

## Peroxidation of proteins and lipids in suspensions of liposomes, in blood serum, and in mouse myeloma cells<sup>★</sup>

Janusz M. Gebicki<sup>✉</sup>, Juan Du, James Collins and Helen Tweeddale

*Department of Biological Sciences, Macquarie University, Sydney, Australia*

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There is growing evidence that proteins are early targets of reactive oxygen species, and that the altered proteins can in turn damage other biomolecules. In this study, we measured the effects of proteins on the oxidation of liposome phospholipid membranes, and the formation of protein hydroperoxides in serum and in cultured cells exposed to radiation-generated hydroxyl free radicals. Lysozyme, which did not affect liposome stability, gave 50% protection when present at 0.3 mg/ml, and virtually completely prevented lipid oxidation at 10 mg/ml. When human blood serum was irradiated, lipids were oxidized only after the destruction of ascorbate. In contrast, peroxidation of proteins proceeded immediately. Protein hydroperoxides were also generated without a lag period in hybrid mouse myeloma cells, while at the same time no lipid peroxides formed. These results are consistent with the theory that, under physiological conditions, lipid membranes are likely to be effectively protected from randomly-generated hydroxyl radicals by proteins, and that protein peroxy radicals and hydroperoxides may constitute an important hazard to biological systems under oxidative stress.

Oxidative stress is the result of formation of reactive oxygen species (ROS) in amounts able to overcome the antioxidant defences of living organisms. The generation of ROS in biological systems has been documented so extensively that it is not possible to list more

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<sup>✉</sup>Correspondence to: Dr. J.M. Gebicki, tel.: (61) 9850 8147; fax: (61) 9850 8245; e-mail: jgebicki@rna.bio.mq.edu.au

**Abbreviations:** ROS, reactive oxygen species; AAPH, 2,2'-azobis(amidinopropane) dihydrochloride; BSA, bovine serum albumin; XO, xylenol orange; Fe-XO, ferric-xylenol orange peroxide assay; DCP, diacetyl phosphate; NaCl/P<sub>i</sub> buffer, phosphate-buffered saline; BHT, butylated hydroxytoluene; TCA, trichloroacetic acid.

than some selected general references [1–8]. ROS are inevitable by-products of normal respiration [1, 9], metabolism and autoxidation of xenobiotics [3], or the product of stresses such as excessive exercise, trauma, ischemia, or infection. In addition, environmental agents such as heat, freezing, radiation, toxins, and ultrasound [10] generate ROS. Because of the high chemical reactivity of most ROS, the experimental link between their excessive formation and exposure to such hazards has led to the view that they are a major source of damage in biological systems. This is supported by the identification of over 50 diseases and debilitating conditions they appear to cause or aggravate [7], including aging, malnutrition, arthritis, some forms of cancer, asbestosis, diabetic cataracts, atherosclerosis, immune injury, damage to the nervous system, and the consequences of iron overload [3, 7, 11].

There is general agreement that the processes initiated by ROS in living organisms and ending in pathology proceed in successive stages:

primary ROS → secondary ROS → primary target molecule → other molecules → cell injury → tissue damage → disease or death

Attempts to inhibit the progress of damaging reactions by interrupting this chain are likely to be most successful in its earliest stages, because the later steps are made up of many components, each able to propagate the damage in different ways and each requiring a separate inhibitor or repair mechanism. It is widely agreed that the superoxide ( $O_2^-$ ) is the most significant general primary ROS because it forms in all aerobic cells and is the end product of many other processes [1–11]. Since its formation cannot be prevented and since it is efficiently converted to other products in most aerobic cells and tissues [6], it is unlikely that any additional control of  $O_2^-$  levels would assist in damage inhibition. Evidence of damage, in spite of the abundance of

superoxide dismutase in aerobic tissues, suggests that effective prevention may need other measures, probably at the point where the  $O_2^-$  is reduced to  $H_2O_2$  by the enzyme, or where the surviving radical is converted to other products. It is well known that the  $O_2^-$  is rather unreactive, and its biological role appears to lie largely in the ability to act as precursor of secondary ROS, with greater potential to initiate chemical changes. This is either the hydroxyl free radical ( $HO^\cdot$ ) formed in the Fenton reaction



