Synthesis and Oxidation of Aminoalkyl-onium Compounds by Pig Kidney Diamine Oxidase

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1. The preparation of a series of compounds derived from diamines by replacing one amino group by a dimethylsulphonium, isothiuronium, trimethylammonium, *NN'*-dimethylimidazolium or *N*-methylpyridinium species is described. 2. The behaviour of these compounds as substrates of pig kidney diamine oxidase is reported. All but the trimethylammonium compounds proved to be substrates. 3. Many of these compounds showed potent inhibition at high substrate concentration and this was studied. 4. On the basis of these and other observations a scheme for enzyme-substrate interaction is suggested.

Pig kidney diamine oxidase (EC 1.4.3.6) oxidizes diamines more readily than monoamines and several explanations of this have been offered.

Zeller (1951) originally suggested that the enzyme was di-negatively charged and attracted a dicationic species of substrate. Subsequently (Zeller, Fouts, Carbon, Lazanas & Voegtli, 1956; Fouts, Blanksma, Carbon & Zeller, 1957) it was found that hydroxy amines were good substrates whereas mono-quaternary derivatives of putrescine and cadaverine were not, and Zeller *et al.* (1956) suggested that diamino substrates acted in a monocationic form as nucleophiles towards an electrophilic residue on the enzyme. The problem was reinvestigated (Bardsley, Hill & Loble, 1970) and with some modifications the former alternative was supported, although non-oxidation of the mono-quaternary compounds was not explained.

It was then considered important to obtain compounds in which one amino group in a diamine was replaced by a group bearing a formal positive charge and to test these compounds as substrates of diamine oxidase. This paper reports the synthesis of such onium congeners of diamines in which the charged group is a dimethylsulphonium, isothiuronium, trimethylammonium, *NN'*-dimethylimidazolium or *N*-methylpyridinium species and the behaviour as substrates is compared with that of parent diamines and other related compounds. The structures for these compounds are illustrated in Table 1.

On the basis of this information and a study of

inhibition by high substrate concentration a revised model for substrate binding is presented.

MATERIALS AND METHODS

Materials

The sources for commercially available chemicals were as reported previously (Bardsley *et al.* 1970). In addition, 3-hydroxyprop-1-ylamine and laboratory reagents were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. 4-Hydroxybut-1-ylamine, 5-hydroxypent-1-ylamine, 6-hydroxyhex-1-ylamine, 4-cyanobut-1-yl bromide, 5-cyanopent-1-yl bromide, 2-aminomethylpyridine, 3-aminomethylpyridine, 4-aminomethylpyridine and 2-(2'-aminoethyl)pyridine were obtained from R. N. Emanuel Ltd., Alpert, Middx., U.K., and *p*-tolunitrile and 1,5-dibromopentane were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.

Protein determination

The biuret method of Itzhaki & Gill (1964) was used to determine protein.

Measurement of substrate oxidation

O₂ uptake was measured by conventional Warburg manometry at 37°C, with air as the gaseous phase. A final volume of 1 ml of buffer (0.1 M-potassium phosphate, pH 7.0) was used, containing enzyme (0.1 unit), catalase (0.2 unit) and appropriate substrate concentrations. The enzyme unit has been defined by Bardsley *et al.* (1970).

Table 1. *Aminoalkyl-onium compounds used in this study*

R is $-\text{[CH}_2\text{]}_n-\text{NH}_2$ (where $n = 1, 2, 3, 4, 5$ or 6) or $-\text{CH}_2-\text{C}_6\text{H}_4-\text{CH}_2-\text{NH}_2$	
Compound	Formula
(I) Dimethylsulphonium compounds	$\text{R}-\overset{+}{\text{S}}(\text{CH}_3)_2$
(II) Isothiuronium compounds	$\text{R}-\overset{+}{\text{S}}=\text{C}(\text{NH}_2)_2$
(III) Trimethylammonium compounds	$\text{R}-\overset{+}{\text{N}}(\text{CH}_3)_3$
(IV) <i>N</i> -Methylpyridinium compounds	(also the 3- and 4-substituted isomers)
(V) 1,3-Dimethyl-4(5)-(2'-aminoethyl)imidazolium bromide (the only <i>NN'</i> -dimethylimidazolium compound)

Enzyme preparation

All operations except steps 1 and 2 were carried out at 4°C. Potassium phosphate buffer, pH 7.0, was used. The preparation is summarized in Table 2.

Step 1. Fresh pig kidney cortices (3.24 kg) were homogenized in 0.1 M buffer (1750 ml) and then centrifuged.

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

Step 3. The supernatant from step 2 was fractionated with $(\text{NH}_4)_2\text{SO}_4$ and the precipitate between 1.2 and 2.5 M collected and dialysed against 0.1 M buffer.

Step 4. The dialysis residue was applied to a hydroxyapatite column (4.5 cm × 21 cm). This was then washed with 0.1 M buffer containing 0.2 M $(\text{NH}_4)_2\text{SO}_4$ and the enzyme eluted by raising the $(\text{NH}_4)_2\text{SO}_4$ concentration to 0.5 M. The active fractions were pooled and the enzyme was collected by raising the $(\text{NH}_4)_2\text{SO}_4$ concentration to 2.5 M.

Step 5. After centrifugation and dialysis against 0.02 M buffer, the dialysis residue was then applied to a DEAE-Sephadex A-50 column (2.5 cm × 33 cm), which was developed by gradient elution. The Varigrad device had 0.02 M buffer (200 ml) in the first compartment, 0.2 M buffer (200 ml) in the second and 0.5 M (200 ml) in the third.

Step 6. The pooled active fractions (47 ml) were then applied to a Sephadex G-200 column (2.5 cm × 92.5 cm) and this was developed with 0.1 M buffer. The active fractions (80 ml) were pooled and concentrated against sucrose. After dialysis the final volume was 13 ml.

Graphical methods

Determination of V and K_m . Plots of v against v/s were used (Eadie, 1952). The slope gives K_m directly, but

where substrate inhibition was pronounced values for K_m were either estimated from the negative slope at low substrate concentration or could not be obtained. V was not obtained by extrapolation; instead, the maximum rate actually obtained was observed over a wide range of substrate concentrations. Where substrate inhibition was weak this was approximately the same as $V_{\max}$.

