CHAPTER IV

ON THE MECHANISMS OF RADIOSENSITISATION BY STABLE FREE RADICAL, TRIACETONAMINE-β-CYL (TAN)

4.1. INTRODUCTION

On irradiation, all the components of a cell are expected to absorb radiation energy in relation to their abundance in the cell. However, the damage to certain critical structures of cell such as, DNA or cell membranes is generally considered more potentially lethal. Numerous events are believed to occur between the absorption of radiation in the cellular constituents and the final biological damage expressed as lethality, genetic changes or somatic abnormalities. In addition, it is well established that some of the initial molecular damage in a cell system can be sustained, repaired or enhanced by a variety of physical, chemical and biological factors (1). Among them, the chemical agents that modify the damage can be divided into two classes; the radioprotectors and radiosensitisers. Radiosensitiser is a substance that enhances the effect of radiation when present during irradiation at non-toxic concentrations. These compounds may have possible applications in radiotherapy of cancer, radiation preservation of food material and radiation sterilisation of medical products.
Several comprehensive reviews on the concepts and mechanisms of cellular radiosensitisation have appeared (2-4). Among the numerous radiosensitisers (5-7) the chemicals with free radical character are of particular relevance to the present work. These include stable organic nitrooxide radicals with the general formula

\[ \text{R}^' \rightarrow \text{N} \rightarrow \text{O} \]

4.2. **STABLE FREE RADICALS (R'R'NO) AS RADIOSENSITISERS**

Oxygen and nitric oxide, both having radical character, have been shown to sensitise bacterial (8,9) and mammalian cells (10) to ionising radiation. Subsequently some substituted organic nitrooxide compounds have been synthesized (11,12) and these have been found to sensitise anoxic cells when present during irradiation (13,14). These compounds did not sensitise cells to radiation in presence of air or oxygen. The chemical structures of some of these nitrooxide compounds have been shown in fig.1 and it can be seen that in all of them nitroxyl group is usually flanked by t-butyl or similar bulky groups. The stability of these radicals is usually ascribed to the steric hindrance (12,15) as well as to the three electron bond nature of the nitroxyl groups (16,17). The stability and low toxicity of the N-oxyl radicals make them promising adjuvant in radiotherapy of cancer.
It is believed that in some tumors a proportion of the cells become deficient in oxygen (hypoxic) because of poor vascular circulation as the tumor grows. Oxygen away from the blood capillary is consumed by fast metabolising cells. A particular region of tumor thus becomes devoid of oxygen and is called hypoxic/anoxic region. These cells are relatively radioresistant and require high radiation doses to be killed. However, the permissible dose of radiation is limited by the tolerance limit of well oxygenated surrounding normal tissues/cells. Since nitrooxide compounds do not sensitise cells in oxygen and enhance radiation killing only of anoxic/hypoxic cells, they may be the drugs of choice for much desired adjuvant in the improvement of radiotherapy of tumors (differential radiosensitising drugs).

Di-t-butylnitrooxide (DTBN) was the first organic nitrooxide radical that was shown to sensitise anoxic bacteria to the level of oxygen sensitisation and it did not show any effect under aerobic conditions of irradiation (15). Since high concentration of this compound was required to observe maximum sensitisation effect, it gave impetus to search for newer compounds in this class which could be efficient sensitisers at lower and hence at non-toxic concentration.

Triacetoneamine-N-oxyl (TAN) or 2,2,6,6-piperidone-4-N-oxyl was found to sensitise bacterial (14) and mammalian (18)
cells at relatively lower concentration to give maximum sensitisation. At these concentrations TAN was not toxic to cells in vitro or to the mice. In some cases TAN has been found to be a better sensitising compound than even oxygen (19). Early investigations suggested that N-oxyls sensitise anoxic cells by the same mechanism as oxygen i.e. by reacting with the radicals produced on vital molecules (13,20).

\[
\begin{align*}
R^* + O_2 & \rightarrow RO_2 \quad (4.1) \\
R^* + TAN & \rightarrow R - TAN \quad (4.2)
\end{align*}
\]

Evidence in support of this hypothesis came from pulse radiolysis experiments when TAN was shown to form adduct with the hydroxythymine (21) and hydroxy DNA radicals (22). Further evidence for such an adduct formation, however, was obtained by irradiation of radioactive labelled TAN with DNA in aqueous solution and extraction of \(^3H\)-TAN-DNA complex on a Sephadex column (23). Rapid mix technique studies however, indicated that under anoxic conditions TAN reacted with at least two transients of DNA.

