RADIATION EFFECTS ON MEMBRANE FUNCTIONS IN CANDIDA ALBICANS

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58 p + fig

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To My Parents

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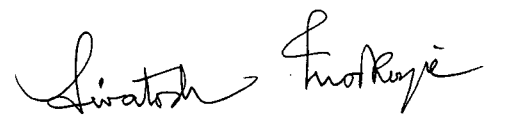
The research work embodied in this dissertation
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is original and has not been submitted in part or
full for any other Degree or Diploma of any university.

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INTRODUCTION

HISTORY

Studies on the radiation damage to membranes started only in early 1950's when Shepphard & Steward reported a leakage of potassium ions from the erythrocytes by the direct effect of radiation. Later, in 1955, Bacq & Alexander proposed that the primary events responsible for cell death are the disturbances of the permeability of certain intracellular structures, notably the mitochondria and microsomes. The 'membrane effect' defined as a disturbance of a barrier between enzyme and substrate in the irradiated cells, was demonstrated in _Rusulla nigricans_ tissue (Bacq & Herve, 1952). The discovery of lysosomes as a subcellular organelle, led Bacq and Alexander (1961) to propose the "Enzyme Release Hypothesis", which suggested that radiation may act by breaking down the lysosomes or other membrane barriers thus allowing the interaction of the proteolytic enzymes and nucleic acid attacking enzymes with their substrates that are ordinarily kept in an intact, viable cell. Cell membranes have, since then, received more attention because, like DNA, this structure has an important property of being present as a single copy whose integrity is essential for normal cell metabolism. Though many changes have been observed in membrane functions after irradiation, yet the cause-effect relationship with the survival has been difficult to interpret.

MEMBRANE STRUCTURE AND FUNCTIONS :

At this stage, it is necessary to consider the structure and functions of the membrane briefly so as to get a precise understanding of the mechanism of radiation damage. Membranes have a number of roles to play in cell metabolism. To fulfill all the functions, Singer and Nicolson (1972) proposed the fluid mosaic model. As seen from Figure (1), it has a lipid bilayer with proteins inserted in between. Proteins are of both types; a) intrinsic and b) extrinsic. Many proteins which are exposed to the outer surface of the membrane, have carbohydrate moieties which have an antigenic importance. Sterol is another important component of eukaryotic membranes which has been shown to play an important role in determining the fluidity of membranes.

The important functions of the membrane are -
a) uptake of nutrients and solutes from the external medium into the cell, b) removal of the residual product in the opposite direction enabling the cell to sustain a definite and constant composition. Membranes are also important for cell to cell attachment and in many organisms e.g. amoeba and paramecium, they help in their movement.

STRUCTURAL DAMAGE TO MEMBRANES :

Membranes are made of water, protein, lipid and carbo-

Medium

Cytoplasm
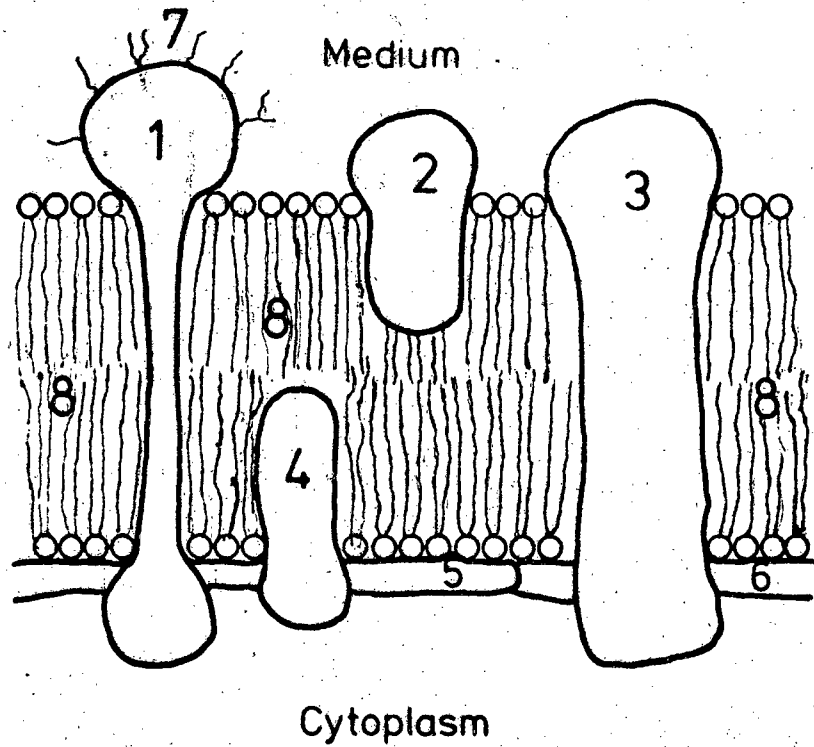
1 *The lipid-globular protein mosaic model with a liquid matrix (the fluid mosaic model).*

1 ± 6 polypeptides; 7 carbohydrates; 8 lipids.

hydrate. Let us briefly consider as to how these
individual components are damaged _in vitro_. Water upon
radiolysis mainly gives hydrogen and hydroxyl free radicals
(.H and .OH respectively). These may reconstitute to give
rise to hydrogen peroxide especially in aerobic condition.
On the other hand, proteins, which form 60% of the membranes
by mass, offer multiple loci for radiation-induced reactions.
Loss of the function of proteins may be due to either
breakage of peptide bond or oxidation of sulphydryl bonds
to disulphide bond and _vice-versa_ or critical change in
the arrangement of side chains. All these lead to a partial
or total unfolding of the peptide chains thus producing a
molecular disorganisation that exposes several unwanted
amino acid side chains. This may change their active
conformation that ultimately alters their properties. The
unfolding of the peptide chains could also cause the different
proteins to aggregate hence producing a decrease in solubility
and an increase in viscosity. The lipids, however, are
mainly attacked at the unsaturations, producing peroxides
in the presence of oxygen. Similarly, sugars in the absence
of oxygen produce highly cytotoxic, alpha-beta unsaturated
carbonyls.

In the membranes these four components are present in
close association with one another and hence the effect

produced after the radiation attack is very different.
One of the primary changes that occurs is the decrease
in electrophoretic mobility (EPM) of cells, which was
shown in yeast (Rink and Teschendorf, 1977), erythrocytes
(Sato et al., 1979B; Sato et al., 1977A; Sato et al.,
1977B), and thymocytes (Miyazawa et al., 1979).

The reduction in the EPM was suggested to be due to the
following reasons:

1. Acidic sugars like sialic acid, hyaluronic acid etc.
   which determine the surface negative charge, were
   dislocated thus decreasing the surface charge
   (Sato et al., 1977A; Sato et al., 1977B).

2. Presence of compounds like concanvalin A, phyto-
   hemagglutinin (Sato and Kojima, 1974) or ion species
   e.g. calcium ions (Sato et al., 1979B) or an enzyme
   (Miyazawa et al., 1979) in the medium, modified the
   surface charge by its binding abilities.

3. Changes in the conformation of the glycoproteins of
   the membrane which led to translocation of the charges
   from peripheral zones to deeper zones (Rink and
   Teschendorf, 1977).

   Along with the decrease in mobility, several other
properties were affected by ionising radiations. Lipid
peroxidation (Yukawa, et al., 1979), free radical formation

(Flossmann and Westhof, 1978), iodination (Rodrigue and Edelman, 1979), decrease in cell proliferation due to the loss of negative charge (Sato et al., 1979A), oxidation of membrane bound thiols in erythrocytes (Shapiro et al., 1969; Sutherland and Pihl, 1968), thymocyte (Chapman and Sturrock, 1974), and yeast (Rink, 1975), loss of antigenic properties of lymphocytes (Facchini et al., 1976), changes in membrane potential (Baisch, 1978) were the primary events which totally disturb the normal metabolism of the cell which may lead to cell death.

However, the above mechanisms were an indirect indication of membrane damage. Direct evidence for a possible radiation damage came from fluorescent probe studies. Lipophilic and amphiphilic fluorescent probes have played a useful role in the study of certain properties of biological membranes such as lipid phase transitions, microviscoity and structural organisation of lipid bilayer. The primary interest in many of these studies has been to establish a relation between changes in the functional state of a membrane and changes in its conformation and dynamics. Yonie and Kato (1978) studied the effect of radiation on the membrane by using a fluorscent probe, 1-amino-8-napthalene sulfonate (ANS). Decrease in the micro-viscosity or increase in fluidity of

the membrane was recorded, which was due to the ⎯ ⁚
disorganisation of the proteo-lipid associations.
Further investigations by intrinsic fluorescence probes
showed that the changes in fluorescence could result
from a change in the environment surrounding tryptophan
residues from being relatively non-polar to being polar,
implying that conformational changes of the membrane
proteins are brought about by low doses of X-rays.

