Biochem. J. (1985) **228**, 615–626
Printed in Great Britain

Studies of the limited degradation of mucus glycoproteins

The mechanism of the peroxide reaction

Brian COOPER,* J. Michael CREETH* and Alastair S. R. DONALD†
**University of Bristol Department of Medicine, Bristol Royal Infirmary, Bristol BS2 8HW, U.K., and †Division of Immunochemical Genetics, M.R.C. Clinical Research Centre, Watford Road, Harrow, Middx. HA1 3UJ, U.K.*

(*Received 23 November 1984/11 February 1985; accepted 26 February 1985*)

1. The reaction between ovarian-cyst glycoproteins and H_2O_2 was investigated in the presence of a number of inhibitors and catalysts. 2. Azide and ^2H_2O were separately found to have little effect, implying that singlet oxygen was not involved. 3. Superoxide dismutase was destroyed by H_2O_2, but mannitol had no effect: thus generalized attack by $OH^{\cdot}$, whether originating from $HO_2^{\cdot -}$ or more directly, is not indicated. 4. The glycoproteins contained trace quantities of Cu and Fe, amounting to about 2 atoms of metal per glycoprotein molecule. 5. Treatment of the glycoproteins with the strong chelator DETAPAC (diethylenetriaminepenta-acetic acid) or Chelex resin eliminated the reaction with H_2O_2; activity could be restored by addition of Cu^{2+} or Fe^{2+} in millimolar quantities. 6. It was concluded that metal-ion catalysis is an essential step in the attack of H_2O_2 on glycoproteins. 7. Spectroscopic and other evidence showed that Cu^{2+} (and probably Fe^{2+}) complexes strongly with poly-L-histidine, and implies that the Cu^{2+} or Fe^{2+} in the glycoproteins is complexed with some of the histidine residues in the glycosylated backbone. 8. Neither polyhistidine nor polyproline reacted with H_2O_2 in the absence of metal ions, but small quantities of Cu^{2+} or Fe^{3+} caused degradation. This was rapid with polyhistidine, which was converted largely into aspartic acid, but slower with polyproline, where limited conversion into glutamic acid occurs. 9. These findings confirm the original hypothesis that peroxide attack on glycoproteins occurs largely at the histidine residues, with simultaneous peptidolysis. 10. The mechanism most probably involves the liberation of $OH^{\cdot}$ by an oxidation–reduction cycle involving, e.g. Cu^{+}/Cu^{2+}: specificity of attack at histidine is due to the location of the metal at these residues only.

In a previous study of the effect of dilute H_2O_2 on some mucus glycoproteins and glycopolypeptides derived from ovarian cysts (Creeth *et al.*, 1983*a*), the reaction at pH 5.6 and 37°C was shown to be slow ($t_{\frac{1}{2}}$ approx. 5h) and to reach a virtual plateau region at approx. 20h. The products of this limited reaction were accordingly presumed to possess some common structural property and were therefore characterized in analytical and physicochemical terms. The carbohydrate composition was found to be little changed from the original, whereas the amino acid composition was altered significantly. Small changes occurred in the proportions of threonine, serine and proline, but the minor constituent histidine was consistently either greatly diminished, or removed altogether, with a concomitant increase in aspartic acid. The molecular masses of the peroxide products were lower than their parent glycoproteins by a factor between 2- and 4-fold.

It appeared, accordingly, that a nearly specific attack on the histidine residues occurred under the conditions described, the histidine being oxidized to aspartic acid by a process that also broke the peptide backbone. Such a reaction clearly has intrinsic interest, in view of the dominant role played by native glycoproteins in the protection of mucosal surfaces. Moreover, the reaction may well have application in sequence studies of the peptide backbone. We have therefore investigated the effect of various inhibitors and other agents, with the purpose of establishing the mechanism of the

reaction, and extended the scope of the work to include polyhistidine and polyproline, which serve as informative models. These studies, reported briefly elsewhere (Creeth *et al.*, 1983*b*), are now described: they show that the reaction is critically dependent upon catalysis by transition-metal ions.

Experimental

Reagents

Reagents were of analytical grade, where obtainable. All solutions were prepared in glass-distilled water; for specific experiments, the water was further purified by passage through a column of Chelex-100 (Na^+ form) (Bio-Rad Laboratories, Richmond, CA, U.S.A.). A sodium acetate buffer solution, pH 5.6 and I 0.10, was used throughout the work.

Glycoproteins

These were the two native glycoproteins 603AmS and 485 used in the earlier work (Creeth *et al.*, 1983*a*), where reference is made to their source. Both these glycoproteins were almost unaffected by Pronase digestion and thus can have little in the nature of naked segment. In this important respect, they were more akin to glycopolypeptides (Donald, 1973). Their weight-average M_r values were both approx. 580000 (Creeth *et al.*, 1983*a*), the number-average M_r values being about 20% less. For convenience, in rough calculations of molar ratios, both M_r values were taken as 500000.

Amino acid homopolymers

Poly-L-histidine of nominal M_r 5000–15000 and poly-L-proline (M_r 10000–30000) were obtained from Sigma Chemical Co., Poole, Dorset, U.K. By ultracentrifuge techniques, the M_r values were found to be somewhat larger (see below).

Reactions with H_2O_2

These were carried out under the same general conditions as used previously (Creeth *et al.*, 1983*a*), the glycoprotein being usually at 2mg/ml in the acetate buffer specified. The reactions were carried out on 1ml quantities in the viscometric equipment described elsewhere (Holt & Creeth, 1972); later experiments were monitored by a similar photo-detection system, but with infrared sources and detectors, the control and timing to 0.01s being programmed by a BBC model B microcomputer (see the acknowledgements).

Since all experiments were of comparative nature, the glycoprotein samples were not dried beforehand. Their moisture content was approx. 10%; hence the initial reduced viscosities calculated when the moisture content was ignored are proportionately low. It is important to realize that the error associated with the reduced viscosities quoted varies during the progress of the reaction: the glycoproteins studied were usually arranged to give at least a 5s increment in flow time, t (relative to the buffer solution, t_0: t_0 was approx. 100s for most experiments). Hence the error in $\eta_{red.}$ produced by, say, 0.05s error in timing was initially about 1%. When $(t-t_0)$ had decreased to, e.g., one-third of its initial value, the corresponding error in $\eta_{red.}$ becomes 3%; however, the error is also increased by the corrections for the increase in total volume and the decrease in glycoprotein concentration consequent upon the introduction of reactants. For these reasons, the terminal values found show pronounced scatter, and little significance should be attached to minor differences in them.

Results

Inhibition studies

Oxidations by H_2O_2 normally involve one or more of the activated species singlet oxygen, the superoxide radical ion or the hydroxyl radical (for review see Singh, 1982). Distinction is made by studying the effects of reagents that either quench or enhance the active species.

