -Iodophenylalanine			6	3.1	52	3.3
<i>m</i> -Iodophenylalanine			20	3.4	17	4.3
<i>p</i> -Iodophenylalanine			7	1.9	27	2.0
<i>o</i> -Hydroxyphenylalanine			8	3.2	40	3.5
<i>m</i> -Hydroxyphenylalanine			19	3.1	16	3.8
<i>p</i> -Hydroxyphenylalanine			9	2.4	27	2.6
L-Leucine	III	526	100	14	14	
L-Phenylalanine			19	1.7	9.0	2.1
L-Tryptophan			33	6.3	19	9.4
4-Methyltryptophan			10	4.4	44	4.9
5-Methyltryptophan			33	13	39	19
6-Methyltryptophan			10	2.1	21	2.3
5-Fluorotryptophan			13	2.0	15	2.3
5-Hydroxytryptophan			18	3.0	17	3.7
5-Benzoxxytryptophan			1.1	3.3	30	3.4

Table 5. *Oxidation of amino acids in the presence of purified Crotalus adamanteus L-amino acid oxidase*

Maximal velocity, V , is expressed with respect to the unit extinction of the venom at 275 m μ and to the actual amount of purified oxidase solution (V^*). Quotients q and q' give the ratio of maximal velocities computed for substituted and unsubstituted amino acids for purified and crude enzyme respectively. The q' values are calculated from results presented in Table 4. See also the legend to Table 2.

Amino acid	Preparation	V	V^*	100 q	100 q'	K_m (mM)
L-Leucine	I (recryst., fresh)	5000	100	100	100	14.3
L-Phenylalanine		950	19	19	19	1.8
L-Phenylalanine	II (recryst., fresh)*		19	100	100	
<i>o</i> -Fluorophenylalanine			25	132	139	2.5
<i>m</i> -Fluorophenylalanine			22	116	90	1.8
<i>p</i> -Fluorophenylalanine			16	84	63	1.2
L-Phenylalanine	III (cryst., 8 days old)	310	20	100		
<i>o</i> -Chlorophenylalanine			12	60		1.6
<i>m</i> -Chlorophenylalanine			16	80		1.2
<i>p</i> -Chlorophenylalanine			13	65		1.0
L-Phenylalanine	III (12 days old)	260	17	100		
<i>o</i> -Iodophenylalanine			8	47	32	3.5
<i>m</i> -Iodophenylalanine			24	141	105	3.6
<i>p</i> -Iodophenylalanine			9	53	37	1.5
L-Phenylalanine	IV (recryst., 5 days old)	580	15	100	100	
<i>o</i> -Hydroxyphenylalanine			6.8	45	42	2.5
<i>m</i> -Hydroxyphenylalanine			13	86	100	1.7
<i>p</i> -Hydroxyphenylalanine			10	67	48	1.3
L-Phenylalanine	III (cryst., 5 days old)	570	18			
L-Tryptophan			36	100	100	5.4
4-Methyltryptophan			9	25	30	3.8
5-Methyltryptophan			36	100	100	10.5
6-Methyltryptophan			9	25	30	1.4

* Extinction too low for reliable reading.

Table 6. *Oxidation of amino acids in the presence of Naja melanoleuca venom*

V_{Leu} 15 μ equiv. of oxygen/hr./mg. of dry venom. See also the legend to Table 2.

Amino acid	100 δ	K_m (mM)	K_e (mM)	K_d (mM)
L-Leucine	100	3.3	3.3	
L-Phenylalanine	91	1.7	1.9	19
<i>o</i> -Fluorophenylalanine	85	2.2	2.6	16
<i>m</i> -Fluorophenylalanine	81	2.2	2.7	12
<i>p</i> -Fluorophenylalanine	67	0.8	1.2	2.4
<i>o</i> -Iodophenylalanine	18	1.6	8.9	2.0
<i>m</i> -Iodophenylalanine	43	0.9	2.1	1.6
<i>p</i> -Iodophenylalanine	35	0.8	2.3	1.2
<i>o</i> -Nitrophenylalanine	34	1.0	2.9	1.5
<i>m</i> -Nitrophenylalanine	116	1.3	1.1	
<i>p</i> -Nitrophenylalanine	48	0.7	15	13
<i>o</i> -Hydroxyphenylalanine	20	4.6	23	5.8
<i>m</i> -Hydroxyphenylalanine	83	1.1	1.3	6.5
<i>p</i> -Hydroxyphenylalanine	82	1.8	2.2	10
L-Tryptophan	53	1.0	1.9	2.1
4-Methyltryptophan	48	7.1	15	14
5-Methyltryptophan	102	2.6	2.6	
6-Methyltryptophan	53	6.7	13	14

arginine) restores the substrate potential (Zeller & Maritz, 1944, 1945). An analogous pair is apparently *p*-aminoethyl- and *p*-guanidinomethyl-phenylalanine (Table 3). 3-Iodo-, 3,5-dibromo- and 3,5-diiodo-tyrosine yielded kinetic data similar to those found for the corresponding *meta*-monohalogen compounds. In exploratory experiments, the Q values for 3mm-3-fluorotyrosine and several venoms were determined, e.g. for *Naja naja* (Q 7) and for *V. russelli* (Q 19). These results suggest that the V values are close to those of unsubstituted phenylalanine. With cobra venom, a much higher rate was observed for *m*-hydroxyphenylalanine than for the *ortho* isomer (Blaschko, 1949), whereas only small differences, if any, were found for the three chlorophenylalanines (Blaschko & Stiven, 1950).

