Biochem. J. (1983) **211**, 323–332
Printed in Great Britain

Studies of the limited degradation of mucus glycoproteins

The effect of dilute hydrogen peroxide

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(*Received 3 June 1982/Accepted 10 February 1983*)

1. The action of dilute H_2O_2 on a series of ovarian-cyst glycoproteins and glycopolypeptides was investigated. 2. Both native glycoproteins and the glycopolypeptides were carbohydrate-rich, of relatively low molecular weight and of simple structure. 3. At pH 5.6 and 37°C, exposure to H_2O_2 for a limited time brought about a partial degradation, the molecular weight being decreased by 2–4-fold. 4. Carbohydrate analysis showed very little change in the oligosaccharide moiety, apart from a small decrease in sialic acid in some samples. 5. Amino acid analysis showed minor changes in serine, threonine and proline contents, but almost total loss of histidine. Concomitantly, there was a small gain in aspartic acid. 6. Myosin, examined at both pH 5.7 and 6.7, exhibited generally similar behaviour, there being losses of other amino acid residues as well as histidine: the viscosity was decreased to a low value, and a range of peptides of widely varying size was produced. 7. It is suggested that attack on the histidine residue, with partial conversion into aspartic acid, is accompanied by scission of the histidyl peptide bond.

The controlled degradation of mucus glycoproteins is of interest for the information on structure that may be obtained and, more particularly, for any inferences that may be drawn concerning the fate of the substances *in vivo*; they constitute the main functional component of those secretions that protect mucous membranes (e.g. Avery Jones, 1978).

As a preliminary to an investigation of the degradation of the very large glycoproteins found in bronchial secretions (see, e.g., Roberts, 1974, 1976; Creeth *et al.*, 1977), we have examined some glycoproteins derived from ovarian cysts, which are of generally lower molecular weight and relatively simpler structure. Characteristically, these substances possess strong A, B, H and Lewis blood-group specificity.

Among the many cyst glycoproteins that have been characterized (see, e.g., Watkins, 1972; Donald, 1973), those from the Le^a cyst denoted 603 are particularly useful. Analytical and physicochemical data are available for the complete range of fractions obtained by solvent extraction (Creeth *et al.*, 1974) and for a similar series obtained by density-gradient fractionation (Bhaskar & Creeth, 1974). Moreover, the effect on the various fractions of a wide-spectrum proteolytic enzyme preparation (Pronase) has been reported (A. S. R. Donald, unpublished work; Creeth, 1977): these Pronase-treated materials generally resembled those obtained from a number of other cyst glycoproteins (Donald, 1973) and probably retain only the glycopolypeptide moiety (Gibbons, 1978) of the original glycoprotein. For the present investigation, two native glycoproteins and three Pronase-treated derivatives were selected, one of the 603 fractions being included in each category.

Preliminary experiments with a variety of oxidative systems showed that H_2O_2 alone brought about a significant decrease in the viscosity of glycoprotein solutions, but, in restricted conditions, did not completely destroy their macromolecular character. Accordingly, the limited degradation brought about by this reagent on the simple types of glycoprotein referred to above was studied and is now reported. A preliminary account of part of this work has been published (Creeth *et al.*, 1982).

Experimental

Reagents

Reagents were of analytical grade. All solutions were made up in glass-distilled water. Except where stated, EDTA was omitted from the buffer solutions

because the iron(II) complex of EDTA is itself catalytically active in some polysaccharide degradations (see the Discussion section). Reactions were carried out in a sodium acetate buffer, pH 5.6, of I 0.10, and this was also used in the physicochemical determinations.

Glycoproteins

These were the substances referred to in earlier publications (Donald, 1973; Creeth *et al.*, 1974) as 485/PI/WS/AmS/50–55% ethanol (A-specific), 603/PI/WS/AmS (Le^a-specific) and the Pronase-treated product, 603/PI/WS/AmS insol/43–50% ethanol/Pr × 4. The other Pronase-treated products, from different cysts, were respectively 531/PI/WI/Pr × 4 (B-specific) and 610/PI/WS/43–50% ethanol/Pr × 4 (Le^a-specific). For brevity, they are referred to below as glycoproteins 485, 603AmS, 603/43–50Pr, 531Pr and 610Pr respectively. The products obtained from them by the action of H_2O_2 are denoted by the addition '/ox' to these designations.

The two native glycoproteins were soluble in saturated $(NH_4)_2SO_4$ solution; this property is determined, among other factors, by molecular size. Glycoprotein 603AmS was the lowest-molecular-weight fraction prepared from this cyst, whereas glycoprotein 603/43–50 was originally of high molecular weight. Glycoprotein 531 was insoluble in water after phenol extraction. Glycoprotein 610 represented a typical substance of moderately high molecular weight, and was included because several of its physical characteristics were known (Donald, 1973). Although the Pronase-treated derivatives possessed compositions distinctly different from their parent fractions, their proportions of peptide, and amino acid profiles, were all similar and closely resembled those of the two native materials specified above. These materials have molecular weights of about 500000.

Reaction with hydrogen peroxide

Reactions were carried out by the addition of small volumes of approx. 5% (w/v) H_2O_2 to 1ml quantities of the glycoproteins at 2.0mg/ml maintained at 37°C in the viscometer assembly described by Holt & Creeth (1972); this procedure enabled the progress of the reaction to be followed conveniently. In general, two lots of 50μl of H_2O_2 solution were added, the second approx. 4h after the first. After 20–28h, the solution recovered from the viscometer was dialysed against several changes of acetate buffer to remove excess peroxide, and used directly for the physical measurements. The quantities specified give approx. 200 molecules of H_2O_2 per oligosaccharide chain of assumed mean length 10 monosaccharide units, or 70–100 molecules of H_2O_2 per peptide bond (taking the glycoprotein composition to be 90% carbohydrate + 10% peptide). When the reaction was followed spectrophotometrically at 220nm (where the peroxide absorption is about 3 times that due to the glycoprotein), it was found that approximately half of the initial 50μl of H_2O_2 had reacted or decomposed at the time of the second addition; thus the H_2O_2 concentration in the mixture never exceeded about 0.08M.

