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SYMPOSIUM ON 'NUTRITIONAL ASPECTS OF FREE RADICALS'

Free radical mechanisms in relation to tissue injury

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The present paper is a broad-ranging account of free-radical biochemistry in general, and of free-radical mechanisms of tissue injury in particular. Because it is broad-ranging within tight constraints of length it is necessarily lacking in detail on some issues of relevance; the following reviews can be consulted for additional coverage: Slater (1972, 1978, 1984), Pryor (1976–84), Mason (1982), Halliwell & Gutteridge (1984).

Free radicals can be defined as molecules or molecular fragments containing a single unpaired electron; this unpaired electron usually gives a considerable degree of chemical reactivity to the free radical: in chemical formulas the unpaired electron is conventionally shown as a 'superscript dot', as with the hydroxyl free radical $OH^{\bullet}$.

Free radicals can be produced in the cells and tissues of our bodies by various processes and reactions; Table 1 divides these into two main sections: (1) formation of free radicals as a result of the impact of radiation, and (2) formation by reduction–oxidation (redox) reactions involving the transfer of an electron. For discussion of the mechanisms that result in the production of free radicals, see Pryor (1966) and Slater (1972). Table 1 also indicates major ways by which free-radical intermediates can be converted to non-radical products by the action of free-radical scavengers; discussion of this important aspect of free-radical biochemistry is at the end of this paper.

As already mentioned, free radicals are usually reactive chemically although some important examples of stable free radicals are known, such as diphenylpicrylhydrazyl ($DPPH^{\bullet}$) and Fremy's salt (potassium nitrosodisulphonate). The

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Table 1. *Major mechanisms resulting in the formation and removal of reactive free-radical intermediates*

Formation
(1) By the impact or absorption of radiation, or both:
  (a) high energy or ionizing radiation
  (b) ultra-violet radiation
  (c) visible light with photosensitizers
  (d) thermal degradation of organic material
(2) By electron transfer ('redox') reactions:
  (a) catalysed by transition metal ions
  (b) catalysed by enzymes

Removal or inhibition of formation, or both
(1) By the action of free-radical scavengers
  (a) hydrophilic scavengers
  (b) lipophilic scavengers
  (c) by spin trapping
(2) By radical–radical interactions
(3) By preventative antioxidants
  (a) metal chelators
  (b) enzymic removal of hydroperoxide and other reactive intermediates

high chemical reactivity, for example, of $OH^{\bullet}$ or the trichloromethylperoxy free radical $CCl_3OO^{\bullet}$, ensures that their half lives ($t_{\frac{1}{2}}$) in the liquid phases of biological materials are very short; the $t_{\frac{1}{2}}$ values for $OH^{\bullet}$ and for $CCl_3OO^{\bullet}$ in a biomembrane, for example, are probably less than 1 ns and 1 μs respectively (see Slater, 1984). Reactive free radicals can be essentially reducing or oxidizing in character; the superoxide anion radical $O_2^{\bullet -}$ is an example of a reducing species when in the presence of ferricytochrome *c*, and $OH^{\bullet}$ is strongly oxidizing:

$$O_2^{\bullet -} + \text{ferricytochrome } c \longrightarrow O_2 + \text{ferrocytochrome } c$$
$$OH^{\bullet} + AH \longrightarrow H_2O + A^{\bullet}$$

These reducing and oxidizing properties can be modified rather easily under experimental conditions such that a reducing species can be 'changed' into another species with oxidizing properties:

$$O_2^{\bullet -} + O_2^{\bullet -} \xrightarrow[-O_2]{+2H^+} H_2O_2 \xrightarrow{+Fe^{2+}} Fe^{3+} + OH^- + OH^{\bullet}$$

$$OH^{\bullet} + HCO_2^- \longrightarrow H_2O + CO_2^{\bullet -}$$

Oxidizing free radicals may initiate (or extend) cell injury by abstracting a hydrogen atom from a polyunsaturated fatty acid (PUFA) to initiate the degradative process known as lipid peroxidation (see p. 6). Free radicals may also add across unsaturated centres in molecules to give covalently bound adducts that may have grossly disturbed biological function.

