Protein Fluorescence: its Generation and Measurement

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Fluorescence can be defined as the immediate emission of light from a molecule or atom after the absorption of radiation. Light is a form of electromagnetic radiation and the exposure of an atom or molecule to this energy excites outer orbital electrons to a higher energy level equivalent to the integral quantum of light absorbed. If the molecule does not decompose as a result of the increase in energy and all the energy is not dissipated by subsequent collisions with other molecules, the electron returns to the lower energy level. As the electron decays back to its ground state it emits a photon of light of lower energy (*i.e.*, higher wavelength), and this form of radiation is termed fluorescence.

Fluorescence is characterised by excitation and emission spectra that are specific to a given molecule, the excitation spectrum being theoretically identical to the absorption spectrum. From this, it would appear that all molecules which absorb light energy should fluoresce; however, this is not so and in fact the fluorescence efficiency of most absorbing molecules is very low because of internal quenching. By virtue of these constraints, few complex molecules fluoresce significantly and fluorescence characterisation and measurement can provide a very powerful analytical tool in biological systems.

Native Protein Fluorescence

All proteins exhibit a native fluorescence spectrum in the ultraviolet region, conferred largely by the presence of aromatic tryptophan and tyrosine residues (Teale, 1960). (This native ultraviolet fluorescence is commonly referred to as tryptophan fluorescence.) However, the spectral characteristics of a protein are not simply a summation of the excitation and emission spectra of constituent amino acids, but rather are modulated by molecular conformation; this allows ultraviolet fluorescence spectra to be used in following structural changes to a given protein.

Extrinsic Fluorescence

Fluorescence can also be conferred on a protein via the binding of intrinsically fluorescent molecules, such as fluorescein, or through the binding of carbonyl groups from fatty acids or glucose to E amino groups of lysine residues. The resultant Schiff bases formed have characteristic fluorescence excitation and emission maxima of 320–360 and 430–460 nm, respectively, in the visible region.

Protein Oxidation and Autofluorescence

Exposure of proteins to a free radical flux alters their composite amino acids, the residues affected depending on both the radical species involved and the nature of the protein. Following mixed oxygen radical attack, a decrease in the native ultraviolet fluorescence has been reported for several proteins, including IgG, gamma lens crystallins of lens proteins, transferrin and superoxide dismutase. Associated with this loss, a dose dependent increase in visible autofluorescence has been described: that is to say, this novel fluorescence is intrinsic to the radical treated protein itself. For IgG this is characterised by an excitation maximum at 360 nm and an emission maximum at 454 nm, and similar changes have been described in proteins isolated from diseased states; both gamma lens crystallins isolated from cataractous lenses and IgG isolated from rheumatoid synovial fluids are described as having increased levels of non-tryptophan autofluorescence in the visible region. Previously, general protein fluorescence has been used as a crude measure of oxygen free radical (OFR) attack *in vivo*; however, such a measurement varies with changes in protein conformation, and is subject to interference from bound lipids, and in haem proteins, from bound metal ions.

It is likely that the macromolecular changes observed following protein oxidation are a manifestation of modifications at the molecular level to specific amino acids. The most susceptible residues are the aromatic amino acids and (where stabilisation of radical intermediates can be achieved by the delocalisation of unpaired electrons around unsaturated ring structures) sulphur containing amino acids, where increasing the valency of sulphur residues facilitates radical intermediate stabilisation. Of these potential sites of attack, it is believed that the oxidation of tryptophan and/or tyrosine residues is most important in conferring visible fluorescence on an oxidised protein.

Tryptophan Fluorophores

The first direct conversion of tryptophan to *N*-formyl kynurenine was achieved by ozonolysis by Witkopp in 1944. Since that time, ultraviolet photolysis of tryptophan has been investigated in a large number of studies: a non-exponential decrease in indole ultraviolet fluorescence has been reported for pure tryptophan (trp) solutions. However, trp photolysis products, when excited at 365 nm, exhibit a new fluorescence emission in the visible region. The isolation and characterisation of tryptophan photoproducts has been extensively undertaken by Singh *et al.*, and they have reported the expected yields of the oxidation products hexahydropyrolloindole, 5-hydroxytryptophan kynurenine and *N*-formyl kynurenine following denaturation of trp by different radical generating systems.

Fluorophores of Tyrosine

The presence of covalent crosslinks with a blue fluorescence was first reported for the protein resilin, found in the ligaments of insects. Later work on the irradiation of poly-L-tyrosine led to the identification of bityrosine, a fluorescent product with an excitation maximum of 290 nm and emission maximum at 410 nm. The synthetic reaction proceeds via the anaerobic dimerisation and enolisation of tyrosine phenoxyl radicals to form a covalent 2,2′-biphenol, which is resistant to proteolysis. This fluorescent crosslink has been suggested to be important in mediating the aggregation and fluorescence of irradiated histones and lens proteins.

Novel Fluorophores in Oxidised Proteins

It has been proposed that the formation of bityrosine from two spatially adjacent tyrosine residues may exhibit the new visible fluorescence induced in an oxidised protein. However, other workers have disputed this and claim that tryptophan oxidation with the concomitant production of kynurenines may account for the major part of fluorescence formation. As neither metabolite would be incorporated into a protein backbone during synthesis, the presence of either in biological fluids is indicative an OFR dependent process occurring *in vivo*.

