Formation of peroxides in amino acids and proteins exposed to oxygen free radicals

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Dilute aqueous solutions of BSA or lysozyme gave positive tests for peroxides after exposure to reactive oxygen species. The reactive species were generated by γ-irradiation, reduction of H_2O_2 with Fe^{2+} ions or thermal decomposition of an azo compound. Peroxides were assayed by an iodometric method. Identification of the new groups as hydroperoxides was confirmed by their ability to oxidize a range of compounds and by the kinetics of their reaction with iodide. The hydroperoxide groups were bound to the proteins and their yields (G values) corresponded to 1.2 -OOH groups per 100 eV of radiation energy absorbed for BSA, and 0.8 for lysozyme. The oxygen free radicals effective in protein peroxidation were the hydroxyl and organic peroxyl, but not superoxide or its protonated form. The efficiency of BSA peroxidation initiated by the hydroxyl radicals was 40%. Protein peroxides decayed spontaneously with a half-life of about 1.5 days at 20 °C. Exposure of the common amino acids to hydroxyl free radicals showed that six of them (glutamate, isoleucine, leucine, lysine, proline and valine) were peroxidized with similar efficiency to the proteins, whereas the rest were inert or much less susceptible. These results suggest that some proteins may be peroxidized by a variety of agents *in vivo* and that their subsequent reactions with protective agents, such as ascorbate or glutathione, may decrease the antioxidant potential of cells and tissues.

INTRODUCTION

The formation of amino acid and protein peroxides was first reported in 1942 by Latarjet and Loiseleur [1] who, probably using an iodometric technique, tested aqueous solutions of a range of compounds after irradiation with X-rays. Horse serum albumin, glycine, alanine, leucine, aspartic acid and lysine gave results indicating the presence of peroxides, but ovalbumin was inert. No peroxide yields were given. There were no further reports of experimental tests of protein peroxide formation for 20 years, but amino acid and peptide peroxides were briefly investigated. A range of amino acids and short peptides exposed to X-rays in air-saturated unbuffered aqueous solutions was assayed for total peroxides by ferrous thiocyanate and iodometric methods, and for H_2O_2 by titanium sulphate [2]. The difference between these results was taken as the amount of organic hydroperoxides produced. Of 13 amino acids tested, isoleucine, leucine, valine, norleucine, glutamate and arginine were peroxidized during irradiation. The di- and tri-peptides also tested positive, provided that they contained one of the sensitive amino acids, but the peroxide yields were decreased to about one-third by the presence of non-peroxidizable glycine or tyrosine.

In the next attempt to detect peroxides in solutions of amino acids and peptides, irradiated this time by γ-rays, two tests were used for the presence of oxidized products: an iodometric assay for peroxides, and the thiobarbituric acid (TBA) reaction, commonly employed for detection of lipid oxidation [3]. Two proteins were also irradiated in this study, cytochrome *c* and haemoglobin, but no conclusions could be drawn on the formation of protein peroxides because the products were only tested for TBA reactivity. However, an unbuffered solution of gelatin irradiated with a large energy dose of γ rays (4000 Gy) tested positive for authentic organic peroxides in an iodometric assay [4].

The two developments that allowed further progress in the studies of protein peroxides were the use of an iodometric assay with improved sensitivity [5] and the destruction with catalase of any H_2O_2 formed simultaneously with the organic peroxides. This approach resulted in detection of peroxides in BSA and serum proteins exposed to ionizing radiation, and in the demonstration that in BSA the peroxidation (defined here as acquisition of peroxide groups) was proportional to the amount of energy absorbed by the solution [6,7]. These findings added to the rapidly growing body of knowledge of other consequences of the interaction between proteins and reactive oxygen species, identified as protein cross-linking and scission [8–10], destruction of amino acids [11–13], increase in proteolytic susceptibility [9,14,15] and loss of biological function [11,16–18]. Many of these changes could be related to processes occurring in living organisms either naturally or in deteriorative or diseased states [19,20]. A finding of particular relevance to our study was the estimate that 10–50% of all antioxidant potential of human blood plasma challenged with peroxyl radicals is due to proteins [21]. This suggests that protein–free-radical interactions may be common and may have considerable significance *in vivo* [22].

