The Effects of Inhibitor Mixtures and the Specific Effects of Different Anions on the Oxidase Activity of Caeruloplasmin

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(Received 20 February 1968)

1. The interpretation of the effects of mixtures of inhibitors on enzymes is considered. 2. The effects of inhibitor mixtures on caeruloplasmin were determined. 3. Fluoride, chloride and cyanate inhibit at one type of site (α), whereas bromide and iodide inhibit at another type (β) present in the same enzyme intermediate. 4. Effects of inhibitor mixtures containing azide or cyanide are consistent with previous indications (Speyer & Curzon, 1968) that these ligands form inhibited complexes with different enzyme intermediates. 5. Isobols of halides or of cyanate with azide indicate that azide inhibits caeruloplasmin by bridging two α sites, these being reduced copper atoms. 6. Iodide and cyanate give hyperbolic plots of $1/v$ against $[I]$. 7. It is suggested that in the cyanate-inhibited complex the inhibitor binds to a reduced copper atom (α site) but that binding of cyanate at another copper atom is sterically prevented. It is suggested that the less bulky α -site inhibitors, fluoride and chloride, cause complete inhibition by binding to both of these copper atoms, which can also be bridged by a single azide group. 8. Each halide shows a pattern of effects on caeruloplasmin that is qualitatively distinct from that of other halides.

Most reports of the effects of mixtures of inhibitors on enzymes describe protection by reversible inhibitors against irreversible inhibitors, e.g. by physostigmine (Koelle, 1946) and butyrylcholine (Cohen, Warringa & Bovens, 1951) against the inhibition of cholinesterases by organophosphorus compounds and by malonate against the inhibition of succinate dehydrogenase by arsenite (Barron & Singer, 1945). However, there has been little systematic work on the effects of mixtures of reversible inhibitors on enzymes except that of Slater & Bonner (1952) on the mutual enhancement by fluoride and phosphate of their inhibitions of succinate dehydrogenase.

In the study of inhibition mechanism it is often of value to know whether different inhibitors bind to the same or to different sites on an enzyme molecule. Further, with complex enzyme mechanisms such as those involving co-operative effects, multiple conformational changes or more than one substrate, many intermediate enzyme forms may occur during enzyme action, and evidence on whether inhibitors bind to the same or to different enzyme intermediates may be important. Such evidence can be obtained by the investigation of the effects of inhibitor mixtures.

Previous work on effects of inhibitors on caeruloplasmin, the copper-containing oxidase in serum,

indicated that inhibitors may act by binding to different sites or to different enzyme intermediates. Thus, whereas the order of inhibitory effectiveness of halide ions against enzymes is usually $F^- < Cl^- < Br^- < I^-$ (e.g. Massey, 1953; Walaas & Walaas, 1956; Rutter & Rolander, 1957; Fridovich, 1963; Warren & Cheatum, 1966), the order of inhibitory effectiveness against caeruloplasmin is remarkable, being $F^- > I^- \approx Cl^- > Br^-$ (Curzon & Speyer, 1967), which may be due to different halide ions binding to the enzyme in different ways. The different inhibitory properties of azide (Curzon, 1966, 1967) and cyanide (Speyer & Curzon, 1968) suggest that they also may bind in different ways. Therefore the effects of mixtures of inhibitors on caeruloplasmin were studied, and the above suggestions of different inhibitor-binding sites were substantiated.

Webb (1963) proposed that the relationships between two inhibitors acting together be described by inhibition isobols (see the Theory section). This has been found a useful form of presentation and examples are given of various types of isobol. In the isobol studies and in further work on halide inhibition it was found that the actions of individual halides on caeruloplasmin were qualitatively distinct, each halide showing a specific group of properties.

THEORY

Inhibition isobols. Loewe (1957) expressed the pharmacological effect of two drugs acting on a tissue at the same time in the form of isobols, i.e. lines showing equieffective combinations of the two drugs plotted on graphs of which the co-ordinates represented the concentrations of the individual drugs in the assay system. Webb (1963) suggested that this graphical method was a convenient way of presenting the effects of mixtures of two inhibitors on an enzyme. According to Webb (1963), when the inhibitors prevent each other's binding to the enzyme a straight-line isobol is obtained (Fig. 1a), and when the inhibitors do not prevent each other from binding they summate and a curved isobol with concave side upwards is obtained (Fig. 1b). This may be readily shown. With a simple Michaelis-Menten system and two non-competitive inhibitors neither of which influences the binding of the other to the enzyme then it can be shown that:

$$V/v = \left(1 + \frac{[I']}{K_i'} + \frac{[I'']}{K_i''} + \frac{[I'][I'']}{K_i'K_i''}\right) \left(\frac{K_m}{[S]} + 1\right) \quad (1)$$

