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The Inhibition of Human Placental Diamine Oxidase by Substrate Analogues

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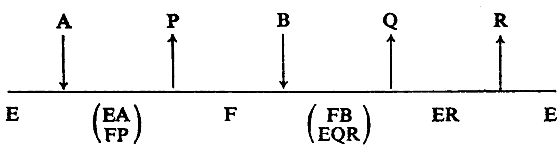
1. The oxidation of *p*-dimethylaminomethylbenzylamine by purified placental diamine oxidase was followed by measuring the change in E_{250} caused by the production of *p*-dimethylaminomethylbenzaldehyde. 2. The inhibition of this reaction by substrate analogues such as isothiouronium, guanidinium, dimethylsulphonium and trimethylammonium compounds was extensively studied. 3. The type and degree of inhibition by mono- and bis-onium compounds is described, and a theory is developed to explain the type of inhibition produced.

The study of enzyme inhibition by substrate analogues is a useful tool in probing structure–function relationships in enzyme action, and has been used to considerable effect with the diamine oxidase isolated from pig kidney (Bardsley & Ashford, 1972). The diamine oxidase (EC 1.4.3.6) which we have isolated and purified from the human placenta has not been studied in such detail, but appears to be of the same type and probably has a protective function in pregnancy, to ensure that concentrations of biogenic amines in the placental microcirculation do not become elevated. It would thus be of interest to the clinician to know if drugs used in pregnancy could modify the activity of the enzyme, and therefore we present results from a study of the effect of substrate analogues on the placental diamine oxidase.

Diamine oxidase catalyses the reaction between two substrates, A and B (diamine and O_2), and three products, P, Q and R (an aminoaldehyde, H_2O_2 and NH_3). A kinetic study of the enzyme from human placenta indicates a Ping Pong Bi Ter mechanism with the following sequence:



where E and F are different enzyme forms (Bardsley *et al.*, 1974).

We have tested both mono- and bis-onium compounds as potential inhibitors of this enzyme, the former corresponding to monoamines (which are poor substrates of this enzyme), the latter to diamines [which are better substrates when the chain of methylene groups between the charged species is of the appropriate length, i.e. 4–5 methylene units (Bardsley *et al.*, 1970, 1971)]. We report values for K_i and ΔG^0, the standard free energy of the enzyme–inhibitor interaction, for a total of 44 substrate analogues.

Experimental

Materials

Preparation of inhibitors. All inhibitors were prepared by conventional methods (Bardsley & Ashford, 1972), the syntheses being unambiguous and proceeding in high yield.

Preparation of enzyme. Enzyme was purified from fresh placentae by the method of Bardsley *et al.* (1974). The purified enzyme, after column chromatography, had an average specific activity (units of enzyme/mg of protein) of 0.6, expressed as *p*-dimethylaminomethylbenzylamine oxidation at 20°C with air as the gaseous phase. The purest preparation we have obtained had a specific activity of 0.9.

Methods

Spectrophotometric method of assay. All experiments were conducted at 20°C in 0.05M-potassium phosphate buffer, pH 7.0, in a final volume of 1.0ml, containing 0.01 unit of enzyme, and appropriate concentrations of substrate and inhibitor. One unit of enzyme activity catalyses the oxidation of 1 μmol of substrate/min at 20°C in 0.05M-potassium phosphate buffer, pH 7.0, with air as the gaseous phase. Change in E_{250} was measured by using a Cary 118C spectrophotometer as described by Bardsley *et al.* (1972). The initial rate of change in E_{250} was proportional to the amount of enzyme used.

Graphical methods and equations. Initial rates were obtained over a period of 15–20min with five substrate concentrations, and double-reciprocal plots were used to obtain K_i values (mM) by using the expression:

$$\text{Slope (or intercept) with inhibitor ([I], mM)} = \text{slope (or intercept) without inhibitor present} \times \left(1 + \frac{[I]}{K_{i\,\text{slope (or intercept)}}}\right)$$

ΔG^0, the standard free energy of the enzyme–inhibitor interaction for $E+I = EI$, was calculated from:

$$\Delta G^0 = RT \cdot \ln K_i$$

Linear double-reciprocal plots were obtained in all cases.