Determination of K'_s . Inhibition by high substrate concentration has been treated by Haldane (1930) and Dixon & Webb (1964, p. 75). According to this theory the enzyme-substrate complex can react with a further molecule of substrate to give an enzyme-bisubstrate complex, which does not lead to further reaction. The maximum observable velocity, V , in such cases bears a relationship to the theoretical maximum velocity in the absence of substrate inhibition, $V_{\max}$, given by:

$$V_{\max}/V = 1 + (K_s/s) + (s/K'_s)$$

where s is the substrate concentration, K_s is the dissociation constant for the enzyme-substrate complex (leading to further reaction) and K'_s is the dissociation constant for the enzyme-bisubstrate complex (causing inhibition). Webb (1963) has shown that a similar expression can be derived without necessarily making the same assumptions (e.g. inactive enzyme-substrate complexes can also be proposed). He suggests plots of v against ps to show substrate inhibition as symmetrical bell-shaped curves, whereas Dixon & Webb (1964, p. 75) recommend plots of $1/V$ against s , which gives K'_s by extrapolation. The variation of K'_s with chain length in a series of related compounds is shown in Fig. 3.

Determination of K_i . Inhibition by bis-onium compounds is shown in Fig. 4 by using double-reciprocal plots. According to Dixon & Webb (1964, p. 327) the intercept

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

Step	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification
1. Homogenate	1900	79800	334	0.00419	100	1
2. Thermal denaturation	800	17440	166	0.00952	49.7	2
3. (NH ₄) ₂ SO ₄ fractionation	163	9050	153	0.01691	45.8	4
4. Column chromatography on hydroxyapatite	27	256	134	0.523	40.1	125
5. Column chromatography on DEAE-Sephadex	47.5	205	124	0.605	37.1	144
6. Column chromatography on Sephadex G-200	13	120	118	0.984	35.3	234

on the base line gives $1/K_m(1+i/K_i)$ for purely competitive inhibition, where i is inhibitor concentration and K_i the enzyme-inhibitor complex dissociation constant.

General methods for the preparation of substrates

General methods used in the preparation of substrates and intermediates are now summarized.

(a) *Preparation of aminoalkyl bromides.* *p*-Aminomethylbenzyl bromide, 3-aminoprop-1-yl bromide, 4-aminobut-1-yl bromide, 5-aminopent-1-yl bromide and 6-aminohex-1-yl bromide were required as hydrobromides for the present study. A simple modification of a method described for 2-aminoethyl bromide (Cortese, 1938) was used to prepare these from the corresponding hydroxyalkylamines. The hydroxy amine (0.05 mol) was refluxed in 60% (v/v) HBr (15 ml) for 1 h. After 10 ml had been distilled off at atmospheric pressure a further quantity of 60% HBr (15 ml) was added and slowly distilled off. The residue was evaporated to dryness under reduced pressure and recrystallized from ethanol-ethyl acetate (yield 75–85%).

(b) *Preparation of S-aminoalkylisothiuronium compounds.* The method of Doherty, Shapira & Burnett (1957) was used to prepare the 3-aminopropyl and 4-aminobutyl compounds by treating the aminoalkyl bromide with thiourea in boiling propan-2-ol. It was extended to prepare the 5-aminopentyl and 6-aminohexyl derivatives and also *S*-(*p*-aminomethylbenzyl)isothiuronium bromide hydrobromide.

(c) *Preparation of aminoalkyldimethylsulphonium bromides.* These were prepared by reaction of the aminoalkyl bromide with dimethyl sulphide. The aminoalkyl bromide (0.025 mol) was dissolved in a mixture of methanol (10 ml) and dimethylsulphide (20 ml) in a stoppered flask and left at room temperature until t.l.c. investigation showed reaction to be complete (3–6 weeks). (It was sometimes necessary to add methanol as the reaction progressed to prevent an oily phase from separating.) The residue was evaporated to dryness and recrystallized from ethanol (yield 70–80%).

(d) *Protection of amino groups from reacting with methyl iodide by prior conversion into phthalimido groups.* Method 1. Equimolar quantities of free amine and phthalic anhydride were thoroughly mixed and heated at 140–160°C until no more water was evolved (5–10 min). The residue was then cooled and recrystallized from methanol (yield 80–90%).

Method 2. Equimolar quantities of amine hydrobromide and phthalic anhydride were thoroughly mixed and heated at 200–220°C until no further HBr was evolved (30–45 min). The cooled residue was then recrystallized from aqueous methanol (yield 75–80%).

(e) *Reaction of phthaloyl derivatives with methyl iodide.* The phthalimido compound (0.01 mol) was dissolved in a mixture of methanol (20 ml) and methyl iodide (5 ml) and refluxed until t.l.c. investigation showed reaction to be complete (1–4 h). The solution was cooled and the deposited solid was filtered off and recrystallized from methanol or aqueous ethanol (yield 90–95%).

(f) *Hydrolysis of phthalimido protecting group from methylated derivatives.* The phthalimido methiodide (0.01 mol) was refluxed in 48% HBr (20 ml) until a copious

precipitate of phthalic acid had formed (2–4 h). The solution was then cooled, water added (20 ml) and phthalic acid filtered off. The filtrate was extracted with ether (2 × 10 ml), evaporated to dryness under reduced pressure and the residue recrystallized from aqueous ethanol (yield 85–95%).

The structures proposed for all new compounds were confirmed by i.r. spectroscopy and, in addition, all new compounds used as substrates gave satisfactory n.m.r. spectra.

Melting points were determined on a Kofler block and are uncorrected.

