A Reinvestigation of the Substrate Specificity of Pig Kidney Diamine Oxidase

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1. The substrate specificity of pig kidney diamine oxidase was reinvestigated with a purer enzyme preparation than has previously been used for this purpose.
2. All substrates were extensively purified before use, and methods of preparation or sources are given, together with R_F values.
3. The substrate specificity determined differed somewhat from that reported by previous workers and, in addition, the behaviour of several compounds not previously used as substrates is described.
4. A model for enzyme–substrate interaction embodying these observations is formulated. It is suggested that a negatively charged substrate-binding group is situated at 6.0–9.0 Å from the oxidizing site. The binding and oxidizing sites are separated by a hydrophobic or methylene-binding site.

The enzyme known as pig kidney diamine oxidase or histaminase [diamine–oxygen oxidoreductase (deaminating), EC 1.4.3.6] was first reported by McHenry & Gavin (1932), and the substrate specificity was extensively investigated by Zeller, Fouts, Carbon, Lazanas & Voegtli (1956), Fouts, Blanksma, Carbon & Zeller (1957), Blaschko & Chrusciel (1959) and Zeller (1963). The enzyme has since been obtained in a homogeneous form (Mondovi *et al.* 1967) and crystallized (Yamada, Kumagai, Kawasaki, Matsui & Ogata, 1967).

It is now known to have a molecular weight of 185 000, to contain Cu^{2+} ions, which do not change in valency as oxidation proceeds, and to contain pyridoxal phosphate, which is involved in substrate binding (Kumagai, Nagate, Yamada & Fukami, 1969). The enzyme catalyses the oxidation of diamines thus (Tabor, 1951):

and also weakly catalyses the oxidation of some monoamines. The mode of enzyme–substrate interaction is usually depicted as in Scheme 1, where an oxidizing site binds one amino group as a cation,

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and where the other amino group is uncharged and acts as a nucleophile towards an electron-accepting site, which is suggested to be a carbonyl group.

However, since this scheme does not fully explain the substrate pattern, we now describe a new model for substrate binding, based on a reinvestigation of the substrate specificity, which more adequately accounts for the known behaviour of this enzyme.

MATERIALS AND METHODS

Table 1 lists all amines and amino acids used in the present study together with sources.

Scheme 1. Interaction between a diamine and diamine oxidase (after Zeller, 1956). One amino group is uncharged and acts as a nucleophile towards an electron-accepting site (EA, which is suggested to be a carbonyl group). The amino group to be oxidized is protonated and is attracted to a proton-accepting group (PA^-), which is negatively charged. Zeller (1951) originally suggested that both sites were negatively charged.

Table 1. *Oxidation of amines and amino acids by pig kidney diamine oxidase*

The rate of oxidation was determined manometrically with a final substrate concentration of 3.3 mM, and is presented as a percentage of the oxidation of cadaverine. A comparison with literature values is given where possible, taken from Zeller (1963), Zeller *et al.* (1956), Fouts *et al.* (1957) and Blaschko & Chrusciel (1959). Here the conditions were varied and precise values not always given. + Denotes a small but definite O₂ uptake. Inter-nitrogen-atom distances in diamines were measured by using Prentice-Hall framework molecular models. All compounds used were purified until only one spot was observed on t.l.c. and *R_F* values refer to one of three systems: *A*, propan-2-ol-aq. NH₃ (sp.gr. 0.88)-water (10:1:1, by vol.); *B*, methanol-aq. NH₃ (sp.gr. 0.88)-water (10:2:1, by vol.); *C*, acetic acid-water (10:1, v/v). Sources of compounds were as follows: *A*, Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A.; BDH, BDH Chemicals Ltd., Poole, Dorset, U.K.; KK, K & K Rare Chemicals, Plainview, N.Y., U.S.A.; KL, Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.; P, prepared by us (see the Materials and Methods section); RNE, R. N. Emanuel Ltd., Alpertown, Middx., U.K.; S, Sigma (London) Chemical Co., London S.W.6, U.K.

