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**APPENDIX 4**

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# EARLY MECHANISMS IN RADIATION-INDUCED BIOLOGICAL DAMAGE

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Because of time limitations, this introduction to the session on mechanisms of radiation action in biological systems cannot deal with all of the important questions that come to mind when one tries to decide what his work is telling him about the nature of the radiation-damage process. Accordingly, I shall choose several that might be used as types and examine briefly the experimental results that should arouse caution in us when we are tempted to write at length "on the basis of these results, ..." There are many areas that will be mentioned only; some are omitted. All are significant.

Recognition of the oxygen effects. An unequivocal demonstration that the "oxygen effect" is really two oxygen effects, at least, was presented 23 years ago for the bacterial spore (Powers et al., Rad. Res. Suppl. 2, 94, 1960). A number of characteristics and properties of each of these effects has been recognized and further, a third and perhaps a fourth oxygen effect have been demonstrated. Some recent publications have given evidence for more than one oxygen effect in other systems, including mammalian cells, but most authors claim that there is only one oxygen effect in cell systems. Since the original papers on oxygen concentration and radiation effects in living cell systems by Howard-Flanders and Alper (Rad. Res. 7, 518, 1957) and by Deschner and Gray (Rad. Res. 11, 115, 1959) a model is used that we have shown to be only a restatement of the widely used Michaelis-Menten function first proposed in 1913 to describe the dependence of the velocity of enzyme catalysed reactions on concentration of substrate. Since 1932 some linear transformations of the original equation have been available; and, because of the uncertainties of dealing with a curvilinear response and especially one that approaches a maximum asymtotically, the use of these linear transforms should be required.

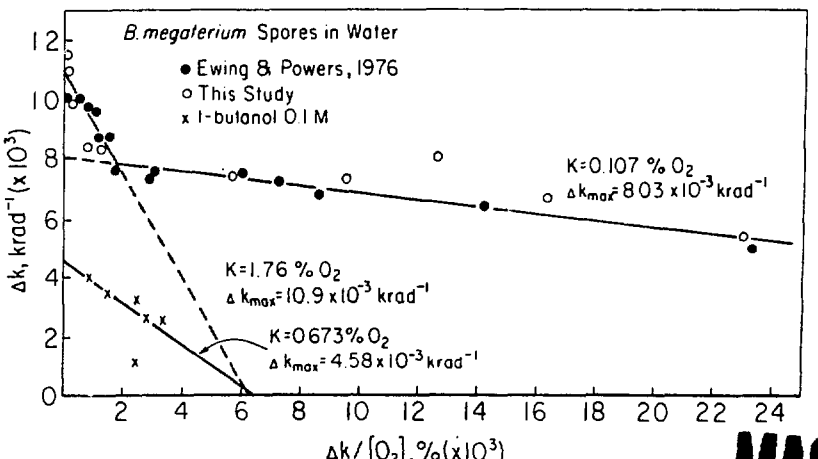


Fig. 1. The dependence of Δk on [$O_2$] in bacterial spores presented as the E-H transform.

Since the experiment involving oxygen concentration and increased radiation sensitivity is of the same form, I have used these transforms to analyse certain numbers that have been described as demonstrating a single oxygen effect (Powers,

Int. J. Rad. Biol. 42, 629, 1982). The first, the Lineweaver-Burk transform, involving a double reciprocal plot is, for unclear reasons, the one most frequently used by the enzymologist and physical chemist. But it is frequently acknowledged that the so-called Eadie-Hofstee transform in which the increment in velocity (in our case sensitivity,) is plotted against the quotient of the increment in velocity divided by the concentration of the substrate (in our case oxygen or other modulator), is far superior because of the distribution of points and the direct recognition of the value of the constants. This we used to analyse literature data on the dependence of sensitivity on oxygen (or other sensitizer) concentration.