In addition, experiments have revealed that TAN could also interfere with the repair process of cells (4). Several redox reactions between TAN and DNA radicals have been suggested. The work presented in this chapter has indicated that reactions of TAN with certain important protective molecules of cell as well as transients of TAN
formed by reaction with water radicals) contribute to the sensitisation mechanism.

4.3. ESR INVESTIGATIONS ON REACTIONS OF TAN

4.3.1. REACTION WITH OXYGEN

The e.s.r. spectrum of TAN (1 mM) in aqueous solution at room temperature exhibits three lines with a separation of 15.3 Gauss and line width 0.46 Gauss (fig. 2a). The observed satellite lines (marked arrow) along with the main spectra arise due to $^{15}$N and $^{13}$C nuclei hyperfine interactions. On removal of dissolved oxygen by bubbling nitrogen gas in the solution, the main feature of the spectrum remained unaltered while intensity of the lines increased by 1.5 times and line width decreased to 0.40 Gauss (fig. 2b). The intensity of the signal, however, came back to original value if the deoxygenated solution was exposed to air for sometime. This indicates that oxygen interacts with TAN radicals leading to loss of their radical character. There has been a doubt, however, whether the decrease in e.s.r. signal in the presence of oxygen is purely physical broadening effect or a chemical reaction.

When oxygen gas was bubbled in a deoxygenated TAN solution, the intensity of e.s.r. lines decreased considerably with concomitant increase in the line width to 0.81 Gauss (fig. 2c). Although it has been suggested from a frozen
Fig. 1. Structures of some N-oxyl radicals: DTBN, di-tert-butyl nitroxide; TAN, triacetoneamine N-oxyl; TMPN, 2,2,6,6-tetramethyl piperidinol N-oxyl; NPPN, norpseudopelletierine N-oxyl.

Fig. 2. E.s.r. signals of TAN (10⁻³ M) prepared in triple-distilled water at room temperature, (a) air equilibrated, (b) N₂-bubbled, (c) oxygenated.

Fig. 3. Decay of radicals of TAN (10⁻³ M in 0.1 M phosphate buffer pH 7.0) in the presence of cysteine under N₂ atmosphere.
system studies that decrease in e.s.r. signal of TAN radicals is due to broadening effect of oxygen (2^+), but results of present study have shown that reduction in the intensity in presence of oxygen could not be accounted in terms of broadening effect alone. Numerical integration of absorption signal as well as line width measurements of esr spectra of TAN in nitrogen and oxygen showed that more than 30% of TAN radicals react with oxygen. Although exact nature of TAN and O_2 complex has not been ascertained, it has been realized that the bonding is weak as it can be easily broken by nitrogen gas bubbling. In fact a kind of bridge formation as shown below has been suggested by other investigators (25): and may explain the present results.

\[ 2 \text{TAN} + \text{O}_2 \rightarrow \text{TAN} - \text{O} - \text{O} - \text{TAN} \quad (4.3) \]

Present observations may partly explain the absence of sensitisation by nitrooxide radicals in aerated/oxygenated cells. The ineffectiveness of nitrooxides in presence of oxygen has been suggested due to relative efficiency of reaction of TAN and O_2 with the radicals formed in cellular molecules. However, above observations indicate that loss of radical character to a considerable extent in presence of oxygen may also make it ineffective.

4.3.2. REACTION WITH SULFYDRYL COMPOUNDS

Reaction kinetics of TAN with cysteine has been
studied in a buffered medium (0.1 M phosphate buffer, pH 7.0) by monitoring the signal intensity of TAN in presence of cysteine.

When 0.25 M cysteine was added to a solution of TAN (1 mM), the esr intensity of the signal decreased with the passage of time. The rate of decay of signal is shown in fig. 3 which was found to be faster at higher concentration (0.5 M) of cysteine. Furthermore, along with the decrease in signal height as a result of reaction, a new signal (arrow marked) was seen appearing superimposed on the low and high field line of the TAN spectrum (fig. 4). The intensity of the new signal increased with time during the course of reaction, reached a maximum and thereafter started decreasing. When reaction was carried out in a deoxygenated condition, the new signal was not observed and reaction proceeded only by disappearance of the TAN signal. It has not been possible to investigate the nature of species giving rise to new esr signal during this work but involvement of oxygen in its formation appears certain. The following tentative reaction scheme can be proposed:

\[
\begin{align*}
\text{TAN} + \text{R - SH} & \rightarrow \text{TAN} - \text{H} + \text{RS}^* \\
\text{In air,} \\
\text{RS}^* + \text{O}_2 & \rightarrow \text{RSO}_2^* \\
\text{RSO}_2^* + \text{R - SH} & \rightarrow \text{RSO}_2\text{H} + \text{RS}^* \\
\text{In nitrogen,} \\
\text{RS}^* + \text{RS}^* & \rightarrow \text{RS} - \text{SR}
\end{align*}
\]
Fig. 4. ESR spectra of air equilibrated solution of $10^{-3}$ M TAN: (a) Spectrum of control TAN solution; and (b) spectrum after the addition of 0.25M cysteine.

