MUTAGENIC AND RECOMBINOGMIC ACTION OF GAMMA - IRRADIATED GLUCOSE SOLUTIONS IN YEAST

By

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A Thesis submitted to the Postgraduate School, Ahmadu Bello University, Zaria, in partial fulfilment of the requirements for the award of degree of Master of Science (Radiation Biophysics).

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DECLARATION

I hereby declare that: (i) this Thesis is an original work carried out by the author under the supervision of Professor Jurgen Kiefer;

(ii) to the best of knowledge no part thereof has been submitted elsewhere for the award of a degree or diploma;

(iii) the work of others is duly acknowledged and referenced.

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CERTIFICATION

This thesis entitled Mutagenic and recombinogenic action of gamma-irradiated glucose solutions in Yeast by Lawal Abdul KADIRI meets the regulations governing the award of the degree of Master of Science (Radiation Biophysics) of Ahmadu Bello University, Zaria, Nigeria, and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

The influence of irradiated glucose solutions on growth, mutation and recombination induction of the yeast, *Saccharomyces cerevisige* was investigated. Aqueous glucose solutions (10%) were irradiated at room temperatures, at dose rates of 19 gray(Gy) per minute, with 10 and 50 kilogray gamma-rays from a cobalt-60 source. The irradiated solutions ware incorporated into heat sterilized components of "the cell's nutrient media.

Two cell strains, kept at exponential growth phase were chronically exposed to the media. No impairment to cell proliferation was observed. Treated media did not influence the induction of mutation or recombination compared to the control media; the measured rates of mutations and recombinations are comparable to the spontaneous level.

The results indicate the possible safety of radappertized foods with high sugar content at screening level.

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ABBREVIATIONS

α	alpha
β	Beta
Can-del	Canavanine delition
Can-I-locus	Canavanine-I-locus
DNA	Deoxyribonucleic acid
DSB	Double strand break
E.Coli	Escherichia Coli
eV	Electron Volt
FAO	Food and Agricultural organisation of the United Nations
F-test	Fisher's test
G-Value	The number of Chemical alterations per 100 ev of absorbed energy.
Gy	gray (1 Joule per Kilogram)
γ	Gamma
IAEA	International atomic energy agency
RNA	Ribonucleic acid
Sc	Synthetic Complete (medium)
S^2	Sample variance
S.Cerevisiae	Saccharomyces cerevisiae.
SSB	Single strand break
t-test	Students' (t) distribution test
u	population mean
UV	Ultraviolet light
$\bar{x}$	sample mean
WHO	World Health Organization of the United Nations.

CHAPTER ONE

I. INTRODUCTION

1.1 General aspects of food irradiation.

Radiation is the transport of energy without a transmitting medium (Kiefer, 1984); with sufficient energy, ionizing radiation ionizes and excites matter resulting into chemical reactions with biomolecules which could alter the normal metabolism of cells. It is on this that food irradiation is based.

The preservation and processing of food, thermally, chemically or by irradiation, fundamentally aims at the reduction, inactivation or killing of the enzymes, insects and micro-organisms that causes the destruction of food components. But the attainment of this objective should be at the minimum expense of the food's wholesomeness.

1.2 Types of radiations.

Ionization radiations with sufficient energy for the ionization of matter could be used. A limitation is that it should not induce radioactivity in the irradiated food (WHO, 1977). These acceptable are:

(1) Gamma radiation from Cs-137 and Co-60 sources. Co-60 emits gamma-rays of energy 1.13MeV and 1.17MeV. Cs-137 emits gamma-rays of 0.66MeV. These energies are less than the energy that may induce radioactivity.

(2) X-rays are produced by machine sources that should be operated at or below an energy of 5MeV - at this level they induce negligible and short lived radioactivity.

(3) Electrons are produced by linear accelarators and van de-Graff generators and could be used provided their energy does not exceed 10MeV (WHO, 1977; WHO, 1981).

1.3 <u>Uniqueness of Ionization radiation.</u>

As a means of preserving food, radiation processing has to compete with other conventional methods such as heating, chemical addition, canning etc; comparison with other processes may be based on safety, economy, social and technical factors. There are instances where irradiation could not prove advantageous compared to other processes and there are objectives that radiation alone may not achieve but only in combination with other food processing methods. Nonetheless ionizing radiation has some unique properties and can achieve effects that cannot be attained by other methods:

(1) Gamma radiation energy is less absorbed, compared to heat, by food substances. It is a "Cold process" and does not appreciably heat the food on treatment. Fresh foods, for example, fruits and vegetables can be processed without damage to the tissues, pathogenic bacteria in frozen products can be killed without thawing.

(2) Ionizing radiations are highly penetrating. Insects and micro-organisms in prepacked food could be eliminated or reduced. This prevents recontamination.

(3) Chemical additives are normally not required as an adjunct to radiation processing; and the latter could replace the need for many chemical preservatives now in use. Chemical burden in diet may thus be reduced.

1.4 Doses applicable.

It is important to apply the minimum necessary dose for a particular objective. This depends on the kind of food; but it should not result in the production of deleterious products in the food. The absorbed dose (herein shortened as the dose) is the amount of energy abosrbed per unit mass, the unit is Gray(Gy). One Gray equals one joule per Kilogram.

The doses (ranges) applicable to various foods are tabulated in Appendix I.

1.5 Wholesomeness.

The irradiation of food may in the process of attaining the desired objectives modify and produce compounds hazardous to the consumer. It could also render the product organoleptically unacceptable (that is due to changes in texture, colour, odour, flavour etc of the treated food) to the consumer, but this is a socio-economic problem and is not considered as an aspect of wholesomeness. The wholesomeness of irradiated food comprises three related aspects.

1.5.1 Nutritional aspects.

Radiation sensitive components may be destroyed. The magnitude of the loss depend on the nature of food, the irradiation conditions etc. Whether or not the loss of a nutrient in an irradiated food is of importance depends on circumstances such as the contribution that this food, or of its components, makes to the total diet. For instance, a partial loss of thiamine in fish would be a concern if that was the key source of thiamine to a particular population. Other relevant factors include the nutritional status and requirements of the population for which that food is intended.

It has been established (WHO, 1981) that at low dose range (up to 1 kGy) nutrient losses are insignificant. At medium dose range (1 - 10 kGy) losses of some vitamins may occur depending on the irradiation conditions and subsequent storage. Irradiation in anaerobic conditions enhances the losses (Basson, 1983; Murray, 1983). In the high dose range (10 - 50 kGy) nutrients and organoleptic quality may both be lost. However, procedures adopted to avoid effects on organoleptic quality, for instance irradiation at temperatures below freezing, even if done in the absence of air, also partially protects nutrients; so that losses may actually be lower than in the medium - dose range if such precautions have not been taken (WHO, 1977).

The accumulation of small losses can lead to serious consequences. Nutrients are also lost in the processing of food by other physical processes for instance heat. In 1976 the joint expert committee of FAO, IAEA and WHO, (WHO 1977) suggested that the reduction of nutritional value produced by irradiation alone should be compared with these produced by other processes and by combinations of irradiation with other processes.

Although there are still some areas of uncertainty that require further investigation, for instance the effects of irradiation on vitamin C levels in foods and folic acid losses, in 1981 the Joint Expert Committee of FAO, IAEA and WHO (WHO, 1981) concluded on the basis of the considerable body of evidence available (at least up to that time) that "there is no cause for particular concern" with regard to to nutritional losses of irradiated foods.

1.5.2 Microbiological aspects.

A radiation process is designed to eliminate or at least to reduce the level of food spoilage by micro-organisms and certain pathogens such as Salmonella present in the food. Processing of food by irradiation from the microbiological point of view falls into two categories:

(i) high dose (more than 10 kGy) treatment for sterilization termed as radappertization and

(ii) low-dose (less than 10 kGy) treatment.

No health hazards related to micro-organisms arise from high-dose irradiation; this process is applied in the sterilization of commercial products.

On the other hand, it is important to consider the possible micro-biological hazards when food is irradiated with a low dose. There are survivors depending on their radiation sensitivity and the dose, but they may require more fastidious growth conditions than undamaged cells and require enough time between treatments for adequate multiplication to prevent their complete destruction. Nonetheless, it is important to consider the potential for selection or genetic alteration that might result in increase of radiation resistance and increased pathogenicity in the residual micro-organisms.

Yeasts and spores of bacillus and Clostridium species of bacteria survive low-dose radiations. Clostridium botulinum is particularly radiation resistant. These radiation resistant micro-organisms are however heat-sensitive and could be reduced, if not eliminated, by pre-irradiation thermal treatment for the inactivation of autolytic enzymes. Soldium Chloride may also be added to increase their sensitivity. Low dose irradiation could prevent the formation of toxins in fish but could not eliminate bacterial spores; subsequent refrigeration below 3°C could hinder the multiplication of the spores (Teufel, 1983).

Repeated irradiation - growth cycles under optimal conditions in the laboratory result in mutated bacteria with an enhanced radiation resistance. However, such an increase may not occur under practical food irradiation conditions.

Virulence of a micro-organism is the summation of its disease-causing capabilities; it could be pathogenic mainly through multiplication and increased infectivity in the host or it may cause diseases mainly through toxin production. Radiation does not

(i) induce enhanced infectivity of salmonella,

(ii) increase toxin formation in bacteria(Teufel, 1983).

The microbiological safety of food irradiation process is fully comparable with conventional food treatments (WHO, 1977; WHO, 1981). No health hazard was established with some of the highly-radiation resistant micro-organisms (Teufel, 1983). Nonetheless combined use of irradiation with heat and/or salt achieves a more efficient reduction in the numbers of the resistant-organisms and thus a greater safety of irradiated foods. Microbiological aspects are not investigated in this work.

1.5.3 Genetic and Toxicological aspects.

These include all aspects of irradiated foods safety and wholesomeness but not nutritional or microbiological. With regard to humans, genetic and carncinogenic effects are particularly important. The direct assessment of the toxic hazards on man is is not feasible. Toxic effects are first appraised from considerations of chemical structure, physiochemical properties and the nature of human enviromental exposure, then through experimental investigation of the biological properties in laboratory animals and other biological systems.

Food is a complex mixture of chemicals, the composition of which may not be defined. Processing further complicates the identification and quantification of every chemical compound produced as a result of processing which might conceivably give rise to health hazards in higher mammals and man.

Toxicological investigations of irradiated food were based on short-term studies extending over 8 - 12 weeks. There were also long-term studies which extended over four successive generations and incorporated carncinogenicity studies. These studies were carried out in rats, mice and dogs, fed with irradiated food. The basic problems in these investigations are due to the complications that arise from nutritional balance. Animal feeding studies are generally expensive, time - consuming and produce results that may not be easily interpreted (Fisbein et al, 1970; Elias, 1983).

Micro-organisms on the other hand are easy to handle ; they multiply into large numbers at shorter times and produce results with relatively higher statistical significance. They are less expensive and more convenient. Their nutrients however vary greatly from foods normally consumed by man, and they lack the digestive and excretory processes present in man. The mutagenic, cytological and physiological effects of irradiated nutrients of these micro-organisms are investigated with the hope of predicting the possible effects on animals and on man.

1.6 Need for further screening.

The Joint Expert Committee of FAO, IAEA and WHO (WHO, 1981) declared the wholesomeness of foods irradiated up to an overall doses of 10 kGy. However a survey of literature on which this conclusion is based indicates that while animal studies established the safety of irradiated foods, (WHO, 1977), screening studies of model food components and extracts fed to drosphila, bacterial and mammalian cellular systems give no conclusive result (Berry et al, 1965; Schubert, 1969; Kasevan and Swaminathan, 1971; WHO, 1977). Chromosomal aberrations were observed in drosphila (WHO, 1966), frame-shift and base-substitution mutations were observed in bacteria (Aiyar and Rao, 1977; Namiki et al, 1973) fed with irradiated sugar solutions.

The apparent grounds for the resolution of the committee are:

(1) Radiation processing of food is a process not an additive hence its wholesomeness should be determined as and on comparative basis with, other physical processing; heat for example. The deleterious products produced are generally of minute amounts and are also produced by other processing methods at higher or equal yields. (Diehl, 1977; WHO, 1977; Ehlerman, 1983).

(2) Mutagenicity or cytotoxicity at cellular level may not be the same as at organised level where the processes of assimilation, catabolism, detoxification and excretion takes place.

(3) Laboratory animal studies and irradiated diets to immunologically incompetent patients showed no effect (WHO, 1981).

Certainly the extrapolation of results from one system to another may raise a false sense of alarm or security but because the evaluation of animal feeding studies are particularly complex (Fishbein, et al, 1970), it was felt that further screening is desirable.

1.7 Objective.

The objective of this work is to investigate the possible biological activity that may be exerted by irradiated aqueous glucose solutions on the yeast S. cerevisiae, as a part of its normal nutrient. the following were taken into consideration:

(1) Most of the previous mutagenic investigations were done with the Ames tester strain - Salmonella typhimurium; it is however a recessive system based on lysine requirement (Zimmerman, 1976). Little consideration was given to the yeast inspite of their importance as an eukaryotic micro-organism and its advantages for genetic investigations (Roman, 1981).

(2) The joint expert committee noted the great similarity of radiolytic products in related foods and the uniformity of reaction to radiation of constituent proteins, lipids and carbohydrates in different foods. It recommends that safety data obtained from the chemical and biological testing of one member of food class be extrapolated to related members of the class taking into consideration the differences in their physical composition. In this respect biological testing of model compounds representing the major classes of foods is important. Glucose is an ideal model compound for the carbohydrates; it is a carbon source for the yeast.

(3) The Joint Expert Committee noted the insufficiency of data on toxicological and mutagenic evaluation of radappertization (food irradiation with doses in excess of 10kGy) and recommends further investigations.

(4) On the realization that carcinogens are also mutagens (Ames *et al*, 1975) and that mutagens also induce recombinations (Zimmerman, 1975) the possibility of inducing recombinations was also investigated.

CHAPTER TWO

LITERATURE REVIEW

2.1 The Radiolysis of aqueous glucose.

In the absence of induced radioactivity and enhancement of microbiological resistance, the wholesomeness of irradiated food is entirely dependent on the radiation induced chemical products. The chemical effects of radiation are dependent on the dose and dose rates applied, the food's chemical nature and physical state, and the irradiation conditions.

Most food substances, except dried ones like pepper, powdered milk, contains a high proportion of water whose radiolytic products play an important role in radiochemical and radiobiological effects.

The yields of the products formed, within 10^{-8} seconds, on irradiating air-free water with gamma-rays are given in Table 2.1.

Table 2.1: The yields of radical and molecular products formed on gamma-radiolysis of air-free water.

