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Free radicals and tissue injury: fact and fiction

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In this lecture I have been asked to give an overview of free radical mechanisms of tissue damage; I will consider some general aspects, outlining a number of fundamental concepts with the aid of specific examples. Moreover, I will try to draw attention, where necessary, to a few situations where uncritical extrapolation, and perhaps inadequate technique, have led to unwarranted conclusions.

There is increasing interest at the present time in the roles of free radicals in relation to a variety of diseases and tissue injuries. In Table I I have put an abbreviated list of types of tissue damage and a variety of diseases where free radical disturbances have been implicated (for more extensive reviews see Slater, 1984, 1986).

The first example shown in Table I is toxic liver injury; examples of substances provoking this type of tissue damage, and where free radical intermediates are believed to be very much concerned are various halogenated materials such as carbon tetrachloride and halothane; a number of nitrogen-containing compounds; various quinones including some of the anti-cancer drugs used to treat certain aspects of that disease; and some polycyclic hydrocarbons. In all of these cases one can quite readily detect free radical intermediates. However, at this stage I want to make a very important caveat; it is one thing to be able to detect the presence of free radical intermediates, and to demonstrate free radical disturbances in a wide range of diseases and type of tissue injury, but it is quite another thing to be able to show that these free radical disturbances have some primary importance to the initial stages of the disease of tissue injury. In many cases the free radical disturbances that can be detected are the secondary consequences of some primary perturbation of metabolism or structure. In consequence, I believe that we really must look at each new report of free radical disturbances in an important disease or type of injury with a certain amount of healthy criticism, and even some scepticism.

I can give one illustration of this potential problem from some recent work of ours on a nutritional disorder known as kwashiorkor, a very important disease, especially in the Third World. It has a number of distressing symptoms associated with it including edema, skin lesions, changes of pigmentation and certainly a very distorted liver metabolism. When we looked at liver samples obtained from patients with this disease we found a very strong free radical signal in the liver. Figure 1 gives the result obtained from a liver sample taken from one baby who died of kwashiorkor; there is a very strong free radical signal located significantly away from the g=2 region. In this particular case we are sure that the free radical disturbance is completely secondary to the initiation of the disease: the free radical change is subsequent to serious liver damage. So having made and briefly discussed that important caveat I can now turn to some examples in depth.

Table I Some major diseases and types of tissue damage believed to involve free radical disturbances in a major way

Some toxic tissue injuries (e.g. CCl_4; acetoaminophen; paraquat)
Alcoholism
Transition metal (e.g. Fe^{2+}, Cu^{2+}) overload
Hyperbaric oxygen
Reperfusion injury
Some examples of nutritional liver disease
Ionizing radiation
Photosensitization
Atherosclerosis
Some CNS – disturbances
Some lung disorders
Some aspects of parasite infection
Phagocytosis and killing of micro-organisms
Arthritis and inflammation
Some examples of tumour initiation and promotion
Ageing

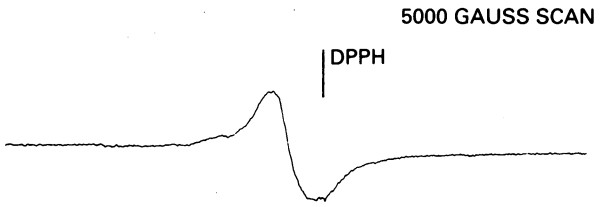

Figure 1 Electron spin resonance spectrum of a sample of liver taken immediately post-mortem from an infant who had died from the complications of kwashiorkor. The sample was analysed at 160°K; spectrometer gain, 1.25×10^5; modulation amplitude, 3.2 Gauss; time constant, 0.5 s; scan time, 500 s; field centre, 3,380 Gauss; scan range 5,000 Gauss; power, 13 db; frequency, 9.54 GH_z; the spectrometer was a Bruker 200D instrument. The spectrum is from unpublished studies of the author with Dr M.J. Davies and Dr M. Golden.

