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Benzofuroxan as a Thiol-Specific Reactivity Probe

KINETICS OF ITS REACTIONS WITH PAPAIN, FICIN, BROMELAIN AND LOW-MOLECULAR-WEIGHT THIOLS

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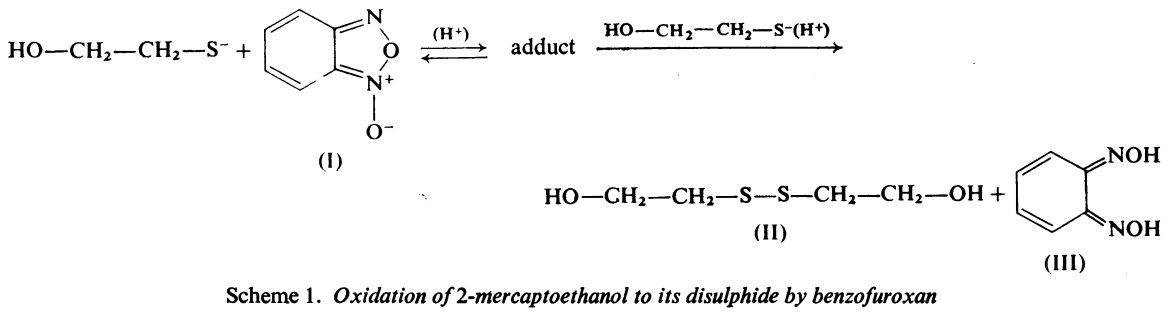
1. The characteristics of benzofuroxan (benzofurazan 1-oxide, benzo-2-oxa-1,3-diazole *N*-oxide) that relate to its application as a reactivity probe for the study of environments of thiol groups are discussed. 2. To establish a kinetic and mechanistic basis for its use as a probe, a kinetic study of its reaction with 2-mercaptoethanol was carried out. 3. This reaction appears to proceed by a rate-determining attack of the thiolate ion on one of the electrophilic centres of benzofuroxan (possibly C-6) to provide a low steady-state concentration of an intermediate adduct; rapid reaction of this adduct with a second molecule of thiol gives the disulphide and *o*-benzoquinone dioxime. 4. The effects of the different types of environment that proteins can provide on the kinetic characteristics of reactions of thiol groups with benzofuroxan are delineated. 5. Benzofuroxan was used as a thiol-specific reactivity probe to investigate the active centres of papain (EC 3.4.22.2), ficin (EC 3.4.22.3) and bromelain (EC 3.4.22.4). The results support the concept that the active centres of all three enzymes either contain a nucleophilic thiolate ion whose formation is characterized by a pK_a of 3–4 and whose reaction with an electrophile can be assisted by interaction of a site of high electron density in the electrophile with the active-centre imidazolium ion of pK_a 8–9, or can provide such ions by protonic redistribution in enzyme–reagent or enzyme–substrate complexes.

In a previous paper (Shipton *et al.*, 1977) we discussed the properties of benzofuroxan (benzofurazan 1-oxide, benzo-2-oxa-1,3-diazole *N*-oxide, I, Scheme 1) and its application as a chromophoric oxidizing agent for thiol groups. The ease with which the reduction of benzofuroxan to *o*-benzoquinone dioxime (III, Scheme 1) can be monitored continuously makes this oxidizing agent suitable for use as a reactivity probe for the study of environments of thiol groups in enzymes and other biological molecules. Byers & Koshland (1975) have emphasized the value of reactivity probes as against spectroscopic probes for the study of conformational states of proteins. The delineation of molecular environments of nucleophilic centres in proteins is facilitated by the presence in the electrophilic-probe molecule of multiple centres of relatively high electron density. Interaction of such centres with acidic groups in the environment of the nucleophilic centre in the protein can have pronounced effects on the observed reactivity of the



Scheme 1. *Oxidation of 2-mercaptoethanol to its disulphide by benzofuroxan*

nucleophile towards the probe and on the form of its pH-dependence. Particularly pronounced effects can be provided by using two-protonic-state electrophiles such as 2,2′-dipyridyl disulphide (see Brocklehurst, 1974; Malthouse & Brocklehurst, 1976; Norris & Brocklehurst, 1976), but other types of probe can also yield valuable information, e.g. anionic alkylating agents (Wallenfels & Eisele, 1968; Chaiken & Smith, 1969) and 4-chloro-7-nitrobenzofurazan [see Shipton *et al.* (1976), Baines *et al.* (1977) and references therein].

Reactivity can be affected by binding effects or by provision of electronic assistance to the reaction itself. The structure of benzofuroxan (I, Scheme 1) suggests that in different cases, reactivity could be enhanced by both of these mechanisms (see the Results and Discussion section). The kinetic study of the oxidation by benzofuroxan of the active-centre thiol groups of papain, ficin and bromelain here reported clearly indicates that this reactivity probe is a sensitive detector of suitably aligned acidic residues.

Detailed kinetic study of the reduction of benzofuroxan by thiols has not been reported hitherto. In the present paper we report a kinetic study of the reaction of benzofuroxan with 2-mercaptoethanol, a typical low-molecular-weight thiol devoid of significantly perturbing intramolecular environment. The mechanism and kinetic characteristics of this reaction provide a basis for the evaluation of environmental influences on the reactions of benzofuroxan with thiol groups in biological molecules. The product of the oxidation of 2-mercaptoethanol by benzofuroxan is a disulphide (Shipton *et al.*, 1976). A disulphide should be the oxidation product also in other cases when the thiol groups are (*a*) in relatively unhindered locations in low-molecular-weight molecules, (*b*) in exposed positions in proteins (e.g. some of the class-I thiol groups of urease; see Norris & Brocklehurst, 1976) such that dimer or polymer formation can occur, and (*c*) juxtaposed in pairs to provide for intramolecular disulphide formation as are cysteine-149 and cysteine-153 in glyceraldehyde 3-phosphate dehydrogenase (see, e.g., Wasserman & Major, 1969).

The reaction of benzofuroxan with 2-mercaptoethanol appears to proceed by a rate-determining attack of the thiolate ion on one of the electrophilic centres of benzofuroxan to form a low steady-state concentration of an adduct. The rapid reaction of this adduct with another molecule of thiol, presumably in the anionic state, provides the disulphide of 2-mercaptoethanol (II, Scheme 1) and the product of a two-electron reduction of benzofuroxan, *o*-benzoquinone dioxime (III, Scheme 1).

When oxidations are carried out at high pH (approx. 11) and when disulphide formation is prevented by steric factors, thiols can be oxidized by benzofuroxan to sulphenic acids (Shipton *et al.*, 1977) and these reactions also probably occur through the intermediacy of a thiol–benzofuroxan adduct. The kinetic characteristics of sulphenic acid formation are compared with those of disulphide formation.