Protein control experiments

Two well-characterized proteins, selected for reasons given below, were treated with H_2O_2 in the same way as the glycoproteins. These proteins were hen's-egg lysozyme (Sigma Chemical Co., Poole, Dorset, U.K.) and rabbit myosin (a gift from Dr. G. R. Offer of the Meat Research Institute, Langford, Avon, U.K.) that had been prepared by the method described by Offer *et al.* (1973).

Conditions for the lysozyme experiments were identical with those described for the glycoproteins, but the reaction could not be followed viscometrically. After reaction for 20h, the mixture was dialysed, and the sample and original were examined spectrophotometrically and by electrophoresis and amino acid analysis. In addition, to allow maximum access of the oxidizing agent to all peptide bonds, a stabilized denatured preparation was made and treated similarly. Reduction of the disulphide bonds was brought about by dithiothreitol (0.04M) at pH 7.2, in the presence of 1% (w/v) sodium dodecyl sulphate at 100°C (2min). After the addition of a 3-fold molar excess of iodoacetamide, the pH being kept constant by the addition of Na_2HPO_4, the solution was dialysed against the acetate buffer, pH 5.6, containing also 1% sodium dodecyl sulphate. The preparation, and its peroxide-treated product, were examined by electrophoresis.

The myosin was supplied in glycerol solution containing 0.5M-KCl at approx. 6mg/ml, and was dialysed, after dilution to 1.6mg/ml, against 0.5M-NaCl, containing no other salts. In our hands, this solution, of pH 6.7, gave a reduced viscosity of 220ml/g, in satisfactory agreement with the accepted value for $[\eta]$ of 234ml/g (Emes & Rowe, 1978). The myosin was treated with H_2O_2 at this pH because it proved to be insoluble in the usual acetate buffer, pH 5.6, containing additionally 0.5M-NaCl. On addition of 50μl of 5% H_2O_2, the relative viscosity fell rapidly until, at 90min, it was little greater than that of the solvent. At this time, a further 50μl of H_2O_2 solution was added. After a total of 20h, the reduced viscosity then being only 5.1ml/g, the solution was chromatographed on Sephadex G10: a 230nm-absorbing peak was eluted at the void volume (fraction 1), well-separated by a non-absorbing region (fraction 2) from a second peak (fraction 3), which was partially included. The second peak contained the excess H_2O_2: after

evaporation to dryness, its u.v. absorption was negligible. The untreated myosin, the treated solution before fractionation and the fractions 1 and 2 were subjected to amino acid analysis; sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was conducted on the original and on the peroxide-treated solution before fractionation. Subsequently, it was found that myosin was sufficiently soluble in 0.5M-KCl buffered to pH 5.7 with 0.01M-phosphate: treatment with H_2O_2 under these conditions was therefore carried out, the products being examined electrophoretically.

N-Terminal analysis

Attempts to determine *N*-terminal residues by the method of Gray (1972) were unsuccessful, owing to the high proportion of carbohydrate in the glycoproteins (90% or more).

Ultracentrifuge methods

Density-gradient experiments were performed with the Centriscan 75 ultracentrifuge, with Beckman 12mm cells with 2°-sector-angle KelF centre sections. The dense electrolyte chosen was Cs_2SO_4, at approx. 30% (w/w) concentration, because the earlier buoyant-density determinations (Creeth *et al.*, 1974) had been made in this medium, which is particularly suitable for density-disperse macromolecules (Creeth & Horton, 1977). U.v.-absorption optics at wavelengths in the range 245–280nm were used to determine the glycoprotein distributions. A check with schlieren optics verified that virtually identical values of ρ_0 were obtained with the two systems.

Molecular-weight determinations were made with the Beckman model E ultracentrifuge, by following the general procedure for sedimentation equilibrium described by Creeth *et al.* (1974), modified by the inclusion of a seven-point cubic smoothing routine (Teller, 1973) applied to the baseline and initial fringe-displacement calculations. Following earlier practice, the results are quoted in terms of the weight-average molecular weight over the whole cell, denoted M_w^0.

Solutions used for molecular-weight determination were prepared by dialysis against the acetate buffer referred to, but containing in addition 0.4M-CsCl, to provide a small stabilizing density gradient at the very low macromolecular concentrations prevailing in the upper parts of the cell.

Analytical composition

Carbohydrates were determined by g.l.c. of the trimethylsilyl derivatives, by the procedure of Clamp (1977). Mannitol, perseitol and arabinitol were used as internal standards.

Amino acids were determined either with a Beckman model 120C or an LKB 4400 amino acid analyser, by the procedure of Donald (1973). Although the absorbances of the glycoproteins in the range 280–288nm were very low, the spectroscopic method of Edelhoch (1967) was used to obtain an indication of the tryptophan content.

Other chemical degradative methods

The two native glycoprotein preparations were subjected to the reductive action of dithiothreitol (0.04M at pH 8.0, 37°C, 24h). These reactions were followed viscometrically. Also, these glycoproteins were subjected to oxidation by performic acid, by the method of Hirs (1967). The reaction mixtures were dialysed free of excess reagent, salts etc. and freeze-dried: the reaction products were then compared with the originals in terms of molecular weight.

Polyacrylamide-gel electrophoresis

Experiments on the glycoproteins were performed with gels containing a gradient of polyacrylamide running from 2.5% at the top to 27% at the bottom (Uniscil, London, U.K.). A phosphate buffer, pH 7.2 and I 0.11, was employed. Detergent was omitted in view of the findings of Holden *et al.* (1971). Solutions for electrophoresis were treated with 0.04M-dithiothreitol, and heated for 2min at 100°C before layering on to the gel. After fixation in methanol/acetic acid/water (8:1:11, by vol.) the gels were stained with periodic acid/Schiff reagent, by following the general procedure of Segrest & Jackson (1972).

