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Research Article

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SOME MOLECULAR CHARACTERISTSICS OF ERYTHROPOIETIN FROM DIFFERENT SOURCES DETERMINED BY INACTIVATION BY IONIZING RADIATION

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The existence of a substance stimulating erythropoiesis in response to anemia or anoxia (erythropoietin, erythropoiesis-stimulating factor. ESF) has been amply confirmed by clinical and experimental data (1). The occurrence of material stimulating erythropoiesis in pathologic conditions such as cerebellar hemangioblastoma (2), renal cysts (3, 4), and renal tumors (5, 6) associated with polycythemia has been noted. Attempts to purify erythropoietin derived from anemic sources have been unsuccessful to date, although extracts of great activity have been made from serum and urine of anemic patients and animals (7–9). The molecular characteristics of the active principle have been studied by using these partially purified concentrates.

Estimates of molecular size and shape of biologically active compounds can be made with unpurified material by an interpretation of the inactivating effects of irradiation upon the biological activity. The theoretical basis for these interpretations was formulated by Lea (10). Pollard, Guild, Hutchinson, and Setlow (11, 12) have noted good correlation between the molecular weight of numerous compounds estimated by these techniques and that estimated by physical tests on pure preparations.

The purpose of this paper is to present the results of efforts to determine the size and shape of the erythropoietin molecule by subjecting crude extracts from the urine of an anemic patient to various doses and qualities of radiation and determining the loss of biological activity by bioassay in transfused polycythemic mice. Fluids containing erythropoiesis-stimulating activity from a renal cyst and from a cystic cerebellar hemangioblastoma were similarly treated, and the results compared with those obtained from the urine-derived material.

METHODS

Erythropoietin-containing material was extracted from the urine of a patient with aplastic anemia by a modification of the technique proposed by Gordon (13). Urine was collected in refrigerated bottles for 24 hours and was acidified to a pH of 4.8 with HCl. Approximately 20 g of acid-washed kaolin was added to the acidified urine and thoroughly mixed. The mixture was filtered with vacuum through Whatman no. 50 filter paper which retained the kaolin. The filtrate was discarded, and the active material was eluted from the kaolin with 100 ml of 1 N NH_4OH. The eluate was dialyzed against tap water overnight in a rocking dialyzer. The dialysate was lyophilized and a light brown, soluble powder resulted which contained about 10 times more erythropoiesis-stimulating activity per milligram than a dialyzed and lyophilized portion of untreated urine. Renal cyst fluid containing erythropoiesis-stimulating activity from a patient with polycythemia and cerebellar hemangioblastoma cyst fluid from a polycythemic patient previously reported (2) were analyzed without extraction.

Samples containing erythropoietin were irradiated either with 2-Mev electrons (low ionization density) to determine the total "vulnerable volume" or with low energy (16 KVcP) X rays (higher ionization density) to ascertain the asymmetry of the target molecule by estimating the total cross-sectional area of the sensitive region.

A beam of 2-Mev electrons was generated in a Van de Graaf electrostatic accelerator and scanned in one axis by an oscillating field coil. The beam was not significantly scattered by the exit window and remained essentially mono-energetic. Output dose-rate was calibrated by means of ionization chambers and gel dosimetry and was maintained at a constant rate while samples to be irradiated were subjected to multiple passages under the beam at a rigidly controlled rate of speed to achieve the desired final doses.

Ten-mg samples of the powdered urinary concentrate were weighed into thin aluminum planchets 2.5 cm in diameter and 0.21 cm deep and evenly spread over the surface of the planchet. One-ml portions of the renal

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and cerebellar hemagioblastoma cyst fluids were dried on similar planchets at 46° C. The planchets were placed on a 2-cm layer of dry ice atop a rotating aluminum disc. The linear rate of progression of each sample under the beam was 3.5 cm per second, and the samples received either 1×10^5 or 5×10^5 rads per pass, depending upon the magnitude of the beam current that was maintained. Dry ice was replaced as necessary during the course of irradiation, so that the samples, resting atop the ice, were at no time subjected to heating. The maintenance of dry-ice temperature was also necessary to exclude liquid or vaporized water, thus avoiding the necessity of irradiation in a completely dry atmosphere.

