Direct Radiation Damage Is Confined to a Single Polypeptide in Rabbit Immunoglobulin G

J. H. Miller,* L. R. Draper,† and E. S. Kempner*
*Laboratory of Physical Biology, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, Maryland 20892; and †Department of Molecular Biosciences, University of Kansas, Lawrence, Kansas 66045

ABSTRACT Frozen rabbit immunoglobulin G was exposed to high-energy electrons. The surviving polypeptide subunits were determined and analyzed by radiation target analysis. Each subunit was independently damaged by radiation whether or not they were bound by disulfide bridges to other subunits, demonstrating that in IgG radiation-deposited energy did not travel across disulfide bonds.

INTRODUCTION

The nature and mechanism of damage to macromolecules caused by the direct action of ionizing radiation has been studied in this and other laboratories for some time. A central unsolved problem has been the mechanism by which damage appears in one region of a radiation-sensitive structure when the original radiation interaction occurred elsewhere. Although damage is found randomly throughout a polypeptide, it is found only locally in single-stranded oligosaccharide and ribonucleic acid (RNA) polymers.

It is assumed that some of the energy deposited by the radiation interaction has been transferred (by some unspecified mechanism) to a distant site. The transfer of radiation-deposited energy along and between polypeptides has been examined in a variety of systems. In oligomeric proteins, radiation damage is usually confined to a single polypeptide. It was reported that energy transfer between the two independent polypeptides comprising ricin required a disulfide bridge (Haigler et al., 1985), whereas the strong noncovalent interactions in the avidin-biotin system did not permit such energy transfer (Kempner and Miller, 1990). Yet other oligomeric proteins showed energy transfer even in the absence of disulfide bridges between polypeptides (Chamberlain et al., 1983; Hymel et al., 1984; McIntyre et al., 1983).

The structure of immunoglobulin G is well known (Carayannopoulos and Capra, 1993). Rabbit IgG is composed of two identical heavy chains, each containing ~52,000 Da total mass of amino acids and two identical light chains, each ~24,000 Da. The IgG molecule contains ~3800 Da of oligosaccharide, generally exclusively on the heavy chains. There are extensive noncovalent interactions between the polypeptide chains, and disulfide bridges have been accurately defined (O'Donnell et al., 1970). Rabbit IgG contains three interchain disulfide bonds: one at the terminus of each light chain linking to a heavy chain, and one weaker disulfide bond between the two heavy chains. This well-defined structure offers an interesting potential for analysis of the direct effects of ionizing radiation. There were previous radiation target analyses of immunoglobulin E (IgE) (Fewtrell et al., 1981) and IgG (Rosse et al., 1967) in which functional reactivity was found to decay exponentially with radiation dose. A radiation study of several biological activities of immunoglobulin M (IgM) has also appeared (Rosse et al., 1967) yielding complex inactivation curves; however, no target size determinations based on its structure have been reported.

MATERIALS AND METHODS

Rabbit IgG from serum was purchased from Sigma (St. Louis, MO) (I-5006) as a lyophilized powder ("essentially salt-free"). The material was dissolved in 50 mM Tris pH 8.6 to 3 mg/ml. In half of the material, disulfide bridges were reduced by treatment with 1 mM dithiothreitol (DTT) (pH 8.6) for 30 min at room temperature, followed by incubation with 2.2 mM iodoacetate for 15 min (Miller and Metzger, 1965). Reduction was ascertained by nonreducing gel electrophoresis: samples were heated at 70°C for 10 min in lithium dodecylsulfate (LiDS) with no reductant and run on 15-well 4–12% Novex Bis-Tris NuPAGE 1-mm gels with the 2-[*N*-morpholino] ethanesulfonic acid (MES) running buffer containing sodium dodecyl sulfate (SDS) and Invitrogen prestained See-Blue molecular weight standards (Carlsbad, CA).