The kinetic characteristics of the reaction of benzofuroxan with 2-mercaptoethanol are compared with those of its reaction with the active-centre thiol groups of papain (EC 3.4.22.2), ficin (EC 3.4.22.3) and bromelain (EC 3.4.22.4). The results suggest that for each enzyme the attack of the thiolate ion on benzofuroxan is rate-determining and, if so, probably occurs at C-6 because the reactions appear to be assisted by interaction of the reagent with the active-centre imidazolium ion. Optimal reaction of the active centres with benzofuroxan appears to utilize the same ionizing groups as those required in the acylation step of the catalytic act.

The results obtained by using this reactivity probe suggest that optimal reactivity in neutral media of the active-centre thiol groups of the thiol proteinases requires attack by thiolate ion assisted by interaction of the electrophilic molecule with imidazolium ion. No evidence was obtained that the reactions of benzofuroxan are affected by the state of ionization of aspartic acid-158 of papain. The concept that optimal thiol reactivity and enzymic activity depend on the presence of both the thiolate anion and imidazolium cation as the transition state is approached does not necessarily require this ion pair to predominate in the cysteine–histidine interactive systems (Brocklehurst, 1974) of the ground states of the thiol proteinases.

Materials and Methods

The preparation of the enzymes and of benzofuroxan, the spectroscopic characteristics of this compound and of *o*-benzoquinone dioxime and the buffers used in the present work have been described by Shipton *et al.* (1977).

Kinetic studies

All reactions were carried out at 25.0°C, in 6.7% (v/v) ethanol, I 0.1, and were monitored spectrophotometrically at 350nm (the absorption maximum of benzofuroxan; $\Delta\varepsilon_{350} = 4300$ litre·mol^{-1}·cm^{-1} in the pH range 8–10) and at 430nm (where the absorption of benzofuroxan is negligible and that of *o*-benzoquinone dioxime is maximal;

$$\Delta\varepsilon_{430} = \varepsilon_{430} \text{ for } o\text{-benzoquinone dioxime} = \varepsilon_{\text{lim.}} + \frac{\tilde{\varepsilon} - \varepsilon_{\text{lim.}}}{\left(1 + \frac{[H^+]}{K_a}\right)};$$

$\tilde{\varepsilon} = 5200$ litre·mol^{-1}·cm^{-1};

$\varepsilon_{\text{lim}} = 3740$ litre·mol^{-1}·cm^{-1} and $pK_a = 6.75$; see Shipton *et al.*, 1977).

Complete progress curves were recorded by using a Cary 16K spectrophotometer. Initial rates were determined by using a Morrow stopped-flow attachment coupled to an Aminco DW2 spectrophotometer with chart recorder output and 600nm as the reference wavelength.

The pH values of reaction mixtures were determined after kinetic runs by using Radiometer equipment.

Results and Discussion

(1) *Mechanistic features of the reaction of benzofuroxan with 2-mercaptoethanol suggested by the nature of the products and the stoicheiometry of the reaction*

Investigation of the reactions of benzofuroxan with L-cysteine and with 2-mercaptoethanol by product isolation and spectral analysis (Shipton *et al.*, 1977) demonstrated that below pH10.5 reactions of 2mol of a low-molecular-weight thiol and 1mol of benzofuroxan provides 1mol of disulphide and 1mol of *o*-benzoquinone dioxime. At pH values above pH10.5 the reactions are complicated by the formation of sulphur oxy acids. The kinetic analysis reported in the present work was therefore confined to a pH range below 10.4.

Entropy considerations make a single-step termolecular reaction involving two molecules of thiol and one molecule of benzofuroxan inherently improbable, and this type of mechanism is precluded by the finding that the rate of formation of *o*-benzoquinone dioxime is first-order in both thiol and benzofuroxan (see Section 2*c*). As shown below, this aspect of the kinetic characteristics of the reaction is in accord with an intermediate-adduct mechanism (see Scheme 1) in which adduct formation is rate-determining. The kinetic data are in accord also with mechanisms involving direct electron transfer from the thiol to benzofuroxan without the formation of a thiol–benzofuroxan adduct. These mechanisms involve thiyl radicals ($RS^{\bullet}$) and sulphenyl ions (RS^{+}). The absence from the present work of detectable side reactions commonly undergone by $RS^{\bullet}$ and RS^{+} under aerobic conditions (see, e.g., Purdie, 1967) suggests that the intermediate-adduct model (Scheme 1) may be more plausible, and this is used below to discuss the kinetic data.

(2) *Kinetic analysis of the reaction of 2-mercaptoethanol and benzofuroxan in terms of the intermediate-adduct model (Scheme 1)*

(*a*) *The model.* The kinetic model of Scheme 1 may be simply and generally represented by eqn. (1) in which, in the present context, S is benzofuroxan, R is 2-mercaptoethanol, X is the intermediate adduct and P represents the reaction products, *o*-benzoquinone dioxime and the disulphide of 2-mercaptoethanol, which are formed in equimolar amount.

$$S + R \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} X \xrightarrow{R;k_{+2}} P \qquad (1)$$

Eqn. (1) differs from the commonly encountered two-step intermediate-complex model in that both the formation of X and the formation of P from X involve R.

(*b*) *The steady-state assumption.* The rate equation for a multistep reaction is usually derived by application of an assumption of steady state around the reaction intermediates. Jencks (1969, pp. 590–591) has discussed the confusion that has sometimes arisen when the steady-state assumption has been applied to non-catalytic reactions. As he points out, the absolute rate of change (in $mol \cdot litre \cdot s^{-1}$) of the concentration of an intermediate that does not accumulate to a significant extent is very small compared with those of the reactants and products, and this is the basis of the steady-state assumption. The rate of change of the concentration of the postulated intermediate (dx/dt) is given by rate of decrease in the concentration of the reactants ($-dr/dt$) minus the rate of increase of the concentration of the products (dp/dt). Experimental observation of near-identity of $(-dr/dt)_{t=0}$ and $(dp/dt)_{t=0}$ therefore must imply that both x and dx/dt are very small.

It is important to point out that when such a reaction occurs under conditions where, within the experimental error, $(-dr/dt)_{t=0} = (dp/dt)_{t=0}$, the kinetic analysis provides no evidence for the existence of the postulated intermediate X. It may be possible in some cases to detect X by changing the conditions such that it accumulates to a significant extent, as was done for oxime formation (Jencks, 1959). In other cases it may be necessary to rely on indirect evidence or even on the 'chemical reasonableness' of the postulate.