In order to state unequivocally that an OFR mechanism is responsible for the oxidative changes that occur to proteins during ageing or disease, the presence of specific modified residues must be demonstrated within the backbone of isolated proteins. We have therefore developed and will describe a sensitive reversed-phase HPLC technique with dual detection

systems (ultraviolet absorbance at 240 nm and fluorescence monitoring) to analyse the products of amino acid oxidation. Aerated amino acids and proteins in solution were exposed to three discrete OFR generating systems: 1, gamma irradiation; 2, gamma irradiation in the presence of formate; and 3, photolysis by ultraviolet light at 254 and 360 nm. OFR denaturated amino acids were chromatographed by use of this HPLC procedure, and systems 1 and 3 resulted in the generation of identifiable fluorescent metabolites of tryptophan, *i.e.*, the kynurenines. Their identity was confirmed by scanning absorption spectroscopy. After complete proteolytic hydrolysis, OFR treated proteins were also analysed by this technique; again, the dose dependent production of kynurenines was detected, although no fluorescent product of tyrosine could be detected by this procedure. Several unidentified fluorophores were found which could not be ascribed to the presence of any one single amino acid, and may reflect the product of two different amino acid radicals.

We will describe the application of this technique to IgG isolated from rheumatoid synovial fluids, IgG from rheumatoid and neonatal sera and proteins isolated from erythrocyte membranes of patients with sickle cell disease.

This work has demonstrated firstly, that specific oxidised products of amino acids are retained in the protein backbone following exposure to OFR generating systems *in vitro*, and secondly, in aerated solutions, that oxidised tryptophan residues confer the major new visible fluorescence on oxidised non-haem proteins, not tyrosine metabolites. In addition, we have shown that the measurement of a specific fluorescent oxidised amino acid product can be applied to biological macromolecules, and may be important in implicating free radical reactions in certain disease processes.

Measurement of Antioxidants and Degradation Products of Peroxides

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The upsurge of interest in damage to living tissue by oxygen derived active molecules has led to the need, among biological scientists and medical researchers, for accurate and reliable methods for the measurement of components of the antioxidant defence mechanism, and of the degradation products derived from lipid peroxides formed as a consequence of attack by activated oxygen on membrane phospholipids.

The primary lipid-soluble antioxidant in biological systems is alpha-tocopherol, which is present in tissues in company with a range of other lipids from which it must be separated in order for it to be measured. Extraction of alpha-tocopherol is normally achieved by homogenisation of the tissue followed by extraction of the total lipid with a standard solvent mixture; serum or plasma are normally extracted directly with hexane. Where the ratio of alpha-tocopherol to other lipids is high, as with plasma or serum, it may be possible to proceed directly to the analytical measurement of the alpha-tocopherol immediately. Frequently, however, this is not possible because the bulk of other lipid is so great that it interferes with the subsequent analysis. In this case it is necessary to introduce a further step that involves saponification of the triglycerides and cholesteryl esters, which removes the bulk of the interfering lipid and provides an "unsaponifiable fraction" which will contain free cholesterol and the fat-soluble vitamins. If the conditions are carefully chosen cholesterol and vitamin A do not interfere with the subsequent analytical procedures. The saponification is carried out in ethanol with a high concentration of added alkali. Alpha-tocopherol is highly susceptible to destruction in alkaline solution, but this can be completely prevented by the addition of an antioxidant; pyrogallol is the agent of choice. The unsaponifiable fraction is then extracted into hexane and washed with water to remove traces of residual alkali. The older methods of separating the tocopherols from each other, and from interfering lipids, which involved thin-layer or gas - liquid chromatography,[1] have now been superseded by high-performance gas - liquid chromatography. The methods used vary but usually involve chromatography with a non-polar solvent to which a somewhat more polar solvent is added in small amounts as modifying agent. The tocopherol peaks are best detected by a fluorescence technique because excitation at 220 nm provides a sharp intense analytical peak at 360 nm. Routine analysis of amounts of tocopherol in the region of 0.5–1.0 ng is readily achieved.

Techniques for the measurement of tocopherols in plasma or tissues were described by Buttriss and Diplock.[2] A modified technique that can be employed for high lipid containing tissues, such as brain, was described by Metcalfe *et al.*[3]

The recent considerable interest in the possible function of carotenoids as agents that, at dietary levels of intake, might be preventative factors in the aetiology of certain forms of cancer, has highlighted the need for reliable methods of assay of these pigments in a wide range of body fluids, tissues and foods. The available methodology has been reviewed by Krinsky and Welankiwar.[4] Oxidation and lability to isomerisation caused by light are hazards that need to be overcome during extraction of the pigment, which is achieved by maceration (where this is needed) with ethanol - water or acetone - water mixtures. The concentration of pigment in the resultant mixture may be high enough to permit extraction into a non-polar solvent, followed by direct measurement of the pigment by spectrophotometric techniques. Saponification is only rarely necessary. Where it is desired to separate the carotenoids in a mixture this can be readily achieved by high-performance liquid chromatography. The use of reversed-phase columns has permitted excellent separation of the carotenoids, using isocratic, step-gradient or continuous gradient solvent mixtures. The recovery of the pigments is usually quantitative and the measurement of each individual compound in the mixture can be carried out by using the known extinction coefficients of the carotenoids concerned. Details of the methodology are to be found in reference 4 above and in the references given in that paper.

