Molecular Mass and Volume in Radiation Target Theory

James C. Osborne, Jr.,* Jay H. Miller,† and E. S. Kempner†
*Beckman Coulter, Fullerton, California 92835, and †Laboratory of Physical Biology, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, Maryland 20892 USA

ABSTRACT Radiation target analysis is based on the action of ionizing radiation directly on macromolecules. Interactions of this radiation with the molecules leads to considerable structural damage and consequent loss of biological activity. The radiation sensitivity is dependent on the size of the macromolecules. There has been confusion and discrepancy as to whether the molecular mass or the molecular volume was the determinant factor in the sensitivity. Some proteins are known to change their hydrodynamic volume at low pH, and this characteristic can be utilized to compare the radiation sensitivities of these proteins in the two states. The results show that the radiation sensitivity of proteins depends on the mass of the molecule and is independent of the molecular volume/shape.

INTRODUCTION

Radiation target analysis is based on the action of ionizing radiation directly on macromolecules (Lea, 1955; Pollard et al., 1955). With the use of gamma rays or high-energy electrons, the method has been used to determine the size of many different proteins (Kempner and Schlegel, 1979; Kempner, 1988).

Crowther's original exposition of target theory (1924) noted the exponential loss of biological activity with ionizing radiation:

$$A = A_0 e^{-IV}$$

where I refers to the number of ionization events caused by the radiation. This was originally measured in terms of roentgens, a unit defined in terms of ionized pairs generated by radiation in a cubic centimeter of air at STP. Thus the V term in the exponent had to have the units of volume. From this it followed that there was a radiation-sensitive "volume" that was associated with the specific biological activity. This concept was used universally for the next 60 years. However, objections to the definition of the roentgen unit arose in the 1920s. A gamma ray or an electron traveling through free space does nothing; only when matter is present can there be an interaction with this radiation, and the more matter that is present, the greater the chance of an interaction. This led to a redefinition of the "roentgen" unit (and its successor units, such as the rad or Gray) in terms of ionizations per gram. However, the idea of a radiation-sensitive volume persisted (Lea, 1955; Pollard et al., 1955; Kepner and Macey, 1968; Kempner and Schlegel, 1979; Jung, 1984). It has been suggested that the volume concept was fundamentally incorrect and a more appropriate description is the following equation:

$$A = A_0 e^{-qmD}$$

where the radiation dose D is in rads (defined as ionizations per gram of material), m is the mass of the radiation-sensitive unit, and q is a constant (Kempner, 1988).

A fundamental assumption in radiation analysis is that each primary ionization (a random event) leads to a loss of activity/structure of that molecular unit. Experimentally one measures the surviving molecular units as a function of radiation dose. According to the Poisson distribution, the frequency of molecules with zero events is e^{-x} where x is the average number of events per molecule. Thus the number of intact molecules (A) is equal to the number of original molecules (A_0) times the negative exponent of the number of primary ionizations (PI) per molecule (m):

$$A = A_0 e^{-(PI/m)} \quad (1)$$

The radiation dose, D, is usually measured in rads, a unit that is defined as that amount of radiation that deposits 100 ergs/g material. Our doses are normally in megarads, therefore,

$$D = 1 \times 10^6 \times 100 \text{ erg/g}$$

Converting ergs to primary ionization events, using the constant q, gives

$$q = 6.24 \times 10^{11}(\text{eV/erg}) \times (\text{PI}/65 \text{ eV})$$

which results in

$$Dq = (6.24 \times 10^{19}/65)(\text{erg/g}) \times (\text{eV/erg} \times (\text{PI/eV})$$

Dividing by Avogadro's number (N), one has

$$Dq/N = (6.24 \times 10^{19}/(65 \times 6.023 \times 10^{23}))$$

$$\cdot (\text{PI/molecule}) \times (\text{mole/g})$$

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Address reprint requests to Dr. Ellis S. Kempner, Laboratory of Physical Biology, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Building 6, Room 140, Bethesda, MD 20892-2755. Tel.: 301-496-6941; Fax: 301-402-0009.

