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Inhibition of Pig Kidney Diamine Oxidase by Substrate Analogues

By W. G. BARDSLEY
Department of Biochemistry, University of Manchester Institute of Science and Technology, Manchester M60 1QD, U.K.

and J. S. ASHFORD
Department of Obstetrics and Gynaecology, University of Manchester, St. Mary's Hospital, Whitworth Park, Manchester M13 0HJ, U.K.

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1. The oxidation of *p*-dimethylaminomethylbenzylamine was followed spectrophotometrically by measuring the change in E_{250} caused by the *p*-dimethylaminomethylbenzaldehyde produced. 2. This reaction was inhibited by substrate analogues such as isothiouronium, guanidino, dimethylsulphonium and trimethylammonium compounds. 3. The inhibition by both mono- and bis-onium compounds has been studied and a comprehensive theory is developed to explain both the type and degree of inhibition produced.

The oxidation of diamines by diamine oxidase from pig kidney is decreased by many reagents. Carbonyl group reagents are thought to inhibit the enzyme action by reacting with pyridoxal, which is a necessary cofactor for the catalytic activity of this enzyme, metal-complexing reagents interfere with copper ions at the active site and many other compounds act as substrate analogues (Zeller, 1963).

Inhibition by substrate analogues has been widely used to interpret the mode of enzyme–substrate and enzyme–inhibitor interaction (Webb, 1966) and the most important compounds in this group are mono-onium compounds corresponding to monoamines (which are poor substrates of this enzyme) and bis-onium compounds corresponding to diamines (which are better substrates when the chain is of the correct length). The effect of chain length of bis-onium compounds in determining the inhibition potency has been studied by Blaschko *et al.* (1951), but only in a semi-quantitative manner (no K_i values were determined), with rather impure enzyme preparations.

The inhibition by several substrate analogues has been shown to be competitive, and double-reciprocal plots in the presence and absence of inhibitors are given for the 2-oxo analogues of putrescine and cadaverine (Macholán *et al.*, 1967) and for *m*-phenylene diamine (Costa *et al.*, 1971). Bulbocapnine also gives competitive inhibition (Chapman & Walaszek, 1962).

We have studied the effect of variation in chain length and other factors on the diamine oxidase reaction and have attempted to interpret the results in terms of a scheme for enzyme–substrate interaction (Bardsley *et al.*, 1970, 1971), in which one positively charged group of the substrate binds to a negatively charged substrate-binding site and the amino group to be oxidized reacts with pyridoxal phosphate at an oxidizing site. From both spectrophotometric (Mondovì *et al.*, 1967*a*) and chemical studies (Kumagai *et al.*, 1969) it is thought that Schiff-base formation occurs between the pyridoxal carbonyl group and the amino groups of the substrate before oxidation takes place. A new spectrophotometric method for following the enzyme-catalysed reaction has been developed and a highly purified enzyme has been obtained (Bardsley *et al.*, 1972). We now report a study of the effect of structure on inhibition potency by using this spectrophotometric method and 44 substrate analogues.

Diamine oxidase catalyses the reaction between two substrates A and B (diamine and oxygen) to produce three products, P, Q and R (an aminoaldehyde, hydrogen peroxide and ammonia). It has been suggested from indirect evidence (Rotilio *et al.*, 1970) that the oxidation reaction involves the production of superoxide anion radicals, and stopped-flow studies (Mondovì *et al.*, 1969) show that addition of substrate anaerobically produces a decrease in E_{500}. This reaction has a rapid phase representing enzyme–substrate-complex formation followed by a slow phase indicating reduction of the enzyme, as high oxygen concentrations caused it to disappear. Incubation of the enzyme with diamine ([^{14}C]cadaverine) under anaerobic conditions gives 0.9 mol of labelled piperidine/mol of enzyme, thus proving that the first substrate, A, can be stoicheiometrically converted into the first product, P, in the absence of the second substrate (Finazzi Agrò *et al.*, 1969). Also, these workers have shown that plots of reciprocal velocity against reciprocal diamine concentration in the presence of several fixed concentrations of oxygen are a family of parallel lines, indicating that only binary

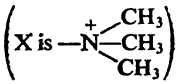
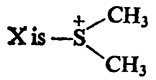
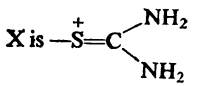
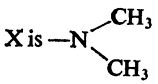
Table 1. *Inhibition by substrate analogues of the oxidation of p-dimethylaminomethylbenzylamine by pig kidney diamine oxidase*

K_I values (mM) were obtained from plots of reciprocal initial velocity against reciprocal substrate concentration by using the expression:

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

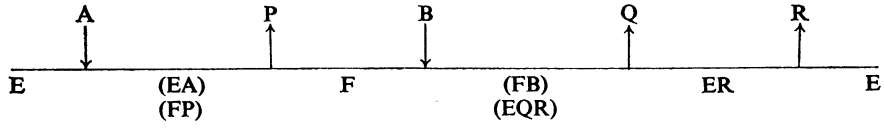
When replots of slope or intercept against [I] were available, K_I was calculated from the intercept on the horizontal axis. Enzyme (0.02 unit) was in a final vol. of 2.8ml and initial rates were determined at 20°C by measuring ΔE_{250}. NC, Non-competitive inhibition; C, competitive inhibition; ΔG^0 is the standard free energy of the enzyme–inhibitor interaction for E+I = EI calculated from $\Delta G^0 = RT \cdot \ln K_I$.

