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(For the period September 78 - July 79)

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on

THE NATURE OF OXYGEN CONTAINING RADICALS IN
RADIATION CHEMISTRY AND PHOTOCHEMISTRY OF AQUEOUS SOLUTIONS

Submitted by

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ANNUAL PROGRESS REPORT

(December 1978 - July 1979)

ON

THE NATURE OF OXYGEN CONTAINING RADICALS IN RADIATION CHEMISTRY AND PHOTOCHEMISTRY OF AQUEOUS SOLUTIONS

INSTITUTIONS

This research will be carried out at the Department of Physical Chemistry and in the Linear Accelerator Department, The Hebrew University of Jerusalem, Israel.

PROJECT ABSTRACT

The proposed research is a continuation of the work conducted under this contract and is outlined. During this year, the main emphasis will be given to study further the properties of HO_2 and O_2^- and $\text{OH}^\cdot$, mainly in their role in biological systems.

We will continue to study and elucidate how O_2^- reacts in biological systems. The toxicity of O_2^- is quite well established but the mechanism is still obscure. The Haber Weiss reaction most probably can not account for the toxicity of O_2^- nor for the formation of singlet oxygen, nor of $\text{OH}^\cdot$.

We will study if reduction of Fe^{3+} complexes by O_2^- in biological systems does catalyze the Haber Weiss reaction and if $\text{OH}^\cdot$ is formed in this mechanism. The role of oxygen, radiosensitizers in radiation damage of bacteriophages and cells will be further studied, as well as on E. Coli and Enzymes.

We will try to elucidate the formation and role of $\text{OH}^\cdot$, O_2^- and O_2 in these systems as well as the relative contribution of endogenous and exogenous damage, and the role of direct and indirect radiation damage to cells.

We intend also to study if SOD (super oxide dismutase) does react only with O_2^- or also with biological peroxides ($RO_2^{\cdot}$) and Hydroperoxides (RO_2H).

Further studies of O_2^- and O_2 with various cytochromes, and Hemoglobins is planned.

RESEARCH PROPOSED ORIGINALLY AND SUMMARY OF ACHIEVEMENTS

DURING THE PREVIOUS YEARS

The research project proposed/ originally was to investigate the physical and chemical properties of Ozonide and Hydro-peroxy radical mainly in aqueous solutions.

We will summarize the results of the problems suggested and studied during the previous years by us.

a. OZONIDE ION

We established the spectrum of this species as well as the decay and formation kinetics of the ozonide. The reaction mechanism and kinetics O_3^- with other solutes were measured and shown to follow by the dissociation of O_3^- .

b. HYDRO-PEROXY RADICAL

We determined the properties of this species. It was shown that it exists as HO_2 and O_2^- with $pK \sim 4.85$. The additional form of the radical $H_2O_2^+$ proposed, seems to be ruled out in solutions down to $pH = 0$. The decay scheme of the radical was well established, including the separate rate constants for the reactions $HO_2 + HO_2$, $HO_2 + O_2^-$ and $O_2^- + O_2^-$. The optical spectrum of HO_2 and O_2^- were determined.

c. COMPLEXES OF HO_2 WITH CATIONS

It was shown that HO_2 forms complexes with many cations yielding MHO_2 . These species are much more stable than the free radical, and they have typical ESR signals. It was found that these complexes undergo readily exchange reactions of the following type.

d. ESR DETECTION WITH IN SITU PHOTOLYSIS USING MODULATED LIGHT

In this method radicals were generated in situ in an ESR cavity by photolysis. The light source was modulated and square pulses with pulse length of 10^{-5} sec were obtained with frequencies of $5H_z - 4KH_z$. By this method, radicals reach

concentrations at the end of pulses which were as low as 10^{-9} M and decay till the next pulse. Using a wave from educator, one can average the ESR signal over many pulses and good signal/noise is obtained although of the very low concentration obtained by this method. In order to prevent depletion, the solutions being changed in the cavity by a slow flow system.

This method was used to follow the decay kinetics of several complexes, such as Th-HO₂.

The method has the advantage to follow reactions of Th-HO₂ at low concentrations, where 2nd order recombination is negligible while with usual techniques one can not prevent the 2nd order decay, as in these methods much higher concentrations of Th-HO₂ are formed.

e. EQUILIBRIA CONSTANTS OF MHO₂ COMPLEXES

We further studied the equilibria of MHO₂ complexes

We determined a few more values of such equilibrium constants with various methods. We also extended the study of the exchange reaction.

and verified and determined some new equilibria constants for this kind of exchange.

f. RATE AND MECHANISM OF COMPLEX FORMATION AND EXCHANGE REACTIONS

We determined the rate of formation of MHO₂ through reaction (1) and the exchange rates (reaction 2) for various cations, such as Th⁴⁺, U⁶⁺, etc. The result indicates that these rates are very fast, and the exchange mechanism (2) may go on either directly (2) or more probably through dissociation of MHO₂(1).

g. COMPLEXES OF Fe³⁺ AND Cu²⁺ WITH HO₂

We continued to establish the existence of complexes of Cu²⁺ and Fe³⁺ with HO₂. Such complexes could be of interest, in view of the biological role of Fe³⁺ and Cu²⁺ compounds in reactions with HO₂ in such systems.

Concerning the existence of CuHO_2^{2+} we got good kinetic evidence, although this complex is short lived and therefore its concentration was too low to record its optical or ESR spectra.

h. OPTICAL SPECTRA OF MHO_2

While the existence of MHO_2 complexes were established and its ESR spectra recorded previously, their optical spectra were not known. Using the pulse radiolysis we were able to observe the optical spectra of ThHO_2^{2+} , UHO_2^{6+} and study these species by absorption spectroscopy.

i. REACTIONS OF H_2O_2 WITH Ce^{4+} , Ti^{3+} , Fe^{2+} and Cr^{2+}

The radical OH formed in photolysis of H_2O_2 or in radiolysis of aqueous solution was well established for years. It was believed that the same radical may be formed by reacting H_2O_2 with Ti^{3+} , Fe^{2+} , or Cr^{2+} . Nevertheless, many experimental results indicated, that OH is not formed in all these cases and we explained the reasons that earlier studies seemed to contradict this finding.