In the absence of products, the reciprocal initial velocity, $1/v$, is given by:

$$\frac{1}{v} = \text{slope} \cdot \frac{1}{[A]} + \text{intercept}$$

In the presence of inhibitor, this expression becomes:

$$\frac{1}{v_i} = \text{slope}\left(1 + \frac{[I]}{K_{i\,\text{slope}}}\right)\frac{1}{[A]} + \text{intercept}\left(1 + \frac{[I]}{K_{i\,\text{intercept}}}\right)$$

where v is the velocity in the absence of inhibitor, v_i is the velocity in the presence of inhibitor, [A] is the concentration of substrate and [I] is the concentration of inhibitor. $K_{i\,\text{slope}}$ and $K_{i\,\text{intercept}}$ cannot be assumed to be simple enzyme–inhibitor dissociation constants, but may be complex constants. True K_i values can also be obtained from replots of slope or intercept against [I], as the intercept of such a replot on the horizontal axis (i.e. zero slope or intercept) is $-K_i$, if the replot is linear. Where such replots were available, a comparison of K_i values obtained by both methods showed them to be practically identical.

Results

Table 1 records the inhibitors used in the present study and the type of inhibition produced, together with the K_i and ΔG^0 values for the effect on the intercept and/or slope.

Typical plots of reciprocal velocity against reciprocal substrate concentration are shown for dimethylsulphonium compounds (Fig. 1), isothiouronium compounds (Fig. 2), guanidinium compounds (Fig. 3) and trimethylammonium compounds (Fig. 4).

Typical replots of the slope of the double-reciprocal graphs against inhibitor concentration are shown in Fig. 5, and Fig. 6 illustrates some intercept replots. Figs. 7, 8, 9 and 10 show how the effect of inhibitor on the intercept and/or slope varies with chain length in the homologous series of polymethylene bis-onium compounds, and also give K_i values for various mono-onium compounds, for comparison.

The experimental results obtained can be summarized as follows.

1. Sulphonium compounds give competitive inhibition (*S*-methyltetrahydrothiophenyl iodide, which gives non-competitive inhibition, is the only exception).
2. Trimethylammonium compounds give competitive inhibition (the $n = 12$ bistrimethylammonium compound, which gives non-competitive inhibition, is the only exception).
3. All other bis-onium compounds give non-competitive inhibition.
4. Most other mono-onium compounds give non-competitive inhibition (see point 6).
5. All *p*-xylylene bis-onium compounds give non-competitive inhibition except for the bisdimethylsulphonium compound.
6. Two compounds give uncompetitive inhibition, *S*-methylisothiouronium iodide ($K_{i\,\text{intercept}} = 0.15$ mM) and ethylamine ($K_{i\,\text{intercept}} = 0.64$ mM).
7. 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 5–8 methylene units for maximum inhibition. A separation of 12 methylene units also gives potent inhibition.
8. In any series, bis-onium compounds are not generally more inhibitory than mono-onium compounds.
9. Replots of intercepts and/or slopes against inhibitor concentration were linear for the following compounds: trimethylsulphonium iodide, pentamethylenebisdimethylsulphonium dibromide, *p*-xylylenebisdimethylsulphonium dibromide, methylisothiouronium iodide, pentamethylenebisisothiouronium dibromide, *p*-xylylenebisisothiouronium dibromide, methylguanidine, pentamethylenebisguanidinium dihydrobromide, tetramethylammonium chloride, pentamethylenebistrimethylammonium dibromide, *p*-xylylenebisdimethylammonium dihydrochloride, ammonium chloride, ethylamine hydrochloride.

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

K_i values (mM) were obtained from plots of reciprocal initial velocity against reciprocal substrate concentration (see the text for details). Assays contained 0.01 unit of enzyme with appropriate concentrations of *p*-dimethylaminomethylbenzylamine and inhibitor in a volume of 1 ml, and initial rates were determined at 20°C by measuring ΔE_{250}. Values for the standard free energy of the enzyme–inhibitor interaction (ΔG^0) were calculated as described in the text. NC, non-competitive inhibition; UC, uncompetitive inhibition; C, competitive inhibition. Values of *n* refer to the number of methylene groups separating the charged species in bis-onium compounds.