Measurement of selenium, and of the selenium dependent enzyme glutathione peroxidase, is being undertaken routinely more frequently in hospital clinical chemistry laboratories as interest in this trace element grows. There are three methods available for the routine measurement of selenium in biological samples; a fluorimetric method, using 2,3-diaminonaphthalene as fluor, atomic absorption using hydride generation and atomic absorption using electrothermal atomisation. The fluorimetric and hydride generation methods require simple digestion with a nitric acid - perchloric acid mixture, which destroys organic matter and converts the selenium to the Se^{IV} oxidation state. However, the technique requires care to avoid the explosive hazard associated with the use of perchloric acid, and also to avoid volatilisation of the selenium. The electrothermal atomisation atomic absorption technique has a high absolute sensitivity but the use of very high temperatures requires the thermal stabilisation of selenium; this can be achieved up to about 1000 °C by the use of several metals, of which nickel is most commonly used. However, the different

oxidation states of selenium that are present in a mixture are more equally stabilised by Cu - Mg mixtures. The three techniques were compared side by side in a recent study, using body fluids, and also some certified standards[5] and details of the methodology can be found in that study. There was no difference between the means of all the methods and the over-all correlation coefficient for hydride generation atomic absorption *versus* fluorimetry was 0.97; all of the methods gave values for the certified material close to the certificated value.

The term glutathione peroxidase refers to the selenoenzyme glutathione: H_2O_2 oxidoreductase (EC 1.11.1.9). Methods for measurement of the enzyme depend upon the following coupled reactions:

$$ROOH + 2GSH \longrightarrow ROH + H_2O + GSSG$$

$$GSSG + NADPH + H^+ \xrightarrow[\text{reductase}]{\text{Glutathione}} 2GSH + NADP^+$$

The original method of Paglia and Valentine[6] requires certain precautions, in particular where blood is concerned, in which case the pseudoperoxidase activity of haemoglobin must be eliminated. The necessary methodology is given in the review of Flohe and Gunzler.[7]

The peroxidation of tissue lipids is normally controlled by a range of antioxidant devices in the absence of which lipid peroxides can accumulate. The measurement of peroxides, or of their degradation products, is therefore of interest in determining the extent to which a system is adequately protected by the antioxidant mechanisms. An early product of lipid peroxidation is the conjugated diene:

$$R{-}CH_2{-}CH{=}CH{-}CH{=}CH{-}\underset{\displaystyle OOH}{\underset{|}{CH}}{-}(CH_2)_n{-}COOH$$

that arises following the formation of a carbon-centred radical in unsaturated fatty acids. The strong absorption maximum at 233 nm may be used as a means of assessing the extent of lipid peroxidation.[8] A rather less sensitive measurement of lipid peroxides can be obtained by taking advantage of the fact that peroxides react with iodide in a 1 : 1 stoicheiometry in acidic solution to liberate iodine.

$$ROOH + 2I^- + 2H^+ \longrightarrow ROH + H_2O + I_2$$

The liberated iodine can be determined by spectrophotometric means,[9] the unreacted iodide which might oxidise during the assay being complexed with cadmium. Alternatively, a reversed-phase high-performance liquid chromatography method may be used which improves the sensitivity of the method by a large factor.[10]

Lipid hydroperoxides undergo spontaneous decomposition to a series of aldehydic products, among which are malondialdehyde [which is one of the products that reacts with thiobarbituric acid to give the well known "thiobarbituric acid reactive substances" (TBARS) reaction, often erroneously referred to as malondialdehyde measurement], hexanal and 4-hydroxynonenal. These may all be measured by high-performance liquid chromatographic techniques. Two different techniques are available for the measurement of malondialdehyde, namely that due to Csallany *et al.*[11] and that due to Esterbauer and co-workers.[12,13] Techniques have been developed for the measurement of hydroxylated unsaturated aldehydes, typified by 4-hydroxy-2,3-*trans*-nonenal.[14,15] In order to detect small amounts of saturated aliphatic aldehydes, such as hexanal, and to reduce the volatility of hydroxyalkenals, derivatisation of the mixture is required prior to its application to the column. Formation of the 2,4-dinitrophenylhydrazone derivatives of these compounds has been used extensively for this purpose.[16,17]

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Measurement of DNA Oxidation Products

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Oxidative DNA damage has been suggested to contribute to the aetiology of inflammatory autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus,[1] as well as to cancer and ageing.[2] A possible mechanism is that certain oxidative modifications to DNA may cause somatic mutations, leading to altered cell function.[3] Oxidative DNA damage may also result in cell death, *e.g.*, via a lowering of energy charge state.[4] From another point of view, oxidatively modified DNA may be antigenic, stimulating the release of anti-DNA antibodies typically found in systemic lupus erythematosus.[5]