No evidence was obtained for the accumulation of an intermediate in the reaction of 2-mercaptoethanol with benzofuroxan. The absence of a detectable initial rapid decrease in A_{350} on admixture of the reactants at several pH values in the range 7.76–10.4 and the apparent identity of the initial rates of benzofuroxan loss (monitored at 350nm) and *o*-benzoquinone dioxime production (monitored at 430nm) suggest that if an intermediate is formed, it accumulates at only very low concentration. The reasons for postulating the existence of an intermediate in this reaction were summarized in Section (1), and the inability to detect the postulated intermediate constitutes argument for the validity of the steady-state assumption in this case.

(*c*) *Order of reaction and rate-determining step.* The formation of *o*-benzoquinone dioxime by reaction of 2-mercaptoethanol and benzofuroxan was investi-

gated by recording both complete progress curves and initial (very early) portions of progress curves (A_{430} versus time in each case). The order of reaction determined from a complete progress curve for a single run is the order with respect to time, and that determined from initial rates at different initial concentrations is the order with respect to concentration (Letort, 1937, 1942); the two orders are not always the same (see, e.g., Laidler, 1965).

(i) Analysis of complete progress curves. For the model given in eqn. (1) the steady-state rate equation is eqn. (2), in which r, s and p are the concentrations of R, S and P respectively at time t.

$$v = \mathrm{d}p/\mathrm{d}t = \frac{k_{+1}k_{+2}r^2s}{k_{-1}+k_{+2}r} \qquad (2)$$

Eqn. (2) shows that this type of reaction is first-order with respect to time in S, but has no simple order with respect to time in R. The reaction may approximate to first-order or to second-order with respect to time in R, depending on the relative magnitudes of k_{-1} and k_{+2}. If r_0 (the initial value of r) is greater than $2s_0$ (s_0 being the initial value of s) and $k_{+2}r \gg k_{-1}$ throughout the reaction, the reaction will appear to be first-order with respect to time in R, i.e. eqn. (2) is closely approximated by eqn. (3).

$$v = k_{+1}r \cdot s \qquad (3)$$

If, however, $k_{-1} \gg k_{+2}r_0$, the reaction will appear to be second-order with respect to time in R, i.e. eqn. (2) is closely approximated by eqn. (4), in which K ($= k_{-1}/k_{+1}$) is the dissociation constant of X.

$$v = \left(\frac{k_{+1}k_{+2}}{k_{-1}}\right) r^2 s = \left(\frac{k_{+2}}{K}\right) r^2 s \qquad (4)$$

If $k_{+2}r_0 \gg k_{-1}$ the reaction order with respect to time can change during the reaction. Initially, the reaction will be first-order in R, but if during the reaction $k_{+2}r$ ceases to be greater than k_{-1} the reaction will tend to second-order in R. Indeed, this change in order with respect to time must occur when $s_0 \geqslant 2r_0$, because then r approaches zero as the reaction approaches completion.

Complete progress curves for the reaction of benzofuroxan (S) with 2-mercaptoethanol (R) were recorded at several pH values by using $s_0 = 2$mM and $r_0 = 50\,\mu$M, i.e. $s_0 \gg r_0$. A typical progress curve (for the reaction at pH8.7) and its analysis by a conventional first-order plot is shown in Fig. 1. The linearity of the first-order plot for the first 98% of reaction indicates that numerically k_{+2} (in units of $\mathrm{M^{-1}\cdot s^{-1}}$) is much greater than k_{-1} (in units of $\mathrm{s^{-1}}$), such that $k_{+2}r$ becomes comparable with k_{-1} only when r becomes less than or equal to approx. 1 μM (i.e. 2% of r_0). Thus at pH8.7, k_{+2} (numerically) $\geqslant$ approx. $10^6 k_{-1}$. Similar results were obtained at pH9.86 and 10.33. These reactions are adequately described, therefore, by eqn. (3), and division of the observed first-order rate constant at a particular pH value by s_0 provides a value of k_{+1}, the second-order rate constant for the formation of X from R and S.

(ii) Initial-rate studies. Extensive kinetic study of slow reactions is most readily carried out by making use of initial rates, and this method is in any case the one that most directly reveals reaction order with respect to concentration.

The steady-state rate equation for the model of eqn. (1) may be written in terms of r_0, s_0 and the initial rate, v_i, as eqn. (5). In deriving eqn. (5) terms in x^2 were neglected. This is permissible because

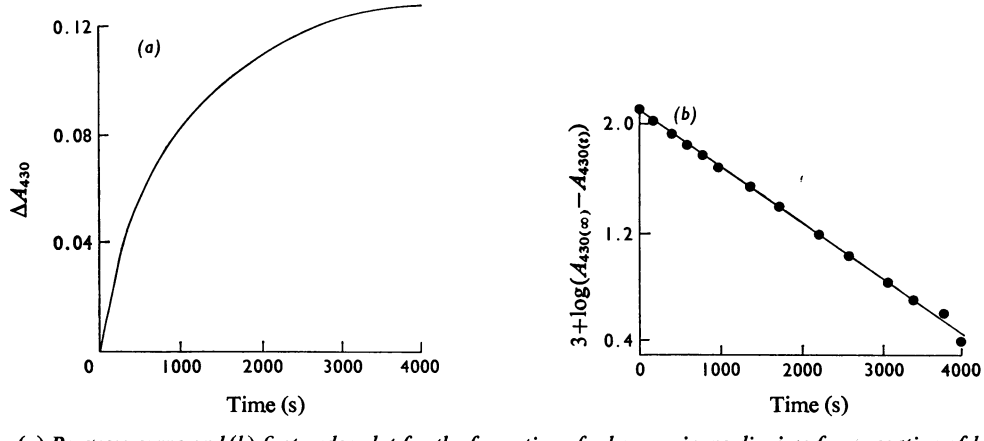


Fig. 1. (*a*) *Progress curve and* (*b*) *first-order plot for the formation of o-benzoquinone dioxime from reaction of benzofuroxan* (2mM) *with 2-mercaptoethanol* (50 μM) *at* 25°C *in* 6.7% (*v*/*v*) *ethanol, pH* 8.7 *I* 0.1
For details see the text. $A_{430(\infty)}$ is A_{430} when the reaction is complete and $A_{430(t)}$ is A_{430} at time t.

$x \ll r_0$ and $x \ll s_0$, and in the steady-state expression the same factors multiply x^2 as multiply xr_0 and xs_0.

$$v_i = \frac{k_{+1}k_{+2}r_0^2 s_0}{k_{-1}+(k_{+1}+k_{+2})r_0+k_{+1}s_0} \quad (5)$$

The possibility that the formation of X or its further reaction to P (see eqn. 2) may be rate-determining may be evaluated in terms of the relative values of k_{-1} and $k_{+2}r$. If the rate of X+R $\rightarrow$ P ($k_{+2}rx$) differs greatly from the rate of X $\rightarrow$ R+S ($k_{-1}x$), either the formation of X from R and S or the formation of P from X and R will be rate-determining.

