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HUMAN KIDNEY DIAMINE OXIDASE: INHIBITION STUDIES

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Abstract—The oxidation of *p*-dimethylaminomethylbenzylamine by purified human kidney diamine oxidase was studied in the presence of substrate analogues such as dimethylsulphonium, trimethylammonium, isothiouronium and guanidinium compounds of various chain lengths. The inhibition by mono- and bis-onium compounds is described. K_i values and ΔG° values are given and the inhibition due to monoamine oxidase inhibitors and certain time-dependent inhibitors is reported. It is concluded that purified diamine oxidase from human kidney resembles the diamine oxidases from hog kidney and human placenta in its inhibitor specificity. In particular, it is inhibited strongly by substrate analogues in the order isothiouronium ≈ guanidinium > dimethylsulphonium > trimethylammonium suggesting a negatively charged substrate binding site. Also, it is weakly inhibited by some monoamine oxidase inhibitors and strongly inhibited in a time-dependent fashion by carbonyl-group reagents.

Diamine oxidase (EC 1.4.3.6.) catalyses the oxidation of a diamine by molecular oxygen yielding an aminoaldehyde, hydrogen peroxide and ammonia as products. Classically, this enzyme was thought to function principally as a histaminase [1, 2] although the best substrates for the enzyme are aliphatic diamines such as putrescine and cadaverine [3]. There is evidence to suggest that cell-free protein synthesis is affected by polyamines such as putrescine in that the magnesium requirement is lowered [4, 5]. Furthermore, spermine enhances the translation of mRNA in cell-free extracts from wheat germ [6]. These and other evidence [7] suggest that polyamines may influence cell division. Indeed, semen, which contains large amounts of polyamines is partly derived from tissues in which there is rapid cell growth and division and recently, a diamine oxidase capable of oxidising spermine and spermidine has been purified from human seminal plasma [8].

The study of enzyme inhibition by substrate analogues is a useful tool in probing the structure–function relationships in enzyme action. This technique has been used to considerable effect with the diamine oxidase from pig kidney and human placenta [9–11], although these are the only sources from which the enzyme has so far been characterised in terms of detailed inhibition studies. Diamine oxidase has been detected in the human kidney [12, 13] and has now been isolated and purified. (Shindler and Bardsley, 1975, unpublished results.)

We have tested both mono- and bis-onium compounds as potential inhibitors, where the former correspond to monoamines, which are poor substrates of this enzyme, and the latter correspond to diamines, which are better substrates, especially when the inter-nitrogen chain of methylene units is of the appropriate length [3, 14].

In the present paper, we report values for K_i and ΔG^0, for a total of thirty-eight substrate analogues and six so-called monoamine oxidase inhibitors. We also report some information about the nature of the time-dependent inhibition of this enzyme by carbonyl-group reagents.

MATERIALS AND METHODS

Preparation of human kidney diamine oxidase

All of the following operations were performed at 4° and in phosphate buffer (0.05 M; pH 7.0) unless otherwise stated.

Step 1. Kidney cortices (400 *g*) were homogenised in a Waring Blender (10 min) with buffer (200 ml) and then centrifuged (8000 *g*; 30 min).

Step 2. The supernatant was heated (55°; 10 min) in a thermostatted water bath, with constant stirring, cooled and centrifuged (8000 *g*; 30 min).

Step 3. To the stirred supernatant from step 2, an ice-cold mixture of ethanol and chloroform (3:1, v/v) was added dropwise until the volume of solvent added was 1/10th that of the supernatant. The resulting suspension was then centrifuged (8000 *g*; 40 min).

Step 4. The supernatant from the previous step was applied to a carboxymethylsephadex column (6 × 25 cm) which was developed by linear gradient elution. The varigrad device had phosphate buffer (0.02 M) in both compartments but the second compartment also contained sodium chloride (0.6 M).

Step 5. The pooled active fractions from step 4 were brought to 2.5 M with the addition of solid ammonium sulphate (30 min) and centrifuged (2000 *g*; 40 min). The precipitate was dissolved in the minimum buffer and applied to a sephadex G 200 column (3 × 70 cm) which was developed by upward elution (4 ml hr^{-1}). The purest fraction had a sp. act. of 0.6 i.u. mg^{-1}, corresponding to an overall purification

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of 1800-fold. The pooled active fractions were dialysed against powdered sucrose and the concentrate stored at −15° in sterile tubes.

