XERODERMA PIGMENTOSUM : SEDIMENTATION AND CELL SURVIVAL STUDIES FOLLOWING IONIZING RADIATION

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ABSTRACT:

In spite of the difficulties involved in molecular and cellular studies of antibiotic-free cultures of primary human cells, investigations of the sedimentation and survival properties of cell cultures derived from Xeroderma Pigmentosum patients were successfully carried out. Emphasis was on the cellular and molecular effects of ionizing radiation; however preliminary data for cell survival after exposure to UV was obtained.

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1. INTRODUCTION

Xeroderma Pigmentosum (X.P.) is an autosomal recessive disorder characterized by a hypersensitivity of the skin to the ultraviolet wavelengths in sunlight. It is often found complicated by a neurological disorder, the de Sanctis Cacchione syndrome, also autosomal recessive (Reed, Landing, Sugarman, Cleaver and Melnyk 1969). The initial symptom of X.P., usually occurring in the first year or so of life, is an erythematous reaction following exposure to natural sunlight; freckling or pigmentation becomes very marked on exposed skin, often accompanied by premalignant, and eventually malignant, lesions, and the progressive changes of senility. Death at an early age, due to malignant skin cancers is not uncommon.

Since X.P. was first described in 1874, (Hebra and Kaposi 1874), many clinical studies have been conducted correlating cutaneous changes to the wavelength of the radiation used to produce them. In the last ten years, investigators have concentrated on the biochemistry and molecular biology of the disease, with tissue cultures derived from X.P. patients providing a very useful tool.

Early attempts to discover a photoactive substance abnormally present in the skin of X.P. patients were centred around porphyrin derivatives as it had been shown that in individuals with the disease hydroa vacciniforme, sensitivity to ultraviolet radiation is correlated with excessive skin concentrations of porphyrins. In this disease sensitization occurs for wavelengths as long as 400 mμ. El-Hefnawi and Cripps (1966) carried out a comprehensive study of the levels of urinary coproporphyrin, uroporphyrins, porphobilinogens, fecal protoporphyrins, coproporphyrins, and blood porphyrins in 12 X.P. patients selected randomly from a group of 46; the results showed that the levels of all metabolites were within the normal range. Reed et al. (1969) also obtained negative porphyrin results, and contrary to the earlier findings of El-Hefnawi (references 5, 6, 7 in Reed et al. 1969), he also found normal levels of serum Cu, blood glutathione and α_2-globulin; 17-ketosteroid and 17-hydroxycorticosteroid levels proved normal. Chromosome studies revealed no abnormalities, and no increased breakage.

Early clinical investigations with X.P. patients involved in vivo exposure of small areas of skin (pigmented and unchanged) to sunlight and artificial UV; follow-up consisted of a qualitative description of resulting erythema and comparison with normal subjects. Lynch (1934) found that for $\lambda \geq 280$ mμ, the X.P. erythema is more rapid, exhibiting greater pigmentation than normals; filtered UV

of 250 mμ evoked a temporal erythematic response similar to normals but again with greater pigmentation; and although exposure to unfiltered radiations resulted in greater pigmentation in X.P. individuals, erythema was less rapid than in normal subjects. The X.P. patient was most sensitive to the wavelength band from 280-310 mμ of which the band 280-297 mμ is absorbed in the atmosphere and doesn't reach the earth's surface.

It was also noticed that for a given X.P. subject, unexposed and chronically exposed skin behaved differently. Consistent with Nakajima's theory that as the disease progresses sensitivity to chronic exposure decreases, Martenstein (1924) observed that previously exposed areas undergo a less severe erythematous reaction than those previously enexposed, exhibiting greater latency and a more rapid recession.

Cripps, Ramsay and Ruch (1971) in the most sophisticated such study to date determined the X.P. and normal action spectra for Minimal Erythema Dose (MED). MED is the minimum dose to cause the development of erythema at a given time following exposure. The skin of normal subjects remained unaffected by $\lambda > 320$ mμ; however for X.P. subjects reactions occurred for λ's as long as 340 mμ. For a given λ, the MED for X.P. individuals was considerably lower than for the control volunteer except at 250 mμ where it was within the control standard deviation. Cripps found the erythema delayed in X.P. individuals (maximum at 72 hours, 24 hours

for normals) but more prolonged (still present at 10 days). The most effective λ at 24 hours in X.P. was 296 mμ (190 ergs/mm^2) and at 72 hours, 293 mμ (110 ergs/mm^2). For normals, the lowest MED for an erythema maximum at 8 hours was 366 ergs/mm^2, at 250 mμ; for a maximum at 24 hours, 250 mμ was again most effective with an MED of 387 ergs/mm^2.

Cleaver (1968) was the first to look at repair replication and unscheduled DNA synthesis after exposure of cultured X.P. fibroblasts to 254 mμ. The term repair replication refers to a non-conservative mode of DNA replication in which damaged bases are replaced. ^{3}H-BUdR is used to substitute for thymidine during DNA replication following UV or ionizing radiation; ^{3}H-DNA of hybrid density results from normal semi-conservative synthesis, whereas ^{3}H-DNA of close to standard i.e. normal density indicates "patches" of repair replication. Unscheduled DNA synthesis describes DNA synthesis which occurs in cells which are not actively replicating their DNA i.e. in non S-phase cells. It is detected as lightly labeled nuclei in autoradiographs after UV- or X-ray-exposed cells have been incubated with radioactive DNA precursors. (Unscheduled DNA synthesis has been shown (Djordjevic et al. 1969) to arise spontaneously but at much lower levels without irradiation.) It is the feeling of many investigators (Painter and Cleaver 1969, and Cleaver 1969a) that these two phenomena, repair replication and unscheduled DNA synthesis, represent the same "repair" mechanism. Although

Cleaver was able to detect increasing levels of repair replication with increasing UV dose in normal human fibroblasts, rebanding of standard density X.P. DNA in CsCl density gradients failed to demonstrate repair replication for doses up to 1000 ergs/mm^2, well above saturation level for normal cells. However, autoradiographic studies showed all non S-phase X.P. cells to be lightly labeled if the photographic emulsions were exposed for 4 weeks. After 100 ergs/mm^2 the grain count over non S-phase X.P. cells was 20% of the count in non S-phase normal cells. On the other hand, after doses of 3 MED at 250 mμ Cripps et al. (1971) observed no significant difference in S-phase semi-conservative DNA synthesis between normal and X.P. cells except perhaps a shorter S-phase in X.P. cells. Following X-irradiation, or UV-irradiation of BUdR-substituted DNA, X.P. cells were found to perform levels of unscheduled DNA synthesis comparable to those found in normal human skin fibroblasts (Cleaver 1969b). The fundamental difference here is that in X-irradiated DNA (McGrath and Williams 1966) and in UV-irradiated BUdR-substituted DNA (Hutchinson and Hales 1970) substantial numbers of strand breaks occur, whereas very few are produced directly by UV in unsubstituted DNA. On the basis of these facts Cleaver (1969b) postulated that X.P. cells lack the UV repair endonuclease responsible for introducing single-strand breaks into damaged regions of the DNA. Such an endonuclease deficiency would not interfere

with the repair response of X.P. cells following treatments inducing DNA strand breakage; however, since it is hypothesized that an endonuclease is necessary for an early stage in the rapair of DNA following UV, cells lacking this endonuclease would perform very low or negligible levels of UV-induced repair synthesis, a characteristic of X.P. cells.

Reed (Reed et al. 1969) cited topical application of 1-5% fluorouracil in propylene glycol as a reasonably effective but limited treatment for X.P.; before resistance to such therapy develops, there is a considerable reduction in the number of premalignant and malignant lesions. Presumably, the fluorouracil could act as a shield as it is a good absorber of the damaging UV.

Studies by Setlow of the excision of UV-induced photoproducts indicated that 70% of pyrimidine dimers had been excised at 24 hours after UV exposure in normal human fibroblasts (Setlow, Regan, German and Carrier 1969); after 48 hours less than 20% had been excised in X.P. cells. Although the unirradiated controls in his alkaline sucrose gradient technique were not reproducible, Setlow claimed to see the appearance and disappearance of single-strand DNA breaks in normal fibroblasts following UV with no such change in the analogous X.P. system. This would support

Cleaver's hypothesis of a faulty or absent repair endonuclease in X.P. The de Sanctis Cacchione X.P. was found by Cleaver et al.(1970)to lack dimer excision at 24 hours.

Bootsma, Mulder and Cohen 1970 and Kleijer, Lohman, Mulder and Bootsma 1970, with access to a highly inbred population localized in a region of Holland, were able to correlate clinical severity of the disease with levels of repair replication and unscheduled synthesis following UV. As expected, more severe symptoms were associated with lower "repair" levels. However, related individuals had identical levels, suggesting the existence of different mutations to the same gene. This characteristic repair level remained constant during in vitro culturing and was therefore not a function of proliferation capacity. Unrelated X.P. patients with different levels of UV-induced unscheduled synthesis had, however, the same level as normal fibroblasts following ionizing radiation. Rejoining of X-ray-induced DNA single-strand breaks occurred at the same rate and to the same extent (to 50-70% of the initial M.W., $1.2\text{-}1.8 \times 10^8$ Daltons) as in normal cells. This rejoining was essentially completed in the first 15' post-irradiation incubation; incubation past 30' caused no further rejoining. In vivo studies (Epstein, Fukuyama, Reed and Epstein 1970) have produced results which are consistent with the idea (derived from in vitro experiments) that defective repair is responsible for the UV response in X.P. individuals. Epstein et al. (1970) found that unscheduled DNA synthesis after exposure to UV

of wavelengths less than 320 mμ revealed a low level of repair (4% of the normal non-X.P. grain count over lightly labeled cells in the upper dermis and 20% in the basal cell layer); little or no repair replication or unscheduled synthesis was found in the tissue cultures of these same patients (Cleaver 1968). A case of X.P. complicated by the de Sanctis Cacchione syndrome showed no sparse labeling whatsoever suggesting a more severe form of X.P. (Of the X.P. cultures used by Bootsma et al. (1970) all of which showed some unscheduled DNA synthesis, none was derived from a patient suffering from this neurological syndrome.) However, later studies (Cleaver 1970) revealed no significant consistent differences in the responses of complicated and uncomplicated forms of X.P. to UV.

Trosko, Krause and Isoun (1970) using cultured human amnion AV_3 cells obtained the same R_F value for sunlight- and UV-induced in vitro photoproducts on Thin Layer Chromatography supporting the suggestion that they represent the same lesion. There was no photoproduct production for sunlight filtered through a 1 cm. glass plate, and exposure to filtered sunlight following 254 mμ did not result in photoreactivation. Five hours of sunlight induced a number of pyrimidine dimers equivalent to a dose of 600 ergs/mm^2 at 265 mμ (.8 MED at 24 hours (Cripps et al. 1971)). Aaronson and Lytle (1970) suggested a close correlation between Host Cell Reactivation (HCR) and capacity for repair replication

in human cells. One of the X.P. strains they tested, which showed 12% of normal HCR for UV-irradiated SV40, was found by Cleaver to have 5% of normal repair replication.

Cleaver (1970) investigated the relationship between extreme in vivo sensitivity and viability on an in vitro cellular level; the X.P. in vivo severity - skin lesions and hyperpigmentation - was found to correspond to little or no in vitro repair replication and reduced colony forming ability (CFA) following UV compared to that for normal fibroblasts. The D_o for X.P. was 9 ergs/mm^2 and that for normals, 29 ergs/mm^2 where D_o is the dose reducing survival to $\frac{1}{e}$ in the exponential portion of the survival curve. Unfortunately, for diagnostic purposes, heterozygotes exhibited normal levels of repair replication; once one X.P. child has been diagnosed in a family, only detection of a fetal homozygote, will be possible by amniocentesis and a repair replication assay. One investigator (Moynahan 1962) stated that the heterozygous state exhibits itself as a tendency to excessive freckling; however, El-Hefnawi, El-Nabawi and Rasheed (1962) found no such tendency in any of 14 X.P. cases, and none of the more recent papers refers to this aspect of the heterozygote.

Patients with a very late onset (age 35 - 40) of clinical symptoms similar to those of X.P. were biopsied for autoradiographic UV studies, by Jung (1970). In vitro repair synthesis was normal; however S-phase synthesis was almost totally depressed. Perhaps (Jung 1970) in these Pigmented

Xerodermoids (P.X.) it is a late repair enzyme also involved in semi-conservative replication that is lacking, or an enzyme not involved in repair at all.

Cleaver (1972) recently found three patients with distinct symptoms of Xeroderma Pigmentosum whose cultured fibroblasts did not respond to UV in the usual way. Cells from these X.P. variants performed normal amounts of repair replication and their single cell survival curves were indistinguishable from those of normal fibroblasts. Defective DNA repair is clearly not implicated in these diagnosed X.P. patients; Cleaver suggests that they represent a biochemically distinct condition and that extrapolations from X.P. studies must be re-evaluated in the light of these recent findings. Cleaver (1972) also found two heterozygotes who don't fit into the pattern of previous experimental results. The heterozygotes, parents of an X.P. child with reduced repair activity, exhibited markedly decreased repair replication as assayed in CsCl gradients after doses of 100-300 ergs/mm^2. Clearly, these three new X.P. subjects, and the atypical heterozygotes represent minority responses at the present level of understanding; however the existence of these variants will necessitate the subdivision of Xeroderma Pigmentosum into distinct forms both for the homozygous and the heterozygote states.

Goldstein (1971) used cultured X.P. cells as a system to study possible causes of in vitro cellular aging. He

was able to conclude that failure in DNA repair is probably not a cause of senescence in vitro, although in vivo, the skin of X.P. patients exhibits an increased rate of carcinogenesis and atrophy, a phenomenon associated with aging in the organs of normal individuals.

The study of mechanisms of radiation damage and repair has been largely restricted to bacterial systems for which many mutants exist, deficient in the different stages of repair. X.P., as a natural human mutant, represents a unique opportunity to investigate such mechanisms in mammalian cells.

In X.P. clinical symptoms arise as the result of sunlight exposure; following UV, unscheduled DNA synthesis and repair replication are considerably reduced, the extent of dimer excision significantly lower than in normal subjects. This evidence together with a normal level and extent of "repair" following damage involving DNA single-strand breaks indicates a high probability that a UV repair mechanism is defective in X.P.

Since it has been postulated but never proven that the repair of ionizing radiation damage and the repair of UV damage share a common pathway (Cleaver 1969b), it was of interest to explore the response of X.P. cells to ionizing radiation.

This dissertation describes such responses at both the molecular and the cellular level. Molecular damage was

assayed as DNA single-strand breaks; the end-point for damage to the whole cell was colony-forming ability.