Ultra-violet radiations (254 nm) were also shown to
damage the membranes of erythrocytes (Konev et al., 1978;
Lordkipanidze et al., 1978; Roshchupkin et al., 1976).
Lipid peroxidation (Lordkipanidze et al., 1978; Roshchupkin
et al., 1976), sulphydryl and disulphide damage (Roshchupkin
et al., 1976), free radical formation (Azizora et al., 1978)
inhibition of enzymes of the membranes (Esteves et al., 1978;
Konev et al., 1978) were the changes due to ultra-violet
radiations.

EFFECT OF RADIATION ON MEMBRANE FUNCTIONS :

Most of the work done regarding radiation damage has
been concentrated on nucleic acids. Only in early 1950's,
scientists started observing that exposure of cells to any
radiations i.e. ionising or non-ionising, produced some
functional damages in the membrane of prokaryotes as well
as eukaryotes.

DAMAGE TO MEMBRANE FUNCTIONS IN PROKARYOTIC SYSTEMS :

Most of the studies of radiation effect: oñ
prokaryotes were done in E. coli (Bhattacharjee and
Samanta, 1978; Sprott et al., 1976; Sprott and Usher,
1977; Joshi et al., 1977; Gholiopour and Yatvin, 1979).
Ultra-violet light, which is known primarily to attack
nucleic acids producing drastic effects like formation
of pyrrimidine dimers (Zelle and Hollaender, 1958), was
also shown to affect membrane processes (Sportt et al.,
1975; Kashket and Brodie, 1962; Jagger, 1972; Bragg, 1971).
An inhibition of the active uptake of several amino acids
was observed, which was different for different wave-
lengths in near ultra-violet and visible range. Hence
presence of three or four photosensitizers, which may be
quinones or cytochromes, was suggested by Sprott et al.,
(1976). Inhibition of oxygen uptake (respiration) was
also observed, which was probably due to some change in
Ca^{2+}, Mg^{2+}, ATPase activity. However, studies with a
ATPase mutant of E. coli proved that ATPase was not involved.

Carbohydrate uptake was also found to be inhibited
following UV-irradiation. Doyle and Kubischek (1976) studied
sorbose uptake and found that its inhibition by near ultra-
violet light was directly related to the inhibition of the
permease specific for its uptake.

Bharadwaj and Lakhchaura (1976) reported no inhibition of respiration by near ultra-violet light in Bacillus cereus cells. Far ultra-violet light (254 nm), however, produced cessation of respiration only in adequately aerated E. coli cells. Specific activity of superoxide dismutase, which scavenages superoxide anion (O_2^-) radical, was high in such conditions, but it was found to play no role in the reduction of respiration and cell death. The inhibition of respiration was actually found to be due to the synthesis of a protein which produced cessation of respiration and whose synthesis got delayed in inadequately aerated conditions (Joshi et al., 1977).

Effect of ionising radiation on the membrane-bound enzymes (Mitchell, 1979) and intracellular enzymes playing a role in the uptake of molecules (Gholiopour and Yatvin, 1979) in prokaryote was also studied. There was no effect of UV radiations on the activation energy of the exonuclease in membrane bound as well as in soluble condition in Micrococcus radiouridans cells. However, some changes were brought about in the intracellular enzyme involved in phospholipid metabolism by ionising radiation in E. coli.

DAMAGE TO MEMBRANE FUNCTIONS IN EUKARYOTIC SYSTEM :

YEAST : Yeast has not been a very popular organism with scientists for the study of radiation damage to membranes

inspite of its having a simple eukaryotic structure.
Scarce data is available and most of it has relevance
to the role of sulfhydryl groups in radiation damage
(Rink, 1975; Brunborg, 1977), potassium and sodium ion
permeability (Rink et al., 1969; Rink et al., 1972; Rink
and Bergeder, 1976) which may lead to inactivation of
certain important intracellular enzyme, whose activity
depends on these ions. Even a small damage to potassium
ion permeability affected the potassium ion dependent
enzyme- aldehyde dehydrogenase, in S. cerevisiae (Rink
and Bergeder, 1976). Alteration in the uptake of these ions
was very much dependent on the membrane bound sulfhydryl
groups. On protecting the sulfhydryl groups by reagents
like glutathione, a simultaneous decrease in the abnormal
efflux of potassium ions was observed (Rink, 1975).

Takashi and Kobayashi (1977) reported an increase in
permeability of S. cerevisiae cells, which was due to the
attack of singlet oxygen and this led to an imbalance
of the intracellular substances which was followed by
death without apparent chromosomal damage.

In general, the yeast cells are comparatively more
resistant than mammalian cells to the lethal effects of
radiations (Myers, 1970).

MAMMALIAN SYSTEM : Erythrocytes have been used extensively to see the effect of various radiations since they posses a functional membrane but no nucleus and other intracellular organelles. Hence, it offers a beautiful system to follow the effect of radiation on membranes alone. Increase in potassium ion efflux and uptake of sodium ion were the first effects of radiations to be noticed by Shepphard and Steward (1952), Buchsbaum and Zirkle (1949), Myers and Bide (1966), Kankura et al. (1969). They noticed a shrinking, then a swelling followed by haemolysis of the erythrocytes after exposure to X-rays. Bresciani et al. (1962) suggested that leakage of potassium ions and uptake of sodium ions was due to the reduction of active transport; however, Shapiro et al. (1966) later observed that there was no reduction in active transport but probably sulfhydryl damage was the main cause of the effect.

Uptake of amino acids were shown to be stimulated in the rat liver cells following whole body exposure to gamma-radiations (Flory and Neuhaus, 1978; Neuhaus and Flory, 1976; Shihabi and Neuhaus, 1971; Yang and Neuhaus, 1971; Kilberg, and Neuhaus, 1975). Only the sodium dependent aminooacids L-system) were found to be stimulated e.g. alpha-amino isobutyric acid (AIB), N-methyl AIB, cycloleucine, L-methionine and glycine. These five amino acids were also mutually inhibitory to each other in normal and irradiated tissues. Hence, this

confirmed the presence of a single, stimulatable transport system in the rat liver which was controlled by hormones like glucogan (Kilberg and Neuhaus, 1978).

Ionising radiations were shown to decrease Na^+K^+ dependent AIB transport system in rat thymocytes (Kwock and Wallach, 1974). Probably the coupling factor was radiosensitive which affected either the rate of substrate turnover or the number of carrier sities.

A decrease in the absorption of glucose and proline by exteriorized ileum (Mohiuddin et al., 1978) and different amino acids like, lysine, α-AIB, methionine, alanine and glycine by an intestinal preparation from rats (Timmermans et al., 1977), were also some of the membrane changes observed following X-rays.

Ultra-violet radiations were also shown to produce membrane permeability changes in both plants (Daughty and Hope, 1976; Roy and Abboud, 1978) and animals (Putvinsky et al., 1977; Konev et al., 1978; Sontag, 1977). In Chara corallina (Daughty and Hope, 1976), a differential effect of wavelengths was observed on membrane properties e.g. hyperpolarized state, action potential and action spectra. The parameter considered for these properties was the active and passive chloride ion permeability. A model was proposed where 254 nm radiation was suggested to

attack proteins with sulfhydryl groups while the possible targets of 285 nm radiation were the aromatic amino acids. Cation permeability (Ca^{++} and Sr^{++}) was also shown to decrease by 900 J/m^2 of ultra-violet radiation in <u>Phaseolous vulgaris</u> (Roy and Abboud, 1978). This effect was reported to be correlated to loss of active transport of the ions as a result of respiratory uncoupling.

The receptor functions of the membrane of lymphocytes (Facchini et al., 1976), known to be one of the most radio-sensitive cell types (Vogel and Ballin, 1955; Salvin and Smith, 1969) and fibroblasts (Koteles, 1976), were also shown to be damaged on exposure to ionising radiation.

<u>POSSIBLE MECHANISM OF RADIATION DAMAGE</u> :

As for the mechanism of radiation damage to membranes, peroxidation of lipids has often been implicated. When cells are irradiated in aerated liquid media, .OH and .H radicals and also hydrated electron ($e_{aq}-$) are produced with radiolysis of water.

$$H_2O \xrightarrow{\quad\quad} H. + OH.$$
$$H_2O \xrightarrow{\quad\quad} H_2O^+ + e^-$$
$$e^- + H_2O \xrightarrow{\quad\quad} e^-aq$$
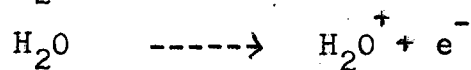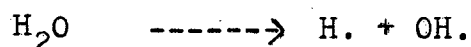
However, when oxygen is present, the following reactions possibly occur -

$$H. + O_2 \longrightarrow HO_2. \text{ (peroxyl radical)}$$

$$HO_2. + HO_2. \longrightarrow O_2 + H_2O_2 \text{ (hydrogen peroxide)}$$

$$e^- aq + O_2 \longrightarrow O_2^- \text{ (superoxide anion)}$$
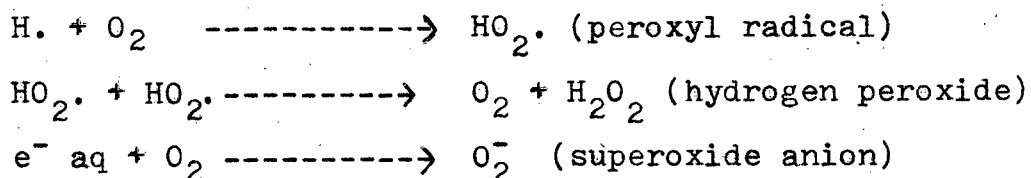
The lethal action of oxygen may be prevented by the action of superoxide dismutase (Petakau and Chelack, 1976) which scavenges O_2^-.