Product	G-Value
e^-_{aq}	2.70
$H^{\bullet}$	0.55
$\dot{O}H$	2.70
H_2	0.45
H_2O_2	0.70

(Source: Allen (1964))

The yields of the products are measured in terms of their G-Value, the number of chemical alterations per 100 eV energy absorbed.

The yields of the products depend on the radiation quality; radiations of high Linear Energy Transfer (LET) produces more radicals products but less molecular products than low LET radiations.

The reactive radicals ($\dot{O}H$, $\bar{e}_{aq}$ and $\dot{H}$) diffuse and react with other components to produce other products some of which are radicals.

In the absence of any solute, the net decomposition of irradiated water is negligible because the molecular products react with the radicals and reform water.

In the presence of Oxygen both $\bar{e}_{aq}$ and $\dot{H}$, but not $\dot{O}H$ react readily with Oxygen: $\bar{e}_{aq} + O_2 \longrightarrow O_2^{\cdot -}$

$$\dot{H} + O_2 \longrightarrow HO_2^{\cdot}$$

The products are essentially the same, since by protonation $HO_2^{\cdot} \rightleftharpoons H^+ + O_2^{\cdot -}$. The hydroxonium anion produced is a mild oxdizing agent, and is less reactive than either $\bar{e}_{aq}$ and $H^{\cdot}$. It under goes reactions which partially regenerate Oxygen and produce more hydrogen peroxide.

The influence of Oxygen depends on the dose and dose rate of the radiation. At ambient temperatures, air saturated water contains 2×10^{-4} Molar oxygen. If food components have similar water content, on irradiation they become anaerobic after a dose of 0.65kGy have been delivered, assuming oxygen is consumed with a G-Value of three.

The presence of a solute, glucose for instance, interferes with the various reaction steps and the yield of the products may change. The direct action of radiation on a solute, in which chemical bonds are raptured, may result into different products. The relative influences of direct compared to indirect actions (that is, those reactions that occur through the radiolytic products of water) depends on the concentration. The radiolytic products of water may play a dominant role in a 10% aqueous solution, and are expected to be the only causes of chemical change in 1% aqueous solutions (Swallow, 1977).

Glucose, $C_6H_{12}O_6$, is a monosaccharide. Its reactions are basically similar to other carbohydrates (von Sonntag, 1980). The solvated electron, $\bar{e}_{aq}$, reacts slowly with carbohydrates-but the hydrogen radical, and to a lesser extent the hydroxyl radical can both abstract carbon bound hydrogen atoms from a carbohydrate solute.

$$RH + {}^{\bullet}H \longrightarrow R^{\bullet} + H_2^{\bullet}$$

$$RH + \dot{O}H \longrightarrow \dot{R} + H_2O$$

Due to its reactivity, the $\dot{H}$ has a lowered selectivity with respect to the site of attack hence a mixture of reaction products including polymeric substances, may be formed. From glucose, for example, six different primary glycosyl radicals are formed at about equal yields (Schüchman and von-Sonntag, 1978). Some of the products that could be produced by gamma-radiolysis of sugar solutions are given in Appendix II.

The organic radical, $R^{\bullet}$, which may also be formed by the direct action of radiation but to a lesser extent, is more stable than the primary radicals. Its reactions are therefore more significant.

(i) It can abstract H-atoms from organic molecules, since the carbohydrates (RH) are also liable, chain reactions particularly at high dose rates may arise. This may be through - disproportionation:

$$R\cdot + R\cdot \xrightarrow{\text{dismutation}} R + R^1$$

(R and R^1 are the original and newly formed compounds respectively).

- and / or dimerization:

$$R\cdot + R\cdot \longrightarrow R - R$$

(ii) Some decompose eliminating water:

$$R\cdot \xrightarrow{\text{dehydration}} R'' + H_2O$$

R'' is a new compound.

(iii) Although organic radicals can slowly add to carbon - carbon double bonds, they are usually reducing agents of minor constituents.

They react readily with oxygen:

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

$$RO_2^\bullet + RH \longrightarrow R\cdot + RO_2H$$

These reactions often give rise to unstable peroxide radicals, mainly - hydroxyalkyl radicals. Subsequent reactions by unstable peroxide radicals result in acidic compounds.

(iv) Organic radicals react with hydrogen peroxide:

$RO_2\cdot + H_2O_2 \longrightarrow -RO - + H_2O + \dot{O}H$ and may slightly lower the yield of hydrogen peroxide (von-Sonntag, 1980).

2.2 *Factors influencing genetic stability.*

In all organisms genetic information is contained in the deoxyribonucleic acid (DNA). The Universality of its role, and its response to radiation and chemical agents is the basis of screening irradiated foods with micro-organisms. As a primary target for radiation action, the effects of external agents on a cell may be explained interms of damages to it's DNA.

The DNA is contained, mainly, in the chromosomes of eukaryotes; in these organisms cell's death may be related to chromosomal breakages. A lesion may not be lethal, it may be removed by repair or recombination correctly or incorrectly. An incorrectly repaired lesion on the DNA may lead to wrong replication and transcription during cell division and may result into one or more of the various genetic alterations depicted in Figure 2.1.

Mutation is a heritable change in the genetic material. An alteration in the DNA is mutagenic only if it results in an irrepairable damage in the daughter cells.

In principle, provided the number of primary lesions per genome is the same, a given mutagen produces the same effect on the DNA irrespective of the DNA source. But the resulting interaction products are handled by various repair and replication enzymes. The enzyme constituents of cells differ, hence the resultant mutagenic effect varies with the cell. Practically this points to the need for good selection of mutational assay system but fundamentally it signifies the influences of DNA repair, replication and recombination in mutation induction.

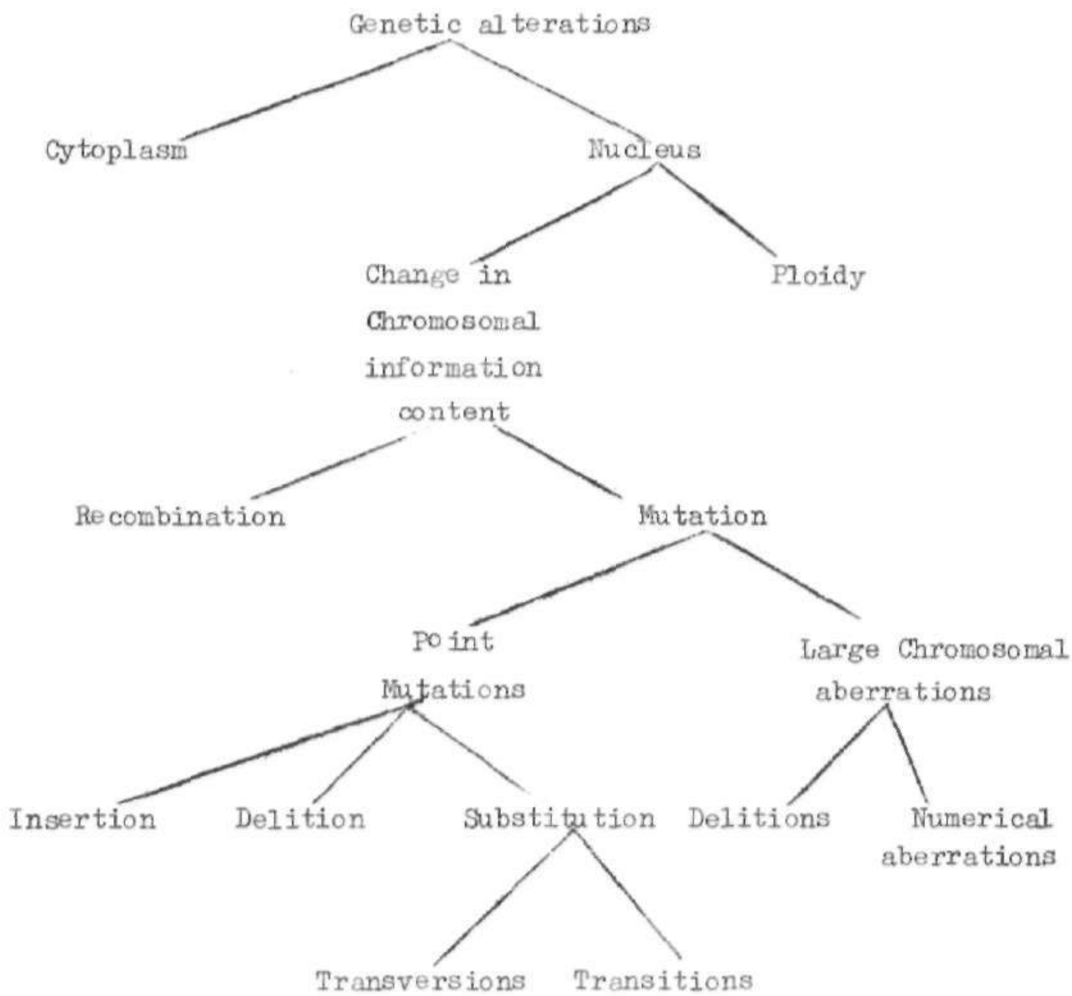

Figure 2.1: Types of genetic alterations

2.2.1 Recombination.

These are mechanisms that result in the rearrangement of nucleotide sequences, constituting a group of related but different genetic exchange reactions between separate segments of DNA. Recombinations are involved in many repair processes; two classes can be distinguished.

In site-specific recombination, exchange occurs at short specific nucleotide sequences on one or both participating DNA strands. The entire exchange is due to a recombinational enzyme. It is not essential in this mechanism for base-pairing between recombining homologous to take place. It is through site-specific recombination that bacteriophages are incorporated into their host genomes.

In general recombination, exchange takes place between homologous DNA sequences, most commonly two copies of the same chromosome as it may happen during meoisis in eukaryotes. This exchange involves DNA - strand exchange intermediates, the exact pathway may vary with organism.

Various models (Halliday, 1964; Esposito, 1978; Espositor and Wagstaff, 1981) have been reported in an effort to describe the pathways. In fungi, viruses and bacteria the processes can be described by the Meselson and Radding (1975) model.

According to the model, general recombination is presumably initiated by single strand breaks in one DNA homologue, which become a site of strand displacement by DNA polymerase. Chemicals and radiation are possible agents that can produce this nick.

Alternatively it may occur spontaneously, for instance during meiosis in eukaryotes when enzymes create nicks to a base-pairing location.

Cross-strand exchange takes place between the two double helices leading to an intermediate structure which is held by the mutual exchange of two of the four strands present, one originating from each of the helices. The point of exchange can be located anywhere between the two homologues, and can migrate rapidly back and forth along the helices spontaneously. This migration extends the region of base pairing between the interacting strands.

The exchange - strand structure can isomerize by a series of rotations presumably by enzymatic reaction. Isomerization alters the positions of the two pairs of strands such that the two non-crossing strands become crossing and vice-versa.

Enzymes cleave the crossing strands; the time this occurs is important. Should cleavage occur prior to isomerization of the cross-strand exchange, the original DNA helices will separate from each other with only short pieces of single strands of DNA being exchanged.

If cleavage, however, takes place after isomerization then one section of each original DNA helix will became linked by staggered joint to another section of the other DNA helix. Thus crossing over takes place.

Yeast cells change from diploid to haploid during meiosis, recombination occurs at three distinct life-cycle stages in the diploid cells (Esposito and Wagstaff, 1981).

(i) Mitotic recombination (intergenic and intragenic) during vegetative cell division.

(ii) Meitotic recombination in diploid cells uncommitted to haploidization following exposure to a meiosis-inducing enviroment.

(iii) Meiotic recombination in cells that complete meiosis and ascosporogenesis.

2.2.2 Repair.

Repair processes are mediated by enzymes, either constitutively or as an inducible process. It is important to distinguish whether repair takes place before, during or after DNA replication. Error-prone in contrast to error-free repair lead to mutations.

Excision repair is basically constitutive and error-free. The exicision repair of pyrimidine dimers is well elucidated in E.Coli (Kelly et al, 1969). In yeast not all the steps are known but at least four loci in the Rad 3 group are concerned with the incision step (Reynolds and Friedberg, 1980). At least seven genes may be required for excision repair in S.cerevesiae, which may act on nuclear DNA and may not be able to remove pyrimidine dimers from mitochondrial DNA (Walters and Moustachi, 1974; Prakash, 1975; Haynes and Kunz, 1981). In mammalian cells it has been established that patients with Xeroderma Pigmentosum have defective nucleotide excision repair . The damages arising from a number of mutagenic chemicals mimic those of UV - radiation and have presumably certain common repair pathways (Kimball, 1980; Setlow, 1980).

Post-replication repair occurs when replication precedes repair. A lesion blocks DNA synthesis at the site. However synthesis continues at distal parts from the site. The gap left opposite the lesion is filled by recombination from the available strand; the used strand is subsequently healed by repair replication. Thus two intact DNA strands are available for the daughter cells. In E.Coli post-replication repair is error-free and essentially constitutive (Rupp et al, 1971). In mammalian cells the gap left by DNA polymerase are not repaired by recombination but by "de-novo" DNA synthesis. Since this occurs with no proper DNA template it is error-prone (Kiefer, 1984).

SOS-repair is inducible and error-prone, it is demonstrated only in E. Coli. It may be evoked by ionizing radiation, UV and alkylating agents (Rupp et al, 1971).

Single - strand breaks (SSB) are a normal stage in DNA replication. UV and gamma radiations, and methyl-metha sulfonate treatment produce single - strand breaks in the DNA. The mechanism through which the breaks arise may be different with each agent; with UV it may occur during the excision repair of pyrimidine dimers. Gamma-rays can induce SSB directly or indirectly following excision of modified bases. Presumably all organisms can repair SSBs. The repair process could be through three stages: radiochemical, enzymatic but without nutrient and a third slower enzymatic process requiring nutrient media.

Double-strand breaks (DSB) are critical structural lesions. They are repairable in E. Coli, yeast (Game et al, 1979) and mammalian cells (Szostak et al, 1983). The repair mechanisms are not completely clear but nucleosomal organization may play a role. In yeast, DSB repair is an efficient process (the rad 52 is the gene responsible for this repair). According to Game at al (1979) excess strand gaps in DNA are themselves recombinational either directly or by the induction of a recombinational repair system.

2.3 Mode of action and type of mutations induced by chemicals.

In the screening of irradiated foods with micro-organisms, the cells are not irradiated but are exposed to irradiated food components. Possible cytotoxic and mutagenic effects are due to radiation induced chemical changes. The chemical composition of irradiated food substances may be complex and can induce various mutations in different ways.