2. MATERIALS AND METHODS

2.1. CELL CULTURES

2.1.1. Human cells

Primary human fibroblast cells from two X.P. patients and from a normal human were kindly supplied to us by Dr. S. Goldstein. The line XP1 was derived from a 20 year old male and the line XP2 from his 21 year old sister. The X.P. cells were obtained by skin biopsy of patients at the Roswell Park Memorial Institute in Buffalo, through the cooperation of Dr. E. Klein. The normal cell line, designated A.N., was obtained from a 3 day old male. All cultures were grown in plastic flasks (Falcon Plastics 3024) in Minimal Essential Medium (MEM F-15 Gibco) supplemented with 14.3% fetal calf serum (FCS 614 Gibco) and a supplement "A". The supplement consisted of 3 ml phosphate buffered saline (PBS), 4.5 gm glucose, 0.33 gm Na pyruvate, 0.3 mg $Fe(NO_3)_3.9H_2O$ in 30 ml double distilled water for 3.5 l of medium. Flasks were seeded at 1-2 x 10^6 cells each and confluent cultures trypsinized and subcultured 5-6 days later (0.25% trypsin Bacto-Difco: 8' at 37°C). Transfers, and all preparation of materials were done in a laminar flow hood (Edgegard Hood, Baker) due to the fact that no antibiotics were used in the culturing

of the X.P. or normal cell lines. Cells were incubated at 37°C in an atmosphere of 5% CO_2, 95% air and 100% humidity.

Doubling time ranged from 24 hours - 50 hours for the fibroblasts (S. Goldstein, private communication)

When the cells were first cultured in our lab, they had already undergone 20 generation times following explant; they were not used for experiments at later than 55 generations. The generation number is as defined by Hayflick (1965).

The karyotypes for both X.P. and A.N. cells were normal during this investigation.

2.1.2. Mouse L-60 cells

Mouse L-60 cells (obtained from G. F. Whitmore, University of Toronto) were used in some experiments as feeder layers for clonal growth. These cells were grown in suspension culture (1×10^5 - 4×10^5 cells/ml) in Minimal Essential Medium (MEM F-13 Gibco) supplemented with 10% undialysed fetal calf serum (FCS 614 Gibco). They were resuspended in MEMA before use in experiments. L-60 cells were chosen for feeders as they did not interfere with the counting of colonies of human cells; A.N.cells on the other hand resembled too closely the cells whose survival was being measured.

2.2. γ-RAY SOURCE

Cells were irradiated in suspension with γ-rays (E = 0.66 MeV) from a 2000 Curie Cs^{137} unit. For alkaline sucrose gradient experiments the dose rate was 2000 rads/min at 0°C in PBS; in γ-ray survival studies the dose rate was 97 rads/min at room temperature in MEMA. The dose rates were measured with a Victoreen Model 570 condenser r-meter. Doses were converted from roentgens to rads using the factor 0.975 rads/roentgen for Cs^{137} γ-rays (Johns 1961). The physical arrangement of the cell suspensions in the Cs unit is shown in Figs. 1a and 1b. Ferrous sulphate dosimetry measurements have confirmed the ionization chamber measurements (B. Palcic, private communication).

2.3. UV SOURCE

Ultraviolet irradiation was performed using an 8 Watt General Electric Germicidal Tube, Number G8T5. Dose rate measurements were taken with a UV intensity meter (Blak-Ray short wave UV meter, model J-225, Ultra-violet Products, Inc., San Gabriel, California). 24-hour cultures of X.P. or A.N. cells in plastic petris (Falcon Plastics 3002) were irradiated at dose rates of 5 ergs/mm^2/sec and 10 ergs/mm^2/sec respectively with the medium removed. Figure 2a shows a dose rate vs. distance curve for the UV source. Figure 2b is a plot of $\frac{1}{r^2}$ vs. dose. To explain the lack of linearity, one must consider that there is UV absorption by the air molecules in the chamber, and reflection by the white

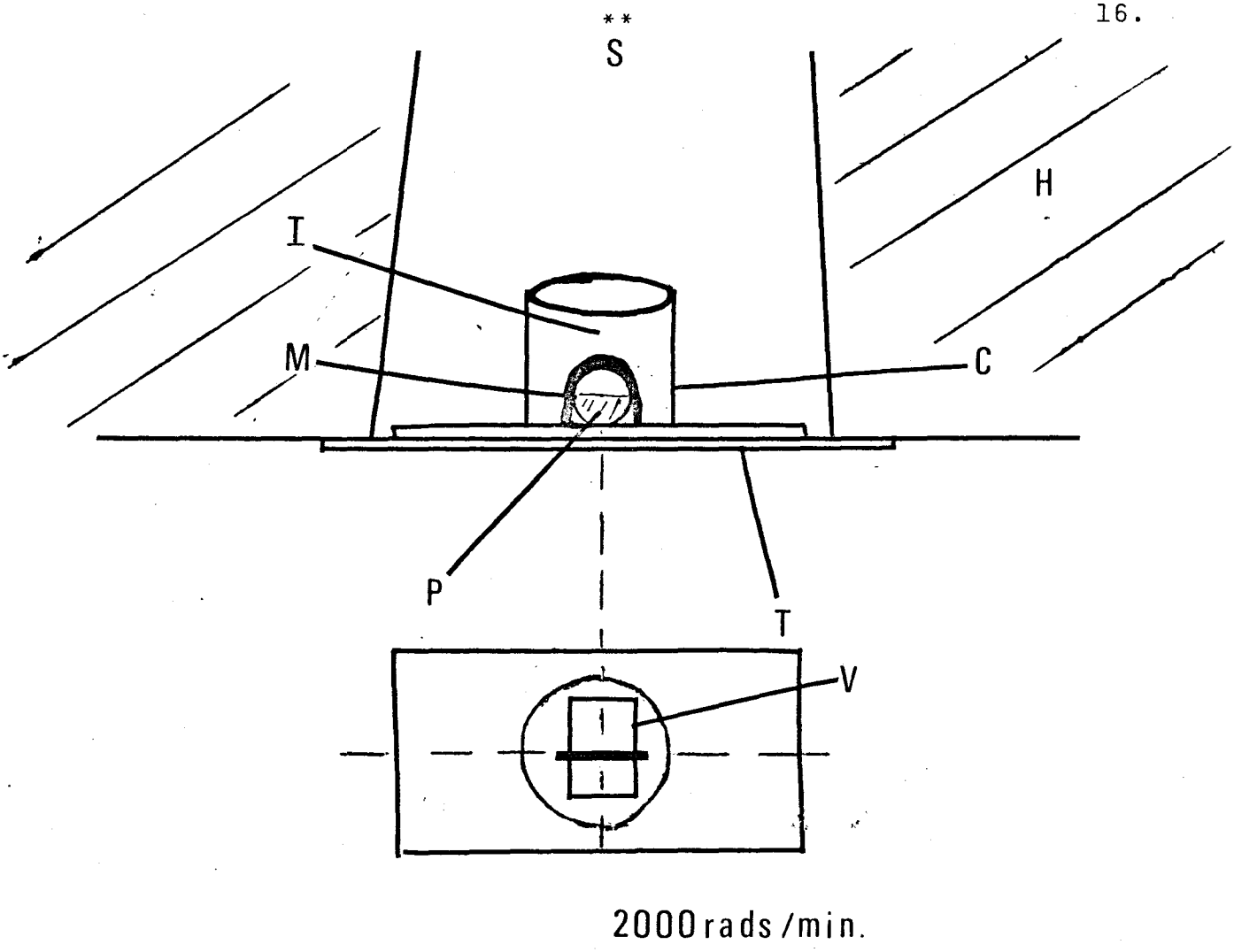


Figure 1a. Cs^{137} unit adapted for γ-irradiation of cell suspension prior to gradient analysis

Plan and side elevation of apparatus used for γ-irradiation of cells prior to gradient analysis. Shown are γ-ray (Cs^{137}) source S, source housing H, metal restraint M, glass cylinder G, glass support T, glass cylinder C, ice-water I, and cell suspension (PBS) P in a cylindrical plastic vessel V.

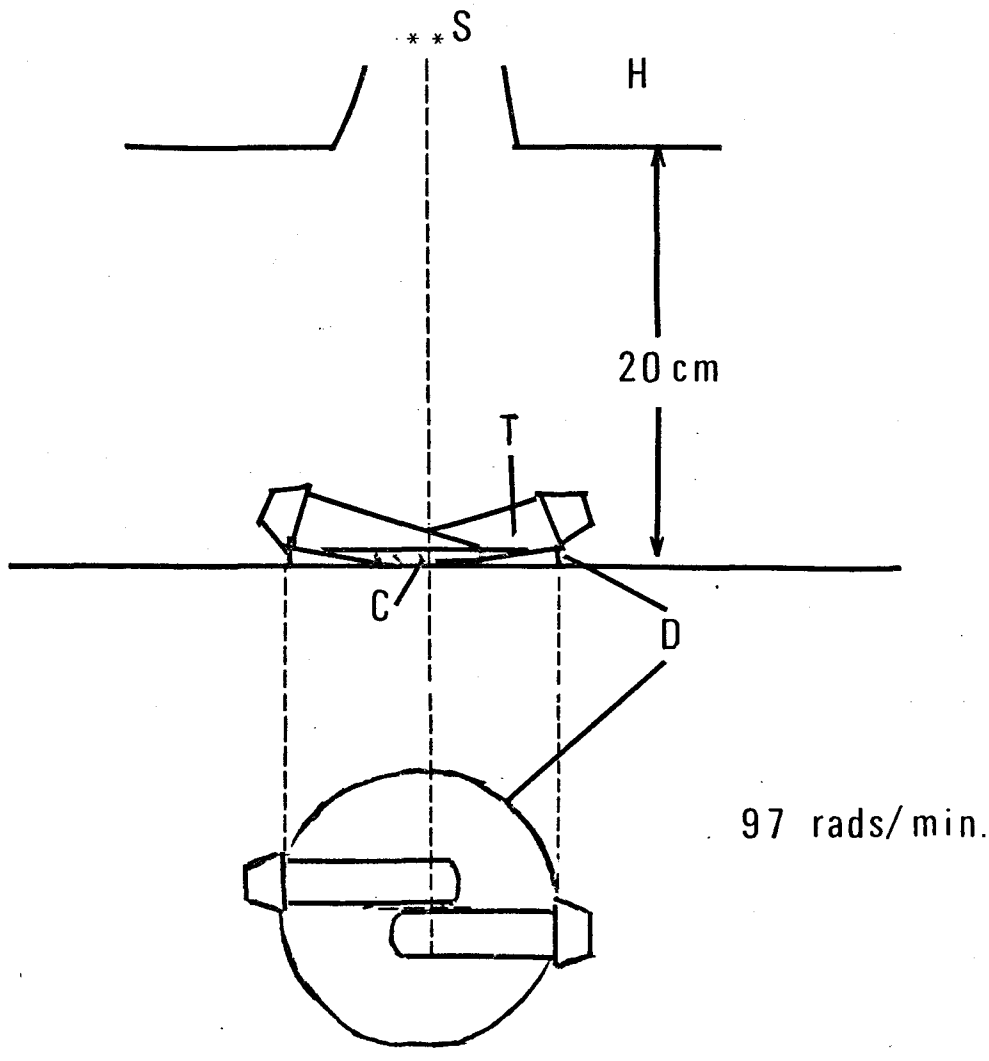


Figure 1b. Cs^{137} unit adapted for γ-irradiation of cell suspension prior to survival studies

Plan and side elevation of apparatus used for γ-irradiation of cells prior to survival studies. Shown are γ-ray (Cs^{137}) source S, source housing H, plastic petri dish D, plastic culture tube T and cell suspension C.

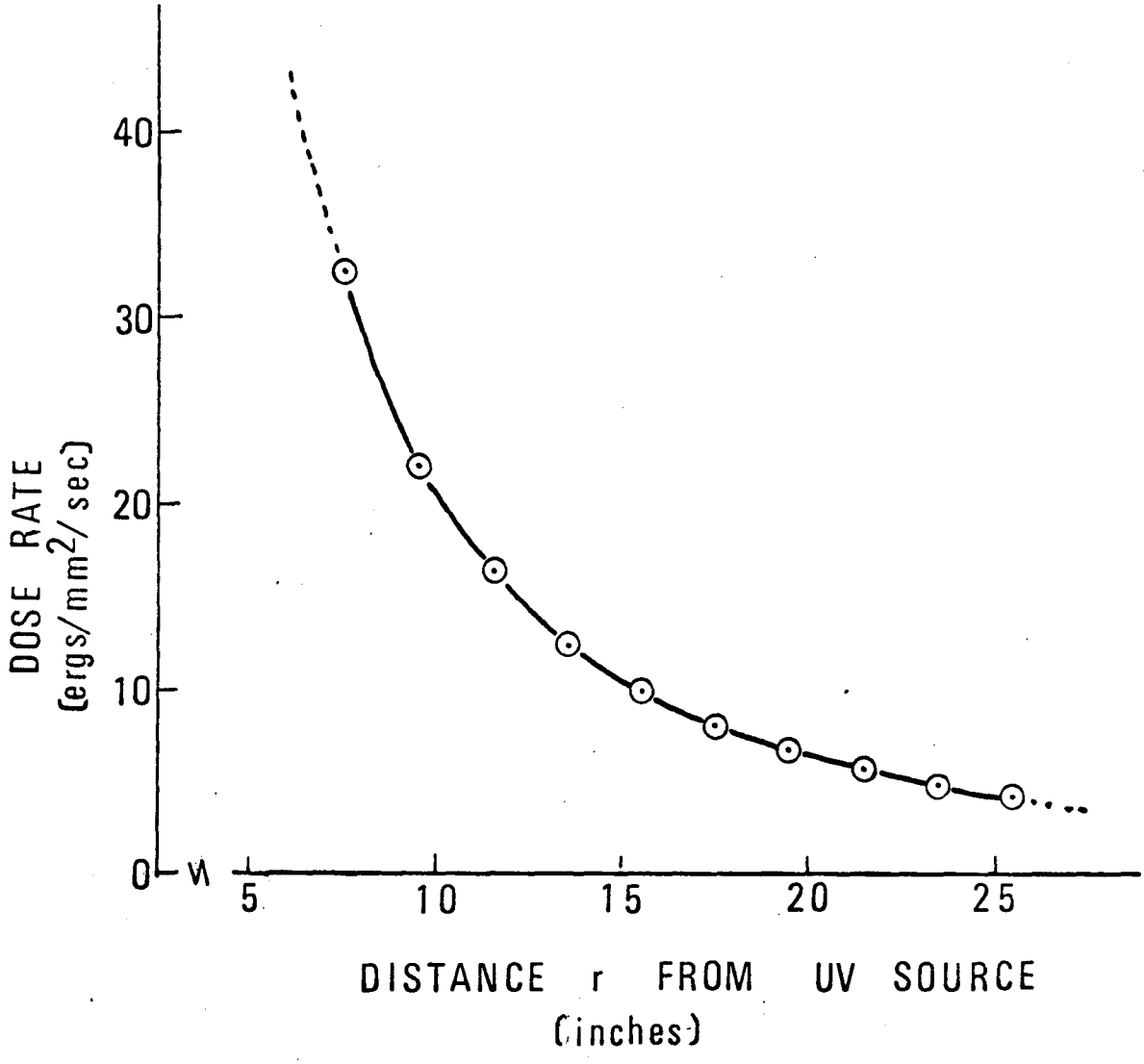


Figure 2a Variation of the UV dose rate with distance (r) from the source

The source of UV irradiation was an 8 watt General Electric Germicidal Tube, number G8T5. Dose rate measurements were taken with a UV intensity meter (Blak-Ray short wave UV meter, model J-225, Ultra-violet Products Inc., San Gabriel, California).