Number	Compound	Source	<i>R_F</i> value and solvent	Rate of oxidation as a percentage of cadaverine oxidation		Inter-nitrogen-atom distance for diamines (extreme values possible in parentheses) (Å)
				Literature value	Present work	
(I)	1,2-Diaminoethane dihydrochloride	BDH	0.25, <i>B</i>	5	4	4.1 (2.7-4.1)
(II)	1,3-Diaminopropane	BDH	0.19, <i>B</i>	25	8	5.5 (3.1-5.5)
(III)	1,4-Diaminobutane (putrescine)	KL	0.08, <i>B</i>	95	96	6.5 (3.0-6.5)
(IV)	1,5-Diaminopentane dihydrochloride (cadaverine dihydrochloride)	KL	0.06, <i>B</i>	100	100	7.0 (3.0-8.0)
(V)	1,6-Diaminohexane	BDH	0.10, <i>B</i>	55	41	8.1 (3.0-9.2)
(VI)	1,7-Diaminoheptane	KL	0.10, <i>B</i>	55	43	9.0 (3.0-10.5)
(VII)	1,8-Diaminooctane	KL	0.14, <i>B</i>	15	16	10.5 (3.0-12.0)
(VIII)	<i>cis</i> -1,4-Bis(aminomethyl)cyclohexane	A	0.14, <i>B</i>	—	0	8.0 (4.3-8.0)
(IX)	<i>o</i> -Bis(aminomethyl)benzene (o-xylylenediamine)	P	0.33, <i>A</i>	+	0	6.3 (3.0-6.3)
(X)	<i>m</i> -Bis(aminomethyl)benzene (<i>m</i> -xylylenediamine)	A	0.36, <i>A</i>	0	+	7.2 (5.1-7.7)
(XI)	<i>p</i> -Bis(aminomethyl)benzene (<i>p</i> -xylylenediamine)	P	0.36, <i>A</i>	0	48	8.0 (7.3-8.1)
(XII)	<i>o</i> -Bis-(2-aminoethyl)benzene	P	0.20, <i>A</i>	—	0	7.9 (3.0-8.1)
(XIII)	<i>m</i> -Bis-(2-aminoethyl)benzene	P	0.27, <i>A</i>	—	7	9.6 (3.0-10.5)
(XIV)	<i>p</i> -Bis-(2-aminoethyl)benzene	P	0.35, <i>A</i>	—	+	10.4 (7.3-11.3)
(XV)	3-Dimethylaminoprop-1-ylamine	KL	0.22, <i>B</i>	—	5	5.5 (3.1-5.5)
(XVI)	4-Dimethylaminobut-1-ylamine	KK	0.13, <i>B</i>	50	32	6.5 (3.0-6.5)
(XVII)	5-Dimethylaminopent-1-ylamine	KK	0.14, <i>B</i>	55	28	7.0 (3.0-8.0)
(XVIII)	6-Dimethylaminohex-1-ylamine	KK	0.19, <i>B</i>	—	8	8.1 (3.0-9.2)
(XIX)	<i>o</i> -Bis-(<i>N</i> -methylaminomethyl)benzene	P	0.51, <i>A</i>	—	0	6.3 (3.0-6.3)
(XX)	<i>m</i> -Bis-(<i>N</i> -methylaminomethyl)benzene	P	0.52, <i>A</i>	—	0	7.2 (5.1-7.7)
(XXI)	<i>p</i> -Bis-(<i>N</i> -methylaminomethyl)benzene	P	0.34, <i>A</i>	—	0	8.0 (7.3-8.1)
(XXII)	<i>o</i> -Bis-(<i>NN</i> -dimethylaminomethyl)benzene	P	0.75, <i>A</i>	—	0	6.3 (3.0-6.3)
(XXIII)	<i>m</i> -Bis-(<i>NN</i> -dimethylaminomethyl)benzene	P	0.44, <i>A</i>	—	0	7.2 (5.1-7.7)
(XXIV)	<i>p</i> -Bis-(<i>NN</i> -dimethylaminomethyl)benzene	P	0.89, <i>A</i>	—	0	8.0 (7.3-8.1)
(XXV)	1-Aminopropane	BDH	0.58, <i>C</i>	25	7	
(XXVI)	1-Aminobutane	BDH	0.63, <i>C</i>	10	9	
(XXVII)	1-Aminopentane	BDH	0.68, <i>C</i>	+	+	
(XXVIII)	Benzylamine	BDH	0.71, <i>B</i>	+	0	
(XXIX)	Phenethylamine	BDH	0.65, <i>B</i>	+	0	
(XXX)	3-Phenylprop-1-ylamine	P	0.50, <i>B</i>	—	0	
(XXXI)	4-Phenylbut-1-ylamine	KK	0.54, <i>B</i>	—	0	
(XXXII)	Histamine dihydrochloride	S	0.52, <i>B</i>	50	51	6.0 (3.5-6.4)
(XXXIII)	Tryptamine hydrochloride	KL	0.74, <i>A</i>	—	0	
(XXXIV)	Tyramine hydrochloride	KL	0.68, <i>B</i>	+	0	
(XXXV)	Noradrenaline bitartrate	KL	0.58, <i>C</i>	—	0	
(XXXVI)	Mescaline sulphate	KL	0.65, <i>B</i>	10	0	
(XXXVII)	3-Aminopropionic acid	KL	0.41, <i>B</i>	—	0	
(XXXVIII)	4-Aminobutyric acid	KL	0.47, <i>B</i>	—	0	
(XXXIX)	5-Aminovaleric acid	RNE	0.43, <i>B</i>	—	0	
(XL)	6-Aminohexanoic acid (6-aminocaproic acid)	KL	0.49, <i>B</i>	—	0	
(XLI)	<i>o</i> -Aminomethylbenzoic acid	P	0.55, <i>B</i>	—	0	
(XLII)	<i>m</i> -Aminomethylbenzoic acid	P	0.65, <i>B</i>	—	0	
(XLIII)	<i>p</i> -Aminomethylbenzoic acid	P	0.71, <i>B</i>	—	0	

