Conf-730952--6

24,271

DNA DAMAGE IN MAMMALIAN CELLS AND ITS RELEVANCE TO LETHALITY

by M. M. Elkind and E. Ben-Hur

Prepared for:

4th Symposium on Microdosimetry,
Verbania Pallanza, Italy, September 24-28, 1973

U of C-AUA-USAEC

ARGONNE NATIONAL LABORATORY, ARGONNE, ILLINOIS

DNA DAMAGE IN MAMMALIAN CELLS AND ITS RELEVANCE TO LETHALITY

M. M. Elkind*
Division of Biological and Medical Research
Argonne National Laboratory
Argonne, Illinois, U.S.A.

and

E. Ben-Hur
Department of Cellular Biochemistry
Hadassah Medical School, Hebrew University
Jerusalem, Israel

ABSTRACT

Cell killing (loss of proliferative capacity) is a principal end point in all radiation effects contingent upon cell viability. Thus, while it occupies the obverse of the coin in the radiation therapy of cancer, cell killing is at least the reverse side in radiation mutagenesis, carcinogenesis, and teratogenesis.

DNA, the molecular carrier of the genetic inheritance, occupies a central position in the affairs of a cell because the properties and characteristics of a cell are dictated by the DNA → RNA → protein axis of information storage, flow, and expression. This, the mutagenic and chromosome-breaking properties of radiation, the biological amplification available to a lesion in DNA, and the fact that DNA molecularly constitutes a very large radiation target, putatively all make DNA the principal target relative to many radiation effects.

To study "Where and what are the sensitive targets in a mammalian cell?" an indirect approach may be useful. This stems from the fact that to kill cells with low LET radiation, sublethal damage must be accumulated. Cells can repair this damage an ability compromised by, for example, the DNA-binding antibiotic actinomycin D. Thus, focussing on DNA, and repair processes in DNA, while indirect, is supported in the instance of cell killing by extensive experimental evidence.

The status of damage registered directly in DNA may be assessed by examining changes in the sedimentation of DNA from irradiated cells. Along with measurements of cell survival, sedimentation data are discussed relative to their bearing on cell killing and their ability to help us understand the organization and replication of DNA in mammalian cells.

---

* Supported in part by a U.S. National Institute of Health Fellowship No. 7 F03 CA52437-02 from the National Cancer Institute.

INTRODUCTION

From the view point of microdosimetry, even in a mammalian cell only 10-15 microns in diameter, many structures considerably smaller in size must be considered as potential radiation targets. As shown in Fig. 1, cytologically a number of structures frequently involving membranous envelopes can be identified (for a brief discussion of cell structures, and their function see ref. 1). The nucleus contains the bulk of the DNA of a cell the organization and state of replication of which depends upon whether or not the cell is still capable of division and where it is in its growth cycle.

The cytoplasm contains a number of structures and organelles whose functions relate in the main to the synthetic and degradative processes that the cell can support. Many of these organelles are essential particularly to cycling cells. In the main, however, nuclear DNA is responsible for the heritable properties of a cell. The proteins manufactured in accord with the information contained in nuclear DNA--as controlled by feedback signals in turn also derived from the genome--dictate the properties of the cell. Hence, the pathway for information expression--the DNA → RNA → protein--inherently possesses an enormous amount of biological amplification. This reason alone has made the DNA of a cell a favored target in respect to many radiation end effects.

Sublethal Damage

Single mammalian cells, exposed to graded doses of low linear energy transfer (LET) radiation, characteristically have a threshold type survival curve. Such a curve for Chinese hamster cells is traced by the circles in Fig. 2. The presence of the threshold means that damage must be accumulated to register a lethal effect. The squares in Fig. 2 show that between dose fractions (see legend) cells surviving a first dose are able to repair sublethal damage. The initial quasi-threshold dose, D_q (= D_o ln n), which is reduced to close to zero by the first exposure, is fully returned by 23 hrs with more than half of it being restored by 2.5 hrs. (For additional details and examples of sublethal damage and its repair, see ref. 2).

To pursue the question where damage related to lethality is registered and of what it consists, the indirect approach of studying the where and

what of the repair of damage can be taken. This is based on the reasonable assumptions that: 1) repair processes take place at, or near, primary sites of damage; 2) repair consist of the reversal or removal of primary lesions; and 3) repair relative to targets and molecules can be effectively pursued via measurements of overall cell function, e.g. survival. However, as reasonable as these assumptions may be, effectively to proceed with studies focused on the DNA of a cell, a justification more compelling than one based only teleology is required. This was sought from evidences of damage interaction between ionizing radiation and drugs which act on DNA.

Nitrogen Mustard and Actinomycin D, DNA Interactive Agents

As sketched in Fig. 3, the bifunctional alkylating agent nitrogen mustard and the antibiotic actinomycin D both interact with duplex DNA. Nitrogen mustard is known to cross-link purines, primarily guanines, in a manner similar to sulfur mustard (3); as such it is only one of a class of agents thought to be lethal to viruses, bacteria, and mammalian cells because it prevents the normal separation of complementary DNA strands during semi-conservative replication (see 4). (Only interstrand cross-links are shown in Fig. 3; however, intrastrand cross-links and single-strand additions are also produced.) Actinomycin D is a duplex DNA intercalator; it is typical of planar two and three ring structures known to intercalate between complementary base pairs (see 5) although, in contrast to other intercalators, actinomycin D strongly binds to DNA; its half residence time in Chinese hamster cells is long, 2.3 hrs (6). In part, at least, actinomycin D is thought to affect cells by interfering with the template properties of duplex DNA presumably by preventing RNA-polymerase from functioning in a normal way (see 5). While these two DNA drugs are known to interact strongly with DNA, they do so in manners quite different from each other and clearly quite different from the frank bond breaking ability of ionizing radiation, Fig. 3. In spite of these differences, the effects produced by nitrogen mustard have been thought to be sufficiently similar to those due to radiation that it is come to be known as a radiomimetic agent. The actions of actinomycin D, on the other hand, are not associated with those of radiation.

Nitrogen Mustard and X-rays

We consider first whether or not nitrogen mustard (NM) interacts with x-ray damage related to lethality. From an extensive series of experiments, the conclusion was reached that these agents act independently (4); an example from this series is in Fig. 4. To avoid possible confusion from mixed population effects associated with the use of asynchronous cells, we show an experiment dealing with cells in their DNA synthetic period, the S phase. Cells in S are most resistant to single x-ray or NM treatments and because most of the cells in a Chinese hamster cell population are in S, results with S cells largely characterize results with asynchronous cells and, in fact, results similar to those in Fig. 4 were obtained with asynchronous cells (4).

Populations of Chinese hamster cells were grown overnight and then synchronized using the hydroxyurea technique of Sinclair (6,7). (The ability of hydroxyurea selectively to kill cells initially in S is responsible for the drop in survival for zero x-ray dose from 1.0 to about 0.4). As a result, the experiment was performed with populations of microcolonies, of average multiplicity $\underline{N} = 3.1$, initially synchronized at the G_1-S border (7), and which 3 3/4 hrs later were at the most resistant period of the S phase. The squares show the x-ray survival of these microcolonies. The uppermost thin line traces the x-ray survival of single S cells after accounting for the influence of multiplicity on the shoulder region of the survival curve in this case by shifting upward, by the appropriate amount (see Chapter 3, ref. 2), the initial portion of the survival curve.

The dashed curves in Fig. 4 involve the combined treatment -- x-irradiation + NM -- delivered in quick succession (see legend). Clearly, the NM treatment must have affected cells since it reduced survival by itself. In spite of this, when account is taken of the residual multiplicities (short segments of thin dashed curves starting from the ordinate), and the single dose combined treatment curves are shifted upward to permit a comparison with the single-dose x-ray curve, it is clear that the x-ray response of cells surviving NM treatment is at most affected in a minor way.

Actinomycin D and X-rays

In Fig. 4, the NM treatment reduced the initial survival by about 1/3. In Fig. 5, we show the effect on the X-ray survival of late S cells of a pretreatment with actinomycin D sufficient to result in an almost equal reduction in initial survival. It is clear that actinomycin D has a major influence of x-ray survival and that this results mainly from a reduction in shoulder width. Accounting for multiplicity, pretreatment with this antibiotic reduces the x-ray survival curve threshold width by a factor of 7 (see legend, Fig. 5) while the $\underline{D}_o$ is reduced only by 26%.

Figure 6 shows that a reduction in capacity for x-ray damage due to actinomycin D is not contingent upon a toxic effect from the antibiotic itself. In this experiment, Chinese hamster cells, near the peak of their resistance in S, were irradiated with graded doses. Survival was assessed in the continuous presence or absence of a low concentration of actinomycin D, 0.002 μg/ml; no concomitant reduction in survival resulted from the drug alone. The data show that this very low concentration of actinomycin D, 1.8×10^{-9}M, resulted in a reduction of shoulder width by 155 rad.

Taken together, the results in Figs. 5 and 6, which are only two examples from extensive studies (6, 8-10), show that actinomycin D potentiates x-ray damage. In Fig. 5, the drug was added prior to irradiation. However, as illustatrated by the potentiation of x-ray killing in Fig. 6 where the drug was added after irradiation, and from a number of other observations (6, 8-10) including the tight binding of actinomycin D in Chinese hamster cells (6), we know that drug treatment before x-irradiation is effective primarily because it is available to act at the sites of damage immediately after exposure when the rate of sublethal damage repair is greatest. Since actinomycin D affects primarily a cell's capacity for sublethal damage, a given treatment with this agent can be equated to a dose of radiation. The final $\underline{D}_o$'s in Fig. 6, being essentially equal, this latter point is quite clear; that is, the actinomycin D experience can be considered as equivalent to 155 rad. We will return again to this point.

Thus, our DNA interactive agents (Fig. 3) affect cell function relative to radiation lesions in ways not predictable from the commonly associated

modes of actions of these drugs. Nitrogen mustard is considered radiomimetic; yet damage due to it does not interact with x-ray damage. Actinomycin D, in the concentration ranges which we have found potentiate x-ray cell killing, has little or no effect on DNA synthesis (9), for example, yet its binding to DNA acts to reduce a cells capacity for sublethal damage.

DNA Sedimentation Experiments

Although the results described to this point illustrate that the ability of a chemical to interact with DNA is not a sufficient condition to insure that it will potentiate x-ray damage, the sequestering ability that duplex DNA has for actinomycin D (see references in 5), and the ability that each agent has to enhance the lethal effect of the other (6,,11), serve to identify as a common locus of action the DNA containing structures of the nucleus. Thus, our focus on DNA is strongly supported by eexperimental evidence and hence, it was proposed that primary radiation lesions, and their repair, involve DNA possibly in structural relationship to its molecular environment (12).

Having arrived at a justification to concentrate on DNA and DNA-containing structures in the context of primary targets, we turned to methods of measuring damage in DNA. For this purpose, the technique of McGrath and Williams for studying the integrity of DNA under alkaline conditions was particularly appealing (13).

Figure 7 shows the essentials of the McGrath and Williams method, as modified for mammalian cells, that we have used (14). Cells are grown in the presence of a specific DNA label like ^{3}H (or ^{14}C) thymidine (TdR). If the intent is to examine long stretches of DNA--so-called template DNA--the label will be made available for periods of time about as long as the growth cycle. Labelled cells are then suspended and an aliquot containing 5-10,000 cells is carefully pipetted on to a lysing solution which in turn is a thin layer on top of a 5-20% high salt, high pH (>12), sucrose gradient. By the use of high ionic strength solutions, electrostatic forces are reduced facilitating the separation of basic proteins from DNA, and by the use of pH's greater than 12, acid proteins are solubilized, the lysis of the cell is assured permitting the DNA contained therein to spread, without mechanical

manipulation, over the surface of the lysing solution. The high pH, however, also implies that complementary hydrogen bonding will be destroyed and hence, the technique is considered as a means of studying single strands of DNA. The principal attraction of the technique, nevertheless, is the ability the method of McGrath and Williams affords of studying DNA that has suffered a minimum amount of shear due to mechanical manipulation. Prior to its advent, the usual chemical extraction of DNA introduced, by itself, damage corresponding to a large dose of radiation, 25,000 rad or more.

Following the lysis of cells, tubes are rotated in an ultracentrifuge and after this the contents of a tube is sampled in order to determine the linear distribution of radioactivity and therefore DNA. From the work of Burgi and Hershey (15) and Studier (16), we know that single-strands sediment according to the 0.4 power of molecular weight providing that other factors, like their viscous drag, are independent of molecular weight and centrifugal force. (The latter qualification comes up again later.)

A balanced presentation of DNA sedimentation data requires us to deal with three aspects of the work. First, we must deal with phenomenology; radiation aside, what is observed when DNA from mammalian cells is sedimented and what does it mean? Second, we must describe and explain the effect that radiation has on this DNA and the ability cells have to alter this effect. And third, we must appraise the strengths and weaknesses of measurements of this type in the context of biological function.

To simplify the presentation of the data relative to DNA damage, we show Fig. 8 in which the dependence on lysis time and dose prior to sedimentation is sketched. These results refer to lysis at ~24° C followed by sedimentation at what we will call "high speed" (i.e., 36-38,000 rpm with a Beckman SW-50.1 or SW-39 rotor respectively). For unirradiated cells, initially--i.e., after lysis for 60 min--the pattern is bimodal. The peak nearest the meniscus, the left one, has been called the "complex" (14), and the one nearer the bottom of the tube the "main peak". Clearly with time, the complex disappears appearing closer to the meniscus with increasing lysis. Because essentially all the DNA loaded on a gradient is accounted for in each case, what is lost from the complex due to progressive hydroxyl

attack, must appear in the main peak. It follows from this that the DNA in the complex is larger in molecular weight than that in the main peak even though it does not sediment as far. (This point we consider again in reference to Figs. 16-18.) Panel (a) in Fig. 9 is an example of progressive resolution of the complex with lysis time.