(*a*) *Singlet oxygen.* The singlet-oxygen species $^1\Delta_g$ is a relatively low-energy active form of oxygen (for review see Wasserman & Murray, 1979) that has been convincingly implicated in the dye-sensitized photo-oxidation of enzymes etc. (Tomita *et al.*, 1969; Matheson *et al.*, 1975). Since the characteristic effect of this reaction is the destruction of histidine and the formation of aspartic acid, together with numerous partly oxidized products of side reactions (Tomita *et al.*, 1969), there is a strong superficial similarity to the glycoprotein reaction with H_2O_2.

Reactions in which singlet oxygen participates are most strongly quenched by the bilirubin anion (Matheson *et al.*, 1975) and by azide (Hasty *et al.*, 1972). However, they are accelerated by ^2H_2O, which prolongs the lifetime of the excited species (Merkel *et al.*, 1972; Ito, 1978). Since bilirubin proved to be totally insoluble in our conditions, the peroxide reaction was carried out both in the presence of azide and with ^2H_2O substituted for ^1H_2O as solvent; the glycoprotein 603AmS was used in the same conditions as before. The results are shown in Fig. 1.

Azide was found to decompose H_2O_2, with the liberation of O_2; this result was confirmed by spectrophotometric determination of the H_2O_2 concentration. Because of the formation of gas bubbles, the viscosity values are less reliable than usual, but the reaction proceeds at essentially the

same rate as the control (see Fig. 1); azide at 20mM is thus without effect. The reaction in ^2H_2O is perhaps slightly slower than the control, but certainly not faster. These observations effectively eliminate any mechanism based on the participation of singlet oxygen. Amino acid analysis of the final product of reaction in ^2H_2O (which is possibly slightly more degraded than in ^1H_2O) gave values indistinguishable from those obtained with ^1H_2O as solvent (see Table 1).

(*b*) *Superoxide.* This radical is recognized as a

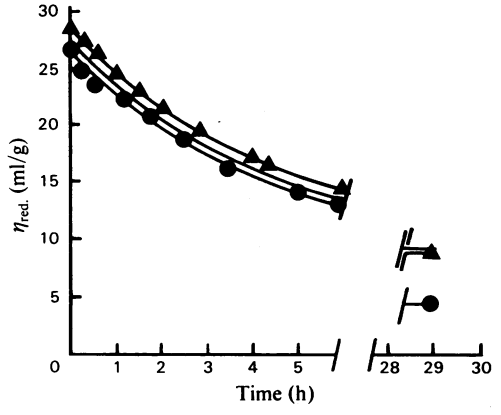

Fig. 1. *Effects of* (*a*) *an inhibitor and* (*b*) *an enhancer of singlet-oxygen-mediated reactions on the glycoprotein reaction with* H_2O_2

(*a*) ●, Reaction under standard conditions + 20mM-NaN_3; (b) ▲, standard conditions except that ^2H_2O replaced ^1H_2O throughout. The curve without symbols represents the control reaction under standard conditions (see the text).

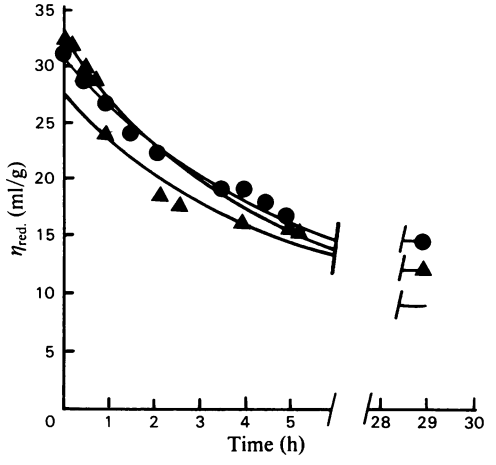

Fig. 2. *Effect of hydroxyl-radical scavengers on the glycoprotein reaction with* H_2O_2

(*a*) ●, Standard conditions + 0.1M-mannitol; (*b*) ▲, standard pH etc., but with formate buffer replacing acetate. The curve without symbols is as for Fig. 1.

Table 1. *Amino acid analysis of native and oxidized glycoproteins*

The first set of values refers to native glycoprotein 603AmS (603n) and this glycoprotein after reaction with H_2O_2 in acetate/H_2O buffer (603/ox./^1H_2O) or acetate/^2H_2O buffer (603/ox./^2H_2O). The second set refers to the native glycoprotein 485 (485n), its oxidation product in standard conditions (485/ox.) and the product after attempted oxidation with H_2O_2 following prior treatment with the resin Chelex-100 (485/Ch/ox.). See the text for details. Units are mol of amino acid/100mol of total amino acids; values are means of duplicate analyses, except where indicated.

	Amino acid composition (mol/100mol)					
	603n	603/ox./^1H_2O	603/ox./^2H_2O	485n	485/ox.	485/Ch/ox.
Asp	4.9	6.2	6.2	2.8	6.3	4.5
Thr	19.0	20.7	20.7	27.1	27.6	31.4
Ser	16.3	17.3	15.7	18.1	17.3	14.4
Glu	4.7	5.0	5.3	4.1	4.1	3.2
Pro	15.1	14.2	14.8	16.0	14.2	14.1
Gly	8.5	8.1	8.1	5.8	7.4	6.0
Ala	12.7	13.2	14.0	9.6	11.1	10.1
Cys	0.0	0.0	0.0	0.0	0.0	0.0
Val	4.3	4.3	4.8	3.7	4.0	4.1
Met	0.3	0.1	0.5	0.4	0.3	0.2
Ile	1.5	1.5	1.4	1.8	1.8	1.9
Leu	2.1	1.9	1.9	1.9	1.4	1.7
Tyr	0.7	0.6	0.4	0.5	0.1	0.2
Phe	1.0	1.1	0.6	1.0	0.5	0.5
His	3.2	0.4	0.7	2.8	0.2	3.2
Lys	1.9	1.9	1.4	1.3	0.9	1.4
Arg	3.2	3.2	3.5	2.6	2.7	2.5
Total peptide content (%)	7.6	7.6	9.0	12.0	7.9	7.1

common intermediate in biological reactions (see e.g., Singh, 1982), and it is detected by the retarding action of superoxide dismutase (Fridovich, 1975). Since this enzyme is itself rapidly attacked by H_2O_2 (Bray *et al.*, 1974; Hodgson & Fridovich, 1975), the likelihood of detecting any effect was not high. Nevertheless an attempt to do so was made, by using a lower initial concentration of H_2O_2 than usual (18mM rather than 80mM). The enzyme was added in excess (135 units), but was found to be without effect on either the glycoprotein at the normal concentration (2mg/ml) or at a higher value (4mg/ml), relative to the control experiments at the lower H_2O_2 concentration. After the reaction had been allowed to proceed for 20h, the solutions were assayed for superoxide dismutase activity by the method of McCord & Fridovich (1969); no activity was found, in agreement with expectation that the enzyme would indeed be destroyed by H_2O_2. Thus no evidence can be obtained directly on the participation of $O_2^{\cdot -}$. However, since the main destructive effect of superoxide is exerted through formation of the intensely active hydroxyl radical, $OH^{\cdot}$, via the Haber–Weiss cycle (see, e.g., Kong & Davison, 1980), the observations in (*c*) below may be relevant.