Preparation of S-aminoalkylisothiuronium bromides

S-(3-Aminoprop-1-yl)- and S-(4-aminobut-1-yl)-isothiuronium bromide were prepared as hydrobromides by the method of Doherty *et al.* (1957). General methods (a) and (b) gave S-(5-aminopent-1-yl)isothiuronium bromide hydrobromide, m.p. 144–145°C (Found: C, 21.9; H, 5.0; Br, 48.9; N, 13.4; C₆H₁₇Br₂N₃S requires C, 22.3; H, 5.3; Br, 49.5; N, 13.0%), and S-(6-aminohex-1-yl)isothiuronium bromide hydrobromide, which crystallized out of the reaction mixture as a di-thiourea complex, m.p. 133–135°C, unchanged by several recrystallizations from propan-2-ol [Found: C, 22.1; H, 5.4; Br, 32.7; N, 20.1; S, 19.6; C₇H₁₉Br₂N₃S₂(CH₄N₂S) requires C, 22.1; H, 5.6; Br, 32.7; N, 20.0; S, 19.7%]. Treatment with picric acid, fractional crystallization of the picrate and regeneration of the hydrobromide gave S-(6-aminohex-1-yl)isothiuronium bromide hydrobromide, m.p. 96–98°C (Found: C, 24.8; H, 5.5; Br, 48.0; N, 12.9; C₇H₁₉Br₂N₃S requires C, 24.9; H, 5.7; Br, 47.4; N, 12.5%).

Aminoalkyldimethylsulphonium bromides

General methods (a) and (c) gave hydrobromides of these compounds that were in all cases hygroscopic and tended to decompose slowly on standing at room temperature. 3-Aminoprop-1-yl dimethylsulphonium bromide hydrobromide had m.p. 87–88°C (Found: C, 21.8; H, 5.2; Br, 56.2; N, 5.0; C₅H₁₃Br₂NS requires C, 21.4; H, 5.4; Br, 56.8; N, 5.0%). 4-Aminobut-1-yl dimethylsulphonium bromide hydrobromide had m.p. 111–112°C (Found: C, 24.3; H, 5.6; Br, 54.5; N, 4.9; C₆H₁₇Br₂NS requires C, 24.4; H, 5.8; Br, 54.2; N, 4.7%). 5-Aminopent-1-yl dimethylsulphonium bromide hydrobromide had m.p. 107–108°C (Found: C, 27.3; H, 6.1; Br, 52.0; N, 4.5; C₇H₁₉Br₂NS requires C, 27.9; H, 6.2; Br, 51.7; N, 4.5%). 6-Aminohex-1-yl dimethylsulphonium bromide hydrobromide had m.p. 143–144°C (Found: C, 29.5; H, 6.3; Br, 49.3; N, 4.7; C₈H₂₁Br₂NS requires C, 29.7; H, 6.5; Br, 49.5; N, 4.4%).

Aminoalkyltrimethylammonium bromides

Reaction of 3-bromoprop-1-ylamine hydrobromide with phthalic anhydride [general method (d) (method 2)] gave 3-phthalimidoprop-1-yl bromide, m.p. 69–70°C (Found: C, 49.4; H, 3.9; Br, 30.2; N, 5.7; C₁₁H₁₀BrNO requires C, 49.3; H, 3.8; Br, 29.8; N, 5.2%). 3-Phthalimidoprop-1-yl bromide (5 g) was dissolved in ethanol (40 ml) and a 33% (v/v) solution of trimethylamine in ethanol added (20 ml).

After the solution had been kept in a stoppered flask for 3 days at room temperature it was evaporated to dryness and the residue recrystallized from propan-2-ol to give 3-phthalimidoprop-1-yltrimethylammonium bromide, m.p. 198–199°C (yield 70%) (Found: C, 51.2; H, 6.0; Br, 24.3; N, 9.0; C₁₄H₁₉BrN₂O₂ requires C, 51.4; H, 5.9; Br, 24.4; N, 8.6%). Hydrolysis (general method f) gave 3-amino-prop-1-yltrimethylammonium bromide hydrobromide, an extremely hygroscopic compound, m.p. 167–168°C (ethanol-anhydrous ether) (Found: C, 25.4; H, 6.4; Br, 56.7; N, 10.2; C₆H₁₈Br₂N₂ requires C, 25.9; H, 6.5; Br, 57.5; N, 10.1%). 4-Aminobut-1-yltrimethylammonium bromide hydrobromide had m.p. 191–192°C (Found: C, 28.9; H, 7.1; Br, 55.1; N, 9.8; C₇H₂₀Br₂N₂ requires C, 28.8; H, 6.9; Br, 54.7; N, 9.6%) as prepared by an extension of the method of Lott & Krapcho (1957). A similar method was used to prepare 5-aminopent-1-yltrimethylammonium bromide hydrobromide, m.p. 209–210°C (Found: C, 31.2; H, 7.5; N, 9.1; C₈H₂₂Br₂N₂ requires C, 31.4; H, 7.2; N, 9.2%). 6-Aminohex-1-yltrimethylammonium bromide hydrobromide had m.p. 152–153°C [Lott & Krapcho (1957) gave m.p. 158–160°C] as prepared via 6-phthalimidohex-1-yl bromide, b.p. 186–188°C/0.4 mm Hg, and 6-phthalimidohex-1-yltrimethylammonium bromide, m.p. 166–168°C (Found: C, 54.9; H, 7.0; Br, 21.4; N, 8.0; C₁₇H₂₆BrN₂O₂ requires C, 55.1; H, 7.1; Br, 21.6; N, 7.6%) by using an extension of the method described for 3-aminoprop-1-yltrimethylammonium bromide hydrobromide.

1,3-Dimethyl-4(5)-(2'-aminoethyl)imidazolium bromide hydrobromide

4(5)-(2'-Phthalimidoethyl)imidazole (N-phthaloylhistamine) was prepared from histamine and phthalic anhydride [general method (d) (method 1)] and had m.p. 175–176°C (Found: C, 64.4; H, 4.5; N, 17.4; C₁₃H₁₁N₃O₂ requires C, 64.7; H, 4.6; N, 17.4). Reaction with methyl iodide was carried out by general method (e) except that at intervals the pH was adjusted to 9.0 with methanolic KOH. 1,3-Dimethyl-4(5)-(2'-phthalimidoethyl)imidazolium iodide had m.p. 255–257°C (Found: C, 45.1; H, 3.9; I, 31.7; N, 10.8; C₁₅H₁₆IN₂O₂ requires C, 45.4; H, 4.1; I, 32.0; N, 10.6%). Hydrolysis by general method (f) gave 1,3-dimethyl-4(5)-(2'-aminoethyl)imidazolium bromide hydrobromide, m.p. 171–172°C (Found: C, 27.8; H, 5.1; Br, 53.1; N, 13.9; C₇H₁₅Br₂N₃ requires C, 27.9; H, 5.0; Br, 53.1; N, 14.0%).