When the total inhibition is constant (isobol conditions) and $[S]$ is constant then $V/v = \text{a constant}$. Eqn. (1) may therefore be rearranged to:

$$\frac{V}{v} \left(\frac{[S]}{K_m + [S]}\right) = \text{a constant} = 1 + \frac{[I']}{K_i'} + \frac{[I'']}{K_i''} + \frac{[I'][I'']}{K_i'K_i''} \quad (2)$$

Thus by plotting $[I']$ against $[I'']$ at constant total inhibition, a line that is concave upwards is obtained except when the complex $EI'I''$ cannot occur, i.e. when both inhibitors bind to the same site. In the latter circumstances the last term in eqn. (2) is zero and a linear equation resulting in a straight-line isobol is obtained. Isobol equations of similar form to eqn. (2) may be derived when I' and I'' are competitive inhibitors, when I' inhibits competitively and I'' inhibits non-competitively, and when I' inhibits non-competitively and I'' gives anti-competitive kinetics, i.e. combining with the enzyme-substrate complex (Ebersole, Guttentag & Wilson, 1944) or with a form of the enzyme produced by its interaction with substrate.

However, when one inhibitor acts by forming a complex only with a form of the enzyme that occurs during enzyme reaction and the other inhibitor acts by forming a complex with the enzyme only in another of its forms, then the isobol is straight whether or not the same site on the enzyme is involved. For example, taking a simple case where I' inhibits by a competitive mechanism, forming a complex with E , but I'' can only form a complex with an intermediate, then it can be shown that:

$$V/v = \frac{K_m}{[S]} \left(1 + \frac{[I']}{K_i'}\right) + 1 + \frac{[I'']}{K_i''}$$

This equation leads to a straight-line isobol.

Complications arise when, as well as forming an inhibited complex, a substance can also bind to the enzyme at non-inhibitory sites. This is likely with charged substances, which potentially can bind electrostatically to any site of opposite charge. Hindering or facilitation of the formation of an inhibited complex with one inhibitor as a result of the non-inhibitory binding of a second inhibitor is then a possibility.

Fig. 1. Inhibition isobols for mixtures of I' and I'' causing constant net inhibition. (a) Inhibitors prevent each other binding to the enzyme at inhibition sites. (b) Inhibitors do not prevent each other binding to the enzyme at inhibition sites. (c) One or both inhibitors also binds to the enzyme at non-inhibition sites, causing hindering of inhibition.

The following guide for the interpretation of inhibition isobols may be stated.

(1) A straight-line isobol indicates that I' and I'' form inhibited complexes at mutually exclusive sites, by binding to either the same intermediate at the same or adjacent sites or to different intermediates.

(2) A curved isobol with concave side upwards and with inhibitions equal to those explicable by summation indicates that I' and I'' form inhibited complexes by binding to the same intermediate at different sites.

(3) A curved isobol with concave side upwards and with inhibitions greater than those explicable by summation indicates non-inhibitory facilitative binding.

(4) A curved isobol with concave side upwards and with inhibitions less than those explicable by summation is difficult to interpret and may be due to non-inhibitory facilitative binding, to inhibitors acting at more than one kind of site or to the conditions of (2) together with non-inhibitory hindering binding.

(5) A curved isobol with concave side downwards (Fig. 1c) indicates that, as well as forming inhibited complexes, one or both of I' and I'' binds to the enzyme at non-inhibitory sites resulting in hindering of inhibitory binding of the other substance.

MATERIALS AND METHODS

Caeruloplasmin. Human caeruloplasmin (American Red Cross batch no. 1995) was used. Treatment and specifications were as described by Curzon & Speyer (1967).

Substrate. *NN*-Dimethyl-*p*-phenylenediamine as its sulphate, bis-(4-dimethylaminophenylammonium) sulphate, was prepared from *NN*-dimethyl-*p*-phenylenediamine dihydrochloride (Eastman Organic Chemicals, Rochester, N.Y., U.S.A.) as described by Curzon & Speyer (1967).

Inhibitors. All inhibitors were the purest available grades: NaF, NaCl, NaN₃, KBr, KI, KCN and KCNO were used. The NaN₃ was recrystallized from water. Cyanide was not detectable (<0.02%) in the KCNO by the copper acetate-benzidine acetate test (Feigl, 1960). Solutions of all in-

hibitors except KCN were brought to pH 5.5 with a pH-meter with dil. NaOH or H₂SO₄ before use.