(*c*) *Hydroxyl radical.* This radical is most commonly generated through metal-catalysed reactions of the Haber–Weiss type, or more directly through Fenton-type reactions (Singh, 1982). It is very widely reactive, but its most effective scavengers are sugars or their derivatives (e.g. mannitol), formic acid, ethanol etc. (see, e.g., Cheng *et al.*, 1981). Reaction mixtures were therefore set up as before, one containing additionally mannitol at 100 or 200mM and the other with formate/formic acid buffer replacing the acetate, but maintaining the pH. Although the formic acid is present at only millimolar concentration, its rate constant is so high that effective scavenging may still be expected (Cheng *et al.*, 1981).

The results are shown in Fig. 2. Mannitol is seen to have little effect, whereas some enhancement of the rate after 1–2h is suggested with formate; clearly neither substance exerts a significant retardation.

To sum up, accordingly, none of the trio of active oxygen species can be detected by recognized scavengers. However, it is known that $OH^{\cdot}$ in particular is so active that it tends to react at the point of generation, and it must be remembered that the peptide backbone of these glycoprotein molecules is heavily glycosylated and thus partly protected from at least the larger inhibitor molecules. In this connexion the effect of EDTA previously reported (Creeth *et al.*, 1983*a*) is suggestive: it was found that EDTA severely retarded the peroxide reaction, but did not stop it completely.

Metal content of the glycoproteins

EDTA does not prevent the participation of Fe^{2+} in the iron-catalysed Haber–Weiss reaction (Halliwell, 1975, 1978*a*; McCord & Day, 1978), but the observations pointed to the involvement of trace amounts of metal impurities in the reaction. Our distilled water and buffer solutions were tested for iron by atomic absorption and for copper, zinc, cadmium and lead by anodic stripping voltammetry (see the acknowledgements); however, no metal was present at more than a few parts per 10^9. Attention was therefore directed towards the glycoproteins themselves: being phenol-extracted, ammonium sulphate-soluble, fractions (Morgan, 1967; Creeth *et al.*, 1974), they had necessarily been exposed to large quantities of these reagents. After being ashed with conc. HNO_3, the residues from both the glycoproteins 485 and 603AmS were analysed for iron by the ferrozine method (Carter, 1971) and for copper by the rubeanic acid (dithiooxamide) method (Center & MacIntosh, 1945). The results were unmistakably positive, giving the values approx. 150p.p.m. for Fe and 50p.p.m. for Cu; these correspond to about 1.5mol of Fe and 0.5mol of Cu per mol of glycoprotein.

Although small, these values show that roughly 2 atoms of these transition metals are sufficiently strongly bound to the glycoprotein molecule to resist the later prolonged water-dialysis steps in the preparation. Moreover, when considering the possible groups in the glycoprotein at which strong binding might be expected, histidine is an obvious candidate, for it is known (Albert, 1950; Dobbie *et al.*, 1955; Martell & Smith, 1974) to bind Cu^{2+}, for example, more strongly than does any other amino acid.

On the basis of these observations, three predictions may be made: (i) since 10–15 histidine residues exist per molecule of glycoprotein and only about 2 can be complexed initially, further catalysis by iron or copper ions should occur; (ii) correspondingly, a chelator that inhibits the participation of metals in oxidoreductive shifts should greatly retard the 'uncatalysed' reaction; (iii) spectral changes are expected when, e.g., Cu^{2+} is liganded to histidine and should be detectable with a histidine-rich macromolecule. It is shown below that all three predictions are realized.

Effect of metals on the glycoprotein – H_2O_2 reaction

(i) *Catalysis by metal ions at low concentration.* We had shown that Fe^{2+}, added to a glycoprotein containing 1mM-EDTA, became an effective catalyst when the EDTA was saturated. The instability of Fe^{2+} and the insolubility of many

Fe^{3+} compounds prompted the use of Cu^{2+} in the present instance: reactions under the standard conditions, but containing additionally 0.1mM-, 0.3mM- and 1.0mM-Cu^{2+}, were set up. All reactions were catalysed, the curves being progressively steeper, and the 20h plateau values were also decreased: the effects are demonstrated by the $t_{\frac{1}{2}}$ values quoted in Table 2. The products at 20h were dialysed and characterized as before, the amino acid contents being quoted also in Table 2. It may be noted that a glycoprotein concentration of 2mg/ml is about 50μM in histidine and 250μM in proline.

It is evident from Table 2 that additional Cu^{2+} does not cause a greater loss of histidine than occurs in its absence, although the conversion into aspartate may be marginally more complete. It is unmistakable, however, that the relatively small loss of proline suggested in the 'uncatalysed' reaction is now a significant feature: some 30% of the proline is destroyed, and an approximately equivalent rise in the content of glutamic acid occurs. No other changes in amino acid composition are strongly evident, although the suspected increase in threonine is confirmed, particularly at 1.0mM-Cu^{2+}. The other notable feature of the analyses is the increase in total peptide content, confirming the earlier report that a small proportion of the carbohydrate component is lost if the reaction is accelerated or, if uncatalysed, very prolonged. Clearly, however, this loss is quite minor.

(ii) *Effect of removal of trace metal ions by chelation.* The very strong chelating agent DETAPAC (diethylenetriaminepenta-acetic acid, as sodium salt) (West, 1969) inhibits the reactions between superoxide and Fe(III) compounds and between H_2O_2 and Fe(II) compounds (the Fenton reaction) (Halliwell, 1978*b*). It thus differs from EDTA. Its effect was tested on the glycoprotein–H_2O_2 reaction by setting up the reactions after prior incubation of the glycoproteins with 2.4mM-DETAPAC for 3 days. As is evident from Fig. 3, virtually no reaction occurs during the 20h period, when a normal decrease to about 25% of the initial viscosity would be expected. At about this time, the first of several additions of 20mM-$CuSO_4$ was made, each addition bringing the Cu^{2+} concentration up by 1mM. No change occurred in the viscosity. At 48h, a second addition of $CuSO_4$ was made, so that the total Cu^{2+} was 2.0mM; again no change resulted. At 72h, a further addition was made, so that the Cu^{2+} should be present in slight excess over the DETAPAC; however, the rate was still low, so at 75h a fourth addition was made, giving a free Cu^{2+} concentration of over 1mM: the reaction was then quite rapid, but a plateau value similar to the 'uncatalysed' reaction was found at approx. 90h.

These results are strong evidence that the

Table 2. *Peroxide oxidation of glycoprotein 603AmS*

Amino acid analyses of products of Cu^{2+}-catalysed reactions, and values of the half-times ($t_{\frac{1}{2}}$) of the reactions. Units of analyses are as for Table 1.