Fig. 5. Reappearance of ESR signals of TAN ($10^{-3}$ M) irradiated in presence of $N_2$ or $N_2O$ and stored in air atmosphere $\circ \circ N_2$; $\Box \Box N_2O$.
On carrying out the reaction in aerated conditions, a white precipitate was obtained after the lapse of a considerable time. However, such a precipitate was not observed when reaction was carried out under nitrogen atmosphere. This may indicate about the difference in the yield of the reaction product in air and nitrogen atmosphere. The rate constant for reaction of TAN with RSH in nitrogen was found to be \( k = 2.5 \pm 0.5 \times 10^{-2} \text{ M}^{-1} \text{ min}^{-1} \). The rate constant in aerated condition could not be calculated due to superposition of the new signal on the TAN signal.

TAN has also been found to react with reduced glutathione. However, neither new esr signal nor any precipitate was obtained as a result of this reaction.

Reaction of nitroxides with sulfhydryls in the cell may partly explain their mechanism of sensitisation. For instance, it has been reported that cells pre-treated with TAN and then washed off show some residual sensitisation (18, 26). This can be understood in terms of possible reaction of TAN with cellular sulfhydryls investigated here. Since -SH compounds are believed to restitute the radiation damaged molecules by donating a hydrogen atom, the reaction,

\[
B^\circ + \text{RSH} \rightarrow \text{BH} + \text{RS}^\circ
\]

(Biomolecule radical)

Therefore, a compound which reacts with -SH groups is expected to show sensitisation. Additionally, TAN may also be fixing
the damage by combining with the radicals.

\[ B^* + T\text{AN} \rightarrow B - T\text{AN} \text{ (adduct)} \]  

4.3.3. **REACTION WITH ASCORBIC ACID**

It has been reported that T\text{AN} does not sensitise \textit{E. coli} B/r in the presence of ascorbic acid (27). However, it was not understood how ascorbic acid makes T\text{AN} ineffective. During the present work it has been found that T\text{AN} reacts with ascorbic acid and thereby radical character of the former is lost.

The nitrooxide moiety of T\text{AN} reacted with ascorbic acid as evidenced by the disappearance of the esr signal upon mixing of equal concentrations of T\text{AN} with reduced ascorbic acid. It was found that the signal height of T\text{AN} reduced to one-fourth of its original value within 5 minutes of addition of ascorbic acid (28) indicating reasonably fast reaction.

4.4. **ROLE OF TRANSIENTS OF T\text{AN} IN RADIOTHERAPY**

Fast radiation chemical studies in aqueous solutions showed that T\text{AN} was able to form co-valent bond with various radicals of cellular molecules (21,22). These observations supported the earlier suggestion that nitrooxide compounds sensitise the cells by fixing the initial molecular damage (fixation hypothesis) on cells in a manner similar to oxygen.
Present work throws some light on the possible involvement of transients of T&N in the sensitisation process.

On irradiation of anoxic solution of T&N, the intensity of esr signal decreased, indicating reaction of water radicals.

\[
\begin{align*}
\text{TAN} + e & \rightarrow \text{TAN}^+ \rightarrow \text{TAN} + H^+ \\
\text{TAN} + \text{OH} & \rightarrow \text{TAN} - \text{OH} \\
\text{TAN} + H & \rightarrow \text{TAN} - H
\end{align*}
\]

The spin concentration progressively decreased with the dose of radiation. The rate constants of reactions of radiolytic water radicals with T&N have been obtained by pulse radiolysis experiments (22).

\[
\begin{align*}
(k_{\text{TAN} + e} = 2.2 \times 10^{10} \text{ M}^{-1} \text{ sec}^{-1}, k_{\text{TAN} + \text{OH}} = 3.3 \pm 0.3 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}, k_{\text{TAN} + H} = 6 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1})
\end{align*}
\]

