PROGRESS REPORT
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BIOLOGICAL EFFECTS OF RADIATION AND RELATED
BIOCHEMICAL AND PHYSICAL STUDIES

Proposal #1: Radiation Studies

1. Free Radical Production in Biologically Significant Compounds
2. Theoretical Investigation of Electron LET Spectra and Dose Relationship for Ionizing Radiations

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RADIATION STUDIES

1. Free Radical Production in Biologically Significant Compounds

A. Irradiated Purine N-Oxides

(In collaboration with Dr. George B. Brown and co-workers of the Division of Biological Chemistry)

Previous progress reports described the measurement of radical intensities in a number of solid UV irradiated purine N-oxides, some of which, such as 3-hydroxyxanthine, have been shown to be carcinogenic in rats by Dr. Brown and co-workers. Attention was now directed to the analysis of the ESR spectra of irradiated 3-hydroxyxanthine (I) and some of its derivatives in an effort to determine the radical structure. The three lines observed in irradiated 3-hydroxyxanthine resembled the anisotropic nitrogen hyperfine spectrum in solid nitroxides although the overall splitting in spectrum of I was 33 Gauss, half the value usually seen in strongly immobilized nitroxide spin label radicals. Values for g ranged from 2.004 to 2.006, which is in the range for nitrogen hyperfine interaction. We postulated that the UV radiation removed the hydroxyl group at the 3 position as in Fig. 1. Then through allylic shifts the odd electron could move to the 5 or 8 position (Fig. I-II-III-IV). Thus we would expect to see hyperfine interaction with the nitrogen at the 3 position and with substituents at the 8 position. A high spin density at the 5 position where there is no hyperfine interaction could be responsible for the relatively narrow overall width.

It would have been desirable to measure the spectra in irradiated single crystals or in solutions of the radicals for the purpose of determining their structure but this has not yet proven technically feasible. Instead a number of N-methyl derivatives of I were irradiated as powdered solids and their spectra compared to see if changes in the hyperfine spectra correlated with the nature of the substituent and the postulated odd electron distribution. Also it was demonstrated that the lines in I did belong to the same (nitrogen) hyperfine multiplet rather than arising from separate radicals. This was accomplished by measuring the spectrum at two different frequencies in the X-band region using cavities constructed in the Institute's machine shop. The frequencies differed by 9% while the separation of the lines remained constant within experimental error ($\leq 2\%$).

The results from the analysis of the N-methyl derivatives support the postulated structure of the radical (Fig. 1) although there are some discrepancies which still must be resolved. The 1-methyl and 7-methyl derivatives showed spectra essentially identical to that of I. This indicates that there is no interaction of the radical with the

hydrogen at N-1 and that methyl substitution at N-7 prevents the resonance exchange $\text{III} \leftrightarrow \text{IV}$ (Fig. 1). The 8-methyl derivative gave a spectrum more complex than I although lines at position corresponding to those in the spectrum I were present. The overall width was about twice that of I and this coupled with the presence of additional lines indicates that the radical is interacting with protons in the C-8 methyl group. Here the electron donating capacity of the 8-methyl group must contribute to stabilizing the radical at C-8 and would explain the greater contribution of the resonance from IV (Fig. 1).

B. Studies on the interaction between the mineral and organic components of bone:

The ESR of irradiated bone and synthetic calcium phosphate systems had already been studied in this laboratory (1). The approach used in the present work differs from the previous work in that transition element ions and stable free radicals (nitroxides) were used as paramagnetic probes in the bonding between calcium phosphate mineral and certain macromolecular species.

It was found possible to obtain vanadyl ion doped mineral by precipitating calcium phosphate from Tris buffered solutions of calcium chloride and disodium hydrogen phosphate (at $\text{Ca} \times \text{P}$ ion product = $132 (\text{mM})^2$) in the presence of VOSO_4 at a level such that $\text{Ca}/\text{VO} = 24$. The precipitates were washed 3 times with distilled water and lyophilized previous to examination in the ESR spectrometer. The spectra were typical of a "powder" spectrum due to random orientation of the vanadyl ions and existing axial symmetry in a clearly resolved hyperfine structure. The following values of magnetic resonance parameters were measured for the doped calcium phosphate:

$$\begin{aligned} g_{11} &= 1.9393 \pm .0005 \\ g_1 &= 1.9989 \pm .0005 \\ g_0 &= 1.9790 \pm .0005 \\ A_{11} &= 194.9 \pm 0.8 \text{ gauss} \\ A_1 &= 62.4 \pm 0.8 \text{ gauss} \\ A_0 &= 106.6 \pm 0.8 \text{ gauss} \end{aligned}$$

Intensity measurements via electronic double integration indicated that about 0.2 μ moles of paramagnetic vanadium species per mg mineral had been incorporated into our samples.

