RADIATION DAMAGE IN MAMMALIAN DNA

SEDIMENTATION STUDIES OF IONIZING RADIATION DAMAGE IN MAMMALIAN DNA

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SCOPE AND CONTENTS:

The production of single strand breaks by ionizing radiation (γ-rays) in mammalian cell DNA was studied by a modified method of alkaline sucrose gradients. The effect of molecular oxygen and 2,4-dinitrophenol on the yield of single strand breaks was examined and the results are reported in this thesis. Also, subsequent rejoining of single strand breaks was studied.

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1. INTRODUCTION

1.1. RADIATION

The interaction of radiation with matter has been of great interest in the past as well as at the present time. Living systems are continuously exposed to various forms of radiation: ultraviolet (UV) radiation from the sun, ionizing radiation from cosmic rays, radioactive decay of unstable atoms, etc.

Since their discovery nearly 80 years ago the use of radiation sources in medical and industrial applications has proliferated rapidly. At present approximately 50% of all patients with tumors are treated with ionizing radiation. Though half of the treated patients are cured successfully, there is still room for improvement which might be facilitated by knowledge of the effects of the radiation particularly at the molecular level.

With regard to molecular effects, radiations are broadly divided into two types:

1) ionizing radiation
2) non-ionizing radiation

When ionizing radiation (x-rays, γ-rays, fast moving charged or uncharged particles) is absorbed in target atoms, it causes the removal of electrons from the target atoms, producing along its path

ionized atoms and free electrons. Non-ionizing radiations are those for which the energy transferred to target atoms in individual interactions is not sufficient to produce ionization (e.g.-ultraviolet (UV) radiation). Both types of radiation have been shown to be capable of killing living cells.

Biological damage arising from ionizing or non-ionizing radiation can be studied at several levels: in whole animals or plants, in cultured cells or in single-cell organisms (i.e. bacteria).

Many end points have been used: gross survival, cytological effects, membrane function, macromolecular synthesis, etc. Techniques also exist which permit the study of molecular damage to proteins and nucleic acids both in vitro, in isolated macromolecular fractions and, more recently, in vivo, in living cells.

In this work I will present the results of studies on molecular damage produced by ionizing radiation on DNA molecules in cultured mammalian cells and I shall compare some of the results to those of survival measurements on the same cells.

For normally dividing cells, the question of whether a cell survives or not after exposure to a certain dose of radiation is defined by its ability to divide indefinitely after the exposure. This is actually measured by irradiating cells and allowing them to proliferate under the most favourable conditions until many divisions have occurred. All cells which maintained their dividing ability after the irradiation, will form with their progeny a colony of cells which can be detected. The number of colonies divided by the initial number of cells plated

represents the surviving fraction S.

1.2. CELLS AND DNA MOLECULES

In most living cells, the genetic information is contained in DNA (deoxyribonucleic acid) molecules. This is true for eukaryotic cells where a nucleus exists and contains the cellular chromosomes, for prokaryotic cells where the chromosomes are not confined to a nucleus, and for most viruses. In some viruses, this function is taken over by RNA (ribonucleic acid). DNA is a major constituent of prokaryotic or eukaryotic chromosomes since it serves as a primary source of information for all molecular synthesis in the cell, for the control of cellular growth and cell division, for the differentiation and specialization of the cell. For normal life and reproduction of cells there must be a continuous flow of information from generation to generation, thus the DNA molecules must be replicated in each generation.

If the DNA is damaged and not repaired, some of this information may be lost and death of the cell may result.

The DNA molecule consists of two linear polymers coiled together to form a double helical structure. Each single stranded polymer is basically composed of alternating sugar-phosphate pairs joined together to form a backbone; to each deoxyribose sugar is attached one of four possible bases: adenosine, cytosine, guanine, and thymine. Each base is involved in a specific hydrogen bonding with a complementary base (e.g. thymine - adenosine) from another single stranded DNA molecule

and thus the two single stranded polymers are joined together in a complementary fashion.

If the hydrogen bonding between all of the bases is broken (by means of high temperature, very high pH, etc.) the two single strands become separated.

In most known bacterial and animal viruses, DNA exists as one single continuous molecule. In general, in viruses, it is in the double stranded form with a few exceptions where it exists as a single stranded molecule. The molecular weight* of viral DNA varies greatly. Polyoma, an animal virus, for example has a molecular weight of $\sim 3 \times 10^6$ daltons, while the bacteriophage T4 is 1.34×10^8 daltons and PBS-1 is $\sim 2 \times 10^8$ daltons.

In prokaryotic cells, for example in bacteria, it is believed that the genome is one single piece of double stranded DNA of molecular weight $\sim 2 \times 10^9$ daltons as based on autoradiographic measurements of chromosomal length in Escherichia coli cells (Cairns 1963). The single stranded molecule then would be one half of that value, $\sim 1 \times 10^9$ daltons. There is some evidence however that the DNA molecule in E. coli is composed of several smaller units which, when in the single stranded form have an average molecular weight of 2.2×10^8 daltons (McGrath and Williams 1966 and 1967). These results of McGrath and Williams were obtained by using the technique of alkaline sucrose gradients which is discussed in greater detail in sections 1.3. and 2.3.

In PPLO M. laidlaw B (also a prokaryotic organism) McGrath and Williams (1967) reported values of 4×10^8 daltons for the molecular

* The term "molecular weight" as used throughout this thesis denotes the mass of a molecule and is expressed in daltons where 1 dalton=1.663×10^{-27} kg.

weight of single stranded DNA using again alkaline sucrose gradients.

In eukaryotic cells where most of the DNA is localized in the cell nucleus the situation is even less clear. If we take the average amount of DNA in mammalian cells to be 0.86×10^{-11} gm in the G1 stage of the cell cycle, distributed then in a cell containing 50 chromosomes, there would be an average of 10^{11} daltons per molecule if each chromosome contains only one double stranded DNA molecule. The single stranded molecule of that duplex would be half of that, that is $\sim 5 \times 10^{10}$ daltons.

Autoradiographic studies in mammalian cells (Huberman and Riggs 1966, Sasaki and Norman 1966) showed that the DNA is replicated in segments as large as $\sim 3.5 \times 10^{9}$ daltons for double stranded DNA; however, Huberman and Riggs (1968) and others (Cairns 1966) showed that these long molecules are really composed of shorter molecules linked in a linear array. For example, Cairns (1966) estimated that in HeLa cells there must be at least 100 such units in a 3 cm long chromosome ($\sim 6 \times 10^{10}$ daltons of DNA) which gives 6×10^{8} daltons per double stranded molecule and 3×10^{8} daltons per single stranded molecule. The size of these units was defined by the amount of DNA which was found between successive replicating sites (points at which replication of DNA is independently initiated) along the chromosome and hence the units were called replicons. Similar studies on mammalian cells were carried out by several other authors (Painter, Jermany and Rasmussen 1966, Taylor 1968, Okada 1968, Coleman and Okada 1968), and the results confirmed the general idea that the DNA molecules in chromosomes are composed of several linearly linked smaller replicons. However, the

replicon size obtained from these studies varied from $\sim 5 \times 10^9$ daltons to only 4×10^6 daltons for double stranded DNA molecules. It should be noted though that these special subunits (replicons) might be present only at the time of replication of the chromosome. They may be subsequently linked to form much longer DNA molecules via the normal phosphate sugar bond which would be indistinguishable from the rest of the internucleotide bonds.

In eukaryotic cells the technique of alkaline sucrose gradients can also be employed to study the size of DNA molecules. In 1967 it was reported by McGrath and Williams that the DNA of slime mold (Physarum polycephalum) has an average single stranded molecular weight of 4×10^7 daltons, and simultaneously another group (Lett, Caldwell, Dean and Alexander 1967) published similar studies on mammalian cells of the L5178Y line. Lett et al. reported that they were unable to band the DNA molecules in the gradients, unless the cells were first exposed to some minimal dose of radiation; without this, the DNA sedimented as a complex to the bottom of the centrifuge tube. It was concluded that the single stranded DNA molecules of unirradiated cells were larger than 5×10^8 daltons and these authors suggested that the DNA in the chromosome might be present in only one piece. Since that time there have been numerous reports describing a whole spectrum of values for the single stranded molecular weight of mammalian DNA obtained by the technique of alkaline sucrose gradients. Some authors (Moroson and Furlan 1970, Lehman and Ormerod 1970b, McBurney, Graham, Whitmore 1971 and 1972, McBurney and Whitmore 1972),

presented evidence that single stranded DNA molecules may be as large as $2\text{-}9 \times 10^9$ daltons. Using the same technique, others have reported values from $2.5 - 5 \times 10^8$ daltons for single stranded DNA (Lohman 1968, Elkind and Kamper 1970, Lett, Klucis and Sun 1970, Elkind 1971, Palcic and Skarsgard 1972a), while lower values in the range of 1×10^8 daltons or smaller were obtained by two additional groups (Humphrey, Steward and Sedita, 1968, Veatch and Okada 1969).

1.3. THE METHOD OF ALKALINE SUCROSE GRADIENTS

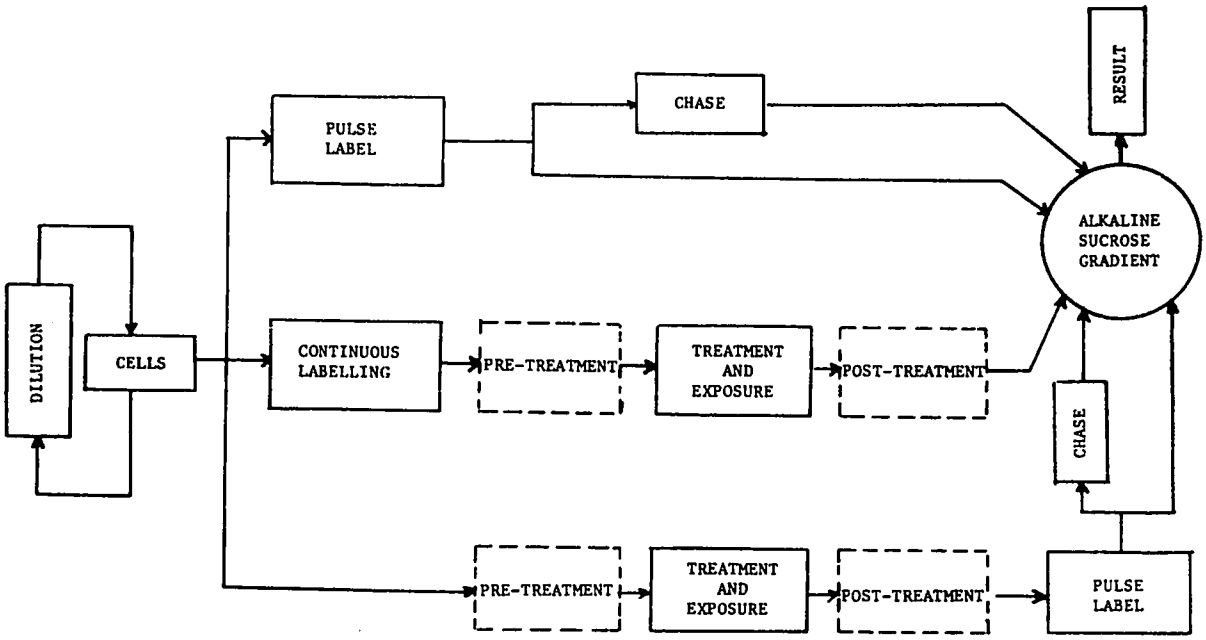
The analysis of whole cell DNA by alkaline sucrose gradient techniques was first introduced by McGrath and Williams (1966), where intact bacterial protoplasts were lysed on the top of a prepared alkaline sucrose gradient (pH = 12, 5 - 20% sucrose). High alkalinity breaks cell walls releasing the cytoplasmic material into solution. RNA molecules are degraded into nucleotides at this high pH, proteins are denatured and DNA molecules, which become free of associated proteins, emerge single stranded since the hydrogen bonding is broken under this highly alkaline condition. If one isolates DNA molecules from cells by standard procedures, the largest molecules which can be isolated without severe damage due to various shearing forces are of the order of $1\text{-}2 \times 10^7$ daltons. When cells are lysed directly on the top of the gradients, the DNA molecules <u>are not</u> handled at all after they are released from their natural milieu and hence the method gives

potentially the largest possible molecules to be assayed.

Usually, the amount of DNA used on the top of a gradient is very small, $\sim$ 0.1 μg, and the only way to detect such a small amount of DNA is to label with a radioactive material which is specifically incorporated into DNA, for example H^3 or C^{14} thymidine may be used.

A typical experimental procedure then consists of preparing cells with radioisotopically labelled DNA and after the cells are treated (with radiation, etc.) they are gently delivered into the lysing solution on the top of an alkaline sucrose gradient. After the cells are lysed on the top for a predetermined time and temperature, the released single stranded DNA molecules are spun in an ultracentrifuge with appropriate centrifugal speed ω and time t. The distance sedimented by the molecules is among other things a function of the molecular weight. After centrifugation, the gradients are fractionated and the radioactivity in each fraction is assayed, which in turn represents the amount of DNA present in that fraction. From the profile of radioactivity in the tube the distribution of molecules in the tube can be calculated and the average molecular weight of the distribution can also be estimated (see also section 2.3.)

A schematic representation of some of the possibilities of the technique of alkaline sucrose gradients can be seen below:



So for example, when the cells are pulse labelled (the upper pathway), steps in the DNA synthesis can be studied. With continuous labelling (middle pathway), either the size of the DNA molecules in different types of cells can be examined or if the cells are exposed to radiation the integrity of the DNA molecules can be checked after such an exposure.

One can also study the effect of combining irradiation with other treatments such as altered temperatures, or exposure to sensitizing agents, protecting agents or metabolic inhibitors. The exposure to chemical agents, for example, can be concurrent with radiation exposure (treatment) or it can take place only before irradiation (pre-treatment) or only after irradiation (post-treatment). As well, various combinations of these treatments are possible. In the work reported in this thesis I have studied the effect of oxygen and 2,4-dinitrophenol (treatment and/or pre-treatment) on the production by ionizing radiation of DNA

single strand breaks in mammalian cells.

The third pathway describes another type of experiment where unlabelled cells are given some particular irradiation and chemical treatment scheme and are then pulse labelled with specific radioactive precursors of DNA synthesis. This allows one to study the newly synthesized DNA which may depend on the state of the primer DNA molecule.

1.4. IONIZING RADIATION DAMAGE OF DNA MOLECULES IN CELLS

Damage produced in DNA by ionizing radiation can be broadly divided into three categories: single strand breaks, double strand breaks, and base or sugar damage. A single strand break occurs in DNA molecules when radiation causes a scission of the phosphate-sugar backbone of one strand only. Double strand breaks appear when both strands are damaged so closely together that the hydrogen bonding between bases on both strands can not hold the strands together. Very little is known about the molecular damage produced by ionizing radiation in the base and deoxyribose when DNA molecules are irradiated while they are still in living cells. Studies of irradiation products in isolated DNA irradiated in aqueous solutions gave some insight into the events which might take place in the DNA molecule in a cell (Kapp and Smith 1970, Ullrich and Hagen 1971).

The method of alkaline sucrose gradients (sections 1.3. and 2.3.) provides an assay for detecting the single strand breaks without the necessity of isolating the DNA from the cells after irradiation. It should be noted that what one measures with this technique is not only those breaks which are produced directly in the phosphate-sugar backbone of the DNA but also those bonds in the backbone which become alkali labile as a consequence of the radiation exposure; together these two types of damage constitute what is generally referred to as single strand breaks. Also, a fraction of the single strand breaks must be due to double strand breaks since the method cannot distinguish between them.

Many workers have investigated the appearance of single strand breaks in various organisms. In virus the DNA molecules are rather small and hence they can be isolated prior to being denatured on the top of an alkaline sucrose gradient without extensive shear due to the isolating procedures. A typical example of such studies in bacterial viruses is that of Freifelder (1966). The same year McGrath and Williams introduced a method whereby whole bacterial protoplasts were lysed on the top of prepared alkaline sucrose gradients. The idea was to prevent any extensive shear of the DNA molecules, once they were released from the cell, by handling them before centrifugation. Many workers (Kaplan 1966, Dean et al. 1969, Lehnert and Moroson 1970, Dugle and Dugle 1971, and others) have used the technique in bacterial cells, and it has also been applied to cells of higher organisms. The production of single strand breaks in

mammalian cells was studied in many laboratories including our own (Lett et al. 1967, Humphrey et al. 1968, Lohman 1968, Terasima and Tsuboi 1969, Matsudaira, Nakagawa and Hishizawa 1969, Dean, Ormerod, Serianni and Alexander 1969, Sawada and Okada 1970, Elkind and Kamper 1970, Moroson and Furlan 1970, Elkind 1971, McBurney et al. 1971, Palcic and Skarsgard 1971, 1972 a and b and many others).

There have also been investigations of double strand scission in DNA molecules from a variety of cell types. For example, using E. coli cells, Kaplan (1966) reported that double strand scissions are principally responsible for the lethal effect of ionizing radiation in those cells. Kitayama and Matsuyama (1971) reported one double strand scission per 15-20 single strand breaks in M. radiodurans. This value is in good agreement with that found in the same organism by Burrell, Feldschreiber and Dean (1971) and also in mammalian cells by Corry and Cole (1968) who reported a ratio of 1 to 10. Lehman and Ormerod (1970b) reported only 1 double strand break for every 70 single strand breaks in comparable studies in mammalian cells.

Dalrymple, Sanders and Baker (1968) proposed that single strand breaks can be caused either directly by radiation or indirectly. They suggested an indirect mechanism by which radiation produces bond abnormalities which are later converted to strand breaks by the system of nucleolytic enzymes. They showed that immediately after irradiation of mammalian cells there is a rapid increase in the number of free 5' phosphoryl termini (Dalrymple, Sanders, Moss, Baker and Wilkinson 1969a and b, Dalrymple, Baker, Sanders, Moss, Nash and Wilkinson 1970) as

measured in the extracted mammalian DNA. This group also demonstrated that if the cells were either held at 0°C after irradiation or treated with 2,4-dinitrophenol prior to and during irradiation, no rapid increase in the number of free 5' phosphoryl termini occurred. If this increase in the number of free 5' phosphoryl termini actually represents new strand scissions, then these observations support the hypothesis that single strand breaks result primarily from the action of nucleolytic enzymes rather than from the direct action of ionizing particles. However, it is possible that the appearance of new 5' phosphoryl termini represents nothing more than a rearrangement of already existing breaks, for example between the 3' and 4' carbon atoms in the deoxyribose (Kapp and Smith 1970). In this case the nucleolytic enzymes need not be involved in producing the scission at all but only in unmasking the 5' end.

The technique of alkaline sucrose gradients offers a more direct method of assaying for single strand breaks. It was used in the present investigations to show that single strand breaks are not the result of enzymatic action following irradiation but rather they are due to a direct or indirect physico-chemical effect of radiation (section 3.2. and 4.3.1.).

1.5. OXYGEN EFFECT ON SINGLE STRAND BREAK PRODUCTION

It is a well known fact that when viral, bacterial or eukaryotic cells are irradiated in the presence of molecular oxygen the survival

at a certain dose of radiation is considerably lower than it is when the same dose is delivered in the absence of oxygen. The oxygen enhancement ratio (OER) is about 3 for mammalian cells; that is, to get the same fraction of cells killed, anoxic cells require 3 times as large a dose as do aerobic cells.

When the oxygen enhancement ratio was studied on single strand break production in viral DNA it was initially reported (Freifelder 1966) that the presence or absence of oxygen did not affect the production of breaks. However, later studies (Boyce and Tepper 1968, Van der Schans and Blok 1970, Johansen, Gurvin and Rupp 1971) showed that there was an oxygen effect on single strand break preparation in viral cells.

Similarly, when isolated DNA molecules were irradiated in the dry state some investigators reported very little, if any, oxygen enhancement (Neary, Simpson-Gildemeister and Peacocke 1970) while others (Lücke-Huhle, Braun and Hagen 1970) did find an oxygen effect in the same system; the OER for the production of single strand breaks in dry DNA was found to be approximately 2.

In bacterial cells there are also conflicting results. Lett et al. (1967) reported an OER of $\sim$ 4 for single strand breaks in M. radiodurans; however in their second paper (Dean et al. 1969) they concluded that no oxygen enhancement exists in M. radiodurans and that the OER observed before was really due to rejoining processes which are going on even at 0°C after irradiation. They claimed that initially

the same number of breaks is produced under aerobic or anoxic conditions, but that some breaks produced in the absence of oxygen are rapidly rejoined at 0°C before the cells are lysed on the top of the gradient. They suggest that when the breaks are produced in the presence of oxygen they are not rejoined at 0°C. This hypothesis was supported by the observation of Dean et al. that when the rejoining process at 0°C was inhibited by addition of 2×10^{-2} M ethylenediaminetetraacetate (EDTA) to the cell suspension there was no oxygen effect. A more thorough study in bacterial cells was done by Lehnert and Moroson (1971) in E. coli. They, too, observed that these cells have a rejoining capacity at 0°C and that this process can be inhibited by the addition of 2×10^{-2} M EDTA. However, in their experiments, even when EDTA was added, an OER of 3 was observed.

Lett et al. (1967) first reported an OER of 2.2 for mammalian cells. In a later paper, however (Dean et al. 1969), this claim was withdrawn on the basis of the shapes of the sedimentation profiles. They claimed that although the profiles for aerobically irradiated cells represented random distributions, anoxic irradiation yielded distributions which were frequently not random. When their molecular weight calculations were based only on random profiles, they claimed that the OER was 1.0.

In our work, we re-examined the effect of oxygen in mammalian cells using 3 different cell lines and two different alkaline sucrose gradients techniques. These data clearly show an OER of approximately 3.0 in mammalian cells (Palcic and Skarsgard 1972a).

1.6. REPAIR OF DAMAGED DNA MOLECULES

In general, when cells are exposed to radiation, alkylating agents, etc., they have the capacity to repair the damaged sites in DNA. Perhaps the best known mechanism is that for the repair of damage produced by UV radiation in the wavelength range 280mμ. The main damage in DNA molecules when cells are exposed to UV light is the formation of pyrimidine dimers, in particular thymine dimers, where two adjacent thymine bases on the same DNA strand become covalently linked (Setlow and Setlow 1962, Setlow 1966).

It has been shown that there are at least 2 mechanism by which these dimers can be eliminated from the DNA. If dimer-containing DNA is subsequently irradiated with UV light of shorter wavelength (240 mμ) most of these dimers can be split (photo-reactivation). Of greater interest in this dissertation is the enzymic mechanism (dark repair) by which thymine dimers can be removed from the DNA molecules. This has been shown to operate in nearly all prokaryotic or eukaryotic cells examined (for review articles see Howard-Flanders and Boyce 1966, Hanawalt and Haynes 1967, Rauth 1970). Briefly, an endonuclease enzyme "scans" the DNA molecule, recognizes the thymine dimer and cuts the phosphate-sugar backbone near the defect. A second enzyme, an exonuclease, then removes the thymine dimer as well as some neighbouring nucleotides, while a third enzyme, DNA polymerase, resynthesizes this region by inserting nucleotides complementary to the opposite strand, which serves as a template; this DNA synthesis is also called repair replication.

Finally, the fourth enzyme, a ligase, joins together the phosphate-sugar backbone after the last nucleotide has been inserted. In mammalian cells this repair replication can occur throughout the cell cycle (not only in S phase) and hence is called unscheduled DNA synthesis (Painter and Cleaver 1969).

Unscheduled DNA synthesis is also observed when mammalian cells are exposed to ionizing radiation (Painter and Cleaver 1967, Spiegler and Norman 1969 and 1970, Brent and Wheatley 1971).

Another indication of repair was reported (Dalrymple, Sanders, Moss, Baker and Wilkinson 1969 a and b, Dalrymple, Baker, Sanders, Moss, Nash and Wilkinson 1970) where the change in the number of free 5' phosphoryl termini was measured. Following irradiation of mammalian cells, Dalrymple et al. observed a rapid increase in the number of free 5' phosphoryl termini (0.5 - 5 minutes after the irradiation). If the cells were further incubated at 37°C, the number of termini decreased and after prolonged incubation approached the control level. The appearance and disappearance of free 5' termini could be inhibited by low temperature or by treatment of the cells with DNP.

Using the method of alkaline sucrose gradients McGrath and Williams (1966) demonstrated rejoining of single strand breaks in E. coli during incubation at 37°C after exposure to ionizing radiation. This effect has been observed by many workers not only in bacterial but also in mammalian systems (Lett et al. 1967, Lohman 1968, Humphrey,

Steward and Sedita 1968, Terasima and Tsuboi 1968, Matsudaira et al. 1969, Elkind and Kamper 1970, Kleijer, Lohman, Mulder and Bootsma 1970, Sawada and Okada 1970, Elkind 1971, McBurney, Graham and Whitmore 1972, and others) and it is found to take place throughout the cell cycle. This process can be inhibited by agents like 10^{-2} M EDTA in bacterial cells (Dean et al. 1969, Lehnert and Moroson 1971) and by 1×10^{-4} M DNP in mammalian cells (Moss et al. 1971, Palcic and Skarsgard 1972b). The rejoining process is temperature dependent and is completely stopped at 0°C (Sawada and Okada 1970, Dean et al. 1969, Elkind and Kamper 1970, Palcic and Skarsgard 1972a).

It was suggested that in M. radiodurans and E. coli, at least, there is a difference in the rate of rejoining of strand breaks depending on whether or not they are produced in the presence of oxygen (Dean et al. 1969, Lehnert and Moroson 1971). We examined this possibility in mammalian cells and the results are reported in this work.

It is believed that in general, double strand scissions cannot be repaired. In mammalian cells, this was shown by Sawada and Okada (1970) and Lehman and Ormerod (1970b). A similar observation was reported for a bacterial system by Kaplan (1966) who employed E. coli cells. However, the bacterium M. radiodurans, which is extremely insensitive to ionizing radiation (by survival measurements) was shown to be capable of rejoining double strand breaks (Kitayama and Matsuyama 1968 and 1971).

2. MATERIALS AND METHODS

2.1. MAMMALIAN CELLS

2.1.1. Culture Technique

Mouse fibroblast cells of the L-60 line (obtained from G. F. Whitmore, University of Toronto) were grown in glass spinner flasks in Minimal Essential Medium (MEM F-13, Gibco) supplemented with 10% undialysed fetal calf serum (FCS, Gibco). The cells were maintained at concentrations ranging between $1 \times 10^5 - 4 \times 10^5$ cells/ml, dilutions were made daily. The average doubling time was 18 hours.

Chinese hamster cells of the $CH2B_2$ cell line were grown as monolayers in glass flasks in an atmosphere of 95% air, 5% CO_2. Medium 1066 was used (Parker, Castor, McCulloch 1957) supplemented with 10% newborn agamma calf serum (North American Biological Inc.) and the cells were subcultured twice per week. The cells were detached from the glass surface using 0.1% trypsin (Bacto-Trypsin, Difco). The doubling time was 12-14 hours.

Human skin fibroblasts of the AN strain obtained from Dr. S. Goldstein, McMaster University, Hamilton (Goldstein 1971) were grown as monolayers in plastic culture flasks (Falcon Plastics) in Minimal Essential Medium (MEM F-15, Gibco) supplemented with 14.3% undialysed fetal calf serum, 2 mg/ml glucose and 1.1×10^{-3} M (final

concentration) of sodium pyruvate (British Drug House, Toronto). The cells were subcultured once or twice per week using 0.25% trypsin.

2.1.2. Labelling Cells with H^3 TdR, C^{14}-L-leucine, and H^3-uridine

L-60 cells were labelled with molecules containing radioactive isotopes for various purposes. In general, sedimentation studies required uniformly labelled DNA molecules and occasionally labelled proteins. When macromolecular synthesis was studied the cells were pulse labelled with a precursor which was specifically incorporated into a particular type of macromolecule: i.e. H^3-uridine into RNA.

For uniform labelling of the DNA molecules, H^3-thymidine (H^3-TdR, specific activity 6.7 Ci/mM, New England Nuclear Corporation, or H^3-TdR, specific activity 15.5 Ci/mM, Amersham-Searle) was used. For labelling purposes, 10 cm plastic petri dishes (Falcon Plastics) were seeded with 5×10^6 cells which were allowed to attach during 1 hour incubation at 37°C. The medium was then replaced with 20 ml of radioactive medium, containing 2μCi/ml of H^3 TdR. These cultures were then incubated at 37°C for 24 hours in an atmosphere 95% air, 5% CO_2. At the end of the labelling period some of the cells were in S phase and many of the DNA molecules were in the initial stages of replication. These molecules would appear as low molecular weight DNA unless their synthesis were allowed to go to completion. Thus the labelling was terminated by an additional 1-2 hour incubation in non-radioactive medium in order to complete the synthesis of those DNA molecules which started their synthesis while radioactive precursor

was still present.

In some experiments where the effect on the DNA of the amount of incorporated H^3-TdR was studied, activities of 0.15 µCi/ml, 1.0 µCi/ml, and 4.0 µCi/ml were used, the rest of the procedure was the same as already described. For the studies on rejoining of breaks, a somewhat different procedure was employed. Plastic tissue culture flasks (250 ml, Falcon Plastics) were seeded with 8×10^6 cells and growth medium was added to a total volume of 50 ml followed by the addition of H^3-TdR, so that the final activity was 0.2 µCi/ml. The flasks were then put into the incubator for 24 hours and the rest of the procedure was the same as for the other experiments.

Radioactive labelling was also used to monitor the rate of DNA and RNA synthesis in cells treated with 2,4-dinitrophenol (see 2.1.3.).

In several experiments the amount of DNA-associated protein was measured. In this case, cells were labelled with C^{14}-L-leucine alone or they were double labelled with C^{14}-L-leucine and H^3-TdR. C^{14}-L-leucine (specific activity 0.24 Ci/mM, New England Nuclear Corp.) was added to medium 1066 lacking L-leucine.
The C^{14}-L-leucine activity was 0.2 µCi/ml. The amount of H^3-TdR in double labelling experiments was 2µCi/ml.

In order to measure DNA synthesis 10 µCi/ml of H^3-TdR was added to a suspension of unlabelled cells (1×10^6 cells/ml) and the cells were incubated at 37°C for 30 minutes. At that time the label

was removed, the cells were washed once in growth medium, once in physiological saline (PBS) and then they were lysed in a 1% sodium dodecyl sulphate (SDS) solution.