In this context, the direct and indirect effects of radiation need to be mentioned. Indirect effect is through the radiolysis of water when the H. and OH. radicals attack any macromolecules of the cell, while when the radiation directly attacks the macromolecules of the cell, it is the direct effect of radiation (Fig. 2).

In the study of the radiation effects of membrane, the two primary macromolecules affected are lipids and proteins. In this context, the role of oxygen in mediating lipid peroxidation due to its reactivity with the radiation induced free radicals, is relevant (Mead, 1952; Raleigh et al., 1977). The reactions which have been presumed to occur during the autooxidation of lipids are shown in Fig. 3.

The peroxidation of the membrane lipids was shown to cause destruction of many susceptible membrane and
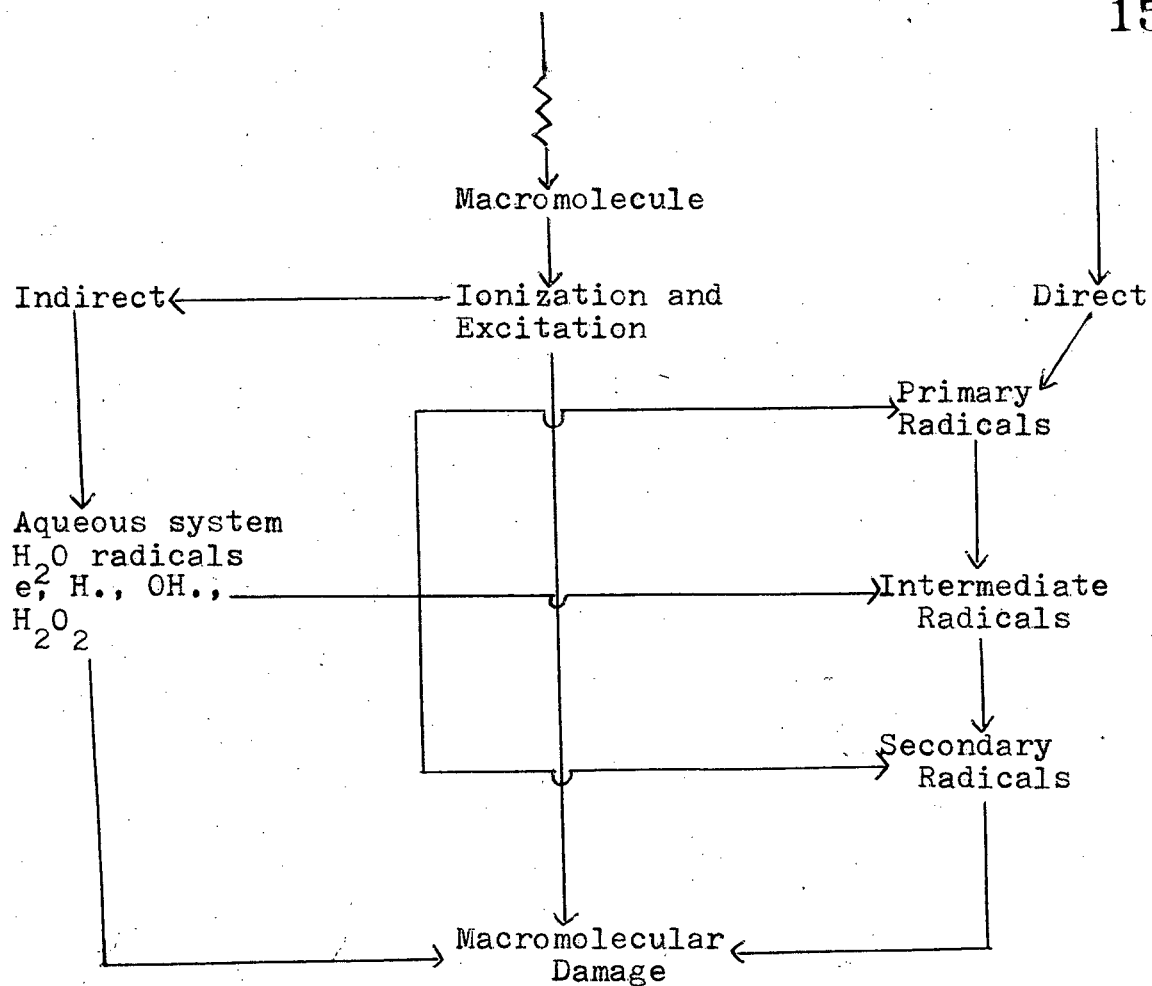
Fig. 2: Figure to demonstrate that a number of reactions take place and a variety of products are formed when a macromolecule of the cell is exposed to ionising radiations.

Initiation

Propogation

$$R \cdot + O_2 \longrightarrow RO_2 \cdot$$

$$RO_2 \cdot + RH \longrightarrow ROOH + R \cdot$$

Termination

$$2R \cdot \longrightarrow RR$$

$$2\ RO_2 \cdot \longrightarrow ROOR + O_2 \quad \Big\} \text{Polymers}$$

$$RO_2 \cdot + R \cdot \longrightarrow ROOR$$
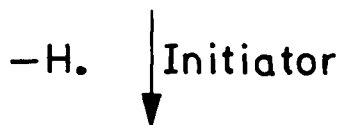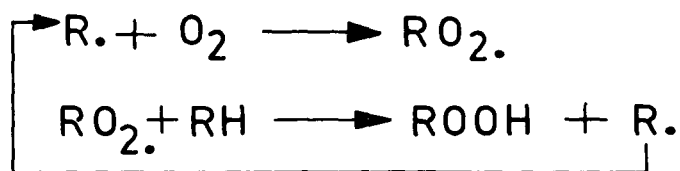
Fig. 3. Auto oxidation of Linoleic acid

cytoplasmic constituents such as oxidisable small
molecules, certain enzymes (Bernheim et al., 1952) and
other membrane structures such as lysosomal membranes
(Tappel, 1968) and cytoplasmic proteins (Tappel, 1966).

Since lipids were shown to play an important role
in the membrane permeability (Gutknecht and Walter, 1979;
Suckling et al., 1979) their structural alteration was
bound to bring about functional changes. Decrease in
microviscosity or increase in the fluidity of the membrane
were the primary effects observed following irradiation
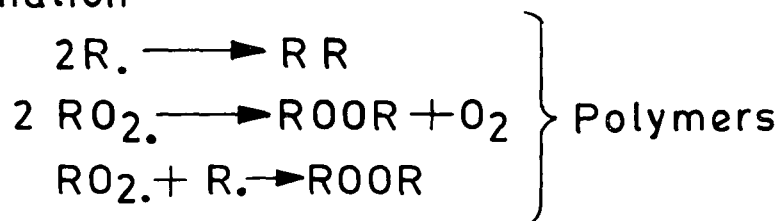(Lekyo et al., 1979). However, these processes were
seen to be repaired to a small extent by the rapid uptake
of some lipids and also due to the presence of an anti-
oxidant α-tocopherol in the membranes (Hansen et al.,
1978; Patternson, 1979).

A change in the composition of the membrane lipids
by growing the E. coli in different media containing
different lipids was shown to affect the radiosensitivity
of the organism (Redpath and Patterson, 1978; Yatvin, 1976;
Suzuki and Akamatsu, 1978; Baldassare et al., 1977). Most
of the experiments done to study the role of lipids were
done on liposomes (Mandal et al., 1978). Whereas Suzuki
(1978) said that the unsaturation was directly related to

radioresistance with respect to K^+ permeability, Yonie
and Kato (1978) reported that it was the disturbance in
the proteo-lipid association that altered the functional
capabilities of the membrane. Ultra-violet radiations were
also shown to produce peroxidation in such systems and
this was directly correlated to leakage of chromate
$(Cr_2O_4^-)$ trapped in the liposome (Mandal et al., 1978).

Forgoing discussion reveals that several reports
have appeared to demonstrate that membranes are the
target of various kinds of radiations. However, the role
of various membrane components e.g. lipid, proteins etc.
in terms of providing radioprotection or radiosensitivity
to membrane is far from clear. In fact literature shows
almost paucity in this respect. The work presented in
this thesis was initiated with this objective i.e. to
assess the effect of radiation (in this case gamma-
radiation) damage following altered composition of plasma
membrane.