(where M is a transition metal ion stabilized by chelation) or a high-valence form of iron [6], or  $H_2O_2$  which can be the source of  $HO^\cdot$ . The  $HO^\cdot$  is able to oxidize all organic materials at close to diffusion-controlled rates [12]. No control at the  $HO^\cdot$  level is possible because, contrary to many claims, its reactions in cells cannot be inhibited by achievable levels of scavenging compounds [13].

The best chance of control of the chain of damage lies in the protection of primary target molecules. These must be vital to the survival of the cell, or be able to transfer the damage to a critical target, such as DNA. Unfortunately, the primary target molecule has not been identified. There are theoretical and experimental reasons against DNA being directly damaged in a cell [12, 14]. Instead, for many years unsaturated lipids were almost universally held to be the crucial cellular targets of the ROS, because *in vitro* lipids can give high yields of oxidized products in a chain reaction. The additional knowledge that this reaction can be inhibited by many antioxidants has generated thousands of studies and a whole industry devoted to the use of lipid antioxidants for disease prevention. Since conditions such as atherosclerosis and coronary heart disease are believed to be caused or aggravated by ROS and are more common in populations with low intake of antioxidant-rich food, many attempts are made to enhance

human health by antioxidant therapy. Most involve the administration of vitamins C and E, carotenoids, and trace minerals such as selenium and zinc, often in pharmacological doses which could never be reached through diet. So far the results have been disappointing, showing benefit in severe deficiencies, but only limited improvement for individuals with normal antioxidant levels [15]. Lipid oxidation may therefore contribute to damage by ROS, but is unlikely to be its only cause [16]. There is also growing theoretical and experimental evidence for the view that lipids are not likely to be a primary target for ROS in living organisms. Instead, it appears that the molecules most likely to be initially attacked by ROS in biological systems are proteins. We have found that such attack can result in formation of reactive protein peroxy radicals and hydroperoxides, able to damage other proteins, DNA, antioxidants, and lipids [17, 18]. In this study, we show that proteins are efficient protectors of lipid membranes against oxidation by radiation-generated HO<sup>•</sup> radicals. We also report on the formation of protein hydroperoxides in human blood serum and in hybrid mouse myeloma cells exposed to hydroxyl radicals generated randomly by ionizing radiation, and discuss the implications of these findings for living organisms.

## MATERIALS AND METHODS

**Chemicals and enzymes** were of highest grade, purchased from BDH Chemicals (Sydney, Australia); Sigma-Aldrich (St. Louis, MO, U.S.A.); Boehringer (Mannheim, Germany); AJAX Chemicals (Sydney, Australia); or Merck, (Darmstadt, Germany). Cell culture media, fetal calf serum, penicillin, streptomycin and heparin were obtained from Trace Scientific, Noble Pk (Victoria, Australia). All glassware was rinsed in detergent, heated in nitric acid for several hours, rinsed repeatedly with water purified in a 4-stage Milli Q (Syd-

ney, Australia) apparatus equipped with a final 0.2  $\mu\text{m}$  filter, and dried at 180°C. These steps were necessary to eliminate contaminants interfering with the peroxide assay.

### The experimental systems

**Liposomes.** A solution of soybean lecithin (Lipid Products, S. Nutfield, Surrey, U.K.) in methanol/chloroform containing 250 mg lipid was mixed with a chloroform solution of diacetyl phosphate (DCP) to give a final lipid/DCP ratio of 25. The solvent was removed under reduced pressure at 37°C and the lipid film dispersed in 6 ml of degassed water under gentle bubbling with argon. The resultant milky suspension of multilamellar liposomes containing 43 mg lipid per ml was divided into 1.5 ml portions and stored at -20°C until use.