Free radicals can be produced in two main ways: (a) radiation, and (b) redox reactions mostly catalysed by transition metals or by enzyme catalysis. I am going to concentrate my talk basically on enzyme catalysis for the production of free radical intermediates.

Table II lists some of the more important classes of free radical that I will be talking about – at least in part – and other speakers will give much attention to. I have also included for the sake of interest, two other molecules: oxygen which is a coupled bi-radical (a molecule in the triplet state) that has 2 unpaired electrons, and singlet oxygen that, although not a free radical, is a reactive chemical species. Probably all of us in this audience recognise that free radicals have important parts to play in normal metabolism and in various types of tissue damage; but it is not so long ago that many people spoke very dismissively about free radical reactions: 'free radicals are unselective and uncontrollable, nasty, fast little things' I can remember people saying. Fortunately, that disparaging view is disappearing. We have only got to look at the progress in synthetic organic chemistry (for example the elegant work of Barton, 1985) or the physiological roles for free radical intermediates as in ribonucleotide reductase (see Williams, 1985), and certainly the various examples of free radical disturbances and diseases, to feel confident about their importance.

However, the old view still exists in a few undisturbed places; last week I was taking part in a PhD *viva* and one of the other examiners asked the student 'Do you really think that free radicals exist, or are they not just a useful theoretical concept?': we passed the student, but we failed the examiner.

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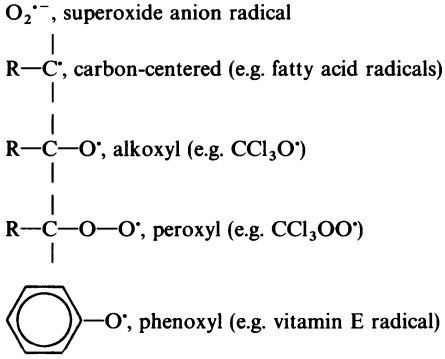
Table II Free radical species that have been implicated in a wide variety of types of tissue damage, or used in studies of such damage

$^3O_2$, ground state oxygen (a molecule with two unpaired electrons; a triplet state molecule)

$HO^{\bullet}$, hydroxyl

$O_2^{\bullet -}$, superoxide anion radical



R—C•, carbon-centered (e.g. fatty acid radicals)

R—C—O•, alkoxyl (e.g. $CCl_3O^{\bullet}$)

R—C—O—O•, peroxyl (e.g. $CCl_3OO^{\bullet}$)

—O•, phenoxyl (e.g. vitamin E radical)

$R—NO_2^{\bullet -}$, nitro-anion radical

R—NO•, nitroxyl (e.g. spin trapped adducts and spin labels)

R—S•, thiyl (e.g. GS• from glutathione)

$^1O_2$, singlet oxygen (not a free radical)

Radicals can, of course, be very difficult to work with; sometimes their study requires very special techniques such as pulse radiolysis (see Willson, 1978). With this technique one can obtain rate constants for free radical reactions in solution; Table III lists some that have been obtained here in Brunel. The rate constants for the hydroxyl radical, by and large, all fall within a very narrow range with whatever substance it interacts. Table III also gives data for an unusual free radical, a derivative of carbon tetrachloride, the trichloromethyl peroxyl radical. Table III shows that the hydroxyl radical reactions are all essentially diffusion-controlled in solution, whereas the trichloromethyl peroxyl radical has rate constants about 10 to 100 times smaller. Rate constants for radical reactions of the kinds shown in Table III can lead on to a very important and fundamental concept: if a very reactive species is produced in a biological environment, for example by an enzyme-catalysed step in the endoplasmic reticulum, the reactive free radical will have a rather restricted or even a *very* restricted radius of diffusion. Very reactive free radicals, such as $HO^{\bullet}$ or $CCl_3OO^{\bullet}$, are essentially trapped in their microenvironment as a consequence of their high chemical reactivity with neighbouring biomolecules: their radius of diffusion is very small. Free radicals, such as $HO^{\bullet}$ or $CCl_3OO^{\bullet}$, have diffusion radii that are very small even in relation to cellular dimensions (see Slater, 1986). This discussion on chemical reactivity leads me on to another point that I want to mention, and concerns some fascinating work done some years ago by Professor Willson with a visitor from Berlin, Dr Hiller (Hiller *et al.*, 1983). In this particular case they were looking at the effects on a bacteriophage of a variety of free radicals. What they found was that, although the hydroxyl radical is generally more reactive than the trichloromethyl peroxyl radical, in this particular biological system the $CCl_3OO^{\bullet}$ radical is more damaging. The reason is that $HO^{\bullet}$ is very unselective, it tends to react with any target that it comes into contact with, and its biological effects are diluted by this relative insensitivity of its types of reaction: the trichloromethyl peroxyl radical, on the other hand, is rather more selective and more damaging, molecule for molecule.