Preparation of inhibitors

All onium compounds were prepared by conventional methods as previously described elsewhere [9]. The drugs used were proprietary preparations obtained from the Pharmacy Department at St. Mary's hospital, Manchester. All other compounds used were of the highest purity obtainable.

Spectrophotometric method

All experiments were performed in potassium phosphate buffer (0.05 M, pH 7.0, 20°) in a final volume of 1 ml containing enzyme (0.003 i.u.) and an appropriate concentration of substrate and inhibitor. The spectrophotometric method of Bardsley *et al.* [15] was used, measurements being determined using a CARY 118C ultraviolet spectrophotometer. One international unit (i.u.) of enzyme is defined as that amount of enzyme that catalyses the oxidation of one μmole of substrate/min at 20° and is equivalent to an absorbance change at 250 nm of 4.08/min when the enzyme is dissolved in 1 ml buffer containing a suitable excess of substrate [$K_{m(app)} \approx 0.05$ mM].

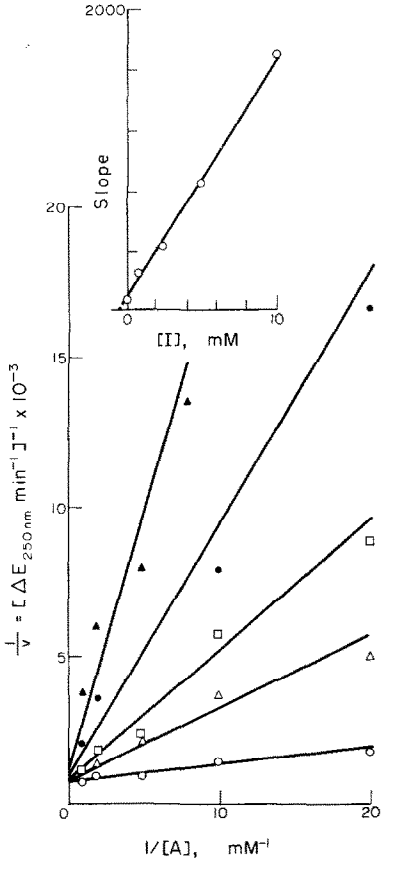

Fig. 1. Inhibition of human kidney diamine oxidase by 1,5-pentamethylene bis-dimethylsulphonium dibromide. ○, *p*-dimethylaminomethylbenzylamine; △, +inhibitor (1 mM); □, +inhibitor (2.5 mM); ●, +inhibitor (5 mM); ▲, +inhibitor (10 mM). Inset: Replot of slopes of double reciprocal plots.

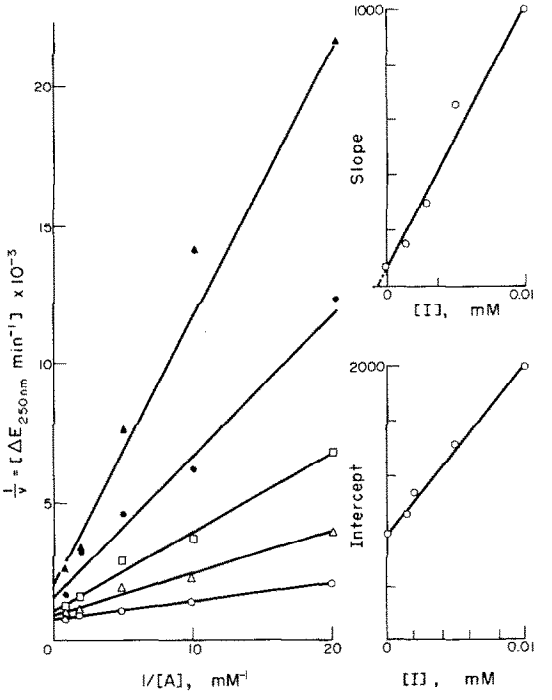

Fig. 2. Inhibition of human kidney diamine oxidase by 1,5-pentamethylene bis-isothiouronium bromide. ○, *p*-dimethylaminomethylbenzylamine; △, +inhibitor (0.0015 mM); □, +inhibitor (0.003 mM); ●, +inhibitor (0.005 mM); ▲, +inhibitor (0.01 mM). Insets: Replot of slopes (top) and intercepts (bottom) of double reciprocal plots.