Earlier work from this laboratory on Candida albicans,
a pathogenic yeast, characterizes the different amino acid
permeases. Thus, Candida albicans has been selected for
present study to follow the effect of γ-radiation on its
cellular permeability (amino acid uptake). This yeast has
also been shown to grow in different-hydrocarbons (alkanes)

and that results in gross membrane lipid composition. This thesis embodies the results pertaining to the effect of γ-radiation on its survival and amino acid transport. An attempt has also been made to alter membrane lipid composition and follow the effect of γ-radiation on amino acid transport in such cells.

Organism and Growth Conditions:

Wild type <u>Candida albicans</u> strain was obtained from National Chemical Laboratories, Poona, India. The cultures were grown for 48 hours on slants containing -

Yeast extract	-	1.0%
Peptone	-	2.0%
Sucrose	-	2.0%
Agar	-	2.0%
Distilled water	-	100 ml

After growth slants were stored at $4^{o}C$ for 3-4 weeks.

Materials:

Of these, agar, peptone and yeast extract were obtained from Difco, USA. Bovine serum albumin (BSA), Folin reagent, cycloheximide, Dithionitrobis (DTNB), 2,5-diphenyloxazole (PPO), 1,4 bis 2-(5-phenyloxazolyl benzene) (POPOP) and amino acid kit were obtained from Sigma Chemical Company, St. Louis, U.S.A. Trichloroacetic acid was from Merck Co., Germany. Uniformly ^{14}C-labelled amino acids were obtained from Bhabha Atomic Research Centre, Bombay, India. Millipore filters (22 cm., 0.45 um pore size) and maxflow filters were obtained from Millipore Corporation, USA and Maxflow, Bombay, India, respectively.

The yeast cells were transferred from a slant into a synthetic minimal medium containing glucose 0.5% (w/v);

KH_2PO_4, 0.3% (w/v); $(NH_2)_2SO_4$, 0.3% (w/v); $MgSO_4$, 0.025% (W/v); $CaCl_2$, 0.025% (w/v) and biotin, 0.001% (w/v). Cells were grown at $30^{o}C$ for about 14-18 hrs and inoculum (2 ml for 100 ml) was then transferred to the same medium. Cell growth was monitored turbidimetrically by reading the absorbance at 460 nm in a Bausch and Lomb Spectronic 20 spectrophotometer. For various studies namely uptake of amino acids and O_2 uptake, cells growing in the mid exponential phase of growth were harvested (7 hours after incubation) by centrifugation (1500 x g for 10 minutes), washed three times with sterile distilled water and suspended in the same.

Irradiation procedure - 3 ml of cells, adjusted to 0.3 O.D. at 460 nm by Bausch and Lomb Spectronic 20 were taken in small petri dishes and then exposed to gamma radiation in a 5600 Ci ^{60}CO-gamma chamber (B.A.R.C.- Bombay, India) at the dose rate of 44 r/sec. (temperature - approximately $25^{o}C$). The dose-rate was determined by ferrous sulfate dosimetry.

Survival studies - The cells were diluted to 10^3 times in sterile distilled water by the simple dilution technique. 0.1 ml of such diluted cells were plated in the agar medium containing 1% yeast extract (w/v); 2% sucrose (w/v); 2% peptone (w/v); 2% agar (w/v). The plates were then incubated

at 30°C for 24 hours and then the number of colonies in the plates were counted.

Uptake of different amino acids - Reaction mixture containing normal or irradiated or proline grown cells were preincubated at 30°C for 10 minutes after the addition of cycloheximide in a rotary shaker. The reaction mixture had 0.7 ml of cells (40-50 ug protein), 0.05 ml cyclohexi-mide (final concentration 200 ug/ml). The reaction was initiated by the addition of ^{14}C-labelled L-amino acids (proline 1 mM; glycine 0.55 mM; lysine, 1.66 mM; glutamic acid, 0.83 mM) to the assay mixture. At pre-set time intervals 0.1 ml aliquots were removed with an Eppendorf pipette and immediately diluted in 2 ml of chilled distilled water. The diluted suspension was rapidly filtered through 0.45 mM millipore filter and washed two times. The filters were dried and radioactivity retained was counted in a Packard Scintillation Counter, using a scintillation fluid containing - 4 gm PPO (2,5 diphenyloxazole)and 0.1 gm POPOP (1,4 bis 2-(5-phenyloxazolyl benzene)) per litre of toluene.

Measurement of Oxygen Uptake:

The rate of oxidation by normal or irradiated cells was measured polarographically at 30°C with an oxygen monitor (yellow spring instruments Co., Ohio) according to Estabrook (1967). The oxygen uptake was assayed in a final

volume of 3 ml. The vessel contained 1 ml of cells
(60-70 ug protein) and 2 ml of distilled water.

Alkane Grown cells:

For such cells, glucose of the synthetic minimal
medium was replaced by 0.25% (v/v) alkanes of different
chain lengths in the growth media. Such cells were grown
for 48 hours to reach the mid exponential phase and then
harvested for further studies.

Preincubation With Proline:

Proline solution was added to the exponentially
growing cells at 6th hour and cells harvested after an
hour of preincubation. Such cells were irradiated and
then used for further studies.

Protein Estimation:

Protein was estimated by the method of Lowry et al.
(1951). Yeast cells were boiled in 10% TCA for 30 minutes
to release tightly bound proteins. After cooling, suspension
was centrifuged and the supernatant discarded. After
complete removal of TCA from the precipitate, it was
dissolved in 0.1N NaOH. To suitable aliquots (containing
50-200 ug protein) 5 ml of protein reagent (mixed 0.5 ml
of 0.5% $CuSO_4.5H_2O$ and 0.5 ml of 1% Na-K tartarate in
50 ml of 2% Na_2CO_3 (made in 1.0 N NaOH), was added. Color
intensity was read after 30 minutes at 560 nm using bovine
serum albumin as standard.

Sulfhydryl Estimation:

The sulfhydryl groups of the membrane were determined by Ellmann's method (1952). The reaction mixture consisted of 5 ml cells, 2 ml of 0.1M phosphate buffer (pH 8.0), 2.5 ml sterile water and 0.5 ml of 5-5' dithiobis (2 nitro benzoic acid) (containing 39.6 mg of reagent in 10 ml of 0.1M PO_4 buffer, (pH 7.0)). After centrifugation (1500 x g, 10 min), the color intensity of the supernatant was measured in Bausch and Lomb Spectronic 20 at 420 nm.

EFFECT OF ⋎-RADIATIONS ON CANDIDA ALBICANS CELLS SURVIVAL

Before investigating any structural or functional
alterations following exposure of cells to gamma radiations,
their survival or colony forming ability was studied. Candida
albicans, cells, grown on minimal media, were exposed to
different doses of ⋎-radiations (5, 10, 20 and 60 kR) as
described in Materials and Methods section. As can be seen
from Fig. 4A, the LD-50 dose (a dose required to kill 50%
cell population) for such cells was 6 kR. As compared to
initial rapid drop in per cent survivors, the survival
drop was rather slow at higher doses of radiations (Fig. 4A).

As discussed in the introduction section of the
thesis Candida albicans cells can grow on various hydro-
carbons (alkanes) of varying chain lengths and as a
consequence to this, membrane lipid composition of such
cells was altered.

Lipid contents of alkane-grown cells - In all the
n-alkanes used, the growth rate of yeast cells was five
to six times slower than the cells grown on glucose medium
(data not shown). The cells were allowed to grow to mid
log phase, harvested and then total lipids extracted. With
the increase in chain length of n-alkanes (C-13 to C-18),
there was a gradual increase in the total lipid contents
(Table I). Furthermore, there was about 4-fold increase

Fig. 4: SURVIVAL OF CANDIDA ALBICANS CELLS FOLLOWING
EXPOSURE TO GAMMA-RADIATIONS

Colony forming ability of the cells was followed
as described in Materials and Methods.

(A) (×) survival of glucose grown cells.

(□) survival of proline preincubated cells.