Inhibitor	Type of inhibition	$K_{i\,slope}$ (mM)	$-\Delta G^0$ (kJ·mol^{-1})	$K_{i\,intercept}$ (mM)	$-\Delta G^0$ (kJ·mol^{-1})
Dimethylsulphonium compounds					
$n=3$	C	0.625	17.97	∞	—
$n=4$	C	0.225	20.46	∞	—
$n=5$	C	0.166	21.20	∞	—
$n=8$	C	0.0495	24.15	∞	—
$n=10$	C	0.0833	22.88	∞	—
$n=12$	C	0.0217	26.16	∞	—
Trimethylsulphonium iodide	C	0.3623	19.30	∞	—
Bisbenzyl-*S*-methylsulphonium iodide	C	0.33	19.53	∞	—
Di-*n*-propyl-*S*-methylsulphonium iodide	C	0.0392	24.71	∞	—
S-Methyltetrahydrothiophenyl iodide	NC	0.138	21.65	0.357	19.33
Isothiouronium compounds					
$n=3$	NC	0.0026	28.89	0.018	26.61
$n=4$	NC	0.00162	32.48	0.033	25.14
$n=5$	NC	0.0031	30.89	0.022	26.124
$n=8$	NC	0.00184	32.17	0.022	26.124
$n=10$	NC	0.0023	31.63	0.142	21.582
$n=12$	NC	0.00172	32.33	0.022	26.124
S-Methylisothiouronium iodide	UC	∞	—	0.015	27.05
NN'S-Trimethylisothiouronium iodide	NC	0.454	18.75	0.0217	26.16
NN'-Bisbenzyl-*S*-methylisothiouronium iodide	NC	0.0166	26.81	0.023	26.02
NNN'N'S-Pentamethylisothiouronium iodide	C	0.277	19.95	∞	—
Guanidinium compounds					
$n=3$	NC	0.0056	29.61	0.011	27.81
$n=4$	NC	0.00175	32.29	0.016	26.90
$n=5$	NC	0.00198	31.99	0.011	27.81
$n=8$	NC	0.033	25.14	0.04	24.67
$n=10$	NC	0.04	24.67	0.05	24.12
$n=12$	NC	0.002	31.96	0.0142	27.19
Guanidine	NC	0.0616	23.61	0.280	19.93
Methylguanidine	NC	0.089	22.72	0.0147	27.106
Trimethylammonium compounds					
$n=3$	C	1.96	15.18	∞	—
$n=4$	C	6.25	12.36	∞	—
$n=5$	C	3.47	13.79	∞	—
$n=8$	C	1.40	16.00	∞	—
$n=10$	C	1.60	15.68	∞	—
$n=12$	NC	0.75	17.53	4.16	13.35
Tetramethylammonium chloride	C	1.25	16.28	∞	—
Aromatic bis-onium compounds					
p-Xylylenebisdimethylsulphonium dibromide	C	0.0266	25.66	∞	—
p-Xylylenebisdimethylisothiouronium dibromide	NC	0.00419	30.16	0.033	25.137
p-Xylylenebisdimethylammonium dihydrochloride	NC	0.2283	20.43	3.33	13.97
Miscellaneous compounds					
o-Xylylenediamine	NC	0.5747	18.175	2.77	14.34
m-Xylylenediamine	NC	0.4504	18.77	1.85	15.32
Imidazole	NC	0.00563	29.46	0.0105	27.93
N-Methylimidazole	NC	0.0065	29.09	0.0097	28.12
Methylamine	NC	0.159	21.31	1.25	16.28
Ethylamine	UC	∞	—	0.64	17.9
Trimethylamine	NC	0.740	17.56	3.125	14.04
Ammonium chloride	C	9.0	11.475	∞	—
Tranylcypramine	NC	0.0874	22.76	0.1618	21.26
Harmine	NC	0.0126	27.48	0.128	21.83

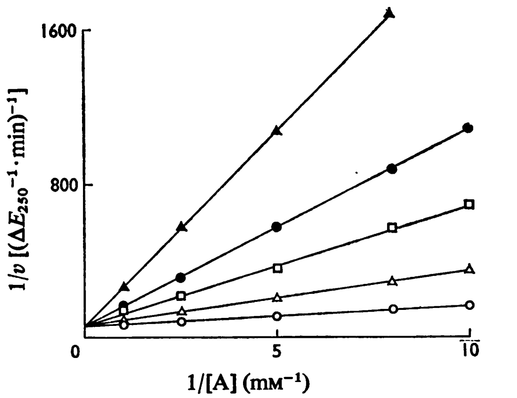


Fig. 1. *Double-reciprocal plots illustrating inhibition of the oxidation of p-dimethylaminomethylbenzylamine by dimethylsulphonium compounds*

For experimental details see the text. ○, *p*-Dimethylaminomethylbenzylamine; △, +trimethylsulphonium iodide (1.0mM); □, +*p*-xylylenebisdimethylsulphonium dibromide (0.08mM); ●, +pentamethylene-1,5-bisdimethylsulphonium dibromide (0.5mM); ▲, +dodecamethylene-1,12-bisdimethylsulphonium dibromide (0.5mM).