We were also able to generate HO_2 from Ce^{4+} and H_2O_2 and from excess of H_2O_2 with either Ti^{3+} , Fe^{2+} or Cr^{2+} . The importance of these studies is in the ability to generate HO_2 and OH chemically.

j. USE OF FLOW METHODS FOR KINETIC AND MECHANISTIC STUDIES

The use of mixtures of Ti^{3+} and H_2O_2 in flow systems was widely employed in studies of radicals by ESR method. The method is based on flowing H_2O_2 with some solute RH and mixing it with Ti^{3+} in front of and ESR cavity.

By this method one obtains high concentrations of radicals in the ESR cavity, and the method is capable to produce many different radicals.

The wide use of the method is due to its advantage in being cheap, yielding high concentrations of various radicals and giving good ESR signals.

As long as the method was used only to characterize the ESR signals of radicals the method proved to be very valuable. Nevertheless, the method was later used to study the kinetics of the radicals formation, decay and other reactions, and also to study reaction mechanisms. In kinetic and mechanistic

studies many results seemed to be in conflict with other studies. We were able to show the reason for these contraversies, as we proved there is an error in the assumptions in using the method for kinetic studies. As in this method, generaaly the radicals observed in the cavity are formed inside the cavity, and are not radicals which are formed out of the cavity (in the mixer), therefore the method is useless for kinetic studies, and may lead aslo to wrong reaction mechanisms.

For this reason, it was shown that double mixing previously used in these studies is completely meaningless.

k. THE CATALYTIC MECHANISM OF MANGANESE-CONTAINING DISMUTASE

The pulse radiolysis method was used to investigate the enzymatic mechanism of HO_2 dismutation. The enzyme studied was the Manganese-containing dismutase of Escheridria coli. The results were interpreted in terms of four oxidation and reduction reactions as follows:

E^+ , E , E^- and E^{2-} may represent forms of the enzyme in which Mn is possibly present in valence I, II, III, IV. It was shown that after about 30 sec of the end of the catalytic process the enzyme activity is completely regenerated.

1. DETERMINATION OF REDOX POTENTIALS OF FREE RADICALS

Most of our effort during 1974-1975 was dedicated to the problem of determination of redox potentials of free radicals, using the pulse radiolysis technique. This study was also extended during the past year⁵. The basic idea of this approach is an obvious one, namely to find an electron acceptor A or a donor D which react with the free radical as follows:

These electron transfer reactions can and were widely studied. If for a given radical R an appropriate acceptor or donor is found, so that one of these reactions reaches equilibrium, then if the equilibrium constant can be measured, one may obtain the radicals' redox potential. There are several assumptions and conditions which have to be fulfilled in order to use this methods for the determination of redox potentials:

- 1) The reaction has to be reversible and equilibrium has to be achieved.
- 2) The equilibrium is not too far neither to the left nor to the right, otherwise the equilibrium constant will not be measurable.
- 3) The redox potential of the respective acceptor or donor is known. It happens that it is not easy to find a system where all these conditions hold. Therefore in some earlier work of Willson and Patel K_{eq} for the electron transfer was measured (conditions 1 and 2 were fulfilled), but the redox potential of R could not have been calculated as it was not known for the acceptor. The method is briefly as follows: The knowledge of K_{eq} for the electron transfer reaction enables to calculate ΔG^0 and ΔE^0 . ΔE^0 is given as the difference of the redox potentials of the radical and the acceptor. Hayon and Coworkers published recently many studies, using this method, in which they claimed to have determined redox potentials of dozens of radicals. We have shown that their method was used wrongly for two reasons and hence their results are meaningless:

- a) In all of their studies, except in the O_2^- case, equilibrium was never achieved and the reaction was not reversible.

b) In all their calculations they used wrong values of the redox potential of the acceptors and donors. Recently they withdrew most of their conclusions in view of our criticism.

The difficulty using this method lies in the fact that most "suitable" acceptors are organic molecules. For organic molecules, oxidation reduction reactions are involving a two electron transfer, for which E^0 is mostly known. Nevertheless in the electron transfer reaction with the radical the *one electron* transfer step is observed and followed.

For the one electron transfer very seldom the E^0 value is known as it involves itself a free radical which is unstable.

After we analyzed the limitations of the method we used it to determine several redox potentials under conditions where the method is justified.

m. THE REDOX POTENTIAL OF THE O_2/O_2^- AND O_2^-/H_2O_2 COUPLES

The redox potential of O_2^- was determined by measuring the equilibria between O_2^- and several semiquibones.

The redox potential of Q/Q^- was not known, but we could calculate it from the values of E^0 (Q/Q_2^-) and from the equilibrium constant of the reaction $2Q^- \rightleftharpoons Q + Q_2^-$, both of these data were available in the literature. Once we had the redox potential of O_2^-/O_2 and the literature value of O_2^-/H_2O_2 , we calculated also the redox potential of O_2^-/H_2O_2 . Our results are given below:

REACTION	E^0 in volts
$O_2 + e \longrightarrow O_2^-$	-0.33
$O_2 + H^+ + e \longrightarrow HO_2$	-0.037
$O_2^- + e \longrightarrow O_2^{--}$	0.102
$O_2^- + H^+ + e \longrightarrow HO_2^-$	0.81
$H^+ + HO_2 + e \longrightarrow H_2O_2$	1.42

These values differ substantially from earlier estimates. These potentials are very important as O_2^- is an important intermediate in many biological processes, such as in the reduction of molecular oxygen. These data were obtained already in 1974-75 and were reported last year. Due to the importance of the value of $E^0 (O_2/O_2^-)$ we extended the study over additional systems. All our latest results confirm the above mentioned value.

n. DETERMINATION OF REDOX POTENTIALS OF OTHER RADICALS

We have determined with the above mentioned method, redox potentials of some additional free radicals, such as several semiquinones. Dr. D. Meisel, now at the Mellon Institute, continued this work there and measured redox potentials of several nitro compounds.

o. THE REACTION OF PEROXY RADICALS FORMED FROM ISOPROPANOL

Isopropanol radical was generated by the pulse radiolysis technique by abstraction of a H atom from isopropanol by an OH. The reaction of O_2 with the radical R was studied.

