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# Evaluation of Benzofuroxan as a Chromophoric Oxidizing Agent for Thiol Groups by using its Reactions with Papain, Ficin, Bromelain and Low-Molecular-Weight Thiols

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1. Benzofuroxan (benzofurazan 1-oxide, benzo-2-oxa-1,3-diazole *N*-oxide) was evaluated as a specific chromophoric oxidizing agent for thiol groups. 2. Aliphatic thiol groups both in low-molecular-weight molecules and in the enzymes papain (EC 3.4.22.2), ficin (EC 3.4.22.3) and bromelain (EC 3.4.22.4) readily reduce benzofuroxan to *o*-benzoquinone dioxime; potential competing reactions of amino groups are negligibly slow. 3. The fate of the thiol depends on its structure: a mechanism is proposed in which the thiol and benzofuroxan form an adduct which, if steric factors permit, reacts with another molecule of thiol to form a disulphide; when the thiol is located in the active site of a thiol proteinase and steric factors preclude enzyme dimer formation, the adduct reacts instead with water or $HO^-$ to form a sulphenic acid; attack on the sulphur atom of the adduct by either a sulphur or oxygen nucleophile releases *o*-benzoquinone dioxime. 4. Benzofuroxan contains no proton-binding sites with $pK_a$ values in the range 3–10 and probably none in the range 0–14; *o*-benzoquinone dioxime undergoes a one-proton ionization with $pK_a = 6.75$. 5. *o*-Benzoquinone dioxime absorbs strongly at wavelengths greater than 410nm, where absorption by benzofuroxan, proteins and simple thiol compounds is negligible; 416nm is an isosbestic point ($\varepsilon_{416} = 5110$ litre·mol$^{-1}$·cm$^{-1}$); $\varepsilon_{430} = 3740 + [1460/(1 + [H^+]/K_a)]$ where $pK_a = 6.75$. 6. The possibility of acid–base catalysis of the oxidation by active-centre histidine residues of the thiol proteinases is discussed.

Many reagents have been used to oxidize thiol groups in enzymes and other proteins (see Webb, 1966, pp. 655–700), but, with the exception of certain aromatic disulphides which effect an oxidation by thiol–disulphide interchange, none of them combines specific thiol oxidation over a wide range of pH with an easily followable and quantifiable reaction.

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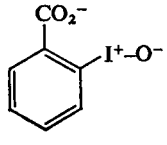
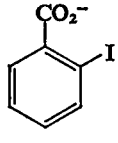
Perhaps the most widely used oxidizing agent has been *o*-iodosobenzoate (I), which is reduced by thiols to *o*-iodobenzoate (II) (see Webb, 1966, pp. 701–728). The extent of reaction is usually measured by determining the quantity of residual *o*-iodosobenzoate by addition of KI and subsequent titration with thiosulphate (Chinard & Hellerman, 1954). The course of the oxidation may be followed also by measuring the decrease in $A_{285}$ due to the loss of the reagent (Leslie & Varricchio, 1968) and Wolf & Wu (1969) used this method to




(I)

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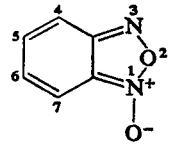


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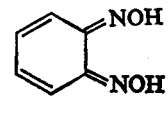



(IV)

study the oxidation of L-cysteine. Spectral analysis at 285nm, however, will generally be difficult when this reagent is used to oxidize thiol groups in proteins.

The present paper reports an evaluation of benzofuroxan (benzofurazan 1-oxide, benzo-2-oxa-1,3-diazole *N*-oxide, III) as an oxidizing agent for thiol groups including those in protein molecules. The reduction of this compound to *o*-benzoquinone dioxime (IV) by thiols is accompanied by a large increase in absorbance at wavelengths far removed from this region where proteins absorb strongly; 416nm is an isosbestic point in the spectrum of *o*-benzoquinone dioxime and the absorbance of benzofuroxan at this wavelength is negligible. Benzofuroxan appears to be a useful oxidizing agent for protein thiol groups. The ease with which the reaction may be monitored continuously makes the reagent suitable for use as a new chemical type of reactivity probe for the study of protein environments of thiol groups.

## Materials and Methods

### *Benzofuroxan*

This was prepared by the method of Boyer & Smith (1963) and purified by chromatography on alumina (type H). A sample (100mg) of crude benzofuroxan was loaded on to an alumina column (25cm×2cm) by using light petroleum (b.p. 60–80°C)/ethyl acetate (4:1, v/v) as solvent. After removal of impurity (predominantly *o*-nitroaniline) by elution with this solvent, pure benzofuroxan was eluted by increasing the ethyl acetate content of the eluent to 1:1 (v/v). The m.p. of samples purified in this way (74–75°C) is similar to the literature value [72–73°C, Boyer & Smith (1963)].

Stock solutions of benzofuroxan (up to 50mM) were prepared in ethanol and were stable for at least 2 weeks when stored at 4°C. The concentration of benzofuroxan was determined as a routine by spectral analysis at 350nm of solutions in 6.7% (v/v) ethanol, pH7.0, by using $\varepsilon_{350} = 6960$ litre·mol$^{-1}$·cm$^{-1}$. The value of $\varepsilon_{350}$ was determined as 6960± 120litre·mol$^{-1}$·cm$^{-1}$ by using solutions of freshly purified benzofuroxan.

### *o-Benzoquinone dioxime*

This was prepared by borohydride reduction of benzofuroxan by the method of Boyer & Ellzey (1960). Recrystallization from water/ethanol (1:1, v/v) gave brown needles (m.p. 148–149°C), which is the value reported by Boyer & Ellzey (1960).

### *Papain (EC 3.4.22.2), ficin (EC 3.4.22.3) and bromelain (EC 3.4.22.4)*

Fully active preparations of these enzymes each containing 1.0mol of thiol groups with high reactivity towards 2,2′-dipyridyl disulphide (2-Py–S–S–2-Py) at pH4.0/mol of protein were prepared by covalent chromatography as described previously (see Stuchbury *et al.*, 1975; Malthouse & Brocklehurst, 1976).

### *Covalent chromatography*

The Sepharose–(glutathione-2-pyridyl disulphide) conjugate was prepared by the method of Brocklehurst *et al.* (1973, 1974).

### *Thiol determinations*

These were performed by using 2-Py–S–S–2-Py as described previously (see Stuchbury *et al.*, 1975). The stoicheiometry of the production of thiopyrid-2-one was determined by using $\varepsilon_{343} = 8080$ litre·mol$^{-1}$·cm$^{-1}$.