	Amino acid composition (mol/100mol)			
	No Cu^{2+}	0.1mM-Cu^{2+}	0.3mM-Cu^{2+}	1.0mM-Cu^{2+} (not duplicate)
Asp	6.2	6.4	6.8	7.0
Thr	20.7	20.0	19.7	23.1
Ser	17.3	16.1	16.5	17.1
Glu	5.0	5.8	8.4	9.1
Pro	14.2	13.9	10.3	9.5
Gly	8.1	8.4	10.3	9.1
Ala	13.2	13.8	13.0	12.7
Cys	0.0	0.0	0.0	0.0
Val	4.3	4.6	4.2	4.1
Met	0.1	0.3	0.2	0.4
Ile	1.5	1.5	1.4	1.6
Leu	1.9	1.8	1.6	1.4
Tyr	0.6	0.5	0.4	0.3
Phe	1.1	1.0	1.0	0.5
His	0.4	0.4	0.4	0.5
Lys	1.9	1.7	2.2	1.7
Arg	3.2	3.4	2.6	1.9
Total peptide content (%)	7.6	9.3	12.4	11.1
$t_{\frac{1}{2}}$ (h)	5	3	1.5	0.75

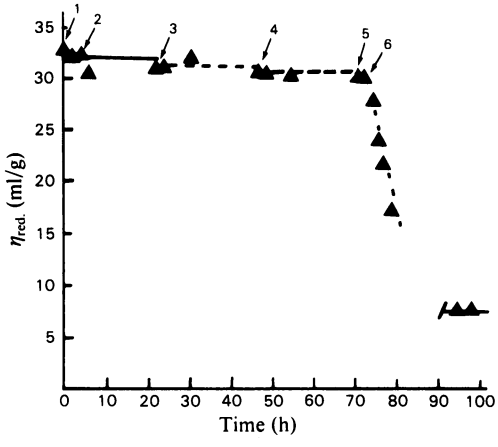

Fig. 3. *Effect of DETAPAC alone and of DETAPAC plus small additions of Cu^{2+} on the glycoprotein reaction with H_2O_2*

The glycoprotein (603AmS) was incubated for 3 days at 37°C with 2.4mM-DETAPAC before addition of 50μl lots of 5% (v/v) H_2O_2 at time zero and 3h (arrows 1 and 2). After 24h, 10μl of mM-$CuSO_4$ was added, making the Cu^{2+} concentration 1.0mM (arrow 3). Three further additions of $CuSO_4$ were made similarly at the times shown (arrows 4, 5 and 6).

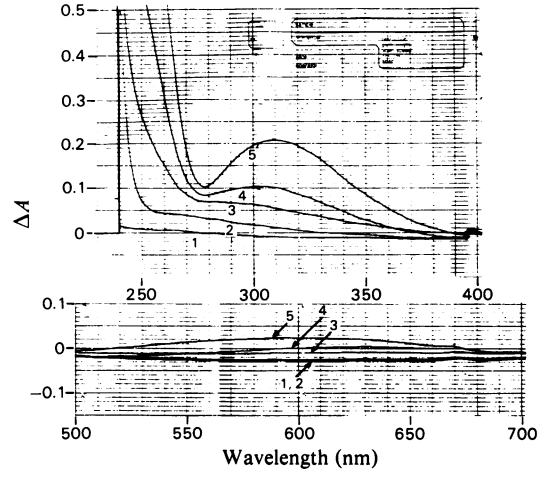

Fig. 4. *Spectra of mixtures of polyhistidine with various concentrations of Cu^{2+} compared with those of the separate components*

Curve 1, buffer blank; curve 2, polyhistidine blank ($Cu^{2+} = 0$); curves 3, 4 and 5, polyhistidine +0.2mM-, 0.5mM- and 0.9mM-Cu^{2+} respectively. For convenience, the 'Cu^{2+} blue' region (approx. 600nm) has been separated and placed below the near-u.v. spectra, but all curves were recorded continuously.

normal reaction proceeds by metal-ion catalysis; a strong chelator can remove the metal ion, and reaction will not then occur until all the chelator is saturated and free metal ions are again present.

An attempt was made to remove the trace metal from the glycoprotein by treatment with the metal-ion-chelating resin Chelex 100, which has a very high affinity for both copper and iron ions. The more-viscous glycoprotein 485 was used: a solution of 2mg/ml in sodium acetate buffer prepared in Chelex-purified water was stirred for 3 days with about one-third of its volume of the water-saturated resin. After low-speed centrifugation, the supernatant was treated as before with H_2O_2: virtually no reaction could be detected viscometrically over the 5h period in which the untreated glycoprotein is strongly affected. This further evidence that metals play an essential part in the apparent uncatalysed reaction was confirmed by the results of the amino acid analyses on the products of the reactions (Table 1); the glycoprotein with prior Chelex treatment before addition of H_2O_2 shows little change from the original (particularly in the histidine content), whereas the untreated substance shows the typical changes in amino acid profile that accompany reaction with H_2O_2. The reactivity of the Chelex-treated glycoprotein was restored on the addition of 50μM-Cu^{2+} (values not shown).

(iii) *Spectroscopic effects.* Since the histidine content of glycoproteins is so low (typically 0.2–0.3% by wt.) spectroscopic changes accompanying the binding of Cu^{2+} are unlikely to be observable directly: this was confirmed experimentally. However, polyhistidine (M_r approx. 30000) is reasonably soluble in acetate buffer at pH5.6, although it has a negative coefficient of solubility and must be prepared at 4°C. A solution containing 2mg/ml is approx. 15mM in histidine. Mixtures were prepared containing 0.2mM-, 0.5mM- and 0.9mM-Cu^{2+} in addition to the above concentration of polyhistidine and examined by difference spectrophotometry, the appropriate Cu^{2+} solution and polyhistidine solutions (in separate cuvettes) being placed in the reference beam. The spectra are shown in Fig. 4. A measurable shift is observed even at the lowest Cu^{2+} concentration, and at 0.9mM a new peak with $\lambda_{max.}$ at 310nm is clearly defined: simultaneously a much broader, shallower, increase can be seen at approx. 600nm, the 'Cu^{2+} blue' region. The 310nm peak (ε very approx. 250$M^{-1}\cdot cm^{-1}$) may reasonably be ascribed to charge transfer from the π_1 and π_2 histidine ring orbitals to Cu^{2+}, in view of the extensive observations by Bernarducci *et al.* (1981) on imidazole and a series of derivatives in methanolic solution. Their values for $\lambda_{max.}$ were all close to 310nm, with ε values ranging from 350$M^{-1}\cdot cm^{-1}$ for imidazole

itself to considerably higher values for the derivatives.