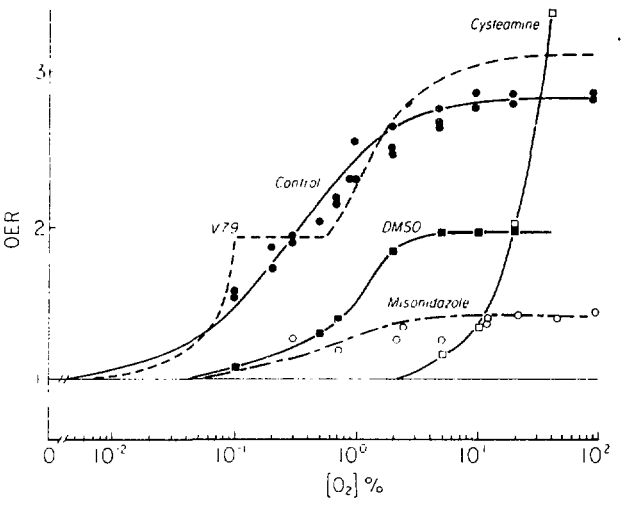


Fig. 2. CHO cells irradiated with $^{60}Co$ under the conditions noted. [DMSO] =1 M.

In the wet state there is evidence for two oxygen effects in spores: from the plot with raw data there are kinetics at low $[O_2]$ different from those at high $[O_2]$ (Ewing and Powers, Science 194, 1049, 1976); and that the one at low concentration could be modified by t-butanol. The E-H transform as shown in Fig. 1 also distinguishes between two sets of experimental points: the originals of Ewing and Powers and those added later. Clearly, on both sets of numbers there are two processes justifying the original interpretation of the directly plotted data that the plateau at intermediate concentrations represents the end of one process and the beginning of another of a different kind. The transform confirms that in t-butanol the low $[O_2]$ component disappears completely. The high $[O_2]$ response is not affected by alcohol at all.

Using data from mammalian cell studies that apparently show one $O_2$ effect, we have demonstrated that several sets of data are resolvable into two components: one being linear at low $[O_2]$ while the other is linear at high $[O_2]$ with separate numerical properties just as in the spore. Fig. 2 is from Whillans (pp. 277-284 in Oxygen and Oxy-Radicals in Chemistry and Biology, Academic Press, New York, 1981) in which the relationship between $[O_2]$ and radiation sensitivity and the effect of added dimethyl sulfoxide are pictured. The averaged numbers are put in the E-H transform style with resulting Fig. 3. There are clearly two sets of numbers in the unmodified oxygen effect, one at low $[O_2]$ and another at high $[O_2]$. DMSO clearly removes the lower leg and changes the slope of the upper one. The effect of

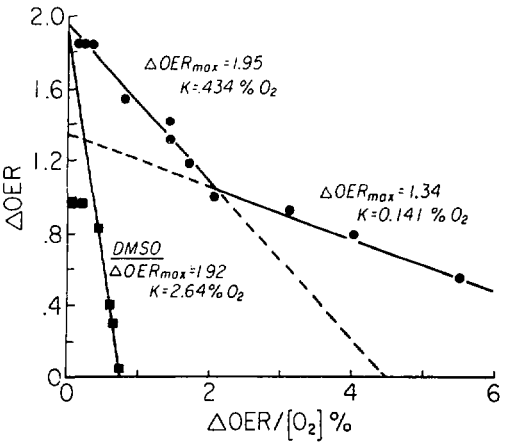


Fig. 3. The Eadie-Hofstee plot of data in Fig. 2.

DMSO at low $[O_2]$ in CHO cells is like that of t-butanol in spores, but the effects of the two are different at high $[O_2]$. We cannot here consider all the various interpretations that these kinds of results suggest. There is an immense algebra generated over the years that has resulted in very fine interpretations of the actions of enzymes and how they can be modified mechanistically. With this demonstration that $O_2$ effects can be analysed in this way, these techniques are open to the radiation biology community. And interactions, competitions, interplays of various sorts amongst the various additives can be used to recognize ways of potentiating and decreasing radiation sensitivity and to suggest mechanisms.

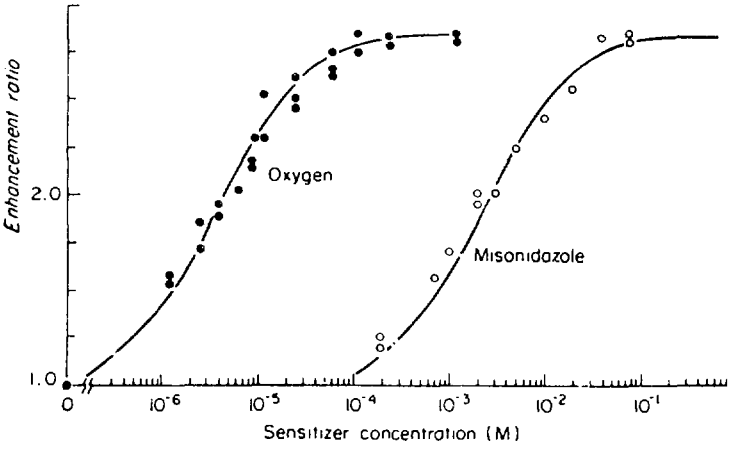


Fig. 4. CHO cells irradiated by $^{60}Co$.