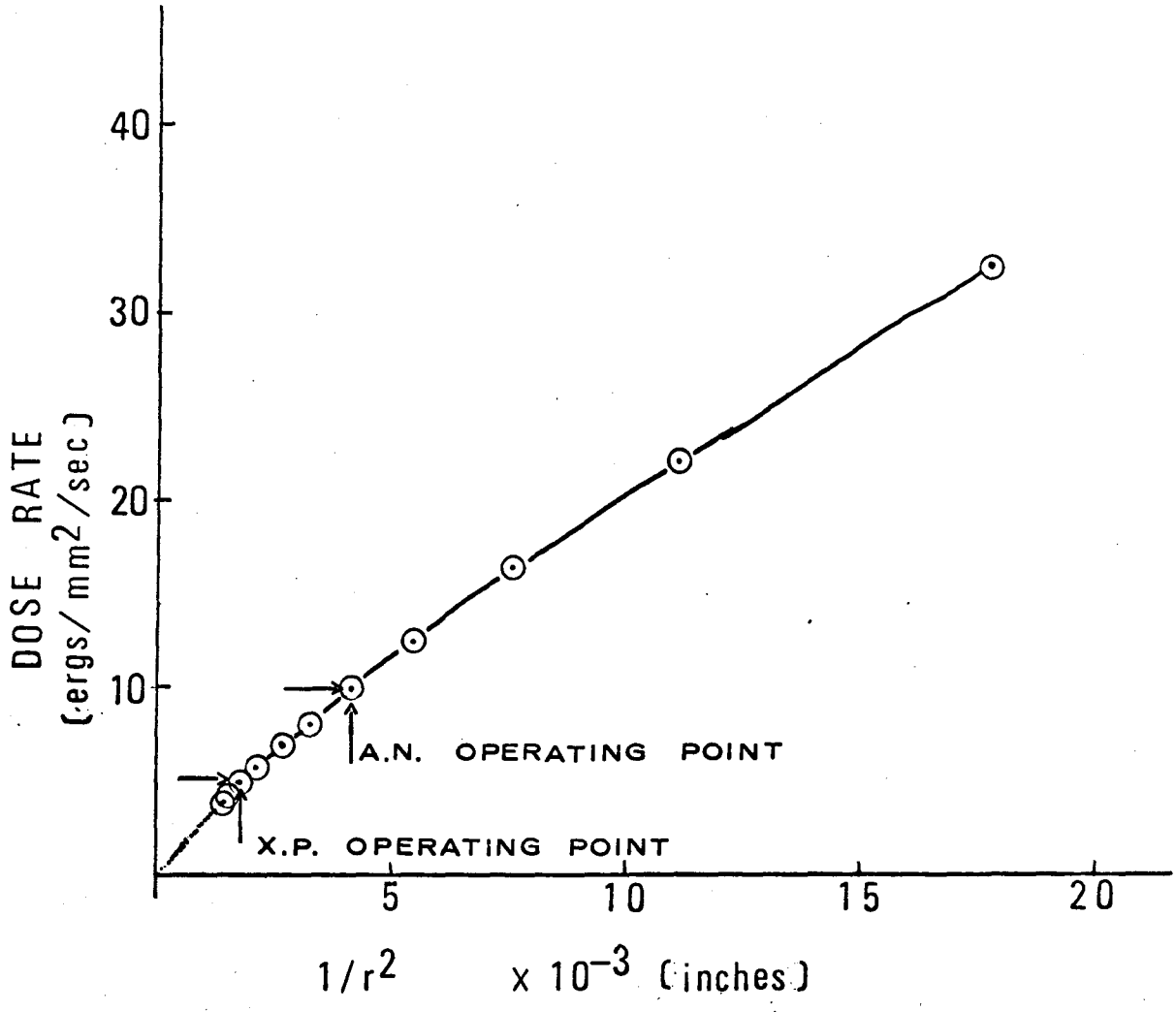


Figure 2b Variation of the UV dose rate with $1/r^2$

Conditions are as for figure 2a.

walls of the chamber. Furthermore the UV radiation enters the chamber through a long narrow slit.

2.4. ALKALINE SUCROSE GRADIENT EXPERIMENTS

2.4.1. Labeling

^{3}H-Thymidine (15.5 C/mM, Amersham-Searle, Don Mills, Ontario) was used to radioactively label the DNA. 1-2 day old cultures were incubated for 24-48 hours in MEMA plus ^{3}H-TdR at 1-2 μC/ml.; this medium was then removed and incubation was continued for about 1 hour in unlabeled medium; the cells were then trypsinized, resuspended in PBS at 0°C and irradiated.

2.4.2. Irradiation

Cells were irradiated in suspension (PBS) at 0°C. For studies of the rejoining of DNA single strand breaks a dose of 10 Krads was used; for investigations of dose response, one suspension received successive doses at 0°C for an accumulated total of up to 30 Krads; after each increment, an aliquot was removed for gradient analysis.

2.4.3. Gradients and Lysing Procedure

2.4.3.1. 5 ml Tubes

An automatic gradient maker (ISCO Model 570) was used to make linear 5 - 20% alkaline sucrose gradients, in 5 ml (1/2" diameter x 2") polyallomer tubes (Beckman). The sucrose solutions contained 0.3N sodium hydroxide (NaOH), 0.001 M ethylenediaminetetraacetic acid (EDTA), 0.01% sodium dodecyl sulphate (SDS) in double distilled water.

A 0.3 ml layer of lysing solution containing 0.5 N NaOH, 0.01M EDTA, and 0.2% SDS in double distilled water was very gently layered on top of the gradient just prior to lysis. 0.02 - 0.04 ml. of cell suspension (2 - 6 x 10^4 cells) was delivered from a microsyringe into the lysing layer over a period of 30 - 45 sec. This critical procedure was facilitated by two mechanical rack-and-pinion drives which operated the syringe. The first of these provided for precise lowering of the syringe until the needle just touched the lysing solution, while the second drive operated the syringe plunger to deliver the aliquot of cells. Cells were left to lyse in the dark and at room temperature for 14-17 hours. This particular range of lysis times per se caused no variation in the sedimentation profiles of irradiated or control DNA (Palcic 1972).

2.4.3.2. 17 ml Tubes

A few experiments were performed using the Beckman SW 27 rotor and 17 Ml (1" diameter x 3 1/2") cellulose nitrate tubes (Beckman). The linear 5 - 20% alkaline sucrose gradients contained 0.3N NaOH, 0.001 M EDTA, 0.01% SDS in double distilled water as in 2.4.3.1. A 0.5 ml layer of lysing solution (0.5N NaOH, 0.01M EDTA, 0.2% SDS in double distilled water) was layered on top of the gradient as in 2.4.3.1. All methods, and lysis times were as in 2.4.3.1.

2.4.4. Centrifugation

All 5 ml gradients were centrifuged at 20°C in a Beckman SW 50.1 rotor at 42,000 r.p.m. for 50 or 65 minutes. The 17 ml gradients were centrifuged at 20°C in a Beckman SW27 rotor at 16,000 r.p.m. for times ranging from 6 hours 45 minutes to 13 hours 35 minutes.

It has been reported that high speeds of centrifugation severely distort the profile of sedimenting molecules if the molecules are larger than 1-2 x 10^8 daltons (Elkind 1971, McBurney et al. 1971 and Palcic 1972), this distortion being due to viscous drag on high molecular weight molecules. Low speeds of centrifugation (<20,000 r.p.m.) result in larger calculated values for the molecular weight of unirradiated DNA. Therefore, low speed centrifugation (16,000 r.p.m.) was used to determine the molecular weights of unirradiated X.P. and A.N. DNA.

2.4.5. Collecting

2.4.5.1. 5 ml Tubes

Using an ISCO Model D fraction collector, 20 fractions of 0.25 ml each were collected from the top for each gradient. The DNA in each fraction was precipitated by 0.5 ml ice cold 20% trichloracetic acid (TCA) before filtration (0.22 μ millipore filter), and washing with ice cold 5% TCA and 75% ethanol. Filters were placed in glass vials and dried; for scintillation counting, 5 ml of a toluene-based scintillation solution (0.315 gm/l POPOP,

4.2 gm/l PPO) were added to each vial and samples were counted for 5 minutes at room temperature in a Beckman 3 channel scintillation counter (LS250). A control aliquot of suspension (0.02 - 0.04 ml, as on the gradient), added directly to 0.3 ml lysing solution in a glass test tube received the same lysis, TCA and counting treatment as above, and as such was used as an indication of the recovery of ^{3}H-counts from the gradients. Recovery was 80% to 110%. For each gradient, the % of total counts for each fraction was calculated (allowing for background) and the % total counts profile plotted.

2.4.5.2. 17 ml Tubes

An ISCO Model D fraction collector was used to collect 25 fractions of 0.75 ml each from the top of each gradient. Collection was directly into glass vials; 0.2 ml of 4N HCl was added to each vial to neutralize the alkaline sucrose solution. For scintillation counting, 5 ml of Aquasol (New England Nuclear) was added to each vial and samples were counted for 5 minutes at room temperature in a Beckman 3 channel scintillation counter (LS 230). A control aliquot of cell suspension (0.02 ml) added directly to 0.5 ml lysing solution in a glass vial received the same lysis, neutralizing and counting treatment as above. Recovery of ^{3}H-counts was 90% - 110%. As in 2.4.5.1. the profile of the % total counts was plotted for each gradient.

2.4.6. Molecular Weight Calculations (Palcic and Skarsgard 1972)

The weight average molecular weight M_w of a distribution of molecules is defined as

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

where n_i represents the number of molecules in the i-th fraction and M_i represents the molecular weight of a molecule which sediments to the middle of the i-th fraction. M_i is related to the sedimentation constant S_i of the molecule (in water at 20°C) by

$$M_i = \left(\frac{S_i}{a}\right)^k \qquad (2)$$

(Burgi and Hershey 1963); Studier (1965) evaluated the constants a and k as 0.0528 and 2.5 respectively. S_i is related to the centrifugation conditions by

$$S_i = \frac{\beta\, d_i}{\omega^2 t} \qquad (3)$$

where β is a constant for a given type of gradient, d_i is the distance sedimented by the molecule during centrifugation, ω is the angular velocity in r.p.m. and t is the time of centrifugation.

Assuming a uniform labeling of the DNA during incubation with ^{3}H-TdR, the percentage total counts (corrected for background) C_i in any fraction i is proportional to both the molecular weight M_i and n_i. Then,

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

where k is a proportionality constant. Using equations (2),(3) and (4),equation (1) becomes

$$M_w = \left(\frac{\beta}{\omega^2 ta} \right)^k \quad \frac{\sum_i C_i (d_i)^k}{\sum_i C_i} \qquad (5)$$

By the time the lysis period is terminated, the starting material has settled to the top of the second fraction for the 5 ml gradients and to the middle of the first fraction for the 17 ml gradients (B. Palcic, 1972). If we use d_f to represent the distance associated with one fraction, then for 5 ml tubes, equation (5) becomes

$$M_w = \left(\frac{\beta\, d_f}{\omega^2 ta} \right)^k \quad \frac{\sum_i C_i (i - 0.5)^k}{\sum_i C_i} \qquad (6a)$$

and the subscript i is 1 for the second fraction collected from the top of the gradient. The molecules of the i-th fraction have a molecular weight M_i at the middle of the fraction, hence the factor $(i - 0.5)^k$ is used in equation (6a). The expression for M_w for DNA layered on 17 ml gradients, is, in analogy with equation (6a)

$$M_w = \left(\frac{\beta\, d_f}{\omega^2 ta} \right)^k \quad \frac{\sum_i C_i\, i^k}{\sum_i C_i} \qquad (6b)$$

Again, the subscript i is 1 for the second fraction collected, due to the position of the starting material at the end of

the lysis period; the factor i^k appears in (6b) because the starting material settles only to the middle of the first fraction collected.

As can be seen in both equations (6a) and(6b), the sedimentation profile alone contributes the summation portion of the M_w expression; the first factor in each of equations (6a) and (6b) is a function of the conditions of centrifugation.

The number average molecular weight M_n of a distribution of molecules is of importance for calculations of the energy requirement (in eV/break) for DNA strand breakage following exposure to ionizing radiation. It is defined as

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

where M_i and n_i are as stated for equation (1). This becomes, in analogy to the expressions for M_w in equations (6a) and (6b),

$$M_n = \left(\frac{\beta d_f}{\omega^2 ta} \right)^k \frac{\sum_i C_i}{\sum_i C_i/(i - 0.5)^k} \qquad (8a)$$

and

$$M_n = \left(\frac{\beta d_f}{\omega^2 ta} \right)^k \frac{\sum_i C_i}{\sum_i C_i/i^k} \qquad (8b)$$

This leads, however, to considerable uncertainty in calculated values of M_n as the contribution of the first few fractions is anomalously large. The expression for M_n is greatly simplified for a random distribution of molecules to (Charlesby 1954)

$$M_n = 0.5 \quad M_w \tag{9}$$

If a molecular distribution is initially uniform, and not random, 5 - 10 strand breaks per molecule will render the distribution random.

2.4.7. Test for Random Distribution of Molecular Sizes

If a population of molecules has a random distribution of molecular sizes, then

$$\frac{\Delta N_i}{\Delta M_i} = K' \quad \frac{1}{M_n^2} \quad e^{-} \quad \frac{M_i}{N_n} \tag{10}$$

where $\Delta N_i = n_i$, 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. Substitution of equation (4) into (10) gives

$$\frac{C_i}{M_i \Delta M_i} = KK' \quad \frac{1}{M_n^2} \quad e^{-} \quad \frac{M_i}{N_n} \tag{11}$$

Taking the logarithm of equation (11) yields

$$\log \frac{C_i}{M_i \Delta M_i} = - \frac{1}{2.3} \frac{M_i}{M_n} + \frac{1}{2.3} \log \frac{KK'}{M_n^2} \tag{12}$$

where

$$M_i = A_{\omega,t} \ (i - 0.5)^k \qquad (13a)$$

or $$M_i = A_{\omega,t} \ i^k \qquad (13b)$$

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

Equations (13a) and (13b) refer to 5 ml and 17 ml tubes respectively.

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

$$k_s = - \frac{1}{2.3 \ M_n}$$

The first few fractions from the top of any gradient are disregarded in fitting straight lines to these data as they are most susceptible to error.

