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RANDOM FACTORS IN THE SURVIVAL CURVE

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This article will begin with some statements on the old topic of survival curves and with some rather well-known arguments. Then an alternative to the conventional treatment will be given followed by some remarks on the different random factors governing the dose-effect relation. A complete analysis is not within the scope of this article but one detailed example may serve to bring out new aspects that have been somewhat neglected up to now.

I. DEFICIENCIES OF THE CONVENTIONAL TREATMENT

First, it is necessary to examine the difficulties involved in the traditional concepts. The sigmoidal survival curves that are observed in experiments with mammalian cells, for example, could be represented by quite a number of different mathematical functions. For historical reasons only a few of these have really been used. The corresponding models go back to target theory but they have survived the classic framework of this theory. The formulas are now relics devoid of any concrete meaning.

The usually applied characteristics of the shape of survival curves are connected with the two models most widely in use. First, there is the so-called multihit model. It produces curves that "bend over", i.e., do not approximate a straight line in the semilogarithmic plot. These curves are conventionally represented by gamma distributions, or

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in less mathematical language, by multihit curves. The order of these curves is called the hit number. It is now known that this is not a real number of hits and that an experimental curve cannot usually be represented uniquely by a multihit curve or a superposition of multihit curves. There are many and various ways of approximating a curve by gamma distributions. Specifically, it can be shown that an arbitrary high mean hit number can be used for this superposition. In fact, a higher mean hit number generally makes a more exact approximation possible. For these reasons, the "hit number" is rarely used anymore. Somewhat heretically one might even suppose that there is a certain bias toward curves that do not bend over and which fit more easily into a formula. This is the case for dose-effect relations that at higher doses approximate exponential shape, and which are commonly treated according to the second of the conventional models.

The classic multitarget model, a very special assumption, has lead to the definition of a "target number." Since, however, this is not a number of targets at all, it is now called the extrapolation number.
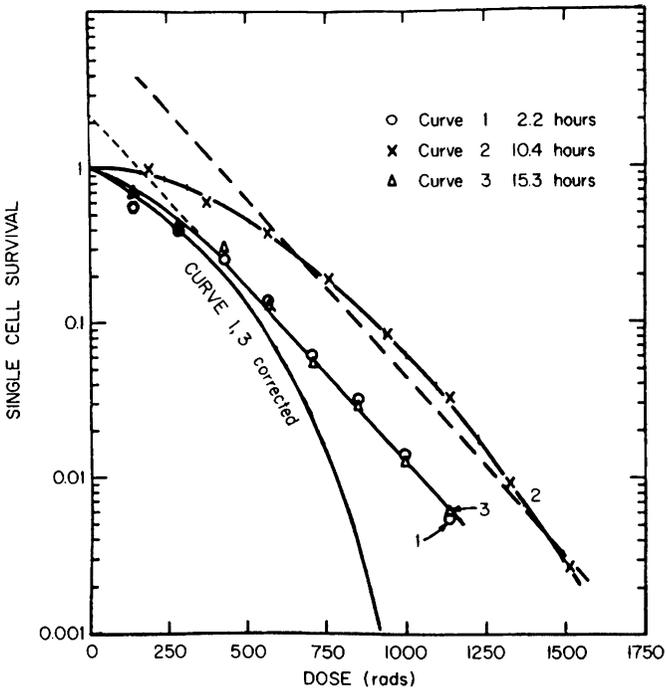
FIG. 1. Survival curves of synchronized Chinese hamster cells in $G_1(o)$, $S(x)$, and $G_2(\Delta)$. (1).

Together with the final slope $1/D_0$ this number allows an easy visualization of the dose-effect curve in a semilogarithmic plot. If in addition one knows the initial slope and D_{37}, the dose for 37% survival, one has a set of numbers that could roughly replace the dose-effect curve. Thus if nothing more is wanted than a shorthand representation of the survival curve, the extrapolation number and D_0 may well be used.