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Multiplying by M, the molecular mass, we obtain the primary ionization per molecule:

$$DqM/N = (6.24 \times 10^{19}/\{65 \times 6.023 \times 10^{23}\}) \cdot (\text{PI/molecule}) \times (\text{moles/g}) \times (\text{g/mole})$$

We can restate Eq. 1 as

$$A = A_0 e^{-(\text{DqM/N})} \quad (2)$$

The unknown in Eq. 2, the molecular mass, is obtained from the slope of a plot of the fraction remaining molecules versus radiation dose. This implied that the volume of the unit per se was not involved. The original volume concept has not been abandoned and some radiation studies still refer to the radiation-sensitive volume (Bradbury and Zammit, 1990; Lidzey et al., 1995).

There are proteins that significantly change their hydrodynamic properties at different pH. Clearly the molecular mass of the protein molecule is unaltered. Presumably there are conformational changes in the molecule with pH, and the quantity of associated solvent molecules is modified. If such proteins were irradiated at two appropriately differing pH values, the radiation sensitivity should change if the hydrodynamic volume is a contributing factor; no change in sensitivity would be observed only if the protein mass were the dependent variable.

Using these criteria, three different proteins at neutral or acid pH were frozen and irradiated. Two proteins were chosen because of their dramatic changes in hydrodynamic volume and shape (Charlwood and Ens, 1957; Shibata and Kronman, 1967; Champagne, 1957; Sogami and Foster, 1968; Tanford et al., 1955; Luzzatti et al., 1961; Raj and Flygare, 1974), and one because it showed no change (Tanford, 1968). Measurement of the amount of surviving monomers in these proteins when irradiated at either neutral or low pH permits quantitative determination of their radiation sensitivity in each condition.

MATERIALS AND METHODS

Chicken egg white lysozyme was purchased from Sigma (L-6876) as a lyophilized powder containing sodium acetate and sodium chloride. Bovine serum albumin from cow blood was obtained from Sigma (A-0281) as a lyophilized powder. Rabbit muscle glyceraldehyde 3-phosphate dehydrogenase from Sigma (G-2267) was supplied as a lyophilized powder from citrate buffer. Each protein was examined for contaminants with a size exclusion column as well as by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Lysozyme was essentially clean of other proteins and was used without further purification. Bovine serum albumin (BSA) and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) were refined by selecting the dominant peak from size exclusion chromatography. The buffer was changed to the appropriate dimethylglutarate buffer by use of Amicon Centricon 10 tubes. The SDS-PAGE of these samples showed only a single Coomassie-stained band.

For ultracentrifugation, proteins were dissolved in 0.15 M dimethylglutarate buffer at 0.15 mg/ml (lysozyme), 0.6 mg/ml (BSA), or 0.39 mg/ml (G3PDH). These concentrations were sufficient to produce an adequate optical density.

Lysozyme activity was measured as described in the Worthington manual.

G3PDH activity was measured as ΔOD_{340}/min in a solution of 0.1 M Tris (pH 8.4), 10 mM sodium arsenate, 1 mM NAD, 9 mM cysteine, and 0.50 μmol glyceraldehyde 3-phosphate at 25°C.

Size exclusion chromatography was performed on a Gilson high-performance liquid chromatography (HPLC) system, using a Pharmacia Superdex 200 column (10 × 300 mm). KPO_4 (0.05 M) + 0.35 M KCl (pH 6.5), at a flow rate of 0.4 ml/min, was used to elute the proteins. Column elution was monitored at 280 nm. Fractions of 0.25 ml were collected, and the peak samples were pooled.

For irradiation, 3,3-dimethylglutaric acid (Sigma D-4379) buffer (0.05 M) at pH 2.5 or pH 7.4 was used to bring each protein to 2.0 mg/ml (Kempner and Miller, 1994). Samples were held at pH 2.5 or 7.4 at 4°C for 4 hours to achieve equilibrium. Aliquots of 0.25 ml were placed in 2-ml glass ampules (Kimble 12010L-2) and frozen rapidly in crushed dry ice. Ampules were sealed with a gas-O_2 torch without thawing of the sample.

