Inactivation of macromolecules by ionizing radiation

Deterministic single-hit or stochastic multievent process?

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A stochastic theory concerning the radiation inactivation of macromolecules such as enzymes or receptors is elaborated. In contrast with the single-hit theory, which assumes a complete inactivation of the target as the result of one hit, the stochastic theory postulates that the degree of inactivation by one hit is a random variable. This distinguishing feature has been considered in order to give a possible interpretation to the observed effect of temperature on the radiation-sensitivity of enzymes. As a consequence of the progressive inactivation during irradiation, the binding affinity of a ligand for the macromolecule is impaired by irradiation. Although this property might discriminate the stochastic theory from the classical single-hit theory on the basis of a statistical analysis of experimentally obtained data, it is shown that the commonly obtained degree of inaccuracy may render the statistical test non-conclusive.

INTRODUCTION

The study of biochemical systems embedded in cell membranes may be approached by using radiation. Indeed, the measure of the biochemical activity after irradiation allows the calculation of the molecular mass of the functional unit *in situ* (Kempner & Schlegel, 1979). For this purpose, the so-called 'target-size analysis' is applied to the data expressed as the logarithm of the fractional residual activity against the radiation dose. The theoretical basis of this method, proposed 40 years ago by Lea (1946), refers to the single-hit concept. This deterministic theory associates the complete loss of activity to an event produced anywhere in the functional unit by a single ionizing particle. Consequently, irradiation partitions the macromolecule population into a subpopulation of naive molecules that have escaped the ionizing radiation and a subpopulation of inactive molecules that have been hit at least once. Thus, if the macromolecule is a receptor for a ligand, the theory predicts that the binding assay should exhibit decreasing maximal binding for increasing radiation dose without any significant modification of the affinity of the ligand for the receptor. Up until now, most experimental observations have supported this property. Therefore the single-hit concept is widely accepted, although its validity has not been directly demonstrated so far.

Pollard *et al.* (1955) reviewed the effect of temperature on the sensitivity of protein activity to irradiation. More recently, Kempner & Haigler (1982) demonstrated that the radiation dose leading to a 37% residual activity exponentially decreases with respect to the absolute temperature at which the sample has been irradiated, and that the change of sensitivity is the same for all the enzymes studied. So far, no interpretation of this observation has been proposed within the context of the single-hit theory. In fact, since this theory assumes that the energy deposited by one hit is sufficient to inactivate the protein completely and since the amount of energy transferred into the molecule is temperature-independent, it seems hard to explain the increase of sensitivity when

the sample is irradiated at higher temperatures. Therefore there is a need to revise the single-hit theory.

In the present paper an alternative working hypothesis on the mode of action of ionizing radiation is considered in order to account for the temperature effect. It is assumed that a single hit changes the conformation of the protein and impairs its activity to an extent that depends especially on the locus of the hit and on the nature of the subsequent chemical modifications. Therefore the complete inactivation of the protein may require more than one hit. Since the ionization of a target leads to a non-predictable modification of the activity of this target, the theory must be essentially statistical. In the context of the binding of a ligand to a receptor, an affinity spectrum for the receptor population must be defined as a function of the radiation dose. *A priori*, such a theory would be invalidated by the data showing no significant modification of the binding affinity. The aim of the present paper is to show that in fact experimentally obtained data presented in the literature are not necessarily capable of rejecting a theory which states that, on average, more than one hit is required for the complete inactivation of the protein.

ELABORATION OF THE THEORY

Ligand–receptor interaction

The theory is developed with the use of ligand binding to a receptor as a biochemical activity sensitive to irradiation. The ligand H binds to a receptor R in a reversible manner:

K_d is the equilibrium dissociation constant. Assuming that the ligand concentration $[H]$ is always much higher than the total concentration of receptor $[R_t]$, the binding activity is equal to:

$$[HR] = \frac{[R_t]h}{1+h} \quad (2)$$

where $h = [H]/K_d$. This equation assumes that the receptor population is homogeneous with respect to the binding affinity.