Low-energy X rays were produced at 16 kv accelerating voltage maintained by a well-filtered, constant, potential apparatus in a beryllium-window tube. Ten-mg samples of urinary concentrate containing erythropoietin were dissolved in 0.8 ml distilled water and evaporated into aluminum planchets at 46° C. Samples were held rigidly 1 cm below the exit window. The sample and tube housing were surrounded with dry ice and air passed over dry ice was circulated between the sample and the exit window. The output was calibrated by using a Victoreen low-energy 2,500 r chamber which had recently been calibrated at the National Bureau of Standards and a rotating absorber wheel from which a sector had been removed. This latter device allowed reliable calibration at extremely high dose-rates. Erythropoietin-containing samples received 5×10^5 r per minute, and the total dose delivered was monitored by electrical integration of beam current for the total exposure time.

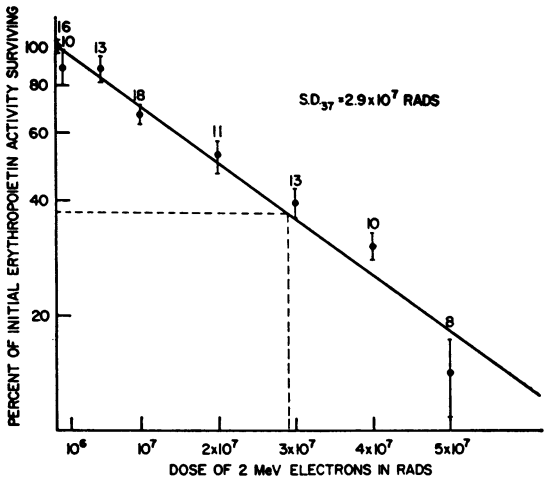

FIG. 1. THE INACTIVATION OF ERYTHROPOITEIN ACTIVITY OF URINE EXTRACTS BY IRRADIATION WITH GRADED DOSES OF 2-MEV ELECTRONS. Each point represents the mean of all animals receiving equally irradiated material; the number of such animals is shown above each point. The standard error of the mean is shown by the lines bracketing the point. The line derived by the least-squares method is superimposed (solid line ———). The dose inactivating 63 per cent of the erythropoietin present initially (SD_{37}) is indicated by the broken line.

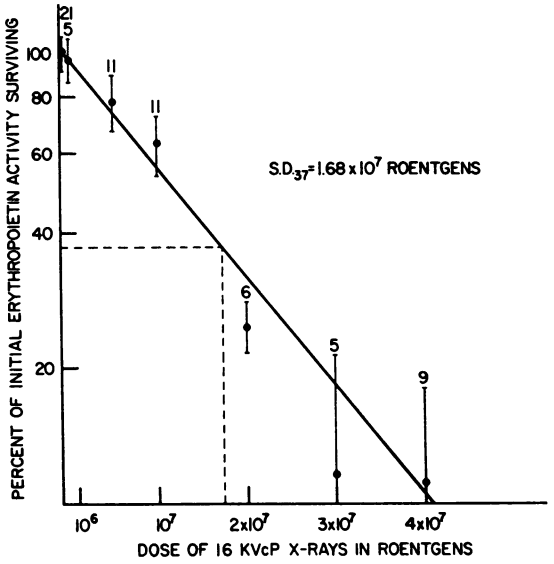

FIG. 2. THE INACTIVATION OF ERYTHROPOIETIN ACTIVITY OF URINE EXTRACTS BY IRRADIATION WITH GRADED DOSES OF KVcP X RAYS. Each point represents the mean of all animals receiving equally irradiated material; the number of such animals is shown above each point. The standard error of the mean is shown by the lines bracketing the point. The line derived by the least-squares method is superimposed (solid line ———). The SD_{37} (the dose inactivating 63 per cent of the initial activity) is indicated by the broken line.

When the samples had been irradiated, the material was dissolved in sufficient physiological saline. Unirradiated control samples were used to assess the initial activity.