**Human blood serum.** Serum was prepared from blood collected from healthy volunteers into Vacutainer tubes (Becton Dickinson, Sydney) containing a clot-inducing gel. The serum was separated by centrifugation at 2200  $\times g$ , and stored in aliquots at -80°C. The storage did not affect any of the measured properties of the serum for several months.

**Cells.** Sp2/0-Ag14 mouse myeloma hybrid cells (Commonwealth Serum Laboratory, Sydney) were grown in suspension in RPMI media containing phenol red, 10% fetal calf serum, 100 units penicillin and 100  $\mu\text{g}$  streptomycin per ml. The incubation was at 37°C under air containing 5% CO<sub>2</sub>. The cells were counted in a hemocytometer, and when the growth reached desired level, they were collected by centrifugation at 250  $\times g$  and resuspended in warm phosphate-buffered saline (NaCl/P<sub>i</sub> buffer) at 3-4  $\times 10^6$  cells/ml.

**Irradiations.** The liposomes, serum, or cells were oxidized by a flux of HO<sup>•</sup> radicals generated in a <sup>60</sup>Co source. For irradiation, the liposomes were suspended in water or 10 mM phosphate at pH 7.4, serum was undiluted, and the cells were in phosphate-buffered saline, pH 7.4. In most experiments, the solu-

tions were saturated with O<sub>2</sub> by bubbling, with the gas flow adjusted to cause minimal foaming, and, in the case of cells, to prevent them from settling and becoming anaerobic. The amounts of HO· radicals generated were calculated from the gamma dose rate, measured by Fricke dosimetry [19]. The average dose rates in these experiments were about 17 Gy/min producing 4.8 μM HO·/min. The exact dose rates were calculated from tables of factors correcting for the normal decay of the <sup>60</sup>Co source. After irradiation, 1 mg/ml of catalase was added to each sample to decompose the radiation-generated H<sub>2</sub>O<sub>2</sub>.

**Hydroperoxide assays.** The solutions used for measurements of protein hydroperoxides contained final concentrations of 25 mM H<sub>2</sub>SO<sub>4</sub> and 150 μM each of Fe<sup>2+</sup> and XO made up in the same solvent [20, 21]. For the measurements of lipid hydroperoxides, 90% methanol acidified with 25 mM H<sub>2</sub>SO<sub>4</sub> was used as solvent. Care was taken not to allow the Fe<sup>2+</sup> to come into contact with solutions above pH 2. After 30 min at room temperature, the absorbance was measured at 560 nm. Protein and lipid hydroperoxide concentrations were calculated with the molar absorption coefficients of 35 500 and 60 000 M<sup>-1</sup>cm<sup>-1</sup>, respectively [21]. Since this assay is very sensitive to pH, where this was in doubt, the absorption coefficient of Fe<sup>3+</sup> in the solutions used was determined by the addition of a known amount of Fe<sup>3+</sup> and measurement of the absorbance of the resulting Fe-XO complex. In all cases, blank solutions contained the appropriate amounts of unirradiated liposomes, serum, or cells.

Liposomes were extracted by vortexing 700 μl of their aqueous suspensions with 750 μl of chloroform, centrifuging at 14 000 × g, collecting samples of the aqueous and organic layers by syringe, and analysing each layer for its hydroperoxide content. For the assay of the total hydroperoxides in serum, the irradiated and control serum samples were first treated with 0.1 units of ascorbate oxidase per ml for 15 min, in order to prevent