Samples were held at −80°C, except during irradiation at −135°C. Radiation exposures of 0–100 Mrads were obtained from 10-MeV electrons produced by a linear electron accelerator (Armed Forces Radiobiology Research Institute, Bethesda, MD) as described (Harmon et al., 1985).

After irradiation, vials were stored unopened at −80°C for several weeks until assay. Vials were opened, and the gas phase was allowed to exchange before the samples were thawed.

SDS-PAGE was performed as described (Miller et al., 1998). Aliquots of the irradiated proteins were electrophoresed and the gels stained with Coomassie blue. Densitometric scans of the gels permitted quantitative determinations of the amount of monomers surviving in each irradiated sample.

RESULTS

Dimethylglutarate buffer was used throughout these experiments because it exhibits pK values permitting it to be used as a buffer at both the neutral (pH 7.4) and acid (pH 2.5) values where two of the enzymes show dramatic changes in sedimentation constants.

The three proteins used in these studies were selected because of their reported sedimentation constants at pH 7.4 and pH 2.5. Each protein solution was frozen and thawed at neutral or at acid pH before sedimentation analysis. These samples confirmed the reported hydrodynamic properties (Table 1).

Lysozyme

Enzymatic activity and surviving monomers were determined in lysozyme samples irradiated at neutral pH. Samples irradiated at pH 2.5 were later thawed for similar determinations; activity measurements were performed at the same neutral pH that was used for the pH 7.4 irradiated samples. There was no loss of enzymatic activity due to either freezing and thawing, or to the freeze-thaw cycle at acid pH.

TABLE 1 Sedimentation analysis of the proteins used in this study

Protein	$s_{20,w}$ pH 7.4	$s_{20,w}$ pH 2.5
Lysozyme	1.71 ± 0.09	1.85 ± 0.11
Bovine serum albumin	4.34 ± 0.02	3.04 ± 0.03
Glyceraldehyde 3-phosphate dehydrogenase	7.86 ± 0.31	2.72 ± 0.36

Multiple independent radiation experiments were performed, each yielding reproducible results. The enzymatic activity of the lysozyme decreased as the same exponential function of radiation exposure at both pH values (Fig. 1). The amount of surviving lysozyme monomers was only slightly affected by exposure to high-energy electrons, regardless of the pH of the buffer (Fig. 2). The radiation target sizes calculated from the individual experiments are given in Table 2.

Bovine serum albumin

Because BSA possesses no enzymatic activity, only the surviving monomers could be monitored in irradiated samples. Multiple independent experiments yielded very reproducible results. In each experiment, parallel samples were irradiated at each pH. Data from four independent experiments were combined; Fig. 3 shows the fraction of initial monomers that remained after exposure to a range of radiation doses. The same results were obtained for samples irradiated at the two pH values. Table 2 gives the target sizes for loss of BSA monomers calculated from the individual experiments.

Glyceraldehyde 3-phosphate dehydrogenase

The enzymatic activity of G3PDH survived freezing and thawing at pH 7.4 without loss, but little or no activity was observed after exposure to pH 2.5, even without freezing. Only surviving monomers could be measured in samples of G3PDH irradiated at pH 7.4 and pH 2.5. Fig. 4 shows the combined results from four independent experiments. The target sizes calculated in each experiment at both pH values (Table 2) show no difference in the enzyme irradiated in the two states.