The preceding trend with lysis time is also indicated in Fig. 8 after a short lysis with increasing doses of radiation. Doses up to ∿1500 rad resolve the complex--see also open circles in panels (b) and (c), Fig. 9--while doses greater than ∿1500 rad lead to the development of a peak to the left of the main peak. As illustrated in panel (d), Fig. 9 (open circles), the latter peak is different in shape from the complex. Also, with increasing dose it grows at the expense of the main peak and progressively appears closer to the meniscus. The dose dependence, plus a number of other lines of evidence from our work and the work of others, confirms that the peak that appears after high doses, $\gtrsim$ 1500 rad, consists of degraded single strands of DNA.

The data in Fig. 9 also show that if cells are incubated after irradiation, the effects produced by the irradiation are reversed to a degree. After 4330 rad, in 20 min, 37°C, essentially all the single-strand breaks are repaired. The results in Fig. 10 indicate that after a somewhat larger dose, 5800 rad, ∿10% of the breaks remain after 70 min at 37°C and further, that while the proportion of breaks which persist is about the same, at 42°C breaks are repaired more rapidly than at 37°C (we return to this point again later).

In addition to repair of single-strand breaks (Figs. 9 and 10), panels (b) and (c) in Fig. 9 show repair of the complex. Further details about this process are to follow; suffice it to note here that after small doses, <1500 rads, incubation for repair results in the reappearance of the complex at the expense of the main peak. Thus, the dose dependent sequence,

<u>complex</u> → <u>main peak</u> → <u>degraded main peak</u>,

is reversed with repair after radiation.

Radioenergetics of DNA Damage

The following properties are worth bearing in mind if DNA break data are to be evaluated in the context of microdosimetry.

A diploid Chinese hamster cell from an asynchronous population on average has $\sim 1 \times 10^{-11}$ gm DNA. This is equivalent to $\sim 6 \times 10^{12}$ daltons. Using the DNA from labelled bacteriophage T4 as a calibration marker, the number average molecular weight $\underline{M}_n$ of main peak DNA has been computed to be $\sim 2 \times 10^8$ daltons (19). This means there are $\sim 3 \times 10^4$ such molecules on average per cell. From measurements of the dose dependence of the productions of single-strand breaks, we estimate that $\sim$55 eV is required per break; thus, $\sim$2500 rad is needed to produce about one break per 2×10^8 daltons. Because about 1/10th of this dose is needed to affect the complex, from target theory considerations we have estimated that the molecular weight of the complex is $\sim$10X larger (19). Indeed, we have recently found that a duplex molecule of about this size can be released from Chinese hamster cells under non-denaturating conditions (18).

Nitrogen Mustard versus DNA Damage and the Nature of the Complex

Experiments dealing with the effect of NM on the sedimentation of the DNA from irradiated cells were performed to help understand: first, the question of the relationship between DNA lesions and cell killing; and second, the nature of the complex.

Figure 11 shows the effect of about a 5% survival dose of NM (4) on the sedimentation of cells irradiated with a dose ordinarily sufficient to lead to a resolution of the complex in 60 min if lysis is at $\sim$24C. NM treated cells lysed immediately after 1440 rad do not display a complex if lysed for 270 min (closed circles); similarly for cells not treated with NM but allowed to repair for 40 min, 37°C before lysis (squares). After 60 min of lysis, we would have observed a complex--e.g., see the effect of 20 min of repair in panel (c), Fig. 9--but the longer lysis in this case has led to the resolution of the complex (Fig. 8). However, when NM treated and x-irradiated cells are also incubated for repair, a prominent complex persists even after lysis for 270 min at $\sim$24°C. (open circles).

A result similar to the preceding but involving a dose now sufficient to produce single-strand breaks is shown in Fig. 12. Here, there is already some indication of an effect of the NM treatment because of the more prominent peak in the region of undamaged single DNA strands than in panel (d), Fig. 9. But the NM treatment has its most dramatic effect following repair. Ordinarily, 40 min at 37°C following 4330 rad would result in the reformation of hardly any complex. In this case, however, almost all of the DNA sediments as a complex. In fact, the pattern is that which would result from about 1/10th the dose without NM.

These data, as do our results with the DNA cross-linking treatment psoralen plus near ultraviolet light (20), support the conclusion that the complex is duplex DNA which, under conditions of denaturation, ordinarily comes apart. The cross-links produced by NM (or by psoralen plus near ultraviolet exposure, 20) when present in sufficient number, serve to hold the complex together. A dose of 1440 rad produces less than one single-strand break per 2×10^8 daltons; hence, most molecules of this size are not hit at all. Ordinarily, the DNA from cells receiving 1440 rad only, resolves to a main peak in 60 min; 270 min is required if NM treatment precedes x-irradiation; that is, longer hydroxyl attack is needed if cross-links are present. If repair also occurs, even 270 min following NM + 1440 rad is inadequate to resolve the complex. Even when a dose of 4330 rad is used, repair leads to a pattern which would have resulted from a much smaller dose if cross-linking precedes irradiation.

Relative to function, the data in Figs. 11 and 12 (and other results with NM to be reported elsewhere) are consistent with our survival results as we will note presently. Since it is likely that DNA cross-linking is the lethal lesion, we point out here that the persistence of the complex is not a prerequisite of viability. Indeed, since duplex DNA must become single-stranded for normal replication, it seems likely that NM exerts its lethal effect by preventing the separation of strands as suggested by the abnormal persistence of the complex under conditions of alkaline hydrolysis which ordinarily would resolve it.

Actinomycin D Versus DNA Damage

We turn now to repair in cells treated with actinomycin D; the results we discuss, Figs. 13 and 14, in part are from a larger study (19,21). In Fig. 13, we show that while pretreatment with actinomycin D does not impede single-strand repair, it does interfer with repair of the complex. In the top panel, we see that the degree of single-strand breakage is not significantly affected by the preirradiation treatment with actinomycin D (see legend). In 20 min at 37°C after irradiation, if anything it appears that cells treated with the antibiotic repaired slightly more rapidly. However, after 90 min it is clear that the actinomycin D treated cells did not repair the complex to the same extent as did cells not treated with drug.

Thus, the data in Fig. 13 are one indication of a differential effect of actinomycin D on the DNA from irradiated cells. However, since our survival results suggest that actinomycin D acts as though it is equivalent to a dose of radiation (e.g., Figs. 5 and 6), the effect of this antibiotic acting by itself is important. To begin with, we note that graded doses of drug have been shown to produce single-strand breaks in a dose dependent fashion as does radiation (19,21). Figure 14 illustrates that this is also true with respect to resolution of the complex. In addition, because of binding ability of actinomycin D to DNA, probably at least some of its interference with repair of the complex after irradiation results from its ability by itself to prevent such repair (21).

Hence, in addition to support for an ability of actinomycin D to act like radiation, we have an indication that repair of single-strand breaks --or DNA damage that leads to single-strand breaks under alkaline conditions--is a process the requirements for which are at least not entirely the same as those for repair of the complex. Since intercalation leads to an untwisting of the DNA duplex, plus the introduction of some single-strand breaks due to its own action or radiation, these data suggest that rotation of the molecule aromnd its long axis reduces repair of the complex because of a lack of register between the bitter ends of a broken strand which otherwise would have been subject to rejoining (i.e., ligase-type action). A result of this type would be expected if repair of the complex involved repair to a covalently closed circle or a structure similar to it (19,21).

Hyperthermia and Sublethal Damage

Hyperthermia during low dose rate irradiation (22), or after acute irradiation, enhances cell killing and this is because for temperatures up to ∿41°C, repair of sublethal damage is suppressed (23). An example of this is shown in Fig. 15. Here the dashed curve refers to the acute exposure, single-dose survival curve of single cells determined in a separate experiment (23). For cells at 37°C, 62% of the D_q has returned in 2.5 hrs, but for 41°C essentially no capacity for sublethal damage is regained. Cells kept at 41.5°C after their first dose suffer a larger amount of potentially lethal damage being cconverted to lethal damage, but their sensitivity to second doses is not appreciably increased.

These data show that hyperthermia, up to 41°C at least, enhances cell killing by suppressing the repair of sublethal damage. In view of this, we examined the effect of hyperthermia on the repair of DNA strand breaks. However, we did this using slow speed sedimentation, 5000 rpm, because of the greater resolution in respect to damage and repair of the complex obtainable in this way (19), and hence, we discuss first the patterns observed after slow speed sedimentation.

When the labelled DNA from Chinese hamster cells is sedimented at 5000 rpm, 0°C, the position of the main peak in the gradient is located in accord with the conventional force x time relationship for a particle at its terminal velocity in an essentially constant gravitational field. In Fig. 16, this means that for unirradiated cells, the main peak should appear at fraction 4. Since we know that a dose of ∿1500 rad resolves the complex into main peak DNA, the last panel confirms the location of the main peak. Hence, for control cells, now we find the complex toward the bottom of the tube where it should be in view of its larger target size compared to main peak DNA. The data show a gradual shift in the complex toward the meniscius and at the same time, the DNA lost from the complex appearing in the position of the main peak. As has been pointed out, this behavior of the label lost from the complex suggests a configurational change akin, for example, to the transition from catenated duplex circles to single-strands and single circles (19). Further, while not critically important to present proposes, we note that it is clear from these data that the sedimentation

behavior of the complex is strongly dependent upon centrifugal force since it is only at low speeds that it has a normal position relative to main peak DNA (14,19).

When examined at slow speed, and for lysis at 3°C as in Fig. 16 and not ∿24°C as in earlier figures, the DNA from cells incubated for repair at 37°C sediments as shown in Fig. 17. In 30 min, only a small proportion of DNA remains in the main peak, and by 90 min, the amount remaining is similar to that for unirradiated cells. That this repair does not return the cell to a normal state is indicated by the fact that by 360 min, the proportion of DNA near the meniscus has increased again as the pathology of dying cells becomes expressed as the degradation of repaired complex.

Figure 18 shows an experiment similar to that in Fig. 16 except that cells were incubated at 41°C after irradiation. Here we can see that the rates of loss of main peak DNA, and reformation of the complex, are slower than at 37°C and in addition a fairly large amount of DNA lies in the region between fractions 8-20 in contrast to the situation for repair at 37°C, Fig. 16.

Thus, we see that even though single-strand repair is more rapid under hyperthermic conditions, Fig. 10, repair of the complex proceeds less favorably at elevated temperatures. This result further supports a connection between damage and repair of the complex as being critical to the cell and also illustrates again the difference between repair of the complex and repair of single-strand breaks.

DISCUSSION

In simple terms, we may surmise what we have described with the aid of Fig. 19. Our experiments dealing with cell function, i.e. cell survival, lead to the "damage-level" diagram on the left. An acute dose of radiation displaces a cell downward from the ground state. If the dose is small, the transition may not go beyond the "sublethal domain." If the dose is large enough, whether or not a cell will survive depends upon repair processes which compete in time with cell processes which result in the expression of, or fixation of, damage. That repair of potentially lethal damage does occur

can be seen from the following. In Fig. 2 we see that a 10% survival dose is about 500 rad. After 500 rad, on average there are ~5000 single-strand breaks in the cell's DNA and about 100 double-strand breaks (data to be presented elsewhere). (About 5000 bond-breaking events would be registered in each 1×10^{-11} gms of mass in the cell.) Clearly, these numbers reduce to probabilities much less than 10% the likelihood that a surviving cell did not suffer any hits in its DNA. While it is a possibility that a cell could survive with some damage, it is unlikely that it would do so with the aforementioned amounts. Hence, the conclusion is forced that an upward transition must occur in time from the lethal to the sublethal domain for cells to express themselves as survivors.

Actinomycin D can be pictured to act by interfering with the upward transition, by shifting upward the threshold between lethal and sublethal damage, or by both. Added just prior to, or just after, a dose of radiation, actinomycin D adds damage similar to x-ray damage, Fig. 5. However, if sublethal and lethal damage differ only in degree, a distinction between the interference with x-ray damage repair between doses and the enhancement of single dose effects may not be possible particularly in view of the persistence of actinomycin D action resulting from its binding to DNA (10).