It was established that $(CH_3)_2C(OH)-O_2$ is formed. Its spectrum and decay kinetics were studied. By catalysis of HPO_4^{2-} , the radical decomposes to yield O_2 and acetone. There were some doubts previously if in reaction (b) HO_2 is directly formed, this study indicates the RHO_2 is formed, but does yield under given condition O_2^- quite fast.

p. THE PEROXY RADICALS OF ACETATE

We extended the studies on MeOH and ProH to glycine. It was found also with this substrate that the glycine radical reacts with O_2 to yield the glycine peroxy radical. This radical also decomposes rapidly ($k=1.25 \times 10^5 s^{-1}$) to yield the O_2^- radical.

q. THE FORMATION OF CrO_2^{2+} IN THE REACTION $\text{Cr}^{2+} + \text{O}_2$

The reduction rate of Cr^{3+} by e_{aq}^- was determined, $k=1.7 \times 10^{10} \text{ M}^{-1} \text{ sec}^{-1}$. The Cr^{2+} thus produced by pulse radiolysis was reacted with O_2 . The same reaction was also studied with the fast flow technique, by mixing Cr^{2+} with and oxygenated acid solution. The rate of the reaction

The interesting feature of this simple reaction is the formation of a complex CrO_2^{2+} whose spectrum and decay kinetics were studied. In contrast to Zn^{2+} and Co^{2+} , which in their reactions with O_2 proceed via electron transfer, this reaction of Cr^{2+} is similar to that of Hemoglobin by a complex rather than electron transfer.

r. OXIDATION OF HEME BY O_2 , O_2^- FERRICYANIDE

In this study we produced heme in the presence of the oxidizing species O_2 , O_2^- and $\text{Fe}(\text{CN})_6^{3-}$. We followed the reactions of the heme with these species. In alkaline solutions the heme reduced O_2 , probably by an outersphere mechanism and did not react appreciably with O_2^- . In neutral solution the heme reacted both with O_2 and O_2^- . At both pH values we could not observe any complex formation. The rate of the reduction of ferricyanide by heme was similar, in neutral and alkaline solutions.

s. THE REACTION OF Mn^{2+} with O_2^- , OH^- , e_{aq}^- and CO_2^-

The reaction rates of Mn^{2+} with reducing agents such as CO_2^- and e_{aq}^- , were measured and were found to be quite slow (2×10^5 and $10^7 \text{ M}^{-1} \text{ s}^{-1}$ respectively).

The oxidations of Mn^{2+} with OH^- was found to be $k=3.4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. The reaction of O_2^- with Mn^{2+} is of interest as Mn constitutes an active site in superoxide dismutase. The reaction of O_2^- with Mn^{2+} has a rate constant of $10^8 \text{ M}^{-1} \text{ s}^{-1}$ and was found to be reversibly $K=1.5 \times 10^4 \text{ M}^{-1}$. The reaction product is either Mn^{3+} or MnO_2^+ .

The surprising fact is that Mn^{2+} does not catalyze the dismutation of O_2^- .

t. THE "HABER WEISS" REACTION AS AN EXPLANATION OF THE TOXIDITY OF O_2^-

In many biological systems, the role of O_2^- in hydroxylation and toxic processes, was assumed to be due to the formation of $OH^\cdot$ radicals. The Haber Weiss reaction (1) - $H_2O_2 + O_2^- \longrightarrow OH^\cdot + O_2$ - was suggested as the origin of this activity. In this study it is shown that this reaction pathway is too slow, and that probably $OH^\cdot$ is formed from the reaction of complexes superoxide with H_2O_2 or/and from the reduction of Fe(III), bound to biological compounds, by O_2^- , the reduced Fe(II) can then react with H_2O_2 as a Fenton reagent, to yield $OH^\cdot$. It is also shown that singlet oxygen cannot be formed in these biological systems neither from the dismutation of O_2^- nor from the reaction of O_2^- with $OH^\cdot$. Singlet oxygen may be formed from the reduction of metal complexes by O_2^- .

u. THE REACTION OF O_2^- and CO_2^- WITH IRON COMPLEXES OF EDTA

The reaction of Fe^{3+} -EDTA and Fe^{2+} with O_2^- and CO_2^- were investigated in the pH range 3.8 - 11.8. Around neutral pH O_2^- reduces Fe^{3+} -EDTA with a rate constant which is pH dependent. At higher pH values this reaction becomes much slower. The CO_2^- radicals reduces Fe^{3+} -EDTA independent of pH. At pH 9 - 11.8, Fe^{2+} -EDTA forms a complex with O_2^- with $k_{Fe^{2+}EDTA + O_2^-} = 2 \cdot 10^6 - 4 \cdot 10^6 M^{-1} \cdot s^{-1}$ which is pH dependent. We measured the spectrum of Fe^{2+} -EDTA- O_2^- and calculated $\epsilon_{max}^{290} = 6400 \pm 800 M^{-1} \cdot cm^{-1}$ in air-saturated solutions. In O_2 -saturated solutions another species is formed with a rate constant of $7 \cdot 10^4 s^{-1}$. This intermediate absorbs around 300 nm but we were not able to identify it.

v. THE EFFECT OF COPPER IONS ON THE REDUCTION OF CIII BY O_2^-

Recently, it was suggested that measured rate of reduction of ferricytochrome C by O_2^- below pH=8, was too high in the presence of high concentrations of formate.

The high values were attributed to the presence of impurities of copper, which compete on O_2^- . This assumption is consistent with either a decrease in the reduction yield of CIII in the presence of copper, or with a very fast reaction of Cu(I) with CIII.

It was previously shown by us and by others that the reduction yield of CIII by O_2^- is 100%. We measured the rate of reduction of CIII by Cu(I) and found that this reaction is slow: $k = (1.5 \pm 0.5) \times 10^3 M^{-1} s^{-1}$.