Table 2. *Summary of the preparation of pig kidney diamine oxidase*

Step	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (unit/mg)	Yield (%)	Purification
1. Homogenate	1650	249000	110	0.0004	100	1
2. Thermal denaturation	1440	26900	73.8	0.0027	67	6
3. Ammonium sulphate fractionation I	210	4700	71.1	0.0151	65	34
4. Column chromatography on hydroxyapatite	125	874	59.3	0.0679	53	154
5. Ammonium sulphate fractionation II	6	268	38.4	0.143	35	323
6. Column chromatography on DEAE-cellulose	6	68	18.5	0.274	17	634

Twice-recrystallized catalase was obtained from Sigma (London) Chemical Co., London S.W.6, U.K., DEAE-cellulose (type DE32) was from W. and R. Balston Ltd., Maidstone, Kent, U.K., DEAE-Sephadex and Sephadex G-200 were from Pharmacia (G.B.) Ltd., London W.13, U.K., semicarbazide hydrochloride, hydrazine hydrochloride, hydroxyammonium sulphate, sodium diethyldithiocarbamate, methylammonium chloride, trimethylammonium chloride and tetramethylammonium chloride were purchased from BDH Chemicals Ltd., Poole, Dorset, U.K. Adsorbent used for t.l.c. was Kieselgel GF 254 (E. Merck A.-G., Darmstadt, Germany).

Measurement of substrate oxidation. Oxygen uptake was measured by conventional Warburg manometry at 37°C with air as the gas phase. A final volume of 3 ml of 0.1M-potassium phosphate buffer, pH 7.0, was used, containing enzyme (approx. 0.1 unit), catalase (46 µg) and a final substrate concentration of 3.3 mM.

One unit of diamine oxidase is defined as the amount of enzyme that catalyses the transformation of 1 µmol of substrate/min under the above standard conditions, and is equivalent to an oxygen uptake of 11.2 µl/min.

Protein determination. Protein was estimated spectrophotometrically from the E_{280} value in 0.1M-potassium phosphate buffer, pH 7.0. An extinction coefficient ($E_{1\text{cm}}^{0.1\%}$) of 1.63 was assumed (Yamada *et al.* 1967).

Enzyme preparation. All steps except 1 and 2 were carried out at 0–4°C and all buffers were saturated with toluene. The enzyme preparation is summarized in Table 2.

Step 1. Fresh pig kidney cortex (1.5 kg) was homogenized in a top-drive macerator (Townson and Mercer) in 0.1M-potassium phosphate buffer, pH 7.0 (1200 ml), and centrifuged.

Step 2. The supernatant was heated at 60°C for 10 min, cooled and centrifuged.

Step 3. The supernatant was fractionated with ammonium sulphate. The precipitate formed between 1.2M- and 2.5M-ammonium sulphate was collected and dialysed against 0.1M-potassium phosphate buffer, pH 7.0.

Step 4. The dialysis residue was applied to a hydroxyapatite column (4.5 cm × 14.5 cm) and washed with 0.1M-potassium phosphate buffer, pH 7.0, containing 0.2M-ammonium sulphate. The enzyme was eluted by raising the ammonium sulphate concentration to 0.5M.

Step 5. Active fractions were combined, and the precipitate formed between 1.4M- and 2.4M-ammonium

sulphate was collected, dialysed against 0.05M-potassium phosphate buffer, pH 7.2, and concentrated to a final volume of 6 ml.

Step 6. The concentrate was dialysed (0.05M-potassium phosphate buffer, pH 7.2) and applied to a DEAE-cellulose column (2.5 cm × 21 cm). Gradient elution was carried out by using a Varigrad device containing 0.05M-potassium phosphate buffer, pH 7.2 (150 ml), in compartment 1, 0.2M-potassium phosphate buffer, pH 7.2 (150 ml), in compartment 2 and 0.5M-potassium phosphate buffer, pH 7.2 (150 ml), in the third compartment. The enzyme was precipitated from the pooled active fractions with ammonium sulphate (2.4M), dissolved in 0.1M-potassium phosphate buffer, pH 7.0 (6 ml), dialysed against the same buffer, centrifuged and stored at 4°C. After storage for 6 months, the activity was half the original value.

Measurement of inter-nitrogen distances in diamines. An approximate measure of the distance between the centres of the two nitrogen atoms in diamine cations was obtained by using molecular models (Prentice-Hall framework molecular models). Table 1 lists estimations made from the most probable conformations adopted in solution, allowing for repulsion between the centres of positive charge and minimizing eclipsed conformations of methylene chains. The values quoted are obviously more accurate for aromatic and short-chain aliphatic diamines than for longer-chain aliphatic diamines.