However, a completely different problem arises if one looks for characteristics that are representative in a mathematical sense and which are open to formal analysis. A few remarks may suffice to show the limitations of concepts such as extrapolation number and D_0 in this respect. An extrapolation number is defined only for dose-effect curves that in a semilogarithmic plot end in straight lines. Even if experimental data suggest that this is the case, the extrapolation to doses not covered by experimental data must remain tentative. Figure 1 shows that the a priori assumption of the existence of an extrapolation number may do violence to a curve; but if, for the moment, we forget this, the fact remains that the extrapolation number as well as D_0 is representative of only that small fraction of the population that survives at highest doses. Even minor changes in the experimental technique can influence this small fraction, as shown in fig. 2. Thus, the characteristics of the curve
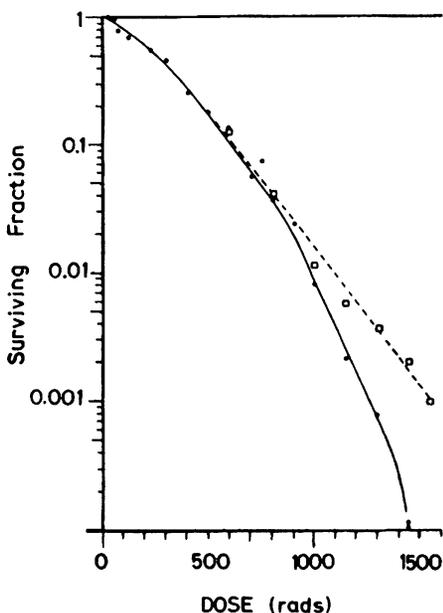
FIG. 2. Survival curve for HeLa cells (2). Medium unchanged (●); medium replaced seven days after plating (□).

are completely altered, while the reaction of the main portion of the population remains unchanged.

Still another example may serve to show that n and D_0 are not truly representative of a survival curve. Figure 1 gives curves for a synchronized population of Chinese hamster cells [as determined by Sinclair and Morton (1)]. The left curve pertains to cells in a sensitive state (G_1 and G_2 phase) while the other curve corresponds to cells in a less sensitive state (S phase). Naturally, the population is not perfectly synchronized. As Sinclair (3) remarks, the final part of the curve is determined by the least sensitive fraction. If this is true, however, one may roughly correct the left curve and eliminate the contribution of the insensitive fraction belonging to the S phase. When this is done, one finds that the corrected curve is not at all characterized by the original extrapolation number and D_0. On the contrary, these numbers refer entirely to the fraction of cells with which the experiment is not concerned. This is a serious limitation, and the conventional characteristics, useful as they may be in qualitative studies, are of little use in the more refined experiments on synchronized populations. If one has to deal with a superposition of different cell states, one should look for characteristics that are additive.

II. The Moments of the Dose-Effect Distribution

The present usage is—as we have seen—not satisfactory. Basic characteristics, however, are easily obtained if one considers the fact that essentially the dose-effect relation is the distribution function of the inactivation dose. It is highly surprising that this fact was never clearly stated. If we recognize that the dose-effect relation is the distribution function of the inactivation dose, it becomes clear that the mean and the standard deviation of the inactivation dose are basic characteristics. This is symbolized in Fig. 3.