The erythropoietin activity of the samples was estimated with a polycythemic mouse assay (14). Female NIH mice weighing 18 to 22 g were injected intraperitoneally on day 1 and day 4 with 0.7 ml of an 80 per cent homologous red cell suspension collected in ACD solution and concentrated by centrifugation. The mice were divided into groups of eight animals, and on days 4 and 5, 0.5 ml of the test material was injected subcutaneously. One-half ml saline was injected into animals used as negative controls. On day 6, 0.5 μc Fe^{59} citrate was injected intraperitoneally. Forty-eight hours later, the mice were bled by decapitation into tared bottles which were reweighed to determine the amount of blood. Duplicate microhematocrits were done, and the data from mice with hematocrits less than 55 were not used. The radioactivity due to Fe^{59} was determined in a well-type gamma ray scintillation counter with a thallium-activated NaI crystal. The percentage of the injected dose of Fe^{59} per milliliter of blood was calculated by using a dilute standard.

RESULTS

In Figure 1 the fraction of the initial erythropoietin activity of the urinary extract remaining

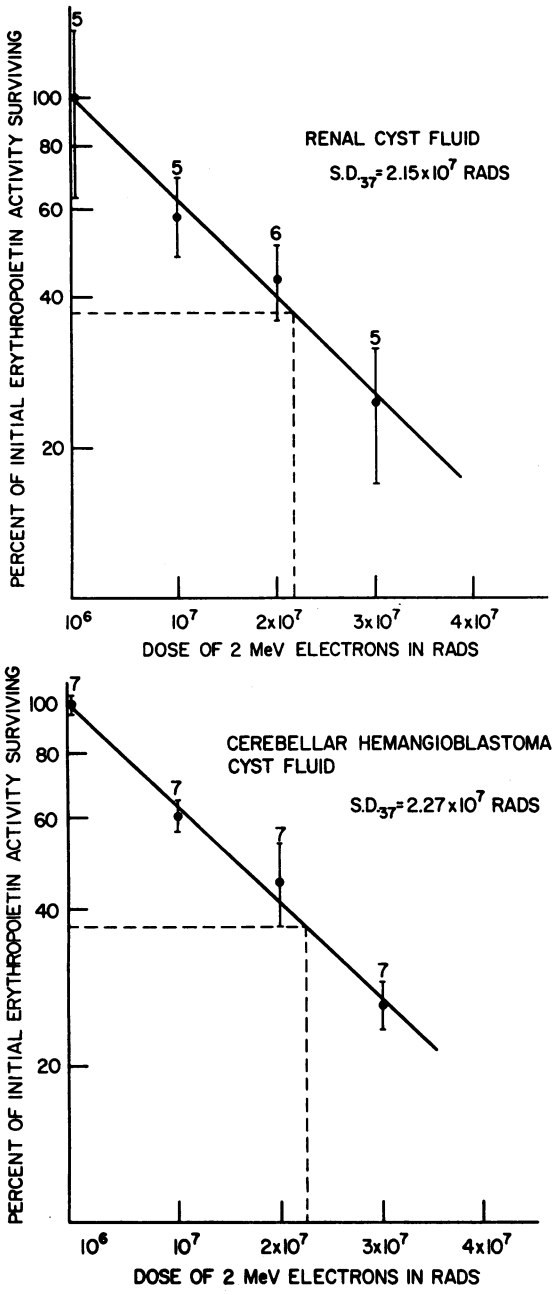

FIG. 3, A AND B. THE INACTIVATION OF THE ERYTHROPOIESIS-STIMULATING ACTIVITY OF RENAL CYST FLUID (A) AND CEREBELLAR HEMANGIOBLASTOMA CYST FLUID (B) WITH GRADED DOSES OF 2-MEV ELECTRONS. Each point represents the mean of all animals receiving equally irradiated material; the number of such animals is shown above each point. The standard error of the mean is shown by the line bracketing the point. The line derived by the least-squares method is superimposed (solid line ———). The SD_{37} (the dose inactivating 63 per cent of the initial activity) is indicated by the broken line.

after irradiation is plotted against the dose in rads of 2-Mev electrons. The least-squares regression line of the means passes near the origin, and its slope is such that 37 per cent of the initial activity remains at a dose of 2.90×10^7 rads (SD_{37} = 37 per cent surviving dose). The coefficient of correlation of this line is 0.979. In Figure 2 similar data is given for irradiation of the same material with 16 KVcP X-rays. The least-squares regression line of the means again passes through the origin and the SD_{37} is 1.68×10^7 roentgens. The coefficient of correlation of this line is 0.975. The analysis of these results according to classical target theory, with tabular data presented by Lea, is shown in Table I.