The control proteins, lysozyme and myosin, were examined by standard methods (Weber *et al.*, 1972), sodium dodecyl sulphate being incorporated in all solutions. For myosin, sample treatment included one series in which dithiothreitol was omitted. The gels were stained with Coomassie Blue.

Results

The time course of a typical reaction between a glycoprotein and dilute H_2O_2 is shown in Fig. 1, in terms of the reduced viscosity of the solution. After 5h, the viscosity, and also its rate of decrease, have fallen to about half their initial values, and after about 20h the rate was quite low: typically the flow-time decreased by approx. 0.5% over the period 20–28h. For this reason the 20h reaction period was taken as standard for all the preparations described here: yields of non-diffusible material averaged about 85%. Prolonged reaction over several days brought about a further decrease in viscosity, and about 75% of the product became diffusible. This reaction, not yet studied in detail, was accompanied by more extensive analytical changes (see below). Unless otherwise specified, all that follows refers to the restricted reaction at pH 5.6.

The reduced-viscosity values characterizing the parent materials and their products are included in the physicochemical data in Table 1. It is evident that H_2O_2 causes a substantial decrease in reduced viscosity, the effect being most marked in the native samples, where the value for the product is about 30–40% of that for the original.

Similar trends are shown by the molecular-weight values: for three of the five samples, the oxidation products have about one-quarter the molecular weight of the original. For glycoproteins 610Pr and 531Pr, however, the viscosity changes are smaller, this correlating satisfactorily with the higher molecular weights found for the products.

The density-gradient distributions are illustrated in Fig. 2, referring to glycoprotein 603AmS, but the major characteristics of all samples were identical: the distributions of both untreated and oxidized glycoproteins are nearly symmetrical, but the oxidation is usually accompanied by a slight increase in buoyant density and a marked broadening of the distribution. Glycoprotein 610Pr is exceptional, no significant change in ρ_0 or zone shape being discernible. The values of the buoyant density are included in Table 1.

The gel patterns observed for lysozyme before and after treatment were identical (Fig. 3*a*). Myosin at pH 6.7 before treatment gave a major sharp band at a position corresponding to a molecular weight of approx. 220000, this band being followed by others (Fig. 3*b*). After treatment, all these bands disappeared completely, and were replaced by an unresolved and rather faint zone in the molecular-weight region 10000–100000. Inclusion of a thiol-reduction step in the sample preparation

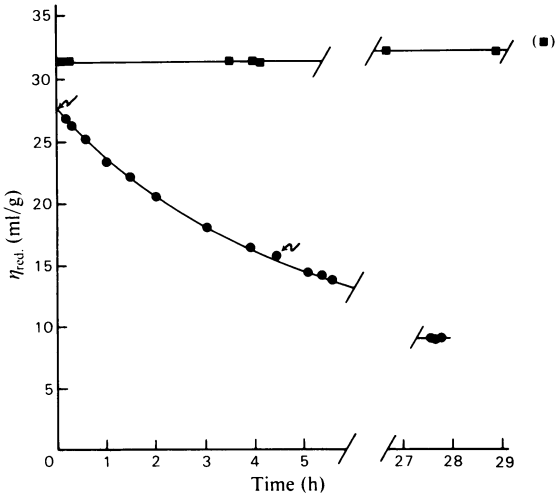

Fig. 1. *Time course* (●) *of oxidative degradation of glycoprotein 603 AmS by* H_2O_2 *in acetate buffer, pH 5.6*
The temperature was 37°C, except during overnight period at approx. 25°C. Arrows indicate times of addition of 50μl quantities of 5% (w/v) H_2O_2. ■, Control experiment, at nominally the same glycoprotein concentration, in which no peroxide was added, but treatment was otherwise identical. Point in parenthesis was determined at 70h.

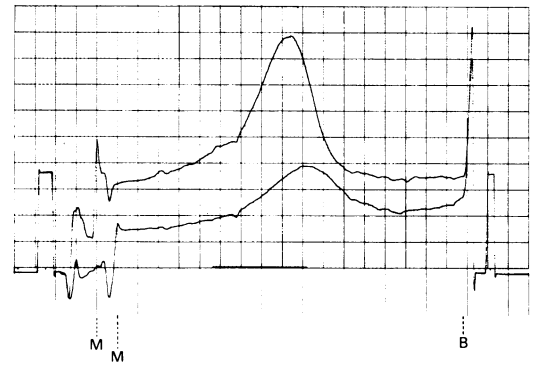

Fig. 2. *Density-gradient patterns for glycoprotein 603AmS (upper curve) and its derivative after treatment with* H_2O_2, *603AmS/ox (lower curve), at initial concentrations of 1.5–2 mg/ml in* Cs_2SO_4 *of initial density 1.353 g/ml*
Note the small difference in meniscus positions, denoted M, between the two cells; positions of cell bottoms (B) were identical. Patterns were recorded at 260nm.

Table 1. *Physical properties of the glycoproteins before and after treatment with* H_2O_2
$\eta_{red.}$ is the reduced viscosity at 2mg/ml, in ml/g; ρ_0 is the buoyant density in Cs_2SO_4 solution, corrected for pressure effects, in g/ml; M_w^0 is the weight-average molecular weight. Pr indicates Pronase-treated (see the text); ox indicates H_2O_2-treated (see the text).