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Table 2. *Some diseases and toxic cell injuries that are known to be associated with free-radical disturbances*

(for background references see Slater (1984))

Nutritional disorders (deficiencies of α-tocopherol and other antioxidants; excesses of free-radical-initiating agents)	Alcoholism
Inflammation	Iron overload of the liver
Rheumatoid arthritis	Many types of toxic liver injury (carbon tetrachloride, other halogeno-alkanes, paracetamol, bromobenzene)
Atherosclerosis	Some types of tumour promotion
Some parasitic infections	Some examples of chemical carcinogenesis
Some lung disorders (paraquat)	Reperfusion injury

It may well be asked at this stage: what is the reason for the considerable and widespread current interest in free-radical mechanisms in tissue injury? A clue to the answer to this question can be obtained from an abbreviated list of cell injuries and diseases that are known to involve free-radical disturbances either in a primary or a secondary way (Table 2). Nutritional disorders associated with free-radical disturbances include (1) deficiencies of natural protective substances such as α-tocopherol and ascorbate; and (2) excess intake of substances that can stimulate free-radical production, such as transition metal salts.

Because very reactive free radicals have short half lives, their concentration in tissues, except in extreme situations, is too low for direct methods of detection by electron-spin-resonance (ESR) spectroscopy. This technique requires relatively high (μM) concentrations to resolve signals satisfactorily from background signals and from broad envelopes of other signals that complicate biological studies (see Borg, 1976). However, free radicals can be trapped as they are produced to give relatively stable adducts that progressively accumulate with time; in this way detectable concentrations of the spin-trap adduct can be obtained (see Janzen, 1980; Rosen & Finkelstein, 1985). Most spin traps that have been used with biological systems to date give nitroxyl-type adducts that have characteristic ESR features. An illustration of this procedure to trap the free radical $CCl_3^{\bullet}$ is shown in Fig. 1.

Reactive free radicals such as $OH^{\bullet}$ often have very high second-order rate constants (K values) with biomolecules likely to be in the immediate environment of where the free radical is produced (see Willson, 1978). The K values for $OH^{\bullet}$ are mostly in the range of 10^9–10^{10}/M per s, which shows that these reactions are essentially diffusion-controlled: this means that the interactions of very reactive species such as $OH^{\bullet}$ are restricted mainly by the diffusion of such species in their environment. Somewhat less reactive than $OH^{\bullet}$ is $CCl_3OO^{\bullet}$ (having K values with thiols, vitamin E and fatty acids in the range 10^6–10^9/M per s; see Slater *et al.* 1985); a relatively unreactive species is $O_2^{\bullet-}$ with K values for fatty acids, etc. in the range 10^{-1}–10^1/M per s (Bielski *et al.* 1983). The very high reactivity of free

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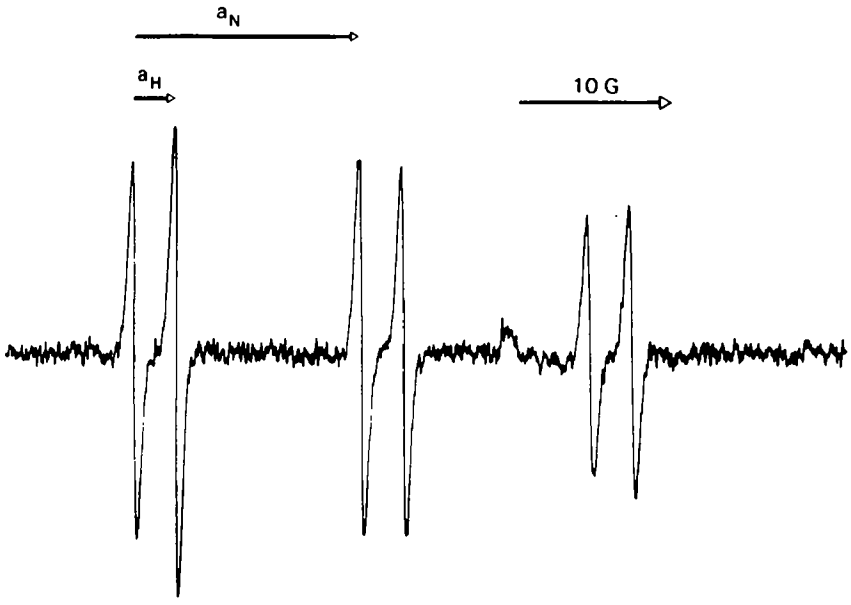