This is not confined to the action of oxygen only, as demonstrated by transforming recent data of Whillans and Hunt (Rad. Res. 90, 126, 1982 [Fig. 4]). The oxygen numbers are those analysed in Fig. 3. The misonidazole data are transformed into the E-H relationship and presented in Fig. 5. And Fig. 6 is the relation plotted in the original form the solid line being the Michaelis function determined by the parametric values of the straight line. This agrees with our earlier demonstration (loco cit.) that the results of Ling et al. (Int. J. Rad. Onc. Biol. Phy. 6, 583, 1980, and Rad. Res. 86, 325, 1981) from CHO cells with misonidazole and $O_2$ are resolvable by the E-H transform into two separate processes,

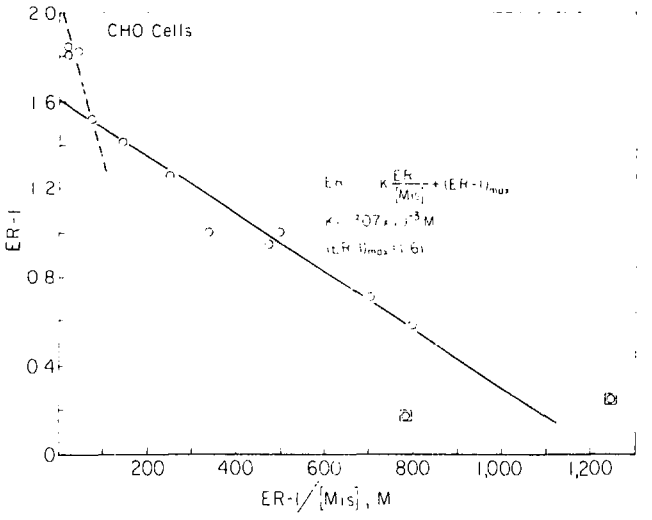


Fig. 5. The data from Fig. 4 in the Eadie-Hofstee form.

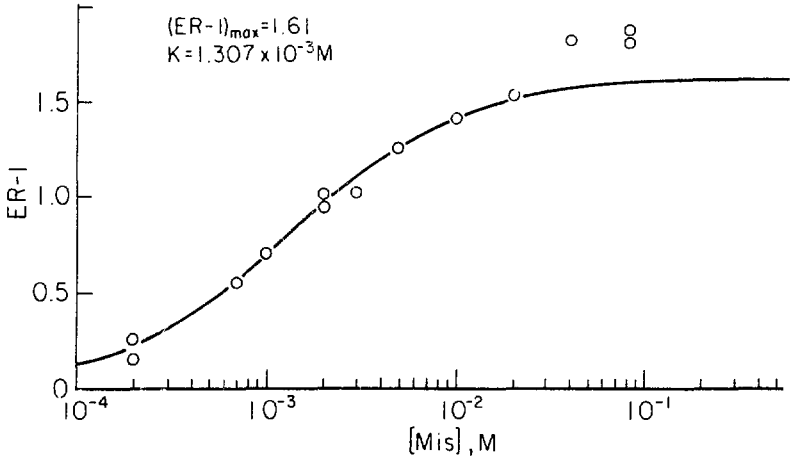


Fig. 6. The Michaelis-Menten plot using data constants from Fig. 5.

one at high and one at low concentration.

But why have these studies not shown two sets of processes in the original numbers, as they show in the spore? It is in the nature of the function itself and the behavior of the response at low and high concentration. Fig. 7 demonstrates the effect of holding hypothetical K constant and methodically increasing maximal increment. It is possible that direct data, even with some variation, might be separable with this behavior. Consider, however, the situation in which systematic change in the values of the two constants occurs as conditions of the experiment change simultaneously. In Fig. 8 there are three responses plotted in the E-H transform, with K and maximal increment in sensitivity changing from one experiment to the next. The three intersect at a common point. Now generate the lines determined by the Michaelis-Menten function and Fig. 9 is the consequence. We see that at top concentrations there might be a prayer of distinguishing among the three different responses, but at the lower concentrations there is little hope. The several points plotted at the low concentrations in this hypothetical situation are systematically separated, but not by much, and a small variability in this system would cause overlap.