For RNA synthesis, 10 μCi/ml of H^3-uridine (specific activity 29 Ci/mM, Amersham-Searle) was used, and for protein synthesis 10 μCi/ml of C^{14}-L-leucine (specific activity 0.24 Ci/mM, New England Nuclear Corporation) was used. The rest of the procedure was the same as described for the measurement of DNA synthesis.

$CH2B_2$ cells are thymidine requiring cells, hence one must supply the minimum amount of thymidine to support their growth. Cells in log phase were trypsinized as described in 2.1.1. 5×10^6 cells were seeded into plastic tissue flasks in the presence of 15 ml growth medium. The cells were incubated at 37°C for 24 hours, the medium was then replaced with 1 ml of complete 1066 medium and 49 ml of 1066 medium from which thymidine and coenzyme were ommitted. H^3-TdR was added to a final activity of 0.25 μCi/ml. The cells were then incubated for an additional 24 hours under conditions identical to those described for L-60 cells. 1 hour prior to the time that the labelled cells were collected, the medium was replaced with non-radioactive growth medium.

AN cells were grown as described in 2.1.1. Two day old cultures ($\sim 5 \times 10^6$ cells) were labelled with H^3-TdR at an activity of 2μCi/ml in fresh growth medium. The labelling period was extended to 48 hours in order to adequately label these slower growing cells. The rest of the procedure was essentially identical to that described

for L-60 cells.

2.1.3. 2,4-Dinitrophenol Treatment of Cells

In order to study the effect of a metabolic inhibitor on single strand break production by ionizing radiation in mammalian cells, the cells were treated with either 1×10^{-4} M DNP (2,4-dinitrophenol) or 5×10^{-4} M DNP. DNP is an inhibitor of oxidative phosphorylation and its presence causes a depletion of the intracellular pool of ATP (adenosine triphosphate). This, in turn slows down and eventually stops many metabolic processes in the cells.

For measurements of macromolecular synthesis (DNA, RNA) suspensions of unlabelled cells were prepared in normal growth medium washed once with PBS to remove glucose and finally resuspended in PBS. 2,4-dinitrophenol (Fisher) was then added to give a final concentration of 1×10^{-4} M or 5×10^{-4} M DNP and the suspensions were incubated at 37°C for 1 hour. Control cultures were incubated in PBS only. The appropriate labelled precursor (see 2.1.2.) was then added and incubation at 37°C continued for 30 minutes. Determination of synthesis rates from the uptake of label proceeded as described in section 2.1.2.

For studies of the effect of DNP on the yield of single strand breaks, H^3-TdR labelled cells were similarly washed and suspended in PBS (controls) or in PBS containing 1×10^{-4} M DNP or 5×10^{-4} M DNP. After incubation at 37°C for 1 hour the

suspensions were quickly cooled to 0°C and irradiated (see 2.1.4.).

2.1.4. Determination of the Relative Level of Intracellular ATP

The relative level of ATP in L-60 cells was determined by the luciferase method (Addanki, Sotos, Rearick 1966; Matsudaira, Furuno, Otsuka 1970). In short, the cells were incubated in PBS or in 1×10^{-4} M or 5×10^{-4} M DNP as described in 2.1.3. After the incubation (1×10^{6} cells/ml) 2.5 ml of cell suspension was introduced into a glass homogenizer and the cells were broken; then cold 20% TCA was added, and the precipitate was spun down. The supernatant (1 ml) was neutralized with NaOH to pH 7 and cooled to 0°C. 0.1 ml of this sample was added to a suspension of 0.2 ml of fire fly extract (Sigma Chemical Company) and 1.7 ml of double distilled water, also at 0°C. After 10 seconds, the fluorescence was measured in a Beckman LS-250 scintillation counter. The number of counts corresponds to the relative level of ATP in the added supernatant.

2.1.5. Irradiation of Cells

In order to prepare experimental suspensions of cells, the radioactively labelled monolayer cultures were treated with 0.1% trypsin (Bacto-Trypsin, Difco) except in the case of human fibroblasts where 0.25% trypsin was used. The cells were exposed to trypsin for 8 minutes at room temperature. The action of trypsin was neutralized by the addition of growth medium containing serum. The cells were then centrifuged, washed once again with medium and finally resuspended in either PBS from which Mg^{++} and Ca^{++} ions

had been omitted, or in growth medium.

Cell suspensions prepared as described in 2.1.4. were loaded into several special glass irradiation vessels ($\sim 1 \times 10^6$ cells/ml, 7 ml each) which were placed in a 0°C bath for the duration of the experiment. O_2 or N_2 (less than 5 parts/million O_2 present, Canadian Liquid Air, Hamilton) was then passed over the suspension for 1 hour prior to and during irradiation. The gas flow rate was 1.0 l/min and the suspensions were agitated with a magnetic stirrer. These conditions were similar to those where cells were irradiated for measurements of survival (Parker, Skarsgard and Emmerson 1969).

For some experiments the cell suspension was loaded into a polystyrene container, made from a 10 ml plastic syringe (Becton, Dickinson) (1 ml/container) and irradiated at 0°C . Oxygen or nitrogen was bubbled through the cell suspension 1 hour prior to and during the irradiation. Occasionally however, the cells were not gassed but the results thus obtained were essentially the same as those where O_2 was bubbled through the cell suspension.

The radiation source was a 2000 curie Cs^{137} unit (0.66 MeV γ-rays). The dose rate at the position of the cells was measured by the methods:

a) Fricke's method (ferrous ammonium sulphate) using the procedures outlined by the American Society for Testing and Materials (Annual Book of ASTM Standards, 1971).

b) Ionization measurements, using a Victoreen Model 570 condenser r-meter.

The measured dose rates were 332 rads/minute and 328.2 rads/minute, respectively for the glass vessels and 2000 rads/minute for samples irradiated in the plastic containers.

2.1.6. Incubation of Cells after Irradiation

After the cells had been irradiated at 0°C they were then kept at this temperature for various periods of time. When we studied the rejoining processes after irradiation, the cells were incubated under the required conditions where the effect of medium, temperature or some other factor was examined.

In general all washing, resuspending, and any other handling of the irradiated cells was done at 0°C, and also the incubation was stopped by placing the cells at 0°C.

Incubation after irradiation was carried out in sterile, non-tissue culture plastic petri dishes (Falcon Plastics) in order to avoid attachment of cells to the culture vessel. Again, incubation was in an atmosphere of 95% air, 5% CO_2. After the incubation period the petris were thoroughly shaken and the suspension pipetted into a test tube at 0°C. The cells were held at 0°C until they were lysed.

2.2. PREPARATION OF LABELLED BACTERIOPHAGE T4 AND T7

Sedimentation measurements of the cellular DNA were normalized using bacteriophage T4 and T7 and Adenovirus 2 as markers. For the

preparation of phage stock, E. coli cells were grown overnight in Antibiotic medium No. 3 (Gibco) to lag phase. They were then diluted 500 times in 50 ml aliquots of the same medium except that sodium chloride at a concentration of 5 g/litre was added to the flasks intended for T4 inoculation. After two hours of further incubation at 37°C, the cells were inoculated with either T4 or T7 bacteriophage at a multiplicity of infection (MOI) of 1×10^{-2}. Simultaneously, H^3-TdR (15.5 Ci/mM) was added so that the final activity was 2μCi/ml.

Two hours after infection, a few drops of chloroform were added to clear the lysates, suspensions were centrifuged for 10 minutes at 700 g and supernatants were filtered through 0.47 μ membrane filters. Filtrates were then centrifuged at 20,000 r.p.m. for 1 hour in a Beckman preparative ultracentrifuge, SW 27 rotor (31 ml tubes). The pellets were washed with 30 ml of PBS, centrifuged again, then resuspended in 0.5 ml of PBS. From this, 0.02 ml aliquots were layered on the top of the gradients.

Adenovirus 2, with its DNA labelled by either H^3-TdR or C^{14}-TdR, was kindly supplied by Dr. S. Mak, McMaster University.

2.3. ALKALINE SUCROSE GRADIENTS

2.3.1. Gradient and Lysing Solutions

Alkaline sucrose gradients were prepared using an automatic gradient former (ISCO Model 570). Gradients were linear, 5-20%, of total volume 4.45 ml when prepared in 5 ml polyallomer tubes (Beckman)

or of total volume 17 ml, when prepared in 17 ml cellulose-nitrate tubes (Beckman). The gradient solutions were prepared with double distilled water and contained 0.3 M sodium hydroxide (NaOH), 0.01% sodium dodecyl sulphate (SDS), 0.001 M ethylenediaminetetraacetate (EDTA) and the appropriate concentration of sucrose. On the top of the prepared gradients a lysing solution was carefully layered just prior to lysis of the cells (0.3 ml on 5 ml tubes and 0.5 ml on 17 ml tubes).

The lysing solution contained 0.5 M NaOH, 0.2% SDS and 0.01M EDTA. All solutions were prepared using double distilled water and were filtered prior to usage through at 0.22μ membrane filter.

A number of our experiments were duplicated following a procedure similar to the alkaline sucrose gradient technique described by Elkind and Kamper (1970), which employs high salt concentrations but no detergent (Section 2.4.).

The gradient technique outlined above evolved as the result of many experiments and it may be of some value to indicate how these proceeded.

At the beginning of our work we used the solutions described by McGrath and Williams (1966). That is, 5% and 20% sucrose solutions were prepared using glass distilled water and adjusted to pH 12 with NaOH. After production of a 5-20% linear gradient in a 5 ml tube, 0.1 ml of lysing solution consisting of 0.5 M NaOH was

layered on the top of the gradient.

Essentially the same solutions were used later by Lett et al. (1967) in their studies of the effect of ionizing radiation on DNA molecules of mammalian cells. Although McGrath and Williams could reproducibly band the DNA molecules of unirradiated bacterial cells (protoplasts), Lett et al.were unable to obtain a sedimentation profile for the DNA from unirradiated mammalian cells. Our initial task then, was to find conditions for which the DNA molecules from unirradiated mammalian cells would band reproducibly.

Our criteria for the selection of a technique were:

a) the profile should sediment to the same average position in the tube in repeated experiments.

b) the shape of the profile should be reproducible

c) the sedimenting DNA should be free of protein and

d) the radioactivity put into the gradient should be recovered in the collected fractions.

Initially then, using the technique of McGrath and Williams we tried a variety of conditions for the lysis of cells on the top of the gradient. The effect of varying the temperature and the duration of lysis was studied as well as the procedure for introducing the cells into the lysing layer and for mixing them with the lysing solution. However, with this technique, we were never able to find a set of conditions which satisfied the four criteria described above. The peak of the distribution sedimented to unpredictable positions, the

shape of the distributions was not reproducible and as well, at the peak position of the DNA distribution one could find a small peak of cosedimenting protein molecules (sometimes up to 10% of the total labelled protein present).

We then tried different concentrations of EDTA a chelating agent which binds metal ions in our lysing and sucrose solutions and simultaneously we studied the effect of increased ionic strength by the addition of various concentrations of NaCl. The presence of EDTA afforded some improvement in terms of reproducible peak position but protein was always found cosedimenting with the DNA. The peak of the DNA distribution was very sharp and often essentially all of the counts were found in one fraction.

It could be noted that the inclusion of EDTA and NaCl corresponds to the method used by Elkind and Kamper (1970) which is described in greater detail in 2.4. Briefly, for unirradiated cells, the Elkind - Kamper technique still did not satisfy the criteria which we had established.

It was not until we added a detergent, sodium dodecyl sulphate (SDS) and mercaptoethanol (MSE) to our solutions, that the criteria were satisfied. These substances promote the denaturation of protein. We then varied the concentration of these agents in order to discover the minimum amounts which would still satisfy the criteria. We found that the addition of MSE was not really necessary and that the minimum amount of SDS required was

0.2% in the lysing solution and 0.01% in the gradient solutions. A typical sedimentation pattern obtained using the final conditions chosen can be seen in figure 1.

Having established the minimum level of SDS required we next varied the concentration of EDTA and NaCl. The concentrations of EDTA selected were 0.01 M EDTA in the lysing solution and 0.001 M EDTA in the gradient solution. We also tested various concentrations of NaCl, up to a maximum of 1M. However NaCl was found to have little effect on the sedimentation properties of the DNA from irradiated or unirradiated cells except that the peaks were somewhat broader and sedimented a little faster due, probably to conformational changes induced in the DNA by the high salt concentration.

2.3.2. Lysing of Cells

A 50 µl microsyringe, pre-cooled to 0°C, was used to dispense the cells into the lysing layer. A mechanical device, employing 2 precision rack and pinion drives, facilitated this procedure. One of the drives was used to lower the loaded syringe until the needle just touched the lysing solution. The other drive was then used to deliver the cells into the lysing layer, a procedure which extended over a period of one minute. Approximately 1×10^4 cells in 0.02 ml were carefully introduced into the lysing layer by this means.

We found that the procedure by which the cells were placed on the top of the gradient was critical. Any lysing solution which comes in contact with the cells prior to the time when they are

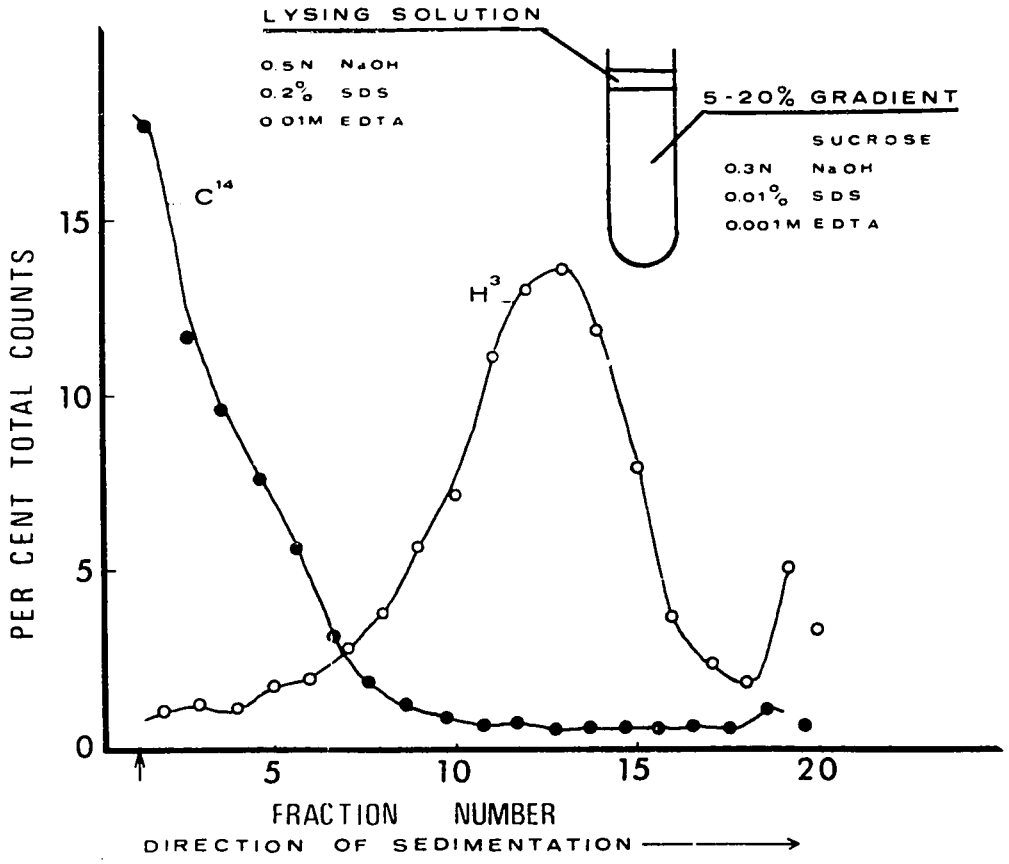


Figure 1. Sedimentation of unirradiated L-60 cell DNA; double labelling experiment.

The cells were uniformly labelled with H^3-TdR in DNA and with C^{14}-L-leucine in protein. 1 x 10^5 cells were lysed for 12 hours at 22°C in a lysing solution (0.5 M NaOH, 0.01 M EDTA, 0.2% SDS) on the top of an alkaline gradient (5-20% sucrose, 0.3 M NaOH, 0.001 M EDTA, 0.01% SDS). Gradients were then spun at ω = 42,000 r.p.m. for 50 minutes at 20°C (SW 50.1 rotor, 5 ml tubes).

-o-o- H^3 counts

-●-●- C^{14} counts

layered on the top of the gradient would lyse some cells and thus release DNA molecules from the cells. This DNA, then, is subject to some shearing forces as the sample is flowed into the lysing layer. The sheared DNA molecules would tend to distort the actual profile.

2.3.3. Temperature of Lysing and Centrifugation

In the final formulae for the lysing solution and gradients which we used in our work we had some detergent (SDS), which limited the choice of the lysing temperatures or centrifugation temperatures to 20°C or above (at lower temperatures SDS precipitates under these conditions). The temperature of the centrifugation was held constant, 20°C, and the lysing temperature was room temperature 22-24°C. The same temperatures were used when the lysing and gradient solutions of Elkind and Kamper (1970) were employed (section 2.4.).

2.3.4. Stability of Gradients

In most of our experiments, lysing times were between 10-14 hours at room temperature though in some cases when we studied the effect of lysing time on degradation of the DNA molecules lysing times were extended to as long as 24 hours. Consequently, it was of interest to determine whether the gradients were stable over these periods. This was done either by using refractometric

measurements or by adding 10^{-3} M uracil to the 20% sucrose solution from which the gradients were made. After the gradients had been collected, either refractive index or optical density (254 nm) measurements were made. In general, the gradients for either 5 ml tubes or 17 ml tubes were found to be quite stable at room temperature; the only changes observed were at the top of the 5 ml gradient. The results are shown in figures 2 and 3.

2.3.5. Centrifugation

All centrifugations were performed at 20°C using an SW50.1 or SW 27 (17 ml) rotor in a Beckman L-65 preparative ultracentrifuge. In order to ensure that the sedimented material obeyed the Burgi - Hershey equation (Burgi and Hershey 1963; equation (2), section 2.3.11.) we routinely checked the DNA sedimentation properties for at least two different values of angular speed of centrifugation ω and time of centrifugation t; ω and t were chosen so that the peak of the distribution sedimented to a position between the 5th and 12th fractions in the 5 ml tube (Beckman SW 50.1 rotor) and between 5th and 17th fractions in the 17 ml tube (Beckman SW 27 rotor). In some experiments with 5 ml tubes ω was varied from 42,000 r.p.m. to 8,000 r.p.m. and t from 50 minutes to 6 hours; for the 17 ml tubes, ω was varied from 20,000 r.p.m. to 8,000 r.p.m. with corresponding times of 5 hours to 36 hours.

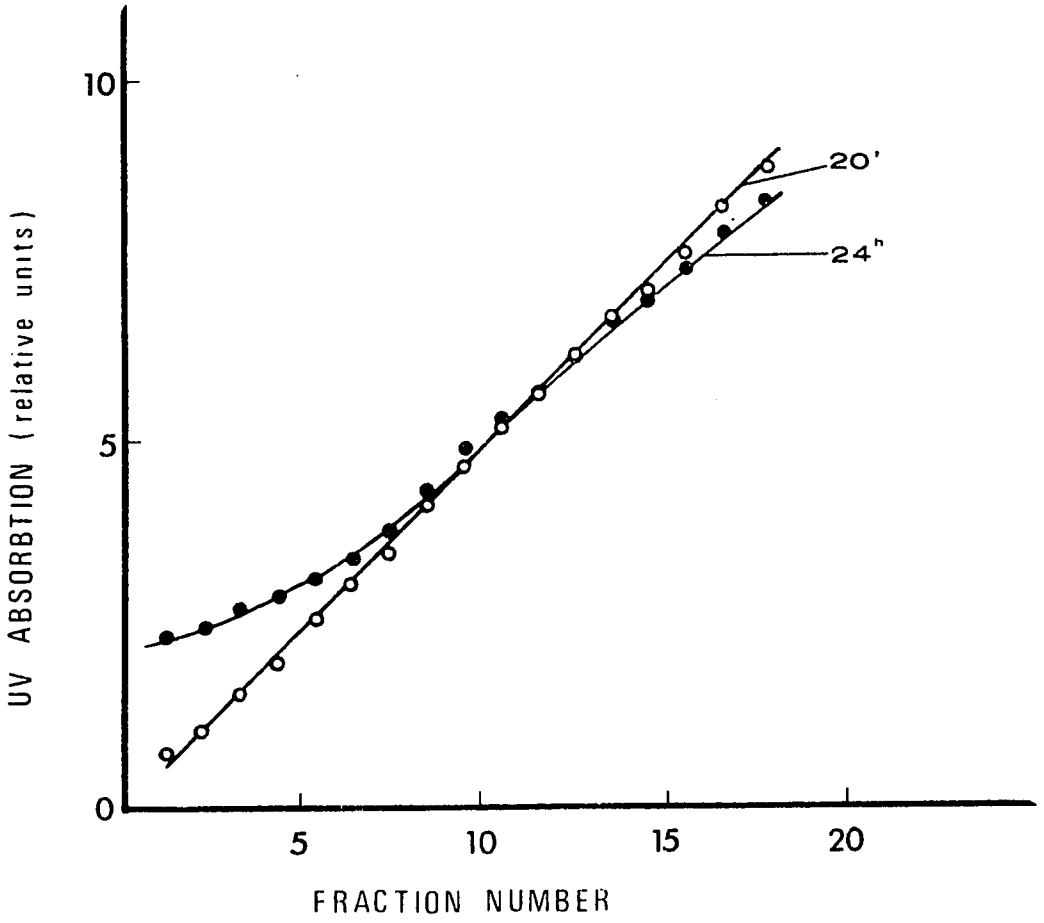


Figure 2. Gradient stability in 5 ml tubes.

10^{-3} M uracil was added to the 20% sucrose solution and gradients were prepared. Centrifugation was begun (Beckman SW 50.1, 42,000 r.p.m., 50 minutes, 20°C).

-o-o- 20 minutes
-o-o- 24 hours after the gradients were prepared.
20 fractions of 0.25 ml each were collected after centrifugation and UV absorbance was plotted against fraction number. The background absorbance due to sucrose is substracted.

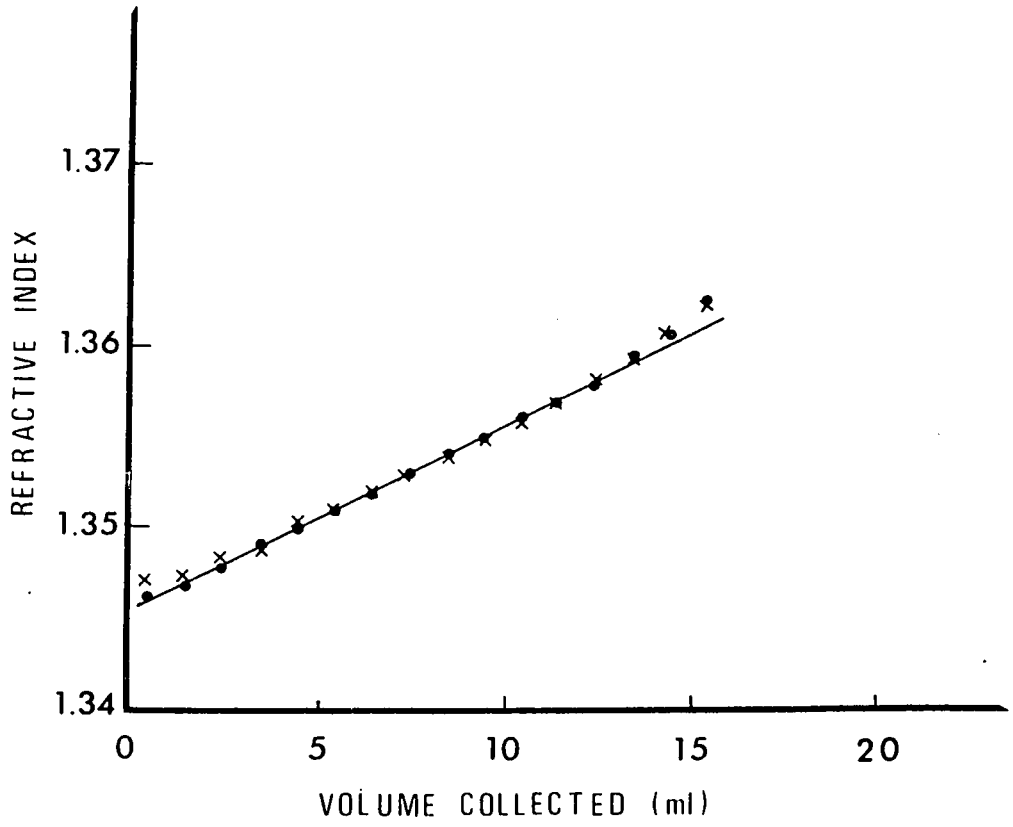


Figure 3. Gradient stability in 17 ml tubes.

Centrifugation was begun (Beckman SW 27, 20,000 r.p.m., 3 hours, 20°C).

-●-●- 20 minutes
-x-x- 24 hours after the gradients were prepared.
16 fractions of 1 ml each were collected after centrifugation, the refractive index was measured and plotted against fraction number.

2.3.6. Interactions between Molecules during Sedimentation

The DNA molecules of unirradiated mammalian cells are rather large and the molecules interfere with each other during sedimentation if their concentration is too high. We examined the sedimentation behaviour when different numbers of cells were used on the top of the gradient and the results for the 5 ml tubes are shown in figure 4. It can be seen that once one uses more than $\sim 2 \times 10^4$ cells per gradient, the DNA molecules appear larger. Furthermore, in figure 5 are shown the profiles obtained when 5×10^3 cells and 1×10^5 cells were used. Not only does the molecular weight appear larger, but also one notices a broadening of the peak with the increased number of cells in the gradient.

When the same effect was studied in the 17 ml tubes, we found no significant differences for cell numbers between 1×10^4 and 5×10^4 per gradient. The higher number of cells, i.e. 5×10^4 still does not show any detectable interactions either in terms of the calculated molecular weight or in the shape of the profiles.

In the rest of our experiments, then, we chose to use 1×10^4 to 2×10^4 cells per gradient in the 5 ml tubes and not more than 3×10^4 per gradient in the 17 ml tubes.

We also studied the possible interaction between two different types of molecules. 5×10^4 unirradiated L-60 cells were lysed on the top of the gradient simultaneously with T4 bacteriophage, adenovirus Ad 2 or irradiated L-60 cells. The profiles were compared

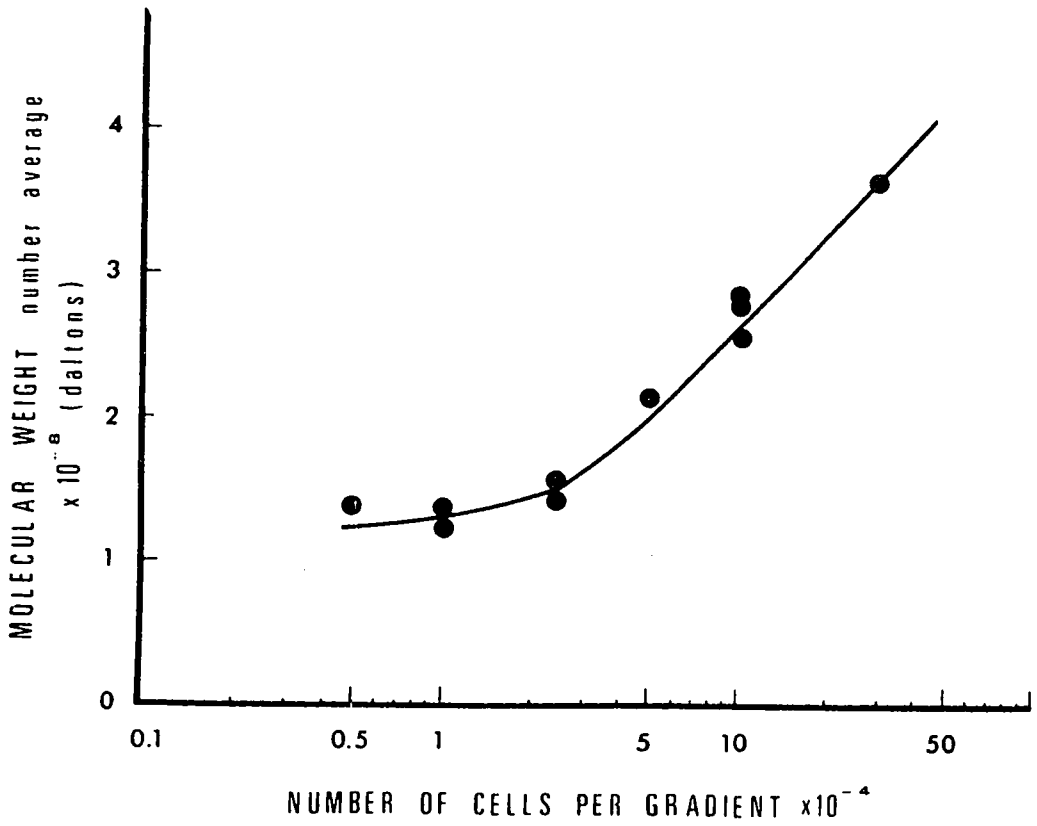


Figure 4. The dependence of the apparent molecular weight (unirradiated cells) on the number of cells.

L-60 cells were lysed for 12 hours at 22°C in a lysing solution (0.5 M NaOH, 0.01 M EDTA, 0.2% SDS) on the top of an alkaline gradient (5 - 20% sucrose, 0.3 M NaOH, 0.001 M EDTA, 0.01% SDS). Gradients were then spun at ω = 42,000 r.p.m. for 50 minutes at 20°C in a Beckman SW 50.1 rotor, 5 ml tubes.