If one assumes that all units of a population are identical and receive the same "local dose," and that no other stochastic factors are involved, one ought to find a well-defined critical dose threshold so that $\sigma^2 = 0$, i.e., the dose-effect curve should be a step function. This, however, is never the case. Whether at a certain dose a cell is inactivated or not depends on a number of different random factors. One of these factors is the variable sensitivity of the cell; another is the variable amount and distribution of absorbed energy; still another factor is the random interplay of the numerous functional components of the biological system with each other and with the exterior parameters. In general it is
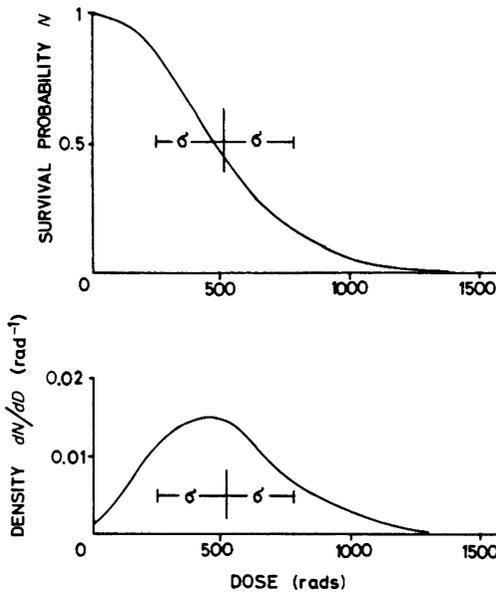
FIG. 3. Survival curve and its derivative for Chinese hamster cells in G_1 and G_2 (*1*). The mean inactivation dose $\bar{D}$ and the standard deviation σ are marked.

difficult to read the individual roles of these different factors from the mere shape of the curve. All random factors, however, have the effect of "broadening" the curve, and therefore the standard deviation of the inactivation dose is a convenient measure of the combined influence of the different random factors.

In mammalian cell culture technique one has to control a large number of factors; usually results are not exactly reproducible in different laboratories. Therefore a comparison is only possible on the basis of characteristics that are not too sensitive to small variations in the experimental setup. $\bar{D}$ and σ^2 fulfill this requirement, while the extrapolation number and D_0 do not, as can be seen from Fig. 2.

Mathematically, the standard deviation is defined in terms of the first two moments of the distribution function, $\bar{D}$ and $\overline{D^2}$

$$\sigma^2 = \overline{D^2} - \bar{D}^2$$

with

$$\bar{D} = \int_0^\infty D \, dN(D)$$

and

$$\overline{D^2} = \int_0^\infty D^2 \, dN(D)$$

If a population is made up of fractions p_i of cells in different states i, then the resulting moments are the weighted mean of the corresponding moments of the dose-effect curves for the pure states i

$$\bar{D} = \sum p_i \times \bar{D}_i$$

and

$$\overline{D^2} = \sum p_i \times \overline{D_i^2}$$

Thus the moments are additive. For σ an analogous relation does not hold. Therefore for actual calculations one should use the moments rather than the variance σ^2. This is important for studies on synchronized cell cultures. Still it is a technical detail only, since from the mean $\bar{D}$ and the variance σ^2 one readily calculates the second moment $\overline{D^2} = \bar{D}^2 + \sigma^2$. Below we will see that one may even modify the above proposal and, instead of the second moment or the variance, use a related dimensionless number to characterize the shape of the dose-effect curve.

III. Two Fundamental Relations

Naturally one may use higher moments to describe a dose-effect curve. In addition to the mean and variance the skewness would be important. As already mentioned, our present concern is not the mere description of the survival curve; moreover, the experimental data are generally not precise enough to warrant exact determination of higher moments. While $\bar{D}$ and σ have simple practical meaning, other less fundamental characteristics may easily lead to complicated but useless models or formulas. We will see that the most simple characteristics allow the most general interpretation.

We have already mentioned the different random factors that are responsible for the variance of the inactivation dose. The main factors are: biological variability, the random nature of energy deposition, and finally, what we call the stochastic nature of vital processes, i.e., all random factors that play a role after the initial disturbance.

The first and the second factor are extensively discussed in the literature, the arguments against target theory being based on the first aspect, while target theory itself has been focused on the second factor. The third point refers to the fact that the interplay of numerous components within a biological system and the influence of the exterior parameters usually prevent an exact predetermination of biological processes.

Later, an example will be given which shows that biological stochastics may well influence the shape of the dose-effect curve.

