Vitamin, selenium, zinc and copper interactions in free radical protection against ill-placed iron

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The possible involvement of highly reactive free radicals in the development of disease, particularly cancer and inflammation, and in tissue injury following heart attack, transplant surgery or certain types of chemical poisoning, are currently attracting considerable interest. Although in the healthy body, carefully controlled reactions catalysed by enzymes continue to be the rule, it now appears more and more likely that in ill-health, non-enzymic free-radical oxidation processes catalysed by iron can sometimes play a critical, if not a central, role. Of course to nutritionists, such interest in non-enzymic oxidations is nothing new. The fact that foodstuffs generally deteriorate on storage, not only in terms of palatability but also in nutritional value, has long been the bane of the food industry. Papers such as 'The effect of heat and aeration upon the fat-soluble vitamine' (Hopkins, 1920), 'Inactivation of vitamin A by rancid fat' (Powick, 1925), 'The autooxidation of fats with reference to their destructive effects on vitamin E' (Cummings & Mattill, 1931) and 'Vitamin E in iron-treated dry rations' (Waddell & Steenbock, 1931), clearly illustrate the early interest in this area.

By the mid-1940s, in spite of the limited techniques available, much of the ground work had already been laid for what we currently know about free radicals, trace metals and vitamins in the development of food rancidity. By then, unsaturated fats were known to be particularly susceptible to oxidation by a metal-catalysed, free-radical chain reaction. Oxygen was known to be consumed in the process and when β-carotene was present a fading of the familiar red–orange colour was observed. Various hydroxylated compounds including vitamin E were known to delay the onset of rancidity and the additional presence of vitamin C could delay the onset even further (Golumbic & Mattill, 1941). Now 40 years later many of these oxidation phenomena are being studied again. This time, however, interest is focused more on the eater than on the eaten. It could be said that medicine and biochemistry have caught up with the food industry. 'We contain unsaturated fat, Fe and O_2: why don't we go rancid?' Or put another way, 'Why doesn't living matter go rancid until it in turn becomes food? Antioxidants such as propyl gallate or butylated hydroxy toluene are often added to biscuits and other fat-containing foodstuffs to prolong shelf-life. 'What are the body's natural antioxidants?' Such questions are providing much of the impetus for the current surge of interest in the role of free-radical reactions, hydrogen peroxide and what has been variously described as 'decompartmentalized', 'ill-placed', 'free', or

'low-molecular-weight chelatable' Fe in general medicine. It is now clear that the healthy body does indeed have a fine armoury of defensive mechanisms, continually alert to minimize the damaging effects of these agents. On the other hand, it is also apparent that such defences may sometimes be overwhelmed, perhaps through the action of light or radiation, a toxic chemical, infection or general stress and the breakdown of the cell's structural compartments. Serious pathological changes may then occur even in the previously healthiest of cells. Indeed, should the normal defensive mechanisms be also weakened by a nutritional or genetic deficiency then the onset of a damaging downward spiral of events may be greatly accelerated.

In our own laboratory three principal types of experiments have been undertaken. Firstly, the reduction or oxidation of various foreign and natural molecules by free radicals generated through the interaction of Fe and the sulphur-containing amino acid cysteine. Secondly, the direct observation by conventional visible absorption spectroscopy (albeit over time-scales of a millionth of a second) of the reaction of free radicals generated by single pulses of ionizing radiation (pulse radiolysis). Thirdly, the irradiation of biological systems for several minutes and the drawing of conclusions from the nature and extent of the chemical, biochemical and biological changes corresponding to a particular known radical concentration (stationary state radiolysis). It cannot be overemphasized that free radicals such as the superoxide radical ($O_2^{\bullet -}$), the hydroxyl radical ($OH^{\bullet}$) or organic peroxy radicals ($RO_2^{\bullet}$) generated by radiation are of thermal energy and are chemically identical to the same free radicals formed biochemically. Such experiments have consequently provided considerable information concerning the manner by which a large variety of free radicals or their subsequent products, or both, interact with biological compounds (see Willson *et al.* 1985).

