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Reoxygenation and Its Potential Role in Radiotherapy[1]

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One interesting development, in current day radiotherapy, is the utilization of high LET radiation in the form of fast neutrons for the treatment of deep-seated tumors (e.g., ref. 1). The principle radiobiological factor arguing in favor of neutron therapy is the reduced oxygen enhancement ratio observed, mainly with single-cell systems, when cell killing with high LET radiation is compared to that with low LET radiation. The evidence seems now generally accepted that significant fractions of at least most solid tumors are hypoxic but still viable (2) and in accord with this, treatment of experimental tumors under conditions which should reduce the proportion of hypoxic cells (e.g., hyperbaric oxygen) usually leads to reduced doses for tumor sterilization (3). In concept, therefore, the low OER associated with high LET radiation could serve to improve the therapeutic ratio by enhancing the degree of damage in tumors compared to normal tissues since the latter are thought to be well oxygenated.

In recent years, several groups of investigators have reported on the state of oxygenation of solid tumors during the course of fractionated irradiation. With some exceptions (4,5), the authors of most of these reports conclude that significant if not complete reoxygenation sets in fairly soon after a moderate dose fraction (6-9). Further, it is reported that many solid tumors have anoxic cell fractions which are initially high, i.e., from 10 to 20% (3,10-13). These estimates come from radiobiological observations and are, therefore, indirect. Nonetheless, if in the natural course of events, appreciable amounts of reoxygenation occur between fractions two implications of considerable importance follow. The first is that reoxygenation should counteract the dose sparing effect that repair of sublethal damage between fractions introduces (14) and thus tend to justify--on purely cell killing grounds--the use of fractionation even when low LET radiations like conventional x rays are used. And, accordingly, the second implication is that the justification for high LET or neutron therapy would be considerably weakened.

In view of these considerations--and the fact that in several instances currently available data in the medical literature do not suggest an appreciable effect due to reoxygenation (14)--it is worthwhile to analyze the experiments dealing with reoxygenation starting from first principles. The objectives of the analysis will be: 1) to delineate the cell biology; 2) to specify criteria which have to be met and, therefore, measurements which should be made in order that reoxygenation data may be interpreted with confidence; 3) to compare animal data on reoxygenation due to fractionation with results on the fractionation treatment of human tumors to see if the latter are consistent with predictions of the former; and 4) to specify the information needed to be able to assess the relevance of radiobiological principles to tumor therapy. Lastly, based upon this analysis, I will propose some clinical trials aimed at determining whether the efficacy of fractionation therapy can be improved by enhancing the reoxygenation in opposition to sublethal damage repair.

Estimating the Hypoxic Fraction

Radiobiologically, the procedure used for estimating the proportion of cells in a tumor which is hypoxic may be described with the aid of Text Figure 1. In this, and the figures to follow, to facilitate tha analysis reasonable values for the parameters involved are assumed. Specifically, in Text Figure 1 these are: aerobic survival parameters $\tilde{n} = 4$, $\tilde{D}_o = 75$ rads; an oxygen enhancement ratio of 3; strict dose modification is assumed for the influence of oxygen so that the hypoxic survival parameters are $\tilde{n} = 4$, $\tilde{D}_o = 225$ rads; and finally a 10% proportion of hypoxic cells.

The survival curve for a population mixed insofar as its oxygen status is concerned can be arrived at as follows. To the aerobic curve plotted from surviving fraction 0.9 is added the hypoxic curve plotted from surviving fraction 0.1. The result is the curve marked "air breathing" which, by way of its inflection, shows that it is composed from at least two curves. The terminal portion of this curve has an hypoxic-like $\tilde{D}_o$; the initial portion of the curve indicates that, at least, a moiety with a smaller $\tilde{D}_o$ is also involved.

Experimentally, the procedure often used for estimating the hypoxic fraction is illustrated by the "N_2 breathing" and "air breathing" curves and is based upon the assay techniques introduced by Hewitt and Wilson (13,15). In one or more donors, tumors are irradiated with graded doses. These animals are alive and breathing air at the time. After exposure, survival is assayed by determining the number of irradiated tumor cells required to give rise to a similar tumor at the site of implantation in an appropriate recipient. Ordinarily, an interpolative method is used to obtain the number required for 50% takes (i.e., the TD_{50}). The ratio of the number required when unirradiated tumors are used to that required after irradiation is taken as the surviving fraction after the particular dose involved. Certain technical questions of importance may come in here--such as the proportion of control cells assayable by this method, differential cell stresses imposed by the manipulations involved in the assay, possible immunological effects, and so on--which in a given instance may minimize the usefulness of the data because, for example, they may not be sufficiently representative of the properties of all the cells of the tumor. I will ignore such technical points and consider only the larger issues which concern essential radiobiological principles. Thus, the curve marked "air breathing" is a result typical of a tumor mixed in respect to state of oxygenation.

The curve in Text Figure 1 marked "N_2 breathing" represents the result when all the cells in the tumor are rendered hypoxic. One way of doing this is first to kill the donors which may be done by nitrogen asphyxiation. Hypoxia sets in as the oxygen locally available at the time of death is depleted. Frequently, the terminal portions of the air and N_2 curves appear parallel and from their relative displacement, the hypoxic fraction is estimated.

Text Figure 1. Schematic representation of the survival of tumor cells when irradiated in a donor under normal conditions of oxygenation, "air breathing," and in killed donors, "N_2 breathing." The latter condition is expected to render all cells hypoxic. The terminal portions of both curves are displaced by a factor of 10 which presumably reflects the fact that 10% of the cells are hypoxic under normal conditions. Survival parameters are: $\tilde{n}$, extrapolation number; $\tilde{D}_o$, the dose to reduce survival by 0.368 along the exponential portion of a curve; and OER = 3. The shoulder width, $D_q = \tilde{D}_o \ln \tilde{n}$.

In Text Figure 1, the <u>measured</u> displacement is indicated by the "air/N_2" ratio. By those who apply this method, the "air/N_2" ratio is assumed to be the <u>hypoxic fraction</u>, i.e., the <u>hypoxic/aerobic</u> ratio. I will distinguish the two by designating the latter by "hyp/aer." Briefly stated, the purpose of this analysis is to define the conditions under which the <u>measured</u> ratio (<u>air/N_2</u>) and the <u>actual</u> ratio (<u>hyp/aer</u>) are equal when the standard test outlined in Text Figure 1 is applied.

Estimating the Degree of Reoxygenation

The procedure for detecting and estimating the extent of reoxygenation is outlined in Text Figure 2. Here the compound "air breathing" curve from Text Figure 1 is also shown and starts from zero dose and surviving fraction 1.0. Immediately after a dose of say 400 rads, the survival curve of those cells which survive is exponential with a $\tilde{D}_o$ = 225 rads and at this time, no change in this curve is expected from rendering the cells hypoxic. At some later time, when tumors still in donor animals are given graded second doses, experimentally it is often found that the "air breathing" and "N_2 breathing" curves become once again displaced as shown in Text Figure 2, for example. The data defining the terminal portions of these curves frequently may be fitted by parallel lines and the air/N_2 ratio is taken once again to be equal to the hyp/aer ratio (10). Usually it is then concluded that in the interval between the conditioning dose and the graded second doses, those cells surviving the conditioning dose were reoxygenated presumably because oxygen that would have been consumed by the initially aerobic cells became available to diffuse to the initially hypoxic cells. For example, from the second dose curves shown in Text Figure 2, since air/N_2 = 0.1 it would be concluded that complete reoxygenation had occurred.

Text Figure 2. Schematic representation of the effect of tumor reoxygenation assayed at some time after a conditioning dose of 400 rads. The conditioning dose is large enough to kill off essentially all oxygenated cells. The displacement in the "air breathing" vs. "N_2 breathing" curves which develops in time is taken to represent reoxygenation of the hypoxic cells which survived the conditioning dose. (See Text Figure 1 for survival curve parameters.)

In passing, I note that as drawn the "N_2 breathing" curve has a shoulder which implies repair of sublethal damage in at least those cells which become oxygenated. This and other points which qualify the preceding interpretation are considered presently.

Conditions for air/N_2 = hyp/aer

With the foregoing background, I inquire now: What are the conditions under which curve displacements as in Text Figures 1 and 2 can be expected accurately to measure initial or reestablished hypoxic fractions? To develop answers to this question, I consider first the variations in survivability that cells experience as they traverse their growth cycles.

Schematically, in Text Figure 3 are shown age-response functions for three well-known, cultured cell lines. As a function of position in the growth cycle--here specified by the customary designations of the phases of the cycle relative to DNA synthesis (G_1, S, G_2, and M)--are shown the variations in survival observed following a fixed dose of low LET radiation like x rays. Broadly speaking, these three cell lines typify three different age-dependent survival characteristics. After a fixed dose, in the case of Chinese hamster cells survival varies mainly because of variation in extrapolation number $\tilde{n}$ or in shoulder width (16). For HeLa cells, survival fluctuates mainly because of final slope variations (17). And in the case of mouse L cells, $\tilde{n}$ and $\tilde{D}_o$ vary inversely through the cycle (18). While these patterns apply to cells cultured *in vitro*, they show nevertheless that first, survival varies through the cycle and second, that the basis for this variation might depend on the cell type and presumably therefore on the tumor type. One factor, however, which is important in cell survival and which current evidence suggest applies in a predictable way from cell-line to cell-line is the dose modifying property of hypoxia. In instances where careful measurements have been performed, survival curves for oxygenated cells are related to those of hypoxic cells by a constant dose factor which is consistent with a constant dose modifying OER through the cell cycle.

Text Figure 3. Age-response functions of three cell lines. In each case, the parameter(s) are indicated which are mainly responsible for the survival variation with age following a fixed dose. An n-type age-response function means that the terminal portions of the survival curves are parallel at different ages and this, in turn, that the shoulder width $\underline{D}_q$ varies through the cycle. A $\underline{\tilde{D}}_o$-type pattern means that $\underline{\tilde{D}}_o$ is the main variable through the cycle with $\underline{\tilde{n}}$ being fairly constant. In this case, $\underline{D}_q$ is proportional to $\underline{\tilde{D}}_o$ (see legend to Text Figure 1).

To analyze the relevance of age-dependent survival variations on the questions at hand, consider the schematics in Text Figure 4. Here, I show several situations relative to cell distributions and, for simplicity, I assume that the age-response properties that apply in the example to follow are similar to those of Chinese hamster cells. However, the conclusions which these examples will make clear are general and not cell-line specific.

Text Figure 4. Schematic drawings of age-density and age-response patterns, the latter based upon Chinese hamster cells. The survival scales for hypoxic vs. oxygenated cells are approximate. Cell number distributions for hypoxic cells only are shown and the number of the latter are assumed to be 10% of the total population. For simplicity, it is assumed that oxygenated cells are uniformly distributed through the cycle. Panel A: hypoxic cells uniformly distributed; panel B, hypoxic cells are mainly in S; and in panel C, hypoxic cells are mainly in G_1.

In Text Figure 4, each panel contains *schema of single-dose survival* variations S(i) and cell number variations u(i) as a function of age or position in the cycle designated by i. No proportionality is intended by the survival scales and the cell number distributions for hypoxic cells only are shown. With respect to the latter, it is assumed that the areas under the curves are the same in each panel and that this corresponds to an hypoxic fraction of 10%. The oxygenated cells are further assumed, for simplicity, to be uniformly distributed through the cycle. Three characteristic cases are shown in panels A, B, and C which I will discuss in that order.

Panel A in Text Figure 4 shows the situation we would expect if the hypoxic cells as well as the aerobic cells are uniformly distributed through the cycle. This is perhaps the simplest case as suggested by the fact that the shapes of the survival patterns for both states of oxygenation are the same. Aside from the fact that I am assuming in this instance a specific relation of hypoxic to oxic cells (i.e., 1 to 9), the sketches in panel A represent what the tissue culturist imagines when he renders his cells hypoxic for an experiment. If his aerobic population was uniformly spread through the cycle, after gassing with nitrogen he would expect his hypoxic cells to be similarly spread. The reason for this is simply that generally in vitro a state of hypoxia can be effected quickly or at least in a period of time, physiologically speaking, short compared to the cycle time of the cells. As a result, cells remain essentially in the same age pattern after gassing that they were in beforehand.

Relative to survival--and what we consider as our standard radiobiological assay of the hypoxic fraction (see Text Figure 1)--the age-density distribution in panel A, Text Figure 4 leads to the result shown in Text Figure 5. Since we have assumed the same relative distribution of hypoxic and aerobic cells, the two curves first, must be parallel in their exponential portions and second, their relative displacement must come about because of the relative sensitivity of the aerobic cells in the "air breathing" case. Thus, we have the situation where the measured ratio "air/N_2" is exactly equal to the actual ratio "hyp/aer" which in this is 0.1 as per my starting assumptions.