With multicellular organisms biological variability is paramount, and with very small objects, like viruses or bacteria, inhomogeneity of energy deposition is the most important factor. In many cases, however, the three different random factors cannot be separated; this is specifically true for mammalian cells irradiated with low LET radiation. Accordingly, formal analysis alone can in no case tell which of the three factors is responsible or mainly responsible for the variance of the inactivation dose. A very general interpretation of the mean and the variance of the inactivation dose is nevertheless possible. This can only be dealt with briefly here. First, a certain modification of the above proposal will be made.

Instead of the variance σ^2 of the inactivation dose a dimensionless number will be used as a characteristic of the dose-effect curve. We choose the expression $\bar{D}^2/\sigma^2$ and call it relative steepness S because its value indicates how nearly the dose-effect curve approximates a step function. As can be easily deduced, $S = 1$ for exponential dose-effect curves. For shoulder curves, $S > 1$, and S increases as the shoulder becomes more prominent. In fact, for the special case of the target theory curves S equals the so-called hit number. It is, however, a characteristic well defined for all kinds of dose-effect relations. The most surprising fact is that this very elementary characteristic permits an exact interpretation that is independent of any hypothetical model. It can be shown that S is a lower limit for the mean number of interacting absorption events necessary to bring about the test effect. If a survival curve has a relative steepness $S = \bar{D}^2/\sigma^2$, the mean number of statistically independent absorption events[1] that interact and bring about the test effect cannot be less than S. Only if there were no other random factors at all except the statistical energy deposition could S be the actual number of interacting absorption events. If a curve has relative steepness S, and if biological variability and biological stochastics play a role, the number of absorption events needed to bring about the test effect must be greater than S. A rigorous proof and a detailed discussion of this relation has been given elsewhere (4, 5).

One can also show that $\bar{D}$ in conjunction with S permits the deduction of a lower limit for the diameter of the sensitive site in the cell. This is a direct application of Rossi's concept of local energy distributions; it is also presented in full detail elsewhere (4, 5). For the case of mammalian cells irradiated *in vitro* with sparsely ionizing radiation, one finds

[1] Absorption event signifies energy deposition by a primary particle and/or its secondaries.

that on the average more than four absorption events must interact over a distance of more than 1 micron to bring about the test effect. The actual value must be greater since, in addition to the inhomogeneity of energy deposition, other random factors play a role.

Thus the values $\hat{D}$ and S allow quite definite statements on the most widely discussed random factor in radiation effect. Whether one would call this a revival of target theory or, on the contrary, a refutation of target theory is a matter of taste.

IV. Biological Stochastics

The above-mentioned relations give lower limits for the number of interacting absorption events and for the diameter of the sensitive site. Just how far these lower limits lie below the actual values is an open question which can only be decided by further analysis of the role of the different random factors.

There are three lines of investigation to be followed. For the analysis of the statistical fluctuations of energy deposition, microdosimetry will be used. Biological variability, i.e., different sensitivity of the cells, will be studied further in synchronized cell cultures. Determination of the moments of the dose-effect curves will be a useful tool especially in these kinetic studies. The third consideration is biological stochastics, i.e., the random fluctuations that occur in biological systems before, during, and after irradiation, and which cause a further uncertainty in the resulting effect. This last factor has been neglected up to now; therefore it will at least be mentioned here.

After irradiation, a highly complicated pattern of cell life and death is observed in the progeny of individual cells. This pattern is not yet well understood, but observations on pedigrees of irradiated cells are now being made in different laboratories. The considerations to be given here, though mathematically different, will lie somewhat along the lines taken by Till *et al.* (6). An explicit discussion is given elsewhere (4).

The development of a cell clone is a series of successful and unsuccessful mitoses, i.e., a birth and death process. This may be symbolized by the graph shown in Fig. 4.