Aminomethyl- and aminoethyl-substituted N-methylpyridinium bromides

These were all prepared as hydrobromides by the sequence of general methods (d) (method 1), (e) and (f) except that reaction times were considerably longer for 2-substituted compounds. 2-Phthalimidomethylpyridine had m.p. 118–119°C (Found: C, 70.7; H, 4.2; N, 11.3; C₁₄H₁₀N₂O₂ requires C, 70.6; H, 4.2; N, 11.8%). 3-Phthalimidomethylpyridine had m.p. 144–145°C (Found: C, 70.7; H, 4.2; N, 11.5; C₁₄H₁₀N₂O₂ requires C, 70.6; H, 4.2; N, 11.8%). 4-Phthalimidomethylpyridine had m.p. 157–158°C (Found: C, 70.4; H, 3.9; N, 12.1; C₁₄H₁₀N₂O₂ requires C, 70.6; H, 4.2; N, 11.8%). 2-(2'-

Phthalimidoethylpyridine had m.p. 90–91°C (Found: C, 71.3; H, 5.1; N, 11.6; $C_{15}H_{12}N_2O_2$ requires C, 71.4; H, 4.8; N, 11.1%). 1-Methyl-2-phthalimidomethylpyridinium iodide decomposed above 210°C (Found: C, 47.2; H, 3.3; I, 33.1; N, 7.9; $C_{15}H_{13}IN_2O_2$ requires C, 47.4; H, 3.4; I, 33.4; N, 7.4%). 1-Methyl-3-phthalimidomethylpyridinium iodide had m.p. 208–210°C (Found: C, 47.2; H, 3.3; I, 33.4; N, 7.4; $C_{15}H_{13}IN_2O_2$ requires C, 47.4; H, 3.4; I, 33.4; N, 7.4%). 1-Methyl-4-phthalimidomethylpyridinium iodide had m.p. 199–200°C (Found: C, 47.2; H, 3.3; I, 32.7; N, 7.8; $C_{15}H_{13}IN_2O_2$ requires C, 47.4; H, 3.4; I, 33.4; N, 7.4%). 1-Methyl-2-(2'-phthalimidoethyl)pyridinium iodide had m.p. 230°C (decomp.) (Found: C, 48.8; H, 3.8; I, 32.9; N, 7.4; $C_{16}H_{15}IN_2O_2$ requires C, 48.8; H, 3.8; I, 32.2; N, 7.1%). 1-Methyl-2-aminomethylpyridinium bromide hydrobromide decomposed above 196°C (Found: C, 29.3; H, 4.0; Br, 56.8; N, 10.0; $C_7H_{12}Br_2N_2$ requires C, 29.6; H, 4.3; Br, 56.3; N, 9.9%). 1-Methyl-3-aminomethylpyridinium bromide hydrobromide had m.p. 226–227°C (Found: C, 29.2; H, 4.1; Br, 56.2; N, 9.8; $C_7H_{12}Br_2N_2$ requires C, 29.6; H, 4.3; Br, 56.3; N, 9.9%). 1-Methyl-4-aminomethylpyridinium bromide hydrobromide decomposed above 230°C (Found: C, 29.4; H, 4.0; Br, 56.5; N, 10.0; $C_7H_{12}Br_2N_2$ requires C, 29.6; H, 4.3; Br, 56.3; N, 9.9%). 1-Methyl-2-(2'-aminoethyl)pyridinium bromide hydrobromide had m.p. 184–186°C (decomp.) (Found: C, 31.7; H, 3.8; Br, 53.1; N, 9.2; $C_8H_{11}Br_2N_2 \cdot \frac{1}{2}H_2O$ requires C, 31.6; H, 4.0; Br, 52.6; N, 9.2%).

Benzylamine derivatives

p-Hydroxymethylbenzylamine. *p*-Cyanobenzyl bromide (10.2 g) prepared by the method of Karrer & von Schmid (1946) was vigorously stirred and refluxed with water (300 ml) and $BaCO_3$ (20 g) for 40 min. Chloroform extraction and distillation gave *p*-cyanobenzyl alcohol, b.p. 114–116°C/0.1 mmHg (Found: C, 71.9; H, 5.2; N, 10.6; C_8H_9NO requires C, 72.2; H, 5.3; N, 10.5%). The 3,5-dinitrobenzoyl ester had m.p. 171–173°C (Found: C, 54.8; H, 2.9; N, 13.1; $C_{15}H_9N_3O_6$ requires C, 55.1; H, 2.8; N, 12.8%). *p*-Cyanobenzyl alcohol (10 g) was reduced with $LiAlH_4$ (5 g) in refluxing anhydrous ether (250 ml) for 3 h. After addition of base and extraction with chloroform, the chloroform layer was extracted with dilute HCl; on evaporation the extract gave *p*-hydroxymethylbenzylamine hydrochloride (3.7 g; 28% yield), m.p. 157–158°C (Found: C, 55.8; H, 6.9; Cl, 20.5; N, 8.2; $C_8H_{12}ClNO$ requires C, 55.4; H, 7.0; Cl, 20.4; N, 8.1%). Treatment with HBr (general method a) gave *p*-bromomethylbenzylamine hydrobromide, m.p. above 300°C (Found: C, 34.4; H, 3.9; Br, 56.2; N, 5.1; $C_8H_{11}Br_2N$ requires C, 34.2; H, 4.0; Br, 56.9; N, 5.0%). Reaction of this with thiourea (general method b) gave *S*-(*p*-aminomethylbenzyl)isothiuronium bromide hydrobromide, m.p. above 300°C (Found: C, 30.2; H, 4.4; Br, 44.7; N, 11.8; $C_9H_{15}BrN_3S$ requires C, 30.3; H, 4.2; Br, 44.8; N, 11.8%), and reaction with dimethyl sulphide for 3 h (general method c) produced *p*-aminomethylbenzylidimethylsulphonium bromide hydrobromide, m.p. above 300°C (Found: C, 34.7; H, 5.2; Br, 47.2; N, 3.9; $C_{10}H_{17}Br_2NS$ requires C, 35.0; H, 5.0; Br, 46.6; N, 4.1%).