### *Buffers*

All reactions were studied at *I* 0.1 and reaction mixtures contained 1mM-EDTA. Buffers were prepared three times more concentrated than as described in King & Sperry (1961) and Dawson *et al.* (1961) and were diluted 1:3 into reaction mixtures. The following buffer systems were used: pH2.8–4.0, formic acid/NaOH; pH4.2–5.4, acetic acid/NaOH; pH5.8–7.8, $KH_2PO_4$/NaOH; pH8.0–9.4, $Na_4P_2O_7$/HCl; pH9.0–10.6, $NaHCO_3$/NaOH.

Although in trial experiments reaction even at high pH of benzofuroxan with buffer salts containing amino groups was shown to be negligible compared with the rates of reaction of this compound with thiols, glycine and Tris buffers were avoided. Borate buffers were also avoided in experiments with benzofuroxan because of the altered spectral characteristics of *o*-benzoquinone dioxime in these buffers, resulting presumably from chelation effects.

### *Spectroscopic measurements*

Electronic spectral measurements were made by using a Cary 16K spectrophotometer and an Aminco DW2 dual-wavelength spectrophotometer with the reference wavelength set to 600nm. Infra-red spectra were recorded by using a Perkin–Elmer 237 grating instrument.

### *Assay of papain esterolytic activity*

This was performed in a 3ml reaction volume at pH6.0 and 25°C by using α-*N*-benzoyl-L-arginine ethyl ester [Sigma (London) Chemical Co., London S.W.6, U.K.] as substrate and a Radiometer titrator as a pH-stat.

### *Separation of protein from low-molecular-weight compounds*

The separation of activated enzyme from reducing agent and modified protein from excess of the modifier and reaction product was performed by using a column (15cm×3cm) packed with Sephadex G-15

(fine). The column was pre-equilibrated in 0.1M-KCl and 1mM-EDTA for 15–20min and the protein samples (≤5ml) were applied and eluted with the same solution by using a flow rate of approx. 70ml/h.

*Preparation of samples of papain modified by reaction with benzofuroxan*

Papain (4.6ml of approx 60$\mu$M-enzyme) in 0.1M-KCl containing 1mM-EDTA were mixed with a solution containing 1ml of 0.25M-$KH_2PO_4$/NaOH, pH6.0, and 0.4ml of 45mM-benzofuroxan in ethanol. The mixture was left for 30min and the protein was then isolated by chromatography on Sephadex G-25 as described above.

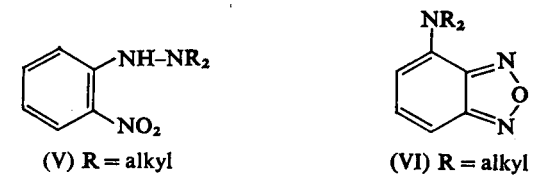
*Preparation of the products of the reaction of benzofuroxan with L-cysteine*

A solution of benzofuroxan (54.4mg, 0.4mmol) in ethanol (10ml) was added to a solution of L-cysteine (96.8mg, 0.8mmol) in water (10ml) and the pH of the solution was adjusted to 9.6 with 1M-NaOH. A fine white precipitate formed and the supernatant became dark red in colour. The reaction mixture was stirred for 1h at room temperature (22°C). The progress of the reaction was monitored by removal of 10$\mu$l samples of reaction mixture each of which were diluted into 3ml of $Na_2CO_3$ buffer (pH9.4, $I$ 0.1). When the value of $A_{430}$, read against a buffer blank, had increased to a constant value the reaction was assumed to be complete. The pH of the reaction mixture was then adjusted to 7.0 with 1M-HCl to ensure complete precipitation of the postulated L-cystine product and the mixture was kept at 4°C for 1h. The precipitate was isolated by gravity filtration by using pre-weighed Whatman no. 1 filter paper and washed with small volumes of ice-cold water and ethanol. The filter paper and precipitate were dried to constant weight at 80°C. The white precipitate weighed 80.5mg. The filtrate and washings were pooled, evaporated to low volume under vacuum and acidified with acetic acid. The solution was kept at 4°C overnight, during which time small dark brown crystals formed. These were isolated by filtration and after drying weighed 45mg. They were then recrystallized from water/ethanol (1:1, v/v). The white precipitate and dark-brown crystals were identified as L-cystine and *o*-benzoquinone dioxime respectively as described in the Results and Discussion section.

## Results and Discussion

*Electrophilic character of benzofuroxan and its selectivity for thiol groups*

In view of the multiplicity of nucleophilic centres in proteins it is important to know something about the electrophilic character and selectivity of potential group-specific reagents. Delocalization of the positive charge on N-1 of benzofuroxan would be expected to confer electrophilic character on N-3, C-4, and C-6, and the limited studies that have been made provide evidence for electrophilic character at N-3 and C-4. Nucleophilic attack by secondary amines has been shown to occur at both N-3 and C-4 (Latham *et al.*, 1972*a*, Latham, 1973). Attack at N-3 is followed by opening of the hetero ring to provide a substituted *o*-nitrophenylhydrazine (V), whereas attack at C-4 is followed by loss of water to provide a 4-substituted benzofurazan (VI). Benxofuroxan reacts with primary nitroalkanes to form 1-hydroxybenzimidazole 3-oxides and Latham *et al.* (1972*b*) have suggested that the reaction proceeds via an initial attack on N-3 by the nitroalkane carbanion.




(V) R = alkyl (VI) R = alkyl

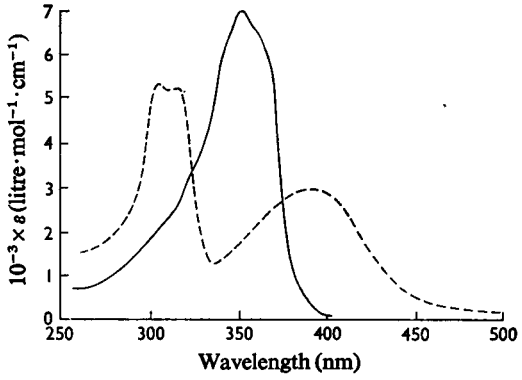



Fig. 1. *Electronic absorption spectrum of benzofuroxan in* 6.7% *ethanol, I* 0.1 *at* 25°C *and pH* 7.0 (——), *and in* 17M-$H_2SO_4$ (– – –)

Study of the reaction of benzofuroxan with thiols has been limited to the aromatic thiols, thiophenol and $\beta$-thionaphthol. Abu El-Haj *et al.* (1972) found that these thiophenols are quantitatively oxidized to their respective disulphides in the presence of excess of benzofuroxan, the latter being reduced to *o*-benzoquinone dioxime.