Metal-ion binding to poly-(amino acids)

Confirmation of the binding of Cu^{2+} to polyhistidine was obtained by determination of the effect of Cu^{2+} on the buoyant density, ρ_0, of the polymer in CsCl solutions. Although the polyhistidine sample was insoluble in either CsCl or Cs_2SO_4 at pH 5.6, buoyant-density measurements are expected to be virtually unaffected (Cox & Schumaker, 1961; Ifft, 1971) and to reflect the value that would have been obtained in solution, at least for simple systems. The results were (in terms of ρ_0 in CsCl solution): polyhistidine alone, 1.32g/ml; +1mM-Cu^{2+}, 1.40g/ml; +2mM-Cu^{2+}, 1.45g/ml; +5mM-Cu^{2+}, 1.45g/ml.

The value for polyhistidine alone is in good agreement with that reported for this pH by Almassy *et al.* (1973). Clearly Cu^{2+} binding increases the buoyant density, as would be expected from the high density of the $Cu^{2+},4H_2O$ ion. (A value of $\bar{v} = 0.21$ ml/g was calculated from the value for SO_4^{2-},H_2O given by Pedersen (1958) and the $\bar{v}$ for the electrolyte as a whole obtained from the density data for $CuSO_4,5H_2O$ given by Washburn (1928). The effect on the buoyant density apparently reaches a maximum at roughly 3mM-Cu^{2+}, or a Cu^{2+}/histidine ratio of approx. 1:7, but a more reliable value is obtainable from M_r measurements in the standard buffer solution.

M_r were determined, by the method previously described (Creeth *et al.*, 1983*a*), on the polyhistidine above, and after mixing with $CuSO_4$ or $FeSO_4$ at a metal ion/histidine molar ratio of 1:3. Calculation of M_r depends on a knowledge of $\bar{v}$ for the species, which of course varies with the extent of binding. However, one may assume two extremes: no binding, when $\bar{v}$ for the polymer alone applies, and complete binding (1:3), when the $\bar{v}$ is easily calculated. The results are given in Table 3, together with those for polyproline.

Even with the limitations imposed by the $\bar{v}$ uncertainty, the results indicate clearly that polyhistidine forms a complex with Cu^{2+} of about 4 times the size of the original polymer. Cu^{2+} binding alone cannot account for this (full 1:3 binding could give an increase of only about 15% in M_r), and so one must conclude that the Cu^{2+} is co-ordinating between different polyhistidine molecules, thus effecting cross-links. Similar conclusions apply to the system polyhistidine–Fe^{2+}.

Polyproline, on the other hand, provides no evidence for complex-formation with either metal ion.

Table 3. *Weight-average M_r values for poly-(amino acids) and their metal-ion complexes*

Two sets of values are given: I refers to the M_r found when no complex-formation is assumed, and the regular $\bar{v}$ applies, whereas II gives the corresponding values assuming a 1:3 poly-(amino acid)/metal ion stoichiometry. In this case the $\bar{v}$ values have been calculated on the basis of additivity, as explained in the text: the method gives $\bar{v}$ 0.2ml/g for $Cu^{2+},4H_2O$ and 0.34ml/g for $Fe^{2+},6H_2O$.

		M_r	
	$\bar{v}$ (ml/g)	I	II
Poly-His	0.67*	31000	—
Poly-His/Cu^{2+}	0.61	130000	110000
Poly-His/Fe^{2+}	0.63	208000	185000
Poly-Pro	0.76*	43000	—
Poly-Pro/Cu^{2+}	0.66	47000	33000
Poly-Pro/Fe^{2+}	0.69	37000	29000

* Values from Cohn & Edsall (1943).

Reaction of proteins and amino acid homopolymers with H_2O_2

Attempts to use various proteins to confirm the central thesis of this work were described earlier (Creeth *et al.*, 1983*a*). However, Levitzki *et al.* (1967) have shown that the third residue of a polypeptide (from the *N*-terminal end) may have a special susceptibility to Cu^{2+}-catalysed oxidation, so that the nature of the side chain may become of subsidiary importance. Accordingly, proteins may not be the most suitable models for understanding the mucus glycoprotein–H_2O_2 system. For this reason, the amino acid homopolymers poly-L-histidine and poly-L-proline, which present obvious simplicities of structure, were investigated.

Poly-L-histidine. Reaction systems with this substance replacing the glycoprotein were set up as described earlier (Creeth *et al.*, 1983*a*), the polyhistidine solution being prepared at 4°C, for the reason stated, before reaction at 37°C. The results are shown in Fig. 5. In the absence of added Cu^{2+} the reaction is very slow, whereas the addition of 1mM-Cu^{2+} causes a rapid decrease in viscosity ($t_{\frac{1}{2}} < 30$min). It is interesting that the reaction could not be observed to completion, for gelation ensued when the reduced viscosity had reached about 30% of its initial value.

The small transient increase in viscosity visible on the addition of the Cu^{2+} proved to be reproducible. Stepwise addition of Cu^{2+} (1mM per step) to a similar polyhistidine solution increased $\eta_{red.}$ by about 1ml/g per step up to a Cu^{2+} concentration of 25mM: this solution was lilac blue. Addition of H_2O_2 caused rapid liberation of O_2 and a change to emerald green. The gelling phenomenon after partial reaction with H_2O_2 was general, but dependent on the Cu^{2+} concentration.

Such gels were found to liquefy ($\eta_{red.}$ approx. 0) after about 2 days at room temperature.

The analytical changes accompanying the reaction were determined by stopping the reaction when the solution was just still liquid by the addition of the stoichiometric amount of Na_2SO_3.

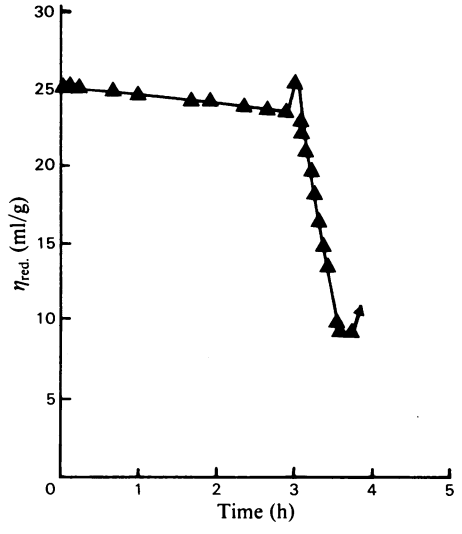

Fig. 5. *Degradation of poly-L-histidine by H_2O_2 and H_2O_2/Cu^{2+}*

H_2O_2 (50μl) was added to 1.0ml of polyhistidine (2.0mg/ml) in the standard conditions at $t = 0$; after 3h $CuSO_4$ was added to 1.0mM. Gelation occurred at about 3.5h.

The product was not dialysed, in order to avoid the loss of minor or small-sized products. The presence of salts did not appear to affect the analyses, the results of which are shown in Table 4. Initially, the homopolymer is about 95% histidine, but in the peroxide reaction product only about 6% of histidine remains, the bulk of the material now consisting of aspartic acid. Small increases in serine and glycine are also apparent. Very similar results were found when Fe^{2+} was used instead of Cu^{2+} as catalyst: here the conversion was marginally less complete, but the main effect is identical. These results, taken with the decrease in viscosity, confirm conclusively the previous hypothesis: H_2O_2, when metal-ion-catalysed, oxidizes the histidine side chain and simultaneously breaks the histidyl peptide bond.

