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IONIZING RADIATION INDUCED ATTACHMENT
REACTIONS OF NUCLEIC ACIDS AND THEIR COMPONENTS

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IONIZING RADIATION INDUCED ATTACHMENT REACTIONS
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INTERNATIONAL SYMPOSIUM

**PROTEIN AND OTHER ADDUCTS TO DNA: THEIR SIGNIFICANCE
TO AGING, CARCINOGENESIS, AND RADIATION BIOLOGY**

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TABLE OF CONTENTS

1. INTRODUCTION
2. MECHANISMS OF IONIZING RADIATION INDUCED SUBSTITUTION
AND ADDITION REACTIONS
3. REACTIONS WITH WATER, FORMATE, AND ALCOHOLS
4. REACTIONS WITH AMINES AND OTHER SMALL MOLECULES
5. NUCLEIC ACID-NUCLEIC ACID REACTIONS
6. REACTIONS WITH RADIATION SENSITIZERS
7. SUMMARY

1. INTRODUCTION

This paper is concerned with reactions which are initiated by ionizing radiations (γ -rays, x-rays, etc.) and which result in formation of new bonds involving nucleic acids or nucleic acid constituents. These reactions may differ from those initiated by near-ultraviolet radiation because identity of the substances affected by the radiation, the nature of the primary chemical species, and their distribution in the irradiated volume all differ. With ionizing radiations the amount of energy deposition in various molecules depends on their electron fraction, not their absorption spectra, and results in formation of significant yields of electrons separated from their parent molecules, cation radicals, and excited states ranging from the lowest excitation levels to superexcited states. These primary species are distributed along the path of each ionizing particle in small clusters in which the probability of interactions within the clusters is comparable to the probability of escape from the clusters. If escape occurs, reaction can occur at a considerable distance (10-1000 Angstroms) from the site of energy deposition. Many of the secondary and subsequent reactions started by the cation radicals and electrons involve free radicals similar to those frequently observed following absorption of near ultraviolet radiation. There is thus a large overlap between ionizing and ultraviolet radiation chemistry in spite of the differences noted above, and the fields contribute to a large degree to one another.

The emphasis in this paper is on non-protein molecules. An extensive review of protein-DNA attachment reactions is given by Yamamoto in an earlier chapter in this book.

Several lines of evidence suggest that attachments of organic molecules to DNA are likely to occur in living systems exposed to ionizing radiations. Among these are the following:

a) Although ionizing radiation is frequently thought of as destroying organic molecules, it is well known that it also can cause increases in size and complexity of molecules. Among important examples are radiation induced polymerization reactions (Dole 1973), and grafting of molecules onto cellulose (Arthur 1970);

b) Increasing information about 1) the microenvironment in the neighborhood of nucleic acids in cells, 2) the patterns of energy deposition by ionizing radiations, 3) the properties of initial chemical products of energy deposition, and 4) the subsequent reactions of these products suggests that in vivo conditions are favorable for formation of covalent bonds between DNA and molecules in the vicinity of DNA. (Myers 1973, a, b, 1974, Ward 1975);

c) Preliminary experiments with isolated nuclei of T. pyriformis have demonstrated that radiation induced binding of small molecules with DNA does indeed occur. In these experiments, ¹⁴C-labelled D-phenylalanine in the medium became attached to DNA during irradiation in such a way that it remained during standard DNA isolation and purification procedures (Byfield et al. 1970).

The significance of attachment reactions to radiation biology arises from the possibilities that they may be important lesions leading to the biological effects of ionizing radiation, and that they may account, at least in part, for the action of substances which modify radiation effects in living organisms. Regarding the former, DNA inactivation,

and phage and cell death are by no means entirely accounted for by the most frequently studied molecular lesions in irradiated DNA, single and double strand breaks (Freifelder 1974). Van der Schans and Bleichrodt (1974) report that 85% of the inactivation of PM2 bacteriophage in frozen solution is not associated with a chain break. Clearly other lethal lesions must be identified. One obvious candidate is base destruction. Another which, in view of the above discussion, must be considered is modification of DNA by attachment of organic molecules to base or pentose moieties. Such modifications might well include formation of DNA-protein crosslinks, intra or intermolecular DNA-DNA crosslinks, and attachment of various non-protein compounds to DNA. With respect to sensitization, several substances (discussed below) which sensitize anoxic cells to ionizing radiation have been shown to form bonds with DNA or DNA constituents (Emmerson and Willson 1968, Ward et al. 1969, Brustad et al. 1971, Cherman et al., 1973b, Willson et al. 1974). It has not been proven that the binding is the cause of the sensitization because other reactions, especially electron transfer also occur; but it may well be.

The general subject of this paper has been reviewed briefly (Myers 1974, Smith 1975).