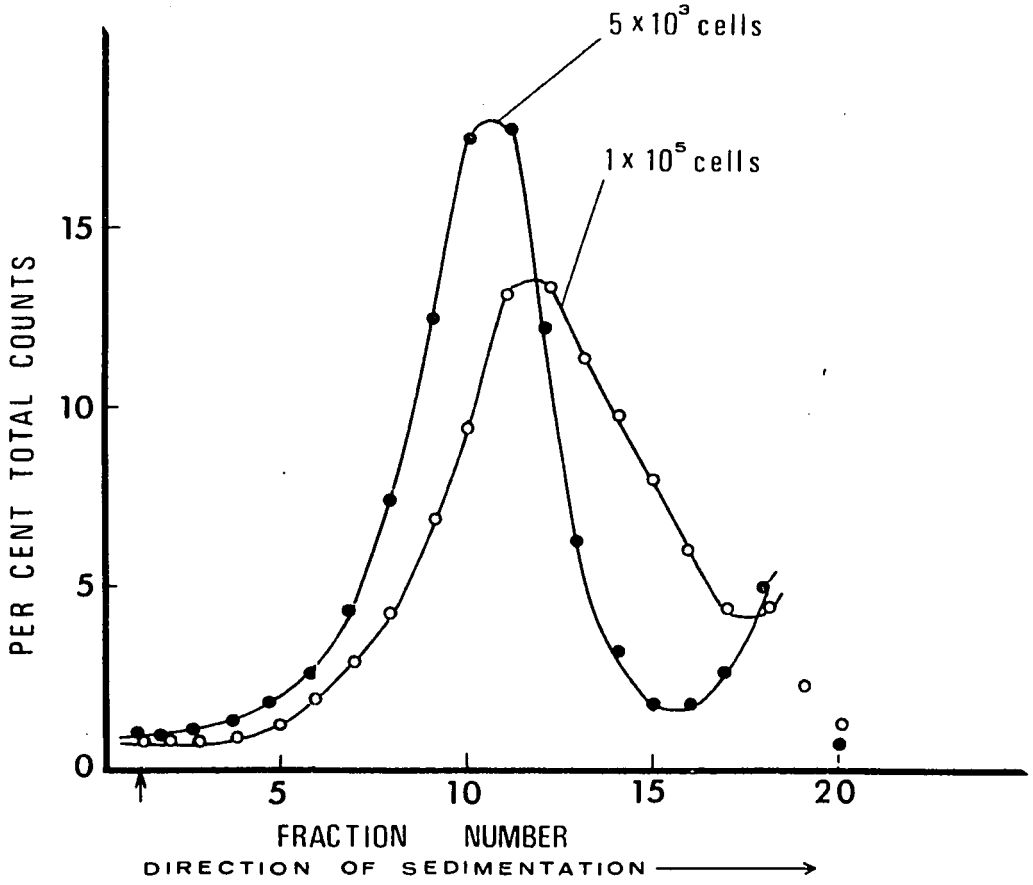


Figure 5. The dependence of the sedimentation profile on the number of cells placed on the gradient.

L-60 cells were lysed for 12 hours at 22°C in a lysing solution (0.5 M NaOH, 0.01 M EDTA, 0.2% SDS) on the top of an alkaline sucrose gradient (5 - 20% sucrose, 0.3 M NaOH, 0.001 M EDTA, 0.01% SDS). Gradients were then spun at ω = 42,000 r.p.m. for 50 minutes at 20°C in a Beckman SW 50.1 rotor, 5 ml tubes.

to those obtained when each of these species were lysed and centrifuged separately. No interactions were observed in either the 5 ml tubes or the 17 ml tubes. Some representative results can be seen in figures 6 and 7. One can see that in both figure 6 and figure 7 the peak position of any distribution is unaffected by the presence of a second sedimenting species.

In some experiments, using 5 ml tubes where 1×10^5 irradiated (10 krads) and 1×10^5 unirradiated cells were lysed together some cosedimentation was observed, which was thus apparently associated with the high concentration of DNA on the gradient. This was manifested as a displacment of the peaks towards each other.

2.3.7. Collecting of Gradients and Counting

After centrifugation, each 5 ml gradient was collected as 20 fractions using an ISCO Model D fraction collector. 17 ml tubes were collected, using the same apparatus, into 25 fractions of 0.75 ml each. The DNA in each fraction was then precipitated with cold 5% TCA (trichloroacetic acid) and collected on membrane filters; these were further washed twice with cold TCA and finally with 75% alcohol, dried and put into scintillation vials. Five ml of toluene based scintillation fluid (toluene with addition of 1,4-Bis-(5-phenyloxazol-2-yl-)-benzene, (POPOP) 0.315 g/l and 2,5-diphenyloxazole (PPO), 4.2 g/l, Aldrich Chemical Company) was added to each vial and the radioactivity was measured in a Beckman scintillation counter (model LS-250 or LS-233).

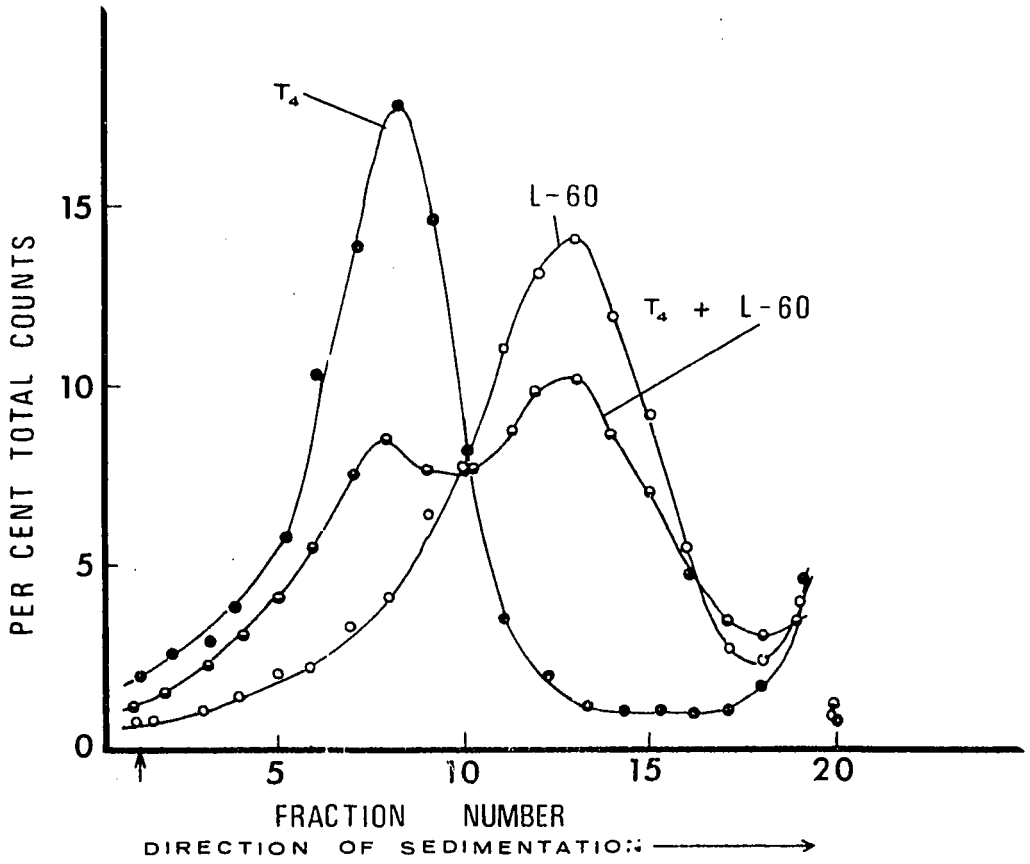


Figure 6. Resolution, 5 ml tubes.

5 x 10^4 unirradiated L-60 cells were either lysed alone -o-o- or together with an equivalent amount of phage T4 -⊙-⊙-. T4 phage were lysed and spun alone under identical conditions -⊙-⊙- 0.3 ml of lysing solution (0.5 M NaOH, 0.01 M EDTA, 0.2% SDS) was layered on the top of an alkaline gradient (5-20% sucrose, 0.3 M NaOH, 0.001 M EDTA, 0.01% SDS). Gradients were then spun at ω = 42,000 r.p.m. for 50 minutes at 20°C in a Beckman SW 50.1 rotor.

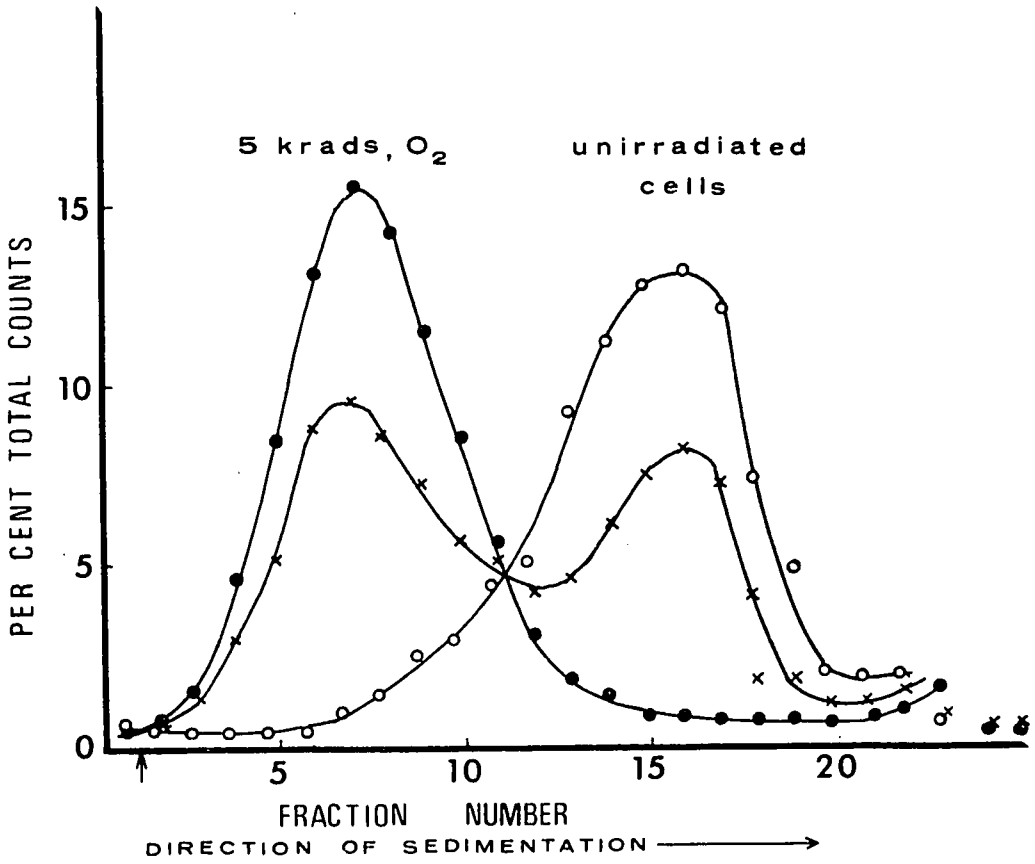


Figure 7. Resolution, 17 ml tubes.

-o-o- , 2 x 10^4 unirradiated L-60 cells lysed alone; -ω-ω- , 2 x 10^4 irradiated L-60 cells (5 krads, O_2) lysed alone; -x-x-, 1 x 10^4 irradiated cells and 1 x 10^4 unirradiated cells lysed together. In all of the gradients the cells were lysed for 12 hours at 22°C in a lysing solution (0.5 M NaOH, 0.01 M EDTA, 0.2% SDS) on the top of an alakaline gradient (5-2% sucrose, 0.3 M NaOH, 0.001 M EDTA, 0.01% SDS). Gradients were then spun at ω = 20,000 r.p.m. for 5 hours and 50 minutes ina Beckman SW 27 rotor, 17 ml tubes.

In some experiments, fractions were collected directly on glass fiber filter papers, which were then washed in cold 5% TCA and 75% alcohol and dried, or just simply dried without washing. The counting proceeded as already described. The results obtained in this way are essentially the same, except that the counting efficiency was somewhat lower.

For each experiment the total counts in all fractions were compared with the total counts in a monitor sample, where a number of cells equal to that lysed on the gradients, was lysed in a tube containing only 0.3 ml of lysing solution.

This lysate was then processed in the same way as were the individual fractions. The recovery of counts, defined as the ratio between total counts in a gradient divided by the monitor counts was always between 0.8-1.2.

The absolute counting efficiency in these experiments, defined as the ratio of the number of counts observed to the expected number of radioactive atoms decayed, was 10%. The number of decays was estimated by measuring the activity of the labelling medium at the beginning and at the end of the labelling period. The fraction of the activity which disappeared from the labelling medium was assumed to be incorporated into the cells present. This yielded an estimate of 10 decays per minute per cell for the labelling conditions used (2μCi H^3-TdR/ml, L-60 cells) whereas typical counting rates were 1 count per minute per cell. This absolute

counting efficiency of 10% is, if anything, an underestimate since it neglects radioactivity that may have remained in cell pools, as well as any cells that were lost in the trypsinization process. Nevertheless, it compares favorably with the counting efficiency of 11% that was obtained when H^3-TdR was deposited directly on a membrane filter along with 0.15 ml of 75% ethanol, 0.15 ml of 5% TCA and 0.15 ml of lysing solution (to stimulate the sample processing procedure) dried and counted as above.

In most of the experiments with the Beckman SW 27 rotor (17 ml tubes) we collected fractions directly into glass vials; the fractions were then neutralized and made acidic by the addition of 0.2 ml of 4 M HCl to the contents of each vial. Subsequently, 5 ml of Aquasol (New England Nuclear) scintillation fluid was added to each vial and after the vials had been thoroughly shaken, the counting proceeded in the scintillation counter as described previously. This procedure (Aquasol) gave a much higher absolute counting efficiency, between 25 and 30%, which allowed us to use lower levels of radioactivity in the labelling of our cells (section 2.1.2.).

2.3.8. Lysing Times. Effect of Prolonged Lysis on Integrity of DNA Molecules

In general, the lysing time used in our experiments was 12 hours. We examined different lysing times with unirradiated mammalian cells and found that at least 6 hours at room temperature

was required to get all of the DNA molecules to sediment to a reproducible position. The lysing time should be sufficient not only to release the DNA from the cells, but also long enough that double stranded DNA molecules become single stranded. Extrapolation of the measurements of the rate of strand separation in bacteriophage (Davison 1966) suggests that a lysing time of 10 minutes is required for strand separation in double stranded DNA of size 5×10^8 daltons.

Some results where different lysing times were used are shown in figure 8. One can see no difference between peak positions for the cells which were lysed for 6 hours and those lysed for 18 hours. Some broadening of the profile is evident at 18 hours, however, and this might result from a diffusion of the DNA molecules prior to the beginning of centrifugation; consequently, the starting point is not the same for all molecules. We conclude that there was no detectable degradation of DNA under highly alkaline conditions over the time span studied. For shorter lysing times e.g. 2.5 hours or 15 minutes the results are shown in figure 9. We found that though some material emerged as a peak of DNA having a somewhat higher molecular weight, most of the radioactivity was located at the bottom of the tube.

The numbers in parentheses in figures 8 and 9 indicate the percentage of counts placed on the gradient (these were

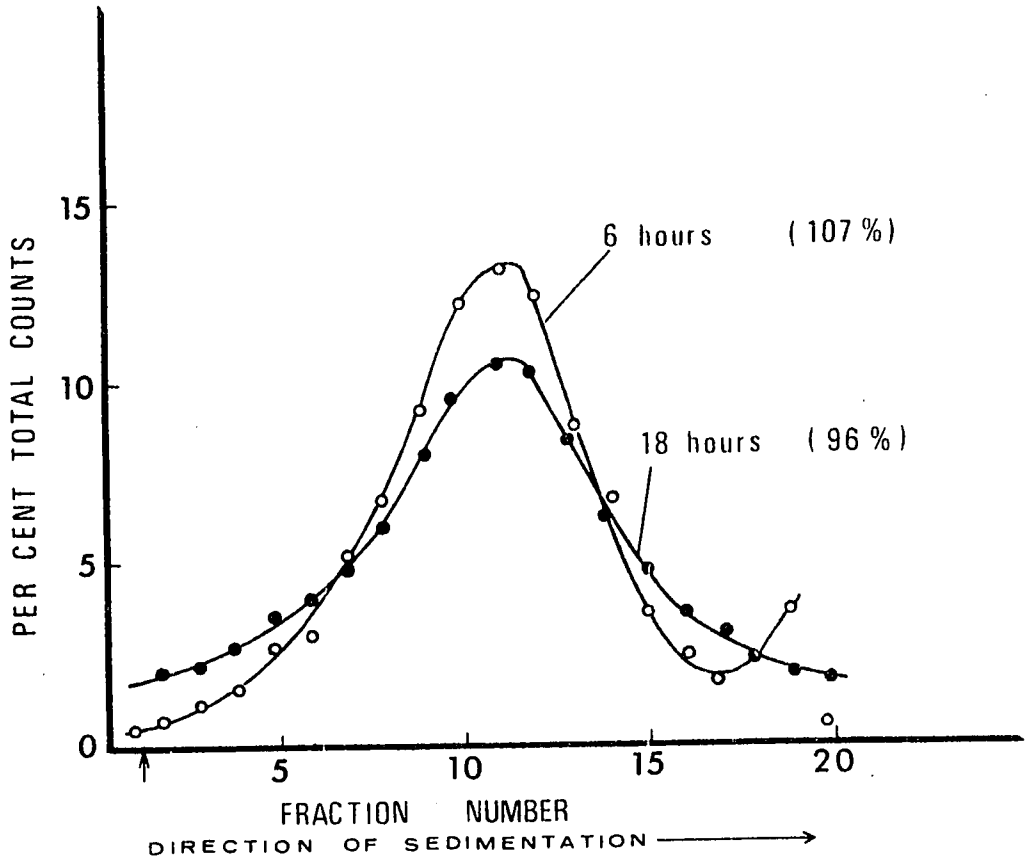


Figure 8. Long lysing time for unirradiated cells, low speed centrifugation.

1×10^4 unirradiated L-60 cells were lysed for 6 hours -o-o-, or 18 hours -●-o-, at 22°C in a lysing solution (0.5 M NaOH, 0.01 M EDTA, 0.2% SDS) on the top of an alkaline gradient (5-20% sucrose, 0.3 M NaOH, 0.001 M EDTA, 0.01% SDS). Gradients were then spun at ω = 12,000 r.p.m. for 7 hours at 20°C in a Beckman SW 50.1 rotor, 5 ml tubes. The per cent values in parentheses indicate the recovery of counts put on each gradient as compared to monitor counts.

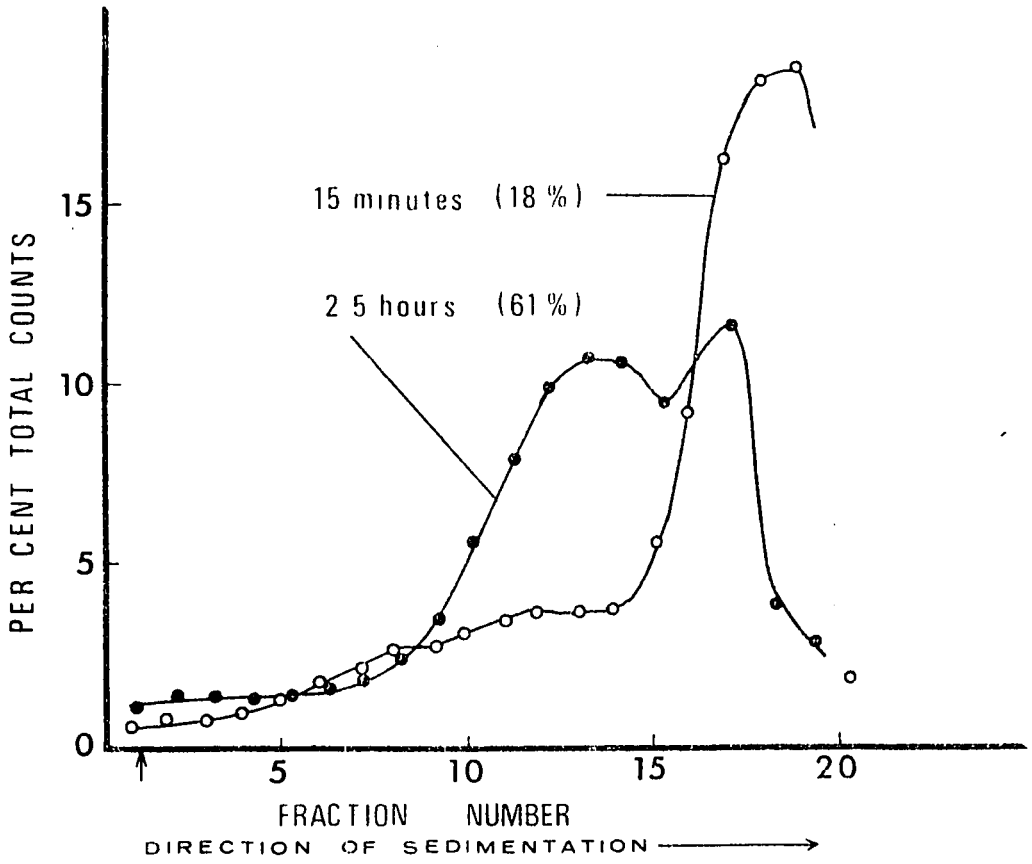


Figure 9. Short lysing times for unirradiated cells, low speed centrifugation.

1 x 10^4 unirradiated L-60 cells were lysed for 2.5 hours -●-●-, or 15 minutes -o-o-, at 22°C in a lysing solution (0.5 M NaOH, 0.01 M EDTA, 0.2% SDS) on the top of an alkaline gradient (5-20% sucrose, 0.3 M NaOH, 0.001 M EDTA, 0.1% SDS). Gradients were then spun at ω = 12,000 r.p.m. for 7 hours at 20°C in a Beckman SW 50.1 rotor, 5 ml tubes. The per cent values in parentheses indicate the recovery of counts put on each gradient as compared to monitor counts.

determined using monitor samples, see section 2.3.7.), which are recovered in the collected fractions. In general, for lysing times of 6 hours or greater the average recovery of radioactivity was 100%. For shorter times, this was not the case. As is shown in figure 9, in a sample receiving 2.5 hours of lysis 61% of the applied radioactivity was recovered while another sample which was lysed for 15 minutes, only 18% of the counts were recovered.

In some preliminary experiments where samples were centrifuged at high speeds (42,000 r.p.m.) we found that even for short lysis times, 3 hours or less, some DNA emerged as a peak at the same position as when unirradiated cells were lysed for 6 hours or more (the rest of the DNA molecules still sedimented to the bottom of the tube). The results are presented in figure 10. This superposition of the peaks, which was not confirmed by low speeds of centrifugation was really an artifact at high rotor speeds. It has been reported (Elkind 1971; McBurney, Graham and Whitmore 1971; Palcic and Skarsgard 1972a; see also section 2.3.9.) that high sedimentation velocities severely distort the sedimentation properties of molecules having a molecular weight of 1-2 x 10^8 daltons or greater.

When the cells were irradiated with a very low dose of ionizing radiation (the lowest tested was 0.5 krads) the lysing time which is necessary in order to have the DNA molecules sedimenting to a reproducible position is much shorter. We examined lysing times

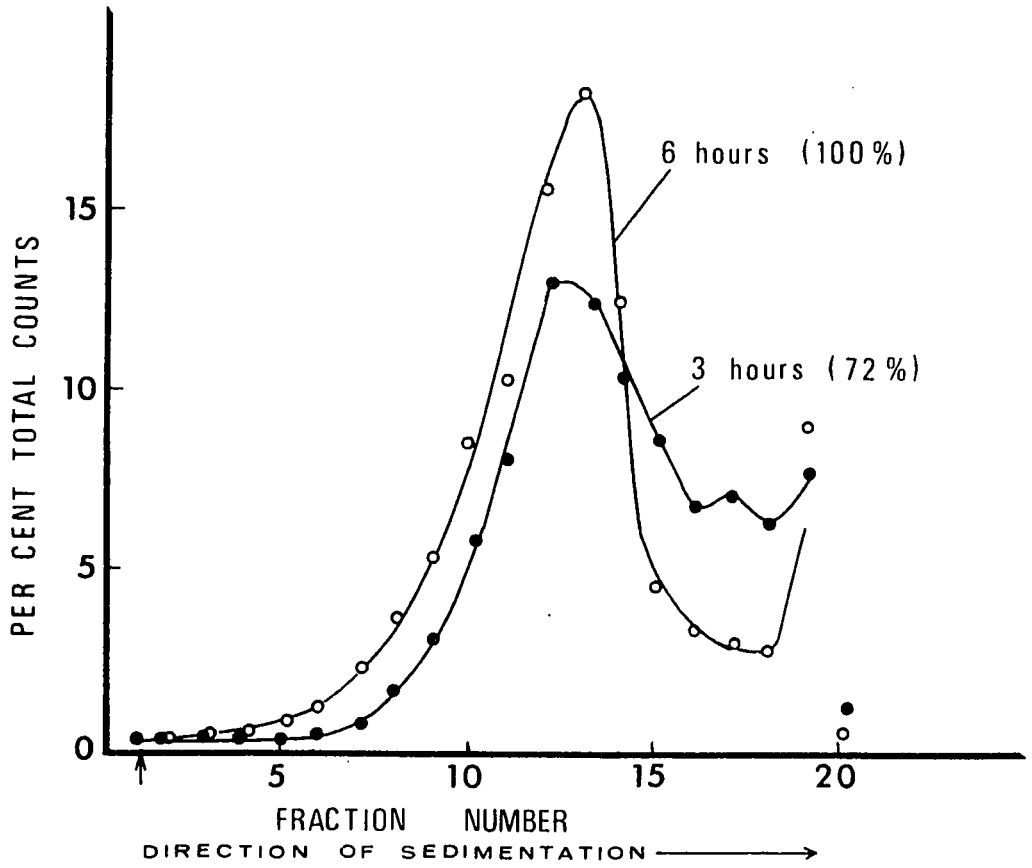


Figure 10. Different lysing times for unirradiated cells, high speed centrifugation.

1×10^5 cells were lysed for 6 hours -o-o-, or 3 hours -o-●- , at 22°C in a lysing solution (0.5 M NaOH, 0.01 M EDTA, 0.2% SDS) on the top of an alkaline gradient (5-20% sucrose, 0.3 M NaOH, 0.001 M EDTA, 0.01% SDS). Gradients were then spun at ω = 42,000 r.p.m. at 20°C for 50 minutes in a Beckman SW 50.1 rotor, 5 ml tubes. The per cent values in parentheses indicate the recovery of counts put on each gradient as compared with monitor counts.

as short as 3 hours and found that the profiles did not differ from those lysed for 12 hours (section 3.1.2.).

One factor which concerned us in the development of our technique was the possibility that our extended lysis periods might lead to degradation of the DNA which could be reflected as a reduced average molecular weight. Figure 8 shows that the average molecular weight is the same for distributions subjected to 6 hours and 18 hours of lysis.

We also explored the possibility of alkaline degradation of DNA using T4 bacteriophage. The results are presented in figure 11. Here again, we cannot detect a significant degradation of the DNA molecules as the peak position values are the same. The broader profile may be the result of the diffusion of the material during lysis on the top of the gradient, as was mentioned before, rather than due to any degradation of the DNA molecules.

2.3.9. The Effect of Rotor Speed on Sedimentation Behaviour of DNA Molecules (Speed Distortion)

It has been reported by Elkind (1971) that high speeds of centrifugation severely distort the profile of sedimenting molecules if the molecules are larger than 1-2 x 10^8 daltons. Similar observations were also reported by McBurney et al. (1971). We studied the effect of speed distortion using both the Beckman SW 50.1 rotor (5 ml tubes) and the Beckman SW 27 rotor (17 ml tubes). The results are shown in figures 12 and 13. The sedimentation properties of low molecular weight material (number average molecular weight Mn = 0.5 x 10^8 daltons or less) were not dependent upon the speed of centrifugation in either rotor over the speed

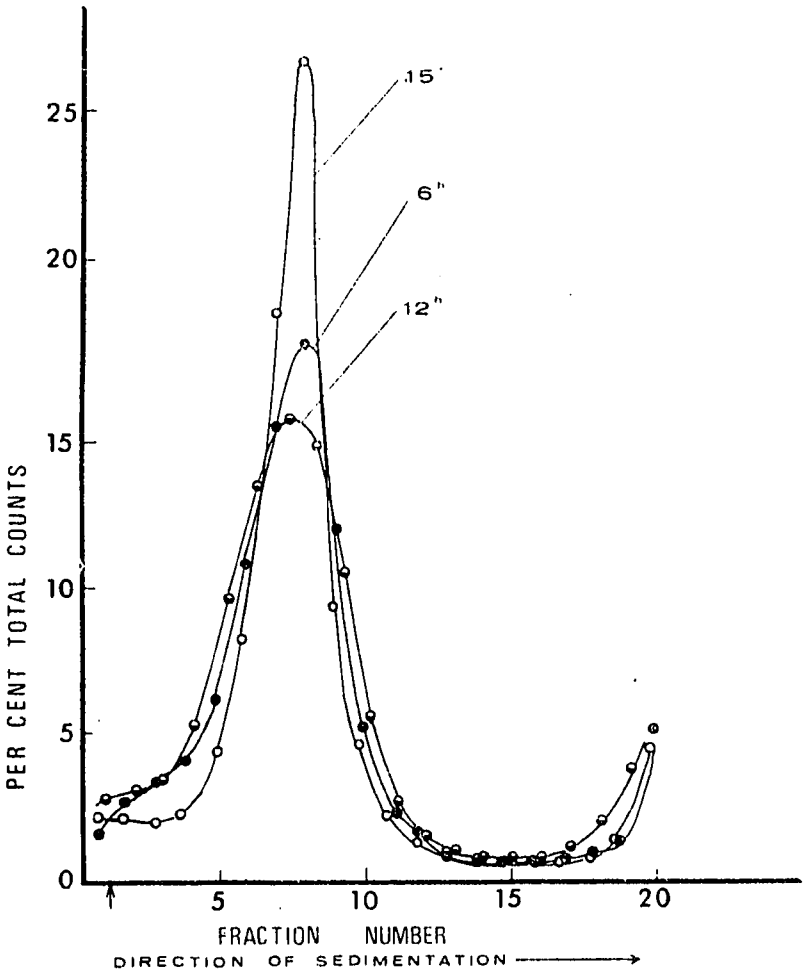


Figure 11. Different lysing times for bacteriophage T4.