While panel A, Text Figure 4 is an example where our standard test gives us exactly the right answer, there are other situations where essentially the right answer would be obtained. First, I should point out that for the standard test to work, the essential requirement relative to population composition is that the distribution of cells through the cycle be the same for both the hypoxic and oxic fractions. When this is the case, the "N_2 breathing" condition creates for the whole population the same age-density distribution that the hypoxic fraction has and as a result, the terminal portions of the survival curves are parallel and displaced by the right amount (e.g., Text Figure 5). Note that it is not required that the age-density distributions be uniform through the cycle (as we assumed in Text Figure 5) but only that they be the same for the hypoxic and oxic fractions.

Text Figure 5. Survival curves resulting from a standard N_2-air breathing test corresponding to the situation in panel A, Text Figure 4.

When this more general criterion is recognized, modifications of it can be identified which permit our standard test to give essentially, if not precisely, the right answer. One of these also can be seen with reference to Text Figure 5 and consists of modifications of the hypoxic cell age distribution in which the percentage of cells in the resistant age interval (shown shaded in Text Figure 5 and panel A, Text Figure 4) remains fixed. Even if the remainder of cells which comprise the 10% hypoxic moiety are distributed differently than shown, the "air breathing" curve will be essentially unaffected and hence, it will remain that $\underline{air/N_2} \simeq hyp/aer \simeq 0.1$. It is also true, however, that the "air breathing" curve would be largely unaffected if the cells in the unshaded age intervals in panel A, Text Figure 4 were removed from the hypoxic moiety. In this circumstance while the ratio $\underline{air/N_2}$ remains about 0.1, it would be larger than the ratio <u>hyp/aer</u>.

Examples Where: air/N_2 ≠ hyp/aer

While, as I have just shown, the population requirements for the N_2-air breathing test to give a reasonably accurate answer are not absolutely rigid, they are nevertheless fairly unique. I illustrate this now with the aid of panels B and C, Text Figure 4 and Text Figures 6 and 7. Panel B is intended to illustrate the case, still involving 10% hypoxic cells, where all of the latter are essentially confined to the most resistant age interval which, in the case of Chinese hamster cells, happens to be the S phase. Now essentially the entire hypoxic cell distribution is shaded and the N_2 fixed dose survival variation is much more peaked than in panel A. As shown in Text Figure 6, the terminal portion of the "air breathing" curve is displaced upward from where it was in Text Figure 5. In a given case, the degree of the latter displacement would depend on the added proportion of resistant cells which, for illustrative purposes, in Text Figure 6 is shown to have a net effect of a factor of 2. Clearly, the effect of such a distribution of cells is to make air/N_2 > hyp/aer even though the latter ratio is still 0.1.

A second example of a lack of equivalence between the observed and actual ratios is sketched in panel C, Text Figure 4 and in Text Figure 7. Once again a 10% hypoxic moiety is assumed but here these cells are mainly confined to a sensitive age interval shown as G_1 in Text Figure 4. As a result, the "air breathing" curve has a terminal portion which is displaced downward from where it should be for our standard test to work and as a result air/N_2 < hyp/aer even though the latter is kept at 0.1.

Text Figure 6. Survival curves resulting from a standard N_2-air breathing test corresponding to the situation described in panel B, Text Figure 4. Here the hypoxic fraction is still 0.1 but the shift in the hypoxic moiety age distribution results in a ratio air/N_2 = 0.2.

Text Figure 7. Survival curves resulting from a standard N_2-air breathing test corresponding to the situation in panel C, Text Figure 4. In this case, the ratio hyp/aer is still 0.1 but the distribution of hypoxic cells leads to air/N_2 = 0.033.

Finally, for completeness, I show an example of failure of the N_2-air breathing technique which should be immediately evident from the survival curves themselves. To begin with, the inset in Text Figure 8 shows: a) an age-density distribution for hypoxic cells (still 10% of the population) confined largely to the S-G_2 region; b) a survival variation curve for oxic cells (presumed to be uniformly distributed in age) similar to that observed after a fairly large dose with mouse L cells (Text Figure 3); and c) a survival variation curve for irradiation under nitrogen weighted by the corresponding age-density distribution. My reason for setting up this example is to illustrate the effect of a $\underline{\tilde{D}}_o$ age variation in contrast to the $\underline{\tilde{n}}$ only variation more characteristic of Chinese hamster cells. The S-G_2 region of the age cycle of mouse L cells has a small $\underline{\tilde{D}}_o$ and large $\underline{\tilde{n}}$ value (18) and as a result, for the air breathing case, the terminal slope will display this minimum $\underline{\tilde{D}}_o$ because the hypoxic cells are in the S-G_2 region. In contrast, for N_2 breathing, the sensitive S-G_2 cells will have little affect on the overall survival curve and hence, the terminal slope will be smaller and $\underline{\tilde{D}}_o$ larger. The result is shown in Text Figure 8 and illustrates that the data would not yield a constant $\underline{air/N_2}$ ratio. This fact by itself indicates that essentially two different populations are effective and therefore that the standard test cannot be used with any reliance at all to estimate the hypoxic fraction. As we indicated in connection with Text Figure 6, there are departures from the requirement of identity of population compositions which may by chance yield $\underline{air/N_2}$ ratios equal to <u>hyp/aer</u> ratios but in general these departures will not work when sensitive moieties constitute most of the hypoxic fraction. Further, when slope changes are involved, inadequacy of the test should be readily evident.

Text Figure 8. An example where the standard N_2-air breathing test already indicates that the hypoxic fraction cannot be estimated because a slope change is involved. The inset, based upon the age-response pattern of mouse L cells, is explained further in the text.

Reoxygenation: Conditions for air/N_2 = hyp/aer

Analogous to Text Figure 1, Text Figure 2 shows the standard test used for a radiobiological determination of the hypoxic fraction at some time after one or more conditioning doses (4,5,10). The presumptions are: first, that at the termination of the conditioning dose sequence, essentially only hypoxic cells survive; second, that the reduced oxygen consumption of killed cells plus possibly shrinkage of the tumor due to cell lysis in time leads to reoxygenation of the surviving cells; and third, a standard N_2-air breathing test then measures the proportion of hypoxic cells which requires that air/N_2 = hyp/aer. I now show that as with the determinations of the hypoxic fractions initially present (Text Figure 1), the standard test for estimating reoxygenation may frequently yield an erroneous result.

Consider first Text Figure 9 where I show schematically age distribution patterns to illustrate a case where the standard test works. In panel A, an age-survival pattern is shown--the shape of which is independent of the state of oxygenation as I noted earlier--as well as the cell number distribution for the total population $\underline{u}_{tot}$ and for the hypoxic moiety $\underline{u}_{N_2}$. Both of the latter have been assumed to be uniform for simplicity. After an acute exposure denoted by "X", the age-density distribution of surviving cells becomes that shown in panel B. (The vertical scale in this panel and in panel C has been increased relative to that in panel A for clarity.) Becuase of the very considerable sensitivity conferred by oxygen, the distribution of surviving cells in panel B is made up almost entirely of hypoxic cells. And further, because of the synchronizing effect of acute exposures which are large enough to surmount, more or less, the shoulder of a survival curve of asynchronous cells, almost all the survivors are in the resistant age interval in the cycle (S for Chinese hamster cells).

Panel C shows the effect of reoxygenation by the fact that now the hypoxic cell age-density distribution is appreciably lower than that for the entire population. However, to illustrate first a case where the standard test works, I have assumed that reoxygenation resulted in only one change; namely, a drop in the proportion of hypoxic cells which are in the resistant age interval.

The sequence shown in Text Figure 9 is illustrated in terms of the pertinent survival curves in Text Figure 10. I have assumed that a conditioning dose of 400 rads produces the population shift from panel A to B, Text Figure 9 insofar as viable cells are concerned. The effect on survival corresponding to the reoxygenation is indicated in the "air breathing" curve in Text Figure 10. This curve is parallel in its exponential part to the corresponding portion of the single-dose curve as it should be since the terminal regions of both curves result from cells of the same age interval and responsiveness (Text Figure 9). The "air breathing" curve is drawn to yield a ratio $\underline{air/N_2} = 0.33$ on the assumption that in the interval between the conditioning dose and the standard test, reoxygenation resulted in two-thirds of the cells becoming oxic. Thus, the lower two curves in Text Figure 10 illustrate the conditions for an accurate radiobiological estimate of the hypoxic fraction.

Text Figure 9. Schematic drawings of age-density and age-response patterns to illustrate the application of the N_2-air breathing test to reoxygenation estimates. In panel A, $\underline{S}$ stands for survival after a fixed dose and the shape of this curve is assumed to be oxygen independent. The age-density distributions are $\underline{u}_{tot}$ for the whole population and $\underline{u}_{N_2}$ for the hypoxic cells which are assumed initially to comprise 10% of the cells. Relative to surviving cells, panel B shows the result of an acute x-ray dose. The ordinate is enlarged arbitrarily for clarity (also in panel C). In panel C, the effect of reoxygenation is represented by a reduction in the shaded area compared to panel B.

Text Figure 10. Application of the N_2-air breathing test to the situation in panel C, Text Figure 9. The change in the ratio air/N_2, depending on repair of sublethal damage, is also shown.

To the foregoing conclusion, unfortunately further qualifications are required. If in time after the 400 rad conditioning dose, those cells surviving did not repair sublethal damage (see Chap. 6, ref. 19) and did not become oxygenated (ignoring for the moment division as well), their survival curve would remain the exponential curve through the second origin in Text Figure 10. Although there is evidence that mammalian cells can repair sublethal damage even when only traces of oxygen are available (14,20) it is conceivable that in a tumor this might not be true or at least the rate would be reduced. If the survivors after 400 rads became oxic with no repair the "air breathing" curve would be the lowermost curve as shown even if those cells which became oxygenated then fully repaired sublethal damage. The reason is that sublethal damage broadens the shoulder on a survival curve whereas oxygen reduces the $\tilde{D}_o$. As the dose is increased, the latter change dominates and hence, the terminal portion of the survival curve remains unaffected. But while oxygen-dependent sublethal damage repair does not affect the terminal slope of the lowermost curve, it would affect the uppermost curve since now all the surviving cells have the same final slope. As shown in Text Figure 10, under these conditions although it might still be true that hyp/aer = 0.33, the observed air/N_2 ratio would be smaller; perhaps, as small as 0.1 if sublethal damage repair shifts the extrapolation number from 1 to 4.5, for example. The net effect would be that the reoxygenation of 67% of the surviving cells after 400 rads would appear to be reoxygenation of 90% of the cells.

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ADDENDA

1. Collins, V.P. The effect of total body irradiation on immunologic tolerance of bone marrow and homografts of other living tissue. Technical Progress Report DASA 1286, Defense Atomic Support Agency, Feb. 1, 1961-Jan. 31, 1962.

2. Collins, V.P. The effect of total body irradiation on immunologic tolerance of bone marrow and homografts of other living tissue. Technical Progress Report (DASA), Defense Atomic Support Agency, Feb. 1, 1962-Jan. 31, 1963.

A final qualification of the situation in Text Figure 10 is required. If sublethal damage repair is independent of oxygen concentration, then it would be possible that the uppermost and lowermost curves could result from the standard test, and therefore that air/N_2 = hyp/aer = 0.1, when other conditions remain as assumed. This is because now once again the air and nitrogen breathing populations are the same in all respects. However, in addition to a knowledge of the composition of the surviving population (panel C, Text Figure 9), now it is also required to know whether sublethal damage repair is independent of oxygen tension.

Reoxygenation: Conditions where air/N_2 ≠ hyp/aer

The age-density distributions in panel C, Text Figure 9 are somewhat artificial since they imply tacitly that no aging of cells occurs during reoxygenation. Except for the fairly unlikely situation that as a result of aging and division the situation sketched in panel C is reestablished, an age-density distribution somewhat more like the inset in Text Figure 11 might be expected. Here, I suggest that while those survivors after 400 rads which remain hypoxic remain in the resistant age interval, those which become oxygenated proceed to age and perhaps divide so that more sensitive age intervals also become populated with viable cells (e.g., G_1, G_2, and M in the case of Chinese hamster cells). With respect to the air breathing data, no significant change in the survival curve occurs and hence, in Text Figures 10 and 11 the lowermost curves are the same. But now since the nitrogen breathing case increases the $\tilde{D}_o$ of cells which are in sensitive instead of resistant age intervals, the upward shift accompanying complete hypoxia would not be as large as in Text Figure 10. Accordingly, the observed ratio air/N_2 might be less than the actual hypoxic fraction.

Text Figure 11. An extension of the case described in Text Figure 10. Here it is assumed that although the fraction of hypoxic cells is the same, the aerobic cells have moved to more sensitive age intervals and as a result they make only a minor contribution to an upward shift in the survival curve for the N_2-breathing case.