Caeruloplasmin oxidase activity method. Except where otherwise stated, 1.0 mM-*NN*-dimethyl-*p*-phenylenediamine (as sulphate) was oxidized by caeruloplasmin at pH 5.5 and 25°. Formation of the red reaction product was measured at 550 m μ in a Unicam SP. 800 spectrophotometer, as described by Speyer & Curzon (1968).

Inhibition isobols. These were determined for 70% inhibition at 25° with 1 mM substrate, an exception being indicated in the text. An inhibition of 70% was chosen so that as wide a range of inhibitor concentrations as possible could be studied consistent with accurate determination of inhibition. The concentration of each of a pair of inhibitors for 70% inhibition in the absence of the second inhibitor was determined on the day on which the isobol was to be obtained. Necessary concentrations fell by 10–20% during the 6-month period in which the work was done. A straight-line isobol joining the concentrations of the two inhibitors as determined above was drawn, and the effect on enzyme activity of inhibitor mixtures with compositions indicated by points on this line was experimentally determined. Inhibitions by these mixtures of 70 $\pm$ 1% were accepted as indicating a straight-line isobol. When deviations larger than $\pm$ 1% were found the concentration of the first inhibitor was varied, that of the second inhibitor being kept constant, until 70 $\pm$ 1% inhibition was obtained. This was repeated at different concentrations of the second inhibitor

so that an experimental 70%-inhibition isobol could be plotted.

Reversibility of inhibition by treatment with Sephadex G-25. This was determined and corrected for losses as described by Curzon & Speyer (1967). High losses occurred the first two or three times that each fresh Sephadex column was used. These results were disregarded.

RESULTS

Reversibility of inhibitions

Reversibility of caeruloplasmin inhibitions by Sephadex treatment was previously shown for azide (Curzon, 1966) and for cyanide, cyanate and benzoate (Curzon & Speyer, 1967). Corrected recoveries of enzyme activity after reversal of other inhibitions by Sephadex treatment were 92% with fluoride, 100% with chloride, 91% with bromide and 94% with iodide. Corrected recovery in the absence of inhibitors was 91% (average of seven determinations).

Effects of inhibitor mixtures

Inhibition by halide-halide mixtures. The iodide-chloride isobol (Fig. 2a) was concave upward. The experimentally determined inhibition at a point on

Fig. 2. Isobols for 70% inhibition of caeruloplasmin with the following inhibitor mixtures: (a) $\circ$, chloride and fluoride; Δ , chloride and bromide; $\square$, chloride and iodide; (b) $\circ$, bromide and iodide; (c) $\circ$, fluoride and bromide; Δ , fluoride and iodide. General conditions were as described in the Materials and Methods section.

Fig. 3. Isobols for 70% inhibition of caeruloplasmin with the following inhibitor mixtures: (a) $\circ$, fluoride and cyanate; Δ , iodide and cyanate; $\square$, azide and cyanate; (b) $\circ$, fluoride and azide; Δ , chloride and azide; $\square$, bromide and azide; +, iodide and azide. Other conditions were as described in the Materials and Methods section.

the curve was 3% less than that calculated from inhibitions due to each inhibitor alone, assuming summation. Therefore iodide and chloride inhibit caeruloplasmin by binding to the same enzyme intermediate but at different sites. Similarly (Fig. 2a) bromide inhibits by binding to the same intermediate as chloride but at different sites, inhibition being the same as that calculated for summation. Thus chloride, bromide and iodide all inhibit by binding to the same intermediate. Therefore the linear bromide–iodide isobol (Fig. 2b) shows that bromide and iodide both inhibit by binding at the same site. The enzyme intermediate therefore has two types of site at which inhibition by halides may occur, one at which iodide and bromide bind and the other at which chloride binds. The fluoride isobols are less easy to interpret since, though the fluoride–bromide and fluoride–iodide isobols (Fig. 2c) were concave upwards, inhibitions were 5% less than if summation occurred. Similarly the fluoride–chloride isobol (Fig. 2a) shows that inhibitory effects were somewhat less than if fluoride and chloride were simply both affecting enzyme activity by binding at the same site. The above results suggest that fluoride and chloride bind at the same kind of site whereas bromide and iodide bind at another kind of site on the same intermediate, but that a slight hindering effect also occurs or that some halides may inhibit by binding at more than one kind of site. The distinct fluoride–chloride and bromide–iodide sites in the same enzyme intermediate are designated α and β respectively.