The phage was lysed for either 15 minutes -o-o- , 6 hours -●-●-, , or 12 hours -⊙-⊙- at 22°C in a lysing solution (0.5 M NaOH, 0.01 M EDTA, 0.2% SDS) on the top of an alkaline gradient (5-20% sucrose, 0.3 M NaOH, 0.001 M EDTA, 0.01% SDS). Gradients were then spun at ω = 42,000 r.p.m. for 50 minutes at 20°C in a Beckman SW 50.1 rotor, 5 ml tubes.

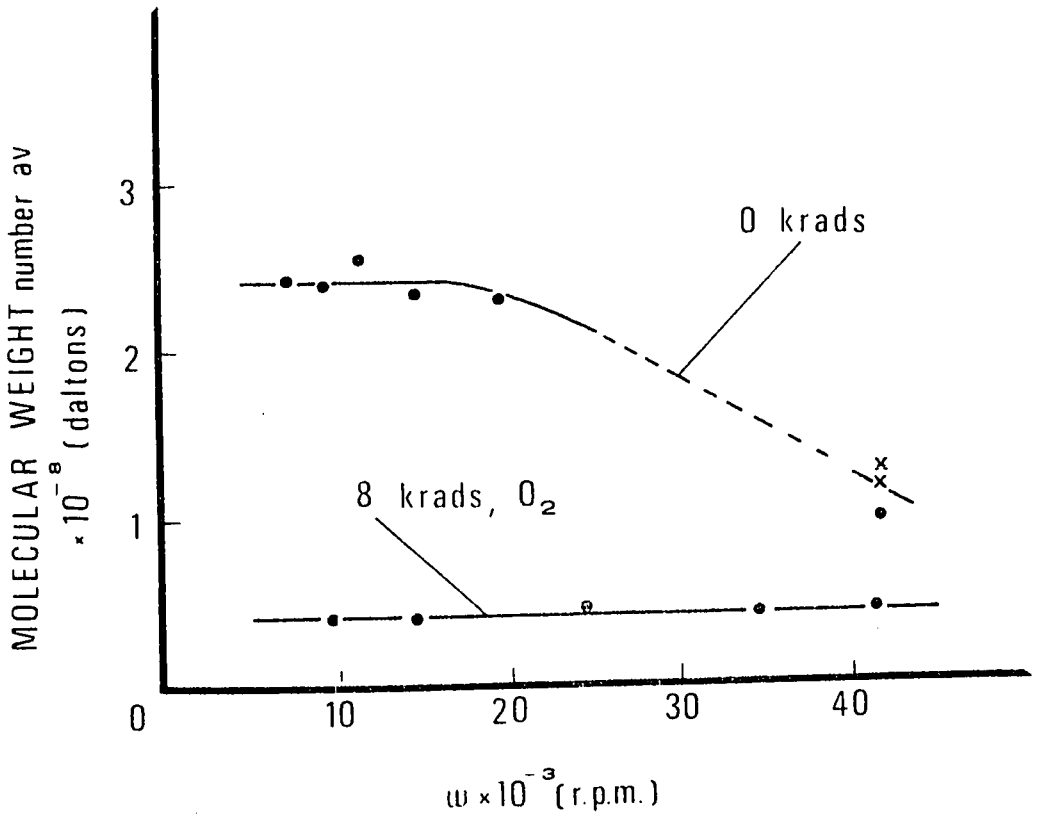


Figure 12. Effect of speed of sedimentation on the apparent molecular weight of irradiated (8 krads) and unirradiated mammalian DNA, 5 ml tubes.

Lysing solution: 0.5 M NaOH, 0.01 M EDTA, 0.2% SDS; gradients: 5-20% sucrose, 0.3 M NaOH, 0.001 M EDTA, 0.01% SDS. 1×10^4 L-60 cells were lysed for 12 hours at 22°C and spun at indicated ω for a time, t, such that the product of $\omega^2 t$ was 1.5×10^9 (r.p.m.)^2 x hour for the cells irradiated with 8 x krads, and for the unirradiated cells spun at 42,000 r.p.m. (crosses); for the rest of the unirradiated cells $\omega^2 t$ was 1.0×10^9 (r.p.m.)^2 x hour. Centrifugation at 20°C, SW 50.1 rotor.

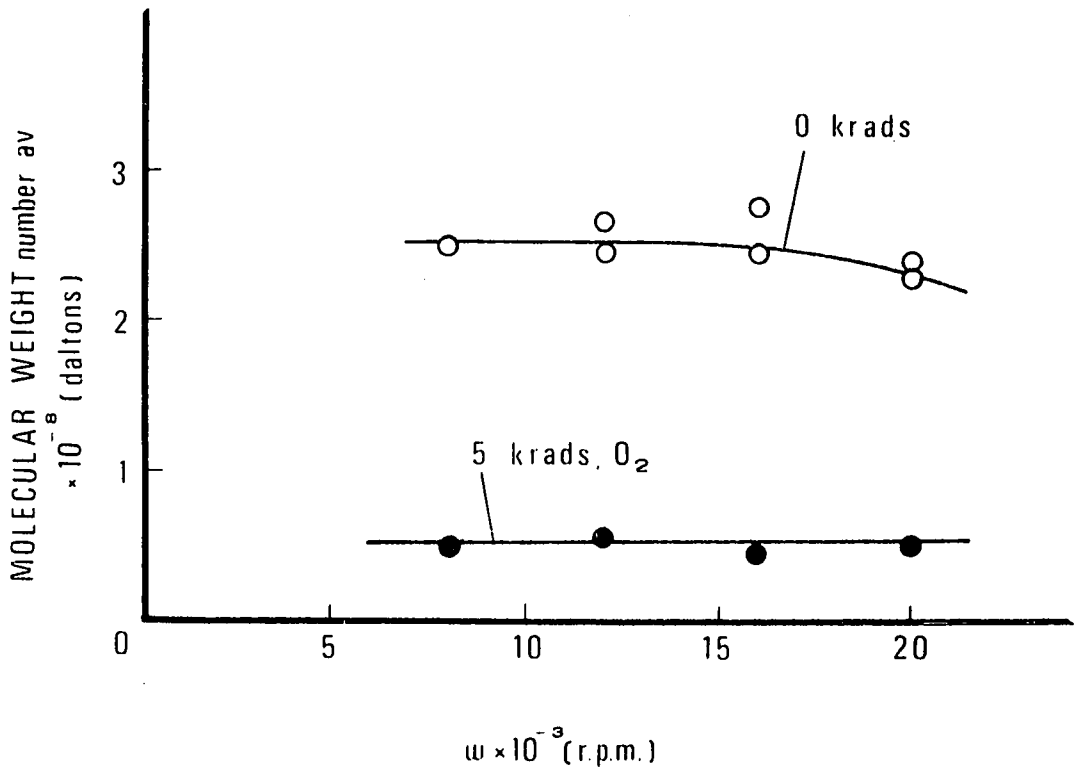


Figure 13. Effect of speed of sedimentation on the apparent molecular weight of irradiated (5 krads) and unirradiated mammalian DNA, 17 ml tubes.

Lysing solution: 0.5 M NaOH, 0.01 M EDTA, 0.2% SDS; gradients: 5-20% sucrose, 0.3 M NaOH, 0.001 M EDTA, 0.01% SDS. 2×10^4 L-60 cells were lysed for 12 hours at 22°C and spun at indicated ω for a time t, such that the product $\omega^2 t$ was 2×10^9 $(r.p.m.)^2$ x hour for both irradiated and unirradiated cells. Centrifugation at 20°C, SW 27 rotor.

range studied. This is true both for the calculated average molecular weight and for the shape of the other profiles. However, it is obvious that for larger molecules (e.g. Mn = 2.5 x 10^8 daltons) the measured value of the molecular weight is dependent upon the angular speed ω at least for speeds greater than 20,000 r.p.m. in both rotors. At angular speeds greater then 20,000 r.p.m. the apparent value of the sedimentation constant $S_{w,20}$ of unirradiated DNA decreases hence reducing the calculated value of the molecular weight. The profiles also become sharper, giving the false impression of a more uniform distribution of molecules. These results are consistent with those reported earlier (Elkind 1971, McBurney et al. 1971).

Consequently, unless the molecular weight of the material was less than 0.5 x 10^8 daltons, angular speeds of ω = 20,000 r.p.m. or less were used in order to avoid this problem of distortion at higher speeds.

2.3.10. Normalization of Gradients.

The measurement of molecular weight by the alkaline sucrose gradient technique is a comparative method and hence for each given combination of gradient solution, temperature of centrifugation, rotor type, tube size, etc., the method has to be normalized with DNA molecules of a known molecular weight.

With the Beckman SW 50.1 rotor (5 ml tubes), we used two different alkaline sucrose techniques, one employing detergent (SDS) in the gradient and the other, using detergent-free high salt conditions as described by Elkind and Kamper 1970, and Elkind 1971; see sections 2.3.1. and 2.4. With the SW 27 rotor (17 ml

tubes), we used only the alkaline sucrose gradient technique which contains detergent (SDS). These three different conditions of sedimentation were normalized by using three DNA markers: bacteriophage T4 and T7 and human adenovirus 2. The molecular weights of the double stranded DNA molecules of these markers are 1.34×10^8, 0.25×10^8 and 0.23×10^8 daltons, respectively.

The $S_{w,20}$ values for these markers under alkaline conditions can be claculated from equation (2) (see section 2.3.11) using the half-molecular weights corresponding to the single stranded forms. These $S_{w,20}$ walues are 70.1, 39.2 and 35.2 Svedbergs, for T4, T7 and Adenovirus 2.

In figure 14 are summarized data obtained when viral molecules were sedimented at different ω and t values in the SW 50.1 rotor (5 ml tubes) with detergent present in the gradients. If the peak in the sedimentation profile is taken as the position to which the DNA molecules sedimented, then equation (3) (see section 2.3.11.) can be used to calculate values of β for each combination of ω and t for each virus. In order to measure accurately the distance sedimented, d_i, (see section 2.3.11.), it was necessary to determine carefully the starting point for sedimenting material. To do this, unirradiated L-60 cells were lysed for the usual 12 hours on the top of the gradient, then without any centrifugation, fractions were collected and assayed for radioactivity. It was found that more than 75% of the total

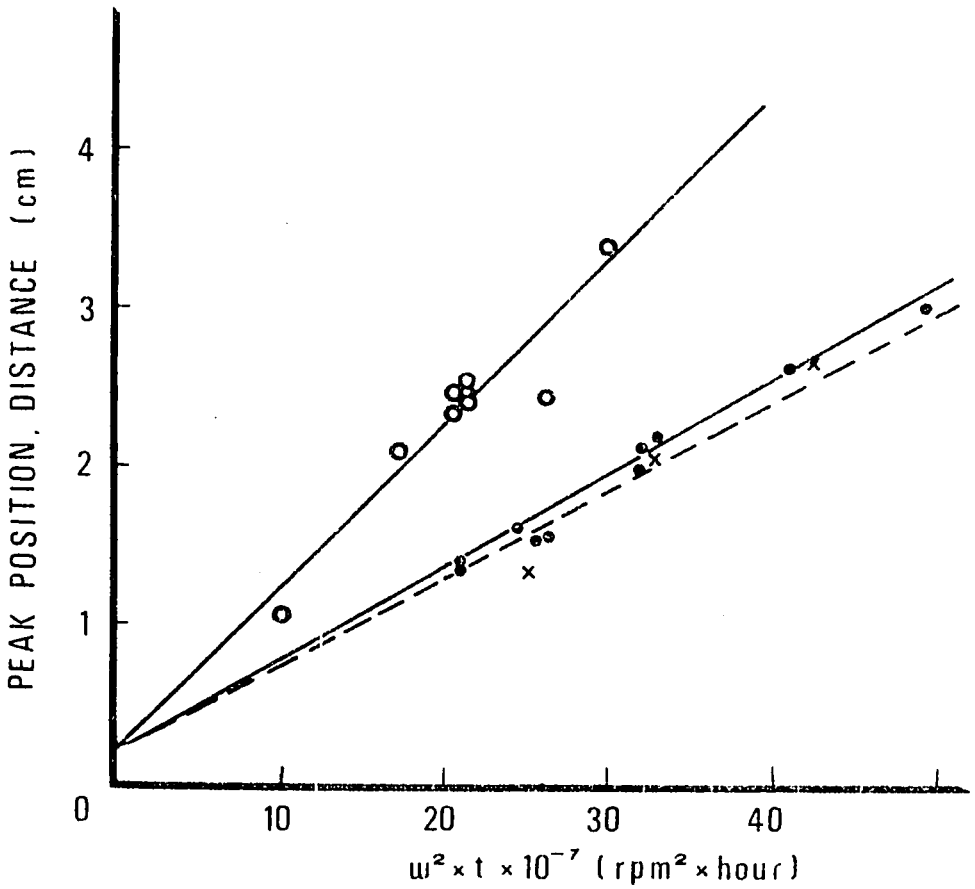


Figure 14. Normalization of gradients containing SDS, Beckman SW 50.1 rotor, 5 ml tubes.

Lysing solution: 0.5 M NaOH, 0.01 M EDTA, 0.2% SDS; gradients: 5-20% sucrose, 0.3 M NaOH, 0.001 M EDTA, 0.01% SDS. Viruses were lysed for 12 hours at 22°C and spun at different ω, and t values at 20°C.

-o-o- Bacteriophage T4
-●-●- Adenovirus 2
-x-x- Bacteriophage T7

Peak position is expressed in terms of the distance from the top of the lysing solution.

counts were equally divided between the first two fractions, in the 5 ml tubes, suggesting that the boundary between these two fractions represented the average starting point for the sedimenting DNA molecules. Since the first fraction contains only the lysing solution, this indicates that during lysis the DNA accumulates at the bottom of the lysing layer.

Consistent with this, one can see in figure 14 that extrapolation of the data to a peak position of 0.25 cm at $\omega^2 t = 0$, gives a reasonably good fit to the measured data. This position, again, corresponds to the boundary between the first and second fractions in the 5 ml tubes.

Values for β were calculated from the slopes of these lines and found to be 6.8×10^{10}, 7.0×10^{10} and 6.1×10^{10} for T4, T7 and Ad 2 respectively, when d is measured in cm, ω in r.p.m. and t in hours. The numerical average of these gives a value $\beta = 6.6 \times 10^{10}$ (To be strictly correct β is not dimensionless; its units are Svedbergs x $(\text{r.p.m.})^2$ x hours).

Figure 15 shows similar data measured in high salt gradients, again in 5 ml tubes (see section 2.4.). For T4, T7 and Ad 2 values for β of 6.3×10^{10}, $5.5. \times 10^{10}$ and 5.3×10^{10} are obtained respectively giving an average β of 5.7×10^{10}.

We also determined the starting point for the distribution in the 17 ml tubes. Here again unirradiated L-60 cells were lysed on the top of the gradient and the gradient was collected without

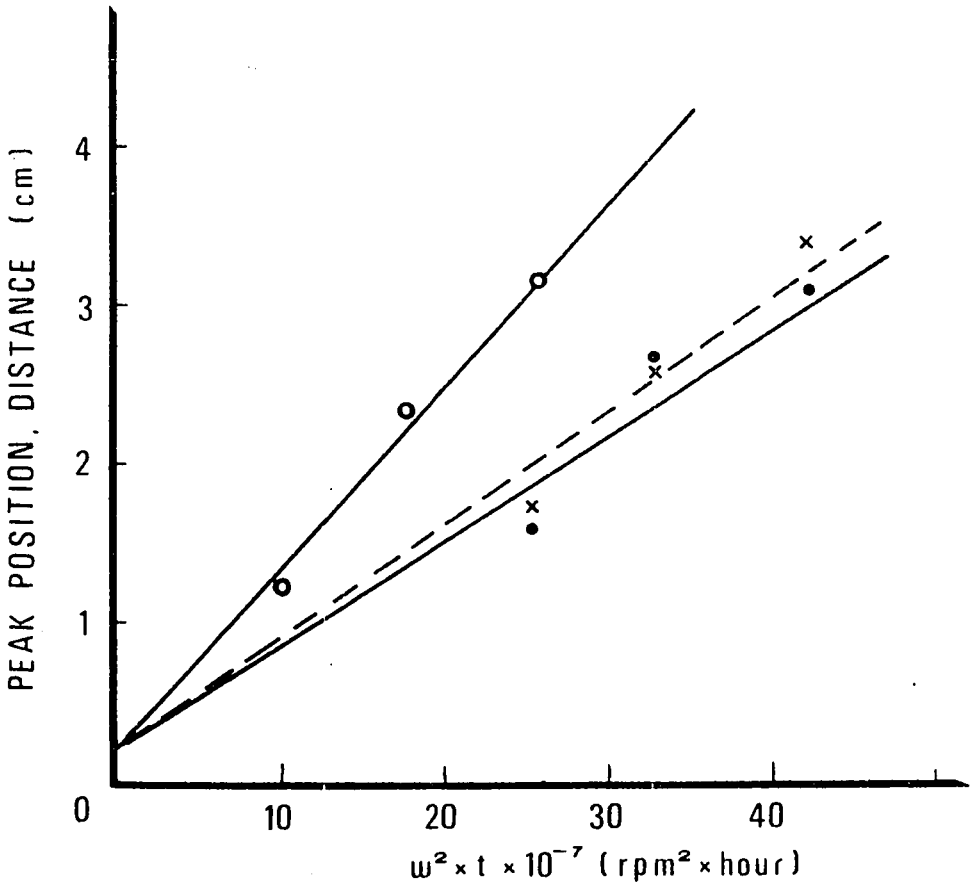


Figure 15. Normalization of high salt gradients, Beckman SW 50.1 rotor, 5 ml tubes.

Lysing solution: 0.45 M NaOH, 0.55 M NaCl, 0.01 M EDTA; gradients: 5-20% sucrose, 0.1 M NaOH, 0.9 M NaCl, 0.003 M EDTA. Viruses were lysed for 12 hours at 22°C and spun at different ω and t values at 20°C.

-o-o- Bacteriophage T4
-●-●- Adenovirus 2
-x-x- Bacteriophage T7

Peak position is expressed in terms of the distance from the top of the lysing solution.

centrifugation after the cells had lysed for 12 hours at 22°C. Here, more than 80% of the counts were found in the first fraction (0.75 ml) so the starting point of the profile was taken to be the middle of the first fraction. Figure 16 represents data measured in 17 ml tubes (SW 27 rotor). The middle of the first fraction corresponds to a peak position of 0.19 cm from the top of the lysing solution. It can be seen in this figure, that this assumption concerning the starting point fits well with the experimental peak positions measured for various combinations of ω and t for each of the three markers.

Values for β calculated from the slopes were found to be 6.7×10^{10}, 6.35×10^{10} and 6.48×10^{10} for T4, T7 and Ad 2, respectively and the numerical average of these values gives $\beta = 6.51 \times 10^{10}$ (ω in r.p.m., t in hours and d in cm).

2.3.11. Calculation of Molecular Weight

Weight average molecular weight is defined as

$$M_{weight} = \frac{\sum_i n_i M_i^2}{\sum_i n_i M_i} \qquad (1)$$

where n_i represents number of molecules in the i-th fraction and M_i the molecular weight of a molecule which sediments to the middle of the fraction. M_i is given by

$$M_i = \left(\frac{S_{i,w,20}}{a} \right)^k \qquad (2)$$

(Burgi and Hershey, 1963)

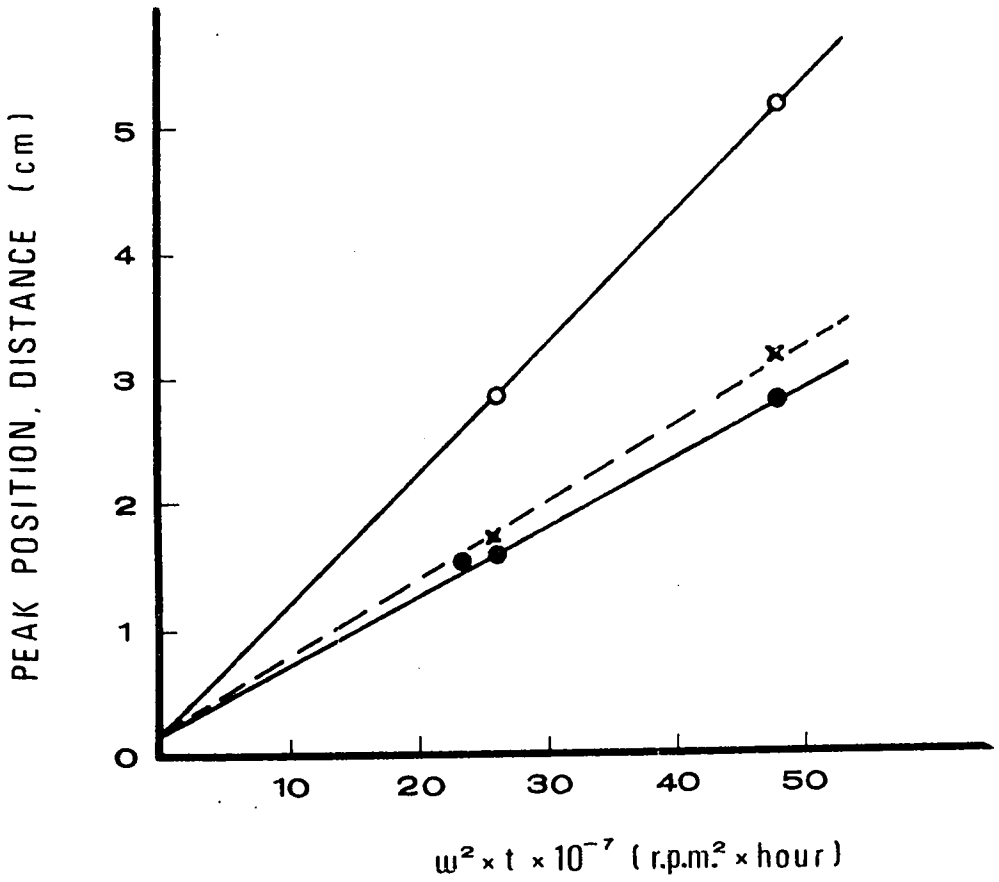


Figure 16. Normalization of gradients containing SDS, Beckman SW 27 rotor, 17 ml tubes.

Lysing solution: 0.5 M NaOH, 0.01 M EDTA, 0.2% SDS; gradients: 5-20% sucrose, 0.3 M NaOH, 0.001 M EDTA, 0.01% SDS. Viruses were lysed for 12 hours at 22°C and spun at different ω and t values at 20°C.

-o-o- Bacteriophage T4
-●-●- Adenovirus 2
-x-x- Bacteriophage T7

Peak position is expressed in terms of the distance from the top of the lysing solution.

where a and k are constants, 0.0528 and 2.5 respectively (Studier 1965) and $S_{i,w,20}$ is the sedimentation constant of that molecule

$$S_{i,w,20} = \frac{\beta\ d_i}{\omega^2\ t} \qquad (3)$$

where d_i represents the distance from the middle of the fraction to the point where the molecule started at the beginning of sedimentation, ω is the angular velocity in revolutions/minute and t, the time of sedimentation. β is a constant which must be measured for a given type of gradient by using DNA of known $S_{w,20}$ value (see section 2.3.10).

The corrected per cent total counts in each fraction is proportional to the number of molecules in that fraction multiplied by the molecular weight of molecules in that fraction. If we assume that all molecules in the i-th fraction are of the same molecular weight, M_i, and that all molecules of molecular weight M_i sediment only to the i-th fraction, then

$$C_i = K\ n_i\ M_i \qquad (4)$$

where K is a proportionality constant. Rearranged equation (4)

$$n_i = \frac{C_i}{K\ M_i} \qquad (5)$$

can be substituted along with equations (2) and (3), into equation (1) giving:

$$M_w = \left(\frac{\beta}{\omega^2 ta}\right)^k \frac{\sum_i C_i (d_i)^k}{\sum_i C_i} \qquad (6)$$

In section 2.3.10. it was shown that the point from which the DNA molecules began sedimenting in the 5 ml tubes was the beginning of the second fraction. Thus, material which sedimented to the middle of the second fraction sedimented 1/2 of the distance associated with one fraction, d_f; material in the third fraction sedimented a distance 3/2 d_f, etc. With this refinement equation (6) becomes

$$M_w = A_{\omega,t} \frac{\sum_i C_i (i - 1/2)^k}{\sum_i C_i} \qquad (6A)$$

where i is the fraction number counted from the second fraction collected, $A_{\omega,t} = \left(\frac{\beta\ df}{\omega^2 ta}\right)^k$ is a constant which takes different values depending on the conditions of sedimentation.

In 17 ml tubes the material is sedimented from the middle of the first fraction and so the material which sedimented to the second fraction travelled the distance associated with one fraction, d_f, material in the third fraction sedimented a distance of 2 x d_f etc., Equation (6) in this case becomes

$$M_w = A_{\omega,t} \frac{\sum_i C_i (i)^k}{\sum_i C_i} \qquad (6B)$$

where again i is the fraction number counted from the second fraction collected. Note that in the expression $A_{\omega,t} = \left(\frac{\beta \, d_f}{\omega^2 ta} \right)^k$ both β and d_f assumed new values for the larger tubes, although the gradient and lysing solutions were identical.

There were three different methods that we could have used to calculate the number average molecular weight. First,

$$M_n = \frac{\sum_i n_i \, M_i}{\sum_i n_i} \qquad (7)$$

where again n_i represents the number of molecules in the i-th fraction and M_i the molecular weight of a molecule which sediments to the middle of that fraction. Secondly, using equations (2), (3) and (5) we could rearrange (7) to give

$$M_n = \left(\frac{\beta}{\omega^2 ta} \right)^k \frac{\sum_i C_i}{\sum_i C_i \, (d_i)^{-k}} \qquad (8)$$

For 5 ml tubes the expression becomes

$$M_n = A_{\omega,t} \frac{\sum_i C_i}{\sum_i C_i \, (i - 1/2)^{-k}} \qquad (8A)$$

and for 17 ml tubes

$$M_n = A_{\omega,t} \frac{\sum_i C_i}{\sum_i C_i (i)^{-k}} \qquad (8B)$$

One can see from equations (8A) and (8B) that relatively few counts in the first few fractions (where i is small) can have a very large effect on the calculated value. Thus small errors in the counts measured in these fractions can contribute very large uncertainty to the value of M_n. This problem is compounded by the fact that the first few fractions (from the top) of any gradient are perhaps the most susceptible to error, because this is where any degradation products or unincorporated label will accumulate. For these reasons, equation (8A) and (8B) were not the method of choice for calculation of M_n in this thesis.

On the other hand, it has been shown by Charlesby (1954) that if the distribution is random (see 2.3.12. equation (11))

$$M_n = 0.5\ M_w \qquad (9)$$

and hence for all random distributions this is a more reliable method of calculating M_n since the first few fractions have little effect on the calculation of M_w (see equations (6A) and (6B) above).

The third method for the calculation of M_n utilizes the slope k_s of a plot of $\log \frac{Ci}{M_i\ \Delta M_i}$ against M_i (ΔM_i is defined in section 2.3.12. equation 14) which is a straight line if the distribution is random (see section 2.3.12.). This is evident from equation (12) in section 2.3.12. From that section equation (15) can be rearranged to give

$$M_n = -\frac{1}{2.3\ k_s} \qquad (10)$$

Equations (9) and (10) offer a more reliable method to obtain M_n for random distributions than does equation (8A) or (8B).

2.3.12. Test for Random Distribution of Molecular Sizes

It has been shown by Charlesby (1954) that regardless of the initial size distribution of any population of macromolecules if these macromolecules are cut by a process which introduces breaks in a random fashion (e.g. breaks produced in DNA by ionizing radiation) one can obtain the equation

$$\frac{\Delta N_i}{\Delta M_i} = K' \cdot \frac{1}{M_n^2}\ e^{-\frac{M_i}{M_n}} \qquad (11)$$

This constitutes a so-called random distribution where $\Delta N_i = n_i$, n_i represents the number of molecules of molecular weight M_i in the i-th fraction, K' is a proportionality constant and M_n is the number average molecular weight (see equation (7) section 2.3.11.). Substituting equation (5) into (11) gives

$$\frac{C_i}{M_i\ \Delta M_i} = KK'\ \frac{1}{M_n^2}\ e^{-\frac{M_i}{M_n}} \qquad (11A)$$

and the logarithm of equation (11A) gives

$$\log \frac{C_i}{M_i \, \Delta M_i} = - \frac{M_i}{2.3 \, M_n} + \frac{1}{2.3} \log \frac{KK'}{M_n 2} \qquad (12)$$

where

$$M_i = A_{\omega,t} \, (i - 1/2)^k \qquad (13)$$

and

$$\Delta M_i = A_{\omega,t} \, [i^k - (i - 1)^k] \qquad (14)$$

from equations (2) and (3). It is very important to note that for the analysis of the randomness of the distribution equations (13) and (14) are satisfied only if the fractions are divided in such a way that the first fraction (i = 1) starts from the point where the material begins sedimentation.

When $\log \frac{C_i}{M_i \, \Delta M_i}$ is plotted against M_i, one should obtain a straight line of slope

$$k_s = - \frac{1}{2.3 \, M_n} \qquad (15)$$

if the size distribution of the DNA is random. As was pointed out in section 2.3.11., the first few fractions from the top of any gradient are perhaps the most susceptible to error, and should be disregarded in the fitting of straight lines to these data.

2.4. HIGH SALT GRADIENTS

One of our basic concerns in this project was to know whether or not the information derived from our studies was peculiar to the sedimentation technique which we used. Of particular relevance here might be the presence of detergent in our lysing and gradient solutions. We therefore repeated the more crucial experiments using the technique developed by Elkind and Kamper (1970) and Elkind (1971) which employs high salt concentrations rather than detergent to dissociate nucleoprotein from DNA.

All of the experiments were done with 5 ml tubes in a Beckman SW 50.1 rotor. The lysing solution contained 0.45 M NaOH, 0.55 M NaCl and 0.01 M EDTA; gradients contained 5 - 20% sucrose, 0.1 M NaOH, 0.9 M NaCl and 0.003 M EDTA. Apart from different constituents in the lysing solution and the gradients, the rest of the conditions (lysing, centrifugation, collecting, etc.) were essentially the same as those described in section 2.3. In short, 1×10^4 cells were lysed for 12 hours at room temperature, 22°C, in a lysing solution on the top of the gradient. The gradients were spun at 20°C at different ω and t, chosen so that the peak of the distribution sedimented to a point somewhere between the 5th and 12th fraction.