A final example of an incorrect conclusion based upon a standard N_2-air breathing test is summarized in Text Figures 12 and 13. Here I start with a condition relative to the hypoxic fraction which I would expect to be more likely than those schematized in Text Figures 4 and 9. When tumor cells outgrow there blood supply, one evidence of this is their inability to be labeled with ^{3}H-thymidine (21). While it is possible that this results from some form of coordinated control of metabolism which may or may not be causally related to hypoxia, it is also quite possible that a lack of DNA synthesis reflects the accumulation of cells in phases other than S. For example, it seems possible that cells might accumulate in G_1 as shown in panel A, Text Figure 12 and even though this is a sensitive phase in the age-response pattern chosen, the sensitivity conferred by oxygen on the rest of the cells could easily leave behind most of the G_1 cells which were hypoxic at the time of the conditioning dose. Diagrammatically, this is shown by the transition from panel A to B with the height of the surviving moiety increased in the latter for clarity. In time, some reoxygenation of these G_1 survivors may occur and concomitantly a portion of these cells might progress into S. This leads to a small reduction of the proportion of cells in G_1 and still hypoxic; however, this does not have much of an effect, as indicated by the "air breathing" curve in Text Figure 13, even though S is a resistant phase. Once again, this is because of the sensitizing effect of oxygen. But when the population is rendered hypoxic, an upward shift in survival could result because now the relative resistance of S cells is expressed. Therefore,

as indicated in Text Figure 13 a small degree of reoxygenation could lead to an air/N_2 ratio considerably larger than the actual hypoxic fraction; in Text Figure 13, we show air/N_2 = 0.1 even though perhaps, only one-third of the cells might have been reoxygenated.

This case illustrates that even if questions relative to a possible oxygen dependence of sublethal damage repair are not implicated, an oxygen-dependent progression into a resistant age interval could lead to an appreciable overestimation of the degree of reoxygenation.

Text Figure 12. Age-density and age-response patterns similar to those in Text Figure 9 except that it is assumed that initially the hypoxic cells are mainly in G_1. The ordinates in panels B and C are increased for clarity. Panel C shows that a small fraction of the surviving cells which were essentially all hypoxic have become oxic and have concomitantly progressed into a resistant age interval.

Text Figure 13. Application of the standard N_2-air breathing test to the situation in panel C, Text Figure 12. Because those cells which have become oxic are also more resistant an overestimate of the hypoxic fraction is obtained.

Requirements for Accurate Hypoxic Fraction Assessments

The preceding examples can be of help in analyzing what in general are the conditions for an accurate assessment of the oxygen status of a tumor either before or during a fractionation series. As will be clear from the following, unfortunately these conditions are not easy to satisfy.

* * * * *

Condition 1--The magnitude of the OER for the particular cells in question must be known as well as whether it is independent of age or position in the growth cycle as current *in vitro* data suggest. The need to know about any age-dependent effects follows from the fact that different population mixtures may be involved. The magnitude of the OER is needed to insure that for the doses (or dose sequences) used, the influence of oxic cells is negligible. When doses of the order of 400 rads or less are used, so that survivals might be in the shoulder region of a curve, it might be necessary to know also the shoulder widths since these will bear on the degree to which oxic cells influence the air breathing data.

Condition 2--The age-response pattern(s) must be known. In the examples presented, I assumed that OER was independent of state of oxygenation. This implies that the shape of the age-response pattern is independent of oxygen. But can we be sure that when cells "grow into hypoxia" as a tumor grows the age-response pattern they assume is related to that of oxygenated cells as would probably be the case when oxic cells are rapidly rendered hypoxic (e.g., as in cell culture experiments)? We need to know therefore whether in general, or at least relative to specific tumors, age-response is a function of degree of hypoxia and therefore, perhaps, the age and/or size of the tumor. The cell biology needed to answer this question is as yet not available.

Condition 3--A requirement related to Condition 2 is whether hypoxic cells grow. If they do, but more slowly, to what extent do their growth phases maintain the same relative lengths that they have when cells are well oxygenated. If they do not growth when hypoxic (or grow quite slowly) are they confined to a particular age interval; for example, are they in G_o? These questions amount to the requirement that we need to know the age-density distribution of particularly hypoxic cells at the time when the N_2-air breathing test is applied.

Condition 4--We need to know whether or not sublethal damage repair is cell age dependent and at an age where oxic cells do repair damage; we need to know if repair depends on the degree of hypoxia or at least if its rate is influenced by hypoxia. Concerning repair under well oxygenated conditions, current evidence indicates that as long as cells have a capacity for sublethal damage, this damage can be repaired although it is conceivable that in some situations, it may be hard to distinguish sublethal damage repair from the assumption of resistance concomitant with progression. But relative to repair vs. oxygen tension, some believe that at least the rate is reduced and as illustrated in Text Figures 9 and 10, differential repair could be responsible for an erroneous estimate of degree of reoxygenation.

* * * * *

In summary, I can state that the conditions which must be met enabling an accurate assessment of the hypoxic fraction lead to the following criteria: a) in general, the age-density distribution of hypoxic and aerobic cells must be the same; b) in general, the OER must be independent of age; c) in the case of estimates of reoxygenation, the conditioning dose(s) must be large enough to render quite small the proportion of initially oxygenated cells still surviving; and d) also relative to reoxygenation, the degree of sublethal damage repair must be the same for the hypoxic and oxic fractions at the time of the N_2-air breathing test. While these criteria are needed for accurate estimates of the ratio hyp/aer, I note that N_2-air breathing survival curves displaced from each other as in Text Figures 1 and 2 for example, indicate at least that the population is mixed with respect to oxygen status and this by itself is biologically important.

Human Tumor Treatment and Reoxygenation

To appreciate the consequences of reoxygenation, particularly complete reoxygenation (10), relative to tumors, I proceed now to outline the consequences. By complete reoxygenation, ordinarily one means that if, for example, a tumor initially consists of 10% hypoxic viable cells (as in Text Figure 1), between dose fractions this percentage is reestablished insofar as concerns those cells which survived the prior irradiation. In Text Figure 14, I show a series of survival curves (from ref. 14) which compares the effect of reoxygenation with that of no reoxygenation while for both situations, full repair of sublethal damage is assumed. In this figure, the ratio aer/hyp is given (instead of the reciprocal as in earlier figures) and by the symbolism aer/hyp = 9 → 0, I mean that no reoxygenation between doses occurs while aer/hyp = 9,9,...9 stands for complete reoxygenation between fractions.

If after successive fraction of size D, no reoxygenation occurs, Text Figure 14 shows that the successive survival curves lie above the single dose curve (the think, continuous line). Thus, repair without reoxygenation requires a greater total dose to reach a given level of survival than would be required by a single dose. On the other hand, the dashed curves below the single dose curve show the effect of complete reoxygenation with repair. Now, after each fraction, the initial curve is replotted from each new origin so that the net effect is that survival drops off more rapidly. Therefore, a given survival level requires a smaller total dose than would be required when a single exposure is used even though I have assumed complete repair of sublethal damage between fractions.

Text Figure 14. Schematic drawing of the effect of repair of sub-lethal damage: (1) when reoxygenation is negligible, curves lying above the thick curve (aer/hyp = 9 → 0); and (2) when reoxygenation returns the mixing ratio to its initial value between each fraction, curves lying below the thick curve (aer/hyp = 9,9,...9). For simplicity, the single fraction dose D was assumed large enough to reach the survival region controlled initially by hypoxic cells. In contrast to earlier figures, here the ratio aer/hyp is used instead of hyp/aer.

Since results with human tumors usually become available as isotreatment plots (14), the predictions of the trends in Text Figure 14 are shown as isosurvival plots in Text Figure 15. The nomenclature here is similar to that in Text Figure 14 and the survival parameters are the same as those in Text Figure 1. As has already been explained in detail (14), the thin sets of lines show the predictions for 100% aerobic cells (lower pair) and 90% aerobic cells with no reoxygenation (upper pair starting from ~4900 rads). No repair (plus no division) between fractions leads to horizontal plots while complete repair for both sets of conditions leads to ascending lines as long as reoxygenation is at most only minorly involved. The thick line which refers to full reoxygenation shows that as the number of fractions is increased, the curve drops to a minimum and then rises. If cells do not repair, this curve drops more rapidly but always lies above the ascending aerobic curve (14).

Text Figure 15. Isosurvival curve for 1×10^{-10} surviving fraction, an initial and repeating mixing ratio of 9, full repair of sublethal damage, and the survival curve parameters used in Text Figure 1. In contrast to earlier figures, here the ratio aer/hyp is used instead of hyp/aer.

Thus, Text Figure 15 shows diagrammatically the opposite trends relative to isosurvival when the extreme situations just outlined are considered. These opposite trends may be characterized as follows. When no reoxygenation occurs, an ascending isosurvival plot is predicted if there is repair of sublethal damage. To a first approximation, the initial steepness of this curve depends upon the OER and the shoulder width of the single-cell survival curve. In contrast, when the amount of repair is moderate and/or the degree of reoxygenation is relatively large, the character of the isosurvival plot changes in that a minimum is evident. Clearly, a family of curves between these extremes is to be expected from various combinations of repair of sublethal damage and reoxygenation but when reoxygenation without repair is involved, a minimum is always predicted.

Several sets of data relative to human tumors show ascending iso-effect trends (14). These include human skin cancers (22-24), carcinoma of the cervix (25), recurrent breast nodules (26), and breast cancer (Primaries, recurrences, and metastases, 27); plots of all of these data are contained in reference 14. In each instance, the importance of sublethal damage seems clear--because the curves are clearly ascending--but in each instance some contribution from reoxygenation cannot be ruled out. Although the data are not extensive enough to establish clear trends, the results of Scott and Brizel (28) in Text Figure 16 for Hodgkin's disease suggest that the initial portion of the isosurvival plot does not go up and may be flat. This could represent a range of numbers of treatments in which reoxygenation is just balanced by repair of sublethal damage before the latter effect, accompanied perhaps by some amount of repopulation between fractions, causes the total dose to increase. It is also possible that the capacity for sublethal damage of these cells, and therefore their ability to repair sublethal damage, is small (29).

The examples from the clinical literature (22-28) illustrate that in general reoxygenation made evident by an early minimum in the isotreatment curve is not found, although Howes and Field (30) believe that a small dip at two fractions is evident in the data on recurrent breast nodules cited above (26). But a lack of a minimum may be likely for these reasons. In the region of say 1 to 10 fractions, each fraction is fairly large. As a consequence, cells (survivors and nonsurvivors) can be expected to age slowly, because they are retarded by the large doses, although general metabolism and attendant oxygen consumption ca be expected to continue. Tumor shrinkage accompanied by the sparing of oxygen due to the breakdown of killed cells may take days to set in and as a result the effects of reoxygenation may be small when the numbers of fractions are relatively few. (I note that frequently after the onset of the treatment of solid tumors, tumor size increases for some number of treatments before it starts to decrease; e.g., see ref. 31). It seems likely to me, therefore, that at the beginning of an isotreatment curve where in terms of the shape of the curve the effect of reoxygenation would be expected to be most evident, the time needed for reoxygenation to have a noticeable effect is inadequate when daily fractions are used. This could be true, I feel, even though N_2-air breathing tests indicate (presumably erroneously) that full reoxygenation can set in within 24 hours.

At the opposite extreme, where the dose size is small and many fractions are used first, cells are delayed less after each fraction so that they may start to express their damage by attempting the replication process sooner. In addition, there is more time for cell breakdown and the effects of oxygen sparing to set in and further, the magnitude of sublethal damage repair may be expected to be less because each dose is not as effective in surmounting the shoulder of the single-cell survival curve as is the case in the high dose region. The net result is a much slower rate of increase of the total dose with number of fractions because in effect, the initial state of oxygenation of the surviving cells is maintained and further, they are treated with small dose fractions.

Thus, for many types of tumors, if the treatments are close to daily, in general I would not expect reoxygenation to be effective when the number of fractions is small (large doses) even though it is in this region where the effect of reoxygenation might be most noticeable. The reason is simply that with a slow growing tumor the growth of which is delayed further by the radiation, enough time is not available for oxygen sparing to set in. And when the number of fractions is large, reoxygenation while important, is not easy to detect in an isotreatment plot because its main effect is to contribute to a lessening of the rate of rise of the curve.

Two qualifications to the foregoing are in order. If, because of particular cell and tumor properties, tumor shrinkage sets in rapidly even after large doses and/or the magnitude of repair of sublethal damage is small, a flat region or a minimum might be observed in an isotreatment plot (e.g., Text Figure 16). On the other hand, if tumor shrinkage and the expected oxygen sparing does not happen soon enough in time when daily fractions are used, it is conceivable that the isoeffect curve might not flatten off as rapidly as otherwise expected even when a large number of fractions is used. The effect in practive of the latter situation could be to fail to achieve cures because the differential advantage that reoxygenation might afford in counteracting sublethal damage repair and/or repopulation does not set in soon enough.

Text Figure 16. A linear plot of the data of Scott and Brizel (28) relative to Hodgkin's disease. Treatments were 5 days per week.