All the protein samples were passed through a Pharmacia PD-10 column (Peapack, NJ) to exchange the buffer to 50 mM Tris pH 7.8. Both native and reduced IgG were adjusted to 2 mg/ml in this buffer. Aliquots of 0.25 ml were frozen in 2-ml glass ampoules (Kimble #12010L-2). Vials were sealed with an oxygen-gas torch and irradiated at −135°C with 13 MeV electrons from a linear accelerator at the Armed Forces Radiobiology Research Institute as described (Harmon et al., 1985).

To separate the surviving light and heavy immunoglobulin chains, the irradiated samples were resolved by denaturing gel electrophoresis (SDS PAGE): samples were heated at 70°C for 10 min in LiDS and DTT and run on a 4–12% gradient Gel (Novex, San Diego, CA). Unirradiated samples at a variety of protein concentrations were also placed on the same gel. Gels were stained with GelCode Coomassie stain (Pierce, Rockford, IL), scanned with a PDSI scanner (Molecular Dynamics, Sunnyvale, CA), and analyzed using Molecular Dynamics software. The stain intensity of the 50,000 M_r and of the 25,000 M_r bands of unirradiated IgG at various protein concentrations were used to calibrate these bands in the irradiated samples.

Additional samples of native and reduced IgG irradiated with 0, 9, or 24 Mrads were treated with 50 mM β-mercaptoethanol at 95°C for 5 min and electrophoresed on a 4–12% gradient gel. Protein was visualized by amido black staining. Western blots of the gels were probed with goat anti-rabbit

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Address reprint requests to Ellis S. Kempner, E-mail: kempnere@exchange.nih.gov.

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IgG (γ heavy-chain specific) conjugated to horse radish peroxidase (Southern Biotech, Birmingham, AL) and detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

Radiation target analyses were as described (Harmon et al., 1985).

RESULTS

Native and reduced IgG samples were analyzed by electrophoresis with SDS but without reductants. In the native IgG samples, a single predominant band was seen (Fig. 1). IgG was only partially reduced by treatment with DTT because one-third of the intact IgG (145 kDa) persisted. Several additional bands of smaller M_r are evident. Because this is a nonreducing gel, movement of bands is not a good estimate of size.

In one experiment, MALDI-TOF mass spectrometry was used to determine the masses in the samples without exposure to SDS: the native IgG revealed peaks at 144.9 kDa and also at 72.0 kDa; the reduced sample showed 145 kDa material remaining; a peak at 72.6 kDa was most likely a single light-heavy chain complex; and peaks near 50 and 23 kDa were presumably the heavy and light chains, respectively. This simply demonstrated that the reduction procedure cleaved interchain disulfide bonds in most of the molecules.

After irradiation, both native and reduced samples were treated with SDS and DTT for denaturing gel electrophoresis (SDS-PAGE). Both samples revealed only two bands, a strong band at M_r 54,000 and a weaker band at M_r 28,000.

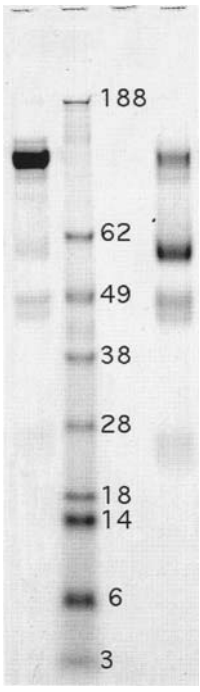

FIGURE 1 Gel electrophoresis of native and reduced IgG. Native IgG (*left lane*) and reduced IgG (*right lane*) were electrophoresed in the presence of SDS but without any reducing agents.

Coomassie blue staining of the 54,000 M_r band decreased in intensity with radiation exposure (Fig. 2, *A* and *B*). Densitometric analysis of these bands revealed the amount of 54,000 M_r material decreasing exponentially with radiation dose (Figs. 3 and 4), leading to a target size of 57 kDa in the native IgG samples and 62 kDa in the reduced samples (Table 1).