2.5. FEEDER CELLS - SURVIVAL STUDIES

Mouse (L-60) cells at 5×10^4 / petri were used as feeder cells in survival studies. Feeder cells are cells which have been heavily irradiated so that they are unable to form colonies but whose continued metabolism conditions the medium for the colony growth of surviving cells. The beneficial effect of feeders on colony formation is most important during the initial stages following plating.

The feeders were suspended in 6-7 ml MEMA (2×10^6 - 5×10^6 cells for an experiment) and administered a dose of 6000 rads.

2.6. SURVIVAL EXPERIMENTS

2.6.1. UV Survival

Log phase cells were trypsinized, resuspended in MEMA, given appropriate dilutions and plated into 6 ml cloning medium (MEMA + 14.3% FCS + 5 x 10^4 feeder cells) in plastic petri dishes (Falcon Plastics 3002). Inocula were initially based on an assumed survival response (10% control plating efficiency, 20 colonies per petri). After 24 hours incubation under the conditions of 2.1, the medium was withdrawn by aspiration and the attached cells were exposed to appropriate UV doses. Fresh medium was added and further incubation allowed for 13-14 days. A medium change was made at day 8. Colonies were then stained with a 2% methylene blue solution, and counted; a minimum of 50 cells was the criterion used for a surviving colony and borderline cases were examined using a dissecting microscope.

2.6.2. γ-Ray Survival

Log phase cells at near confluence were trypsinized, resuspended in 7-8 ml MEMA at 2 x 10^5 cells/ml and placed in a plastic test tube for irradiation. After doses of 0, 125, 250, 375, 500, 625, 750 rads, aliquots of suspension were removed, and after appropriate dilutions, cells were plated in 5 cm plastic petri dishes containing 6 ml cloning medium (as in 2.6.1). This plating was completed within 1 1/2 hours of trypsinization to minimize the effects of suboptimal growth conditions. Incubation was at 37°C under the conditions of 2.1 for 13-14 days; medium was changed at day 7-9. Colonies were stained and counted as in 2.6.1.

3. RESULTS

3.1. SEDIMENTATION STUDIES

3.1.1. Molecular Weights : 5 ml Tubes

Fig. 3 shows typical sedimentation patterns for DNA from X.P. cells after exposure to doses of 0 and 10 Krads of ionizing radiation. Cells which had been grown to almost a monolayer, their DNA ^{3}H-labeled during the last 2 days of culture, were trypsinized, irradiated and lysed on an alkaline sucrose gradient following the procedure outlined in section 2.4. The gradient and lysing solution compositions are illustrated in the upper right hand corner of the figure.

McGrath and Williams (1966), working in a bacterial system, introduced the technique of lysing cells on the top of an alkaline sucrose gradient, the purpose of this technique being to minimize shear damage to the DNA. In the alkaline environment, (pH 12, McGrath and Williams (1966)) the DNA is denatured and sediments as single strands. The decreased sedimentation distance for irradiated DNA was interpreted as evidence of radiation-induced DNA breaks. The procedure has been adapted for mammalian cells in several laboratories (Lett et al. 1967, Humphrey et al. 1968, Lohman 1968, Terasima and Tsuobi 1968, Matsudaira et al. 1969, Dean et al. 1969, Sawada and Okada 1970, Elkind and

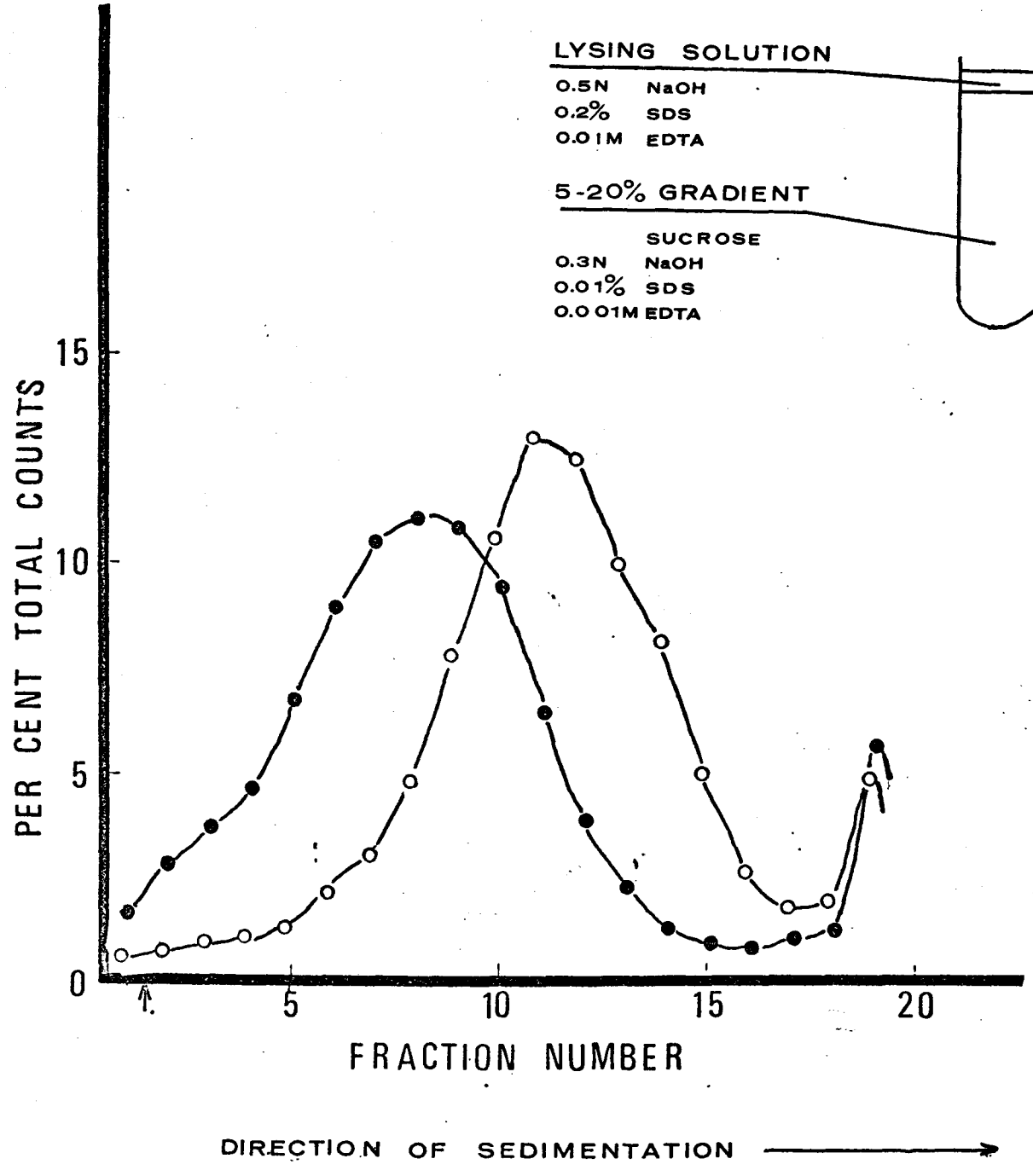
Figure 3 Production of DNA single strand breaks by ionizing radiation (XP1 cells)

XP1 cells, labeled for 48 hours with ^{3}H-TdR were administered doses of 0 Krads or 10 Krads of γ-rays at 0°C. 6×10^{4} cells were then lysed for 16 hours at room temperature in a lysing solution (0.5 N NaOH, 0.01M EDTA, 0.2% SDS) on the top of an alkaline sucrose gradient (5 - 20% sucrose, 0.3N NaOH, 0.001M EDTA, 0.01% SDS). Gradients were spun immediately following this lysis period, at ω = 42,000 r.p.m. for 50 minutes at 20°C (SW 50.1 rotor, 5 ml tubes). The lysing solution and gradient compositions are illustrated in the upper right hand corner of the figure.

(o) ^{3}H counts, 0 Krads, $M_w = 2.2 \times 10^{8}$ daltons;

(●) ^{3}H counts, 10 Krads, $M_w = 0.84 \times 10^{8}$ daltons

The recovery of radioactive counts was 100% for unirradiated (0 Krads) cells and 98% for irradiated (10 Krads) cells.


LYSING SOLUTION
0.5N NaOH
0.2% SDS
0.01M EDTA
5-20% GRADIENT
SUCROSE
0.3N NaOH
0.01% SDS
0.001M EDTA
PER CENT TOTAL COUNTS
0
5
10
15
5
10
15
20
FRACTION NUMBER
DIRECTION OF SEDIMENTATION

Kamper 1970, Moroson and Furlan 1970, Elkind 1971, McBurney et al. 1971 and many others); our experimental conditions are a variation of those originally outlined by McGrath and Williams (1966) and are described in detail elsewhere (Palcic and Skarsgard 1972).

The profiles in fig. 3, obtained from 5 ml gradients, correspond to single strand DNA weight average molecular weights of 2.2×10^8 and 0.84×10^8 daltons for 0 and 10 Krads respectively. These M_w values were calculated as shown in equation (6a)

$$M_w = \left(\frac{\beta \, d_f}{\omega^2 t a} \right)^k \frac{\sum_i C_i \, (i - 0.5)^k}{\sum_i C_i}$$

The constant β was calculated to be 6.6×10^{10} (Palcic and Skarsgard, 1972) using T4, T7 and Adenovirus markers; d_f, the distance sedimented by a molecule which sediments from the middle of one fraction to the middle of the next fraction is 0.249 cm; ω is the angular velocity of the rotor during sedimentation and is given in revolutions/minute; t is the time of centrifugation in hours; a is 0.0528; k is 2.5; C_i is the % total ^{3}H-counts in the fraction i (corrected for background).

3.1.2. Molecular Weights : 17 ml Tubes

M_w values for 17 ml tubes are calculated using equation (6b)

$$M_w = \left(\frac{\beta \, d_f}{\omega^2 t a} \right)^k \frac{\sum_i c_i \, i^k}{\sum_i c_i}$$

Again, the gradients were normalized using T4, T7 and Adenovirus markers; new values of 6.51×10^{10} and 0.378 cm were found for β and d_f respectively (Palcic 1972). Unirradiated XP1, XP2 and A.N. DNA when sedimented at 16,000 r.p.m. in 17 ml tubes yielded calculated M_w values of 3.8×10^8, 3.5×10^8 and 3.4×10^8 daltons respectively. The results obtained from both 5 ml (42,000 r.p.m.) and 17 ml (16,000 r.p.m.) tubes are discussed at greater length in section 3.1.3.

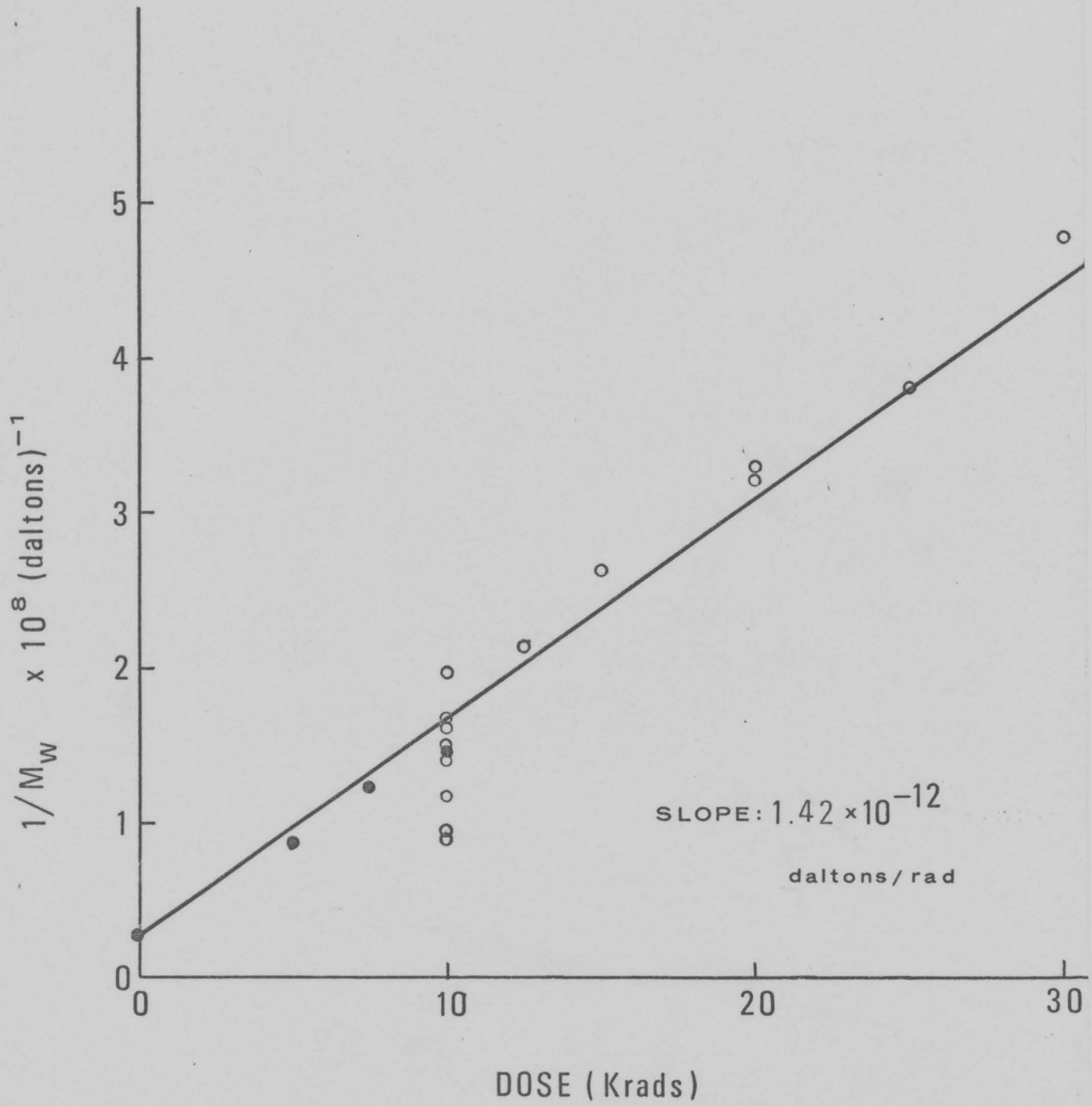
3.1.3. Efficiency of DNA Single Strand Breakage (eV/break)

In order to determine, in terms of numbers of radiation-induced DNA breaks, the radiation response of the different cell strains to increasing doses of ionizing radiation, cell suspensions were exposed at 0°C to doses up to 30 Krad. and lysed immediately following irradiation. Figures 4 and 5 are radioactivity profiles typical of such an experiment for X.P. and A.N. cells respectively. As the dose of radiation increases, the DNA profile shifts towards the top of the tube, indicating progressively smaller pieces of DNA. For random distributions one can calculate the efficiency of the radiation-induced DNA breakage from the data of figures 4 and 5 by plotting them in terms of $\frac{1}{M_w}$ vs. dose; figures 6 and 7 represent the data from several dose response experiments, and the corresponding efficiencies in eV/break given later in this section have been calculated from the straight lines which represent least squares fits to these data. If the size distribution of unirradiated molecules is random, the

Figure 4 Dose response for DNA single strand break induction : alkaline sucrose gradient profiles (XP1 cells)

XP1 cells, labeled for 48 hours with ^{3}H-TdR were administered doses of 0, 5, 10, 12.5, 15 and 20 Krads ionizing radiation at 0°C. 4.5×10^{4} XP1 cells were lysed for 16 hours at room temperature (lysing solution contained 0.5N NaOH, 0.01M EDTA, 0.2% SDS; gradients contained 5 - 20% sucrose, 0.3N NaOH, 0.001M EDTA, 0.01% SDS); gradients were spun at 42,000 r.p.m. for 50 minutes at 20°C. An SW 50.1 rotor was used for centrifugation of the gradients (5 ml.)