In present experiments, the spectrum of T&N was found to reappear when the irradiated solution was left open in the air atmosphere. The rate of reappearance of esr signals of T&N was faster when it was irradiated in nitrogen than in nitrous oxide gas atmosphere as can be seen in fig. 5 (N\textsubscript{2}O converts hydrated electrons to hydroxyl radicals). In addition, the percentage of T&N radicals recovered after irradiation by \(\gamma\)-rays (720 krad) in nitrogen atmosphere was much higher when the irradiated solution was sealed in a capillary than when it was left open in air atmosphere. The reappearance of signals can be attributed to the re-formation of T&N radicals.
by transfer of electron from TAN-anions to oxygen molecules
and by breaking of the unstable TAN-hydroxyl radical adducts
formed in N$_2$ and N$_2$O irradiations respectively.

\[ TAN + e \rightarrow TAN \text{ or } TAN - H \]  
\[ TAN + O_2 \rightarrow TAN + TAN - O - O - TAN + O_2 \]

It was of interest to see further if electron can be
transferred from TAN anion to some other molecule. p-benzo-
quinone was chosen for this purpose since it is known to
have high electron affinity. The rate of appearance of esr
signal increased when p-benzoquinone (1 mM) was added to an
irradiated solution of TAN (fig. 6) presumably by transfer
of electron from TAN anion to p-benzoquinone.

\[ TAN + BQ \rightarrow TAN + BQ \]

The expected benzoquinone radical ion as a result of electron
addition was therefore detected spectrophotometrically
(\(\lambda_{max} = 430\) nm). The optical density at 430 nm due to
formation of benzoquinone radical (29) increased with
concomitant reappearance of TAN signals. The amount of
benzoquinone radicals formed was however considerably reduced
when TAN solution was irradiated in the presence of electron
scavengers like KNO$_3$ (1.0 M) and N$_2$O (fig. 7). Similarly
electron transfer process from TAN anion to thymidine and
menadione was observed. These results show a possibility
of TAN acting as energy transporting agent to the critical
Fig. 7. Formation of radical anion on addition of 10^{-3} moles of p-benzoquinone to \( \gamma \)-irradiated TAN solution. \( \bullet - \bullet \), irradiated under N\(_2\) atmosphere; \( \bigcirc - \bigcirc \), irradiated under N\(_2\).O atmosphere.

Fig. 8. Effect of K\(_4\)Fe(CN)\(_6\) on the post-irradiated appearance of ESR signal of TAN from its gamma-irradiated solution (dose-820 krad). \( [\bigcirc \bigcirc \bigcirc \bigcirc \bigcirc ] \) irradiated solution of TAN under nitrogen atmosphere and then left exposed to air; \( [\bigstar \bigstar \bigstar \bigstar \bigstar ] \) 10^{-2} M K\(_4\)Fe(CN)\(_6\); \( \bigstar - \bigstar \), 0.1M K\(_4\)Fe(CN)\(_6\); and \( \bigcirc - \bigcirc \), 0.5M K\(_4\)Fe(CN)\(_6\)
targets in the cell. The long life of T&N-anion and its ability to transfer the electron to biological molecules may be considerably important in the expression of its sensitising effect.

The esr signal of irradiated T&N was also found to reappear on addition of potassium ferrocyanide. The rate of appearance was dependent on the concentration of ferrocyanide added (fig. 8). This is possibly due to transfer of electron from ferrocyanide to an OH adduct of T&N which alternatively results in breaking of the adduct giving back T&N radicals.

\[
\text{T&N} + \text{OH} \rightarrow \text{T&N-OH} \xrightarrow{\text{Fe(CH)\text{\textsubscript{6}}}} \text{T&N} + \text{OH}^- + \text{Fe(ON)\text{\textsubscript{6}}}^- \quad (\text{4.16})
\]

A similar mechanism for T&N-OH abstracting electron has been suggested in pulse radiolysis studies (30). These results indicate that hydroxyl radical adducts of T&N may act as an oxidizing agent and they can abstract electron from radiation generated ion pairs on the vital cellular molecule and thereby inhibit the possibilities of repair/restitution of the damaged sites and thereby leading to increased radiation damage.
4.5. FUTURE WORK

4.5.1. TAN AS MOLECULAR PROBE TO MONITOR CELL INTERIOR

In addition to their use as potential radiosensitiser, nitroxide radicals find application as molecular probes in various biological and other systems. The usefulness of nitroxide radicals, particularly TAN, to measure cytoplasmic viscosity of bacterial cell has been recently demonstrated (31). Efforts will be made in future to monitor cell interior using TAN in various pathological tissues and cells.

