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CURRENT PROBLEMS IN RESEARCH

Molecular Basis for Action of Ionizing Radiations

A simple model describes the inactivation by ionizing radiations of molecules in the living cell.

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From an early date in its development, radiation biology has tried to give explanations in molecular terms of various phenomena observed. Indeed, in their attempts in this direction radiation biologists must be reckoned as pioneers in opening up the explosively developing field of modern molecular biology.

The Target Theory

It was recognized early that certain simple biological systems, such as a suspension of bacteria, when irradiated, lost a measurable property—for example, the ability to multiply and form colonies—as an exponential function of radiation dose (see Fig. 1). Usually in these cases the effect was also found to be independent of dose rate. Such results were quickly correlated with a knowledge of the physical properties of ionizing radiations to produce the target theory of radiation action, as first propounded by Blau and Altenburger (*1*), Dessauer (*2*), Crowther (*3*), and Condon and Terrill (*4*).

The important characteristic of ionizing radiations in the target theory is the localized release of comparatively large amounts of energy, as graphically illustrated in the cloud chamber photograph shown in Fig. 2. Each water droplet represents the expenditure of 30 to 100 electron volts of energy, as may readily be determined by counting the number formed from the absorption of a known amount of radiation. Such an energy is large as compared to the energies of a few electron volts associated with even the strongest chemical bonds, and it is reasonable to assume that the immediate neighborhood of an ionization will be disrupted sufficiently to prevent the structure from carrying out a highly specific biological process such as an enzymatic reaction.

If the volume of a biological structure or target which must be intact to carry on a process is V, and the dose D is expressed in terms of primary ionization events per unit volume, then the mean number of inactivating events per target is easily seen to be VD. Some targets will receive a number of "hits" close to the mean number, others will receive more or fewer hits because of the random nature of the process. The most interesting class is that comprising the targets which have received no hits. The fraction of such targets is readily calculated from statistical considerations to be e^{-VD}, and this relationship immediately provides a simple explanation for the exponential survival curve. The independence of dose rate follows from the all-or-nothing nature of the assumed mechanism.

A plot of the survival of more complex organisms as a function of dose is usually a sigmoid (so-called from its shape when plotted on linear graph paper) with a shoulder at low doses, as shown in Fig. 1. This is readily interpreted by a theory postulating either multiple targets or multiple hits in the same target. The target theory received its full expansion in two books which appeared just after World War II, *Actions of Radiations on Living Cells*, by D. E. Lea (*5*), and *Das Trefferprinzip in der Biologie*, by N. Timofeeff-Ressovsky and K. G. Zimmer (*6*).

The Diffusion Theory

Even as the target theory was approaching its prime, a second theory began to arise. An offshoot of nuclear energy studies after 1945 was the extensive development of the understanding of the radiation chemistry of water, a development sparked particularly by strong research groups at the various Atomic Energy Commission laboratories. It became increasingly clear that the action of ionizing radiation on water resulted in the formation of the highly reactive free radicals H and OH.

$$H_2O \rightarrow H + OH$$

Since a living cell is 80 percent water, it is reasonable to assume that most of the absorbed energy will be used to form such water radicals, which can diffuse about the cell to produce other free radicals on organic molecules. These organic radicals again will diffuse before they form an even less reactive set of radicals, thus spreading the effects of the radiation further and further. This point of view received a tremendous impetus from the early work of Dale (*7*) and others (*8*),

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Table 1. Variation in radiosensitivity (relative to that in the intact cell) of the enzyme invertase with stage of purification (*31*).

Stage of procedure	Radiosensitivity on purification by the method of		
	Dieu	Sumner	Fisher
In cell		1 ± 0.05	
Crude extract	1.48	1.43	0.83
Dialyzed extract	0.80		0.87
Precipitate	0.63		1.05
Final product		0.78	0.53

in which a dose of the order of a few hundred roentgens was found to destroy the activity of sufficiently dilute enzyme systems as effectively as it destroyed individual living cells. Clearly, the effects on dilute enzyme systems were entirely the result of diffusion of radiation-produced radicals, and the presumption was that the same process might occur also in cells.