p-Methylaminomethylbenzylamine. A mixture of *p*-cyanobenzyl bromide (10.3 g), dioxan (40 ml) and 33% (v/v) methylamine in ethanol (18 ml) was kept in a stoppered flask at room temperature for 1 day. Evapora-

tion to dryness and distillation of the residue gave *p*-methylaminomethylbenzonitrile, b.p. 156–160°C/0.1–0.2 mmHg (3 g; 39% yield). A sample of this material (2.75 g) was reduced with Raney nickel (approx. 6 g) in acetic anhydride (75 ml) at 50°C for 2 h. After filtration and removal of solvent, *NN'*-diacetyl-*p*-methylaminomethylbenzylamine was obtained as a viscous oil (4.6 g; 97% yield). A sample of this (4.2 g) was hydrolysed by refluxing with 25% (w/v) KOH in methanol (100 ml) to give *p*-methylaminomethylbenzylamine, b.p. 130–131°C/0.3–0.4 mmHg (1 g; 37% yield). The *dipicrate* had m.p. 200–202°C (Found: C, 41.5; H, 3.2; N, 18.7; $C_{21}H_{20}N_8O_{14}$ requires C, 41.5; H, 3.3; N, 18.4%), and the *dihydrochloride* has m.p. above 300°C (Found: C, 48.6; H, 7.3; Cl, 31.3; N, 12.7; $C_9H_{16}Cl_2N_2$ requires C, 48.4; H, 7.2; Cl, 31.8; N, 12.6%).

p-Dimethylaminobenzylamine. This was prepared from *p*-cyanobenzyl bromide and dimethylamine by a modification of the previous method. It had b.p. 96–100°C/0.4–0.6 mmHg and formed a *dipicrate*, m.p. 181–183°C (Found: C, 42.4; H, 3.5; N, 17.8; $C_{22}H_{22}N_8O_{14}$ requires C, 42.5; H, 3.6; N, 18.0%), and a *dihydrochloride*, m.p. 242–244°C (Found: C, 50.4; H, 7.7; Cl, 29.7; N, 11.7; $C_{10}H_{18}Cl_2N_2$ requires C, 50.6; H, 7.7; Cl, 29.9; N, 11.8%).

p-Aminomethylbenzyltrimethylammonium bromide hydrobromide. *p*-Dimethylaminomethylbenzylamine and phthalic anhydride [general method (d) (method 1)] gave *NN*-dimethyl-*p*-phthalimidomethylbenzylamine, m.p. 75–76°C (Found: C, 73.5; H, 6.0; N, 9.6; $C_{18}H_{18}N_2O_2$ requires C, 73.5; H, 6.2; N, 9.5%). This reacted with methyl iodide (general method e) to produce *p*-phthalimidomethylbenzyltrimethylammonium iodide, m.p. 242–243°C (decomp.) (Found: C, 52.3; H, 4.8; I, 29.7; N, 6.5; $C_{19}H_{21}IN_2O_2$ requires C, 52.3; H, 4.9; I, 29.1; N, 6.4%). Hydrolysis by general method (f) gave *p*-aminomethylbenzyltrimethylammonium bromide hydrobromide (decomposed above 225°C) (Found: C, 38.6; H, 5.9; Br, 46.6; N, 8.2; $C_{11}H_{20}Br_2N_2$ requires C, 38.9; H, 5.9; Br, 47.0; N, 8.2%).

p-(2'-Aminoethyl)benzylamine. To *p*-cyanobenzyl bromide (10 g) in ethanol (180 ml) was added dropwise a solution of KCN (3.4 g) in water (25 ml) over 30 min. After a further 45 min at 50°C the solvent was removed to give *p*-cyanobenzyl cyanide, which was purified by sublimation at 58–60°C/1.0 mmHg (5.9 g; 81% yield). A sample of this (5 g) in methanol (50 ml) was added to Raney nickel (approx. 15 g) in methanol (250 ml) saturated with NH_3 . After 3 h at 80°C under pressure (80 atm H_2) with mechanical agitation the autoclave was cooled and the contents were filtered, evaporated to dryness and partitioned between chloroform and dilute HCl. The basic material was distilled to give *p*-(2'-aminoethyl)benzylamine, b.p. 84–86°C/0.1–0.2 mmHg (1.5 g; 29% yield). The *dipicrate* had m.p. 220–224°C (Found: C, 41.6; H, 3.3; N, 18.5; $C_{21}H_{20}N_8O_{14}$ requires C, 41.5; H, 3.3; N, 18.4%), and the *dihydrochloride* had m.p. above 300°C (Found: C, 47.9; H, 7.1; Cl, 31.5; N, 12.4; $C_9H_{16}Cl_2N_2$ requires C, 48.4; H, 7.2; Cl, 31.8; N, 12.6%).

RESULTS

The u.v.-absorption spectrum of the final enzyme solution corresponded to that reported for the crystalline enzyme by Yamada, Kumagai, Kawasaki,

Table 3. *Oxidation of substrates by pig kidney diamine oxidase*

V ($\mu\text{l}\cdot\text{min}^{-1}$) is the maximum initial rate of O_2 uptake actually observed experimentally. K_m (mM) is the apparent Michaelis constant obtained from plots of v against v/s (where no value is given this is due to insufficient results (—), substrate inhibition (+) or no appreciable oxidation ($\times$)). K'_s (mM) is the apparent substrate-inhibitor constant obtained from plots of $1/v$ against s (N is given where no substrate inhibition was observed over a large range of substrate concentrations).