In all these studies, modification of the proteins by free radicals was regarded as the significant event, with little attention given to the possibility that the altered molecules may themselves be initiators of further reactions. However, this could be an important process. The acquisition of new chemical groups by proteins exposed to free radicals would result in effective trapping of some of their chemical energy, if the new groups proved to be reasonably stable and selectively reactive. We have recently reported that this can occur with at least two proteins (BSA and lysozyme) which acquire both oxidizing and reducing groups [23]. In this paper we report on the identity of the oxidizing groups, the free radicals responsible for their formation, and their stability. Formation of peroxides in the whole range of common amino acids was also studied. In a later paper we will show that several biological systems generating free radicals can

Abbreviations used: AAPH, 2,2′-azobis(2′-amidinopropane) hydrochloride; SOD, superoxide dismutase; TBA, thiobarbituric acid.

also peroxidize proteins. Taken together, the results suggest that formation and reactions of amino acid and protein peroxides may constitute significant biological events.

MATERIALS AND METHODS

Materials

Glassware used in the handling and storage of solutions was cleaned by heating in concentrated nitric acid, washing in distilled and deionized particle-free water and drying. Solutions were made up in distilled water purified by passage through a four-stage Milli Q system (Millipore–Waters, Sydney, Australia) with a 0.2 μm final filter. Except for phosphate buffer, all solutions were made up on the day of use. BSA (Cohn fraction V, fat-free), lysozyme, catalase and superoxide dismutase (SOD) were supplied by Boehringer-Mannheim or by Sigma. Chromatographic standard-grade amino acids were from Pierce Chemical Co.; other chemicals, solvents and chromatographic materials were of AnalaR or h.p.l.c. grade, obtained from Merck, Malinckrodt, BDH, Pharmacia or Aldrich. 2,2′-Azobis(2′-amidinopropane) hydrochloride (AAPH) was supplied by Polysciences (Warrington, PA, U.S.A.).

Irradiations

Dilute solutions of proteins or amino acids, usually buffered with 20 mM sodium phosphate at pH 7.4, were irradiated in a ^{60}Co facility at the University. The energy dose rate was measured by Fricke dosimetry [24] and expressed in Gy/min [1 gray (Gy) = 1 J/kg]. To ensure saturation with air, the irradiations were periodically interrupted and the solutions stirred for 1 min. After irradiation, sufficient catalase was added to destroy radiation-generated H_2O_2. This did not interfere with assays for any other peroxides.

Peroxide assays

The iodometric method developed for lipids [5] was applied to the proteins as described recently [23].

Other methods

Protein concentrations were measured either by the dye-absorption method (Bio-Rad) or by absorbance at 280 nm after determination of the molar absorption coefficient. Separation of protein from low-M_r materials was carried out by filtration on Sephadex G-50 M (Pharmacia) columns, in Centricon 30 (Amicon) microconcentrator filters or by dialysis. The secondary structure of BSA was analysed with the software package Peptistructure (Version 7) [25].

RESULTS

Oxidation by reactive oxygen species

The oxidized derivatives of proteins and amino acids were produced by exposure of dilute aqueous solutions of the parent compounds to reactive oxygen species generated by γ-irradiation, by the reduction of H_2O_2 or by decomposition of a peroxyl radical-generating compound. Each of these techniques has been used extensively to produce reactive oxygen intermediates and each gives products which differ in yields and sometimes in nature.

The use of γ-radiation offers the best control of the nature and quantities of free radicals generated. Under our conditions, all the energy absorbed in the irradiated solutions led to decomposition of the water and, for every 100 eV absorbed, to the formation of 2.8 hydroxyl ($HO^{\cdot}$) and 3.2 superoxide ($O_2^{-\cdot}$) free radicals and 0.7 H_2O_2 molecules [24].

The second method used to generate reactive oxygen species was the reduction of H_2O_2 by Fe(II), which leads to formation of $HO^{\cdot}$ radicals or to oxidizing iron complexes [26]. In this system, the yield of the oxidant depends on the conditions. In contrast, thermal decomposition of AAPH produces well-defined peroxyl radicals at predictable rates [27].

Initial experiments showed that after exposure to γ-radiation, dilute solutions of BSA and lysozyme oxidized iodide under conditions indicative of the presence of peroxides. Irradiation in the absence of oxygen produced no peroxides, even when air was re-admitted to the samples after the irradiation.

When a solution of BSA irradiated under the standard conditions for 20 min was eluted from a column (18 cm × 2 cm) of Sephadex G-50 and the collected fractions were tested, a single protein (Figure 1, curve C) and two peroxide-positive peaks (Figure 1, curve A) were found. Treatment with catalase removed the trailing peak, without affecting the size or position of the leading peak (Figure 1, curve B).