Thus in a "diffusion" theory, as opposed to the "target" theory, radiation action was ascribed solely to the widespread diffusion of radiation-produced radicals. Specific phenomena of radiobiology, such as the effect of oxygen concentration and of various protective compounds, were interpreted in terms of changes in radical concentrations. This mechanism was sometimes referred to as "indirect action," as contrasted with the "direct action" underlying the target theory.

Although both of these theories describe experimental results in terms of molecular events, relatively little direct evidence, on the whole, was available for the mechanisms at work at the atomic level in the cell. Many workers, particularly D. E. Lea, saw the need for such data, but the largest single accumulation of data was inspired by a somewhat different viewpoint.

Direct Action on Molecules

Ernest C. Pollard, at Yale, pointed out that with sufficient knowledge of the physical and chemical processes taking place, ionizing radiations could be used to study the living cell (*9*). This concept was foreshadowed by several investigators, notably Lea and Zimmer, but Pollard and his associates proceeded to amass a considerable number of the data necessary to make the idea a usable one (*10*).

Briefly, a large number of biological molecules were irradiated in the dry state with ionizing radiations. The survival of a specific biological property, such as enzymatic activity, was found to be exponential with dose, and independent of dose rate. From the slope of the survival curve, the target volume could be calculated. This required a knowledge of the energy needed to produce a primary ionization, which was taken to be 100 electron volts, the value deduced from measurements of ionization in gases (*11*). The use of this figure leads to a simple relation between the dose in rads (or roentgens) necessary to reduce activity to 37 percent of its initial value, D_{37}, and the mass M of the target volume, expressed in molecular weight units:

$$D_{37} \times M = 0.7 \times 10^{12}$$

It is clear that this relation predicts the result one intuitively expects—that a large dose will be needed to inactivate a small target, and vice versa.

Figure 3 illustrates some of the results. The known molecular weights of a number of molecules are plotted horizontally, and the equivalent molecular weights of the target volume, vertically. The straight line was drawn on the assumption that the two volumes are the same. The agreement over four

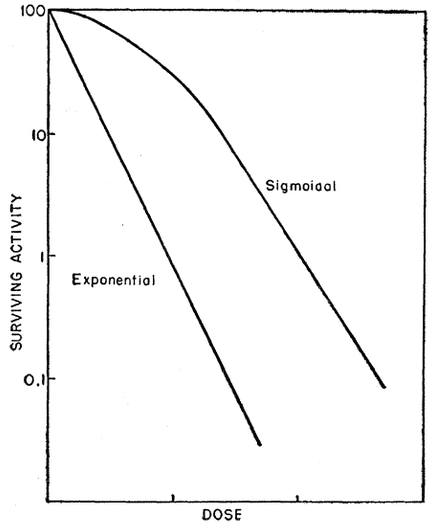

Fig. 1. Typical curves for the survival of biological activity, plotted vertically on a logarithmic scale against radiation dose on a linear scale.

orders of magnitude is impressive, and it is apparent that the target theory hypothesis is well supported.

The variations of the points from the line are larger than the experimental errors, showing that other factors are at work. The four points connected by a dashed vertical line are for the enzyme catalase at temperatures (reading from the bottom up) of —180°, 20°, 80°, and 112°C (*12*). Another sort of change is shown in Table 1, in which is listed the radiation sensitivity of the enzyme invertase at various stages of purification from yeast cells. However, it is clear that the changes in the sensitive volume are of the order of a factor of 2. This corresponds to energy transfer over a distance only a fraction of the dimensions of the sensitive volume, so that the target concept is still essentially valid.