Data Needed to Assess Tumor Fractionation

Recognizing that in tumor therapy a principal objective is to achieve a maximal differential response between malignant and normal tissue, it remains nevertheless important to assess to what extent the radiobiology of cells is applicable. Therefore, I undertake to briefly describe first, the main cell properties of a tumor that should be measured and second, the main tumor properties which should be determined for a reasonably definitive assessment. Since the data I will call for are hard to get, I will also indicate some limited observations which might be obtainable in the clinic and which might still permit inferences of value. In doing this, I will not attempt an evaluation of feasibility. While many of the observations may not be possible, ways of measuring or inferring others may be worked out in time if at least we know what is required.

Cell Properties

1. Starting perhaps with biopsy specimens, it would be desirable to know if histology can be used to predict survival properties but in any event, for a given tumor we need to know: a) the aerobic survival curve; b) the OER; and c) whether either a) or b) strongly depend upon the region of the tumor sampled. Presumably, cell culture techniques would be used for these measurements since in principle, they ought to work. In the limit, we would want to know the age-response properties of the oxic and hypoxic regions of the tumor as well as the age-density distributions but hopefully, the need for data as extensive as this could be avoided.

2. Using cell culture methods and asynchronous cells, we would need to know: a) what is the fractionation response relative to repair of sublethal damage; b) whether this is significantly affected by hypoxia; and c) whether a) and b) depend on the region of the tumor sampled.

3. We would need to know the dose and oxygen dependence of population growth (for single and fractionated doses) and particularly when cell breakdown starts. It would be desirable to know if and to what extent oxygen consumption is maintained and the relation between changes in this and the onset of cell breakdown. If the cells in question can grow fairly actively under hypoxic conditions, we may find at most a weak dependence of sublethal damage repair on state of oxygenation.

Tumor Properties

We would want to know:

1. What proportion of the tumor mass consists of viable tumor cells and how they are distributed with respect to the availability of oxygen.

2. Are hypoxic, viable cells in cycle.

3. After one or more dose fractions what are the growth kinetics of the tumor and hos is this dependent upon the interval between fractions.

4. How and when does the distribution of oxygen in the tumor change during fractionation.

5. To what extent are host-tumor interactions involved like immunological reactions of the host against small residual fractions of viable cells and to what extent, if at all, is this mediated by tumor shrinkage.

Clinical Data

While animal tumor systems can, and in part are, being used to develop insights concerning the foregoing properties, it is desirable to gain information relative to given types of human tumors where possible. For this purpose, it seems justified to limit the scope of an experimental inquiry in order to effect a reasonable compromise with practicability. The question I put, therefore, is: To what extent does reoxygenation play a role in the treatment of tumors and how might this role be made more effective?

While the body of data available for analysis on the fractionation response of human tumors is limited (e.g., refs. 22-28), when plotted on coordinates essentially the same as in Text Figure 15, ascending curves are obtained. This means, to begin with, that repair of sublethal damage is effective since the universality of this process is established (e.g., ref. 19), and a reasonable explanation of such results on other grounds is not available. In some instances, perhaps, it is possible that essentially only oxic cells populated the tumors (e.g., the results of Friedman and Pearlman on recurrent breast nodules, 26), but in general it seems hard to believe that significant fractions of viable hypoxic cells were not involved. Thus, it appears that either all or at least an appreciable part of the cells which have hypoxic cell sensitivities do repair sublethal damage at least rapidly enough to lead to ascending isoeffect curves when daily treatments are involved.

As a guide to clinical studies, therefore, I propose the thesis that tumors can be treated differentially more effectively if contributions to cell killing from reoxygenation can be increased simply by affording more time between fractions for cell lysis and tumor shrinkage to set in. To illustrate this, consider two extreme situations aimed at testing this thesis.

* * * * *

Case I. A tumor type is selected known to shrink relatively rapidly after modest doses delivered, for example, daily. Since, radiobiological reasoning indicates that the effect of reoxygenation might be most effective when about 10 or fewer treatments are involved (Text Figure 15), the experiment could be started by selecting say 6 (e.g., Monday through Saturday) and a total dose in agreement with current practive. A comparison would then be made between daily treatment, treatment every second day, every third day, and so on. At the same time, a careful record should be kept of tumor size in order to correlate tumor growth and shrinkage with the presence or absence of enhanced effectiveness and a larger number of cures with increasing fractionation interval. The prediction from my thesis is that the net benefit would be small from increasing the interval between fractions in this case.

Case II. A tumor type is selected which is radioresistant in that its growth rate is usually observed to be only slowly altered after the onset of daily treatment. Once again, 10 or fewer fractions are chosen and using an accepted total dose, controlled series of treatments are initiated with the principal variable being the interval between doses.

To facilitate the choice of treatment times, some preliminary observations of tumor growth after irradiation should be made (if not already available) in order that intervals can be selected for the study which straddles the time for the onset of tumor shrinkage. Further, if possible, biopsy specimens as a function of time after irradiation should be sought in order to confirm the onset of cell degeneration and to seek evidence for reoxygenation. For the latter purposes, the scoring of pycnotic cells in relation to capillaries could be used as an indication of cell degeneration. Observations of the spatial distribution of cells that can incorporate ^{3}H-thymidine may yield information of the extent to which cells initially out of cycle (presumably hypoxic) are brought back into cycle as oxygen becomes available.

In the foregoing protocols, constant treatment intervals are implied. In addition to varying the number of treatments and the total dose, consideration should be given to using an interval sequence which might be changed as treatment progresses. For example, if it takes about 5 days for the onset of tumor shrinkage after a given dose, an initial interval of about this time would be reasonable to use but thereafter depending on the rate of shrinkage and/or the indications from biopsy measurements, the interval might be decreased.

* * * * *

Recapitulation

In the foregoing clinical trials, I have kept the proposals relatively simple in an effort to effect a reasonable compromise with practicality. To test the applicability of radiobiological principles and isosurvival theory, it is easy to propose approaches considerably more complex and for such purposes, more complete measurements of cell as well as tumor properties could be made along the lines I suggested. However, current evidence leads me to believe that two opposing radiobiological factors control to a large extent the response of many solid tumors. These are repair of sublethal damage and the oxygen effect. Having as they do essentially opposing effects relative to tumor sterilization, what I have proposed in essence is a simple way, at a clinical level, of increasing the influence of the sensitizing effect of oxygen in order to shift the balance in the direction of a smaller total dose for a given effect. The procedures I have outlined depend upon host mediated reoxygenation although, as I have pointed out, the evidence for this from animal tumor systems is good in a qualitative sense but might be quite poor quantitatively. Once tumor shrinkage has set in, hyperbaric oxygen could also add an additional benefit.

An underlying assumption in pitting the oxygen effect in opposition to sublethal damage repair is that normal tissue is well oxygenated and that any success in increasing tumor cell killin with the same or a smaller total dose than otherwise used would constitute an improved differential effect. Certainly this would be expected to apply to normal cell renewal systems, which are also known to repair sublethal damage, but it could also apply to essential normal tissues whose function does not involve cell division and which may not be able to repair sublethal effects. It is also noteworthy that a reduced OER for high LET radiation might still be worth taking advantage but its usefulness would decrease as methods for enhancing reoxygenation improve.

Finally, I should note that effecting a greater measure of reoxygenation between doses does not mean that the survival enhancing effects resulting from a greater degree of sublethal damage repair would work against the greater killing expected from reoxygenation. Even if it is true that cells suffering extreme conditions of hypoxia repair damage only after they become oxygenated, when large dose fractions are involved there would be little if any net survival increase. The reason is that oxygenation also means a decrease in $\tilde{D}_o$ by 2 to 3 times and as a result, the effect of repair relative to shoulder width would very likely be overshadowed by the sensitization due to oxygen.

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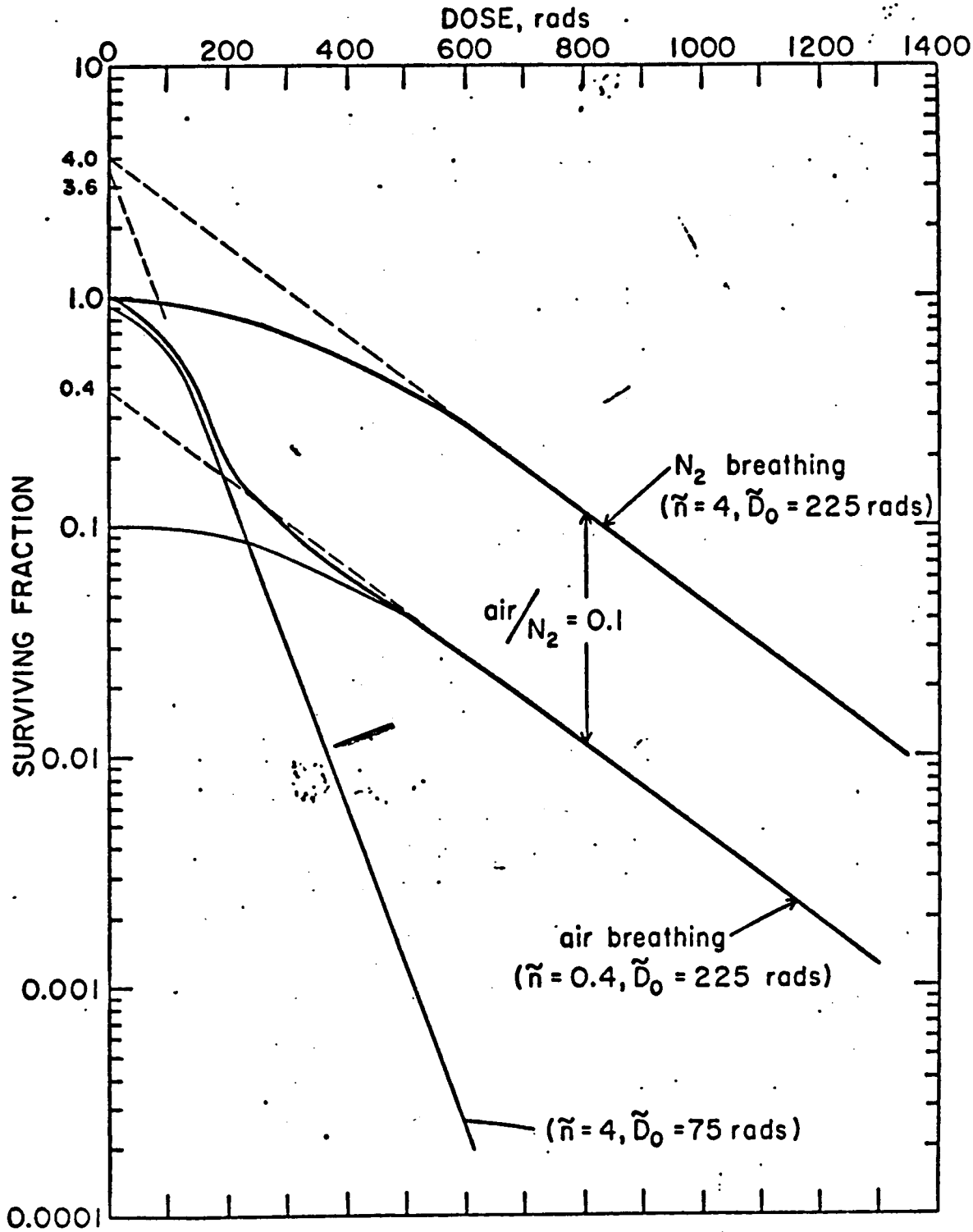