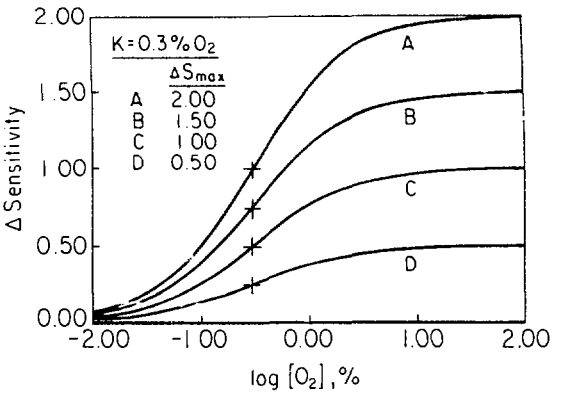


Fig. 7. Hypothetical competition curves with constant K and varying $\Delta S_{max}$.

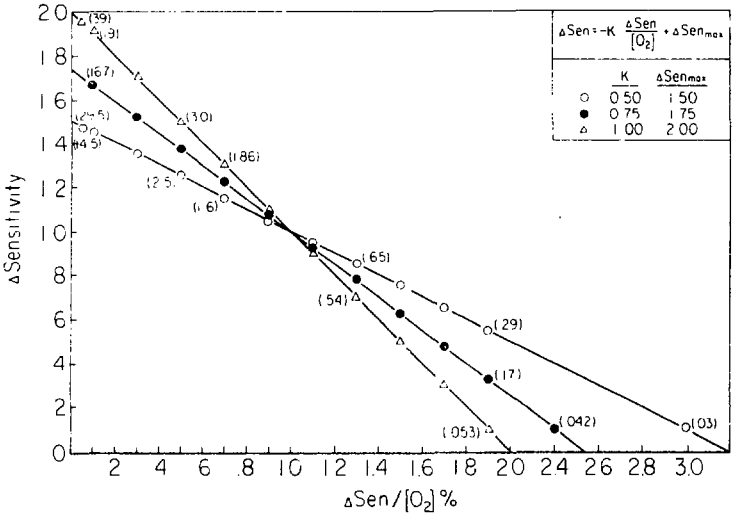


Fig. 8. Hypothetical E-H plots of data with varying K and $\Delta S_{max}$. Numbers in () = $\%O_2$.

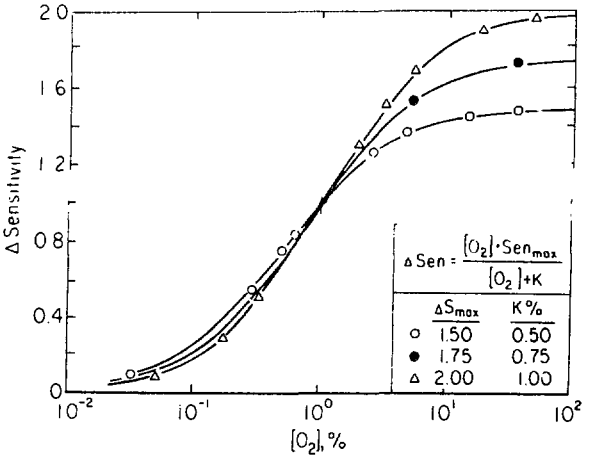


Fig. 9. The Michaelis-Menten plot of data of Fig. 8.

These methods of examination of the data not only tell us that the relationship between sensitizer and radiation sensitivity is much more complicated than much of the literature suggests. But they will tell also what data are required in a set to determine the response, and what experiments have to be done to understand the mechanisms by which the sensitizer operates; and allow the use of the in-depth algebraic

considerations of competition experiments of the enzyme kineticists to radiation biology. For instance, it should be possible to methodically change the concentration of the hydroxyl radical scavenger and modify the lower leg of the E-H transform progressively to yield a series of numbers that would give us a competition rate that should be equivalent to the reaction rate of the hydroxyl radical with the scavenger, if, indeed, it is the removal of •OH that is occurring.

Is the hydroxyl radical important? There should be an easily gained answer to that question. The literature in radiation chemistry cites many compounds that can remove •OH rapidly, and some that increase its yield. Use these on the cell. But reduction in cell sensitivity by alcohols, for instance, does not necessarily mean involvement of •OH in the radiation effect. What has to be considered?