The basis for these results might still be questioned on the grounds that ionizing radiation produces large numbers of excitations, as well as ionizations. The question is, Why are these not counted in as inactivating events? However, Setlow (*13*) has measured the number of enzyme molecules inactivated per photon absorbed, or excitation produced. For most ultraviolet radiation, the yield stayed within the order of 10^{-2} to 10^{-3} even well down into the vacuum ultraviolet. However, for photon energies of the order of 10 electron volts, the yield increased sharply toward unity. Similarly, irradiating monomolecular layers of protein with very low

Table 2. Radiation doses needed to inactivate enzymes in cells (*32*).

Enzyme (cell)	Dose (Mrad) to reduce to 37% of original activity		Yield (in molecules per 100 electron volts) in dilute solution (G)	Diffusion distance ρ(A)
	Dry	Wet		
Invertase (yeast)	12	6	0.15	29
Alcohol dehydrogenase (yeast)	28	1.3	3.0	31
Coenzyme A (yeast)	200	3	2.7	35
Coenzyme A (*E. coli*)		15	2.7	17
Coenzyme A (peas)		5	2.7	30
Coenzyme A (beef heart)		>100	2.7	<5
Coenzyme A (beef liver)		>100	2.7	<5
Acetylcholinesterase*	4.8	4.8		∽0

*See Cotzias and Serlin (*33*).

voltage electrons produced little loss in activity until the electron energy exceeded 10 electron volts, when the efficiency rose rapidly (*14*). In both these cases the presumption is that the removal of an electron is, for practical purposes, the effective event in biological action of ionizing radiations, and this justifies the use of 100 electron volts as the measure of the inactivating event.

The utility of this information for biological studies is very great. For example, one can use this method to determine the approximate molecular weight of a substance which has some specific assayable property. The unique feature is that the substance does not have to be purified in any way, since the measured radiation-sensitivity does not depend greatly on the surrounding medium, provided only that the substance be dried to prevent diffusion of water radicals.

The magnitude of the direct action on molecules such as enzymes in cells can be determined by the irradiation of *dry* cells. Table 2 lists the doses necessary to reduce the activity of some enzymes irradiated in dried cells to 37 percent of the original activity. The results are in good agreement with the target hypothesis, the larger enzymes (such as invertase) requiring lower doses than do small molecules such as coenzyme A.

If the cells are now irradiated in the normal wet state, the doses needed to inactivate drop greatly, as shown in Table 2, column 3. The most obvious assumption is that the diffusion of water radicals is contributing to the inactivation process. The relative proportions of the two processes, of indirect to direct action, can be seen to vary enormously from one molecule to another. For the first three entries, for different enzymes in yeast cells, for example, the ratio of dry to wet dose varies from 2 to 1 for invertase, to 20 to 1 for alcohol dehydrogenase, to 100 to 1 for coenzyme A.

A Simple Theory

This information can be fitted into a simple conceptual scheme in the following way. In the first place, different molecules have different sensitivities to inactivation by water radicals. Such sensitivities can be measured by determining the number of molecules inacti-