Oxidants such as the superoxide anion radical ($O_2\cdot^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^\cdot OH$) can be generated by ionising radiation and as a product of normal

aerobic metabolism, *e.g.*, mitochondrial and endoplasmic reticulum electron transport chains.[1] Increased oxygen radical production, decreased levels of antioxidant enzymes (*e.g.*, superoxide dismutase, catalase and glutathione peroxidase) or defective DNA repair mechanisms might lead to increased levels of DNA damage. This could, in turn, lead to an increased probability of somatic mutation, resulting in altered self antigens and autoimmunity. It is noteworthy that there is an association between sites of chronic inflammation, where neutrophils are sequestered and stimulated to generate oxygen radicals, and an increased incidence of malignancy.[6]

DNA Oxidation Products in Biological Systems

Various forms of damage to DNA by oxygen radical generating systems have been described. These include strand breaks, inter-strand and DNA-protein crosslinks, base modifications and apurinic and apyrimidic sites.[7]

DNA Strand Breaks

One method of determining DNA strand breaks is based on the rate of DNA unwinding in dilute alkali solution, using the double-stranded DNA binding dye ethidium bromide.[8] This method has recently been modified to allow accurate and reproducible analysis of DNA strand breaks in isolated DNA as well as circulating cells.[9] DNA strand breakage is an early event after exposure of cells to oxidants such as H_2O_2.[6] The appearance of DNA damage is rapidly followed by activation of the enzyme poly-ADP-ribose polymerase as part of the DNA-repair process. This enzyme consumes NAD^+, and if the degree of DNA damage exceeds a critical level then the process of NAD^+ and ATP depletion could become irreversible with resultant cell death. Carson *et al.*[4] have suggested a role for DNA strand breakage and the programmed synthesis of poly(ADP-ribose) in the genesis of lymphocyte dysfunction induced by exposure of resting lymphocytes to oxygen radicals. The latter workers have termed this process "NAD^+-dependent programmed cell death" and have suggested that this phenomenon represents a "suicide response" of cells with extensively damaged DNA, which might prevent the early emergence of somatic mutants with malignant potential.

DNA Base Modifications

More than 20 products of oxygen radical attack on DNA have been identified by gas chromatography - mass spectrometry (GC - MS). An important mechanism is site specific $^{\cdot}OH$ generation, catalysed by iron bound to cellular DNA.[10] Amongst the products which have been identified are: thymine glycol, thymidine glycol, 8-hydroxyadenine, 4,6-diamino-5-formamidopyrimidine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine, 5-hydroxycytosine and 5-hydroxyuracil.[7]

Another product formed on exposure of DNA to ionising radiation has been identified: the $^{\cdot}OH$ adduct of the DNA nucleoside deoxyguanosine, which can be isolated from enzymatically digested DNA as 8-hydroxydeoxyguanosine (8-OHdG).[11] By using GC - MS with selected-ion monitoring, 8-hydroxyguanine (the base adduct corresponding to 8-OHdG) was one of the major products formed when isolated DNA was exposed to oxygen radicals generated by either a hydrogen peroxide - iron system[12] or phorbol myristate acetate-stimulated neutrophils.[13] Later we will discuss the measurement of 8-OHdG by high-performance liquid chromatography with electrochemical detection (HPLC - EC) and the significance of the results obtained thus far.

Products of Bleomycin-catalysed DNA Degradation

Many chemotherapeutic drugs, *e.g.*, bleomycin and streptonigrin, appear to be "radiomimetic" in that they produce biological effects similar to those of ionising radiation.[14] Bleomycin is thought to act as an anti-tumour agent by virtue of its ability to form a ternary complex with iron and DNA. This complex can be activated by the cytochrome P450 system and damage DNA in an oxygen dependent reaction, possibly involving $^{\cdot}OH$. It has been demonstrated in cell-free systems that the DNA damage includes induction of single and double strand breaks and release of free bases and base propenals, which can decompose to form malonaldehyde.[15] The measurement of the malonaldehyde - thiobarbituric acid chromophoric adduct is the basis of an assay for "catalytic" iron in biological fluids.[16] In addition, the bleomycin - iron complex will catalyse the hydroxylation of deoxyguanosine to 8-OHdG in isolated DNA.[17] However, the relative *in vivo* importance of these various reactions, if any, has not been defined.

Measurement of 8-Hydroxydeoxyguanosine by HPLC - EC

Nucleosides and bases are readily separated by reversed-phase HPLC. A number of nucleosides and bases are readily electro-oxidisable,[18,19] the ease of oxidation being dependent upon the number of substituent OH groups in the molecule. The oxidisable bond is the C4–C5 bond of the base structure.