In early studies Johansen and Howard-Flanders (Rad. Res. 24, 184, 1965) and Sanner and Pihl (Rad. Res. 37, 216, 1969) used vegetative bacterial cells and numerous •OH scavengers to build only an approximate correlation between •OH scavenging efficiency and protective capability. These are not critical demonstrations, but they pointed up the problem.

This problem is even more difficult in mammalian cells because of their fragility. Chapman et al. (Rad. Res. 56, 291, 1973) used DMSO that is tolerated by the cells; and because it protects and also reacts well with •OH, removal of •OH was called important by these authors.

However, in contrast, DMSO in spores is a sensitizer. Also, DMSO extends the lifetimes of oxygen sensitive radicals in spores in aqueous suspension (Ewing, Int. J. Rad. Biol. 41, 563, 1982), just as it extends the lifetimes of organic radicals detected and measured by EPR spectrometry (Sridhar, R., Personal Communication). The sensitizing action of DMSO in anoxia has been observed independently at Austin, Texas. The suspicion must be that the actions of DMSO are many and complex, and choosing just one should not be allowed.

Extensive and methodical inquiry into •OH action in radiation damage has been made with the bacterial spore, a cell that tolerates extreme and toxic conditions, and responds to radiation as do vegetative cells. There are some lessons in the results obtained.

Nitrous oxide doubles •OH yield in irradiated water. Alcohols react rapidly with •OH. In spores $N_2O$ sensitizes and alcohols reduce this sensitization to near $N_2$ levels (Powers and Cross, Int. J. Rad. Biol. 17, 501, 1970) as expected if •OH is important. With a series of low ethanol concentrations, we demonstrated exhaustion of alcohol protection at different and serial radiation doses; but at doses much higher than expected. Just later we proposed a two step oxidation model involving •OH and $H_2O_2$ to explain their behavior and relation to lethality in our spore experiments (Int. J. Rad. Biol. 24, 207, 1973). This was based on removal of •OH and $H_2O_2$ separately. That model seemed competent for spores irradiated anoxically, although not in high [$O_2$] environments. $N_2O$ has been used also by several other laboratories in similar studies: with E. coli by Samuni and Czapski (Rad. Res. 76, 624, 1978); in Pseudomonas by Watanabe et al. (Rad. Res. 89, 325, 1982 and 88, 557, 1981); and in mammalian cells by Roots et al. (Rad. Res. 92, 245, 1982).

A modification of the oxidation scheme has been proposed by Ewing (Rad. Res. 94, 171, 1983) after extending the observations to include superoxide dismutase. He proposes that the relation $H_2O_2 + {}^{\bullet}OH \rightarrow H_2O + {}^{\bullet}O_2^- + H^+$ is important because SOD reduces sensitivity if, under anoxia, reagent $H_2O_2$ is added to sensitize, or irradiation is done under low $[O_2]$. (N.B. Under both conditions electrons are removed [i.e. we are in "sensitizer" conditions].)

These results (insufficiently described here in this brief communication) might to some justify firm belief that ·OH is an important species in cellular radiation damage, and that an alcohol, nitrous oxide or DMSO experiment or two can demonstrate it. However, there are complications that must be considered. Because in anoxia some alcohols protect, others do not. While very high concentrations of one alcohol (glycerol) were found to be protective in anoxia (Webb et al., Int. J. Rad. Biol. 7, 481, 1963), in the early experiments others gave no reduction in anoxic sensitivity in spores. Later, in the extended study of Ewing (Rad. Res. 68, 459, 1976) we learn that alcohols can be divided into two groups--those that form radicals with unpaired spins on the α-carbon (as methyl, ethyl and allyl in Table 1), and those that do not (as t-butyl, t-amyl and benzyl in the table). The first three can protect the spores in anoxia; the second three do not. The conceivable importance of the reducing capacity of the organic radical formed after reaction with ·OH was pointed out by Richmond et al. (Rad. Res. 63, 140, 1975), and Ewing's experiments almost surely demonstrate it. Simple removal of ·OH--or removal of ·OH per se --is not the reason for protection under these experimental conditions. The reducing species must be produced. Is it a competitor of some damaging (oxidizing?) species other than ·OH?