(B) Survival of cells grown in different alkanes;

(○) C-13;   (●) C-14;   (△) C-15;

(▲) C-16;   (◻) C-18.
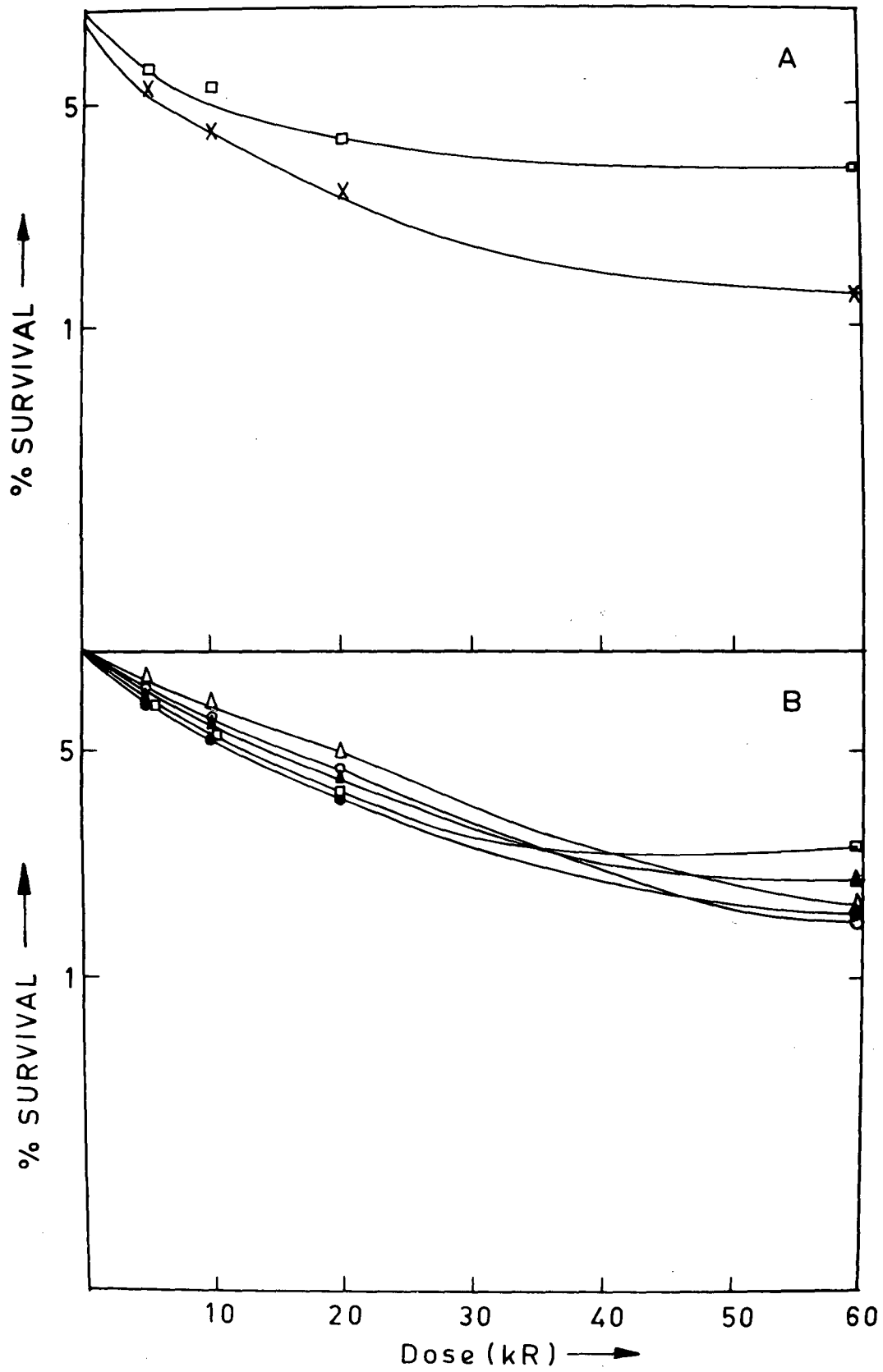
TABLE I

TOTAL LIPID CONTENTS OF GLUCOSE AND N-ALKANE-
GROWN CANDIDA ALBICANS CELLS

Carbon source	Total Lipid (mg lipid/mg protein)	Fold increase	Phospholipid
Glucose	0.01	-	15
Tridecane	0.16	1.6	36
Tetradecane	0.20	2	35
Pentadecane	0.25	2.5	22
Hexadecane	0.38	3.8	14
Heptadecane	0.45	4.5	8
Octadecane	0.47	4.7	12

Lipid extraction was done as described by Singh et al. (1978).

in the total lipid content in C-17 and C-18 grown cells as compared to glucose grown cells.

There was no significant change in total ergosterol in cells grown on C-17 and C-18 n-alkanes. However, it was 20-40% more in n-alkanes of lower chain lengths (C-13 to C-16) (Table II), as compared to glucose grown cells. There was also no significant difference in total glyceride content (Table II).

Phospholipid composition in alkane grown cells - In contrast with C-16, C-17 and C18 grown cells, the total phospholipid contents were about 2-fold higher in C-13, C-14 and C-15 cells as compared with the glucose grown cells (Table I). The differences observed in total phospholipid contents became more clear when the lipids of alkane grown cells were analysed for their individual phospholipids (data not shown).

Mishina et al. (1977) have recently analysed lipids of C. tropicalis and C. lipolytica grown on n-alkanes and glucose. The increase in total lipid contents in C. tropicalis cells have been due to an increase in the phospholipid content. But in C-16, C-17 and C-18 cells, the increase in total lipids may be due to different reasons as the contents of ergosterol and glyceride are not enough to explain the

TABLE II

ERGOSTEROL AND GLYCERIDE CONTENT OF CANDIDA ALBICANS

CELLS GROWN IN GLUCOSE AND N-ALKANE CONTAINING MEDIUM

Carbon sources	Ergosterol Content (mg ergosterol/mg protein)	Glyceride Content (mg glyceride/mg protein)
Glucose	.015	.01
Tridecane	.022	.09
Tetradecane	.020	.012
Pentadecane	.017	.011
Hexadecane	.018	.09
Heptadecane	.015	.013
Octadecane	.015	.012

Ergosterol and glyceride contents were estimated as described by Singh et. al. (1978).

observed lipid accumulation. Therefore, the differences observed between the two strains of Candida may reflect their varying metabolic capacities. The preparation of membrane vesicles from glucose and alkane grown cells have revealed that such lipid changes observed with the whole cells are mainly associated with membrane lipids. Therefore, altered lipid composition of whole cells indirectly reflects changes associated with the plasma membrane, justifying our approach to use such cells to assess their radiosensitivity.

In order to assess if aforementioned lipid changes in alkane grown cells would in any way affect the survival of Candida albicans cells, these cells were also irradiated to γ -radiations at similar doses. It was observed that LD-50 dose for different alkane grown cells was significantly higher (LDH50, 12-20 kR) as compared to glucose grown cells (Fig. 4B). This would suggest that probably the lipid accumulation or changes in alkane grown cells offers a slight protection towards γ-radiation exposure effect as was judged by cell survival.

Effect of γ-Radiations on Amino Acids Transport:

Uptake of various solutes is an index of cellular metabolism of a normal cell. Therefore, any change in cellular permeability would indirectly reflect subsequent metabolic changes too. Recently, membranes have been

discussed as a primary site of radiation interaction (introduction part of this thesis), therefore uptake of various amino acids was selected as an index of membrane function to follow the damage to transport after exposing the cells of γ-radiations.

Uptake of L-proline, glycine, L-lysine and L-glutamic acid was studied in glucose and alkane grown Candida albicans cells exposed to γ-radiations. These amino acids have already been shown to be transported by carrier mediated active uptake system (Jayakumar et al., 1978). Fig. 5 (A-D), reveals the uptake of four different amino acids in normal Candida albicans cells. Following γ-irradiations, a decrease in the transport of all amino acids was observed. However, the inhibition was more for proline and lysine (80-85% inhibition), as compared to glycine and glutamic acid uptake (70% and 60% inhibition respectively). For each amino acid different dose of γ-radiations was required to get 50% reduction in their total accumulation.

Amino Acids	Dose	
Proline	6	
Glycine	25	Data taken from Fig. 5 (A-D)
Lysine	17	
Glutamic acid	23	

Fig. 5: TRANSPORT OF PROLINE, GLYCINE, LYSINE AND
GLUTAMIC ACID IN CANDIDA ALBICANS CELLS GROWN
IN GLUCOSE CONTAINING MEDIUM

Uptake of amino acids was followed as described
in Materials and Methods.

(×) uptake of amino acids in glucose grown cells.

