CONF-720115--2

The hydroxyl radical and the hydrated electron
in radiation effects in cells.

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Symposium

"Molecular Basis of Radiation Biology"

3-5 January 1972

Department of Physical Chemistry

The Hebrew University of Jerusalem

Work supported in part by U.S. Atomic Energy Commission
Contract AEC At-(40-1)-3408 and National Institutes of
Health Grant PHS GM 13557.

The hydroxyl radical and the hydrated electron
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Our first paper on the modification of radiation effects by extraction of water appeared in 1953, 19 years ago this year.¹ Our first paper on the bacterial spore system was published in 1957, 15 years ago this year.² Prior to the publication of that, and since, many persons have worked steadily to develop the spore story. Among these have been Charles Ehret, Robert Webb, a number of skillful research assistants at Argonne, Alan Tallentire working independently and at times with us, Tamiko Iwasaki, Marian Cross, John Wilson, several graduate students--Bob Richmond, Bruce Kimler, Skip Johnson, David Ewing, Nelson Schiller--and a number of devoted research assistants at The University of Texas. We are fortunate now to count Michael Simic as part of our team.

The interest originally was the radiation sensitivity of the dry system and how it can be modified by the gradual addition of water. The dry system has been described well.³ Its characteristics have been delineated quite completely, although not yet understood completely. Most recently our interest has been in the fully wetted spore, to describe the properties of it in terms of recent radiation chemistry.

Today I shall give a brief description of the general role of water in radiation sensitivity as we see it now, and then describe in particular the recent experiments (last two years) that have suggested to us a model for the participation of water in radiation damage in fully wetted cells, as well as for the mode of action of some sensitizing compounds.

The General Role of Water in Radiation Damage.

First the analytical method (Fig. 1).⁴ We plot surviving fraction against dose for each experimental condition. When irradiation of dried spores is in nitrogen followed by water treatment (condition A), the least radiation sensitivity is observed. The slope is smallest. We call that k_I in the general algebraic expression for the response. Curve B, which shows the next higher sensitivity, happens when irradiation is in nitrogen followed by exposure to oxygen prior to immersion in water. In this case, an element of sensitivity has been added, an element that we call k_{III} . This is removed by water treatment before O_2 exposure, as in Curve A. Almost certainly k_{III} is a measure of the contribution of oxygen-dependent long-lived free radicals which operate in the dry spore to bring about part of the radiation damage. In Curve C where the sensitivity is the highest (irradiation in oxygen), we see that another element of sensitivity has been added; so that now k_{O_2} is $k_I + k_{III} + k_{II}$, the last being observed only when oxygen is present during irradiation. It has been our goal to describe the properties of each of the elements separately. Obviously this is required if any one of these properties changes in a way different from any others as changes in environmental conditions occur--water content, temperature, and so forth.

The first complete study of the role of water in the spore was described by Tallentire and Powers (1963).⁵ In Figure 2, we see that each element is separated from the others: k_I is water-independent up to approximately 10^{-6} Torr water vapor pressure; k_{III} , the free radical fraction, operationally disappears as the cells are increasingly wetted, with an "immediate" oxygen effect being the only O_2 element remaining at

saturation. With respect to the oxygen effects, the cells are very much more sensitive in the dry state than they are in the wet. Water is a protector. Note also, however, that for the anoxic response water is a sensitizer, for at saturation an increase in radiation sensitivity in k_I is observed.

Our analysis at the time allowed for several interpretations of the immediate oxygen effect at saturation. In Figure 3, several analytical schemes are presented.⁶ According to one scheme, the k_{III} element disappears completely when the cells are fully wetted (Scheme A), in which case k_{II} is the oxygen effect in the wet cell. Another scheme (D) says that k_{II} disappears completely during the process of wetting and, therefore, k_{III} is a measure of the damage that occurs in the fully wetted cell. The other two systems are conceptually more attractive. These say that k_{II} and k_{III} decrease together as water is added, and that the oxygen effect being observed in the fully wetted state is a combination of k_{II} and k_{III} . That is, there are two oxygen effects in the wet cell as we have clearly demonstrated for the dry cell. Note that in one system the partitioning is 60-40. This may be of some importance in the interpretation of certain sensitization results you will hear about in this conference.