FIGURE 1

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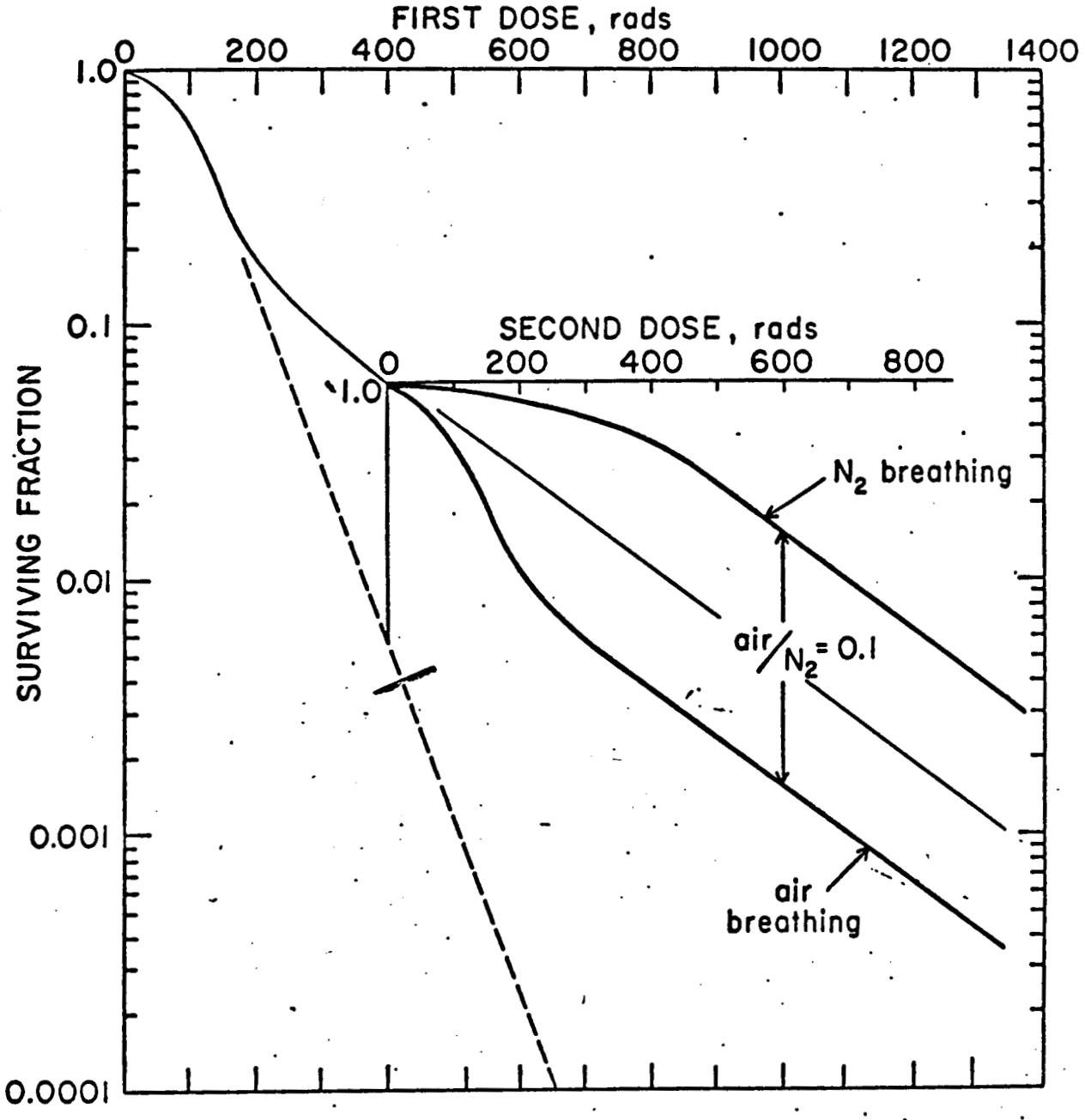

FIGURE 2

Neg. 9-212-69

AGE RESPONSE FUNCTIONS

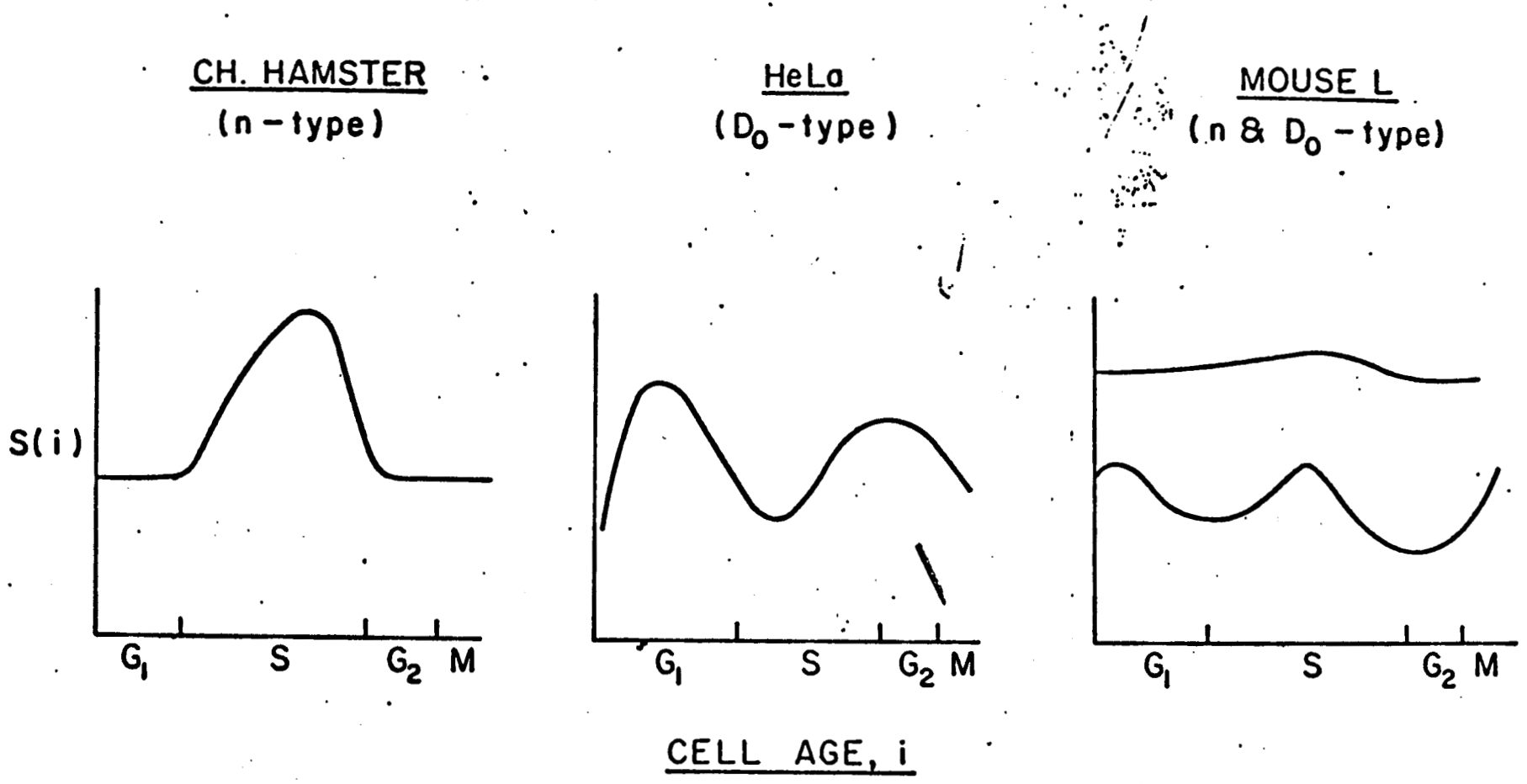

FIGURE 3

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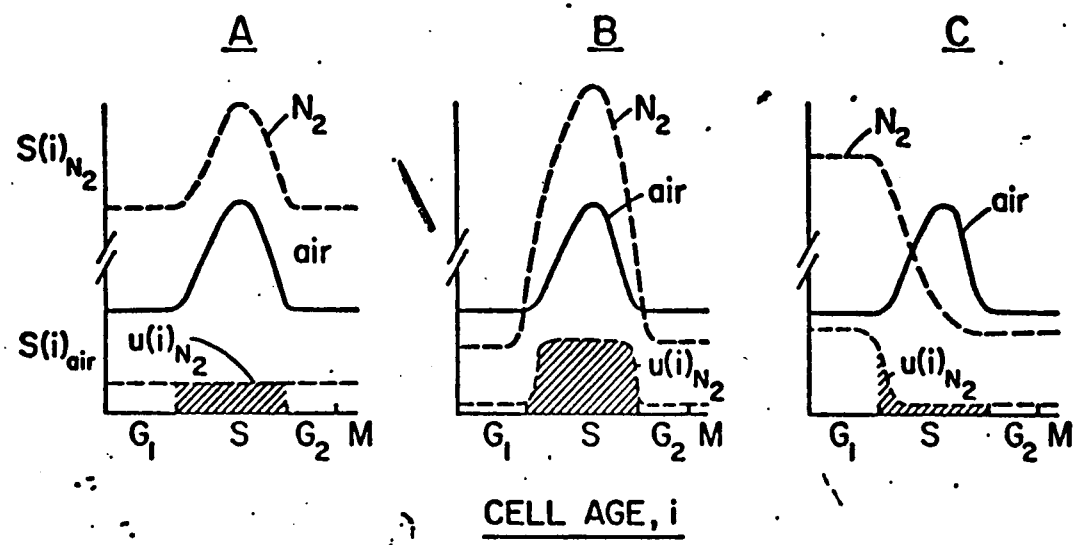

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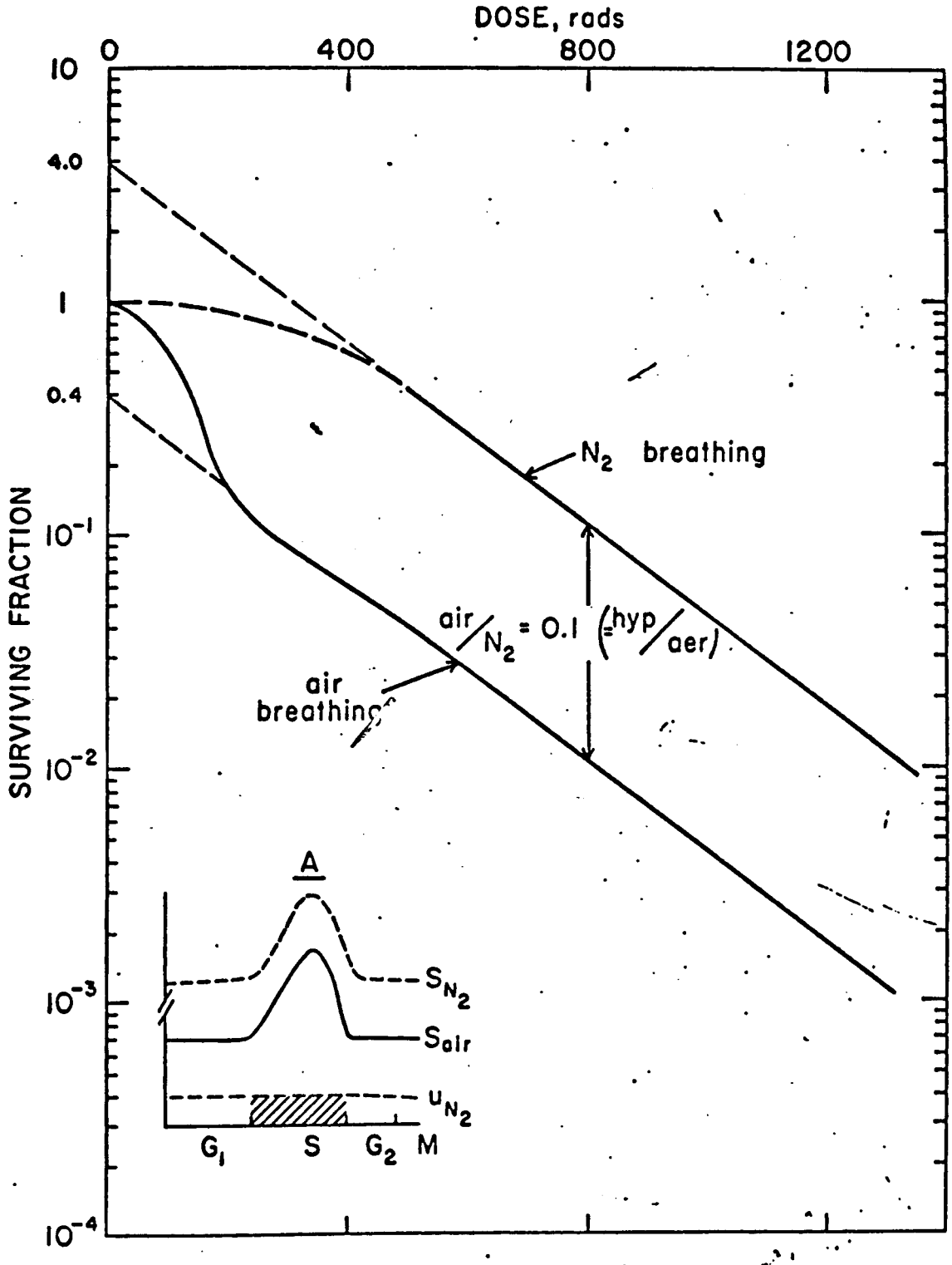