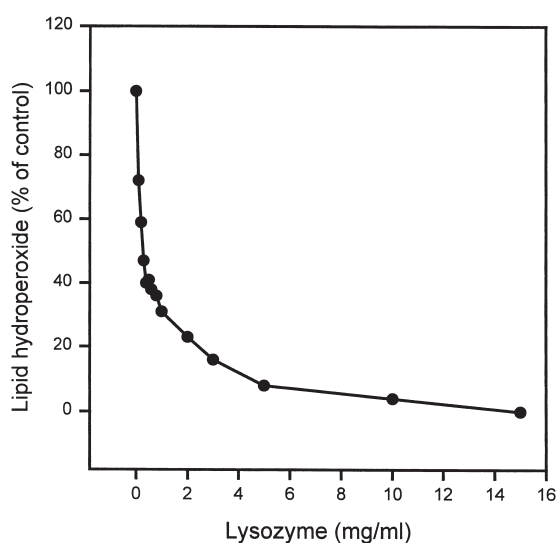
the interference of ascorbate with the hydroperoxide assay. Control experiments showed that this resulted in complete oxidation of the serum ascorbate. Each of triplicate samples contained 100 μl of serum and was made up to 1 ml with the Fe-XO assay solution. Serum proteins were freed of lipids by the addition of 200 μl of 6 M guanidine hydrochloride to 150 μl of the irradiated or control serum, followed by extraction of the lipids with 500 μl of chloroform. The mixture was centrifuged for 5 min at 4 000 × g, the top 320 μl of the aqueous layer made up with the Fe-XO assay solution to 500 μl and its absorbance measured at 562 nm in an Eppendorf BioPhotometer. Peroxide measurements on the serum lipid fraction were carried out by the addition of 1.2 ml of chloroform/methanol (2:1, v/v) solution containing 0.02% butylated hydroxytoluene (BHT) to 300 μl of serum. After centrifugation, 600 μl of the organic layer was removed with a microsyringe and used for measurement of the hydroperoxide content. Cell lipids and proteins were separated by precipitating a known number of cells with 10% trichloroacetic acid (TCA), followed by centrifugation and a second TCA wash. The final precipitate was washed with 2 ml of isopropanol and 4 ml of hexane, each containing 0.2% BHT. The organic extracts were combined, evaporated under argon, and redissolved in 300 μl of chloroform for hydroperoxide measurements. Protein hydroperoxides were measured directly by the addition of the Fe-XO assay solution to the cell TCA precipitate.

**Ascorbate assay.** Serum ascorbate levels were measured by HPLC with electrochemical detection [22].

## RESULTS

Initial tests of the effect of proteins on the oxidation of liposomes by the HO· radicals consisted of irradiation of liposome suspensions in 10 mM phosphate buffer, pH 7, in the

presence of increasing concentrations of bovine serum albumin (BSA). Subsequent measurements of hydroperoxides showed apparent complete inhibition of lipid oxidation by BSA concentrations around 1 mg/ml. However, when the liposomes were oxidized in the absence of BSA and subsequently mixed with the protein, the lipid hydroperoxides were removed within a few minutes. Tests with 3 other albumins and with casein, pepsin, lactoglobulin and apotransferrin showed that at 1 mg/ml they also reduced the liposome hydroperoxides. The effect was less pronounced at pH 4, but at neutral pH, 70–80% of the hydroperoxides were decomposed by these proteins, and 98% by BSA. Of the several proteins tested, only ribonuclease A and lysozyme had little effect on the peroxides. We therefore irradiated liposomes in the presence of increasing concentrations of lysozyme to study its effect on the formation of lipid hydroperoxides. The results (Fig. 1) show that



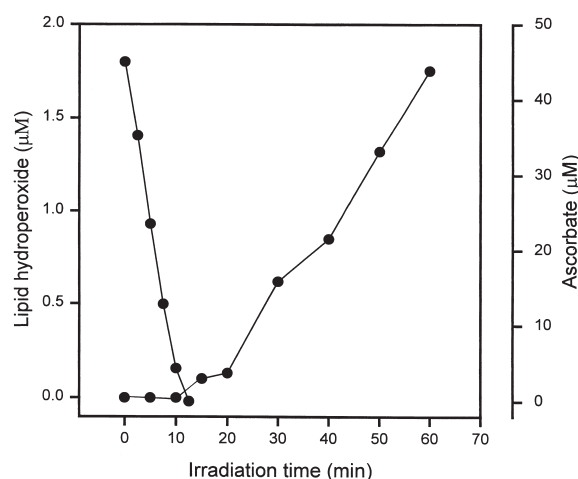
**Figure 1. Inhibition of lipid peroxidation in liposomes by lysozyme.**

Suspensions of soybean liposomes in phosphate buffer containing various concentrations of lysozyme were exposed for 30 min to radiation-generated hydroxyl radicals, and lipid hydroperoxides measured after extraction with chloroform.