HPLC - EC has allowed the determination of 8-OHdG in femtomole amounts[20] and increased levels were shown in cellular DNA when cell lines were exposed to ionising radiation[21] or hydrogen peroxide.[6] 8-Hydroxydeoxyguanosine is mutagenic, causing an increase in the frequency of misincorporation of DNA bases both at the damaged base and at bases adjacent to it.[22] There is indirect evidence that DNA repair mechanisms exist for this lesion.[21]

By using HPLC - EC, Ames and colleagues[23] studied the oxidation state of the nuclear DNA and the mitochondrial DNA of rat liver. 8-Hydroxydeoxyguanosine was present at a level of 1 per 130 000 bases in nuclear DNA and 1 per 8000 bases in mitochondrial DNA and it was suggested that mitochondrial DNA might be exposed to greater fluxes of oxygen radicals due to uncoupling of the respiratory chain. The HPLC - EC method has also been applied to human urine, in which the concentration of 8-OHdG is relatively high. Preliminary results indicate a trend towards lower levels in chronic granulomatous disease patients (whose abnormal neutrophils cannot produce $O_2^{\cdot -}$) than are found in normal control subjects.[24] However, the results were not statistically significant, and a larger population needs to be studied before firm conclusions can be drawn. Furthermore, it is not known if 8-OHdG in urine is derived exclusively from DNA via repair enzyme processes or whether dietary intake and/or gut microflora make a significant contribution.

In view of, firstly, the difficulties in interpreting the significance of measurements of 8-OHdG in human urine, and secondly, the high levels of other electrochemically active compounds in urine, we set out to ascertain whether base-line levels of 8-OHdG could be measured in normal human peripheral blood cells. We also wished to establish whether bleomycin, as a radiomimetic agent and putative carcinogen, induced the formation of 8-OHdG in cultured cells.

Human mononuclear cells and granulocytes were isolated from approximately 40 ml of fresh heparinised human blood.[25] Human and rat hepatocytes were isolated as described earlier.[26] DNA was isolated by phenol extraction and enzymatically digested to the nucleoside level with deoxyribonuclease I, *N. crassa* endonuclease, phosphodiesterase I (*Crotalus atrox*) and *E. coli* alkaline phosphatase.[27] The 8-OHdG standard was synthesised by using the Udenfriend system[28] and the structure confirmed by fast atom bombardment mass spectrometry (courtesy of Dr P. Farmer, MRC Toxicology Unit, Carshalton, Surrey).

The method for reversed-phase HPLC of the DNA nucleosides was modified from the procedure of Floyd *et al.*[20] 8-Hydroxydeoxyguanosine was determined by using a BAS Model LC-4B amperometric EC detector (Biotech, Luton, Bedfordshire) with a glassy carbon working electrode operated at an applied potential of +0.8 V *versus* silver - silver chloride

and typically 1 nA full scale deflection. Deoxyguanosine was determined by using an ultraviolet detector (A_{290}) arranged in series. This enabled us to express the results as moles of 8-OHdG per mole of deoxyguanosine in the hydrolysate. The mobile phase was 50 mM ammonium acetate - acetic acid buffer, pH 4.1, to which was added 50 ml of methanol per litre. A Techsphere 5 ODS column (0.46 × 25 cm, HPLC Technology, Macclesfield, Cheshire) was used with a flow-rate of 1 ml min^{-1}. Occasional overnight flushing of the system with an EDTA solution (1 mM) improves the base-line noise. Other groups report the use of isocratic elution with a citric acid - sodium acetate - sodium hydroxide - acetic acid buffer containing 10%, or even 15%, of methanol. In our hands, two other unidentified electrochemically active compounds co-chromatographed with 8-OHdG under these conditions.

The detection limit of the method was approximately 20 fmol of 8-OHdG at S/N = 3. This is about a 1000-fold improvement on the sensitivity of ultraviolet detection, using the wavelength maximum of 245 nm for 8-OHdG.[20] Thus, we have found that it is possible to detect base-line levels of oxidative DNA damage in cells from blood, a readily accessible human tissue.[29] Our *in vitro* studies have shown that although both bleomycin and gamma irradiation induce 8-OHdG formation in isolated DNA, no increase in 8-OHdG above control levels could be detected in intact human or rat hepatocytes exposed to bleomycin *in vitro* at 4 or 37 °C.[30] Bleomycin does, however, induce unscheduled DNA synthesis in isolated, non-permeabilised hepatocytes ([^{3}H]thymidine incorporation), implying that 8-OHdG formation is not a major determinant of bleomycin-induced DNA repair.[31]

Evidence that DNA Oxidation Plays a Role in Autoimmune Diseases

Human granulocytes produce high levels of oxygen radicals on activation at sites of inflammation. Activation of granulocytes with the tumour promotor tetradeconylphorbolacetate (TPA) resulted in an increased cell content of 8-OHdG (about 1 8-OHdG per 600 guanine bases in their DNA) compared with non-activated cells.[32] The increase was prevented by the presence of superoxide dismutase during exposure of the cells to TPA. This observation suggests that levels of DNA strand breaks and 8-OHdG might be increased in the DNA of circulating cells from patients with various inflammatory diseases.

Recently, the rate of DNA unwinding (a measure of DNA strand breaks) was determined in circulating mononuclear cells.[33] It was found that the rate of DNA unwinding was significantly increased in rheumatoid arthritis cells, compared with normal control cells and osteoarthritis cells. Lesions such as strand breaks result in relaxation of the supercoiling, causing reduced density of nuclear material, as assessed by sucrose gradient centrifugation. The latter method was used to show that nuclear material from the lymphocytes of many patients with autoimmune disease was lighter in density,[25] possibly indicating the presence of unrepaired DNA damage. Furthermore, Emerit *et al.*[34] have suggested that the chromosomal aberrations seen in lymphocytes from scleroderma patients are due to oxygen radical induced DNA damage.

