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Free-radical reaction in biological systems

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Summary

Free radicals are a highly reactive chemical species which differ from all other species in possessing an unpaired electron. Although free-radical activity in living systems is energetically improbable, recent work suggests that a number of important biological processes depend on it. In contrast to enzymic metabolism these free-radical processes tend to be non-cyclic, irreversible, non-homoeostatic, and energetically wasteful. For the organism as a whole they may nevertheless have considerable survival value. Free radicals are too short-lived to be demonstrable in tissues; but methodological advances are leading to the detection and measurement of characteristic free-radical reaction products. The findings suggest that free-radical activity is not only a potential cause but also a common, perhaps invariable, consequence of cell damage. It is possible that some of the secondary products of free-radical reactions may help to regulate the body's local and systemic response to injury.

Introduction

For a hundred years biochemistry and chemical pathology have been firmly rooted in enzymology. Perhaps too firmly. Sir Frederick Gowland Hopkins epitomised the catalytic nature of enzymes by stating that 'an enzyme is where it acts'. To prove their presence, in other words, we need only demonstrate their accelerating action on a specific biochemical change. The aphorism is true; but like most aphorisms it is open to distortion. It is *not* true that in biological systems where there is action there must be an enzyme; yet it is this distorted doctrine that has become tacitly accepted.

By and large enzymes are Nature's instruments for maintaining the constancy of life. This applies both to structure and to function. Although enzymes can act in free solution, they are at their best when forming part of organised structures. Conversely, the maintenance of living structures depends on the ceaseless activity of enzymes. Functionally enzymic pathways maintain a balance between energy-consuming and energy-producing pathways. Energy generated enzymically can be stored as chemical fuel: energy consumed enzymically can be used to accomplish biological work. It is a perfect homoeostatic system, most tellingly represented by interlocking cycles. The cycles have no beginning and no end just as enzymic charts have no top and no bottom. But if life as portrayed by enzymic biochemistry goes on smoothly and indefinitely life in reality does not.

All living individuals decay and die; and even their relative longevity depends on the continuous decay and death of the units of which they are composed. There are, moreover, countless biological processes which are essential for the survival of complex organisms but which are yet non-homoeostatic, non-cyclic, irreversible, and wasteful in terms of energy. These processes could be — indeed they undoubtedly are—influenced by enzymes; but, like death itself, they do not fit into the general pattern of enzymic biochemistry. To understand them we need to recognise a different *kind* of biochemical reaction, and more and more evidence suggests that these depend on the generation and activity of free radicals.

The concept of free radicals

Before considering free radicals in a biological context it may be useful to recall some facts of physical chemistry. All conventional chemical species have one characteristic in com-

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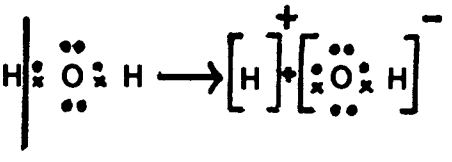
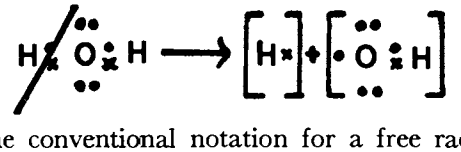
mon: they have a paired electron complement. Electrons can be conceived as negative charges circling around a positive nucleus. The reason why they need to be paired is that, in addition to orbiting around the centre, they also spin around their own axis; and to maintain stability every electron that spins to the right must be balanced by an electron that spins to the left. An ordinary chemical bond, therefore, represents *two* electrons, each side contributing one; and when the bond splits it splits in such a way that one fragment retains both electrons and the other retains neither. Water, for example, can be represented by the simplified electronic structure:

where the dots are the electrons derived from the oxygen atom and the crosses are the electrons derived from the two hydrogen atoms. When the molecule ionises one fragment keeps two bonding electrons and thereby acquires a formal negative charge (OH^-); the other fragment keeps none and thereby acquires a formal positive charge (H^+):



But it is *theoretically* possible for a bond to split in such a way that the two fragments retain one bonding electron each—that is, by 'homolytic' fission. *If* that happened and *if* one of the resulting fragments maintained a separate existence for long enough to react independently with another molecule or ion, then the fragments would qualify as 'free radicals' and the reaction would be 'free-radical reaction'. In the case of water this type of fission would generate a hydrogen atom (as distinct from a hydrogen ion) and a hydroxy free radical:



(The conventional notation for a free radical is a single dot. The hydroxy radical is therefore $OH^{\cdot}$.)