Inhibition by cyanate or azide plus halides. It is convenient to consider cyanate and azide together as the inhibition kinetics obtained with these inhibitors are identical, Lineweaver–Burk plots being parallel (Curzon, 1966, and below). Cyanate summates with iodide but gives a straight-line isobol with fluoride (Fig. 3a). This is consistent with

cyanate inhibiting by binding at α sites. But azide gives essentially straight-line isobols with fluoride, chloride and cyanate, which inhibit at α sites, and also with bromide and iodide, which inhibit at β sites (Figs. 3a and 3b). The significance of the difference between azide and fluoride, chloride and cyanate is considered in the Discussion section.

Inhibition by cyanide plus another inhibitor. Cyanide gives a straight-line inhibition isobol with either azide or fluoride (Fig. 4a). This indicates that cyanide inhibits (i) at α sites, (ii) at the azide-binding site, or (iii) to a different enzyme intermediate to those binding azide or fluoride.

Isobols of cyanide with chloride, bromide or iodide (Figs. 4b and 4c) are clearly different from isobols of these halides with the other inhibitors under consideration. Thus, though concave-downward isobols indicating non-inhibitory hindering have been observed in the absence of cyanide (e.g. Fig. 2a, and also the chloride–benzoate isobol), these effects are negligible in comparison with the hindering of cyanide inhibition by chloride and bromide (Fig. 4b). Further, the cyanide–iodide isobol (Fig. 4c) is of a different shape from that obtained with iodide with other inhibitors, and is also the only 70%-inhibition isobol obtained in which inhibition is significantly greater than explicable by summation (+14%), indicating facilitation of cyanide inhibition by iodide. Facilitation is apparent at lower iodide concentrations only. Thus the 40%-inhibition iodide–cyanide isobol, for which only lower iodide concentrations were necessary, shows facilitation throughout (Fig. 4d) with inhibition 8% greater than that corresponding to summation. Whatever the reason for these effects, the marked differences of the cyanide–halide isobols from those of halides with other inhibitors strongly suggests that explanation (iii) is correct and that cyanide inhibits caeruloplasmin by binding to a

Fig. 4. Inhibition isobols (70% inhibition except where otherwise stated) for caeruloplasmin with the following inhibitor mixtures: (a) $\circ$, azide and cyanide; Δ , fluoride and cyanide: (b) $\circ$, chloride and cyanide; Δ , bromide and cyanide: (c) $\circ$, iodide and cyanide: (d) $\circ$, iodide and cyanide (40% inhibition). Other conditions were as described in the Materials and Methods section.

different intermediate to that binding the other inhibitors.

Specific effects of halide and other inhibitory anions

Effect of time on inhibition. In general, inhibitors reach equilibrium with caeruloplasmin at 25° within the first 2 min. of enzyme action (Curzon & Speyer, 1967), though cyanide requires 5–15 min. At lower temperatures a number of inhibitors equilibrate more slowly, e.g. azide and chloride (Curzon, 1966). Therefore the time-courses of halide inhibitions were compared at 5° . When the standard procedure was used, the enzyme being added last to the incubation mixture, fluoride, bromide or iodide inhibition rapidly reached equilibrium, but about 8 min. was required for chloride inhibition to reach equilibrium. Pre-incubation of chloride with the enzyme for 30 min. resulted in rapid equilibration on addition of sub-

strate. Benzoate required more than 30 min. for equilibration in the presence of substrate under standard conditions at 7.5° , though salicylate equilibrated within 2 min. The surprisingly specific effect of chloride is considered in the Discussion section.

Effect of inhibitor concentration on inhibition. When inhibitor concentrations are plotted against the reciprocals of enzyme velocities then straight lines are obtained if saturation with inhibitor causes complete inhibition. When the enzyme-inhibitor complex has some activity or an alternative enzyme reaction path is available, then hyperbolic curves are obtained, which may tend asymptotically to zero slope at saturation with inhibitor; these have been termed 'hyperbolic inhibition' (Cleland, 1963a). Fluoride gives a straight line when results are plotted by the above method (Fig. 5), chloride gives a curve of increasing slope as chloride concentration increases, and bromide, iodide and

Fig. 5. Inhibition of caeruloplasmin by halide ions: ○, fluoride; △, chloride; □, bromide; ●, iodide. Other conditions were as described in the Materials and Methods section.