The band near 28,000 M_r gave more complex responses to radiation. After low doses of radiation there was a small increase in stain intensity (Figs. 3 and 4). After greater radiation exposures of both native and reduced IgG samples, the survival of these polypeptides decreased as an apparent exponential function of radiation dose. This yielded target sizes of 19 kDa (native) and 22 kDa (reduced) (Table 1).

The latter complex radiation data can be analyzed by target theory models (Kempner, 1995). The model assumes that the final exponential slope is due to the destruction of the monomer subunit; the initial portion of the inactivation curve is dominated by the destruction of a larger mass structure (Kempner, 1995). The curve is analyzed as the difference between two exponentials. In the present experiments, the mass of the larger structure was obtained by ''curve stripping'' (Kempner, 1995) and gave 112 kDa (native samples) and 120 kDa (reduced samples) (Table 1). The final slope of all the complex curves extrapolate at the intercept to an average value of 1.5 $\pm$ 0.5. In this radiation target model, this implies that one or two units of ~20 kDa are released by every radiation interaction with a structure of ~120 kDa.

In view of the known structure of IgG, interpretation of the 120 kDa value is difficult. Because there is a very large error in its determination, a model based on the known IgG structure was developed (vide infra). Only heavy and light chains are present in the sample, and only damage to the heavy chains could possibly result in the appearance of additional polypeptides of ~28,000 M_r. The model predicts

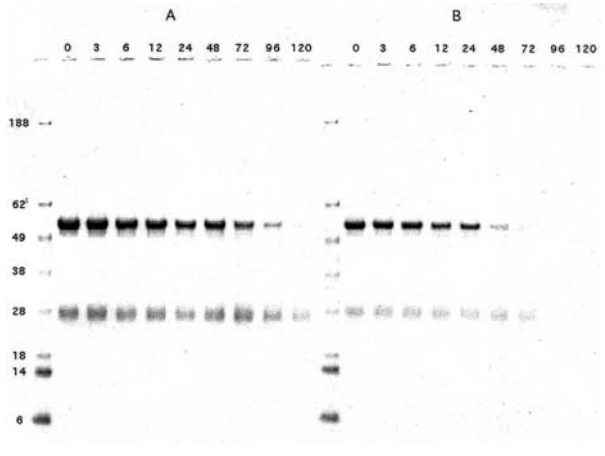

FIGURE 2 Denaturing gel electrophoresis of irradiated native (*A*) and reduced (*B*) IgG. Individual lanes show the radiation doses (in Mrads) received by the sample. Coomassie staining intensity is a measure of the amount of surviving subunits. Data from a single experiment.

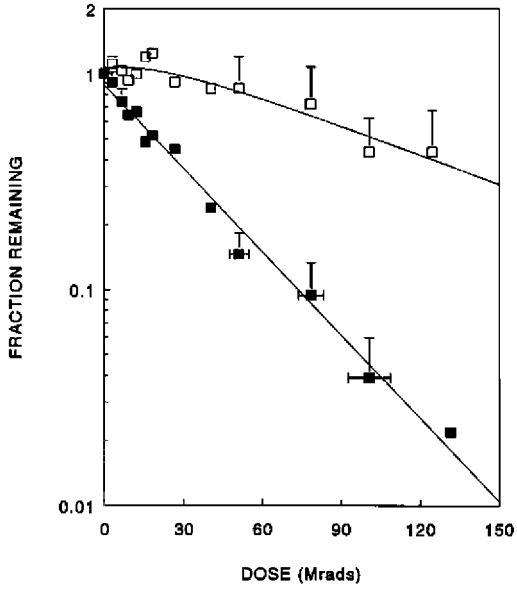

FIGURE 3 Radiation inactivation of monomers in native IgG samples. Data from three independent experiments shown as averages + SD after each radiation dose. In each irradiated sample, the μg protein detected by Coomassie staining was expressed as a fraction of that observed in the unirradiated sample. The 54,000 M_r band (*closed symbols*) decreased as a simple exponential function of radiation dose. The 28,000 M_r band (*open symbols*) showed a slight increase after small doses of radiation, but decreased after further exposure.