Preparation of hydrochlorides. All compounds employed as potential substrates in this study, except compounds (XXXV)–(XLIII) inclusive, were used as hydrochlorides. These were prepared by cautious addition of a slight excess of conc. HCl to an ethanolic solution of the amine, followed by evaporation to dryness under reduced pressure. The residues were then repeatedly recrystallized until seen to be homogeneous on t.l.c. and pure as judged by melting points.

Melting points. A Kofler block was used and the values are uncorrected.

Spectroscopic measurements. All compounds prepared were identified unambiguously by i.r. and n.m.r. spectroscopy. In addition, new compounds were further identified by mass spectrometry and elemental analysis of derivatives.

Preparation of amines and amino acids. *o*-Bis(amino-methyl)benzene (IX). *o*-Bis(phthalimidomethyl)benzene

(3g), prepared by the method of Strassmann (1888), was refluxed in a solution of 100% hydrazine hydrate (2ml), ethanol (25ml) and chlorobenzene for 3 h. *o*-Bis(amino-methyl)benzene was then extracted with acid, and obtained as a white crystalline hydrochloride (1.14g, 72% yield).

p-Bis(aminomethyl)benzene (XI). This was prepared by a similar method from *p*-bis(bromomethyl)benzene, and obtained as a *hydrochloride*, m.p. > 360°C (Found: C, 46.0; H, 6.8; N, 13.4; $C_8H_{14}Cl_2N_2$ requires C, 46.0; H, 6.7; N, 13.4%).

o-Bis-(2-aminoethyl)benzene (XII). *o*-Bis(cyano-methyl)benzene (3.9g) was reduced with H_2 at atmospheric pressure and at 60°C in acetic anhydride (75ml) by using Adams platinum catalyst (0.4g). The cool filtered solution was evaporated to give *o*-bis(acetamidomethyl)benzene (5.87g, 95% yield). A recrystallized sample of this (2.1g) was hydrolysed with refluxing methanolic KOH (25% KOH in methanol) to give *o*-bis-(2-aminoethyl)benzene (1.24g, 87% yield), b.p. 88–90°C/0.15 mmHg.

m-Bis-(2-aminoethyl)benzene (XIII). This was prepared by the method of Ruggli & Prijs (1945) and had b.p. 104–108°C/0.2 mmHg.

p-Bis-(2-aminoethyl)benzene (XIV). This was prepared by the method of Ruggli & Prijs (1945) and had b.p. 104–106°C/0.15 mmHg.

o-Bis-(*N*-methylaminomethyl)benzene (XIX). A mixture of *NN*'-dimethylhydrazine dihydrochloride (10g), dimethylformamide (20ml) and anhydrous K_2CO_3 (20g) was agitated at 100°C until no further CO_2 was released. *o*-Bis(bromomethyl)benzene (10g) was added and the solution refluxed for 20 min. After filtration and acidification, the solution was evaporated to dryness under reduced pressure and the basified residue extracted with ether to give a clear oil, which on distillation gave 1,2-dimethyl-4,5-benzo-1,2,3,6-tetrahydropyridazine, b.p. 64–66°C/0.05 mmHg (3.7g, 30.2% yield). The n.m.r. spectrum in $CdCl_2$, with tetramethylsilane as the internal reference, showed six *N*-methyl protons (7.6 τ , singlet), four ring-methylene protons (6.15 τ , singlet) and four aromatic protons (2.85 τ , multiplet). Junge & Staab (1967) quote b.p. 63–65°C/0.001 mmHg and ring-methylene protons (6.19 τ , singlet) for this compound.

A sample of this distillate (3.12g) was reduced with zinc powder (30g) and 5M-HCl (150ml) for 2 h at 100°C to give *o*-bis-(*N*-methylaminomethyl)benzene [3.2g, quantitative yield; the n.m.r. spectrum in trifluorodeuteroacetic acid, with tetramethylsilane as internal standard, showed six *N*-methyl protons at 6.96 τ (triplet), four methylene protons at 5.36 τ (triplet) and four aromatic protons at 2.32 τ (singlet)]. The material was purified by conversion into the *dipicrate*, m.p. 182–185°C (Found: C, 42.5; H, 3.9; N, 18.4; $C_{22}H_{22}N_8O_{14}$ requires C, 42.4; H, 3.6; N, 18%), which was recrystallized and reconverted into the free base (overall yield 80%).