cyanate (Fig. 6a) give curves of decreasing slope. Cyanide, azide and benzoate give straight lines (not shown). With bromide, iodide or cyanate it is not clearly apparent that zero slope is attained. However, Cleland (1963a) showed that with hyperbolic anti-competitive inhibition:

$$1/v_i = \frac{1}{V} \left(\frac{1 + [I]/K_i}{1 + [I]V'/K_iV} \right) \quad (3)$$

where v_i is the velocity of the enzyme reaction in the presence of $[I]$ and excess of substrate and V' is the velocity in the presence of excess of inhibitor and excess of substrate. Subtracting $1/V$ from both sides and rearranging:

$$\frac{1}{1/v_i - 1/V} = \frac{VK_i}{[I](1 - V'/V)} + \frac{V'}{(1 - V'/V)} \quad (4)$$

Therefore by plotting $1/(1/v_i - 1/V)$ against $1/[I]$ a straight line is obtained. The vertical intercept equals $1/(1/V' - 1/V)$, from which V' can be calculated. The horizontal intercept equals $-V'/VK_i$, from which K_i can be calculated. Results with cyanate or iodide give straight lines when plotted by this method (Fig. 6b). Also, theoretical values for $1/v_i$ calculated by substituting the derived constants in eqn. (3) lie on the same curves as experimental values (Fig. 6a). Therefore these anions cause hyperbolic inhibition.

Inhibitions were also plotted by using the method of Johnson, Eyring & Williams (1942), the slopes of

the logarithmic plots obtained giving the number of moles of inhibitor bound/mole of sensitive sites (Fig. 7). Most slopes were in the range of 0.8–1.1, indicating 1 mole of inhibitor/mole of sites. Chloride gave a slope of 1.3. Cyanate, bromide and iodide gave slopes of about 1 at lower inhibitions, and these decreased as inhibition increased.

Effect of substrate concentration on inhibition. Caeruloplasmin gives curved Lineweaver–Burk plots (Osaki, 1966; Curzon, 1967; Walaas, Løvstad & Walaas, 1967) and therefore, except when these are clearly competitive, as with cyanide (Speyer & Curzon, 1968), or clearly parallel, as with azide (Curzon, 1966) and cyanate (Fig. 8), they are not easy to interpret visually. Therefore in Fig. 9 percentage inhibitions are plotted against the negative logarithm of the substrate concentration. Results are shown for a single concentration of each inhibitor. Curves at two other inhibitor concentrations were similar in form and are not shown. Positive slopes are obtained when Lineweaver–Burk plots are parallel (anti-competitive inhibition) as with azide and cyanate, and positive slopes of lower gradient are obtained when Lineweaver–Burk plots, though not parallel, are less convergent than for non-competitive inhibitions, suggesting some anti-competitive character. Competitive and non-competitive kinetics lead to negative and zero slopes respectively. Benzoate, bromide and chloride inhibitions are of clearly anti-competitive character (Fig. 9a), though not completely so. Anti-competition is suggested by the curves for fluoride and iodide at higher substrate concentrations (Fig. 9b), though the forms of the curves are consistent with the indications from isobol experiments that some halides may inhibit by binding at more than one kind of site.

DISCUSSION

Protein geometry and charge distribution impose restrictions on the binding of ions to proteins that may result in specificity of ion binding or action. Thus, for example, gelatin has 17 sites/mol. of mol.wt. 10^5 that bind chloride ion but only 12 sites/mol. of mol.wt. 10^5 that bind the slightly larger bromide ion (Hörmann & Ananthanarayanan, 1967). Specific effects are reported frequently for cations but only rarely for anions. A well-known specific effect of an anion is the activation of amylase by chloride (Myrbäck, 1926), and the unusual order of activatory effectiveness of halides on glycerate dehydrogenase ($F^- < I^- = Cl^- < Br^-$) (Holzer & Holldorf, 1957) suggests differences in the ways in which different halides bind to this enzyme. When ions bind to the metal atoms of metalloproteins the above restrictions are reinforced by those imposed by the electronic character of the

