Radiation Inactivation of Biological and Immunological Activities of Beef Thyrotropin

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The molecular weight of bovine thyrotropin has been estimated by several investigators. In 1955, Pels, Simpson, and Evans (1) prepared bovine thyroid-stimulating hormone of 1 to 4 units per mg by a method of trichloroacetic acid fractionation and obtained a sedimentation coefficient of 1.02 by ultracentrifugation. They estimated the molecular weight to be 10,000. In 1956, Pierce and Nyc (2) by means of purification on Amerlite IRC-50 prepared bovine TSH with a potency of 5 U.S.P. units per mg, and by means of ultracentrifugation estimated the sedimentation coefficient to be 2.5 to 2.9. Condiffe and Bates (3) in 1957 by means of chromatography on both IRC-50 and carboxymethyl-cellulose prepared bovine TSH with a potency of 15 U.S.P. units per mg, and later (4) with a potency of 20 to 50 units per mg. The sedimentation coefficient was estimated to be 2.6 (5). In 1960, Carsten and Pierce (6) by chromatography on DEAE-cellulose and by starch gel electrophoresis, prepared bovine TSH with 30 to 60 units per mg. By using the technique of micro-electrodialysis they estimated the molecular weight to be 26,000 to 30,000. Fontaine and Condiffe (7) in 1963 with density gradient centrifugation estimated the sedimentation coefficient to be 2.82 S, and the molecular weight to be 26,000 to 31,000. Since completely purified preparations of bovine TSH have not been prepared, these molecular weights should not be viewed as firmly established (5).

The molecular volume of protein and polypeptide hormones may be estimated by means of the rate of inactivation of biological activity on exposure to ionizing radiation of low energy transfer (8). The major advantage of this method is that purification is not required.

We have studied the inactivation of bovine TSH by 2.0 m.e.v.-electron beam generated in a Van de Graaff electrostatic accelerator. The dose per pass was measured by oxidation of ferrous sulfate to ferric sulfate in solutions 1 cm deep (9) and was extrapolated to surface dosage from the data of Trump and Van de Graaff (10). Multiple samples were tested throughout the field to insure homogeneity. Intensity of the beam was adjusted so that 1.0 × 10^6 rads were absorbed in the ferrous sulfate solution per pass, and dose was varied by varying the number of passes through the beam. The planchets were placed on slabs of Dry Ice to keep heating to a minimum during radiation. Two independent radiations were performed and a total of eight planchets were studied at each of eight radiation doses (0, 22.3, 44.5, 66.8, 89.0, 133.5, 155.8, and 178 × 10^6 rads). The hormone was eluted from the planchets with at least 20 ml of phosphate-buffered saline (pH 7.8).

Bioassay of Bovine TSH—Biological assays were performed by the method of Bates and Cornfield in the day-old chick (11). This method utilizes the TSH-stimulated release of radioiodine from the thyroid glands of chicks treated with propylthiouracil and thyroxine. The percentage of radioiodine remaining in the gland is inversely related to the log dose of TSH. Bovine TSH from each planchet was assayed at 4 to 6 dilutions with 4 to 6 chicks for each dilution. Bioassays were performed on bovine TSH radiated at 0, 22.3 × 10^6, 44.5 × 10^6, and 66.8 × 10^6 rads. The activity remaining on planchets radiated at higher doses was too little to be conveniently detected by this bioassay.

Immunooassay of Bovine TSH—The assay utilized depends upon competition between the sample of TSH being assayed and radioiodinated TSH for combination with anti-TSH. Antibody-bound TSH is separated from nonantibody-bound (free) TSH, and the amount of radioactivity appearing in either fraction is a function of the amount of TSH in the unknown sample. Rabbit antisera to purified bovine TSH previously prepared were utilized (12). Radioiodinated bovine TSH was prepared by the method of Greenwood, Hunter, and Glover (13). The specificity of this immunooassay was previously demonstrated. U.S.P.-bovine TSH, Thytropar, and the purified bovine TSH in equivalent biological amounts had identical immunological activity. Furthermore, the antisera were shown to give a single line against purified bovine TSH by double diffusion in agar gel and to be capable of neutralizing the biological activity of bovine TSH (12). The radioimmunoassay was performed as described (12) with the exception of the following modification. The antibody-bound TSH was separated from free TSH in one series