Base analogs hinder DNA replication through the inhibition of DNA strands separation. Halogenated bases and uridine derivatives, like 5-Bromodeoxyuridine (BUdR) can replace thymidine or cytosine during DNA synthesis resulting into transitions and transversions of bases.

The Alkylating agents, Nitro and Sulfur mustards are the best known mutagens in human enviroment (Ames, 1983). Monoalkyl agents cause intra strand links; bi - and polyfunctional alkylating agents on the other hand are more likely to produce interstrand covalent links which by blocking strand separation prevent DNA replication.

The N-7 atom of guanine is highly susceptible to alkylation by the sulfates presumably because it is sterically available by its location in the wide groove of the DNA double helix. A modified guanine may lead to mispairing, alternatively, the alkyl group on N-7 of guanine labilizes the β-Glylosidic bond resulting into depurination, subsequent filling of the delition with a base, may result into both transition and transversions. Recent studies (Prakash and Parakash, 1980) suggest that in alkylation by nitroso-compounds, misreplication of O-6 alkyl guanine and error prone repair of the gap left opposite to it may account for most induced mutations.

Nitrous acid oxidatively deaminates adenine. cytosine and guanine, free amino groups are lost and replaced by hydroxyl groups converting adenine to hypoxanthine, guanine to xanthine, cytosine to uracil. The new forms lead to mispairing due to their affinity to other bases. Nitrous acid may also be involved in intranstrand cross-links within the DNA molecule. This may vary with cells but could presumably inhibit replication at distal positions. In yeast, according to Snow (1967) four of ultraviolent light sensitive loci (at least 19 loci are known - (Lemontt, 1980)) are also hypersensitive to nitrous acid.

Some compounds interact with DNA and RNA so as to induce structural alterations with the nucleic acid molecule but without formation of covalent bonds. Acridines and phenanthridines have a planar ring system which is presumably intercalated between adjacent base-pairs of the DNA double helix. This effectively changes inter-base separation and cause frame-shift mutations (Freese, 1971). Polynuclear aromatic hydrocarbons and Nitrogen-containing carcinogens may act and mutagenize in the same manner. However antibiotics and mycotoxins, such as Actimomycin D, turns off DNA and RNA synthesis; it is rather lethal than mutagenic. Other antibiotics have varying effects. Mitomycin C, a cross-linking agent, produce unusual chromatid exchange in human leukocytes chromosomes. Streptonigrin induces reversions in yeast (Fishbein <u>et al</u>, 1970).

The radical producing compounds depend on oxygen with which they react, transition metals usually catalyses the reaction, and produce organic radicals which attack DNA base-rings. The 5 - 6 double bond of pyrimidine dimers are particularly vulnerable.

Bases could be removed, and the rupture of the ring-system labilizes the sugar - phosphate backbone giving rise to strand breaks. Radicals could also break phosphodiester bonds directly and cause depolymerization and manifest chromosomal and chromatid breaks.

The effects of hydrazine, hydroxylamide and its derivatives are mainly through their degradation products, which may include hydrogen peroxide and other radical producers rather than the compounds themselves (Kimball, 1980). The primary radicals and the hydroxonium ion are short lived and could attack only the nearby DNA strands. However their reaction with other organic molecules produces more stable organic - peroxy radicals. Organic peroxides, aldehydes and phenols are in this category.

Free radicals are produced in many chemical and metabolic reactions and may therefore contribute significantly to spontaneous mutations. Radical scavengers and inhibitors moderate their effects. In E. Coli hydrogen peroxide is detoxified by catalase and peroxidase. Thacker (1975) demonstrated that hydrogen peroxide can be destroyed by hydroperioxidase in yeast, provided the peroxide concentration is not too high.

2.4 Mutational assay at can-1 locus.

In S.cerevisiae, the uptake of all exogenous arginine into the cell is controlled by the means of arginine permease specific system under normal conditions of ammonia repression, when general amino-acid-permeases are inactive. This same permease controls the uptake of the toxic arginine analogue L-canavanine which in a wild type cell competes with arginine uptake.

$$H_2N-\overset{\overset{NH}{\|}}{C}-NH-CH_2-CH_2-CH_2-CH(NH_2)-COOH$$

arginine

$$H_2N-\overset{\overset{NH}{\|}}{C}-NH-O-CH-CH-CH(NH_2)-COOH$$

L-canavanine

Alteration of can-I locus, located on chromosome V, produces mutants carrying single recessive alles. The mutants are resistant to the highly toxic arginine analogue L-canavanine. All such canavanine - resistant mutants map at the same genetic locus (Greenson et al, 1966; Whelan, et al, 1977).

Can-I codes for a membrane protein, the arginine permease, that must be synthesized, presumably on the cytoplasmic ribosome and subsequnently be transported to its functional location in the cell membrane. For mutations studies its distinguishing feature is the involvement of a non-soluble enzyme activity. It is a selective forward mutation - system sensitive to many physical and chemical agents (Brusick, 1972; Lemontt, 1977; Larimer, 1978; Lemontt and Lair, 1982; Gocke and Manney, 1979).

Mutations and recombinations are assessed (the basis of the recombination assay is discussed in section 4.3) by plating on solid media containing canavanine for haploid and diploid cells, respectively, provided the canavanine concentration is not too high. However, not all cells are viable, hence survival is determined with media containing arginine.

The quantification of recombination and mutation in any assay system have to take into consideration the spontaneous mutants or recombinants present in the test system even in the absence of treatment.

2.5 Spontaneous mutations and recombinations:

A small fraction of spontaneous mutants or recombinants may arise from background radiation, it is however mainly due to intracellular mechanism (Sargentini and Smith, 1985). DNA replication, recombination and repair may all be involved in spontaneous mutagenicity. Cellular DNA metabolism plays an important role in the induction and expression of mutations and recombinations. In this investigation the genetic effects on cells due to media treatment are evaluated relative to the levels of spontaneous mutations and recombinations already existing.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Cell Strains.

Two strains of *Saccharomyces cerevisiae* were used:

(1) C420 - 3B a lysine dependent haploid obtained from Dr. A. James (Canada).

(2) CD 364 a diploid which was constructed from a wild haploid (A 364 A) and mutant haploid. The mutant haploid, C 364 - Can-del, contains a canavanine deletion and was obtained from Dr. M. Whiteway (USA). The construction was done at the Radiation Centre of Justus - Liebig University, Giessen, West Germany. The diploid has no nutrient dependency.

3.2 Media.

All chemicals used for the preparation of media are of analytical grade. Unless otherwise stated the underlisted are the compositions of each Medium made up with deionized water to one litre and then autoclaved; un autoclaved solutions were made in sterilised water.

3.2.1 Complete Medium

Table 3.1: Complete Medium

Component	Weight(g)
Yeast extract	10.0
Bacto Peptone	20.0
B - Glucose	20.0
Agar	20.0

One litre solutions autoclaved

3.2.2 Sc-Medium

One litre of Sc - Medium was made up of the following(Table 3.2) separately prepared solutions.

Table 3.2 Sc - Medium

Component	Concentration (g/1)	Remark	Volume (ml/1 of Sc-Medium)
Amino-acids (stock solution)	Table 3.2.1	Autoclaved	10.0
Threonine	10g/1	Autoclaved	10.0
Arginine OR	2g/1	Autoclaved	10.0
Canavanine sulfate	10g/1	Not Autoclaved	10.0
'The non-amino-acid components'.	Table 3.2.2	Autoclaved	970.0

One litre of the amino-acids stock solution was made up of the following:

Table 3.2.1 Amino-acids stock solution for Sc-medium.

Amino-acid	Weight(g)
Histidine	10.0
Tryptophan	2.0
Adenine	2.0
Methionine	2.0
Leucine	3.0
Lysine	3.0

The 970ml of the "non-amino-acid components of the Sc-Medium" was made up of the following.

Table 3.2.2 Non-Amino-acid Components of Sc-Medium.

Component	Weight(g)
Difco Yeast Nitrogen Base	6.7
Agar(for Solidification)	20.0
Glucose	20.0

The other components were added to this autoclaved solution while it was warm.

3.2.3 Wickerham medium.

One litre of Wickerham medium was made of the following separately prepared solutions.

Table 3.3 Wickerham medium

Component	Concentration (g/l)	Remark	Volume (ml/l of Wickerham medium)
Vitamins stock Solution	Table 3.3.1	Autoclaved	10.0
Ferric Chloride	24.2	Not Autoclaved	10.0
Trace elements			
(Boric acid	0.571	Not Autoclaved	1.0
and Zinc Chloride)	0.145		
Potassium Iodide	0.1	Not Autoclaved	0.1
The "main-components"	Table 3.3.2	Autoclaved	978.9

The cell strain C420-3B requires lysine for its growth. In this case, 10 ml of lysine solution (3g/l) separately autoclaved was added to each litre of autoclaved Wickerham medium.

The composition of one litre of vitamins stocks solution for the Wickerham medium is given in Table 3.3.1.

Table 3.3.1 Vitamins - stock solution for Wickerham medium:

Vitamin	Weight(g)
Thiamine	0.04
Pyridoxine	0.04
Nicotinamide	0.04
Pantothenic acid	0.04
Inositol	0.20
Riboflavin	0.02
4-Aminobenzo acid	0.02
Biotin solution (1g/l)	2.0ml

Autoclaved vitamins stock solutions were kept in a refrigerator.

The 978.9ml solution of the "main components" of Wickerham medium was made-up of the following.

Table 3.3.2 The main-components of Wickerham's medium.

Component	Weight(g)
Glucose	10.000
Asparagine	1.000
Potassium monophosphate	0.125
Potassium diphosphate	0.875
Magnesium sulfate	0.500
Calcuim chloride	0.100
Soldium chloride	0.100

The vitamins and other solutions, listed in Table 3.3, were added to this autoclaved solution while it was warm. The preparation of the treated Wickerham media was given in Section 3.5.2.

3.3 Autoclaving.

It is usual to sterilize the nutrient media of the cells. This elliminates bacteria - that otherwise contaminate and grow faster than the yeast cells. All the autoclaving was done in this investigation at 120°C, one atomospheric pressure for 20 minutes.

3.4 Irradiation and dosimetry.

3.4.1 Fricke - dosimetry.

The Fricke dosimeter is based on the oxidation of an aerated ferrous sulfate solution by radiation. It is valid only in the dose range of 40- 400Gy (Fricke and Hart, 1968). This range is well below the doses used in this investigation. Hence the dosimeter was used only for the purpose of determining the dose rates at a particular position in the irradiation facility.

The composition of the dosimeter is given in Table 3.4.

Table 3.4 Fricke dosimeter solution.

Chemical	Weight(g)
Ferrous sulfate	0.392
Sodium chloride	0.058

The salts were dissolved in 12 ml of 0.8N sulfuric acid. The solution was made-up to a litre with triply distilled Water. Fresh solutions were prepared before use. Stock solutions were kept in refrigerator.

Dose rates were determined by irradiating 3ml aliquots of the dosimeter solution for known duration, 10-15 minutes, for these durations the doses delivered are within the valid range of the Fricke dosimeter, in the irradiation facility.

The absorbance of the irradiated solutions were then compared to those of unirradiated solutions (controls). Absorbance were measured at 3040 Å, the wave length at which the optical absorption coefficient of the ferrous ions is less than 0.05% of that of ferric ions. These measurements were done in a I-cm quartz absorption cell.

At 25°C the G-value of ferric ions is 15.6 and the molar extinction coefficient of ferric and ferrous ions are 2197M^{-1} cm^{-1} and 1M^{-1} cm^{-1} (at 3040 Å) respectively. Under the above stated conditions the dose, according to Fricke and Hart (1968), is given as D(Gy) = 2.756 x 10^2 x DOD where DOD is the difference in optical density between irradiated solution and control.

The dose - rates is therefore

$$D(\text{Grays per minute}) = \frac{2.756 \times 10^2 \times DOD}{\text{irradiation time(minutes)}}$$

A plot of the differences in optical density as a function of irradiation time was made, from the slope the dose rate was determined.

The dose rate was 19.0 ± 1.0 Gy/minute. The samples were therefore exposed for 8.77 and 43.86 hours in order to attain the doses of 10kGy and 50 kGy respectively. The uniformity of the doses delivered were frequently checked with radiochromic-dye film detectors (McLaughin, et al, 1977). The films were externally attached to the solutions containers during irradiation.

3.4.3 Irradiations:

All irradiations were carried out in the "Ring Schieber". It is a metal box facility into which samples can be exposed to γ-rays from a Co-60 source. Irradiations were done at room temperatures and normal atmospheric pressure. Solutions were irradiated in glass bottles "Seromed" measuring 5cm in diameter and 10cm high. The bottles were tightly covered with plastic.

For all treatments 10g of glucose dissolved in 100ml of sterilized distilled water was used.

3.5 Preparation of growth media:

3.5.1 Control Medium.

For all investigations Wickerham media were used. Control media were prepared in the normal way-as listed in Table 3.3.

3.5.2 Treated Media.

A 10ml of 10% aqueous glucose solution was irradiated either with 10kGy or 50kGy. Immediately after irradiation it was added to the other autoclaved components of one litre Wickerham media (In this case the Wickerham media composition was as listed in Table 3.3.2 except the omission of glucose and the reduction of the deionized water used for dissolving the medium to 968.9ml before autoclaving). Usually the irradiated glucose was added into the medium together with the vitamins - when the autoclaved medium was still warm.

3.6 Cell cultures.

3.6.1 Semi-continuous Cultures.

Cells were first grown on solid complete medium for 3 days in a 30^{o}C incubator, cell suspensions were then made in sterilized deionized water. On suitable dilutions they were counted microscopically with a haemotocytometer. Appropriate dilutions were then made for inoculation into growth media and plating on arginine and canavanine.

In all cases cells were diluted such that a 1.0ml suspension was inoculated into 99-ml aliquots of each treated and control growth media, each contained in 300ml flat-base conical flasks. Thus in each 100ml of treated growth medium (that is containing 10 kGy or 50 kGy irradiated glucose) or control media (that is the normally autoclaved glucose) there are 2 x 10^3 cells/ml immediately after the inoculation of the fungi. At this density the micro-organisms have enough nutrients as may be required for their multiplication over the next 24 hours. Covered cellular suspensions, in their treated or untreated nutrient media were grown in rotatory shaker, 100 revolutions per minute, at 30°C for 24 hours.