Glycoprotein ...	603AmS	603AmS/ox	485	485/ox	603/43–50/Pr	603/43–50/Pr/ox	531/Pr	531/Pr/ox	610/Pr	610/Pr/ox
$\eta_{red.}$ (ml/g)	27.6	8.8	53.7	22.3	29.5	17.1	41.1	28.8	42.5	31.0
ρ_0 (g/ml)	1.356	1.362	1.306	1.310	1.346	1.365	1.341	1.348	1.321	1.319
$10^{-3} \times M_w^0$	585	169	580	175	470	119	477	309	416	220

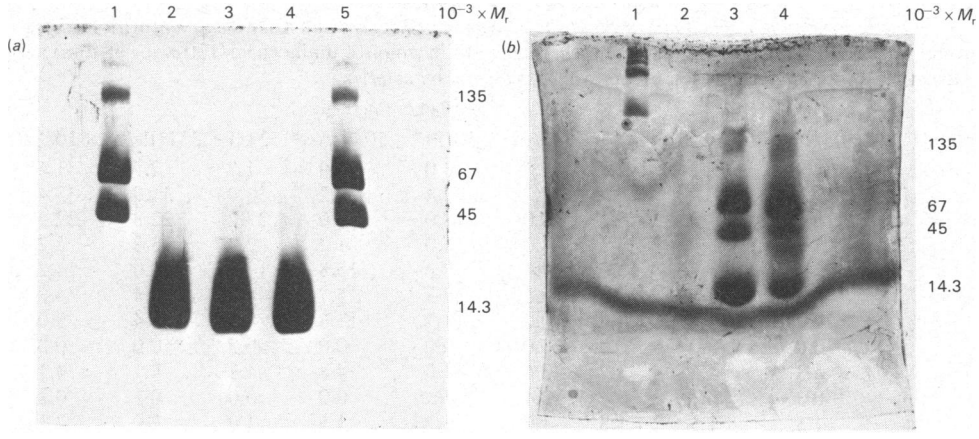

Fig. 3. *Sodium dodecyl sulphate/polyacrylamide-gradient-gel electrophoresis of control proteins before and after treatment with H_2O_2*

(*a*) Lysozyme. Channels 1 and 5, standards (hen ovalbumin and bovine serum albumin); channel 2, lysozyme, after reduction and alkylation (see the text), after treatment with H_2O_2 for 20h at 37°C; channel 3, procedural control, lysozyme after reduction and alkylation, treated identically as sample in channel 2 but with omission of the H_2O_2; channel 4, lysozyme untreated, except for standard preparation for electrophoresis. The lysozyme channels were slightly overloaded to facilitate detection of faster components. (*b*) Myosin. Channel 1, untreated myosin; channel 2, myosin treated with H_2O_2; channels 3 and 4, standards (hen ovalbumin, serum albumin and lysozyme). Standards in channel 4 were thiol-reduced; in all others thiol treatment was omitted.

Table 2. *Relative carbohydrate compositions of the glycoproteins before and after treatment with H_2O_2*

Glycoprotein designations are as in Table 1. The monosaccharide content is given as residues/6.0 residues of galactose (see the text). The values for total peptide are given as g/100g of glycoprotein and were obtained by summing the values for the individual amino acids (Table 3).

Glycoprotein ...	603AmS	603AmS/ox	485	485/ox	603/43–50/Pr	603/43–50/Pr/ox	531/Pr	531/Pr/ox	610/Pr	610/Pr/ox
Fucose	3.6	4.1	4.9	4.8	3.6	3.3	4.0	4.1	2.2	2.0
Galactose	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0
N-Acetyl-glucosamine	6.5	5.7	4.8	5.2	7.2	6.5	3.4	2.8	5.3	5.1
N-Acetyl-galactosamine	1.6	1.2	4.9	4.8	2.3	1.8	2.6	2.0	3.2	2.9
N-Acetyl-neuraminic acid	0.5	0.1	0.4	0.4	1.2	1.0	0.3	Trace	0.9	0.4
Total peptide	7.6	7.6	12.0	10.7	8.0	6.3	14.0	13.7	17.2	14.1

removed most of the higher polymers of myosin, but did not affect the pattern from the oxidized material.

In the experiment at pH5.7, the effect of H_2O_2 was similar, but less complete, a small proportion of the original protein being visible. The molecular-weight range of the peptides produced was again 10000–100000.

Carbohydrate composition

The results of the g.l.c. sugar analyses of the glycoproteins before and after treatment with H_2O_2 are summarized in Table 2. Because recoveries from the analyses were variable, the results are given in terms of the molar ratios of each sugar relative to 6mol of galactose. This procedure, which follows previous practice (see, e.g., Fraser & Clamp, 1975), does not allow comparisons of galactose content or total carbohydrate, but provides a reliable basis for comparisons of all the other sugars. Indirect evidence that the total carbohydrate content varies between quite narrow limits is provided by the values for the peptide content, quoted in Table 2, together with the values for the buoyant density given earlier. It was shown previously that the buoyant density is a sensitive index of the ratio of carbohydrate to peptide (Creeth *et al.*, 1974). In general, it is clear

Table 3. *Amino acid composition of the glycoproteins before and after treatment with H_2O_2*
Glycoprotein designations are as in Table 1. All values are in μmol of amino acid/100 μmol of total amino acids, and are averages of duplicate analyses, except where indicated by asterisk.

Glycoprotein ...	603AmS	603AmS/ox	485	485/ox	603/43–50/Pr*	603/43–50/Pr/ox*	531/Pr	531/Pr/ox	610/Pr	610/Pr/ox
Asp	4.9	6.2	2.8	3.7	3.0	3.9	1.7	2.5	1.6	2.9
Thr	19.0	20.7	27.1	27.7	27.4	29.2	33.0	31.9	33.4	30.1
Ser	16.3	17.3	18.1	20.0	18.0	20.0	23.2	22.8	22.9	23.0
Glu	4.7	5.0	4.1	4.3	1.0	1.2	2.4	3.8	2.2	4.5
Pro	15.1	14.2	16.0	14.7	18.3	15.8	13.7	12.0	14.2	11.9
Gly	8.5	8.1	5.8	6.2	6.3	5.7	4.8	6.4	4.8	7.0
Ala	12.7	13.2	9.6	10.0	11.7	11.6	8.5	8.4	9.0	8.8
CyS	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.2	0.0
Val	4.3	4.3	3.7	3.6	4.0	4.8	4.1	4.1	4.1	4.0
Met	0.3	0.1	0.4	0.4	Trace	0.0	0.0	0.1	0.1	0.1
Ile	1.5	1.5	1.8	1.7	1.3	1.6	1.9	2.0	2.1	2.1
Leu	2.1	1.9	1.9	1.7	1.4	1.7	1.1	1.4	1.3	1.6
Tyr	0.7	0.6	0.5	0.3	0.3	0.3	0.3	0.5	0.1	0.5
Phe	1.0	1.1	1.0	0.9	0.4	0.8	0.4	0.7	0.2	0.8
His	3.2	0.4	2.8	0.8	2.2	0.1	1.1	0.3	1.2	0.2
Lys	1.9	1.9	1.3	1.3	1.8	1.5	1.5	1.5	1.1	1.1
Arg	3.2	3.2	2.6	2.2	2.7	2.7	1.9	1.7	1.8	1.6