	V ($\mu\text{l}\cdot\text{min}^{-1}$) (Substrate concn. giving this value in parenthesis)	V (% of that of parent diamine)	K_m (mM)	K'_s (mM)
1,3-Diaminopropane	0.40 (5 mM)	100	0.5	196
3-Aminopropan-1-ol	0.26 (100 mM)	65	27.2	N
3-Aminoprop-1-yl bromide	0.16 (100 mM)	40	20.9	N
1-Aminopropane	0.19 (50 mM)	47.5	15.6	N
3-Dimethylaminoprop-1-ylamine	0.18 (30 mM)	45	38.2	—
3-Aminoprop-1-yldimethylsulphonium bromide	0.22 (1 mM)	55	+	7
<i>S</i> -(3-Aminoprop-1-yl)isothiuronium bromide	0.16 (1 mM)	40	+	7
3-Aminoprop-1-yltrimethylammonium bromide	0	0	$\times$	$\times$
1,4-Diaminobutane (putrescine)	1.08 (5 mM)	100	0.5	751
4-Aminobutan-1-ol	0.28 (75 mM)	25.9	23.6	N
4-Aminobut-1-yl bromide	0.14 (50 mM)	13	6.3	N
4-Aminobutane	0.12 (30 mM)	11	+	27
4-Dimethylaminobut-1-ylamine	0.89 (30 mM)	82.4	11.1	—
4-Aminobut-1-yldimethylsulphonium bromide	0.99 (50 mM)	91.7	1.5	295
<i>S</i> -(4-Aminobut-1-yl)isothiuronium bromide	0.42 (3 mM)	38.9	0.04	14
4-Aminobut-1-yltrimethylammonium bromide	0	0	$\times$	$\times$
1,5-Diaminopentane (cadaverine)	1.28 (20 mM)	100	1.2	1200
5-Aminopentan-1-ol	0.45 (100 mM)	35.2	36.5	N
5-Aminopent-1-yl bromide	0.47 (100 mM)	36.7	58	N
5-Aminopentane	0.10 (30 mM)	7.8	+	10
5-Dimethylaminopent-1-ylamine	0.56 (30 mM)	43.8	15.1	—
5-Aminopent-1-yldimethylsulphonium bromide	0.56 (10 mM)	43.8	0.5	396
<i>S</i> -(5-Aminopent-1-yl)isothiuronium bromide	0.57 (3 mM)	44.5	0.4	24
5-Aminopent-1-yltrimethylammonium bromide	0	0	$\times$	$\times$
1,6-Diaminohexane	0.79 (20 mM)	100	1.3	174
6-Aminohexan-1-ol	0.25 (100 mM)	31.6	43.6	N
6-Aminohex-1-yl bromide	0.21 (100 mM)	26.7	48.5	N
6-Aminohexane	0	0	—	—
6-Dimethylaminohex-1-ylamine	0.13 (30 mM)	16.5	2.3	—
6-Aminohex-1-yldimethylsulphonium bromide	0.78 (3 mM)	98.7	0.9	50
<i>S</i> -(6-Aminohex-1-yl)isothiuronium bromide	0.23 (1 mM)	29.1	+	13
6-Aminohex-1-yltrimethylammonium bromide	0	0	$\times$	$\times$
<i>p</i> -Bis(aminomethyl)benzene (<i>p</i> -xylylenediamine)	0.86 (10 mM)	100	0.3	125
<i>p</i> -Hydroxymethylbenzylamine	0.35 (70 mM)	40.7	63.7	450
<i>p</i> -Methylbenzylamine	0.15 (30 mM)	17.4	10.3	—
<i>p</i> -Methylaminomethylbenzylamine	1.13 (20 mM)	131.4	3	83
<i>p</i> -Dimethylaminomethylbenzylamine	1.03 (50 mM)	119.8	2	1400
<i>p</i> -Aminomethylbenzylidimethylsulphonium bromide	0.76 (3 mM)	88.4	1.2	100
<i>S</i> -(<i>p</i> -Aminomethylbenzyl)isothiuronium bromide	0.39 (1 mM)	45.3	+	36
<i>p</i> -Aminomethylbenzyltrimethylammonium bromide	0	0	$\times$	$\times$
<i>p</i> -(2'-Aminoethyl)benzylamine	0.58 (20 mM)	67.4	0.8	100
4(5)-(2'-Aminoethyl)imidazole (histamine)	0.37 (5 mM)	100	0.3	37
1,3-Dimethyl-4(5)-(2'-aminoethyl)imidazolium bromide	0.16 (7.5 mM)	43.2	0.3	36
2-Aminomethylpyridine	0.12 (10 mM)	100	16.3	—
1-Methyl-2-aminomethylpyridinium bromide	0.15 (10 mM)	125	—	—
3-Aminomethylpyridine	0.20 (10 mM)	100	4.5	—
1-Methyl-3-aminomethylpyridinium bromide	0.17 (10 mM)	85	—	—
4-Aminomethylpyridine	0.21 (10 mM)	100	4.6	—
1-Methyl-4-aminomethylpyridinium bromide	0.21 (10 mM)	100	—	—
2-(2'-Aminoethyl)pyridine	0.14 (10 mM)	100	—	—
1-Methyl-2-(2'-aminoethyl)pyridinium bromide	0.14 (10 mM)	100	—	—

Matsui & Ogata (1967) and did not have the absorption peak at 405nm found by Mondovi *et al.* (1967). In our hands preparations with lower specific activities have shown this band, which may be due to a contaminant, as Goryachenkova & Ershova (1965) suggest.

Table 3 shows that, when one amino group in a diamine is substituted by a hydroxy group or a group bearing a formal positive charge, V is somewhat decreased, except for trimethylammonium compounds, which were not oxidized. The apparent K_m was not appreciably altered for onium compounds, but hydroxy, dimethylamino or bromo substitution caused a marked increase in K_m .

Inhibition by high concentrations of substrate is shown in Table 3 and Figs. 1, 2, 3 and 4. From these it is apparent that substrate inhibition depends on both the structure of the groups involved and the chain length. Hydroxy amines, bromo amines, putrescine and cadaverine gave almost no inhibition; 1,3-diaminopropane, 1,6-diaminohexane and dimethylsulphonium compounds gave weak substrate inhibition; histamine and the isothiuronium compounds showed strong substrate inhibition. In any given series the substrate inhibition is minimal for four and five methylene units, rising on either side of this. *p*-Xylylenediamine showed strong substrate inhibition, but this decreases considerably as one amino function is dimethylated, monomethylation having little effect.