Degradation of heterocyclic amino acids. There are reports of high Q values for many heterocyclic amino acids containing simple or fused rings (Zeller *et al.* 1946; Zeller & Maritz, 1944, 1945; Meister, Levintow, Kingsley & Greenstein, 1951; Bender & Krebs, 1950; Frieden, Hsu & Dittmer, 1951). Histidine, in fact, served as the first substrate of ophidian L-amino acid oxidase. These experiments have been extended by determinations of K_m and V . Replacement of the benzene ring by a heterocyclic ring led to a marked increase of V in several instances (Tables 2–6). With histidine, maximal velocity was diminished by the introduction of a methyl group in the 1-position and even more so in the 3-position (the latter being analogous to the *ortho* position in a six-membered ring). The effect of ring-methylation of tryptophan on V values followed the pattern: 5-methyl $\geq$ 6-methyl $\geq$ 4-methyl. Substitution in the 5-position appears to be of critical importance (Tables 2, 4, 5 and 6), since residues capable of entering proton bonding depress V and since the ratio of maximal velocities for tryptophan and 5-methyltryptophan varies significantly from one venom to another.

Effects of D-amino acids on the degradation of L-amino acids. The maximum velocity of the oxidation of L-phenylalanine by a partially purified enzyme from *C. adamanteus* was unaffected by the presence of 20mm-D-phenylalanine. Similarly, it has been observed that a tenfold amount of D-leucine did not affect the oxidation of 10mm-L-leucine (Zeller, 1951).

DISCUSSION

The present results extend previous specificity studies of ophidian L-amino acid oxidases (Zeller *et al.* 1946; Zeller & Maritz, 1944, 1945; Zeller, 1948, 1951; Meister & Wellner, 1963; Bender & Krebs, 1950; Frieden *et al.* 1951; Greenstein, Birnbaum & Otey, 1953; Suzuki & Iwanaga, 1960), by adding 40 amino acids to the number of known

substrates, and by giving the constants of the Michaelis-Menten relationship. Previous reports only list rates measured at arbitrary substrate concentrations. According to our results, V and K_m are strongly influenced by the locus and the size of the ring substitution. Maximum velocity, for example, is diminished by the introduction of any residue (except fluorine) into the *ortho* position of phenylalanine, the 3-position of the imidazole nucleus of histidine and the 4-position of the indole nucleus of tryptophan. Since the rate depression caused by *ortho*-halogens grows larger as the atomic number increases, the importance of the size of the substituent on V is apparent. On the other hand, there is no obvious relation between the chemical nature of substituents and the maximal velocities. Substituents that have opposite effects on electron distribution, either by induction or resonance, or that display various degrees of polarization etc., exert essentially the same influence. Our observations therefore cannot be ascribed to the intramolecular transmission of forces from the substituent to the α -carbon (D-C-B-A in Scheme 1), the site of the primary enzymic event (A-a).

Since all but the smallest *ortho* substituents decrease V (without necessarily increasing K_m), steric effects might be considered responsible for this phenomenon. Waite & Niemann (1962) suggest such an explanation for the results of their experiments on the hydrolysis of a series of acylated L-valine esters by α -chymotrypsin, and they conclude that large residues attached to the substrate provide considerable steric shielding of the reactive carbonyl group. This type of steric hindrance is precluded as a general interpretive principle for ophidian L-amino acid oxidase, because it does not account for the depression of V in *para*-substituted phenylalanines or in 6-methyltryptophan.

Ring substitutions (e.g. residue D in Scheme 1) could conceivably induce conformational changes in the peptide chains, and these in turn could prevent a correct fit between enzyme and substrate (Scheme 1b). This would result in a slow-down of the enzyme-substrate interaction. This conclusion

Scheme 1. Linear model of the enzyme-substrate complex. The capital letters stand for the various parts of the substrate, the small ones for the corresponding parts of the enzyme to which the former are bound (Zeller, Fouts, Carbon, Lazanas & Voegtli, 1956). The primary enzymic event is supposed to take place between A and a. See the text for further discussion.

is not supported by the observation that in some instances we find almost identical V values for substrates of entirely different shape and size, e.g. for isopentylamine and benzylamine (monoamine oxidase) (Zeller, 1963b), or for leucine, phenylalanine and 5-methyltryptophan (Table 6).