Therefore, our results rule out the possibility that below pH=8 copper impurities affect the measured rate constant of the reduction of CIII by O_2^- .

The rate of reduction of Cu(II) by CO_2^- was also determined $k = (1.5 \pm 0.3) \times 10^8 M^{-1} s^{-1}$.

w. ONE ELECTRON REDUCTION OF METMYOGLOBIN & METHEMOGLOBIN AND THE REACTION OF THE REDUCED MOLECULE WITH O_2

We have used the pulse radiolysis technique to reduce with solvated electrons (e_{aq}^-) a single Fe(III) site in methemoglobin and metmyoglobin. The reduction process was followed spectrophotometrically and the reactions rate constants were measured: $k_{e_{aq}^- + \text{methemoglobin}} = 6.5 \pm 1 \times 10^{10} M^{-1} sec^{-1}$. $k_{e_{aq}^- + \text{metmyoglobin}} = 2.5 \pm 3 \times 10^{10} M^{-1} sec^{-1}$. Approximately 60% of the e_{aq}^- have probably reacted with the globin moiety.

We followed the reaction of the reduced proteins to yield the oxyderivatives and measured the rate constants of the oxygenation processes k_{reduced} . $\text{methemoglobin} + O_2 = 2.6 \pm 0.6 \times 10^7 M^{-1} sec^{-1}$ and $k_{\text{myoglobin} + O_2} = 1.8 \pm 0.2 \times 10^7 M^{-1} sec^{-1}$. All the rate constants were measured at pH = 6.8² = T = 22⁰ ± 2⁰ C.

The high rate constant for reduced methemoglobin indicates that one-site reduced methemoglobin is probably in the R state, as predicted for methemoglobin from x-ray analysis.

The spectra of the reduced and oxygenated species were measured under similar conditions at $\lambda = 450 - 650 \text{ nm}$. We were able to follow slight changes in the microsecond time scale, these changes were attributed to conformational changes.

We were not able to detect any reaction between the radicals O_2^- and the heme group (which would result in a complex such as heme $-O_2$). This may be due to kinetic reasons.

PUBLICATIONS ASSOCIATED WITH THIS CONTRACT DURING THE YEARS 67 - 79

The publications associated with this project include 60 technical progress reports of which 5 were submitted during the last year. In addition, out of this research 45 papers appeared in the various journals of which 4 came into the journals in the last year. Further 2 papers were submitted, 1 of them is in print.

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GRADUATE STUDENTS TRAINED :

1.	B. Halperin	-	received his M.Sc. degree
2.	A. Samuni	-	" " " "
3.	R. Yelin	-	" " " "
4.	I. Duchovni	-	" " " "
5.	A. Samuni	-	received his Ph.D. degree
6.	D. Behar	-	" " " "
7.	E. Peled	-	" " " "
8.	D. Meisel	-	" " " "
9.	A. Gaaton	-	" " " "
10.	Y.A. Ilan	-	" " " "
11.	Y. Ilan	-	" " " "
12.	M. Pick	-	started Ph.D. in 1974

ACHIEVEMENT OF RESEARCH PROJECT DURING THE LAST YEAR

The project was continued along some of the lines proposed last year.

During this year, Progress Report Nos. 55-60 were submitted.

Four publications appeared and two further publications have been submitted to Journals, of them one is in print.

The work carried out during the last two years dealt with the following problems:

A. EXPERIMENTAL RESULTS

Radiation damage to bacteriophage T4 and E. Coli

Oxygen is well known to enhance the radiation damage of cells. (O.E.R. - oxygen enhancement ratio). This effect is generally attributed to damage to the DNA.

We have chosen to study this problem first with Bacteriophage T4 and then extended it to E. Coli and to Penicillinase.

In this study we wanted to elucidate the following problems:

- a) Is the radiation damage an endogenous or exogenous effect.
- b) Is the damage direct or indirect effect.
- c) Which radicals are mainly responsible for the radiation damage.
- d) What is the role of oxygen in enhancing damage.
- e) What is the radiosensitizing mechanism of oxygen and other radiosensitizers.

1) DETERMINATION OF THE RELATIVE ROLE OF EXOGENOUS AND ENDOGENOUS EFFECTS

We developed a technique to determine the relative contributions of the endogenous & exogenous effects. By irradiating the organisms in the presence of Polyethylene glycol, with molecular weight of 4000 in the presence of N_2O we are scavenging all the radicals formed in the solution. On the other hand this high molecular weight scavenger does not penetrate into the organism and therefore does not effect any endogenous damage. Using this technique, we have shown that with Bacteriophage T4 almost all the radiation damage is by an exogenous effect. Our results show that with E. Coli the majority of the effect is endogenous.

2) THE IDENTIFICATION OF THE SPECIES CAUSING THE RADIATION DAMAGE AND THE RELATIVE ROLE OF DIRECT AND INDIRECT RADIATION DAMAGE

The radiation forms mainly $\text{OH}^\cdot$ radicals and solvated electrons. In the presence of oxygen O_2^- is formed from e_{aq}^- .

In order to determine the toxicity of these radicals we carried out the following irradiations:

- Solutions with formate and oxygen. In this system only O_2^- is present, as both $\text{OH}^\cdot$ and e_{aq}^- are converted into O_2^- .
- N_2O saturated solutions. In this case all radicals are converted to $\text{OH}^\cdot$.
- Helium saturated solutions. Here we get roughly equimolar concentrations of $\text{OH}^\cdot$ and e_{aq}^- .
- Oxygen saturated solutions. Here we get roughly equal amounts of $\text{OH}^\cdot$ and O_2^- .
- N_2O saturated solution with some Oxygen. Here all radicals are converted to $\text{OH}^\cdot$ but O_2^- is present!

Using these systems we were able to show that with bacteriophage T4 practically all the damage is due to $\text{OH}^\cdot$. Neither O_2^- nor e_{aq}^- does damage the phage. The apparent damage caused by O_2^- can be implicated due to the enhancement formed by O_2 . The lack of any damage when only O_2^- are formed (case a) rules this out. In the next paragraph this point will be extended.