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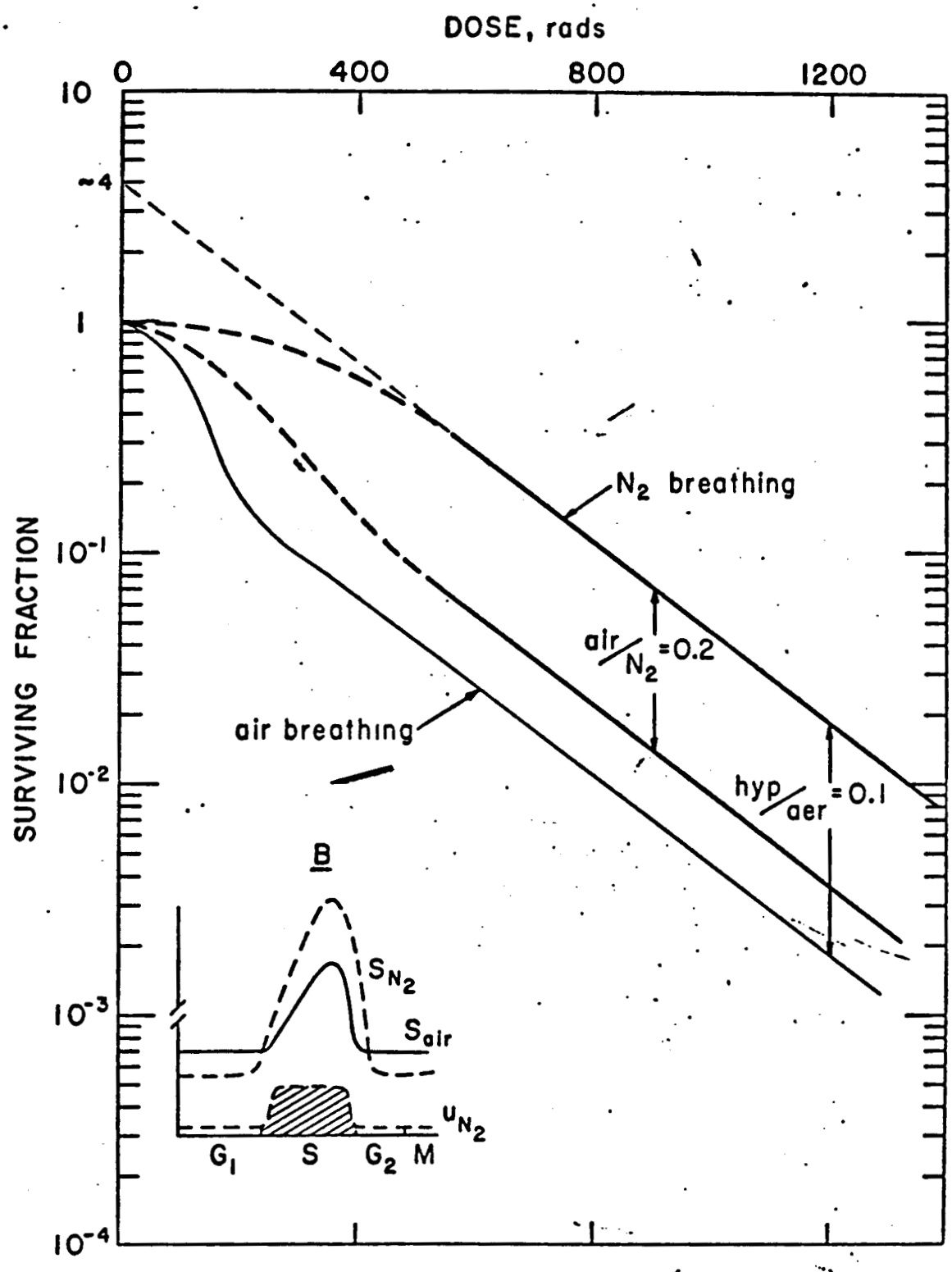

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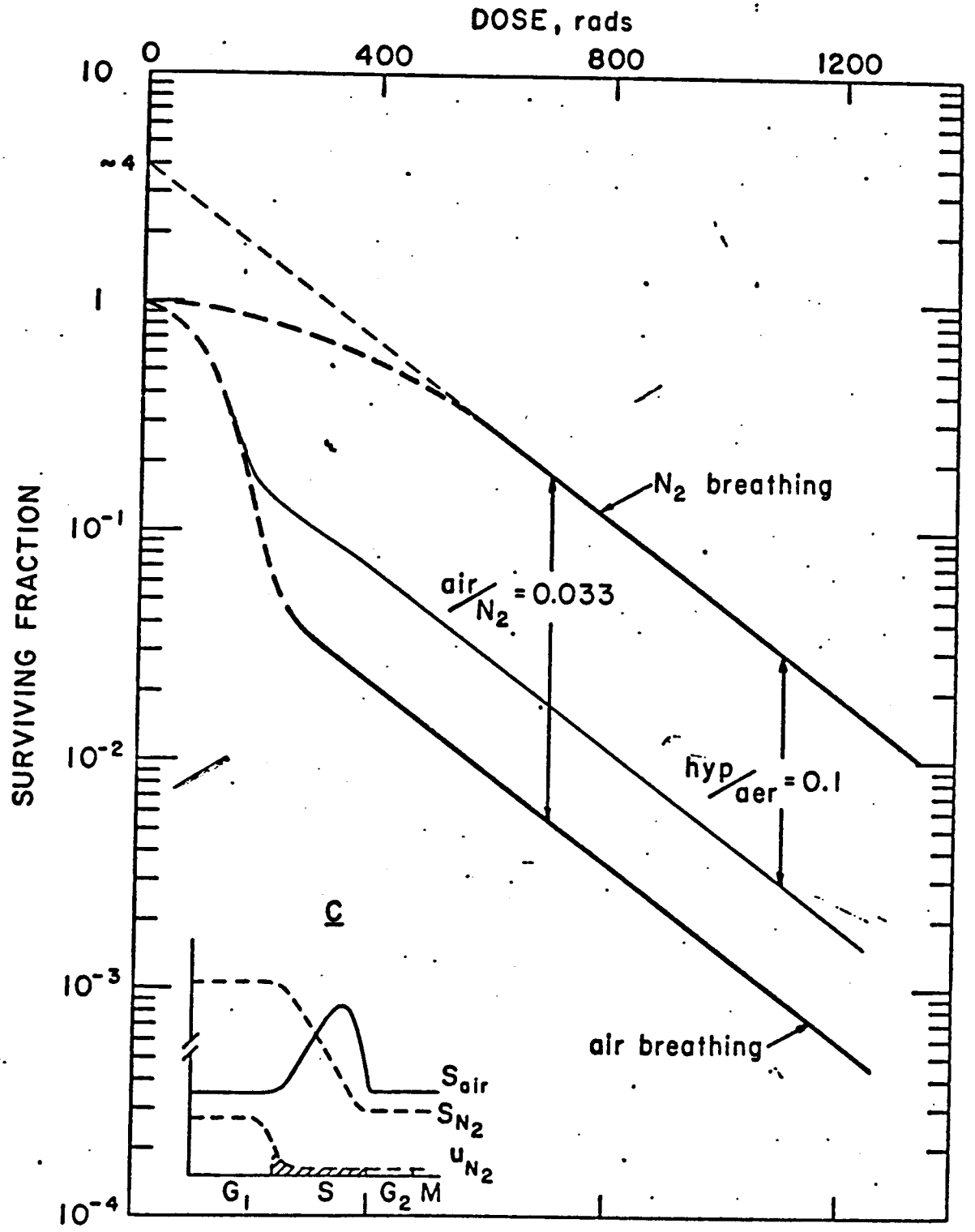

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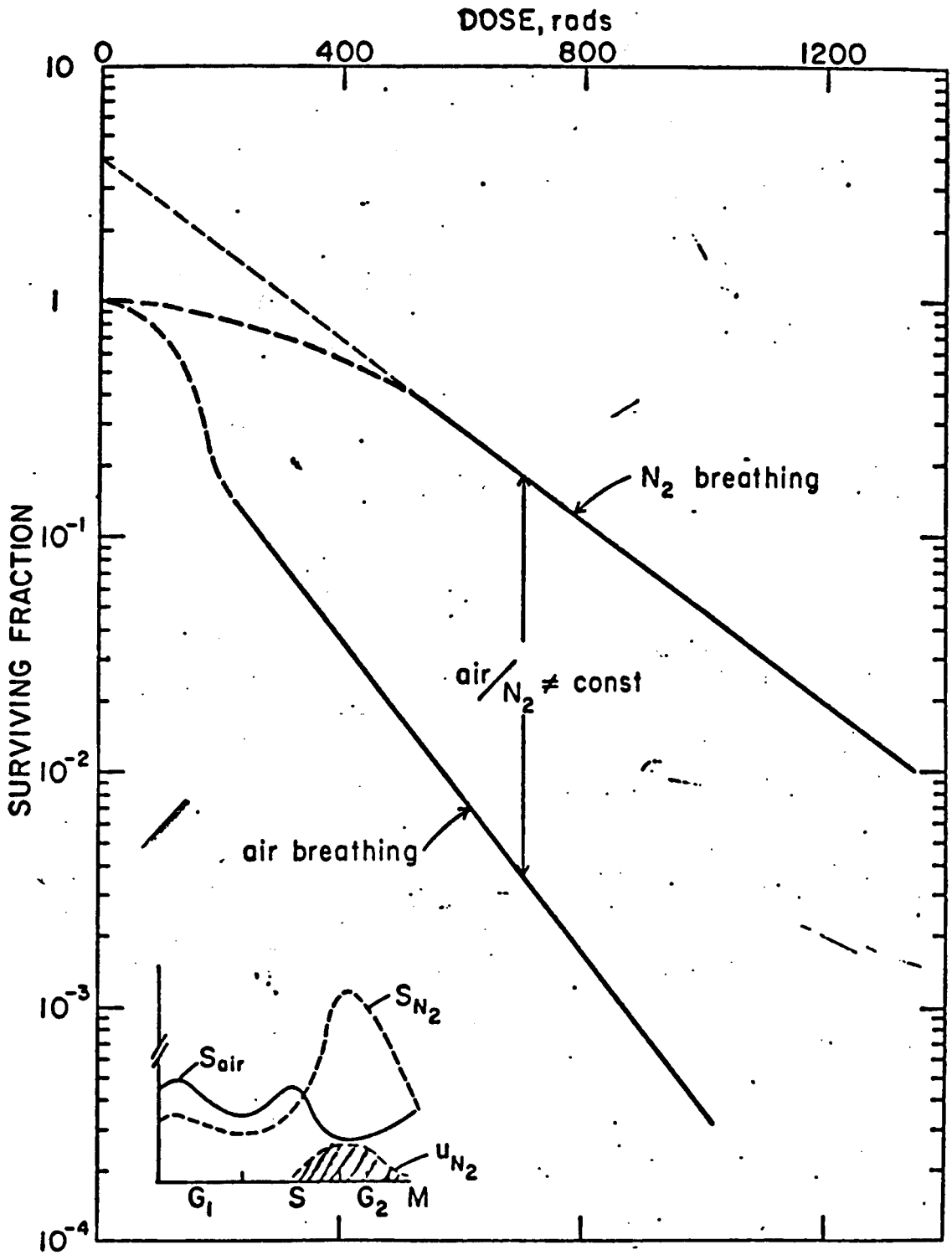

FIGURE 8

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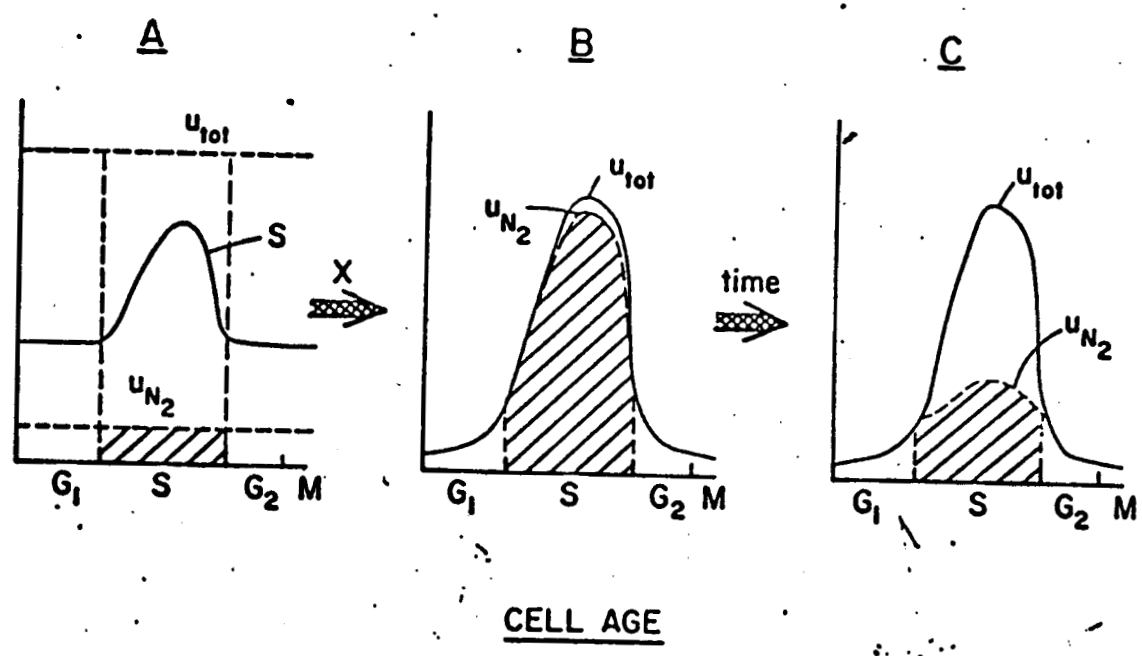