Whereas there are still other interpretations of our experimental results, we stress one that appears to be particularly simple. It is based on the observation that certain substances, although they appear to fulfil all the requirements to be excellent substrates for a given enzyme and although they are efficiently bound to the active site, do not undergo chemical changes (dystopic complex). Such an example is given by *o*-iodobenzylamine, which is not attacked by ox-liver monoamine oxidase (Zeller, 1963a,b); ox-plasma amine oxidase, however, catalyses the oxidation of this substance. Similarly, 'non-productive' complexes have been postulated for α -chymotrypsin (Hein & Niemann, 1962). We assume that the rate of an enzyme reaction, as indicated by V , depends not only on the previously recognized reaction parameters, but also on the relative production of eutopic and dystopic complexes (eqns. 1 and 2). By taking the existence of dystopic complexes into consideration one can

derive new relationships between reaction rates and substrate concentrations (eqns. 3–5); subscripts e and d refer to the terms dystopic and eutopic, and V and K_m are the conventional symbols. The terms δ , K_e and K_d of eqn. (3) are defined by eqns. (4)–(6).

$$-d[S]/dt = \delta V_e[S]/(\delta K_e + [S]) = V[S]/(K_m + [S]) \quad (3)$$

$$1/\delta = 1 + \sum_{i=1}^n K_e/K_d \quad (4)$$

$$K_e = (k_{-1} + k_{+2})/k_{+1} \quad (5)$$

$$K_{d,i} = k_{-3,i}/k_{+3,i} \quad (6)$$

According to eqn. (3), two substrates that respond identically to the action of a given enzyme, except for the degree of formation of a dystopic complex, should yield parallel lines in the Lineweaver–Burk plot. In testing groups of closely related substrates, parallelism or near parallelism has been often observed for L-amino acid oxidase (Fig. 1) and for monoamine oxidase (Zeller, 1963b). Identical slopes for the Lineweaver–Burk lines, however, are not a necessary consequence of our hypothesis, because the binding energies for the various com-

plexes are not necessarily the same. To obtain a numerical value for δ , and thus for K_e and K_d , a numerical value for V_e has to be assumed. Since leucine led to the highest maximal velocities (Tables 2–6), and since the Q values for leucine were among the highest reported for many amino acids and for the venoms of eight additional species (Zeller *et al.* 1946; Zeller & Maritz, 1944, 1945; Frieden *et al.* 1951; Greenstein *et al.* 1953), we have tentatively set $V_e = V_{\text{Leu}}$. One apparent exception was the rate of degradation, Q , of phenylalanine in the presence of *Naja haje* venom, which was observed to be higher than that of leucine (Zeller *et al.* 1946). By repeating and extending our previous determinations with the same venom preparation, we obtained: for L-leucine, V 10 and K_m 5.6; for L-phenylalanine, V 10 and K_m 3.3. The results are very similar to those given for *N. melanoleuca*, another African cobra (Table 6). In view of the possibility that maximal velocities ($V' \geq V$) higher than those given for leucine may yet be found, the values of K_e and K_d listed in Tables 2–5 must be regarded as lower limits for the true values; accordingly we set $K'_e \geq K_e$ and $K'_d \leq K_d$. By assuming that only one dystopic complex is formed and by using eqns. (7) and (8), one arrives at the numerical values for K_e and K_d

$$\delta = V/V_e = K_m/K_e; K_e = K_m/\delta \quad (7)$$

$$K_d = \delta K_e/(1 - \delta) \quad (8)$$

listed in Tables 2, 3, 4 and 6. These constants permit a better insight into the interaction between enzyme and substrate than the conventional data. As one example, we mention the correlation between V and the atomic radii of halogens, as seen in the results obtained with *Daboia* venom (Table 2). According to our hypothesis, the fall in V with increasing atom radius may not be due to a decrease in the rate of breakdown of the complex, but rather due to an increase in the binding energy between the substrate and the dystopic position.

Our results also throw some light on the process of the formation of enzyme–substrate complexes. Previous results obtained with D-amino acids and L-tyrosine ethyl ester (Zeller & Maritz, 1944, 1945) and with *N*-methylated L-amino acids (Zeller, Maritz & Iselin, 1945) seem to indicate that, although the carboxylic and amino groups do not contribute much to the binding energy, they are essential for the preliminary orientation of the substrate molecule to the binding site. By analogy with monoamine oxidase (Zeller, 1955, 1956) one may think of coulombic forces bringing the substrate within range of short-distance forces. As the results with the pairs glycine–phenylglycine and alanine–phenylalanine (Table 1) and the fairly uniform K_d values for phenylalanine with various

venoms suggest (Tables 2-6), the benzene ring contributes a great deal to the binding energy between amino acid oxidase and substrate. These results suggest the presence of an aromatic ring in the active site to which the aromatic ring of the substrate may be attached by London forces and overlapping π -orbitals; as the work of Némethy & Scheraga (1962) on the interaction of apolar side chains or proteins indicates, strong hydrophobic forces do arise between the two aromatic rings or between a benzene ring and the isobutyl residue of leucine.

This study was supported by a U.S. Public Health Service Research Career Award, K6-GM-14,200, and by U.S. Public Health Service Research Grants GM 05927-05 from the Division of General Medical Sciences and NB01348-07 from the National Institute of Neurological Diseases and Blindness. Paul F. Palmberg assisted in the experimental work.

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