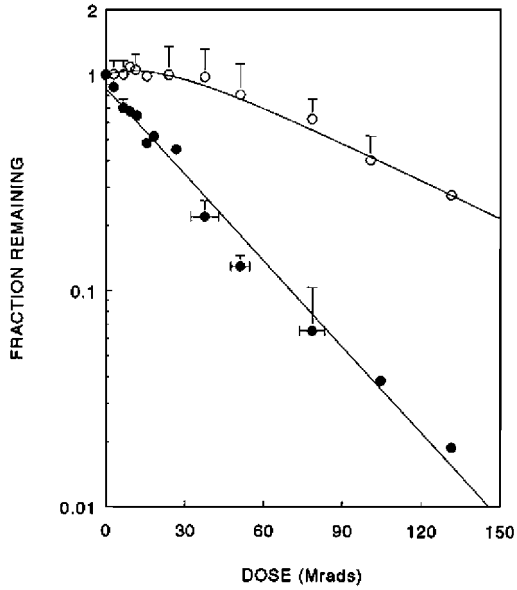

FIGURE 4 Inactivation of monomers in reduced IgG samples. Data averaged from three independent experiments as described in Fig. 3 legend. Data from the 54,000 M_r band are shown as closed symbols; data from the 28,000 M_r band are shown as open symbols.

that the surviving fraction of the ~28,000 M_r material will be $e^{-.0134D} + (1 - e^{-.0290D})e^{-.0134D} = 2e^{-.0134D} - e^{-.0424D}$.

The inactivation curves of native (Fig. 3) and reduced IgG (Fig. 4) are indistinguishable. The 28,000 M_r data from the two sets were combined and plotted in Fig. 5 together with the equation developed in this model. This equation, based entirely on the known structure of IgG, fits the data quite well.

The fundamental assumption of this model is that fragments of the heavy chain are responsible for the increase seen in the lower band. MALDI-TOF of irradiated samples showed new fragments near the light chain. To determine their origin, Western blots of SDS PAGE gels were developed with goat anti-rabbit IgG ((γ heavy-chain specific) antibody (Fig. 6). In unirradiated IgG samples (both native and reduced) only the 50,000 M_r band showed any reactivity with the antibody; this confirms the specificity of the antibody. However, samples exposed to 9 or 24 Mrads showed the appearance of heavy-chain specific antibody binding to material electrophoresing as a smear down the gel with some suggestion of discrete bands in the mixture. Therefore, some of the material near the light-chain band was derived from the irradiated heavy chains.

DISCUSSION

The detailed structure of IgG is firmly established. The native form of IgG showed masses of 145 and 72 kDa by MALDI-TOF; these sizes correspond to those expected for intact IgG and the half-molecule (one light and one heavy chain). The reducing treatment used in this study was evaluated by nonreducing gel electrophoresis; the presence of intact IgG molecules indicates that some of the molecules were not reduced. If the results of irradiation had been different, it would have been important to insure that the reduction was complete. MALDI-TOF analysis of the reduced form also showed 145 kDa material remaining, as well as masses of 72, 50, and 23 kDa; these are interpreted as the intact, half-molecule, heavy and light chains, respectively.

Both the native and reduced IgG were resolved on denaturing gel electrophoresis, revealing two principal bands at 54,000 and 28,000 M_r; the accepted values are 52,000 and 24,000.

Radiation inactivation curves from native and reduced samples are indistinguishable. The larger monomer disap-

TABLE 1 Target size analysis of surviving rabbit IgG subunits

		28,000 M_r Band	
	54,000 M_r band (kDa)	Smaller target (kDa)	Larger target (kDa)
Native IgG	57 ± 8	19 ± 5	112 (n = 2)
Reduced IgG	62 ± 9	22 ± 3	120 ± 32

Irradiated IgG samples were denatured, reduced, and electrophoresed on SDS PAGE. Coomassie stain intensity of individual bands was converted to μg protein. Target sizes were calculated from data of each experiment. Values given as averages ± SD from three independent experiments.