It is demonstrated below that aliphatic thiol groups, both in low-molecular-weight molecules and in the enzymes papain, ficin and bromelain, are quantitatively oxidized by benzofuroxan with concomitant production of *o*-benzoquinone dioxime. These reactions appear to be much faster than possible competing reactions with other nucleophiles,

which indicates that benzofuroxan may possess a high degree of specificity for thiol groups in proteins.

*Spectral and ionization characteristics of benzofuroxan*

The electronic absorption spectrum of benzofuroxan at pH7.0 and 25°C ($I$ 0.1) in 6.7% (v/v) ethanol is shown in Fig. 1. The spectrum is characterized by a complex asymmetric absorption band ($\lambda_{max.} = 350$nm; $\varepsilon_{350} = 6960$litre·mol$^{-1}$·cm$^{-1}$). This band remains unchanged in the pH range 1–13 and is not changed even in 4M-NaOH or 5M-HCl. This indicates that benzofuroxan probably does not possess proton-binding sites that protonate in the pH range 0–14. In 17M-$H_2SO_4$, however, the spectrum is markedly changed (see Fig. 1 and consists of a complex band with maxima at 305nm and 315nm ($\varepsilon_{315} = 5320$litre·mol$^{-1}$·cm$^{-1}$) and a broad band with $\lambda_{max.} = 387$nm ($\varepsilon_{387} = 2980$litre·mol$^{-1}$·cm$^{-1}$). These spectral changes are consistent with the observations of Gripper-Gray (1966) who suggested that protonation of either N-3 or the *N*-oxide oxygen atom was characterized by $pK_a = -8.3 \pm 0.1$ (see Boulton & Ghosh, 1969).

Potentiometric titration of solutions of benzofuroxan [2mM in 6.7% (v/v) ethanol] showed that no uptake or loss of protons occurred in the pH range 3–10, outside which titration of solvent precluded such experiments.

*Spectral and ionization characteristics of o-benzoquinone dioxime*

The electronic absorption spectrum of *o*-benzoquinone dioxime at various pH values (25°C, $I$ 0.1) in 6.7% (v/v) ethanol is shown in Fig. 2. In weakly acidic media the spectrum is characterized by one major absorption band, $\lambda_{max.} = 395$nm ($\varepsilon_{395} = 6450$litre·mol$^{-1}$·cm$^{-1}$). In alkaline media this band shifts to longer wavelengths ($\lambda_{max.} = 430$nm, $\varepsilon_{430} = 5200$litre·mol$^{-1}$·cm$^{-1}$), which is in accord with the spectral changes reported by Thompson & Foley (1972).

The pH-dependence of $\varepsilon_{430}$ is shown in Fig. 3 and is described by eqn. (1) in which $\tilde{\varepsilon} = 5200$litre·mol$^{-1}$·cm$^{-1}$, $\varepsilon_{IIm.} = 3740$litre·mol$^{-1}$·cm$^{-1}$ and $pK_a = 6.75$.

$$\varepsilon_{430} = \varepsilon_{IIm.} + \frac{\tilde{\varepsilon} - \varepsilon_{IIm.}}{1 + \frac{[H^+]}{K_a}} \quad (1)$$

Potentiometric titration of solutions of 10mM-*o*-benzoquinone dioxime in 6.7% (v/v) ethanol showed that this spectral change is accompanied by a change in proton stoicheiometry of 1mol of $H^+$/mol. In view of the various possibilities that exist in this molecule for zwitterion formation,

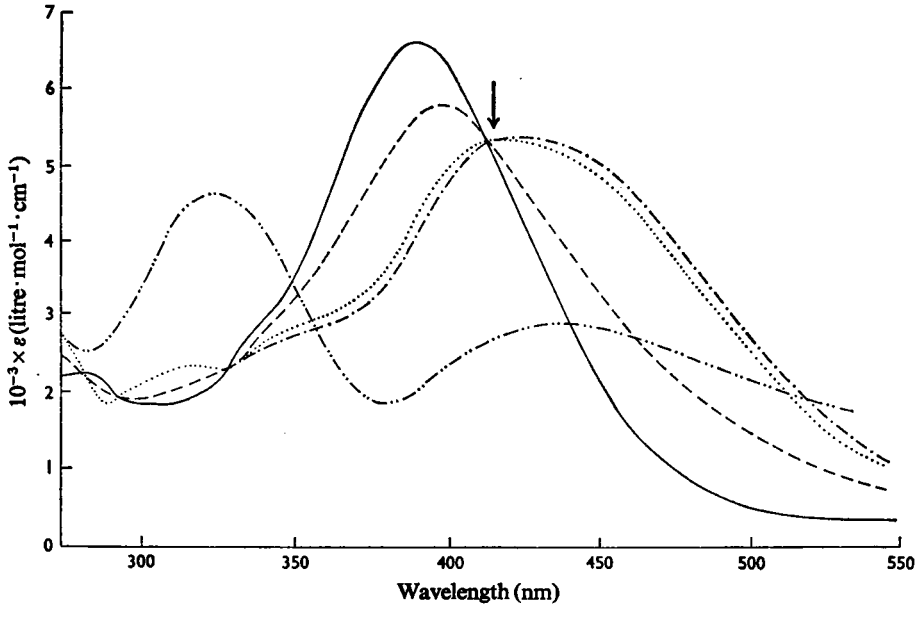



Fig. 2. *Electronic absorption spectrum of o-benzoquinone dioxime in* 6.7% *ethanol, I* 0.1 *at* 25°C
——, pH4.02; – – –, pH7.62; ····, pH7.90; –·–·, pH11.95; –··–, 5M-HCl. The arrow at 416nm shows the isosbestic point ($\varepsilon_{416} = 5110$ litre·mol$^{-1}$·cm$^{-1}$).