(○) 0 Krads;
(◐) 5 Krads;
(●) 10 Krads;
(Δ) 12.5 Krads;
(X) 15 Krads;
(▼) 20 Krads.


5
4
3
2
1
0
0
10
20
30
$1/M_W \times 10^8$ (daltons)$^{-1}$
DOSE (Krads)
SLOPE: 1.42×10^{-12}
daltons/rad

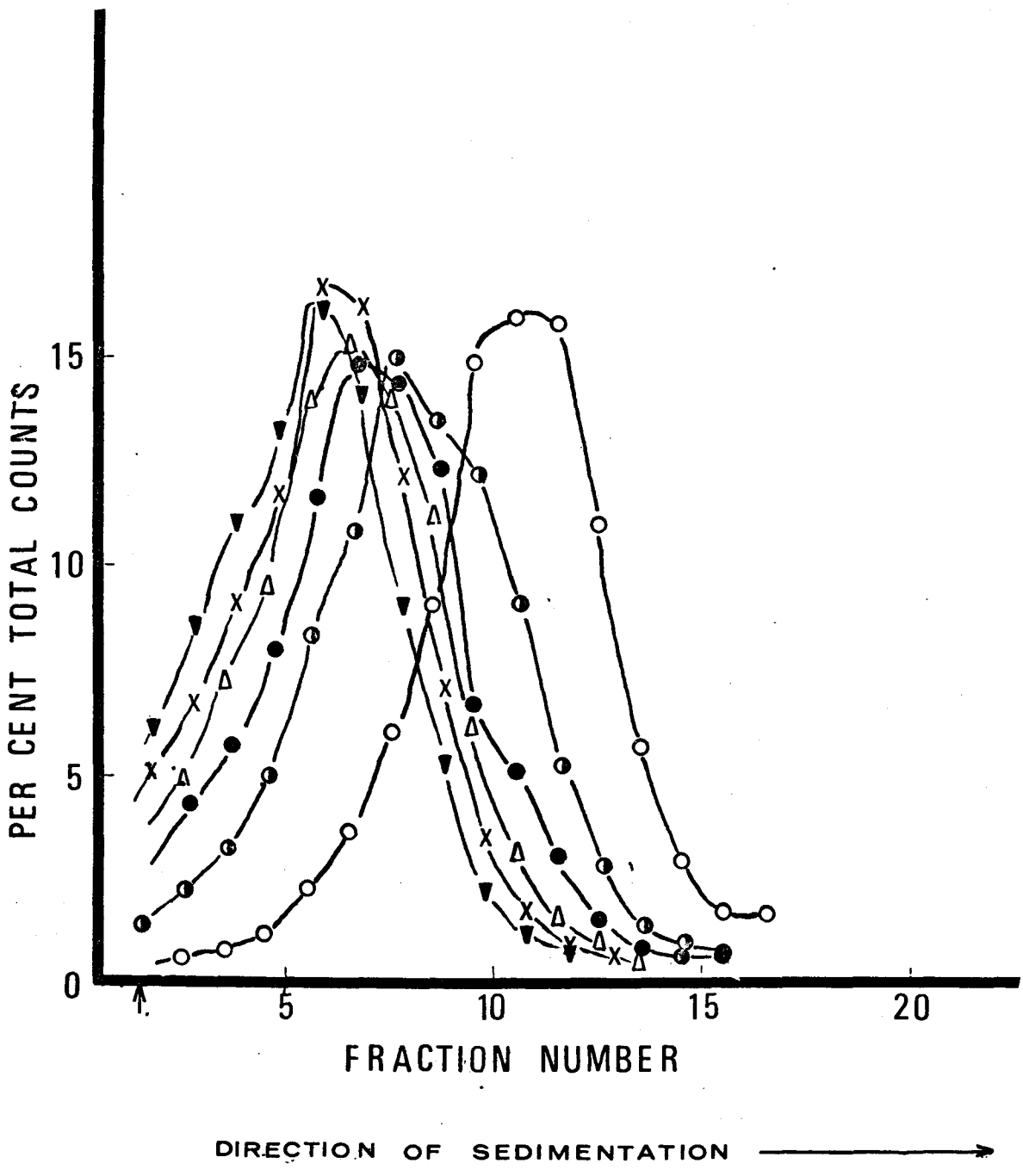

PER CENT TOTAL COUNTS
0
5
10
15
5
10
15
20
FRACTION NUMBER
DIRECTION OF SEDIMENTATION

Figure 5 Dose response for DNA single strand break induction : alkaline sucrose gradient profiles (A.N. cells)

A.N. cells, labeled for 48 hours with ^{3}H-TdR were administered doses of 0, 4, 10, 16 and 25 Krads ionizing radiation at 0°C. 6×10^{4} cells were lysed for 16 hours at room temperature (lysing solution contained 0.5N NaOH, 0.01M EDTA, 0.2% SDS; gradients contained 5 - 20% sucrose, 0.3N NaOH, 0.001M EDTA, 0.01% SDS); gradients were spun at 42,000 r.p.m. for 50 minutes at 20°C. An SW 50.1 rotor was used for centrifugation of the gradients (5 ml.).

(○) 0 Krads;
(X) 4 Krads;
(●) 10 Krads;
(Δ) 16 Krads;
(◑) 25 Krads.

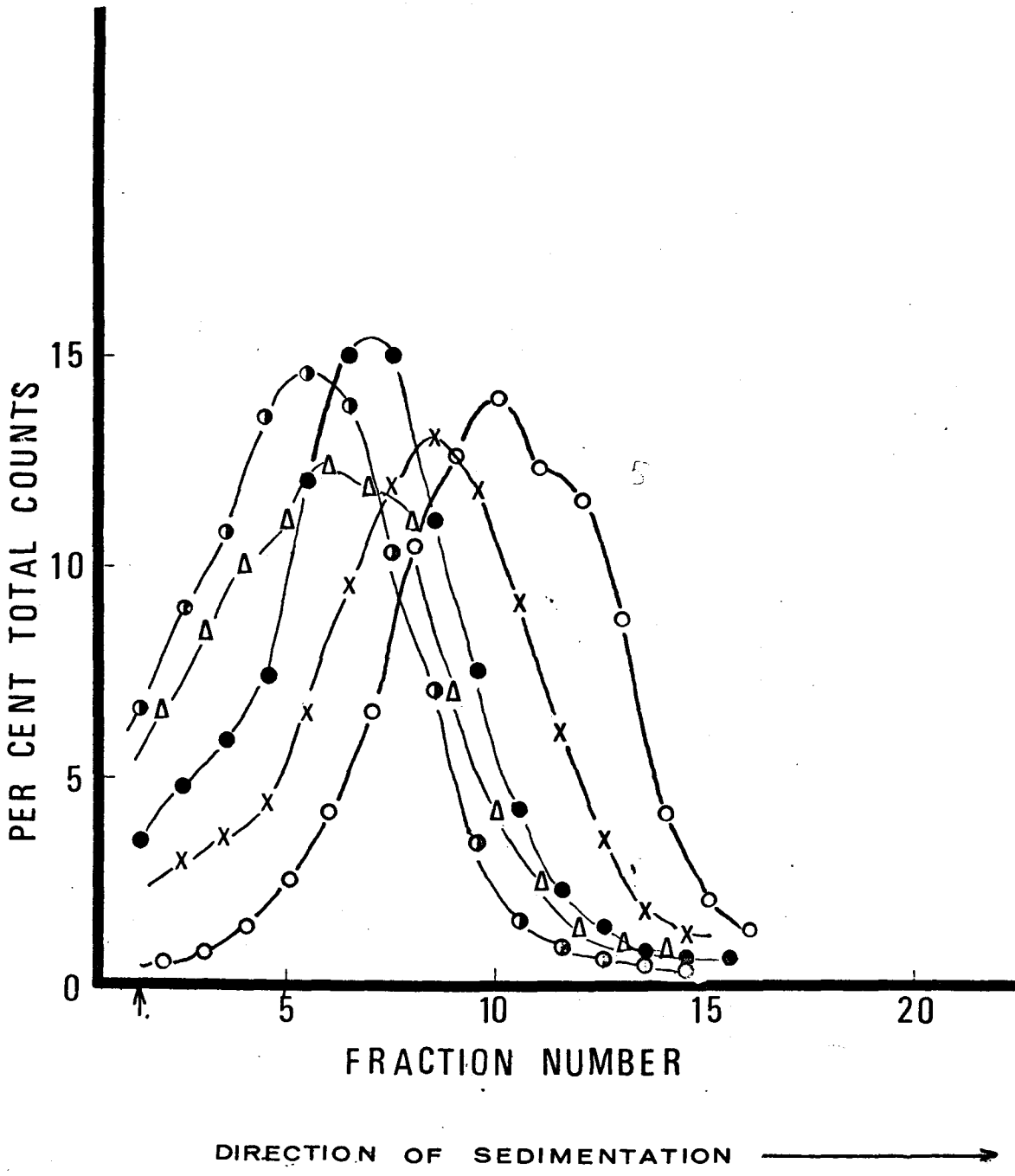

PER CENT TOTAL COUNTS
0
5
10
15
5
10
15
20
FRACTION NUMBER
DIRECTION OF SEDIMENTATION

Figure 6 Dose response for DNA single strand break induction : $1/M_w$ vs. dose (X.P. cells)

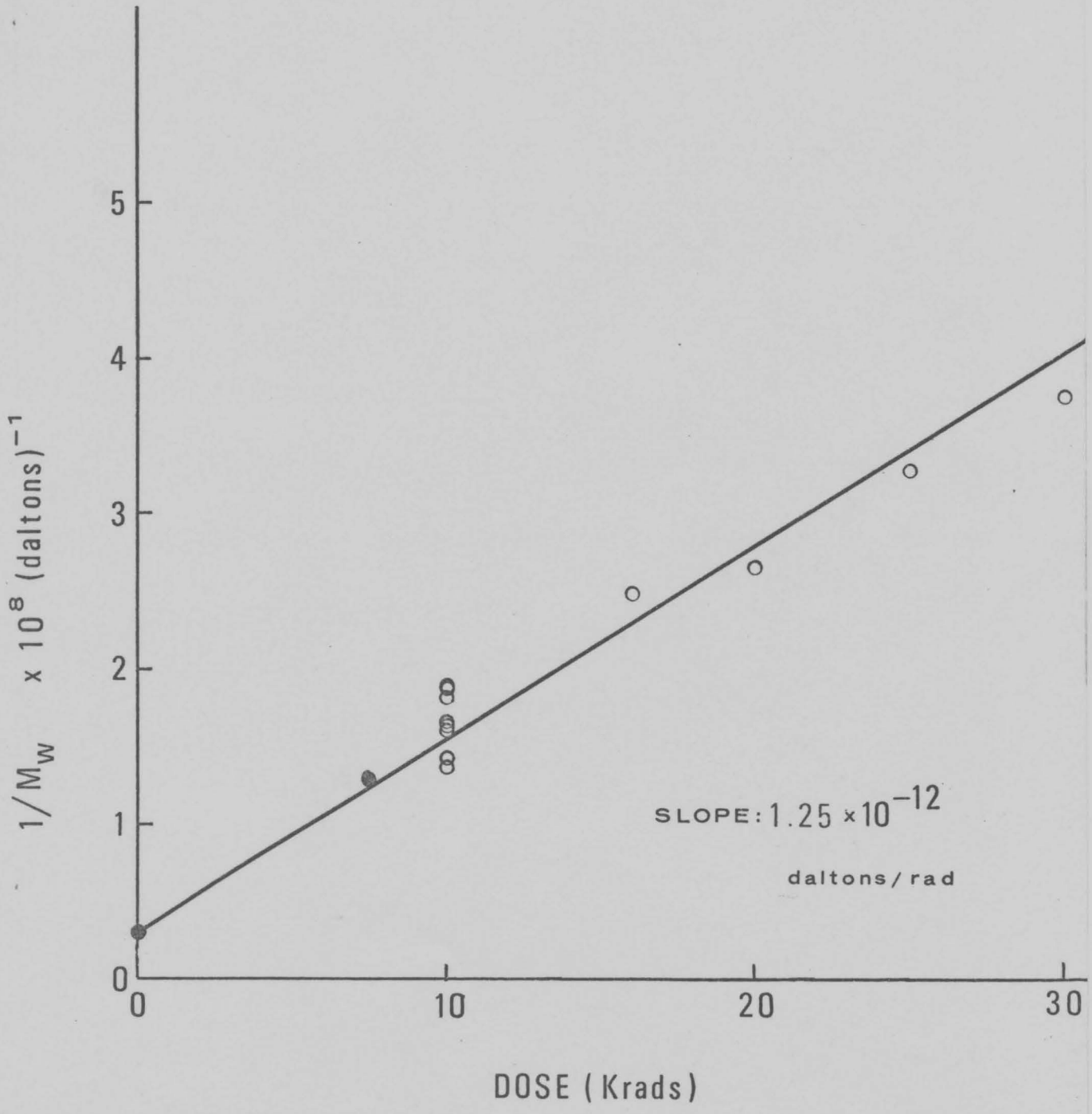
X.P. cells labeled for ∿ 48 hours with ^{3}H-TdR were administered, at 0°C, doses of ionizing radiation from 0 Krads to 30 Krads. Lysing solution contained 0.5N NaOH, 0.01M EDTA, 0.2% SDS. Gradients contained 0.3N NaOH, 0.001M EDTA, 0.01% SDS. Lysis at 22°C for 14 - 17 hours. Centrifugation was at 42,000 r.p.m. (5 ml tubes, SW 50.1 rotor) for 50 or 65 minutes, or at 16,000 r.p.m. (17 ml tubes, SW 27 rotor) for 6 hours 45 minutes - 13 hours 35 minutes. Weight average molecular weights were calculated according to equation (6a) or (6b). The line is the least squares fit for a straight line through a fixed point (for unirradiated cells, $1/M_w = 0.26 \times 10^{-8}$ dalton^{-1} gives the intercept).