In the right half of Fig. 19, we summarize in an operational way what we know from our DNA studies. From data not presented here, we know that a large fraction of a cell's lipid cosediments with the complex and as the complex is resolved, this cosedimentation is lost (24). Further, repair of damage to the complex results in cosedimentation of these two materials again. While it seems likely that some of the lipid behaving in this way nonspecifically condenses on the DNA as a concomitant of cell lysis, this observation supports the possibility that DNA-membrane (lipid is a principal constituent of membrane) has an integral association in mammalian cells as it has in bacteria (see also the electron microscopic evidence of Davies and Small, 25). We picture DNA as attached in discrete places to the membrane after Comings and Okada (26) although we are not able to specify the length of the duplexes between attachment points. Further, the tertiary or higher order structure between attachment points cannot be specified from our measurements (i.e., circles or catenates of circles could be

attached to membrane at discrete points). Radiation, having as it does a primary ability to break bonds, renders damaged the DNA-lipid complex, and following this cells repair single-strand breaks, damage to the DNA complex, and, in fact, double-strand breaks (23). After a supralethal dose--e.g., 4330 rad in Fig. 9--essentially all the single-strand breaks are repaired. Even after a dose following which fewer than 1 cell in 1000 survive--e.g., 1700 rad in Fig. 17--in 90 min cells repair damage to the complex sufficiently that its sedimentation is hard to distinguish from that of the DNA from unirradiated cells. As revealing as sedimentation studies of this type are in view of the opportunity they afford us to examine DNA with a minimum of artifically introduced breaks, they do not permit us to distinguish between DNA that is "repaired" and functional, and DNA that is "repaired" and not. This is clearly a limitation of the methods. Repair as observed by us might in fact represent lesion fixation rather than functional repair. Still, as shown in Fig. 16, doses of radiation after which there are comparable assortments of surviving and non-surviving cells in a population of irradiated cells--i.e., survival after 100 rad, ~85%; after 200 rad, ~60%; after 400 rad, ~25%; and after 800 rad, ~2%--are the doses which resolve the main peak from the complex. Even though we are not able to identify a surviving from a non-surviving cell on the basis of the sedimentation properties of its DNA after repair, we are encouraged to associate integrity of the complex with survival because damage to the complex is demonstrable in the range of dose corresponding to relatively high survival. Further, the DNA data with actinomycin D support this view as do results, not described here, obtained with irradiated cells in which a proportion of the thymidine in the DNA had been replaced by the sensitizer 5-bromodeoxyuridine (27).

In spite of limitations noted, our results with radiation and the DNA interactive agents nitrogen mustard and actinomycin D support the following If the DNA duplex is sufficiently cross-linked, a cell will be killed but in a way that does not enhance x-ray cell killing because the latter results from quite another form (or other forms) of damage. We propose that x-ray lethality results from configurational changes in the DNA and/or the DNA and its molecular environment (12,14,19,21,24). The radiation induced sedimentation discontinuities we observe under alkaline conditions--e.g., Fig.

16--and the selective inhibition by actinomycin D of repair of the complex as opposed to repair of single-strand breaks, supports the notion that the configurational change is at the level of at least tertiary structures and involves the opening of a duplex circle (or a catenate of circles). Since evidence for such structures in mammalian cells has not as yet been forthcoming--although the circularity of the bacterial chromosome is well established and circularity is now proposed for dinoflagellates (28)--we are faced with the ultimate problems of elucidating chromosome structure and function in eukaryotes as inescapable requirements of elucidating the relevance of DNA damage to lethality in mammalian cells.

REFERENCES

1. Elkind, M. M. "An Introduction to the biology of the mammalian cell," to appear in the Proceedings of the Conference on Physical Mechanisms in Radiation Biology, October 1972, Airlie, Virginia, USA.
2. Elkind, M. M. and Whitmore, G. F. The Radiobiology of Cultured Mammalian Cells, Gordon & Breach Science Publishers; New York, 1967.
3. Lawley, P. D., Lethbridge, J. H., Edwards, P. A., and Shooter, K. Y. J. Mol. Biol., 39, 181-198 (1969).
4. Sakamoto, K. and Elkind, M. M. Biophys. J., 9, 1115-1130 (1969).
5. Elkind, M. M., Kano, E., and Sutton-Gilbert, H., J. Cell Biol., 42, 366-377 (1969).
6. Elkind, M. M., Sakamoto, K., and Kamper, C., Cell & Tissue Kinetics, 1, 209-224 (1968).
7. Sinclair, W. K., Cancer Research, 27, 297, (1967).
8. Elkind, M. M., Kamper, C., Moses, W. B., Button-Gilbert, H., Brookhaven Symp. Biol., 20, 134 (1967).
9. Elkind, M. M. Radiation Research, 1966, G. Silini (ed.), pp. 558-586, North-Holland Amsterdam, 1967.
10. Elkind, M. M. Sutton-Gilbert, H., Moses, W. B., and Kamper, C. Nature, 214, 1088 (1967).
11. Elkind, M. M. and Kano, E. Radiation Research, 44, 484-497 (1970).
12. Elkind, M. M., Moses, W. B., and Sutton-Gilbert, H., Radiation Research, 31, 156-173 (1967).
13. McGrath, R. A., and Williams, R. W. Nature, 212, 534 (1966).
14. Elkind, M. M. and Kamper C., Biophys. J. 10, 237 (1970).

15. Burgi, E. and Hershey, A. D. Biophys. J., 3, 309 (1963).
16. Studier, F. W. J. Mol. Biol., 11, 373-390 (1965).
17. Elkind, M. M. and Ben-Hur, E. Israel J. Chem., 10, 1255-1272 (1972).
18. Ben-Hur, E. and Elkind, M. M. Thermally enhanced radioresponse of Chinese hamster cells; damage and repair of single-stranded DNA, double-stranded DNA, and a DNA complex. Submitted.
19. Elkind, M. M. Biophys. J., 11, 502-520, (1971).
20. Ben-Hur, E. and Elkind, M. M. Biophys. Biochem. Acta, in press.
21. Elkind, M. M. and Chang-Liu, C.-M. Int. J. Radiat. Biol., 22, 313-324 (1972).
22. Ben-Hur, E., Bronk, B., and Elkind, M. M. Nature, New Biology, 238, 209-210 (1972).
23. Ben-Hur, E., Elkind, M. M. and Bronk, B. Thermally enhanced radioresponse of cultured Chinese hamster cells. Inhibition of repair of sublethal damage and enhancement of lethal damage. Submitted.
24. Elkind, M. M. and Chang-Liu, C.-M. Int. J. Radiat. Biol., 22, 75-90 (1972).
25. Davies, H. G. and Small, J. V. Nature, 217, 1122-1125 (1968).
26. Comings, D. E. and Okada, T. A. Exptl. Cell Res., 62, 293-302 (1970).
27. Shipley, W. V. and Elkind, M. M. Radiation Research, 48, 86-94 (1971).
28. Haapala, O. K., Soyer, M.-O. Nature, New Biology, 244, 195-197 (1973).

FIGURE LEGENDS

Figure 1. Skematic cross section of a mammalian cell. Structures and organelles are, in places, exaggerated for clairty. (From ref. 1.)

Figure 2. Single and fractionated dose survival of single Chinese hamster cells, line V79-1. Radiation: 55kV x-rays, 722 rads/min. Survival, colony formation; standard errors one shown where large enough relative to the data points. $\underline{n}$ stands for extrapolation number, the ordinate to which the terminal portion of a curve extrapolates, and $\underline{D_o}$ the dose capable of reducing survival by the factor $1/\underline{e}$ along the exponential part of a curve.

Figure 3. Skematic representation of the effects on duplex DNA of x-rays, nitrogen mustard, and actinomycin D. X-rays produce single-strand breaks, double-strand breaks, and base damage. Nitrogen mustard treatment results in mono-additions and intrastrand cross links as well as interstrand alkylations. Actinomycin D is thought to act by intercalating.

Figure 4. Survival of microcolonies of Chinese hamster cells, line V79-753B. Cells synchronized at the G_1-S border with hydroxyurea (2 mM, 3 1/2 hrs) were allowed to progress for 3 3/4 hrs into the most resistant period of the DNA synthetic phase. They were then treated with x-rays (as for Fig. 2) or nitrogen mustard NM just prior to or after each x-ray dose. The reduction in survival from 1.0 to about 0.4 was due to the lethal action of the hydroxyurea on those S phase cells initially in the population (7) since they are rendered non-colony formers. The reason for the small displacement in the two combined treatment curves is not known but unimportant to the interpretation of the data. (From ref. 4.)

Figure 5. Comparative survival curves for late S phase Chinese hamster cells (details as in Fig. 4). The data shown are for the survival of a population of microcolonies, starting multiplicity $\underline{\bar{N}} = 3.6$. Accounting for multiplicity, the residual shoulder widths are 359 rad and 51 rad, open and closed circles respectively. (From ref. 8.)

Figure 6. Potentiation of x-ray cell killing of Chinese hamster cells by 0.002 μg/ml of actinomycin D present in the medium from the time of irradiation until colonies were scored (details as in previous figures). (From ref. 8.)

Figure 7. The McGrath and Williams (13) alkaline sucrose sedimentation technique as applied to mammalian cells (see text).

Figure 8. Skematic representation of sedimentation patterns obtained at high speed (36-38,000 rpm) with DNA from labelled Chinese hamster cells. For a given time of lysis at ∿24°C, the dose dependence is shown vertically. For a given dose, the dependence on lysis time is shown horizontally. (From ref. 17.)

Figure 9. The effect of lysis time (a) and increasing doses of x-rays on resolution of the complex, (b) and (c), and the x-ray breakage of single-stranded DNA (d) as observed with sedimentation in alkaline gradients. The closed circles in (b), (c), and (d) show the effect of incubation for repair prior to sedimentation (60 mm lysis, ∿24°C.) Sedimentation was at high speed, 38,000 rpm. (From ref. 14.)

Figure 10. The time course of the repair of single-strand breaks at 37°C and 42°C. Following incubation for repair, cells were lysed and then sedimented for 17.1 hrs at 11,000 rpm, 3°C. (From ref. 18.)

Figure 11. High speed sedimentation in alkaline gradients (as for Fig. 9) of labelled DNA from Chinese hamster cells treated or not with nitrogen mustard, NM. Lysis was for 270 mm at ∿24°C. P.Y. stands for percent yield, the percent of the starting radioactivity collected in the 30 fractions.

Figure 12. As for Fig. 11 except for the larger NM and x-ray doses.

Figure 13. High speed sedimentation of the DNA from two separate stocks of Chinese hamster cells. Cells labelled with ^{3}H-TdR were irradiated only while those pretreated with actinomycin D prior to irradiation had been labelled with ^{14}C-TdR. Just before lysis (as for Fig. 9), the cell stocks were mixed. Thus, DNA from both types of cells were sedimented in the same tube. (From ref. 21.)

Figure 14. High speed sedimentation of DNA from Chinese hamster cells treated for 30 min with actinomycin D (no irradiation). Other details as for Figs. 9 and 11.

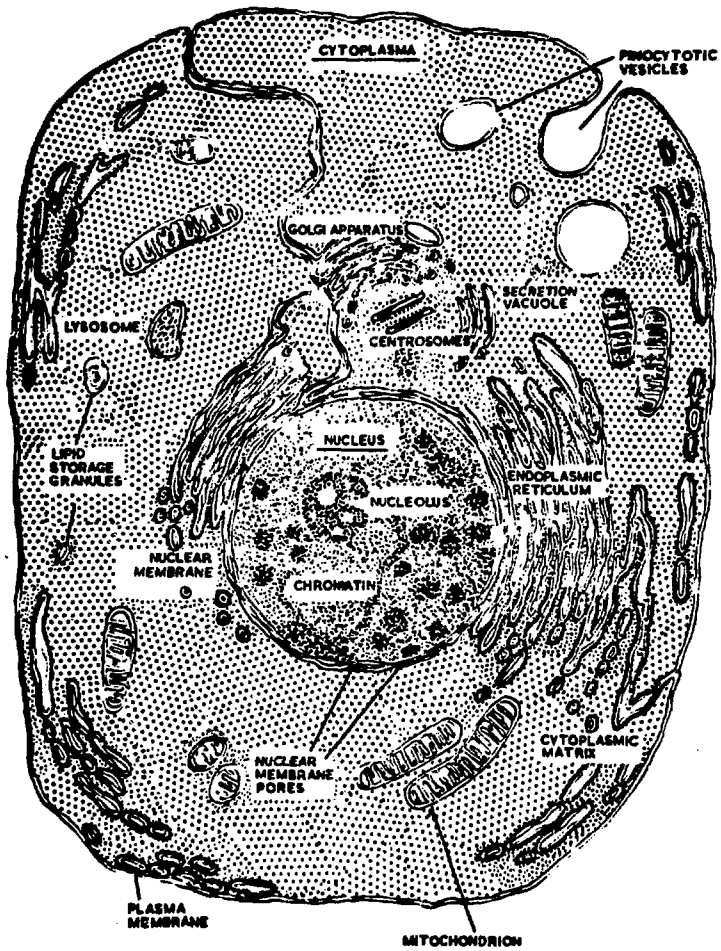
Figure 15. Fractionation survival curves of Chinese hamster cells for 3 temperatures between acute irradiations (250 kV x-rays, 360 rads/min) at 0°C. In each case, cells were suspended in a buffer containing 20% medium and maintained at the temperatures shown for 2.5 hrs. (From ref. 23.)