Table III Some second-order rate constants ($M^{-1}s^{-1}$) obtained by the Free Radical Research Group in Brunel University. For background details see Packer *et al.* (1978), Slater *et al.* (1987*b*) and Willson (1978)

Reactant	*HO•*	*$CCl_3OO^{\bullet}$*
Tryptophan	10^{10}	1.2×10^8
Glutathione	10^{10}	1.4×10^7
Arachidonic acid	10^{10}	7×10^6
Promethazine	10^{10}	4.5×10^8
Propyl gallate	1.2×10^{10}	2×10^8
α-tocopherol	10^{10}	5×10^8
β-carotene	10^{10}	1.5×10^9

A few examples of free radical disturbances in relation to tissue injury will now be given; the first example concerns the hepatotoxic activity of CCl_4 (for general background see Slater, 1972). In the middle 1960s we found, independently with Recknagel's group in America, that carbon tetrachloride is metabolised in the liver, probably to a free radical product; these studies on metabolic activation opened up a new field of study in biochemical pathology. In this early work it was speculated that CCl_4 is metabolized by the cytochrone P450 electron transport chain in the endoplasmic reticulum to a trichloromethyl radical and that this produced a variety of types of tissue damage. It proved quite difficult to demonstrate unequivocally that $CCl_3^{\bullet}$ is produced under conditions in the liver cell or *in vivo*; in fact, with esr techniques then available it was not possible. However, with the development of spin trapping techniques, it became possible in the late 1970s, first by Poyer *et al.* (1978) and by Lai *et al.* (1979) using the spin trap phenyl-α-t-butyl nitrone, and followed shortly after by ourselves (Albano *et al.*, 1982). Earlier attempts in our laboratory using another spin trap, methylnitrosopropane, had given equivocal results (Ingall *et al.*, 1978). These studies with spin traps demonstrated that CCl_4 is metabolised *in vitro* and *in vivo* to $CCl_3^{\bullet}$.

We had written, repeatedly, as had many other groups, that the trichloromethyl radical is a very reactive species that initiates liver damage; in retrospect, one can conclude that it does not really matter how many times you say the same thing or how authoritatively you say it, such statements are really no substitute for fact! In this particular case a real surprise awaited us, and was provided during the visit of John Packer to our laboratory in 1978. He studied the reactivity of $CCl_3^{\bullet}$ using pulse radiolysis and, much to our surprise, found it relatively unreactive under the conditions in the pulse radiolysis set-up; in fact, it goes very slow indeed with most of the molecules we look at except oxygen. With oxygen it reacts (Mönig *et al.*, 1983) at diffusion controlled rates to yield $CCl_3OO^{\bullet}$; this derived radical is much more reactive chemically than $CCl_3^{\bullet}$, by several orders of magnitude (Packer *et al.*, 1978; see Table III). In consequence, using pulse radiolysis, we were able to show that this peroxyl radical is really a much more reactive species than the CCl_3 radical itself, and is probably the main initiating species for lipid peroxidation. This was a surprise to us and led to a number of other important developments that have been discussed elsewhere (see Slater, 1984, 1986, 1987*a*, *b*).