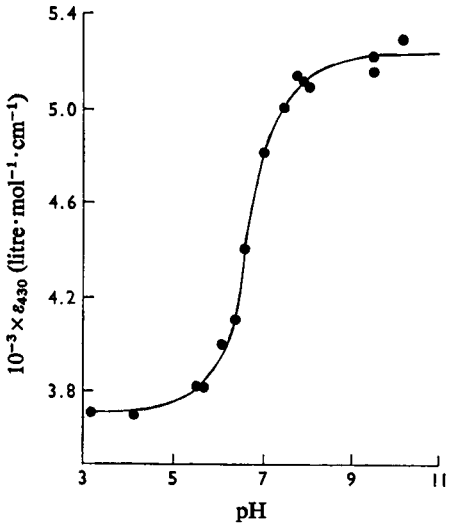



Fig. 3. *pH-Dependence of $\varepsilon_{430}$ of o-benzoquinone dioxime in 6.7% ethanol, I 0.1 at 25°C*

The line corresponds to

$$\varepsilon_{430} = \varepsilon_{11m.} + \frac{\tilde{\varepsilon} - \varepsilon_{11m.}}{1 + \frac{[H^+]}{K_a}}$$

$\tilde{\varepsilon} = 5200$ litre·mol$^{-1}$·cm$^{-1}$, $\varepsilon_{11m.} = 3740$ litre·mol$^{-1}$·cm$^{-1}$ and $pK_a = 6.75$.

intramolecular hydrogen bonding and geometrical isomerism a precise interpretation of the spectral changes is not obvious. Protonation of one oxime nitrogen atom might be predicted to result in a bathochromic shift by increase in chromophore polarity and likewise protonation of the second nitrogen atom might give rise to a subsequent hypsochromic shift. An additional reversible spectral change is observed in 5M-HCl in which the main absorption band is shifted to shorter wavelengths (see Fig. 2). Benzofuroxan does not absorb appreciably at wavelengths above approx. 410nm and the production of *o*-benzoquinone dioxime is conveniently monitored either at 430nm by making use of eqn. (1) or at 416nm, which is an isosbestic point ($\varepsilon_{416} = 5110$ litre·mol$^{-1}$·cm$^{-1}$) (see Fig. 2).

*Reaction of benzofuroxan with low-molecular-weight thiols*

1. *Identification of reaction products.* The electronic absorption spectrum of a solution of benzofuroxan (100$\mu$M) at pH9.6 is shown in Fig. 4. Addition of a negligible volume (50$\mu$l) of a solution of *N*-acetyl-L-cysteine or 2-mercaptoethanol to give a concentration of thiol approx. 100 times the benzo-

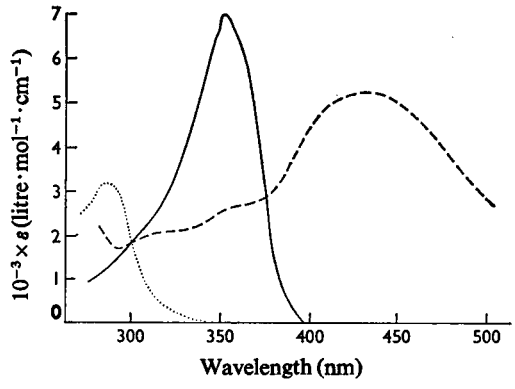



Fig. 4. *Changes in the electronic absorption spectrum of benzofuroxan resulting from its reaction with* L-*cysteine at pH9.6 in 6.7% ethanol (I 0.1M) and 25°C*

——, Spectrum of benzofuroxan (100$\mu$M); ----, spectrum approx. 3min after addition of L-cysteine to give a concentration of 10mM; ····, spectrum of the reaction mixture 16h after the addition of the L-cysteine.

furoxan concentration resulted in a decrease in $A_{350}$ and the formation of a new absorption band with $\lambda_{max.} = 430$nm (see Fig. 4). This spectral change was complete after approx. 3min. The spectrum of the product in the reaction mixture is closely similar to that of *o*-benzoquinone dioxime (Fig. 2) and $\Delta\varepsilon_{430}$ for the reaction is equal to $\varepsilon_{430}$ for *o*-benzoquinone dioxime.

When the reaction mixture was kept at pH9.6 and room temperature (approx. 22°C) the spectrum underwent a slow additional change during a period of 16h. The absorption band at 430nm collapsed and a new band formed with $\lambda_{max.} = 287$nm and $\varepsilon_{max.} = 3014$ litre·mol$^{-1}$·cm$^{-1}$ (see Fig. 4). This second slow spectral change occurred less rapidly still if either a lower concentration of the thiol was used or if the reaction was performed at lower pH values.

It is demonstrated below that the product derived from benzofuroxan formed in the initial rapid reaction with thiol is *o*-benzoquinone dioxime. The subsequent slow spectral change is consistent with the conversion of *o*-benzoquinone dioxime into *o*-phenylenediamine [$\lambda_{max.} = 289$nm; $\varepsilon_{max.} = 2890$ litre·mol$^{-1}$·cm$^{-1}$ in 0.2M-$Na_2CO_3$, Anno & Sado (1956)] in the presence of excess of thiol. This is the product of electrochemical reduction of *o*-benzoquinone dioxime (Thompson & Foley, 1972).

To establish the nature of the products of the reaction of benzofuroxan with a low-molecular-weight thiol, a reaction was performed on a semi-microscale, (see the Materials and Methods section). For this experiment L-cysteine was used as the thiol because the low solubility of the expected product, L-cystine,

facilitated its isolation. It was predicted that adduct formation involving the attack of the amino group of L-cysteine with benzofuroxan (Ruggli & Buchmeier, 1945; Latham *et al.*, 1972*a*) would not be a competing reaction, because reactions of amines with benzofuroxan generally require more forcing conditions (Ruggli & Buchmeier, 1945). Also, no reaction of glycine with benzofuroxan was observed under the same conditions as those used in the L-cysteine reaction over the same time period.

The white precipitate formed by the reaction of benzofuroxan with a 2-fold excess of L-cysteine at pH9.6 was identified as L-cystine by the close similarity of its i.r. spectrum (nujol mull) to that of an authentic sample of L-cystine. The yield was 83% based on L-cysteine.

The brown solid, isolated by evaporation from the supernatant after removal of the L-cystine was identified as *o*-benzoquinone dioxime by comparison with an authentic sample of this compound obtained by borohydride reduction of benzofuroxan. The two samples had closely similar m.p. values, u.v. spectra, i.r. spectra, and p$K_a$ values; potentiometric titration gave the mol.wt. of the unknown product as 134±2 (mol.wt. of *o*-benzoquinone dioxime is 138).

The yield of *o*-benzoquinone dioxime isolated was 83% based on benzofuroxan.

2. *Reaction stoicheiometry and possible outline mechanism.* The above results suggest a reaction stoicheiometry at pH9.6 of two molecules of thiol to one molecule of benzofuroxan. More accurate results were obtained by spectral analysis of reactions of 2-mercaptoethanol (47.6–286 μM). The formation of *o*-benzoquinone dioxime was followed as an increase in $A_{430}$ recorded against benzofuroxan-buffer blanks and its final concentration was calculated in each case by using $\varepsilon_{430} = 5300\,\text{litre}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ (see eqn. 1). A plot of final *o*-benzoquinone dioxime concentration versus initial thiol concentration (Fig. 5) is linear with slope 0.48±0.02, which demonstrates that, under the conditions used, the production of one mol of *o*-benzoquinone dioxime by reduction of one mol of benzofuroxan requires the consumption of two mol of thiol; by analogy with the L-cysteine reaction, these become the corresponding disulphide.