Inhibitor		Type of inhibition	$K_{I\ \text{slope}}$ (mM)	$-\Delta G^0$ (kJ/mol)	$K_{I\ \text{intercept}}$ (mM)	$-\Delta G^0$ (kJ/mol)
Bis-onium compounds of the type X—$[CH_2]_n$—X						
Bisdimethylsulphonium compounds (X is $-\overset{+}{S}(CH_3)_2$)	$n=3$	—	∞	—	∞	—
	$n=4$	C	3.85	13.6	∞	—
	$n=5$	C	4.2	13.3	∞	—
	$n=6$	C	1.7	15.5	∞	—
	$n=8$	C	2.2	14.9	∞	—
	$n=10$	C	4.5	13.2	∞	—
	$n=12$	C	4.1	13.4	∞	—
Bisisothiouronium compounds (X is $-\overset{+}{S}=C(NH_2)_2$)	$n=3$	NC	0.03	25.4	0.05	24.2
	$n=4$	C	0.042	24.6	∞	—
	$n=5$	NC	0.03	25.4	0.08	23.0
	$n=6$	NC	0.03	25.4	0.08	23.0
	$n=8$	NC	0.02	26.4	0.05	24.2
	$n=10$	NC	0.05	24.2	0.11	22.2
	$n=12$	NC	0.02	26.4	0.07	23.3
Bisguanidinium compounds (X is $-NH-C(=\overset{+}{N}H_2)-NH_2$)	$n=3$	NC	0.03	25.4	0.06	23.7
	$n=4$	C	0.03	25.4	∞	—
	$n=5$	NC	0.04	24.7	0.14	21.6
	$n=6$	NC	0.04	24.7	0.29	19.9
	$n=8$	NC	0.45	24.4	0.92	17.0

	$n = 10$	NC	0.43	24.5	0.60	18.1
	$n = 12$	C	0.01	28.1	∞	—
Bistrimethylammonium compounds 	$n = 3$	C	37	8.0	∞	—
	$n = 4$	C	126	5.1	∞	—
	$n = 5$	NC	54	7.1	183	4.1
	$n = 6$	NC	41	7.8	141	4.8
	$n = 8$	NC	15	10.2	24	9.1
	$n = 10$	NC	21	9.4	265	3.2
	$n = 12$	C	7.3	6.4	∞	—
Bis-*p*-xylylene compounds of the type X—CH_2—pC_6H_4—CH_2—X						
X is 		C	0.04	24.7	∞	—
X is 		NC	0.054	24.0	0.02	26.4
X is 		NC	5.2	12.8	8.1	11.7
Miscellaneous compounds						
o-Bis(aminomethyl)benzene (*o*-xylylenediamine)		NC	4.2	13.3	2.0	15.1
m-Bis(aminomethyl)benzene (*m*-xylylenediamine)		NC	6.3	12.4	0.04	24.7
Imidazole		NC	0.02	26.4	0.12	22.0
1-Methylimidazole		C	0.03	25.4	∞	—
S-Methylisothiouronium iodide		C	0.09	22.7	∞	—
S-Ethylisothiouronium bromide		C	0.26	20.1	∞	—
Trimethylsulphonium iodide		C	1.7	15.5	∞	—
Ethyldimethylsulphonium iodide		C	0.47	18.7	∞	—
Ammonium chloride		C	33.8	8.3	∞	—
Methylammonium chloride		C	2.5	14.6	∞	—
Ethylammonium chloride		C	9.3	11.4	∞	—
Trimethylammonium chloride		C	99	5.6	∞	—
Tetramethylammonium chloride		C	99	5.6	∞	—

enzyme–substrate complexes are involved. As ammonia gives competitive inhibition against diamine, as shown by a replot of the slope effect being linear (the present paper), it must be the last product to be released, i.e. R. Although hydrogen peroxide destroys this enzyme (Mondovì *et al.*, 1967*b*), we have shown that at low concentrations (up to 3mM) it gives uncompetitive inhibition against diamine as variable substrate, proving that it is released after the second substrate has added (W. G. Bardsley & J. S. Shindler, unpublished work).

There seems to be only one scheme that is consistent with these product-inhibition patterns and partial reactions at the molecular level and that is a Ping Pong Bi Ter reaction with two enzyme forms E and F and ordered release of products as in the following scheme (Cleland, 1963*a*,*b*,*c*) where A is a diamine, B is oxygen, P is an aminoaldehyde, Q is hydrogen peroxide and R is ammonia:



We have determined the full range of product inhibition patterns and substrate patterns and although these generally agree with this scheme (e.g. ammonia against oxygen at fixed amine concentration is uncompetitive) there are certain complications. Both substrates give substrate inhibition and, whereas this is normal for Ping Pong mechanisms (Cleland, 1970), it limits the usefulness of product inhibition studies, especially in this case, as *p*-dimethylaminomethylbenzaldehyde promotes substrate inhibition by *p*-dimethylaminomethylbenzylamine, and hydrogen peroxide promotes substrate inhibition by oxygen (W. G. Bardsley & J. S. Shindler, unpublished work).