2. MECHANISMS OF IONIZING RADIATION INDUCED SUBSTITUTION AND ADDITION REACTIONS

In considering the effects of ionizing radiations on nucleic acids in cells, it is important to keep in mind that the nucleic acids are in more or less intimate contact with many other kinds of substances. These are reasonably certain to include water, proteins, especially histones or protamines, membrane components, various other organic compounds, and metal counter-ions. When such a system is exposed to ionizing radiation,

a single 'hit', or act of energy deposition is very likely to involve two or more of the substances. A brief discussion of typical reactions follows.

If energy is deposited in a water molecule the initial reaction is believed to be separation of an electron with formation of the highly reactive cation radical, H_2O^+ (the dot signifies an unpaired electron). The H_2O^+ reacts almost immediately with H_2O to give a hydroxyl free radical ($\cdot\text{OH}$). The electron may interact with water molecules to give a hydrated electron, e_{aq}^- , or with a compound which has an appreciable electron affinity. In dilute aqueous solutions yields of $\cdot\text{OH}$ and e_{aq}^- per 1000 rad, with x- or γ -rays, are about 2.7×10^{-6} mol/liter. Small yields of hydrogen atoms, H_2 , and H_2O_2 ($\sim 0.5 \times 10^{-6}$ mol/liter) also are observed. With α -radiation, yields of H_2 and H_2O_2 are larger, and of $\cdot\text{OH}$ and e_{aq}^- , smaller.

Analogous reactions occur if energy is deposited in any of the organic compounds, including DNA. These may be written formally

The cation radicals may react with other molecules, or may undergo deprotonation reactions, possibly with water, to form neutral free radicals.

The fate of the electrons is the same as that of the electron from water.

Secondary reactions include a) capture of hydrated electrons by organic molecules, b) transfer of electrons from substances of lower electron affinity to substances with higher electron affinities (Adams et al., 1968),

c) reaction of hydroxyl free radicals with organic compounds to produce organic free radicals, and d) interactions involving the organic free radicals to give nucleic acid with organic substances incorporated as adducts or substituents. Items a) and b) will not be discussed further. The hydroxyl free radical (item c) reacts by addition to unsaturated bonds of organic compounds, or by abstraction of hydrogen atoms from saturated C-H bonds. With nucleic acids, it has been shown to add to the 5,6-double bond of pyrimidine bases (illustrated for thymine) and to the 4, 5, or possibly the 7,8-double bond of purine bases.

The radical shown will be referred to as the Thy- $\cdot\text{OH}$ adduct radical in the rest of this paper. The $\cdot\text{OH}$ free radical abstracts hydrogen from the deoxyribose or ribose part of nucleic acids, but the site of attack is not certain. The reaction with ethyl alcohol is illustrative:

On absorption by aqueous DNA solutions of several thousand rad it has been shown that about 80% of the $\cdot\text{OH}$ reacts with base and about 20% with the deoxypentose moieties (Scholes et al. 1960). Evidence is accumulating, however, which is consistent with the notion that the proportion of attack on the bases as compared with that on the pentose in native double stranded DNA is less until after the molecule has become partially uncoiled as a result of strand breakage (Ward 1975). The proportion of attack on the various bases is in proportion to the amount of each base in the DNA (Scholes et al. 1969, Myers et al. 1973b).

The rate constants for reaction of $\cdot\text{OH}$ with nucleic acids and the organic compounds in the vicinity of nucleic acids are very large, of the order of $10^8 - 10^9 \text{ liter mol}^{-1} \text{ sec}^{-1}$, or close to the limit imposed by the rate of diffusion. It may be anticipated, therefore, that $\cdot\text{OH}$ will react at a site close to its site of formation with almost any organic compound which is available.

Relatively little is known about structure and reactivity of cation radicals formed by deposition of energy in nucleic acids or of their deprotonated derivatives. Cation radicals are reported to have been observed by ESR spectroscopy at 77°K of irradiated thymine, cytosine, and DNA (unpaired spin localized on cytosine or guanine (Gräslund et al., 1971)), and several neutral free radicals that could have been formed by deprotonation of cation radicals have been observed at higher temperatures (see Myers 1974 for a review). Among the latter is a substituted methyl radical observed in both irradiated thymine and DNA (Hartig and Dertinger 1971).

(2-7)

Interactions involving the organic free radicals may lead to formation of covalent bonds between nucleic acids and organic compounds by several reaction paths. These include reactions between a) organic molecules and nucleic acid radicals (neutral or charged):

b) organic radicals (neutral or charged) and nucleic acid molecules:

and c) organic radicals and nucleic acid radicals:

In the remainder of this paper experimental results, obtained for the most part with model systems, will be reviewed. These will show that each of the three pathways may play a role in radiolysis of DNA in vivo.

