NUCLEOTIDE RADICAL OXIDATION AND ADDITION REACTIONS WITH CELLULAR RADIOSENSITIZERS

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Received March 8,1973

SUMMARY

The yields of inorganic phosphate (iPO_4) released from deoxygenated solutions of irradiated purine 5'-mononucleotides are enhanced in the presence of low concentrations of oxygen, or such electron-affinic cellular radiosensitizers as p-nitroacetophenone and nitrofurans. The degree of radiosensitization increases with the sensitizer's electron-affinity, oxygen showing an approximately two-fold sensitization. Other electron-affinic cellular radiosensitizers, including the stable free radical triacetonamine-N-oxyl (TAN), protect against iPO_4 release, the degree of protection increasing with the sensitizer's electron-affinity. These findings are consistent with competition between nucleotide free radical oxidation and addition to sensitizer. Their relevance to mechanisms of radiosensitization is discussed.

Recently, electron-affinic compounds have been shown to mimic oxygen in their ability to radiosensitize a wide variety of chemical and biological systems[1-6]. Several types of chemical damage to DNA and its components, including destruction of bases[3], single-strand breaks[7] and release of iPO_4 from mononucleotides[1,2], are enhanced by the addition of radiosensitizers, and in each case the degree of radiosensitization is in good agreement with the results for in-vitro cellular systems. In all these experiments, radiosensitization only occurs in the absence of oxygen or of hydroxyl-radical scavengers, and is maximal at low concentrations of sensitizer (0.5 to 2 x 10^{-4} M). The degree of radiosensitization generally increases with increasing electron-affinity of the sensitizer, although the structures of both the substrate and radiosensitizer influence the effect[1]. We have studied the radiation-induced release of iPO_4 from the purine

mononucleotide guanosine 5'-monophosphate (5'-GMP), and the effect of a wide range of potential electron-affinic radiosensitizers, as means of investigating molecular mechanisms of cellular radiosensitization. The radiosensitizers, p-nitroacetophenone and the nitrofurans, enhance the yield of iPO_4 release from 5'-GMP in a manner analogous to that found previously by other workers for O_2[8]. Other radiosensitizers, however, including TAN, protect against iPO_4 release at concentrations well below those required for competitive scavenging of OH free radicals. In both redox processes, sensitizer molecules compete for nucleotide radicals.

METHODS

Unbuffered solutions of 5'-GMP (10^{-3} M) containing different concentrations of radiosensitizer (10^{-6} to 10^{-3} M) were prepared in triply distilled water. Freshly prepared solutions were degassed by bubbling with high-purity nitrogen and N_2O before and during irradiation in a γ-cell at a dose rate of 15 krad/min, as measured by Fricke dosimetry[1]. The yield of radiation-induced release of iPO_4 was measured immediately after irradiation with a Technicon Autoanalyser, using an ammonium molybdate procedure[1]. The efficiency of radiosensitized iPO_4 release in deoxygenated solution is defined for the optimal sensitizer concentration $[C]_o$, as a percentage of the oxygen effect:

$$\text{Sensitization Efficiency} = \left\{ \frac{G(-iPO_4)[C]_o - G(-iPO_4)_{N_2}}{G(-iPO_4)_{O_2} - G(-iPO_4)_{N_2}} \right\} \times 100\%$$

By this definition, a sensitization efficiency of 100% equals the oxygen effect. For the electron-affinic compounds which protect against iPO_4 release, their efficiency is expressed as a percentage of the control G value (number per 100 eV of energy absorbed) for iPO_4 release in deoxygenated solutions:

$$\text{Protection Efficiency} = \left\{ \frac{G(-iPO_4)_{N_2} - G(iPO_4)[C]_o}{G(-iPO_4)_{N_2}} \right\} \times 100\ \%$$

According to this expression, 100% protection occurs when $G(-iPO_4)[C]_o = 0$

RESULTS AND DISCUSSION

The yield of iPO_4 released from γ-irradiated aqueous solutions of 10^{-3} M 5'-GMP depends upon the presence or absence of oxygen. Figure 1 shows