Poly-L-proline. Proline forms about 15% of the peptide backbone of the mucus glycoproteins, and small losses (1–2%) were observed in the reaction with H_2O_2. These were increased in the reactions that were actively metal-ion-catalysed (Table 2). Accordingly, the Cu^{2+}-catalysed oxidation of polyproline was studied in the same way as the polyhistidine, giving the results shown in Fig. 6 and Table 4. The reaction with peroxide is undetectable before the addition of the catalyst (1mM-Cu^{2+}), when a much slower ($t_{\frac{1}{2}}$ approx. 2h) degradation is initiated. Higher Cu^{2+} concentration produces a faster reaction (result not shown). Gelling did not occur in this system, and so the reactions could be followed to virtual completion. When Fe^{2+} was substituted for Cu^{2+}, insoluble

Table 4. *Amino acid analyses of poly-(amino acids) and their oxidation products*

Values are given for poly-L-histidine and poly-L-proline, and their respective products ('ox.') of reaction with Cu^{2+}- or Fe^{2+}-catalysed H_2O_2. Units are as for Table 1.

	Amino acid composition (mol/100mol)				
	Poly-His	Poly-His/ox. (Cu^{2+})	Poly-His/ox. (Fe^{2+})	Poly-Pro	Poly-Pro/ox. (Cu^{2+})
Asp	0.9	80.3	67.6	1.2	2.9
Thr	0.0	1.9	5.5	0.0	0.6
Ser	1.1	4.8	3.4	1.6	1.1
Glu	0.0	0.0	1.2	0.6	16.0
Pro	0.0	0.0	0.0	94.8	77.2
Gly	1.4	5.8	4.9	0.6	1.3
Ala	0.0	0.0	1.5	0.0	0.0
Cys	0.0	0.0	0.0	0.0	0.0
Val	0.7	0.0	5.2	0.0	0.0
Met	0.0	0.0	0.0	0.0	0.0
Ile	0.0	0.0	0.0	0.0	0.0
Leu	0.0	0.0	1.6	0.0	0.8
Tyr	0.0	0.0	0.0	0.0	0.0
Phe	0.0	0.0	0.5	0.0	0.0
His	94.5	6.1	7.3	0.0	0.0
Lys	0.8	1.1	0.0	1.3	0.0
Arg	0.0	0.0	1.3	0.0	0.0

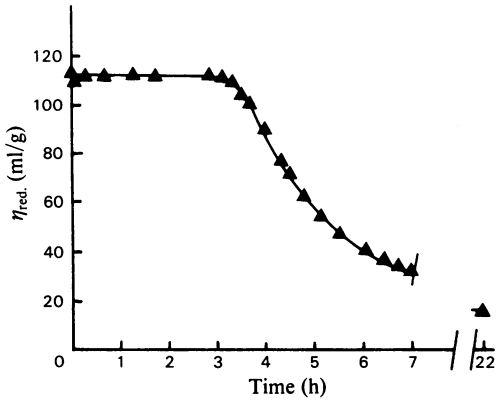

Fig. 6. *Degradation of poly-*L*-proline by H_2O_2 and H_2O_2/Cu^{2+}*

Conditions were as indicated in Fig. 5 legend, except that the initial concentration of polyproline was 5.0mg/ml. No gelation occurred.

products were obtained, which were not further characterized.

The analyses in Table 4 show that the initial homopolymer is about 95% proline, which is indeed partly oxidized to glutamic acid by the action of H_2O_2. However, the conversion is only partial, the products, obtained in the same way as with polyhistidine, still retaining a high proportion of proline. A slight increase in aspartic acid is the only significant change in addition to the unmistakable increase in glutamic acid. The reaction proceeds to a slightly greater extent at the higher Cu^{2+} concentration, but the general picture is the same.

In contrast with the polyhistidine, the addition of Cu^{2+} to polyproline alone produced no increase in viscosity. This implies the absence of complex-formation, in agreement with the M_r values given in Table 3. We conclude from these results that proline in peptide linkage is slightly susceptible to metal-ion-catalysed oxidation by H_2O_2, but to a much smaller extent than histidine in peptide linkage. Thus the results obtained with glycoproteins are seen to be in agreement with expectation.

It is also apparent from these results that histidine and Cu^{2+} form strong complexes, probably in the approximate ratio 3:1. This is in agreement with the radiotracer work by A. Levitzki [unpublished work, cited by Pecht *et al.* (1967)], in which the association constant for this system is given as approx. 10^{18}. Iron as Fe(II) is probably similarly bound, but the added complication of oxidation to insoluble Fe(III) complexes prevents quantitative interpretation.

For polyproline, however, there is no evidence that complex-formation occurs with either metal ion, and indeed it is difficult to formulate any scheme that would allow it.

Discussion

The results on glycoproteins and polyhistidine described above suggest a probable mechanism for the reaction with H_2O_2. Since the evidence that transition-metal ions play an essential part in the reaction is so strong, it is assumed that all the glycoproteins and glycopolypeptides previously described contain the metals as trace impurities, although some have still to be tested. Furthermore, the assumption is made that the metal ions are complexed to residues in the glycosylated polypeptide backbone: this is necessary because the glycoproteins used here contained little naked segment. Because experimental studies of the redox behaviour of iron may be complicated by the irreversible precipitation of Fe(III) complexes, the following discussion is specialized to copper, but it is virtually certain that iron could play an entirely equivalent role.

The Cu^{2+}-catalysed oxidation of glycoproteins bears some resemblances to the degradation of DNA by the Cu^{2+}–phenanthroline complex studied by Gutteridge & Halliwell (1982); H_2O_2 was shown to be an essential intermediate. The essence of this work was the demonstration that recognized scavengers of the hydroxyl radical were ineffective in retarding the reaction, but a mechanism involving $OH^{\bullet}$ was nevertheless probable: the lack of effect of the scavengers was attributed to the reaction of the $OH^{\bullet}$ virtually at its point of generation.

Later, Rowley & Halliwell (1983) made the even more pertinent observation that free histidine, or serum albumin, lowered the bulk concentration of $OH^{\bullet}$ in a system in which the radical was generated by a Cu^{+}/Cu^{2+} couple; it was suggested that the radical was still produced, but was not liberated because it attacked the histidine, free or in peptide linkage. Furthermore, this reaction would account for the well-known destruction of proteins by the Cu^{2+}/ascorbate system (Orr, 1967*a*,*b*; Samuni *et al.*, 1981, 1983).