Ping Pong kinetics have been demonstrated for the following amine oxidases: pig plasma (Taylor & Knowles, 1971), pig brain (Tipton, 1968), tyramine oxidase of *Sarcina lutea* (Kumagai *et al.*, 1971), bovine liver (Oi *et al.*, 1971) and bovine plasma (Oi *et al.*, 1970).

Materials and Methods

Preparation of enzyme

Enzyme was prepared as described by Bardsley *et al.* (1972); the purest preparations had a specific activity of 1.73 units/mg at 37°C and the average specific activity was 1.48. As these units are some 20% larger than units expressed as cadaverine oxidation (cadaverine is a better substrate than *p*-dimethylaminomethylbenzylamine) and as the purest enzyme used previously for kinetic work (Costa *et al.*, 1971) had a specific activity of only 1.25 expressed as cadaverine oxidation, it seems that the enzyme used in the present work must be regarded as the purest yet obtained.

Spectrophotometric method

All experiments were conducted at 20°C in 0.1M-potassium phosphate buffer, pH 7.0, in a final volume of 2.8ml containing enzyme (0.02 unit) and an appropriate concentration of substrate and inhibitor. Change in E_{250} was measured as described by Bardsley *et al.* (1972).

Preparation of inhibitors

These were all prepared by conventional methods, the syntheses being unambiguous and proceeding in high yield. All compounds were crystallized until melting points were constant and the t.l.c. behaviour showed them to be pure.

Dimethylsulphonium compounds. The alkyl halide was dissolved in methanol, a large excess of dimethylsulphide was added and the mixture was kept in a stoppered flask at room temperature until t.l.c. showed that the reaction was complete (at least 3 weeks for dibromides but considerably less for alkyl iodides and *p*-xylylene dibromide). The solution was then evaporated to dryness and the residue recrystallized from ethanol.

Isothiouronium compounds. Stoicheiometric quantities of alkyl halide and thiourea were dissolved in ethanol and refluxed for 2h. After cooling, the crystalline product was filtered off and recrystallized from ethanol.

Trimethylammonium compounds. The alkyl dibromide was dissolved in ethanol and an equal weight of anhydrous trimethylamine was added. The mixture was left for 24h at room temperature in a stoppered flask and then the solution was evaporated to dryness and the residue was recrystallized from ethanol.

Guanidinium compounds. A solution of *S*-ethylisothiouronium bromide in ethanol was added dropwise to a stoicheiometric amount of diamine and the mixture was left at room temperature in a stoppered flask for 5 days. After evaporation of the solution to dryness, the residue was recrystallized from ethanol.

Results

Table 1 records the inhibitors used in the present work 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).

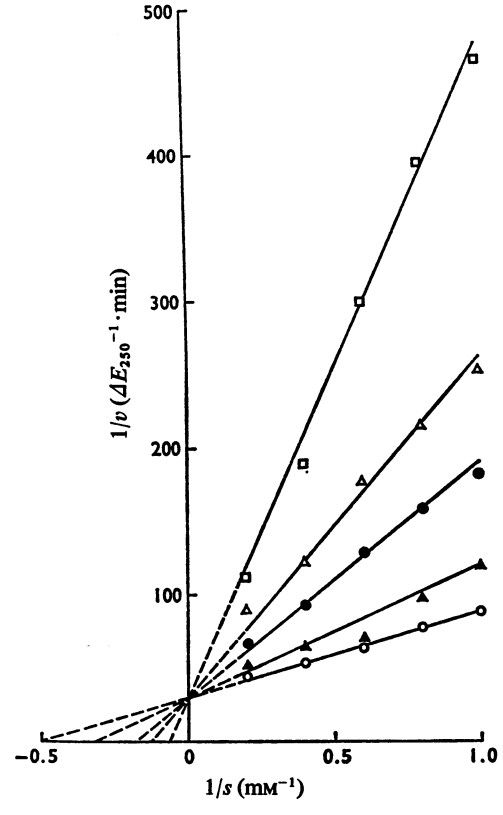


Fig. 1. *Double-reciprocal plots illustrating inhibition of the oxidation of p-dimethylaminomethylbenzylamine by dimethylsulphonium compounds*

For experimental details see the text. ○, *p*-Dimethylaminomethylbenzylamine; △, *p*-dimethylaminomethylbenzylamine + 1,4-tetramethylenebisdimethylsulphonium dibromide (7.5 mM); □, + 1,8-octamethylenebisdimethylsulphonium dibromide (10 mM); ●, + *p*-xylylenebisdimethylsulphonium dibromide (0.1 mM); ▲, + ethyldimethylsulphonium iodide (0.2 mM).

Replots of the slope against the inhibitor concentration are given for ammonium (Fig. 5*a*), trimethylenebisisothiouronium and tetramethylenebisguanidinium cations (Fig. 5*b*). Intercept replots are shown in Fig. 6 for trimethylenebisisothiouronium dibromide and *p*-xylylenebisisothiouronium dibromide.