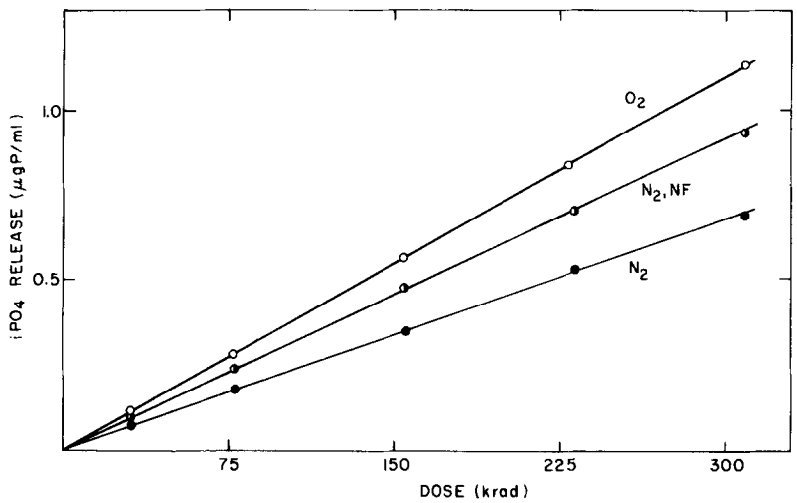

Figure 1: Radiation induced release of iPO_4 from 10^{-3} M 5'-GMP in deoxygenated (●) and oxygenated (○) solutions. The radiosensitizing effect of 10^{-4} M nitrofurazone (NF) in deoxygenated solution is also shown (◑)

that in all cases the amount of iPO_4 released increases linearly with dose. The G value for iPO_4 release $\{G(-iPO_4)\}$ in oxygen-saturated solutions is 0.21, compared with 0.13 in deoxygenated solutions. Other electron affinic compounds, including nitrofurazone (NF), also sensitize the release of iPO_4 when present in low concentrations in deoxygenated solution (Figure 1). The degree of sensitization depends upon the sensitizer's electron affinity and is independent of dose[1]. No additional sensitization occurs in the presence of electron-affinic oxygen suggesting a competition for the same nucleotide free radical.

For a fixed high concentration of 5'-GMP, the degree of radiosensitization of iPO_4 release by a particular radiosensitizer depends upon its concentration. The results are shown for nitrofurazone in Figure 2a and 2b, in N_2 saturated and N_2O saturated solutions, respectively. The effect of oxygen is

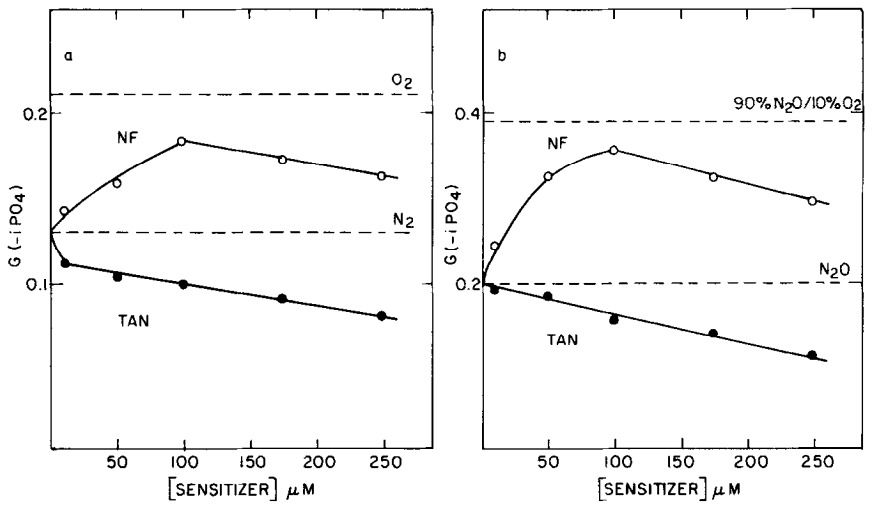

Figure 2: G values for iPO_4 release from 10^{-3} M 5'-GMP as a function of sensitizer concentration for a) deoxygenated, and b) N_2O saturated solutions. The dotted lines show the effect of oxygen.