Irradiation of solutions containing various concentrations of BSA or lysozyme showed that, up to 4 mg/ml, the yields of the oxidizing moieties increased with protein concentration (Figure 2). Above this concentration no further increases were seen, indicating that, for both proteins, 4–5 mg/ml was necessary and sufficient to scavenge all the free radicals responsible for generation of the reactive groups. The results shown were obtained from duplicate measurements in separate experiments, with the individual readings within the range of ± 1.5 μM of the average peroxide concentration, or just outside the heights of the data points shown in Figure 2. The yields of the protein-bound reactive groups, expressed in units of G values (number of reactive groups formed per 100 eV of energy absorbed) com-

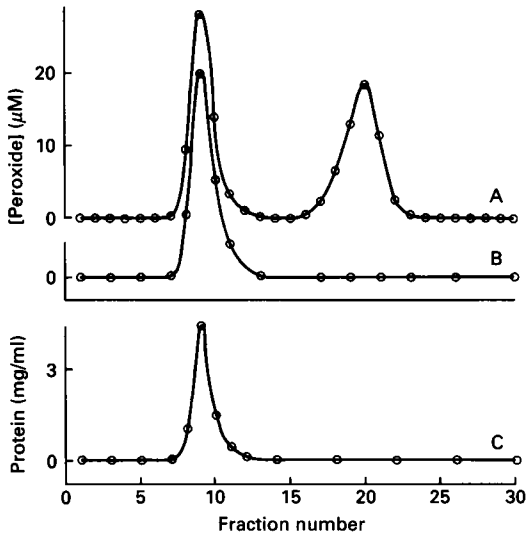

Figure 1 Formation of peroxide groups in BSA

BSA (10 mg/ml) in 20 mM air-saturated phosphate, pH 7.4, was irradiated with γ-rays to a dose of 1000 Gy. Part of the solution was fractionated on a Sephadex G-50 column and all fractions were tested for the presence of peroxides by the tri-iodide assay (curve A) and for protein by absorbance at 280 nm (curve C). The remaining sample was treated with 350 units of catalase/ml and chromatographed on the same column (curve B). Results shown are from a single experiment, with other similarly treated samples showing different peak heights but identical peak positions.

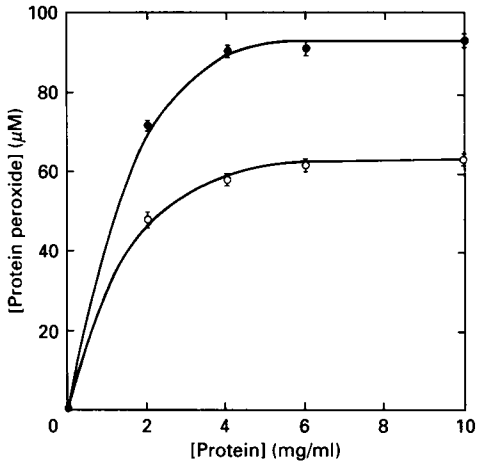

Figure 2 Effect of protein concentration on yields of peroxides

The proteins in 20 mM phosphate, pH 7.4, were irradiated with γ-rays to a dose of 900 Gy. Air saturation was maintained. Catalase (325 units/ml) was added and each sample was tested for the presence of peroxides. The data points were obtained in duplicate experiments. ●, BSA; ○, lysozyme.

Table 1 Reduction of protein peroxide groups

Protein solutions (10 mg/ml) in 20 mM phosphate, pH 7.4, were irradiated with γ-rays to a dose of 1000 Gy. After removal of H_2O_2 with catalase (350 units/ml) the protein peroxides were measured and the reducing agents were added to give the final concentrations indicated. After 1 h at 22 °C, rapid dialysis was performed under conditions which removed all traces of the reductants. Concentrations of the proteins and remaining peroxides were then measured in the dialysed samples.

Reducing agent	Concn. (mM)	Protein peroxide reduced (%)	
		BSA	Lysozyme
Borohydride	1	63	80
Borohydride	100	99	91
Dithionite	1	58	92
Triphenylphosphine	100	93	88
Ascorbate	1	31	90
Glutathione	1	66	76

Table 2 Lowering of the yields of protein peroxides by free radical scavengers

Protein solutions (10 mg/ml) in 20 mM phosphate, pH 7.4, were irradiated with γ-rays to a dose of 1000 Gy. Control solutions contained only the protein, whereas test samples were irradiated in the presence of 100 mM solute, except for mannitol, which was present at 500 mM. After rapid dialysis to remove the scavenger solutes and H_2O_2, all samples were assayed for the presence of protein peroxides.

Radical scavenger	Inhibition of peroxide formation (%)	
	BSA	Lysozyme
Benzoate	81	66
t-Butanol	68	68
Dimethyl sulphoxide	95	83
Formate	99	89
Mannitol	93	77
Tris buffer (pH 7.4)	64	55

monly used in radiation research, were 1.2 for BSA and 0.8 for lysozyme. These yields were directly proportional to the energy dose absorbed, to at least 1500 Gy, as long as air saturation was maintained [23].