A necessary component of all these mechanisms was a reducing agent by which the Cu^{+} ion could be produced as the first step in the cycle. Gutteridge & Wilkins (1983) (see also Barb *et al.*, 1951) suggest that H_2O_2 itself can produce sufficient Cu^{+} to allow the cycle to commence: their results on the degradation of proteins by H_2O_2 and iron or copper salts showed consistently that the lower oxidation state of the metals was much more active, but nevertheless both Cu^{2+} and Fe^{3+} induced reaction.

Convincing evidence that the metal must be

reduced in order to take part in the reaction was obtained through e.p.r. measurements by Shinar *et al.* (1983) in their study of the role of Cu^{2+} and ascorbate in the inactivation of acetylcholinesterase; moreover they obtained, by the same means, proof that the metal ion complexed to the enzyme.

The explanations advanced to account for the destruction of proteins have clear application to our observations on mucus glycoproteins, in spite of the high proportion of carbohydrate in these substances and their consequently very different physical properties. In summary, the scheme is as follows.

(i) The solvent-fractionated mucus glycoproteins contain trace metallic impurities: in particular, some Cu^{2+} is complexed to a few of the histidine side chains.

(ii) In the presence of H_2O_2, some Cu^+ is produced:

$$\text{Complex–}Cu^{2+} + H_2O_2 \rightarrow \text{Complex–}Cu^{+} + HO_2^{\cdot} + H^+ \quad (1)$$

(iii) After the loss of its proton, the superoxide radical (pK4.8; Behar *et al.*, 1970) may react with more H_2O_2 (the Haber–Weiss reaction):

$$O_2^{\cdot -} + H_2O_2 \rightarrow O_2 + OH^- + OH^{\cdot} \quad (2)$$

This reaction is more accurately represented as metal-ion-catalysed (McCord & Day, 1978; Samuni *et al.*, 1981):

$$\text{Complex–}Cu^{2+} + O_2^{\cdot -} \rightarrow \text{Complex–}Cu^{+} + O_2 \quad (3)$$

$$\text{Complex–}Cu^{+} + H_2O_2 \rightarrow \text{Complex–}Cu^{2+} + OH^- + OH^{\cdot} \quad (4)$$

The last stage (4) is the copper analogue of the Fenton reaction; it could, of course, occur immediately after eqn. (1) but the scheme as written emphasizes the site-specific generation of $OH^{\cdot}$ by both pathways.

(iv) Destruction of the histidine residue at which the $OH^{\cdot}$ is formed is now envisaged, so liberating the Cu^{2+} and enabling it to co-ordinate at a further histidine residue and continue the cycle. In detail, a mechanism favouring aspartic acid formation and peptidolysis must be sought. The C=C bond of the imidazole ring must be a susceptible, electron-rich, target at which $OH^{\cdot}$ could be added, so the production of aspartic acid is not surprising; moreover, Tomita *et al.* (1969) found urea derivatives in their study of the oxidation of histidine by singlet oxygen. This moiety accounts for all but the remaining imino group. It is also noted that Shaltiel & Patchornik (1963) used the oxidizing agent *N*-bromosuccinimide to obtain nearly quantitative peptidolysis at the histidine carboxy group; their mechanism called for the formation of a new *N*-terminal at the bond originally linked to the histidine carboxy group. Our failure to find a new *N*-terminal may partly have been due to the linkage of histidine in these glycoproteins to a wide variety of other amino acids, in keeping with the very weak and ill-defined fluorescence observed in the test. However, it is relevant to note that pyruvoyl analogues were isolated by Levitzki *et al.* (1967), arising from oxidation of the peptide $\diagdown$CH–$\diagup$CO group; their subsequent decomposition would lead to an amide rather than a free amino group.

The occurrence of ligand-to-metal charge transfer (inferred from the observation of the 310nm absorption band described above), implying the excitation of an electron from a ligand-centred orbital to a metal-centred orbital, must be regarded as facilitating the crucial cycle-initiating reaction (1); some co-ordination complexes of Cu(II) are known that undergo spontaneous reduction to the Cu(I) state (Birker & Reedijk, 1983). In the protein system described above, a specific reducing agent, usually ascorbate, was necessary: in our system, in which the H_2O_2 was initially at 80mM, ascorbate at 10mM not surprisingly produced little effect. It seems likely that the high concentration of H_2O_2 eliminates the need for a more powerful reducing agent, but it would clearly be of interest to determine if a similarly specific oxidation occurs at much lower H_2O_2 concentrations in the presence of ascorbate. Detailed e.p.r. spectroscopy of the Cu^{2+}/polyhistidine/H_2O_2 system should also be revealing.

An alternative mechanism, essentially an extension of that developed by Shaltiel & Patchornik (1963), which is not dependent on free radicals, may be advanced: further details are given by Cooper (1984).

It is difficult to extend either mechanism to the oxidation of proline to glutamic acid, despite its apparent similarity to the histidine–aspartic acid reaction, and this observation remains unexplained. A detailed investigation of the effect of scavengers etc. is clearly desirable. It must be noted, however, that the proline–glutamic acid conversion could not occur without peptidolysis, in contrast with the histidine–aspartic acid conversion; the latter would, in principle, be restricted to the side chain alone.

Two other aspects of the glycoprotein/H_2O_2 system deserve comment. Cross *et al.* (1983) have shown that the large disulphide-linked glycoprotein aggregates in gastric mucus are degraded by mixtures of H_2O_2, Fe^{2+} and ascorbate. This is explicable in the terms we have outlined above, it being recognized that these glycoproteins possess residues additional to histidine that are susceptible to peroxide attack (cysteine, tryptophan). In addition, however, Cross *et al.* (1984) have shown

that the smaller glycopolypeptides are effective scavengers of $OH^{\cdot}$ when this is generated by pulse radiolysis, i.e. not in a site-specific manner. Such scavenging action, they point out, is to be expected from the high sugar content of the glycopolypeptides, and the well-known effectiveness of sugars in general in this respect. They suggest that this scavenging action may be of primary importance in protecting the respiratory and gastrointestinal tracts from adventitious radicals in smoke etc. or those produced by normal cellular action. This concept may well be important in understanding the function of mucus, but clearly it is not related to the site-specific action of H_2O_2 with which we have been concerned.

Finally, an attempt has been made to link normal and pathological bronchial function to the now-proven susceptibility of bronchial glycoproteins to H_2O_2 in the presence of trace amounts of copper (J. M. Creeth & B. Cooper, unpublished work). The argument (Clamp & Creeth, 1984) is speculative, but hinges on the role of lactoferrin as a copper-carrier: if correct, it provides a pointer to a route whereby massive amounts of glycoproteins could accumulate, thereby contributing to obstructive airways disease.

We are greatly indebted to Dr. Barry Halliwell, of King's College, London, U.K., who pointed out to us the probable significance of the site-specificity that we had observed. We thank also Mr. Ian Elliott of the University of Bristol, who constructed the computer-controlled viscometric equipment and wrote the appropriate software. The glycoprotein samples were generously given to us by Dr. W. M. Watkins and Professor W. T. J. Morgan of the Clinical Research Centre. Dr. G. Nickless of the Department of Chemistry, University of Bristol, very kindly performed the trace-metal analyses on the buffer solutions. We acknowledge the financial support of the Medical Research Council and the Lister Institute of Preventive Medicine.