0.3 mg/ml of the protein gave 50% and 10 mg/ml 96% reduction of the amount of hydro-

peroxide generated by HO<sup>•</sup> radicals in unprotected liposomes, with progressive protection as the protein concentration increased.

A series of classical studies of the formation of lipid peroxides in human blood plasma showed that the pattern of the oxidation of the phospholipids, cholesterol esters, and triglycerides was determined by the presence and concentration of ascorbate [23, 24]. In these experiments, plasma lipids were oxidized by peroxy free radicals only after a lag period, during which ascorbate was completely depleted. Interestingly, none of the other water or lipid soluble plasma antioxidants played any part in the inhibition of lipid peroxidation. We also found that serum lipids were initially protected from oxidation by the radiation-generated HO<sup>•</sup> radicals (Fig. 2). When the ascorbate was removed by dialysis or by ascor-

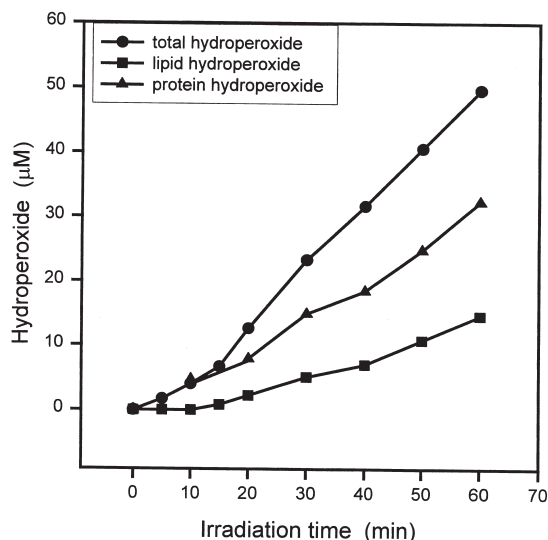


**Figure 2. The kinetics of oxidation of ascorbate and lipids in serum.**

Human blood serum was irradiated by  $\gamma$  rays in the presence of oxygen and the loss of ascorbate and formation of lipid peroxides measured in samples removed at intervals.

bate oxidase, the lipid peroxides formed immediately at a constant rate during the irradiation. Evidently, the presence of high albumin concentrations in serum did not interfere with the formation and persistence of lipid peroxides, unlike their rapid removal from liposomes by most proteins reported above.

The explanation may lie in differences in the extent of interaction between proteins and lipids in the two systems, and the accessibility of lipid peroxides to agents external to the membranes and lipoproteins. In contrast, neither ascorbate nor any other endogenous substance present in serum was able to prevent or delay the oxidation of the proteins (Fig. 3). Formation of carbonyl residues on the serum



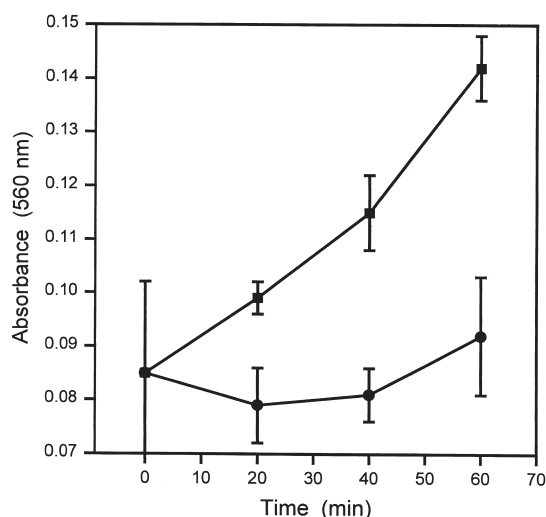
**Figure 3.** The kinetics of formation of protein and lipid hydroperoxides in serum.