In these experiments we followed Elkind's technique directly except that we generally used a lysis time of 12 hours where they generally used a lysis time of 4 hours. However,

Shipley and Elkind (1971) showed that lysis times in the range 4 hours to 24 hours all gave the same result. When unirradiated cells from either the L-60 or the Chinese hamster line were used with this technique, the DNA sedimented irregularly and most often was found at the bottom of the tube. Occasionally, however, the DNA would band in a very sharp peak (usually one fraction) similar to the complex peak of Elkind and Kamper. Some protein remained associated with the DNA even when cells were lysed for 18 hours at 25°C. A small amount of radiation, however, released the DNA from the complex yielding protein-free DNA which sedimented reproducibly. The distribution of the DNA molecules of irradiated cells was somewhat broader and it sedimented further than in the detergent-containing gradient. Consistent with this, β values determined by marker DNA molecules for these gradients were lower (section 2.3.10., figures 14 and 15).

This observation is also consistent with the reports (Studier 1969, Rosenberg and Studier 1969, Triebel and Reinert 1971) that single stranded DNA molecules show a pronounced folding which is dependent on the ionic strength. A decrease in the ionic strength is accompanied by an expansion of the DNA molecule while increasing ionic strength yields a more tightly folded molecule. Thus the addition of high concentrations of salt makes the molecules smaller and the sediment faster under otherwise identical conditions.

3. RESULTS

3.1. EFFECT OF OXYGEN ON SINGLE STRAND BREAK PRODUCTION BY IONIZING RADIATION IN MAMMALIAN CELLS

3.1.1. Effect on the Sedimentation Profiles of Different Doses of Radiation, Delivered under Aerobic or Anoxic Conditions

When the efficiency of the production of single strand breaks in DNA is studied in either bacterial cells or eukaryotic cells it is most important that none of the breaks are rejoined before they can be detected. It was reported by Lett et al.(1967) that when mammalian cells were exposed to ionizing radiation, the molecular weight of single stranded DNA molecules became smaller with increasing radiation dose, presumably due to the appearance of single strand breaks. It was also found that the irradiated cells were capable of rejoining these breaks under favorable conditions.

In all experiments which are reported in this section, we irradiated cells at 0°C since it had been suggested (Matsudaira et al. 1969, Dean et al.1969, Sawada and Okada 1970, Elkind 1971) that this temperature completely inhibits the rejoining process of single strand breaks in mammalian cells (see also sections 1.6 and 3.3.).

In figure 17 a and b some typical sedimentation profiles of single stranded DNA molecules are shown. L-60 cells were

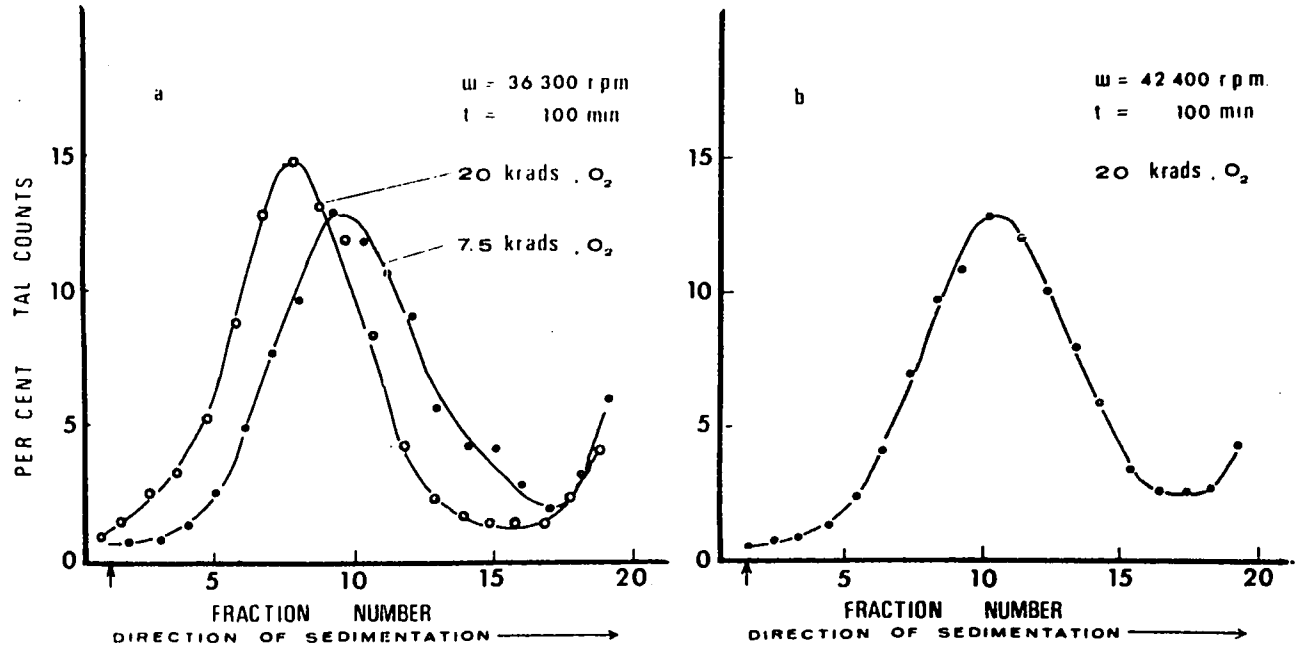


Figure 17; a, b, DNA sedimentation profiles of cells irradiated in the presence of oxygen; detergent containing gradients.

1×10^4 L-60 cells were lysed for 12 hours at 22°C in a lysing solution (0.5 M NaOH, 0.01 M EDTA, 0.2% SDS) on the top of an alkaline gradient (5-20% sucrose, 0.3 M NaOH, 0.001 M EDTA, 0.01% SDS). The indicated values of ω and t were chosen so as to obtain a peak position near fraction 10. The arrows indicate the position from which the DNA began sedimenting. Centrifugation in a Beckman SW 50.1 rotor (5 ml tubes) at 20°C.

irradiated in an oxygen atmosphere that is, 0_2 was flowed over the cell suspension as described in section 2.1.5. The cells were kept at 0°C until they were lysed on the top of the alkaline sucrose gradients. Figure 17 represents the profiles which were obtained by using 5 ml tubes (SW 50.1 Beckman). The samples were spun at values of $\omega^2 \times t$ chosen so that the peak position came near the 10th fraction. In figure 17 a are shown the profiles of cells irradiated with 20 krads and 7.5 krads; both samples were centrifuged under identical conditions. DNA from the cells receiving the lower dose sedimented much further indicating a larger molecular weight than the sample receiving the higher dose. In figure 17 b is shown the DNA sedimentation of cells receiving 20 krads as in figure 17 a except that in order to sediment the DNA to approximately the same position as for 7.5 krads (figure 17 a), a much higher centrifugal speed ω was required (t unchanged). This shows again the existence of smaller pieces of DNA molecules in the cells irradiated with the higher doses of radiation. The same effect can be seen in figure 7 (section 2.3.6.) where 17 ml tubes (Beckman SW 27) were employed. There, the comparison between the profiles of the DNA molecules of unirradiated and irradiated cells (5 krads) can be examined.

It should be noted that the results were the same if no oxygen was flowed above the stirred cell suspension during the

irradiation, that is, only air was present above the cell suspension.

The results of an experiment where high salt rather than detergent was used in the lysing and gradient solutions are presented in figure 18. The general description of the profiles in 17 a also applies here.

We also employed other cell lines; Chinese hamster cells $CH2B_2$ and human skin fibroblasts of the AN strain. The results were essentially the same as for L-60 cells.

When L-60 cells were irradiated under conditions similar to those used for survival measurements (Parker, Skarsgard and Emmerson 1969, Agnew and Skarsgard 1972) in an atmosphere free of oxygen we found much fewer single strand breaks produced as compared to the samples receiving the same dose in the presence of oxygen. Anoxic conditions were obtained by flowing pre-purified nitrogen (5 ppm 0_2 or less) over the stirred cell suspension for at least 15 minutes prior to and during irradiation.

In figure 19 a two sedimentation profiles are shown of L-60 cells irradiated with the same dose (7.5 krads) under aerobic and anoxic conditions. It can be seen that the DNA molecules from the cells irradiated in the presence of oxygen sedimented much more slowly thus indicating a lower molecular weight than the DNA from the cells which were irradiated under anoxic conditions. Figure 19 b shows the results obtained when two different doses were used to irradiate L-60 cells under

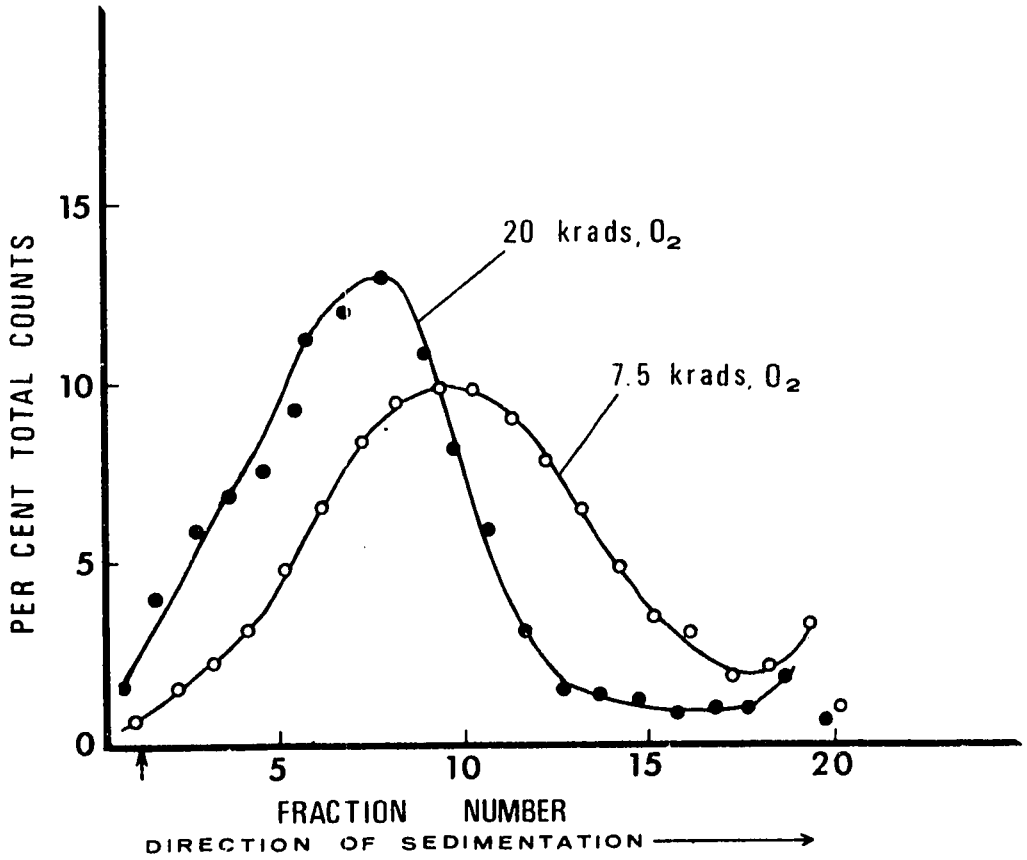


Figure 18. DNA sedimentation profiles of cells irradiated in the presence of oxygen; high salt gradients.

1×10^4 of L-60 cells were lysed for 12 hours at 22°C in a lysing solution (0.45 M NaOH, 0.55 M NaCl, 0.01 M EDTA) on the top of an alkaline gradient (5-20% sucrose, 0.1 M NaOH, 0.9 M NaCl, 0.003 M EDTA). Centrifugation at 31,900 r.p.m. for 100 minutes at 20°C in a Beckman SW 50.1 rotor (5 ml tubes).

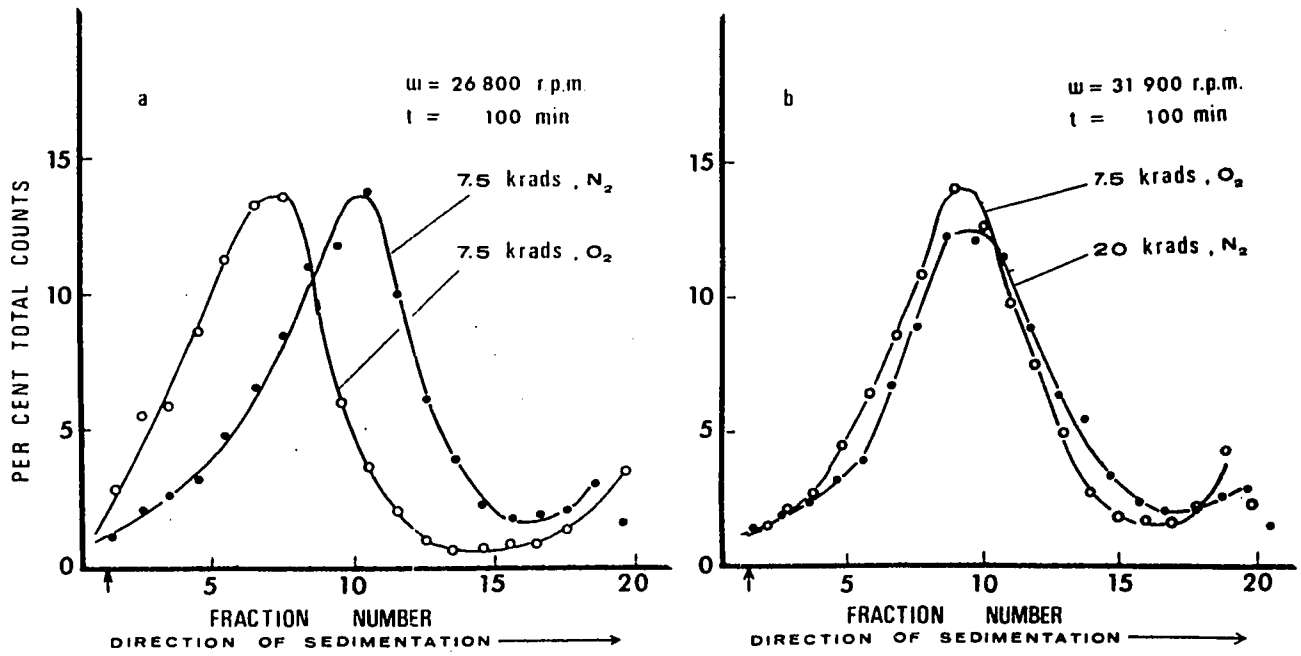


Figure 19; a, b. DNA sedimentation profiles of cells irradiated in the presence or absence of oxygen; detergent containing gradients.

1×10^4 L-60 cells were lysed for 12 hours at 22°C in a lysing solution (0.5 M NaOH, 0.01 M EDTA, 0.2% SDS) on the top of an alkaline gradient (5-20% sucrose, 0.3 M NaOH, 0.001 M EDTA, 0.01% SDS). The indicated values of ω and t were chosen so as to obtain a peak position between fractions 6 and 12. The arrows indicate the position from which the DNA began sedimenting. Centrifugation in a Beckman SW 50.1 rotor (5 ml tubes) at 20°C.

aerobic and anoxic conditions. Doses of 7.5 and 20 krads were used for aerobic and anoxic irradiation respectively, and the samples were then lysed on the top of two identical gradients. The gradients were then spun under the same conditions of centrifugation. Both the peak position and the shape of the sedimentation profiles are very similar for the two gradients though the ratio of the doses was 2.67; this suggests an oxygen enhancement ratio (OER), of that order of magnitude.

Figure 20 shows the same phenomenon when high salt containing gradients were used, rather than detergent. Here too, the ratio between the doses was 2.67, and the results again indicated an OER close to this value (see also section 3.1.3.).

Similar results were obtained when $CH2B_2$ or AN cells were used instead of L-60 cells.

In some prelimiary experiments of this type, we irradiated cell suspension in polystyrene containers (see section 2.1.5.). Here, nitrogen or oxygen was bubbled through the suspension for 1 hour prior to as well as during irradiation. In these experiments we did not find any difference between the oxygen and nitrogen samples which received the same dose.

It has been reported (Chapman, Sturrock, Boag and Crookall 1968, Boag 1969, Davies and Baker 1970) that radiobiologically significant amounts of molecular oxygen are released from polystyrene vessels so that systems which are supposedly anoxic may not

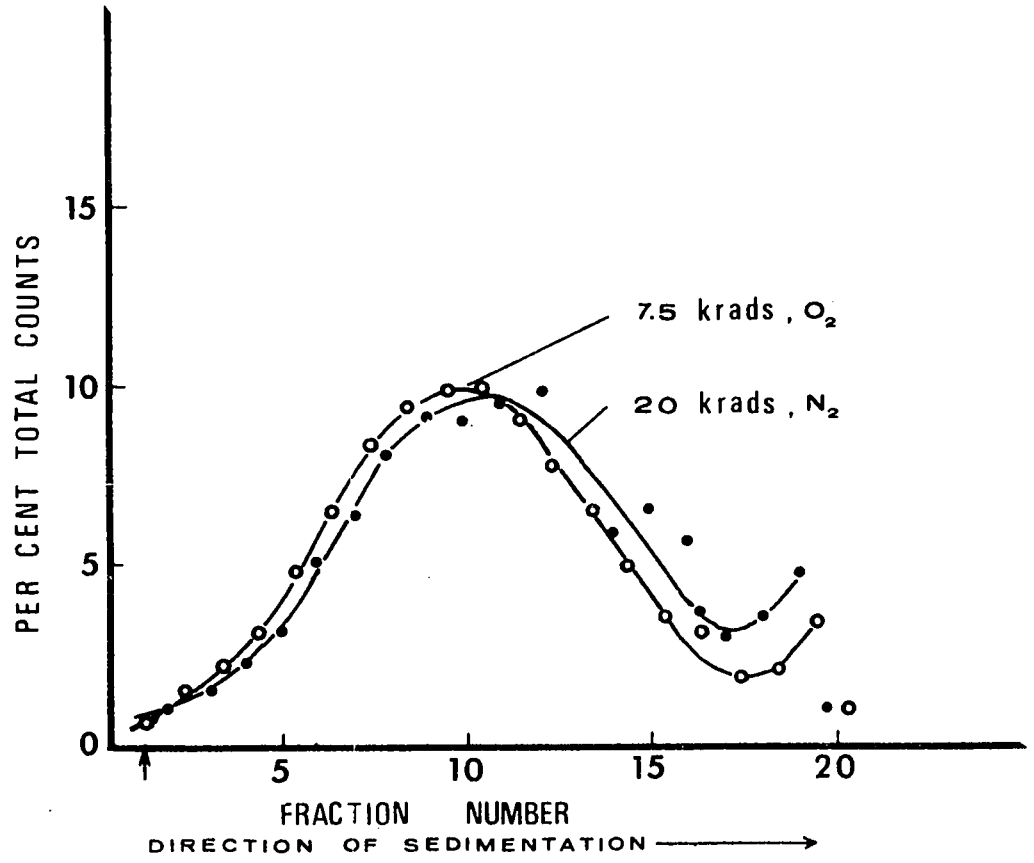


Figure 20. DNA sedimentation profiles of cells irradiated in the presence or absence of oxygen; high salt gradients.

1×10^4 L-60 cells were lysed for 12 hours at 22°C in a lysing solution (0.45 M NaOH, 0.55 M NaCl, 0.01 M EDTA) on the top of an alkaline gradient (5-20% sucrose, 0.1 M NaOH, 0.9 M NaCl, 0.003 M EDTA). Centrifugation at 31,900 r.p.m. for 100 minutes at 20°C in a Beckman SW 50.1 rotor (5 ml tubes).

actually be sufficiently free of oxygen to allow one to achieve a true anoxic response.

Similarly, we found that if tygon tubing was used in the gas lines in combination with the glass irradiation vessels, no oxygen enhancement was observed.

3.1.2. eV per Break Calculation and Oxygen Enhancement Ratio (OER)

In the previous chapter it was shown that the presence of O_2 caused a substantial increase in the yield of single strand breaks in the DNA of cells exposed to ionizing radiation. Figure 21 is a summary of the experiments done with L-60 cells in 5 ml tubes (Beckman SW 50.1 rotor) using the method which employs detergent in the lysing and gradient solutions. These experiments were done under both aerobic and anoxic conditions. In this figure, the inverse of the weight average molecular weight (M_w) is plotted against dose. Each plotted point is the result of one gradient. A straight line passing through $1/M_w = 0.2 \times 10^{-8}$ dalton $^{-1}$ at zero dose was fitted to the data by the method of least squares. The slopes of these lines are 1.70×10^{-12} and 0.58×10^{-12} dalton^{-1} rad^{-1} for O_2 and N_2 respectively. The oxygen enhancement ratio (OER) is the ratio of these two values, 2.9.

The dose (measured in rads, where 1 rad represents 100 ergs (0.625×10^{14}eV) of energy absorbed in 1 gm of the target) which doubles the value of $1/M_n$, produces one break in the DNA molecule. By using the equation (9) (see section 2.3.11.) the values for

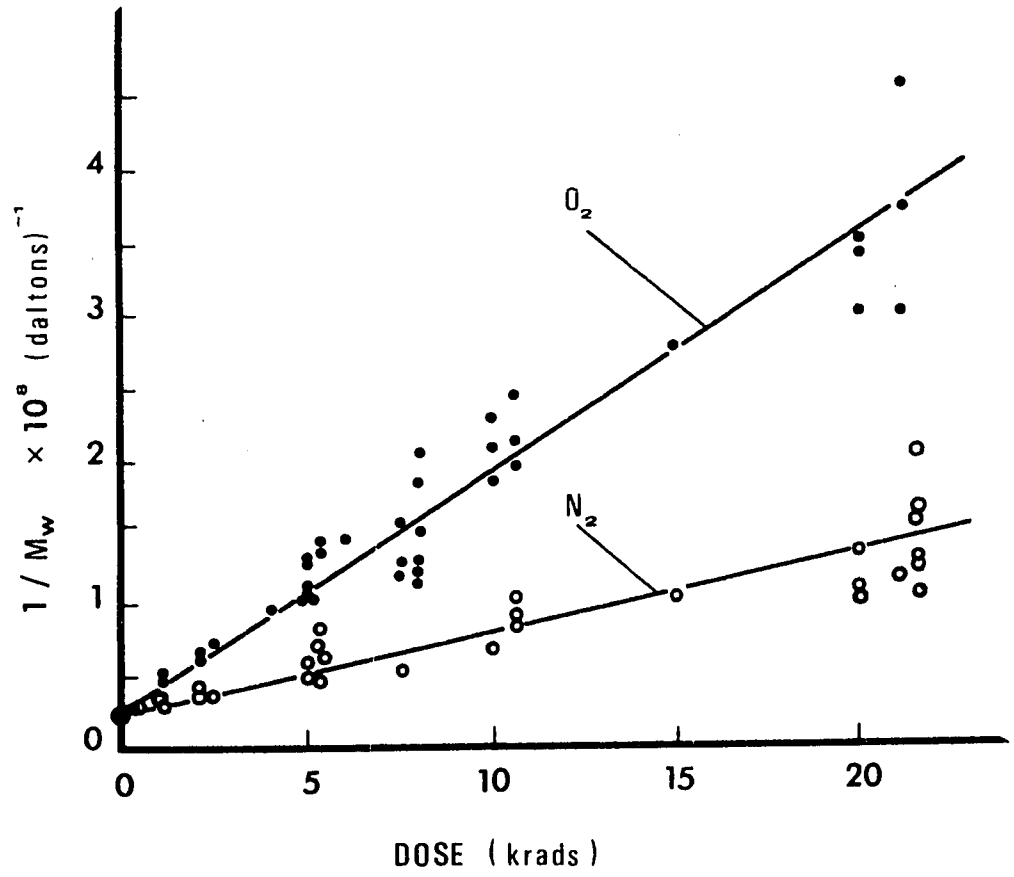


Figure 21. Oxygen effect and DNA single strand breaks, 5ml tubes; detergent containing gradients.

L-60 cells were irradiated under aerobic or anoxic (N_2) conditions and then 1 x 10^4 cells were lysed for 12 hours in a lysing solution (0.5 M NaOH, 0.01 M EDTA, 0.2% SDS) on the top of an alkaline gradient (5-20% sucrose, 0.3 M NaOH, 0.001 M EDTA, 0.01% SDS) at 22°C. Following this, the gradients were spun for various times at appropriate ω's at 20°C (SW 50.1 rotor). The weight average molecular weight was calculated according to the equation (6A); the lines are the least squares fit for a straight line through a fixed point (for unirradiated cells $1/M_w = 0.2 \times 10^{-8}$ dalton^{-1} gives the intercept).

$1/M_n$ can be calculated from $1/M_w$. From the slopes then, the energy which must be released in the DNA molecule to produce a single strand break was computed and the values obtained were 31 eV/break and 90 eV/break for aerobic and anoxic conditions, respectively.

The value of 31 eV/break in the presence of oxygen is lower than most other reported values in mammalian cells (Lett et al. 1967, Dean et al. 1969, Elkind and Kamper 1970, Lehman and Ormerod 1970a, McBurney, Graham and Whitmore 1971). The reports quote values which average about 60 eV/break.

We also carried out this experiment using 17 ml tubes in a Beckman SW 27 rotor. The results are presented in figure 22. The slopes of the curves, both under aerobic and anoxic conditions were again determined by the best fit of a straight line through a fixed point (0.2×10^{-8} daltons^{-1} for single strand DNA molecules of unirradiated cells). The values of these slopes are 1.48×10^{-12} and 0.475×10^{-12} dalton^{-1} rad^{-1}. The ratio of these two numbers was calculated, giving an OER of 3.1. From the slopes eV/break values were computed to be 35.2 eV/break when oxygen is present and 110 eV/break in the absence of oxygen (N_2 atmosphere). Though the value of 35 eV/break is a little higher than that obtained with 5 ml tubes, the eV/break value in the presence of oxygen is still in the same range as that obtained with 5 ml tubes. Though the results obtained with the 17 ml tubes were perhaps more reliable, there were more measurements done with the 5 ml tubes; the

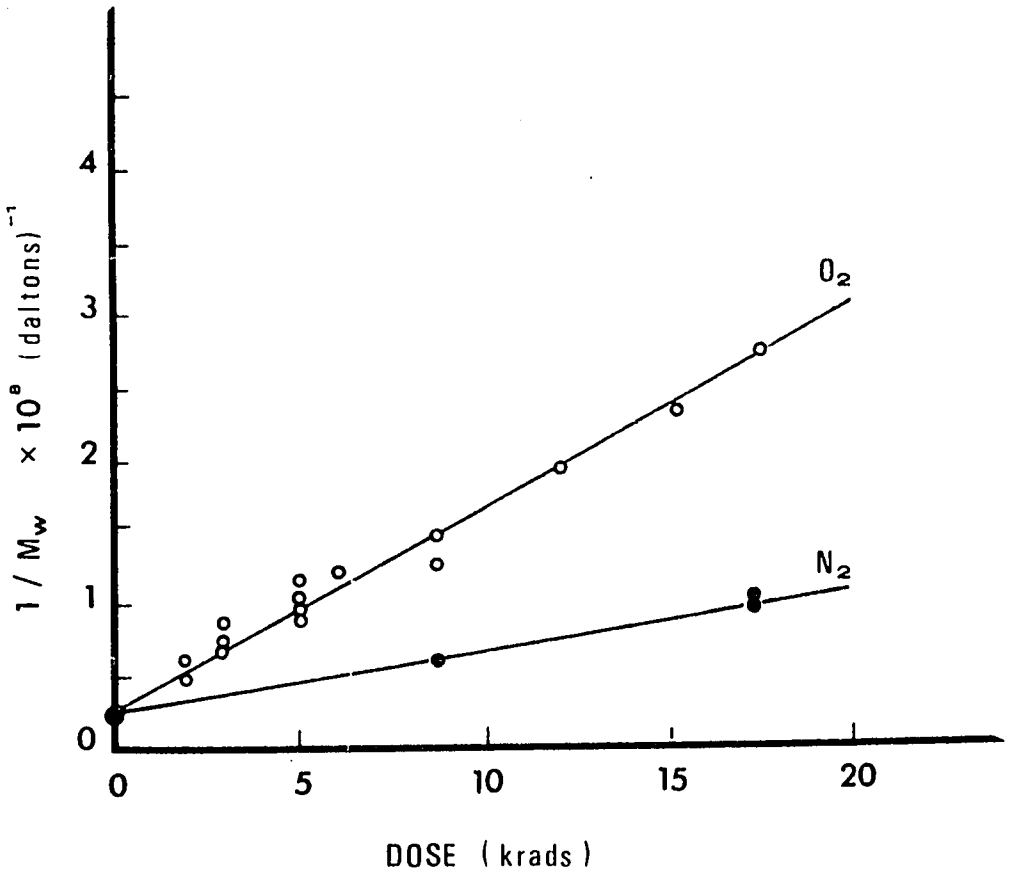


Figure 22. Oxygen effect and DNA single strand breaks, 17 ml tubes; detergent containing gradients.

L-60 cells were irradiated under aerobic (o) or anoxic (N_2) conditions (•) and then 2 x 10^4 cells were lysed for 12 hours in a lysing solution (0.5 M NaOH, 0.01 M EDTA, 0.2% SDS) on the top of an alkaline gradient (5-20% sucrose, 0.3 M NaOH, 0.001 M EDTA, 0.01% SDS) at 22°C. Following this, the gradients were spun for various times at appropriate ω's at 20°C (Beckman SW 27 rotor). The weight average molecular weight was calculated according to the equation (6B); the lines are the least squares fit for a straight line through a fixed point (for unirradiated cells $1/M_w = 0.2 \times 10^{-8}$ dalton^{-1} gives the intercept).

average value of 33 eV/ break was calculated from the results obtained with both centrifuge tubes and this value was further used.

In figure 23 are plotted the results obtained when cells were irradiated under aerobic conditions and then lysed for 3 hours or 6 hours, as opposed to the standard lysing time of 12 hours. The number of breaks measured is the same for all lysis times in that the points plotted in this figure (representing) 3 hours and 6 hours of lysis) coincide quite closely with the straight line which is a replot of the 0_2 line in figure 22 (12 hours lysis).