Close examination of the anoxic part of the response reveals a sharp increase in radiation sensitivity as D_2O is added above approximately 5×10^{-1} Torr EVP and approximately 1 Torr when H_2O is the added water (Fig. 4).⁷ Note the interesting difference between H_2O and D_2O in these experiments. It is clearly a water-dependent anoxic kind of damage occurring in this spore. When hydrogen sulfide is present during irradi-

ation (Fig. 5) the anoxic response is sub-divisible into 2--one which is removed when hydrogen sulfide is present during irradiation, the other which persists.³ We call these k_{Ia} and k_{Ib} . As cells are wetted in the presence of and in the absence of hydrogen sulfide, the responses are such that we can recognize a water-dependent and a water-independent component for each of these two kinds of damage.

So, if we allow for two oxygen effects and four anoxic effects in the fully wetted state, there are at least six operationally separable kinds of damage occurring in the spore in suspension, of which two are certainly associated with water presence. Our final explanation of the participation of water in radiation damage in the spore system must account for these two (and perhaps more) water elements. Also, it will require that three water compartments must also be taken into consideration (Fig. 6).³ In this generalized model, we have recognized these compartments on the basis of the response of the spore when the compartment is occupied by D_2O on the one hand, as compared to its response when the compartment is occupied by H_2O on the other, in spores of varying water content in O_2 and in its absence. So, in general we recognize two (maybe more) water associated kinds of radiation damage that may occur in any one of three water compartments in spores. This is the territory we must explore in radiation chemistry terms in the future.

Evidence for Hydroxyl Radical Participation in Radiation Effects in Spores.

1. The N_2O Series

The reaction $N_2O + e^-_{aq} \rightarrow \cdot OH + OH^- + N_2$ doubles the hydroxyl radical yields in water. Believing this, we have constructed a series

of survival curves under various experimental conditions utilizing N_2O .⁸ Figure 7 demonstrates that, indeed, when N_2O is present the sensitivity observed is intermediate between that seen in oxygen and that in nitrogen. This can be evidence that an increase in hydroxyl radical concentration results in an increase in radiation sensitivity. The reduction of this sensitizing action seen when ethanol--a strong $\cdot OH$ scavenger--is present supports this view (Fig. 8). Several other $\cdot OH$ scavengers (t-butanol and N-methylacetamide) can reverse N_2O sensitization.⁹ Note that the nitrogen line in 1 M EtOH is approximately the same as that observed in the absence of EtOH. It is important to recognize that the sensitization seen after N_2O addition that can be removed by EtOH appears here not to relate to that seen in nitrogen, since EtOH in high concentration does not affect the nitrogen line. (But, see model below that accounts for this.) In oxygen (Fig. 9) there is no effect seen after addition of any additive. That is, the oxygen effect is unaffected by either N_2O or EtOH.⁸

In low concentrations of EtOH different effects are observed. Figure 10 is the survival curve seen in saturated N_2O at 10^{-4} M EtOH. This is a well defined two-component, or biphasic, response. The low dose limb is between the N_2O line on the one hand ^{and} the nitrogen line on the other. Figure 11 shows the results seen at three different EtOH concentrations. Here the low dose limb points are accumulated, demonstrating a single response curve that is independent of alcohol concentration at these low concentrations. In Figure 12 we see that the second components are also independent of concentration. It is the position of the break point on the dose axis that varies with EtOH concentration. In two of the instances presented the doses at the break points are too

high to be consistent with known chemical yields as they are measured in chemical systems.