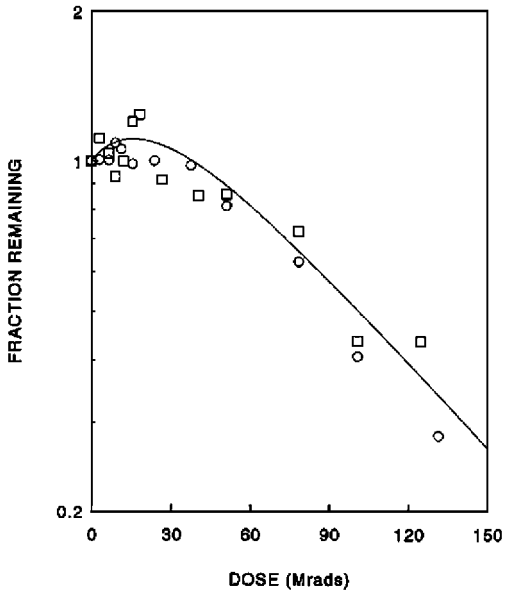

FIGURE 5 Inactivation of 28,000 M_r monomers in native (□) and reduced (○) IgG irradiated samples. Curve drawn is from the model described in the text.

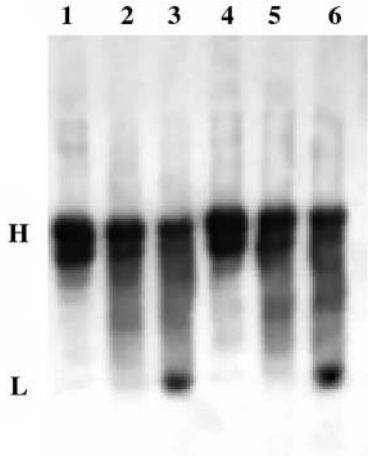

FIGURE 6 Gel electrophoresis of native (*lanes 1–3*) and of reduced (*lanes 4–6*) IgG samples exposed to 0 (*lanes 1, 4*), 9 (*lanes 2, 5*), or 24 (*lanes 3, 6*) Mrads of high-energy electron radiation. Position of the heavy and of the light chain (determined from amido black staining of the gel) are indicated by H and L. A Western blot of the samples was probed with heavy-chain specific goat anti-rabbit IgG antibody coupled to horse radish peroxidase. In unirradiated samples, reaction is seen only in the heavy-chain band. In irradiated samples, additional antibody-reactive material is seen as a smear overlapping the light-chain band, indicating the appearance of fragments from the heavy chain.

pears as a simple exponential function of radiation dose, yielding target sizes of 57 and 62 kDa in the two cases. The smaller monomer shows a complex radiation response. All the previously reported examples of such complex results involved surviving biological activity; in those, the larger structure was interpreted as a unit that prevented expression of activity by the smaller unit.

In the present study, however, only surviving structures were detected, independent of any biological function. Because the irradiated samples contained only IgG molecules, the complex inactivation curve is interpreted as a larger structure that decomposes on irradiation to smaller units: a ''parent'' unit that, on irradiation, results in the appearance of polypeptides of ~28,000 M_r. Although less likely, it is remotely possible that destruction of some larger structure led to an increase in the amount of Coomassie stain bound by the smaller subunits, altering the calibration of the stain intensity.