Table 4. *Amino acid compositions of lysozyme and myosin and their products before and after treatment with H_2O_2*
Units are as in Table 3; values refer only to the amino acids specified (see the text) and are not corrected for tryptophan content. Values in the last column refer to peroxide-treated myosin before chromatographic fractionation.

Protein ...	Lysozyme*	Lysozyme/ox*	Myosin	Myosin/ox (fraction 1)	Myosin/ox (fraction 2)	Myosin/ox (total)
Asp	22.7	21.8	10.1	13.1	8.1	12.8
Thr	5.7	5.9	5.1	4.7	4.8	5.5
Ser	8.3	8.7	5.2	5.9	9.2	6.4
Glu	4.3	4.5	18.6	22.1	15.8	20.7
Pro	2.7	3.0	2.7	1.7	21.1	2.2
Gly	10.1	10.3	5.2	4.2	13.4	5.8
Ala	9.7	9.9	9.0	9.8	8.2	9.7
CyS	0.8	0.6	1.3	0.0	0.0	0.4
Val	4.6	4.6	4.8	5.8	4.5	4.9
Met	1.5	1.5	2.7	1.5	0.0	0.0
Ile	4.2	4.3	4.2	4.0	1.7	3.9
Leu	6.2	6.2	8.8	8.7	1.5	7.9
Tyr	2.6	2.6	2.4	1.0	0.0	0.4
Phe	2.6	2.5	3.4	2.2	0.0	2.9
His	1.0	1.1	1.8	0.6	0.0	0.6
Lys	4.5	4.2	9.9	9.6	7.7	10.6
Arg	8.4	8.6	4.9	5.0	3.9	5.1

* Lysozyme and its derivative were reduced and alkylated, leading to very low values for cyst(e)ine and spuriously high values for aspartic acid, from which *S*-carboxymethylcysteine is unresolved.

that H_2O_2 produces very little effect on the carbohydrate composition, although in the glycoproteins other than glycoprotein 485 a slight trend towards lower values, relative to galactose, is suggested.

Amino acid composition

The results for the glycoproteins and their peroxide products are given in Table 3, and for the control proteins in Table 4. The glycoproteins present a reasonably consistent picture: of the three amino acids that make up about half the total peptide, threonine and serine are slightly increased and proline is slightly decreased. The changes, however, are proportionately rather small. Histidine, on the other hand, is unquestionably considerably decreased by peroxide treatment, the loss

ranging from almost complete to some 65% for glycoprotein 485. Simultaneously, there is an increase in aspartic acid, by a small proportion that might escape notice but for its consistency throughout the series. The changes in the other amino acids determined by the analyser are small and within the accepted errors of the procedure. The tryptophan analyses were inconclusive. Spectra recorded on the Pronase-treated parent materials showed that the absorbances in the 280–288 nm range were virtually zero. The native glycoproteins, however, had measurable absorbances in this range, and Edelhoch's (1967) method gave values of 0.7 mol/100 mol for glycoprotein 603AmS and 0.2 mol/100 mol for glycoprotein 485. These values, which depend critically on the baseline correction, are below the level of significance, but indicate that the possibility of the existence of a few tryptophan residues (not more than 4) per peptide chain cannot be eliminated. All peroxide products had negligible absorbance in the range quoted.

The prolonged reaction of one of the glycoproteins (603AmS) with H_2O_2 (6 days at pH 5.6) caused greater losses in proline, together with other indications of non-specific oxidative breakdown.

The results for the control proteins showed differences in behaviour between them. Lysozyme, which had been selected because of its single histidine residue (Imoto *et al.*, 1972), was apparently unaffected by the standard treatment with H_2O_2; this was true of both the native protein and the reduced, alkylated and stabilized form. Thus histidine and aspartic acid contents are seen (Table 3) to be unchanged, whereas the tryptophan content, initially 6.0 residues/molecule as expected (Imoto *et al.*, 1972), became 5.5 residues/molecule after treatment, this value being uncorrected for possible losses during dialysis. Thus tryptophan is certainly less affected than in the alkaline conditions employed by Hachimori *et al.* (1964), and the constancy of amino acid composition agrees with the electrophoretic behaviour. Long-term (14 days) treatment of native lysozyme with H_2O_2 at pH 5.6 led to the loss of approx. 50% of the protein as peptides non-fixable in gel electrophoresis, the remainder retaining the mobility of the control enzyme.

Myosin, on the other hand, is significantly changed in several respects: in addition to the loss of histidine and gain in aspartic acid, there is a loss of methionine and tyrosine and possibly also of cyst(e)ine and proline. In the unfractionated sample the apparently large increase in aspartic acid is partly spurious, because an unidentified interfering peak was incompletely resolved from aspartic acid in the chromatograms. Tryptophan analyses were not attempted with this protein, but integration of the A_{280} values for fraction 1 of the chromatographic separation described above suggested negligible losses of tryptophan, after correction for tyrosine.

Thus, allowing for the possibility that traces of tryptophan (if they exist) may also be affected, one must conclude that oxidation of histidine, with partial conversion into aspartic acid, occurs as the major reaction with the glycoproteins studied in the present investigation. The density-gradient distributions are consistent with only a very minor change in the proportion of peptide to carbohydrate, in agreement with the analytical values.