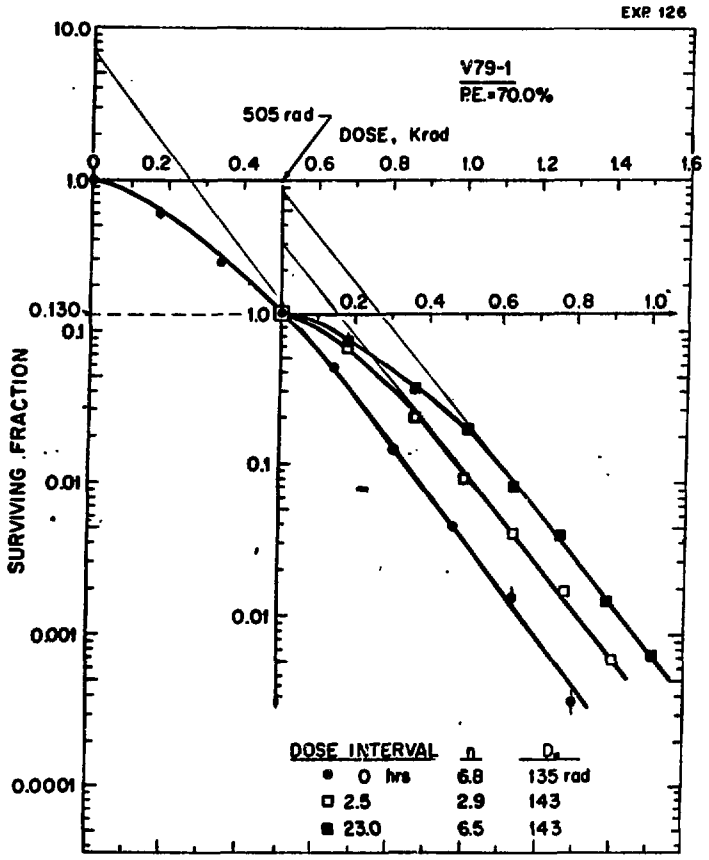
Figure 16. Slow speed sedimentation of labelled DNA from Chinese hamster cells after graded doses of x-rays. Lysis: 60 mm, 3°C. Sedimentation: 5000 rpm, 16.25 hrs., 0°C. Other details similar to Fig. 9. (From ref. 19.)

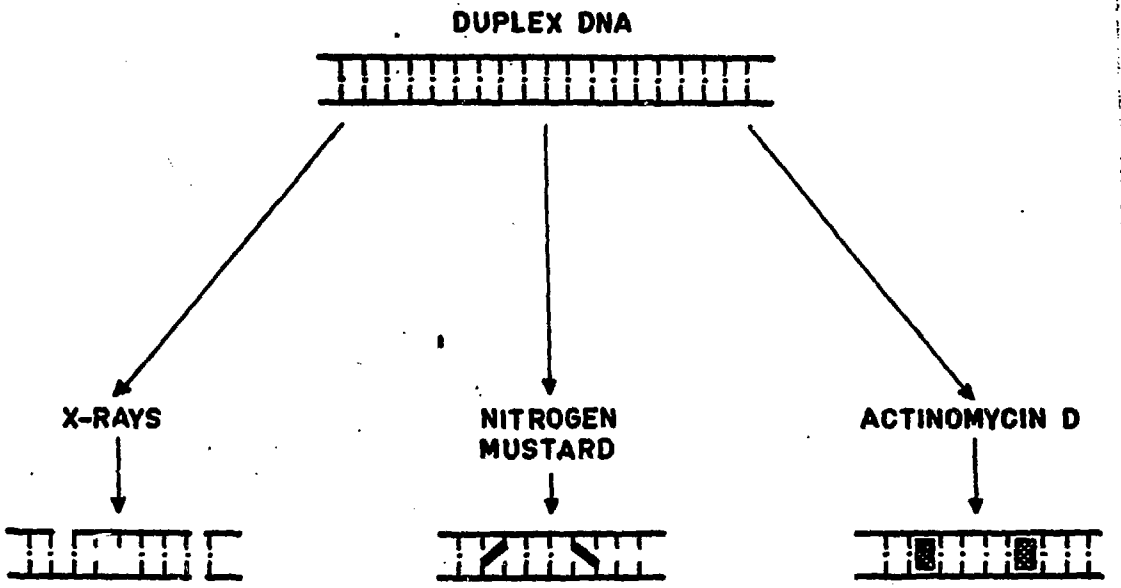
Figure 17. Slow speed sedimentation of labelled DNA from Chinese hamster cells incubated for repair at 37°C after 1700 rad. Lysis and sedimentation as for Fig. 16. (From ref. 23.)

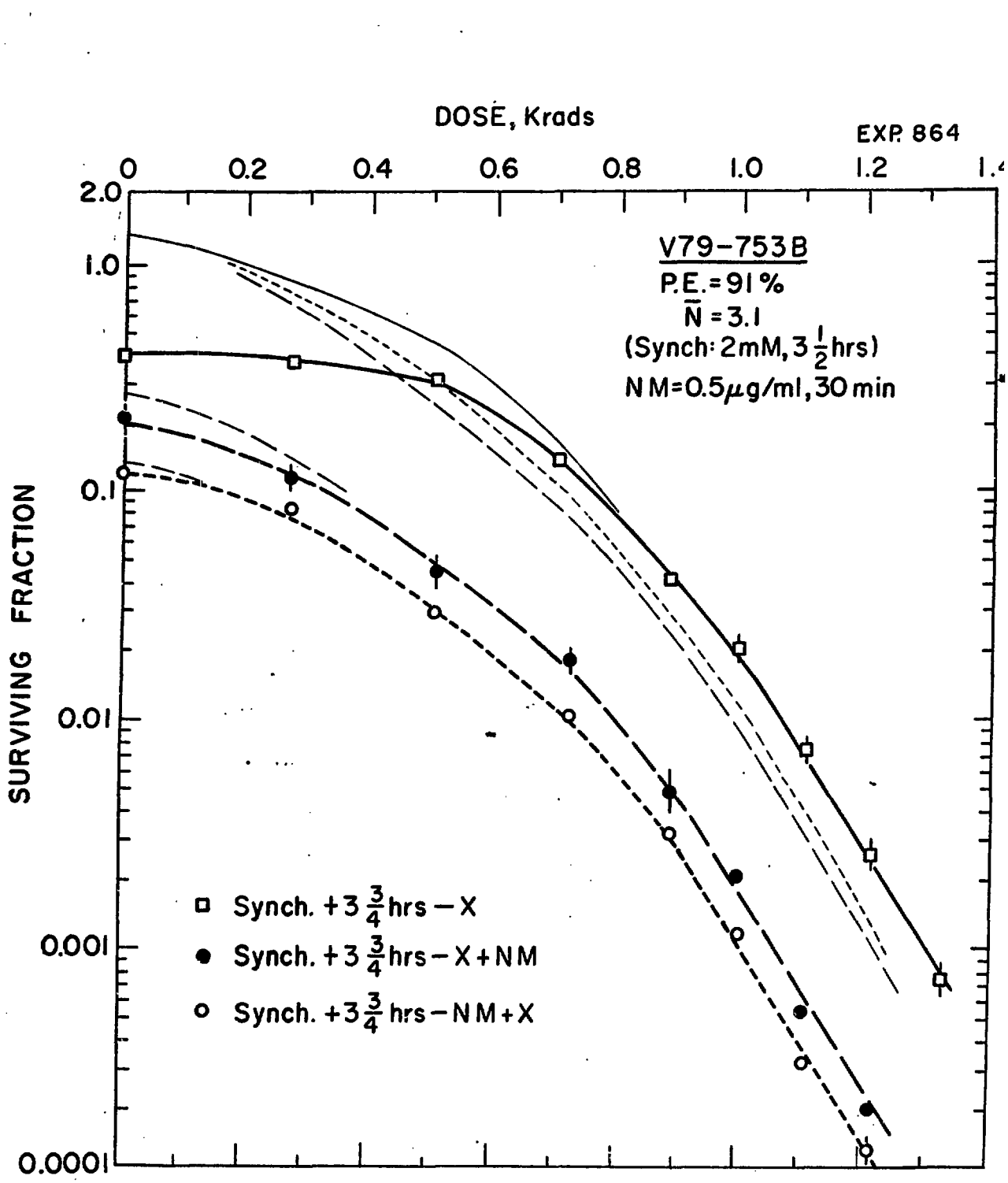
Figure 18. An experiment similar to that in Figure 17 in all details except that cells were incubated at 41°C instead of 37°C after 1700 rad. (From ref. 23.)

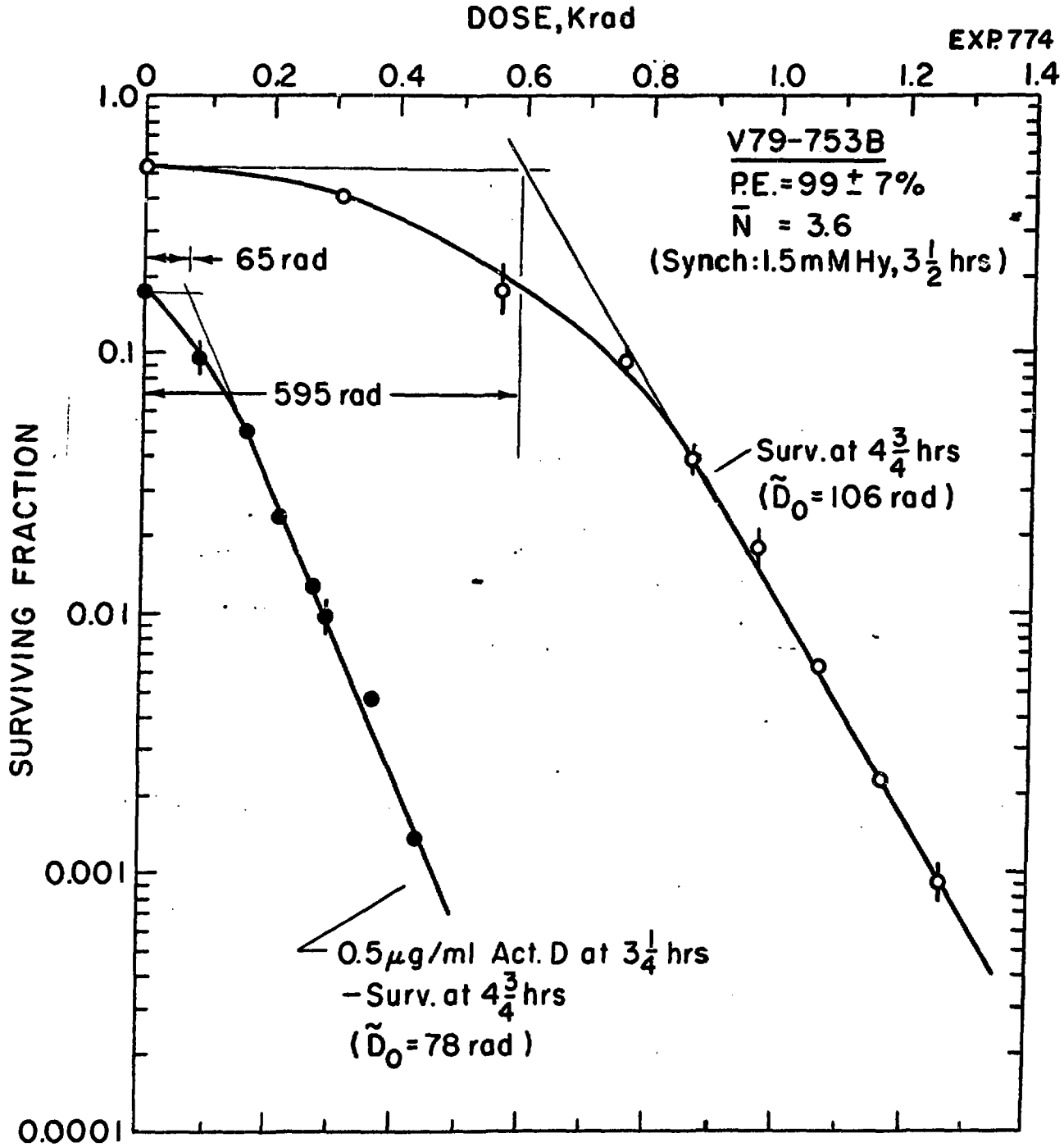
Figure 19. Schematic representations of the effect of radiation and actinomycin D on cell survival (left) and the effect of radiation on DNA damage (right). (From ref. 17.)