Human blood serum was exposed to  $\gamma$  rays under a stream of oxygen and the total, protein, and lipid hydroperoxides measured in samples removed at intervals.

proteins was also initiated immediately on exposure to the radiation (results not shown). The sum of the amounts of protein and lipid hydroperoxides was close to the total measured independently and is shown in the top curve in Fig. 3. The results shown were generated in a single experiment, which was typical of at least 6, but which showed subtle variations in peroxide yields and points of inflection of the curves, presumably due to variations in the ascorbate content of different serum samples. However, the overall behavior of all specimens was similar.

It was recently reported for the first time that protein hydroperoxides are generated in cultured U937 cells exposed to peroxy radi-

cals [25]. A significant additional observation was the simultaneous complete lack of lipid oxidation. The results shown in Fig. 4 demonstrate that protein peroxidation in cells is not confined to a particular cell line or to one type of radical. Here, protein hydroperoxides were generated by gamma irradiation of mouse

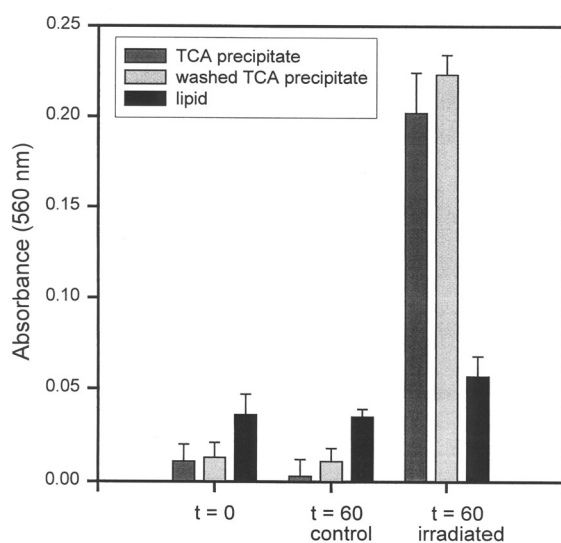


**Figure 4.** Formation of protein hydroperoxides in mouse myeloma cells.

Cell suspensions were exposed to radiation-generated hydroxyl radicals, precipitated and washed with trichloroacetic acid, and analysed for the presence of hydroperoxides.

myeloma hybrid cells. The radical presumed to be responsible for the oxidation was the  $\text{HO}\cdot$ . The irradiated and control cells were oxygenated during the experiment. The concentrations of protein hydroperoxides generated by 60 min irradiation, calculated with the absorption coefficient of  $35\,500\text{ M}^{-1}\text{cm}^{-1}$  determined for BSA [21], were between 1 and  $2\text{ }\mu\text{M}$ . As in the study with U937 cells, no significant amounts of lipid peroxides formed during the course of the 60 min irradiation. When the TCA precipitates from the irradiated cells were washed with methanol and hexane, there was no loss of the 560 nm absorbance characteristic of hydroperoxides (Fig. 5). Measurements of the absorbance of the extracted cell lipids at 560 nm in the normal peroxide assay showed that no lipid per-

oxides formed in the time of considerable protein oxidation (Fig. 5).



**Figure 5.** The effect of delipidation on the levels of peroxides in oxidized cells.

The amounts of hydroperoxides were measured in cell precipitates, before and after a wash with isopropanol and hexane, and in the washings.

## DISCUSSION

The results of this study support the broad proposition that damage to proteins may constitute an early and vital step in the transmission of biological damage induced by ROS. Until relatively recently, this was not a widely shared view; in fact, it was generally accepted that proteins act as passive antioxidants, protecting living organisms from oxidative stress by scavenging the ROS and by sequestering metals, which could catalyze the formation of HO<sup>•</sup> from H<sub>2</sub>O<sub>2</sub> [6, 26]. Any collateral damage sustained by the proteins would make them susceptible to proteolysis, followed by replacement through normal biosynthesis. This theory was challenged by the finding that many proteins exposed to ROS can acquire new chemical reactivity [17, 18]. One of the active residues is the -OOH group, which was shown to have the capacity to initiate several processes with potential to cause damage, if they should occur in living organisms. They in-

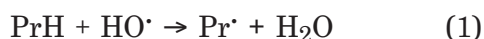
clude the destruction of GSH and ascorbate, generation of a range of new radicals, formation of covalent crosslinks with DNA, and inactivation of GSSG reductase [27].