The *dihydrochloride* of *o*-bis-(*N*-methylaminomethyl)benzene had m.p. 263–264°C in a sealed tube (Found: C, 50.8; H, 7.6; Cl, 30.2; N, 11.7; $C_{10}H_{18}Cl_2N$ requires C, 50.6; H, 8.0; Cl, 29.9; N, 11.8%).

m-Bis-(*N*-methylaminomethyl)benzene (XX). This was prepared by the method of Sander & Burmeister (1962), and had b.p. 120–122°C/4.5 mmHg. The *hydrochloride* was hygroscopic, and had m.p. 132–134°C (sealed

tube) (Found: C, 50.7; H, 8.0; Cl, 29.7; N, 11.7; $C_{10}H_{18}Cl_2N_2$ requires C, 50.6; H, 8.0; Cl, 29.9; N, 11.8%).

p-Bis-(*N*-methylaminomethyl)benzene (XXI). A mixture of *p*-bis(bromomethyl)benzene (10g) and ethanolic 30% methylamine (100ml) was maintained at 70°C for 4 h in an autoclave. After removal of solvent, basic material was extracted with acid and distilled to give *p*-bis-(*N*-methylaminomethyl)benzene, b.p. 110–112°C/4.5 mmHg (1.3g, 22% yield). The *hydrochloride* had m.p. 293–295°C in a sealed tube (Found: C, 50.6; H, 7.6; Cl, 30.5; N, 11.9; $C_{10}H_{18}Cl_2N_2$ requires C, 50.6; H, 8.0; Cl, 29.9; N, 11.8%).

o-Bis-(*NN*-dimethylaminomethyl)benzene (XXII). The method of von Braun & Cahn (1924) for preparing this compound gave, in our hands, low yields of impure material, and also proved hazardous. A simple modification is now described. *o*-Bis-(*NN*-dimethyl-*N*-phenethylaminomethyl)benzene dibromide (18g), prepared as described by von Braun & Cahn (1924), was suspended in 4M-NaOH (200ml). After being stirred for 45 min at 100°C the cooled solution was partitioned between 3M-HCl and chloroform. The acid layer was then basified, and extracted with chloroform to yield a clear oil, which on distillation gave *o*-bis-(*NN*-dimethylaminomethyl)benzene, b.p. 64–68°C/0.2 mmHg (5.8g, 95% yield). The *hydrochloride* was hygroscopic and had m.p. 216–218°C (sealed tube) (Found: C, 54.5; H, 8.4; Cl, 26.9; N, 10.3; $C_{12}H_{22}Cl_2N_2$ requires C, 54.5; H, 8.4; Cl, 26.8; N, 10.6%).

m-Bis-(*NN*-dimethylaminomethyl)benzene (XXIII). This was prepared as described for compound (XXI) from *m*-bis(bromomethyl)benzene and dimethylamine in 66% yield, and had b.p. 54–56°C/0.1 mmHg. The n.m.r. spectrum in carbon tetrachloride, with tetramethylsilane as internal reference, showed twelve *N*-methyl protons (7.84 τ , singlet), four methylene protons (6.64 τ , singlet) and four aromatic protons (2.87 τ , multiplet). The *dipicrate* had m.p. 205–206°C (Found: C, 44.2; H, 4.1; N, 17.5; $C_{24}H_{26}N_8O_{14}$ requires C, 44.3; H, 4.0; N, 17.2%).

The *hydrochloride* was hygroscopic, and had m.p. 150–152°C (sealed tube) (Found: C, 54.1; H, 8.3; Cl, 26.7; N, 10.6; $C_{12}H_{22}Cl_2N_2$ requires C, 54.4; H, 8.4; Cl, 26.8; N, 10.6%).

p-Bis-(*NN*-dimethylaminomethyl)benzene (XXIV). This was prepared by the method of Fusco, Chiavarelli, Palazzo & Bovet (1948), and had b.p. 94–96°C/0.4 mmHg.

3-Phenylprop-1-ylamine (XXX). Cinnamaldoxime was reduced by the method of Ruggli & Prijs (1945) to give phenylpropylamine, b.p. 62–65°C/1.4 mmHg (42.5% yield).

o-Aminomethylbenzoic acid (XLI). *o*-Phthalimidomethylbenzoic acid (8.4g), prepared by the method of Bornstein, Drummond & Bedell (1958), was refluxed in ethanol (100ml) and hydrazine hydrate (2ml) for 3 h. The solid, filtered off from the reaction mixture, was extracted with acid to give *o*-aminomethylbenzoic acid hydrochloride (2.5g, 66% yield). Neutralization with ammonia gave *o*-aminomethylbenzoic acid.

m-Aminomethylbenzoic acid (XLII). *m*-Cyanobenzylphthalimide, prepared by the method of Reinglass (1891), was hydrolysed by refluxing with acetic acid (34%, v/v) and conc. HCl (66%, v/v) for 3 days. Phthalic acid was extracted with ether, and *m*-aminomethylbenzoic acid was isolated as the hydrochloride.

p-Aminomethylbenzoic acid (XLIII). This was prepared by the method of Albert & Magrath (1944).

RESULTS

Table 1 lists the R_F values for purified amines and amino acids, along with an approximate evaluation of the inter-nitrogen distance for diamines. The rates of oxidation of these potential substrates are presented as a percentage of the rate with cadaverine, the best-known substrate, and a comparison is given with values reported by previous workers, where this is available.