FIGURE 9

Neg. 9-222-69

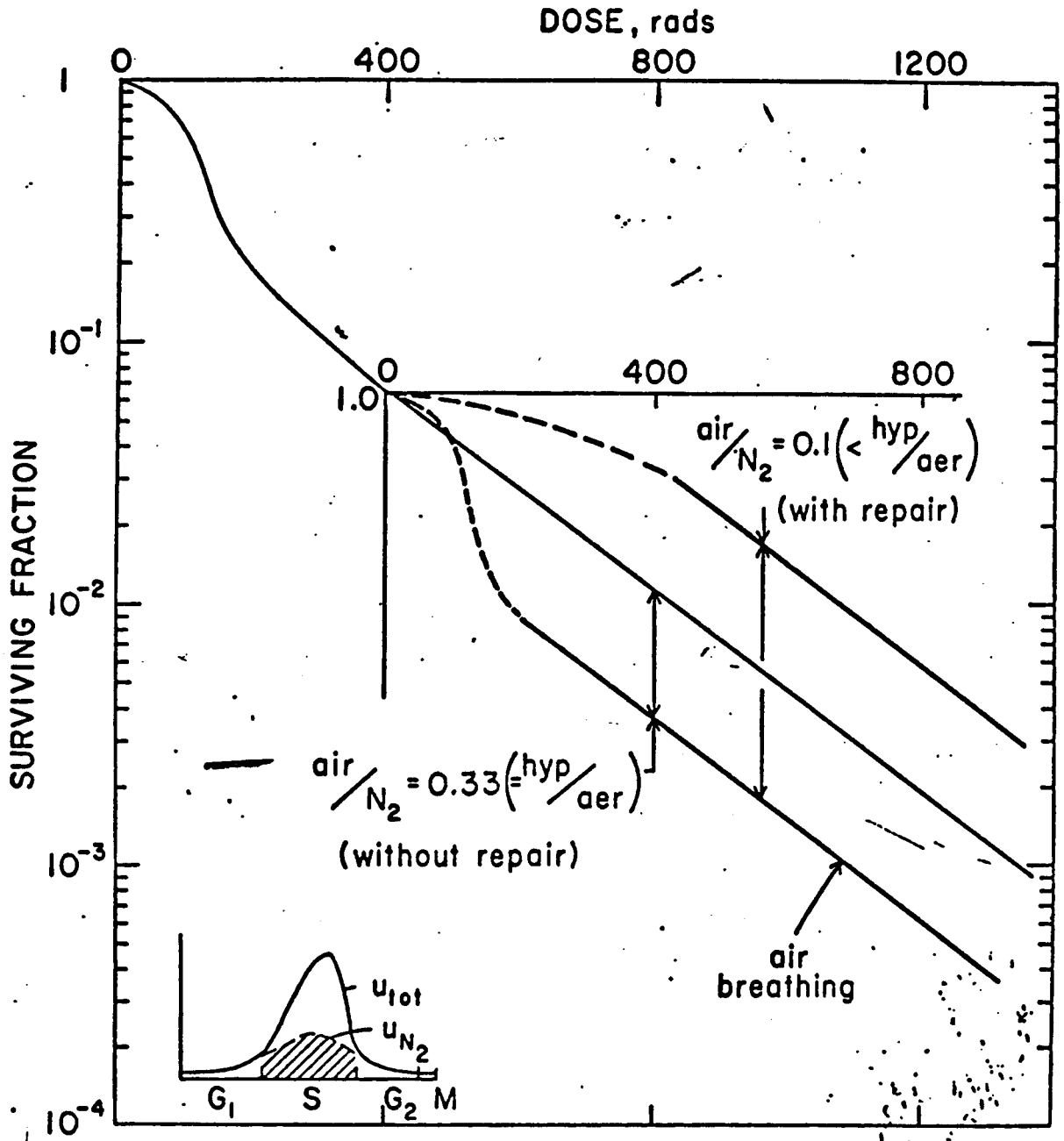

FIGURE 10

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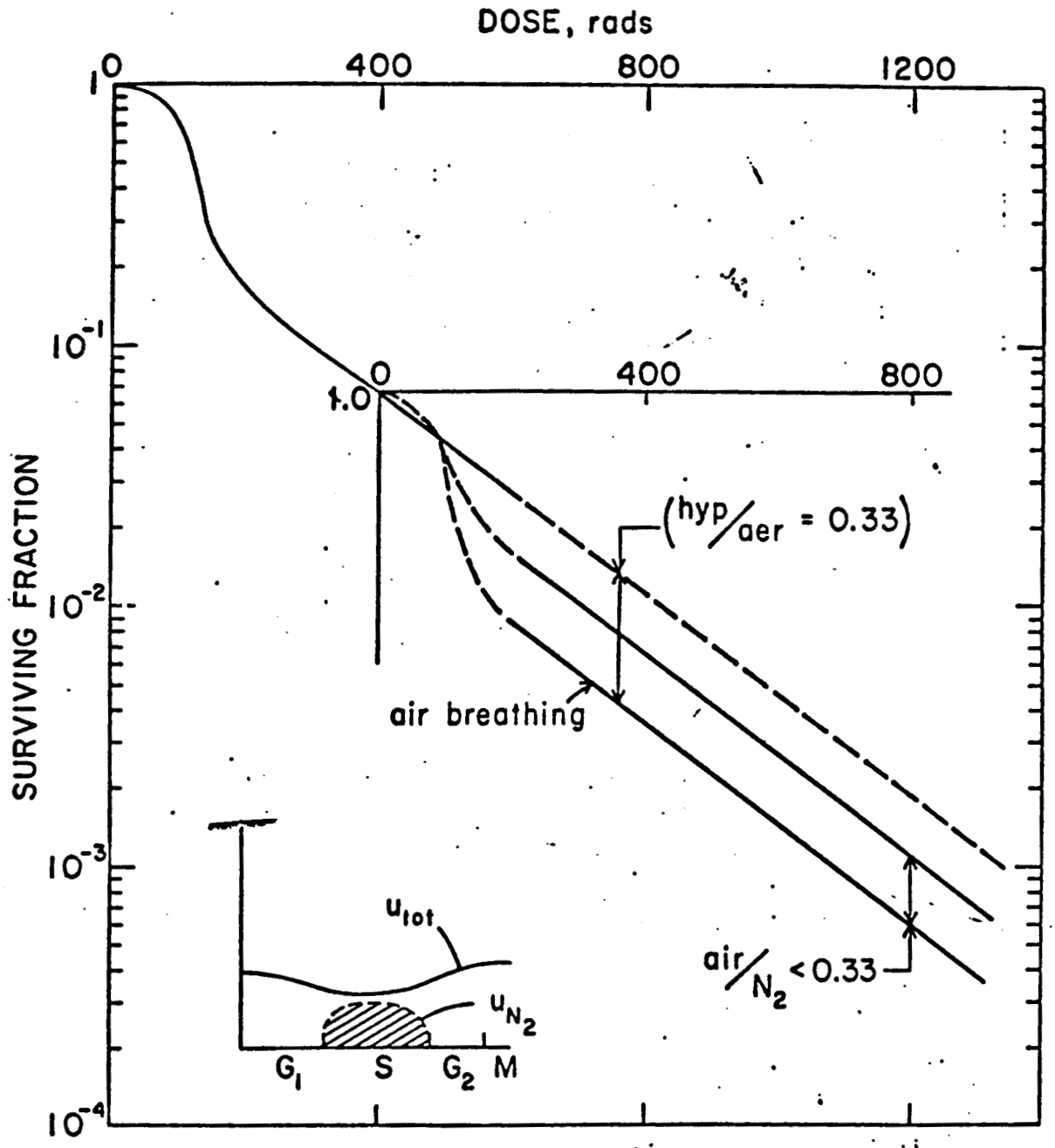

FIGURE 11

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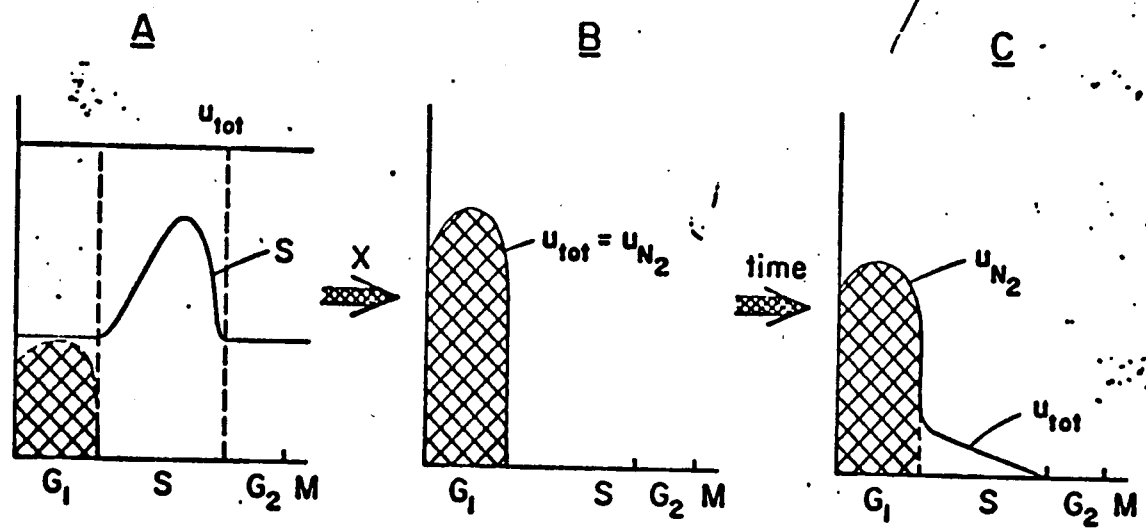