Fig. 6. Hyperbolic inhibition of caeruloplasmin; $1/v_i$ is plotted against $[I]$. Iodide was at 10 mm $[S]$: (a) $\circ$, experimental; $\bullet$, calculated from Fig. 6(b) by using eqn. (3) (see the text). Cyanate results were extrapolated to $[S] = \infty$: Δ , experimental; $\blacktriangle$, calculated as above. Other conditions were as described in the Materials and Methods section. (b) Results of Fig. 6(a) plotted as eqn. (4) (see the text) ($V = 0.172$ extinction unit): $\circ$, iodide (experimental); Δ , cyanate (experimental). DPD^+ is the first free-radical oxidation product of *NN*-dimethyl-*p*-phenylenediamine. From these lines the following constants were derived: cyanate: $V' = 9.4\text{ mm-DPD}^+/\text{g. atom of Cu/sec.}$; $K_i = 0.032\text{ mm}$; iodide: $V' = 13.1\text{ mm-DPD}^+/\text{g. atom of Cu/sec.}$; $K_i = 7.5\text{ mm}$. Extinction units were converted into velocities in $\text{mm-DPD}^+/\text{g. atom of caeruloplasmin Cu/sec.}$ by multiplying by 560. Owing to the complex relationship between substrate concentration and velocity in the presence of iodide, v_i could not be found by extrapolation and therefore values of v in the presence of iodide at 10 mm -substrate were used to obtain an approximate value for V' . Under these conditions the enzyme is 88% saturated (Curzon, 1967).

Fig. 7. Inhibition of caeruloplasmin by various anions. Results are plotted by the method of Johnson *et al.* (1942); α is the fractional activity. Inhibitors were: $\circ$, cyanide; Δ , azide; $\square$, cyanate; $\bullet$, benzoate; $\blacktriangle$, fluoride; $\blacksquare$, chloride; $\bigcirc$, bromide; $\blacksquare$, iodide. Other conditions were as described in the Materials and Methods section.

metal atoms (Pearson, 1963) and by the directivity of the valence bonds that bind ligands to metal atoms. Marked specificity is shown in the effects of

halide ions on the metalloprotein caeruloplasmin, no two halides exhibiting the same qualitative pattern of effects (Table 1).

Fig. 8. Effect of substrate concentration on inhibition of caeruloplasmin by cyanate. Lineweaver-Burk plots are shown at the following concentrations of cyanate: $\circ$, none; Δ , 0.05 mM; $\square$, 0.10 mM; $\bullet$, 0.80 mM. Other conditions were as described in the Materials and Methods section.

The anomalously high inhibition of caeruloplasmin by iodide (Curzon & Speyer, 1967) may be related to the deduction from isobol experiments that the enzyme has two kinds of site, α and β , fluoride and chloride inhibiting at α sites and bromide and iodide at β sites. At α sites fluoride is more inhibitory than chloride and at β sites iodide is more inhibitory than bromide. Though the inhibition of caeruloplasmin by azide and cyanide can be assumed to involve ligand-copper interaction (Curzon, 1966; Speyer & Curzon, 1968) it is less apparent that the halide binding sites contain copper, as halides at concentrations comparable with those used affect enzymic and other properties of many non-metal-containing proteins (Fridovich, 1963; Warren & Cheatum, 1966). Previously (Curzon, 1960; Peisach & Levine, 1965) it was suggested that chloride inhibited caeruloplasmin by forming a caeruloplasmin-copper-chloride complex. That the α sites at which chloride and fluoride inhibit may contain copper is indicated as, though fluoride is by far the least inhibitory halide ion against enzymes in general, it is the most inhibitory halide against caeruloplasmin, and many metal-dependent enzymes are strongly inhibited by

Fig. 9. Effect of substrate concentration on inhibition of caeruloplasmin. Inhibitors were: (a) $\circ$, 4 μ M-azide; Δ , 0.1 mM-cyanate; $\square$, 3 mM-benzoate; $\bullet$, 5 mM-salicylate; $\blacktriangle$, 17 mM-chloride; $\blacksquare$, 32 mM-bromide: (b) $\circ$, 1 μ M-cyanide; Δ , 6 mM-fluoride; $\square$, 15 mM-iodide. Other conditions were as described in the Materials and Methods section.

Table 1. *Specific effects of halide ions on caeruloplasmin*

	Fluoride	Chloride	Bromide	Iodide
Inhibition site	α	α	β	β
$1/v-[I]$ plot	Linear	Increasing slope	Decreasing slope	Decreasing slope
Isobol with cyanide	Linear	Concave downwards	Concave downwards	Complex
Rate of inhibition at 5°	Fast	Slow	Fast	Fast