In the previous paragraph 'theoretically' and 'if' are italicised because for many years it was axiomatic that, apart from the gas state and a few freak instances, homolytic fission could never happen. The twin foundations of modern chemistry, the concepts of valency and of molecular weight, seemed to forbid it. Not until the 1930s was this article of faith disproved. Workers in the oil industry then showed that, even though free radicals might have an independent lifespan of only a few microseconds, free-radical activity accounted for some of the most important and most spectacular chemical transformations known. The rancidification of fats and oils in particular was shown to be a free-radical chain reaction in which each step is activated by the uptake of an oxygen molecule. This was a liberating discovery. Within a few years plastics and polymers of every shape and kind, all based on free-radical technology, were transforming everyday life. Only on biological thinking did the new species make little impact. There were probably two reasons for this.

Because all chemical systems tend toward stability and because stability demands the pairing of electrons a large single dose of energy is generally required to generate a reactive free radical. The purpose of intermediate metabolism on the other hand—in so far as biological transactions can be said to have a purpose—and the *ideé fixe* of classical biochemistry is the parcelling up of large lumps of energy into minute doses. It could be argued, in other words, that free-radical generation along normal metabolic pathways is intrinsically improbable. Not that such rational (if misguided) arguments were ever advanced. Free-radical activity was not seriously considered in a biological context because of the forbidding image of free-radical processes in non-biological systems. These processes, it seemed, could be delayed or prevented, but once they had been triggered off they were unstoppable, uncontrollable, and irreversible. Many were highly destructive. Some were literally explosive. To biochemists, conditioned for half a century to the subtle interplay and self-regulating equilibria of enzymic cycles, the pattern seemed profoundly irrelevant to life, positively unbiological. Exceptions were of course recognised — the biological conse-

quences of high-energy irradiation was one—but not until the past decade did free-radical activity emerge as a biological phenomenon perhaps as important and as ubiquitous as enzymic catalysis.

The chemical pathology of free radicals

Such a new subject cannot, of course, be reviewed in a historical perspective; and indeed advances are now so fast and in so many directions that any kind of perspective is bound to prove illusory. (Carcinogenesis and thrombosis are among the fields which are being invaded by free-radical concepts and which can barely be mentioned in the present review. No doubt there are others.) Nevertheless, it may be helpful to group current developments around three complex questions. First, what are the physical and chemical factors which promote free-radical generation in living organisms? Second, how do living organisms protect themselves against free-radical damage? And third, what are the biological consequences of free-radical activity?

FACTORS WHICH PROMOTE FREE-RADICAL ACTIVITY

Free-radical activity may still appear uncontrollable in comparison with metabolic regulation; but we now recognise a diversity of physical and chemical variables which promote or inhibit it.

1) Molecular oxygen is a critical ingredient of most biological free-radical processes, so critical that we tend to use the terms 'free radical' and 'oxygen free radical' almost interchangeably. (Similarly we tend to equate 'antioxidants' with 'free-radical scavengers'.) There are several reasons for this, all of them related to the unique properties of the oxygen molecule. Because in some respects the molecule behaves not like a molecule but like two unstable free radicals strung together it will avidly latch on to other unstable molecular sites. This may bring about complete homolytic bond disruption. Under certain conditions, moreover, the molecule itself can undergo step-wise (single-electron) reduction, generating in the process a number of intensely reactive free-radical intermediates. It must be assumed that it is these free-radical intermediates which are responsible for clinical 'oxygen damage' of all kinds, from retrolental fibroplasia to pulmonary fibrosis, as well as for the modulating influence of oxygen tension on the effects of high-energy irradiation. But, less obviously, there can be little doubt that these free radicals also play a role in phagocytosis, in prostaglandin generation, and perhaps in immune-complex formation *(1)*.

2) Transitional-metal ions—ions which can lose or gain a single electron as they change from one valency state to another—have long been recognised as powerful initiators of free-radical oxidations in industrial chemistry and they are no less powerful in biological mixtures. The rate of free-radical oxidation in many tissue homogenates is largely determined by their free iron content *(2)*, and tissue damage due to iron overload in vivo probably depends on free-radical reactions catalysed by the metal *(3,4)*.

3) Organic solvents such as carbon tetrachloride are acute liver poisons, and Slater laid the foundations of free-radical biochemistry in Britain by demonstrating the free-radical nature of the damage *(5)*. The wide implications of his findings — for example, in relation to alcohol and drug toxicity in man—still need to be fully explored.

4) The degree of unsaturation of the material is an important variable, susceptibility to free-radical attack in homologous series increasing with the number of double bonds in the molecule. This means that in cells the most 'essential' polyunsaturated lipids are also the most vulnerable. It may also account for the central role of arachidonic acid in so much current physiological, pharmacological, and pathological research.

5) High-energy irradiation, both with X rays and with ultraviolet light, undoubtedly generates free radicals; its therapeutic and destructive effects largely depend on this.