A summary chart for the metabolism of CCl_4 is given in Figure 2 to draw together a few points that I have made. First of all CCl_4 is an example of a compound that is metabolically activated in the liver. It goes to a primary radical species, the trichloromethyl radical, and this can undergo a variety of secondary reactions. It can for example lose another chloride to go to the dichlorocarbene and then to carbon monoxide, a minor pathway. It can add to unsaturated compounds in its environment resulting in covalent binding; this has been well worked out. It has some oxidising ability and can abstract hydrogen atoms thereby producing chloroform; an old experimental finding in studies with CCl_4 is the formation of $CHCl_3$. Then, as already mentioned, CCl_3 can react very quickly with oxygen to form the peroxyl species that is more reactive chemically; this can certainly hydrogen-abstract much more rapidly than trichloromethyl and can thereby initiate lipid peroxidation. So one can see even from this very simple illustration that, by and large, there is a variety of very early reactions that

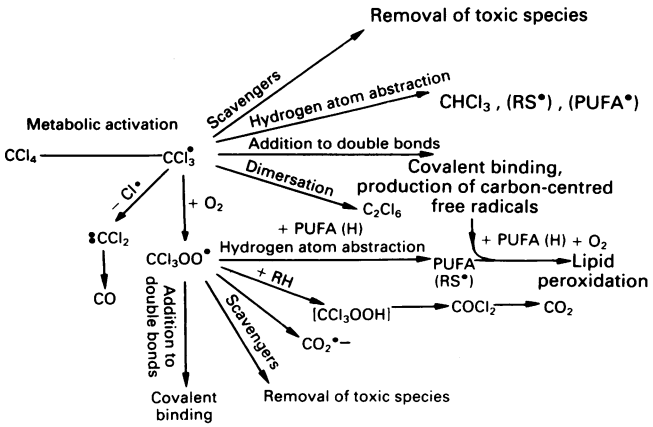


Figure 2 A summary chart for the activation of CCl_4 in rat liver endoplasmic reticulum. For background references see Slater (1972, 1984, 1985) and Connor *et al.* (1986).

have to be considered in the case of a molecule like CCl_4. From the range of studies illustrated in Figure 2 it has become apparent that a number of reactive intermediates and products have to be considered in the first few mseconds to seconds after introducing CCl_4 into a liver cell cystem. There is the lipid solvent itself, the primary free radical species, the rapidly formed peroxyl-species, there is evidence now of the alkoxyl species, there are derived fatty acid radicals, there is some evidence of activated oxygen species. Moreover, lipid peroxidation produces a whole variety of products and these have considerable biological activities. In addition, there are disturbances to the prostaglandin cascade that complicate the picture metabolically very rapidly indeed. Trying to decide which intermediate or pathway is of primary importance becomes very difficult against such a backdrop of complex products and the short time-scales involved (see Slater 1986, 1987*a*, *b*).