Identity of the protein-bound reactive groups and radicals responsible

Although the ability of irradiated proteins to oxidize iodide under standard assay conditions suggests that they had acquired peroxide or hydroperoxide functional groups, this evidence should not be regarded as conclusive. There is no specific reaction that could be used to characterize peroxide groups under the conditions of our study, where typically less than one oxidizing group is formed per protein molecule. We therefore exposed the oxidized BSA and lysozyme to a range of reducing agents known to react with peroxides, in order to test the nature of the groups generated. The agents and their effects are listed in Table 1. The results are consistent with the presence of hydroperoxide (-OOH) groups in the irradiated proteins. Additional identification of the reactive groups as -OOH was provided by the kinetics of their reaction with I^-. Oxidized proteins reacted rapidly, with over 80% of reaction completed in 10 s, whereas dialkyl peroxides require much stronger acid conditions and, frequently, heating [28].

Irradiation of dilute aqueous solutions containing oxygen leads to the production of both $HO^\bullet$ and $O_2^{-\bullet}$ free radicals in similar amounts. To test which one of them was responsible for protein peroxidation, we irradiated the proteins in the presence of scavenger solutes, known to react rapidly with the $HO^\bullet$ radicals. Results summarized in Table 2 show that competition for the $HO^\bullet$ radicals between the added solutes and BSA or lysozyme protected the proteins from peroxidation. None of these solutes react with $O_2^{-\bullet}$, and in fact its yield in the presence of formate is doubled, so that the $O_2^{-\bullet}$ was ineffective in initiating protein peroxidation. However, active SOD present during irradiation decreased the yields of protein peroxide, suggesting that $O_2^{-\bullet}$ is involved in reactions leading to the stable peroxide end products (these observations will be reported separately).

Identification of $HO^\bullet$ as the radical responsible for initiation of protein peroxidation suggested that the phenomenon should be relatively insensitive to pH. This was tested with solutions containing 5 mg/ml BSA and 20 mM phosphate, one at pH 7.4 and the other at pH 5.2. A 10 min irradiation under standard conditions resulted in the formation of 70.8 μM BSA peroxide at pH 7.4 and 72.2 μM at pH 5.2. These values are not significantly different. The result confirms the role of $HO^\bullet$ radicals in protein peroxidation and shows that $HO_2^\bullet$, a much stronger oxidant than its conjugate base $O_2^{-\bullet}$ and 105 × more abundant at pH 5.2 than at pH 7.4, was incapable of inducing detectable peroxidation of BSA.

Amino acid peroxides

It seems likely that the amino acid composition of a protein should be an important determinant of its susceptibility to peroxidation. We therefore measured the ability of the common amino acids to form peroxides. Dilute aqueous solutions of amino acids buffered with 20 mM phosphate, pH 7.4, were irradiated and any peroxides formed were measured by the method used for proteins. Early tests showed that some amino acids were readily peroxidized. We selected proline to find the concentration necessary to scavenge the $HO^\bullet$ free radicals, which were presumed to be the agents of peroxidation by analogy with the proteins (Figure 3). Other amino acids were tested by

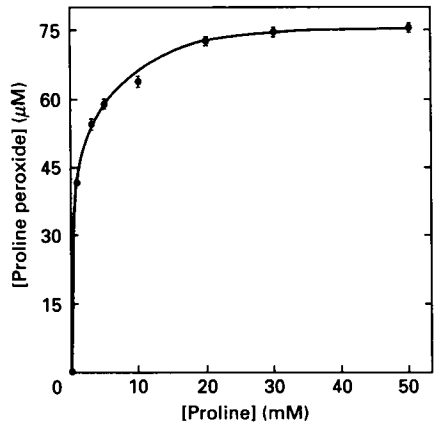

Figure 3 Effect of concentration of proline on yield of peroxides

Proline in 20 mM air-saturated phosphate, pH 7.4, was irradiated to a dose of 500 Gy, treated with catalase and assayed for peroxides. Results shown are from triplicate experiments.