When the present data are analyzed as the difference of two exponentials, the final portion of the inactivation curve yields target sizes close to 20 kDa, comparable to the mass of that monomer. Resolution of the initial region yields larger target sizes, roughly estimated at ~110–120 kDa. Analysis of compound curves in this manner leads to considerable error in target size determination because only a few of the data points are used in resolving the components. A simple model was developed based upon realistic expectations from the IgG structure: 1) the samples contain only IgG large and small subunits; 2) appearance of additional ~24 kDa structures can only come from radiation-damaged 50 kDa subunits; and 3) a single radiation interaction in the large subunit cleaves the polymer. The model proposes that radiation interactions in heavy chains lead to the appearance of polypeptide fragments, some of which move on electrophoresis with the light chain. Fragments that are of similar mass to the light chain should be lost at a similar rate. Consistent with the fundamental assumption of the model, MALDI-TOF showed the appearance of such fragments in irradated IgG and electrophoresis showed material near the light band, which is reactive with heavy-chain specific antibody. Thus this model adequately describes all the observations reported here for IgG.

These results agree with the general observations in other proteins that radiation damage occurs randomly throughout a polypeptide, except when irradiated in the absence of oxygen (Le Maire et al., 1990). The indication of some discrete heavy-chain fragments in the general array of products is consistent with the suggestion that the radiation sensitivity of immunoglobulins might be related to domains (Fewtrell et al., 1981). A rabbit IgG heavy chain contains several domains, two of which are linked by a ''hinge'' region. This stretch of amino acids is particularly susceptible to mild enzymatic proteolysis, resulting in fragments of ~20 kDa (Fleishman et al.,1963; Stanworth and Turner, 1978). These regions may also be sensitive to radiation.

Earlier radiation inactivation studies of IgE (Fewtrell et al., 1981) reported the loss of IgE binding activity to its receptor; a target size of 117 kDa was reported. An even earlier radiation study of air-dried rabbit IgG (Rosse et al., 1967) reported the loss of complement-mediated hemolytic activity

of an anti-RBC (red blood cell) antibody followed a simple exponential function of dose. The required temperature correction (Kempner et al., 1986) for their irradiations at −80°C was not known at that time. When this factor is included in analyzing their data, the target size for hemolysin activity becomes 76 kDa, consistent with the mass of a half molecule of IgG. This result most likely indicates that the loss of function of a half IgG molecule precludes the antibody from inducing complement-mediated hemolysis. The present study of the radiation inactivation of IgG clearly demonstrates that the individual polypeptide subunits are damaged one at a time. Target sizes of 57 and 20 kDa mean that one and only one polypeptide is cleaved by a single radiation hit, even in cases where they are linked to each other by single disulfide bridges in a four-chain structure. There was a previous radiation study of ricin, a dimeric molecule linked by a single disulfide bridge (Haigler et al., 1985). It was reported that the target size for biochemical function was equal to the sum of the masses of the two disulfide-linked polypeptides A and B. In the reduced state, function disappeared only when the A polypeptide was hit. From these results, it was concluded that radiation-deposited energy could transfer across a disulfide bridge. Unfortunately, no structural damage was directly measured. The present conclusions are completely opposite those of the earlier work. Several possible explanations for this discrepancy are obvious. The mechanism or efficiency of energy transfer across a disulfide bridge may be influenced by the structure of the two polypeptides. Alternatively, results from loss of function may involve conformational changes not involving covalent bond breakage: a radiation interaction with the B chain of ricin might ultimately result in a conformational change of the A chain with consequent loss of function but no cleavage of the A chain backbone. The mechanism of energy transfer has been assumed to be through some covalent bond structure; the validity of this assumption must be established.

Whatever the explanation for the discrepancy, it is now clear that in IgG radiation energy did not travel across a disulfide bond.