The relationships of the rate constants that determine the order of reaction with respect to concentration (the 'true' order of reaction; see Letort, 1937, 1942) and the rate-determining step are revealed by considering the denominator of eqn. (5). It is helpful to consider the condition $r_0 \gg s_0$ separately from other conditions of concentration.

(A) $r_0 \gg s_0$. If this condition obtains, the denominator of eqn. (5) becomes $k_{-1}+(k_{+1}+k_{+2})r_0$.

The condition that R+S $\rightarrow$ X be rate-determining is that $k_{+2}r_0 \gg k_{-1}$, and when this condition also is applied, the model of eqn. (1) becomes that of eqn. (6) and the rate equation, eqn. (5), becomes eqn. (7).

$$S \xrightarrow{(R);\,k_{+1}} X \xrightarrow{(R);\,k_{+2}} P \quad (6)$$

$$v_i = \frac{k_{+1}k_{+2}r_0 s_0}{k_{+1}+k_{+2}} \quad (7)$$

This analysis, culminating in eqn. (7), appears to show that the apparent second-order rate constant is an assembly of k_{+1} and k_{+2}, which can approximate to either of these rate constants depending on their relative magnitudes. This is illusory, because for the conditions under discussion, the requirement of a negligible steady-state concentration of X throughout the course of the reaction determines that $k_{+2} \gg k_{+1}$ (see below), and thus the apparent second-order rate constant approximates closely to k_{+1} (eqn. 8):

$$v_i = k_{+1}r_0 s_0 \quad (8)$$

For the model of eqn. (6) the maximum value of x/s_0 during a particular kinetic run is given by eqn. (9) (see Frost & Pearson, 1961, p. 168) in which $\mathscr{R} = k_{+2}/k_{+1}$.

$$\left(\frac{x}{s_0}\right)_{max.} = \mathscr{R}^{\mathscr{R}/(1-\mathscr{R})} \quad (9)$$

The value of $(x/s_0)_{max.}$ decreases as k_{+2}/k_{+1} increases, and $(x/s_0)_{max.}$ becomes negligible only when $k_{+2} \gg k_{+1}$.

The condition that X+R $\rightarrow$ P be rate-determining is that $k_{+2}r_0 \ll k_{-1}$, which provides for the quasi-equilibrium condition. Then eqn. (5) becomes eqn. (10), in which $K = k_{-1}/k_{+1}$.

$$v_i = \frac{k_{+2}r_0^2 s_0}{K+r_0} \quad (10)$$

The inability to detect X implies that $r_0 \ll K$, and eqn. (10) becomes eqn. (11).

$$v_i = \frac{k_{+2}}{K} r_0^2 s_0 \quad (11)$$

The reaction is thus second-order with respect to concentration in r_0, and the experimentally determined third-order rate constant is k_{+2}/K (in units of $M^{-2} \cdot s^{-1}$).

(B) r_0 not $\gg s_0$. Inspection of the denominator of eqn. (5) shows that a reaction that exhibits kinetics that are first-order with respect to concentration in both S and R requires that k_{-1} and $k_{+1}s_0$ be negligible. When r_0 is not $\gg s_0$ this implies that $k_{+2} \gg k_{+1}$,

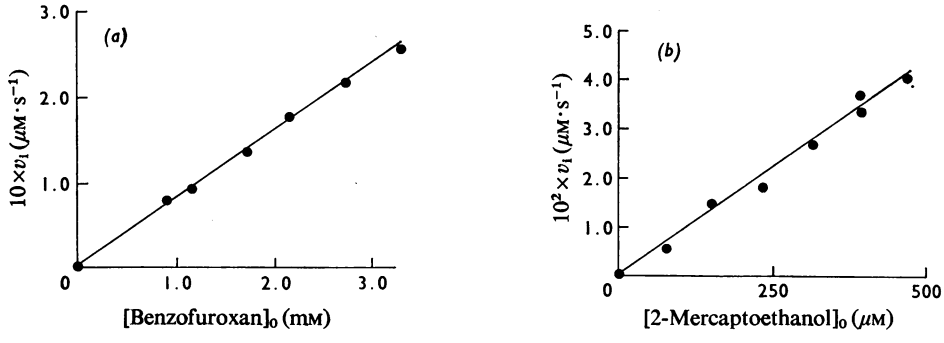


Fig. 2. *Dependence of initial rate of o-benzoquinone dioxime formation on reactant concentration*
(*a*) Dependence of v_i on [benzofuroxan]$_0$ by using [2-mercaptoethanol]$_0$ = 1.76mM, pH7.74, 6.7% (v/v) ethanol, I = 0.1 and 25°C. (*b*) Dependence of v_i on [2-mercaptoethanol]$_0$ by using [benzofuroxan]$_0$ = 1.95mM, pH7.70, 6.7% (v/v) ethanol, I = 0.1 and 25°C. Initial rates were calculated from A_{430}–time data and initial concentrations.

and the rate is given by eqn. (8). As discussed above, when third-order kinetics are exhibited, the rate is given by eqn. (11).

The reaction of benzofuroxan with 2-mercaptoethanol was shown to conform to eqn. (8) by experiments at several values of pH. Typical plots of v_i versus s_0 and r_0 are given in Figs. 2(*a*) and 2(*b*) respectively. The values of second-order rate constants at particular pH values as determined by analysis of complete progress curves and as determined by initial-rate studies were found to be closely similar, e.g. at pH8.7, $k_{+1} = 0.48\,M^{-1}\cdot s^{-1}$ (from the analysis of the progress curve given in Fig. 1) and $k_{+1} = 0.43 \pm 0.03\,M^{-1}\cdot s^{-1}$ (from initial-rate measurements at 12 different values of s_0 and r_0). Thus the reaction of benzofuroxan with 2-mercaptoethanol is first-order with respect to concentration in both reagents, and adduct formation is rate-determining.

(3) *pH-dependence of* k_{+1}

Values of k_{+1} were calculated from initial rates determined in the pH range approx. 6.8–10.3 by using $s_0 = 0.36$mM and $r_0 = 1.47$mM. The pH-dependence of k_{+1} is shown in Fig. 3 and conforms closely to eqn. (12), in which the pH-independent rate constant $\tilde{k}_{+1} = 2.9\,M^{-1}\cdot s^{-1}$ and $pK_a = 9.65$; this pK_a value corresponds closely to that of the thiol group of 2-mercaptoethanol [9.6, Danehy & Noel (1960); 9.72, Irving *et al.* (1964)].

$$k_{+1} = \frac{\tilde{k}_{+1}}{1 + \dfrac{[H^+]}{K_a}} \qquad (12)$$

The most obvious interpretation of the data in Fig. 3 is that the intermediate adduct is formed by unassisted nucleophilic attack of the thiolate ion of 2-mercaptoethanol on one of the electrophilic centres of benzofuroxan, characterized by a second-order rate constant of approx. $3\,M^{-1}\cdot s^{-1}$.