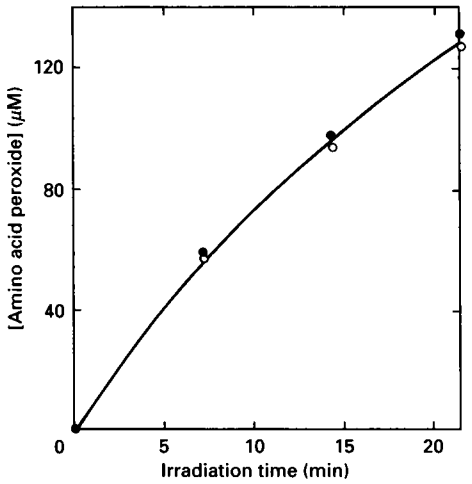

Figure 4 Effect of energy dose on peroxide yields in proline and valine

The amino acids (20 mM) in air-saturated phosphate, pH 7.4, were irradiated at 60 Gy/min. Peroxides were measured after treatment with catalase. ○, Valine; ●, proline. The data points shown are from a single irradiation for each amino acid.

irradiation of 20 mM solutions for 5 min at 63 Gy/min. All solutions were saturated with air. The results are shown in Table 3. In addition to the concentration of peroxides formed, the Table shows the fraction of HO• radicals produced by the γ-rays which were effectively converted into amino acid peroxides. The amino acids fell into three groups: those giving no significant amounts of peroxides, those giving high yields, and an intermediate group.

Unlike the two proteins tested, amino acid peroxidation was not linear with the duration of irradiation (Figure 4). The reason for this is not clear, but may be related to progressive degradation of the irradiated molecules. However, this should not affect the peroxide yields shown in Table 3 significantly, because the short irradiations used ensured that the main factor determining the amount of peroxide produced was the structure of the amino acid.

Peroxidation by other reactive oxygen species

The ability of other reactants to induce protein peroxidation was tested by the use of the Fenton reaction and thermal decomposition of AAPH. The former gives HO• radicals at acid and mainly Fe(IV) or Fe(V) at neutral pH [29], whereas the latter decomposes to peroxyl radicals [27]. In the Fenton oxidation, H_2O_2 was added to BSA (final concentrations 100 mM and 10 mg/ml) in solution buffered at pH 7.4 with 20 mM phosphate. A slightly acid solution of Fe(II) was added slowly with stirring over a 5 min period to a final concentration of 2 or 4 mM Fe. In one case, the Fe(II) was complexed with twice its concentration of EDTA before addition. After incubation with catalase to destroy unchanged H_2O_2, the samples were assayed for protein peroxides. Results in Table 4 show that the oxidant produced in reduction of H_2O_2 was a good initiator of BSA peroxidation. Experiments with lysozyme (not shown) gave qualitatively similar

Table 3 Peroxide yields in amino acids oxidized by free radicals

Amino acids (20 mM) in 20 mM phosphate, pH 7.4, were irradiated with γ-rays to a dose of 315 Gy. H_2O_2 was removed with catalase and the remaining peroxides were measured. Peroxidation efficiency is the number of peroxide groups formed per HO• radical generated (× 100). The G values listed (defined as number of peroxide groups formed per 100 eV of energy absorbed) had standard deviations of up to 0.04.

Amino acid	Peroxidation efficiency	G value
Alanine	11	0.31
Arginine	13	0.37
Asparagine	2	0.06
Aspartic acid	6	0.17
Cysteine	0.4	0.01
Glutamine	16	0.44
Glutamic acid	28	0.77
Glycine	3	0.07
Histidine	4	0.12
Hydroxyproline	2	0.06
Isoleucine	43	1.21
Leucine	44	1.22
Lysine	34	0.96
Methionine	0	0
Phenylalanine	5	0.14
Proline	44	1.24
Serine	0	0
Threonine	0	0
Tryptophan	18	0.51
Tyrosine	3	0.08
Valine	49	1.37

Table 4 Peroxide formation in BSA exposed to oxidizing species

Concentrations used were BSA (10 mg/ml) in 20 mM phosphate, pH 7.4, 5 mM H_2O_2 and 8 mM EDTA. Fe(II) in dilute acid solution was added slowly to the protein to the final concentration shown and then left for 30 min. AAPH (50 mM) was added to the protein and kept at 50 °C for 1 h. Irradiation of the protein with γ-rays was carried out to a dose of 945 Gy. BSA peroxides were assayed after removal of H_2O_2 with catalase.

System	BSA peroxide formed (μM)	BSA peroxide / BSA
BSA	0	0
BSA + H_2O_2	0	0
BSA + Fe (4 mM)	0	0
BSA + H_2O_2 + Fe (2 mM)	63.8	0.43
BSA + H_2O_2 + Fe (4 mM)	82.7	0.55
BSA + H_2O_2 + EDTA + Fe (4 mM)	92.8	0.62
BSA + irradiation	98.3	0.66
BSA + AAPH	123.5	0.82

Table 5 Decay of protein peroxides during storage

BSA (10 mg/ml) and lysozyme (5 mg/ml) were peroxidized by γ-irradiation, H_2O_2 was removed by catalase, and protein peroxides were measured before and after storage under the conditions shown.