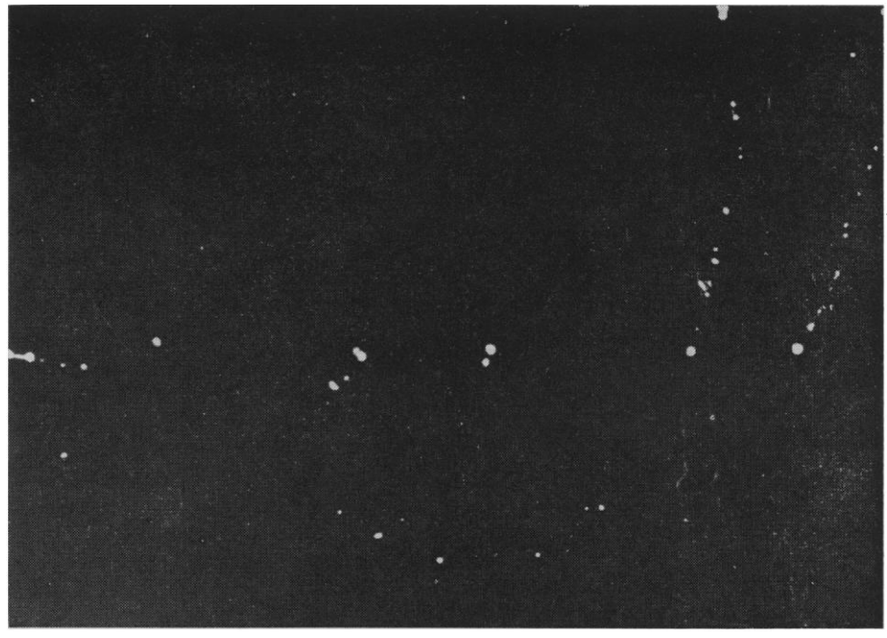

Fig. 2. A cloud-chamber photograph of ionizations produced by x-rays. [From P. Auger, *Ann. phys.* **6**, 183 (1926), as reproduced from *An Atlas of Typical Expansion Chamber Photographs*, W. Gentner, H. Maier-Leibnitz, W. Bothe, Eds. (Pergamon Press Limited, London, 1954), reproduced with permission].

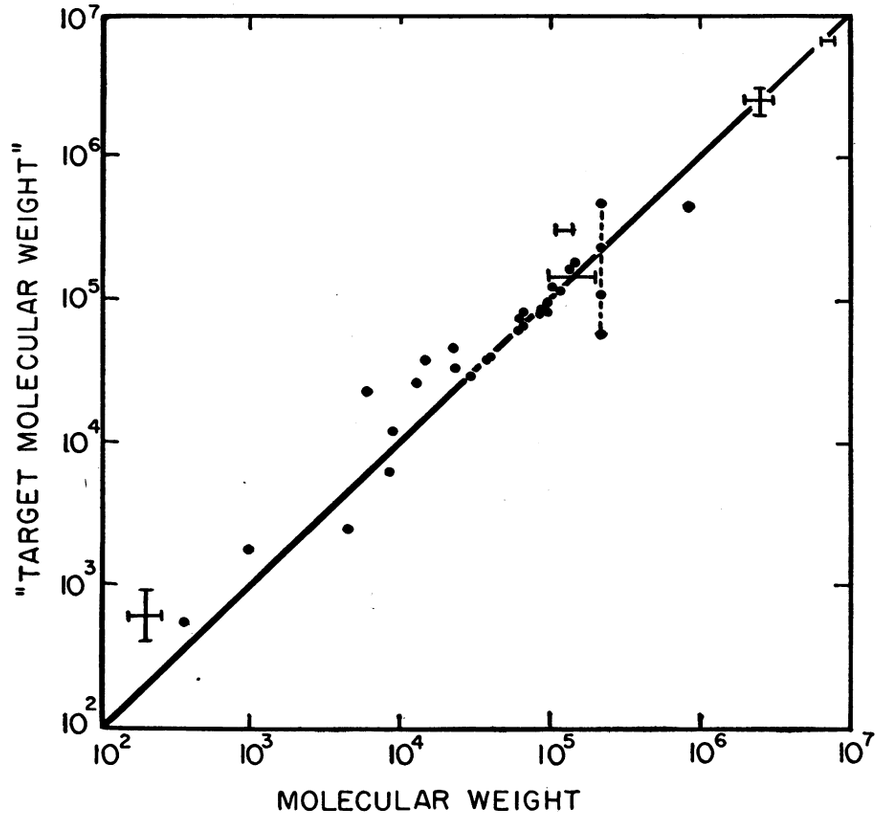

Fig. 3. The radiation target size, given in molecular-weight units for a variety of biological molecules irradiated in the dry state, plotted against the known physicochemical molecular weight. The straight line is the expected relation if the two molecular weights are equal. The data plotted are those given in Table 2 of E. C. Pollard *et al.* (*10*). The dashed vertical line connects four points representing the target size of the enzyme catalase at different temperatures (see text).