CELL AGE

FIGURE 12

Neg. 9-221-69

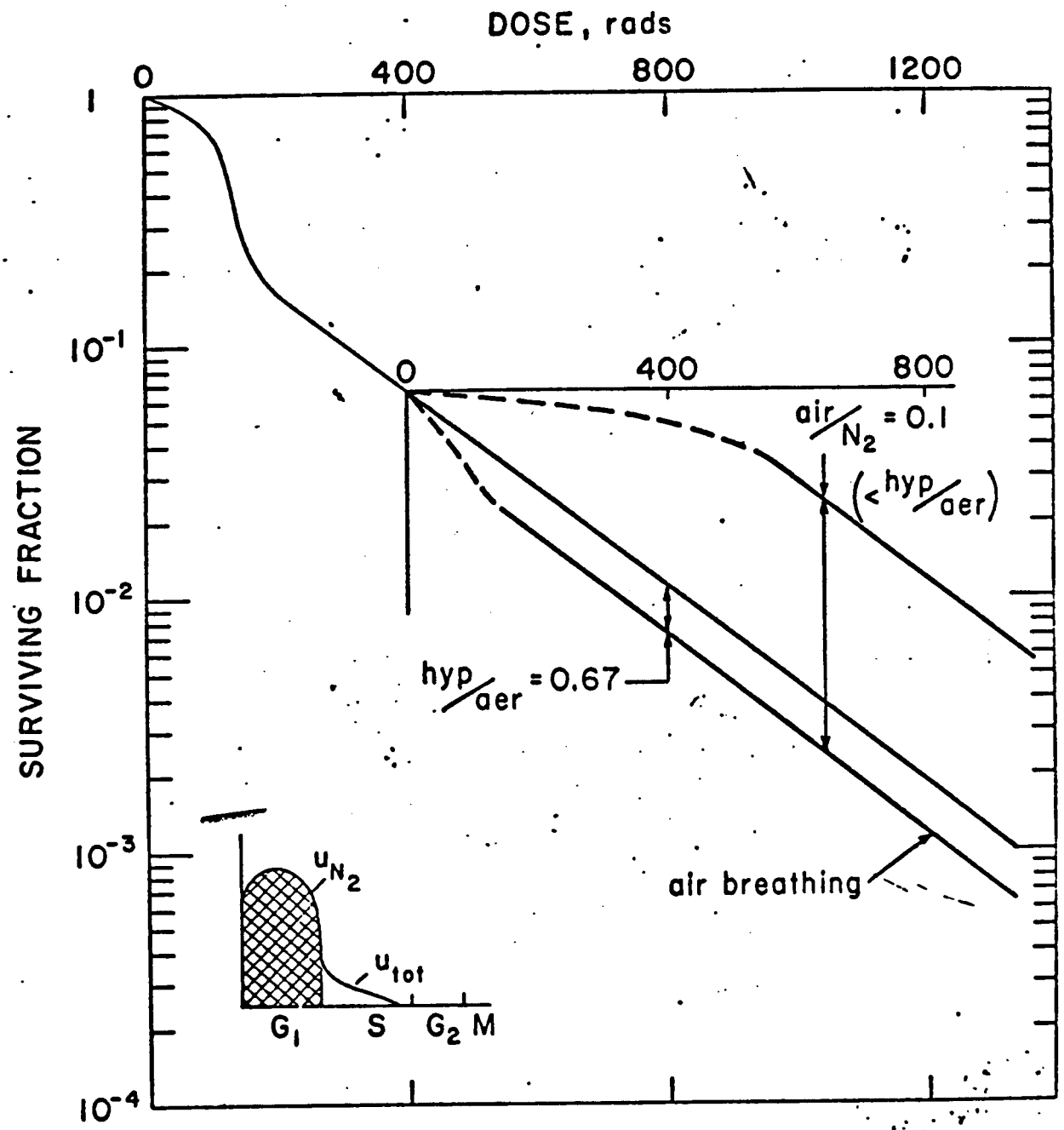

FIGURE 13

Neg. 9-218-69

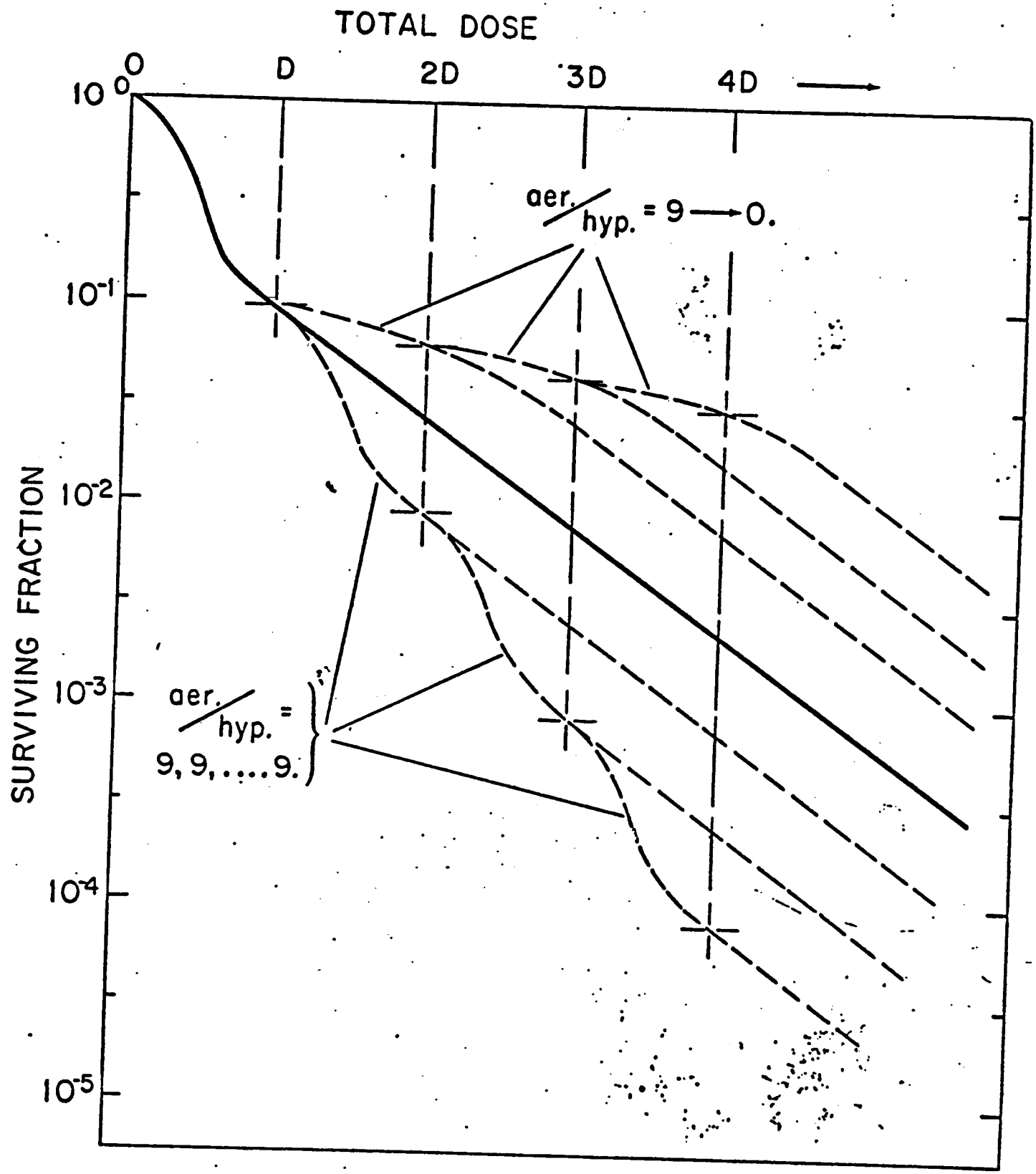
TOTAL DOSE
0
D
2D
3D
4D
10 0
10-1
10-2
10-3
10-4
10-5
SURVIVING FRACTION
aer./hyp. = 9 → 0.
aer./hyp. = 9, 9, ...9.

FIGURE 14

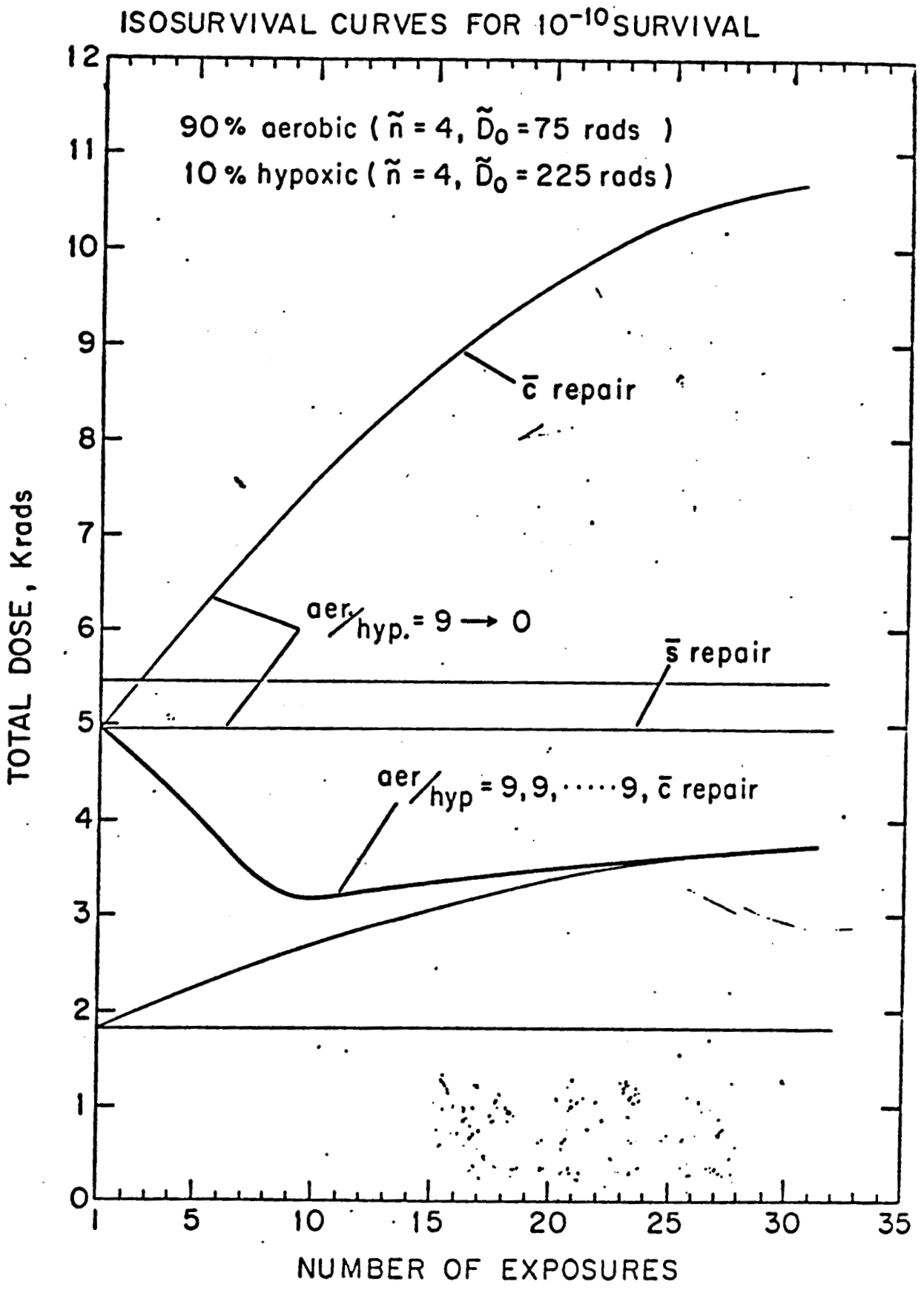
ISOSURVIVAL CURVES FOR 10^{-10} SURVIVAL
90% aerobic ($\tilde{n}$ = 4, $\tilde{D}_0$ = 75 rads)
10% hypoxic ($\tilde{n}$ = 4, $\tilde{D}_0$ = 225 rads)
$\bar{c}$ repair
aer./hyp. = 9 → 0
$\bar{s}$ repair
aer/hyp = 9, 9, ····· 9, $\bar{c}$ repair
TOTAL DOSE, Krads
NUMBER OF EXPOSURES
0
1
2
3
4
5
6
7
8
9
10
11
12
1
5
10
15
20
25
30
35

FIGURE 15

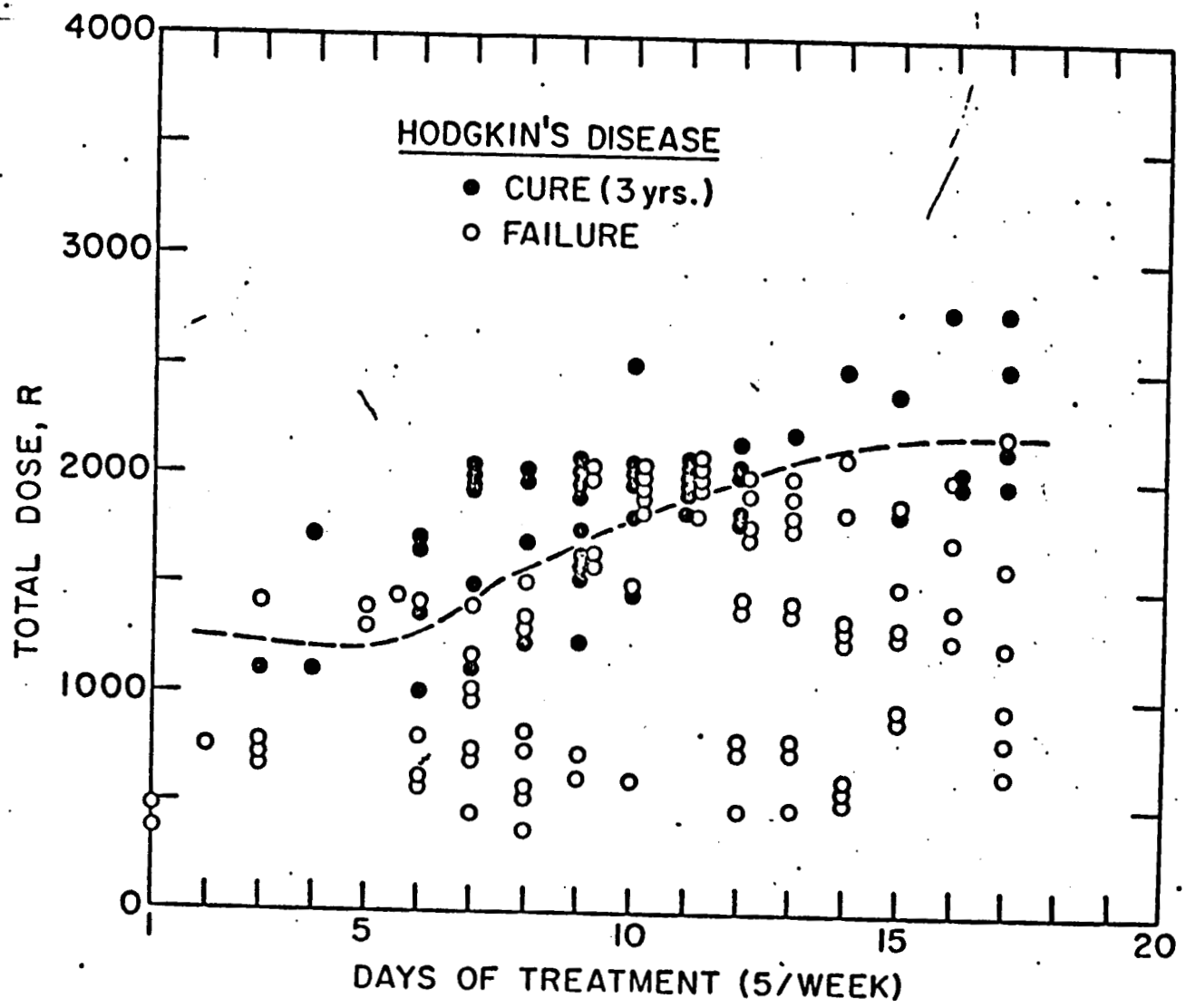

FIGURE 16

Neg. 9-211-69