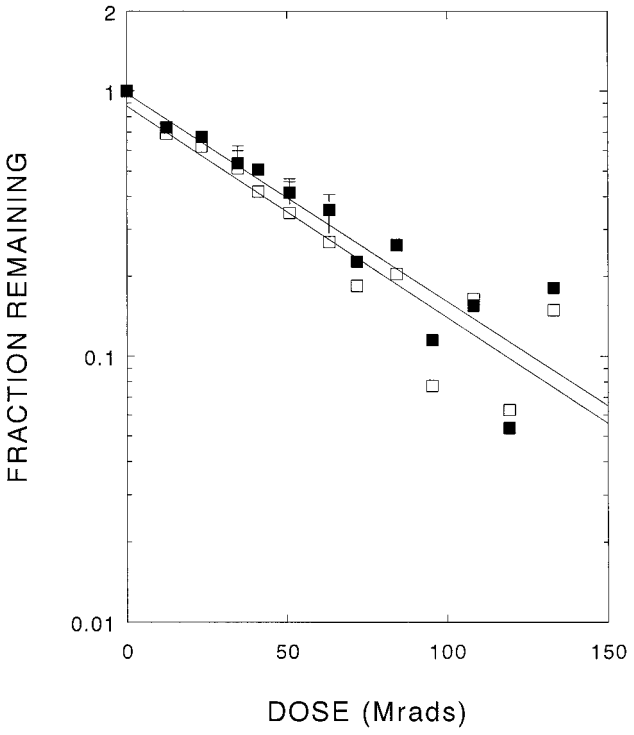

FIGURE 1 Surviving lysozyme activity in samples irradiated frozen at pH 2.5 (□) or pH 7.4 (■). Data from four independent experiments are shown as mean + SD.

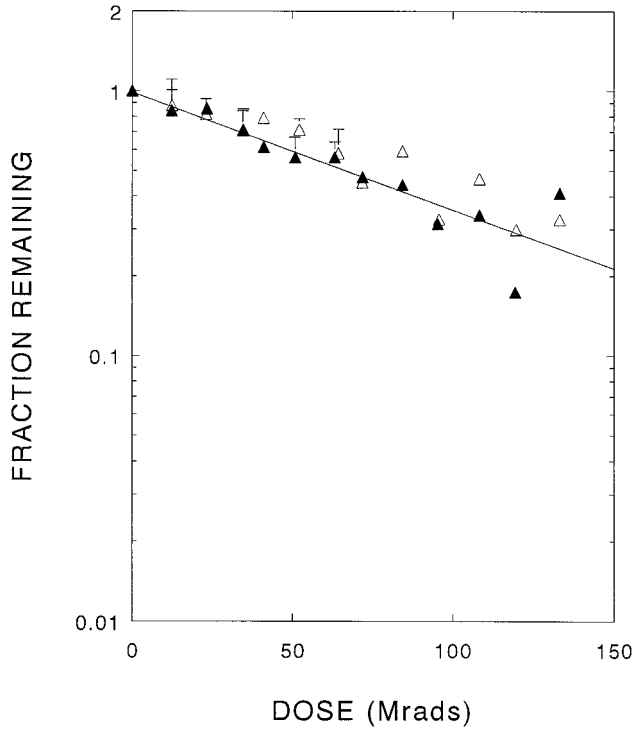

FIGURE 2 Lysozyme monomers surviving irradiation at pH 2.5 (△) or pH 7.4 (▲). Data from three independent experiments in Fig. 1 are shown as the mean + SD. The solid line is a least-squares fit to the pH 7.4 data.

DISCUSSION

The hydrodynamic properties of proteins are dependent on their mass as well as that of their associated solvent molecules. The shape of the molecule is also a factor. Some proteins have been shown to display a change in sedimentation constant in going from neutral to acid pH. Because the monomeric mass of the protein is constant, this variation is ascribed to changes in protein conformation and associated solvent molecules. In the case of G3PDH, there is a profound decrease in sedimentation

TABLE 2 Radiation target sizes based on loss of monomers

Protein	Target size (kDa)	
	pH 7.4	pH 2.5
Lysozyme	20.5 ± 7.0	14.0 ± 5.8
Bovine serum albumin	59.6 ± 8.4	74.7 ± 22.8
Glyceraldehyde 3-phosphate dehydrogenase	68.0 ± 21.4	55.4 ± 18.8