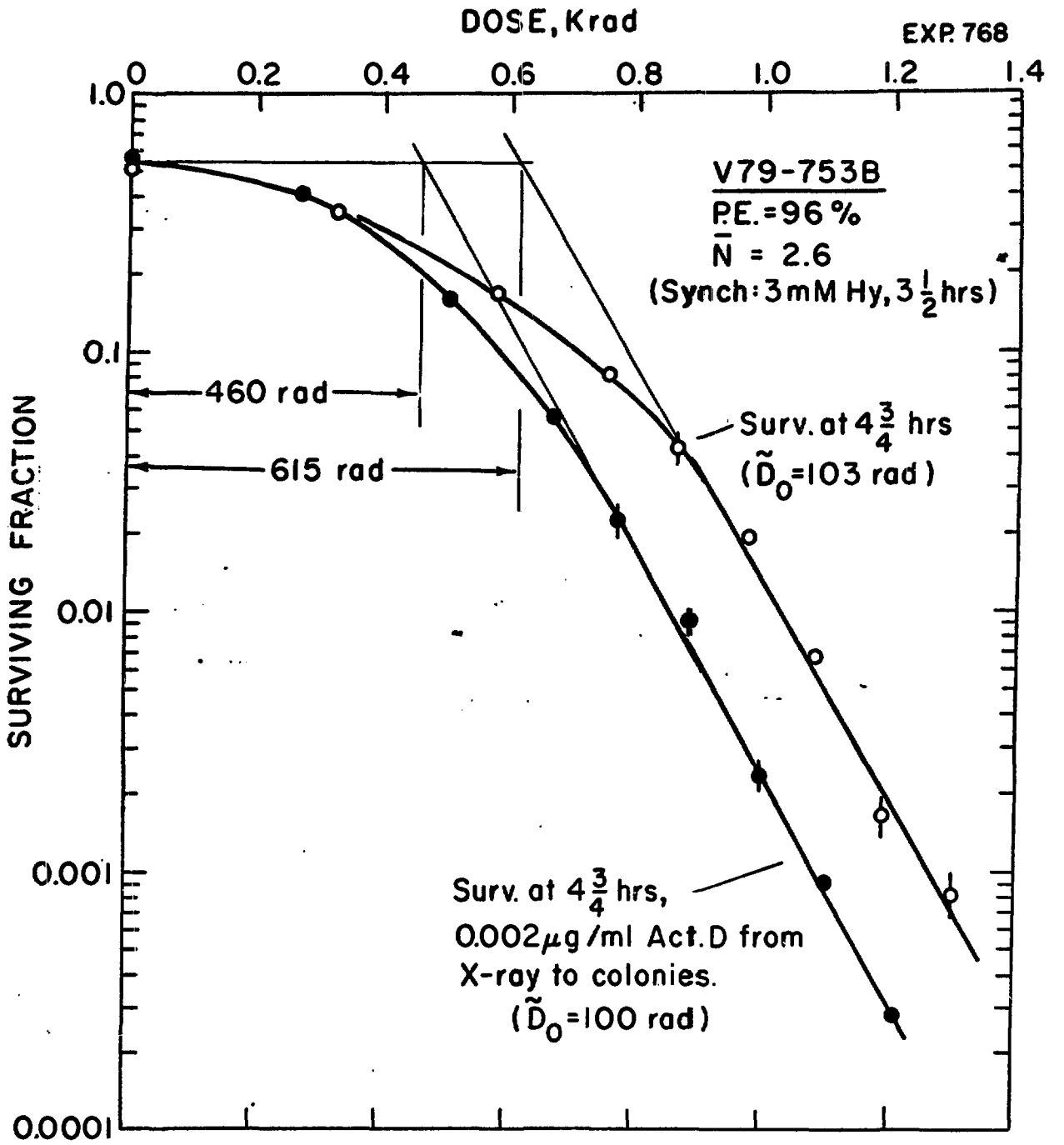

CYTOPLASMA
PINOCYTOTIC VESICLES
GOLGI APPARATUS
SECRETION VACUOLE
LYSOSOME
CENTROSOMES
NUCLEUS
LIPID STORAGE GRANULES
ENDOPLASMIC RETICULUM
NUCLEOLUS
NUCLEAR MEMBRANE
CHROMATIN
CYTOPLASMIC MATRIX
NUCLEAR MEMBRANE PORES
PLASMA MEMBRANE
MITOCHONDRION

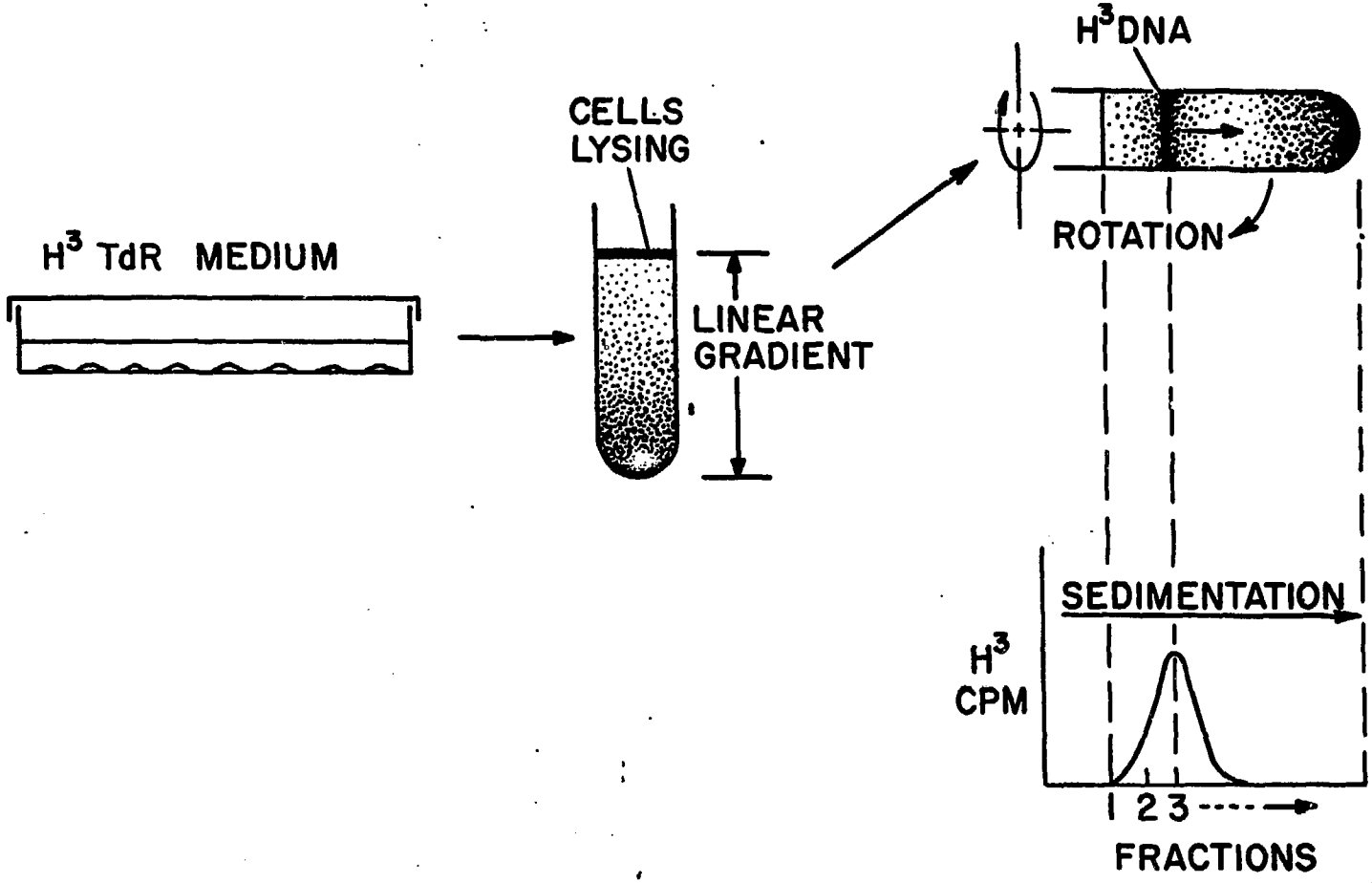

EXP 126
V79-1
P.E.=70.0%
505 rad
DOSE, Krad
SURVIVING FRACTION
DOSE INTERVAL
n
D_o
0 hrs
6.8
135 rad
2.5
2.9
143
23.0
6.5
143

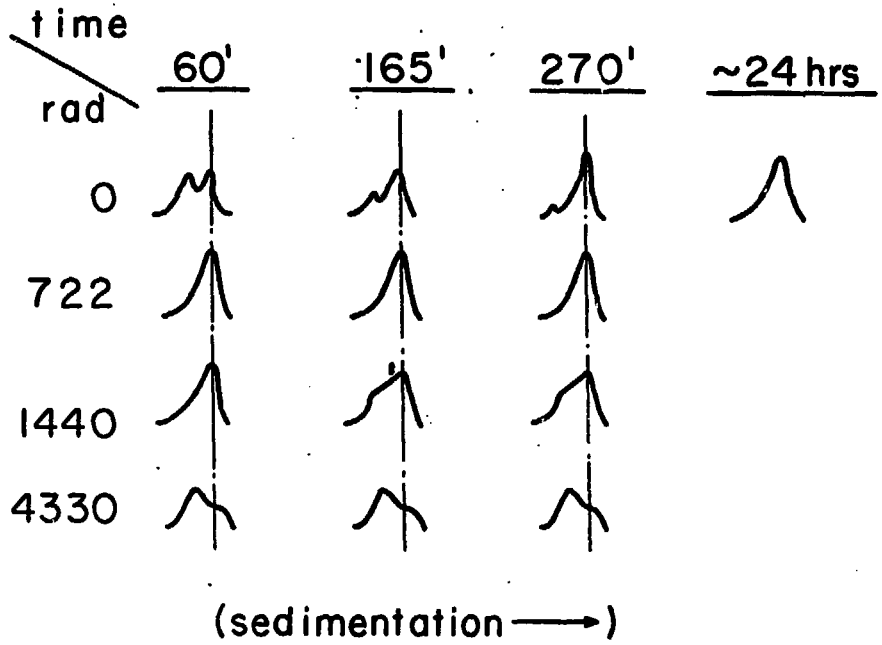

DUPLEX DNA
X-RAYS
NITROGEN MUSTARD
ACTINOMYCIN D

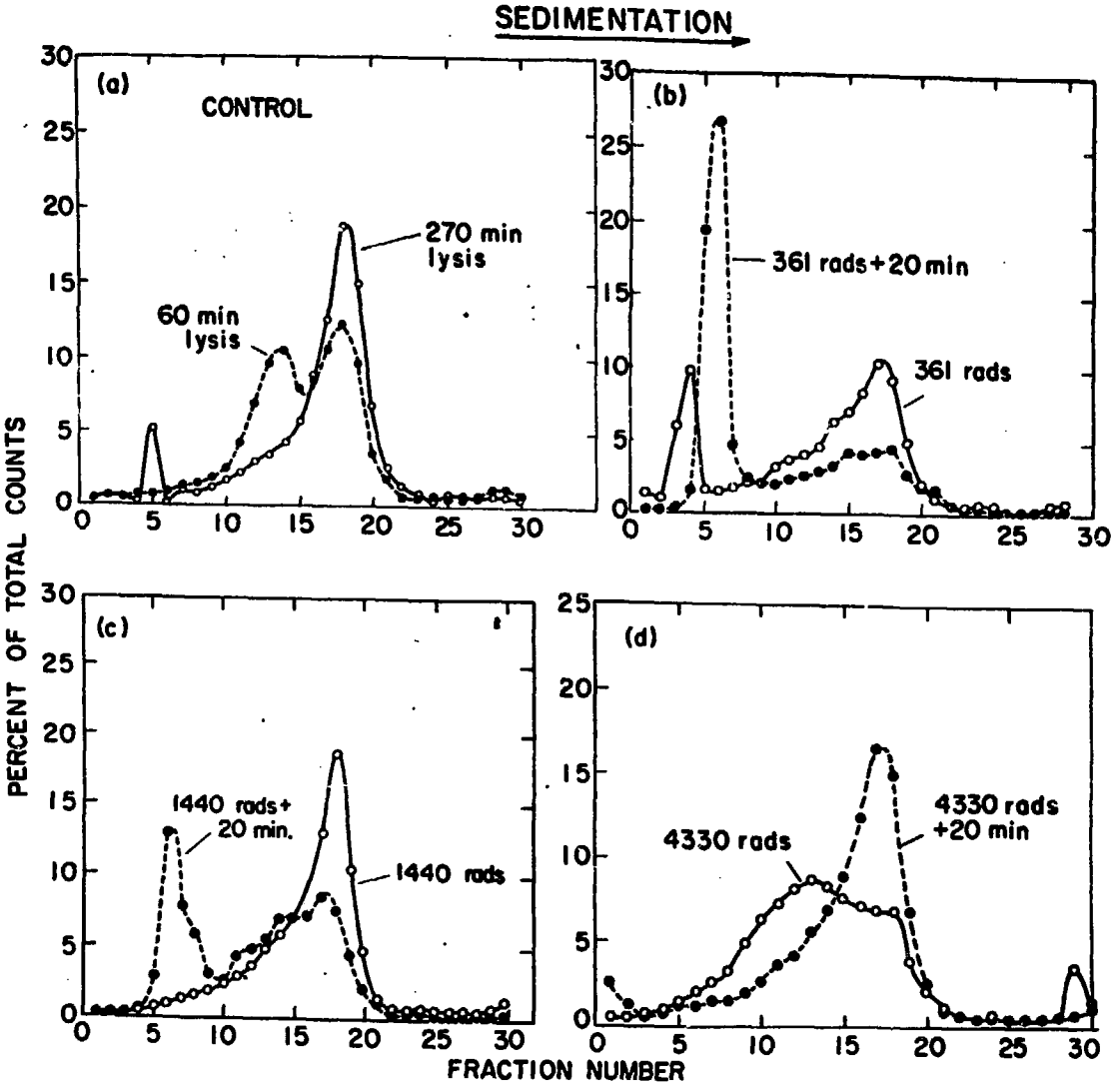

DOSE, Krads
EXP. 864
0
0.2
0.4
0.6
0.8
1.0
1.2
2.0
1.0
0.1
0.01
0.001
0.0001
SURVIVING FRACTION
V79-753B
P.E.=91%
N̄=3.1
(Synch: 2mM, 3 1/2 hrs)
NM=0.5μg/ml, 30 min
Synch. +3 3/4 hrs – X
Synch. +3 3/4 hrs – X+NM
Synch. +3 3/4 hrs – NM+X