shown for comparison as a dotted line. Optimum sensitization for nitrofurazone occurs at a concentration $[C]_o$ of 10^{-4} M, which is much lower than the nucleotide concentration. Under these conditions, the primary reactive species (G values in parenthesis) formed in the radiolysis of water, $\{e^-_{aq}$ (2.8), OH (2.8), H (0.6)$\}$ react almost entirely with the nucleotide[4]. Consequently, sensitization involves a secondary reaction between the nucleotide free radicals and sensitizer molecules[9]. Since sensitization takes place in N_2O saturated solutions[1], where e^-_{aq} are converted to OH as the only reactive species, and since scavengers for OH such as alcohols completely eliminate the effect[4], we suggest that nucleotide free radicals formed by OH attack are the principal reactive species.

When these experiments were extended to other electron-affinic compounds, it was found that they fell into two categories - those, like nitrofurazone and oxygen, that enhanced the yield of iPO_4, and those that protected against iPO_4. Figures 2a and 2b show the protective action of TAN as a function of TAN concentration in deoxygenated and N_2O saturated solutions. The dose independent protection increases with TAN concentration. However, a high degree of protection occurs at very low TAN concentrations, where all the primary species react with

5'-GMP. Hence protection, like sensitization, involves a reaction between nucleotide radicals, and not with the primary species e^-_{aq} and OH. In oxygen-saturated solutions, the protection against iPO_4 release by low TAN concentrations is suppressed. This indicates that O_2 is again competing for the same nucleotide free radical, and that the reaction with O_2 is more efficient.

The efficiencies of sensitization and protection of iPO_4 release from 5'-GMP irradiated in deoxygenated solution are shown in Table 1 for a wide variety of electron-affinic cellular radiosensitizers and potential radiosensitizers arranged in order of increasing redox potential. The efficiencies, calculated for sensitizer concentrations $[C]_o = 10^{-4}$ M, generally increase with increasing redox potential. Of the well-known cellular radiosensitizers studied, only p-nitroacetophenone, the nitrofurans, and oxygen (and to a much smaller extent N-ethylmaleimide), consistently radiosensitize. Inorganic ions, including Fe^{3+} and Cu^{2+}, also enhance iPO_4 release to approximately the same extent as oxygen. Other compounds which radiosensitize bacterial and mammalian cells, including TAN and menadione only protect. Diamide and nitrobenzene show no effect.

PROPOSED MECHANISM

The following mechanism is proposed:

$$OH + \text{5'-GMP} \longrightarrow \text{5'-GMP}\cdot \text{ (radicals)} \longrightarrow iPO_4 \text{ release} \qquad (1)$$

$$\text{5'-GMP}\cdot + \text{sensitizer} \begin{cases} \nearrow \text{5'-GMP radical oxidation} \rightarrow \text{5'-GMP}^+ + S^- + iPO_4 \text{ release} & (2a) \\ \searrow \text{addition} \rightarrow \text{5'-GMP-S radical adduct} & (2b) \end{cases}$$

The results directly show that radiosensitizers (S) act by two different mechanisms in this system. For the stable free radical TAN, nucleotide radical-sensitizer adduct formation is favoured, as it is for menadione. The sensitizers N-ethylmaleimide, p-nitroacetophenone and the nitrofurans are efficient radical oxidants. Both processes involve a redox reaction and a competition of the sensitizer with electron-affinic oxygen for nucleotide free radicals. Optimum results in both cases are obtained with low concentrations of radiosensitizer. These observations,

TABLE 1

Sensitization and Protection Efficiencies for various Electron-Affinic Compounds

Radiosensitizer*	Sensitization Efficiency (%)	Protection Efficiency (%)	E^1_o (V vs SCE)†
N-ethylmaleimide	23	-	- 0.79
Nitrobenzene	0	0	- 0.47
5-nitrouracil	-	15	-
Triacetonamine-N-oxyl	-	23	- 0.32
p-Nitroacetophenone	84	-	- 0.28
Nitrofurazone	77	-	- 0.25
Menadione	-	30	- 0.22
Diamide	0	0	- 0.15
1-(2,4-dinitrophenyl) pyridinium chloride	91	-	- 0.10
Cu^{2+}	90	-	-
Oxygen	100	-	- 0.08
$Fe(CN)_6^{3-}$	79	-	0.00
Fe^{3+}	109	-	-