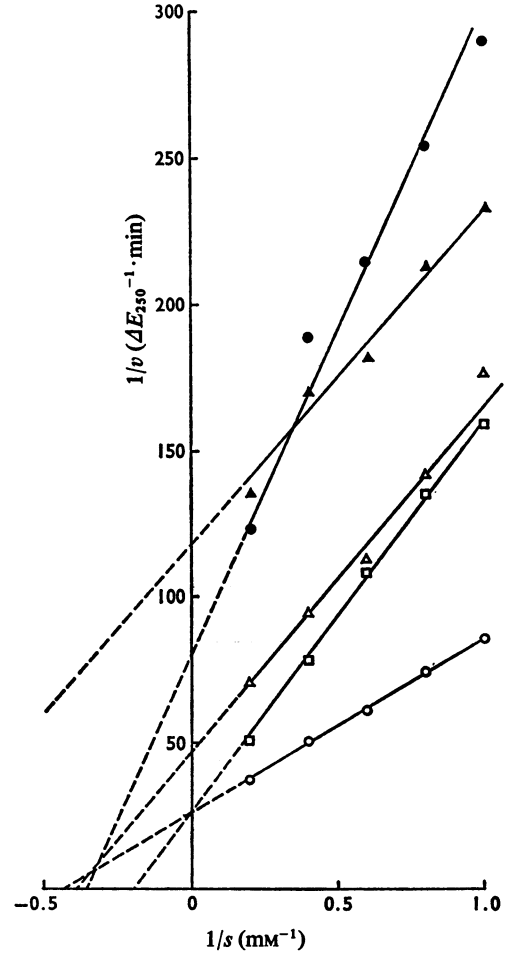


Fig. 2. *Double-reciprocal plots illustrating inhibition of the oxidation of p-dimethylaminomethylbenzylamine by isothiouronium compounds*

For experimental details see the text. ○, *p*-Dimethylaminomethylbenzylamine; △, *p*-dimethylaminomethylbenzylamine + 1,3-trimethylenebisisothiouronium dibromide (0.05 mM); □, + 1,4-tetramethylenebisisothiouronium dibromide (0.05 mM); ●, + 1,8-octamethylenebisisothiouronium dibromide (0.1 mM); ▲, + *p*-xylylenebisisothiouronium dibromide (0.025 mM).

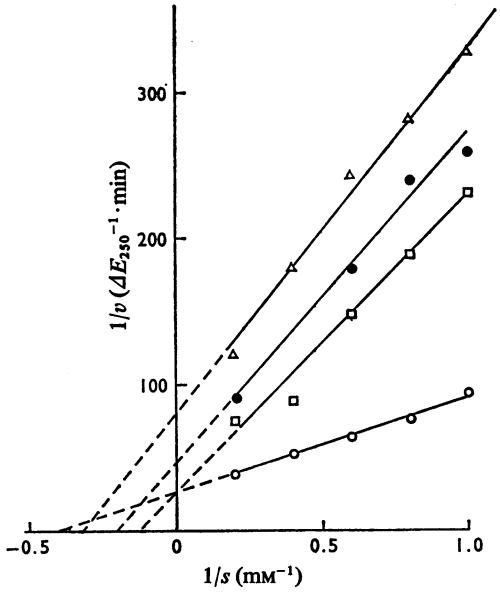


Fig. 3. *Double-reciprocal plots illustrating inhibition of the oxidation of p-dimethylaminomethylbenzylamine by guanidinium compounds*

For experimental details see the text. o, *p*-Dimethylaminomethylbenzylamine; △, *p*-dimethylaminomethylbenzylamine + 1,3-trimethylenebisguanidinium dibromide (0.1 mM); □, + 1,4-tetramethylenebisguanidinium dibromide (0.075 mM); ●, + 1,5-pentamethylenebisguanidinium bromide (0.1 mM).

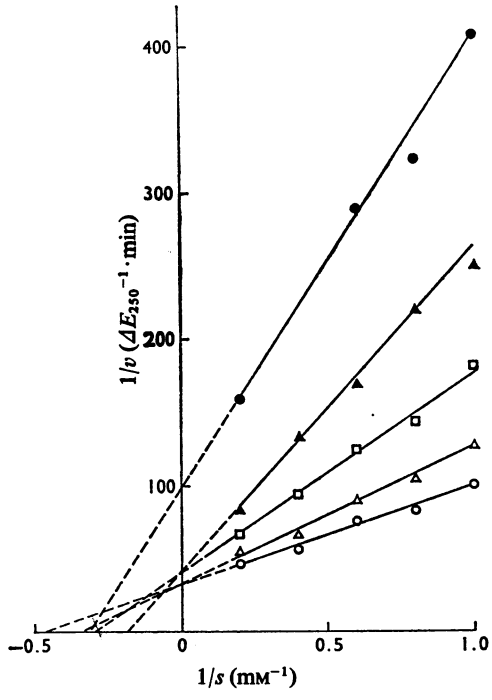


Fig. 4. *Double-reciprocal plots illustrating inhibition of the oxidation of p-dimethylaminomethylbenzylamine by trimethylammonium compounds*

For experimental details see the text. o, *p*-Dimethylaminomethylbenzylamine; △, *p*-dimethylaminomethylbenzylamine + 1,4-tetramethylenebistrimethylammonium dibromide (50 mM); □, + 1,5-pentamethylenebistrimethylammonium dibromide (50 mM); ●, + 1,8-octamethylenebistrimethylammonium dibromide (50 mM); ▲, + 1,10-decamethylenebistrimethylammonium dibromide (50 mM).