Because adduct formation is rate-determining, the kinetics of these reactions provide no information about the reaction of the adduct with 2-mercaptoethanol. It seems likely, however, that this reaction also involves attack of the nucleophilic thiolate ion of 2-mercaptoethanol.

(4) *Structure of the intermediate adduct, X*

The simplicity of the kinetic form of the reaction of benzofuroxan with 2-mercaptoethanol suggests that the rate-determining attack of the thiolate ion on benzofuroxan occurs at only one electrophilic centre, unless more than one such centre have closely similar reactivities. The *N*-oxide moiety at N-1 of benzofuroxan would be expected to confer electrophilic character particularly on C-6, C-4 and N-3, and thus there are three possible candidates (IV–VI, Scheme 2) for the structure of the adduct.

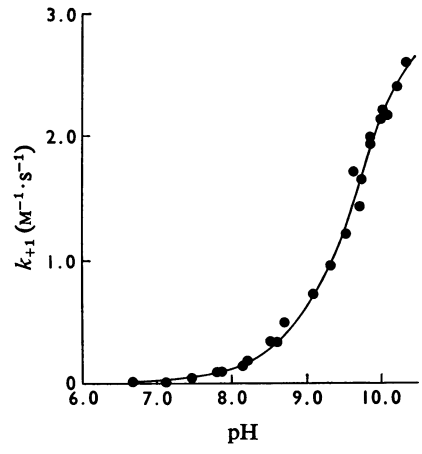


Fig. 3. *pH-dependence of the second-order rate constant* (k_{+1}) *for adduct formation by benzofuroxan and 2-mercaptoethanol at 25°C, in 6.7% ethanol, I 0.1*

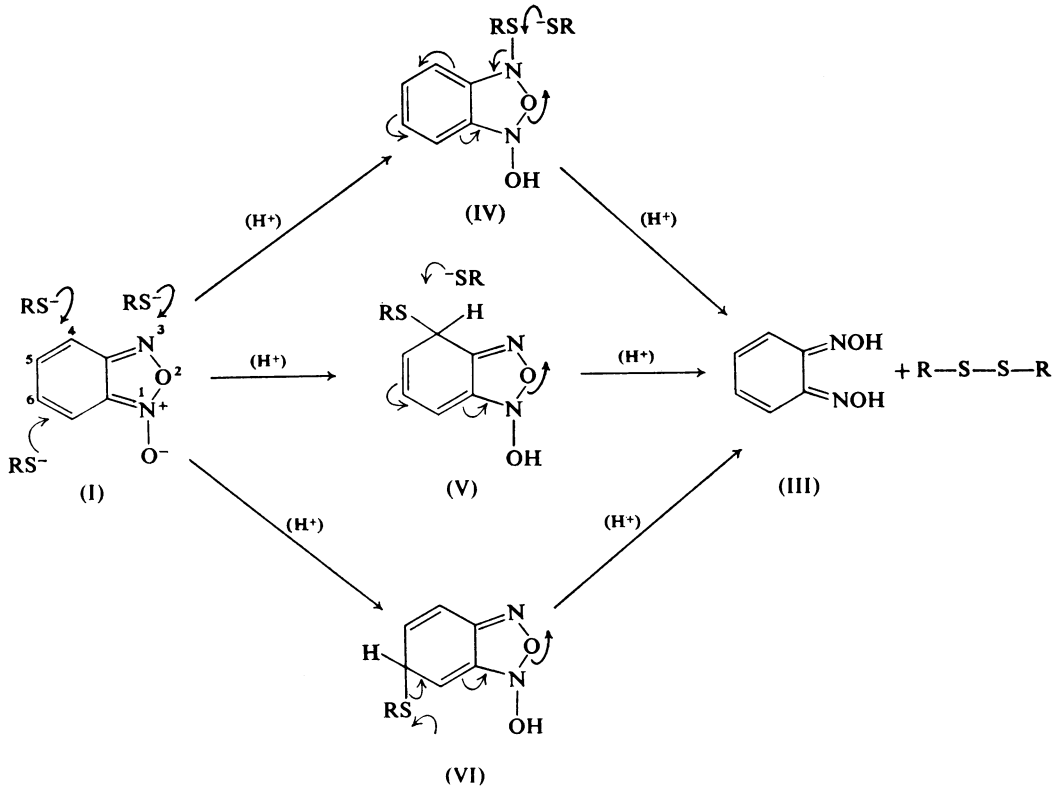
Values of k_{+1} were calculated from initial rates of increase in A_{430} (see the text). The curve is theoretical for

$$k_{+1} = \tilde{k}_{+1}/\left(1 + \frac{[H^+]}{K_a}\right)$$

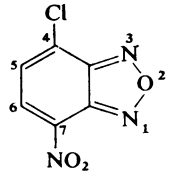
i.e. eqn. (12) in which $\tilde{k}_{+1} = 2.9\,M^{-1}\cdot s^{-1}$ and $pK_a = 9.65$. The parameters were evaluated by linear regression of the reciprocal transform of eqn. (12).

Evidence has been presented for nucleophilic attack by nitrogen and carbon nucleophiles at N-3 and C-4 (Latham *et al.*, 1972*a*,*b*; Latham, 1973). Although reactions of nucleophiles at C-6 of benzofuroxan have not been reported, analogy with the electrophilic character of 4-chloro-7-nitrobenzofurazan (VII) (Di Nunno *et al.*, 1975; Baines *et al.*, 1977) and kinetic analysis of the reactions of benzofuroxan with the thiol-proteinases (see Section 5*c*) suggests that C-6 might be the preferred electrophilic centre.

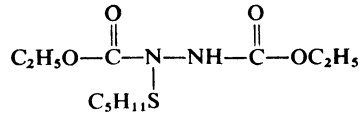
On the other hand, the observation of Ingold (1953) that the atom situated at the terminus of a conjugated electrophilic system is often more susceptible to nucleophilic attack than are atoms at intermediate loci would suggest N-3 of benzofuroxan as the preferred site of attack, and attack at N-3 by secondary amines has been shown to result in the opening of the hetero ring. Oxidation of thiols involving their reaction at electrophilic nitrogen is not without precedent. Kosower *et al.* (1969) proposed that the oxidation of cysteine to cystine by phenyldiazenecarboxylate proceeds via an intermediate formed by



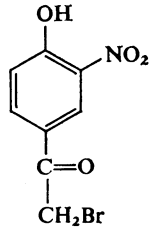
Scheme 2. *Three versions of the intermediate-adduct mechanism for the oxidation by benzofuroxan of a thiol (RSH) to a disulphide (R–S–S–R)*



(VII)



(VIII)



(IX)

Formulae (VII)–(IX)

attack at an azo-nitrogen atom. In an analogous reaction (the oxidation of 1-mercaptopentane by diethyldiazenecarboxylate) Mukaiyama & Takahashi (1968) have isolated the intermediate adduct (VIII).