Fig. 1. Electron spin resonance spectrum of the adduct formed by the reaction of trichloromethyl ($CCl_3^{\bullet}$) free radicals with the spin trap phenylbutylnitrone (PBN). The $CCl_3^{\bullet}$ free radicals were produced by photolysis of CCl_3Br at room temperature. The instrumental settings were: gain $1{\cdot}25\times10^6$, modulation amplitude 0·25 G, time constant 0·5 s, scan time 500 s, power 10 mW, field centre 3475 G, scan 60 G, frequency 9·77 GHz. The CCl_3Br and PBN were in water; the CCl_3Br was present as a saturated solution with PBN present at 10 mM. a_N and a_H are the hyperfine splitting constants for nitrogen and hydrogen respectively (see Borg, 1976). Magnetic field strength in Gauss (G) is along the horizontal axis; a scale of 10 G is indicated by the length of the arrow.

radicals such as $OH^{\bullet}$ means (Slater, 1976) that they cannot diffuse very far in a biological environment before interacting; the diffusion radius is thus very small in contrast to $O_2^{\bullet-}$ that can diffuse appreciable distances (see Fig. 2). Although $O_2^{\bullet-}$ is rather unreactive (its protonated form $O_2H^{\bullet}$ is considerably more reactive; dissociation constant (pK_a) $\sim$ 4·5) and can thereby diffuse, it can undergo 'conversion' to much more reactive species (see eqn (1)) and thus produce biologically damaging reactions at a distance from the initial site of formation:

$$O_2^{\bullet-} \xrightarrow{\text{diffusion}} O_2^{\bullet-} \longrightarrow H_2O_2 \xrightarrow{Fe^{2+}} OH^{\bullet} \longrightarrow \text{damage} \qquad (1)$$

Reactive free radicals can produce disturbances to cells and tissues in many different ways: Fig. 3 summarizes some of the major pathways that can result in damage. For extensive discussions of these pathways see Slater (1972, 1982, 1984) and Mason (1982). In the present paper only one aspect of Fig. 3 will be discussed in more detail: the role of lipid peroxidation in free radical-mediated cell injury.

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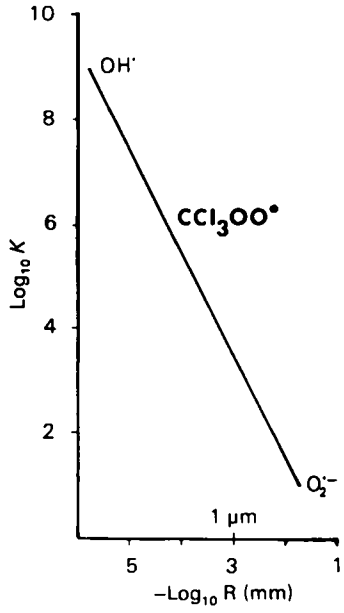

Fig. 2. The relation between the chemical reactivity (shown as the logarithm of the second-order rate constant (K) of the free radical with a biomolecule such as an unsaturated fatty acid) of a free-radical species and its diffusion radius (R) from its site of formation (see Slater, 1976, 1984).