We also used other mammalian cells: Chinese hamster $CH2B_2$, and human fibroblasts of the AN strain. The eV/break value was of the same magnitude as with L-60 cells. Figure 24 shows the results for Chinese hamster cells. Both lines are again the best fit through a fixed point from figure 22 for L-60 cells.

In figure 25 results are shown where L-60 cells were irradiated under aerobic or anoxic conditions,and then lysed on the top of alkaline gradients. Here, however, the lysing and gradient solutions contained a high salt concentration rather than detergent. The points in the figure represent experiments

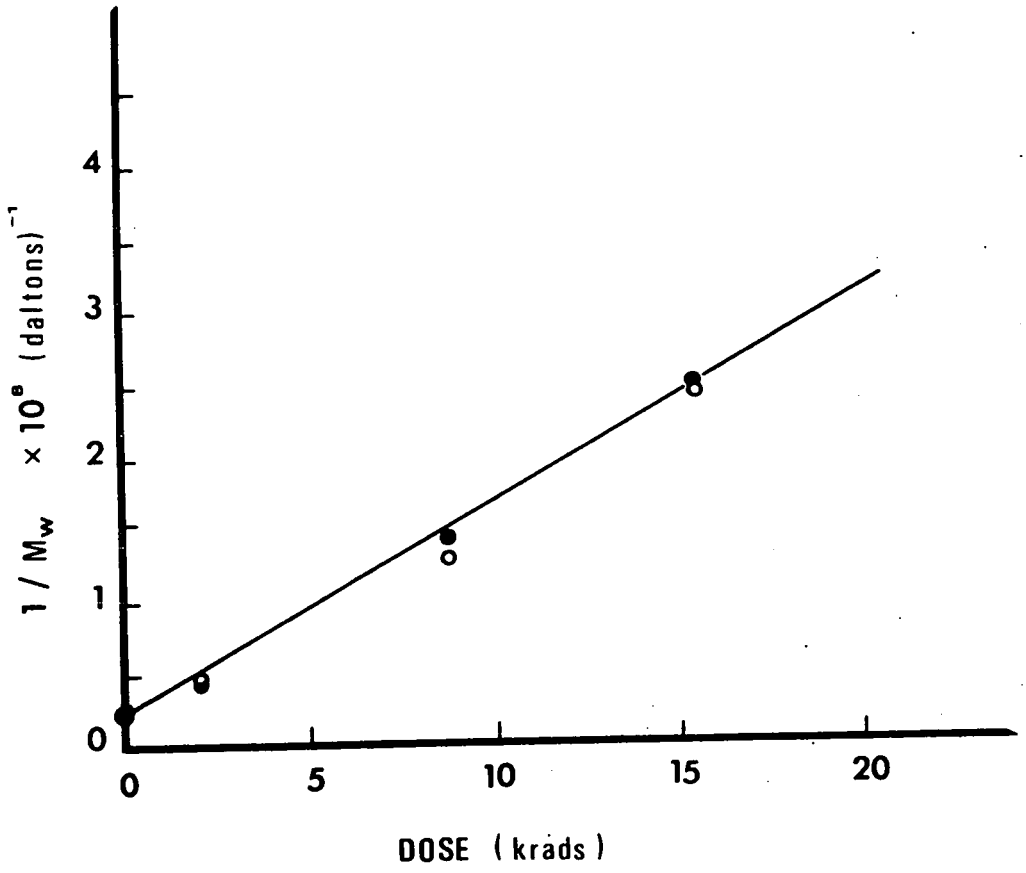


Figure 23. Short lysing times and single strand breaks, after irradiation under aerobic conditions; 17 ml tubes, detergent containing gradients.

L-60 cells were irradiated in the presence of oxygen and then 2 x 10^4 cells were lysed for 6 hours (o) or 3 hours (•) in a lysing solution (0.5 M NaOH, 0.01 M EDTA, 0.2% SDS) on the top of an alkaline gradient (5-20% sucrose, 0.3 M NaOH, 0.001 M EDTA, 0.01% SDS) at 22°C. Following this, the gradients were spun for various times at appropriate ω's at 20°C (Beckman SW 27 rotor). The weight average molecular weight was calculated according to the equation (6B); the line represents that one of O_2 from figure 22.

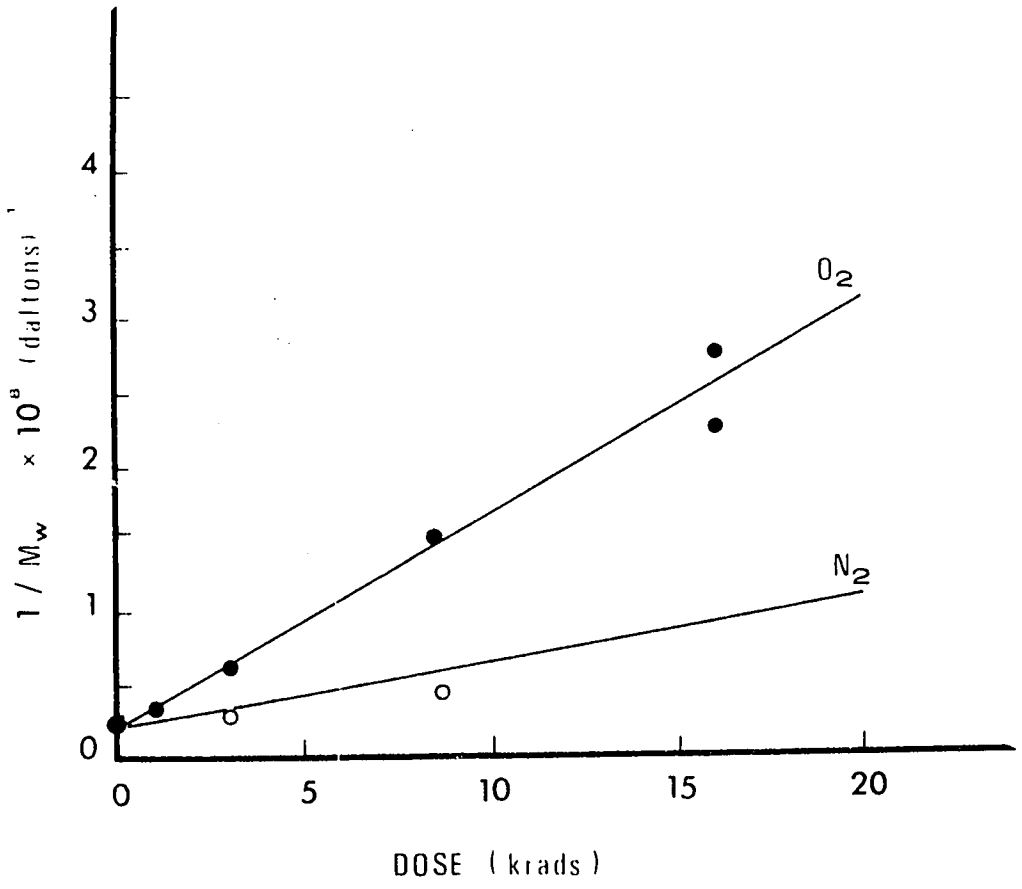


Figure 24. Oxygen effect on DNA single strand breaks in Chinese hamster cells, 17 ml tubes; detergent containing gradients.

$CH2B_2$ cells were irradiated under aerobic (•) or anoxic (N_2) conditions (o) and then 2 x 10^4 cells were lysed for 12 hours in a lysing solution (0.5 M NaOH, 0.01 M EDTA, 0.2% SDS) on the top of an alkaline gradient (5-20% sucrose, 0.3 M NaOH, 0.001 M EDTA, 0.01% SDS) at 22°C. Following this, the gradients were spun for various times at appropriate ω's at 20°C (Beckman SW 27 rotor). M_w was calculated using equation (6B). The lines are those from equivalent experiments with L-60 cells in figure 22.

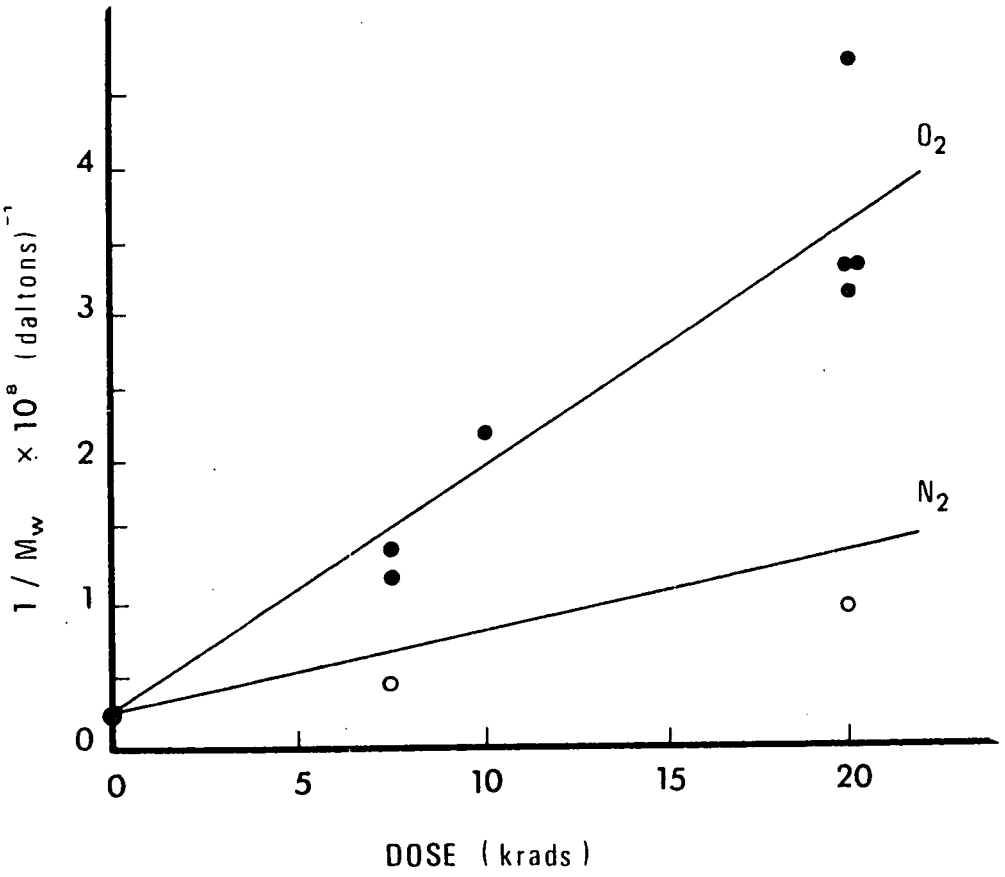


Figure 25. Oxygen effect and DNA single strand breaks, 5 ml tubes; high salt gradients.

L-60 cells were irradiated under aerobic (•) or anoxic (N_2) conditions (°) and then 1 x 10^4 cells were lysed for 12 hours in a lysing solution (0.45 M NaOH, 0.55 M NaCl, 0.01 M EDTA) on the top of an alkaline gradient (5-20% sucrose, 0.1 M NaOH, 0.9 M NaCl, 0.003 M EDTA). Following this, the gradients were spun for various times at appropriate ω's at 20°C (Beckman SW 50.1 rotor). The weight average molecular weight was calculated according to the equation (6A). The broken lines are the least squares fit for a straight line through a fixed point (for unirradiated cells, $1/M_w = 0.2 \times 10^{-8}$ dalton^{-1} gives the intercept). Solid lines are those from similar experiments in detergent containing gradients in figure 21.

done with 5 ml tubes (Beckman SW 50.1 rotor) and the lines are those from figure 21 where the experiments were performed under identical conditions except that detergent rather than high salt concentration was used. Though the points are somewhat scattered they do not deviate significantly from the straight lines. When a straight line is fitted to the oxygen points the resulting efficiency of breakage is again 31 eV/break, the same as we found in 5 ml gradients containing detergent.

Both nitrogen points are lower than expected, perhaps indicating an even larger OER. However, it should be noted, that in this particular experiment, both the detergent and high salt gradients gave lower than usual $1/M_w$ values. The detergent points are nevertheless included in figure 21.

3.1.3. Test for Random Distribution of Molecular Sizes

Dean, Ormerod, Serianni and Alexander (1969) pointed out the need to determine whether measured sedimentation profiles adequately follow Poisson statistics when cells are irradiated with ionizing radiation. A linear-linear plot of the profiles in the form of equation (12) in section 2.3.12. (i.e. $\log \frac{C_i}{M_i \Delta M_i}$ vs. M_i) should yield a straight line, if the size distribution of molecules is random.

As an example in figures 26 a, b, c, and d the profiles of figures 19 a and b are analyzed by this method. For all

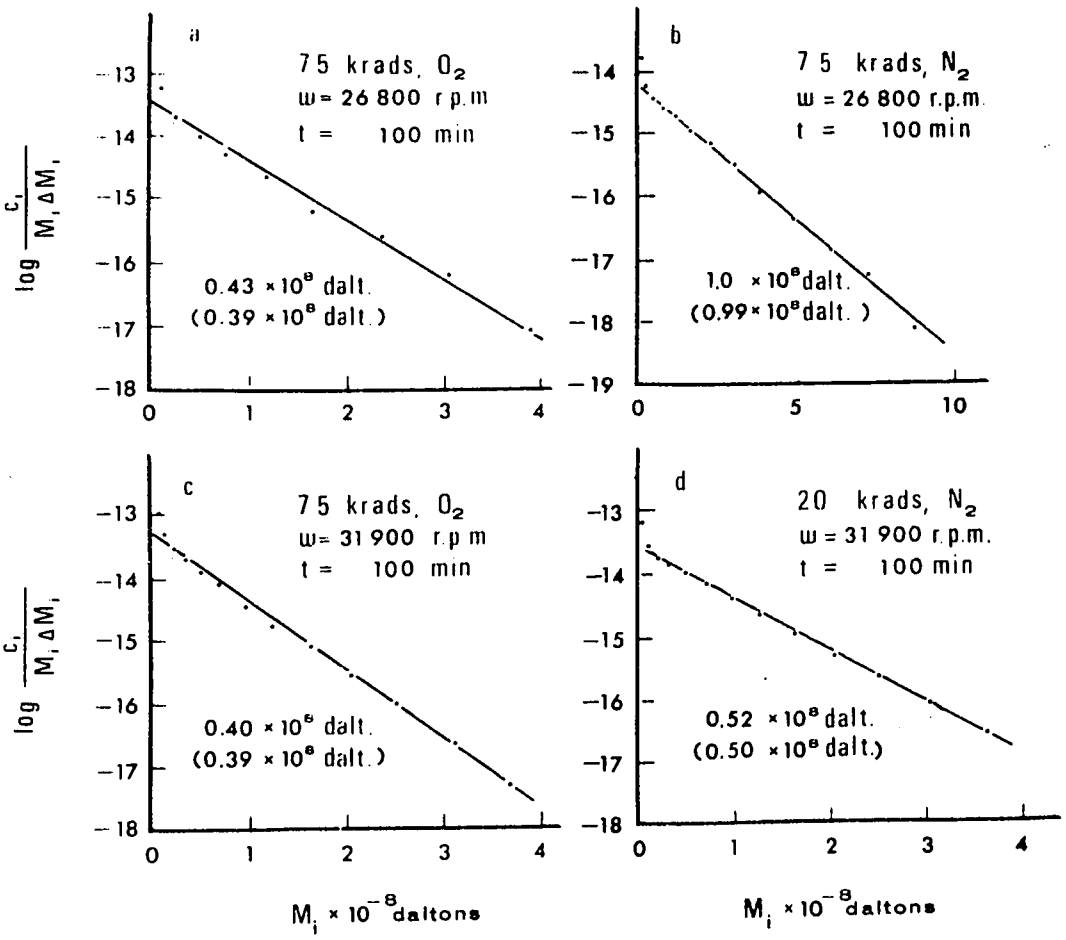


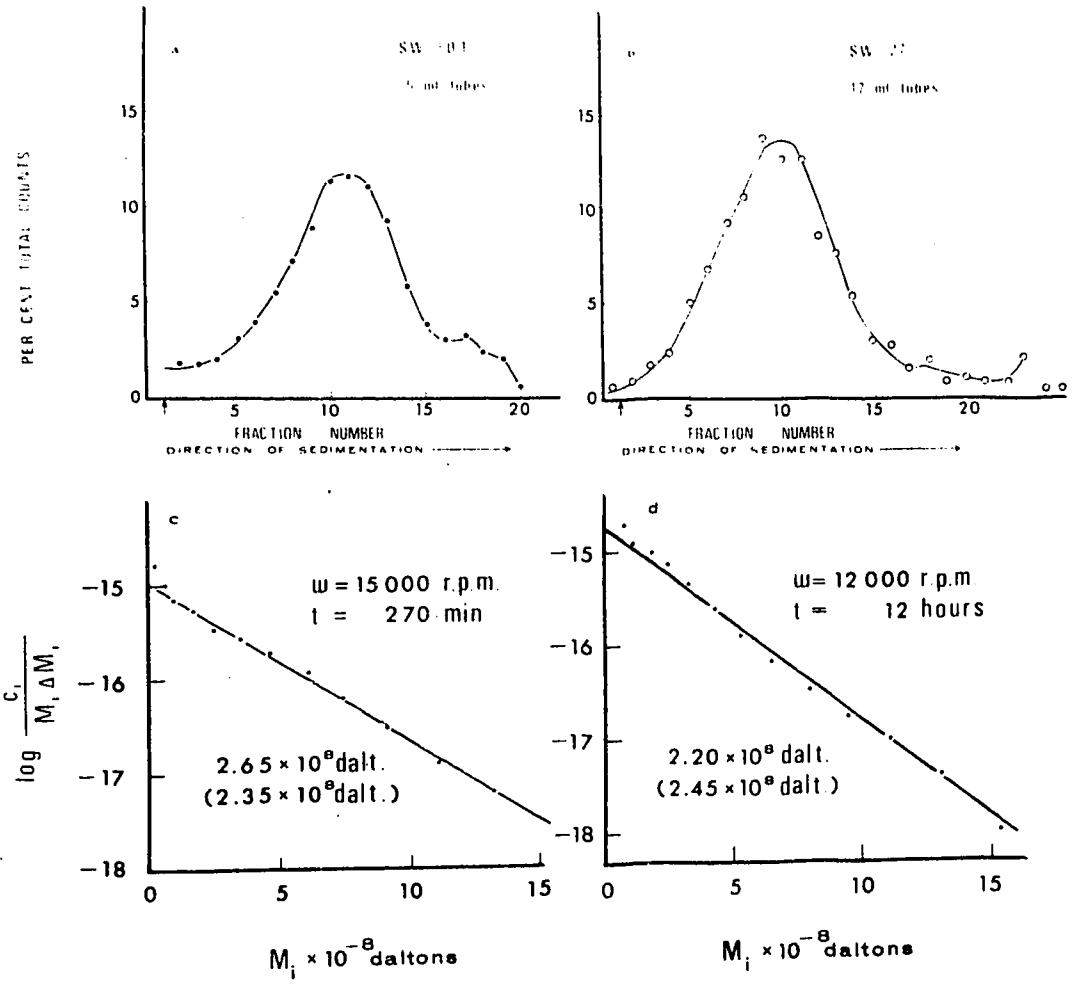
Figure 26. Test for random distribution of DNA molecular sizes from irradiated cells.

Profiles of figures 19 a, b were analyzed for randomness by equation (12). Values for $M_n = -1/2.3k_s$ are given for each profile. The comparison, values of $M_n = 0.5\ M_w$ are shown in parentheses.

points except the first 2 to 3 points (corresponding to the first 2 to 3 fractions), the data are quite adequately represented by a straight line, indicating then, that the DNA profiles represent random distributions. As was pointed out in 2.3.11. the first few fractions in the gradient are anomalously sensitive to error and it is justifiable to ignore them.

It is important to note that this is true for profiles of DNA molecules of cells irradiated under aerobic or anoxic conditions. The values for the number average molecular weight obtained from the slopes of the lines in figure 26 were compared and found to be in good agreement with those obtained by using equations (6A) and (9) and are given in parenthesis in the figure.

The same test for the randomness of the distribution of DNA molecules can be applied on the profiles obtained from unirradiated cells. In figure 27 a and b, two typical profiles from L-60 cells are plotted, one obtained with 5 ml tubes and the other with 17 ml tubes. The two profiles were then analyzed for randomness in figure 27 c and d. One observes that even in unirradiated cells the profiles appear to represent a random distribution of molecules. Again, the number average molecular weights which were obtained either from the slopes of figure 27 or by using equations (6A or 6B) and (9) were not significantly different.



Figures 27 a, b, c, d. Test for random distribution of DNA molecular sizes in unirraidated cells.

L-60 cells were lysed in a lysing solution (0.5 M NaOH, 0.01 M EDTA, 0.2% SDS) on the top of an alkaline gradient (5-20% sucrose, 0.3 M NaOH, 0.001 M EDTA, 0.01% SDS) in either 5 ml tubes or 17 ml tubes and were lysed for 12 hours at 22°C. Gradients were then spun at 15,000 r.p.m. for 4.5 hours in a Beckman SW 50.1 rotor, 5 ml tubes (a) or 12,000 r.p.m. and 12 hours in Beckman SW 27 rotor, 17 ml tubes (b). Both profiles were then analyzed for randomness using equation (12). Values for $M_n = -1/2.3k_s$ are given for each profile in (c) and (d) and are compared with values of $M_n = 0.5\ M_w$, shown in parentheses.

3.1.4. Effect of the Presence of EDTA during Irradiation

It was first reported by Dean et al. (1969) using bacterial cells M. radiodurans that some single strand breaks could be rejoined even when the cells were kept at 0°C. These results were later confirmed by others (Lehnert and Moroson 1971) in E. coli cells. The same authors found, however, that a high concentration of EDTA, e.g. 2×10^{-2} molar or higher, inhibits this rejoining process regardless of the temperature of the post irradiation incubation.

Any rejoining of single strand breaks which takes place during or after irradiation (until the cells are lysed on the top of a gradient) would seriously interfere with the observations and cause considerable error in the calculated eV/break values (see section 3.1.2.). Thus we also tested the effect of the presence of 2×10^{-2} M EDTA in some of our experiments. Figure 28 shows a comparison of two profiles in one of which the cells were treated with 2×10^{-2} M EDTA in PBS. The cells were then irradiated with 10 krads under aerobic conditions with EDTA still present. We found no differences in the sedimenting profiles (or calculated molecular weights) indicating that no rejoining had taken place at 0°C in L-60 cells. This is consistent with results reported in the literature (Matsudaira et al. 1969, Dean et al. 1969, Sawada and Okada 1970, Elkind 1970), and also with our own observations. No rejoining of single strand breaks was found to take place at 0°C,

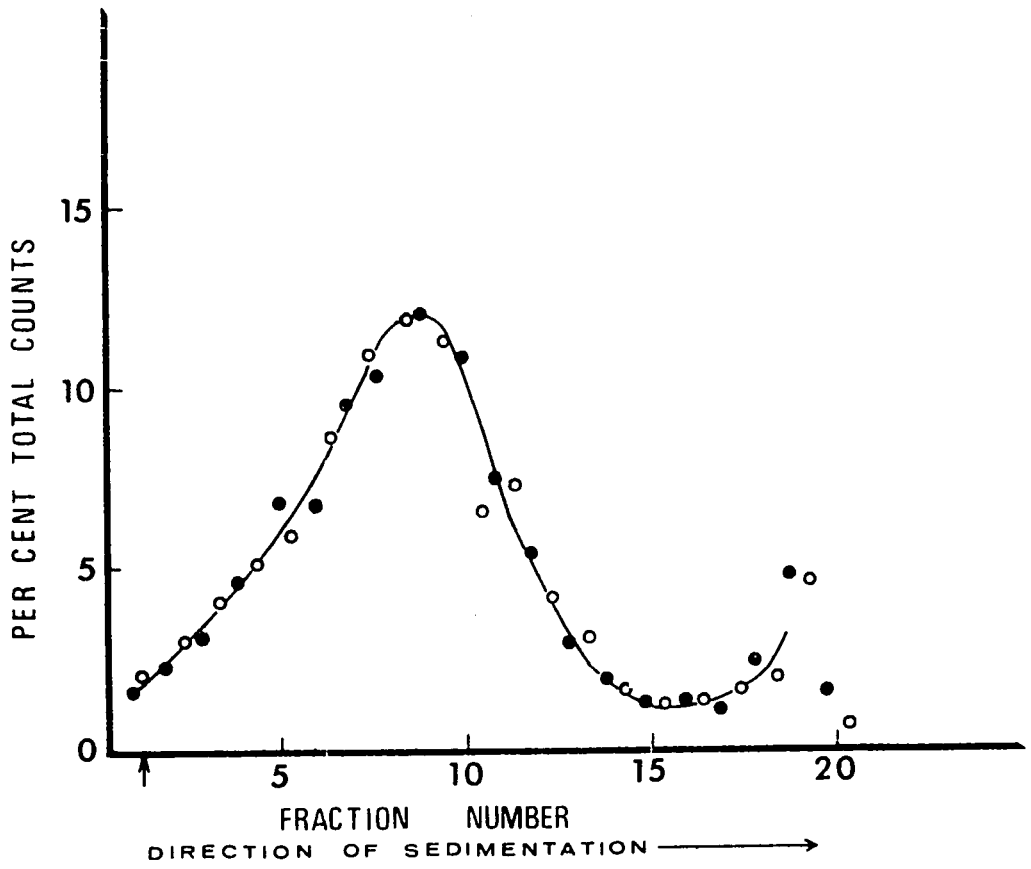


Figure 28. DNA sedimentation profiles of irradiated cells (10 Krads, aerobic conditions) with EDTA treatment.

L-60 cells were suspended in PBS containing 2×10^{-2} M of EDTA (•) or in PBS alone (o); the cells were then irradiated at 0°C, and kept at this temperature until they were lysed in a lysing solution (0.5 M NaOH, 0.01 M EDTA, 0.2% SDS) on the top of an alkaline gradient (0.3 M NaOH, 0.001 M EDTA, 0.1% SDS) for 12 hours at 22°C. The gradients were then spun for 1.5 hours at 30,000 r.p.m. at 20°C in a Beckman SW 50.1 rotor (5 ml tubes).

in any of the three mammalian cell lines which we used in our experiments; this was true even for periods longer than 4 hours (see sections 3.3.1. and 3.3.2.).

3.2. EFFECT OF THE METABOLIC INHIBITOR 2,4-DNP ON THE PRODUCTION OF SINGLE STRAND BREAKS IN MAMMALIAN CELLS.

3.2.1. Effect of 2,4-DNP on DNA, RNA and Protein Synthesis and on the ATP Level in Treated L-60 Cells.

In order to test the hypothesis of Dalrymple et al. 1968 and 1970, we treated mammalian cells with 2,4-dinitrophenol (DNP) prior to and during the irradiation. Their hypothesis proposes that single strand breaks in DNA are generally not produced by direct action of ionizing radiation, but rather, they result primarily from the action of nucleolytic enzyme(s) which act after irradiation. DNP, which inhibits metabolic processes by uncoupling oxidative phosphorylation, can, according to these authors (Dalrymple et al. 1969 a, b, Dalrymple et al. 1970), inhibit the action of the nucleolytic enzyme(s) and hence a treatment of the cells with DNP should prevent the appearance of single-strand breaks after irradiation.

In figure 29 are shown the rates of DNA, RNA and protein synthesis in the presence and absence of DNP. L-60 cells were exposed to either 1×10^{-4} M DNP or 5×10^{-4} M DNP in PBS or just to PBS (controls) for 1 hour at 37°C prior to these measurements.

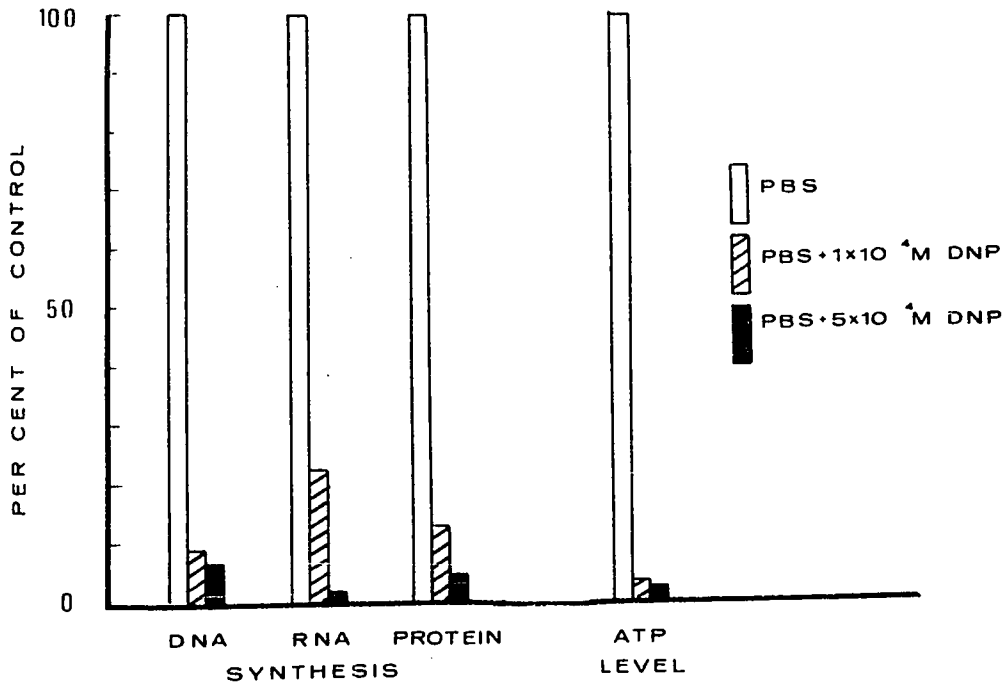


Figure 29. Effect of DNP on macromolecular synthesis and ATP level.

L-60 cells were washed and suspended in PBS (1×10^6 cells/ml) or in PBS containing DNP. After incubation for 1 hour at 37°C, the cells were pulse-labelled for 30 minutes with H^3-TdR, H^3-uridine or C^{14} L-leucine in order to measure DNA, RNA, or protein synthesis respectively. The ATP level was measured by the luciferase method.