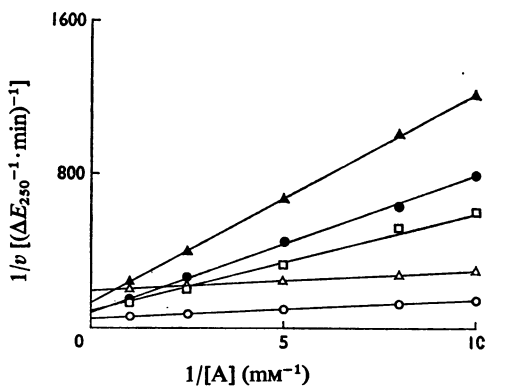


Fig. 2. *Double-reciprocal plots illustrating inhibition of the oxidation of p-dimethylaminomethylbenzylamine by isothiouronium compounds*

For experimental details see the text. ○, *p*-Dimethylaminomethylbenzylamine; △, +*S*-methylisothiouronium iodide (0.04mM); □, +*p*-xylylenebisisothiouronium dibromide (0.02mM); ●, +pentamethylene-1,5-bisisothiouronium dibromide (0.02mM); ▲, +dodecamethylene-1,12-bisisothiouronium dibromide (0.02mM).

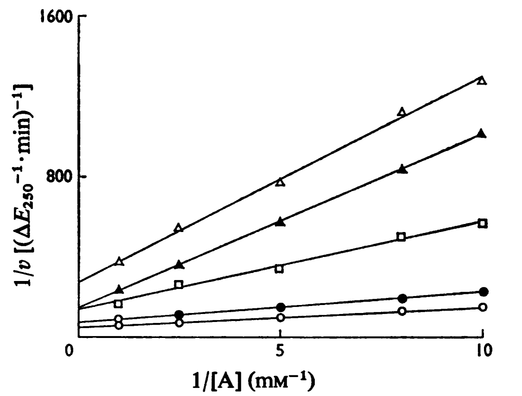


Fig. 3. *Double-reciprocal plots illustrating inhibition of the oxidation of p-dimethylaminomethylbenzylamine by guanidinium compounds*

For experimental details see the text. ○, *p*-Dimethylaminomethylbenzylamine; △, +methylguanidine (0.2mM); □, +trimethylene-1,3-bisguanidinium dihydrobromide (0.02mM); ●, +octamethylene-1,8-bisguanidinium dihydrobromide (0.02mM); ▲, +dodecamethylene-1,12-bisguanidinium dihydrobromide (0.02mM).

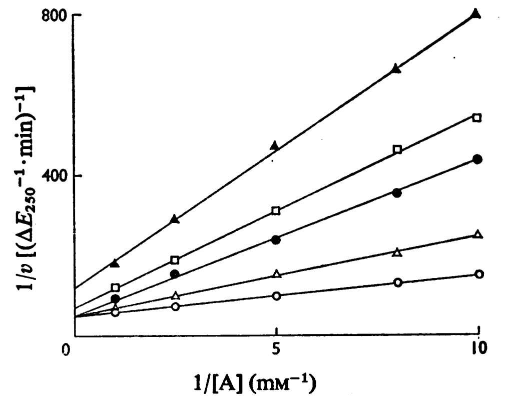


Fig. 4. *Double-reciprocal plots illustrating inhibition of the oxidation of p-dimethylaminomethylbenzylamine by trimethylammonium compounds*

For experimental details see the text. ○, *p*-Dimethylaminomethylbenzylamine; △, +tetramethylammonium chloride (0.5mM); □, +*p*-xylylenebisdimethylammonium dihydrochloride (1.0mM); ●, +octamethylene-1,8-bistrimethylammonium dibromide (5.0mM); ▲, +dodecamethylene-1,12-bistrimethylammonium dibromide (5.0mM).