My next example concerns lipid peroxidation, a free radical-mediated reaction already mentioned above. The attack of an oxidising free radical species such as $CCl_3OO^{\bullet}$ on a polyunsaturated fatty acid (e.g. arachidonate) can result in a covalently-bound adduct, or in hydrogen atom abstraction thereby initiating peroxidation. The latter process can result in a complex variety of products as shown in Table IV. Some of these products have very interesting properties. For instance, as recent work by Dormandy's group has shown, there are fascinating changes in dienes in alcoholism and certain other diseases (see Dormandy, 1985). The lipid hydroperoxides affect the prostaglandin cascade at micromolar concentrations; the epoxy fatty acids affect the release of some hormones at nanomolar concentrations. In addition, there is a large family of aldehydes, ketones and alkanes that are products of lipid peroxidation; we have concentrated our work on the unsaturated aldehydes, especially the 4-hydroxy-alkenals, in collaboration with Esterbauer's group in Austria and Dianzani's group in Italy. Some of the aldehydes detected as products of the peroxidation of rat liver endoplasmic reticulum are butanal, hexanal, hexenal, octanal, 4-hydroxy-hexenal, nonanal, nonenal, 4-hydroxy-nonenal, 4,5-dihydroxy-decenal, and malonaldehyde. Of these, one of the most biologically active is 4-hydroxy-nonenal, first identified by Benedetti, Comporti and Esterbauer in 1980 (Benedetti *et al.*, 1980). The biological properties of this compound, and related hydroxy-alkenals, are notable. Hydroxy-alkenals inhibit DNA synthesis at really quite low levels, much lower than their effects on protein or RNA synthesis; Dianzani's group have shown effects on adenyl cyclase at micromolar levels: they react rapidly and non-enzymically with thiols. We have found an effect on platelet aggregation in the micromolar range (Slater *et al.*, 1986), and they affect white cell movement, again Dianzani's work, in the nanomolar range; so that these products of lipid peroxidation have a wide range of biological activities at low concentration. This leads to another concept (Slater, 1976): if a reactive free radical is produced in a membrane we know from the preceding discussion that its diffusion radius is limited; however, by initiating secondary processes such as lipid peroxidation the derived products can have a longer lifetime, larger radii of diffusion and can diffuse really quite appreciable distances and thereby extend the cellular damage. So, in this way, what starts off as a highly localised event in a biological membrane can result in severe metabolic perturbances at a considerable distance. One other comment on lipid peroxidation can be given here. It is often said that lipid peroxidation is inhibited by the addition of metal chelating agents. Well, of course, those who work in the field know that this is a generalisation that does not always apply. You have only got to look at the long-standing distinguished work of Aust and his group in the States to recognise that point. Another example can be obtained from data that we reported a few years ago now for some metal chelators (Cheeseman *et al.*, 1981) with o-phenanthroline; as the concentration of the chelator is increased lipid peroxidation also increases. What is more, the metabolic activation and covalent binding of CCl_4 to microsomes also increase. The example just given is one of several that are now known; other chelators that also stimulate lipid peroxidation under appropriate conditions are αα-dipyridyl and trifluoro-thenoyl-butane dione. On the contrary, desferal is a very strong general inhibitor of lipid peroxidations of the type discussed above although some pro-oxidant effects have been reported (Slater and Sawyer, 1971). It is the result of changes in the redox potential of the chelated transition metal ion (usually Fe^{2+} or $Fe^{3+}-$) thereby either facilitating or inhibiting one electron movements.

Table IV Major products of peroxidation of unsaturated fatty acids such as arachidonate ($C_{20:4}$)

Lipid hydroperoxides (e.g. HPETE's)
Hydroxy fatty acids (e.g. HETE's)
Epoxy fatty acids
Alkanals
Alkenals
4-hydroxy-alkenals
Dienals
Alkanes

Now I want to turn to another main example, to leave the liver and go to uterine cervix, to mention a problem that has interested me for nearly 25 years, and a problem that is reaching an interesting state of development. Cancer of the cervix can, of course, be completely cured if the early signs are recognised and treated; however in some parts of the world the early signs are not consistently recognised or treated, and cancer of the cervix in some of these areas is now a major cause of death by cancer.

In this country and the Western world early lesions of the cervix are increasing in incidence. Some work done by Margaret Wolfendale (see Wolfendale *et al.*, 1984) a few years ago in the Oxford region showed that the incidences of early lesions of the cervix in women that had either been screened or not previously been screened over 5-year periods increased quite strikingly from 0.5 to 3.7, and 3 to 9 per thousand smears examined respectively. In relation to invasive cancer of the cervix, some data from the National Cancer Institute, Bangkok with whom we collaborate, shows that the number of patients with advanced cancer of the cervix seen in one of the Institute clinics has increased considerably over the last few years. Taken together with the increased incidence of early lesions seen in parts of the Western world, there is cause for some apprehension.