6) Structural disintegration is still the least recognised and may yet prove to be the most important cause of biological free-radical activity. None of the factors mentioned so far dents the argument that free-radical generation in biological systems *is* intrinsically improbable on energetic grounds. What does dent it is the fact that it is based almost entirely on knowledge gained from studying only two types of biological material—intact structures (tissues, cells, microsomes) or structureless solutions and homogenates. It ignores the crucial event

that separates these two stable states, structural breakdown itself. It is true that we know extraordinarily little about the chemical reactions peculiar to this transition; but we can nevertheless make a few generalisations. The build-up of organised biological structures is highly energy-consuming and much of the energy consumed probably remains latent in structural organisation itself. This means that structural breakdown is, potentially at least, an immensely exergonic happening. Of course some of the energy released may be dissipated. But the picture of stricken cells simply disintegrating is almost certainly false. Their demise probably more closely resembles a submicroscopic explosion. The force of such an explosion could disrupt stable electron pairs, and perhaps it almost invariably does.

PROTECTION AGAINST FREE RADICALS

Turning from promotion to protection, food chemists have shown that, however hard we try to preserve unsaturated lipids exposed to air, light, moisture, and trace-metal catalysts, they will sooner or later rancidify. This encapsulates a biological as well as an industrial problem *(6)*. Living cells are shot through with unsaturated lipids. (We would instantly set solid if they became saturated—as we do in rigor mortis.) They contain numerous transitional-metal complexes. They are drenched in oxygen. We incubate ourselves at 37°C and we are in continuous if not always purposeful motion. Why then do we not go rancid? The answer is far from clear. Circulating antioxidants such as vitamin E are essential safeguards in many animal species; but, except perhaps in the perinatal period, they do not seem to be indispensable in man *(7,8)*. A whole family of enzymes — catalase, various peroxidases, the superoxide dismutases—can forestall or extinguish oxygen free radicals, but they are probably only second-line, emergency mechanisms *(6,9)*. The copper-protein caeruloplasmin is a powerful antioxidant, but it is an extracellular protein *(10,11)*. Probably the most important single factor which protects organised structures from free-radical injury is structural organisation itself; so long as this is preserved the ingredients of free-radical oxidation remain insulated from each other *(9)*.

THE CONSEQUENCES OF FREE-RADICAL ACTIVITY

For some years free-radical pathology faithfully reflected its origins in industrial chemistry. What concerned food chemists was loss of food by rancidification. What concerned biochemists was loss of cell lipids by peroxidation. It had of course been known for many years that lipids disrupted by free radicals undergo tortuous secondary fragmentation; but the possibility that free radicals might not only destroy but also generate biologically active metabolites was barely envisaged until the 1960s. A group of Austrian workers then showed that many free-radical products were cytotoxic, especially to certain strains of neoplastic cells, and that some of the active fragments could be separated and partially characterised chromatographically *(12)*. Since then watery extracts and water-soluble free-radical products of increasing purity have been prepared from autoxidising lipids and they have proved to be extraordinarily potent in an astonishing range of in-vitro systems. The mixture includes antibacterial agents *(13)*, compounds which affect platelet aggregation, intermediates which influence the intrinsic and extrinsic clotting mechanism *(14)*, and many, perhaps most, of the prolific kinship of the prostaglandins.

METHODOLOGY

At this point we must pause to make the somewhat trite reservation that in-vitro potency is no proof of an in-vivo role. Free radicals have a lifespan of the order of microseconds and except in special cases, using special instrumentation, their presence in tissues cannot be detected. Evidence, therefore, of biological free-radical activity has always rested on more remote effects; and for 20 years it has rested on a single chemical reaction.

In 1944 Kohn and Liversedge described a new 'aerobic metabolite' which appeared in brain homogenate exposed to air and which, on heating in acid, formed a coloured complex with thiobarbituric acid (TBA) *(16)*. It was subsequently shown that the metabolite, malonyldialdehyde (MDA), is a distant offshoot, or rather a mixture of distant offshoots, of the free-radical peroxidation of polyunsaturated lipids *(17,18)*. Methods based on

the reaction were rapidly and widely adopted in the food industry and, further refined, they helped to establish the ground-rules of free-radical biology. They could even be applied to clinical diagnosis. Human red blood cells generate MDA when exposed in vitro to oxidant stress *(19)* and their susceptibility to free-radical oxidation correlates with their susceptibility to haemolysis *(20,21)*. (Currently the MDA assay enjoys an Indian summer as a quick measure of prostaglandin synthesis, though the implications of MDA generation are often ignored.) But although the MDA/TBA reaction provided ample proof that human tissues are *vulnerable* to free-radical attack and even suggested that vulnerability may be related to disease, it never provided proof that such an attack ever occurs in vivo. Red blood cells, for example, can be induced to autoxidise under stress, but even in severe haemolytic states fresh cells contain no significant amounts of MDA. The probable explanation crystallised only during the past few years as a result of the work of Tappel and his group. They showed that MDA and related compounds readily interact with proteins and other molecules which possess reactive amino groups. The Schiff-base-type complexes formed in this way no longer give a positive TBA reaction, but they have distinctive fluorescent spectra *(22,23)*. The latter finding suggested a new methodological approach.