Also given in figure 29 is the intracellular ATP level in treated and untreated cells. At the concentrations of 1×10^{-4} M and 5×10^{-4} M DNP, DNA synthesis falls respectively to 9% and 6% of the level in control cells, RNA synthesis to 22.5% and 2%, and protein synthesis to 13% and 4%, respectively. The ATP level was reduced to 3% and 2%, respectively. These results are comparable to those obtained in similar studies (Baker, Dalrymple, Sanders and Moss 1970, Moss, Dalrymple, Sanders, Wilkinson and Nash 1971).

3.2.2. Sedimentation Profiles from Irradiated or Unirradiated Cells Treated with 2,4-DNP

In figures 30 and 31 the DNA sedimentation profiles of untreated cells are compared to those of cells treated with 1×10^{-4} M DNP. The cells were exposed to 0, 2, 10 or 20 krads under aerobic conditions. At any given dose, the presence of DNP had no effect on either the peak position or the shape of the profile.

When the cells were treated with 5×10^{-4} M DNP the results were exactly the same as those described for cells treated with 1×10^{-4} M DNP (see also figure 32, section 3.2.3.).

3.2.3. eV per Break and OER in DNP Treated Cells

In section 3.1.3. we described the procedure whereby we determined the eV/break and the OER for irradiated cells. The data from figure 21 provides the eV/break value.

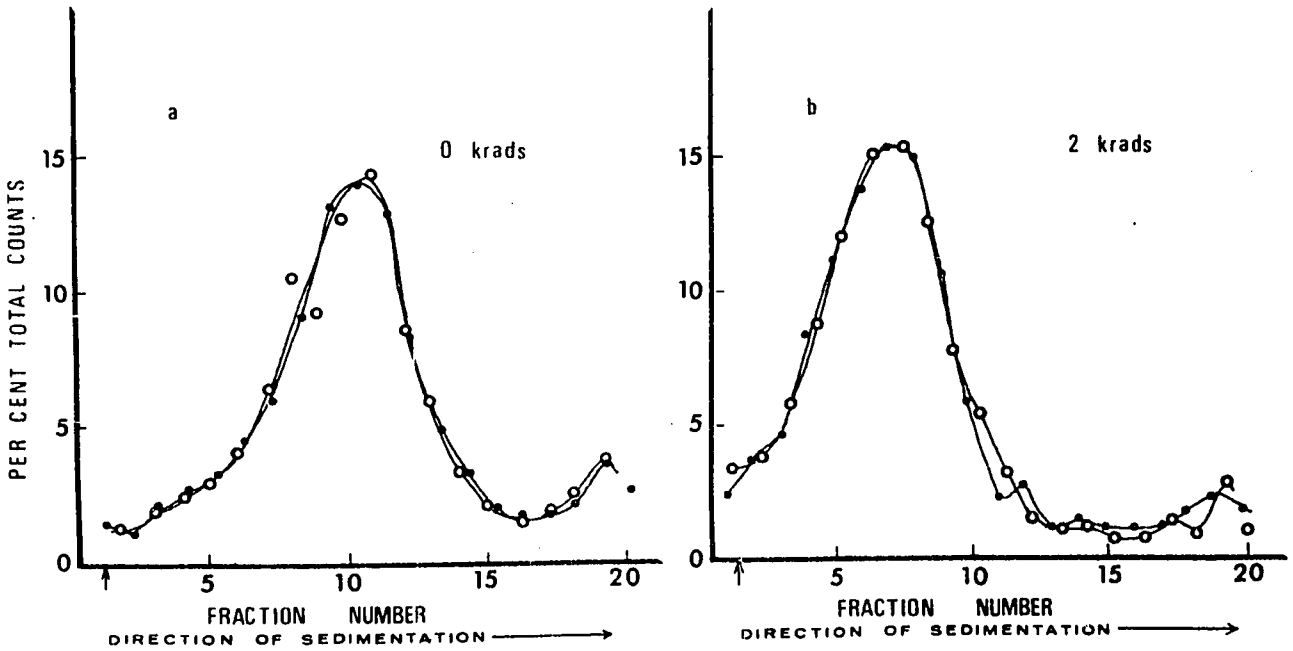


Figure 30 a, b. DNA sedimentation profiles of irradiated and unirradiated cells treated with 1 x 10^{-4} M DNP.

L-60 cells were suspended in PBS containing 1 x 10^{-4} M DNP (•) or in PBS alone (○). The samples were then incubated for 1 hour at 37°C, cooled to 0°C and exposed to (a) 0 krads or (b) 2 krads under aerobic conditions. Following this, 1 x 10^4 cells were lysed for 12 hours at 22°C in a lysing solution (0.5 M NaOH, 0.01 M EDTA, 0.2% SDS) on the top of an alkaline gradient (5-20% sucrose, 0.3 M NaOH, 0.001 M EDTA, 0.01% SDS). The gradients were centrifuged in a Beckman SW 50.1 rotor (5 ml tubes) at 20°C and ω = 18,000 r.p.m., t = 180 minutes.

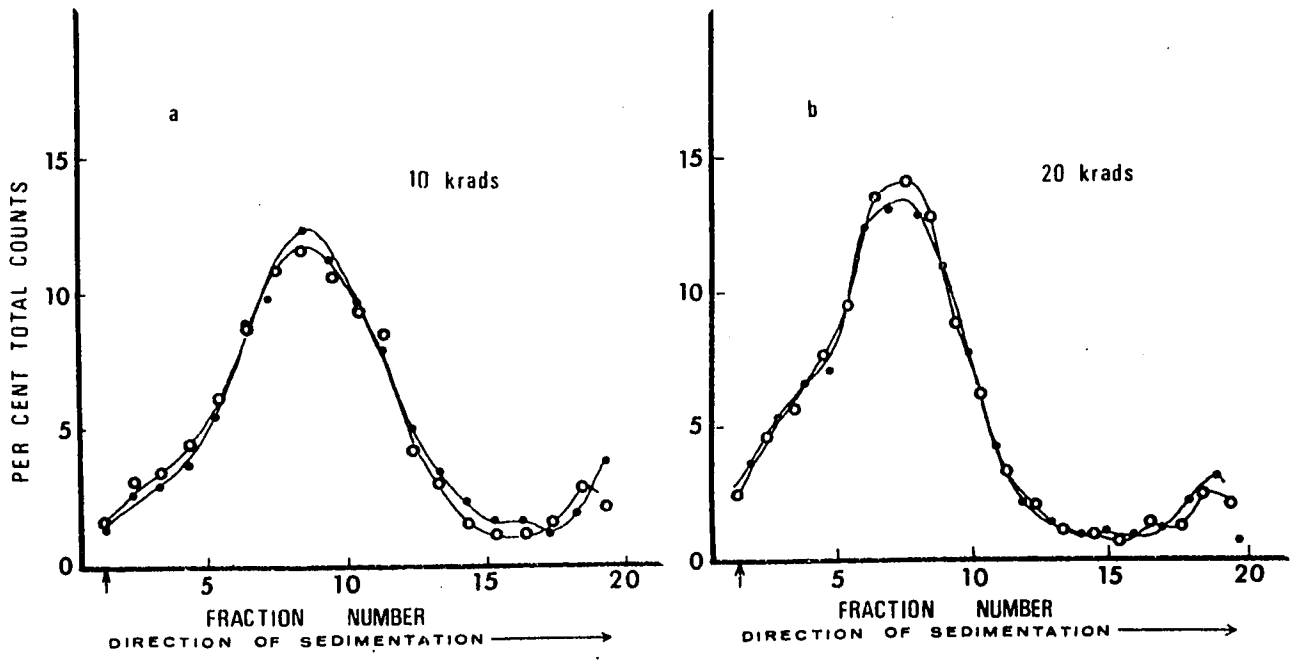


Figure 31 a, b. DNA sedimentation profiles of irradiated cells, treated with 1 x 10^{-4} M DNP.

L-60 cells were suspended in PBS containing 1 x 10^{-4} M DNP (●) or in PBS alone (o). The samples were incubated for 1 hour at 37°C, cooled to 0°C and exposed to (a) 10 krads or (b) 20 krads under aerobic conditions. 1 x 10^4 cells were then lysed for 12 hours at 22°C in a lysing solution (0.5 M NaOH, 0.01 M EDTA, 0.2% SDS) on the top of an alkaline gradient (5-20% sucrose, 0.3 M NaOH, 0.001 M EDTA, 0.01% SDS). The gradients were centrifuged in a Beckman SW 50.1 rotor (5 ml tubes) at 20°C and ω = 35,000 r.p.m., t = 100 minutes.

Figure 32 shows a similar plot of $1/M_w$ vs dose for cells treated with DNP for 1 hour prior to and during irradiation. In this figure, the solid lines are actually those fitted to the data of figure 21. It can be seen that the data points in figure 32 are quite adequately represented by these lines and hence we again conclude that DNP has no effect on strand breakage in either aerobic or anoxic conditions. Thus the eV/break values and the OER for DNP treated cells are unchanged.

3.2.4. Effect of DNP on Single Strand Break Production in Different Cell Lines

Chinese hamster cells ($CH2B_2$) and human fibroblasts (AN) were also treated with either 1×10^{-4} M DNP or 5×10^{-4} M DNP and again we found that the presence of DNP had no effect on the number of breaks produced by radiation in either line. The results are shown in figures 33 a and b.

Experiments on the effect of DNP described to this point were all done with detergent containing gradients. We also did some runs using the high salt technique (see section 2.4.) and a typical profile obtained with L-60 cells is shown in figure 34. Again, the peak position, the shape of the profile and the calculated molecular weight were unaffected by the presence of DNP.

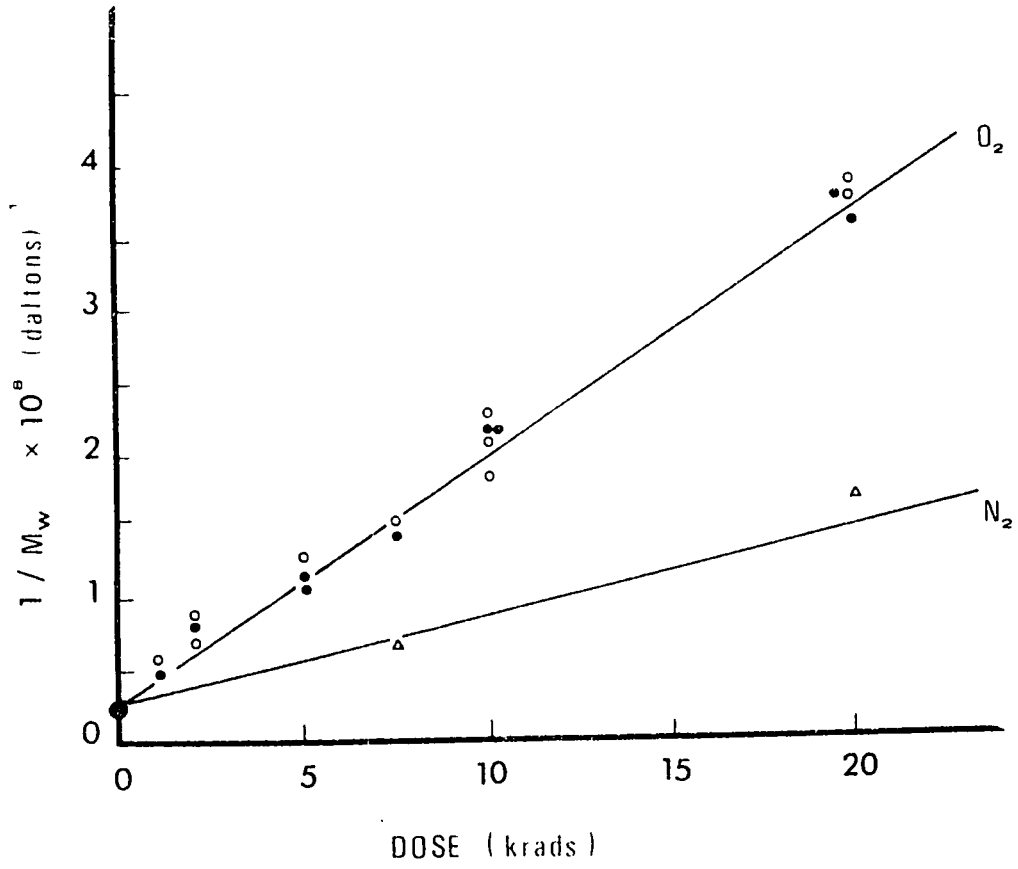


Figure 32. Dose dependence of molecular weight and the effect of DNP, 5 ml tubes; detergent containing gradients.

L-60 cells were suspended in PBS containing 1 x 10^{-4} M DNP (o, Δ) or 5 x 10^{-4} M DNP (●). The samples were incubated for 1 hour at 37°C, cooled to 0°C, and irradiated under aerobic (o, ●) or anoxic (Δ) conditions. They were then lysed for 12 hours at 22°C in a lysing solution (0.5 M NaOH, 0.01 M EDTA, 0.2% SDS) on the top of an alkaline gradient (5-20% sucrose, 0.3 M NaOH, 0.001 M EDTA, 0.01% SDS). Following this, the gradients were centrifuged in a Beckman SW 50.1 rotor at 20°C using appropriate values of ω and t. The weight average molecular weight M_w was calculated according to equation (6A) (section 2.3.11.). The solid lines represent measurements made under identical conditions except that DNP was absent (figure 21., section 3.1.2.).

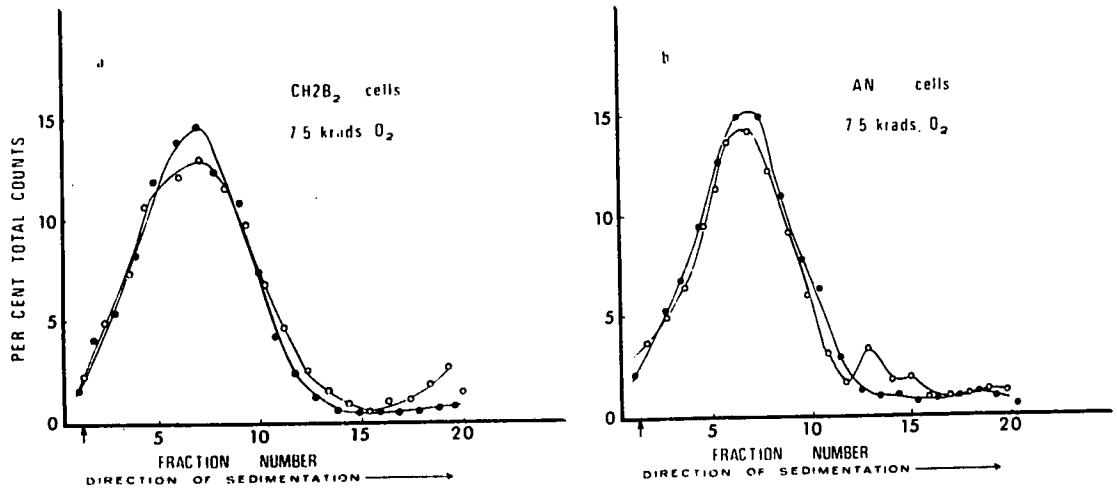


Figure 33 a, b. DNA sedimentation profiles of irradiated $CH2B_2$ and AN cells treated with 1×10^{-4} M DNP.

Either cells were suspended either in PBS containing 1×10^{-4} M DNP (o) or PBS alone (●). The samples were incubated for 1 hour at 37°C, cooled to 0°C and irradiated with 7.5 krads under aerobic conditions. 1×10^4 were then lysed for 12 hours at 22°C in a lysing solution (0.5 M NaOH, 0.01 M EDTA, 0.2% SDS) on the top of an alkaline gradient (5-20% sucrose, 0.3 M NaOH, 0.001 M EDTA, 0.01% SDS. Following this, the gradients were centrifuged in a Beckman SW 50.1 rotor (5 ml tubes) at 20°C and ω = 30,000 r.p.m., t = 100 minutes.

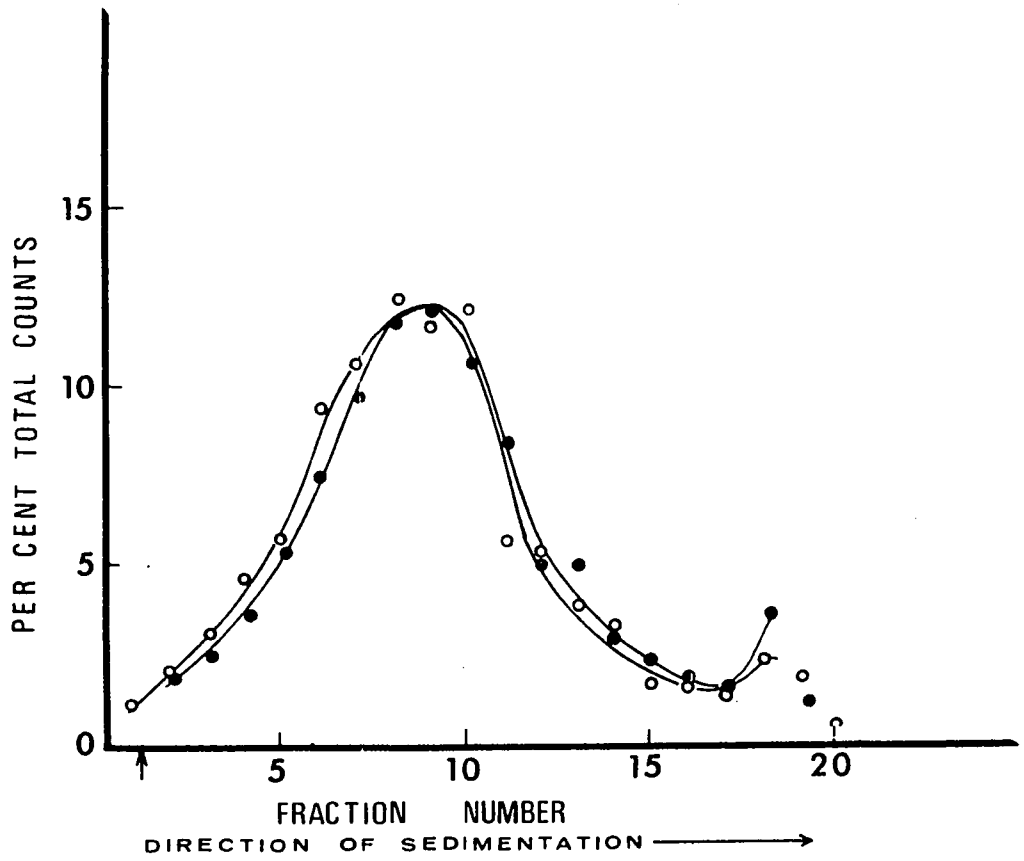


Figure 34. DNA sedimentation profiles of L-60 cells treated with 1×10^{-4} M DNP; high salt containing gradients.

Cells were suspended in PBS containing 1×10^{-4} M DNP (●) or in PBS alone (o). The samples were incubated for 1 hour at 37°C, cooled to 0°C and irradiated with 7.5 krads under aerobic conditions. 1×10^4 cells were then lysed for 12 hours at 22°C in a lysing solution (0.45 M NaOH, 0.55 M NaCl, 0.01 M EDTA) on the top of an alkaline gradient (5-20% sucrose, 0.1 M NaOH, 0.9 M NaCl, 0.003 M EDTA). Centrifugation in a Beckman SW 50.1 rotor (5 ml tubes) at 20°C, ω = 30,000 r.p.m., t = 100 minutes.

3.3. KINETICS OF REJOINING IN MAMMALIAN CELLS

3.3.1. The Rejoining Process Studied in Cells Irradiated in the Presence of O_2.

It was stated earlier that all mammalian cell lines which have been tested so far, have a capacity to rejoin DNA single strand breaks under certain conditions (see section 1.6.). We tested L-60 cells for the rejoining potential after the breaks were introduced by exposing the cells to ionizing radiation. The kinetics of this process are described in figure 35.

First, it can be seen that there is no rejoining detectable at 0°C up to 2 hours after irradiation. This was found to be the case even when the irradiated cells were incubated at 0°C for as much as 5 hours after irradiation (5 hours was the longest time tested, results are not shown in the figure). Secondly, the rejoining process was temperature dependent; it was found to be more rapid at 37°C than at 24.4°C. Thirdly, not all of the breaks were rejoined during a 1 hour period at 37°C in L-60 cells under the conditions described in the caption to figure 35; that is, the cells were irradiated in growth medium and then incubated in the same medium after the irradiation.

We found that the rejoining of single strand breaks also took place when the L-60 cells were irradiated in PBS and then were either resuspended in growth medium and incubated at 37°C, or were just incubated at 37°C.

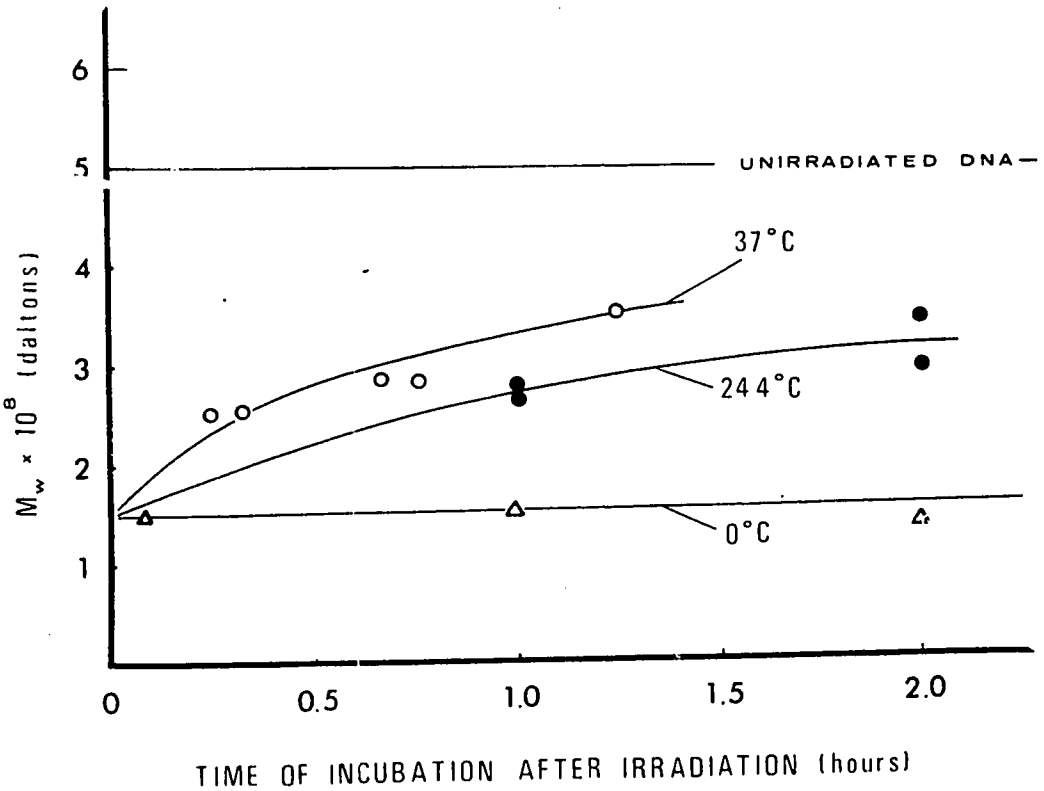


Figure 35. Kinetics of rejoining of breaks produced under aerobic (O_2) conditions in L-60 cells; the effect of temperature.

L-60 cells were irradiated with 3 krads (O_2) in growth medium and incubated at 37°C -o-o- , 24.4°C -●-●- , or 0°C -Δ-Δ- . The incubation was carried out in the same growth medium after irradiation for various periods of time. The cells were then lysed on the top of an alkaline gradient, the gradients were sedimented and M_w of single stranded DNA was calculated. Each point represents one gradient.

Consistent with these results, we found that no rejoining took place at 0°C in either Chinese hamster cells (see also figure 37, section 3.3.2.) nor in human fibroblast cells though both cell lines are capable of rejoining at 37°C.

3.3.2. Rejoining of the Breaks Produced when L-60 Cells are Irradiated in the Absence of O_2.

It was shown in section 3.1. that the presence of oxygen at the time of irradiation increases the number of single strand breaks. Consequently, it was of interest to see whether the kinetics of rejoining of the breaks produced in the absence of oxygen are comparable to the kinetics of rejoining of breaks produced with O_2 present. Figure 36 shows the results of these studies; the solid curve is that of figure 35 for 37°C, i.e. for L-60 cells which were irradiated with 3 krads in the presence of oxygen and then incubated for various times in growth medium before lysis. We exposed an identical suspension to a dose which produced and equivalent number of single strand breaks in an N_2 atmosphere; the dose used was 8.7 krads. After irradiation the cells were incubated under aerobic conditions at 37°C until they were lysed. These results are plotted in figure 36 as closed circles and it can be seen that they correspond quite closely to the curve for oxygen. Thus it appears that for equivalent amounts of strand breakage the kinetics of rejoining are the same for damage produced either in O_2 or N_2. We have already shown that the

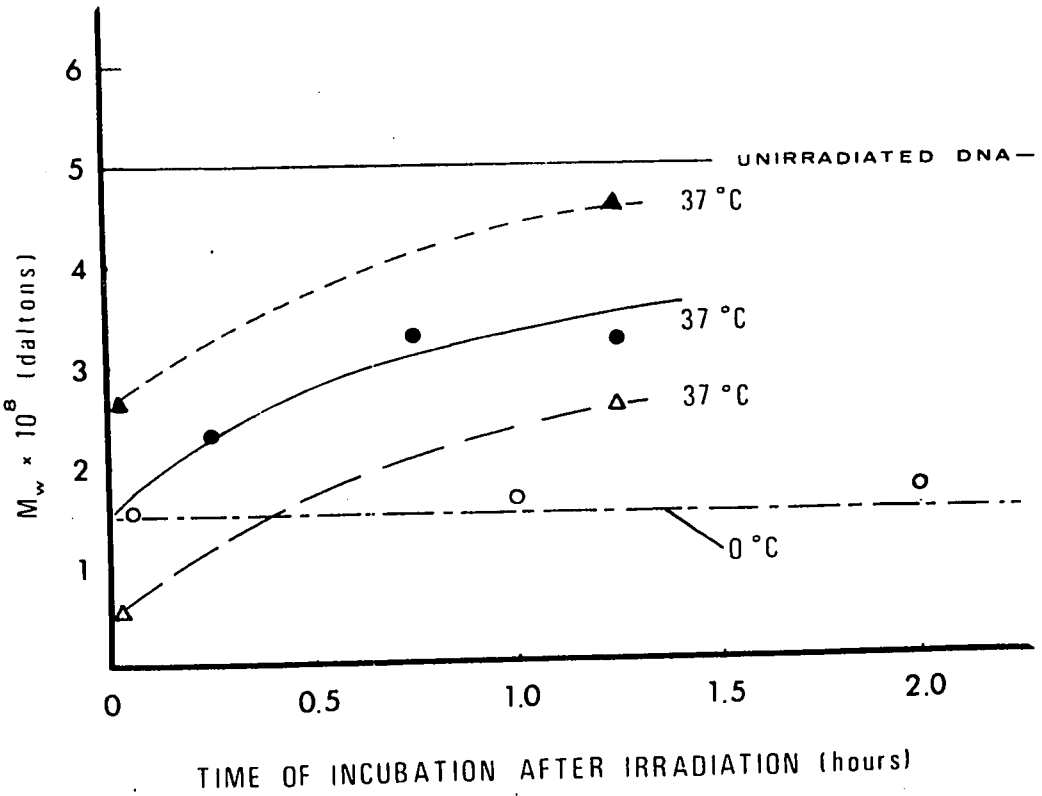


Figure 36. Kinetics of rejoining of breaks produced under anoxic (N_2) conditions in L-60 cells.

L-60 cells were irradiated with either 8.7 krads (o, ●) or 3 krads (▲) in an anoxic (N_2) atmosphere, or they were irradiated by 8.7 krads under aerobic conditions (Δ). After irradiation the cells were incubated for various times at 37°C in growth medium. The cells were then lysed on the top of an alkaline sucrose gradient, the DNA was were then sedimented and M_w was caluclated. Each point represents the result of one gradient. The solid curve is the one for 37°C from figure 35.

OER for cell survival can be explained in terms of the OER for single strand breaks and figure 36 suggests, as we would expect, that the OER for survival is not dependent upon the rejoining efficiency. One argument that needs to be discounted, however, is the possibility that there is a different rejoining efficiency for breaks produced in N_2 but that the larger dose used (8.7 krads) obscured this in our experiment by producing a qualitatively different rejoining process. Thus it was necessary to determine whether or not the nature of the rejoining process was dose dependent. The results of an experiment designed to check this are included in figure 36. The closed triangles describe the rejoining process in cells receiving 3 krads in N_2 and the open triangles show the rejoining in cells receiving 8.7 krads in O_2. One can see that the responses for cells receiving 3 krads and 8.7 krads in O_2 or in N_2 are all qualitatively similar, though they reflect different levels of initial strand breakage. Therefore it seems unlikely that the possiblity of differential rejoining efficiency between damage produced in N_2 or O_2 is significant.

In figure 36 it can also be seen that the single strand breaks produced under anoxic conditions are not repaired at 0°C (open circles).