Recent results seem to indicate as if H atoms causes radiation damage similar to that of $\text{OH}^\cdot$. This effect is swept out somewhat by the presence of phosphate buffer. The buffer may convert a substantial amount of e_{aq}^- into H atoms.

3) THE EFFECT OF OXYGEN OER

The fact the O_2 roughly doubles the radiation damage seemed to indicate that $\text{OH}^\cdot$ and O_2^- are toxic while e_{aq}^- does not do any damage. The fact that in N_2O saturated solutions the radiation damage is similar to that in O_2 saturated systems seemed to support this hypothesis.

We found that adding some O_2 to N_2O does double the damage caused in N_2O or O_2 saturated systems. This indicates that the radiation damage is only due to $\text{OH}^\cdot$. The role of O_2 is to increase the radiation damage caused by $\text{OH}^\cdot$. Apparently $\text{OH}^\cdot + \text{RH} \rightarrow \text{R}^\cdot$ which reacts with O_2 to form $\text{RO}_2^\cdot$. This last reaction fixates and enhances the damage.

4) RADIATION EFFECT ON PENICILLINASE

Our preliminary results indicate that the radiation damage to this enzyme is due to H and OH mainly. The e_{aq}^- seems to cause less damage than H and OH, whose efficiency seems to be similar.

The O_2^- does not damage at all the enzyme, but Oxygen fixated the damage. Therefore the presence of Oxygen seems to enhance the damage. This effect is due to scavenging e_{aq}^- and H but at the same time O_2 fixates the OH damaged sites.

5) THE SPECIFICITY OF SUPEROXIDE DISMUTASE

The wide scale occurrence of SOD in practically all the cells, is well established. One clear role of SOD is to catalyze the dismutation of O_2^- .

Many questions on the toxicity of oxygen and the protective role of SOD could be clarified if we would be sure that SOD does only react on O_2^- and not on biological peroxides or hydroperoxides.

We studied whether SOD or Cu^{2+} do react with peroxides. The substrates we studied are RO_2 , where R are alcohol radicals of CH_3OH and CH_3COHCH_3 . We found that with this RO_2 radicals neither Cu^{2+} nor SOD had any catalytic effect of the destruction of RO_2 . We plan to study other biological peroxides, such as those from DNA, Lipids and Linoleic acids.

6) REDUCTION MECHANISM OF CYTOCHROME C, AND OTHER REDUCTANTS

In order to understand better the reduction mechanism of cytochrome C we studied the reduction of acetylated, fully succinylated and dicarboxymethyl horse cytochromes C by the radicals $CH_3\dot{C}H\ OH$, CO_2^- , O_2^- , and e_{aq}^- , and the oxidation of the reduced cytochrome C derivatives by $Fe(CN)_6^{3-}$ were studied using the pulse radiolysis technique. Many of the reactions were also examined as a function of ionic strength. By obtaining rate constants for the reactions of differently charged small molecules redox agents with the differently charged cytochrome C derivatives at both zero ionic strength and infinite ionic strength, electrostatic and conformational contributions to the electron transfer mechanism were effectively partitioned from each other in some cases. In regard to cytochrome C electron transfer mechanism, the results, especially those for which conformational influences predominate, are supportive of the electron being transferred in the heme edge region.

7) PULSE RADIOLYSIS STUDY OF THE BINDING OF O₂ TO
REDUCED METHEMOGLOBIN A

Using the pulse radiolysis, valence hybrids may be formed from methemoglobin. These valence hybrids are characterized by that they contain a single-reduced heme in the tetramer. We studied the reaction of molecular oxygen with the valence hybrid in the absence and presence of organic phosphates as a function of pH.

It is concluded that human adult stripped methemoglobin resides in an R quaternary structure while the presence of IHP stabilizes the T structure at pH below 7.5. This transition between the quaternary structures of methemoglobin cannot be accounted for by the switch between the high-spin and the low-spin states of the ferric iron. This switch of spin state takes place at pH > 8.2.

B. DEVELOPMENT OF EXPERIMENTAL METHODS

1) COUPLING THE PULSE RADIOLYSIS TO A COMPUTER

The advantage of coupling the computer to the pulse set up is obvious, and this is done recently in different laboratories. We are doing the same thing in a somewhat different way.

In addition, to using the computer to control the whole experimental set up; (synchronization of the pulse, the light pulse, pulse length, delays etc.) we developed the following principles for data storage. For relatively slow signals, when every 5×10^{-6} sec a point is recorded, the output of the photomultiplier is fed through an A- to D converter into a NOVA computer. For fast signals we developed the following method. The P.M. signal is displaced on an oscilloscope screen which serves as a temporary memory and is capable to display very fast pulses, depending on the oscilloscope speed (less than 10^{-8} sec/cm). The trace on the screen is taken by a T.V. camera which output is fed into the computer. The data analysis thereafter are treated by the computer. The computer programs are already working and the use of the A to D converter for slow events does operate. In addition we purchased a Biomation which will enable us to record fast processes. We still continue to develop

the fast T.V. camera technique. We hope to finish this development within the next three to six months.

NEW OBJECTIVES OF CONTINUATION OF THE PROJECT

Part of the objectives as proposed previously were accomplished, as described earlier, while other objectives will be further studied. Our main effort during the previous year was dedicated to the problem of the determination of redox potentials of free radicals with special priority to the redox potential of O_2^- .

During the last year our main interest and emphasis was given to the role of O_2^- in biological systems. This mainly in studying the possibility of formation of $OH^\bullet$ from O_2^- , and the role of $OH^\bullet$, O_2^- and oxygen in radiation damage to cells, and the mechanism of oxygen toxicity in biological processes.

From the progress of our studies throughout this last year, we intend to give priority in the future to the following problems, most of them proposed last year. The proposal of objectives outlined here are for the next 2-3 years of our research.