TABLE 1

| Alcohol | $k_{OH} \times 10^8$ $M^{-1} s^{-1}$ pH7 | Product |
|---|---|---|
| Methyl | 6.0 | $\dot{C}H_2OH$ |
| Ethyl | 11.0 | $CH_3\dot{C}HOH$ |
| Allyl* | 20.0 | $CH_2{:}CH\dot{C}HOH$ |
| t-Butyl | 4.2 | $CH_3\overset{\displaystyle \dot{C}H_2}{\overset{\displaystyle \vert}{\underset{\displaystyle \vert}{COH}}}\underset{\displaystyle CH_3}{}$ |
| t-Amyl | 18.5 | $CH_3CH_2\overset{\displaystyle \dot{C}H_2}{\overset{\displaystyle \vert}{\underset{\displaystyle \vert}{COH}}}\underset{\displaystyle CH_3}{}$ |
| Benzyl | 84.0 | $HO\dot{C}_6H_5CH_2OH$ |

*12% of product

In the case of the action of some sensitizers, however, simple removal of ·OH does appear to be the reason the alcohol reduces the increment in damage caused by the sensitizer. Such appears to be the case in the $N_2O$ experiments cited above. Another example is Fig. 10 (Ewing, Rad. Res. 73, 121, 1978) in which the reduction in sensitivity is strictly correlated to the hydroxyl radical scavenging efficiency of the alcohols tested--including alcohols of the two kinds above. This latter kind of evidence is required, if one claims that reduction of effect by alcohols is evidence for direct participation of ·OH in radiation effects. The same kind of evidence applies to sensitization by PNAP and to low concentrations of $O_2$--there is a ·OH component in the sensitization process that can be prevented by the per se removal of ·OH. (Always remember this is accompanied by a non ·OH component.)
A brief summary of the ·OH questions:
1. Removal per se of ·OH is responsible for reduction of some sensitizers' actions; so, the ·OH is involved in the sensitization process. This is established after tests of a variety of ·OH scavengers, and the showing of good correlation with scavenging efficiency.

2. But removal per se of •OH is not responsible for protection demonstrated by several alcohols in anoxically irradiated spores. Rather it is the production of certain alcohol radicals after •OH reaction: if the unpaired spin is on the α-carbon, it is protective; if it is on the β-carbon it is not protective. The OH radical generates a protective radical that interferes with the action of a third party.

3. A variant of the action of the second kind operates for one sensitizer--cobalt hexammine chloride ($Co[NH_3]_6Cl_3$).

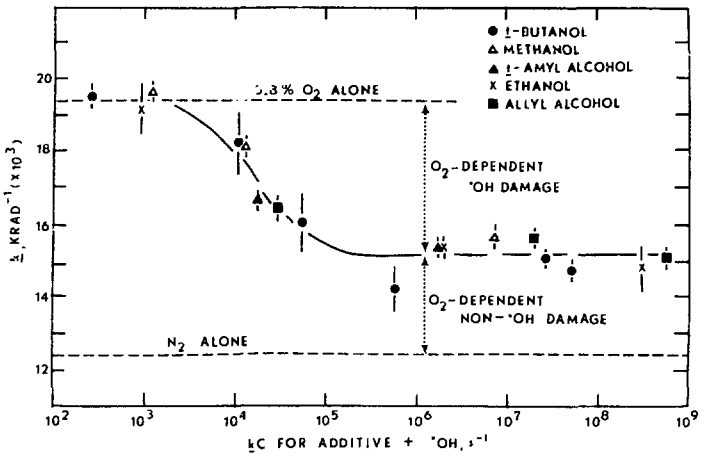


Fig.10. Reduction of sensitivity in spores.

Ethyl alcohol reduces sensitization caused by $CoA_6$ and if mechanism 1 above operates, so should t-butanol. But, instead, it sensitizes. Here the ethanol radical may interfere directly with $CoA_6$, perhaps by reducing it, which t-butanol radical would not.

4. Then, there is the further problem of the possibility of at least two actions as we show above using transforms of the Michaelis-Menten equation. Upon which of the pair of processes does the •OH act?