3. REACTIONS WITH WATER, FORMATE, AND ALCOHOLS (TABLE 1)

3.1. Addition of Water or Parts of Water

Reactions of nucleic acid constituents and nucleic acids with water have been studied extensively (for reviews see Scholes 1968 , Ward 1975) and only sufficient information will be given here to provide examples.

On radiolysis of oxygen-free dilute aqueous solutions of thymine, the thymine is converted in less than stoichiometric amounts to compounds with elements of water added to the 5,6- double bond or substituted into the methyl group. Among the products are the hydrate, the glycol, and dihydrothymine (Teoule et al., 1972, Teoule and Cadet 1975, Infante et al., 1973). Similar products are obtained with uracil (Smith and Hays, 1968).

These substances, in turn, appear to have H_2O , H_2O_2 , and H_2 added to the base. They are probably formed by disproportionation reactions of the electron or $\cdot\text{OH}$ -adduct radicals. For example,

Analogous reactions with inclusion of protonation steps would give dihydrothymine and the hydrates. Formation of these products by consecutive reactions with two $\cdot\text{OH}$, 2e^-_{aq} , or $\cdot\text{OH} + \text{e}^-_{\text{aq}}$ can be excluded by kinetic considerations.

Yields for thymine disappearance are well below the amounts of $\cdot\text{OH}$ and e_{aq}^- produced by radiolysis of water. This was recognized many years ago (Scholes et. al. 1960) and it was proposed that reactions occur which regenerate the parent molecule. The disproportionation reactions for formation of products are among these. These reactions reduce the maximum yield to half the initial amount of $\cdot\text{OH}$ and e_{aq}^- ; yields are lower than this, and variable, however, so that other reconstitution reactions must occur. Recently it has been shown that the glycol can be converted to thymine by radiation (Namiki and Hayashi 1970) and it is well known that the hydrates reconstitute the parent molecule. Further, the yields of the disproportionation reactions may be reduced by reactions such as

If oxygen is present a different set of reactions occurs. First, the oxygen reacts with e_{aq}^- to give the anion radical of HO_2

The anion radical is the stable form at physiological pH. The reaction prevents e_{aq}^- from reacting with Thy (or DNA) but $\cdot\text{OH}$ can still add to give the Thy- $\cdot\text{OH}$ adduct radical. Oxygen adds to this radical to give the 5-hydroxy-6-hydroperoxy radical (Scholes et al., 1960).

(3-5)

This radical is reduced by electron transfer from $\cdot\text{O}_2^-$ to give the anion of the hydroxy hydroperoxide.

This addition of oxygen blocks the restitution reactions and increases the yield for thymine destruction to the yield of $\cdot\text{OH}$ produced by radiation. Several products, especially the glycol, are formed in addition to the hydroxy hydroperoxide.

Radiation induced reactions of other bases, and of nucleosides and nucleotides have not been studied as extensively as those of thymine. The reactions appear to be similar to those of thymine in that $\cdot\text{OH}$ adds to unsaturated bonds of all the bases, restitution reactions occur, and O_2 adds to the base- $\cdot\text{OH}$ adduct radicals and with nucleosides and nucleotides, to radicals on the pentose moiety. Some of the secondary reactions leading to final products differ, however (Scholes 1968, Cadet and Teoule 1972, Ward 1975). The hydroxyhydroperoxides of thymine and cytosine have been observed in DNA irradiated in vitro (Schweibert and Daniels 1971) and of thymine in vivo (Cerutti 1974).

3.2. Addition of $\cdot\text{COO}^-$ Ion Radical and Related Reactions

Formate ion in oxygen free irradiated solutions can also block restitution reactions (Kamel and Garrison 1965). In solutions of

cytosine (the diagrams show only the 5,6-double bond of cytosine)

the following occurs.

This is followed by the addition reactions:

These radicals undergo disproportionation to give cytosine and

The effect is to increase the yield for cytosine destruction, just as does oxygen. Other substances which accomplish the same result are ethanol (Kamal and Garrison 1965), cysteine, and ascorbic acid (Holian and Garrison 1969). The latter two compounds are believed to react by transfer of an H-atom to the base anion radical, i.e., the H-atom adds. In the absence of any additive the restitution reaction can be written

In the presence of cysteine, RSH, this reaction is stopped by

The yield of BH_2 is equal to the yield of e_{aq}^- . This reaction illustrates very well a non-protective reaction of sulfhydryl compounds.