I. ROLE OF O_2 AND OF O_2^- IN RADIODAMAGE & RADIOSENSITIZERS

The contributions of molecular oxygen and of various water-radicals to the biological radiodamage in T4 bacteriophage were studied. We will extend these studies to E. Coli B. and various mutants of E. Coli. The test organisms are γ -irradiated in buffered suspensions and the spectrum of the water-radicals is controlled by a careful choice of the saturating gases and the radical-scavengers added. Thus, it is possible to distinguish between the contributions of the various radicals as well as between the effects of O_2 and O_2^- .

The use of radical scavengers which do not penetrate the cell will enable a better distinction between the endogenous and exogenous effects involved. The results showed that for a T4 bacteriophage the biological damage is predominantly caused by $OH^\bullet$ while e_{aq}^- as well as O_2^- do not contribute to the radiodamage. The results also indicated that the oxygen enhancement in the systems studied cannot be attributed to the formation of O_2^- from e_{aq}^- and O_2 , but rather to the direct interaction of O_2 with the target bio molecules.

Another conclusion which is supported by our results is that in T4 bacteriophage the radiation effect is predominantly indirect.

We plan the following studies in this direction:

- 1) To extend our preliminary studies with E. Coli. B. and further establish the identity of the radicals responsible for the radio damage and the role of O_2 . This in addition to determine the contribution of direct effect and the relative role of endogenous damag.
- 2) Comparison of the radiosensitivity of E. Coli. mutants (C600 and HB101 for example) one of which cannot repair damaged DNA. This study can help to distinguish between the oxygen effects on the bacterial DNA as compared to other cellular component.
- 3) Comparison of the above two mutants as hosts for irradiated T4 viruses. The results of such a study may shed more light on the role of oxygen and the water radicals in the inactivation of the viral DNA.
- 4) The effect of radiosensitizers ("electron affinic", "free radicals") as mimic compounds for the oxygen effect.

It is aimed to extend the previous study further and check the effect and sensitization mechanism of the radiosensitizers on the biological systems previously studied.

II. RADIATION DAMAGE TO ENZYMES

We will study the effect of radiation on Enzymes. We started this study on Penecilinase.

This study will try to determine the relation efficiency of OH, H and e_{aq}^- on the radiation damage. In addition the radiation damage of O_2^- will be studied.

III. EFFECT OF O_2 & RADIOSENSITIZERS ON THE RADIATION DAMAGE TO ENZYMES

As enzymes are to some extend model compounds to radiation damage, we will try to determine the effect and mechanism of sensitization by O_2 and radiosensitizers.

We will try to isolate if the sensitization is limited to radiation damage caused by H, OH or e_{aq}^- .

IV. EFFECT OF pH ON RADIATION DAMAGE

As radiation damage is generally studied around neutral pH, and frequently in buffered solutions, it may be that the buffer affects the damage. In phosphate buffer e_{aq}^- may react with the buffer.

therefore possibly H atoms will cause some of the radiation damage.

In order to elucidate the role of e_{aq}^- and H in radiation damage to phages, E. Coli and enzymes, we will study the effect of phosphate in the presence and absence of N_2O . N_2O will compete with $H_2PO_4^-$ for e_{aq}^- and therefore this scavenger may be used to isolate these effects.

V. PULSE RADIOLYSIS STUDY OF FETAL HUMAN METHEMOGLOBIN

Fetal hemoglobin that is stripped of organic phosphates shows a reduced affinity towards oxygen when compared to stripped adult hemoglobin (hemoglobin A). In the presence of organic phosphates, like inositol hexaphosphate or 2,3-diphosphoglycerate, the situation is reversed. This explains the mechanism for oxygen supply to the fetus from his mother.

We intend to study the reaction of molecular oxygen with valence hybrids that will be formed radiolytically from human fetal methemoglobin. The reaction will be studied in the presence and absence of organic phosphate as a function of pH.

VI. THE REACTION O_2^- WITH HEMOGLOBIN & METHEMOGLOBIN

O_2^- is known to react both as a reducing agent (i.e., $cyt\ c(III) + O_2^- \rightarrow O_2 + cyt\ c(II)$) and as an oxidizing species (i.e., $Fe(CN)_6^{4-} + O_2^- \rightarrow Fe(CN)_6^{3-} + O_2$). By analogy, O_2^- could, in principle either reduce oxyhemoglobin (IV) to

methemoglobin(III), or in turn, it could oxidize the heme-iron(II) in deoxy-hemoglobin to methemoglobin.

We intend to study the mechanism of and the effect of the allosteric modifier, IHP, on the reaction between hemoglobin and superoxide radical.

VII. PULSE RADIOLYSIS STUDY OF RADICALS WITH ISOURAMIL AND RELATED PYRIMIDINES

Favism is the acute hemolytic crisis following the ingestion of broad beans (*Vicia faba*) in glucose-6-phosphate dehydrogenase deficient individuals. Isovramil (2,4,5 trihydroxy pyrimidine) and divicine 2,6 diamino - 4,5 dihydroxy pyrimidine), two substances found in the bean, are implicated as the toxic constituents. In addition to their role in the etiology of Favism, these two compounds are pyrimidines of similar structure to those pyrimidines found in DNA, i.e., Cytosine and Thymine.

We intend to study the reactions of these pyrimidines and their derivatives with e_{aq}^- , $OH^\bullet$ and O_2^- .

VII. PRODUCTION OF STABLE SOLUTIONS OF O_2^- AND STUDIES OF REACTIONS OF O_2^-

We will construct a system based on milipore-CO Cartridge to purify alkaline solutions. It is well known that in the purified solutions the life time of the O_2^- is pH dependent, and at high pH's it can exist for a few hours.

We intend to use a flow system and mix these alkaline solutions of O_2^- with various solutes in buffered solutions, and thus will be able to study the reactions of O_2^- around neutral pH. In our flow system we will use both optical and ESR detection.

a) Catalyst for the Haber Weiss reaction

We intend to study the effect of various metal complexes as catalysts on the Haber Weiss reactions:

b) Oxidation Reduction Potentials of HO_2 and MHO_2 Complexes

In order to understand further the reactions of HO_2 , and complexes of HO_2 with cations or with metal porphyrins, it will be very useful to determine their oxidation reduction potentials in the reactions

This will be done in a similar way done by Hayon et al and by us, by choosing reagents X with suitable oxidation reduction potentials.

c) Reaction Rates of HO_2 and O_2^- with Various Solutes

We will study the rate of HO_2 radical with various solutes. These reactions may occur in biological processes and it would be interesting to study these rates. Furthermore, HO_2 and O_2^- may react both as reducing and oxidizing agents.