There is good agreement with literature values, except for compound (XI), which we found to be a good substrate, but which was reported by Blaschko & Chrusciel (1959) to be unoxidized, and compounds (XVI) and (XVII), which were not oxidized as well as is reported (Zeller *et al.* 1956). Of the monoamines tested, only propylamine and butylamine were appreciably oxidized under the conditions used, there being no measurable oxygen uptake with tryptamine, tyramine, mescaline, nor-adrenaline and the phenylalkylamines (XXVIII)–(XXXI). The reported oxidation of some of these compounds may be due to the use of crude enzyme preparations.

It has been reported that *C*-terminal hydroxylation of alkylamines improves their substrate potency (Fouts *et al.* 1957), but we found that this effect is only small. 4-Aminobutan-1-ol, 5-aminopentan-1-ol and 6-aminoheptan-1-ol were demonstrably oxidized only if the substrate concentration was raised tenfold. The same behaviour was shown by the series of alkylamines from hexylamine to dodecylamine, which showed a slow oxygen uptake only in concentrated solutions, and here the insolubility of the amines becomes a limiting factor, making accurate measurement difficult.

Table 1 also shows that none of the ω -amino acids tested was oxidized at all. Also, it is apparent that the best substrates have inter-nitrogen distances of approx. 6.0–9.0 Å.

Table 3 shows the effective inhibition of the enzyme by reagents specific for carbonyl groups and the copper-chelating agent diethyldithiocarbamate, and also the marked inhibitory power of compounds (IX), (X) and methylamine. The amino acids, ammonium chloride, trimethylammonium chloride, tetramethylammonium chloride and other methylated amines tested were only weak inhibitors.

Table 3. *Inhibition of pig kidney diamine oxidase*

The substrate was 1,5-diaminopentane (3.3 mM) except in experiments marked *, where *p*-bis(aminomethyl)-benzene (3.3 mM) was used.

Inhibitor	Concn. (mM)	Oxygen uptake (μ l/min)		Relative rate (%)
		No inhibitor	With inhibitor	
KCN	1.0	1.04	0.11	11
KCN*	1.0	0.55	0	0
Semicarbazide	0.01	1.17	0	0
Semicarbazide*	0.01	0.55	0	0
Hydrazine	0.01	1.04	0	0
Hydrazine*	0.01	0.55	0	0
Hydroxylamine	0.01	1.04	0	0
Hydroxylamine*	0.01	0.55	0	0
Diethyldithiocarbamate	1.0	1.04	0.25	24
(IX)	3.3	0.87	0.06	7
(X)	3.3	0.87	0.03	3
(XX)	3.3	1.09	1.04	95
(XXI)	3.3	1.09	0.90	83
(XXIII)	3.3	1.09	1.04	95
(XXIV)	3.3	1.09	0.90	83
NH ₄ Cl	3.3	0.66	0.63	95
CH ₃ ·NH ₂ Cl	3.3	0.66	0.36	55
(CH ₃) ₃ NHCl	3.3	0.66	0.62	95
(CH ₃) ₄ NCl	3.3	0.66	0.63	95
(XXXVII)	3.3	1.23	1.12	91
(XXXVIII)	3.3	1.23	1.09	89
(XXXIX)	3.3	1.23	1.05	85
(XL)	3.3	1.23	1.11	90
(XLI)	3.3	1.23	1.17	95
(XLII)	3.3	1.23	1.08	88
(XLIII)	3.3	1.23	1.10	89

DISCUSSION

Any attempt to rationalize the mode of action of this enzyme must satisfy certain general observations, listed below, which will then explain the behaviour of any compound as a substrate or inhibitor.

Observation 1. Only primary amines with free α -hydrogen atoms are oxidized. Substitution on the nitrogen atom or methylene group causes loss of activity.

Observation 2. Diamines are oxidized more readily than monoamines, and, in the best substrates, the amino groups are separated by a four- or five-membered methylene chain, i.e. with an inter-nitrogen separation of 6.0–9.0 Å.

Observation 3. The enzyme is completely inhibited by carbonyl-group-specific reagents and copper-chelating agents, and is strongly inhibited by alkylguanidines, alkylguanidines and some primary amines. Secondary and tertiary amines and ω -amino acids are weak inhibitors.

Observation 4. Oxidation of alkylamines is slow and can be demonstrated only by using concentrated solutions of enzyme and substrate. There is a definite increase in rate of oxidation from methylamine to butylamine and a decrease from pentylamine onward. Substitution of the terminal methyl group with a hydroxy substituent produces slightly better substrates with butylamine, pentylamine and hexylamine.

Observation 5. Aliphatic ω -amino acids and *o*-, *m*- and *p*-aminoethylbenzoic acids are not oxidized.