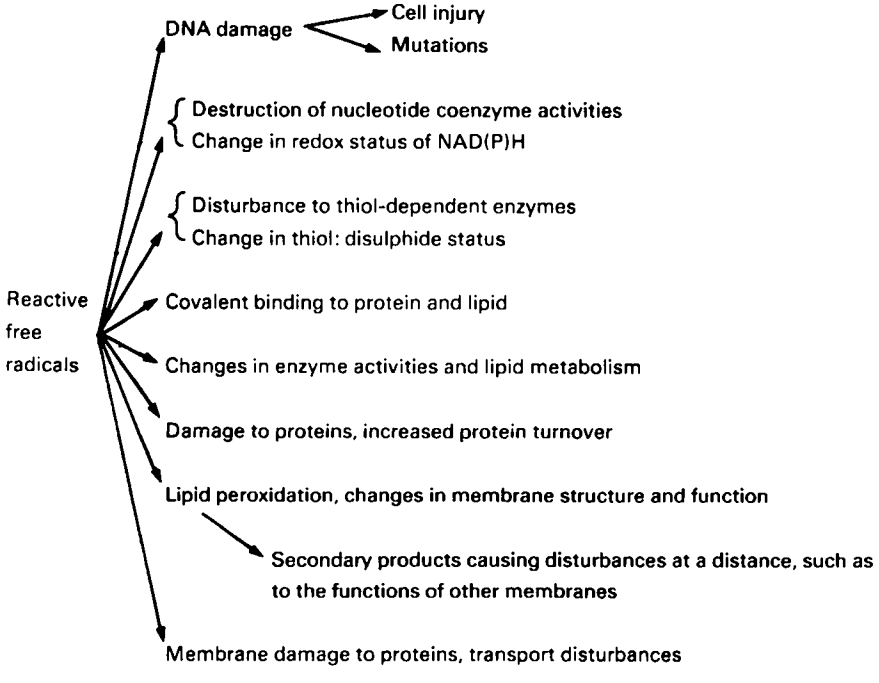

Fig. 3. Some major types of damaging reactions that can result from the production of reactive free radicals in biological material.

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Lipid peroxidation

Biomembranes (plasma membrane, endoplasmic reticulum, mitochondrial membrane, etc.) contain substantial amounts of PUFA that can undergo peroxidative breakdown. The initial step is hydrogen-atom abstraction followed by addition of oxygen to give a lipid peroxy free radical (LOO$^\bullet$), which may enter a complex series of reactions that yield a variety of products. In the following scheme the unsaturated lipid is shown as LH and metal ions as M^{n+}:

$$LH \xrightarrow{-H^\bullet} L^\bullet \xrightarrow{+O_2} LOO^\bullet \xrightarrow{LH} LOOH \qquad (2)$$

$$LOOH \xrightarrow{M^{n+}} LO^\bullet \qquad (3)$$

$$LOO^\bullet, LO^\bullet, LOOH \longrightarrow \text{products including aldehydes, unsaturated aldehydes, alkanes, epoxy-fatty acids, hydroxy-fatty acids} \qquad (4)$$

Lipid peroxidation has been the subject of numerous investigations in relation to cell injury (see Dianzani & Ugazio, 1978; Dormandy, 1978; Bus & Gibson, 1979) especially with respect to the peroxidation of PUFA. Generally speaking, the most extensive changes in PUFA that occur during peroxidation of biomembranes involve the highly unsaturated acids $C_{20:4}$ (arachidonate) and $C_{22:6}$ (docosahexaenoate) (for example, see May & McCay, 1968; Ahmed & Slater, 1981). Peroxidation of lipid material in biomembranes can also involve cholesterol (Smith, 1981) but the biological implications of this have not been so extensively evaluated as have those for PUFA peroxidation. Extensive peroxidation of a biomembrane can affect the structure and function in several important ways as indicated in Table 3. One consequence of the peroxidation of a biomembrane is a decrease in the fluidity of the lipid phase of the membrane assembly (Dobretsov *et al.* 1977; Slater, 1979): this can be expected to have important consequences in relation to many of the major metabolic functions dependent on membrane structure (see Houslay & Stanley, 1982). Membrane fluidity can be studied in various ways but one that is particularly useful and widely applicable is based on ESR-spin labelling (Smith, 1972). In this procedure a molecule labelled with a stable free-radical marker (often containing a nitroxyl-group) is inserted into the membrane; analysis of the ESR spectrum subsequently obtained can give information about the motion of the spin label in the lipid, and hence of the fluidity of the membrane. Fig. 4 gives some information on the use of spin labels. From these particular experiments (W. Xin, M. J. Davies and T. F. Slater, unpublished results) an interesting fact emerged: different ways of stimulating liver microsomes to peroxidize had different effects on membrane fluidity even though each method was controlled to give the same amount of malonaldehyde production.