On incubation for 24 hours, the eukaryotes were microscopically connted. Suitable dilutions were again made, as described above, such that 1 ml of yeast suspensions, containing 2 x 10^5 cells, were inoculated into fresh media. The celullar suspensions in refreshed nutrient media were again grown for a day and the same process was repeated. By this semi-continuous growth culture 99% of the previous day's nutrient medium, in which the cells were grown, was removed and replaced with fresh medium. The eukaryotes are therefore continuously grown in media with enough nutrients. Growth impairement due to nutrient exhaustion (when the fungi multiples) may not occur. The method keeps the micro-organism continuously in exponential phase and cytotoxic, mutagenic and recombinogenic effect could be better expressed than in stationary phase.

Normally one litre of each treated and control media were prepared at a time, and can be used for 4 - 5 days provided for each treated and control two cultures were made per day.

3.6.2 Batch Cultures.

In some cases cellular suspensions were incubated in unchanged nutrient media for some days. The cells were counted each day.

3.7 Plating on Sc-Media containing Canavanine or Arginine.

Two hundred cells were plated on arginine plates in all cases, 2×10^4 cells were plated on canavanine for the diploid strain. Whenever possible, 2×10^7 haploid cells were plated on canavanine. The growth of the haploid in most cases could not enable the plating of the desired cell density. Hence the micro-organisms were concentrated by filtering fixed volumes of known cell density. The filtered yeast were then plated on canavanine. The platting of above stated cell concentrations was to enable 100 - 200 colonies be formed on each plate. Plated fungi were incubated for 3-days at 30°C. Mutants, survivors and recombinants were then scored.

Cells were plated on Sc-medium on each day, they were also counted, diluted and reinoculated into fresh nutrient media. On the first day the liquid cultures were made, the micro-organisms were all inoculated from a suspension made from cells grown on a single solidified complete media plate, hence the mutants and recombinants recorded for that day are spontaneous. But on subsequent days mutants, recombinants and survivors are not necessarily spontaneous, they could be due to the culturing system or due to the treatment of the nutrient media. This could only be discussed in comparison to what was observed in the control. At least two plating on arginine and canavanine were made for each of the yeasts grown on the treated media and on the control medium.

The variation in spontaneous mutants and recombinants were monitored by plating the cell strains on Sc-media at frequent but irregular days.

3.8 pH Measurements.

The acidity of unirradiated and irradiated glucose, as well the acidity of the treated and control media were measured with a pH-meter. Measurements were done immediately after irradiation of the glucose (to avoid contamination such pH-measured glucose solutions were not incorporated into Wickerham media), and were monitored over subsequent days. The acidity of treated and control media were measured immediately after preparation and on storage, at 30°C in an incubator, over a period of few days.

CHAPTER FOUR

RESULTS AND DISCUSSIONS

All the results presented here are the means of at least three separate experiments. The inter experimental error, the standard error, of each plotted point was indicated. General aspects of the data treatment were discussed in appendix III.

4.1 Growth.

The daily growth of the haploid strain in the different media were presented in Figures 4.1 and 4.2. There are random variations of the cell's proliferation within each growth medium. Statistical analysis (t- and F- distributions, appendix IV) indicates no significant difference between the growth rates in the control media and those in nutrient media into which was incorporated 10kGy and 50 kGy irradiated glucose solutions respectively.

The growth pattern of the diploid were depicted in Figures 4.3 and 4.4. The average growth rate of this strain was better than that of the haploid. Again, as with the haploid, statistically, - appendix IV, there are no differences in the growth rates observed in the control saparately compared to those observed in the treated media.

In order to produce energy and undertake cellular activities yeast requires a carbon source, a nitrogen source, some vitamins and few mineral ions. Growth and cellular reproduction respond in an orderly manner to the availability of nutrients. Under aerobic conditions, the assimilation of glucose produces optimal growth;

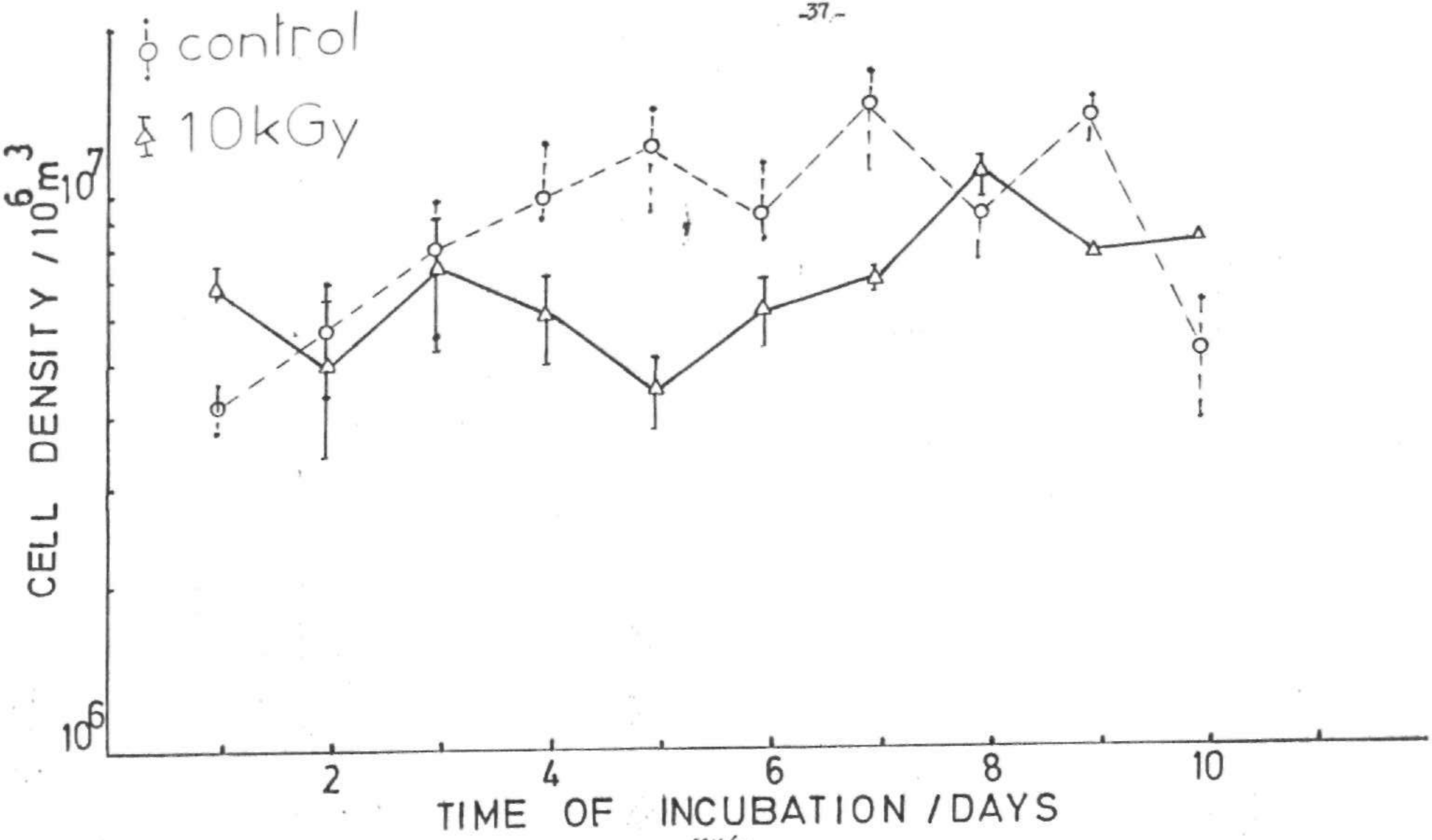

Figure 4.1 Growth of haploid cells C-420-3B in 10kGy treated and control media.

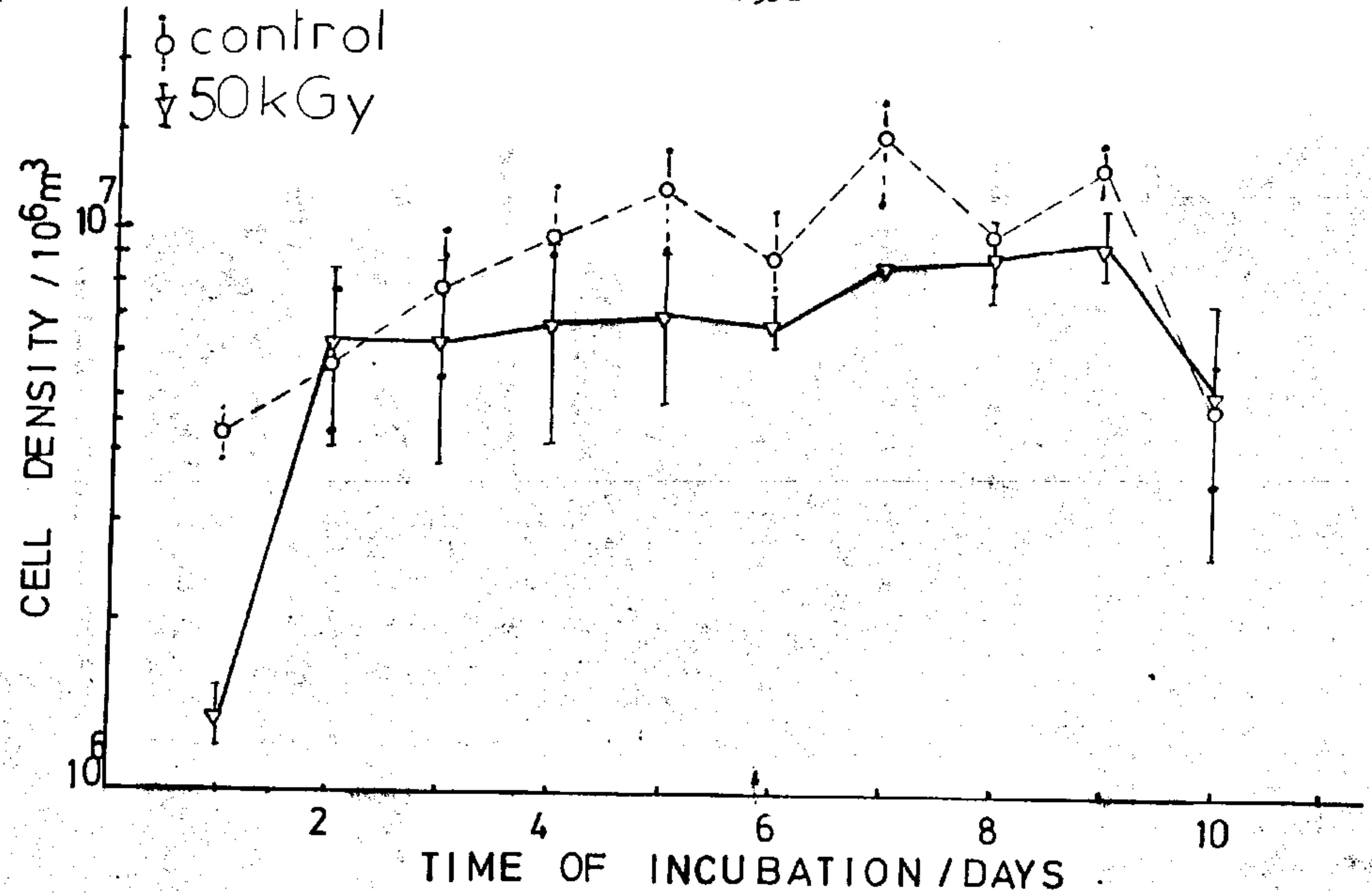

Figure 4.2 Growth of haploid cells C 420-3B in 50kGy treated and control media.

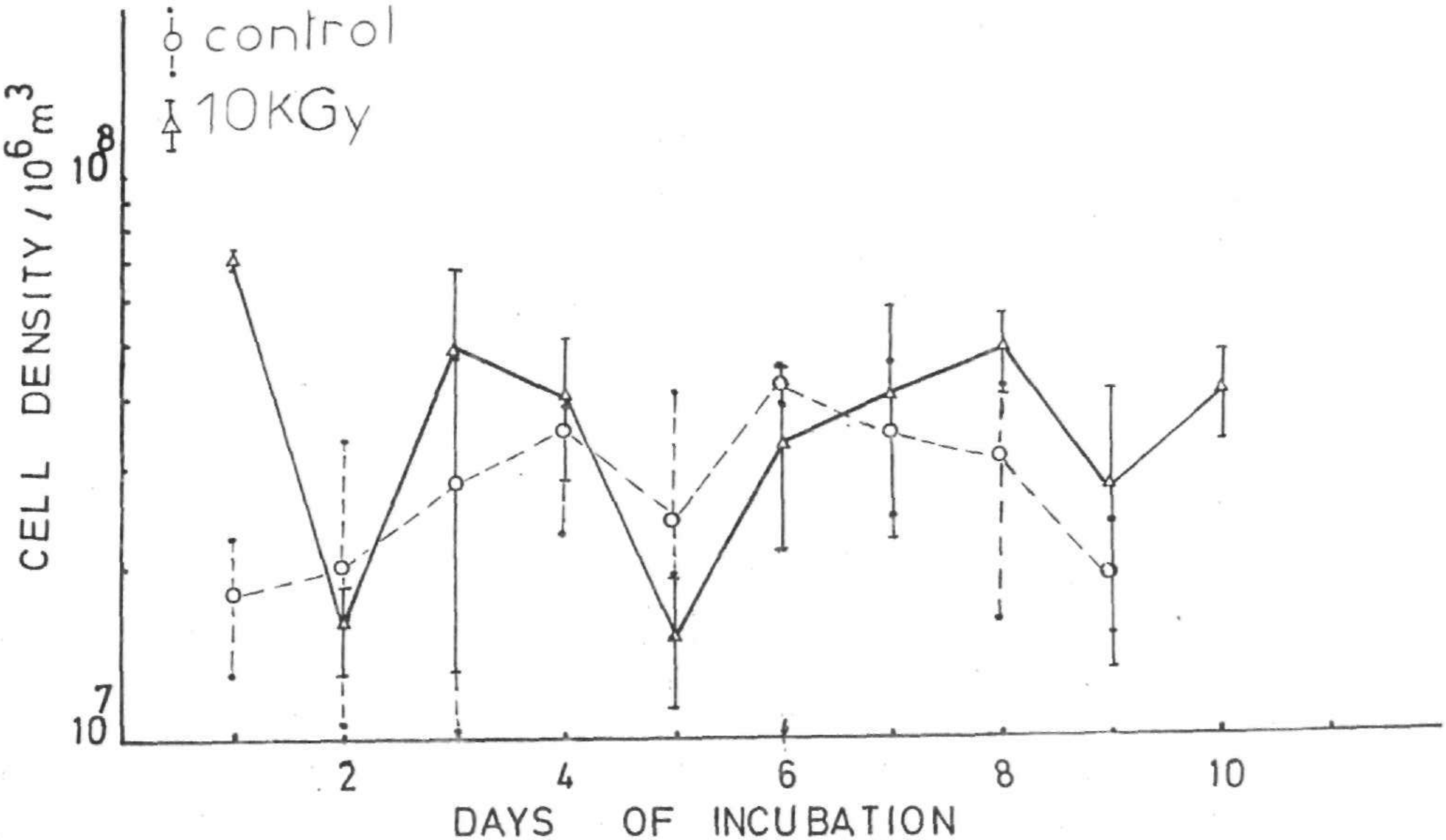

Figure 4.3 Growth of diploid cells CD 364 in 10KGy treated and control media.