Measurement of time-dependent inhibition

Enzyme solution (0.003 i.u.) was placed in a cuvette. At zero time, a portion of inhibitor (0.1 ml) was added and mixed manually. After incubation for the required length of time, substrate was added (0.9 ml of 1.1 mM *p*-dimethylaminomethylbenzylamine) and the change in absorbance recorded at 250 nm.

Determination of K_i *and* ΔG^0

Initial rates were obtained over a period of 15 min at five different substrate concentrations and double reciprocal plots and replots of slope and intercept against inhibitor concentration were used to obtain an estimate of K_i [16] using the relationships: $S(I)$, double reciprocal slope as a function of inhibitor concentration; $\mathrm{Int}(I)$, double reciprocal intercept as a function of inhibitor concentration; [I], inhibitor concentration; [A], *p*-dimethylaminomethylbenzylamine concentration; [B], oxygen concentration (saturating at 0.14 mM in air); $K_b = 0.043$ mM; K_{is}, $K_{i\,\mathrm{Int}}$, Inhibition constants according to E + I = EI, $K_i = [E][I]/[EI]$ for linear slope and intercept effects with [1/A] varied and [B] fixed were found from $S(I) = (1 + [I]/K_{is})\ K_a/V$ $\mathrm{Int}(I) = (1 + K_b/[B] + K_b[I]/[B]\ K_{i\,\mathrm{Int}})/V$ by replotting slopes and intercepts against $[I]$; Standard free energies were then calculated from $\Delta G^0 = R\ T \ln K_i$, K_i being expressed in M. Over the range of substrate used, double reciprocal plots against 1/[A] with [B] fixed (air) were approximately linear.

RESULTS

Typical plots of reciprocal velocity against reciprocal substrate concentration are shown for dimethylsulphonium compounds (Fig. 1), isothiouronium compounds (Fig. 2), guanidinium compounds (Fig. 3), trimethylammonium compounds (Fig. 4) and monoamine oxidase inhibitors (Fig. 5). Typical replots of slopes and intercepts of these double reciprocal plots against inhibitor concentration are also shown as insets to these figures.

The experimental results may be summarised as follows:

1. Sulphonium and trimethylammonium compounds generally give competitive inhibition.
2. Isothiouronium and guanidinium compounds give non-competitive inhibition.
3. The inhibitory potency in any series is approximately isothiouronium = guanidinium ≫ dimethylsulphonium > trimethylammonium. The optimum chain length separating the charged species in bis-onium compounds appears to be five to eight methylene units, though longer chain compounds are also inhibitory.
4. In any series, bis-onium compounds are not generally more inhibitory than mono-onium compounds.
5. Replots of slopes and/or intercepts against inhibitor concentration were linear for bis-onium compounds.

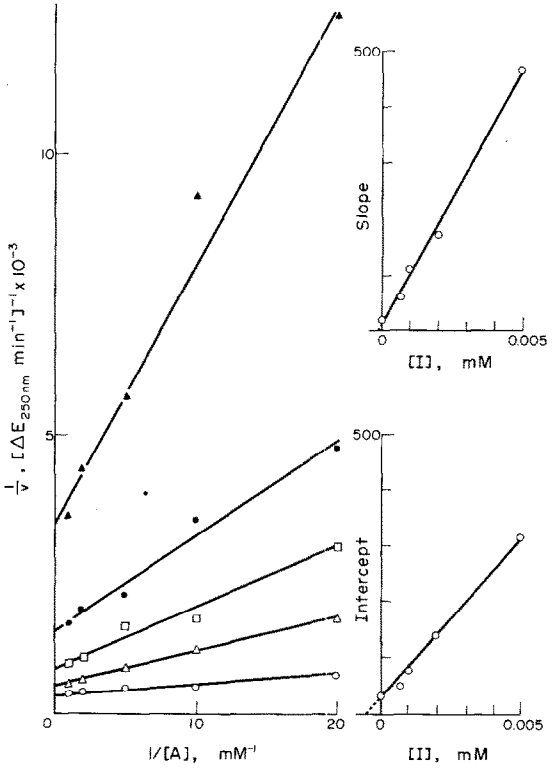

Fig. 3. Inhibition of human kidney diamine oxidase by 1,5-pentamethylene bis-guanidinium dibromide. ○, *p*-dimethylaminomethylbenzylamine; △, +inhibitor (0.00075 mM); □, +inhibitor (0.001 mM); ▲, +inhibitor (0.002 mM); ■, +inhibitor (0.005 mM). Insets: Replot of slopes (top) and intercepts (bottom) of double reciprocal plots.