Whichever electrophilic centre of benzofuroxan is the preferred site of attack by low-molecular-weight thiolate ions in solution, specific binding of the reagent to particular proteins may in some cases dictate that one of the other centres is attacked instead.

(5) *Benzofuroxan as a reactivity probe for thiol groups in proteins*

(*a*) *Characteristics of the probe reagent.* Benzofuroxan fulfills the requirement of one general type of active-centre probe in possessing hydrophobic character together with centres of relatively high electron density. The *N*-oxide oxygen atom could associate with an acidic side chain so as to assist in the orientation of one of the electrophilic centres of benzofuroxan with an active-centre thiolate ion, and

this could help to determine the value of k_{+1} and the form of its pH-dependence. Alternatively the ring oxygen atom could associate with an acidic side chain, and this might be able to provide general acid catalysis of the formation of *o*-benzoquinone dioxime from intermediate adducts such as (IV), (V) or (VI) of Scheme 2, in cases where this step of the reaction was rate-determining (see below). These qualities augment the even more general and necessary characteristic of very high selectivity for one type of functional group, in this case a thiol group. The reactions of benzofuroxan with other types of nucleophile referred to in Section 4 are much slower (see Shipton *et al.*, 1977).

(*b*) *Thiol groups in proteins: effect of environment on the kinetic form of their reaction with benzofuroxan.* As discussed in the introduction, thiol groups in proteins can be classified as (i) exposed thiol groups, which can undergo intermolecular disulphide formation, (ii) juxtaposed thiol groups, which can undergo intramolecular disulphide formation, and (iii) thiol groups that cannot undergo disulphide formation for steric reasons and whose initial oxidation product is a sulphenic acid. In addition, there are so-called 'buried' thiol groups that do not react with electrophilic reagents until exposed by a conformation change (see, e.g., Malthouse & Brocklehurst, 1976). After exposure, these thiol groups could conceivably fall into any of the three classes given above; their apparent reactivities, however, might be determined, at least in part, by the rate of the conformational change.

Reaction of some thiol groups of type (i) with benzofuroxan might be expected to conform to eqn. (8) and provide values of k_{+1}. In other cases, however, the relatively severe steric constraints involved in reactions of two protein molecules might make the formation of *o*-benzoquinone dioxime from the adduct rate-determining and the kinetics second-order in protein (i.e. eqn. 11).

In these reactions, as in all reactions of proteins, it is important to bear in mind the possibility of adsorptive complex-formation before covalency changes. In such cases, even if high dissociation constants make such complexes kinetically undetectable, an apparent second-order rate constant such as k_{+1} should be regarded as a ratio of a first-order rate constant and a dissociation constant.

Reaction of thiol groups of type (ii) would be expected usually to conform to eqn. (8) and provide values of k_{+1}, since the intramolecular attack of the second thiolate ion on the adduct would not normally be expected to be rate-determining. Such reactions could not of course be second-order in R; the appropriate kinetic model is eqn. (13), but without H_2O or OH^- in the step.

When dealing with the reactions of benzofuroxan with thiols of type (iii) also the kinetic model (eqn. 1) must be modified, minimally to the model of eqn. (13).

$$S + R \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} X \xrightarrow{H_2O;\ OH^-;\ k_{+2}} P \qquad (13)$$

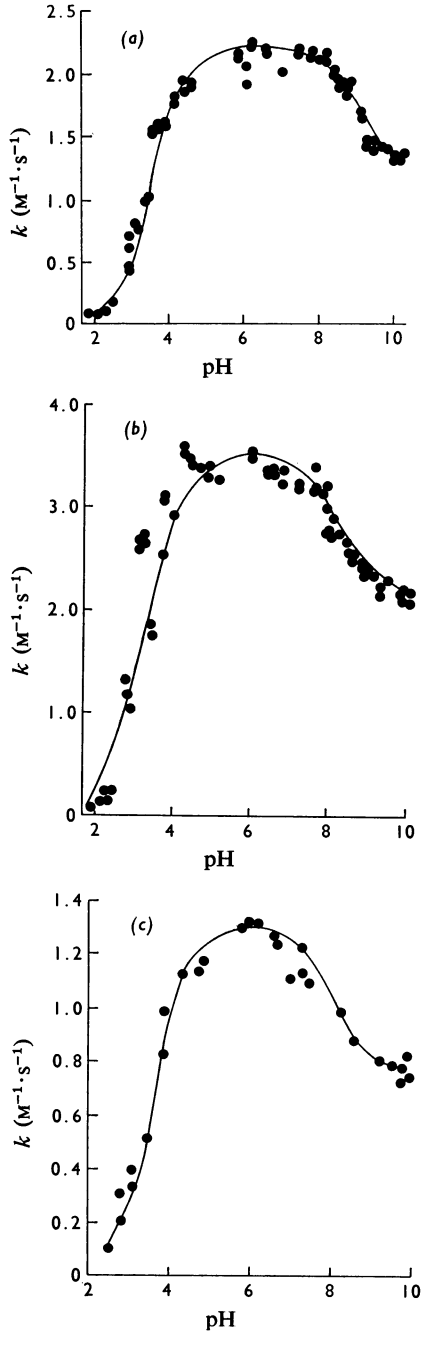
Thiols of type (iii) are readily identified by the stoicheiometry of the reaction (Shipton *et al.*, 1977). In eqn. (13) the sulphur nucleophile R is replaced by an oxygen nucleophile (H_2O or OH^-) in the second step of the reaction. One consequence of this change is that the rate-determining step can no longer be identified by determination of the order of reaction in thiol unless adduct breakdown is rate-determining and X can be made to accumulate to a sufficient extent.

(*c*) *Kinetics of the reactions of benzofuroxan with papain, ficin and bromelain.* The active-centre thiol groups of these thiol proteinases correspond to type (iii) in the classification discussed in subsection 5(*b*). Thus in all three cases, one molecule of enzyme thiol reacts with one molecule of benzofuroxan to produce one molecule of *o*-benzoquinone dioxime and one molecule of inactivated enzyme, which is probably, initially, the sulphenic acid (see Shipton *et al.*, 1977).