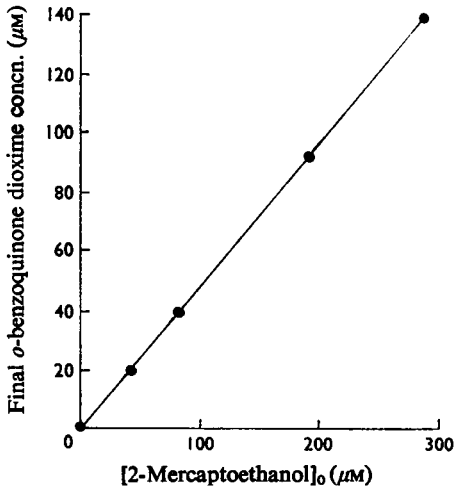



Fig. 5. *Stoicheiometry of the reaction of 2-mercaptoethanol with excess of benzofuroxan at pH9.6 in 6.7% ethanol (I 0.1) at 25°C*

[Benzofuroxan]$_0$ = 1 mM; the slope of the line is 0.48±0.02.

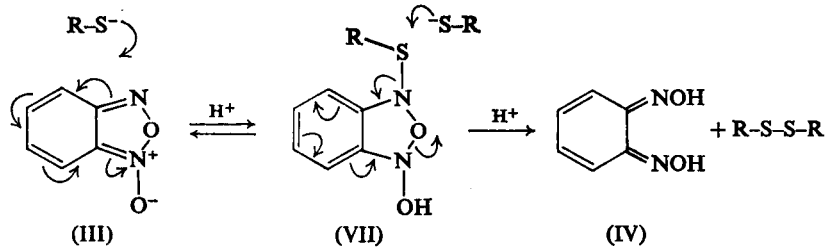
Entropy considerations make a single-step termolecular reaction inherently improbable and a more complex mechanism is suggested by the finding that the rate of production of *o*-benzoquinone dioxime is first order in both thiol and benzofuroxan (M. Shipton & K. Brocklehurst, unpublished work). In view of the known ability of benzofuroxan to react with other nucleophiles to form adducts, one possible mechanism could involve a rate-limiting attack of the thiol on one of the electrophilic centres of benzofuroxan (see above) to form an adduct. This could then subsequently react relatively rapidly with another molecule of thiol (RSH) to provide the disulphide (R–S–S–R) and *o*-benzoquinone dioxime (eqn. 2).

$$\text{RSH} + \text{benzofuroxan} \rightleftharpoons \text{adduct} \xrightarrow{\text{RSH}} \text{R–S–S–R} + o\text{-benzoquinone dioxime} \qquad (2)$$

One version of eqn. (2) is shown in Scheme 1; the atom situated at the terminus of a conjugated electrophile system is often more susceptible to nucleophilic attack than atoms at intermediate loci (see Ingold, 1953) and attack at N-3 by nitrogen nucleophiles has previously been shown to result in the opening of the hetero ring (see above). It must be pointed out, however, that mechanisms for the model represented by eqn. (2) could be written instead in terms of adduct formed by nucleophilic attack at either C-4 or C-6. For convenience, subsequent discussion is given in terms of the mechanism shown in Scheme 1.

The second step of eqn. (2) and Scheme 1 must be essentially irreversible, because (*a*) *o*-benzoquinone dioxime is produced in amount stoicheiometric with either the [benzofuroxan]$_0$ or ½[RSH]$_0$, which ever is the smaller (the subscript 0 denotes initial concentrations), and (*b*) no loss of *o*-benzoquinone dioxime was observed on its admixture with 2-mercaptoethanol disulphide (both approx. 300 μM) at several pH values in the range 3–10.

Benzofuroxan is stable at room temperature in 4M-NaOH. The variation in the stoicheiometry of




Scheme 1. *Possible mechanism for the oxidation of thiols to disulphides by benzofuroxan*

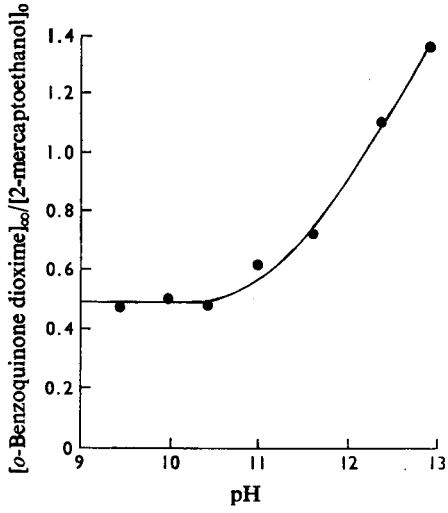



Fig. 6. *pH-dependence of the stoicheiometry of the reaction of* 47 $\mu$M-*2-mercaptoethanol with* 2.0 mM-*benzofuroxan in* 6.7% *ethanol* (*I* 0.1) *at* 25°C

its reaction with 2-mercaptoethanol at high pH (using $[RSH]_0 = 47\,\mu M$ and $[\text{benzofuroxan}]_0 = 2\,mM$) therefore suggests the formation of an intermediate [such as compound (VII) in Scheme 1] that is more susceptible to nucleophilic attack (by $HO^-$) than benzofuroxan itself. Fig. 6 shows how the ratio $x = [o\text{-benzoquinone dioxime}]_\infty/[2\text{-mercaptoethanol}]_0$ varies with pH; at pH values above 10.4 the increase in the ratio $x$ to values greater than 0.5 can be explained by participation of $HO^-$ instead of $RS^-$ in the breakdown of the adduct (VII) in the second step of Scheme 1. Such a reaction could still result in the production of *o*-benzoquinone dioxime, but sulphenic acid (RSOH) would be formed instead of disulphide. Values of $x > 1$ observed at pH values >12.2 could result from disproportionation of the sulphenic acid to regenerate thiol and produce sulphinic and sulphonic acids. An alternative explanation is that the sulphenic acid might itself form an alkali-labile adduct with benzofuroxan. The ability of sulphenic acids (presumably as their anions) to act as nucleophiles has been reported (Kice & Cleveland, 1973; Kukolja & Lammert, 1973).

It is possible to rationalize all the results given above in terms of direct electron-transfer mechanisms involving thiyl radicals ($RS^\bullet$) and sulphenyl ions ($RS^+$). The absence of detectable side reactions commonly undergone by $RS^\bullet$ and $RS^+$ under aerobic conditions suggests, however, that the intermediate adduct model (eqn. 2 and Scheme 1) may be more plausible and this is used below to discuss oxidation of protein thiol groups by benzofuroxan.