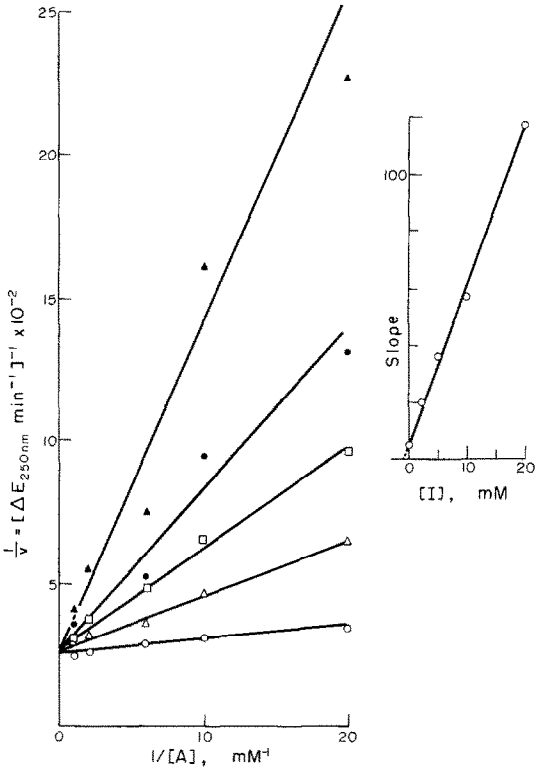

Fig. 4. Inhibition of human kidney diamine oxidase by 1,5-pentamethylene bis-trimethylammonium dibromide (pentamethonium). ○, *p*-dimethylaminomethylbenzylamine; △, +inhibitor (2.5 mM); □, +inhibitor (5 mM); ●, +inhibitor (10 mM); ▲, +inhibitor (20 mM). Inset: Replot of slopes from double reciprocal plots.

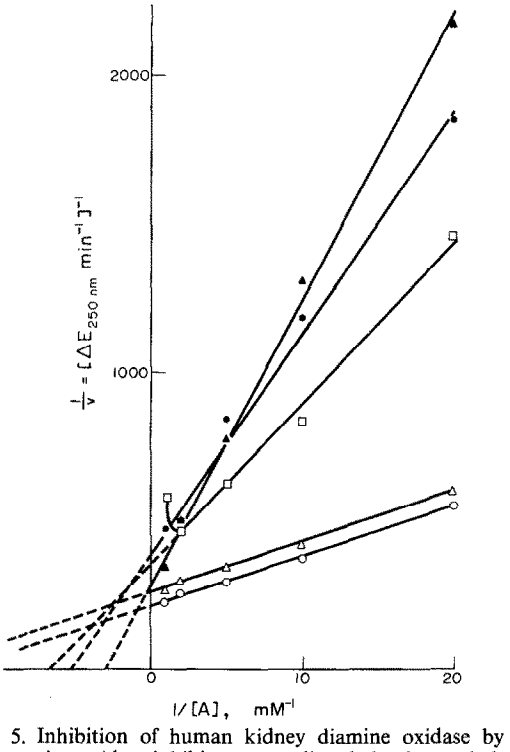

Fig. 5. Inhibition of human kidney diamine oxidase by monoamine oxidase inhibitors. ○, *p*-dimethylaminomethylbenzylamine; △, +pargylline (1.0 mM); □, +isocarboxazid (Marplan) (0.75 mM); ●, + hydrallazine (apresoline) (0.0005 mM); ▲, phenelzine (0.25 mM).

Table 1. Inhibition by substrate analogues of the oxidation of *p*-dimethylaminomethylbenzylamine by purified human kidney diamine oxidase.