References

Albert, A. (1950) *Biochem. J.* **47**, 531–538
Almassy, R., Zil, J. S. V., Lum, L. G. & Ifft, J. B. (1973) *Biopolymers* **12**, 2713–2729
Barb, W. G., Baxendale, J. H., George, P. & Hargrave, K. R. (1951) *Trans. Faraday Soc.* **47**, 591–616
Behar, D., Czapski, G., Rabani, J., Dorfman, L. M. & Schwarz, H. A. (1970) *J. Phys. Chem.* **73**, 3209–3213
Bernarducci, E., Schwindinger, W. F., Hughey, J. L., Krogh-Jespersen, K. & Schugar, H. J. (1981) *J. Am. Chem. Soc.* **103**, 1686–1691
Birker. P. J. M. W. L. & Reedijk, J. (1983) in *Copper Coordination Chemistry: Biochemical and Inorganic Perspectives* (Karlin, K. D. & Zubieta, J., eds.), pp. 409–424, Adenine Press, New York
Bray, R. C., Cockle, S. A., Fielden, E. M., Roberts, P. B., Rotilio, G. & Calabrese, L. (1974) *Biochem. J.* **139**, 43–48
Carter, P. (1971) *Anal. Biochem.* **40**, 450–458
Center, E. J. & MacIntosh, R. M. (1945) *Ind. Eng. Chem. Anal. Ed.* **17**, 239–240
Cheng, L., Kellogg, E. W. & Packer, L. (1981) *Photochem. Photobiol.* **34**, 125–129
Clamp, J. R. & Creeth, J. M. (1984) *Ciba Found. Symp.* **109**, 121–136
Cohn, E. J. & Edsall, J. T. (1943) *Proteins, Amino Acids and Peptides*, p. 370, Reinhold Publishing Co., New York.
Cooper, B. (1984) Ph.D. Thesis, University of Bristol
Cox, D. J. & Schumaker, V. N. (1961) *J. Am. Chem. Soc.* **83**, 2439–2444
Creeth, J. M., Bhaskar, K. R., Donald, A. S. R. & Morgan, W. T. J. (1974) *Biochem. J.* **143**, 159–170
Creeth, J. M., Cooper, B., Donald, A. S. R. & Clamp, J. R. (1983*a*) *Biochem. J.* **211**, 323–332
Creeth, J. M., Cooper, B. & Donald, A. S. R. (1983*b*) *Proc. Int. Symp. Glycoconjugates 7th* 593–594
Cross, C. E., Allen, A., Pearson, J. P. & Halliwell, B. (1983) *Proc. Int. Symp. Glycoconjugates 7th* 578–579
Cross, C. E., Halliwell, B. & Allen, A. (1984) *Lancet* **i**, 1328–1330
Dobbie, H., Kermack, W. O. & Lees, H. (1955) *Biochem. J.* **59**, 246–257
Donald, A. S. R. (1973) *Biochim. Biophys. Acta* **317**, 420–436
Fridovich, I. (1975) *Annu. Rev. Biochem.* **44**, 147–159
Gutteridge, J. M. C. & Halliwell, B. (1982) *Biochem. Pharmacol.* **31**, 2801–2805
Gutteridge, J. M. C. & Wilkins, S. (1983) *Biochim. Biophys. Acta* **759**, 38–41
Halliwell, B. (1975) *FEBS Lett.* **56**, 34–38
Halliwell, B. (1978*a*) *FEBS Lett.* **92**, 321–326
Halliwell, B. (1978*b*) *FEBS Lett.* **96**, 238–242
Hasty, N., Merkel, P. B., Radlick, P. & Kearns, D. R. (1972) *Tetrahedron Lett.* **1**, 49–52
Hodgson, E. K. & Fridovich, I. (1975) *Biochemistry* **14**, 5294–5299
Holt, J. C. & Creeth, J. M. (1972) *Biochem. J.* **129**, 665–676
Ifft, J. B. (1971) *C. R. Trav. Lab. Carlsberg* **38**, 315–338
Ito, T. (1978) *Photochem. Photobiol.* **28**, 493–508
Kong, S. & Davison, A. J. (1980) *Arch. Biochem. Biophys.* **204**, 18–29
Levitzki, A., Anbar, M. & Berger, A. (1967) *Biochemistry* **6**, 3757–3765
Martell, A. E. & Smith, R. M. (1974) *Critical Stability Constants, vol. 1: Amino Acids*, pp. 61–62, Plenum Press, New York
Matheson, I. B. C., Etheridge, R. D., Kratowich, N. R. & Lee, J. (1975) *Photochem. Photobiol.* **21**, 165–171
McCord, J. M. & Day, E. D. (1978) *FEBS Lett.* **86**, 139–142
McCord, J. M. & Fridovich, I. (1969) *J. Biol. Chem.* **244**, 6049–6055
Merkel, P. B., Nilsson, R. & Kearns, D. R. (1972) *J. Am. Chem. Soc.* **94**, 1030–1031
Morgan, W. T. J. (1967) *Methods Immunol. Immunochem.* **1**, 75–81
Orr, C. W. M. (1967*a*) *Biochemistry* **6**, 2995–2999
Orr, C. W. M. (1967*b*) *Biochemistry* **6**, 3000–3006
Pecht, I., Levitzki, A. & Anbar, M. (1967) *J. Am. Chem. Soc.* **89**, 1587–1591
Pedersen, K. O. (1958) *J. Phys. Chem.* **62**, 1282–1290

Rowley, D. A. & Halliwell, B. (1983) *Arch. Biochem. Biophys.* **225**, 279–284
Samuni, A., Chevion, M. & Czapski, G. (1981) *J. Biol. Chem.* **256**, 12632–12635
Samuni, A., Aronovitch, J., Godinger, D., Chevion, M. & Czapski, G. (1983) *Eur. J. Biochem.* **137**, 119–124
Shaltiel, S. & Patchornik, A. (1963) *J. Am. Chem. Soc.* **85**, 2799–2806
Shinar, E., Navok, T. & Chevion, M. (1983) *J. Biol. Chem.* **258**, 14778–14783
Singh, A. (1982) *Can. J. Physiol. Pharmacol.* **60**, 1330–1345
Tomita, M., Irie, M. & Ukita, T. (1969) *Biochemistry* **8**, 5149–5160
Washburn, E. W. (1928) *International Critical Tables*, p. 67, McGraw-Hill Book Co., New York
Wasserman, H. H. & Murray, R. W. (1979) *Org. Chem. (N.Y.)* **40**
West, T. S. (1969) *Complexometry*, pp. 138–139, BDH Chemicals Ltd., Poole