Now this leads me on then to an example where a report

in the literature turned out to be the result of an artefact but, nevertheless, resulted in some interesting developments. In the mid 1960s we were trying to find a chemical method for screening for cancer of the cervix, which could be automated and used on large numbers of patients. My attention was then caught by a report that showed an esr spectrum of a mouse tumour (Brennan *et al.*, 1965): normal cells gave a single line and the tumour cells gave a triplet. I thought at the time, if this difference also occurred in cancer of the cervix, it might be helpful in directing us to some early biochemical changes in cancer of the cervix. However, when we examined human cervix using esr we got a result that was completely different from the original report of Brennan *et al.* (1965): what we had hoped to see was a qualitative difference, in one case a triplet, and in the other case a singlet. In fact, what we found was something really completely unexpected: we found (Slater, 1971; Benedetto *et al.*, 1981) that in the normal human cervix there was a very strong signal, in fact as far as I know the strongest esr signal reported in biological material, whereas in cancer of the cervix the signal was very much reduced or absent. We were of course puzzled as to why our result was so different from the changes reported earlier in mouse tumour but then it turned out that the results presented in the early study on mice had been caused by changes occurring during the preparation of the tissue. What we had found in fact, with human cervix, turned out to be more or less a consistent finding with many tumours: that is, tumour tissue tends to have a low esr signal compared with normal. Recently we have taken up this story again and have been able to show that the strong signal in normal human cervix is a lipid peroxyl-radical species. Moreover, the normal human cervix has very active cyclo-oxygenase and lipoxygenase activities. We thought that lipid peroxyl radicals might have an interesting effect on aspects of redox balance within the cells and so we set up an investigation in collaboration with groups in Austria, Turin and Bangkok to look at human cancer of the cervix. The example reported here relates to reactive protein thiol groups. These can be stained in sections of the cervix or in isolated cells, and the intensity of stain measured quantitatively. In sections of cervix, a marked difference in intensity of staining is seen between epithelium and stroma. Moreover, the ratio of staining intensity in epithelium: stroma is very different in normal tissue compared with tumour tissue (see Nohammer *et al.*, 1986). A number of interesting developments have come out of these studies; for example, similar changes can be seen around the lesion almost like a field effect. To cut short a rather complicated and long story, we now have good procedures for sections that allow discrimination between normal and abnormal tissue samples. Our current activity is directed at trying to turn these rather sophisticated, time-consuming measurements on sections into simpler methods for cervical smears.