Effect of other degradative reagents

Many native glycoproteins are dissociated by thiols, which cleave the disulphide bonds joining the smaller units [see, e.g., Dunstone & Morgan (1965), Snary *et al.* (1970), Creeth *et al.* (1977) and Pearson *et al.* (1981)]. Similarly, performic acid is believed to oxidize these bonds specifically (Meyer, 1977) and to perform a more thorough cleavage than thiol reduction.

Although the amino acid composition of the glycoproteins studied in the present work showed very small proportions of cyst(e)ine, it was thought desirable to establish the action of these reagents. Reduction by dithiothreitol produced barely significant changes in viscosity; e.g. for glycoprotein 485 the values of $\eta_{red.}$ were 53.7 and 51.9 ml/g before and after treatment respectively. For glycoprotein 531Pr, where the cyst(e)ine content was just significant, a slight increase in viscosity (from 43.0 to 46.3 ml/g) occurred on reduction. It was concluded that intermolecular disulphide bridges are essentially absent. The products of performic acid oxidation gave weight-average molecular weights approx. 10% lower than those for the parent materials (the values were 513000 and 519000 for the products from glycoproteins 603AmS and 485 respectively) but the carbohydrate composition was essentially unchanged. It is clear, therefore, that the performic acid reaction, in virtually anhydrous formic acid, is quite unrelated to the action of dilute aqueous H_2O_2 at pH 5.6.

Sodium ascorbate, in the presence of H_2O_2, is well known as a degradative agent for many macromolecules (see the Discussion section). However, at 10 mM-ascorbate the reaction was slightly slower than normal and the final viscosity slightly higher. EDTA, which could influence catalysis by any trace amounts of metallic impurities in the reagents, was found to have a dual effect (Fig. 4). In the absence of H_2O_2, EDTA at 2.0 mM produced a slow decrease in viscosity. When H_2O_2 was added, at 21 h, the fall in viscosity then being about 10% of its usual final value, the rate of degradation increased, but did not approach the rate observed in the absence of EDTA. Since the glycoprotein alone was quite stable, for at least 70 h, under these conditions,

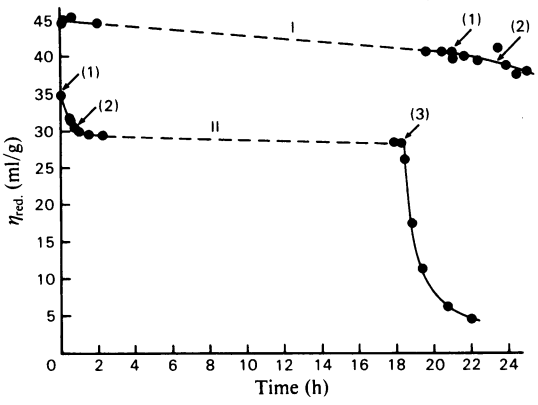

Fig. 4. *Effect of EDTA on the peroxide attack on glycoproteins*

Curve I: glycoprotein A485, 2mg/ml, in acetate buffer pH5.6, containing 2.0mM-EDTA. Determinations of $\eta_{red.}$ were made during 0–2h and 19.5–25h. At 21h and 23.5h (arrows 1 and 2) additions of 50μl of 5% (w/v) H_2O_2 were made. The rate of reaction is much lower than in the absence of EDTA. Curve II: glycoprotein 603AmS, same nominal conditions except that EDTA was not incorporated in the initial solution. At zero time (arrow 1) 50μl of 5% H_2O_2 was added: the subsequent reaction proceeded with typical velocity (see Fig. 1). At 1h (arrow 2) EDTA (50μl of 44mM) was added to 2.0mM. At 18h (arrow 3) $FeSO_4$ was added to 3.0mM, giving a free Fe^{2+} concentration of 1.0mM.

the EDTA appears to be initiating a reaction that is negligible in its absence: however, the EDTA appears to protect the glycoprotein from the normal peroxide attack. Verification of this conclusion was obtained by addition of the EDTA (2mM) after the glycoprotein–H_2O_2 reaction had proceeded for 1h: the reaction was immediately retarded, and then proceeded at a much lower rate, similar to that found when EDTA was present throughout. In the presence of both free Fe(II) at 1mM and EDTA–Fe complex at 2mM a very rapid reaction ensued, leading to a very low value of the reduced viscosity.

Range of serological specificity

The glycoproteins for which detailed results are reported in the present paper include those of blood-group specificities A, B, and Le^a. This range embraces the main structural differences between the terminal sugar residues in the oligosaccharide chains (Watkins, 1972), but for completeness a Pronase-treated glycoprotein of H-specificity (glycoprotein H602/Pr; Donald, 1973) was given brief study. Its viscosity was decreased by about 30% (from 29.3 to 20.7ml/g) and its carbohydrate analysis was unchanged by the peroxide treatment; thus it resembled glycoprotein 610Pr more closely than, for example, the glycoprotein 603 derivatives.

Discussion

The two native glycoprotein fractions studied in the present work belonged to the category characterized by (i) solubility in hot saturated $(NH_4)_2SO_4$ solution, (ii) moderate molecular weight, almost unchanged by thiol reduction or performic acid oxidation, and (iii) a correspondingly low or negligible content of cystine. Such materials were found (Donald, 1973) to be virtually unaffected by the action of Pronase, and, most significantly, their amino acid profiles were very similar to those of the Pronase-treated glycoproteins produced from materials initially of quite different composition. Since proteolytic-enzyme treatment of glycoproteins is recognized (Donald, 1973) to remove the unglycosylated region of peptide (the naked segment; Masson, 1973), it must be concluded that the native materials, glycoproteins 485 and 603 AmS, themselves possessed little in the way of naked segment and thus closely resembled the three Pronase-treated glycoproteins used also.