*Reactions of papain with benzofuroxan*

1: *General.* The course of the reaction of benzofuroxan with thiol groups in proteins might be expected to vary with the environment of the thiol group. Reaction with exposed thiol groups might be predicted to result in intermolecular disulphide formation, i.e. formation of protein dimers or polymers if steric considerations permit. Proteins containing suitably juxtaposed thiol groups might be expected to undergo intramolecular disulphide formation.

It was decided to study the effect of benzofuroxan on papain, because the environment of the single thiol group of this enzyme is reasonably well defined and the formation of papain dimers remains unlikely on steric grounds, despite the work of Kazanskaya & Nicolskaya (1975) who claim to have prepared such dimers.

2: *Spectral changes accompanying the reaction of papain with benzofuroxan.* Reaction of papain (approx. 50 $\mu$M) with excess of benzofuroxan (approx. 3 mM) at pH 8.2 and 25°C (*I* 0.1) in 6.7% (v/v) ethanol produced an increase in $A_{430}$, which reached a stable value after 10 min; $\Delta\varepsilon_{430}$, based on $[RSH]_0$ (R = papain) was 5400 litre·mol$^{-1}$·cm$^{-1}$. A similar reaction performed at pH 4.0 resulted in an increase in $A_{395}$; $\Delta\varepsilon_{395}$, based on $[RSH]_0$ was

6300 litre·mol$^{-1}$·cm$^{-1}$. These values of the difference extinction coefficients correspond closely to the values of $\varepsilon$ for *o*-benzoquinone dioxime: pH 8.2, $\lambda_{max.} = 430$ nm, $\varepsilon_{max.} = 5200$ litre·mol$^{-1}$·cm$^{-1}$; pH 4.0, $\lambda_{max.} = 395$ nm, $\varepsilon_{max.} = 6450$ litre·mol$^{-1}$·cm$^{-1}$. These spectral data suggest therefore that papain reacts readily with benzofuroxan in both weakly acid and weakly alkaline media to produce one mol of *o*-benzoquinone dioxime/mol of enzyme thiol. This is in contrast with the reaction of benzofuroxan with simple low-molecular-weight thiols in which two mol of thiol are required to produce one mol of *o*-benzoquinone dioxime under similar reaction conditions.

The nature and stoicheiometry of formation of the *o*-benzoquinone dioxime product in the papain reaction was confirmed by an extensive study of the pH-dependence of $\Delta\varepsilon_{430}$ (based on $[RSH]_0$). In the pH range 3.8–10.0 the $\Delta\varepsilon_{430}$–pH profile was closely similar to the $\varepsilon_{430}$–pH profile for *o*-benzoquinone dioxime shown in Fig. 3.

3: *Nature of benzofuroxan-modified papain.* The electronic absorption spectrum at pH 6.0 of a sample of benzofuroxan-modified papain (prepared free from low-molecular-weight material, as described in the Materials and Methods section) was identical with that of native papain at pH 6.0. This provides further evidence that the increase in $A_{430}$ observed during the reaction of papain with benzofuroxan is due to production of a non-protein bound, low-molecular-weight chromophoric species. The proposition that benzofuroxan reacts specifically with the active-centre thiol group of papain is supported by the finding that no increase in $A_{430}$ was observed on admixture of benzofuroxan with papain –S–S–2-Py prepared by reaction with 2-Py–S–S–2-Py.

Samples of papain modified by reaction with benzofuroxan at pH 3.3, 6.0 and 9.8 exhibited no esterolytic activity towards $\alpha$-*N*-benzoyl-L-arginine ethyl ester in the absence of reducing agents. Incubation of the inactive papain derivative with 20 mM-L-cysteine and 5 mM-dithiothreitol at pH 8.0 for 5 min at 25°C ($I$ 0.1), or inclusion of these concentrations of the reducing agents in the assay mixture, resulted in the restoration of 15±2% of the esterolytic activity of the native enzyme. Activity was not increased further by incubation with the reducing agents for longer times.

Since *o*-benzoquinone dioxime is formed from benzofuroxan by reduction, the papain thiol group must become oxidized during the reaction. Oxidation to the disulphide state does not occur. Papain possesses only one free thiol group, that of cysteine-25 (see, e.g., Glazer & Smith, 1971) so intramolecular disulphide-bond formation cannot occur. Also, as was expected, papain dimers are not formed in the benzofuroxan reaction: the elution patterns for benzofuroxan-modified papain and native papain from a Sephadex G-100 column (26 cm×2.5 cm) cannot be distinguished, which demonstrates that the molecular weights of papain and its oxidation product do not differ by a factor of 2.

The stoicheiometry of the oxidation demands a net loss of two electrons from the thiol sulphur atom to provide a sulphenyl derivative, and the most likely candidate is papain sulphenic acid.

Addition of an excess of a low-molecular-weight aliphatic thiol (RSH) to papain sulphenic acid (papain–SOH) might be expected to restore enzymic activity completely by stoicheiometric regeneration of the active-centre thiol group as in eqns. (3) and (4).

$$\text{Papain–SOH} + \text{RSH} \rightarrow \text{papain–S–S–R} + \text{H}_2\text{O} \quad (3)$$

$$\text{Papain–S–S–R} + \text{RSH} \rightarrow \text{Papain–SH} + \text{R–S–S–R} \quad (4)$$

Sulphenic acids have been shown to possess the type of electrophilic character required by eqn. (3) (Kukolja & Lammert, 1973). If papain sulphenic acid is the initial product of the reaction of papain and benzofuroxan, therefore, it is necessary to propose a further process to account for the 85% irreversible loss of activity. Apart from a structural change that makes the sulphenic acid moiety inaccessible to the aliphatic thiol, the most obvious further process that a sulphenic acid could undergo is oxidation by molecular $O_2$ to sulphinic and/or sulphonic acids (see, e.g., Sokolovsky *et al.*, 1969). Regeneration of active papain from either of these oxidation states would not be achieved by addition of an aliphatic thiol (see, e.g., Glazer & Smith, 1965; Drenth *et al.*, 1975). The 15% activity that is regenerated by this treatment may therefore derive from papain sulphenic acid that has not undergone this further oxidation.