EXPERIMENTAL PROCEDURE

Preparation and Radiation of Samples—Bovine TSH (Armour Thytropar) was dissolved in distilled water in a concentration of 10,000 U.S.P. millunits per ml. Aliquots of 0.1 to 1 ml were pipetted onto stainless steel planchets which were then either immediately frozen and lyophilized or alternatively dried under vacuum at 4°C. The planchets were stored in desiccators until needed for radiation.

Samples were passed at constant speed through a 2.0 m.e.v.-electron beam generated in a Van de Graaff electrostatic accelerator. The dose per pass was measured by oxidation of ferrous sulfate to ferric sulfate in solutions 1 cm deep (9) and was extrapolated to surface dosage from the data of Trump and Van de Graaff (10). Multiple samples were tested throughout the field to insure homogeneity. Intensity of the beam was adjusted so that 1.0 × 10^6 rads were absorbed in the ferrous sulfate solution per pass, and dose was varied by varying the number of passes through the beam. The planchets were placed on slabs of Dry Ice to keep heating to a minimum during radiation. Two independent radiations were performed and a total of eight planchets were studied at each of eight radiation doses (0, 22.3, 44.5, 66.8, 89.0, 133.5, 155.8, and 178 × 10^6 rads). The hormone was eluted from the planchets with at least 20 ml of phosphate-buffered saline (pH 7.8).

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1 The abbreviations used are: bovine TSH, bovine thyroid-stimulating hormone; TSH, thyroid-stimulating hormone.
were studied and loss of immunological activity was calculated and $178 \times 10^6$ rads. Bovine TSH from each planchet was measured in a well-type scintillation counter. All procedures except counting were performed at 4°C. Immunoassays were performed on bovine TSH radiated at 0, 22.3, 44.5, 66.8, 89.0, 133.5, 155.8, and 178 $\times 10^6$ rads. One hour later, 2.3 ml of 12.8% NaCl solution were added and the tubes were again stored for 1 hour. Samples were then centrifuged at $500 \times g$ for 20 minutes and the supernatant radioactivity of an aliquot measured. In a second series, this separation was achieved by an alcohol precipitation method modified from the TSH extraction procedure described by Bates and Condliffe (5). In this method, 4 ml of 100% ethanol were added to 1 ml of the reaction mixture. One hour later, 2.3 ml of 12.8% NaCl solution were added and the tubes were again stored for 1 hour. Samples were then centrifuged at $500 \times g$ for 20 minutes and the supernatant radioactivity of an aliquot measured in a well-type scintillation counter. All procedures except counting were performed at 4°C. Immunoassays were performed on bovine TSH radiated at 0, 22.3, 44.5, 66.8, 89.0, 133.5, 155.8, and 178 $\times 10^6$ rads. Bovine TSH from each planchet was assayed at 4 to 6 dilutions. Antisera prepared in two rabbits were studied and loss of immunological activity was calculated independently for each.

Calculations—Calculations of percent activity remaining as measured by immunosassay and bioassay were performed as previously described (14). Calculations of molecular weights were performed with the formula presented by Hutchinson and Pollard (15)

$$M = \frac{0.72 \times 10^6}{D_t} \text{ (rads)}$$

where $M$ is molecular weight and $D_t$ is radiation dose in rads required to reduce activity to 36.8%. It should be emphasized that this development with radiation dose in rads makes calculations of molecular weight independent of density (15). Linear regression coefficients were calculated as $Y$ on $X$ by the method of least squares; the dose of radiation necessary to reduce hormonal activity to 36.8% ($D_{36.8}$) was calculated directly from the regression coefficient.

RESULTS

Biological Activity—Radiation of bovine TSH resulted in loss of biological activity. Log of percent activity remaining was plotted against radiation dose in rads and the linear relationship shown in Fig. 1 was obtained. The radiation dose required to reduce activity to 36.8% was 22.5 $\times 10^6$ rads (95% limits = 19.8 to 26.1). The molecular weight calculated from these data is 32.0 $\times 10^6$ (27.6 to 36.4).