It has been demonstrated that blood lymphocytes from patients with certain autoimmune diseases, *e.g.*, rheumatoid arthritis, systemic lupus erythematosus and Behcet's syndrome, show increased sensitivity to the toxic effects of the alkylating agent *N*-methyl-*N*-nitrosourea compared with normal subjects and patients with other disorders.[35] The autoimmune disease cells are also relatively deficient in the DNA repair of O^6-methylguanine. Furthermore, lymphocytes from patients with a wide variety of autoimmune inflammatory conditions (*e.g.*, rheumatoid arthritis, systemic lupus erythematosus) have a higher susceptibility to X-irradiation.[25] In view of these findings, it is noteworthy that there is increasing evidence that oxygen radicals may play a part in DNA damage induced by a wide variety of environmental mutagens.[2] We suggest that oxygen radical-mediated DNA damage may also play a role in somatic mutation, leading to autoimmune diseases.

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Food Irradiation and Free Radicals

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The topic of food irradiation is currently receiving considerable attention in the press, owing to a bill now passing through Parliament that will legalise the sale of irradiated food in the United Kingdom. In the minds of many people irradiation is associated with radioactivity and the recent disaster at Chernobyl has done nothing to allay the fears of the public. Irradiation of food does not cause contamination with radioactive substances, but is merely another process, like cooking or pasteurisation, that inevitably causes some change in the food. On the other hand, like any new process, it requires careful regulation to prevent abuse. Consequently, considerable time and effort is being spent on finding a suitable test for irradiated food. It is precisely because irradiation produces very little change in food and because the changes that are observed are also caused by other processing methods, that a specific, universal test is not yet available. One of the major effects of ionising radiation is the formation of free radicals, and since electron spin resonance (ESR) is the ideal method for detection and identification of free radicals, it is consequently a prime candidate for detecting irradiated food. However, radicals are generally very reactive and short lived, particularly when produced in aqueous solution. They are also formed as short lived intermediates in many normal metabolic processes. So, in moist tissues such as meat, fruit or vegetables, any radiation induced radicals will have decayed within minutes of irradiation or will be indistinguishable from the endogenous radicals. Our attention should focus on dried materials such as seeds, spices or bone. ESR examination of spices shows the presence of a weak signal of free radicals present prior to irradiation. Some of these radicals are due to organic components, such as semiquinones, occurring naturally, while other radicals are induced by grinding. It is not possible to assign the signal to specific free radicals owing to its broad, featureless nature. Irradiation enhances the endogenous ESR signal and so it is possible to infer from the ESR spectrum that the material has been irradiated. Much plant material contains traces of manganese, which gives a characteristic ESR signal and which is not influenced by irradiation. This signal can act as an internal standard, the ratio of free radical and Mn^{2+} peak heights giving an indication that the food has been exposed to ionising radiation. However, the free radical signal decays, often within several days, to the level in the unirradiated material. Consequently this non-specific free radical signal cannot provide positive proof of irradiation. On the other hand, a specific, radiation induced signal, assigned to a cellulose radical, has been observed in many seeds, and while this decays within about two weeks in the intact fruit, its presence is a clear indication of irradiation. The signal has also been detected in some spices, where its rate of decay is dependent on the moisture content of the sample. However, as the rate of decay cannot be determined, other than in carefully controlled test samples, the magnitude of the signal cannot be used to determine the dose of radiation received.

Irradiation of bone produces a stable free radical that is only observed after irradiation and which has a readily identifiable ESR spectrum. Treatments such as prolonged grinding or heating give free radical signals that are clearly different from the radiation induced signal. Moreover, a major proportion of the radiation induced signal is indefinitely stable, even withstanding cooking. Thus, ESR can provide a quick and easy method of detecting whether any food containing bone has been exposed to a dose of ionising radiation in the relevant range, which is 1–10 kGy. All that is required is a fragment of bone that has been roughly dried and weighs a few milligrams. The same ESR signal is seen in bones from all types of meat, poultry, fish and even frogs. A similar signal is also observed in the calcified shells of crustacea and shellfish, making the method applicable to a wide range of foodstuffs.

Quantification of the dose of radiation requires considerably greater effort. The response of the ESR spectrometer varies over the volume of the sample with the result that quantification requires either a "point" sample or a large uniformly shaped, homogeneous sample. This is best achieved by powdering the sample. It is also necessary to dry the samples to a uniform extent, because increasing amounts of water in the sample greatly reduce the sensitivity of the spectrometer. The dose response of bone and other calcified tissues varies with the degree of calcification and of crystallinity. For example, for a given dose of radiation, the signal in mussel or crab shell is 4 or 5 times greater than that in beef or chicken bones, which in turn is greater than that in fish bones or prawn cuticle. The lower limit for detection of irradiation was found to be about 50 Gy for beef or chicken, but as low as 5 Gy for mussel shell and as high as 250 Gy for prawn cuticle. These doses are well below those likely to be used commercially. Within a species there may be considerable variation in dose response, the hard leg bones of a chicken giving a much greater signal than the soft rib or breast bones. There is even considerable variation between similar bones within a species. Consequently, while it would be possible to estimate the dose received by a given sample on the basis of the average dose response of a particular bone or shell, a more accurate value can be obtained by repeated re-irradiation of each sample with known doses and extrapolation of the height of the radiation induced signal to zero.