MODEL DEVELOPMENT

The model proposes that the 24 kDa light chains initially present will decrease exponentially with radiation dose at a rate given by $e^{-.0134D}$. (The exponent is obtained from $\exp(-[24/1792]D)$ where the radiation dose, D, is given in Mrads and the constant 1792 includes a temperature correction of 2.8 for irradiations at −135°C, the value of 60 eV per primary ionization, and various factors to convert from one system of units to another to express the target mass in kDa.) The heavy chains of 52 kDa will decrease at a rate determined by its mass: ($e^{-.0290D}$). The fraction of 52 kDa units destroyed will be $1 - e^{-.0290D}$, each molecule of which gives rise to a polypeptide of q kDa, which electrophoreses with the M_r 28,000 band. The survival of these generated polypeptides will be given by $(1 - e^{-.0290D})\,e^{-[q/1792]D}$. Thus, the surviving fraction of total observed smaller polypeptides will be $e^{-.0134D} + (1 - e^{-.0290D})\,e^{-[q/1792]D}$. If $q \sim 24$ kDa, the surviving fraction will be $e^{-.0134D} + (1 - e^{-.0290D})\,e^{-.0134D} = 2e^{-.0134D} - e^{-.0424D}$.

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REFERENCES

Carayannopoulos, L., and J. D. Capra. 1993. Immunoglobulins: structure and function. *In* Fundamentals of Immunology. W. E. Paul, editor. Raven Press, New York. 283–314.

Chamberlain, B. K., C. J. Berenski, C. Y. Jung, and S. Fleischer. 1983. Determinaton of the oligomeric structure of the Ca^{2+} pump protein in canine cardiac sarcoplasmic reticulum membranes using radiation inactivation analysis. *J. Biol. Chem.* 258:11997–12001.

Fewtrell, C., E. S. Kempner, G. Poy, and H. Metzger. 1981. Unexpected findings from target analysis of immunoglobulin E and its receptor. *Biochemistry*. 20:6589–6594.

Fleischman, J. B., R. R. Porter, and E. M. Press. 1963. The arrangement of the peptide chains in gamma-globulin. *Biochem. J.* 88:220–228.

Haigler, H. T., D. J. Woodbury, and E. S. Kempner. 1985. Radiation inactivation of ricin occurs with transfer of destructive energy across a disulfide bridge. *Proc. Natl. Acad. Sci. USA*. 82:5357–5359.

Harmon, J. T., T. B. Nielsen, and E. S. Kempner. 1985. Molecular weight determinations from radiation inactivation. *Methods Enzymol.* 117: 65–94.

Hymel, L., A. Maurer, C. J. Berenski, C. Y. Jung, and S. Fleischer. 1984. Target size of calcium pump protein from skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* 259:4890–4895.

Kempner, E. S. 1995. The mathematics of radiation target analyses. *Bull. Math. Biol.* 57:883–898.

Kempner, E. S., and J. H. Miller. 1990. Direct effects of radiation on the Avidin-Biotin system. *J. Biol. Chem.* 265:15776–15781.

Kempner, E. S., R. Wood, and R. Salovey. 1986. The temperature dependence of radiation sensitivity of large molecules. *J. Polym. Sci. B: Polymer Physics*. 24:2337–2343.

Le Maire, M., L. Thauvette, B. de Foresta, A. Viel, G. Beauregard, and M. Potier. 1990. Effects of ionizing radiations on proteins. *Biochem. J.* 267:431–439.

McIntyre, J. O., P. Churchill, A. Maurer, C. J. Berenski, C. Y. Jung, and S. Fleischer. 1983. Target size of D-β-hydroxybutyrate dehydrogenase. *J. Biol. Chem.* 258:953–959.

Miller, F., and H. Metzger. 1965. Characterization of a human macroglobulin. *J. Biol. Chem.* 240:4740–4745.

O'Donnell, I. J., B. Frangione, and R. R. Porter. 1970. The disulphide bonds of the heavy chain of rabbit immunoglobulin G. *Biochem. J.* 116: 261–268.

Rosse, W. F., H. J. Rapp, and T. Borsos. 1967. Structural characteristics of hemolytic antibodies as determined by the effects of ionizing radiation. *J. Immunol.* 98:1190–1195.

Stanworth, D. R., and M. W. Turner. 1978. Immunochemical analysis of immunoglobulins and their subunits. *In* Handbook of Experimental Immunology, 3rd ed. D. M. Weir, editor. Blackwell Sci. Publ., London, 6.1–6.102.