We start with one cell. Each successful mitosis is a step to the right, each death of a cell a step to the left. The probability for successful mitosis is called p, the probality for cell death q. If the absorbing barrier $n = 0$ is reached, the process is terminated, and all cells are dead. This process is well known in probability theory. Historically one of the oldest problems, it is called the "gambler's ruin problem."
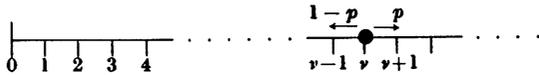
$$p_K = \frac{1 - \frac{q}{p}}{1 - \left(\frac{q}{p}\right)^K}$$

FIG. 4. Graph for the development of a cell clone according to the so-called ruin problem.

The formula for the probability p_K that a single cell will grow into a group of at least K cells is given below Fig. 4 and is represented by the curves of Fig. 5.

What does that mean for the dose-effect relation? As an example, let us assume that the probability p decreases exponentially with dose and is constant after irradiation. One then obtains the dose-effect curves shown in Fig. 6. The experimental end point is the ability of the single cell to generate a group of at least K cells. Obviously the curves get more and more exponential if K is decreased. Thus the shape of the survival curve may well be strongly influenced by the experimental end point.
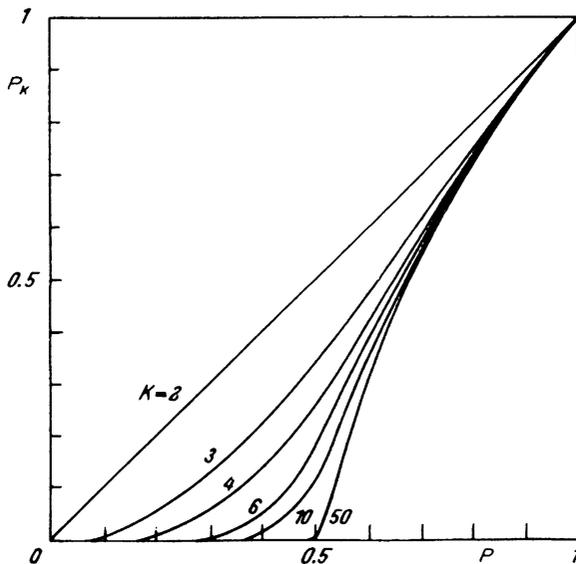

FIG. 5. The probability p_K for formation of at least K cells as a function of the success probability p in a single mitosis.
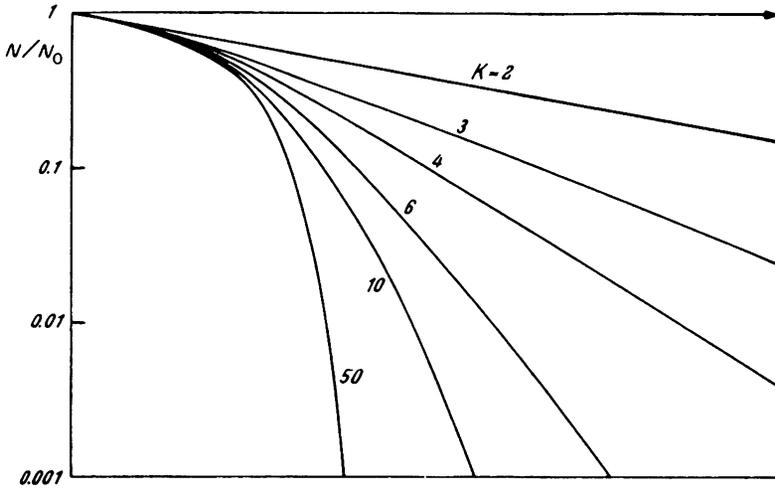
FIG. 6. Survival curves that result if p decreases exponentially with dose and if the formation of at least K cells is taken as the experimental end point.