In order to investigate the mineral interaction with the organic phase, soluble macromolecules were added either to the calcium or phosphate solutions and were allowed to co-precipitate with the mineral phase in the presence or vanadyl ions as before.

Macromolecules used:

1. Collagen - chick skin, neutral salt soluble protein obtained as a gift from Dr. J. Termine of the NIDR at Bethesda. This is the chief protein of bone.
2. Gelatin - Bacto gelatin from Difco Company - a denatured form of collagen.
3. Lysozyme - a basic protein of well characterized structure known to act as a nucleating agent in synthetic calcium phosphate systems.
4. Bovine Serum Albumen - an acidic protein of known amino acid composition.
5. Phosvitin - a phosphoprotein with phosphate groups attached at serine residues.
6. Chondroitin - SO_4 - a mucopolysaccharide typical of the ground substance of bone
7. Sodium polyacrylate - a non-nitrogen containing polycarboxylic polymer
8. Polyamino acids - to provide various specific protein side chain residues in isolation from other kinds of residues:

acidic: polyglutamic acid, sodium salt

basic: polylysine, polyarginine

neutral: poly-alanine

peculiar to collagen: polyproline (Sigma type II)
polyhydroxyproline

All macromolecular additives were used at 10 mg in a 50 ml system of CaP product = 132, Ca/P = 1.1 of reacting calcium and phosphate ions. From recorded ESR spectra studies were done on the perturbation of magnetic parameters due to presence of the macromolecule.

For example, it was possible to measure the Fermi isotropic contact term (K) to within ± 0.007 error. This number is a measure of the amount of unpaired electron density at the vanadium nucleus. The results indicate a value of $K = 0.761$ for mineral with no additive. Addition of collagen leaves this value unchanged ($K = 0.760$) as might be expected, the vanadyl ion finding the mineral and collagen environments very similar. However, it is interesting to note that gelatin shows a value of $K = 0.783$, significantly lower than for mineral alone indicating increased delocalization of the electron from the vanadium nucleus.

Another somewhat surprising result shows up in comparing polyproline with polyhydroxyproline. For the former, the K value is 0.766, the same as for pure mineral in experimental error limits. The latter however shows $K = 0.736$, a significant increase in delocalization of unpaired electron from vanadium nucleus comparable to that seen in gelatin.

Studies of a similar nature were performed for manganese doped calcium carbonate, though much less bonding information was obtained here, since the only purpose in looking at this system was to develop a probe for carbonate to be used in later work on the influence of carbonate on calcium phosphate systems. The manganese hyperfine lines are much sharper and better resolved than the vanadium lines and should prove to be a sensitive probe in carbonate systems. However, manganese does not yield spectra as well resolved as vanadium in phosphate systems.

Further work was done using nitroxide stable free radicals to label the macromolecular species. Some solid state spectra have been studied but interpretation is difficult and is still underway.

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2. Theoretical Investigation of Electron LET Spectra and Dose Relationship for Ionizing Radiation

A. High Intensity Pulsed Radiations

This investigation aims at a theoretical analysis and explanation of certain biophysical and biological phenomena observed when bacterial cells are irradiated at extremely high dose rates (kilorads to megarads in a few nanoseconds, as obtained from field emission units). These rates, which sharply contrast to conventional ones, result in a qualitative modification of the radiation response of the irradiated cells: the survival curves exhibit a transition in their shape at a certain dose, from the slope for oxygenated to that for anoxic conditions (1). The transition is attributed to the onset of anoxia at and above the transition dose, as a result of the intense radiochemical reactions initiated by the irradiation. The rate of oxygen rediffusion into the cells from the gas above the cell suspension, and the time-scale over which diffusion occurs are directly controlled by the physical properties of the cell suspension and provide a guide to the possible mechanisms of oxygen rediffusion, including processes that may either promote or inhibit oxygen diffusion. The particular object has been to develop a physical model, sufficiently simple and involving as few arbitrary assumptions as possible, that will explain the experimental data (Epp, Division of Physical Biology). The experimental diffusion curve exhibits a semiplateau that spans two time decades (1 to 100 milliseconds), with saturation occurring a few seconds after the pulse. The diffusion process covers about five time decades. This behavior cannot occur with simple diffusion, and the data strongly suggests the presence of an inhibiting, or absorbing agent, partially removing the rediffusing oxygen and delaying saturation.