Yet, recent experiments show that exhaustion of alcohol as well as accumulation of products can explain these abrupt changes in the response line.¹⁰ In Figure 13 the open triangles define the biphasic response in N_2O -EtOH. The open circles describe a control experiment showing that manipulation of the suspension is allowed. The X's represent the response when 0.9 of a suspension is replaced with fresh alcohol after irradiation to 240 krad. In fresh EtOH the low dose response line is seen. When, instead of EtOH, water is used as the diluent, an intermediate response is observed (solid circles).

In another experiment, an alcohol solution (10^{-4} M) was pre-irradiated with 240 krad (the break point), and this solution was used as the suspending medium. Figure 14 shows that the response line is linear over 5 decades with an inactivation constant like that of the high dose line of the biphasic response.

In irradiated solutions of EtOH saturated with N_2O , two products expected from secondary reaction of $CH_3\dot{C}HOH$ (formed by reaction of $\cdot OH$ with EtOH) are acetaldehyde (CH_3CHO) and the dimer $(CH_3CHOH)_2$. Both acetaldehyde and the dimer are expected to react with $\cdot OH$ and operate as protectors. Figure 14 shows also that the inactivation constant in 5×10^{-5} M acetaldehyde saturated with N_2O is $1.5 \times 10^{-2} \text{ krad}^{-1}$, a value close to the N_2 value. If anything, accumulated acetaldehyde will act to decrease sensitivity rather than increase it.

One other stable product is H_2O_2 . Figure 15 shows that additions of small amounts of H_2O_2 cause concentration-dependent increases in

sensitivity. In N_2 , H_2O_2 at equivalent concentration is without effect, probably because of its fast exhaustion (Fig. 16).

Clearly, H_2O_2 at those low concentrations ($< 10^{-4}$ M) in the presence of N_2O is a sensitizer to radiation, but not in its absence. Also, it was shown by H_2O_2 measurements, that in N_2O solutions with high concentrations of EtOH in which the N_2 response is observed, H_2O_2 is produced in concentrations that are effective when EtOH is not present. Or, H_2O_2 sensitizes but not when $\cdot OH$ is removed.

The following has been proposed as a model that accounts for the action of both $\cdot OH$ and H_2O_2 . If RH_2 is a "target" molecule, and $\underline{R}$ is a lethally damaged one, then (Fig. 17)

Reaction (2) has been measured in $(CH_2OH + H_2O_2; k_2 = 9.5 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1})$.¹¹

This scheme allows for sensitization of cells to radiation by H_2O_2 in the presence of $\cdot OH$ but not in its absence. The action of alcohol in disturbing the sequence by removal of $\cdot OH$ is obvious.

The reactions of e_{aq}^- with two products of water taken with this model

suggest that since e_{aq}^- acts to reduce both H_2O_2 (equation 3) and $\cdot OH$ (equation 4), ordinarily e_{aq}^- acts as a protective agent. Removal of e_{aq}^- would increase the concentrations of H_2O_2 and $\cdot OH$. (5 and 6). In other words, agents with high affinity for electrons increase local concentrations of $\cdot OH$ and H_2O_2 and, according to our model, will act as sensitizers.

These considerations explain the following experimental observations:

- 1) Sensitization by N_2O : it increases $\cdot OH$ concentration and allows H_2O_2 build up by removal of all electrons that would prevent this build up.
- 2) Inhibition of this sensitization by alcohols: removal of $\cdot OH$, one necessary species. H_2O_2 concentrations are sufficiently high but it is not effective in the absence of $\cdot OH$.
- 3) Absence of protection by alcohols in N_2 : H_2O_2 build up is not allowed by electrons, and local $\cdot OH$ concentrations are reduced by electrons. Therefore, addition of EtOH has no effect.