Fe control and free-radical prevention

Although contamination by metals, particularly Fe and copper, has long been known to hasten the deterioration of food, it is only in the last 10 years that the critical role of Fe in the development of disease has begun to be seriously appreciated. Although Fe is vital, its presence in the wrong place at a particular time may be extremely damaging. The background to our own interest in the role of Fe in disease has been extensively documented elsewhere (Willson, 1976, 1977*a*,*b*, 1983). In summary, studies with the drug metronidazole (Flagyl) and subsequent reading of the literature led us to the inevitable conclusion that the safe compartmentalization of Fe is critical to the health of the cell. If decompartmentalization of Fe occurred (either due to the action of a foreign agent, possibly a chemical, a virus, or changes induced by radiation) free-radical reactions might be initiated. This would be particularly so if the Fe, or a reactive complex, $Fe^{2+}(X)$, came into contact with H_2O_2 or sites containing labile sulphur such as those present in the amino acid cysteine.

$$Fe^{2+}(X) + H_2O_2 \longrightarrow Fe^{3+}(X) + OH^- + OH^{\bullet} \qquad \text{Fenton reaction}$$

$$OH^{\bullet} + RH \longrightarrow R^{\bullet} + H_2O \qquad \text{Damage}$$

$$\begin{matrix} RS^- \\ RS^- \end{matrix} \diagdown\!\diagup Fe^{2+} + O_2 \longrightarrow \begin{matrix} RS^- \\ RS^- \end{matrix} \diagdown\!\diagup Fe^{3+} + O_2^{\bullet -} \qquad \text{Damage}$$

Subsequent experiments in other laboratories have reinforced the arguments presented, not only in relation to cancer and anti-cancer drugs such as metronidazole, misonidazole and bleomycin, but also in relation to inflammation and diseases such as malaria (Lown & Sim, 1977; Bahnemann *et al.* 1978; Gutteridge, 1979; Blake *et al.* 1981; Clark *et al.* 1984; Halliwell & Gutteridge, 1984; Samuni *et al.* 1986). Indeed the observation that the levels of decompartmentalized Fe (low-molecular-weight chelatable Fe) are increased substantially in the brains of animals subject to cardiac arrest and subsequent resuscitation, is currently causing particular excitement amongst those interested in ischaemic injury and reperfusion (Bulkley *et al.* 1983; McCord, 1985; Nayini *et al.* 1985).

Zinc and protection against Fe–thiol interactions

As a corollary to the proposal of the importance of Fe status in disease it was suggested that Zn may play a vital role in masking labile S sites so reducing damage from radical processes at critical times during the life of the cell (Willson, 1976, 1977*a*,*b*). Again, other work added possible support for this proposal (Chvapil, 1973; Searle & Tomasi, 1982; Coppen *et al.* 1986).

$$\begin{matrix} RS^- \\ RS^- \end{matrix} \diagdown\!\diagup Zn^{2+} + O_2 \longrightarrow \text{No reaction}$$

A Zn–aspartate complex has been shown to afford remarkable protection against alcohol as well as radiation-induced injury (Floersheim, 1985; Floersheim & Floersheim, 1986). In New Zealand a marked reduction in the incidence of poisoning of sheep by fungal toxin sporidesmin has been observed following the suggestions by a farmer, Mrs. Gladys Reid, that Zn supplements be introduced (Towers & Smith, 1978; Munday, 1984). The masking of thiol groups by Zn through mercaptide formation is only one possible free-radical-related mechanism. Zn deficiency could also lead to a deficiency in the Zn–copper-containing enzyme superoxide dismutase (*EC* 1.15.1.1; SOD) or to a lowering of the normal levels of metallothionein (metSH). This protein, which is induced by Zn, contains an abundance of thiol groups and might be expected to protect by simple $O_2^{\bullet}$

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scavenging or possibly by repairing organic free radical lesions in a similar manner to glutathione.