A number of other compounds were studied and found to be poor substrates. Maximum rates of oxidation for these compounds, together with the concentration at which this was observed are as

follows: ethylamine (O_2 uptake $0.122 \mu\text{l} \cdot \text{min}^{-1}$ at 10mm); *S*-(2-aminoethyl)isothiuronium bromide (O_2 uptake $0.168 \mu\text{l} \cdot \text{min}^{-1}$ at 3.0mm); benzylamine (O_2 uptake $0.052 \mu\text{l} \cdot \text{min}^{-1}$ at 10mm); phenethylamine (O_2 uptake $0.047 \mu\text{l} \cdot \text{min}^{-1}$ at 10mm); 3-phenylpropylamine (O_2 uptake $0.034 \mu\text{l} \cdot \text{min}^{-1}$ at 10mm); 4-phenylbutylamine (O_2 uptake $0.040 \mu\text{l} \cdot \text{min}^{-1}$ at 10mm); *p*-methoxybenzylamine (O_2 uptake $0.102 \mu\text{l} \cdot \text{min}^{-1}$ at 10mm); *p*-methoxyphenethylamine (O_2 uptake $0.078 \mu\text{l} \cdot \text{min}^{-1}$ at 10mm); spermine (O_2 uptake $0.036 \mu\text{l} \cdot \text{min}^{-1}$ at 30mm); spermidine (O_2 uptake $0.108 \mu\text{l} \cdot \text{min}^{-1}$ at 30mm); lysine, tyramine and mescaline (no detectable oxidation).

Several of the benzylamine derivatives prepared (particularly *p*-dimethylaminobenzylamine) have been useful for assay of the enzyme, u.v. absorption by the aldehyde produced on oxidation being determined spectrophotometrically.

Fig. 1. Typical plots of v against v/s (Eadie, 1952) for cadaverine (O) (showing no substrate inhibition), *S*-(5-aminopent-1-yl)isothiuronium bromide (Δ) and histamine ($\square$) (showing inhibition by high substrate concentration).

Fig. 2. Methods used for illustrating substrate inhibition after Dixon & Webb (1964, p. 75) (a) and Webb (1963) (b) for cadaverine (O), *S*-(5-aminopent-1-yl)isothiuronium bromide (Δ), histamine ($\square$) and *p*-xylylenediamine ($\bullet$). $ps = -\log s$.

Fig. 3. Variation of $\log K'_s$ (where K'_s is the substrate inhibitor constant; see the text) with chain length with homologous series of diamines ($\circ$), *S*-aminoalkylisothiuronium compounds (Δ) and aminoalkyldimethylsulphonium compounds ($\blacktriangle$).

DISCUSSION

It is known that diamines interact under anaerobic conditions with pig kidney diamine oxidase causing an alteration in the u.v. spectrum of the enzyme (Yamada *et al.* 1967; Mondovì *et al.* 1967). A derivative of a Schiff base between histamine and pyridoxal phosphate has been isolated after borohydride reduction (Kumagai, Nagate, Yamada & Fukami, 1969), seeming to implicate Schiff-base formation between diamine and pyridoxal phosphate at the site of oxidation. The valency state of copper during the reaction is not settled (Mondovì *et al.* 1967, 1969), but Ping Pong kinetics have been suggested (Finazzi-Agro, Rotilio, Costa & Mondovì, 1969).

A reliable scheme for discussing the mode of

Fig. 4. Double-reciprocal plots (Dixon & Webb, 1964, p. 327) illustrating inhibition of cadaverine oxidation by pentamethylene bis-onium compounds. $\circ$, Cadaverine; Δ , Cadaverine + 1,5-pentamethylene-bis-dimethylsulphonium dibromide (1.0 mM); $\blacksquare$, cadaverine + 1,5-pentamethylene-bis-guanidine dihydrobromide (0.1 mM).

action of this enzyme would be as a series of reversible stages: stage 1, association of enzyme and substrate; stage 2, where favourable association has occurred, Schiff-base formation between one amino group of the substrate and the aldehyde group of pyridoxal phosphate at an oxidizing site on the enzyme; stage 3, a prototropic shift converting the first Schiff base into a Schiff base between pyridoxamine and an aldehyde; stage 4, hydrolysis and dissociation into the aldehyde (the first product) and an altered enzyme (containing pyridoxamine instead of pyridoxal); stage 5, complex-formation between this altered enzyme and O_2 (the second substrate); stage 6, oxidation reaction leading to a complex of ammonia, hydrogen peroxide and enzyme; stage 7, dissociation into enzyme and second products (ammonia and hydrogen peroxide).

Cleland (1963) has shown that in bisubstrate reactions of this type (Bi Ter Ping Pong mechanism) the apparent K_m is related to the concentration of both substrates and reaches a limiting value as saturation with substrate occurs. In our case the concentration of the fixed substrate (O_2) was constant throughout, but the final stages were rendered irreversible by the presence of catalase removing hydrogen peroxide as it was formed.

States 1 and 2 will obviously depend on the relationship between substrate structure and the nature of the substrate-binding region of the enzyme. Rate constants for the steps involved will be very much influenced by changes in substrate structure.

Stage 3 involves ionization of a proton from the methylene group adjacent to the substrate amino group involved in Schiff-base formation, and is not likely to be much influenced by changes in substrate structure where these are confined to more distant regions of the substrate molecule. This will be a slow rate-limiting step.

Stage 4 is similar to stage 2.

Stages 5, 6 and 7 will be identical in all substrates and, in the present work, did not reach equilibrium, so the formulations suggested by Cleland (1963) are not directly applicable in this case.

However, it seems reasonable to conclude that the overall velocity of the reaction will be mostly affected by substrate structure at stages 1 and 2, and that the apparent K_m determined in this work is an approximate inverse measure of the affinity of the enzyme for substrate.

Under equilibrium conditions Oi, Inamasu & Yasunobu (1970) have suggested that the K_m for ox plasma amine oxidase approximates to K_s , the substrate dissociation constant, whereas Oi, Shimada, Inamasu & Yasunobu (1970) have proposed that for ox liver mitochondrial amine oxidase K_m is a kinetic constant. In both cases benzylamine was the substrate.

Nature of the substrate-binding site. The results obtained with compounds containing one amino group and one onium group exclude the possibility that the substrate acts as a nucleophile towards an electrophilic residue on the enzyme (Zeller *et al.* 1956) since these onium groups could not act as nucleophiles. Non-oxidation of the trimethylammonium compounds remains to be explained.