Lipid peroxidation can be of major significance to cell injury produced by free-radical mechanisms but it is often very difficult to decide if the contribution of peroxidation is at a primary stage leading to injury, or at a later stage resulting

Table 3. *The effects of lipid peroxidation on aspects of membrane structure and function*

1. Decrease in relative content of $C_{20:4}$ and $C_{22:6}$ fatty acids
2. Formation of lipid hydroperoxides that may stimulate or inhibit specific enzymes associated with biomembranes
3. Oxidation of thiol-groups that may affect enzyme activities in the membrane; and protein conformation that is related to protein–lipid associations
4. Decrease in lipid fluidity of the biomembrane
5. Liberation of breakdown products from the site of lipid peroxidation to produce damaging effects elsewhere

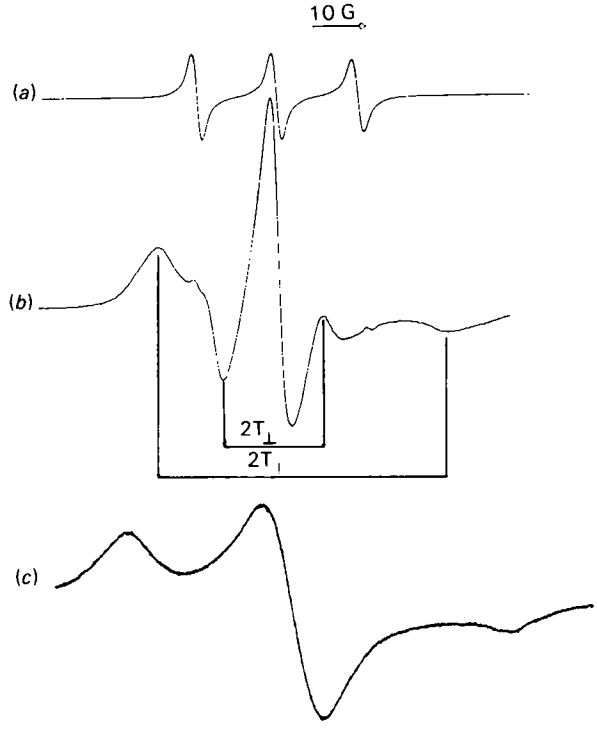

Fig. 4. Electron spin resonance spectra of the spin label 5-doxyl-stearic acid under different conditions: (*a*) in chloroform at room temperature, (*b*) incorporated into liver microsomal lipid at room temperature, (*c*) in chloroform at 77°K. The instrument settings were: modulation amplitude 0·125 G, time constant 0·1 s, scan time 500 s, field scan 100 G, power 10 mW and (*a*) gain $1{\cdot}25\times10^4$, field centre 3475 G, frequency 9·72 GHz, (*b*) gain 1×10^5, field centre 3475 G, frequency 9·72 GHz, (*c*) gain 2×10^5, field centre 3370 G, frequency 9·42 GHz. Magnetic field strength in Gauss (G) is along the horizontal axis; a scale of 10 G is indicated by the length of the arrow.

The spectra show characteristic differences due to (*a*) isotropic tumbling, (*b*) anisotropic motion, (*c*) the frozen 'powder' spectrum. By making measurements $T_\perp$ and $T_\parallel$ (see Borg, 1976) on spectrum (*b*) the order parameter 'S' can be calculated that is a measure of the membrane fluidity; when the environment around the label is completely fluid S = 0, when completely frozen S = 1.