Figs. 7, 8, 9 and 10 show how the effect of inhibition on the intercept and/or slope varies with chain length in the homologous series of polymethylene bis-onium compounds.

Any comprehensive theory for the effect of substrate analogues as inhibitors must satisfy the experimental evidence now summarized.

1. All dimethylsulphonium compounds give competitive inhibition.

2. All tetramethylene bis-onium compounds give competitive inhibition.

3. Most other bis-onium compounds give non-competitive inhibition (the $n = 12$ bisguanidinium and bistetramethylammonium compounds give competitive inhibition, as does the $n = 3$ bistrimethylammonium compound).

4. Most of the mono-onium compounds give competitive inhibition (imidazole gives non-competitive inhibition and is the only exception).

5. All *p*-xylylene bis-onium compounds give non-competitive inhibition except for the bisdimethylsulphonium compound (1).

6. No uncompetitive inhibition was observed.

7. The inhibitor potency in any series is approximately isothiouronium = guanidinium ≫ dimethylsulphonium ≫ trimethylammonium. Bis-onium compounds are not generally more inhibitory than the mono-onium compounds and alteration of chain length or increased alkylation does not have a particularly marked effect.

8. Replots of intercepts and/or slopes against inhibitor concentration were linear for the following compounds: ammonia, trimethylsulphonium, tetramethylenebisdimethylsulphonium, methylisothiouronium, trimethylenebisisothiouronium, tetramethylenebisguanidinium, *p*-xylylenebisdimethylammonium, *p*-xylylenebisisothiouronium and tetramethylenebistrimethylammonium cations.

Discussion

We shall follow the definitions and excellent methods of Cleland (1963*a*,*b*,*c*) in attempting to analyse the slope and intercept effect produced on double-reciprocal plots. According to this author the reciprocal initial velocity, $1/v$, in the absence of products and presence of inhibitor, [I], is given by

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

In the present case [A] is the concentration of substrate, V is the maximal velocity and K_a is the apparent Michaelis constant for A at fixed oxygen concentration (air). $K_{i\,\text{slope}}$ and $K_{i\,\text{intercept}}$ are not assumed to be enzyme–inhibitor dissociation constants, but may be complex constants. The true K_i values can be obtained from replots of slope or intercept against [I], as the intercept of such a replot with the horizontal axis (zero slope or intercept) is $-K_i$, if the slope or intercept replot is linear. Parabolic replots could occur if the inhibitor reacted twice with the enzyme, and hyperbolic replots or replots with slopes of higher complexity could be produced by partial inhibitors.

Where $K_{i\,\text{intercept}}$ is very large there will only be slope effects (competitive inhibition); where $K_{i\,\text{slope}}$ is very large there will only be intercept effects (uncompetitive inhibition) and all other cases will be referred to as non-competitive inhibition (slope and intercept effects). Slope effects will be assumed to be due to the variable substrate and inhibitor competing for the same binding site and intercept effects will be assumed to be due to the reaction of the inhibitor with enzyme forms other than those with which the variable substrate combines.