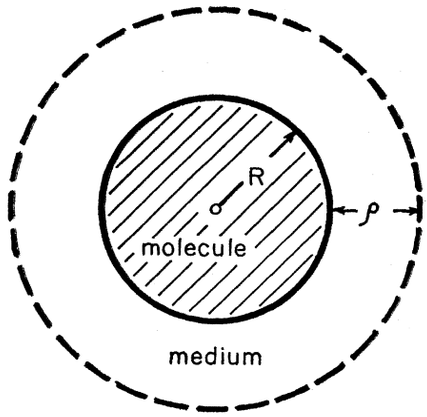

Fig. 4. A simple picture of radiation action on a molecule in a cell. The molecule, shown schematically as a sphere of radius R, is surrounded by a water layer of average thickness ρ. Water radicals from this water layer contribute to the inactivation of the molecule.

vated per unit dose delivered to a dilute solution, where the only process is the indirect effect. This yield G, in molecules per 100 electron volts of energy absorbed, is given in Table 2, column 4.

The requirement for large doses to inactivate enzymes in wet cells, as compared to the doses needed in dilute solutions, results from the removal of radicals by reaction with materials in the cell. Let ρ be the mean distance that the radicals diffuse through the cell before they disappear. Then, by a simple theory first worked out by Zirkle and Tobias (*15*), the magnitude of the indirect effect and the yield in dilute solution may be used to calculate the value of ρ, with only the additional assumption that the radical reacts on first encounter with the molecule being assayed. Values of ρ are listed in Table 2, column 5. The fact emerges that many of the measured values are remarkably constant and of the order of magnitude of 30 angstroms.

The assumption made in this calculation—that the radiation-produced radicals react on essentially all collisions with the target molecule—is reasonably supported by two lines of evidence. The most convincing is the recent set of measurements, by Harold Schwarz at Brookhaven (*16*), of the absolute reaction rates between hydroxyl radicals and H_2 and H_2O_2. From these measurements and a number of previously determined ratios of rate constants (*17*) it can be determined that the probability that the hydroxyl radical will react with most organic molecules is between .1 and 1.0. This value is in good agreement with earlier, less reliable, data obtained by a pulse technique (*18*). Since the calculated value of ρ depends directly on the square root of the probability, the change in the value of ρ will not be large.

The other evidence is that the observed radical diffusion distance is roughly the thickness of water which must surround the average macromolecule in a cell to account for the 80 percent of water included. The calculation here is one of order of magnitude only. From the known macromolecular composition of cells, the sizes of these molecules, and the known distribution of carbohydrate in cell walls and lipoproteins in membranes, we find that the average thickness of water in the cytoplasm is of the order of 15 to 50 angstroms about each macromolecule. The range of thicknesses reflects widely different assumptions about the ways in which the macromolecules are arranged.

The whole situation then reduces to the rather simple picture shown in Fig. 4. A particular molecule, shown as a sphere of radius R, will be inactivated if a primary ionization occurs within its volume. Radicals formed in the volume of water [equal to about $4\pi R^2\rho(1 + \rho/R)$] immediately surrounding the molecule, will also cause inactivation. The efficiency will be determined by the measured yield G. Thus the effective inactivation volume, on a target theory basis, will be

$$4\pi R^2\rho G(1 + \rho/R) + \frac{4}{3}\pi R^3$$

The application to molecules of other shapes, such as long thin rods, follows directly from the discussion. The parameters R (molecular radius) and G (the yield for radical attack) are properties of the molecule, to be determined by suitable experiments. The parameter ρ, measuring the distance a radical can diffuse, is a function of the cell environment. It can vary from essentially zero for a molecule so located that it comes in contact with no water, as appears to be the case for acetylcholinesterase (see Table 2), to a value of the order of 30 angstroms.