Figure 3, A and B, presents data derived from the irradiation of renal cyst fluid and cerebellar cyst fluid, respectively, with 2-Mev electrons. These results are compared with those from the irradiation of erythropoietically active urine extract in Table II.

DISCUSSION

The molecular weight of erythropoietin from an anemic source calculated from the present data supports the contention of Rambach, Cooper, and Alt (7) and White, Gurney, Goldwasser, and Jacobson (8) that erythropoietin is a small molecule. The determination of the molecular weight by this technique has the major advantage that purity of the compound is not necessary for the analysis. This is of special importance, since chemically and physically pure erythropoietin has not been produced despite great efforts. Goldwasser and his associates have concentrated the erythropoietin activity of large quantities of anemic sheep plasma nearly 50,000 times, but the small amount of material which they obtained was not pure when chromatographed (8).

The technique of irradiation inactivation enabled us to compare erythropoiesis-stimulating material from renal and cerebellar cysts with that produced in response to anemia. Since only milliliter quantities of these cyst fluids were available, concentration or purification were not possible. These studies indicate that the erythropoiesis-stimulating material from these sources are all molecules of the same or nearly the same size.

Gordon (1) has suggested that erythropoietin may be a small molecule that is associated with a

TABLE I

Target-theory analysis of radiation inactivation data for erythropoietin in urine extracts

Radiation	37% Inactivation dose (SD_{37})	Apparent target diameter (2r)	Apparent molecular weight *	Approximate 95% confidence limits of estimation of molecular weight †
2 Mev electrons	$2.9 = 10^7$ rads	3.9 mμ	27,000	24,000–30,000
16 KVcP X-rays	1.68×10^7 roentgens	5.2 mμ	66,000‡	52,000–77,000

* Assuming a density of 1.35 g per cc (see text).
† See appendix.
‡ Assuming a spherical shape (see text).

glycoprotein moiety. The present studies, however, indicate that the unit necessary for biological activity has a molecular weight of about 30,000; this is compatible in size with a small protein. The enzymatic inactivation of erythropoietin by proteolytic enzymes such as trypsin, chymotrypsin, aminopeptidase, and papain also suggests that the protein structure is essential for biological activity (15).

Since the inactivation characteristics of irradiation measure the volume of the biologically active molecule, the extrapolation to molecular weight depends on an assumption of density for the material (10). Simple proteins have a density of 1.35 g per cc; carbohydrate-containing proteins have greater density in proportion to their carbohydrate content. The erythropoietin-rich material concentrated by Goldwasser and his associates contained 29.2 per cent carbohydrate (16). If we assume a density of 1.60 g per cc for the carbohydrate portion, the density of the erythropoietin molecule is probably not greater than about 1.4. No appreciable difference in the results of the calculations is seen if this figure is used as the density of the material.

The apparent inconsistency between the high-energy electron and low-energy X-ray data might be explained by the difficulty in obtaining absolute dosimetry with low-energy X rays due to the broad spectrum of secondary electron energies produced; however, a more likely explanation is that a target that departs markedly from spherical form will show increasingly larger apparent target radii as the ionization density of the radiation is increased (10). Multiplicity of sensitive "target" sites within a single erythropoietin molecule could give similar results (16), but this hypothesis can be dismissed, since the shape of the inactivation curve with sparsely ionizing, 2-Mev electrons is of the "single-hit" or pure exponential variety. A multiple target system would give a sigmoidal dose-response to radiation of this ionization density. Hence, realizing the limitations of the data obtained in these experiments, we feel that urine-derived erythropoietin probably is an elongated molecule up to ten times as long as its breadth, as calculated from the tables of Lea (10).