$$OH^{\bullet} + RH \longrightarrow R^{\bullet} + H_2O \quad \text{Damage}$$

$$R^{\bullet} + metSH \longrightarrow RH + metS^{\bullet} \quad \text{Repair}$$

$$\text{or } R^{\bullet} + O_2 \longrightarrow RO_2^{\bullet}$$

$$RO_2^{\bullet} + RH \longrightarrow RO_2H + R^{\bullet} \quad \text{Damage}$$

$$RO_2^{\bullet} + metSH \longrightarrow RO_2H + metS^{\bullet} \quad \text{Protection}$$

Whether free-radical interactions inhibited by Zn play any, let alone major, roles in the amelioration of the previously described conditions remains an open question. Zn is known to be a constituent of some hundred enzymes. Clearly any overall deficiency will have enormous repercussions on cell biochemistry generally and free-radical-initiated changes may not be amongst the first to be expressed pathologically.

Selenium and glutathione and protection against Fe–peroxide interactions

As we have seen, Fe can also interact readily with H_2O_2 to form hydroxyl free radicals ($OH^{\bullet}$) directly. Fe can also react with organic peroxides to form alkoxy free radicals which are also strong oxidants. Such peroxides may be formed metabolically through the action of enzymes such as xanthine oxidase (*EC* 1.1.3.22), as a result of lipid peroxidation, or through the diversion of electrons from normal enzymic electron transport pathways. Clearly the efficient and safe removal of peroxides by non-free radical mechanisms is of vital importance. Although catalase has long been known to rapidly remove H_2O_2 in such a manner, there is now good evidence that in many instances the Se-containing enzyme glutathione peroxidase (*EC* 1.11.1.9) also has a key role in this respect.

The importance of Se in muscle integrity has been known to veterinary medicine for some time. In parts of China a dramatic reduction in the incidence of Keshan disease, with its severe heart degeneration and often early death, has occurred following mass medication with sodium selenite. Again in both these instances it is thought that tissue injury is at least partly associated with decreased levels of the Se-containing peroxidase (*EC* 1.11.1.7) enzyme (see Diplock, 1981, 1984). Peroxides generally are reduced by the peroxidase with the simultaneous oxidation of glutathione (GSH) to its disulphide GSSG. A ready supply of GSH is also of paramount importance. Fortunately it can be readily generated from GSSG by a reaction with NADPH catalysed by the enzyme glutathione reductase (*EC* 1.6.4.2). The formation of NADPH from $NADP^+$ is itself dependent on the efficient functioning of the enzyme glucose-6-phosphate (G6P) dehydrogenase (*EC* 1.1.1.49).

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$$RO_2H + 2GSH \xrightarrow{\text{Peroxidase}} GSSG + H_2O + ROH$$

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

$$NADP^+ + G6P \xrightarrow[\text{dehydrogenase}]{\text{G6P}} NADPH + H^+ + 6\text{-phosphogluconate}$$

In certain Mediterranean areas a considerable proportion of the population have a genetic disorder (Favism) resulting in a deficiency in this enzyme. The erythrocytes of such persons are particularly susceptible to haemolysis, should they eat the broad bean (*Vicia faba*). The bean is known to contain the chemical divicine which readily autoxidizes with the formation of free radicals (Rotruck *et al.* 1971; Clark *et al.* 1984).

Copper and free-radical protection by SOD

The fact that the Cu-containing protein, SOD, affords protection to a variety of biochemical systems in vitro is undisputed. Although some beneficial anti-inflammatory effects of the enzyme have been reported after injection directly into the synovial cavity, no double-blind clinical trials seem yet to have been undertaken. The actual protective mechanism of the enzyme continues to be a matter of debate. One possibility is that the superoxide free radical assists in keeping 'ill-placed' Fe in the reduced state thus enabling it to enter into the Fenton reaction (see Halliwell, 1987). The reaction with SOD prevents this.