Kinetic study of the reaction of benzofuroxan with papain at pH values approx. 3.5, 7 and 9–10, by using initial-rate measurement and analysis of complete progress curves, showed it to be first-order with respect to both time and concentration (up to 20 μM) in papain thiol and first-order with respect to concentration (up to 3 mM) in benzofuroxan. The monophasic nature of the progress curves of A_{350} versus time and the close similarity in the initial rate of increase in A_{430} and the initial rate of decrease in A_{350} provide no evidence for either adsorptive or covalent intermediates. If such intermediates exist, they must therefore be present in very low concentrations.

Second-order rate constants (k) for the reaction of papain with benzofuroxan in the pH range 2–10 were determined by measurement of the initial rates of *o*-benzoquinone dioxime production (ΔA_{430}) by using initial concentrations of 10.8 μM-papain and 1.5 mM-benzofuroxan. The pH–k profile for the papain reaction (Fig. 4*a*) differs markedly from that for the reaction of 2-mercaptoethanol with benzofuroxan (Fig. 3). Whereas Fig. 3 is a simple sigmoidal curve (pK_a 9.65), Fig. 4(*a*) is a combination of a sigmoidal curve similar to that of Fig. 3 but with pK_a 9.0 and a bell-shaped curve characterized by pK_a values of 3.6 and 9.0. The two reactive protonic states suggested by the profile of Fig. 4(*a*) are designated X and XH to indicate their relative stoicheiometries in protons; the X state corresponds to the reaction at pH $\gg$9 and the XH state to the region of the optimum of the bell at pH values around 6. Fig. 4(*a*) suggests that the XH_2 state approached at pH $\ll$4 has negligible reactivity. The interesting features of Fig. 4(*a*) are: (i) two pK_a values are displayed, one about 4 and one about 9; (ii) the reactivities of both the X and XH

state are of similar magnitiude to that of the only reactive protonic state (the X state) of the reaction of 2-mercaptoethanol with benzofuroxan (Fig. 3); (iii) the reactivity of the XH state is greater than that of the X state.



Evidence is accumulating that reactions involving nucleophilic attack by the thiol group of cysteine-25 of papain depend on the state of ionization of the enzyme controlled by two molecular pK_a values, one about 4 and one about 9. Reactions that are thus controlled include acylation by substrate during the catalytic act (see, e.g., Glazer & Smith, 1971), alkylation by both anionic alkylating agents such as chloroacetate (see, e.g., Chaiken & Smith, 1969) and uncharged alkylating agents such as chloroacetamide and methyl iodide (Polgar, 1973; Halász & Polgar, 1976) and thiol–disulphide interchange with 2,2′-dipyridyl disulphide [see, e.g., Brocklehurst (1974) and Shipton *et al.* (1975)]. Although all these reactions were studied under conditions that might reveal pK_a values of ionizing groups in the enzyme molecule, the difficulties inherent in inferring such group pK_a values from observed molecular pK_a values are considerable (see Brocklehurst & Dixon, 1976; Knowles, 1976). Accordingly it is difficult to be sure that, for any one reaction, molecular pK_a values reflected by profiles of pH against second-order rate constant approximate closely to pK_a values characteristic of ionizing groups in the enzyme molecule. However, the fact that reactions of the papain thiol group with several different types of electrophilic centre are controlled by molecular pK_a values of 4 and 9 seems to provide evidence that these pK_a values may indeed be characteristic of the papain molecule. The demonstration in Fig. 4(*a*) that the reaction of papain with benzofuroxan depends on pK_a values close to 4 and 9 may be additional evidence for this postulate. If the pK_a values of Fig. 4(*a*) do characterize ionizations of the free enzyme, the attack of the papain thiol group on benzofuroxan to form a postulated intermediate adduct could be rate-determining, as in the reaction of benzofuroxan with 2-mercaptoethanol (see Section 2). The fact that the pH-independent

Fig. 4. *pH-dependence of the second-order rate constant (k) for the formation of o-benzoquinone dioxime by reaction of benzofuroxan with (a) papain, (b) ficin and (c) bromelain at 25°C in 6.7% ethanol, I 0.1*

Values of k were calculated from initial rates of increase in A_{430} (see the text). The theoretical curves were calculated by using

$$k = \frac{\tilde{k}_{XH}}{1+\frac{[H^+]}{K_I}+\frac{K_{II}}{[H^+]}} + \frac{\tilde{k}_X}{1+\frac{[H^+]}{K_{II}}}$$

an dfor (*a*) $\tilde{k}_{XH} = 2.2\,M^{-1}\cdot s^{-1}$, $\tilde{k}_X = 1.3\,M^{-1}\cdot s^{-1}$, $pK_I = 3.6$, $pK_{II} = 9.0$, for (*b*) $\tilde{k}_{XH} = 3.5\,M^{-1}\cdot s^{-1}$, $\tilde{k}_X = 2.1\,M^{-1}\cdot s^{-1}$, $pK_I = 3.2$, $pK_{II} = 8.2$, and for (*c*) $\tilde{k}_{XH} = 1.3\,M^{-1}\cdot s^{-1}$, $\tilde{k}_X = 0.75\,M^{-1}\cdot s^{-1}$, $pK_I = 3.6$, $pK_{II} = 8.0$. The values of the parameters were obtained by using the optimization procedure described previously (Shipton *et al.*, 1976).

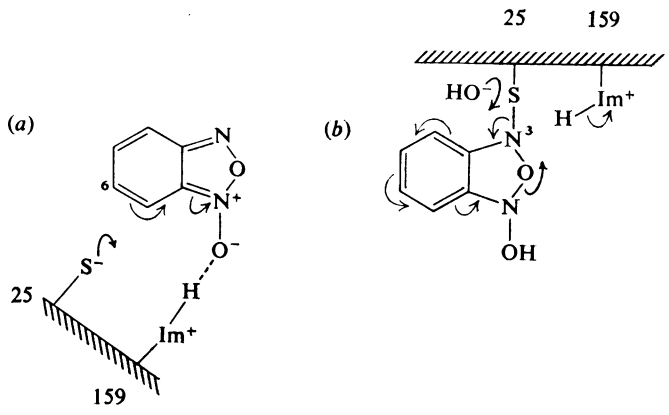
rate constants for both the X and XH states of the papain reaction are similar to that for the X state of the 2-mercaptoethanol reaction (see Table 1) is compatible with a common rate-determining step for both reactions. The available evidence suggests that the papain active centre can exhibit nucleophilicity associated with the thiol group in two protonic states, and the reactions of particular interest are those in which the XH-state reactivity is greater than the X-state reactivity. This is because the acylation step of the catalytic act is characterized by a reactive XH state and X and XH_2 states of negligible reactivity. Fig. 4(*a*) shows that the reaction of the papain thiol group with benzofuroxan is such a reaction. As in the reactions of papain with anionic alkylating agents [see, e.g., Chaiken & Smith (1969) and Jolley & Yankeelov (1972)] the most obvious interpretations of the relatively high reactivity of the XH state is that it results from interaction of a site of high electron density in the reagent (for benzofuroxan, presumably the *N*-oxide oxygen atom) with the imidazolium ion of the postulated active-centre ion pair (cysteine-25 thiolate–histidine-159 imidazolium) (see Scheme 3*a*). The interactive cysteine-25–histidine-159 system is detected most readily by using the two-protonic-state electrophilic probe, 2,2′-dipyridyl disulphide (see Brocklehurst, 1974), and Polgar's (1974) data could mean that this interactive system could contain at least 50% of the ion pair (Malthouse & Brocklehurst, 1976). The active-site geometry of papain suggests that interaction of benzofuroxan with the imidazolium ion could result in attack of the cysteine-25 thiolate ion at C-6 of benzofuroxan (see Scheme 3*a*) to provide an intermediate adduct analogous to Compound (VI) of Scheme 2. This is the electrophilic centre of benzofuroxan that might be predicted to be the preferred site for nucleophilic attack based on analogy with the electrophilic character of 4-chloro-7-nitrobenzofurazan (VII) (see Section 4).