The basic postulate of the theory is that the interaction of an ionizing particle with a receptor molecule induces a random loss of binding affinity. Defining the loss by LRT (where R is the gas constant and T the absolute temperature), one obtains:

$$L = \ln(K_{d, \text{ after the hit}}/K_{d, \text{ before the hit}}) \quad (3)$$

The random variable L thus varies between 0 and $+\infty$. If $\mathcal{N}(L)$ represents the distribution of the receptors hit at least once with respect to their residual affinity, the concentration of hit receptor sites filled at a certain ligand concentration is given by:

$$[HR]_{\text{hit}} = [R]_{\text{hit}} \int_0^\infty \mathcal{N}(L) \cdot \frac{h}{e^L + h} \cdot dL \quad (4)$$

Determination of the affinity spectrum

In order to compute the binding activity expressed by the hit receptors (eqn. 4), the theory must provide the affinity spectrum $\mathcal{N}(L)$ as a function of the radiation dose. The determination of $\mathcal{N}(L)$ requires the definition of a transition probability describing the chance that one hit provokes a certain loss of affinity. Thus $P_{\text{tr}}(L) \cdot \Delta L$ is the probability that, as the result of one hit, the decrease of the receptor affinity is characterized by a value comprised between L and $L + \Delta L$. The density of probability $P_{\text{tr}}(L)$, which is defined for any non-negative values of L , must fulfil the normalization constraint:

$$\int_0^\infty P_{\text{tr}}(L) \cdot dL = 1 \quad (5)$$

and thus $P_{\text{tr}}(L)$ must decrease to zero as L increases to infinity. A monotonously decreasing exponential function has been arbitrarily chosen:

$$P_{\text{tr}}(L) = \lambda \cdot e^{-\lambda L} \quad (6)$$

where $1/\lambda$ is related to the efficiency of radiation to impair the receptor affinity. The maximal efficiency corresponds to a zero λ value. Computations show that the fraction of receptors for which the K_d is increased at most by one order of magnitude as the result of one hit (i.e. $L = 2.3$) is equal to 0.9 and 0.99 when λ is equal respectively to 1 and 2. Thus such values ensure that at least these fractions of hit receptors express a significant fraction of binding activity in spite of the action of one hit.

It is accepted that the probability of having i ionizations in a molecule submitted to a radiation dose D follows a Poisson distribution, and thus the fraction of molecules hit i times is equal to:

$$\frac{N_i}{N_{\text{tot}}} = e^{-\sigma D} \cdot \frac{(\sigma D)^i}{i!} \quad i = 0, 1, 2, \dots \quad (7)$$

where σ is the sensitivity of the molecule to be hit. This sensitivity is essentially associated to the number of electrons in the target molecule and consequently to the molecular mass of the target. Thus the fraction of molecules that have escaped the irradiation is equal to:

$$\frac{N_0}{N_{\text{tot}}} = e^{-\sigma D} \quad (8)$$

When $D = 1/\sigma$, 37% of the molecules have not been hit.

The affinity spectrum $n(L, i)$ for the fraction of

receptors that have been hit i times is determined as follows. If $i = 0$, the distribution with respect to L is by definition equal to:

$$n(L, 0) = \begin{cases} \infty & \text{for } L = 0 \\ 0 & \text{for } L > 0 \end{cases} \quad (9)$$

Indeed, the population of intact receptors is supposed to be homogeneous. For $i > 0$, the distributions must be computed by using the transition probability defined by eqn. (6), i.e.:

$$n(L, i) = \frac{1}{\theta_i} \int_0^L n(L, i-1) \cdot \lambda \cdot e^{-\lambda L} \cdot dL \quad (10)$$

where θ_i is a normalization factor such that:

$$\int_0^\infty n(L, i) \cdot dL = N_i \quad (11)$$

Using eqns. (9), (10) and (11), one obtains for $i = 1, 2, \dots$:

$$n(L, i) = N_{\text{tot}} \cdot e^{-\sigma D} \cdot \frac{(\sigma D)^i}{i!} \cdot e^{-\lambda L} \cdot \frac{\lambda^i L^{i-1}}{(i-1)!} \quad (12)$$

Finally, the affinity spectrum of the receptors hit at least once is equal to:

$$\mathcal{N}(L) = \frac{1}{N_{\text{tot}}} \cdot \sum_{i=1}^{\infty} n(L, i) \quad (13)$$

Unfortunately, it is not possible to express the analytical function equivalent to this series. Therefore $\mathcal{N}(L)$ must be numerically evaluated for each L value. Using eqns. (4), (12) and (13), one can compute the binding activity for a sample in which a dose D has been deposited:

$$B_D = [R] \cdot e^{-\sigma D} \cdot \left(\frac{h}{1+h} + \sum_{i=1}^{\infty} \frac{(\lambda \sigma D)^i}{i!(i-1)!} \cdot \int_0^\infty \frac{L^{i-1} \cdot e^{-\lambda L} \cdot h}{e^L + h} \cdot dL \right) \quad (14)$$

This equation is the sum of two terms. The first one is a monoexponential function of D , which does not depend on λ . This term reflects the contribution of the receptors that have escaped the irradiation and thus corresponds to the residual activity as obtained in the deterministic single-hit theory. The second term may be considered as a correction to this deterministic theory. In other words, the proposed stochastic theory contains the deterministic theory as a particular case obtained when λ decreases to zero.