The above model is, of course, an oversimplification. The labilization of the cell that accounts for the decrease in p will be transient and not constant after irradiation. Certainly the other random factors will play a role, too. Thus it remains to be determined what fraction of the variance of the inactivation dose is brought about by the different random factors. Nevertheless, we will give a practical example.

Haefner (7) followed the pedigrees of yeast cells. He did not consider the above stochastic interpretation at all, but if we take his results and compare them with the outcome of the theoretical model, we obtain rather good agreement as shown in Fig. 7.

Four different classes of cell fates are distinguished. First, there are cells with no failures at all in the first four generations. Second, there are cells that produce a colony but have some dying cells among their progeny. Third, there are cells with some successful mitoses which do not produce a colony. Fourth, there are cells that die after irradiation without any mitosis. Obviously the correspondence between theoretical and experimental curves is quite good, so that the simplified model given above may not be completely unrealistic. The main difference between the curves is that experimentally one finds only a few cells that die immediately after irradiation. This, however, is easily understood; even heavily damaged cells may undergo an abortive division.

One should be very careful not to assume that this model is really adequate just because it leads to the right results. This would be to
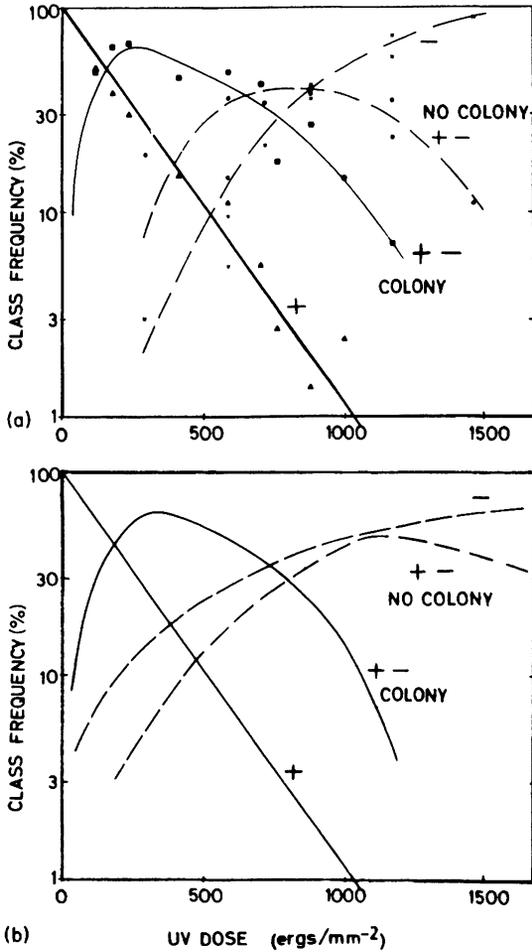
FIG. 7. UV-dose dependence of the frequencies of four cell classes distinguishable in an irradiated population of haploid yeast cells. (a) Experimental curves (7); (b) theoretical curves.

repeat an old mistake in a new direction. In fact, sigmoidal survival curves are neither pure multihit curves, nor distribution functions of the sensitivity, nor direct records of the cells' stochastic behavior in a series of mitoses. They are a mixture of all three, and this is the main reason for the treatment proposed in this article. The preceding example may merely serve to show that this one factor, biological stochastics, must not be overlooked.

A last remark may illustrate the situation in mammalian cell culture studies. Here the difficulties are still quite obvious and one arrives at results that would seem to exclude each other.

According to Elkind (8), two doses work independently on the cell if separated by an interval of about 20 hours. If dose D leads to survival $N(D)$, this dose followed by a second dose of equal size yields survival $N(D) \times N(D)$. In other words, sublethal damage from the two doses if separated by 20 hours does not interact to become lethal.