Thus, it would appear that *both* the earlier theories are required to explain radiation action on a molecular basis and that a synthesis of the two appears to satisfy the experimental data. However, in essence the final result most closely resembles the target theory,

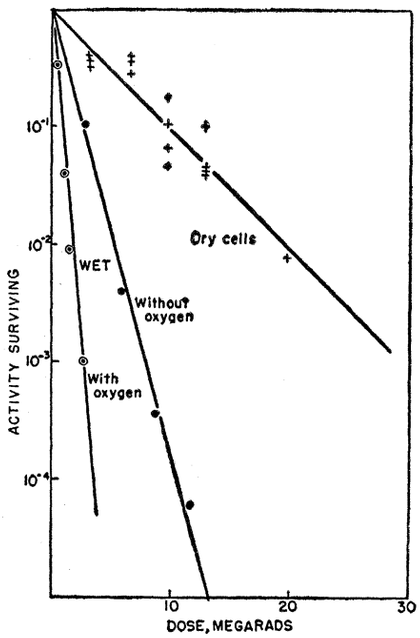

Fig. 5. The inactivation of a property (ability to transform) of DNA when irradiated under various conditions in a cell (*21*).

except that now the target has fuzzy edges.

The major point of this article has now been made. A large number of radiobiological data can readily be fitted into this scheme. There are also data which do not fit in so readily. Let us discuss a case which at first sight appears to be inconsistent but which, when the details are examined, is actually in complete agreement.

Oxygen Effect in Cells

A well-established phenomenon in radiobiology is the ability of oxygen to increase the radiation sensitivity of a wide variety of living cells by a factor of 2 to 4 (*19*). This enhancement is so general that the most logical conclusion is that the process operates at the molecular level. Indeed, the radiation sensitivities of molecules in cells, such as the enzyme invertase in yeast cells (*20*) and the deoxyribonucleic acid (DNA) transforming principle in pneumococcus cells (*21*), are increased by a factor of about 3 in the presence of oxygen.

From the picture formulated it is clear that this increase must be either in the direct action, in the indirect action, or in both. An increased sensitivity in direct action—that is, enhancement of radio-sensitivity by about

a factor of 3 for materials in the dry state—has been demonstrated for a number of enzymes (*22*). However, the effect of oxygen on the indirect action of water radicals has been carefully measured for several enzymes in dilute solution (*23*), and only for a single one, deoxyribonuclease, has an enhancement with oxygen been reported. Deoxyribonucleic acid extracted from pneumococcus cells has been studied with extreme care in dilute solution (*24*). When a specific biological property of the DNA—the ability to transform (*25*)—was measured, no effect of oxygen on the radiosensitivity in dilute solution could be found.

Figure 5 shows that for DNA irradiated in pneumococcus cells, about one-third the total effect is presumably direct action occurring in dry cells, and that the indirect effect accounts for more than half the total inactivation in wet cells. Yet a full threefold increase in radiosensitivity is found in the presence of oxygen.

If the indirect effect does not increase in the presence of oxygen, then how is the oxygen effect on DNA in the cell to be explained? The answer is shown in Fig. 6. If compounds containing sulfhydryl groups, —SH, are added to dilute solutions of DNA or enzymes, the radiosensitivity is found to increase when oxygen is bubbled through the solution. This, of course, provides an interesting bit of information on the mechanism of the oxygen effect. In the present context, the significance is that since living cells contain sulfhydryl groups, the magnitude of the oxygen effect in vivo can be well correlated with the effects in vitro and there is no disagreement with the concept developed in this article.

Difficulties with Proposed Model

The model of radiation action that has been presented is so easy to visualize that it is too bad nature will not let us take it literally. Several well-verified experimental results show that certain factors have been left out.