2. Biacetyl

The effect of biacetyl ($CH_3COCOCH_3$) on spores is to sensitize maximally (at 40% of the O_2 effect) at $\sim 3 \times 10^{-3}$ M, with decreasing effectiveness as concentration is either increased or decreased (Fig. 17).¹² In terms of the model above involving $\cdot OH$ and its reaction with e_{aq}^- , we propose the following: at low concentrations biacetyl competes well for e_{aq}^- ($k = 3.2 \times 10^{10} \text{ M}^{-1} \text{ sec}^{-1}$) sparing $\cdot OH$ (equation 4), with effectiveness increasing as concentration goes up because of increasingly effective local concentrations of $\cdot OH$. At 3×10^{-3} M, the local instantaneous e_{aq}^- population is depleted, and further increases in concentration are meaningless for this effect. But in these concentrations $\cdot OH$ is now removed by biacetyl ($k = 2.4 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}$) and a protective action is exhibited. At very high concentrations the N_2 response is seen.

Thus biacetyl has two actions--one in low concentrations that increases local concentrations of $\cdot\text{OH}$ and one at high concentrations that decreases $\cdot\text{OH}$ concentrations locally. We note here and save for later use that $\underline{k_c}$ at the peak is $3 \times 10^{-3} \times 3.2 \times 10^{10} = 9 \times 10^7 \text{ sec}^{-1}$ (Table 2).

3. NO_3^-

If the model and its use in explaining the peaked biacetyl effect is correct, an electron acceptor with a very low reaction rate with $\cdot\text{OH}$ should not show protection at high concentrations. NO_3^- is such a species ($k_{e\text{aq}}^- = 1 \times 10^{10}$; $k_{\text{OH}} > 10^5$). In Figure 18 are the results.¹³ Curve A is the effect of increasing NO_3^- concentrations in anoxic preparations. The rise in sensitivity to a maximum is followed by a long plateau extending over a 100X increase in concentration. This effect agrees with the explanation offered for the biacetyl effect; in this instance NO_3^- is not removing $\cdot\text{OH}$ at high concentrations. The sensitization is to 40% of the O_2 effect as in biacetyl. The $\underline{k_c}$ at maximal effectiveness is $10^{10} \times 7 \times 10^{-3} = 7 \times 10^7 \text{ sec}^{-1}$, a value not far from that seen above for biacetyl ($9 \times 10^7 \text{ sec}^{-1}$).

This sensitizing effect can be competed for by addition of *t*-butanol (Curve C) and by iso-butanol (Curve B), with iso-butanol's being a little more effective at low concentrations, in line with its slightly higher rate with $\cdot\text{OH}$ ($k_{\text{OH} + i\text{-BuOH}} = 2 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}$; $k_{\text{OH} + t\text{-BuOH}} = 6 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}$). These results support the proposition that in lower concentrations electron acceptors act as sensitizers by increasing local $\cdot\text{OH}$ concentrations.

The effect in O_2 (Curve D) is different from any observed up to

now in that an "over oxygen effect" emerges. This result is not now explainable in any mechanism that we have considered, although there appears to be a $\cdot\text{OH}$ component (solid circle is NO_3^- at 3×10^{-4} M--the peak--in t-BuOH, and the X is k_{O_2} with t-BuOH only).

4. Ag^+

Some metal ions have high affinities for electrons. Our test of Ag^+ , as silver sulfate, is shown in Figure 19. There is a concentration-dependent enhancement of radiation sensitivity to a peak at 2×10^{-3} M Ag^+ . At concentrations higher than this, the sensitivity falls. (There is a complexity at high concentrations of Ag^+ that cannot be discussed here.) This sensitizing action which equals that of O_2 is successfully competed against by t-butanol; in Figure 20 the medium is 2×10^{-3} M in Ag^+ , the concentration yielding highest sensitivity in N_2 . These results support the proposition that increases and decreases in $\cdot\text{OH}$ concentrations are the causes of the rises and falls in radiation sensitivity above the N_2 level. At peak effectiveness the k_c of electrons for Ag^+ is $3.2 \times 10^{10} \text{ M}^{-1} \text{ sec}^{-1} \times 2 \times 10^{-3} \text{ M} = 6.4 \times 10^7 \text{ sec}^{-1}$, in good agreement with NO_3^- and biacetyl.