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from injury. An example of the former situation is the hepatotoxic action of CCl_4; this type of liver injury is dependent on the metabolic activation of CCl_4 to the trichloromethyl free radical ($CCl_3^{\bullet}$) by an enzymic process in the endoplasmic reticulum (for review, see Slater, 1982). This primary product $CCl_3^{\bullet}$, can bind covalently to lipid and protein but is relatively unreactive in abstracting hydrogen atoms from PUFA to initiate lipid peroxidation. However, in the presence of O_2 the $CCl_3^{\bullet}$ species is converted rapidly (K value approximately 10^9/M per s) to the much more oxidizing species $CCl_3OO^{\bullet}$ that readily abstracts H from arachidonate (K value, 7×10^6/M per s; Forni *et al.* 1983). A substantial peroxidation of the liver endoplasmic reticulum is a major feature of the early damage caused by CCl_4 to the liver.

An example of lipid peroxidation being associated more with secondary rather than primary mechanisms of damage concerns the initiating action of the ferrous ion. Generally, the cell is adequately protected against damaging effects of Fe^{2+} by effective complexing of the iron (as in transferrin, haemosiderin, ferritin, etc.; Bothwell *et al.* 1979) and by compartmenting these 'pools' of Fe away from susceptible membrane sites. If the cell structure is damaged such that Fe is 'decompartmentalized' (Willson, 1977) or so that excess Fe can get access to intracellular organelles then extensive and rapid peroxidation may ensue. Free Fe can stimulate free-radical reactions in various ways as follows: (a) by entering a Fenton-type reaction to give $OH^{\bullet}$:

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + OH^- + OH^{\bullet} \qquad (5)$$

(b) by catalysing a redox-shuttle whereby electrons pass from NAD(P)H to oxygen:

$$\text{NADPH} \longrightarrow \text{flavoprotein} \xrightarrow{Fe^{3+}\text{–chelate}} Fe^{2+}\text{–chelate} \qquad (6)$$

$$Fe^{2+}\text{–chelate} \xrightarrow{O_2} Fe^{3+}\text{–chelate} + O_2^{\bullet -} \qquad (7)$$

(c) by reacting with lipid hydroperoxide (LOOH) to yield the generally reactive lipid alkoxy-species:

$$\text{LOOH} \xrightarrow{Fe^{2+}} \text{LO}^{\bullet} \qquad (8)$$

The metal-catalysed degradation of LOOH can be followed by ESR spin trapping that allowed the identification of both $LOO^{\bullet}$ and $LO^{\bullet}$ species (Davies & Slater, 1986).

Examples where Fe-overload is associated in vivo with tissue damage are in kwashiorkor (see Golden, 1987) and in the hind-limb paralysis observed in vitamin-E-deficient piglets subsequently given an Fe–dextrose injection (Patterson *et al.* 1971).

Lipid peroxidation can be stimulated also in cells and tissues by a direct action

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of radiation; the potentially lethal effects of ionizing radiation are, of course, well known and made use of in radiotherapy. Although in such cases the reactive radicals are formed throughout the cell and can damage the cell in many ways (see Fig. 3), lipid peroxidation is an important component. With much-less-energetic radiation, such as visible light, the main mechanism of cell injury can be peroxidation of biomembranes as in photosensitized damage to skin (Slater & Riley, 1966). With domestic animals the photosensitization may arise by toxic contaminants in the food (e.g. fungal toxins) causing liver injury, followed by accumulation in the blood and tissues of porphyrin photosensitizers that are normally excreted in bile (see Slater *et al.* 1964). Other ways in which dietary changes can increase peroxidative damage include increases in the PUFA content of biomembrane (see Hammer & Wills, 1978) and induction of the NADPH–cytochrome P_{450} electron transport chain that can supply electrons for redox-cycling. Some inducers are antioxidants and are used as food additives (Hanssen & Marsden, 1984).