In figure 37 are presented data on the rejoining process in Chinese hamster cells and they show essentially the same situation

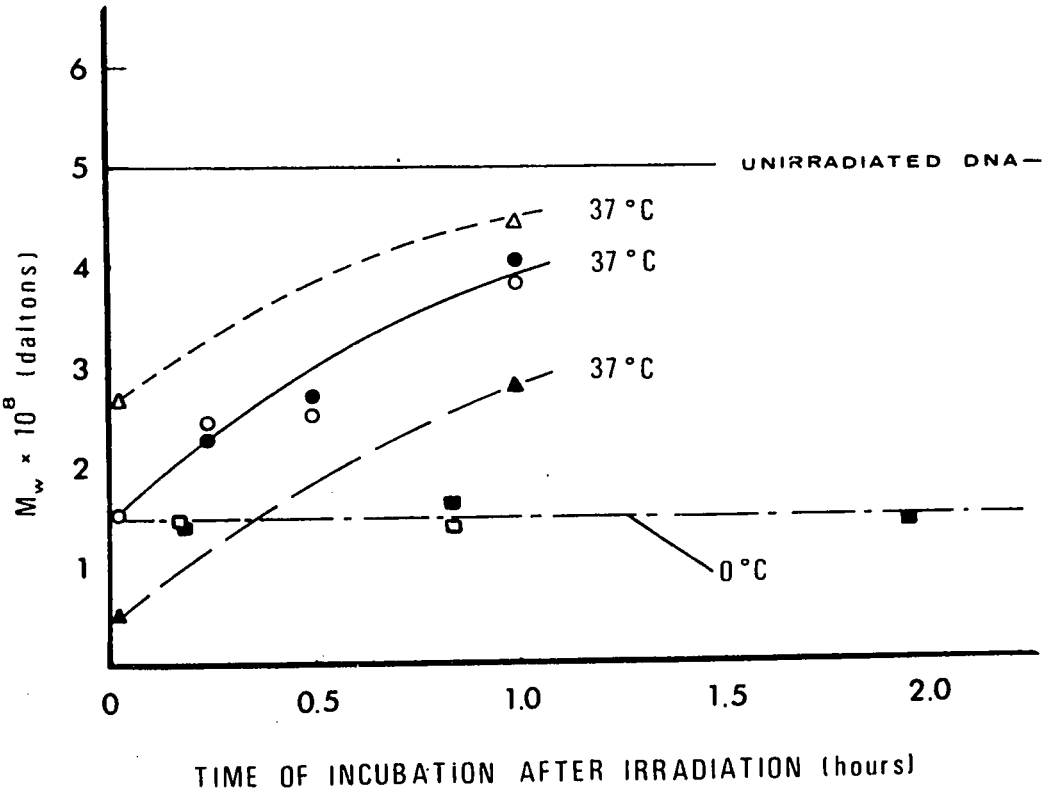


Figure 37. Kinetics of rejoining of breaks produced under aerobic (O_2) or anoxic (N_2) conditions in $CH2B_2$ cells.

The cells were irradiated under aerobic conditions with 3 krads (●,■) and 8.7 krads (▲) or under anoxic (N_2) conditions with 8.7 krads (○,□) or 3 krads (Δ). After the irradiation the cells were incubated for various times at 37°C or 0°C in growth medium. The cells were then lysed for 12 hours at 22°C on the top of an alkaline sucrose gradient. Gradients were spun in a Beckman SW 27 rotor (17 ml tubes) and M_w was calculated from the sedimentation profiles according to equation (6B). Each point represents the result of one gradient.

as was observed for L-60 cells in figures 35 and 36. There is no detectable difference in the kinetics of rejoining of the single strand breaks whether they are produced under aerobic or anoxic conditions; again, no rejoining takes place at 0°C.

3.3.3. Effect of 2,4-Dinitrophenol on Rejoining

It was shown in section 3.2. that when L-60 cells were treated for one hour with 2,4-dinitrophenol at either 1×10^{-4} M or 5×10^{-4} M DNP in glucose free PBS, the macromolecular synthesis was essentially stopped and the intracellular pool of ATP was depleted. We then examined the effect of DNP on the kinetics of rejoining of single strand breaks.

The results are presented in figure 38. The cells were treated with 1×10^{-4} M DNP in PBS for 1 hour prior to and during irradiation and the rejoining was followed for 1 hour. In a parallel experiment the DNP was omitted. As can be seen in this figure, the cells in PBS were capable of rejoining the single strand breaks with kinetics comparable to those in full growth medium, shown in figure 35 (section 3.3.1.). In cells which were treated with DNP, and where the drug was not removed after irradiation, there was no rejoining of breaks; however, if the drug was washed away at some time after the irradiation and the cells were resuspended in growth medium, some rejoining took place. The kinetics of this rejoining seemed to be slower than in untreated cells. This observation was also confirmed by Moss et al. (1971).

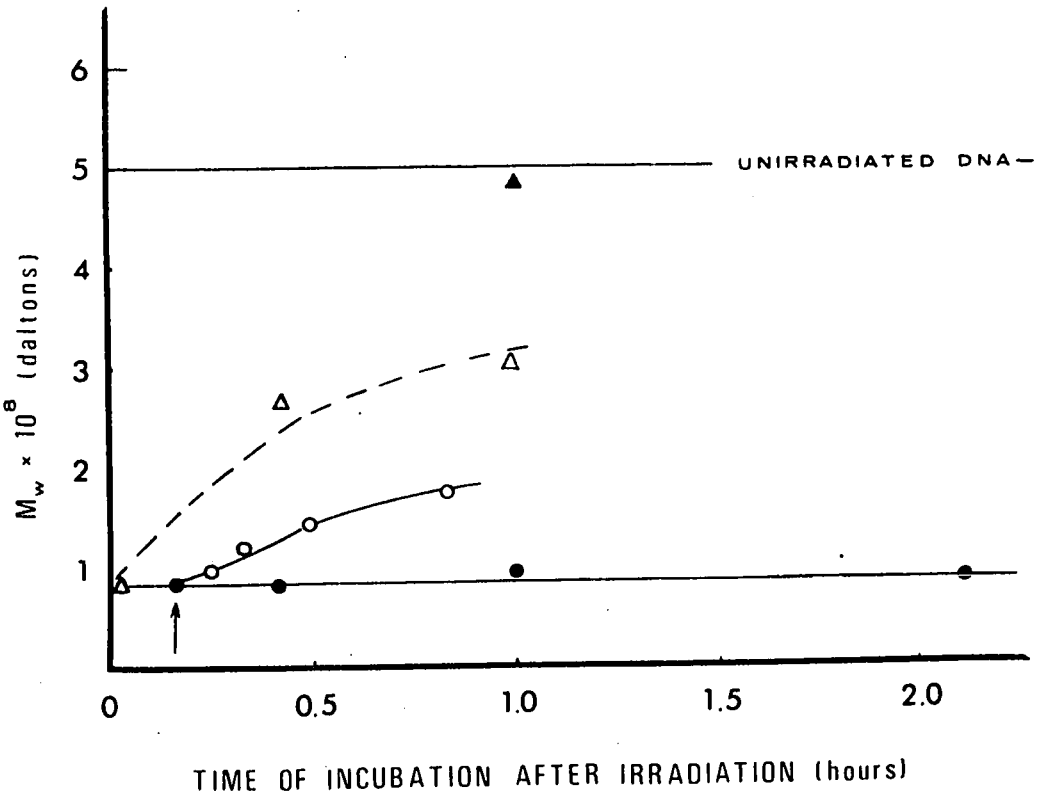


Figure 38. Kinetics of rejoining of breaks produced under aerobic conditions in L-60 cells treated with 1 x 10^{-4} M DNP.

The cells were suspended in PBS containing 1 x 10^{-4} M DNP (●, ▲, o) or in PBS alone (Δ) and incubated for 1 hour at 37°C, cooled to 0°C and irradiated with 5 krads (Δ,●,o) under aerobic conditions, or not irradiated at all (▲). After the irradiation they were incubated for various times at 37°C. In some samples the drug was washed away after 10 minutes of incubation at 37°C after the irradiation (o, see also the arrow) and the cells were further incubated in PBS alone. After incubation cells were lysed on the top of an alkaline sucrose gradient for 12 hours at 22°C, and then the gradients (5 ml tubes) were spun in a Beckman SW 50.1 rotor. M_w was calculated according to the equation (6A). Each point represents the result of one gradient.

Sawada and Okada (1970) reported that treatment of mammalian cells with 1×10^{-4} M DNP did not prevent the rejoining process. However, these authors added the drug only after irradiation and their cells were incubated in complete growth medium throughout the experiment. In some preliminary studies where the cells were treated with 1×10^{-4} M DNP in complete growth medium, we observed that DNA synthesis remained at essentially the same level as that in untreated cells even 1 hour after DNP was added to the growth medium. The medium contains glucose (1 mg/ml), and since DNP is really a specific inhibitor of oxidative phosphorylation, treatment with the drug in the presence of glucose does not prevent the cells from obtaining the necessary minimum supply of ATP via the glycolytic pathway.

In order to determine whether this was, in fact, the explanation of the observations of Sawada and Okada, we repeated the experiment in our system under conditions identical to those of Sawada and Okada. That is, the untreated cells were irradiated in growth medium and immediately after irradiation, DNP was added to the cell suspension to a final concentration of 1×10^{-4} M. The cells were then incubated at 37°C, the rejoining was measured and found to be comparable to that of cells not treated with DNP.

4. DISCUSSION

4.1. THE RELIABILITY OF THE MODIFIED METHOD OF ALKALINE SUCROSE GRADIENTS

4.1.1. Long Lysis Time; Effect of Labelling

In the search for conditions which would best satisfy our criteria (section 2.3.1.) we increased considerably the time of lysis of the cells. It was shown in section 2.3.8. that prolonged lysis does not affect the calculated molecular weight of DNA molecules in unirradiated mammalian cells or in bacteriophage T4. Under the conditions described, the single stranded DNA is very stable. These results are consistent with those reported by Shipley and Elkind (1971) where high salt concentrations were used rather than detergent in the lysing and gradient solutions, and times of lysis up to 24 hours were examined.

It should be pointed out that in the degradation studies of Shipley and Elkind and in our own studies on degradation, the molecular weightof the DNA was 2.5×10^8 daltons or less. Some authors who have reported much larger molecules in unirradiated mammalian cells, have presented evidence that these DNA molecules can be degraded to smaller pieces. This will be discussed in section 4.2.

In all experiments reported in this thesis the mammalian DNA was labelled with H^3 TdR, with the tritium located in the methyl group of carbon 5 in the thymine base. The decay of tritium to helium is accompanied by the emission of a β^- particle (negatively charged electron) which gives rise to the measured scintillation. However, these low energy β^- particles (E_{max} = 0.018 MeV) emitted on each disintegration have a high probability of being absorbed near the point where they originate. They are thus able to produce strand breakage even after the cells are lysed.

Rosenthal and Fox (1970) reported that H^3 decay produces single strand breaks with an efficiency of 30%. These experiments were done with bacterial cells containing H^3 labelled DNA. The cells were stored at -30°C to accumulate breaks produced by H^3 decay. Single strand breaks were then measured by the method of alkaline sucrose gradients.

If we assume the same efficiency of single strand break production when the DNA is in the lysing solution on the top of our gradient, then one would expect approximately 2.2×10^3 single strand breaks per cell during our 12 hour lysing time. This calculation is based on the highest specific activity used in our experiments for molecular weight determination; this labelling gave $\sim$ 10 decays per minute per cell (see section 2.3.7.). If the molecular weight of DNA from unirradiated cells is taken to

be 2.5 x 10^8 daltons, then we have $\sim$ 3 x 10^4 molecules of this size per cell which together experience a total of 2.2 x 10^3 breaks during the 12 hours of lysis. This means that an average of 0.075 breaks per molecule occurs during lysis and using Poisson statistics one calculates that 93% of all molecules receive no breaks. If, in fact, only 7% of the molecules had sustained breaks in this manner, detecting them by this method would be difficult.

It is unlikely that the efficiency of strand breakage measured by Rosenthal and Fox for double stranded DNA in frozen cells applies to the single stranded DNA in our lysing solution. Evidnece for this is the fact that when we labelled L-60 cells with activities ranging from 0.15μCi/ml to 4μCi/ml, no changes in the DNA sedimentation profiles of unirradiated cells were observed.

4.1.2. Validity of the Burgi-Hershey Equation in these Studies.

It can be seen in section 2.3.11. that the calculation of molecular weight depends on equations (2) and (3). The relationship in equation (3) was first established by Burgi and Hershey (1963) who used neutral sucrose gradients and isolated double stranded DNA molecules of bacteriophage T2 and T5. Later, this relationship was confirmed using analytical zone sedimentation under alkaline conditions by

Studier (1965), who established constants a and k in equation (2) for single stranded DNA molecules. He again used isolated DNA molecules from bacteriophage ϕx, λ_{dg}, T7 and T2 where the largest single strand DNA molecule was that of T2 phage, 0.67 x 10^8 daltons. It is questionable how reliably one can extrapolate Studier's data to molecular weights as high as 2.5 x 10^8 daltons and even much higher than that (since for calculation of an average molecular weight one takes into account molecules much larger than the average).

In our experiments with mammalian DNA we frequently changed the values of ω and t. Thus it was possible to determine whether the relationship in equation (3) was valid for our technique and we did this by plotting the distance of sedimentation (peak position) vs $\omega^2 t$. A linear relationship was observed for both the 5 ml tubes and the 17 ml tubes indicating that equation (3) is applicable in our sedimentation technique.

4.2. SIZE OF DNA MOLECULES IN MAMMALIAN CELLS

4.2.1. Is DNA in Chromosomes Composed of Subunits?

Currently, it is an open question whether the chromosome in mammalian cells, and for that matter in bacterial cells, contains only one very large double stranded DNA molecule or is composed of smaller subunits (see section 1.2.). In our experiments with unirradiated cells we measured an average molecular weight of

2.5×10^8 daltons for single stranded DNA, which gave 5×10^8 daltons for the corresponding double stranded structure. Since there is as much as 10^{11} daltons of double stranded DNA per chromosome, we would have to argue that DNA in mammalian cells is composed of smaller subunits, a few hundred per chromosome.

Similar results were reported by many other authors (Lohman 1968, Elkind and Kamper 1970, Lett, Klucis and Sun 1970, Elkind 1971), however, it should be pointed out that this molecular weight was obtained only when the cells were lysed for rather long times at room temperature. In all cases the cells were placed directly into the lysing solution on the top of a gradient.

4.2.2. The Size Distribution of Subunits

If we accept the idea that the DNA in the chromosome is composed of subunits, the next question is, what is the nature of the size distribution? It was shown in section 3.1.3. that the size distribution of unirradiated L-60 cells closely resembles a so called random distribution; that is, a distribution which one would expect to obtain by the introduction of breaks in much larger molecules by a random process (Charlesby 1954).

One possible explanation of the random size distribution which we observed for unirradiated cells is that the distribution was simply the result of random degradation of DNA by the highly alkaline conditions during our rather long lysing times, in which

case it provides no information about the subunit size. However, if we accept such an explanation we then must postulate a peculiar phenomenon, namely, that the DNA (single stranded) is alkali labile until a distribution with an average molecular weight of 2.5×10^8 daltons is obtained and then the DNA molecules become rather resistant to further alkali exposure. For instance, there is no essential difference between 6 hours and 18 hours of lysis for our unirradiated cells (figure 8) or between 4 hours and 24 hours in experiments by Shipley and Elkind (1971). DNA molecules smaller than this (for example from irradiated mammalian cells or from bacteriophage T4 (figure 11)) are also insensitive to prolonged exposure to alkali.

Some other workers (Lett et al. 1970) who also used prolonged lysis at room temperature found pieces of molecular weight 5×10^8 daltons. The profile in their experiments appeared to represent a uniform distribution and this is the basis for their argument that all subunits are of uniform size. Similar profiles have been seen both by Elkind (1971) and by ourselves under high speeds of centrifugation where a distortion of the high molecular weight side of the peak occurs (Elkind 1971, McBurney, Graham and Whitmore 1971, Palcic and Skarsgard 1972a, see also section 2.3.9.). The effect produces an apparent sharpening of the peak. This is a possible explanation of the profile shape observed by Lett, Klucis and Sun (1970), though confirmation of this is impossible since

neither the speed nor the time of centrifugation is given in their paper.

4.2.3. One Molecule per Chromosome?

McBurney, Graham and Whitmore (1972) developed a technique for very gentle lysis of mammalian cells. Alkaline gradients were overlaid with 2% sucrose in water and the cells were placed directly into this layer. The authors suggested that as the cells settled towards more alkaline conditions, lysis took place but the disruption of cells was much more gentle than when the cells were placed directly into the lysing solution. The cells were then lysed for 16-20 hours at 4°C and centrifugation followed at the same temperature. They showed that this gentle lysis yielded DNA molecules of molecular weight $\sim 9 \times 10^9$ daltons. They also showed that at least three different processes could break these large molecules to lower molecular weight material:

a) Placing the cells directly into the lysing solution rather than allowing them to settle slowly through 2% sucrose gave a profile with much lower molecular weight and they contend that this is the result of shearing forces associated with direct lysis.

b) They also obtained a lower molecular weight profile when they raised the temperature of lysis to room temperature.

c) Differences in the profiles were observed according to whether the DNA was labelled with H^3 TdR or C^{14} TdR, indicating

that the higher specific activity required with H^3 labelling may lead to greater radiation damage to the DNA.

From their experiments (McBurney et al. 1972, McBurney and Whitmore 1972) they concluded that the entire DNA complement of a chromosome may exist as a single molecule.

4.2.4. Membrane Attachment Model

The observations by Comings and Kafekuda (1968) led Comings (1968) and Ormerod and Lehman (1971) to propose a model whereby the DNA molecules are attached, at least during some part of the cell cycle, to various points on the nuclear membrane, forming loops of double stranded DNA between these sites. This model could explain to a great extent the various data on the DNA sedimentation properties of unirradiated mammalian cells (and also bacterial cells) reported so far. The sites at which the DNA is attached to the membrane are presumably associated with the replication, the transcription and perhaps the repair of the DNA molecules and the number of sites, their spacing and the subsequent length of the DNA loops could depend on the cell type and on the stage in the cell cycle.

Applying this model to explain our results, where we used the detergent SDS and lysis at room termperature for at least 6 hours, we could conjecture that we might have broken the DNA molecules at nearly all attachment sites. Similarly, high salt concentration and lysis at room temperature for at least 3-4 hours

(Elkind and Kamper 1970, Elkind 1971, Shipley and Elkind 1971) might have accomplished the same end in Chinese hamster cells.

However, much shorter times of lysis (20 minutes) under the condition described by Elkind and Kamper (1970) and Elkind (1971), left most of the DNA in a complex, which was associated with some protein and lipid. This complex was estimated to be of a molecular weight larger than 5×10^9 daltons. The amount of DNA in the "complex" was dependent on the temperature of lysis, the exposure to visible light during lysis and time of lysis (Elkind 1971). It was suggested by Elkind (personal communication) that the "complex" may actually represent DNA which is still attached to the nuclear membrane.

The observations of McBurney et al. (1972) and McBurney and Whitmore (1972) discussed in section 4.2.3. can perhaps also be explained by this model. If, in fact, the DNA exists as a long continuous molecule, attached at various points to the membrane, it is possible that their gentle lysis technique may detach the entire molecule from the membrane without causing any single- or double-strand ruptures. Alternatively, though McBurney et al. showed that less than 1% of the total cell protein and lipid remained associated with the DNA, we think it is possible that some membrane structure might have been still associated with the DNA, sufficient to hold together the DNA loops to form a larger structure. The second alternative, then, does not require

one to postulate one continuous DNA molecule but rather, is consistent with the idea that many molecules or subunits may be held together via protein or protein-lipid linkers. This might also explain the "complex" (Elkind and Kamper 1970).

We have some results which support the latter hypothesis. We attempted to repeat the experiment of McBurney et al. using the gentle lysis technique. We labelled DNA L-60 cells with H^3-TdR and protein with C^{14} L-leucine, which allowed us to examine the amount of protein associated with the DNA with higher resolution than was possible in McBurney's experiment. In all our experiments we still observed a small peak of protein cosedimenting with the peak of DNA. The amount of protein varied between 2% and 4% of the total cell protein. In parallel experiments, the DNA peak (which was frequently recovered in one fraction) sedimented to different positions, the protein co-sedimented with it. Thus it appears that the protein was closely associated with the DNA.

It may be possible to test the validity of membrane attachment model by studying the sedimentation profile of DNA taken from cells at different stages in the cell cycle, since presumably at least during mitosis, the DNA membrane association is either altered or non-existent.

4.3. SINGLE STRAND BREAKS

4.3.1. Mechanism of Production of Single Strand Breaks

One of the primary objectives of our work was to determine whether single strand breaks in DNA which are produced by ionizing radiation are the result of a direct and/or indirect action of ionizing radiation, or rather the product of nucleolytic enzyme(s) operating after the irradiation, as has been proposed by Dalrymple et al. (1968); this hypothesis has been discussed in greater detail in section 1.4.

Their proposal was backed by the observation of free 5' phosphoryl termini in the DNA molecules after irradiation, which was shown to be an enzymatic process which could be inhibited by either 0°C or treatment with 2,4-DNP. Also, when they studied the appearance of single strand breaks (Moss et al. 1971) they observed that the production of single strand breaks was decreased by a factor of 6.7 when the cells were treated with DNP.

Our data clearly do not support Darlrymple's hypothesis, First, when we irradiated L-60 cells in the presence or absence of 2,4-DNP we found no difference in the initial number of breaks. Secondly, if the cells were incubated after the irradiation, at no time did we observe a decrease in the molecular weight. On the contrary, when the cells were incubated in the absence of DNP, the rejoining of single strand breaks took place.

Treatment of the cells with DNP, as described in

section 2.1.3. prevented rejoining. However, as soon as the DNP was washed away, the capacity for rejoining was restored.

These results were confirmed by using three different mammalian cell lines and two different techniques of alkaline sucrose gradients. We concluded that in general in mammalian cells the presence of DNP has no effect on the initial number of breaks. Furthermore, all of the breaks which are observed with the technique of alkaline sucrose gradients are produced at the time of irradiation by the direct and/or indirect action of ionizing radiation.

We believe that the appearance of free 5' phosphoryl termini represents the unmasking of the 5' phosphoryl group or groups at positions along the phosphate-sugar backbone where breaks occur. This unmasking process may be inhibited with 2,4-DNP via the depletion of ATP in the cells (if we exclude the possibility that DNP is a specific inhibitor of the "unmasking enzyme").

We cannot explain the data of Moss et al. (1971) where they showed that treatment of L-cells with DNP decreased the number of breaks as measured by the method of alkaline sucrose gradients, unless their cells are very different than other mammalian cells. One difference in their technique is that they lysed their cells in a tube, not on the top of the gradient, and after lysis for 60 minutes at room temperature, the lysate

was pipetted onto the top of the alkaline gradient. One possible though unlikely explanation of their results is that DNA from cells treated with DNP is less susceptible to the shearing forces introduced by the handling of the DNA molecules after they are released into the lysing solution.

4.3.2. eV per Break Value

It has been shown in section 3.1.3. that for each 33 eV of energy released by radiation in the DNA molecules of L-60 cells, one single strand break was produced when the cells were irradiated in the presence of oxygen. The value of 33 eV/break is the average of the values obtained using the two different rotors: 31 eV/break for the SW 50.1 (5 ml tubes) and 35 eV/break for the SW 27 (17 ml tubes). A similar efficiency was found for Chinese hamster cells excluding the possibility that the rather low eV/break value found for L-60 cells is a peculiarity of the cell line.

The first value reported for mammalian cells was 70 eV/break (Lett et al. 1967). This was later corrected by Lehman and Ormerod (1970) to 44 eV/break. Values of 65 eV/break (Lett et al. 1970) and 72 eV/break (McBurney et al. 1971) have also been reported for mammalian cells.

We considered the possibility that the low value of eV/break measured in our laboratory was due to the fact that we were using detergent in our lysing and gradient solutions. Thus,

L-60 cells were examined using the technique of alkaline sucrose gradients containing high salt and no detergent (figure 25). Again, a value of 31 eV/break was found, eliminating the possibility that the presence of detergent has an effect on the number of breaks observed.

The possibility that the prolonged lysing time has an effect on the eV/break value was also explored. In figure 23 we showed that the same number of breaks are found when the irradiated cells are lysed for 3, 6 or 12 hours (12 hours was the standard lysing time). Therefore, at least over this range of lysing times, the eV/break value does not vary.

When L-60 cells were treated with either 1×10^{-4} M DNP or 5×10^{-4} M DNP the eV/break value was the same as in cells not treated with DNP (figure 32).

In bacterial cells eV/break values have also been measured. Dean et al. (1969) reported 50 eV/break in M. radiodurans. Since that time, a value of 40 eV/break was measured by Burrell, Feldschreiber and Dean (1971).

A very sensitive method for assaying for single strand break formation is possible in some bacterial cells where a part of the bacterial genome exists in the form of covalently bonded circular DNA. Some bacteriophage display the same molecular form in bacterial cells after the cells have been infected with the virus. The introduction of one single strand break into such a molecule result in two single stranded DNA molecules after

alkaline denaturation: one rod shaped molecule, and one circular molecule which separate on denaturation. Both sediment at approximately the same speed in an alkaline sucrose gradient. If the covalently bonded circular genome has no single strand break, then on denaturation the two strands cannot separate but remain entangled. The resulting collapsed and supercoiled molecule sediments much faster than expected, 3-4 times faster in fact, than the disentangled circular or rod shaped single stranded molecules. This property provides a method whereby single strand breaks can be measured with very high resolution.

This technique was used by Johansen, Gurvin and Rupp (1971) to assay for single strand breaks in bacteriophage λ. They found a value of 24 eV/break in the presence of oxygen. Preliminary results indicated that the same value is to be expected for bacterial DNA molecules (E. coli). This value is considerably lower than that previously reported for E. coli cells, ∿ 80 eV/break (Lehnert and Moroson 1971).

From physical measurements it was shown by Rauth and Simpson (1964) that, on the average, 60-70 eV are expended per absorbtion event. If only the direct effect of ionizing radiation contributed to the production of single strand breaks, we would then expect a value of 60-70 eV/break. Our finding of 33 eV/break in mammalian cells, together with the value of 24 eV/break reported for λ bacteriophage by Johansen et al., suggest

that there is perhaps a considerable contribution due to the indirect effect of ionizing radiation in the production of single strand breaks. For example, the free radicals OH· and H· which are two of many products of the irradiation of water, can react with the DNA molecule. One possible consequence of such a reaction is the formation of a single strand break.

4.3.3. Oxygen Enhancement

In section 3.1. we demonstrated that the presence of oxygen during the irradiation of mammalian cells enhances the number of breaks by a factor of 2.9. It is important to note that this was true for three different mammalian cell lines and for two methods of alkaline sucrose gradients.

The analysis of the sedimentation profiles for the randomness of the size distribution showed that the profiles from cells irradiated under either aerobic or anoxic conditions constitute so called random distributions.

Dean et al. (1969) reported that there is no oxygen effect for the production of DNA single strand breaks in mammalian cells. Our work presented here does not support this observation; rather, it suggests that in general in mammalian cells the oxygen enhancement ratio is approximately 3. It is of interest that this value is the same as that obtained by survival measurements in mammalian cells.

Adams and Dewey (1963) and Adams and Cooke (1969) proposed

a mechanism by which molecular oxygen participates in the radiochemical processes. In short, the radiation produces an electron and a positive ion in the target molecule. If a molecule of very high electron affinity such as oxygen is present in the immediate environment, transfer of the electron from the polarized target molecule to oxygen could occur. The positive ion could then decay to a neutral free radical, which can also react with molecular oxygen. These reactions can lead to the formation of a single strand break as seen with the method of alkaline sucrose gradients.

4.4. REJOINING OF SINGLE STRAND BREAKS

It was shown in section 3.3.1. that the rejoining process is dependent on temperature of incubation, being slower at lower temperature. It was completely stopped at 0°C. The number of breaks which remained after one hour was dependent on the initial number of breaks present, both in L-60 and $CH2B_2$ cells. From the presented data, however, it appears that the rejoining process is faster in Chinese hamster cells than in L-60 cells.

It was of interest to determine whether the kinetics of rejoining were different for damage produced under aerobic and anoxic conditions, since a difference in rejoining would imply a difference in the quality or nature of the single strand breaks produced in the presence or absence of oxygen. However,

when we introduced the same number of breaks into the DNA molecules by different doses of radiation under aerobic or anoxic conditions, we found that the kinetics of the rejoining processes were the same. These results, nevertheless, do not conclusively demonstrate that the breaks produced under anoxic and aerobic conditions are qualitatively identical.

In section 3.3.3. was shown the effect of 2,4-DNP on the rejoining process in L-60 cells. The treatment of cells with DNP completely inhibited the rejoining process; however, when DNP was washed away, the capacity of the cells to rejoin at least some of the breaks was restored.

5. CONCLUSIONS

In this work we have developed and used a modified technique of alkaline sucrose gradients which allows reliable measurement of DNA molecular weights in mammalian cells. With this technique we have shown:

(1) the DNA from unirradiated asynchronous cells has a molecular weight $\sim 2.5 \times 10^8$ daltons and that the size distribution is random.

(2) when mammalian cells are irradiated in the presence of molecular oxygen an oxygen enhancement ratio (OER) of 2.9 for the production of single strand breaks is observed. This value is remarkably similar to that obtained in measurements of survival (OER $\sim$ 3) in the same cells.

(3) the rejoining of single strand breaks is independent of whether these breaks are produced in the presence or absence of oxygen.

(4) the rejoining process is temperature dependent; it is completely inhibited at 0°C.

(5) the treatment of mammalian cells with 2,4-DNP in glucose-free PBS completely inhibits the rejoining process.

(6) in general, in mammalian cells, all of the single strand breaks produced by ionizing radiation as observed by the

technique of alkaline sucrose gradients are produced by the direct and/or indirect physico-chemical action of radiation. There are no observable breaks due to the action of nucleolytic enzymes which act after the irradiation. This was concluded from the following:

a) at no time after irradiation at 0°C did we observe a further degradation of DNA molecules on subsequent incubation at 37°C, on the contrary only an increase in molecular weight was observed.

b) the treatment of cells with 2,4 DNP did not change the inital number of breaks produced; if DNP was removed after irradiation and the cells incubated at 37°C, only rejoining of breaks rather than further degradation of DNA was ever observed.

(7) the energy which must be released in the DNA in order to produce one single strand break in mammalian cells is 33 eV.

APPENDIX

List of Publications

1. L. D. Skarsgard, B. Palcic and D. A. Agnew. 1969. Sensitization of Anoxic Mammalian Cells to Radiation by Organic Free Radicals: ESR Studies. Abstract. Radiat. Res. 39, 455.

2. B. Palcic and L. D. Skarsgard. 1971. The Effect of 2,4-Dinitrophenol, Temperature and O_2 on Single-Strand Breaks Produced in DNA by Ionizing Radiation. Abstract. Radiat. Res. 47, 315.

3. B. Palcic and L. D. Skarsgard. 1972. The Effect of Oxygen and DNA-Single-Strand Breaks Produced by Ionizing Radiation in Mammalian Cells. Int. J. Radiat. Biol., 21, 417.

4. B. Palcic and L. D. Skarsgard. 1972. DNA Single-Strand Breaks Produced in Mammalian Cells by Ionizing Radiation After Treatment with 2,4-Dinitrophenol. Int. J. Radiat. Biol., 21, 535.

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