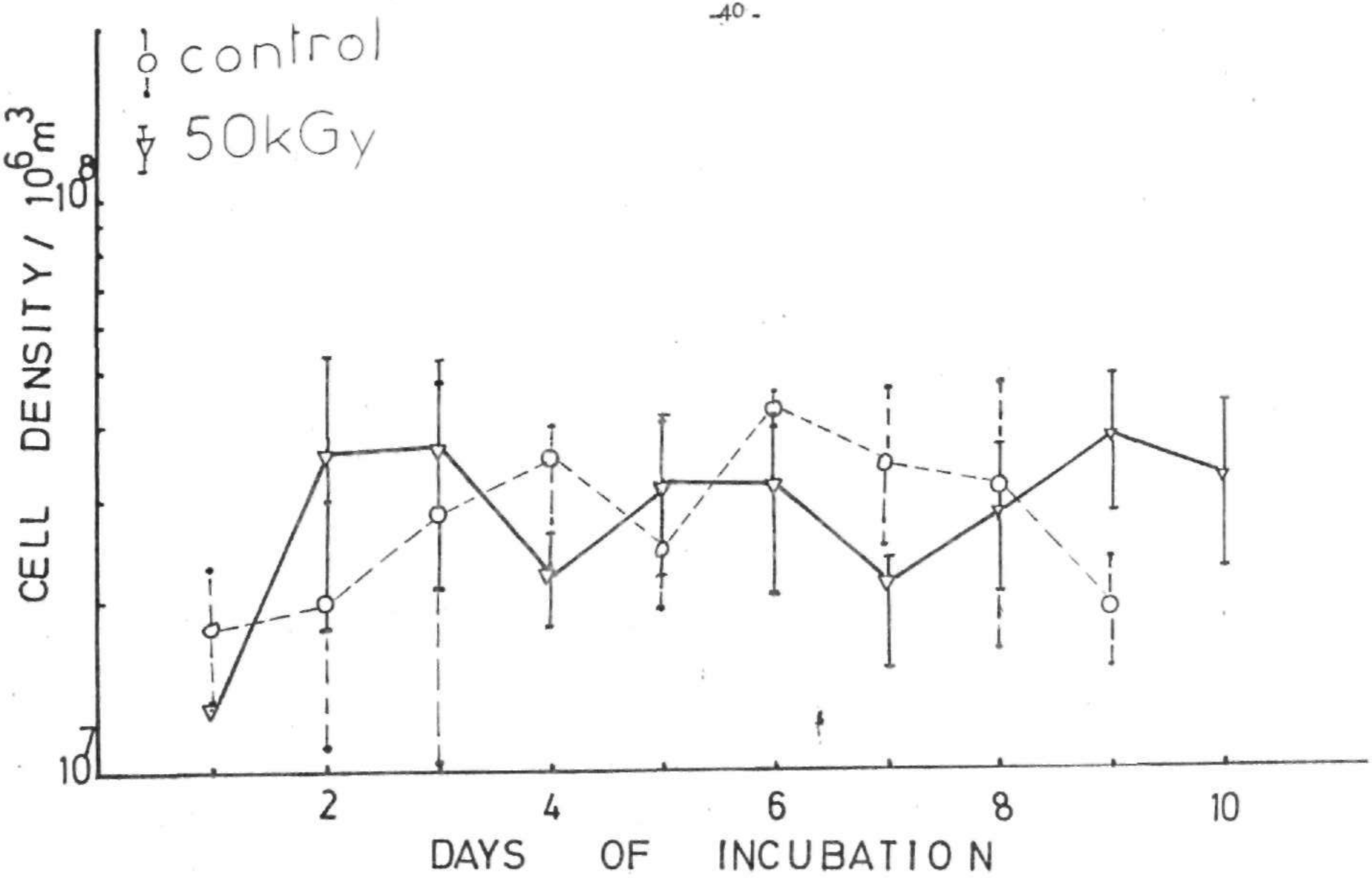

Figure 4.4. Growth of diploid cells CD 364 in 50 KGy treated and control media.

for S.cerevesiae under these conditions the growth rates are uniform with a doubling time of approximately two hours. Yeast could however utilize other carbon-sources, but less efficiently and growth rates may vary (Fraenkel, 1982). In the absence of adequate nutrients, cells are arrested in the unbudded phase of the cell cycle. Thus with nutrient limitation the fungi are in stationary phase. It is important to determine when this occurs under the experimental conditions.

The growth of the haploid and diploid strains in batch culture were plotted in Figures 4.5 and 4.6. Clearly by the second day the cells are in stationary phase. At least for the first day, the cell's growth is exponential, hence the incubation procedure used in this investigation is suitable for determining possible cytotoxic effect on chronic exposure of cells to treated media.

The method may not however determine 'brief periods' growth impairement that may affect few cells. The exponential growth of unaffected cells may overshadow the non-growth of a few cells. Such subtle effects could perhaps be determined through visual quantification of the ratio of budding to non-budding cells on exposure to treated media (Kiefer, Personal communication).

4.2 Mutations.

The mutants per survivor determined daily for the haploid strain grown in control media, 10kGy and 50 kGy - irradiated glucose incorporated into the Wickerham media were presented in Figures 4.7 and 4.8. With each growth medium, the observed mutants per survivor varies considerably from day to day. Hence net accumulation or otherwise of the mutants could not be determined.

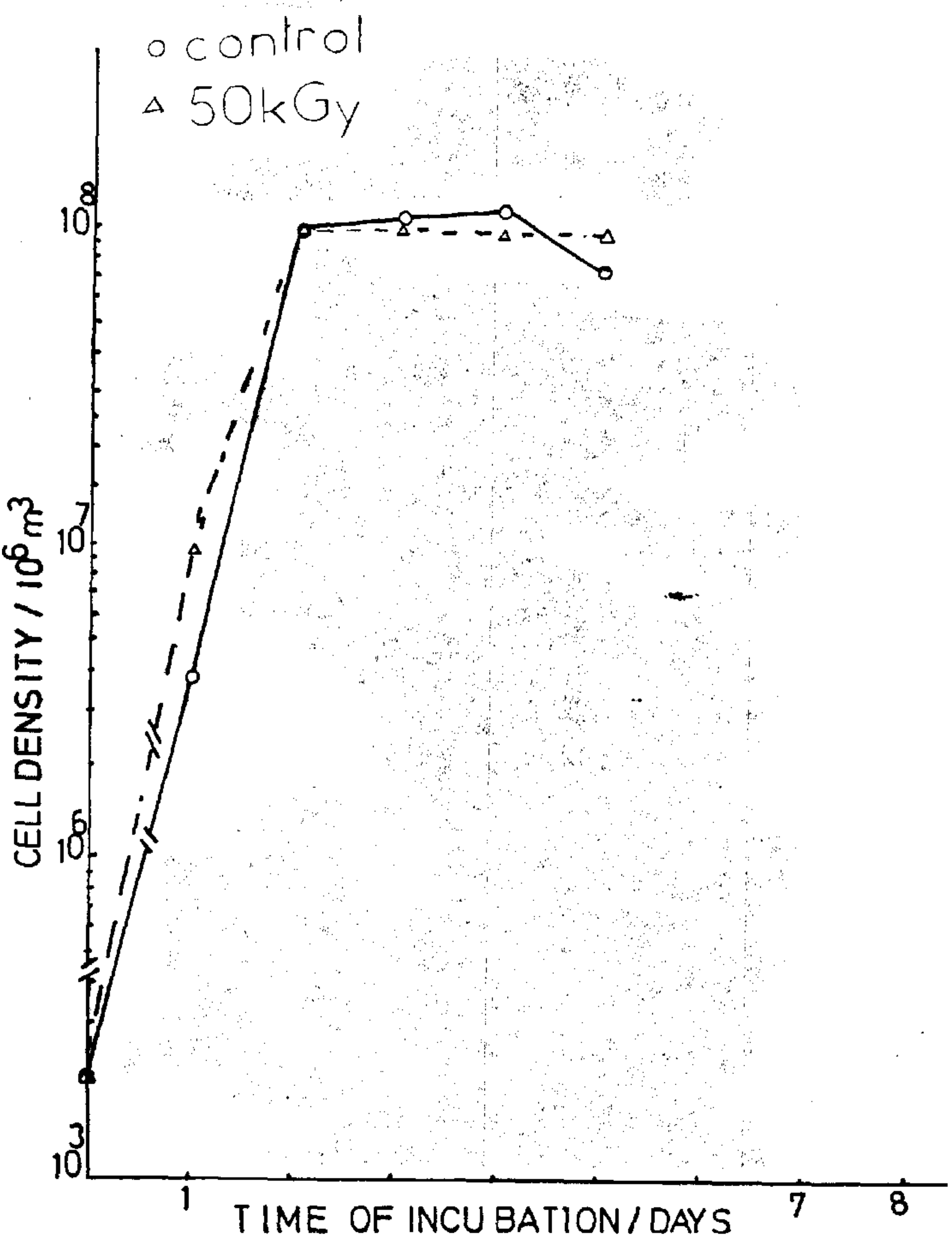

Figure 4.5: Batch growth of haploid cells C 410-3B in 50kGy treated and control media.

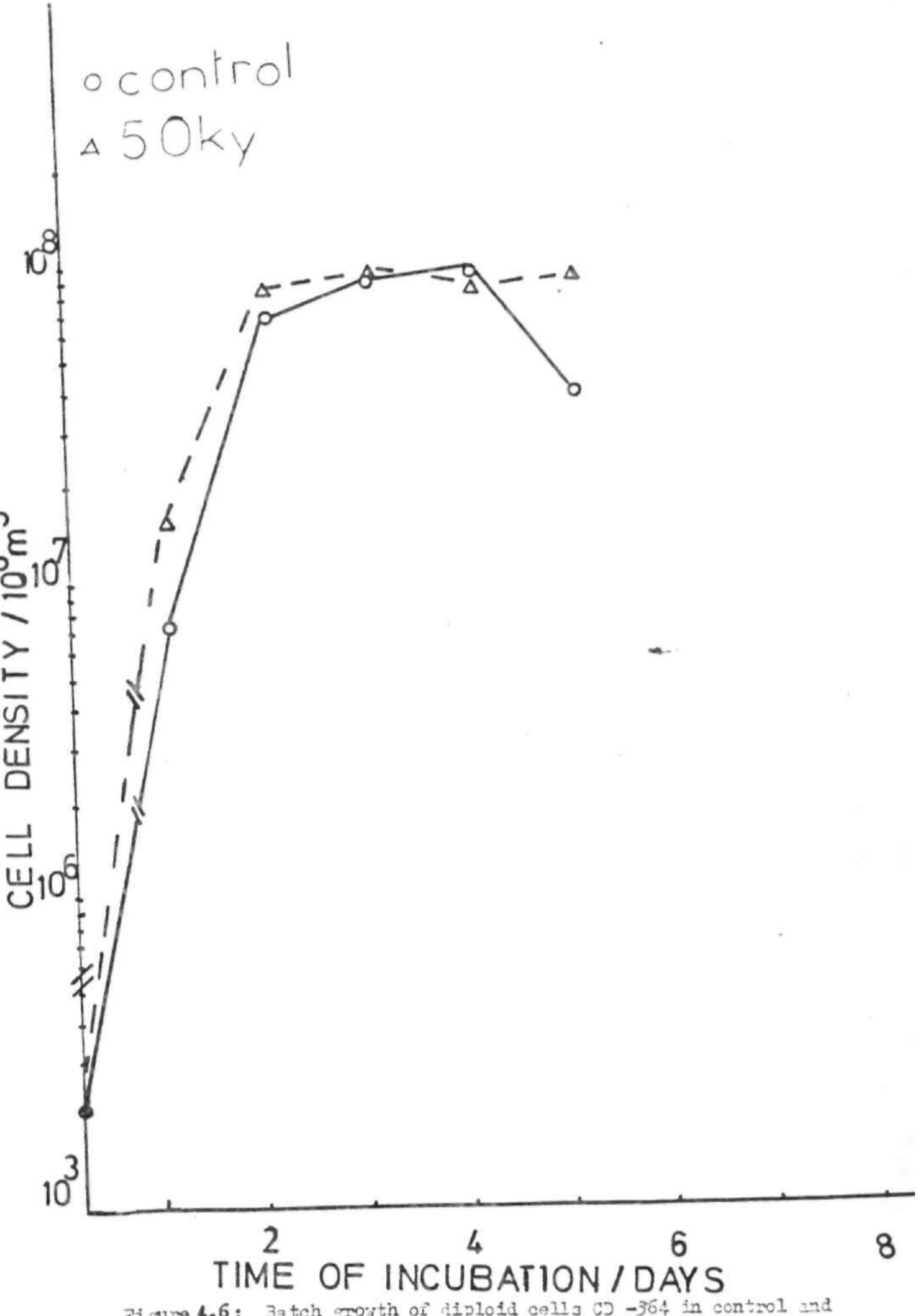

Figure 4.6: Batch growth of diploid cells CD -364 in control and 50kGy treated media.