Models involving various types of absorption processes (constant rate of absorption, proportional rate of absorption, etc.) were considered and translated into mathematical terms. The solutions of the resulting diffusion equations did not exhibit the experimental plateau and, in general, differed from the data.

On further analysis it was concluded that the rate of oxygen absorption could not be assigned or approximated mathematically before solving the diffusion equation, but, at any time, it was fundamentally dependent in a specific way on the concentration of available absorbing agents at that time and on the flux of oxygen at the same time. The model was supplemented by making explicit the simple but necessary assumption that the interaction of an oxygen molecule with a molecule of the absorbing agent results in the removal of both from

Further consideration in the diffusion process. In addition, the interaction cross-section, σ (a priori numerically unknown), appears in the formulation. The time-evolution of the concentration, R , of the absorbing species was first derived:

$$R = R_0 \left(1 + \sigma D \int_0^t \nabla c \, dt' \right) \quad (1)$$

where R_0 is the initial concentration (a priori numerically unknown), ∇c is the gradient of the oxygen concentration, c , and D is the diffusion coefficient. For a flux of oxygen molecules, $-D \nabla c$, and an interaction cross-section, σ , the number of interactions per cm^3 per second, that is, the rate of oxygen absorption is:

$$-\sigma R D \nabla c = -\sigma D \nabla c R_0 \left(1 + \sigma D \int_0^t \nabla c \, dt' \right) \quad (2)$$

The geometry corresponding to the experimental set-up is one dimensional involving a thin wet layer of cells exposed to oxygen-containing gas on one surface. The cells were assumed to be about one micron below the surface, and resting on a wet millipore filter, a few hundred microns thick (effectively infinitely thick).

The diffusion equation with the absorption term (2) above, is highly non-linear and a direct analytic solution is not possible. It is solved by introducing for the concentration in the absorption term the solution one obtains without absorption. The solution one obtains is:

$$\frac{c(x,t)}{c_0} = \operatorname{erfc}\left(\frac{x}{2\sqrt{Dt}}\right) - \frac{\sigma R_0}{2\pi} \int_0^t d\tau \int_{x_{\min}}^{\infty} dx' \frac{\exp\left(-\frac{(x')^2}{4D\tau}\right)}{\sqrt{D(t-\tau)}} \cdot \left\{ 1 - \sigma c_0 \left(2\sqrt{D\tau} \exp\left(-\frac{(x')^2}{4D\tau}\right) - x'\sqrt{\pi} \operatorname{erfc}\left(\frac{x'}{2\sqrt{D\tau}}\right) \right) \right\} \cdot \left\{ \exp\left(-\frac{(x-x')^2}{4(t-\tau)D}\right) - \exp\left(-\frac{(x+x')^2}{4(t-\tau)D}\right) \right\} \quad (3)$$

(additional iterations can be done, if necessary). The first term is that of no absorption, and second term, subtracted from the first, describes the effect of absorption. This term, when plotted, is bell-shaped, as intuitively expected. It contains two parameters, σc_0 , σR_0 , to be determined. This was carried out by subtracting the experimental curve from the first term of eq. (3) to obtain the experimentally derived absorption curve. We then place the theoretical maximum near the experimental one. This fixes the values of σc_0 , σR_0 , and this completely determines the theoretical curve. Both curves are exhibited in Fig. 1 and agreement is seen to be definitely good. The concentrations themselves are shown in Fig. 2, with agreement being good again. A plateau is evident in both curves.