The cytoplasmic viscosity of cells is undoubtedly important to various cellular processes and is believed to be controlled by the internal membranes and other macromolecular structures. Evidence has been obtained that the
aqueous interior of cells is more viscous than the outside aqueous medium (31-36). However, excepting a few (35, 36), most of the methods measure bulk or macroviscosity. The stable free radical nitroxides particularly TAW, on the other hand, offers a great advantage to explore the internal environment of cells at molecular scale. The information on the diffusion and rotational motion of a nitroxide molecule in a medium provides what is called microviscosity and it may not mimic bulk viscosity of a solution on the cytoplasm. The rotational motion of a nitroxide molecule is measured in terms of rotational correlation time ($\tau_c$). It is defined as the time required for a molecule to forget its previous state of motion and is related to the bulk viscosity of a medium by stokes relation,

$$\tau_c = \frac{4\pi \eta a^3}{3 kT} \quad (4.17)$$

where $a =$ particle radius, $T =$ absolute temperature, and $k = \text{Boltzmann's constant}$, $\eta = \text{viscosity}$.

It becomes obvious from above equation that higher the value of $\tau_c$, larger the microviscosity which may be an indication of higher internal membranous structures and other macromolecules.

A few important observations have already been made in recent years using TAW as a probe. It has been
reported from an experiment on endoplasmic reticulum vesicles that the surface of membrane produces an order on the interior aqueous medium (37). Also, the arguments have been presented that the high membranous structures may produce such a high microviscosity in aqueous regions that the diffusion along the plane of membrane or within the membrane may be faster and more efficient than the three dimensional viscous aqueous protoplasm (31). The lateral diffusion in membrane may be important to vital cellular logistic processes. Thus physical studies on cellular internal environment may provide valuable information on various cell functions.

As mentioned above, the microviscosity of a cell interior can be measured by estimating rotational correlation time (\( \tau_c \)) of the TAN molecules in the cellular cytoplasm. The methodology consists in allowing the TAN molecules enter the cell interior by passive diffusion and by broadening the probe molecules outside the cell by dipolar interaction of paramagnetic metal or salts which do not diffuse in the cell. Usually ferricyanide or nickel chloride at appropriate concentrations are used. Analysis of esr spectra of TAN molecules inside the cell provide information on the rotational motion of the probe, polarity and order of the medium (56). Rotational correlation, \( \tau_c \), is calculated from the following well known formula (38):
\[ \tau_c = k \frac{\Delta \beta}{\frac{h_0}{h-1} - 1} \] (4.18)

where \( k = 6.5 \times 10^{-10} \) sec, \( \Delta \beta \) = line width of the middle line, \( h_0 \) and \( h-1 \) are the intensities of middle and high field lines of the triplet.

During the present work internal viscosity of red blood cells has been measured using TAN as spin probe. The \( \tau_c \) value of TAN in cell cytoplasm is an order of magnitude higher than in aqueous phosphate buffer saline. Similar observation has been recently reported (39).

\[ \tau_c \text{ in buffer} : 2.12 \pm 0.15 \times 10^{-11} \text{ sec} \]
\[ \tau_c \text{ in 50\% suspension} : 1.68 \pm 0.005 \times 10^{-10} \text{ sec} \]

of red blood cells

The signal intensity of TAN radicals decreased with time indicating reaction with some cellular molecule or enzymes. However, \( \tau_c \) value remained unchanged during the time of measurement. If the cells reacted with TAN for a reasonable time were loaded with fresh initial concentration of TAN, almost original signal intensity could be obtained. This indicates that the radicals were not reacting to the sites which were easily saturated. Possibly decay of TAN radicals was due to enzymatic redox reactions. Implications of these observations in cell functions are being presently investigated.
An effort was made to find if cellular cytoplasmic order varies from cell to cell or from normal to pathological abnormalities and whether probe method could provide information on such variations. \( T_c \) value in *E. coli* B/r and fibrosarcoma were measured as \( 2.019 \pm 0.1219 \times 10^{-10} \) sec and \( 3.0493 \times 10^{-10} \) sec respectively. However, many more measurements are required before any conclusion can be drawn.

It is hoped that spin probes may be helpful in studying cytoplasmic response of cells in drug treatments, radiation exposure and other abnormalities including malignancy. Investigations are being continued to explore the possibilities of application of this technique in various studies of biological relevance.

4.6. REFERENCES


38(a) Keith, A. D., and Snipes, W., Biophys. J., 10, 618 (1970)

38(b) Keith, A., Snipes, W., and Chapman, D., Biochemistry, 16, 634 (1977)