(o) 5 ml tubes; (●) 17 ml tubes

Figure 7 Dose response for DNA single strand break induction : $1/M_w$ vs. dose (A.N. cells)

A.N. cells labeled for ∿ 48 hours with ^{3}H-TdR were administered, at 0°C, doses of ionizing radiation from 0 Krads to 30 Krads. Lysing solution contained 0.5N NaOH, 0.01M EDTA, 0.2% SDS. Gradients contained 0.3N NaOH, 0.001M EDTA, 0.01% SDS. Lysis at 22°C for 14 - 17 hours. Centrifugation was at 42,000 r.p.m. (5 ml tubes, SW 50.1 rotor) for 50 or 65 minutes, or at 16,000 r.p.m. (17 ml tubes, SW 27 rotor) for 6 hours 45 minutes - 13 hours 35 minutes. Weight average molecular weights were calculated according to equation (6a) or (6b). The line is the least squares fit for a straight line through a fixed point (for unirradiated cells, $1/M_w = 0.30 \times 10^{-8}$ dalton^{-1} gives the intercept).

(O) 5 ml tubes; (●) 17 ml tubes


$1/M_w \times 10^8$ (daltons)$^{-1}$
5
4
3
2
1
0
0
10
20
30
DOSE (Krads)
SLOPE: 1.25×10^{-12}
daltons/rad

distribution remains random regardless of the dose administered, and $M_w = 2M_n$ is valid for all points. If, however, the initial distribution is assumed to be uniform, then $M_w = M_n$ for controls; only for doses of more than 3 or 5 Krads (> 5 breaks per initial number average molecule) is the distribution random and the expression $M_w = 2 M_n$ valid. These considerations are of importance for the eV/break calculations, as analysis of our gradient profiles gives us M_w (equation (6a) or (6b)) whereas efficiency estimates require values of M_n.

Experiments in our laboratory with cells of the L-60 line (Palcic and Skarsgard, 1972) indicated a random distribution of DNA molecules in unirradiated cells ($M_w \sim 4.9 \times 10^8$ daltons) for centrifugation speeds lower than 20,000 r.p.m. These same molecules when spun at 42,000 r.p.m. (as in some experiments described in this dissertation) suffered significant speed distortion; use of equation (6a) yielded weight average molecular weights in the range $1.4 - 2.6 \times 10^8$ daltons for control (unirradiated) DNA. No such distortion occurred for irradiated cells (dose > 5 Krad), which showed random distributions at speeds as high as 42,000 r.p.m.

In view of this fact, sedimentation experiments were carried out on unirradiated DNA using the 17 ml tubes and lower speeds of centrifugation for both X.P. and A.N. cells. Cells were spun at 16,000 r.p.m. in the SW27 rotor at 20°C, conditions which had been shown to eliminate the speed distortion problem (Palcic and Skarsgard, 1972).

The zero-dose points in both figure 6 and figure 7 are the result of this slow speed centrifugation, as are the points for doses of 5 Krad and 7.5 Krad. The control (unirradiated) weight average molecular weight of single stranded X.P. DNA (3.8×10^8 daltons) and that of A.N. DNA (3.4×10^8 daltons) found for 16,000 r.p.m. centrifugation are more similar to the value obtained by Palcic for L-60 cells (4.9×10^8 dalton). These values are substantially larger than those obtained at 42,000 r.p.m. (see section 3.1.1.) indicating that for human cells, too, there is a significant speed distortion for high molecular weights.

The slopes of the X.P. and A.N. dose response curves (see figures 6 and 7) are calculated to be 1.4×10^{-12} dalton^{-1} rad^{-1} and 1.3×10^{-12} dalton^{-1} rad^{-1} respectively yielding the values 37eV/break for X.P. DNA and 42eV/break for A.N. DNA.

To be compared with these eV/break values are those in other mammalian systems: 33eV/break in Mouse L-60 cells irradiated in O_2 (Palcic, 1972); 44eV/break (Lehman and Ormerod 1970); 65eV/break (Lett et al. 1970); 72 eV/break (McBurney et al., 1971). A very sensitive alkaline sucrose gradient technique has enabled Johansen, Gurvin and Rupp (1971) to report a value as low as 24 eV/break in λ phage.

Assuming 10 μμgm DNA/cell, our eV/break values correspond to 17 breaks/rad/cell and 2.1×10^3 breaks/D_o/cell for X.P. For A.N. cells we obtain 15 breaks/rad/cell and

1.8×10^3 breaks/D_o/cell. The D_o values for X.P. and A.N. cells are 125 rads and 118 rads respectively, calculated from the γ-ray survival studies to be described in section 3.2.2.

3.1.4. Rejoining of DNA Single Strand Breaks

If, preceding lysis on the gradient, irradiated cells are first incubated at 37°C, in culture medium or physiological saline, the corresponding sedimentation profile is found to shift, with increasing incubation time, back toward the control (zero dose) position. McGrath and Williams (1966) and later, others (Lett et al. 1967, Lohman 1968, Humphrey et al. 1968, Terasima and Tsuobi 1969, Matsudaira et al. 1969, Elkind and Kamper 1970, Kleijer et al. 1970, Sawada and Okada 1970, Elkind 1971, McBurney et al. 1972, Palcic and Skarsgard 1972 and others) believed that this indicated rejoining of DNA breaks. A kinetic study of such rejoining is shown in figure 8 for X.P. cells and in figure 9 for A.N. cells. The fact that the profile of irradiated DNA does not shift back completely to the position of unirradiated DNA indicates that for the incubation times considered, rejoining does not proceed to 100% completion. Indeed, no investigators have claimed to see 100% rejoining in mammalian cells, which could indicate that there are different types of γ-ray-induced breaks, only some of which can be rejoined. The DNA repair ligase, implicated in the rejoining process, requires for activity, adjacent 3'-OH and 5'-P moieties in the DNA backbone;

Figure 8 Rejoining of radiation-induced DNA single strand breaks : gradient profiles (XP1 cells)

XP1 cells were irradiated with 10 Krads γ-rays. After irradiation, they were incubated for various times in PBS (at 37°C). The cells were then lysed for 15 hours, (22°C) on the top of an alkaline sucrose gradient (5 ml) and sedimented at 42,000 r.p.m. for 50 minutes.

(○) 0 Krads;
(●) 10 Krads, 0' at 37°C;
(Δ) 10 Krads, 5' at 37°C;
(▼) 10 Krads, 10' at 37°C;
(+) 10 Krads, 15' at 37°C;
(⊖) 10 Krads, 20' at 37°C.

This represents a sample experiment.

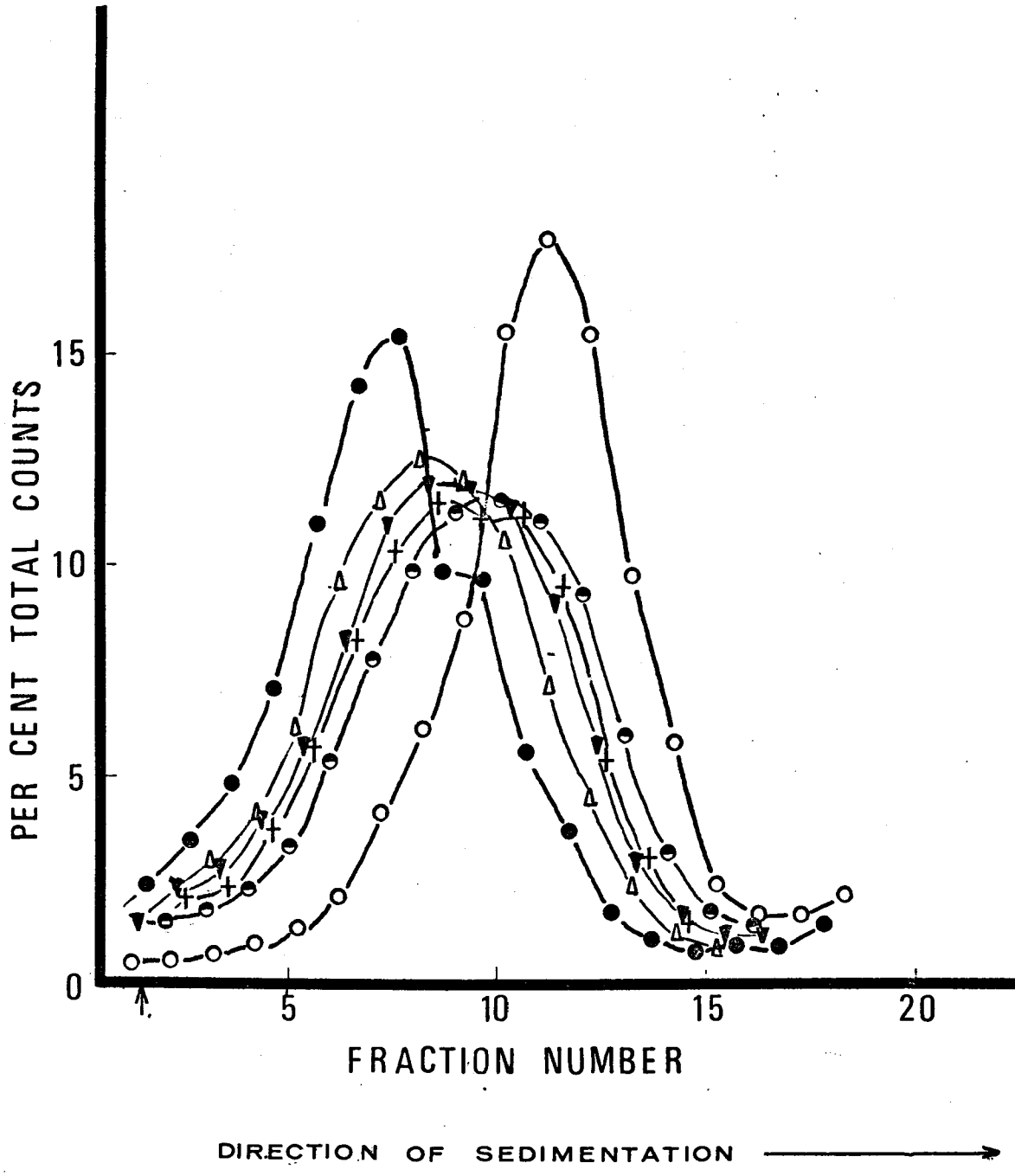
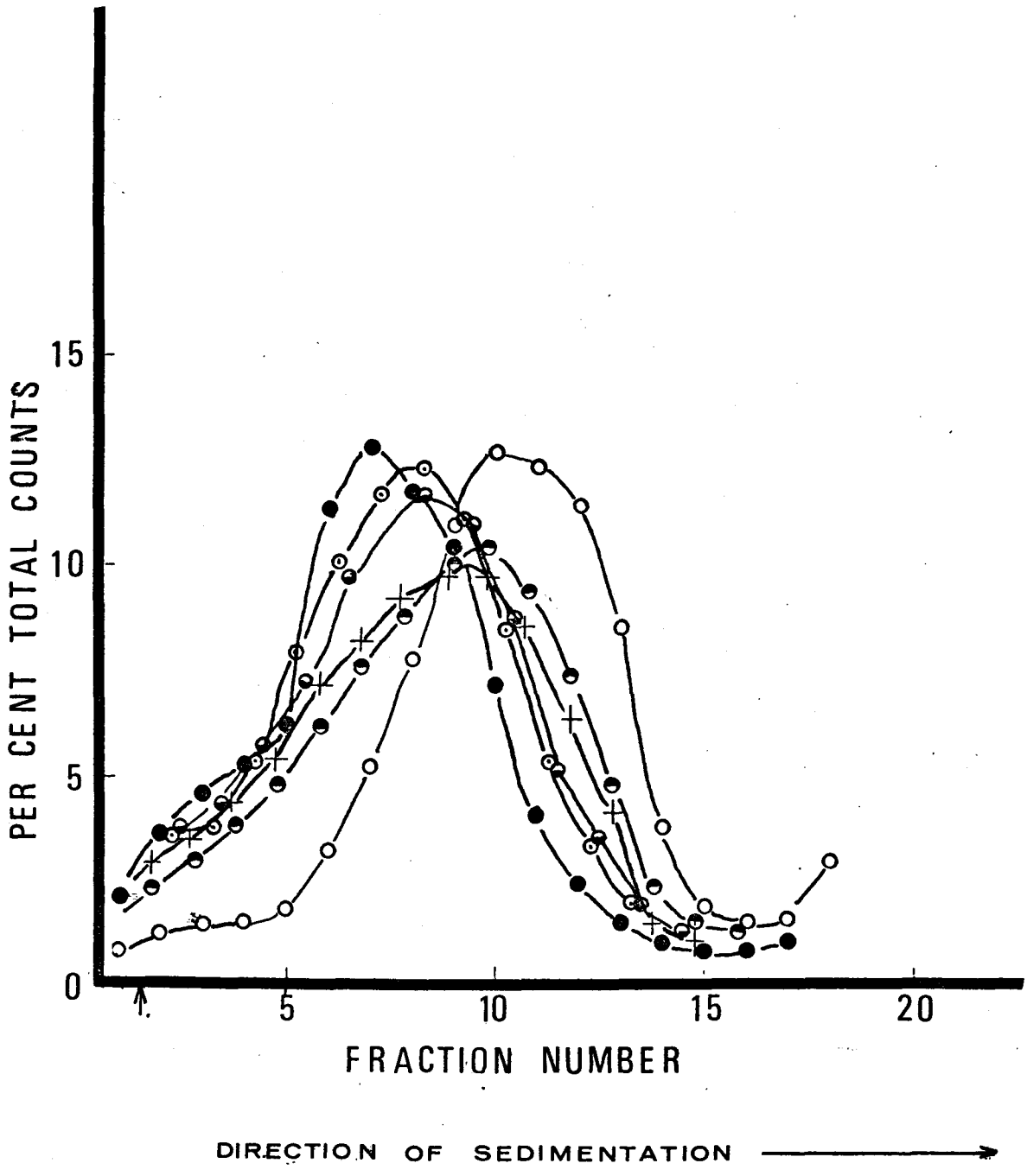

PER CENT TOTAL COUNTS
0
5
10
15
5
10
15
20
FRACTION NUMBER
DIRECTION OF SEDIMENTATION

Figure 9 Rejoining of radiation-induced DNA single strand breaks : gradient profiles (A.N. cells)

A.N. cells were irradiated with 10 Krads γ-rays. After irradiation, they were incubated for various times in PBS (at 37°C). The cells were then lysed for 15 hours, (22°C) on the top of an alkaline sucrose gradient (5 ml) and sedimented at 42,000 r.p.m. for 50 minutes.

(○) 0 Krads;
(●) 10 Krads, 0' at 37°C;
(⊙) 10 Krads, 2' at 37°C;
(◒) 10 Krads, 5' at 37°C;
(+) 10 Krads, 20' at 37°C;
(◓) 10 Krads, 30' at 37°C.