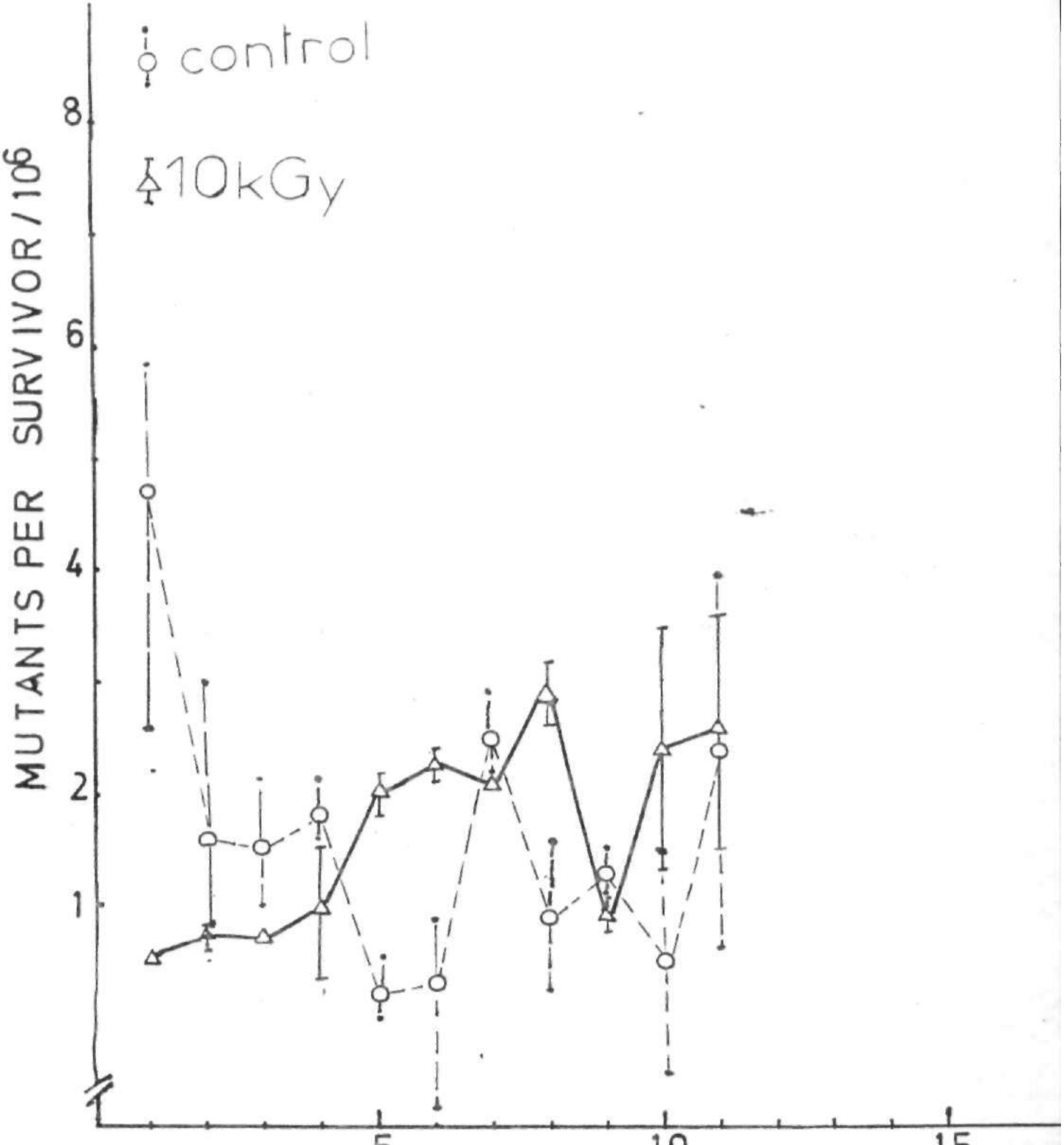

Figure 4.7: Mutants per survivor of haploid cells in 10kGy treated media and control media.

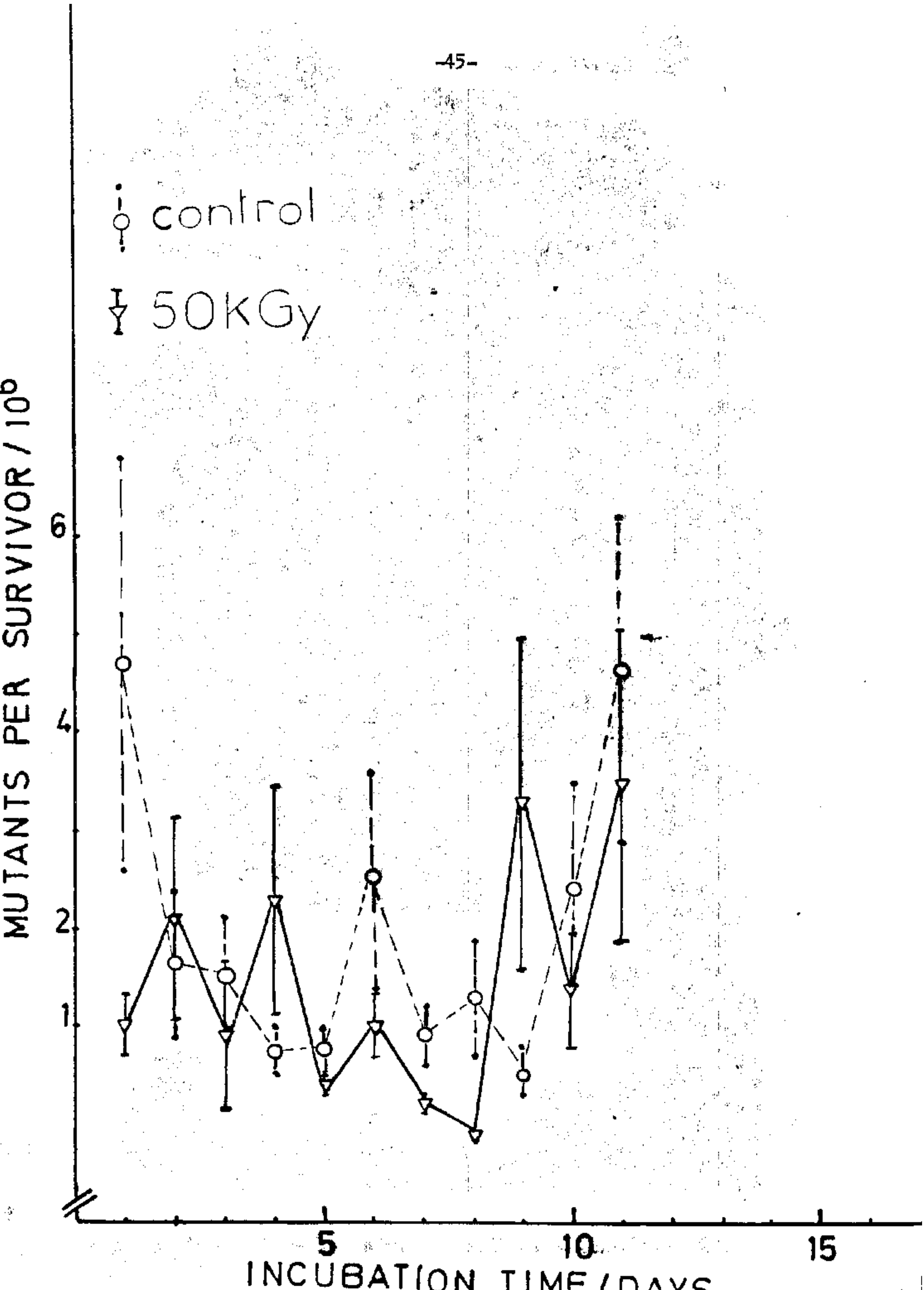

Figure 4.8.: Mutants per survivor of haploid cells in control and 50KGy treated media.

The spontaneous mutants per survivor of the strain were determined at randomly selected dates; result was given in Figure 4.9. The spontaneous mutants per survivor vary in the range (1-7) x 10^{-6} (neglecting the unusual number of mutant / survivor observed in 19 July). This variation and range corresponds to that of control and treated media (Figures 4.7 and 4.8) it may therefore be deduced that the day to day variations in the observed number of mutants per survivor was not due to the incubation procedure. Variations were also observed in each of the three growth media, hence the mutants per survivor in the treated media were compared to that in the control; t- and F-distributions - appendix V, indicates no difference in the three media. Thus, the incorporation of 10kGy and 50kGy irradiated glucose into Wickerham media does not enhance or reduce the average magnitude of mutants per survivor at least compared to a heat treated control medium.

4.3 Recombination.

In a normal diploid cell, autosomal information carried by the DNA is homologous. The diploid strain used in this investigation was constructed from a wild type haploid and a mutant-type haploid (C364-can-del) with a large canavanine delition. Although the two alles still code for the same gene, the presence of a large delition can be considered to have created a non-homologous site.

A non-homologous site is important for mutational studies as the mutation - rate of such a site is of the order of 10^{-6}. But in the diploid, the mutation rate at the homologous site is the square of this, that is of order 10^{-12}, which makes

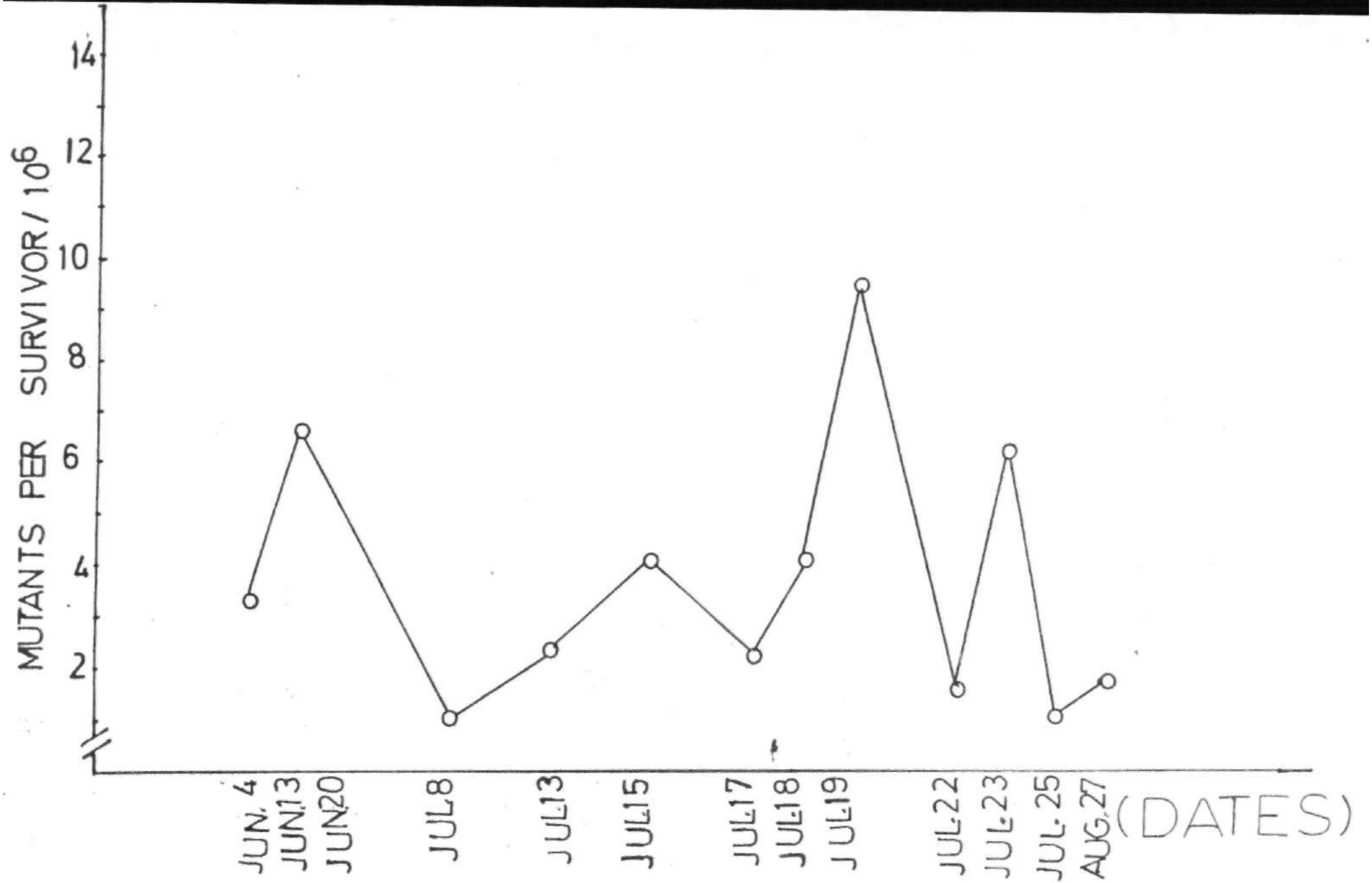

Figure 4.9 Spontaneous mutants per survivor of the Haploid cells C420-3B.

mutational studies impractical. However due to the common homologous regions left in the arginine permease-site, in the diploid strain, there is a high probability for recombination. Gene conversion, the non-reciprocal transfer of information from one DNA duplex to another, may take place between the homologous chromosomes. This is the basis of scoring recombinations, instead of mutations, at the canavanine locus of the diploid cells.

The determined recombinants per survivor for the diploid were drawn in Figures 4.10 and 4.11. Similar problems were encountered as in the scoring of mutations of the haploid hence the data were analysed in a similar manner. Figure 8.12 indicates the variation in the spontaneous recombinants per survivor monitored at irregular intervals. The range of the spontaneous recombinants per survivor is (2 - 8) x 10^{-4} which corresponds to the range observed in the control, 10 kGy and 50 kGy irradiate glucose ... included into the growth media of the cells. Again, statistically (t- and F- distributions appendix VI), the recombinants per survivor are not different in the treated media compared to the control.