Finally, I want to consider some work we have done recently on lipid peroxidation in liver tumour cells. It has been known for quite a long time that many types of liver tumour peroxidise much more slowly than do samples of normal liver (for references, see Cheeseman *et al.*, 1986*a*). Our studies have concentrated so far on the Novikoff tumour, the Yoshida tumour, and ethionine- and aflatoxin-induced tumours. With each of these types of liver tumour there is a much diminished rate of lipid peroxidation compared with normal liver. This conclusion applies whether the type of peroxidation studied is enzyme-catalysed, or is non-enzymic such as that initiated by ionising radiation. The conclusion we have reached in such cases is that the major contributory factor to the reduced rate of peroxidation is an increase in the tumour's content of lipophilic antioxidant. In fact, direct measurements of lipid soluble, chain-breaking antioxidant in the Novikoff and Yoshida tumours clearly shows a considerably increased content compared with normal. Moreover, the major contribution to this lipid antioxidant activity is α-tocopherol (Cheeseman *et al.*, 1986*a*). A question that quickly occurred to us was: is the change in lipid peroxidation just referred to a distinctive feature of liver tumour cells or is it a reflection of cell division that in normal liver is present to a very small extent? To study this question we have turned to the normal regenerating liver where cell division is considerably stimulated. In this study (see Cheeseman *et al.*, 1986*b*), cyclical changes in DNA-synthesis have been found, as previously reported (Hopkins *et al.*, 1973) and accompanied by corresponding inverse fluctuations in lipid peroxidation. Thus, when DNA-synthesis is at a maximum lipid peroxidation is suppressed, and *vice-versa*. These changes in lipid peroxidation appear to result from movements of lipid soluble antioxidant, in and out of the regenerating liver, to ensure corresponding changes in lipid peroxidation. One can immediately ask: why does this occur? One answer may be that the genetic material, relatively uncovered during mitisis, becomes susceptible to peroxidative and free radical damage: to combat this danger, cells have evolved a mechanism for changing their antioxidant content. Another and inter-related possibility is that lipid peroxidation acts as a coarse control of cell division through the biological activity of some of its products (e.g. 4-hydroxy-alkenals; see Slater, 1973); this mechanism, also, requires changes in antioxidant content at different stages of the cell cycle. This latter possibility, when considered together with recent findings on the biological activity of such peroxidative products as epoxy-fatty acids (Capdevila *et al.*, 1983) and lipid hydroperoxides suggests that a physiological role for lipid peroxidation may well be of considerable significance.

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Discussion

Cramp: One of the difficulties in radiation work if you are looking for lipid peroxidation is that you very often have to give very big doses compared to those used in cell survival studies. Can you give us some idea of what your concentration of attacking species are with respect to their ability to kill cells or cause tissue malfunction?

Slater: Well, actually that is an almost impossible question to answer at the present time. It is, however, a very important question and one that we are in fact in the process of tackling. The reason why it is difficult to answer your question is that, by and large, the methods that are available for measuring lipid peroxidation in biological environments are not very good. You can get round that objection to some extent by using a variety of different methods. For example, following changes of substrate, following oxygen utilisation in certain situations, looking for diene conjugations or alkane gas evolution, or malonaldehyde or lipid hydroperoxide, by chemiluminescence or whatever. But, in general, it is not very easy to be sure about the levels of low rates of lipid peroxidation in organised systems. We have to realise also that many of those components that I mentioned as indicators of lipid peroxidation also undergo metabolism in organised systems so that if you are only going to produce them at low rates you may well see no change in their steady state concentration since they are metabolised as fast as they are produced. For example, malonaldehyde is readily metabolised and also binds to neighbouring structures by straightforward chemical means. Lipid hydroperoxides are metabolised; diene conjugates are metabolised, and so on. This all adds up to a very difficult situation in trying to decide if, in a particular type of tissue disturbance, a low rate of lipid peroxidation has any primary importance. It is not so difficult to be sure in cases where you have a high rate of lipid peroxidation because you can detect this disturbance even with the rather insensitive methods that I mentioned. But the real problem is associated with a low rate of lipid peroxidation that results, perhaps, in some local injury, but one cannot be completely sure because the currently available methods are inadequate. I can add that some of the breakdown products of lipid peroxidation have rather profound biological effects. For example, the epoxy fatty acids which Capdevila's group have shown have effects on receptor function at 10^{-9} M. Also the hydroxy alkenals have effects at 10^{-9} M on chemotaxis so this really does make interpretations of the significance of low levels of lipid peroxidation not only difficult but potentially of great importance.

Ward: You mentioned the specificity of the $CCl_3O^{\bullet}_2$ radical. What is the specificity and why are phage more susceptible to $CCl_3O^{\bullet}_2$ than to the hydroxyl radical?

Slater: This is Robin Willson's work with Karlo Hiller on phage.

Willson: We are not sure but it looks as if it could be damage to protein. $CCl_3O^{\bullet}_2$ is unlikely to react very rapidly with DNA for example, and it is known to react with considerable specificity with proteins. Methionine, cysteine