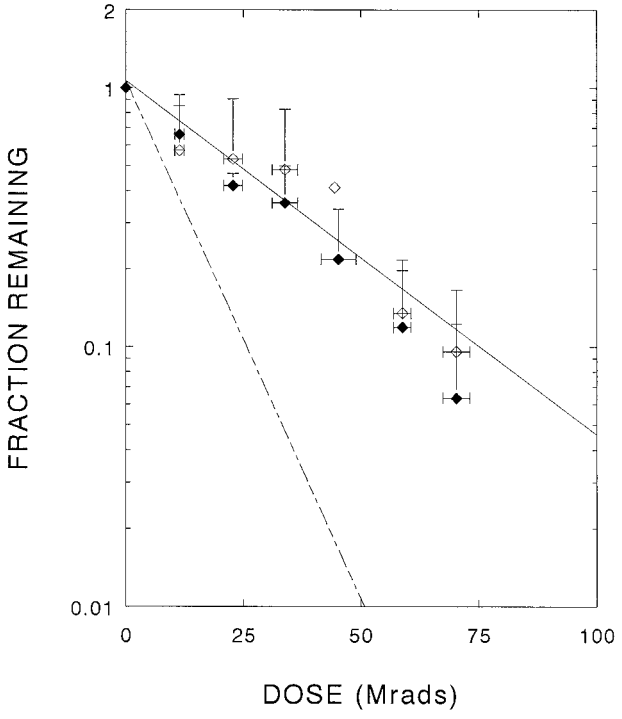

FIGURE 3 Bovine serum albumin monomers surviving irradiation at pH 2.5 (♦) or pH 7.4 (◇). Data from four independent experiments are shown as mean + SD. The solid line is a least-squares fit to the pH 7.4 data; the dashed line is a theoretical fit, assuming radiation sensitivity at low pH changes in inverse proportion to $s_{20,w}$ and assuming spherical geometry.

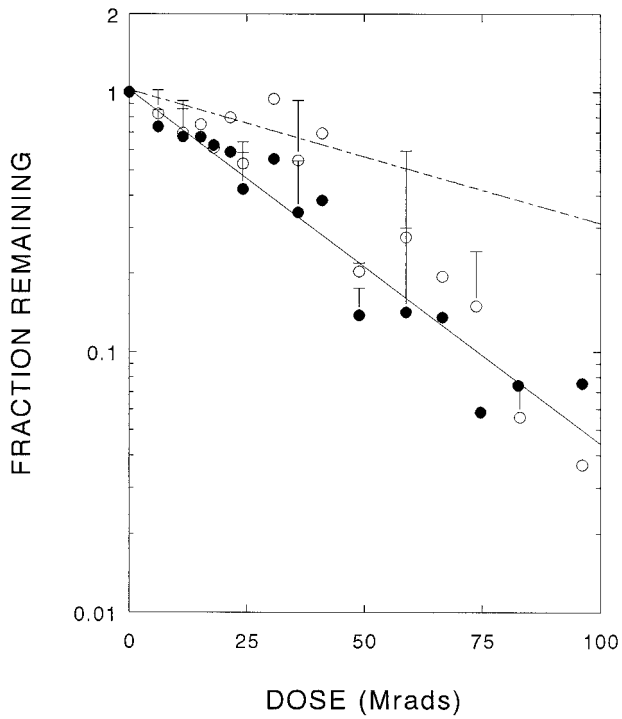

FIGURE 4 Glyceraldehyde 3-phosphate dehydrogenase monomers surviving irradiation at pH 2.5 (○) or pH 7.4 (●). Data from four independent experiments are shown as mean + SD. The solid line is a least-squares fit to the pH 7.4 data; the dashed line is a theoretical fit, assuming radiation sensitivity at low pH changes inversely with $s_{20,w}$ and assuming spherical geometry and dissociation into monomers.

constant at low pH due to the release of monomers from the tetramer (Shibata and Kronman, 1967).