Discussion

In analysing the results summarized, we shall consider the importance of slope and intercept effects on double-reciprocal plots. Where $K_{i\,\mathrm{intercept}}$ is very large, only slope effects can be detected (competitive inhibition); where $K_{i\,\mathrm{slope}}$ is very large, only intercept effects are observed (uncompetitive inhibition); all other cases will be referred to as non-competitive inhibition (both slope and intercept effects). Slope

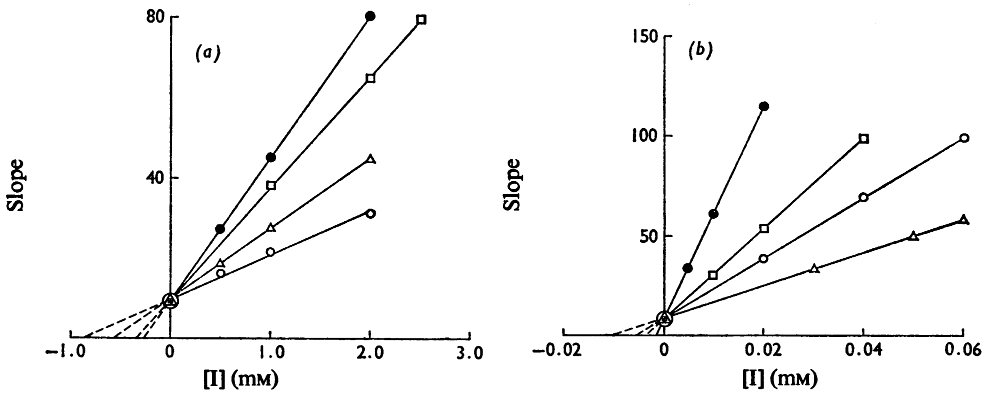


Fig. 5. *Variation of the slope of double-reciprocal plots* (1/*v against* 1/*s*) *with concentration,* [*I*] (*m*M), *of inhibitor*

For experimental details see the text. (*a*) ○, Tetramethylammonium chloride; △, trimethylsulphonium iodide; □, *p*-xylenebisdimethylammonium dibromide; ●, methylguanidine. (*b*) ○, *p*-Xylylenebisisothiouronium dibromide; △, pentamethylene-1,5-bisdimethylsulphonium dibromide; □, pentamethylene-1,5-bisisothiouronium dibromide; ●, pentamethylene-1,5-bisguanidinium dihydrobromide.

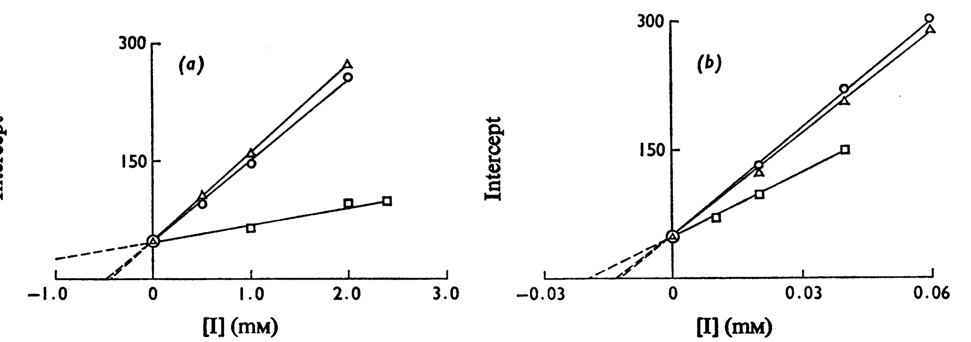


Fig. 6. *Variation of the intercept of double-reciprocal plots* (1/*v against* 1/*s*) *with concentration,* [*I*] (*m*M), *of inhibitor*

For experimental details see the text. (*a*) ○, Ethylamine; △, methylguanidine; □, *p*-xylylenebisdimethylammonium dibromide. (*b*) ○, *p*-Xylylenebisisothiouronium dibromide; △, *S*-methylisothiouronium iodide; □, pentamethylene-1,5-bisisothiouronium dibromide.

effects are assumed to be due to the variable substrate and inhibitor competing for the same binding site, whereas intercept effects are assumed to be due to the reaction of the inhibitor with enzyme forms to which the variable substrate does not bind.

Slope effects

The results obtained previously (Bardsley *et al.*, 1974) and in the present paper lead us to postulate a negatively charged substrate-binding site on the enzyme surface, similar to that found with pig kidney diamine oxidase, to which one positively charged group of the substrate binds for oxidation.