Inhibitor	Type of inhibition	$K_{i(slope)}$ (mM)	$-\Delta G^0$ (J mole^{-1})	$K_{i(intercept)}$ (mM)	$-\Delta G^0$ (J mole^{-1})
Dimethylsulphonium compounds					
n = 3	C	0.90	17.1	∞	—
n = 4	C	0.35	19.4	∞	—
n = 5	C	0.30	19.7	∞	—
n = 6	C	0.04	25.0	∞	—
n = 8	C	0.02	26.4	∞	—
n = 10	C	0.01	28.2	∞	—
n = 12	C	0.004	30.1	∞	—
Trimethylsulphonium iodide	C	0.21	20.6	∞	—
Isothiouronium compounds					
n = 3	NC	0.00062	34.8	0.0025	31.5
n = 4	NC	0.00046	35.5	0.0015	32.7
n = 5	NC	0.00045	36.6	0.0014	32.8
n = 6	NC	0.00037	36.1	0.0021	31.9
n = 8	NC	0.00013	38.6	0.0011	33.4
n = 10	NC	0.00078	34.3	0.0018	32.2
n = 12	NC	0.0002	37.7	0.0016	32.5
S-methylisothiouronium iodide	NC	0.012	27.6	0.069	23.3
Guanidinium compounds					
n = 2	NC	0.002	31.9	0.0017	32.4
n = 3	NC	0.00027	36.8	0.00045	35.6
n = 4	NC	0.00023	37.3	0.00064	34.7
n = 5	NC	0.00015	38.3	0.00010	39.2
n = 6	NC	0.00023	37.3	0.00064	34.7
n = 8	NC	0.0052	29.7	0.0015	32.7
n = 10	NC	0.0101	28.0	0.0022	31.1
n = 12	NC	0.00017	38.0	0.00076	34.3
Guanidine	NC	0.0112	27.8	0.0018	32.2
Methylguanidine	NC	0.0176	26.7	0.042	24.6
Trimethylammonium compounds					
n = 3	C	1.15	16.5	∞	—
n = 4	C	2.3	14.8	∞	—
n = 5	C	1.5	15.8	∞	—
n = 6	C	0.54	18.3	∞	—
n = 8	C	0.3	20.5	∞	—
n = 10	C	0.17	21.2	∞	—
n = 12	C	0.025	25.8	∞	—
Tetramethylammonium chloride	C	2.28	14.8	∞	—
ammonium chloride	C	4	13.5	∞	—
Aromatic bis-onium compounds					
p-Xylylenebisdimethyl-sulphonium dibromide	C	0.013	27.5	∞	—
p-Xylylenebisdimethyl-isothiouronium dibromide	NC	0.0011	33.4	0.0041	30.2
p-Xylylenebisdimethyl-ammonium dihydrochloride	NC	0.068	23.4	0.96	16.9
Mono-amine oxidase inhibitors*					
Isocarboxazid	NC	0.38	19.2	0.26	20.1
*Hydrallazine	NC	0.0011	33.4	0.00017	38.0
Phenelzine	C	0.027	25.6		—
*Iproniazid	NC	0.8	17.4	0.27	20.0
*Pargylline	UC		—	0.34	19.5
*Nialamide	NC	0.33	19.5	0.24	20.3

Values of n refer to the number of methylene groups separating the charged species in bis-onium compounds. NC, non-competitive inhibition; UC, uncompetitive inhibition; C, competitive inhibition. *These drugs gave non-linear slope and intercept replots; values of K_i and ΔG^0 are, therefore, only approximate and refer to K_{ii} for E + I = E I.

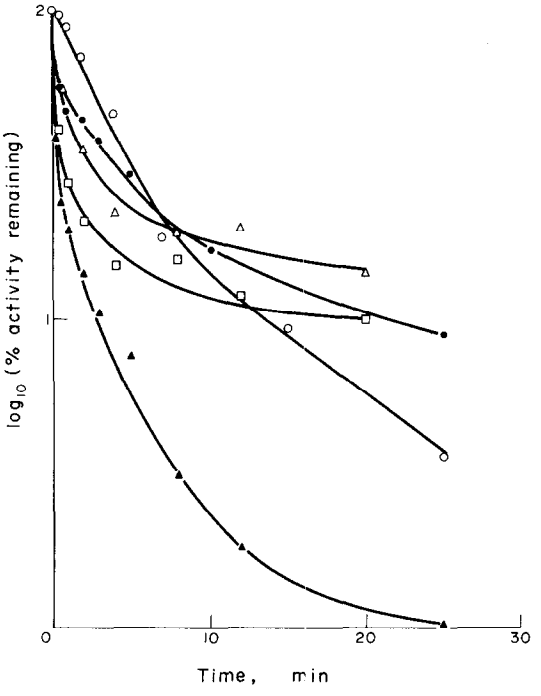

Fig. 6. Time-dependent inhibition of human kidney diamine oxidase by carbonyl-group reagents. For experimental details, see text. Final concentrations of inhibitors were as follows: ○, semicarbazide (10 μM); △, Girard's reagent T (5 mM); □, Girard's reagent T (10 mM); ●, hydroxylamine (10 μM); ▲, aminoguanidine (10 μM).