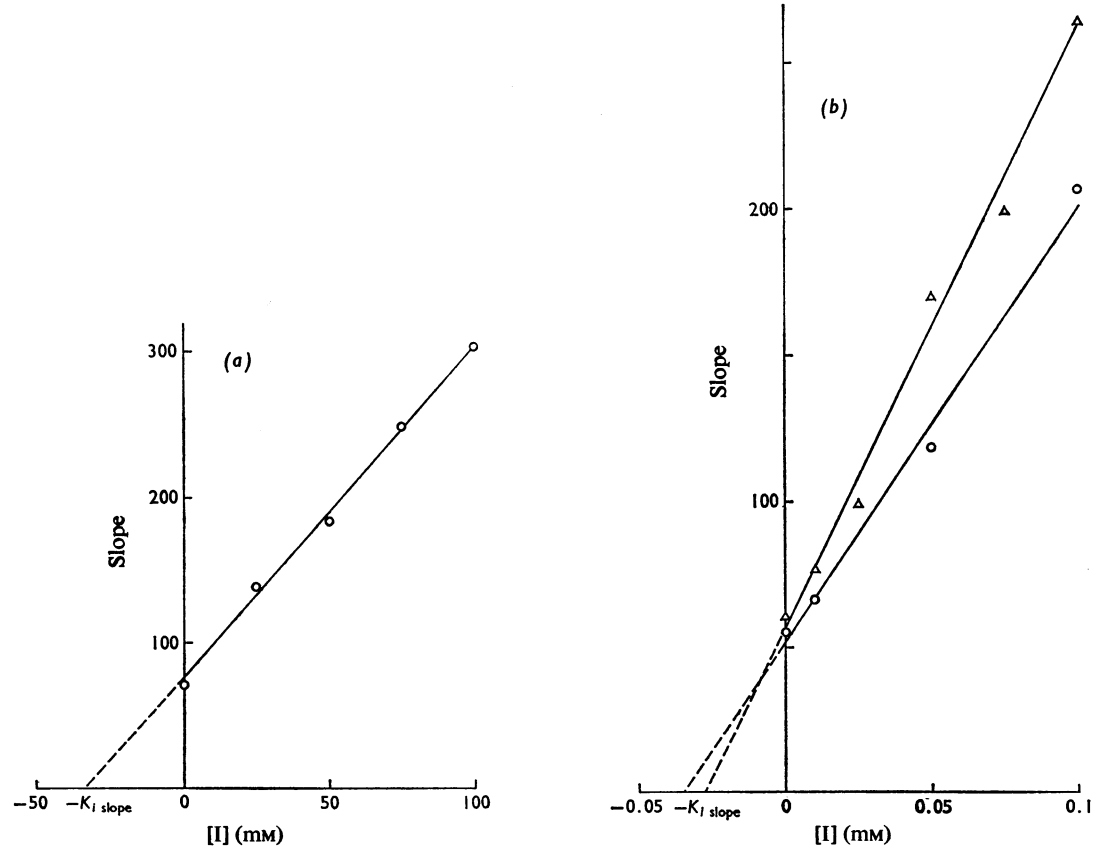


Fig. 5. *Variation of the slope of double-reciprocal plots* ($1/v$ *against* $1/s$) *with concentration,* [*I*] (*m*M), *of inhibitor*

For details see the text. (*a*) Ammonium chloride; (*b*) 1,3-trimethylenebisisothiouronium dibromide (○) and 1,4-tetramethylenebisguanidinium dibromide (△).

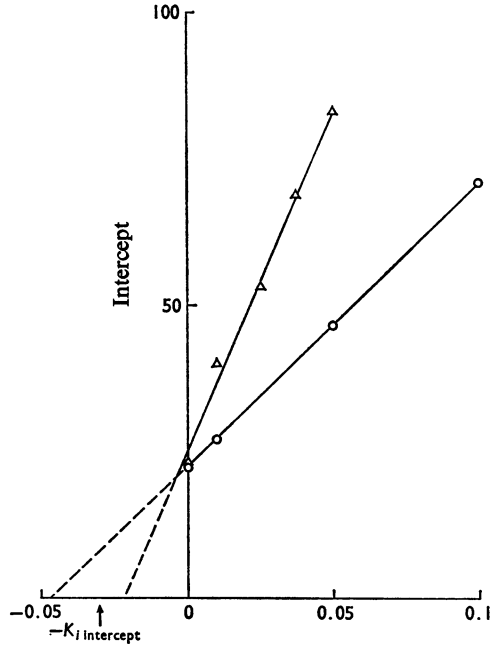


Fig. 6. *Variation of the intercept of double-reciprocal plots* (1/*v* *against* 1/*s*) *with concentration,* [*I*] (*m*M), *of inhibitor*

For details see the text. ○, 1,3-Trimethylenebisisothiouronium dibromide; △, *p*-xylylenebisisothiouronium dibromide.

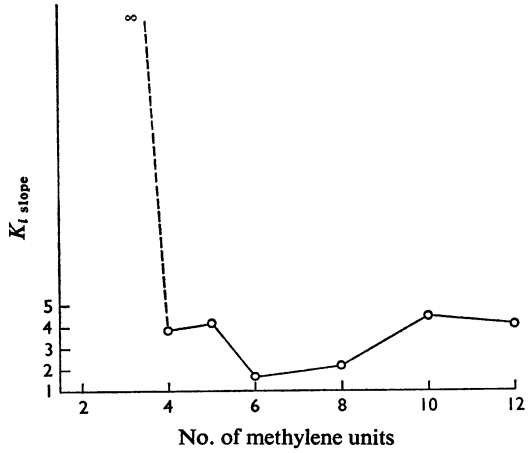


Fig. 7. *Variation of* $K_{i\ slope}$ (*m*M) *with chain length for a homologous series of bisdimethylsulphonium compounds*

For details see the text.

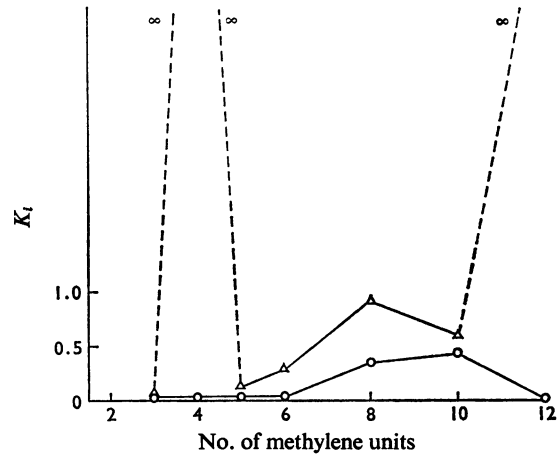


Fig. 8. *Variation of* $K_{i\ slope}$ (*m*M) (○) *and* $K_{i\ intercept}$ (*m*M) (△) *for a homologous series of bisguanidinium compounds*

For details see the text.