* Radiosensitizer concentrations 10^{-4} M

† Redox potentials measured at pH 7 in 0.05 M tris buffer

together with the fact that sensitization only occurs in deoxygenated or N_2O saturated solutions and not in the presence of OH scavengers[4], are consistent with the proposed scheme (reactions 1,2): the nucleotide radicals formed by OH attack undergo secondary reactions with sensitizers, either leading to oxidation of the nucleotide radicals with a subsequent hydrolysis and increased yield of iPO_4, or involving radical-sensitizer addition blocking any iPO_4 release from nucleotide radicals. Pulse-radiolysis studies demonstrate the efficiency of electron-transfer oxidation of biological target free radicals[4,9,10], particularly those of nucleic acid bases, nucleosides and nucleotides (reaction 2a), by electron-affinic radiosensitizers.

Indirect evidence of a competing free radical-sensitizer addition reaction (reaction 2b) has been reported for the sensitizers TAN[11], N-ethylmaleimide[12], quinone[13] and nitrofurans[9]. Also steady-state studies show that radioactively labelled sensitizers bind to the acid-insoluble fraction of irradiated DNA[4,14,15] in deoxygenated or N_2O saturated solutions, in the absence of OH scavengers. Maximal binding occurs at low sensitizer concentrations where DNA scavenges the majority of primary reactive species, but it is not known which DNA radicals are responsible.

Since nucleotide radical oxidation should lead to strand breakage, whereas adduct formation leads to sensitizer binding to target molecules, it should be possible to extend these studies to cellular systems. Although adduct formation leads to "protection" against iPO_4 release in these model chemical studies, nevertheless, the resulting damage in a cell may be potentially lethal, the extent depending upon cellular repair. Consequently, a small chemical effect may prove significant biologically.

REFERENCES

1. Raleigh, J. A., Greenstock, C. L. and Kremers, W., Int. J. Rad. Biol., submitted.
2. Greenstock, C. L., Raleigh, J. A., Kremers, W. and McDonald, E., Int. J. Rad. Biol., 22, 401 (1972).
3. Greenstock, C. L. and Banerjee, C., Rad. Res., 47, 217 (1971).
4. Chapman, J. D., Greenstock, C. L., Reuvers, A. P., McDonald, E. and Dunlop, I., Rad. Res. (1973) in press.
5. Chapman, J. D., Reuvers, A. P., Borsa, J., Petkau, A. and McCalla, D. R., Cancer Res., 32, 2616 (1972).
6. Chapman, J. D., Raleigh, J. A., Borsa, J., Webb, R. G. and Whitehouse, R., Int. J. Rad. Biol., 21, 475 (1972).
7. Dugle, D. L., Chapman, J. D., Gillespie, C. J., Borsa, J., Webb, R. G., Meeker, B. E. and Reuvers, A. P., Int. J. Rad. Biol., 22, 545 (1972).
8. Daniels, M., Scholes, G. and Weiss, J. J., J. Chem. Soc., 3771 (1956).
9. Greenstock, C. L. and Dunlop, I., Int. J. Rad. Biol., 23, 197 (1973).
10. Adams, G. E., Greenstock, C. L., Van Hemmen, J. J. and Willson, R. L., Rad. Res., 49, 85 (1972).

11. Willson, R. L. and Emmerson, P. T., "Radiation Protection and Sensitization", eds. Moroson, H. L. and Quintiliani, M. (Taylor and Francis Ltd., London), 73 (1970).

12. Hayon, E. and Simic, M., Rad. Res., 50, 464 (1972).

13. Simic, M. and Hayon, E., Int. J. Rad. Biol., 22, 507 (1972).

14. Johansen, I., Ward, J. F., Siegel, K. and Sletten, A., Biochem. Biophys. Res. Commun., 33, 949 (1968).

15. Brustad, T., Jones, W. B. G. and Naaken, K. F., Int. J. Rad. Phys. Chem., 3, 55 (1971).