	Protein peroxide remaining (%)	
Storage	BSA	Lysozyme
Immediate assay	100	100
24 h at −20 °C	78	78
24 h at 5 °C	57	58
24 h at 22 °C	40	50

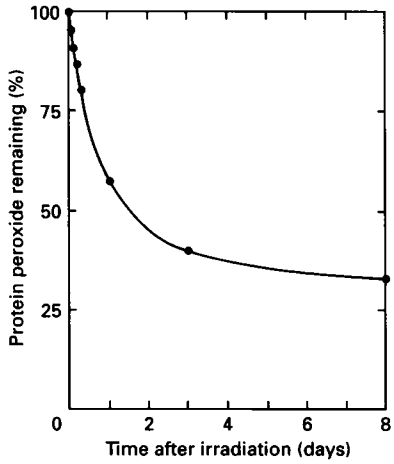

Figure 5 Effect of storage on the decay of BSA peroxides

BSA oxidized by γ-irradiation was periodically assayed for peroxides during storage at room temperature. The data points shown combined results obtained in the first half day after irradiation (with most points omitted for clarity), with readings at days 1, 3 and 8 from duplicate measurements, the values of which cannot be distinguished on the graph.

results. Incubation with 50 mM AAPH at 50 °C for 1 h also produced significant peroxidation of the protein. In these experiments the activity of AAPH was confirmed by its ability to peroxidize soya bean phosphatidylcholine liposomes. The AAPH was removed from the oxidized protein by gel filtration, because it interfered with the peroxide assay.

The effectiveness of the Fenton reaction in initiating protein peroxidation led us to test the possibility that trace metal impurities, virtually impossible to exclude from aqueous solutions, may play a role in determining the amounts of protein peroxides formed by radiation. Normally the solutions were buffered with phosphate, because it is unreactive towards $HO^\cdot$ and $O_2^{-\cdot}$. However, phosphate forms sparingly soluble complexes with metals and could therefore alter any trace-metal-dependent yield of peroxides. We compared BSA peroxide yields in irradiated phosphate and in unbuffered aqueous solutions, between pH 6 and 7.4. There was no significant difference in the levels of peroxide formed, showing that any trace-metal impurities in the solvent did not affect the process of peroxidation.

Stability of protein peroxides

An important aspect of any potential biological role of protein peroxides is their long-term stability. We investigated the effect of temperature on the persistence of BSA and lysozyme peroxides stored in 20 mM phosphate solutions. The peroxides were produced by $HO^\cdot$ radicals, measured immediately and then re-measured in samples kept for 24 h under the conditions listed in Table 5. There was loss of the peroxides in all samples, but a low storage temperature decreased their rate of decay. A more detailed examination of the loss of BSA peroxides in samples stored at room temperature is presented in Figure 5. Analysis of the rate of decay showed it to be a complex process which did not follow either first- or second-order kinetics.

DISCUSSION

The results reported here are part of a systematic investigation of the formation and properties of reactive moieties generated in proteins by the action of free radicals [23], made possible by the modification of the tri-iodide assay [5] for use with water-soluble macromolecules. As reported here, the technique is fairly sensitive, requires no expensive equipment, has reasonable specificity for the hydroperoxy group and is, most importantly, quantitative. However, it needs to be applied with some care. With proteins, oxygen must be completely excluded. Secondly, other oxidants and reductants capable of reacting with I^- or I_2 interfere. In this work, the particular problem of H_2O_2 generated during radiolysis of aqueous solutions was solved by the use of catalase (Figure 1). The final caution concerns any possible iodination of substances present in the assay which would lower the amount of I_3^- measured. The problem can be overcome by deliberate iodination of the peroxidized molecules before application of the assay [30] or by the use of a high initial concentration of I^-.

The binding of the reactive groups to the oxidized proteins was repeatedly demonstrated by their survival during all procedures used to separate large and small molecules (Figure 1). Measurements of the yields of peroxides generated in many experiments showed that they were a major product of the attack of radiation-generated free radicals on the two proteins tested. For every 2.8 $HO^\cdot$ radicals formed, 1.16 BSA and 0.82 lysozyme peroxide groups were produced, provided that the protein concentration was sufficient to scavenge all of the $HO^\cdot$. This qualification is important in calculating the number of radicals interacting with the BSA; some values published previously are likely to be in error because of assumption of complete scavenging of radicals by 0.33 mg of protein/ml [31]. According to our results (Figure 2), at this concentration 70% of $HO^\cdot$ radicals escape reaction with the BSA. At the lowest concentration giving complete scavenging (4 mg/ml), in a 10 min irradiation, each BSA molecule was hit by 3 $HO^\cdot$ radicals and acquired 1.23 peroxide groups. Thus the efficiency of conversion of $HO^\cdot$ into peroxides was 41%.