Titration of benzofuroxan-modified papain with excess of 2-Py–S–S–2-Py results in the formation of 0.14±0.03 mol of Py-2-SH (thiopyrid-2-one)/mol of protein, which correlates well with the 15% activity that can be regained. It is important to reiterate that during the benzofuroxan modification of papain, *o*-benzoquinone dioxime had been released stoicheiometric with the total thiol content of the protein and not merely with 85% of it. The reaction of 14–15% of the oxidized papain preparation with 2-Py–S–S–2-Py may be accounted for in terms of thiosulphinate formation (eqn. 5):

$$\text{Papain–SOH} + \text{2-Py–S–S–2-Py} \rightarrow \text{papain–S(=O)–S–2-Py} + \text{Py-2-SH} \quad (5)$$

The ability of sulphenic acids to exhibit such nucleophilicity in addition to the electrophilicity discussed above has been noted previously (Kice & Cleveland, 1973). The small values of $\Delta A_{343}$ (approx. 0.01) for the 2-Py–S–S–2-Py titrations of

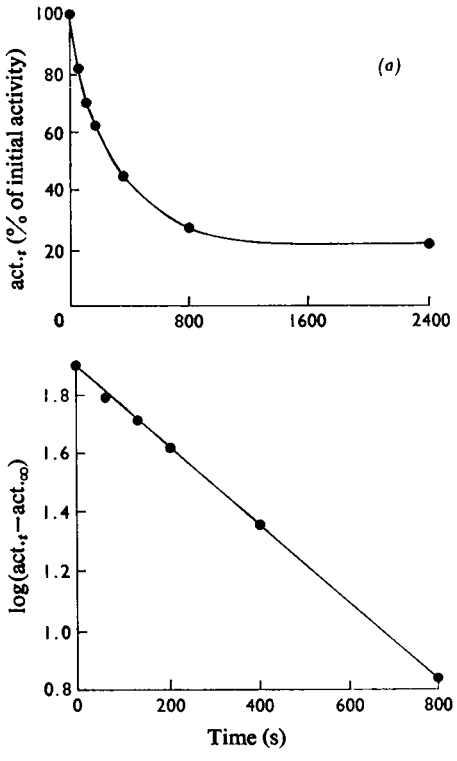



Fig. 7. *Kinetics of activity loss by papain* (18.8 $\mu$M) *consequent on reaction with benzofuroxan* (1 mM) *in* 6.7% *ethanol, pH* 7.92, *I* 0.1 *at* 25°C

Activity at time $t$ (act.$_t$) was determined by using α-*N*-benzoyl-L-arginine ethyl ester as substrate in reducing media as described in the text; the linearity of the plot demonstrates adherence of the irreversible loss of catalytic activity to first-order kinetics and provides $k_{obs.}$, the pseudo-first-order rate constant for the process.

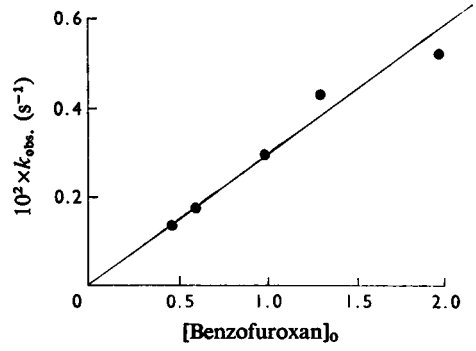



Fig. 8. *Determination of the second-order rate constant* ($k_I$) *for the irreversible inactivation of papain consequent on reaction with benzofuroxan*

Reaction conditions are those given in the legend to Fig. 7. The slope of the $k_{obs.}$–[benzofuroxan]$_0$ plot gives $k_I = 2.86 M^{-1} \cdot s^{-1}$.

oxidized papain precluded the accurate calculations of second-order rate constants, but it was possible to establish that release of Py-2-SH was much faster at pH8.0 than at pH4.0 using the same values of [2-Py-S-S-2-Py]$_0$ and [protein]$_0$ for each titration. This confirms that the release of Py-2-SH was not due to residual native papain that had not undergone oxidation, because it is a characteristic of the papain active centre that it reacts much more rapidly with 2-Py-S-S-2-Py at pH4 than at pH8 (Brocklehurst & Little, 1970; Brocklehurst, 1974).

Formation of the papain sulphenic acid may be envisaged in terms of Scheme 1 in which a postulated intermediate adduct such as compound (VII) is attacked by a water molecule or $HO^-$ ion instead of by another thiolate ion which is prevented by steric considerations.

4: *Kinetic study of the modification of papain by benzofuroxan: does 'papain' form more than one type of sulphenic acid?* Reaction mixtures were prepared at pH7–9.2 in 6.7% (v/v) ethanol (*I* 0.1) at 25°C containing papain (15.3 $\mu$M) and benzofuroxan (0.48–2 mM). Reactions were initiated by addition of benzofuroxan at zero time and samples of the reaction mixtures were assayed for esterolytic activity after various times as described in the Materials and Methods section. Assays were performed at pH6.0 in the presence of 20 mM-L-cysteine plus 5 mM-dithiothreitol (i.e. a large excess of low-molecular-weight thiol over both protein and residual benzofuroxan).

For each run a plot of residual catalytic activity versus time was constructed. A typical example is shown in Fig. 7(*a*) and the adherence of the activity loss to first-order kinetics is demonstrated in Fig. 7(*b*). Plots like that in Fig. 7(*a*) always reached a constant value equal to 15–20% of the initial activity.

A plot of the observed first-order rate constants for a series of such runs versus the initial benzofuroxan concentration (Fig. 8) provided a second-order rate constant for the inactivation process ($k_I$) of $2.86 M^{-1} \cdot s^{-1}$. Since the assays were performed in reducing media $k_I$ presumably relates to irreversible activity loss. The similarity of $k_I$ ($2.86 M^{-1} \cdot s^{-1}$) to the value of the second-order rate constant for the formation of *o*-benzoquinone dioxime by papain/benzofuroxan mixtures under similar conditions ($2.2 M^{-1} \cdot s^{-1}$) suggests that the supposed subsequent oxidation of 80–85% of the papain sulphenic acid by $O_2$ is fast relative to the rate of sulphenic acid formation.

In marked contrast with the rapid oxidation of 80–85% of the papain sulphenic acid proposed above,

15–20% of the esterolytic activity can still be recovered by treatment with low-molecular-weight thiol compound even after leaving for 2h at pH7.0 and 25°C in the presence of air. The apparent difference in stability of 15–20% of papain sulphenic acid may reflect a hitherto unreported heterogeneity of papain, at least as prepared by covalent chromatography. It is interesting in this connexion that approx. 20% of the papain thiol group seems to possess much lower reactivity towards 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole than the remaining 80% (Stuchbury *et al.*, 1975). The stability of some sulphenic acids has been attributed to microenvironments that protect the sulphenic acid moiety from solvent and thus from solute oxygen.