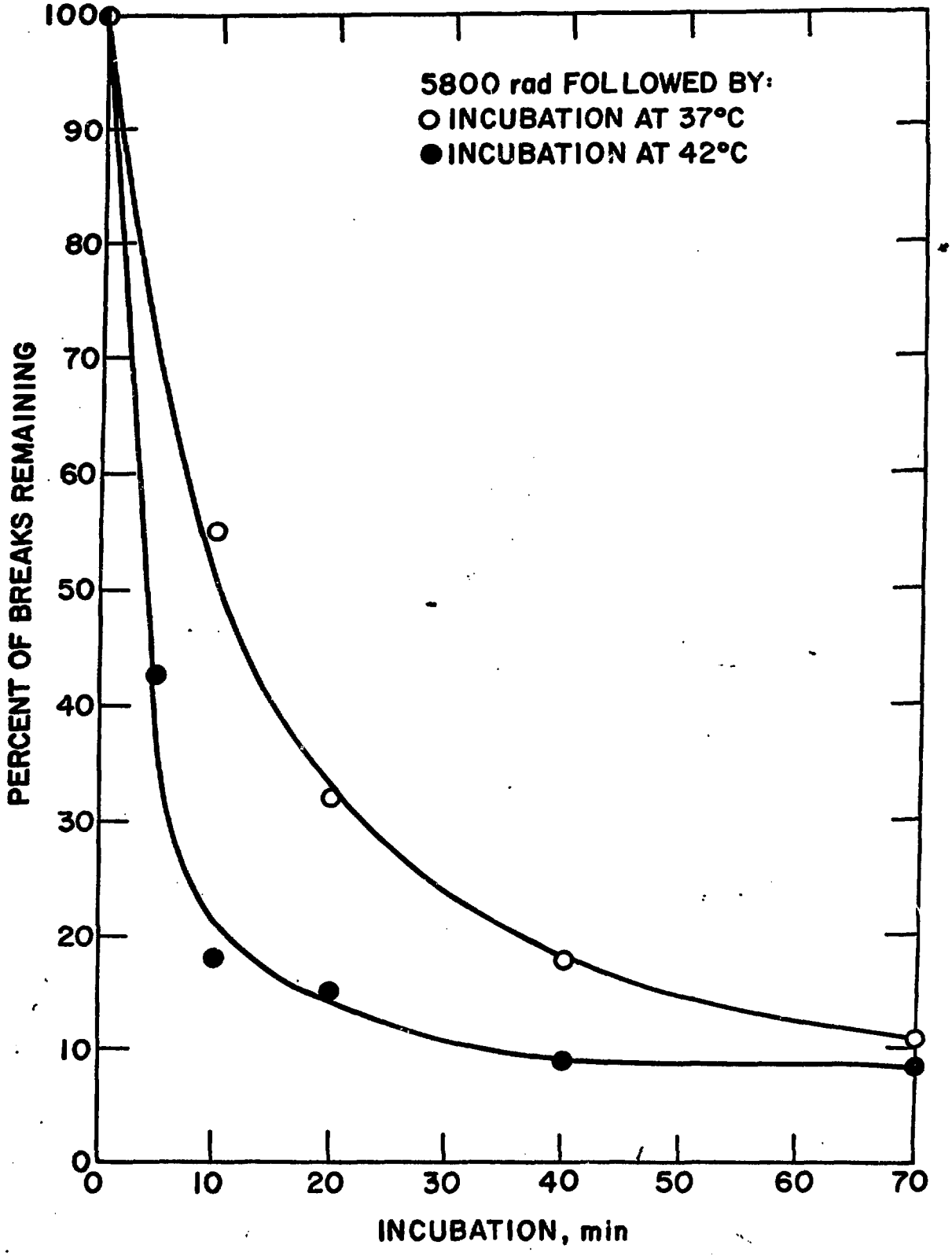

DOSE, Krad
EXP. 774
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.0
0.1
0.01
0.001
0.0001
SURVIVING FRACTION
V79-753B
P.E. = 99 ± 7%
N̄ = 3.6
(Synch: 1.5 mM Hy, 3½ hrs)
65 rad
595 rad
Surv. at 4¾ hrs
(D̃0 = 106 rad)
0.5 μg/ml Act. D at 3¼ hrs
– Surv. at 4¾ hrs
(D̃0 = 78 rad)

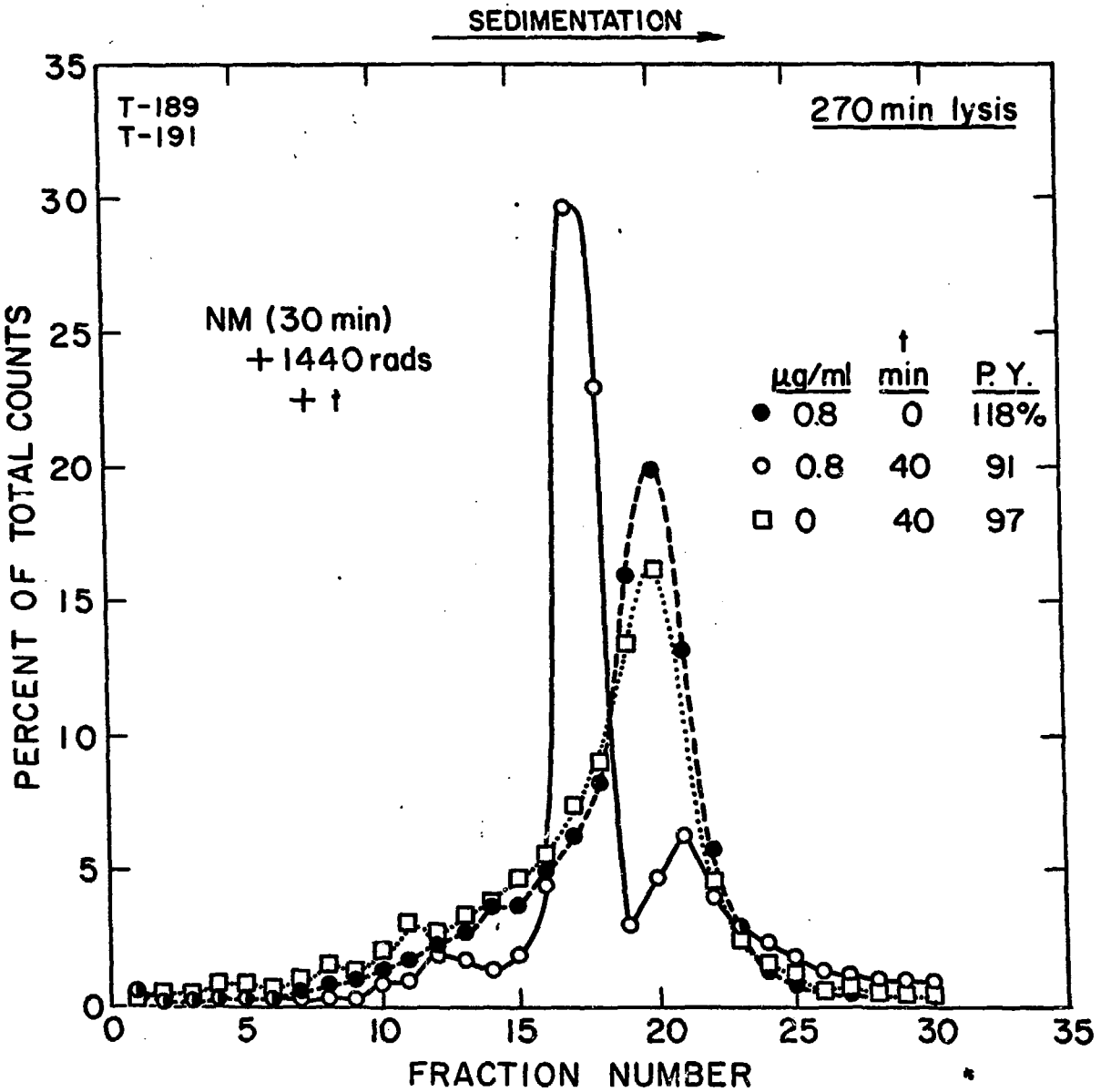

DOSE, Krad
EXP. 768
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.0
0.1
0.01
0.001
0.0001
SURVIVING FRACTION
V79-753B
P.E. = 96 %
N̄ = 2.6
(Synch: 3mM Hy, 3 1/2 hrs)
460 rad
615 rad
Surv. at 4 3/4 hrs
(D̃0 = 103 rad)
Surv. at 4 3/4 hrs,
0.002 μg/ml Act. D from
X-ray to colonies.
(D̃0 = 100 rad)