The only other measurement of the yields of peroxides in irradiated proteins known to us was reported by Garrison et al. [4], who found 0.38 peroxide groups in irradiated collagen for every 2.8 $HO^\cdot$ generated. Compared with BSA or lysozyme this is a low value, probably due to the unusual structure and composition of collagen.

The nature of the group generated by the reactive oxygen species on the compounds used in this study needs some comment. All of the reagents listed in Table 1, together with I^-, would reduce peroxide groups. The high reactivity of these groups towards I^- suggests that they are hydroperoxides rather than peroxides or peroxy acids. Additional evidence against the latter lies in the large variations in the yields of reactive groups on irradiated amino acids (Table 3), all of which possess the carboxy group potentially convertible into -COOOH.

We propose the following simple mechanism for the formation of protein hydroperoxides by the oxidizing free radicals:

$$PrH + HO^{\bullet} \rightarrow Pr^{\bullet} + H_2O \quad (1)$$

$$Pr^{\bullet} + O_2 \rightarrow PrOO^{\bullet} \quad (2)$$

$$PrOO^{\bullet} + e^{-} \rightarrow PrOO^{-} \quad (3)$$

$$PrOO^{-} + H^{+} \rightarrow PrOOH \quad (4)$$

$$PrOO^{\bullet} + PrH \rightarrow PrOOH + Pr^{\bullet} \quad (5)$$

$Pr^{\bullet}$ is a carbon-centred radical with a lifetime long enough for reaction (2) to occur. Our results show that in BSA about 40% of $Pr^{\bullet}$ radicals ultimately form PrOOH; the rest decompose, are rearranged or react faster with other species than with oxygen. The origin of the electron (reaction 3) is unknown, but we have evidence that at least some electrons can be supplied by $O_2^{-\bullet}$. The probability of direct abstraction of H from other parts of the protein molecule by $PrOO^{\bullet}$ (reaction 5) is real, in view of the ability of AAP-$OO^{\bullet}$ to induce protein peroxidation. Reaction (5) would regenerate a free radical which might enter into steps 2–5, but the chain would terminate quickly by formation of a protein radical which cannot react with oxygen.

Protection from peroxidation given by the free-radical scavengers (Table 2) confirms that the process was initiated by the $HO^{\bullet}$.

The yields of peroxides in amino acids exposed to free radicals (Table 3) agree quite well with the smaller range tested by Okada, except for arginine [2]. We have confirmed that susceptibility to peroxidation was high for amino acids possessing a tertiary carbon or at least two adjacent CH_2 groups.

At present there is no information on the relative abilities of individual amino acids in proteins to become peroxidized. It is likely that the two main factors determining the location of peroxide groups in proteins are the 'peroxidizability' and surface location of the constituent amino acids. Peroxidizability may be affected by incorporation into a polypeptide [2] but the list of susceptible and resistant free amino acids (Table 3) is probably not much altered in proteins. The second factor, surface location, determines the probability of reaction between a protein residue and the free radical generated in bulk solution. In the case of radiation-generated $HO^{\bullet}$ radicals, only the first organic groups in the protein surface are attacked, because the high reactivity of $HO^{\bullet}$ makes its mean free path very short in solutions [32]. Information on all the amino acids in BSA [25] shows 15 sequences containing two to seven residues with surface probabilities [33] greater than 10. This compares with an overall average value of 4.6, in a range of 0.2–62.6, for the 607 residues. Since 46% of the amino acids making up these 15 surface regions belong to the group of six identified as being readily peroxidizable (Table 3), there is no shortage of sites at which peroxide groups are likely to form. In the case of peroxidation catalysed by transition metals at the protein surface, the location of any peroxide groups formed will depend on the position of the metal-binding site as well as the proximity of susceptible residues.

The effectiveness of the Fenton reagents in inducing protein peroxidation (Table 4) was expected, since the system generates $HO^{\bullet}$ radicals or other highly oxidizing species and is known to give other indices of oxidation in proteins [34,35]. Reaction with AAP-$OO^{\bullet}$ radicals means that other peroxyl radicals, widely believed to be important agents of biological damage, are also potentially capable of protein peroxidation [21,36,37].