Observation 6. Dimethylaminoalkylamines and aminoalkylguanidines, e.g. agmatine, are oxidized at a rate about half that for the corresponding diamines.

Observation 7. Arylalkylamines, from benzylamine to phenylbutylamine, tyramine, noradrenaline, mescaline and tryptamine, are very poor substrates. Histamine is readily oxidized.

Observation 8. *p*-Bis(aminomethyl)benzene is a good substrate, but the *meta* and *ortho* isomers, though not oxidized, are potent inhibitors.

Scheme 2 is suggested as a model for substrate binding that satisfies these observations.

The binding site (BS) is best regarded as a formal negative charge on the enzyme surface, rather than a carbonyl group as suggested by Zeller (1963), since at the optimum pH for oxidation (pH 6.4–7.8) diamino substrates with pK_a values of approx. 10.0 would be present as dications and could not therefore act as nucleophiles. The negative-charge hypothesis would then explain observation 2 directly and observation 6 indirectly, since the dication from dimethylaminoalkylamines or aminoalkylguanidines could align in two possible ways, only one of them leading to oxidation. The guani-

Scheme 2. Proposed scheme for enzyme-substrate interaction. BS is a negatively charged substrate-binding site. Hy is a substrate-binding or amino-binding site. OS represents the site for oxidation where Schiff-base formation between the amino group and a pyridoxal phosphate derivative is envisaged. ::::: denotes strong electrostatic attraction between BS and the second basic group of the substrate, which is positively charged. illustrates the weak attraction between Hy and the hydrophobic region of the substrate.

dino portion of an aminoalkylguanidine would certainly be completely protonated at pH 7.0, whereas the pyridine portion of 2-(2-aminoethyl)pyridine (pK_a 3.8) would not be. This explains why, though agmatine is a good substrate, being bound to BS by attraction between the negative charge and the positive charge of the substrate, 4- and 2-(2-aminoethyl)pyridine are not oxidized, being present as monoamines with a nucleophilic pyridine group. Blaschko, Friedman, Hawes & Nilsson (1959) suggest that histamine is oxidized as the dication, since the pH optimum for oxidation was 6.3. Observation 5 is most easily explained if the binding site is negatively charged, since here the mutual repulsion between the similarly charged enzyme and substrate carboxylate anion would be considerable, completely preventing oxidation. Also, this hypothesis would explain why, after increasing as the chain length is extended from methylamine to butylamine (observation 4) the rate falls off, since further portions of the alkyl chain suffer repulsion from the adjacent negative charge. This repulsion would be lessened by *C*-terminal hydroxylation.

The oxidizing site (OS) is presumed to contain the cofactors. It seems likely that Schiff-base formation occurs here, followed by oxidation, and this would explain observations 1 and 3 (cf. Kumagai *et al.* 1969). Primary amines, e.g. methylamine and 2-(2-aminoethyl)pyridine, could then be acting as inhibitors by forming Schiff bases which, for some reason, are not oxidized. Groups may be present in the enzyme that assist in the formation of an imine between the pyridoxal phosphate and the primary amino groups, e.g. a basic group could remove a proton from the $-NH_3^+$ group, or an acidic group could make the aldehyde more reactive. The

combination may, however, be adequately facilitated by the proximity into which the groups are placed as the substrate is bound. We also suggest that the binding and oxidizing sites are separated by a distance of 6.0–9.0 Å, since this corresponds to the inter-nitrogen separation in the best diamine substrates (observation 2).

The hydrophobic binding site (Hy) is envisaged as binding methylene groups more strongly than aromatic rings (observations 4 and 7). Thus the increase in substrate potency from methylamine to butylamine is due to progressively stronger enzyme–substrate interaction, whereas, when phenylalkylamine side chains are sufficiently long to interact adequately, the aromatic ring then suffers repulsion from the negative charge at the amino-group-binding site (BS).

Several diamines (compounds VIII, IX, X and XII) with inter-nitrogen distances favourable for oxidation are not oxidized, and this information can also be used to illuminate the nature of the hydrophobic binding site. The case of the xylylene-diamines (observation 8) is particularly important here. Oxidation of the *para* compound (XI) shows that there can be little steric hindrance to aromatic rings. Non-oxidation of the *ortho* and *meta* compounds, coupled with their considerable inhibitory power, is difficult to explain, since here the inter-nitrogen distance is favourable for oxidation. An explanation is offered in Scheme 3. Here it is suggested that, after interacting with the amino-group-binding site strongly and the hydrophobic binding site weakly, it is only in the *para* compound that the other amino group is juxtaposed to the oxidizing site, allowing eutopic complex-formation and oxidation to occur (cf. Zeller, 1963). With the *meta* and *ortho* isomers, dystopic complex-formation

would result from substrate binding, since the second amino group would be held away from the oxidizing site, allowing inhibition but not oxidation. A similar explanation can be given for compound (XII). Compound (VIII) probably binds to the amino-group-binding and hydrophobic binding sites in such a way that the free amino group is held away from the oxidizing site, as a result of the bulky cyclohexane ring preventing the substrate from lying close to the enzyme surface.