Papain sulphenic acid has been suggested as the product of oxidative inactivation of papain by reagents other than benzofuroxan [phenylhydrazine in the presence of $O_2$ (Allison & Swain, 1973); β-(2-hydroxy-3,5-dinitrophenyl)ethanesulphonic acid sultone (Campbell & Kaiser, 1973)]. Lin *et al.* (1975) studied the inactivation of papain by $H_2O_2$ and proposed that the inactivation is due almost entirely to the formation of papain sulphenic acid. In marked contrast with papain inactivated by treatment with benzofuroxan, papain inactivated by $H_2O_2$ can be reactivated to a considerable extent by treatment with low-molecular-weight thiol compound even after the inactive papain has been exposed to air for approx. 4h. Further, peroxide-inactivated papain does not react with the disulphide, 5,5′-dithiobis-(2-nitrobenzoic acid) at pH8.

*Reactions of ficin and bromelain with excess of benzofuroxan*

Reaction of ficin (25μM) or bromelain (25μM) with benzofuroxan (3.44mM) in the pH range 3–10 resulted in increases in $A_{430}$ indicative of the production of 0.96±0.03mol of *o*-benzoquinone dioxime/mol of enzyme thiol. These results suggest that, like papain, both ficin and bromelain are oxidized to sulphur oxyacid derivatives by benzofuroxan.

*Possible active-centre assistance to the reaction of benzofuroxan with the thiol proteinases*

The second-order rate constants for the reactions of benzofuroxan with the essential thiol groups of papain, ficin and bromelain in approximately neutral media (approx. $2M^{-1}\cdot s^{-1}$) are about 100 times greater than the corresponding rate constant for the reaction of benzofuroxan with 2-mercaptoethanol even though the enzymic reactions appear to make use of water or $HO^-$ as a nucleophile in neutral media. This suggests possible assistance to the oxidation by another active-centre residue. In view of reports by Malthouse & Brocklehurst (1976) and Shipton *et al.* (1976) that ficin (and possibly bromelain) may not possess a carboxyl group conformationally equivalent to that of aspartic acid-158 of papain, the similarity in the rates of oxidation of benzofuroxan by all three enzymes suggests that assistance is not supplied by a carboxyl group. The only acid–base system common to the three active centres, other than the thiol group, appears to be the essential histidine imidazole group. If benzofuroxan oxidations can be assisted by general acid or general base catalysis, this reagent may prove to be a valuable reactivity probe as well as a chromophoric, thiol-specific, modifying reagent.

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## References

Abu El-Haj, M. J., Dominy, B. W., Johnston, J. D., Haddadin, M. J. & Issidorides, C. H. (1972) *J. Org. Chem.* **37**, 589–593

Allison, W. S. & Swain, L. C. (1973) *Arch. Biochem. Biophys.* **155**, 405–410

Anno, T. & Sado, A. (1956) *Bull. Chem. Soc. Jpn.* **29**, 620–625

Boulton, A. J. & Ghosh, P. B. (1969) *Adv. Heterocycl. Chem.* **10**, 1–41

Boyer, J. H. & Ellzey, S. E. (1960) *J. Am. Chem. Soc.* **82**, 2525–2528

Boyer, J. H. & Smith, P. A. S. (1963) *Org. Synth. (Rev. Collect. Vol.)* **4**, 75–78

Brocklehurst, K. (1974) *Tetrahedron* **30**, 2397–2407

Brocklehurst, K. & Little, G. (1970) *FEBS Lett.* **9**, 113–116

Brocklehurst, K., Carlsson, J., Kierstan, M. P. J. & Crook, E. M. (1973) *Biochem. J.* **133**, 573–584

Brocklehurst, K., Carlsson, J., Kierstan, M. P. J. & Crook, E. M. (1974) *Methods Enzymol.* **34B**, 531–544

Campbell, P. & Kaiser, E. T. (1973) *J. Am. Chem. Soc.* **95**, 3735–3741

Chinard, F. P. & Hellerman, L. (1954) *Methods Biochem. Anal.* **1**, 1–26

Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. *Data for Biochemical Research*, pp. 475–508, Oxford University Press, Oxford

Drenth, J., Swen, H. M., Hoogenstraaten, W. & Sluyterman, L. A. AE. (1975) *Proc. K. Ned. Akad. Wet. Ser. C* **78**, 104–110

Glazer, A. N. & Smith, E. L. (1965) *J. Biol. Chem.* **240**, 201–208

Glazer, A. N. & Smith, E. L. (1971) *Enzymes 3rd Ed.* **3**, 501–546

Gripper-Gray, A. C. (1966) Ph.D. Thesis, University of East Anglia

Ingold, C. K. (1953) *Structure and Mechanism in Organic Chemistry*, p. 697, Connell University Press, New York

Kazanskaya, N. F. & Nicolskaya, T. I. (1975) *Vestn. Mosk. Univ. Chiv. Khim.* **16**, 49–54

Kice, J. L. & Cleveland, J. P. (1973) *J. Am. Chem. Soc.* **95**, 104–109
King, E. J. & Sperry, W. M. (1961) *Biochemists Handbook* pp. 30–40, E. and F. N. Spon, London
Kukolja, S. & Lammert, S. R. (1973) *Angew. Chem. Int. Ed. Eng.* **12**, 67–68
Latham, D. W. S. (1973) Ph.D. Thesis, University of Salford
Latham, D. W. S., Meth-Cohn, O. & Suschitzky, H. (1972*a*) *Tetrahedron Lett.* **52**, 5365–5368
Latham, D. W. S., Meth-Cohn, O. & Suschitzky, H. (1972*b*) *J. Chem. Soc. Chem. Commun.* 1040–1041
Leslie, J. & Varrichio, F. (1968) *Can. J. Biochem.* **46**, 624–627
Lin, W. S., Armstrong, D. A. & Gaucher, G. M. (1975) *Can. J. Biochem.* **53**, 298–307
Malthouse, J. P. G. & Brocklehurst, K. (1976) *Biochem. J.* **159**, 221–234
Ruggli, P. & Buchmeier, F. (1945) *Helv. Chim. Acta* **28**, 850–863
Shipton, M., Stuchbury, T. & Brocklehurst, K. (1976) *Biochem. J.* **159**, 235–244
Sokolovsky, M., Harell, D. & Riordan, J. F. (1969) *Biochemistry* **8**, 4740–4745
Stuchbury, T., Shipton, M., Norris, R., Malthouse, J. P. G., Brocklehurst, K., Herbert, J. A. L. & Suschitzky, H. (1975) *Biochem. J.* **151**, 417–432
Thompson, C. D. & Foley, R. T. (1972) *J. Electrochem. Soc.* **119**, 177–183
Webb, J. L. (1966) *Enzyme and Metabolic Inhibitors*, vol. 2, chap. 6, Academic Press, New York
Wolf, W. & Wu, T. (1969) *J. Pharm. Sci.* **58**, 491–493