Has a range of concentrations been tested? The occurrence of peaked efficiency is not uncommon, and its existence can lead to much confusion. Nitric oxide was first reported a sensitizer (Howard-Flanders, Nature 180, 1191, 1957); later in another system it was revealed a strong protector (Powers et al. Rad. Res. Suppl. 2, 94, 1960); later it was shown to sensitize at low [NO], and, passing through a peak, to protect at high [NO], first by Dale et al. (Int. J. Rad. Biol. 4, 1, 1961) and repeated by Johansen et al. (Rad. Res. 24, 184, 1965); then to have two simultaneous actions--one protective and the other sensitizing (Powers et al. PNAS 46, 984, 1960). Also, biacetyl is a sensitizer of spores at low concentration and a protector at high concentration (Tallentire et al. Int. J. Rad. Biol. 14, 397, 1968); and this property was used as the basis for the "electron sequestration" theory of chemical sensitization to radiation (Powers, Isr. J. Chem. 10, 1199, 1972). The peak is the consequence of high reaction rates of the compound with both $e^-_{aq}$ and •OH. Also in the spore the sensitizers p-nitroacetophenone and acetone sensitize in a "peaked" fashion (with maximal efficiencies over short concentration ranges) and acetophenone suggests one (Tallentire et al. Isr. J. Chem. 10, 1185, 1972). The strong dependence of the action of a variety of sensitizers on concentration is discussed elsewhere in this report. In those instances in which a saturation at high concentration is observed, mechanical errors are not important; but for peaks that is not true. Further, even in saturation cases, the mechanism at high concentration is not that at low necessarily. An interesting particular instance is maleate, a compound called inactive with respect to sensitization some years ago, but found later to be a sensitizer at the high concentration predicted by its redox potential (Simic and Powers, Int. J. Rad. Biol. 26, 87, 1974).

Are you allowed to pick the mechanism you like from an array? The obvious answer is no, but not everyone believes that. At one time not so long ago

(1950's) when the big thing in radiation biology was protection by chemical agents, cysteamine was a much used reducer of radiation effect--because it is a sulfhydryl that could chemically repair radicals. Ten years later cysteamine--because of its high reaction rate ($1.5 \times 10^{10}$ $M^{-1}$ $s^{-1}$) with $^{\bullet}OH$--is used to support the proposal that hydroxyl radical is important in vegetative bacterial cells. Ten years later the same argument is used to support $^{\bullet}OH$ involvement in mammalian cells. But the only thing measured is reduction in sensitivity. The uncertainties involved in the use of DMSO because of its several effects are detailed elsewhere in this report. The action chosen as the important one is sometimes dictated by the politics of the times.

Other considerations. Understanding of physical and chemical mechanisms involved in radiation damage in cells should be expected only after rigorous consideration of all possibilities. In the several examples cited above, we note: a single compound has two chemical actions and either can be protective, and the choice between the two is sometimes a modish one; sensitizers can have protective actions as well, depending on the concentration; sensitizers can have two sensitizing actions, depending on the concentration; competitive protectors can act in one manner on one sensitizing action and in another manner on the other; sensitizers have double actions that cannot be recognized with the algebra in vogue today; protectors can act by $^{\bullet}OH$ removal, but only when a sensitizer is present; the same protectors can protect in the absence of sensitizers but only if after $^{\bullet}OH$ reaction a certain class of organic radical is produced, and it is the protector. The minimal requirements for model construction should be that it should be testable, subject to attack; and it should attempt to rationalize what is known, or it must be restricted explicitly to designated parts of the body of knowledge such as that above. Unfortunately, these conditions are infrequently met by our collegiate model builders.

There are several additional questions, facts, and problems that deserve examination and discussion in this context for which there are no time and space here. Among them we can mention briefly the following: where is the site of action--inside or outside the cell, in or out of the nucleus, is DNA involved, and what kind of evidence is required for these questions; do the so-called "$O_2$-mimics" really act like $O_2$ (in some ways yes, and in others no--and use of "$O_2$-mimic" is prejudicial); does water have a role other than acting as a source of free radicals--is the state of water and its relation to biomolecules important (the answer is "yes" to each question); to what extent are solvent effects operating when we use high concentrations of radiation modifiers; why do we frequently see breaking survival curves (biphasic survival curves to some) that cannot be explained by known radiation chemistry; what is the importance of the molecular excited state in high energy radiation biology, and, in this connection, the role of energy transfer at the macromolecular level; when shall we apply knowledge from non-polar chemical systems to the response of the cell, consisting as it does of many non-polar elements of critical importance? We are only at the beginning of inquiry into fundamental mechanisms in radiation biology.

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DEDICATION: To Michael Ebert.