The ESR method has now been tested in a number of trials conducted both nationally and internationally. From these it is clear that ESR can provide a reliable answer to whether or not foodstuffs containing calcified tissue have been irradiated. The method can also provide a determination of the dose accurate to within a factor of 2. Further collaboration is underway to remove possible sources of error and improve the accuracy, but for most purposes the present accuracy would appear to be adequate since most commercial irradiation facilities would only guarantee the dose received by all parts of a consignment to within limits of 1–1.7.

Free Radical Production in Irradiated Pharmaceuticals and Confectionery and its Application to Radiation Accident Dosimetry

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As the use of radioactivity in medicine increases, there is a general need for dosimetric systems that can be applied to continuous monitoring and accident situations. In an accident, the exposure of radiation workers can be found from personal monitors. However, for personnel not equipped with such devices, an alternative approach must be relied upon. The interaction of ionising radiation with organic matter generates free radicals, which in a solid matrix may become trapped and hence are detectable by electron spin resonance (ESR) spectroscopy. In this work, materials were sought which could be used as accident dosimeters, *i.e.*, solid, probably organic substances found on or about an accident victim and which could be used with ESR spectroscopy to give a rapid and reliable dose assessment.[1]

Experimental

Electron spin resonance spectra were recorded using a Bruker ER 200 X-band spectrometer which was connected to a data system (Bruker ESP 1600). Unless stated otherwise, all measurements were carried out at ambient temperature. The pharmaceuticals were in tablet form and covered a wide range of medicinal applications. Virtually all the samples had "background" pre-irradiation signals and these were unaffected by grinding.

Results and Discussion

Spectral Assignment, Sensitivity and Fading

All the samples showed induced ESR signals following 15 kGy of γ-radiation (see Table 1). Pre-irradiation signals resulting from the presence of metal centres showed no loss of intensity, which indicates that irradiation did not effect a change in the paramagnetic states. The pre-irradiation signals that originated from organic radicals were overlaid by similar resonance lines, which shows that irradiation induces more of the same radical centres. On examination of the ESR spectra obtained from the confectionery and pharmaceuticals, three distinct groups appeared. Piriton, triazolam, propranolol and frusemide [group (a) in Table 1] gave identical spectra [Fig. 1(*a*)]. The Polo mint, chewing-gum and Sanatogen [group (b) in Table 1] showed the same induced signals [Fig. 1(*b*)]. Bolvidon and the multi-vitamin tablets [group (c) in Table 1] each gave the spectrum shown in Fig. 1(*c*).

In order to facilitate the assignment of the above ESR spectra, powdered samples of various sugars were irradiated with subsequent ESR analysis. The spectrum obtained from lactose was identical with the group (a) signals [Fig. 1(*a*)] and that obtained from sucrose was identical with the spectra seen in group (b) [Fig. 1(*b*)]. No match was found for group (c) [Fig. 1(*c*)].

Study of the fading of the ESR intensity at ambient temperature shows which items would be most useful in an accident situation when the samples in question may have to be despatched some distance to the nearest ESR laboratory. Clearly the fading will be considerably reduced at lower temperatures, but storage in dry ice or liquid nitrogen may not always be practicable. The terms "fading" and "radical decay" are used here to denote the loss of ESR intensity in irradiated samples with the passage of time under ambient conditions. The confectionery and pharmaceuticals, with the exception of paracetamol, all maintained 50–90% of the original signal after 4000 h. In particular, glyceryl trinitrate, and members of the sugar-containing groups [(a) and (b) in Table 1] show a remarkable stability. Lorazepam, multi-vitamin and paracetamol show a rapid decrease within the first 3 h (25%) followed by a slow decline between 100 and 4000 h (*ca.* 10%). This implies the existence of two radical centres differing in stability.

In order to determine the sensitivity, weighed portions of the samples were irradiated with a considerably lower dose (100 Gy). The results are presented in Table 1 as spins g^{-1} Gy^{-1}. This can be regarded as a pseudo-*G* value and is used solely for comparative purposes. An examination of the pseudo-*G* values gives an indication of which samples are the most susceptible to free radical production and hence which might be more useful at lower doses.

Combining the results from the sensitivity and fading experiments allowed the elimination of several samples before the more stringent task of dose calibration was carried out. The samples retained for further investigation included the chewing-gum, Polo mint, Ceporex, glyceryl trinitrate and Piriton.

Table 1. Sensitivity to irradiation

Sample	Pseudo-*G*/ 10^{12} spins g^{-1} Gy^{-1}
(a) Frusemide	6.0
Piriton	12.5
Triazolam	12.1
Propanolol	10.8
Lorazepam	8.5
(b) Sanatogen	5.2
Chewing-gum	8.7
Polo mint	12.2
(c) Bolvidon	1.4
Multi-vitamin	1.4
(d) Glyceryl trinitrate	10.4
(e) Ceporex	4.7
(f) Paracetamol	1.4

Dose Calibration

Weighed amounts of the chosen samples in fragment form were irradiated over a range of absorbed doses (2–150 Gy) and their ESR responses determined by double integration and peak-height measurements. Calibration graphs were plotted for each sample and the error (95% confidence level) involved in the spectral collection and measurement was determined. The best straight-line fit for each data set with its associated linear regression coefficient (*r*-value) was also found.