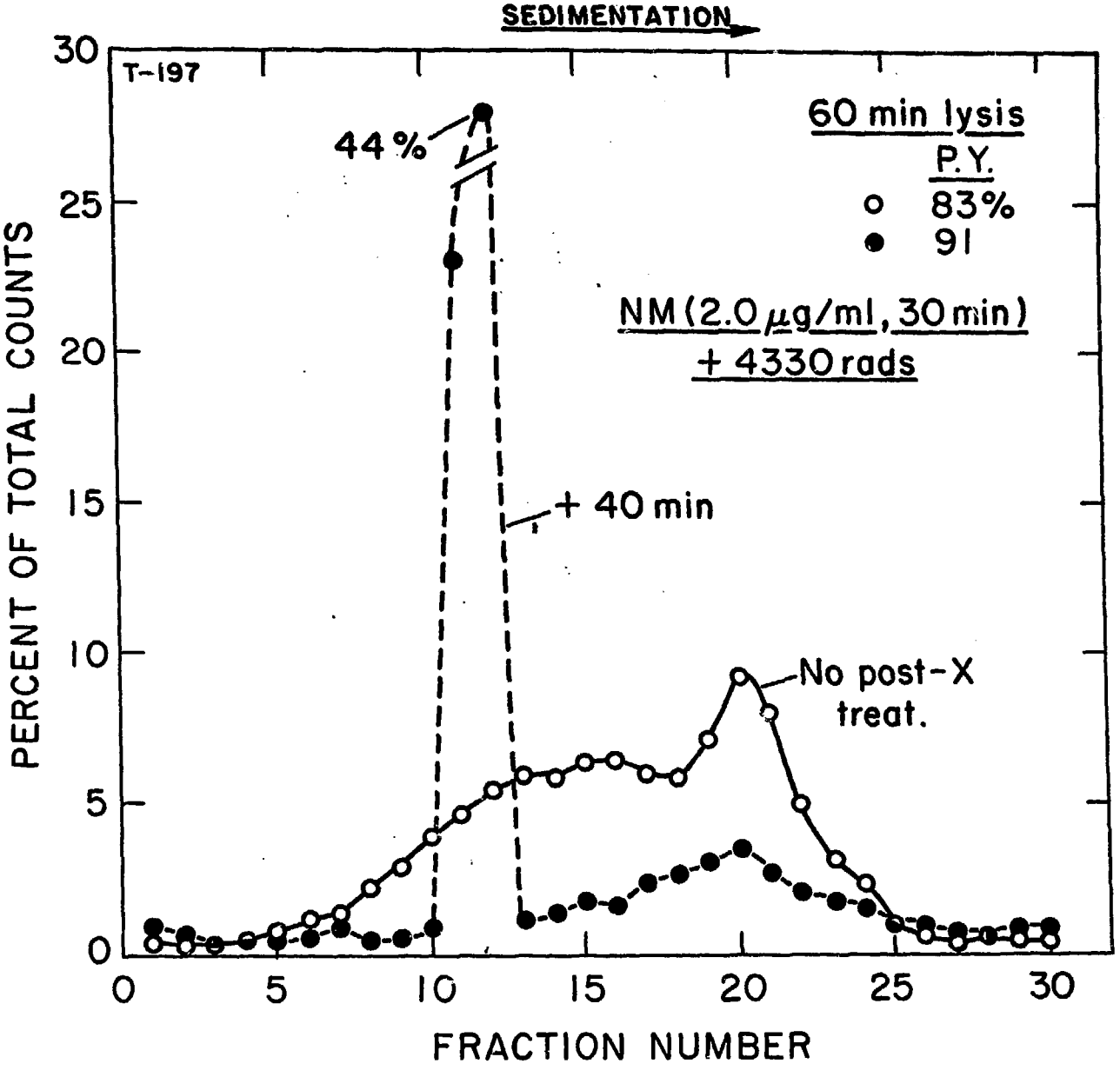

H³ TdR MEDIUM
CELLS LYSING
LINEAR GRADIENT
H³DNA
ROTATION
SEDIMENTATION
H³ CPM
1 2 3
FRACTIONS

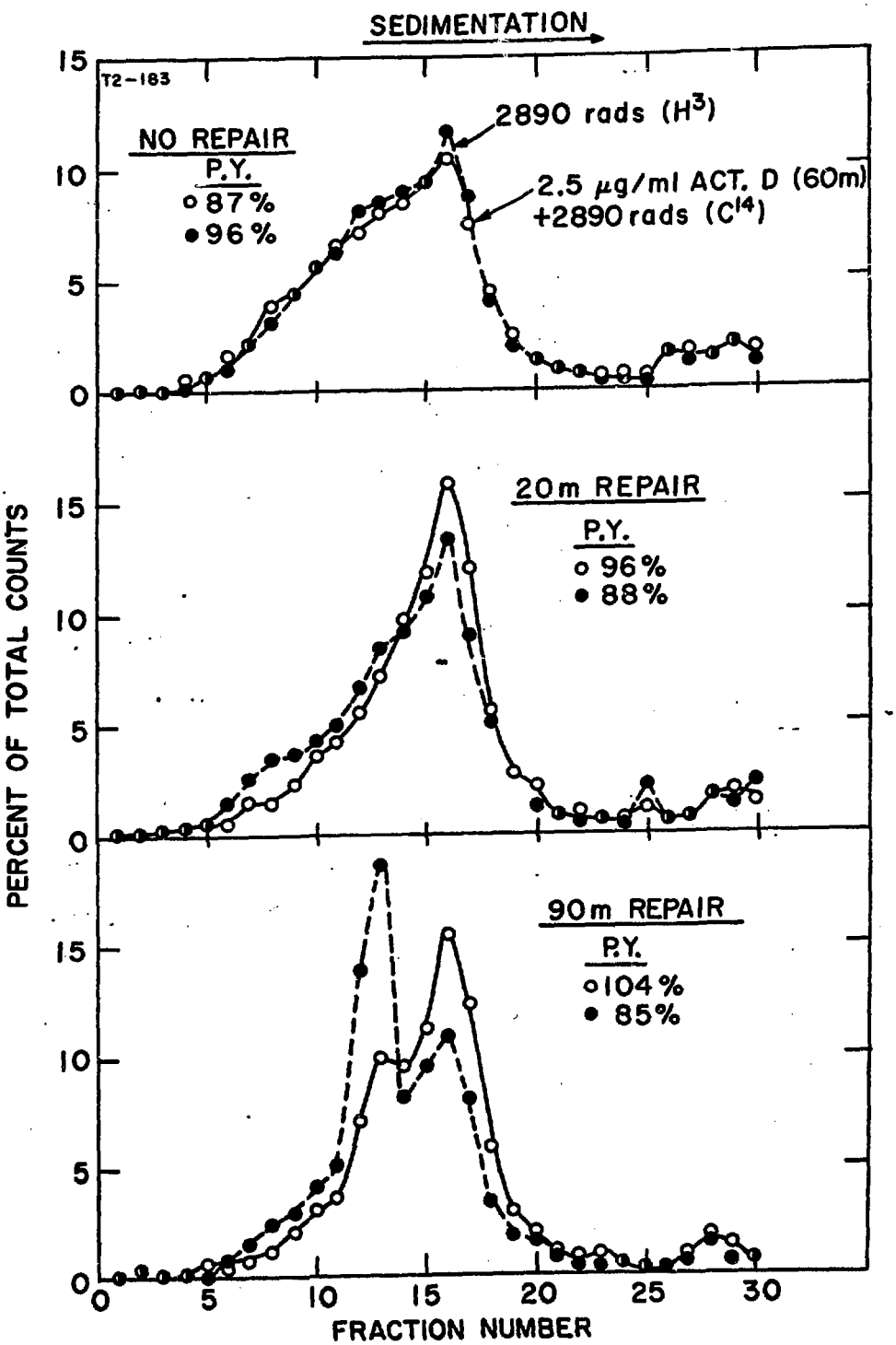

time
rad
60'
165'
270'
~24 hrs
0
722
1440
4330
(sedimentation ⟶)

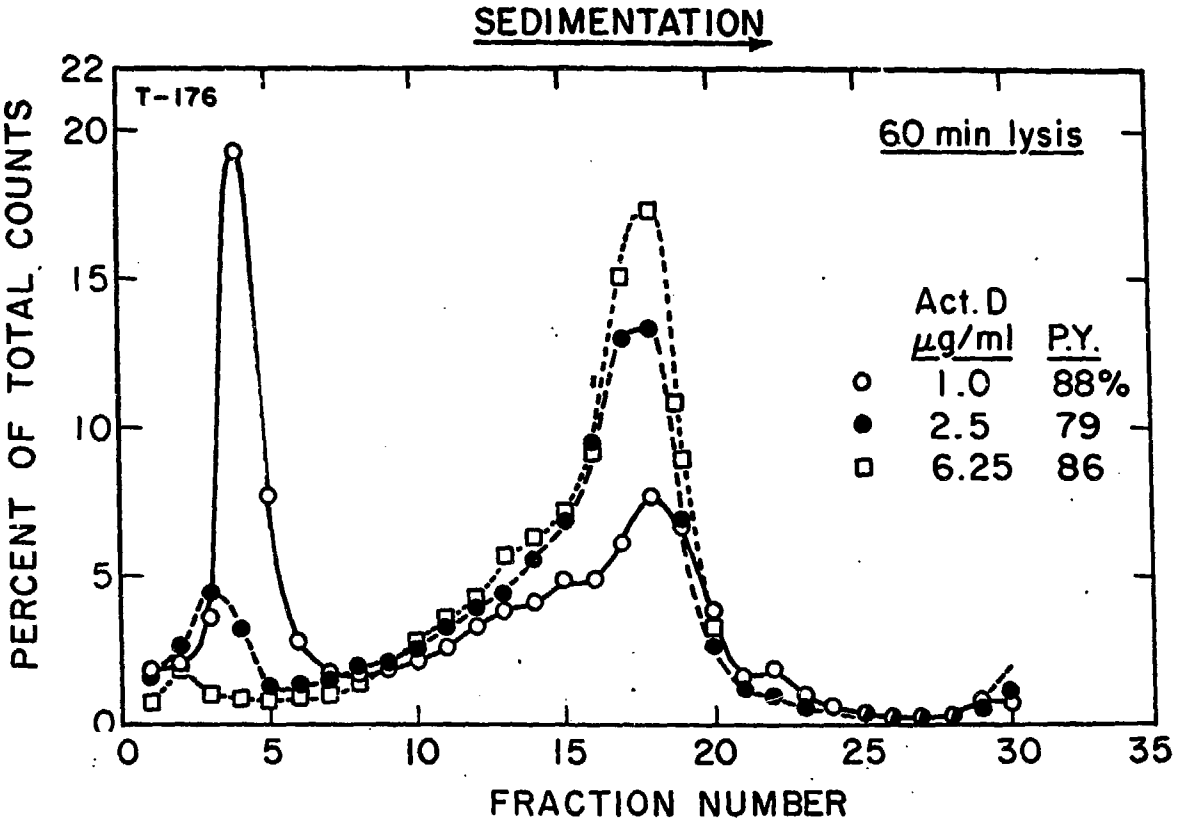

SEDIMENTATION
(a)
CONTROL
270 min lysis
60 min lysis
(b)
361 rads+20 min
361 rads
(c)
1440 rads+ 20 min.
1440 rads
(d)
4330 rads
4330 rads +20 min
PERCENT OF TOTAL COUNTS
FRACTION NUMBER

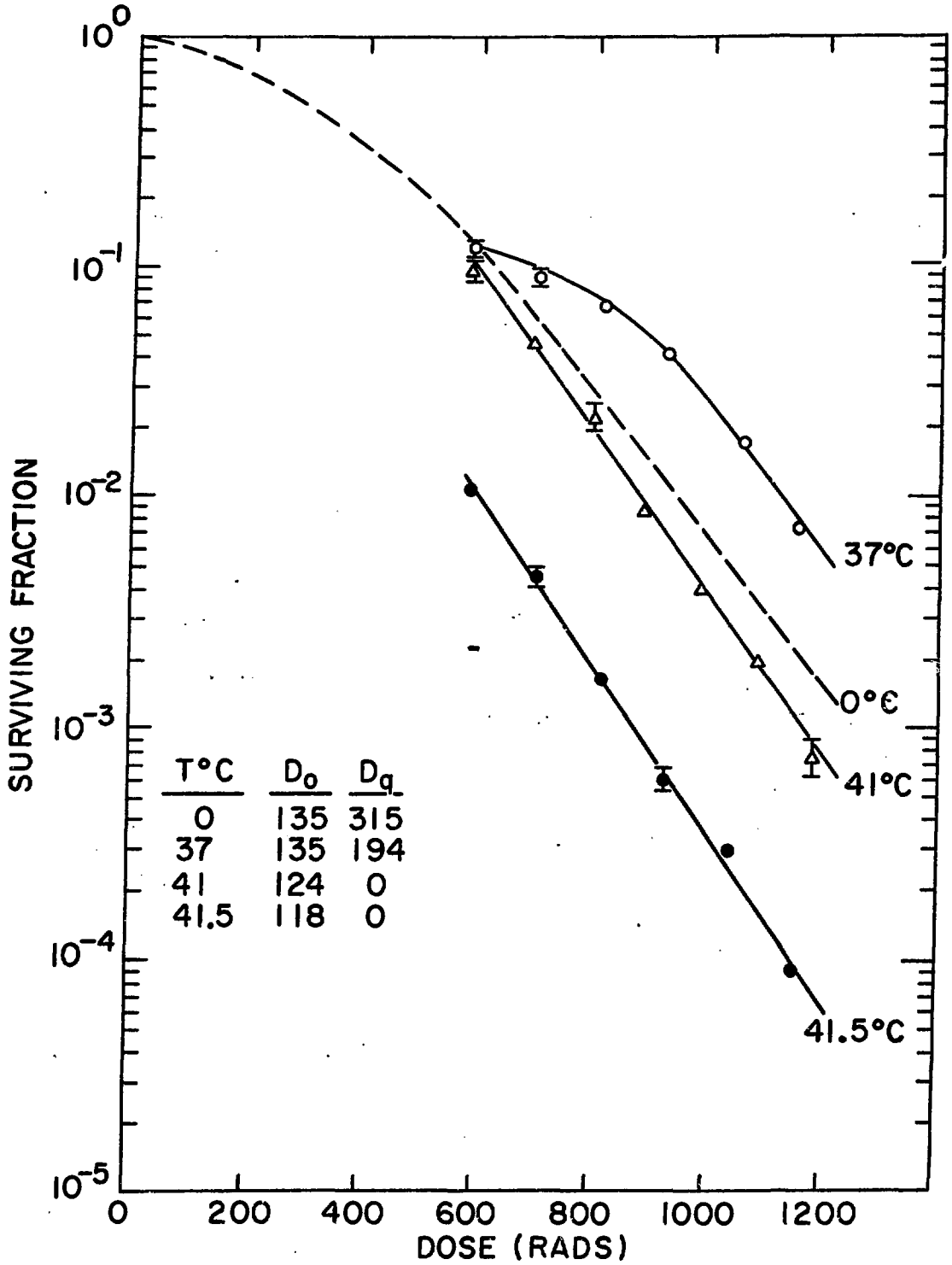

5800 rad FOLLOWED BY:
O INCUBATION AT 37°C
● INCUBATION AT 42°C
PERCENT OF BREAKS REMAINING
INCUBATION, min
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70

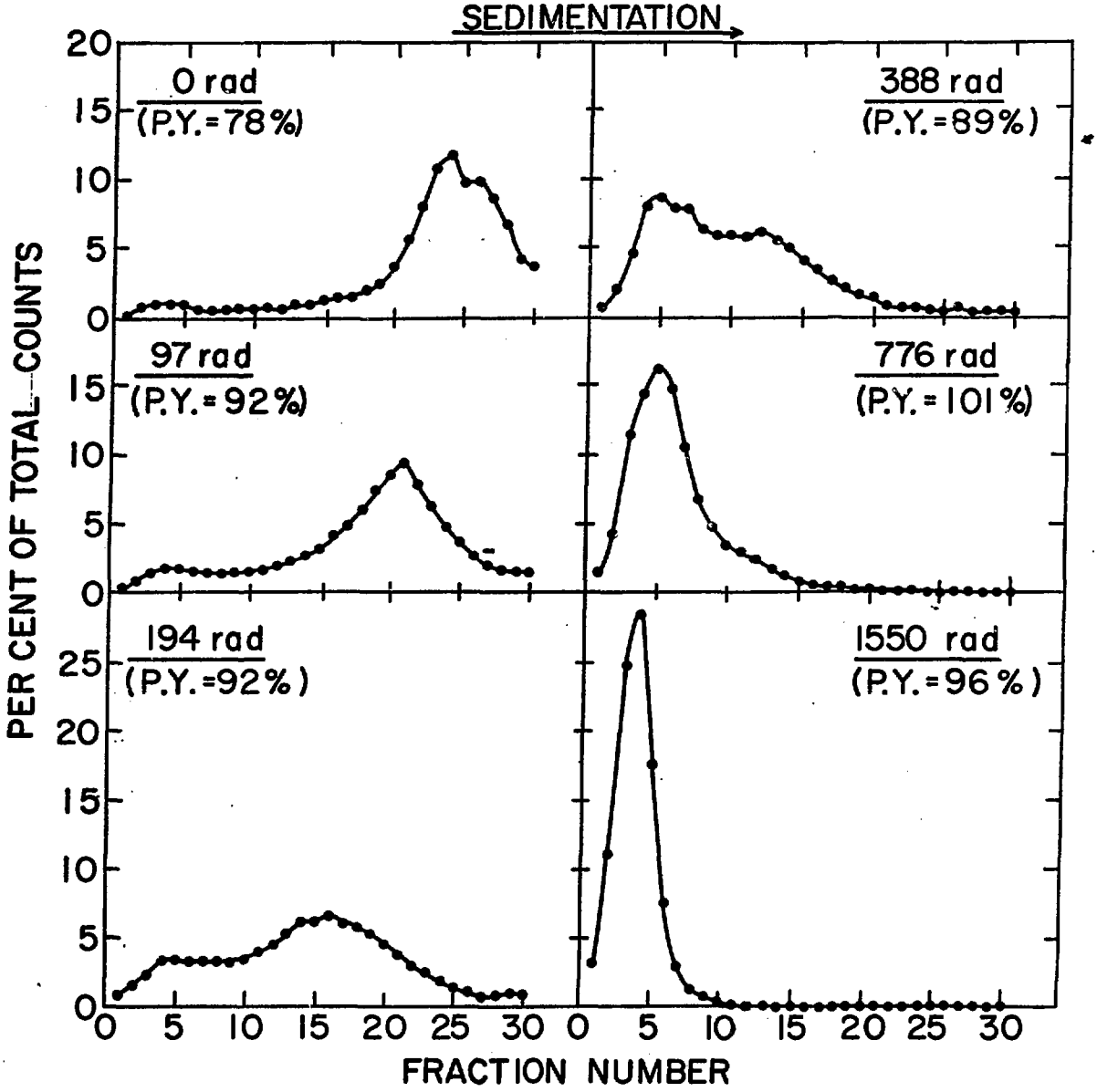

SEDIMENTATION
T-189
T-191
270 min lysis
NM (30 min)
+1440 rads
+ t
µg/ml
t min
P.Y.
● 0.8 0 118%
○ 0.8 40 91
□ 0 40 97
PERCENT OF TOTAL COUNTS
FRACTION NUMBER
0
5
10
15
20
25
30
35