3.3. Reactions with Alcohols

Ethyl alcohol has been shown to add to thymine by reactions which involve the hydroxyethyl radical (Brown et. al. 1966). In oxygen free aqueous solutions containing about 5×10^{-3} M thymine and 0.1M EtOH nearly all $\cdot\text{OH}$ produced by radiolysis of water react with the alcohol to give the previously shown hydroxy ethyl radical (Eq. 2-6). The product isolated is

(3-12)

Both cis and trans isomers are formed. These compounds are probably formed by a disproportionation reaction between the thymine anion radical (formed by capture of the hydrated electron) and the thymine hydroxyethyl adduct radical. The yield is about one-third of the yield of $\cdot\text{OH}$. N,N'-dimethylthymine undergoes a similar reaction with ethyl alcohol with of yield nearly equal to the $\cdot\text{OH}$ yield. The O,O'-dimethylthymine does not undergo the addition reaction.

The thymine-alcohol adduct compound undergoes a secondary reaction on continued irradiation of oxygen-free nitrogen-saturated solutions which leads to formation of 6-ethylthymine, in which the 5,6-double bond has been restored (Zarebska and Shugar, 1972).

(3-13)

Both $\cdot\text{OH}$ and electrons appear to be essential for this reaction, but the mechanism is not known. It should be noted, however, that while it appears that substitution of ethyl for hydrogen has occurred, the mechanism is not that of a simple substitution reaction. Confirming that ethylthymine is a secondary product, the yield (amount formed per unit radiation dose) increases with increasing radiation.

Purine bases have been shown to undergo radiation-induced reactions with alcohols which result in substitution at the C(8) position. The reaction is shown for caffeine (Elad et al., 1969).

Isopropyl alcohol forms the analogous product plus 8-ethylcaffeine in a 1-1 ratio.

Substitution of 2-propanol into adenine, adenosine, guanosine, 2-deoxyguanosine, and 4-deoxypyrimidine has also been observed (Steinmaus, et al. 1969, 1971).

These particular experiments were carried out under very different conditions from those for the pyrimidine reactions described above. Unfortunately, experiments have not been carried out under comparable conditions with both purines and pyrimidines. Steinmaus et al. dissolved small amounts of the purine compound in the alcohol or a solution of

approximately equal amounts of the alcohol and acetone. In some experiments a little water was present, but in all cases most of the energy was absorbed by the alcohol and acetone. The apparent substitution is believed to occur by addition of alcohol free radicals and $H\cdot$ (or $e^- + H^+$) generated by the radiation, to the 8-9 double bond of caffeine or the 7-8 double bond of other purines, followed by an oxidation step during isolation of the compound which gives the observed product. Yields range from 15 to 80% conversion of the initial material after radiation doses of the order of 10^7 rad, and for the reaction of 2-propanol with caffeine, are larger by a factor of about two if acetone is present in the solution.

The relative reactivity of purines and pyrimidines was investigated by Leonov et al. (1973). Mixtures of about equal amounts of thymine and adenine (~ 3 mmol each) in 2-propanol were irradiated. Although both bases react with alcohol when irradiated separately, in the mixture, all of the reaction was with the purine. A similar result was obtained with a mixture of adenosine and thymine. Leonov and Elad (1974a) suggest that the suppression of the reactivity of the pyrimidines may be related to association of the pyrimidines with purines in alcohol solutions. An alternative explanation may be that the rate constants for reactions between the alcohol radical and the two kinds of bases differ.

4.1 REACTIONS WITH AMINES AND OTHER SMALL MOLECULES (TABLE 2)

Many aliphatic amines react with purines to give substitution of an N -alkenyl or an alkyl group for the hydrogen atom at C(8) of the purine. Irradiation of deoxygenated 5M aqueous amine solutions containing caffeine is reported to give the alkyl substituent (Stankunas, et al. 1971).

Thus ethyl-, diethyl-, and triethylamine all give 8-ethylcaffeine. Normal propylamine gives 8-n-propylcaffeine, and isopropylamine gives 8-isopropylcaffeine. Tertiary-butylamine does not react with caffeine, suggesting that a hydrogen atom on the α -carbon atom is necessary for reaction. The composition of the solutions used for these experiments is such that 25-50% of the energy from the radiation is deposited in the amine, and the rest in water. Thus all of the products from both water and amine radiolysis are available for the reaction, and it is impossible with the available data to suggest a mechanism. It may be suggested by analogy with the alkylation of thymine and purines by alcohol that the reactions proceed by addition to the 8-9 double bond, and that secondary reactions lead to formation of the substituted compound.

In similar experiments in which energy deposition was almost entirely in the amine, i.e., the solutions contained much less water, Elad and Salomon (1971) and Salomon and Elad (1974) obtained α -aminoalkyl substituents of purines as well as the alkoyl substituents. Thus radiolysis of iso-propylamine solutions containing adenine gave 8- α -amino-isopropyladenine. Under similar conditions piperidine formed 8-(2' piperidyl)-caffeine and 8-(2'piperidyl)-adenine.