(□) uptake of proline in proline preincubated cells.
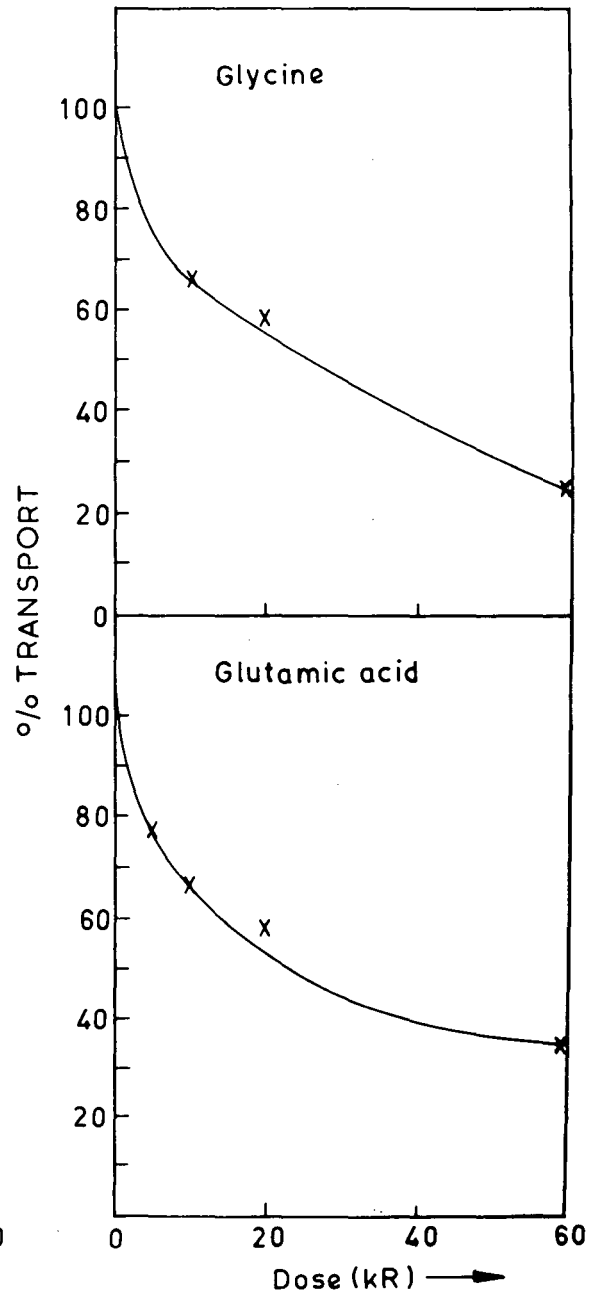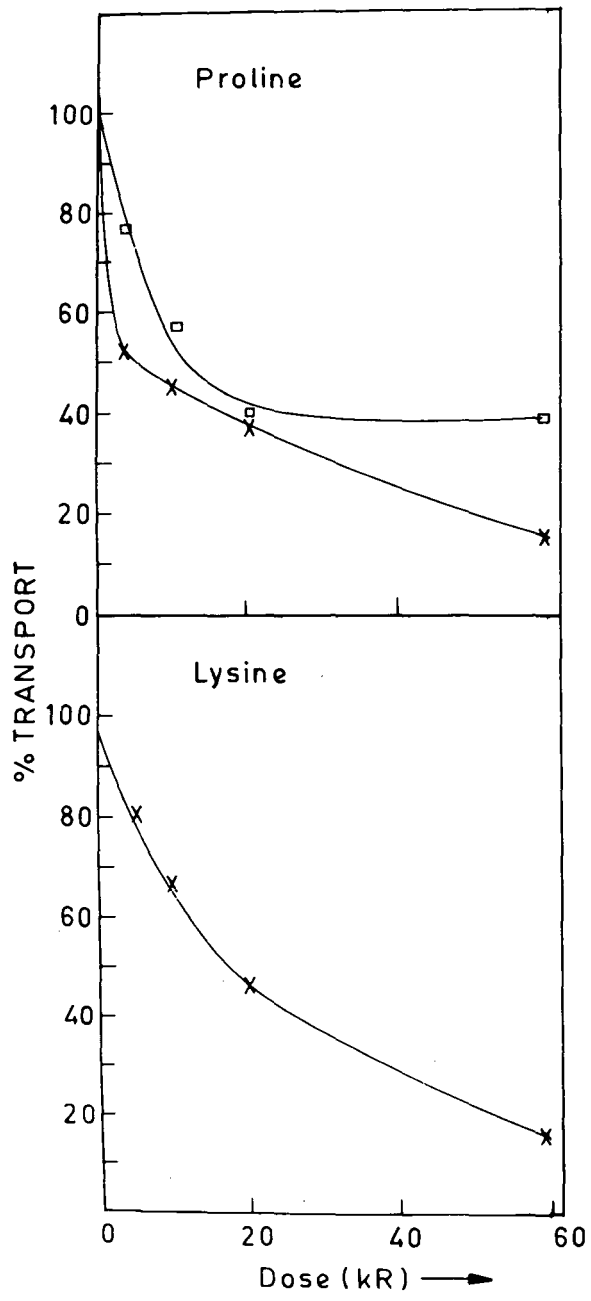
It had been earlier shown that <u>Candida</u> <u>albicans</u>
took up less proline and lysine when grown in alkanes as
compared to glucose grown cells (Singh <u>et</u> <u>al</u>., 1978).
However, there was no effect on the uptake of glycine
and glutamic acid. It was, therefore, suggested that
different amino acids permease respond differently to
changed lipid environment. In order to investigate, if
the lipid changes observed in alkane grown cells had any
effect on the amino acid uptake systems following exposure
to γ-radiations, the transport of proline, glycine, glutamic
acid and lysine was followed in these cells (Fig. 6 A-D).
The transport after low and high doses of radiations in
alkane grown <u>Candida</u> <u>albicans</u> cells is shown in Table III.
As shown there, the transport of proline and glutamic
acid was less severely affected in alkane grown cells as
compared to glucose grown cells. In other words, a
resistance was observed in proline and glutamic acid uptake
by alkane grown cells at all doses. However, when the
transport of lysine was studied, such resistance was
seen only at high doses. Glycine uptake on the other hand,
exhibited a wide variation within the different alkanes at
all doses studied.

Effect of γ-Radiations on Oxygen Uptake:

As observed from the above results, the transport of
four amino acids was significantly affected by γ-radiations.
Earlier studies, from this laboratory have demonstrated that

Fig. 6: TRANSPORT OF PROLINE, GLYCINE, LYSINE AND
GLUTAMIC ACID IN CANDIDA ALBICANS CELLS GROWN
IN ALKANE MEDIUM

Uptake of amino acids was done as described
in Materials and Methods.

(o) C-13;   (●) C-14;   (△) C-15;

(▲) C-16;   (□) C-18.
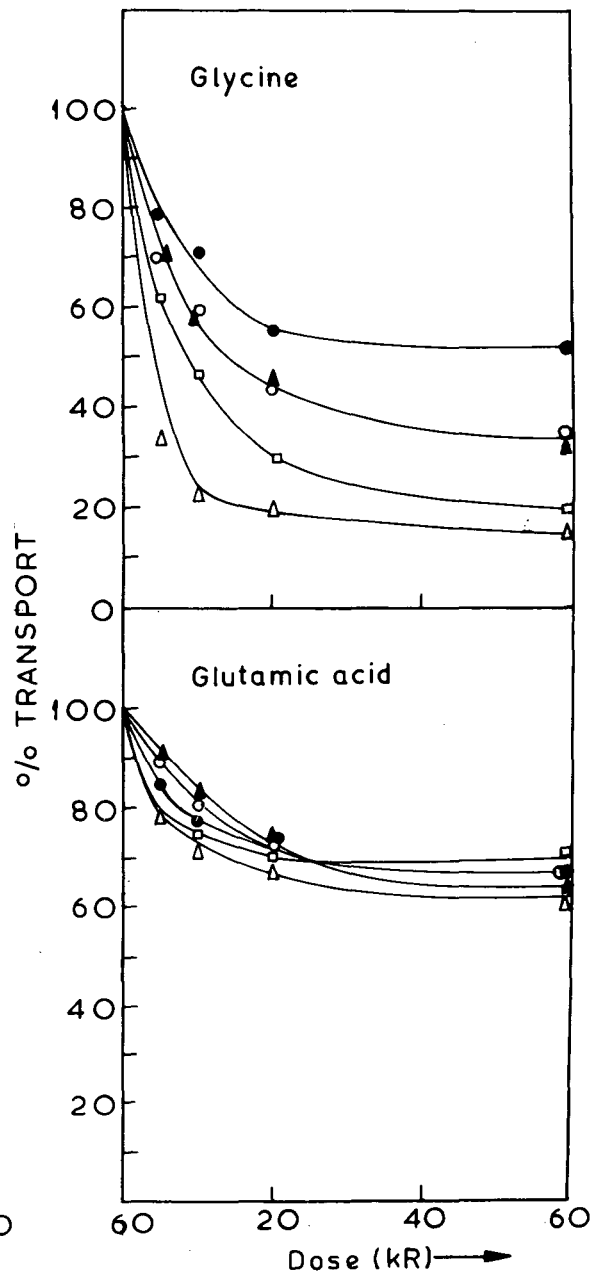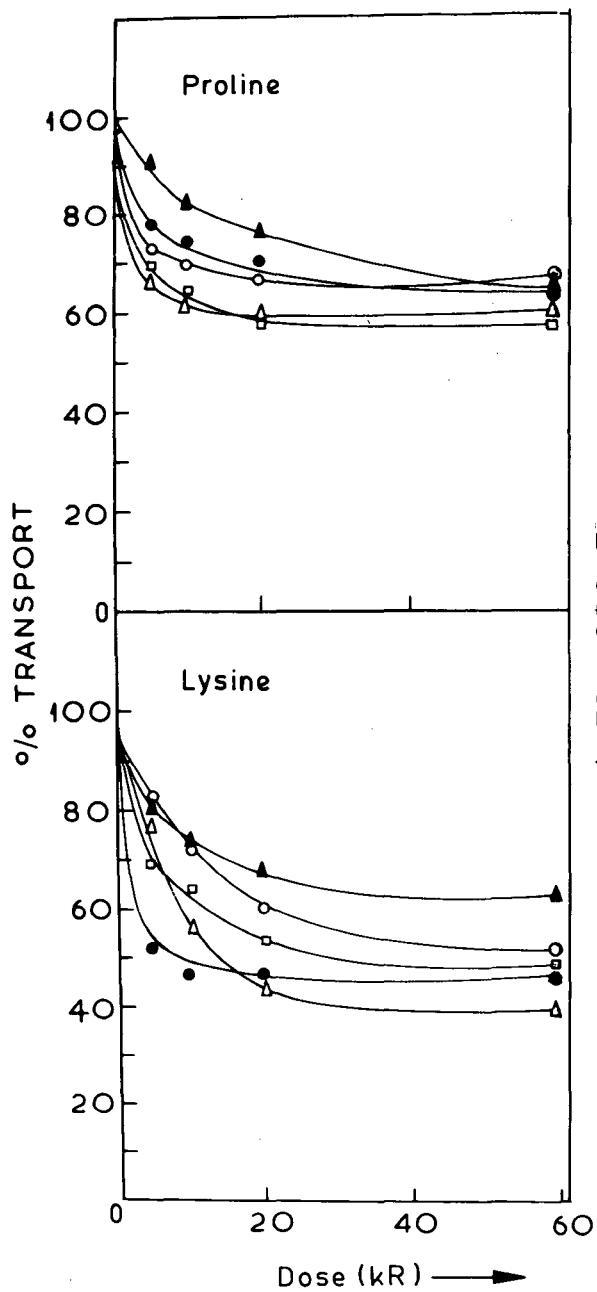
TABLE III