Potentially, fluorescence spectroscopy is a highly sensitive tool whose value in biological research depends on the relative specificity and definition of the fluorescent properties of the compounds studied. On the face of it free-radical products, a large, unstable, and incestuous tribe, hardly qualify. Yet for once the difficulties are proving less debilitating than anticipated, and characteristic complexes both of lipid and of non-lipid origin are now being identified and measured in fresh tissues and tissue fluids *(24)*.

But fluorescence spectroscopy has not been the only recent methodological advance. Ultraviolet spectroscopy can be used to measure 'diene conjugation', a characteristic double-bond pattern of damaged fatty-acid chains *(24,25)*. An entirely different 'non-invasive' technique depends on the formation of highly volatile hydrocarbons as a result of free-radical fragmentation. These — for example, ethane and pentane—are eliminated by living organisms in expired air; and, although they are present only in picomolar concentration and should perhaps be expressed in 'micro-soupçons', they can be identified and measured by gas–liquid chromatography *(26–28)*. At a different experimental level subtle structural changes which may follow free-radical attack on cells and subcellular organelles can be monitored by chemilluminescence, polarisation, and deformability *(29–33)*.

SUPEROXIDE AND INFLAMMATION

Paradoxically perhaps further and persuasive proof of in-vivo free-radical activity has come from a new family of enzymes, the superoxide dismutases *(34)*. Unlike other protective enzymes (for example, catalase, glutathione peroxidase), the dismutases are true free-radical scavengers—that is, their specific substrate is not a stable or relatively stable molecule but the superoxide-ion free radical ($O^{\cdot-}$). This probably accounts for the lateness of their discovery. One cannot prepare a solution of free radicals as one can prepare a solution of starch or lactic acid; the free-radical substrate has to be continuously generated as a transient intermediate in the course of another enzymic reaction. By capturing the intermediate the dismutase aborts the first reaction, and it is this inhibition which is measured.

The superoxide dismutases are widely distributed and it has been suggested that the presence of powerful free-radical scavengers in most cells hints at widespread in-vivo free-radical activity. Whether or not this is a respectable argument, the enzymes have provided a much-needed tool for identifying free-radical steps in complex biological processes. Inflammation is an immediate challenge. It is a basic biological response whose local and systemic components have been described in minute detail but whose underlying trigger and regulating mechanisms have remained elusive. We can, of course, indulge in name-dropping, talk of 'amines', 'kinines', 'prostaglandins', and this, that, and the other 'factor'; but names provide no answers to a long series of rhetorical questions. What triggers off the capillary dilatation? What regulates the astonishingly precise and concerted migration of phagocytes, the stepping-up of leukopoiesis in dis-

tant sites, the rise in body temperature, the immune response? The specific inhibition of some of these events by superoxide dismutase points to the generation of oxygen free radicals as a key event. It also suggests that some of the products of free-radical activity play a role as essential messengers and mediators *(35–41)*.

Changing concepts

Experimental advances are turning long-held concepts upside down. Fifteen years ago biological free-radical reactions were envisaged—in so far as they were envisaged at all—as a rare potential *cause* of cell death. It now appears that free-radical activity may be the *sequel* of most forms of structural damage. For centuries rancid fat was regarded as a source of lethal toxins. It now appears that some products of rancidification may play a role in protecting the organism as a whole against the effect of local tissue destruction. But many conceptual difficulties remain, and one in particular could become a stumbling block.

It is possible to draw too sharp a distinction between enzymic and free-radical processes: in living systems the two are inextricably linked. (On the one hand enzymes can both beget and extinguish free radicals; on the other free radicals can both stimulate and destroy enzymes.) The distinction is nevertheless valid. Enzymes have become familiar because enzymic activity means a succession of replicate moves which extend over a long period of time. Free-radical reactions cannot be paraded in this way. A single free radical may be the trigger which sets in motion an extended sequence of events, but the initiating species may exist only for a split second. Some free-radical products may be characteristic, but others are not. When reaction products can be generated either by a free-radical or by an enzymic route (for example, many of the prostaglandins) the unquestioning tendency at present is to assume the enzymic alternative. Not only are enzymes familiar and ubiquitous, but their action can also be replayed experimentally without an 'unrealistic' input of energy. Yet the assumption may be wrong. Enzymic activity is the foundation of every biological system; but when systems themselves change the underlying mechanism is more likely to be an act of free radicals.

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