In these preliminary calculations the value of the depth, x , was one and a half micron; the value for the diffusion coefficient was that of water. Other values may be more realistic and will be used in subsequent calculations. The value of the cross-section σ was about 10^{-6} (micron)² and R_0 was about $20 C_0$, where C_0 is the saturation level of oxygen concentration in the layer. This indicates that the absorbing species are of a different type than the radicals that tied up the oxygen during the pulse, since the latter are estimated from the experimental set-up to be of the order of $3 C_0$. These findings are preliminary and are subject to modification on subsequent calculations.

B. LET and RBE Studies in Water for Cyclotron-Produced Neutron Beams

The current availability of relatively high neutron fluxes from installations such as the cyclotron at our laboratory, and elsewhere, has made possible the use of high energy neutron beams for biological experimentation and clinical applications. Such beams possess the deep penetration ability of x-rays and have a radiobiological effectiveness (RBE) well above that of x-rays; in addition the oxygen enhancement ratio is about 1.5 compared to about twice this for x-rays. Thus, neutron beams can be particularly effective in cases of deep-seated anoxic (radioresistant) tumors. The elevated values of RBE are due to the high values of the linear energy transfer (LET) of the recoil protons (tens of Kev per micron) and the still higher LET of the heavier ion recoils (hundreds of Kev per micron).

LET spectra have been obtained experimentally and theoretically in several cases involving neutron and charged particle beams. Furthermore, the resulting RBE of these irradiations and its dependence on LET has been of particular interest. Results on this point by various investigators (2) show qualitative agreement; quantitative differences are partly due to differences in experimental set-ups and methods. From a mathematical standpoint, the method to compute the biologically effective average LET is not known, and thus averages of various types (dose, track-averages) have been considered as a guide to RBE. RBE vs. LET studies today, however, consistently show an increase in RBE with LET up to about 100 Kev/ μ (maximum proton LET) and a decline at higher LET (3, 4, 5).

The particular concern of this investigation has been to analyze the variation of RBE vs. neutron penetration depth. More broadly the aim is to analyze and obtain the RBE range of variation on a few selected basic physical factors, which, in addition to depth are: 1) the average proton LET and the average heavy ion LET (on a dose basis), separately considered, 2) the relative dose fraction of proton and heavy ion recoils and x-rays. These factors were assigned a range of variations that cover most practical irradiation cases. A change in one of these factors results, in turn, in a specific change in RBE.

The analysis assumed that the RBE for mono-LET radiation increased with increasing LET up to $LET \sim 100$ and then decreased for still higher values of LET. Employing simple increasing and decreasing functions (linear and inverse linear, or exponentials), the trend in RBE with variations of the above-mentioned factors was obtained and tabulated. It should be noted that these factors are not necessarily independent for a given irradiation: changing depth in general results in

changed LET spectrum (i.e., changed LET averages) and modified proportions of proton and heavy recoil particle doses. But adequate spectral knowledge at depths of interest is often lacking and can change seriously with changed incident neutron spectrum. For this reason, the range of possible LET averages and dose fractions was considered at a few depths to obtain the corresponding ranges of RBE variations. This analysis shows the limits of RBE increase or decrease due to changes of spectra of most neutron beams of practical interest.

In particular, it was found that as a rule RBE tends to increase or remain practically constant with increasing depth. The increase was small, however, about ten percent or less. There are situations where a very small decrease in RBE is obtained, but this was for assumed LET averages and dose fractions that are unlikely to be practically encountered.

The work so far excluded the dose due to X-rays of the spectrum. This is substantial and may modify the RBE trend. This is indicated in Table I, which contains an example of current preliminary calculations, with and without X-ray component, based in part, on the depth-dose calculations of Snyder and Neufeld (6) for 5 MeV neutrons incident on water and utilizing average LET's in the range 10-20 KeV/ μ for the proton component and around 300 KeV/ μ for the heavy recoil components. A curve of RBE vs. LET similar to that of Barendsen, et al. (7) was used.

TABLE I

<u>Depth (cm.)</u>	<u>Without X-ray Component</u>	<u>With X-ray Component</u>
2	2.94	2.80
8	3.16	2.83
16	3.30	2.81

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FIGURE 1. Comparison of Experimental and Theoretical Oxygen Absorption Distributions

FIGURE 2. Comparison of Experimental and Theoretical Oxygen Diffusion Distributions

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