PERCENTAGE TRANSPORT OF AMINO ACIDS FOLLOWING GAMMA-IRRADIATION OF CANDIDA ALBICANS CELLS.

Medium	% Transport							
	Proline		Glycine		Lysine		Glutamic acid	
	5 kR	60 kR	5 kR	60 kR	5 kR	60 kR	5kR	60 kR
Glucose	52	15	75	25	80	15	77	34
C-13	74	67	71	34	84	52	90	67
C-14	78	63	80	52	54	47	85	67
C-15	66	60	46	15	78	40	79	62
C-16	89	64	71	34	80	63	92	64
C-18	69	57	62	20	70	49	80	70

The above values are calculated from Fig. 6.

oxidation is a prerequisite for the amino acid uptake. Studies were, therefore, undertaken to examine if there was any effect of ϒ-radiations on cellular oxidation.

Normal cell exhibited a respiration rate of 0.167 umoles of oxygen per mg of cellular protein per minute which was decreased to 54% (0.074 umoles/mg protein/minute) following 5 kR of ϒ-radiation exposure. There was even more decrease in oxygen utilization at higher doses of irradiation. The oxygen uptake was reduced to 22% (0.037 umoles/mg protein/minute) of its normal rate at 60 kR of ϒ-dose (Fig. 7).

When Candida albicans cells grown on different alkane media were irradiated, it was observed that there was no change in the oxygen uptake between normal versus irradiated cells at low doses (Fig. 7). However, at higher doses (60 kR), slight decrease in oxygen uptake was observed and this decrease varied for different alkanes (0.02 umoles/mg protein/minute). It is pertinent to mention here that oxygen consumption of alkane grown cells is drastically reduced as compared to glucose grown normal Candida albicans cells (Singh et al., 1978). The effect of radiation on oxidation reported here, are on the left over oxidation rate.

Effect of Radiation on SH Groups:

The sulfhydryl groups form one of the most reactive and ubiquitous ligands in biological systems. It is

Fig. 7:   OXYGEN UPTAKE IN CANDIDA ALBICANS CELLS GROWN

IN GLUCOSE AND ALKANE MEDIUM.

·Details regarding the measurement of oxygen

uptake are described in Materials and Methods.
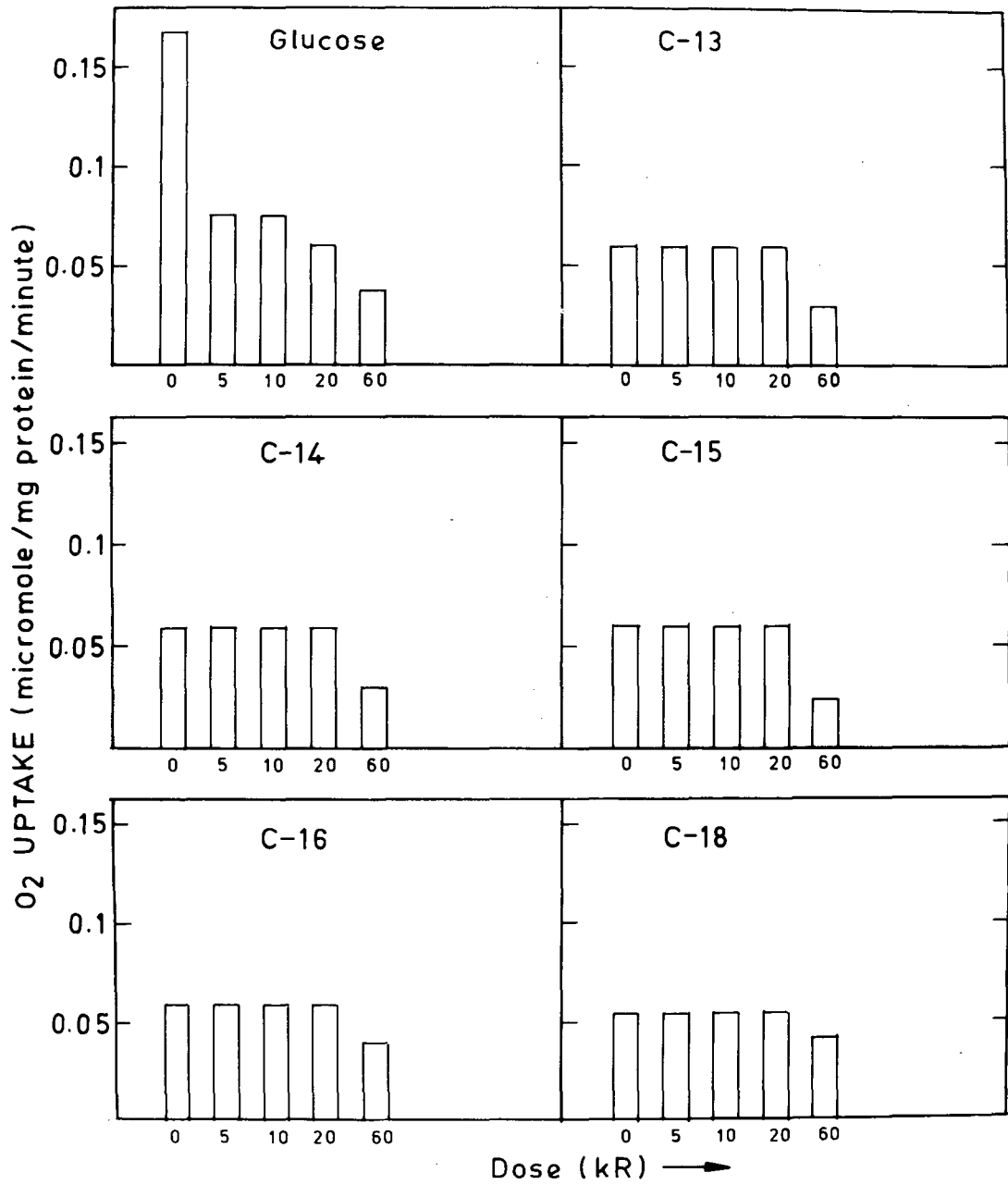
involved in many membrane functions. Different sulfhydryl
reagents e.g. N-ethylmaleimide (NEM), p-chloromercuribenzene
sulphonic acid (pCMBS) have been shown to alter proline
uptake and oxygen consumption at different concentrations
(Jayakumar et al., 1978) which suggested the involvement
of SH groups in the transport process. Candida albicans
cells were exposed to γ-radiations and the molar concentra-
tion of exposed SH groups was determined. No significant
variation in SH concentration was observed following
irradiation at all doses. The normal unirradiated cells
had 0.375×10^{-4}M of -SH groups that were slightly decreased
to 0.325×10^{-4}M per mg protein.