Change in direct effect with surrounding medium. The data in Table 1 clearly indicate that the radiation sensitivities of dry molecules are a function of the surrounding medium. This could be the result of energy transfer into certain molecules, causing increased radiation sensitivity; energy transfer out, to give protection; or changes in the physical state of a given molecule which make it more or less sensitive to a given amount of energy released within its structure. At the moment the effect is not an enormous one, but it would be worth while to try to understand the factors involved.

The mechanism of the oxygen effect in direct action. It has been assumed above that every molecule receiving a primary ionization is inactivated. The increase in radiation damage in the presence of oxygen is then hard to understand. Although other explanations are possible, the simplest is one given by Howard-Flanders (*19*)—that the actual number of primary events is higher than calculated and that only a fraction of them are manifested in the absence of oxygen. Whatever the true explanation, at least one more assumption will have to be added to the model suggested in this article.

The variation in radiation sensitivity with temperature. The radiation sensitivity of a number of enzymes in the dry state varies with temperature in much the same way that the sensitivity of catalase does (see Fig. 3). It is possible that this phenomenon, and the

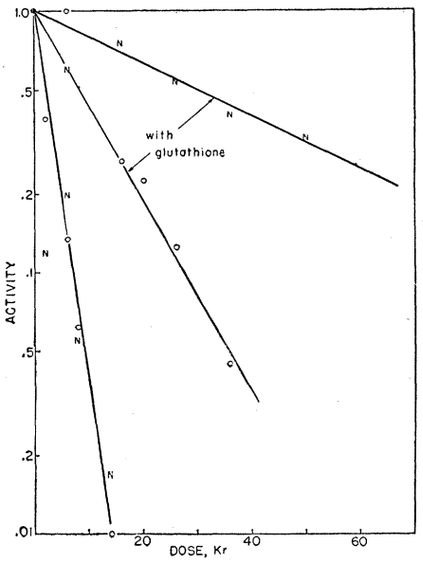

Fig. 6. Inactivation in dilute solution of the ability of pneumococcal DNA to transform. The steep curve to the left shows that inactivation is the same when the irradiation is carried out under a nitrogen (N) or an oxygen (O) atmosphere. When glutathione is added, the decrease in sensitivity is caused by a relatively trivial event, the removal of water radicals by reaction with glutathione. The significant point is that the radiosensitivity is then different in oxygen and in nitrogen (*23*).

two others also, involve energy transfer over distances of perhaps 10 to 30 angstroms. In the absence of accurate knowledge, the factors just discussed represent uncertainties of the order of a factor of 2 in the effects to be expected from a given dose of radiation.

Areas of Inadequate Information

Whereas these factors are known to complicate the simple picture, there are others whose importance cannot be determined because of lack of sufficient information.

Change in the direct effect in the presence of water. Since the direct effect does change with the nature of the surrounding medium, it is quite possible that the presence of water may change the response of a structure to an ionization within it. Closely related to this is the result of energy release in water molecules which are firmly bound to a macromolecule. There are some indications that these considerations are important. Okada (*26*) has found that the yield (number of molecules inactivated per 100 electron volts of energy absorbed) drops when an equal mass of water is absorbed onto a dry enzyme preparation. It has also been reported that the degradation of polysaccharides is less efficient in the presence of a small amount of water than in the dry state (*27*). Suitable experiments to measure any change in direct effect with hydration are badly needed.

Energy required per primary ion cluster. The figure of 100 electron volts which has been used for energy required per primary ion cluster was obtained from gas data only. Recent considerations have cast doubt on the validity of using gas data for effects taking place in condensed (liquid or solid) phases. On the experimental side, modern techniques (*28*) have made it possible to pass a monoenergetic beam of 5- to 20-kilovolt electrons through a foil so thin (approximately 100 angstroms) that the average electron makes only a single interaction, and to analyze, in energy, the transmitted beam with sufficient accuracy to detect losses of even an electron volt or so. A typical curve of numbers plotted against energy loss is shown in Fig. 7. The analyzers used so far for these purposes accept electrons only within a very narrow angle of scattering, so that the total