Although the simplest interpretation of Fig. 4(*a*) is that given above, the possibility remains that the similarity in the reactivities of the various protonic states of the reactions of benzofuroxan with papain and with 2-mercaptoethanol is fortuitous and the release of *o*-benzoquinone dioxime from the adduct is rate-determining in the papain reaction. If this is so, the relative reactivities of the XH and X states of the papain reaction could be accounted for in terms of general acid catalysis of adduct breakdown by the imidazolium ion of histidine-159 if the adduct were formed by attack at N-3 (see Scheme 3*b*).

Table 1. *Characteristics of the reactive protonic states of the reactions of benzofuroxan with 2-mercaptoethanol, papain, ficin and bromelain*

Details are given in the text and in the legends to Figs. 3 and 4; $\tilde{k}_{XH}$ and $\tilde{k}_X$ are the apparent pH-independent second-order rate constants that characterize the XH and X states respectively of the reactions; the pK_a values given in parentheses are those that characterize the formation of the particular protonic states.

Thiol	$\tilde{k}_{XH}$ ($M^{-1}\cdot s^{-1}$)	$\tilde{k}_X$ ($M^{-1}\cdot s^{-1}$)
2-Mercaptoethanol	—	2.9 (pK_a 9.65)
Papain	2.2 (pK_a 3.6)	1.3 (pK_a 9.0)
Ficin	3.5 (pK_a 3.2)	2.1 (pK_a 8.2)
Bromelain	1.3 (pK_a 3.6)	0.75 (pK_a 8.0)



Scheme 3. *Possible origins of the high-reactivity of the XH state of the reaction of papain with benzofuroxan*

In (*a*) adduct formation is rate-determining and in (*b*) elimination of *o*-benzoquinone dioxime is rate-determining. Im^+, imidazolium ion.

When a process involving the papain active centre is shown to depend on a state of ionization characterized by a pK_a of 4, an ambiguity in molecular interpretation arises because the papain active centre is characterized by two pK_a values of 3–4 (Shipton *et al.*, 1975). One of these is probably associated with the carboxyl group of aspartic acid-158 and the other with the cysteine-25–histidine-159 interactive pair.

One approach to the possible resolution of this ambiguity is to compare the pH–*k* profile for the reaction of a given reactivity probe with the papain thiol group with the profile for the analogous reaction of the ficin thiol group. This approach was suggested by a study of papain, ficin and bromelain by using 2,2′-dipyridyl disulphide and 4-chloro-7-nitrobenzofurazan as reactivity probes (Malthouse & Brocklehurst, 1976; Shipton *et al.*, 1976). Whereas both of these reactivity probes detect two pK_a values around 3–4 in papain, they detect only one in ficin and probably only one in bromelain, and this is most reasonably associated with the cysteine–histidine interactive system common to all three enzymes. The pH–*k* profiles for the reactions of benzofuroxan with ficin and bromelain are shown in Figs. 4(*b*) and 4(*c*) respectively. The similarity of these profiles to that for the papain–benzofuroxan reaction (Fig. 4*a*) supports the interpretation suggested above that the high XH-state reactivity in each of these reactions derives from assistance provided by the active-centre imidazolium ion. The precise requirements for an XH-state reactivity ($\tilde{k}_{XH}$) to be greater than an X-state reactivity ($\tilde{k}_X$) remain to be determined. Interaction with the active-centre imidazolium ion clearly seems to require a centre of relatively high electron density in an appropriate location in the electrophilic reactant molecule. This requirement could account for values of $\tilde{k}_{XH}/\tilde{k}_X > 1$ for the reactions of papain with chloroacetate and benzofuroxan, but does not seem able to account for the difference in the pH–*k* profiles for the reactions of papain with chloroacetamide (Polgar, 1973) and 1-bromo-4-hydroxy-3-nitroacetophenone (IX) (Furlanetto & Kaiser, 1973). Whereas for the chloroacetamide reaction $\tilde{k}_{XH}/\tilde{k}_X < 1$, this ratio is >1 for the reaction of the bromoketone reagent (IX), and the reaction appears to be unaffected by the state of ionization of the phenolic hydroxyl group. One possibility is that when an electrophilic molecule does not contain a formal negative charge, a centre of relatively high electron density (such as the carbonyl oxygen atom of structure IX) can interact effectively with the active-centre imidazolium ion only if the reagent is relatively hydrophobic. This might account for values of $\tilde{k}_{XH}/\tilde{k}_X > 1$ for structure (IX) and benzofuroxan, but values <1 for chloroacetamide.

It is important to emphasize that the possibility must still remain that the interactive cysteine–histidine systems of these enzymes may contain virtually no ion pair and that protonic redistribution to provide the thiolate ion and imidazolium ion required for effective reactivity may be promoted by the binding of a suitable electrophilic molecule. Whereas an interactive system can be detected with reasonable certainty by using two-protonic-state electrophiles such as 2,2′-dipyridyl disulphide (Brocklehurst, 1974), the location of protons within the system cannot be unambiguously determined either kinetically or by spectroscopic probes (see Dixon, 1976).

It is the presence in benzofuroxan of a suitable site of high electron density that suggests that this particular reactivity probe might be most effectively bound if a proton were located between the *N*-oxide oxygen atom and the imidazolium nitrogen atom as in Scheme 3(*a*). Even if this plausible representation of the adsorptive complex is correct, however, its existence does not permit an assignment of the exact structure of the interactive cysteine–histidine systems in the native enzyme molecules.

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