This property of proteins can be utilized to resolve a question that has been recurrent in the development of radiation target theory. The concept of a "radiation-sensitive volume" was inherent in the first description of target theory some 75 years ago (Crowther, 1924). It arose because the unit of radiation dose, the roentgen, was defined at that time in terms of ionizations (or more correctly, electrostatic units of charge) per unit volume. Subsequently, the great development of target theory by Lea (1955) was focused on the sensitive volume concept, and this was perpetuated by others in the field (Pollard et al., 1955; Kempner and Schlegel, 1979; Jung, 1984). The conceptual model imagines a primary ionization occurring in a molecule at some point in space, and radiolytic products then diffusing to and reacting with some biologically active structure that was thereby inactivated. Refinement of the radiation target technique showed that irradiation of frozen samples was the best and most reliable method. Under such conditions, diffusion of radiation products is effectively eliminated. It was specifically stated that mass was the appropriate parameter (Steer et al., 1980; Beauregard et al., 1987), and a theoretical physics explanation for this was put forth (Kempner and Haigler, 1985). Nevertheless, the "volume" concept has persisted (Bradbury and Zammit, 1990; Lidzey et al., 1995).

If the "radiation-sensitive volume" refers to the volume of the polypeptide chain alone, the difference between the uses of mass or volume would be trivial: if V in in the first equation above refers to the molar volume, V/M, which is equivalent to the partial specific volume v/g, then

$$IV = (\mathrm{PI}/V) \times (V/\mathrm{g}) = Dq$$

But if the hydrodynamic volume or shape is involved, there can be significant deviations compared to that of the polypeptide chain alone. Now an experiment has been designed to specifically address this question. At acid pH, some proteins show altered sedimentation in the ultracentrifuge; this has been described as a change in hydrodynamic volume. Because there is no change in the inherent mass of the monomeric polypeptides, this phenomenon can be utilized to test whether the radiation sensitivity of frozen proteins depends on the mass or the volume/shape of the molecule.

The reported effects of pH on the sedimentation constant of three proteins were confirmed in the present samples, which were acidified, frozen, and thawed. The sedimentation of lysozyme is independent of pH (Tanford, 1968) and was chosen as an internal control. As expected, both the enzymatic activity and the amount of surviving monomers of lysozyme showed no change in radiation sensitivity at the

TABLE 3 Potential structures contributing to the radiation target size

Case	Volume	Mass
1	Volume occupied by the polypeptide chain excluding waters of hydration	Molecular mass
2	Volume occupied by the polypeptide chain and waters of hydration	Hydrated mass
3	Volume occupied by three-dimensional shape of polypeptide	Solution mass including "bulk" solvent within the 3-D envelope

acid pH. The quantitative change in sedimentation constants between neutral and acid pH of bovine serum albumin (Charlwood and Ens, 1957) and G3PDH (Shibata and Kronman, 1967) were reproduced in the present frozen and thawed samples. It is specifically assumed that during and after freezing there was no change in the hydrodynamic volume, which was then reversed during thawing.

Table 3 describes the expected target size for each of the three possible radiation-sensitive structures. Results in the literature (Charlwood and Ens, 1957; Champagne, 1957; Sogami and Foster, 1968; Tanford et al., 1955; Luzzatti et al., 1961; Raj and Flygare, 1974) indicate changes in both the hydration and shape of BSA (cases 2 and 3 of Table 3) below pH 4.5. If the radiation sensitivity corresponded to cases 2 or 3 (Table 3), then inactivation of BSA would be dependent on pH. As this was not observed, our studies indicate that the radiation target size corresponds to case 1 in Table 3. As already noted, volume and mass in case 1 (Table 3) are interconvertible. These results show that "target size" for BSA does not depend on degree of hydration or shape of the polypeptide chain.

The enzymatic activity of G3PDH at pH 7.4 is maintained through freezing and thawing, but acidification to pH 2.4 irreversibly destroys enzymatic activity. However, the radiation sensitivity of the monomers could be followed. As with the monomeric BSA, G3PDH (a tetramer) showed no change in radiation sensitivity of the monomers, even though the sedimentation properties of both proteins had been altered.

These results show that, in agreement with the theoretical argument, the radiation sensitivity of frozen proteins is directly dependent on their mass and is independent of their hydrodynamic volume or shape.

The radiation target sizes of a large number of proteins have been shown to agree well with the mass determined from the known structure (Kempner and Schlegel, 1979; Kempner, 1988). The present experiments indicate that the same measurements would be obtained even in samples in which these proteins were denatured.

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