Immunological Activity—Radiation of bovine TSH also resulted in loss of immunological activity, but the rate of loss was considerably less than for biological activity. Independent assays were performed with two antisera (anti-bovine TSH A2B and B2B). When immunological activity was measured with antisera A2B, the dose required to reduce activity to 36.8% was 36.2 $\times 10^6$ rads (33.1 to 40.0). Molecular weight calculated from these data is 19.9 $\times 10^6$ (18.0 to 21.8). When immunological activity was measured with antisera B2B, the radiation dose required to reduce activity to 36.8% was 38.3 $\times 10^6$ rads (31.5 to 48.9). The molecular weight calculated from these data is 18.8 $\times 10^6$ (14.7 to 22.9). A semilog plot of percent activity remaining and radiation dose with antisera A2B is shown in Fig. 1.

DISCUSSION

When biologically active proteins are exposed to ionizing radiation, the biological activity decreases. If the radiation utilized produces primary ionizations spaced widely apart in relation to the thickness of the molecule and a single ionization is sufficient to inactivate the molecule, then the following relation holds

$$-dn = n_0VdI$$

where $n_0 = \text{total number of molecules}$ and $n = \text{number of molecules in which no ionization has occurred at } I$ (ionizations per volume), and $V = \text{target volume}$. Integrating,

$$\ln \frac{n}{n_0} = -VI$$

where $n/n_0 = \text{fraction of molecules in which an ionization (hit) has not occurred}$. When 36.8% of activity remains (15)

$$\ln \frac{n}{n_0} = -1 \text{ and } V = \frac{1}{I}$$

This development is applicable when high energy electrons or $\gamma$-rays are used and molecules the size of protein hormones are studied. The average energy transfer event (primary ionization) is said to release about 100 e.v. Hutchinson and Pollard (15) have estimated that 60 e.v. may be expended in producing the ion cluster. The remaining energy is transferred to secondary electrons (or $\delta$ rays). A portion of the energy transferred as $\delta$-rays will be released within the confines of the original ion cluster; the residual energy may produce new clusters. It has been estimated that each 100 e.v. will produce an average of 14 separate ion clusters or 75 e.v. per ion cluster.

Dosage of radiation is usually expressed in terms of rads ($R$). Formula 3 can be restated in terms of rads on the basis of the following relations

![Fig. 1. Radiation inactivation of immunologic and biologic activities of bovine thyrotropin. The solid lines indicate the calculated regression lines; the dashed lines and shaded areas indicate the 95% confidence limits of these regression lines. Immunologic activity was obtained with antisera A2B (see text).](image-url)
of molecule. If one ion cluster per g = 1.2 x 10^{-12} rads, then a straight line of slope $-V$ is obtained. If more than one ionization is required to inactivate a single molecule, a curve having a shoulder (concave downward) is obtained. When only two or three ionizations are required to inactivate a molecule, the slope of the linear portion of such a curve can be calculated and utilized to estimate molecular weight. Such estimates will contain a small error when few ionizations are required but increasingly large errors when larger numbers of ionizations are required to inactivate the molecule.

Relatively little information is available on the mechanism by which radiation produces inactivation. Most investigators believe the ionization is the major energy transfer event and initiates the process of inactivation. Others have suggested that small amounts of energy may be transferred from the charged particle to the protein and result in excitations which in turn inactivate the molecule. For a critique of the target theory, the reader is referred to the review of Augenstein (17). The efficiency of excitations in inactivating proteins has been shown to be low (about 1%) (15). Furthermore, when thin foils of varying composition were radiated with monoenergetic electrons, the peak energy loss occurred at about 23 e.v., and few electrons lost less energy than this (18). This indicates that relatively few excitations occurred independent of ionizations. However, the molecular weight determined by radiation is at times dependent upon the temperature of the samples during irradiation (16). One explanation of this effect is that at higher temperatures excitations become increasingly more important in causing inactivation. Because of this possibility, we have attempted to keep heating to a minimum by placing all samples on Dry Ice during irradiation.