fluoride. In particular, fluoride is the most inhibitory halide against two other copper-containing enzymes, tyrosinase (Krueger, 1955) and laccase (Peisach & Levine, 1965). The sensitivity of the α site to cyanate also suggests this site contains metal. The α -site inhibitors are either anti-competitive or have an anti-competitive component showing similarity to azide, which gives completely anti-competitive kinetics. Formation of inhibited complexes with an intermediate form of an enzyme commonly leads to anti-competitive kinetics (Cleland, 1963b). Results thus correlate with the suggestion (Peisach & Levine, 1965) that halide ions inhibit caeruloplasmin by binding to reduced copper atoms and preventing their reoxidation. Further, inhibition kinetics with azide are consistent with azide groups bridging copper atoms of half-reduced or fully reduced forms of caeruloplasmin (Curzon, 1966; Speyer & Curzon, 1968). It is suggested that α sites are single reduced copper atoms and that azide binds across two α sites, which must therefore be in a specific arrangement. Hence straight-line isobols are found with mixtures of azide and α -site inhibitors. The hyperbolic plot of $1/v$ against $[I]$ for cyanate shows that the enzyme has some activity when cyanate is in excess. If this were due to some enzyme molecules not being inhibitable then slopes of reciprocal plots would increase at high inhibitions. Since this is not found enzyme molecules inhibited by cyanate probably have some activity. This suggests that when fluoride or chloride are bound at one reduced copper atom these ions can bind at a second reduced copper atom with similar facility. When cyanate is bound at one copper atom then binding of more cyanate may be prevented by steric interference. Cyanate ions are larger than fluoride or chloride ions and the two reduced copper atoms are assumed to be close enough to be bridged by azide. As the binding of a single azide group to an enzyme molecule causes complete inhibition, the fully reduced enzyme probably contains a cluster of four reduced copper atoms that are reoxidized together by the same oxygen molecule (Curzon, 1966). Caeruloplasmin still has 10% of its original enzymic activity when saturated with cyanate, and therefore an enzyme mechanism with greatly decreased activity involving reoxidation of a part of the cluster of copper atoms may be possible. The mechanism under these conditions might be analogous to that of fungal laccase, which has considerable similarities to caeruloplasmin both spectrally (Mosbach, 1963) and in substrate specificity (Mosbach, 1963; Peisach & Levine, 1965), but, unlike caeruloplasmin, has only two reducible copper atoms/mol. (Broman, Malmström, Aasa & Vänngård, 1962; Mosbach, 1963).

Iodide forms a complex with cuprous copper

(Sillén & Martell, 1964). However, inhibition of caeruloplasmin by iodide is not evidence that β sites contain copper. Many properties of non-metal-containing proteins, including such gross phenomena as helix-coil transitions (Hippel & Wong, 1964), are more sensitive to iodide than to other halides. The hyperbolic plot of $1/v$ against $[I]$ for iodide shows that here also the enzyme has some activity when saturated with inhibitor. As iodide does not inhibit at the α sites, the linear iodide-azide isobol indicates that iodide may cause reorganization of enzyme structure (Warren & Cheatum, 1966) so that reduced copper atoms can no longer be bridged by azide, though they are still available to fluoride, chloride and cyanate, which are assumed not to form bridged structures. If bromide and iodide alter the orientation of the reduced copper atoms this would explain the inhibition by these ions.

The inhibitory properties of azide against caeruloplasmin (Curzon, 1966, 1967) are qualitatively different from those of cyanide (Speyer & Curzon, 1968). Thus azide gives anti-competitive kinetics and 1 azide group is bound/mol. of caeruloplasmin in the inhibited complex, but cyanide gives competitive kinetics and 2 cyanide groups are bound/mol. The linear azide-cyanide isobol (Fig. 4a) shows that these inhibitors do not act by binding at different sites of the same enzyme intermediate. The marked qualitative difference between halide-azide and halide-cyanide isobols strengthens the suggestion (Speyer & Curzon, 1968) that azide and cyanide inhibit caeruloplasmin by binding to different intermediates. Protection against cyanide inhibition by chloride and bromide is not due to that interaction of the enzyme with these latter ions that results in inhibition, as protection is prominent at halide concentrations that cause negligible inhibition. If access to sites at which this protective binding occurs is sterically restricted then the lack of protection by iodide might indicate that a crevice in which the cyanide or protector is bound is too narrow to accommodate an iodide ion. The lack of protection by fluoride might then be due to it being too small to interfere sterically with cyanide binding. Alternatively, if protective binding strengths are in the order $F^- < Cl^- < Br^- < I^-$ (Fridovich, 1963; Warren & Cheatum, 1966) then fluoride might be too weakly bound at the relevant site to be effective in the limited concentration range imposed by the isobol conditions.