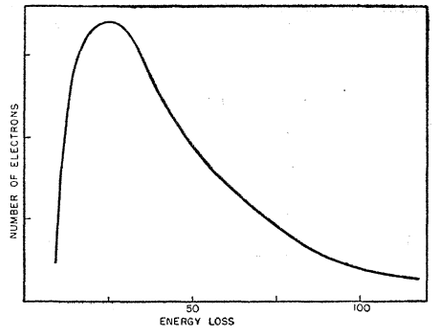

Fig. 7. A typical plot of number of electrons against the energy (in electron volts) lost by the electrons in passing through a very thin plastic foil (*28*).

number of electrons losing a given amount of energy can be determined only by summing data over many angles.

The data obtained reveal two interesting facts. One is that in solids very few electrons lose an amount of energy between 2 and 10 electron volts, whereas in gases losses of energy (corresponding to excitation of molecules) in these amounts are the most probable loss events. Second, from crude data taken on Formvar films and integrated over all angles, it appears possible that the mean energy loss per interaction event may be considerably smaller than the 100 electron volts determined from the gas data.

On the theoretical side, in a recent paper Fano (*29*) has proposed a possible explanation of both these effects, based on the mutual interaction of excited states of molecules which are located sufficiently close together, as in the solid state. Better understanding of this matter, and particularly a reliable value for the mean energy per primary interaction event, is clearly needed. Experiments along these lines are underway at present.

The fate of the ejected electron. In direct action, an almost mystical significance is ascribed to the act of ionization—that is, the actual separation of an electron from the parent molecule. This significance is based on sound enough reasoning, since the vacuum ultraviolet and the very low energy electron experiments both tend to indicate a rapid rise in inactivation efficiency as available energy passes the level of ionization energy—about 10 electron volts for many organic molecules. A better understanding of why this is so probably hinges on knowing what happens to the ejected electron.

In indirect action the ejected electron is usually believed to interact with water in some way to form the species customarily referred to as the H atom. The spatial relationships of the OH and H radicals at formation are hotly in dispute at the moment. If the electron returns to the parent ion, as some maintain, the OH and H radicals will be formed close together, and probably in about the same yields within the cell and in dilute solutions. On the other hand, if the electron wanders away from the ion, it may be captured 10 to 100 angstroms away from the OH radical formed near the positive ion. In fact, in the cell it may not even be captured by a water molecule, but may be captured by part of the solid phase, so that few of the species classed under the name "H radical" would be formed at all in the cell.

The biological effect of any low-energy free electrons formed either from water or from the direct effect is also of interest. The one bit of information available about this is that electrons of energies below 10 electron volts, as mentioned before, seemed to have very little effect on protein monolayers (*14*).

Over and above these points there is the consideration of the basic mechanism of inactivation. If the target volume is the whole molecule, does this mean that all of the molecule is necessary for a specific biological action, or is energy transferred into the active site? If the latter supposition is right, why is the energy transferred to the active site and not to some unessential part? Possibly a knowledge of the effects of ionizing radiation will contribute to an understanding at the molecular level of the whole problem of biological specificity.

Conclusion

While it is clear that much more needs to be known about detailed processes, it seems likely that the general picture presented in this article will provide a convenient framework within which to order many of the phenomena treated in radiobiology. Not only does this picture incorporate many of the concepts found useful in earlier theories but it also has the value of being simple, concrete, and easily visualized. From the theory, a reasonable estimate may be made of the effect of a given dose of radiation on a specific kind of molecule in a cell. Conversely, from the immediate effects produced by a certain dose, some estimate may be made of the mass of intracellular material which must be involved in the processes which are assayed for. Perhaps these advantages will suffice to carry the theory over its many difficulties and deficiencies until another and more encompassing viewpoint can be reached (*30*).

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