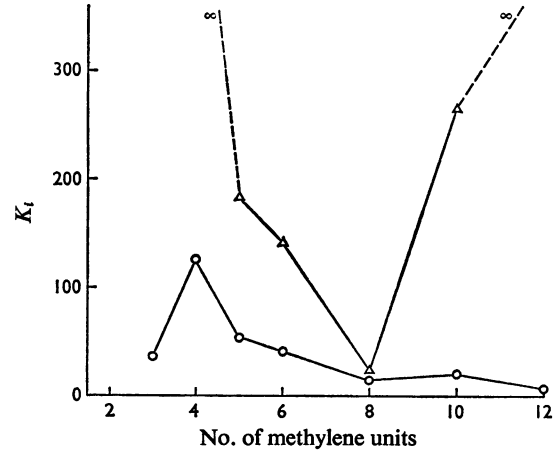


Fig. 9. *Variation of* $K_{i\ slope}$ (*m*M) (○) *and* $K_{i\ intercept}$ (*m*M) (△) *with chain length for a homologous series of bistrimethylammonium compounds*

For details see the text.

Slope effects

Presumably all the compounds discussed here, being positively charged, can form enzyme–inhibitor (EI) complexes by complexing with the substrate-binding site negative charge. As bis-onium compounds can only react with one-onium group, $K_{i\ slope}$ should be fairly constant within any family

of bis-onium compounds and similar to the value for the related mono-onium compound (Table 1 and Figs. 7, 8, 9 and 10). Thus we have explained generalization 6 and the slope effects described in generalizations 1, 2, 3, 4 and 5. Thus, the slope effect is simply produced by competition between the substrate and the mono-onium cation (or only one end of a bis-onium cation) for the negative charge on the enzyme and we have no need to postulate any other binding sites.

From the $K_{i\,slope}$ results, we can obtain a very approximate value for the free energy of interaction between a positively charged -onium group and the negative charge at the substrate-binding site as follows: guanidinium group 24.4–28.1kJ/mol, isothiouronium group 20.1–26.4kJ/mol, dimethylsulphonium group 13.2–24.7kJ/mol and trimethylammonium group 5.1–10.2kJ/mol.

From generalization 7 we conclude that no complexes of the type:

$$E+2I = EI_2$$

are formed and that none of the compounds tested were partial inhibitors. Fig. 11 illustrates the competition between substrate and -onium compounds producing the slope effect.

Intercept effects

It can be assumed that in enzyme form F the oxidizing site now contains pyridoxamine instead of pyridoxal. Therefore, the net charge at the oxidizing site at pH7.0 will be altered by +1. It is clear that the substrate-binding site now has a decreased free energy of interaction with -onium groups because of the increased positive charge at the oxidizing site and therefore complexing cannot occur so readily. Thus dead-end complexes are not easily formed between F and mono-onium compounds and so intercept effects are not seen, explaining generalization 4.

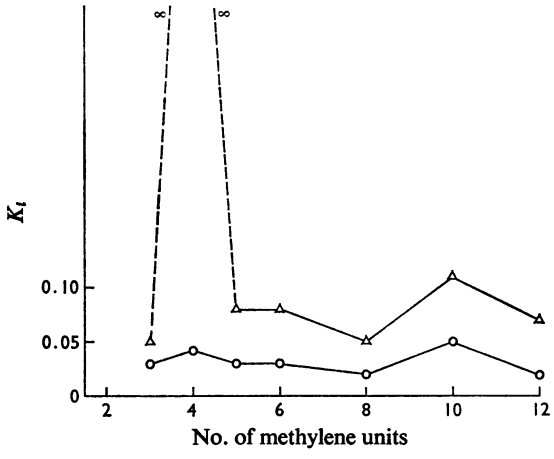


Fig. 10. *Variation of $K_{i\,slope}$ (mM) (○) and $K_{i\,intercept}$ (mM) (△) with chain length for a homologous series of bisisothiouronium compounds*

For details see the text.

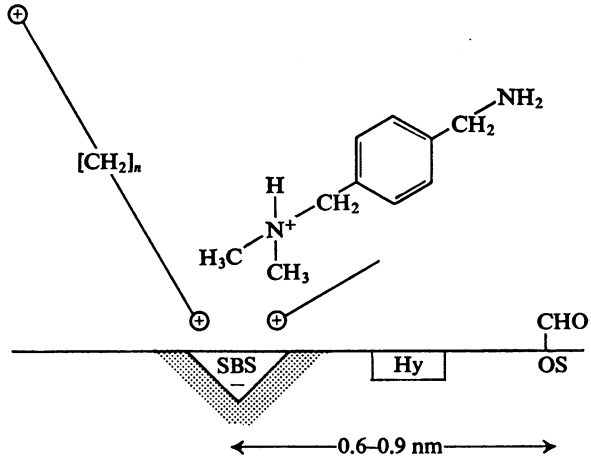


Fig. 11. *Competitive inhibition (slope effect) caused by dead-end complexing between enzyme form E and inhibitor*

SBS is the substrate binding site (a negative charge in a hydrophilic region, stippled); Hy is the hydrophobic binding site; OS is the oxidizing site (containing the pyridoxal carbonyl function as illustrated). ⊕——, a mono-onium compound, and ⊕——$[CH_2]_n$——⊕, a bis-onium compound, competing with substrate (dimethylaminomethylbenzylamine) for the substrate binding site.