We intend to study these reactions with cytochrome C and other metal porphyrins.

d) Oxidation Mechanisms With O_2

We intend to study the oxidation mechanism of simple Ions such as Cr^{2+} or V^{2+} with O_2 . These reactions may be one electron transfer reaction in which case O_2^- would be formed. This study may be extended to oxidations of the reduced metal porphyrins by O_2 , such as Ferrocyclochromes at appropriated pH.

e) Properties of MHO_2 Complexes

We have shown that many cations $M(M=Ce^{+3}, Ti^{+4}, U^{+6}, Th^{+4}, Zr^{+4}, Nb^{+4}, La^{+3}, V^{+5}, Hf^{+4}, \text{etc.})$, form complexes with HO_2 . We intend to find out if O_2^- also forms such complexes.

Moreover, we will study some of the main properties of these complexes, also with metal porphyrins such as:

- 1) Their ESR spectra and optical spectra;
- 2) The rate of their formation in the reaction HO_2 or $O_2^- + M \longrightarrow MHO_2$;
- 3) The pK of the equilibrium $M + HO_2 \rightleftharpoons MHO_2$;
- 4) The decay kinetics of MHO_2 ;
- 5) Reactivities of MHO_2 with other solutes such as cations as well as organic solutes, both as reducing and as oxidizing agents.

These properties are interesting as such, as well as necessary in order to understand the exchange mechanism of

These studies will be carried out using flow system with ESR detection, as well as by using pulse radiolysis with optical detection.

f) Possible Implications to Biological Systems

We will try to extend the work of M - HO₂ complexes, their exchange reactions, etc., where M and M' are not bare cations but substances of biological interest, such as cytochromes, for instance.

SIGNIFICANCE OF THIS RESEARCH PROJECT

This research project concerns the chemical and physical properties of the peroxy radicals. Three radicals, OH , e_{aq}^- and H , are formed in the radiolysis of water but in oxygenated solutions the principal radicals are OH and O_2^- . The same radicals are formed in the photolysis of certain aqueous solutions in many chemical reactions. Any understanding of the radiation chemistry and photochemistry of such systems is impossible without the knowledge of the properties of these radicals that we are attempting to gain.

These oxygen radicals are also of great importance in radiation biology and photobiology. The toxicity of O_2^- and its role in biological processes is of major interest.

Therefore, the study of the properties of O_2^- and OH is of major importance in understanding the biological function of O_2^- .

The main significance of this project is in the following senses:

- 1) The extension of generation of HO_2 or O_2^- by chemical reactions to study properties of O_2^- at neutral pH which is of greater interest from the biological point of view.
- 2) The study of complexes of HO_2 with metal ions and such exchange reactions of HO_2 between several metal ion complexes is of great interest.

The ease with which the HO_2 radical complexes with metal ions is somewhat surprising. The significance of this point to radiation biology and other biological chemical processes is unknown but it would seem that further investigation of these complexes is desirable. The comparison of our studies in the properties of MHO_2 complexes with the enzymatic catalysis of HO_2 .

This effort may lead to have biological interest as such a reaction may play a role in physiological processes as H_2O_2 decomposition, biosynthesis or other biological oxidation reduction reactions.

- 3) To understand further the oxidation reduction reactions of HO_2 or its complexes both in biological and nonbiological systems, the thermodynamical information of these species is crucial.

We intend to determine not only pK values of HO_2 in its complexes, but also E° -the redox potential of both HO_2 and its complexes.

In order to demonstrate the importance and the use of these data for biological problems, we will bring two examples:

In studies of the enzymatic mechanism of the action of superoxide dismutase it was argued in the literature whether a complex between the enzyme and O_2^- is first formed. We were able to show that such a complex is most probably not formed - this was possible due to the knowledge of the redox potentials of the enzyme and that of the O_2^- .

Another example is the question if in the reaction of O_2 with cytochrome oxidase O_2^- may be formed? It was argued against this possibility on grounds of thermodynamical arguments, with our value of E^0 of the O_2^- we were able to show that one cannot rule out the possible existence of O_2^- in this system. The last example demonstrates the application of such data for analyzing the possible mechanism of some enzymatic reactions on grounds of the thermodynamical data.

4) The toxicity of O_2^- and its role in biological systems is quite obscure. This study is aimed to understand how O_2^- functions in biological system. We have shown that O_2^- does not react through the "Haber Weiss" reaction, and we search for alternative mechanisms. This study has also implication on the formation and function of singlet oxygen, and the mechanism of its toxicity.

5) In our studies with hemoglobins, we may get better understanding on the spicifity of the various conformations in chemical reactions such as O_2 binding as well as other redox-oxidation reactions.

6) The effect of O_2 as radiosensitizer is well established. Our studies may enable to determine the answer to the following questions:

- a) What are the radicals responsible for radiodamage?
- b) Does O_2^- cause any radiation damage?
- c) What is the mechanism of O_2 on the sensitization process.
- d) How do radiosensitizers react.

7) We will try to establish if SOD is specific and reacts with O_2^- only or also with other organic biological peroxides and Hydroperoxides. This is an important question as to the role of SOD in the cell.

SCIENTIFIC PERSONNEL

Prof. G. Czapski will be responsible for the project and will spend all his time in research on it.

Mrs. Yael Ilan (Ph.D) will continue to work with fast reaction flow system on reactions HO_2 and O_2^- using both optical and ESR detection. Mr. and Mrs. Ilan will extend this study also on complexes of HO_2 with metal ions and metal porphyrins, mainly on various derivatives of Cytochrome C. They will also study the formation of O_2^- and $\text{OH}^\bullet$ in biological systems. Mr. E. Brener will finish the coupling of the accelerator and pulse radiolysis technique to our new NOVA computer, also for fast events.