METHODS

The theoretical model was numerically simulated in order to mimic roughly a typical experiment as obtained by Shorr *et al.* (1984): 11 ligand concentrations varying between $K_d/4$ and $10K_d$, and nine radiation doses ranging from 0 to 80 Mrad. The parameters $[R]$ and σ are equal respectively to 1 (arbitrary unit) and 0.1 Mrad^{-1} . For each value of h and D , the concentration of specifically bound ligand is computed by using eqn. (14). Total binding is obtained by adding to the specific binding a non-specific component equal to αh , where α is equal to 0.01. This non-specific binding thus represents about 10% of the total binding at a ligand concentration equal to $10K_d$. A random error is then introduced by generating a random number issued from

Fig. 1. Numerical simulation of the stochastic model as defined by eqn. (14)

The inactivation curve is represented for different situations. The value of the parameter λ is indicated by the number associated to each curve. $\cdots$, $h = 1$; $---$, $h = 10$; $- \cdot - \cdot -$, $h = 100$; $---$, borderline case independent of h (single-hit model).

a normally distributed population with the total binding as the mean and with a dispersion characterized by a 5% coefficient of variation.

The computer-generated data were analysed according to the procedure used by Shorr *et al.* (1984). At each

radiation dose, the model, consisting of one saturable binding site and one non-specific linear component, is fitted to the pseudo-experimental saturation binding isotherm by weighted non-linear regression (Mendel & Mendel, 1985). Thus the three parameters of the model, namely the maximal specific binding, the equilibrium constant K_d and the parameter α of the non-specific binding, are determined without any hypothesis on the mode of action of irradiation and at each dose independently. For each parameter, the Spearman rank correlation coefficient is then computed to test for the existence of an association between this parameter and the radiation dose (Siegel, 1956). The inactivation curve is then plotted, where the residual binding activity is defined as the maximal specific binding determined by the fitting to the binding isotherm. A straight line is fitted by linear regression to the inactivation curve according to the well-known target-size analysis. A runs test is performed on the residuals in order to test for a systematic deviation of the inactivation curve from linearity (Siegel, 1956).

RESULTS

Fig. 1 shows the inactivation curves generated by the stochastic model for different radiation efficiencies and ligand concentrations. For a maximal efficiency ($\lambda = 0$), the curve is linear and corresponds in fact to the single-hit model. In this case, the fractional survival activity does not depend on the ligand concentration. For decreasing efficiencies ($\lambda > 0$), the inactivation curve exhibits a curvature with a downward concavity, reminiscent of a classical multi-hit process. At a given λ value, this curvature is more pronounced when the ligand concentration is high.

Fig. 2(a) shows the saturation binding isotherms

Fig. 2. Numerical simulation of an experimental system obeying the stochastic theory with $\lambda = 1$ and with a random error of 5% (see the Methods section for further details)

Panel (a), binding saturation isotherms at different radiation doses. The numbers represent the data points and are equal to the dose expressed in 10^7 rad. The curves were obtained by weighted non-linear regression. Panel (b), inactivation curve deduced from the same data. The straight line was obtained by linear regression.

Table 1. Results of the weighted non-linear regression of the data presented in Fig. 2(a)

D (Mrad)	$[R_d]$	K_d	α
0	0.95	0.97	0.012
10	0.61	0.92	0.028
20	0.48	1.52	0.032
30	0.42	1.8	0.015
40	0.42	3.6	0.013
50	0.11	1.5	0.024
60	0.10	2.4	0.018
70	0.03	1.22	0.020
80	0.03	2.5	0.016
r^*	-0.97	0.53	0.033

$P^\dagger < 0.005$ Non-conclusive Non-conclusive

* Spearman rank correlation coefficient

† One-tailed test with $n = 9$.

obtained at different radiation doses with $\lambda = 1$ and when a random error is introduced. The numerical results of the weighted non-linear regression are presented in Table 1. The Spearman rank correlation test rejected the non-correlation with the radiation dose at a 5% level of confidence only for the maximal specific binding. Neither the specific binding affinity nor the α parameter describing the non-specific binding can be demonstrated to be associated to the radiation dose. Thus the residual maximal specific binding was used to draw the inactivation curve (Fig. 2b), which was then fitted by linear regression. The runs test was not able to reject the linearity of the inactivation curve.