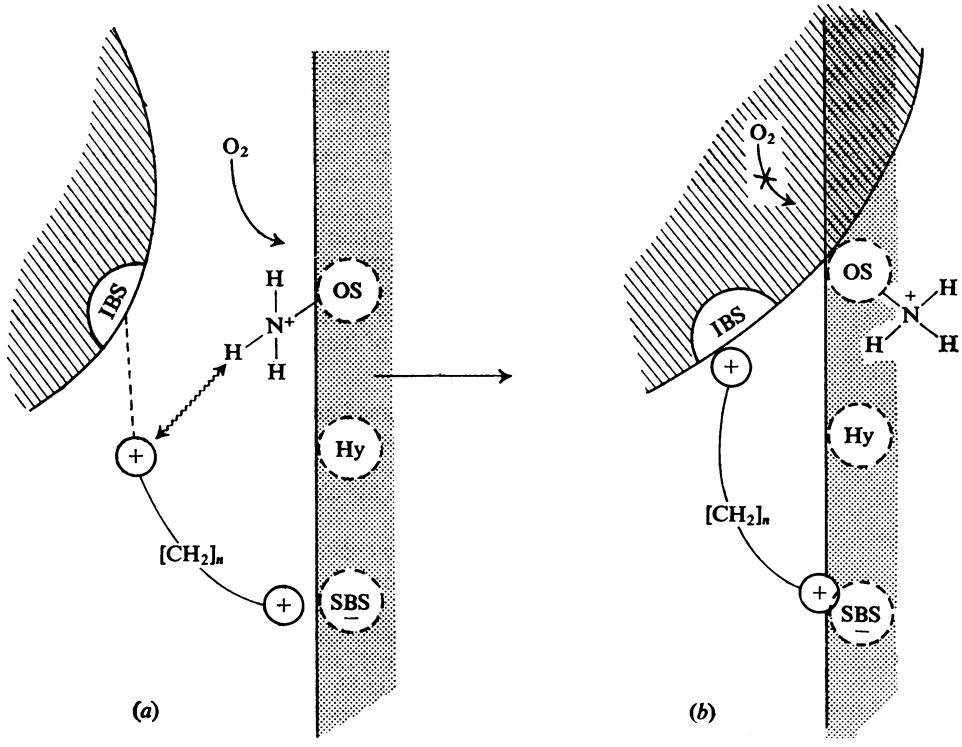


Fig. 12. *Explanation for intercept effects by dead-end complexing between F and inhibitor*

The oxidizing site (OS) is now binding pyridoxamine instead of pyridoxal and this has generated a positive charge. ⊕——$[CH_2]_n$——⊕ is a bis-onium compound complexing between the substrate-binding site (SBS) and the substrate-inhibitory-binding site (IBS). - - - - - - represents attraction between the -onium group and the IBS site, which has an affinity for isothiouronium > guanidinium > amino > dimethylsulphonium groups. ⟿ represents repulsion between the positively charged OS and the -onium group. When $n = 3$ and especially 4, this repulsion is sufficient to offset the attraction between the -onium groups and the IBS, which causes movement of the peptide chain bearing the IBS shown in (*a*). (*b*): This movement is shown as interfering with the interaction between the OS and oxygen, which would normally occur here to convert pyridoxamine into pyridoximine.

If complexing between some bis-onium compounds and the negative charge does slow the forward reaction down, then:

$$F + I = FI$$

would only be inhibitory (FI a true dead-end complex) when I is a suitable bis-onium compound. We have previously argued (Bardsley *et al.*, 1971) that adjacent to the substrate-binding site there is a substrate–inhibitor-binding site, which has an affinity for isothiouronium, guanidinium and aromatic systems, but has a much lower affinity for amino groups and dimethylsulphonium groups. If we conclude that complexing between both this substrate–inhibitor-binding site and the substrate-binding site is necessary for dead-end-complex formation with F, then, generalizations 1, 3, 4, 5 and 6 are satisfactorily explained and only generalization 2 remains to be explained.

Since we have argued that the oxidizing site has now become positively charged in F relative to E and as compounds with four or five methylene units are the best substrates, it is likely that the distance between the oxidizing site and the substrate-binding site corresponds to a separation of four or five methylene units. When the tetramethylene bis-onium compounds complex with form F they would tend to complex with the substrate-binding site, but would immediately suffer repulsion from the positive charge generated by the substitution of pyridoxamine for pyridoxal at the oxidizing site.

An approximate measure of the free energy of

interaction between inhibitor and form F would be: isothiouronium 22.2–26.4kJ/mol, guanidinium 17.0–23.7kJ/mol and trimethylammonium compounds 3.2–9.1kJ/mol.

This scheme for the production of intercept effects is illustrated in Fig. 12.

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