This represents a sample experiment.


PER CENT TOTAL COUNTS
0
5
10
15
5
10
15
20
FRACTION NUMBER
DIRECTION OF SEDIMENTATION

however, Kapp and Smith (1970) have shown that ionizing radiation can induce DNA lesions in regions other than the phosphate esters of the sugar-phosphate DNA backbone i.e. in the sugar residues.

On the other hand, it is generally assumed that in competition with repair processes there is a so-called fixation process (it could be DNA replication or some part of it) which fixes damage. Presumably those lesions which are fixed would not rejoin though initially they may have been identical to others which did rejoin.

It would be useful to look at the data of figures 8 and 9 in terms of the numbers of breaks remaining in the DNA as a function of the time of postirradiation incubation at 37°C. However, due to the fact that high molecular weight DNA (control DNA or DNA which has undergone lengthy postirradiation incubation) does not sediment randomly at 42,000 r.p.m., the data of figures 8 and 9 cannot be used to obtain reliable quantitative estimates of the numbers of single strand breaks present in X.P. and A.N. DNA. Examination of all of the X.P. and A.N. rejoining experiments performed at 42,000 r.p.m. showed qualitatively, at least, that there were not marked differences in the extent and rate of rejoining in these two cell lines. This is in agreement with the findings of Kleijer et al. (1970).

In order to make better determinations of the numbers of breaks remaining in irradiated DNA, rejoining experiments

were repeated using the 17 ml tubes and a centrifugation speed of 16,000 r.p.m. was performed.

One rejoining experiment for each of the XP1 and XP2 cell lines using 16,000 r.p.m. centrifugation yielding the results shown in figure 10. We were unable at the time, to perform a similar experiment for A.N. cells. The number of breaks per gram DNA (b) was calculated according to

$$b = N\left(\frac{1}{M_n} - \frac{1}{M_o}\right) \qquad (16)$$

where b is the number of breaks/g, N is Avogadro's number, M_w is the number average molecular weight of the DNA and M_o is the number average molecular weight of unirradiated DNA. Most of the rejoining is completed in the first 20 minutes of postirradiation incubation, in agreement with the results of Kleijer et al. (1970). The control weight average molecular weights, however (3.8×10^8 daltons for XP1 DNA; 3.5×10^8 daltons for XP2 DNA), are considerably larger than those of Kleijer's 3 X.P. patients (1.17×10^8 daltons to 1.84×10^8 daltons).

3.2. SURVIVAL STUDIES

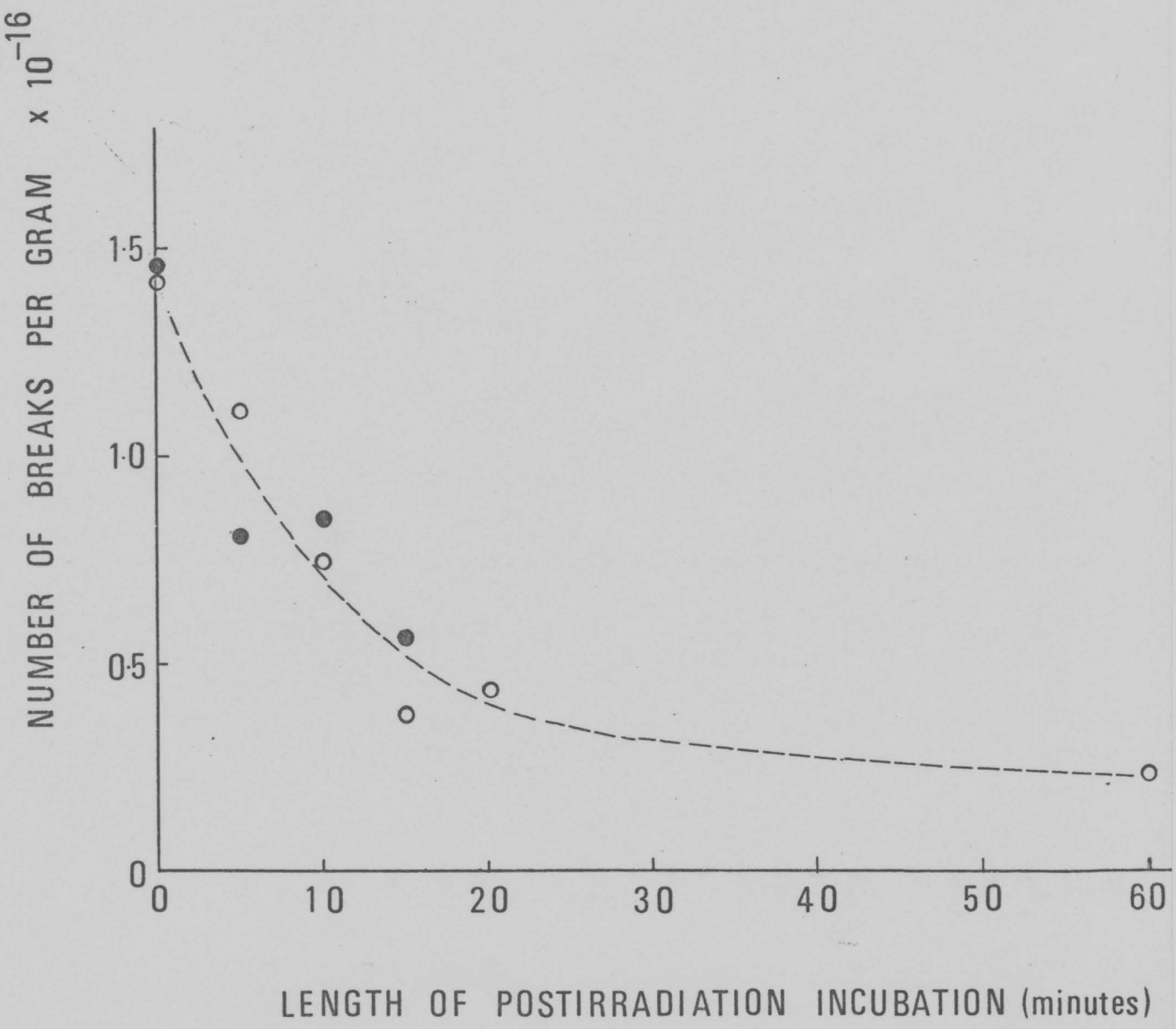
3.2.1. UV Survival

Xeroderma Pigmentosum has been associated with increased sensitivity to UV radiation both in vitro (Cleaver 1968) and in vivo (Cleaver 1968 and Reed et al. 1969); in vitro, this increased sensitivity has been correlated with little

Figure 10 Kinetics of rejoining of DNA single strand breaks produced by ionizing radiation : effect of post-irradiation incubation at 37°C on the number of single strand breaks remaining in the DNA (XP1 and XP2 cells)

XP cells were irradiated with 10 Krads γ-rays (0°C). Following irradiation they were incubated for various times at 37°C (in PBS) and then immediately lysed on the top of an alkaline sucrose gradient (17 ml). The DNA was sedimented (SW 27 rotor, 16,000 r.p.m.) after 15 hours lysis, and the M_w for each gradient was calculated. The number of DNA single strand breaks/g was calculated according to equation (16). Each point refers to a separate gradient.

(●) XP1;
(o) XP2.


NUMBER OF BREAKS PER GRAM x 10^{-16}
1·5
1·0
0·5
0
0
10
20
30
40
50
60
LENGTH OF POSTIRRADIATION INCUBATION (minutes)

or no repair replication (Cleaver 1968 and Cleaver 1970), diminished levels of unscheduled DNA synthesis (Cleaver 1968, Reed et al. 1969 and Bootsma et al. 1970), greatly reduced C.F.A. following UV exposure (Cleaver 1970 and Goldstein 1971), and insignificant excision of UV-induced pyrimidine dimers (Cleaver and Trosko 1970 and Setlow et al. 1969). We looked at the post-UV colony-forming ability of our X.P. and normal strains to confirm that in X.P. we indeed have a UV-sensitive strain. Figure 11 shows the results of our investigation. For the A.N. data the computed surviving fractions refer to a single experiment. In the case of X.P. the surviving fractions were calculated using a control (zero dose) plating efficiency obtained from a separate UV experiment; this plating efficiency was within the limits normally found in X-ray survival studies (see 3.2.2.). For the X.P. experiments the D_o and $\tilde{n}$ values[1] were 7.6 ergs/mm^2 and 1 respectively; for the single A.N. experiment, D_o and $\tilde{n}$ were 29.5 ergs/mm^2 and 1 respectively. For the least squares fitting of the A.N. data (only 3 doses) an exponential survival was assumed as is found in Cleaver (1970).

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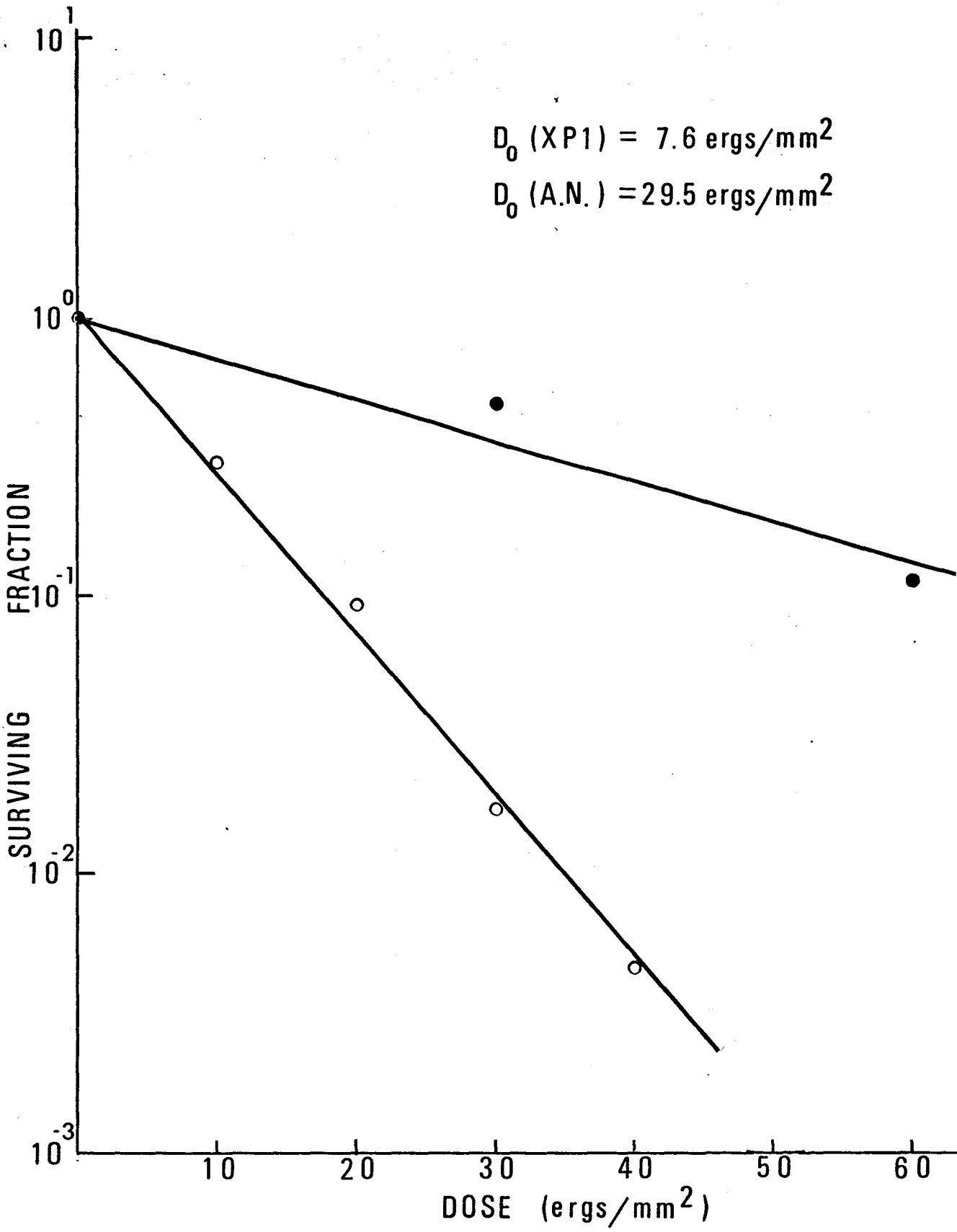
[1] D_o is the dose reducing survival to 1/e in the exponential portion of the survival curve; the extrapolation number $\tilde{n}$ refers to the zero dose intercept of the exponential portion of the survival curve. D_o is a measure of the intrinsic radio-resistance of a cell line whereas $\tilde{n}$ is regarded by most as an indication of the cell's capacity to accumulate sublethal damage without losing its capacity to divide.

Figure 11 UV survival (XP1 and A.N. cells)

24-hour cultures (XP1 or A.N. cells + L-60 feeders at 5×10^4 cells per petri) were exposed at 22°C (with MEMA removed) to various doses of UV. Fresh MEMA was then added and further incubation was allowed for 14 days, with a change of medium on day 8. Colonies were stained for counting on day 14.

(O) XP1;
(●) A.N.


D_0 (XP1) = 7.6 ergs/mm^2
D_0 (A.N.) = 29.5 ergs/mm^2
SURVIVING FRACTION
10^1
10^0
10^{-1}
10^{-2}
10^{-3}
10
20
30
40
50
60
DOSE (ergs/mm^2)

Cleaver (1970) stated a D_o of 9 ergs/mm^2 for his X.P. strain and 29 ergs/mm^2 for his normal cells; both X.P. and normal survival curves were exponential. Goldstein's XP1 and XP2 had D_o's of 2 ergs/mm^2 and exponential survival; his normals had D_o's ranging from 18 - 24 ergs/mm^2 and an extrapolation number $\tilde{n}$, of 3 - 4 (Goldstein, 1971).