Available evidence indicates that the cyclic ether, tetrahydrofuran (THF), and dioxane react with purines under the influence of ionizing radiation by reactions analogous to those of the amines and alcohols discussed above (Leonov and Elad, 1974b). THF gives the appropriate 8-substituted caffeine and adenine derivatives, and dioxane the 8-substituted derivatives of caffeine. The substituant is attached to the

purine C(8) atom by a bond to the carbon alpha to a ring oxygen. The substitution reaction is believed to take place by means of free radical addition reactions followed by secondary reactions which give the observed product.

Several small molecules have been observed to become attached to DNA or RNA on irradiation of aqueous solutions. These include thyroxine, glyceraldehyde, leucine, and other amino acids (Byfield et. al. 1970). The attachment of C¹⁴-leucine, as determined by acid insolubility, is proportional to DNA concentration (0.05% to 0.2%) and to radiation dose to 10⁶ rad. On hydrolysis and paper chromatographic separation of components of the irradiated DNA, the C¹⁴ is not localized, suggesting that several products may have been formed.

What appears to be an entirely different kind of reaction has been observed with polycyclic aromatic compounds such as benz(a)pyrene (BP) and pyrene (P) (Chan and Ball, 1971). When a physical complex between either of these compounds and calf thymus DNA is irradiated in a dilute cacodylate solution, the hydrocarbon becomes covalently bonded to the DNA. The degree of binding is negligible to a dose of about 8000 rad, but then increases rapidly. After a dose of 17,000 rad, the amounts of binding of BP and P respectively are 1 mol/100 and 1 mol/270 DNA nucleotides (initial ratios 1 to 48 and 1 to 27). The criterion for binding are unextractability by ethanol or cyclohexane, and shifts in the ultra-violet absorption spectra. The template activity of DNA containing bound BP (1 mol/370 nucleotides) in an in vitro RNA synthesizing system at short (15 min.) incubation times is the same as that of control DNA

(no BP) exposed to the same amount of radiation (10,000 rad) - about 45% of that of unirradiated DNA. After 30 minutes of incubation, the activity is only 80% of that of the control DNA. The results suggest that the covalently bound BP does not block binding of the RNA polymerase or interfere with initiation of transcription, but that it does result in retardation or termination of transcription when a growing RNA chain encounters a bound BP molecule.

Irradiation of a 0.1% calf thymus DNA solution containing fluorescein has been reported to result in binding of the dye to the macromolecule (Andersson 1969).

5. NUCLEIC ACID-NUCLEIC ACID INTERACTIONS (TABLE 3)

5.1. Base-Base Attachment Reaction to Give Dimers on Radiolysis of Uracil

In spite of many investigations of the radiolysis of pyrimidine bases in the absence of oxygen, a satisfactory understanding of the reactions has not been obtained. It has recently been reported (Shragge et al. 1974), however, that several major dimeric products of uracil radiolysis have been overlooked. The products formed at pH 5 in N_2 -saturated solutions have structures such as

(5-1)

The corresponding 6-6 and 5-6 dimers are also possible. The dimers are believed to be formed by the radical combination reactions $\text{Ura}^-\cdot\text{OH} + (\text{Ura})^-(\text{H}^+)$ and $2 \text{Ura}^-\cdot\text{OH}$ respectively. At pH 8.5, also in N_2 , dimers have been obtained in which the 5,6 bonds of the rings are unsaturated, for example

The 2-pyrimidone derivative is probably formed, as well.

Dimers of the cyclobutane type produced by photolysis are not reported to be formed by ionizing radiation.

5.2. Intramolecular Attachment of Pentose and Base Moieties of Nucleotides.

Keck et. al. (1966) reported that irradiation of adenosine monophosphate (AMP) in deoxygenated solutions resulted in attachment of the ribose to the base at the C(8) position, giving a cyclonucleotide. Raleigh and Kramers (1975) have identified the compound as the 8,5' cyclo-compound. The yield is equal to the yield for inorganic phosphate release over a wide pH range and is doubled when N_2O is substituted for oxygen (N_2O converts e_{aq}^- to $\cdot\text{OH}$), suggesting that $\cdot\text{OH}$ brings about the reaction. Oxygen inhibits formation of the cyclonucleotide. Attack on the base by the lesion on the pentose moiety may be a significant event in the radiolysis of intact DNA, in which bases appear to be at least partly shielded from attack by $\cdot\text{OH}$ (Ward 1975).