4.4 Discussion:

In this investigation the possible induction of cytotoxic, mutational and recombinational effects by gamma-irradiated glucose solutions incorporated into normally heat sterilized growth media of the cells are investigated. The procedure used could determine the influence of gamma-irradiated nutrient components if and only if as a result of the treatment the biological-end points are statistically and consistently different from their initial conditions-namely the spontaneous mutations/recombinations. But since variations

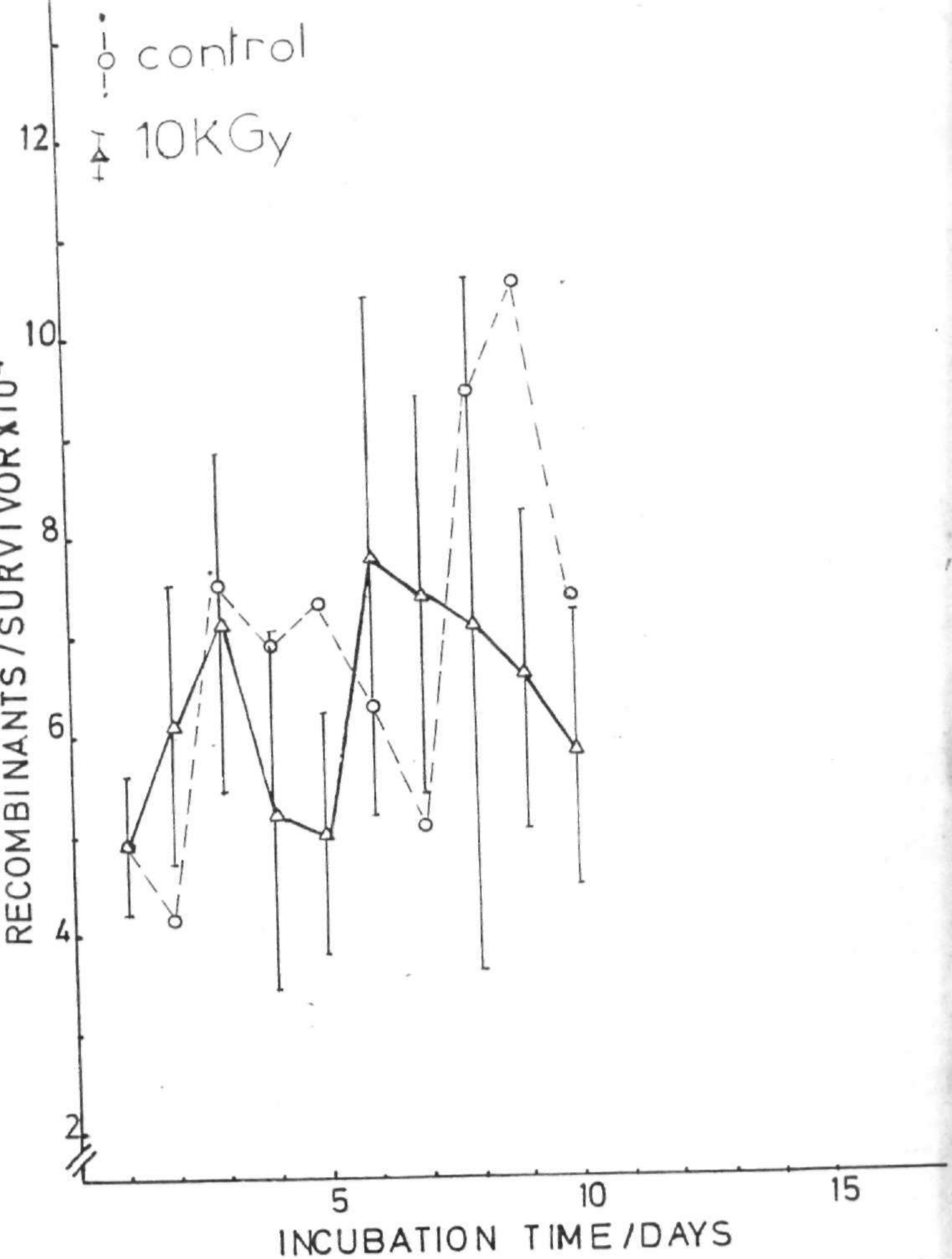

Figure 4.10: Recombinants per survivor of diploid cells in treated (10KGy) and control media.

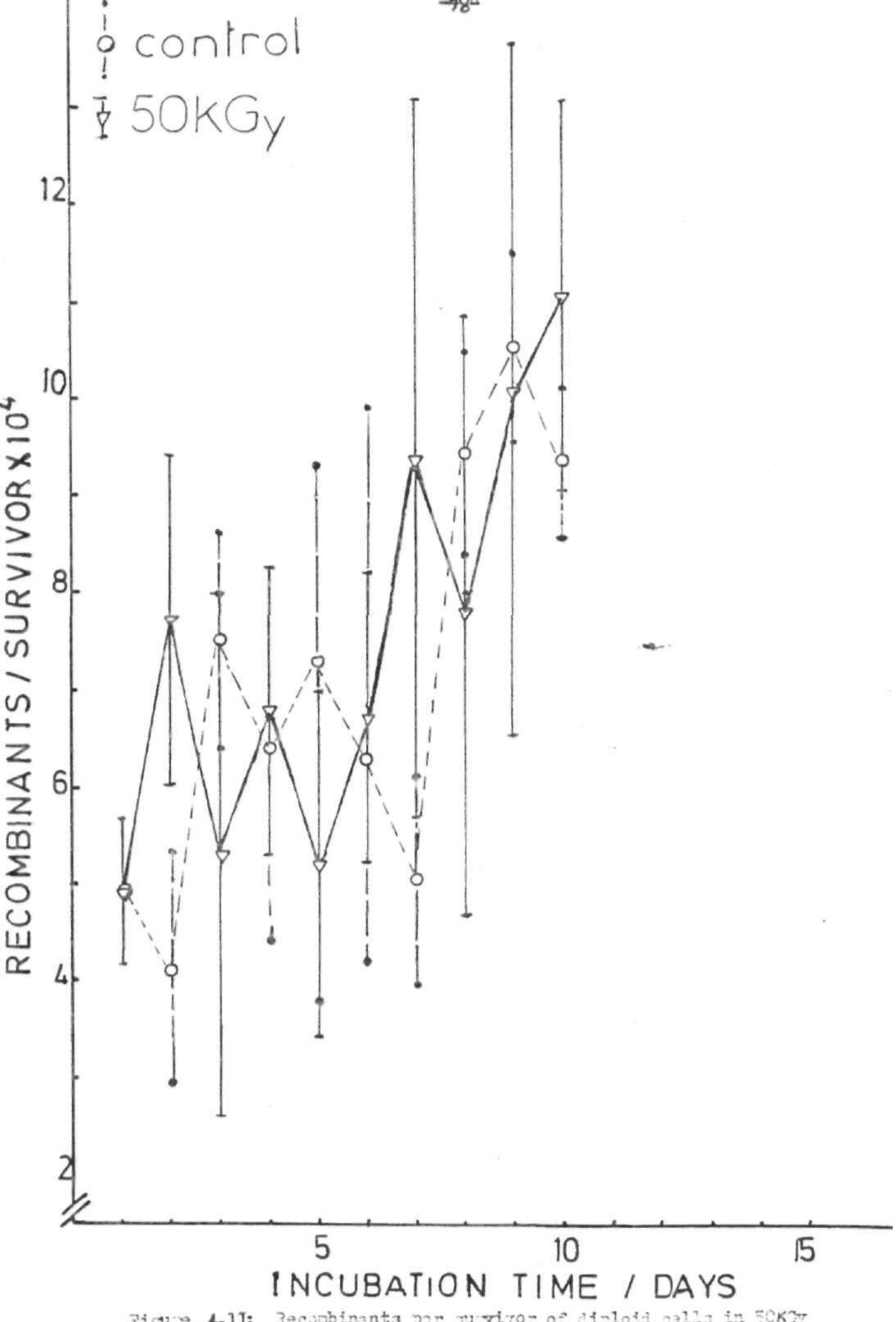

Figure 4.11: Recombinants per survivor of diploid cells in 50KGy treated and control media.

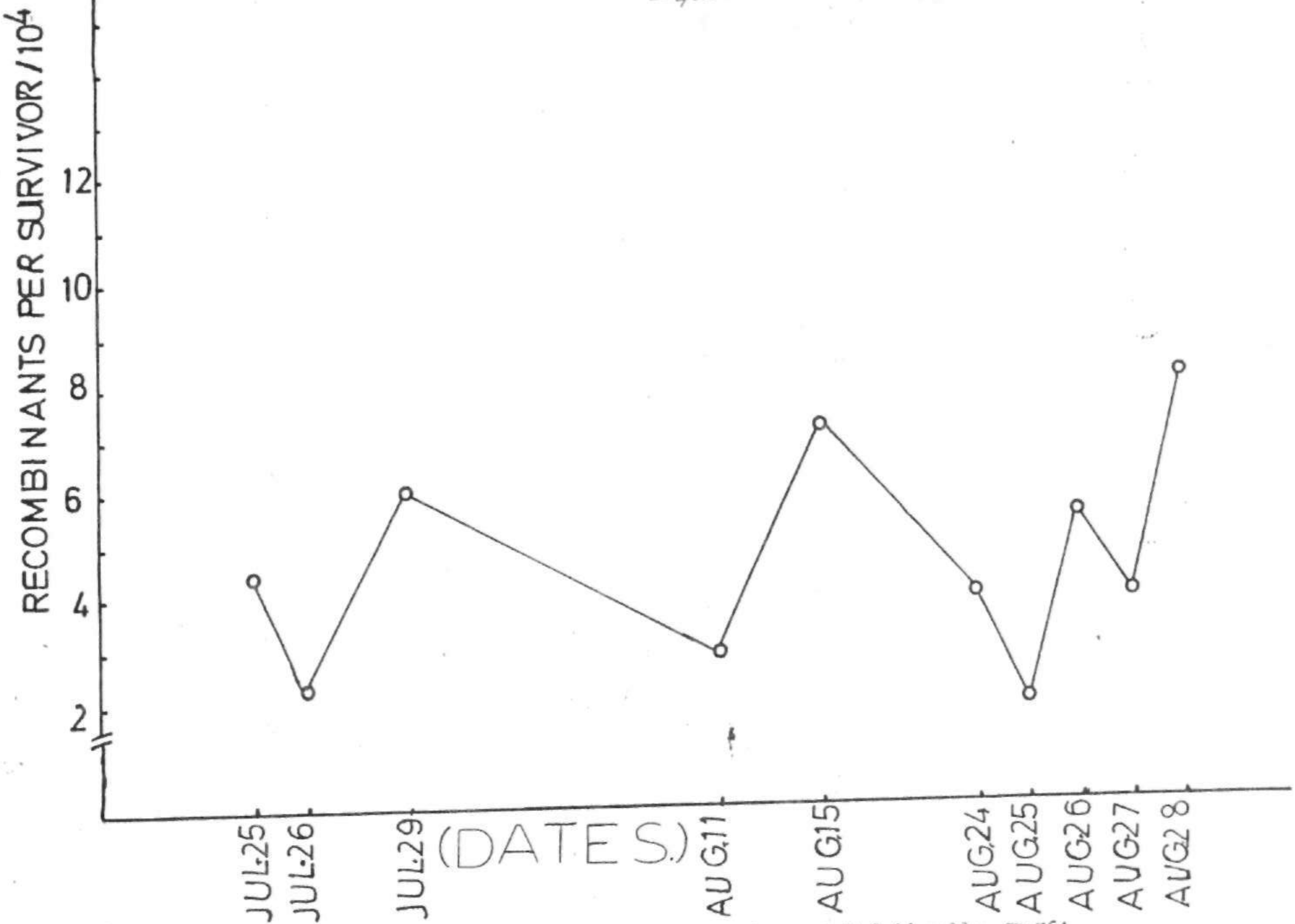

Figure 4.12 : Spontaneous recombinants per survivor of diploid cells CD 364.

could also be due to the experimental procedure, the incubation method for instance, possible effects were compared with the control.

Although these results indicate that no biological activity was exerted, at least on comparison with the control, the possible production of compounds which could exert biological activity may not be ruled out. The measurements of the irradiated solutions pH immediately after irradiation indicates an average value of 2.79 ± 0.06 and 3.04 ± 0.32 for the 50kGy and 10kGy irradiated glucose solutions, respectively; and the pH values remain unchanged on storage of the solutions for some days. The pH of unirradiated glucose solutions was 6.66 ± 1.08. And for comparison: the pH of separately autoclaved glucose solutions was 2.80 ± 1.06 (In one instance a value of 5.17 was measured). In each case the concentration of the glucose solutions was 10%.

pH measurements could only give an indication of the overall acidity of the end products. Nevertheless, it is worth noting that in the complete growth media, the pH of the control, 10kGy and 50kGy irradiated glucose incorporated into the media were 4.56 ± 0.82, 4.74 ± 0.60 and 4.33 ± 0.70 respectively and the acidity hardly varies with time. The formation of hydrogen peroxide, an acidic radiolytic product, has been attributed as responsible for the observed biological activity of irradiated sugar solutions (Schubert, 1969; Kesavan and Swaminathan, 1971). Hydrogen peroxide was determined as a growth inhibitor and cellular inactivator, and mutagenic to adenine auxotropic yeast (Thacker, 1975). But concentrated solutions of the peroxide were used. Moreover the same investigator demonstrated that the cytotoxic agent can be destroyed by cellular hydroperioxidase provided that the peroxide concentration is not too high.

Under deoxygenated conditions the yield of acidic products are generally low. According to Beyers et al, (1983) the yield of H_2O_2, under gamma-radiolysis of sugar solutions, is G = 1.3 ± 0.2 and 0.05 ± 0.01 for irradiation under oxygenated and deoxygenated conditions. Under the partly anaerobic conditions of this investigation the yield of hydrogen peroxide may be very low. Schubert and Sanders (1971) discounted any possible role of hydrogen peroxide or other organic peroxides on the basis of low yield.

Not all radiolytic products are acidic. However the fact that mutagenicity is enhanced by the presence of oxygen (Aiyar and Rao, 1977) leads to attribution of lethal, cytotoxic and mutagenic effects to acidic products. Low molecular weight compounds, aldehydes, glyoxal etc are also produced in the gamma-radiolysis of sugar solutions (von Sonntag, 1980) and are known as enviromental mutagens (Ames, 1983; Auerbach et al, 1977). Deoxy sugar (Sherz, 1970) or low molecular weight carbonyls such as malonalydehyde (Sherz, 1970), formaldehyde (Holsten et al, 1965), glyoxal (Berry et al, 1965) were all suggested as responsible for the various observed effects. Schubert and Sanders (1971) discounted all these on grounds of low concentrations in gamma-irradiated, unbuffered, oxygen-free sugar solutions. They suggested that cytotoxic products of such solutions could be only due to α, β -unsaturated carbonyl sugars derived from radiolytically produced dicarbonyl sugars (hexasuloses). Whatever may be the cytotoxic principles, acidity certainly plays a role. The particular sensitivity of α,B - unsaturated carbonyl sugars to alkaline media was noted by Beyers, et al,(1983). According to the investigators the mutagenicity of glyoxal, is optimal at a concentration of 1000 ug per plate, at this level it increases the mutation frequency in Salmonella 2.5 fold. Hence, it may be concluded that in this investigation even if cytotoxic or mutagenic compounds were produced their concentrations may not be high enough as to produce an observable effect compared to the control.

No attempt was made, in this investigation to oxygenate the solutions during irradiation, or to buffer the glucose solutions, or to isolate the various radiolytic products and determine their individual effects. This was to enable our conditions to resemble, as much as possible, the practical conditions likely to be encountered in the food industry. For the same reason a 10% solution of glucose, which reflects the sugar content of most foods, was irradiated instead of the 1% solutions used in other investigations. This may enable a more reliable prediction of the possible hazards of irradiated food components fed to animals; at a research level its disadvantage is that comparison with other investigations is difficult.

Furthermore, this investigation involves the chronic exposure of cells to treated media, such that consistent effects on exponentially growing cells could be determined. In other investigations of media effects cells were briefly exposed to the media. Since yeast cells may undergo a lag-phase on exposure to a new nutrient media (Bijerk and Hall, 1977) the author felt this incubation procedure was proper.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion.