Dr. A. Samuni, senior lecturer in the department of Microbiology, will work on the role of O_2^- in radiation damage of cells. He will study the role of O_2 in the oxygen enhancement ratio (OER) as well as the mechanism of various radiosensitizers and protectors.

The Department of Physical Chemistry of the Hebrew University will contribute towards the Project, both in personal and other expenses, considerably in excess of the funds requested under this contract. (For example, in 1967-1978 about \$400,000 was spent for the linear accelerator and experimental equipment, in addition to another \$100,000 for construction of the accelerator building, and lately additional \$150,000 were spent for computer being coupled to the pulse equipment and to the accelerator).

QUALIFICATIONS OF PRINCIPAL INVESTIGATOR

Professor G. Czapski, the Principal Investigator, studied at the Hebrew University, where he received his M.Sc. and Ph.D degrees in Physical Chemistry. From 1960 to 1962 he was research associate at Brookhaven National Laboratory, N.Y., U.S.A., working on radiation chemistry of aqueous solutions with Drs. A.C. Allen and H.A. Schwarz. During the summer of 1962 he was invited for approximately a month as guest of the Radiation Chemistry Department at the "Boris Kidrich Institute", Vinca, Yugoslavia. In the summer of 1963 he spent five months as resident research associate at Argonne National Laboratory, Illinois, U.S.A., working with Dr. L.M. Dorfman on pulse radiolysis. In 1964 he was guest scientist at the Nuclear Research Center Democritus in Athens,

Greece, and in the summer of 1965 stayed as guest scientist at Brookhaven National Laboratory. At the present, Professor Czapski is the Director of the Linear Accelerator Laboratory. In 1970 he was guest Professor at Carnegie Mellon University. Since 1964 Professor Czapski is also guest professor at Tel-Aviv University. For the years 1975-1978 he was elected as Rector of the Hebrew University.

Professor Czapski's interest was centered in the properties and identity of radicals produced by radiation in aqueous systems. The study of reactions of hydrogen atoms was carried out by producing them in the gas phase by an electrodeless discharge and introducing them into aqueous solutions. The different oxidation mechanisms were investigated, and evidence for H_2^+ and hydride intermediate was given in several systems which were studied. By using the kinetic salt effect, it was first proven that one form of the reducing radical has a unity negative charge - thus being solvated electron - an assumption which was previously postulated. In other studies, using fast flow system techniques coupled with high intensity radiation, he found that formation of HO_2 , O_2^- radicals and of H_2O_3 .

The formation of HO_2 radical was studied with fast kinetic techniques in the reaction of Ce^{+4} and H_2O_2 .

In flash radiolysis of aqueous solution, he studied the decay and formation rates and the spectra and pK of the HO_2 radicals. In addition new intermediates in alkaline solutions were found, one of which probably is the ozonide ion (O_3^-).

The formation of complexes of HO_2 with metal cations M were established ($M-HO_2$). The properties and possibly role of such complexes were studied.

Lately he was involved in the determination of redox potentials of free radicals and determined the redox potential of the O_2/O_2^- couple as well as that of several semiquinones.

In the last years the research of the properties of O_2^- and its complexes was extended to biological problems.

The studied reactions of O_2^- with cytochrome C and some derivatives, reactions of O_2^- and of O_2 with Hemoglobin, Methemoglobin and Oxyhemoglobin, the role of O_2^- in biological processes and its toxicity were studied. This is recently extended to the radiation damage of bacteriophages and E.Coli.

The role of O_2^- in the radiation damage is being elucidated.

Professor Czapski constructed and set up a fast reaction flow system, a flash photolysis apparatus and pulse radiolysis laboratory. The last equipment is now being coupled directly to a computer.

A list of publications is attached.

OTHER FINANCIAL ASSISTANCE

The principal investigator has no other research contracts. He had for the last year a small grant for collaboration with the radiation biology group in the GSF Neuherberg, Germany. This grant was mainly for covering mutual visits of scientists between these laboratories.

LIST OF PUBLICATIONS

1. Czapski, G., Stein, G. and Vogel, U., The Oxidation of Fe^{2+} in Aqueous Solutions by H atoms, *Bull. Res. Council Israel*, 7A, 231-232 (1958).
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GENERAL INFORMATION

a. FACILITIES AVAILABLE

1. Cesium γ -ray source (2000 curie).
2. Flash Photolysis apparatus. (Built in the department).
3. Several spectrophotometers.
4. Flow system for measuring fast reactions. (Built in the department).
5. An ESR Varian instrument.
6. All general equipment in the physical chemistry laboratory.
7. Complete pulse Radiolysis set up coupled to computer.

b. CONTRACTOR'S CONTRIBUTION TO PROJECT (no charge)

The Hebrew University constructed a flow system and flash photolysis apparatus at an approximate cost of \$25,000 for each in addition to acquiring all equipment mentioned above. The Hebrew University also covers Dr. Czapski's salary (who will devote 50% of his time to this project) as well as that of part-time technicians and junior assistants. Dr. Czapski will spend part of this year in the Department of Radiology of Stanford University, U.S.A.

c. LINEAR ACCELERATOR LABORATORY

A laboratory for a linear accelerator has been built, (above 3,000 sq.ft.). The budget for the accelerator with its auxiliary experimental equipment is \$250,000. The accelerator arrived in November 1968 and is operating fully with its auxiliary pulse radiolysis equipment, with detection times down to 10^{-8} sec.

In the last three years additional \$150,000 were invested in purchasing a Biomation, a NOVA computer, Disc, display, hard copy unit and fast printer. These components together with the development of fast A to D converters, enable the direct control of the accelerator and pulse radiolysis set up by the computer. In addition, this set is capable of storage and evaluation of the measured data directly.

To the present time the development reached the stage that events as long as 10^{-6} sec. can be handled directly by the computer. Faster processes as short as 10^{-8} sec. will be soon handled also directly through the computer. (probably within six months.)