Replacement of one amino group of a diamine by a dimethylamino group usually alters V slightly, but usually causes a large increase in K_m , and it is difficult to see why introduction of a further methyl group should have such a pronounced effect. The explanation cannot be that the formal positive charge renders the substrate-binding site and substrate incompatible, since a large number of compounds with formal positive charges are oxidized. Also, steric hindrance is rendered less likely by the oxidation of bulky heterocyclic, aromatic and isothiuronium compounds although, of all the compounds tried that carried a positive charge, only the trimethylammonium compounds present a severely hindered close approach to the charged centre itself. The aminoalkyltrimethylammonium compounds were not inhibitory and pentamethonium had weak inhibitor potency, so the conclusion must be that the binding site has a low affinity for trimethylammonium groups. Bardsley & Hill (1970) tentatively suggested that a possible explanation could be that the negative charge on the enzyme is located in a hydrophilic region. It is then envisaged that a critical balance exists between the attraction of a

cation to the enzyme negative charge and repulsion due to interaction between the hydrophobic methyl groups of the substrate and the hydrophilic region surrounding the enzyme negative charge. With diamines, dimethylaminoalkylamines, dimethylsulphonium compounds, isothiuronium compounds, quaternary heterocyclic and *p*-xylylenediamine derivatives the overall effect of the balance resulting from attraction between positively charged substrate and negatively charged enzyme, repulsion between the hydrophilic region surrounding the enzyme negative charge and hydrophobic regions of the substrate, together with the operation of steric factors is affinity, since the group contributing to binding is positively charged and either solvated by water molecules or has a flat structure owing to trigonal hybridization. With trimethylammonium compounds there is no overall affinity, since the positively charged nitrogen atom is tetrahedrally hybridized and, being surrounded by hydrophobic methyl groups, is probably not appreciably solvated by a solvent cage of water molecules. This scheme also accommodates the hydroxyalkylamines, which would be good substrates because of attraction between the hydroxy group and this hydrophilic region, although this attraction would be less than that between formal positive and negative charges, and higher substrate concentrations would be needed for saturation (cf. the high values for K_m for hydroxy amines in Table 3).

Inhibition by high substrate concentrations. Several possible explanations can be advanced for this phenomenon. (a) Substrate molecules at high concentration might crowd on to the active site in an end-on position instead of the parallel alignment needed for the forward reaction (Zeller, 1951). This explanation cannot be upheld, since monoamines and mono-onium compounds would be strongly inhibitory, which they are not (Zeller, 1951; Blaschko, Fastier & Wajda, 1951). (b) Substrate molecules might be able to bind on to the enzyme so that the amino group is adjacent to the substrate-binding site and the other group (e.g. the imidazole ring in histamine) is juxtaposed to the oxidizing site. This is untenable, since 1,3-diaminopropane and 1,6-diaminohexane show weak substrate inhibition and *p*-xylylenediamine shows strong inhibition. (c) The substrate might be able to complex with the enzyme or cofactor, e.g. histamine might react with pyridoxal (Mondovi, Scioscia-Santoro, Rotilio & Costa, 1964). This cannot be the explanation, since it is unlikely that the wide variety of structural types showing inhibition could have common mechanisms of reaction with either the enzyme or a cofactor. (d) It is now suggested that there is, in addition to the negatively charged substrate-binding site, an inhibitory substrate-binding site, which has a comparable affinity for substrate groups

containing delocalized π -electron systems (e.g. aromatic and heterocyclic rings, isothiuronium and guanidinium groups), a much decreased affinity for dimethylsulphonium and amino groups and negligible affinity for alkyl and hydroxy groups. Obviously in the equilibrium between enzyme, substrate, correctly orientated enzyme-substrate complex and enzyme-substrate complex with the substrate in the inhibitory position, chain length is important, determining the goodness of fit between substrate and the two receptor sites on the enzyme. With a chain length of four or five methylene units the substrate has maximum affinity for the enzyme in the oxidizing position. When the number of methylene units is less than four or greater than five then there is more tendency for substrate molecules to complex in the inhibitory position. (e) A more complicated explanation could be put forward, e.g. a combination of the previous types could occur concurrently, adsorption on a distant site might alter the three-dimensional structure of the active site or there may be a substrate-inhibitor-binding site and another negative site such that reaction here alters the rate of substrate oxidation.

To settle these alternatives a preliminary study of the inhibition by 1,5-pentamethylene bis-onium compounds was undertaken. The bis-guanidinium and bis-isothiuronium compounds gave strong mixed inhibition, the bis-trimethylammonium compound weak competitive inhibition (K_i 1.0 mM) and the bis-dimethylsulphonium compound strong competitive inhibition (K_i 0.27 mM). This kinetic behaviour may best be interpreted in terms of mechanism (d) by assuming that the bis-guanidinium and bis-isothiuronium compounds inhibit by one onium group interacting with the inhibitory-substrate-binding site and the other with the substrate-binding site negative charge, whereas the bis-dimethylsulphonium compound inhibits competitively with one onium group adjacent to the substrate-binding site negative charge and the other next to the oxidizing site.

This assumes that the oxidizing site is also negatively charged (which is distinctly possible, since pyridoxal phosphate is probably located here) or has affinity for onium groups, and also explains why, even though the rate of oxidation of the diamines decreases markedly as chain length exceeds six methylene units, inhibition by bis-onium compounds does not decrease so rapidly (Blaschko *et al.* 1951).

This may be because the long-chain bis-onium compounds can inhibit with one onium group at the inhibitory-substrate-binding site and one at the oxidizing site.

Fig. 5 illustrates the structure of the active site embodying the structural features suggested by these conclusions. It is to be regarded as a working

Fig. 5. Proposed scheme for substrate binding in pig kidney diamine oxidase using information from this and previous work (Bardsley *et al.* 1970). OS represents the oxidizing site where the cofactors are located and where the amino group to be oxidized is involved in Schiff-base formation. Hy is a hydrophobic binding site. SBS is the negatively charged substrate-binding site situated 6.0–9.0 Å from the oxidizing site. This is surrounded by a hydrophilic region shown shaded in. IBS denotes the proposed inhibitory-substrate binding site., Van der Waals type of interaction between methylene groups and hydrophobic binding site., Electrostatic attraction between oppositely charged groups., Attraction between group with delocalized π -electron system and inhibitory-substrate-binding site (see the text for details). (a) Histamine bound in a position before Schiff-base formation, i.e. in the correct alignment for oxidation. (b) Histamine aligned in the inhibitory position.

hypothesis rather than as excluding a dynamic or induced-fit model.

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