For the pig kidney enzyme, the amino group to be oxidized then reacts with pyridoxal phosphate at the oxidizing site to form a Schiff base, after which oxidation of the amine takes place.

All the compounds used in this study, being positively charged, can presumably form enzyme–inhibitor (EI) complexes by combining with the negative charge on the substrate-binding site. As $K_{i_{slope}}$ is fairly constant within any one family of bis-onium compounds, and similar to the value for related mono-onium compounds (Table 1 and Figs. 7, 8, 9 and 10), it would appear that the bis-onium compounds react end-on, with only one onium group competing with the substrate for the negative charge

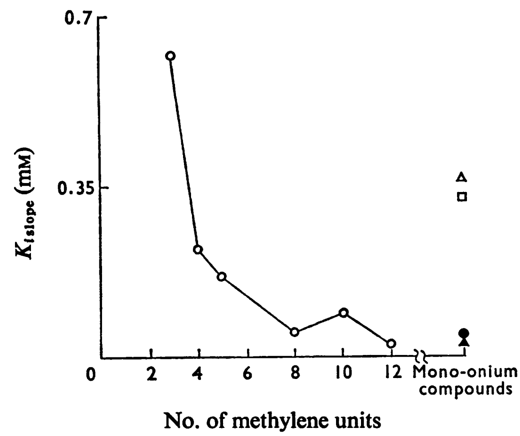


Fig. 7. *Variation of $K_{i\,slope}$ (mM) with chain length for a homologous series of dimethylsulphonium compounds*

For experimental details see the text. ○, Bisdimethylsulphonium series; △, trimethylsulphonium iodide; □, bisbenzyl-*S*-methylsulphonium iodide; ●, di-*n*-propylmethylsulphonium iodide; ▲, *p*-xylylenebisdimethylsulphonium dibromide.

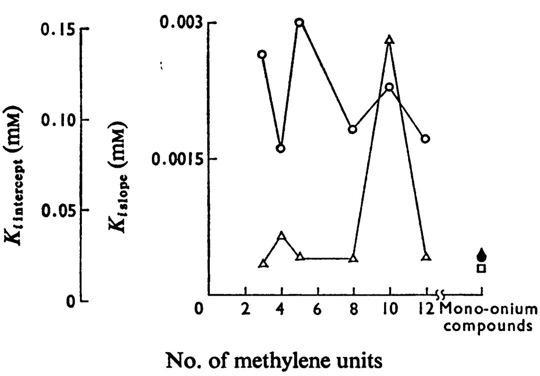


Fig. 8. Variation of $K_{i\,slope}$ (mM) *and* $K_{i\,intercept}$ (mM) *with chain length for a homologous series of isothiouronium compounds*

For experimental details see the text. ○, $K_{i\,slope}$, bisisothiouronium series; △, $K_{i\,intercept}$, bisisothiouronium series; □, $K_{i\,intercept}$, *S*-methylisothiouronium iodide; ●, $K_{i\,intercept}$, *NNN'N'S*-pentamethylisothiouronium iodide; ▲, $K_{i\,intercept}$, *NN'*-bisbenzyl-*S*-methylisothiouronium iodide.

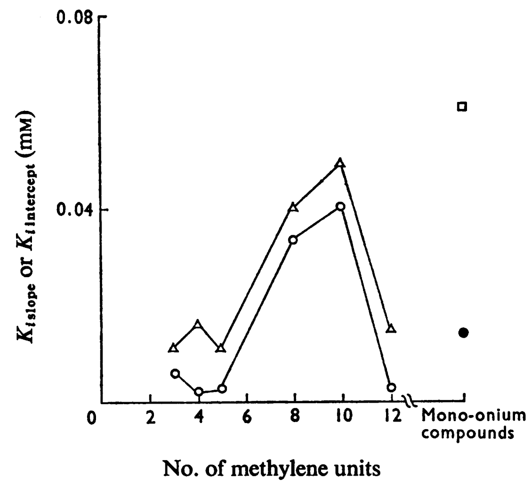


Fig. 9. *Variation of $K_{i\,slope}$ (mM) and $K_{i\,intercept}$ (mM) with chain length for a homologous series of guanidinium compounds*

For experimental details see the text. ○, $K_{i\,slope}$, bisguanidinium series; △, $K_{i\,intercept}$, bisguanidinium series; □, $K_{i\,slope}$, guanidine; ●, $K_{i\,intercept}$, methylguanidine.