Similar conclusions may be obtained for lower values of λ , i.e. when the deviation of the residual binding activity from a pure mono-exponential law is less pronounced (details not shown). On the other hand, if λ is increased to 2, the linearity of the inactivation curve cannot be rejected in general, but a significant increase of K_d is observed for increasing values of radiation dose, suggesting that the binding affinity is impaired by irradiation (details not shown). However, the accuracy of the data has a prime importance concerning the conclusions of the statistical tests, since a 10% coefficient of variation for the binding data renders the Spearman test non-conclusive in what concerns the correlation between K_d and D when $\lambda = 2$ (details not shown).

DISCUSSION

The modification of proteins and the consequent impairment of their activity have been widely studied by using various techniques [see Rakitzis (1984) for a review]. Most of them involve modifications of the protein at specific loci, leading to a complete or partial loss of activity. Inactivation by irradiation has the particularity that the attack by the ionizing particle occurs randomly in the protein. Up until now, it has been assumed that one hit is sufficient for a complete inactivation of the protein. It has been argued that the existence of this deterministic process is the consequence of the large amount of energy deposited in the protein (Kempner & Schlegel, 1979). However, Simic & Dizdaroglu (1985) have shown that more subtle changes may occur between macromolecule components irradiated

by the commonly used ^{60}Co γ -radiation source, suggesting that the radiation-induced modifications are confined to narrow domains of the proteins. Although it is certainly possible that such modifications may drastically affect the activity, especially when they occur in the vicinity of the active site, there is no reason to believe that any hit at any place will systematically lead to a complete inactivation of the protein. It is the role of the proposed stochastic theory to describe the progressive inactivation by irradiation. Within this framework, the effect of temperature on the radiation-sensitivity of proteins may be tentatively interpreted. Indeed, it is conceptually acceptable that the gravity of the physicochemical modifications subsequent to a primary ionization is temperature-dependent, and thus the inactivation could be, on average, more pronounced at higher temperatures. In an all-or-none process, as depicted by the single-hit concept, the efficiency of the hit is maximal. Hence a temperature increase cannot induce a further increment of efficiency.

In a mathematical point of view, the stochastic theory adds a corrective term to the law of activity loss obtained in the single-hit theory (eqn. 14). This term results from the introduction of a transition probability (eqn. 6) that describes the chance that the K_d of the binding protein is increased by a certain multiplicative factor when the protein is hit. In the absence of any knowledge concerning the nature of the radiation-induced modifications, the function describing this probability has been arbitrarily defined. Although the shape of the inactivation curve may depend on the choice of this function, the present study shows that such functions may be found, leading to apparently linear inactivation curves. With a λ coefficient equal to 1 and a random error of 5% in the binding assay, the data obtained by simulation are very similar to those reported by Shorr *et al.* (1984) on the β -adrenergic receptor. On the basis of the usual statistical tests, it would have been concluded that the single-hit model is acceptable, although the data were generated by the stochastic theory. Now, a λ value equal to 1 means that one hit produces at most a 10-fold increase of K_d in 90% of the cases. In these conditions and for a ligand concentration 10 times the native K_d , the average number of hits per receptors (σD) required to reach a 37% residual activity is equal to 4 (Fig. 1). At this ligand concentration, the mean efficiency of one hit is thus 4 times lower than the one characterizing the single-hit process. Thus the attempt at distinguishing between the two theories is not a purely academic problem.

CONCLUSION

In this study, the generally accepted single-hit theory is challenged by a new theory, which postulates that the radiation efficiency for inactivating the protein is not maximal. Indeed, the local nature of the radiation-induced modifications in the protein and the correlation between the radiation-sensitivity and the temperature during irradiation suggest that a complete inactivation of the unit may not be systematically due to a single hit. As proposed in the stochastic theory, besides the probability that a protein is hit (eqn. 7), the efficiency of the hit to inactivate the protein must be taken into account in the evaluation of the radiation-sensitivity. Nothing indicates that the mathematical description of this efficiency in the proposed theory is correct (eqn. 6). However, if it is true

that this efficiency is not maximal, and the current data cannot reject this assumption, it has to be experimentally demonstrated that the efficiency is independent of the protein nature. If this is not the case, the protein with a very different efficiency cannot be adequately characterized by the target-size analysis. Up until now, the discrepancies between the molecular masses determined by the target-size analysis and by other techniques were generally attributed to the existence of a functional unit consisting of a part of the macromolecule or of an aggregation of units.

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