CONCLUSIONS

A crude, urine-derived, erythropoietin-containing preparation was subjected to inactivation by high-energy electrons and low-energy X rays. The molecular weight was calculated to be about 27,000 by use of the "target theory" computations of Lea (10). Inactivation characteristics to low-energy X rays suggest that the molecule is asym-

TABLE II

Comparison of data derived from irradiation inactivation for erythropoiesis-stimulating material from three sources

Source	37% Inactivation dose (SD_{37})	Apparent molecular weight	Approximate 95% confidence limits of estimation of molecular weight *
Urine of anemic patient	2.90×10^7	27,000	24,000–30,000
Cerebellar hemangioblastoma cyst fluid	2.27×10^7	34,500	29,000–40,000
Renal cyst fluid	2.15×10^7	36,500	30,000–46,000

* See Appendix.

metric and is about ten times as long as it is wide. The result of irradiation of erythropoiesis-stimulating material from renal and cerebellar hemangioblastoma cysts from patients with polycythemia suggests that the biologically active molecule from these sources has the same or nearly the same size as that derived from urine of an anemic patient.

APPENDIX

Basis of calculations and estimation of error. The calculations for the determination of molecular size from the inactivating effects of ionizing irradiation are based on the assumptions and calculations of Lea (10). The area within which an ionization, a "hit," must be produced to obtain inactivation of the molecule in the "target." It is assumed that a single hit in a given target will inactivate the target molecule. For low doses of radiation, the number of inactivations is equal to the number of "hits." As the dose is increased, however, some of the ionizations will occur in targets already hit, and hence the number of inactivations will be less than the number of hits. Therefore, for given increments of radiation dose, the same fraction of the remaining targets will be inactivated rather than the same absolute number of targets. Consequently, the curve of surviving activity (a measure of the number of surviving targets) plotted against dosage of radiation is exponential. This relationship is expressed by the formula:

$$-dn/n = dD/D_0, \qquad [1]$$

which integrates to

$$ln\ (n/n_0) = -D/D_0, \qquad [2]$$

which may be rearranged to

$$n = n_0 e^{-D/D_0}. \qquad [3]$$

In these equations, n_0 is the initial number of targets, n is the number of targets surviving a dose D, D_0 is the dose required to score an average of one hit per target, and e is the base of the natural logarithms. In order to find the dose D_0, D is set equal to D_0 and Equation 3 becomes

$$n = n_0 e^{-1}. \qquad [4]$$

Since $e^{-1} = 0.368$, the number of targets surviving the dose D_0 is 36.8 per cent of the initial number of targets, i.e., at the dose of radiation at which approximately 37 per cent of the initial biological activity remains, the statistical average of one ionization per molecule has occurred. This is referred to as the mean lethal dose, or 37 per cent surviving dose (SD_{37}).

Tables and charts have been calculated by Lea relating the SD_{37} to the molecular size and by extrapolation to the molecular weight. The calculations on which these charts are based correct for overlapping of the effects of more than one ionization cluster and for δ-rays (low-energy radiation produced during ionization). Such calculations are 'tedious to describe and tedious to perform' (10). An approximation for high-energy radiation is given by the equation

$$SD_{37} \times M = 0.7 \times 10^{12}, \qquad [5]$$

where M is the molecular weight (12).

The error in the estimation of the molecular diameter or molecular weight due to random variability in the technique of irradiation or in the bioassay of the biological activity is thus related to the error in the estimation of the SD_{37} due to these causes. The SD_{37} in these experiments has been calculated from the least-squares line (Figures 1–3). The calculation of the standard error of the coefficient of regression of that line gives an estimate of the variability of that line due to random processes. Thus, the ranges of error given for the molecular weights are calculated by adding and substracting respectively twice the standard error of the coefficient of regression to and from that coefficient in order to give approximate 95 per cent confidence limits. The SD_{37} for the line described by these expressions was calculated and the molecular weights corresponding to these values of SD_{37} were estimated from the charts of Lea.

The error in the estimation of molecular weight due to inadequacies of the theory of radiation inactivation is difficult to evaluate. A fairly close correlation between molecular weights derived by this theory and those calculated from purified products has been shown (12). Thus the application of the target theory gives a good approximation of the molecular size especially when high-energy, sparsely ionizing radiations are used.

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