The low rate of inhibition of caeruloplasmin by chloride but not by other halides may be distinguished from the slow inhibition by azide (Curzon, 1966) or cyanide (Speyer & Curzon, 1968), as only with chloride is the period of gradual increase of inhibition eliminated by preincubation of enzyme with inhibitor. Since chloride inhibition is largely

anti-competitive the inhibited complex is presumably formed with an enzyme intermediate. Hence the slow step that occurs during preincubation is probably not a slow formation of the inhibited complex but a chloride-dependent conformational change as a result of which inhibition by chloride occurs during cycling. The gradually increasing slope of the $1/v$ versus $[I]$ plot and the steep Johnson *et al.* (1942) logarithmic inhibition plots for chloride may therefore be due to a shift of equilibrium from chloride non-inhibitable conformers to inhibitable conformers as chloride concentration increases. Marriott (1965) also noted a slow chloride-dependent change of caeruloplasmin in the protection by chloride against gradual decolorization by EDTA.

We thank the American Red Cross for supplies of caeruloplasmin. The work was supported by a grant from the Medical Research Council.

REFERENCES

- Barron, E. S. G. & Singer, T. P. (1945). *J. biol. Chem.* **157**, 221.
- Broman, L., Malmström, B. G., Aasa, R. & Vänngård, T. (1962). *J. molec. Biol.* **5**, 301.
- Cleland, W. W. (1963a). *Biochim. biophys. Acta*, **67**, 173.
- Cleland, W. W. (1963b). *Biochim. biophys. Acta*, **67**, 188.
- Cohen, J. A., Warringa, M. G. P. J. & Bovens, B. R. (1951). *Biochim. biophys. Acta*, **6**, 469.
- Curzon, G. (1960). *Biochem. J.* **77**, 66.
- Curzon, G. (1966). *Biochem. J.* **100**, 295.
- Curzon, G. (1967). *Biochem. J.* **103**, 289.
- Curzon, G. & Speyer, B. E. (1967). *Biochem. J.* **105**, 243.
- Ebersole, E. R., Guttentag, C. & Wilson, P. W. (1944). *Arch. Biochem.* **3**, 399.
- Feigl, F. (1960). *Spot Tests in Organic Analysis*, 6th ed., p. 365. Amsterdam: Elsevier Publishing Co.
- Fridovich, I. (1963). *J. biol. Chem.* **238**, 592.
- Hippel, P. H. von & Wong, K. Y. (1964). *Science*, **145**, 577.
- Holzer, H. & Holldorf, A. (1957). *Biochem. Z.* **239**, 292.
- Hörmann, H. & Ananthanarayanan, S. (1967). *Hoppe-Seyl. Z.* **348**, 995.
- Johnson, F. H., Eyring, H. & Williams, R. W. (1942). *J. cell. comp. Physiol.* **20**, 247.
- Koelle, G. B. (1946). *J. Pharmacol.* **88**, 232.
- Krueger, R. C. (1955). *Arch. Biochem. Biophys.* **57**, 52.
- Loewe, S. (1957). *Pharmacol. Rev.* **9**, 237.
- Marriott, J. (1965). Ph.D. Thesis: University of London.
- Massey, V. (1953). *Biochem. J.* **53**, 67.
- Mosbach, R. (1963). *Biochim. biophys. Acta*, **73**, 204.
- Myrbäck, K. (1926). *Hoppe-Seyl. Z.* **159**, 1.
- Osaki, S. (1966). *J. biol. Chem.* **241**, 5053.
- Pearson, R. G. (1963). *J. Amer. chem. Soc.* **85**, 3533.
- Peisach, J. & Levine, W. G. (1965). *J. biol. Chem.* **240**, 2284.
- Rutter, W. J. & Rolander, B. (1957). *Acta chem. scand.* **11**, 1663.
- Sillén, L. G. & Martell, A. E. (1964). *Spec. Publ. chem. Soc. no. 17: Stability Constants of Metal Ion Complexes*, p. 338.
- Slater, E. C. & Bonner, W. D. (1952). *Biochem. J.* **52**, 185.
- Speyer, B. E. & Curzon, G. (1968). *Biochem. J.* **106**, 905.
- Walaas, E., Løvstad, A. & Walaas, O. (1967). *Arch. Biochem. Biophys.* **121**, 480.
- Walaas, E. & Walaas, O. (1956). *Acta chem. scand.* **10**, 122.
- Warren, J. C. & Cheatum, S. (1966). *Biochemistry*, **5**, 1702.
- Webb, J. L. (1963). *Enzyme and Metabolic Inhibitors*, vol. 1, p. 495. New York and London: Academic Press Inc.