$$O_2^{\cdot -} + O_2^{\cdot -} \xrightarrow[2H^+]{\text{SOD}} H_2O_2 + O_2 \qquad \text{Protection}$$

$$O_2^{\cdot -} + Fe^{3+}(X) \longrightarrow Fe^{2+}(X) + O_2$$

$$Fe^{2+}(X) + H_2O_2 \longrightarrow Fe^{3+}(X) + OH^- + OH^{\cdot} \qquad \text{Fenton reaction}$$

$$OH^{\cdot} + RH \longrightarrow R^{\cdot} + H_2O \qquad \text{Damage}$$

To add further confusion, recent studies in our own laboratory indicate that the enzyme may reduce the damaging action of some organic peroxy radicals ($RO_2^{\cdot}$); for example those derived from the DNA base thymine. The apo protein (prepared by dialysis of the native enzyme against cyanide) affords little protection but does so in the presence of as little as 500 nM Cu ions. Since such peroxy radicals may damage proteins containing accessible cysteine, tryptophan and methionine residues, this latest possible mechanism of action of the Cu protein is particularly intriguing (see Gee *et al.* 1985; Willson, 1985; Willson *et al.* 1985).

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Provitamin A (β-carotene) and protection against organic peroxy radicals ($RO_2^{\bullet}$)

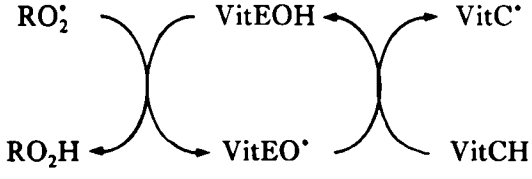
Considerable success has been claimed for the use of β-carotene as an oral photoprotective agent in disorders such as pophyria. More recently it has also been suggested that the provitamin may play a role in the prevention of cancer. Excited O_2 and photosensitizer molecules which, like free radicals, can be highly damaging, have long been known to be quenched by β-carotene. Recent pulse radiolysis studies have shown that the peroxy radical derived from carbon tetrachloride can also be rapidly scavenged (Packer *et al.* 1981). Whilst this highly damaging species, $CCl_3O_2^{\bullet}$, can be hardly considered a natural peroxy radical, these observations do raise the possibility of a more general role for both vitamin A and β-carotene in free-radical protection.

$$RO_2^{\bullet} + \beta\text{-carotene} \longrightarrow \text{products} \qquad \text{Protection}$$

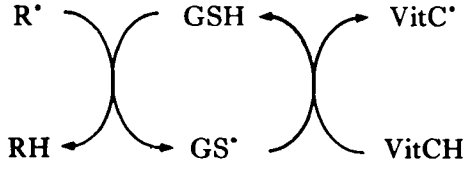
$$RO_2^{\bullet} + RH \longrightarrow RO_2H + R^{\bullet} \qquad \text{Damage}$$

Regeneration of vitamin E and GSH by vitamin C

It is now widely accepted that vitamin E (α-tocopherol) and a water-soluble derivative, Trolox C (Roche, Welwyn Garden City), react rapidly with the wide variety of organic peroxy radicals and, as such, can act as chain-breaking antioxidants. Indeed, recent studies indicate that vitamin E is the major, if not the only, lipid-soluble chain-breaking antioxidant in human blood plasma (Burton *et al.* 1983). Other recent studies have also confirmed the early indications that vitamin C, whilst acting as a water-soluble antioxidant in its own right, can also act in cooperation with vitamin E (Nathans & Kitabchi, 1975; Packer *et al.* 1979; Chen *et al.* 1980; Leung *et al.* 1981; Niki *et al.* 1982, 1983, 1984; Barclay *et al.* 1983; Fukuzawa *et al.* 1985). Vitamin E (VitEOH) can be continuously regenerated at the expense of vitamin C (VitCH) by the following cycling sequence:



Related pulse radiolysis studies have shown that GSH might also be regenerated in a similar manner (Forni *et al.* 1983).



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Concluding remarks

Hopefully the previously described examples of recent studies of free-radical reactions will have illustrated how radiation techniques are being used to investigate processes of possible relevance to the role of nutrition in health and disease. Pulsed fast kinetic absorption spectroscopy, electronic pressure sensors and O_2 electrodes are indeed a far cry from the painstaking chemical analysis, manometric methods and dietary assays of 50 years ago. However, it is hoped that these, in their turn, will prove as valuable and provide a useful foundation for further study.

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