4. Other Sensitizers

Other than our diacetyl work, investigation of organic sensitizers has been done in other laboratories. Working with the spore system, Tallentire and Jacobs¹⁴ have results with PNAP, AP and acetone. Characteristics of the sensitizations are:

1. They are concentration dependent and show peaks of effectiveness, as do biacetyl and Ag^+ . All of these have intermediate

$k_{\text{e-}}^{\text{aq}} / k_{\text{OH}}$ ratios (Table 1).

2. They all sensitize only to about 40% of the O_2 effect, like NO_3^- and biacetyl, but unlike Ag^+ that sensitizes to the full O_2 effect.
3. The concentrations for peak effectiveness are in the order acetone > AP > PNAP, the order of their electron affinities as pointed out by Tallentire and Jacobs, and also apparently in order of their reaction rates with e_{aq}^- . With respect to the last, these compounds almost fall in line with NO_3^- , Ag^+ and biacetyl. Considering all of them together we see that the order of concentration at which they show maximal effectiveness (Table 2) is, with one exception, in inverse order of their reactions rates with e_{aq}^- .

Within an order of magnitude the kc values at maximally effective concentration are the same for NO_3^- , biacetyl, Ag^+ and PNAP. AP and acetone appear to require excessively high concentrations.

Another kind of sensitizer, TAN, is effective in bacteria¹⁵ and in mammalian cells.¹⁶ While the $k_{e_{aq}^-}/k_{OH}$ ratio of 5.5 (Table 1) indicates a "peaked" action, this has been observed only in mammalian cells. The concentration at the peak is 1 mM, indicating a kc of $2.2 \times 10^7 \text{ sec}^{-1}$ ^{fair} in agreement with the values above, even though different cells are being compared.

Models Involving Organic Molecules

Several schemes are at hand now for the action of sensitizers. One of these (Adams)¹⁷ involves the removal of an electron from a target molecule by a compound--the sensitizer--with a high affinity for electrons. This prevents charge recombination that is likely if the electron is not removed. This scheme involves direct action of radiation on the target molecule and direct transfer of an electron from it to the sens-

itizer. It does not include a provision that allows for 1) our results showing that certain sensitizing actions can be competed against successfully by $\cdot\text{OH}$ scavengers; and 2) that H_2O_2 build-up can be shown to be important at low concentrations for sensitizing action.

The simple picture suggested by the experiments described by us for anoxic sensitization implicates electron removal that allows $\cdot\text{OH}$ and H_2O_2 build-up, the two required species, and the model must account for this. The time of sequestration of the electron can be after the target molecule has become involved. Utilizing this, the scheme of M. Simic (private communication) can partly account for our results. It also by-passes one argument against our "electron sequestration" explanation, an argument based on instantaneous concentrations of $\cdot\text{OH}$, H_2O_2 and e_{aq}^- that are to be expected. According to this model, $\cdot\text{OH}$ and e_{aq}^- form a pair on the target molecule that can repair itself. The electron accepting sensitizer can interfere with the recombination (equation 7), or it or another sensitizer can react on the $-\text{OH}$ site (equation 8).

This scheme allows for two reactions in the sensitizing sequence and for the competition by alcohols.

Conclusions

The evidence here that $\cdot\text{OH}$ and H_2O_2 are important in sensitization of anoxic spores prescribes some characteristics of the perfect sensitizer, for this system at least. It should react well with e_{aq}^- , but poorly with $\cdot\text{OH}$, so that exact concentrations above certain levels become less important. The evidence here, and elsewhere, that some of these sensitize to only 40-50% of the oxygen effect might indicate that there are indeed two oxygen effects, as we have suspected for spores for some time, only one of which involves e_{aq}^- removal. The other may be the $\text{R}^\cdot + \text{O}_2 \longrightarrow \text{RO}_2^\cdot$ reaction described by us years ago. If this turns out

to be true for mammalian cells, then the good sensitizer should react with $R^{\cdot}$ in an O_2 -mimicking way. The indication is that many of the ones being tested currently do not have this action.