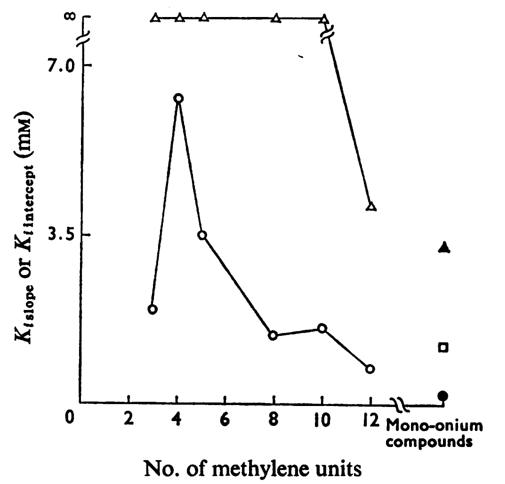


Fig. 10. *Variation of $K_{i\,slope}$ (mM) and $K_{i\,intercept}$ (mM) with chain length for a homologous series of trimethylammonium compounds*

For experimental details see the text. ○, $K_{i\,slope}$, bistrimethylammonium series; △, $K_{i\,intercept}$, bistrimethylammonium series; □, $K_{i\,slope}$, tetramethylammonium chloride; ●, $K_{i\,slope}$, *p*-xylylenebisdimethylammonium dibromide; ▲, $K_{i\,intercept}$, *p*-xylylenebisdimethylammonium dibromide.

on the substrate-binding site. By postulating another negative charge on the enzyme surface situated at a distance of 5–8 methylene units from the substrate-binding site, and a further negative charge at a distance of 12 methylene units, with a hydrophobic region between them, the variation of K_i with chain length of bis-onium compounds (Figs. 7, 8, 9 and 10) is explained.

From the $K_{i\,\mathrm{slope}}$ results, we can obtain an approximate value for the free energy of interaction between a positively charged onium species and the negative charge at the substrate-binding site: guanidinium species, 22.72–32.29 kJ·mol^{-1}; isothiouronium species, 18.75–32.48 kJ·mol^{-1}; dimethylsulphonium species, 17.97–26.16 kJ·mol^{-1}; trimethylammonium species, 12.36–17.53 kJ·mol^{-1}.

As all replots of slopes and intercepts were linear, we conclude that no complexes of the type:

$$E+2I = EI_2$$

can occur, and that none of the compounds tested were partial inhibitors, i.e. giving alternative reaction pathways.

Intercept effects

If we assume that intercept effects are due to the combination of inhibitor with form F of the enzyme in the Ping Pong Bi Ter sequence, then $F+I = FI$ would only be inhibitory (FI being a true dead-end complex) when I was an onium compound with the appropriately charged onium species. It would appear that sp^2 hybridized groups, i.e. isothiouronium, guanidinium and aromatic species, fulfil this requirement rather better than dimethylsulphonium and trimethylammonium species. This is in accordance with the results from the pig kidney diamine oxidase (Bardsley & Ashford, 1972).

An approximate measure of the free energy of interaction between inhibitor and form F would be: isothiouronium species, 21.58–27.05 kJ·mol^{-1}; guanidinium species, 24.12–27.81 kJ·mol^{-1}.

A surprising discovery was the effect of the so-called monoamine oxidase inhibitors, harmine and tranylcypramine, both of which were potent inhibitors of the purified placental diamine oxidase. Because of this, we examined the effect of tranylcypramine on purified pig kidney diamine oxidase, and found it to be a potent inhibitor of this enzyme also ($K_{i\,\mathrm{slope}}$ = 0.35 mM, $-\Delta G^0 = 19.38$ kJ·mol^{-1}; $K_{i\,\mathrm{intercept}}$ = 0.06 mM, $-\Delta G^0 = 23.68$ kJ·mol^{-1}). Similarly other so-called monoamine oxidase inhibitors (e.g. phenelzine, isoniazid and mebanazine) cause inhibition of diamine oxidase both from human placenta and pig kidney (M. J. C. Crabbe & W. G. Bardsley, unpublished work). We therefore conclude that compounds that were once regarded as specific inhibitors of monoamine oxidase can no longer be thought of in this light, owing to their potent effect on two enzymes of the diamine oxidase type.

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