While these results indicate that proteins may be significant targets of many ROS and could act as conduits for the transmission of damage in living organisms, much additional information is needed to test this hypothesis. Perhaps most importantly, we need to know whether the reactive protein hydroperoxides are a product of the action of ROS under physiological conditions, and whether their formation precedes damage to other cell components. There are considerable technical difficulties in obtaining answers to these questions, because of the limited lifetimes of protein hydroperoxides and the low sensitivity of current methods available for their measurement. A useful, if technically complex method, proved to be the detection of the products of reduction of protein hydroperoxides, which gave the first evidence for protein peroxide formation *in vivo* by identifying hydroxy-leucine and hydroxyvaline in advanced human atherosclerotic plaques [28]. Such plaques are believed to be associated with high levels of oxidative stress. In this study, we were primarily concerned with the sequence in which proteins and lipids are peroxidized in mixed heterogeneous systems and in cultured cells exposed to HO<sup>•</sup> radicals.

The use of ionizing radiation allows precise control over the nature of the radicals, the quantities produced, and the effects of modifying factors such as oxygen. One of the questions we were able to address was the relative efficiency of formation of protein and lipid hydroperoxides by the HO<sup>•</sup> radicals in heterogeneous systems consisting of lipids and proteins. Such mixtures constitute useful models for biological systems made up of cells enclosed by membranes rich in lipids, bathed in aqueous solutions containing large amounts of proteins. In homogeneous solutions, the randomly generated HO<sup>•</sup> radicals attack individual organic components largely according

to their relative masses. However, the fractions of radicals reacting with different components in heterogeneous solutions, containing soluble proteins and insoluble lipids, are much harder to calculate. The results summarized in Fig. 1 show that lysozyme was able to protect the liposomes from peroxidation almost completely at concentrations far smaller than those typical of biological situations. These observations suggest that biological membranes and lipid-rich lipoproteins are likely to be resistant to radiation-generated radicals under physiological conditions. However, oxidation of lipids by radicals generated in metal-catalysed site-specific processes may not be inhibited by proteins. We are currently studying this possibility.

The kinetics of the oxidation of the major serum components (Figs. 2, 3) shows that, unlike the lipids, proteins were not protected by any of the antioxidants present. While the formation of carbonyl residues may not constitute a hazard to other serum molecules, the products of protein oxidation are likely to initiate further potentially damaging reactions. These can involve protein hydroperoxides [27], or their precursor, protein peroxy radicals:



Here PrH is the native protein, Pr<sup>•</sup> a carbon-centred protein radical, PrOO<sup>•</sup> a protein peroxy radical, and RH a compound scavenging the PrOO<sup>•</sup>. RH may be a protein, an antioxidant, or any molecule containing a labile H. Reactions of class (1) have very high rate constants [29, 30]. The rate constants of reaction (2) and (3) have not been measured with proteins, but analogous reactions of other molecules have *k* values of around  $2 \times 10^9$  and  $2 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$  respectively [13]. The results with liposomes (Fig. 1) show that even low protein concentrations easily outcompeted lipids for the HO<sup>•</sup> radicals. In the case of serum, this

suggests that the initial target of the HO<sup>•</sup> radicals is its most abundant component, the proteins. The loss of ascorbate cannot be ascribed to a direct reaction with HO<sup>•</sup> (Fig. 2), since the ratio of the average masses of proteins/ascorbate in serum is about  $10^4$  (Table 1) [31]. Instead, ascorbate most probably reacts with the initial relatively long-lived product of the attack of HO<sup>•</sup> on protein, the PrOO<sup>•</sup>. Once the ascorbate is oxidized, the unprotected lipids (LH) react with the protein radicals, resulting