This should not be the case according to the above considerations, as can be seen from Fig. 5 and 6. A simple special case should make this clear. After irradiation with dose D one finds some cells that undergo division, while one of the daughter cells dies and the other one divides and goes on to form a colony. This is symbolized in Fig. 8a.
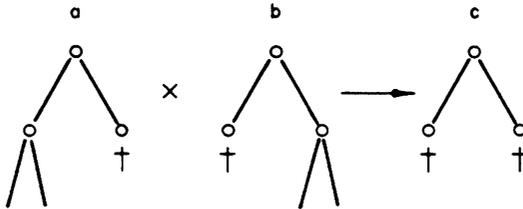

FIG. 8. Interaction of nonlethal damage.

An analogous case is shown in Fig. 8b. If one assumes that two doses separated by 20 hours work independently, and that Fig. 8a represents the result of the first dose and Fig. 8b the result of the second dose, then the outcome would be Fig. 8c. The assumption is realistic; because of the mitotic delay induced by the first dose there may be no mitosis during the interval. In this case, survival times survival yields failure; in contrast to Elkind's finding, sublethal damage adds up to lethal damage even if separated by an interval of 20 hours.

There are several possible explanations for this contradiction. Either Elkind's results are only approximately valid, or they pertain to experiments in which the random pattern of success and failure in the first few mitoses after irradiation is of little importance. Still another possibility may be that the interaction of "nonlethal" damage is just balanced by an opposite effect. Either by selection or by actual change in the biological state the first dose may render the surviving fraction more resistant to the second dose. This question must be answered by further observations on the development of single cell clones.

APPENDIX

The determination of the moments of a distribution function is usually done numerically. A graphic procedure which is perhaps the easiest method will be presented here.

By partial integration one can deduce the following relations

$$\bar{D} = \int_0^\infty D \, dN\,(D) = \int_0^\infty N(D) \, dD$$

and

$$\overline{D^2} = \int_0^\infty D^2 \, dN\,(D) = \int_0^\infty 2D \times N(D) \, dD$$

Thus one does not really need to differentiate the survival curve $N(D)$. One merely determines the area under it in the linear plot; this yields the mean inactivation dose $\bar{D}$ (area below $N(D)$ in Fig. 9).
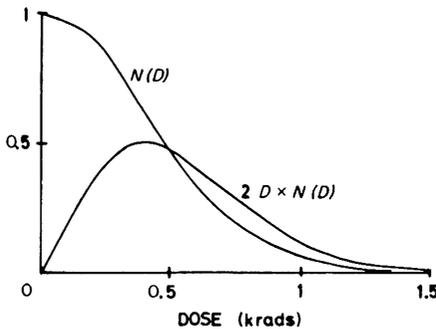

FIG. 9. Graphic determination of the moments of the survival curve $N(D)$ (*1*).

Then one multiplies the dose-effect curve by $2D$. The area under this curve is equal to the second moment $\overline{D^2}$ (area below $2D \times N(D)$) in Fig. 9). From $\bar{D}$ and $\overline{D^2}$ one readily obtains the variance $\sigma^2 = \overline{D^2} - \bar{D}^2$ and the relative steepness $S = \bar{D}^2/\sigma^2$. In the example given in Fig. 9, one obtains $\bar{D} = 520$ rads, $\sigma = 270$ rads, and $S = 3.9$.

There is, incidentally, a certain connection between the extrapolation number n and the relative steepness S. A high value of one variable usually indicates a high value of the other. This is apparently what is meant if one states that a high extrapolation number means a large shoulder.

The statement, however, is not strictly true; a large shoulder means a high extrapolation number while the opposite is not necessarily the case. An almost exponential curve may still have a high extrapolation number. In terms of S one may state that a high value of S is always connected with a high value of n, while a high value of n may also occur with a small S. For sigmoidal curves (i.e., curves with no point of inflection in the semi-logarithmic plot) one deduces the relation

$$S \leqslant (1 + \log n)^2$$

As the proof of this relation is rather simple, it will be omitted here. However, the relation itself is important insofar as it shows that S is a measure for the shoulder while n is not, or only in a very restricted way.

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