5.3. Attachment of bases to Nucleic Acids.

Nucleic acid bases become attached to RNA (Sigma Type XI from baker's yeast) when aqueous solutions (about 1% RNA, 5×10^{-4} M bases) are exposed to γ -rays at near neutral pH (Yamamoto 1973). The criterion for attachment was precipitation of the bases (^{14}C -labeled) with RNA by 5% trichloroacetic acid. Binding yields in the presence of air were less than 1% of the starting materials for doses up to 10^6 rad, and were less than yields for binding to serum albumin (25-45%) under comparable conditions. In the absence of air, binding yields were likewise small when an $\cdot\text{OH}$ scavenger such as methanol or thiocyanate was present. With a nitrogen atmosphere, and no $\cdot\text{OH}$ scavenger binding yields of about 1.5% were obtained for pyrimidine bases, and about 0.5% for purine bases. With N_2O , which converts e_{aq}^- to $\cdot\text{OH}$, in solution, binding yields were increased to about 2% (4.8×10^4 rad) for pyrimidines and 1% for purines. This results suggest that the binding reaction is initiated by $\cdot\text{OH}$, but the reaction mechanisms and product structures are unknown.

5.4. Crosslinking of Nucleic Acids.

Crosslinking of DNA with DNA has been observed on irradiation of isolated salmon or herring sperm DNA containing 0 to 300% of its weight in water (Lett, et al, 1961, Lett and Alexander 1961). The criteria used to establish occurrence of crosslinking was a combination of measurements of viscosity, radius of gyration, and determination of molecular weight by light scattering. The radiation was 1-2 MeV electrons; doses were of the order of a megarad. The crosslinkage reaction competes with chain scission. In dry DNA or DNA containing 25% of its weight in water crosslinking predominates slightly with the

result that branched molecules are formed. Oxygen decreases the yield of crosslinks slightly. Increasing the water content to 200% of the weight of DNA increases the amount of crosslinking in the absence of oxygen, and results in formation of a gel by a dose of 10^6 rad. Oxygen has a greater inhibition effect in moist than in dry DNA. Further increase in water content gives a relative increase in the scission reaction; with water contents greater than 300% no gel is formed. It is hypothesized that absorption of energy from the radiation by DNA causes a rupture of the chain with formation of "active centers," probably free radicals. Under sterically favorable conditions, two of these combine to give a crosslink if no oxygen is present. Small amounts of water increase the probability of the active centers coming together, but larger amounts increase the efficiency of degradation processes.

Radiolysis of DNA from E. coli bacteriophage T1 in dilute aqueous solution (200 μ g DNA/ml, 0.165 M NaCl) gives a small yield of crosslinks to doses of about 2000 rad. No further crosslinkage occurs as the dose is increased (Coquerelle et al., 1969). When the intact phage is irradiated under conditions in which most of the products of water radiolysis are scavenged it was found that per inactivated phage, 0.03 DNA-DNA crosslinks were formed. This yield is small compared with other lesions: 3.5 single strand breaks, 0.17 double strand breaks, and 0.06 DNA-protein crosslinks (Bohne et al., 1970).

6. REACTIONS WITH RADIATION SENSITIZERS (TABLE 4)

The sensitizers considered here are substances which increase the sensitivity of living cells, usually relatively anoxic, to the effects

of ionizing radiation. They must not be confused with the sensitizers used in photochemistry.

6.1. 5-Bromo uracil (BU)

Under certain conditions, this substance can be made to replace some of the thymine in DNA. Cells in which such replacement has been made are generally more radiosensitive than normal cells. A chemical mechanism to explain the increased sensitivity had been proposed (Adams, 1967, Zimbrick et al. 1969 a, b). BU reacts with electrons by a rapid dissociative attachment reaction to give a uracilyl radical.

The electron source may be a hydrated electron, an anion radical such as $\text{COO}^\bullet$, or some other substance capable of transferring an electron. The uracilyl radical is highly reactive and will react with hydrogen donors to form uracil and another organic free radical.

The donor may well be a neighboring pentose moiety on the DNA main chain. An alternative reaction of the uracilyl radical is addition of oxygen (Gilbert et al., 1971, Gilbert and Wagner 1972). The rate constant for oxygen addition is 24 times greater than that for H-abstraction from methanol (which should behave kinetically somewhat like the deoxyribose moiety of DNA). Which reaction will occur will depend on the local oxygen concentration. If the abstraction reaction occurs, the DNA will have a foreign base, uracil, and a lesion on a deoxyribose moiety which may lead to a strand break or other chemical change. If oxygen adds, a peroxyradical will be present in the DNA where a thymine base ought to be. Either event would be expected to cause difficulty in DNA function.

6.2. N-ethylmalimide (NEM).

This compound, when present in solution during irradiation, sensitizes anoxic cells to the lethal effects of radiation. It undergoes several kinds of reactions, including binding to DNA, which may contribute to its effects. The binding is dose dependent and appears to involve a reaction between DNA radicals and NEM molecules. Oxygen competes with NEM for binding sites, and in oxygen saturated solutions, binding of NEM does not occur (Johansen et al 1968, Ward et al. 1969).