On the other hand, it is entirely possible that anoxic sensitization by added chemicals and sensitization by O_2 are not the same. Our "over oxygen" effects suggest that O_2 sensitization and Ag^+ sensitization, for example, are different in nature. Experimental ways must be found to interfere with the actions of oxygen in cellular systems.

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FIGURE 2

Fig. 7 (Slide 282)

FIGURE 3

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Fig. 8 (Slide 283)

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Fig. 9 (Slide 286B)

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FIGURE 7

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FIGURE 8

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Fig. 15 (Slide 453)

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Fig. 16 (Slide 484)

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Equations 1&2 (Slide 485)

Equations 3&4 (Slide 483)

TABLE I

X	$k \text{ (M}^{-1}\text{sec}^{-1}) \times 10^{-9}$		
	$X + e_{aq}^-$	$X + \cdot\text{OH}$	$e_{aq}^-/\cdot\text{OH}$
Diacetyl	32.0	.24	~ 100
Acetone	5.9	.068	87
TAN	22.0	4.0	5.5
NEM	32.0	5.9	5.4
NPPN	24.0	4.0	6.0
PNAP	> 30.0	~ 6.0	~ 4.0
AP	28.0	~ 6.0	~ 4.0
Ag^+	32.0	11.0	3.0

529

Table I

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Fig. 17(Slide 254)

TABLE II

X	CONC (M)	$k \times 10^{-10} (e_{aq}^-)$ $\text{M}^{-1} \text{sec}^{-1}$	k_c
Acetone	8.0	0.6	5×10^9
AP	3×10^{-2}	2.8	8×10^8
NO_3^-	7×10^{-3}	1.0	7×10^7
Diacetyl	3×10^{-3}	3.2	9×10^7
Ag^+	2×10^{-3}	3.2	6×10^7
PNAP	1×10^{-3}	> 3.0	> 3×10^7

530

A = NO_3^- in N_2 , D = NO_3^- in O_2
 B = A + t-BuOH, C = A + Iso-BuOH
 at 10^{-2} M NO_3^-

X = 10^{-1} t-BuOH in O_2
 ● = X + 3×10^{-4} NO_3^-
 ○ = Iso-BuOH in N_2

Fig. 18 (Slide 454)*

Table II

TABLE I

$k \text{ (M}^{-1} \text{sec}^{-1}) \times 10^{-9}$		
$X + \text{c}^{\text{eq}}$	$X + \text{OH}$	$\text{c}^{\text{eq}}/\text{OH}$
32.0	.24	≈ 100
5.9	.068	87
22.0	4.0	5.5
32.0	5.9	5.4
24.0	4.0	6.0
> 30.0	≈ 6.0	≈ 4.0
28.0	≈ 6.0	≈ 4.0
32.0	11.0	3.0

529

Fig. 17 (Slide 254)

2.54

Table I

M)	$k \times 10^{-10} (\text{c}^{\text{eq}})$ $\text{M}^{-1} \text{sec}^{-1}$	k c
	0.6	5×10^9
2	2.8	8×10^8
3	1.0	7×10^7
	3.2	9×10^7
	3.2	6×10^7
	> 3.0	$> 3 \times 10^7$

530

A = NO $_3^-$ in N $_2$, D = NO $_3^-$ in O $_2$
 B = A + t-BuOH, C = A + Iso-BuOH
 at 10^{-2} M NO $_3^-$

X = 10^{-1} t-BuOH in O $_2$

● = X + 3×10^{-4} NO $_3^-$

⊙ = Iso-BuOH in N $_2$

Fig. 18 (Slide 454)

2

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Fig. 19 (Slide 522)

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Model I

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Model II