Lea (8), Pollard et al. (20), Hutchinson (15, 21), and others have utilized the target theory to estimate the molecular weight of numerous enzymes by radiation inactivation. Good agreement exists between estimates by radiation inactivation and estimates by more conventional techniques. In addition, molecular weights of several hormones have been estimated with radiation techniques. These include insulin (22), adrenocorticotropic hormone (23, 24), erythropoietin (25), human chorionic gonadotropin (14), and human luteinizing and follicle-stimulating hormone (20). One of the interesting aspects of this method is that only the “radiation-sensitive” volume is measured. Generally, the entire molecule has been the volume estimated when loss of activity has been measured by biological or enzymatic assays, but certain exceptions exist. One exception is adrenocorticotropic, which was radiated with deuterons. The molecular weight was estimated to be 2400 ± 800, which is approximately half the molecular weight of the entire molecule (23). We have also exposed purified adrenocorticotropic hormone to 2.0 m.e.v. electrons (24). The molecular weight estimated from our radiation inactivation data was 3.6 x 10^{6}, which is in better agreement with the accepted molecular weight. Reasons for the differences in results are not apparent.

In the studies reported herein, the molecular weight estimated with depletion of radiiodine from the thyroid of the day-old chick was 32.0 x 10^{6}. This molecular weight estimate is consistent with the sedimentation coefficients of Carsten and Pierce (6) and Bates and Condliffe (5) and with the molecular weight estimate by microelectrodialysis (6). However, when radiation inactivation was measured by the radioimmunoassay, the rate of inactivation was considerably less. The molecular weight calculated from these data averaged 18.6 x 10^{6}. Thus an ionization occurring anywhere within the entire molecule is sufficient to destroy biological activity, whereas an ionization must occur in a considerably smaller volume to destroy immunological activity. An ionization occurring within the molecule, but outside this sensitive volume, will destroy biological activity but not effect immunological activity.

Previous studies on other proteins have also revealed such differences. Human chorionic gonadotropin was radiated with 2.0 m.e.v. electrons (27). When the molecular weight was estimated by prostate or testes weight in the immature hypophysectomized male rat, similar values which averaged 27.0 x 10^{6} were obtained. This value agrees well with the best estimate reported by ultracentrifugation and light scattering (28). However, when radiation inactivation was measured by hemagglutination inhibition or by a radioimmunoassay similar to that described in this report, the molecular weights were 10.7 x 10^{6} and 11.6 x 10^{6}, respectively. Similarly, invertase was radiated with deuterons and residual activity measured by enzymatic and immunologic assays (19). The molecular weight estimated from immunological data was 27,000 while the molecular weight calculated from loss of enzymatic activity was 118,000.

SUMMARY

Bovine thyrotropin was irradiated with 2.0 m.e.v. electrons. Biological activity of the bovine thyroid-stimulating hormone was measured by depletion of radiiodine from the thyroids of day-old chicks. Immunological activity was measured by a radioimmunoassay. Irradiation caused loss of activity as measured in each assay system, but the rate of inactivation was considerably less. The molecular weight calculated from loss of enzymatic activity was 18.6 x 10^{6}.

$M = V_p N$

where $p$ = density, $N$ = Avogadro's number, and $V$ = volume of molecule. If one ion cluster per g = 1.2 x 10^{-12} rads, then

$$M = \frac{0.72 \times 10^{23}}{R}$$

It may be noted from examination of Formula 2 that a linear relationship exists between log of percent activity remaining and radiation dose. In the instances that the premises upon which the theoretical development was based are not true, the experimental data will deviate from those predicted. If log of percent activity remaining is plotted against radiation dose, a straight line of slope $-V$ is obtained. If more than one ionization is required to inactivate a single molecule, a curve having a shoulder (concave downward) is obtained. When only two or three ionizations are required to inactivate a molecule, the slope of the linear portion of such a curve can be calculated and utilized to estimate molecular weight. Such estimates will contain a small error when few ionizations are required but increasingly large errors when larger numbers of ionizations are required to inactivate the molecule (16).
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REFERENCES

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