Antioxidants

Antioxidants can be classified as (1) preventative or (2) chain-breaking antioxidants (see Burton *et al.* 1983*a*). In the former category are metal chelators, superoxide dismutase (*EC* 1.15.1.1), catalase (*EC* 1.11.1.6) and glutathione (GSH) peroxidase (*EC* 1.11.1.9). In the second category are lipid-soluble, chain-breaking agents such as α-tocopherol, ubiquinone, retinoic acid and β-carotene; and water-soluble substances such as ascorbate, GSH and urate.

Dietary constituents that can affect the preventative antioxidant status of cells include selenium (a necessary trace metal for GSH peroxidase; Flohé *et al.* 1973) and Cu^{2+}, Zn^{2+} and Mn^{2+} that are involved in the action of superoxide dismutases (Fridovich, 1982). Metal chelators can be used clinically, rather than as nutritional components, to chelate out Fe and Cu; for example, the use of penicillamine in Wilson's disease and desferrioxamine in haemochromatosis. The chain-breaking antioxidant status of the body can clearly be considerably influenced by the dietary content of substances such as vitamin E, ascorbate, cysteine (for GSH synthesis) and so on.

The major lipophilic chain-breaking antioxidant in human plasma, in rat liver, in rat liver tumours and in rat endoplasmic reticulum is α-tocopherol (Table 4). Under normoxic conditions other lipophilic constituents do not play a major role although at low O_2 tensions β-carotene can be significantly active (Burton & Ingold, 1984).

The total antioxidant capacity of plasma has recently been evaluated by Wayner *et al.* (1985) who found that (in percentage terms) the contributions to total activity were: α-tocopherol 5, ascorbate 15, urate 25, protein-thiols 50.

It is noteworthy that synergistic interactions can occur between different scavengers and this may be of great importance biologically. For instance, Golumbic & Mattill (1941) reported that vitamin C 'protected' vitamin E from destruction in foods; Tappel (1968) suggested that this was due to a repair of

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Table 4. *The content of α-tocopherol and of total lipid-soluble chain-breaking antioxidant ('total antioxidant') in human plasma, human erythrocyte membranes, normal rat liver, microsomal suspensions prepared from normal rat liver or Novikoff tumour cells, and Novikoff tumour cells*

(The human results are from Burton *et al.* (1983*b*) and the rat results are from Cheeseman *et al.* (1986) and unpublished results of K. H. Cheeseman, K. U. Ingold and T. F. Slater. Values are means and standard deviations.)

	α-Tocopherol (nmol/mg lipid)		Total antioxidant (nmol/mg lipid)	
Sample	Mean	SD	Mean	SD
Normal human plasma	7·6	2·8	8·0	2·5
Human erythrocyte membranes	2·6	0·9	2·7	0·8
Normal liver (adult, male rat)	2·30	0·53	2·99	0·51
Normal liver microsomes (adult, male rat)	1·67	0·31	2·30	0·31
Novikoff tumour cells	5·62	0·78	6·19	1·18
Novikoff microsomes	4·87	0·37	6·54	1·73
Normal isolated hepatocytes (adult, male rat)	0·76	0·05	0·93	0·06
Yoshida liver tumour (adult rat)	4·2	0·6	4·2	0·3

vitamin E$^{\bullet}$ by vitamin C; Packer *et al.* (1979) determined the kinetic features of these reactions using pulse radiolysis; and Doba *et al.* (1985) have reported similar interactions of vitamins E and C in lipid micelles. Thus, 'protective' chains of antioxidants may function *in situ* (Packer *et al.* 1979). It is interesting to speculate that other free-radical scavengers that occur naturally and may be used as food additives or drugs (e.g. the flavonoids) might also synergistically interact with vitamins E and C. Some flavonoids, for example, are effective free-radical scavengers in biological systems (Slater & Eakins, 1975). For detailed discussion of the mechanisms of protection against free radical-mediated damage see Slater (1981); Slater *et al.* (1985).

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