A suggestion as to the mechanism of binding has been obtained in model experiments with thymine and other bases. These show that NEM binds to the Thy- $\cdot$ OH adduct radical. It also is expected to bind at a radical site formed by H $\cdot$ abstraction from the pentose moiety of DNA. At either site, competition with oxygen would be expected.

The relation between NEM binding and sensitization of DNA is not certain because NEM undergoes other reactions. It reacts rapidly with e_{aq}^- and $\cdot OH$ ($k_{(e_{aq}^- + NEM)} = 3.2 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$, $k_{(\cdot OH + NEM)} = 5 \times 10^9$), with $\text{Thy-}\cdot OH$ adduct in part by electron transfer, and with other base radicals. It is certain, however, that in cellular systems, NEM will react with organic radicals produced by radiation, thus altering yields and types of radiation-induced products.

6.3. Nitrofurans.

Various derivatives of nitrofuran appear to have good potential as sensitizing agents in anoxic or hypoxic cells. Many of these compounds have the general structure

Nitrofurazone is R-CH=NNHCONH_2 .

Metronidazole has the somewhat

different structure:

Structures of related compounds are given by Chapman et. al. (1973a).

These compounds undergo several reactions in irradiated systems. Among them are reactions with e_{aq}^- , with $\cdot OH$, with organic free radicals (observed with metronidazole), binding with DNA, and binding with protein (observed with nitrofurazone), (Chapman et al. 1972, Chapman et al. 1973b, Willson et al. 1974). The binding reaction with DNA is decreased by

oxygen, i.e., the sensitizer and oxygen appear to compete for the same site. Conditions for binding indicate that the sensitizer molecule reacts with a DNA free radical generated by $\cdot\text{OH}$. The binding efficiency is small, only about 4% with metronidazole, but since the amount of binding required for cell death is unknown, it is possible that the reaction may play an important role in sensitization.

6.4. TAN (2,2,6,6,-tetramethyl-4-piperidone-N-oxyl).

Since the report by Emmerson and Howard-Flanders (1964) of the ability of nitroxides to sensitize anoxic cells to radiation, this family of compounds has been studied extensively. The compounds differ from the other sensitizing agents discussed above in that they are "stable" free radicals, i.e., they have an unpaired electron. The structure of TAN, for example, is

This compound reacts with the Thy- $\cdot\text{OH}$ adduct with the large bimolecular rate constants $k = 3.5 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}$ (Emmerson and Willson, 1968). Similar values have been obtained for reaction with Cyt- $\cdot\text{OH}$ and Gua- $\cdot\text{OH}$, but the adenine adduct appears to react much more slowly ($k < 10^{-7} \text{ M}^{-1} \text{ sec}^{-1}$). (Brustad, et al. 1972) The adenosine- $\cdot\text{OH}$ adduct reacts normally (Wold and Brustad, 1973). The major products of the reaction with thymine- $\cdot\text{OH}$ adduct (Cadet et al. 1975) have been identified as the cis- and trans-isomers of TAN-Thymine- $\cdot\text{OH}$ adducts in which attachment has occurred by radical-radical reactions.

A minor product was the product of a reaction with the methyl group, also a radical-radical reaction.

Radiolysis of thymidine in the presence of TAN gives a mixture of pyrimidine and nucleoside products. Binding of TAN to sugar free radicals seems to be a minor process. The main thymidine products were identified as the (+) and (-) diastereoisomers of cis- and trans-TAN-Thy-[•]OH adducts with the TAN on the 6-position of the pyrimidine ring.

In experiments with DNA (calf thymus) it has been found that TAN binds with DNA radicals (Brustad et al. 1971) induced primarily by reactions of [•]OH with the DNA. In addition to binding, TAN reacts with a transient DNA species by electron transfer, to give reduced TAN.

Binding of TAN to DNA during and immediately after irradiation of E. coli K-12 has been observed and compared with the degree of sensitization (Wold and Brustad, 1974). These experiments make use of the long lifetimes of DNA radicals. It was found that binding can occur without sensitization, but radiosensitization is always accompanied by some binding. It may be that binding is necessary, but not sufficient, for sensitization. It also was noted that sensitization is delayed compared with entrance of TAN into the bacterium, suggesting that the lethal event may be interaction of TAN with sterically hindered DNA radical sites in base pairs.

7. IMPORTANCE OF ATTACHMENT REACTIONS IN RADIATION BIOLOGY

In the foregoing material it has been shown that explanations of effects of ionizing radiations on living organisms are by no means complete, and that mechanisms for modifying radiation effects are even less well understood. It has also been pointed out on the basis of fundamental concepts that the environment within a cell is such that complex radiation induced reactions between different substances should be expected and that these should include attachment reactions. The possibility of such reactions has been confirmed by studies with model systems which have shown that attachment and other reactions occur between nucleic acid constituents and numerous compounds related to those existing in the vicinity of nucleic acids in cells. Studies of reaction mechanisms have shown that many of the reactions take place by radical-molecule reactions, with the radical sometimes initially on the nucleic acid constituent and sometimes on the other molecule. Other attachment reactions have been shown to occur by radical-radical reactions. Radiation modifiers have been shown to become attached to nucleic acids in vitro and in vivo, and there are indications that attachment may be necessary for the action of certain sensitizers.