In general, the samples gave a linear response down to 10–25 Gy, below which the scatter was larger than the experimental error. Contributing factors to this scatter include the background signal from each sample, the small amount of sample (typically 10–70 mg) and the variation in fragment shape. In order to avoid these problems, larger powdered samples would have to be used, whenever possible, and a pre-irradiation value found for each.

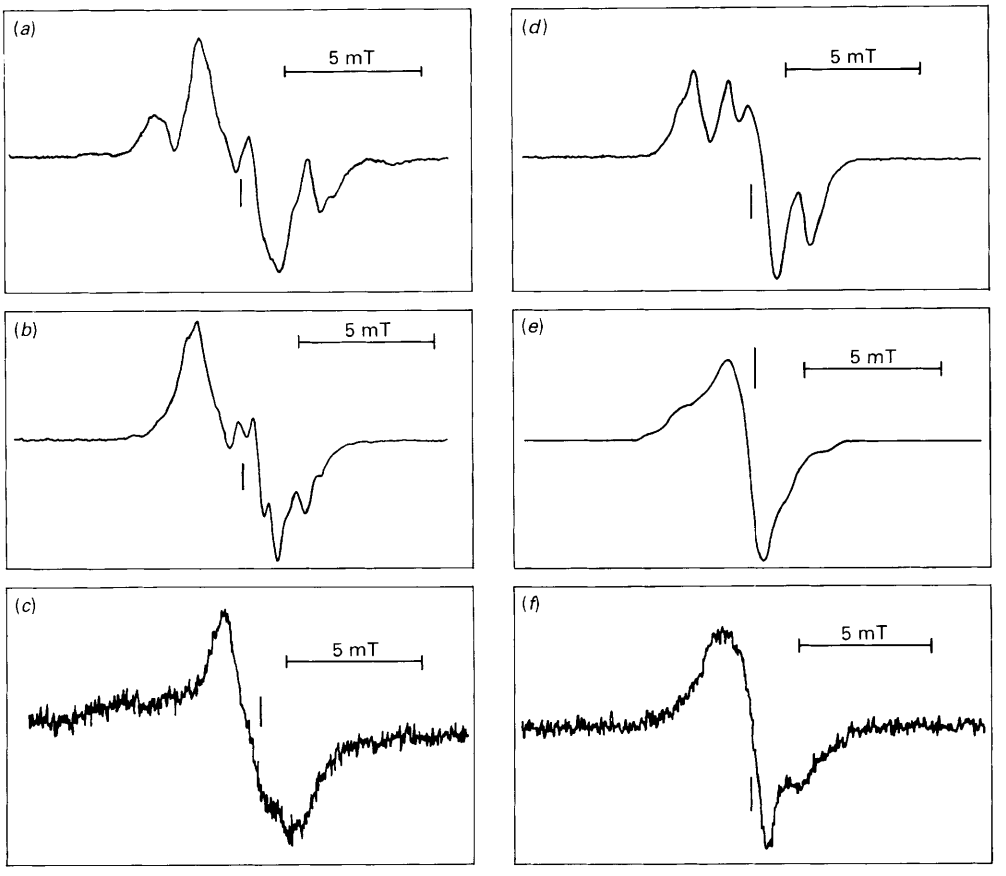

Fig. 1. Radiation-induced ESR spectra of the confectionery and pharmaceuticals. (*a*) Lactose-containing group: Piriton, frusemide, triazolam, propranolol and lorazepam [for lorazepam, the dominant spectrum (lactose) was overlapped by a weak resonance line, which faded over a period of *ca.* 700 h]; (*b*) sucrose-containing group: Polo mint, chewing-gum and Sanatogen; (*c*) Bolvidon and multi-vitamin; (*d*) glyceryl trinitrate; (*e*) Ceporex; and (*f*) paracetamol. All spectra were recorded from powdered samples at ambient temperature (295 K); the microwave power was 1 mW. Marker position corresponds to the centre of the DPPH (diphenyl picryl hydrazyl) resonance line (g = 2.003 7)

Conclusions

A number of materials have been shown to be potentially useful for ESR dosimetry, although in some instances only one component of a mixture is suitable. The pseudo-G value as defined herein gives a useful indication of the sensitivity of a potential dosimeter.

Samples containing sugars (*e.g.*, sucrose and lactose) give a high sensitivity to radiation and the induced radicals are relatively stable.

Although slow signal fading is an advantage, if the time interval between irradiation and ESR measurement is known it should still be possible to determine the dose using substances whose ESR signals decay rapidly, by extrapolation of the fading curves.

For routine dosimetric measurements, a major requirement of the dosimeter would be uniformity of sample, and accurate placement in the ESR cavity would have to be ensured for reproducibility of results. In the accident situation it would be important to carry out post-accident calibration of an irradiated sample by using very similar, if not identical, samples before any dosimetric result could be given for an irradiated individual.

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