Ten per cent glucose solutions were irradiated with respective doses of 10 kGy and 50 kGy at a dose rate of 19Gy/min. On incorporation into autoclaved components of Wickerham media, the solutions did not impair growth or induce mutation and recombination in yeast saechromyces cerevisiae

The determination of genetic effects was based on the sensitivity of the can-I locus to many mutagenic and recombinogenic agents. Neither the precise nature and yields of the radiolytic products under our irradiation conditions nor their fate on mixing with other components of the cell's nutrients media was known. Nevertheless the involvement of an essential enzyme activity in a foward mutational assay assured us that our system was more sensitive than the recessive, nutritional dependent Ames test.

No single test system could provide a conclusive assessment of irradiated food's wholesomeness. This work, using eukaryotic cells indicates the possible safety of glucose containing food's radappertization, at least at a screening level.

5.2 Recommendations.

The conclusions of this work support the resolutions of the Joint FAO/IAEA/WHO Expert Committee on the Wholesomeness of Irradiated Food (WHO, 1981). It may therefore be suggested that further screening of irradiated foods with micro-organisms is not necessary.

The main research problem that remains, in food irradiation, relate to large scale and commercial size applications. However many of the proposed application of radiation processing of food also demand well established and reliable handling systems in the food trade such as refrigeration and freezing (Ehlermann, 1983). Certainly food irradiation is a powerful tool for developing countries like Nigeria, and in many cases no alternative to it may be envisaged. The sprouting of yams could be prevented by a radiation dose of 50 - 100 Gy while various chemical treatments were not found to be effective (Adesuyi and Mackenzie, 1973). Nonetheless, the potentials of radiation-processing should be investigated in the context of local customs and conditions.

Wholesomeness may be extrapolated from one food to another within the same class, taking into consideration the differences, for instance water content, that may exist between them. It may be recommended that this should be substantiated by physical and chemical analysis and evaluation. The homogenates centrigulates, distillates and powders from untreated and irradiated whole foods should be tested. The evaluations may include gas chronotography, mass spectrometry, UV-absorption spectrometry etc. The non-volatile compounds are particularly important.

The limitation imposed on the sources and energies of radiations that may be used in food irradiation is to ensure that the threshold energy for nuclear transformations in any of the important isotopic constituents of food would not be exceeded. It has been established (Becker, 1983) that induced radioactivity, if present, is less than 0.1% of radioactivity normally present in food (viz. K-40. C-14, H-3 and other naturally-occuring nuclides). The three isotopic constituents of food that have the lowest threshold energy, namely the less abundant isotopes H-2, C-13 and O-17, all yield reaction products that are not radioactive.

This should not be taken for granted. Natural radioactivity may vary with location. In Nigeria, Bauchi State has some history of radioactive mineralisation. It has been established that there is a preferential enrichment of Uranium to Thorium and Potassium in the Sokoto basin (Uwah, 1984). The Harmattan winds blow north-east across Niger Republic (a country with Uranium deposits) and then to Nigeria. It may therefore be recommended that the level of radioactivity in various enviromental samples be established as a core-and pre-requisite for the evaluation of Food irradiation.

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APPENDIX I

Main Objective	Means of attaining the Objective	Food	Dosage (KGy)
1) To minimize contamination of food to which the ingredients are added.	Reduction of population of microbes in the special ingredient.	Spices and special food ingredients.	10-30
2) Extension of Storage life	Inhibition of sprouting	Tubers(for example potatoes), bulbs(for example Onions)and other underground organs of plants.	0.05-0.15
3) Improvement of Keeping properties.	Reduction of population of moulds and Yeast and/ or in some instances delay of maturation.	Fruits and certain vegetables	1 - 5
Prevention of loss of stored food or spread of peats	Killing or sexual sterilization of insects.	Cereals,fresh and dried fruits and other products liable to infestation.	0.1-0.5
Prevention of Parasitic diseases transmitted through food(Radicidation)	Destruction of Parasites such as Trichinella Spiralis and Taenia Saginata	Meat and other foods carrying pathogenic parasites.	0.1-0.3

(appendix I contd.)

APPENDIX I (contd)

Main Objective	Means of attaining the Objective	Food	Dosage (KGy)
Prevention of food poisoning (Radicidation).	Destruction of Salmonellae.	Frozen meat and other foods liable to contamination with pathogens.	3 - 10
Extension of refrigerated storage below 3°C (Radicidation).	Reduction of population of microorganisms capable of growth at these temperatures.	Meat, fish and other perishable foods.	0.5-10
Safe long term preservation without refrigerated storage (Radappertization).	Destruction of spoilage organism and many other pathogens present particularly clostridium botulinum.	Meat, fish and other perishable and protein products.	10-50

Source: WHO (1966), Rowley and Bryniolfsson (1980) and Ehlerman (1983).

APPENDIX II

Products (and Their Initial G-Values) from the γ-Radiolysis of Deoxygenated, N_2O - saturated or 4:1 N_2O/O_2-Saturated Aqueous Solutions of D-Glucose at a Dose Rate of 0.18Gy/s at Room Temperature

Product	G-value N_2O	G-value N_2O/O_2
D-Gluconic acid	0.15	0.90
D-arabino-Hexos-2-ulose	0.15	0.90
D-ribo-Hexos-3-ulose	0.10	0.57
D-xylo-Hexos-4-ulose	0.075	0.50
D-xylo-Hexos-5-ulose	0.18	0.60
D-gluco-Hexodialdose	0.22	1.55
2-Deoxy-D-arabino-hexonic acid	0.95	absent
5-Deoxy-D-threo-hexos-4-ulose	} 0.08	absent
5-Deoxy-D-xylo-hexonic acid	} 0.08	absent
2-Deoxy-erythro-hexos-5-ulose	} 0.08	absent
5-Deoxy-D-xylo-hexodialdose	} 0.08	absent
3-Deoxy-D-erythro-hexos-4-ulose	} 0.25	absent
3-Deoxy-D-erythro-hexos-2-ulose	} 0.25	absent
4-Deoxy-L-threo-hexos-5-ulose	} 0.25	absent
6-Deoxy-D-xylo-hexos-5-ulose	0.05	absent
2-Deoxy-D-erythro-hexos-3-ulose	a	absent
4-Deoxy-D-threa-hexos-3-ulose	a	absent
D-Arabinose	0.01	} 0.10
D-Arabinonic acid	absent	} 0.10

APPENDIX II (contd)

Product	N_2O	N_2O/O_2
D-Ribose	0.005	absent
D-Xylose	0.005	0.08
xylo-Pentodialdose	absent	
2-Deoxy-D-erythro-pentose	0.04	absent
D-Erythrose	0.01	0.02
D-Erythronic acid	absent	
L-Threose	0.003	absent
L-threo-Tetrodialdose	absent	0.20
3-Deoxytetrulose	0.02	absent
1,3-Dihydroxy-2-propanone	0.03	absent
D-Glyceraldehyde and glyceric acid	absent	0.13
Glyoxal	b	0.11
Glyoxylic acid-glycolic acid	b	0.4
Formaldehyde	b	0.12
Formic acid	b	0.6
D-Glucose consumption	5.6	5.6

a Products identified (no- G-values given) in Namiki et al (1973). They are expected to be included in the G-values of the other deoxyhexosuloses given in the Table.

b Not determined, probably absent

Source: Van Sonntag (1980).

APPENDIX III

PRINCIPLES OF THE APPLIED STATISTICS

I. Inter-experimental errors.

The semi continuous cultures were maintained for 10-15 days, growth, survival and recombinants or mutants were scored daily. Each experiment may be repeated atleast three times. The mean and standard error of a parameter observed on the first-day off each of repeated set of experiments is determined. The same was done for the second day, third day etc of the set of experiments. If for the measurement of a particular biological-end point $X_{i,j}$ is observed in experiment i on incubation j then of the mean of the parameter determined for the particular set of experiment on incubation day j is $\bar{X}_j = \sum_{i=1}^{N} X_{i,j}$. The standard error, $S.e_j = \sqrt{\frac{\sum_{i=1}^{N}(X_{i,j} - \bar{X}_j)^2}{N(N-1)}}$

Usually N is 3

This procedure may indicate consistent accumulation or reduction of biological activity as a function of incubation time.

Microscopic counting may not be precise, cells may stick to the sides of a test tube and suspensions may not be homogenous inspite of the usual rigorous shaking. Normally, at least two samples are carefully taken from each culture (two cultures are made for each treated and control). These are counted and averaged. Even with these precautions, an error of 10% may be allowed. Such intra-experimental errors may amplify the inter-experimental errors.

II. The student's t-test.

If $\bar{X}$ and S^2 are the mean and variance, respectively, of a random sample of size n taken from a normal population having the mean u and unknown variance σ^2, then $t = \frac{\bar{X} - \mu}{S} \sqrt{n}$ is a value of a random variable T having t distribution with n-1 degrees of freedom.

The t-test could be applied to determine the difference between two means for small size samples with unknown standard deviations. For two samples of means $\bar{X}_1$ and $\bar{X}_2$ the value of the random difference between the samples is given by the difference between the means divided by the standard error of the differences. The calculated value is them compared with tabulated t-distribution at n-1 degress of freedom, where n is the size of each of the samples.

In the following appendices the null hypothesises Ho of equal means: $U_c = U_t$ and $U_c = U_f$ are tested against the alternative hypothesis of unequal means. U_c, U_t and U_f are the means of the measured biological -end-points in the control, 10KGy and 50KGy treated media respectively. The two tailed tests were done at 5% level of significance of rejecting the null hypothesis and n-1 degress of freedom.

III. The F-test.

This test the equality of variances. If S_1^2 and S_2^2 are the variances of independent random samples of size n_1 and n_2 taken from normal populations with variances σ_1^2 and σ_2^2, respectively, then $f = (S_1^2 / \sigma_1^2) / (S_2^2 / \sigma_2^2)$ is a value of a random variable F having f distribution with n_1-1 and n_2-1 degrees of freedom.

We are interested in determining if the incubation length could also have an effect. The two -way classification was used to analyse both the treatments and incubation crederia. In this the variances of all the observations are split into parts each of which measures variability attributed to some specific source. Basically, the total sum of squares of the data is split into three components: the sum squares for the rows and column , and the error sum of squares. The equality of both the row means and the column means are tested.

In the following appendices the null hypothesis of equality of (1) rows means (2) Column means are tested against the alternative hypothesis of unequal means, at 5% level of significance of rejecting the null hypothesis. The degrees of freedom are the number of rows less one and the number of column less one . Rows refers to the incubation days and columns refer to growth, mutants per survivor or recombinants/survivor, as the case may be in the control, 10KGy and 50KGy treated media.

APPENDIX IV

RESULTS OF STATISTICAL ANALYSIS OF HAPLOID CELLS C420-3B GROWTH

1. The t-test

	Growth in control compared to 50KGy treated media	Growth in control compared to 10KGy treated media
Difference between means	1.19	2.08
Standard error	0.63	1.19
Computed t	1.89	1.75

From statistical tables t at 5% and 9 degrees of freedom is 2.262. Null hypothesis of equality of means may be accepted.

2. The ANOVA

Source of variation	Sum of squares	Degrees of freedom	Mean square	Computed f
Row means	95.60	9	10.62	2.54
Column means	30.23	2	15.12	3.63
error	75.16	18	4.17	
total	200.99	29		

From statistical tables, $F_{0.05}$ (9,18)= 2.46 and $F_{0.05}$ (2,18)=3.55
But $F_{0.01}$ (9,18) = 3.60 and $F_{0.01}$ (2,18)= 6.01

There are differences of the growth in the three media and in the day's of incubation at 5% level of significance, but not at 10% level of significance. The differences may be ignored in that the stronger two-tailed t-test indicates no difference.

APPENDIX V

RESULTS OF STATISTICAL ANALYSIS OF DIPLOID CELLS CD 364 GROWTH.

1. The t-test

	Growth in control compared to 50KGy treated media	Growth in Control compared to 10KGy treated media
Difference between means	−0.22	−0.98
Standard error	0.42	0.60
Computed t	−0.52	−1.63

From statistical tables t at 5% and 9 degrees of freedom is 2.262. The hypothesis of equality of means may be accepted. These results indicate that growth in the treated media may better than in control.

2. The ANOVA

Source of variation	Sum of squares	Degrees of freedom	Mean square	Computed f
Row means	6.88	9	0.76	0.44
Column means	6.86	2	3.43	2.01
error	30.71	18	1.71	
Total	44.45	29		

$F_{0.05}\ (9,18) = 2.46$ and $F_{0.05}\ (2,18) = 3.55$.

The null hypothesis of equality of means and variances may be accepted at 5% level of significance.

RESULTS OF STATISTICAL ANALYSIS OF
MUTANTS PER SURVIVOR

1. The t-test

	Growth in control compared to 50KGy treated media	Growth in control compared to 10KGy treated media
Difference between means	0.31	0.32
Standard error	0.51	0.50
Computed t	0.61	0.64

From statistical tables t at 5% and 10 degrees of freedom is 2.228. Hence the hypothesis of equality in growth may be accepted.

2. The ANOVA

Source of variation	Sum of squares	Degrees of freedom	Mean square	Computed f
Row means	14.85	10	1.49	1.15
Column means	0.71	2	0.36	0.28
error	26.15	20	1.30	
Total	41.71	32		

From Statistical tables, $F_{0.05}(10,20) = 2.35$ and $F_{0.05}(2,20)=3.49$.

The null hypothesis of equality of variances and means may be accepted.

APPENDIX VII

RESULTS OF STATISTICAL ANALYSIS OF RECOMBINANTS PER SURVIVOR

1. The t-test

	Growth in control compared to 50KGy treated media	Growth in control compared to 10KGy treated media
Difference between means	-0.87	0.45
Standard error	0.78	0.74
Computed t	-1.12	0.59

From statistical tables t at 5% and 10 degrees of freedom is 2.228. The null hypothesis of equality of means may be accepted at 5% level of significance. The growth in 50KGy treated media may be better than in the control.

2. The ANOVA

Source of Variation	Sum of squares	Degrees of freedom	Mean square	Computed f
Row mean	102.92	10	10.29	3.67
Column mean	9.85	2	4.93	1.76
Error	36.05	20	2.80	
Total	148.82	32		

From statistical tables, $F_{0.05}(10,20) = 2.35$ and $F_{0.05}(2,20)=3.49$. The null hypothesis of equality of recombinants per survivor may accepted but the recombinants per survivor varies with the incubation days. This may be of little significance.