The most direct interpretation of the results reported above is that H_2O_2 at pH5.6 and 0.05–0.08M breaks the peptide backbone of the glycoproteins by specific attack at the histidine residues, which are partially converted into aspartic acid in the process. Strong support for this conclusion is provided by the physical evidence: the extent of change, as manifested by the difference in molecular weight, is sharply correlated with the histidine content of the parent glycoprotein (cf. glycoproteins 485, 531Pr and 610Pr). Thus it appears likely that the histidyl bond, rather than any other, is cleaved by the oxidation, although direct proof of this is lacking. Moreover, since the products usually have molecular weights within the range 100000–200000, some regularity of occurrence of susceptible histidine along the peptide chain is implied, if histidine is indeed the point of attack. The density-gradient distributions of the oxidized materials show that the fragments produced by oxidation are all essentially of similar peptide–carbohydrate composition, no disproportionation having occurred. The small changes in buoyant density are compatible with the losses in histidine and other very minor losses of amino acids. All results imply that the complex oligosaccharide chain structure of these glycoproteins (Watkins, 1972; Rovis *et al.*, 1973) is unchanged, except perhaps for the loss of some sialic acid: this sugar always occurs in terminal positions.

A rather specific attack upon a molecule as complex as a glycoprotein is at first sight unexpected, but with both proteins and carbohydrates some

conditions are recognized in which the reactions appear to be very restricted. Neumann (1972) has reviewed the action of H_2O_2 on proteins, recognizing attack on cyst(e)ine, methionine, tyrosine, tryptophan and histidine; the reactions vary widely in extent, depending on the conditions. Similarly, the well-known degradation of hyaluronic acid by ascorbate [see, e.g., McCord (1974), Greenwald & Moy (1980) and Wong *et al.* (1981)], which involves H_2O_2 as an intermediate (Schmut & Hofmann, 1975), proceeds by random cleavage of glycosidic bonds (Cleland *et al.*, 1969).

Oxidations by H_2O_2 usually involve free radicals (hydroxyl, hydroperoxide or superoxide) or singlet-oxygen molecules (Ebsworth *et al.*, 1973). Iron-catalysed reactions in particular tend to produce the very reactive hydroxyl radical $^{\bullet}OH$ by a modified Haber–Weiss cycle [see, e.g., Kong & Davison (1980) and Halliwell (1978)]. EDTA does not inhibit the action of $^{\bullet}OH$ on biological polymers because the iron-complex reacts more rapidly with superoxide to produce $^{\bullet}OH$ than does free iron (Harris *et al.*, 1972; Halliwell, 1978). Ascorbate in the absence of iron is a scavenger of superoxide (Nishikimi, 1975; Blake *et al.*, 1981), and in the presence of iron a Fenton-type reaction may occur (Norman & Radda, 1962). The photosensitized oxidation of proteins in the presence of dyes involves the loss of histidine and the appearance of a proportion as aspartic acid; this reaction (Tomita *et al.*, 1969; Matheson *et al.*, 1975) is now known to involve singlet oxygen as the active species.

In the light of these observations, it seems likely that the rapid degradation of glycoproteins by H_2O_2 in the presence of iron proceeds by way of a Fenton-type mechanism, whereas the small effect of EDTA alone may represent the initiation of such a metal-catalysed reaction (from trace impurities in the reagents). The slow and limited reaction between H_2O_2 and the glycoproteins reported in the present paper, in which decrease in the histidine content is the most significant analytical feature, bears a strong resemblance to the photosensitized protein oxidations referred to, but identification of the active oxygen species must await a detailed study of the effect of a range of quenching agents (see, e.g., Kong & Davison, 1980).

The apparently specific nature of the attack must be due to the pH chosen and the virtual absence from the backbone of those other amino acids known to be susceptible, particularly tryptophan.

The results with myosin provide support for the general mechanism suggested. Thus, together with the loss of histidine (and other susceptible residues) demonstrated analytically, the virtually total loss of viscosity and the electrophoretic demonstration of peptides in the molecular-weight range 10000–100000 provide conclusive evidence that attack by H_2O_2, at both pH 5.7 and 6.7, entails destruction of peptide linkages in addition to any modification of side-chain residues. The action on glycoproteins is seen therefore to be in general agreement with expectation. The fact that some histidine remains unattacked (e.g. in glycoprotein 603AmS) may suggest differences in susceptibility arising from particular local sequences, a possibility that receives some support from the results on myosin, where 30% of the histidine fails to react, and lysozyme, where the single histidine residue did not react.

The ability to obtain fragments of glycoproteins smaller than the products of proteolytic digestion, whose carbohydrate content is virtually unchanged, may prove helpful in studies of the amino acid sequence of the peptide backbone, only short stretches of which are now known (Goodwin & Watkins, 1974). However, there is, as yet, no evidence as to whether or not the subunits are identical.

Comparison of the molecular-weight values now found for the parent glycoproteins with those determined when the substances were first prepared reveals significant differences. Thus for glycoproteins 603AmS and 610Pr the values reported earlier (Creeth *et al.*, 1974; Donald, 1973) were 840000 and 740000 respectively, whereas 590000 and 420000 are now observed. The method has been considerably refined in the meantime, but remains the same in principle. It may be significant that the histidine content of one of these two substances has also changed slightly, that of glycoprotein 603AmS decreasing from 4.1 to 3.2%. Slow atmospheric oxidation during storage at room temperature, unprotected from daylight, is probably implicated, and accordingly more stringent control of storage conditions is desirable. In this respect, it is noteworthy that the two glycoproteins on which peroxide had minimal effect, namely glycoproteins 531Pr and 610Pr, were initially very low in histidine content (1.1–1.2%).

We are grateful to Dr. W. M. Watkins, F.R.S., of the Clinical Research Centre, for supplying the original glycoproteins and for many discussions. We thank also Dr. John Williams and Dr. Paul Wood of the University of Bristol for their help and advice, and Dr. Gerald Offer, of the Meat Research Institute, for the gift of the myosin sample. Dr. Richard Davies, of the Department of Rheumatology, Hope Hospital, Manchester, U.K., took part in some of the preliminary experiments. We thank the Medical Research Council for a supporting grant (to B. C.).