Other evidence can be presented to support the presence of a hydrophobic binding site, e.g. the low rate of oxidation when the methylene chain of diamines is hydroxylated (Zeller *et al.* 1956; Macholán, Rozprimová & Sedláčková, 1967) would be due to the resultant decrease in hydrophobic character. The increased rate of oxidation when the methylene chain is methylated, as in 1,3-diaminobutane and 1,3-diaminopentane (Zeller, 1963), would be caused by an increase in the hydrophobic character of the methylene chain of the substrate. Also, the failure of trimethylammonium chloride and tetramethylammonium chloride to inhibit the enzyme by ion-pair formation with the amino-binding site, suggests that, for successful inhibition, it is necessary for binding to occur also at the hydrophobic binding site, as with the alkylguanidines and alkyldiguanidines. Finally, long-chain diamines can, of course, accommodate to the requisite inter-nitrogen distance. The poor rate of oxidation here could be due to decreased methylene binding in the looped conformation adopted to form a suitable enzyme–substrate complex, in addition to the decreased population of appropriately spaced dications.

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Scheme 3. Plan of the interaction between bis(amino-methyl)benzenes and diamine oxidase. A eutopic enzyme–substrate complex, and thus oxidation, can only occur with the *para* isomer, where the amino groups are adjacent to the binding site (BS) and oxidizing site (OS), and where the aromatic ring is interacting with the hydrophobic binding site (Hy), promoting satisfactory alignment of substrate and enzyme active site. Despite a favourable inter-nitrogen distance, the *meta* isomer can only form a dystopic complex leading to inhibition but no oxidation.

REFERENCES

- Albert, A. & Magrath, D. (1944). *J. chem. Soc.* p. 678.
- Blaschko, H. & Chrusciel, T. L. (1959). *Br. J. Pharmac. Chemother.* **14**, 364.
- Blaschko, H., Friedman, P. J., Hawes, P. & Nilsson, K. (1959). *J. Physiol., Lond.*, **145**, 384.
- Bornstein, J., Drummond, P. E. & Bedell, S. F. (1958). In *Organic Syntheses*, vol. 38, p. 81. London and New York: John Wiley and Sons Inc.; London: Chapman and Hall.
- Fouts, J. R., Blanksma, L. A., Carbon, J. A. & Zeller, E. A. (1957). *J. biol. Chem.* **225**, 1025.
- Fusco, R., Chiavarelli, S., Palazzo, G. & Bovet, D. (1948). *Gazz. chim. ital.* **78**, 951.
- Junge, B. & Staab, H. A. (1967). *Tetrahedron Lett.* no. 8, p. 709.

- Kumagai, H., Nagate, T., Yamada, H. & Fukami, H. (1969). *Biochim. biophys. Acta*, **185**, 242.
- McHenry, E. W. & Gavin, G. (1932). *Biochem. J.* **26**, 1365.
- Macholán, L., Rozprimová, L. & Sedláčková, E. (1967). *Biochim. biophys. Acta*, **136**, 258.
- Mondovi, B., Rottilio, G., Costa, M. T., Finazzi-Agro, A., Chiancone, E., Hensen, R. E. & Beinert, H. (1967). *J. biol. Chem.* **242**, 1160.
- Reinglass, P. (1891). *Ber. dt. chem. Ges.* **24**, 2416.
- Ruggli, P. & Prijs, B. (1945). *Helv. chim. Acta*, **28**, 674.
- Sander, M. & Burmeister, D. (1962). *Chem. Ber.* **95**, 964.
- Strassman, H. (1888). *Ber. dt. chem. Ges.* **21**, 576.
- Tabor, H. (1951). *J. biol. Chem.* **188**, 125.
- von Braun, J. & Cahn, R. S. (1924). *Justus Liebigs Annln Chem.* **436**, 262.
- Yamada, H., Kumagai, H., Kawasaki, H., Matsui, H. & Ogata, K. (1967). *Biochem. biophys. Res. Commun.* **29**, 723.
- Zeller, E. A. (1951). In *The Enzymes*, vol. 2, part 1, p. 536. Ed. by Sumner, J. B. & Myrbäck, K. New York: Academic Press Inc.
- Zeller, E. A. (1956). In *Ciba Found. Symp. Histamine*, p. 339. Ed. by Wolstenholme, G. E. W. & O'Connor, C. M. London: J. and A. Churchill Ltd.
- Zeller, E. A. (1963). In *The Enzymes*, vol. 8, p. 314. Ed. by Sumner, J. B. & Myrbäck, K. New York: Academic Press Inc.
- Zeller, E. A., Fouts, J. R., Carbon, J. A., Lazanas, J. C. & Voegtli, W. (1956). *Helv. chim. Acta*, **39**, 1632.