3.2.2. γ-Ray Survival

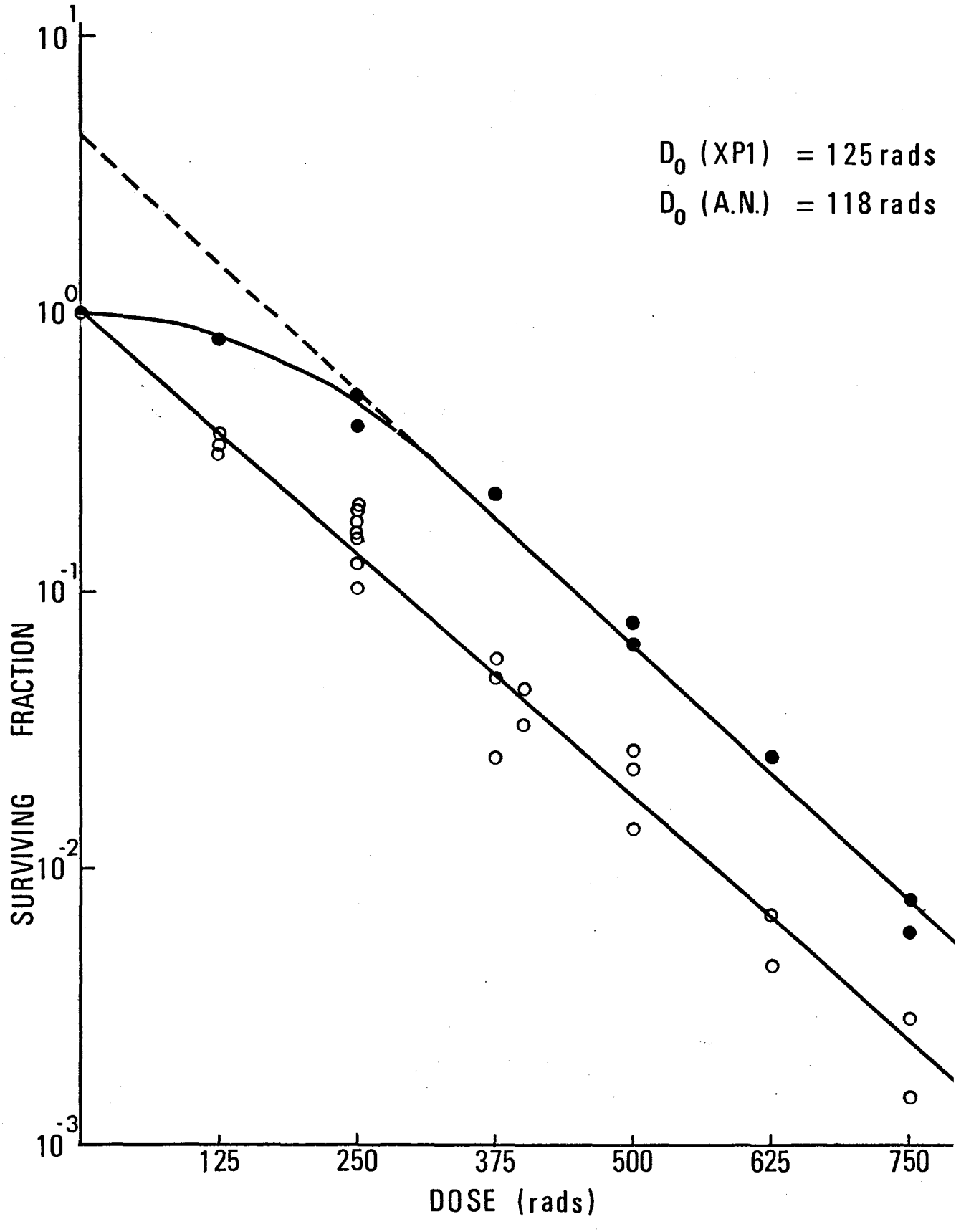
While the results of the previous section show that the colony-forming ability of X.P. cells is remarkably sensitive to UV damage, no one has, to the present time, measured the γ-ray response of X.P. cells. Since the X.P. cells, being deficient in UV repair, represent a unique opportunity to test the hypothesis that mammalian cells have a common mechanism for the repair of UV-produced damage, we proceeded to measure their γ-ray survival response. If the genetic change present in X.P. (a change which interferes with UV repair) were in no way involved with recovery from sublethal γ-ray damage, then we might expect X.P. cells to demonstrate a γ-ray survival similar to A.N. cells.

Figure 12 shows survival curves for X.P. and A.N. cells following γ-ray doses up to 1000 rads. Cells were irradiated in suspension in MEMA, aliquots were removed and diluted appropriately, and single cells were plated in petris (see section 2.6.2) containing 6 ml of cloning medium. The petris were incubated for 14 days and then stained with methylene blue and analyzed for surviving colonies. In some

Figure 12 γ-ray survival (XP1 and A.N. cells)

Cells (XP1 or A.N.) were irradiated in MEMA at room temperature at a concentration of 2×10^5 cells/ml. After appropriate dilutions, cells were then plated in cloning medium containing 5×10^4 L-60 feeder cells per petri. The petris were fed on day 7 - 9; colonies were strained for counting on day 14.

(○) XP1;
(●) A.N.


D_0 (XP1) = 125 rads
D_0 (A.N.) = 118 rads
SURVIVING FRACTION
10^1
10^0
10^{-1}
10^{-2}
10^{-3}
125
250
375
500
625
750
DOSE (rads)

experiments the medium was removed from the petris and replaced with fresh medium (i.e. the cultures were 'fed') at day 7 - 9. The effect of this feeding was to increase in identical fashion the plating efficiencies of the unirradiated and surviving irradiated cells; the slope of the survival curve remained unchanged. Both A.N. and L-60 cells were initially used (separately) as feeders in survival studies; however, the A.N. cells as a feeder background resembled too closely the cells whose survival was being measured (X.P. or A.N.) and identification of colonies was hindered. L-60 cells were selected as preferred feeders as they didn't interfere with colony counting. It can be seen that, although the two survival curves have very similar slopes in the exponential portions, at a given dose, A.N. exhibits a higher surviving fraction. From these responses one obtains values for $\tilde{n}$ and D_o as follows: for X.P., D_o = 125 rads and $\tilde{n}$ = 1; for A.N., D_o = 118 rads and $\tilde{n}$ = 4.33. These are listed in Table 1.

Thus, X.P. and A.N. have essentially the same intrinsic cellular radiation response re CFA following ionizing radiation: in the exponential survival regions, a given dose kills the same fraction of X.P. cells as A.N. cells. However, the 'shoulder' or non-linear portion of the A.N. curve ($D_o \leq$ 250 rads) indicates that, at low doses, normal cells can tolerate accumulated damage before killing results. The presence of such a shoulder is normally assumed to indicate that the

Strain	UV Survival		γ-Ray Survival	
	D_o(ergs/mm^2)	$\tilde{n}$	D_o(rads)	$\tilde{n}$
Our results (XP1)	7.6	1	125	1
Cleaver[1] (XP)	9	1	-	-
Goldstein[2] (XP1,XP2)	2	1	-	-
Our results (A.N.)	29.5	1	118	4.33
Cleaver[1] (normal)	29	1	-	-
Goldstein[2] (normal)	22.7_{av}	3-4	-	-
Little[3] (Chang liver)	-	-	152	2.1

1 Cleaver (1970)
2 Goldstein (1971)
3 Little (1969)

Table 1 UV and γ-ray survival parameters (X.P. and normal human cells)

cell possesses a mechanism for the repair of certain so-called sublethal lesions and only when this repair mechanism is saturated does the survival response become exponential with dose. In this context, the absence of a shoulder in the X.P. γ-ray survival response must be taken as an indication that X.P. cells possess no such repair mechanism.

Thus, our X.P. cells which are deficient in UV repair (XP1 has 9% and XP2 25% normal unscheduled DNA synthesis after 220 ergs/mm^2 (Cleaver 1970)) are also deficient in the repair of damage produced by ionizing radiation. And, it should be acknowledged, this observation is made in spite of the fact that X.P. cells appear to rejoin γ-ray induced single strand DNA breaks in a normal fashion.

This apparent inconsistency is discussed in the following chapter.

4. DISCUSSION

It has been postulated (Cleaver 1969b) that the repair of DNA lesions, present as DNA base damage, requires enzymatic scission of the polynucleotide chain, excision of the damage, and repair replication followed by ligase action to close the resulting gap. As X-rays induce single strand DNA breaks (McGrath and Williams 1966), repair of X-ray induced lesions does not require the initial enzymatic incision; the later stages may or may not be involved in the repair of these breaks.

Various investigators have shown that X.P. fibroblasts are unable to repair UV-induced lesions: repair replication (Cleaver 1968 and Cleaver 1970), unscheduled DNA synthesis (Cleaver 1968, Reed et al. 1969 and Bootsma et al. 1970) and the excision of pyrimidine dimers (Cleaver and Trosko 1970 and Setlow et al. 1969) are absent or reduced in comparison with normal fibroblasts. These three 'repair' processes require as a first step the action of a repair endonuclease; however, normal levels of repair synthesis (Cleaver 1969b), - both repair replication and unscheduled DNA synthesis, - result following radiation-induced single strand breaks in X.P. DNA. These results prompted Cleaver to postulate that X.P. fibroblasts are deficient in an early repair step involving base damage recognition and enzymatic chain scission. This was supported by the alkaline sucrose gradient studies of

Kleijer (1970) and by those in this investigation (Figures 8 and 9) which indicate that following ionizing radiation, (20 Krad for Kleijer's experiments and 10 Krad in this investigation) the rate and extent of rejoining of single strand breaks by X.P. DNA are not significantly different from those of normal fibroblasts. This normal level of 'repair' synthesis and rejoining in response to ionizing radiation occurs for fibroblasts from patients with large variations in both the clinical severity of their Xeroderma Pigmentosum symptoms and in the molecular responses of their fibroblasts to UV in terms of repair synthesis (Bootsma et al. 1970). In no system has rejoining been found to proceed to 100% completion; however, this could be due to the fact that not all of the radiation products are substrates for the chain degradation enzyme required to excise the damaged region (Kapp and Smith 1970) or perhaps the damage is fixed before repair is possible.

In the experiment of figure 10, complete elimination of radiation-induced single strand breaks would involve the rejoining of 1 break per 4.2×10^7 daltons or 9.1 breaks per control M_w of 3.8×10^8 daltons for X.P. DNA. For A.N. these figures are 1 break per 4.0×10^7 daltons and breaks per M_w of 3.4×10^8 daltons. These A.N. figures are for a control M_w found for 16,000 r.p.m. and a M_w 0.65×10^8 after 10 Krads for 42,000 r.p.m. Kleijer et al. (1970) report 1.03 - 1.95 breaks per 10^8 daltons after 20 Krads and 49 - 70%

of these breaks rejoined after 60 minutes (repair) at 37°C. Our results indicate a rejoining efficiency of ~80% for X.P. DNA. Most of the rejoining was found to occur in the first few minutes of repair incubation (figure 10) and rejoining saturated by 60' (figure 10, and Kleijer et al. (1970)).

Values for the efficiency of DNA single strand breakage were found from slopes of the curves in figure 6 and figure 7. Using $M_n = 0.5\ M_w$ valid for a random distribution of molecular sizes (Charlesby 1954) where M_n is the number-average molecular weight and M_w the weight-average molecular weight of a distribution of molecules, values of 37eV/break for X.P. and 42eV/break for A.N. were found. These correspond favourably with values found for other mammalian systems (see section 3.1.3. for references).

In an attempt to correlate the UV responses of X.P. at the molecular, cellular and whole body levels, Cleaver (1970) investigated the CFA of X.P. cells following different UV doses. He found X.P. fibroblasts remarkably more sensitive to the lethal effect of UV ; both normal and X.P. cells exhibited exponential survival; however the D_o's differed by a factor of 3.2 (29 ergs/mm^2 for normal cells; 9 ergs/mm^2 for X.P. cells). Goldstein (1971) using the same XP1 and XP2 as in this investigation found an X.P. D_o value of 2 ergs/mm^2. Our own values are summarized in table 1 along with those cited above. They are taken from the survival curves illustrated in figure 11.

The possibility of a common repair pathway for the repair of both UV-induced and ionizing radiation-induced damage suggested to us the need to look at the cellular-level response of X.P. fibroblasts following ionizing radiation. Figure 12 illustrates the response of X.P. and normal CFA to doses of γ-rays up to 1000 rads. The survival parameters are included in table 1 with those for UV survival response. The distinct lack of a shoulder (pure exponential survival curve) in the X.P. curve as opposed to the A.N. curve for which the extrapolation number is 4.33 suggests that X.P. fibroblasts are deficient in the repair of sublethal damage following ionizing radiation. Had the X.P. and A.N. survival responses been indistinguishable, it would have suggested that (i) the repair systems for UV-induced and ionizing radiation damage are independent and X.P. has a completely functional ionizing radiation repair system or that (ii) if the different repair systems share common steps in their respective repair pathways, the type of damage produced following ionizing radiation eliminates the need for the step which is defective in repair of UV damage in X.P. cells. However, the responses are <u>not</u> indistinguishable and although X.P. and A.N. fibroblasts exhibit the same inherent γ-ray sensitivity i.e. their D_o's are very similar, not only are the X.P. cells lacking in a UV repair system, but they are deficient in the repair of sublethal γ-ray damage as well.

In order to pursue further this lack of repair of

sublethal damage following ionizing radiation it would be necessary to perform split-dose experiments with both X.P. cells and A.N. cells. In such a split dose experiment, cells are exposed to a dose of ionizing radiation, after which they are left to incubate at 37°C for a given time interval; a second dose is then administered, and the cells are plated for survival analysis. Cells which are capable of repairing sublethal damage, do so during the incubation period after the first dose of radiation; for time intervals of the order of a few hours, the amount of repair of sublethal damage increases with time of incubation. As a result, the survival of cells exposed to split doses of radiation is greater than that of cells exposed to the same total dose delivered at one time. It is a general characteristic of mammalian cell lines to have enhanced survival following split doses of ionizing radiation; if X.P. cells were to fail to demonstrate a split-dose recovery they would be unique among log phase mammalian cells in that respect. It is also worthwhile to point out that the lack of a shoulder in the ionizing radiation response of X.P. cells makes this strain unique; the presence of a shoulder is a general property of all asynchronous log phase mammalian cells.

It is debatable whether the rejoining of DNA single-strand breaks can be related to survival in mammalian cells (Sawada and Okada 1970). Certainly, in this dissertation, the doses used in studies of DNA single strand breaks (5 - 30 Krads)

both for breakage efficiency and for rejoining, are such that there is negligible survival. It would indeed be questionable to try and extrapolate the results of section 3.1.4. to lower doses and relate rejoining parameters to D_o values for cell survival. It would be of interest to look at rejoining in X.P. and normal cells for γ-ray doses approaching those used in survival studies; however, the resolution of our technique in that dose range would make comparisons very difficult.

5. CONCLUSIONS

In this investigation we were able to show that

(i) X.P. cells are much more sensitive to UV than normal cells when assayed for colony-forming ability (D_o = 7.6 ergs/mm^2, XP1; D_o = 29.5 ergs/mm^2, A.N.).

(ii) although X.P. cells (D_o = 125 rads) and A.N. cells (D_o = 118 rads) exhibit similar inherent sensitivities in γ-ray survival studies, the X.P. survival curve is without a shoulder (n = 1) whereas A.N. cells exhibit a shoulder of ∿ 250 rads (n = 4.33). This is evidence that X.P. cells are deficient in the repair of sublethal damage following ionizing radiation.

(iii) following exposure of cells to ionizing radiation, rejoining of radiation-induced DNA single strand breaks occurs at similar rates and to similar extents in X.P. and A.N. cells.

(iv) our alkaline sucrose gradient technique gives values of 37 eV/break and 42 eV/break for X.P. DNA and A.N. DNA respectively.

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