References

Avery Jones, F. (1978) *Br. Med. Bull.* **34**, 1–4

Bhaskar, K. R. & Creeth, J. M. (1974) *Biochem. J.* **143**, 669–679

Blake, D. R., Hall, N. D., Treby, D. A., Halliwell, B. & Gutteridge, J. M. C. (1981) *Clin. Sci.* **61**, 483–486
Clamp, J. R. (1977) *Biochem. Soc. Trans.* **5**, 1693–1695
Cleland, R. L., Stoolmiller, A. C., Roden, L. & Laurent, T. C. (1969) *Biochim. Biophys. Acta* **192**, 385–394
Creeth, J. M. (1977) *Mod. Probl. Paediatr.* **19**, 34–45
Creeth, J. M. & Horton, J. R. (1977) *Biochem. J.* **161**, 449–463
Creeth, J. M., Bhaskar, K. R., Donald, A. S. R. & Morgan, W. T. J. (1974) *Biochem. J.* **143**, 159–170
Creeth, J. M., Bhaskar, K. R., Horton, J. R., Das, I., Lopez-Vidriero, M.-T. & Reid, L. (1977) *Biochem. J.* **167**, 557–569
Creeth, J. M., Cooper, B., Donald, A. S. R. & Clamp, J. R. (1982) *IRCS Med. Sci.* **10**, 548–549
Donald, A. S. R. (1973) *Biochim. Biophys. Acta* **317**, 420–436
Dunstone, J. R. & Morgan, W. T. J. (1965) *Biochim. Biophys. Acta* **101**, 300–314
Ebsworth, E. A. V., Connor, J. A. & Turner, J. J. (1973) in *Comprehensive Inorganic Chemistry* (Bailar, J. C., Emeleus, H. J., Nyholm, R. & Trotman-Dickenson, A. F., eds.), vol. 2, pp. 771–777, Pergamon Press, Oxford
Edelhoch, H. (1967) *Biochemistry* **6**, 1948–1954
Emes, C. H. & Rowe, A. J. (1978) *Biochim. Biophys. Acta* **537**, 110–124
Fraser, D. & Clamp, J. R. (1975) *Clin. Chim. Acta* **59**, 301–307
Gibbons, R. A. (1978) *Br. Med. Bull.* **34**, 34–38
Goodwin, S. D. & Watkins, W. M. (1974) *Eur. J. Biochem.* **47**, 371–382
Gray, W. R. (1972) *Methods Enzymol.* **25B**, 121–138
Greenwald, R. A. & Moy, W. W. (1980) *Arthritis Rheum.* **23**, 455–463
Hachimori, Y., Horinishi, H., Kurihara, K. & Shibata, K. (1964) *Biochim. Biophys. Acta* **93**, 346–360
Halliwell, B. (1978) *FEBS Lett.* **96**, 238–242
Harris, M. J., Herp, A. & Pigman, W. (1972) *J. Am. Chem. Soc.* **94**, 7570–7572
Hirs, C. H. W. (1967) *Methods Enzymol.* **11**, 197–199
Holden, K. G., Yim, N. C. F., Griggs, L. J. & Weisbach, J. A. (1971) *Biochemistry* **10**, 3110–3113
Holt, J. C. & Creeth, J. M. (1972) *Biochem. J.* **129**, 665–676
Imoto, T., Johnson, L. N., North, A. C. T., Phillips, D. C. & Rupley, J. A. (1972) *Enzymes 3rd Ed.* **7**, 666–862
Kong, S. & Davison, A. J. (1980) *Arch. Biochem. Biophys.* **204**, 18–29
Masson, P. L. (1973) in *Cervical Mucus in Human Reproduction* (Elstein, M., Moghissi, K. S. & Borth, R., eds.), pp. 82–92, World Health Organisation, Scriptor, Copenhagen
Matheson, I. B. C., Etheridge, R. D., Kratowich, N. R. & Lee, J. (1975) *Photochem. Photobiol.* **21**, 165–171
McCord, J. M. (1974) *Science* **185**, 529–531
Meyer, F. A. (1977) *Biochim. Biophys. Acta* **493**, 272–282
Neumann, N. P. (1972) *Methods Enzymol.* **25**, 393–400
Nishikimi, M. (1975) *Biochem. Biophys Res. Commun.* **63**, 463–468
Norman, R. O. C. & Radda, G. K. (1962) *Proc. Chem. Soc. London* 138
Offer, G. R., Moos, C. & Starr, R. (1973) *J. Mol. Biol.* **74**, 653–676
Pearson, J. P., Allen, A. & Parry, S. (1981) *Biochem. J.* **197**, 155–162
Roberts, G. P. (1974) *Eur. J. Biochem.* **50**, 265–280
Roberts, G. P. (1976) *Arch. Biochem. Biophys.* **173**, 528–537
Rovis, L., Anderson, B., Kabat, E. A., Greuzo, F. & Liao, J. (1973) *Biochemistry* **12**, 5340–5354
Schmut, O. & Hofmann, H. (1975) *Biochim. Biophys. Acta* **411**, 231–235
Segrest, J. P. & Jackson, R. L. (1972) *Methods Enzymol.* **28**, 54–63
Snary, D., Allen, A. & Pain, R. H. (1970) *Biochem. Biophys. Res. Commun.* **40**, 844–851
Teller, D. C. (1973) *Methods Enzymol.* **17D**, 346–441
Tomita, M., Irie, M. & Ukita, T. (1969) *Biochemistry* **8**, 5149–5160
Watkins, W. M. (1972) in *Glycoproteins: Their Composition, Structure and Function* (Gottschalk, A., ed.), 2nd edn., pp. 830–891, Elsevier, Amsterdam
Weber, K., Pringle, J. R. & Osborn, M. (1972) *Methods Enzymol.* **26C**, 3–27
Wong, S. F., Halliwell, B., Richmond, R. & Skowroneck, W. R. (1981) *J. Inorg. Biochem.* **14**, 127–134