This accumulated evidence suggests that further investigation of attachment and related reactions which may be reasonably expected to occur in cells will be an important and critical part of our continuing efforts to understand radiation effects and develop ways to modify them.

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TABLE 1

ATTACHMENT REACTIONS OF NUCLEIC ACIDS INDUCED BY IONIZING RADIATIONS:
WATER, FORMATE, AND ALCOHOLS

<u>Compound</u>	<u>Nucleic Acid Derivative</u>	<u>Reference</u>
H ₂ O	Thymine	Teoule, et al., 1972
		Infante, et al., 1973
H ₂ O	Uracil	Smith and Hays, 1968
O ₂	Base- [•] OH adduct	Scholes et al., 1960
formate	Cytosine	Kamel and Garrison, 1965
ethanol	Cytosine	Kamel and Garrison, 1965
ethanol	Thymine	Brown, et al., 1966
"	N,N'-dimethylthymine	"
"	Thymine	Zarebska and Shugar, 1972
"	Caffeine	Elad, et al., 1969
2-propanol	Caffeine	"
"	Adenine	Steinmaus, et al., 1969, 1971
"	Adenosine	"
"	Guanosine	"
"	2-deoxyguanosine	"
"	6-ethoxypurine	"
"	Thymine plus adenine	Leonov, et al., 1975
"	Thymine plus adenosine	"

TABLE 2

ATTACHMENT REACTIONS OF NUCLEIC ACIDS INDUCED BY IONIZING RADIATIONS:

AMINES AND OTHER SMALL MOLECULES

<u>Compound</u>	<u>Nucleic Acid Derivative</u>	<u>Reference</u>
D-phenylalanine	DNA <u>in vivo</u>	Byfield, et al., 1970
ethylamine	Caffeine	Stankunas, et al., 1971
diethylamine	"	"
triethylamine	"	"
<u>n</u> -propylamine	"	"
<u>iso</u> -propylamine	"	"
t-butylamine	" (no reaction)	"
<u>iso</u> -propylamine	Adenine	Salomon and Elad, 1974
piperidine	"	"
"	Caffeine	"
tetrahydrofuran	Caffeine	Leonov and Elad, 1974
"	Adenine	"
dioxane	Caffeine	"
thyroxine	DNA <u>in vitro</u>	Byfield, et al., 1970
glyceraldehyde	"	"
leucine	"	"
benz(a)pyrene	DNA	Chan and Ball, 1971
pyrene	"	"
fluorescein	"	Andersson, 1969

TABLE 3

ATTACHMENT REACTIONS OF NUCLEIC ACIDS INDUCED BY IONIZING RADIATIONS:

NUCLEIC ACID-NUCLEIC ACID INTERACTIONS

<u>Compound</u>	<u>Nucleic Acid Derivative</u>	<u>Reference</u>
uracil	Uracil	Shragge et al., 1974
adenosine mono- phosphate	(Intramolecular)	Keck et al., 1966, Raleigh and Kramers, 1975
uracil	RNA	Yamamoto, 1973
thymine	"	"
cytosine	"	"
adenine	"	"
guanine	"	"
DNA	DNA	Lett et al., 1961, Lett and Alexander, 1961
DNA	DNA	Coquerelle et al., 1969
"	DNA in phage	Bohne et al., 1970

TABLE 4

ATTACHMENT REACTIONS OF NUCLEIC ACIDS INDUCED BY IONIZING RADIATIONS:
RADIATION SENSITIZERS

<u>Compound</u>	<u>Nucleic Acid Derivative</u>	<u>Reference</u>
electrons	5-bromouracil (BU)	Adams, 1967, Zimbrick et al., 1969a.
"	BU-DNA	Zimbrick et al., 1969b.
N-ethylmaleimide	Thy- $\cdot$ OH adduct radical	Ward et al., 1969
"	DNA	Johnsen et al., 1968
Nitrofurazone	DNA	Chapman et al., 1972 Chapman et al., 1973
metronidazole	DNA	Willson et al., 1974
TAN	Thy- $\cdot$ OH adduct	Willson and Emmerson, 1968, Cadet et al., 1975
"	Base- $\cdot$ OH adducts	Brustad et al., 1972, Wold and Brustad, 1973
"	DNA <u>in vitro</u>	Brustad et al., 1971
"	DNA <u>in vivo</u>	Wold and Brustad, 1974