The rate of loss of hydroperoxides from the oxidized proteins (Table 5, Figure 5) shows that their lifetimes are long enough for reactions even at sites quite remote from their point of formation. Oxidation of important biological antioxidants such as ascorbate or GSH (Table 1) may have severe consequences for an organism exposed to an oxidant stress. It remains to be shown whether such processes operate *in vivo*. However, our study suggests that the biological consequences of protein peroxidation may be quite significant. The probability of interaction between free radicals generated in biological systems and proteins is high. After water, which is unreactive, proteins are the most abundant constituent of cells and extracellular fluids by weight. They are readily attacked by the very free radicals likely to be generated *in vivo*. In the process, the protein molecules trap the energy of the radicals, acting as antioxidants. For serum proteins exposed to peroxyl radicals, this radical-trapping antioxidant parameter is very high, between 10 and 50% of the total, second only to urate [21]. However, the price of this protection is damage to the proteins. Many studies of such damage under physiologically plausible conditions have been carried out, but none have so far considered protein peroxides to be a major product. What is known is that, in the presence of oxygen, serum albumins exposed to oxyradicals suffer polypeptide chain scission [8,9,22,31,38–40] leading to the formation of discrete fragments [8,38]. With γ-irradiation, all constituent amino acids of BSA are affected, but some show especially high damage; in one study [12] modification of 50–80% of aspartate/asparagine, cysteine, histidine, lysine, proline and tyrosine was recorded after high doses of radiation. Polypeptide chain scission and modification of amino acids results in the formation of new groups on albumins such as carbonyl and amino [9,41,42]. These, in general, increase with the damage sustained by the albumin. Increased susceptibility to proteolysis is also a common consequence of protein oxidation. For irradiated BSA, this was first reported by Okada et al. [14], and has been confirmed in several more recent studies [15,22,31,40]. A current report has shown that, in spite of increased susceptibility to proteinases, some fragments of radiation-damaged BSA accumulate in macrophages [43]. This may explain the finding of undegraded oxidized proteins in old or diseased tissues [11,19].

Many reactive oxygen species are able to cause damage to BSA and other albumins. Of the radicals generated by the radiolysis of water in the presence of oxygen, $HO^{\bullet}$ is active but $O_2^{-\bullet}$ and e^{-} are inert [8,9,15,31,42]. Systems containing H_2O_2 and Fe or Cu ions generate potent agents of protein modification [9,22,35,39,42]. Other effective reagents include Cu with ascorbate, horseradish peroxidase [19] and peroxyl radicals derived from lipid and other hydroperoxides [22,37,39] or from azo compounds [21,36]. Presumably this list would include many, if not all, of the other agents and conditions used to oxidize a wide range of proteins [19], but they have not yet been tested with albumins.

Until now [23], no study has shown that modification of proteins by free radicals can confer on them new chemical reactivity towards common biochemicals. Indeed, it has been suggested that oxyradical damage to plasma albumin is biologically insignificant because of the abundance of this protein and its relatively short half-life of 20 days [44]. According to this theory, the molecule acts as a sacrificial antioxidant, being able to protect other cell constituents from damage, but it is itself unprotected by the usual plasma antioxidant arsenal of urate, ascorbate, tocopherol and bilirubin. In plasma challenged with peroxyl radicals, this antioxidant contribution of proteins is crucial and exceeded only by the non-renewable ascorbate [36]. Our results indicate that BSA and lysozyme are not inert end-targets of oxyradical reactions. The formation of relatively stable hydroperoxide and other groups on molecules exposed to $HO^{\bullet}$ or $ROO^{\bullet}$ radicals in the presence of oxygen effectively traps some of

their chemical energy, extending their lifetime and radius of operation. It is likely that other intra- and extra-cellular proteins can also be peroxidized by free radicals and that this can turn them into second toxic messengers in cells and tissues. One potential result of their action is the oxidation of GSH and ascorbate by protein peroxides (Table 1), which could cause depletion of these vital antioxidants *in vivo*. This in turn can remove the normal barriers to the oxidation of other biomolecules. One can take as an example plasma, where ascorbate is the principal antioxidant, and where the loss of this compound allows the peroxidation of the constituent lipoproteins even in the presence of urate and tocopherol [36]. Formation of lipid peroxides in low-density lipoprotein particles and their subsequent breakdown can modify the apolipoprotein B protein, leading to controlled uptake of the cholesterol-rich particles by macrophages. In humans and some animal species this process can lead to atherosclerotic damage and death [45]. Since there is much other evidence that loss of GSH or ascorbate has deleterious effects on living organisms, our results suggest that peroxidized proteins may be an important link between the formation of reactive oxygen species and cell or tissue damage. We are at present investigating this theory.

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