6. Although the monoamine oxidase inhibitors tested were generally only weakly inhibitory, phenelzine and hydrallazine were potent inhibitors. Replots for these compounds were generally non-linear.

Table 1 summarises the inhibition data obtained with these compounds.

Examples of the time-dependent inhibition of human kidney diamine oxidase by carbonyl group reagents are illustrated in Fig. 6. In every case, a plot of $\log_{10}$ (% activity remaining) against time resulted in a smooth curve.

DISCUSSION

Inhibition of enzyme activity produces slope and/or intercept effects on double-reciprical plots in which slope effects are assumed to be due to the variable substrate and inhibitor 'competing' for the same binding site and where intercept effects are assumed to be due to the reaction of the inhibitor with enzyme forms to which the variable substrate does not bind. The product inhibition pattern with ammonia competitive, aldehyde non-competitive and hydrogen peroxide uncompetitive (Shindler and Bardsley, unpublished results) is consistent with a ping-pong mechanism similar to that of the pig kidney and human placental enzymes. The experiments described in this paper were conducted with oxygen fixed (air) and amine varied over such a range that the double reciprocal plots were approximately linear since our aim was to probe the nature of the substrate binding site.

All the onium compounds tested were inhibitors of human kidney diamine oxidase producing slope effects, supporting the previous contention [9, 10] that the positively charged onium group competes with substrate for a negatively charged binding site on the enzyme. Since each mono-onium compound and its corresponding bis-oniums give comparable values of $K_{i(slope)}$, it is reasonable to suppose that only one of the two onium groups binds to the enzyme in an end-on manner.

Since replots of slopes and intercepts were linear, we can assume that no complexes of the type $E + 2I = EI_2$ can occur and that none of these inhibitors were partial inhibitors, i.e. giving alternative reaction pathways.

From the $K_{i(slope)}$ results we can obtain an approximate value for the free energy of interaction between positively charged onium species and the negative charge at the substrate-binding site: guanidinium species, 28–38 J $mole^{-1}$; isothiouronium species, 34–38 J $mole^{-1}$; dimethylsulphonium species, 17–30 J $mole^{-1}$; trimethylammonium species, 14–26 J $mole^{-1}$.

An approximate free energy of interaction producing intercept effects would be: isothiouronium species, 31–33 J $mole^{-1}$; guanidinium species, 32–39 J $mole^{-1}$.

Monoamine oxidase inhibitors. Work in this laboratory has indicated that certain monoamine oxidase inhibitors are also inhibitors of diamine oxidase [10, 11]. We have tested several of these drugs as inhibitors of human kidney diamine oxidase and have found them to be inhibitory. Slope/intercept replots for these compounds were generally non-linear and this fact, coupled with their great structural variation, makes it difficult to assign a mode of inhibition, though these findings, coupled with the knowledge that some of these compounds produce time-dependent inhibition of DAO, confirm our suspicions that they can no longer be regarded as specific inhibitors of monoamine oxidase.

Time-dependent inhibition. We have recently shown that inhibition of hog kidney and human placental diamine oxidases by carbonyl group reagents gives non-linear semi-logarithmic plots [17] and a general theory for this has been developed [18]. Figure 6 shows that the human kidney enzyme gives similar time-dependent inhibition by carbonyl group reagents.

CONCLUSION

The inhibition studies described in this paper lead us to conclude that the enzyme that we have isolated and purified from human kidney is closely similar to the well characterised enzymes isolated from hog kidney and human placenta. It seems to have a negatively charged substrate binding site which interacts with mono- and bis-onium compounds in a similar way to the other diamine oxidases and, in addition, would appear to have an active site carbonyl group. Once again the appreciable diamine oxidase inhibition by monoamine oxidase inhibitors was demonstrated.

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cology, University of Manchester for support and laboratory facilities, to the staff of the Department of Surgical Histology, Manchester Royal Infirmary for the provision of kidneys and to the Medical Research Council for a grant towards the purchase of a Cary 118C spectrophotometer used in this study. We also acknowledge the helpful comments of Dr. K. F. Tipton, Department of Biochemistry, University of Cambridge, during the preparation of this manuscript.

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