**Table 1. Probability of reaction of randomly-generated hydroxyl radicals with serum components.**

Serum composition given according to data [31].

| Component | Content (g/l) | Probability (%) |
|-----------|---------------|-----------------|
| Proteins  | 75            | 92.7            |
| Lipids    | 6             | 7.4             |
| Urate     | 0.045         | 0.056           |
| Vitamin E | 0.01          | 0.012           |
| Ascorbate | 0.008         | 0.006           |
| Bilirubin | 0.005         | 0.006           |

in the well-known lipid chain oxidation. The oxidation of serum components consists therefore of 2 phases, characterised by the initial presence and final absence of ascorbate:

#### Phase 1 (ascorbate present)

Reactions (1) and (2) followed by:



#### Phase 2 (ascorbate oxidized)

Reactions (1) and (2) followed by:



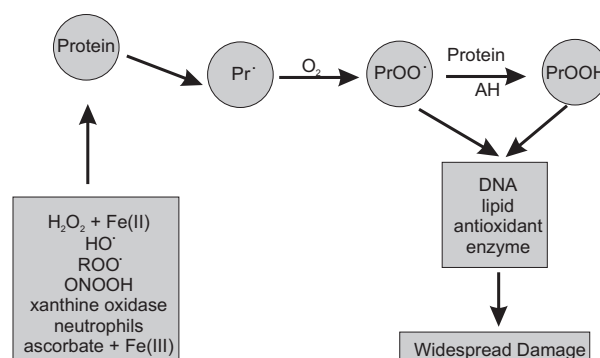


Although this scheme can account for our results with serum, it remains tentative, pending the determination of the rate constants of reactions (2), (4) and (5). These measurements are currently under way.

Results obtained in this study with the mouse myeloma cells confirm earlier findings with a different cell line and different radical oxidant [25]. In the earlier work, exposure of U937 cells to chemically generated peroxy radicals led to the formation of protein hydroperoxides. No lipid peroxides formed under conditions producing about  $1 \mu\text{M}$  PrOOH per mg cell protein. The results clearly indicated that the protein hydroperoxides were an early product of the reactions of the radicals with U937 cells, and that their formation was not delayed by intracellular antioxidants. Results shown in Figs. 4 and 5 show that protein peroxidation is not restricted to a particular cell line or free radical oxidant. As in the previous study, protein rather than lipid hydroperoxides were the initial products of the action of the radicals, with apparently little if any lag in their formation. We have also found, in agreement with the earlier study, that peroxy radicals produced by the decomposition of AAPH generated protein hydroperoxides in the mouse myeloma as well as in the human U937 cells (results not shown). These results demonstrate directly that peroxidized proteins can form in living cells. At present, this required the application of a large cumulative amounts of radicals, because the existing analytical methods are incapable of detecting small amounts of hydroperoxides.

A tentative proposal for the role of proteins in the transmission of biological damage is shown in Fig. 6. Most of the agents known so far to have the ability to generate protein hydroperoxides are shown in the first box. Likewise, the known reactions of the hydroperoxides with components of cells are also listed. Direct reactions of PrOO $\cdot$  radicals have not so far been demonstrated, but, at least in the case of serum, the argument for the oxida-

tion of ascorbate by these radicals seems compelling. The assumption that at least some of the changes induced in the secondary target molecules by PrOO $\cdot$  and/or PrOOH also seems reasonable. This damage may be slow to develop; the viability of mouse cells was not significantly affected by the accumulation of



**Figure 6.** Proposed role for proteins in the transmission of biological damage initiated by reactive oxygen species.

The symbol Pr represents protein.

$1\text{--}2 \mu\text{M}$  of protein hydroperoxides (results not shown). Clearly, much more research is required to supplement the current knowledge of the formation of protein hydroperoxides and their effects *in vivo*. However, it is already possible to suggest that attempts to reduce or eliminate excessive damage by ROS in living organisms should concentrate on the neutralization of protein peroxy radicals and on the harmless decomposition of their hydroperoxides.

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