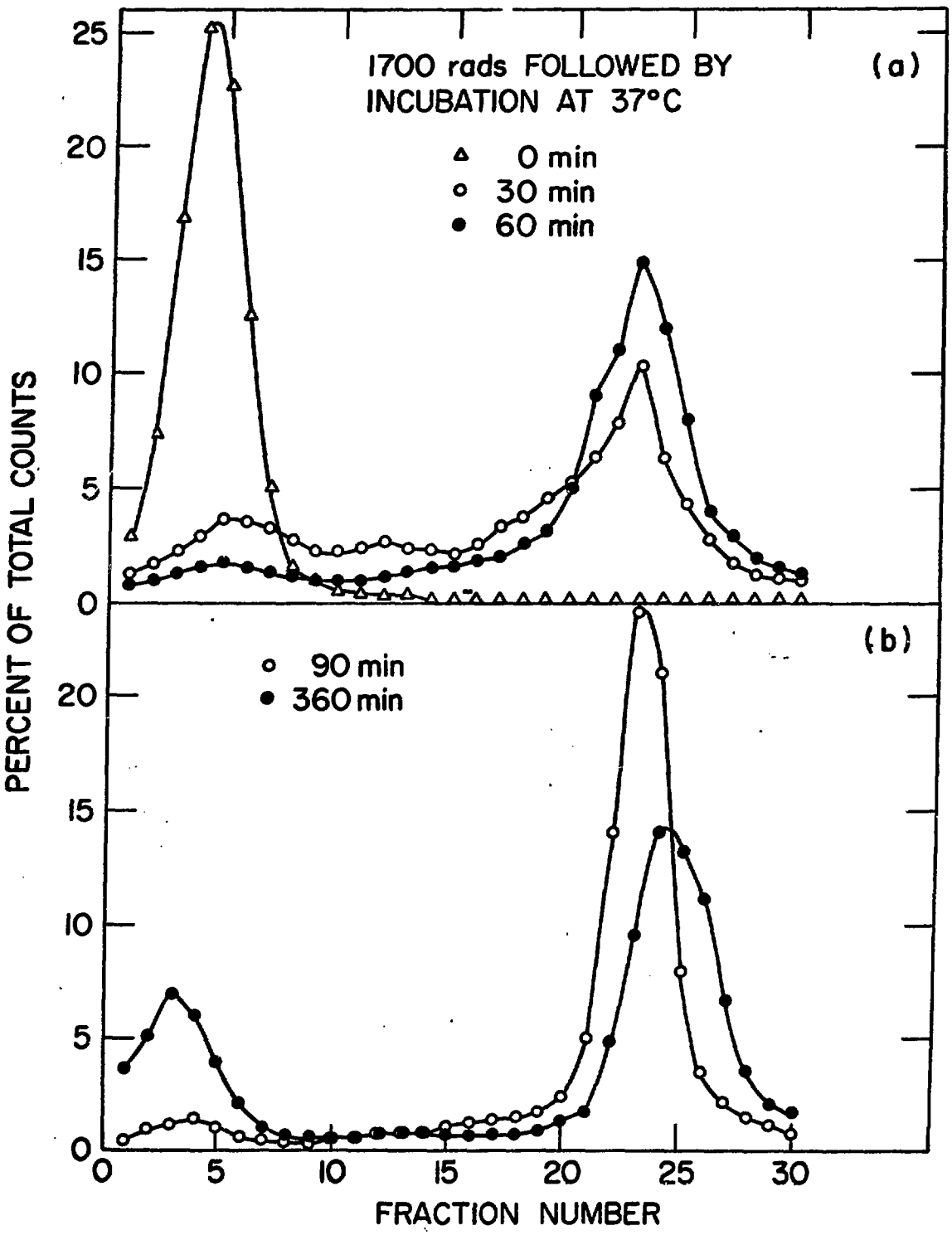

SEDIMENTATION
T-197
60 min lysis
P.Y.
83%
91
NM (2.0 μg/ml, 30 min)
+ 4330 rads
44%
+ 40 min
No post-X
treat.
PERCENT OF TOTAL COUNTS
FRACTION NUMBER
0
5
10
15
20
25
30

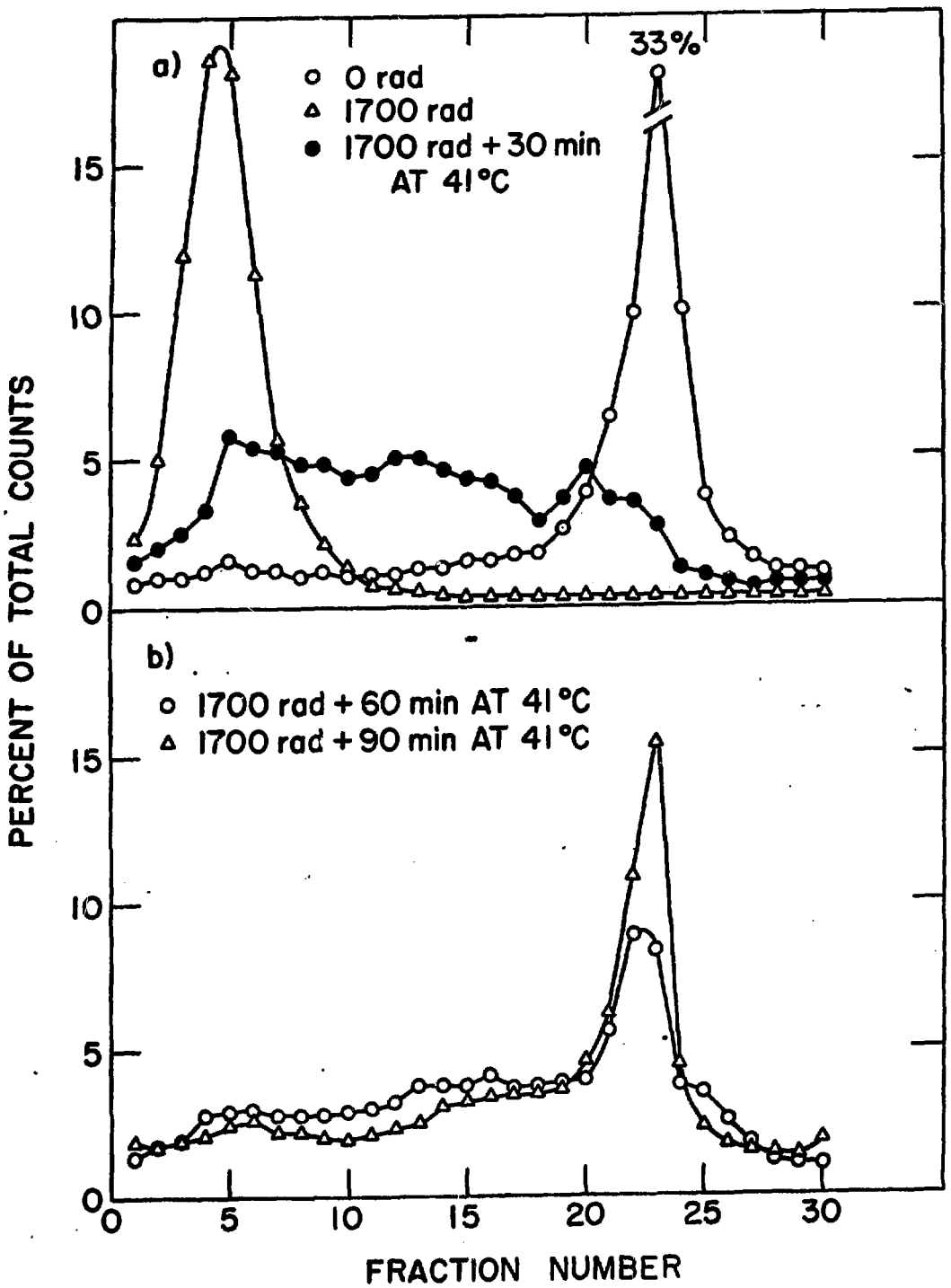

SEDIMENTATION
T2-183
NO REPAIR
P.Y.
○ 87%
● 96%
2890 rads (H3)
2.5 μg/ml ACT. D (60m)
+2890 rads (C14)
20m REPAIR
P.Y.
○ 96%
● 88%
90m REPAIR
P.Y.
○ 104%
● 85%
PERCENT OF TOTAL COUNTS
FRACTION NUMBER
0
5
10
15
20
25
30

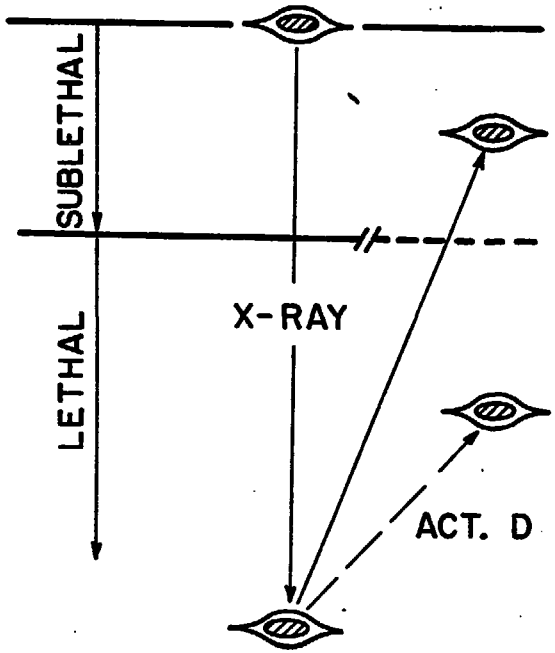

SEDIMENTATION
T-176
60 min lysis
Act. D
µg/ml P.Y.
1.0 88%
2.5 79
6.25 86
PERCENT OF TOTAL COUNTS
FRACTION NUMBER
0
5
10
15
20
25
30
35
22

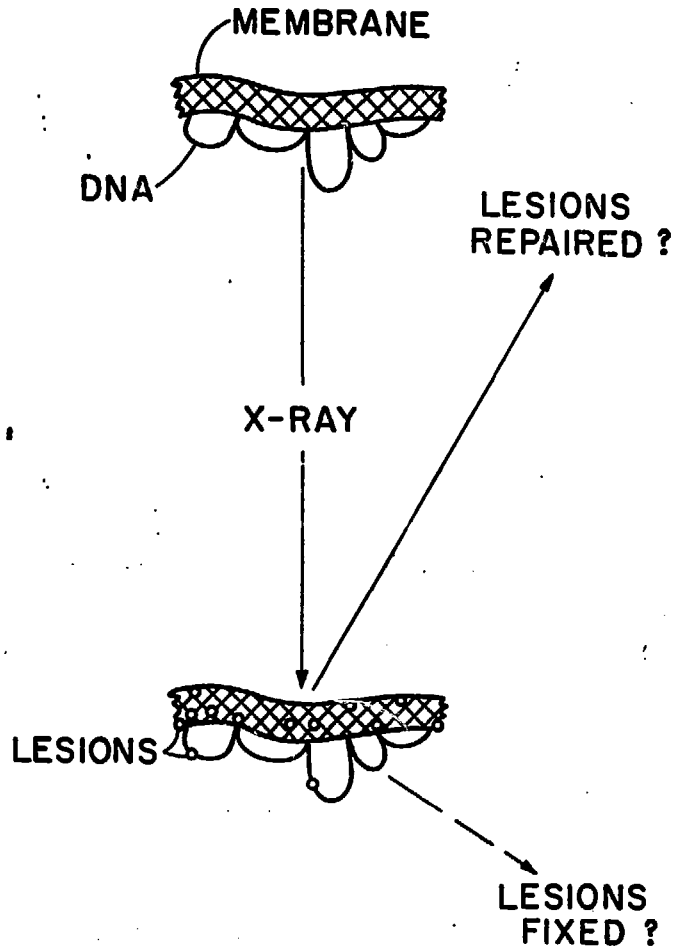

SURVIVING FRACTION
DOSE (RADS)
37°C
0°C
41°C
41.5°C
T°C Do Dq
0 135 315
37 135 194
41 124 0
41.5 118 0


SEDIMENTATION
0 rad
(P.Y.=78%)
388 rad
(P.Y.=89%)
97 rad
(P.Y.=92%)
776 rad
(P.Y.=101%)
194 rad
(P.Y.=92%)
1550 rad
(P.Y.=96%)
PER CENT OF TOTAL COUNTS
FRACTION NUMBER


1700 rads FOLLOWED BY
INCUBATION AT 37°C
(a)
0 min
30 min
60 min
(b)
90 min
360 min
PERCENT OF TOTAL COUNTS
FRACTION NUMBER
0
5
10
15
20
25
30


a)
○ 0 rad
△ 1700 rad
● 1700 rad + 30 min AT 41°C
33%
b)
○ 1700 rad + 60 min AT 41°C
△ 1700 rad + 90 min AT 41°C
PERCENT OF TOTAL COUNTS
FRACTION NUMBER
0
5
10
15
20
25
30


SUBLETHAL
LETHAL
X-RAY
ACT. D


MEMBRANE
DNA
LESIONS REPAIRED ?
X-RAY
LESIONS
LESIONS FIXED ?