Preincubation Studies:

Apart from the constitutive system of uptake an
inducible proline transport system has also been shown to
be present in Candida albicans cells (Jayakumar et al. 1979).
The inducer was found to be proline and no other amino acids
was able to induce the system. Such induced cells were
irradiated and their survival and proline transport studied.

The LD-50 value for such cells was shown to be 10 kR
(Fig. 4A), which was higher than the normal glucose grown
cells suggesting a slight resistance towards radiation
exposure. Such resistance for proline uptake was also
observed at all studied doses (Fig. 5A). Table IV shows the
percent transport in normal and preincubated cells following
5 kR and 60 kR of radiation exposure.

TABLE IV

PERCENTAGE TRANSPORT OF PROLINE FOLLOWING GAMMA-
IRRADIATION OF CANDIDA ALBICANS CELLS

Dose	Transport	
	Normal cells	Preincubated cells
5 kR	52	76
60 kR	15	38

The above values are calculated from Fig. 5.

DISCUSSION

There is overwhelming experimental data to readily implicate the radiation-induced lesions of DNA in bringing about the observed loss of survival, chromosomal aberrations, mutations and cell death. The experimental data showing that the chemicals which affect the replication and transcription of DNA also modify the radiosensitivity, lend massive support to the view that DNA is the primary target. The demonstration of a relationship between the interphase chromosomal volume (ICV) and radiosensitivity is also an additional support for implicating DNA as the major target of the action of radiation. In recent years, however, the possibility that the radiation damage to membranes could be as much important and relevant in elucidating the mechanism(s) of radiation damage and then acheiving a control over radioprotection and radiosensitization, is becoming increasingly evident. One evidence in this regard is that membrane fractions exhibit a much higher oxygen enhancement ratio (OER) than the DNA. Not withstanding a few scanty reports (Rink, 1975; Myers, 1970; Sprott, et al., 1976; Mitchell, 1979) in the literature, there is still no clear demonstration of radiation effects on membrane structure and function and consequently the role of membranes in radiobiological damage.

In this dissertation, an attempt has been made using a pathogenic yeast, <u>Candida</u> <u>albicans</u>, as a model system, to follow changes in membrane related phenomena e.g. uptake of amino acid, cellular oxidation, conformation of sulfhydryl groups etc. following the exposure of cells to γ-radiations.

One of the first things studied was the possible relationship between the loss of survival (Fig. 4A) and the uptake of four amino acids (Fig. 5). It was observed that following irradiation, the accumulation of these amino acids was reduced but the extent of inhibition of accumulation of these amino acids was variable. These amino acids have been shown to be transported via different permeases present in the membrane. Conformational changes of the permease by UV radiations has been shown in <u>E</u>. <u>coli</u> by Doyle and Kubitschek (1976) and the same could possibly happen with regard to γ-radiations. The observed variations with regard to the inhibition of accumulation of these amino acids may be due to the difference in the accessibility of the different permeases to γ-radiations and hence this asymmetric localization of various permeases may affect their radiosensitivity towards γ-radiations.

Loss of -SH-groups may be one of the primary reasons for the molecular disorganisation of proteins of the membranes which leads to transport changes as shown by Rink (1975), in

<u>Saccharomyces</u> <u>cerevisiae</u>. However, our results do not
demonstrate any noticeable change in total available-
-SH groups during and after irradiation of <u>Candida</u>
<u>albicans</u> cells to different doses. Hence, unlike <u>E</u>. <u>coli</u>
(Doyle and Kubitschek, 1976), the decrease in the accumu-
lation may not be solely due to change in conformation of
proteins but may depend on the breakage of certain weak
bonds like hydrogen bonds and the disturbance of the
proteolipid associations as also suggested by Yonie and
Kato (1978).

A parallel decrease in respiration or oxygen uptake
and transport of amino acids by UV radiation has been
reported in <u>E</u>. <u>coli</u> (Sprott et al., 1976). Earlier studies
from our laboratory (Jayakumar <u>et</u> <u>al</u>., 1978) establish
the fact that cellular oxidation is necessary for active
amino acid uptake in <u>Candida</u> <u>albicans</u> cells eventhough there
is no correlation between the two processes. So, we have
investigated the effect of γ-radiations on oxygen uptake.
Like survival and transport, this parameter has also been
found to decrease. This may be due to the inhibition of
the respiratory enzymes. However, this would not explain
the decrease in amino acid transport.

While discussing the mechanism of radiation damage
to the functions of membrane, we cannot leave aside the

cellular lipids which form almost half of the mass of the membranes. These materials, some of which contain allyl moieties with highly labile H atoms, are particularly sensitive to oxygen mediated radiation damage (Tappel, 1973). Hence, lipid peroxidation may contribute to the radiation-induced damage to membrane transport in Candida albicans cells by disturbing the proteolipid associations.

It has been found in our laboratory that membrane permeability changes on altering the lipid composition of the membranes,(Singh et al. (1978). Now by changing the lipid composition of Candida albicans cells (Tables I and II), the question was asked if there was any effect of this alteration on the radiosensitivity of Candida albicans cells. It is pertinent to mention here that alkane grown cells transport these amino acids but the rate and level of accumulation was affected differently (decreases) in such cells which is primarily due to the suppression of the functional protein related to the transport of amino acids. Here, we have assumed the data on membrane transport to be a measure of structural integrity of the membranes. The tenability of this assumption has been recently demonstrated by Huijbers et al. (1979) who has performed electron microscopic, histochemical and cytochemical studies in liver tissue of ducklings to study the structural damage to membranes

following X-irradiation. This data confirms the fact that alteration of membrane structure is related to permeability changes on exposure to radiations. Our studies reveal a resistance in the inhibition of glutamic acid, proline and lysine (Fig. 6). The percentage reduction of these amino acids was much less in alkane-grown cells as compared to glucose grown cells at similar doses of radiation. However, a difference in the uptake is observed between the amino acids following irradiation which may be due to the fact that different carrier(s) responsible for amino acid transport respond differently to the changes in the lipid ienvironment and become more or less accessible to radiations. The difference in lipid composition between the two cell types may account for the differential radiosensitivity of cells grown in different alkanes. The rate of oxygen utilization of alkane grown cells was only affected at higher doses of radiation in contrast to glucose grown cells where the oxygen uptake was severely reduced even at lower doses (Fig. 7). Such a change in inhibition pattern of oxidation could be attributed to lipid changes. The change in lipid composition offers a kind of protection of oxygen utilization.

It has been shown that oxidation of highly organised lipid bilayers can damage DNA and the species of oxidising lipid responsible for this damage is unknown but it could

be free radicals or non-radical compound (Pietronigro et al., 1977). Moreover, the alteration of membrane lipids has been shown to influence the damage to DNA (Yatvin, 1976; Redpath and Patterson, 1978). With this in mind, we investigated the damage to survival of alkane grown Candida albicans cells following exposure to Υ-radiations. Like for transport, a resistance is observed here also (Fig. 4B). Since the lipid changes in such alkane grown cells are mainly associated with the plasma membrane fraction of Candida albicans cells, therefore, a relationship between the DNA and membrane damage, owing to the changes in the membrane, is implicated. Our results support the fact that alteration of membrane structure influences the radiosensitivity of the Candida albicans cells to Υ-radiations. Hence, the DNA dmage is mediated through membranes.

Survival and transport of proline have also been studied in the proline preincubated Candida albicans cells. The induced uptake of proline is due to new synthesis of permease responsible for proline uptake in the membrane (Jayakumar et al., 1979). Effect of radiations on such induced Candida albicans cells show a radioresistance with regards to survival and proline transport. The resistance may be due to the fact that the dose of radiations,

which produced permeability alterations by affecting a
certain number of permeases in the normal membranes,
now act on enhanced number of permeases. Hence, the
radiation effect gets diluted. However, such changes
in the membrane also bring about a resistance in the
survival as compared to glucose grown cells thus
confirming the suggestion, we discussed above, that
BNA damage is correlated to the structural damage of
cellular membranes.

However, our knowledge regarding the radiation
effect on biopolymers such as lipoproteins, lipopoly-
saccharides and lipopolysaccharide-protein of complexes
which are important consituents of the cytoplasmic
membrane and plasmalemmae, is very limited. Studies
are now underway in our laboratory to investigate any
other possible conformation change(s) following irradiation
with the use of various fluorescent dyes.

In the present study, the lipid changes observed
are not very specific, therefore, the involvement of a
specific lipid in radiosensitivity could not be ascertained,
but it is very clear that even such a gross lipid change
does affect Candida albicans radiosensitivity. During
the course of this work, a similar observation was reported
for E. coli cells (Yatvin, 1978), where a change in lipid

composition was demonstrated to affect the radio-
sensitivity of the organism. Efforts are underway
in our laboratory to specifically alter the membrane
lipids by genetic or environmental manipulations of
Candida albicans cells to elucidate the involvement
of various lipid components in radiosensitivity.

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