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## A radioimmunoassay which specifically measures human chorionic gonadotropin in the presence of human luteinizing hormone

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*With antiserum to the  $\beta$ -subunit of human chorionic gonadotropin (HCG), we have developed a radioimmunoassay which selectively measures HCG in samples containing both human pituitary luteinizing hormone (HLH) and HCG. High HLH levels observed in samples obtained at the midcycle peak or from castrate patients do not cause significant inhibition in the specific HCG radioimmunoassay. The sensitivity of the assay is sufficient for distinguishing HCG from follicular and luteal phase HLH levels. This specific HCG radioimmunoassay is ideal for following serum HCG levels in patients undergoing chemotherapy for HCG-secreting tumors as well as for follow-up of patients after termination of molar pregnancies. In addition, the sensitivity of the assay will permit earlier diagnosis of pregnancy which, in turn, would permit earlier therapeutic intervention if desired.*

ANTISERA generated to the  $\beta$ -subunit of human chorionic gonadotropin (HCG) discriminate between human pituitary luteinizing hormone (HLH) and HCG,<sup>1-3</sup> while most of those produced following immunization with intact hormone do not. One of those sera produced following a single immunizing dose of 50  $\mu$ g of the  $\beta$ -subunit of HCG was used to develop a radioimmunoassay which specifically measures HCG in plasma samples containing both HLH and HCG. The development of that radioimmunoassay and some applications which re-

fect the potential clinical usefulness of the specific HCG assay are discussed herein.

### Material and methods

**Antigens.** Highly purified HCG (Roussel) and the  $\beta$ -subunit of HCG (CR-100 $\beta$ ) were prepared by Drs. Robert Canfield and Francis Morgan.<sup>4</sup> The highly purified HCG preparation was bioassayed by the ventral prostate weight assay<sup>5,6</sup> and immunoassayed by a double-antibody-immunoassay procedure outlined below. The Second International Standard for HCG, generously provided by Dr. Derek Bangham (World Health Organization), served as reference preparation for those assays. Highly purified human pituitary hormones—HLH (LER 960 and LER 1417), human pituitary follicle-stimulating hormone HFSH (LER 1366), and HTSH (human thyrotropin, Pierce Fraction 4), and a cruder extract, LER 907, containing both HFSH and HLH—were provided by the National Pituitary

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Agency through the Hormone Distribution Officer, National Institute of Arthritis and Metabolic Diseases.

**Antisera.** Specific antisera to the  $\beta$ -subunit of HCG were generated by the multiple intradermal injection technique described elsewhere.<sup>3</sup> One antiserum (SB6) with greatest sensitivity and specificity for HCG was selected for further study.

**Iodination.** A modification of the method of Greenwood, Hunter, and Glover<sup>7</sup> was used for labeling the  $\beta$ -subunit with Na <sup>125</sup>I (Union Carbide Corp., New York, New York). To 4  $\mu$ g of the  $\beta$ -subunit dissolved in 0.01 ml. of distilled water was added 2 mCi. of Na <sup>125</sup>I, 0.025 ml. of 0.4M Na phosphate buffer (pH 7.5), and 0.01 ml. (25  $\mu$ g) of chloramine T, and this was allowed to react for 2 minutes at room temperature. The reaction was stopped with addition of 0.025 ml. (62.5  $\mu$ g) of sodium metabisulfite. The reaction mixture was immediately layered onto a 5 by 0.5 cm. column bed of Biogel P-60 (50 to 100 mesh). The column bed had been equilibrated with 0.01M Na phosphate with 0.15M NaCl, pH 7.8 (phosphate-buffered saline (PBS) after having been washed with 1.5 ml. of 2 per cent bovine serum albumin (Armour) in phosphate-buffered saline. A simulated <sup>125</sup>I standard (New England Nuclear, Boston, Massachusetts) was used to calculate the specific activity of the iodinated glycoprotein, assuming that the mass of iodinated hormone transferred from the reaction vessel to the column was proportional to total counts transferred onto the column. Specific activities attained ranged from 50 to 100  $\mu$ Ci per microgram. After each iodination, the maximum immunoreactivity of the iodinated subunit was measured by treating <sup>125</sup>I- $\beta$  with excess first antibody. When maximum immunoreactivity ranged from 65 to 85 per cent, the iodoprotein was suitable for our radioimmunoassay.

**Specificity.** The cross-reaction of the highly purified human pituitary preparations, HFSH, HLH, and HTSH, was studied by competitive inhibition in a system containing <sup>125</sup>I- $\beta$  and the antiserum to the  $\beta$ -sub-

unit of HCG. In addition to the highly purified pituitary hormonal preparations, highly purified HCG, Second International Standard HCG, Second International Reference Preparation HMG, and LER 907 were studied. The antibody was used in a final serum dilution of 1:100,000 in all assays.

**Assay procedure.** All assays were carried out by the double-antibody technique as described previously,<sup>8</sup> but the conditions for incubation were changed. The assay was initially incubated at 37° C. for 2 hours, then transferred to 4° C. for an additional 15 to 17 hours. Timed studies confirmed that equilibrium had been attained under those conditions. Second antibody (sheep anti-rabbit gamma globulin) was added at the end of that time, and incubation was continued for a minimum of 6 hours at 4° C. before separating bound and free hormone by centrifugation.

**Clinical assays.** Highly purified HCG (see above) served as reference preparation for all clinical assays. Varying concentrations of HCG were added to outdated human male blood bank plasma and aliquots were stored at -16° C. All assay tubes including blank and standard curve tubes contained an equal volume of human plasma in order to obviate the nonspecific effect of plasma proteins observed with the assay system. The nonspecific counts in assays run with and without normal human serum were identical, but maximum counts bound in the assay tubes containing labeled ligand alone were lower in the presence of normal human serum or plasma, reflecting interference of plasma proteins on the antigen-antibody reaction. A series of control plasma samples were run in replicate in all assays and served as the basis on which intra- and inter-assay variation was calculated.

Daily plasma samples obtained from women with presumptively ovulatory menstrual cycles were assayed for immunoreactive HLH and HCG. Our routine HLH radioimmunoassay measured both HLH and HCG in a given plasma sample since the antiserum used did not discriminate between HLH and HCG.

Plasma samples obtained from castrated

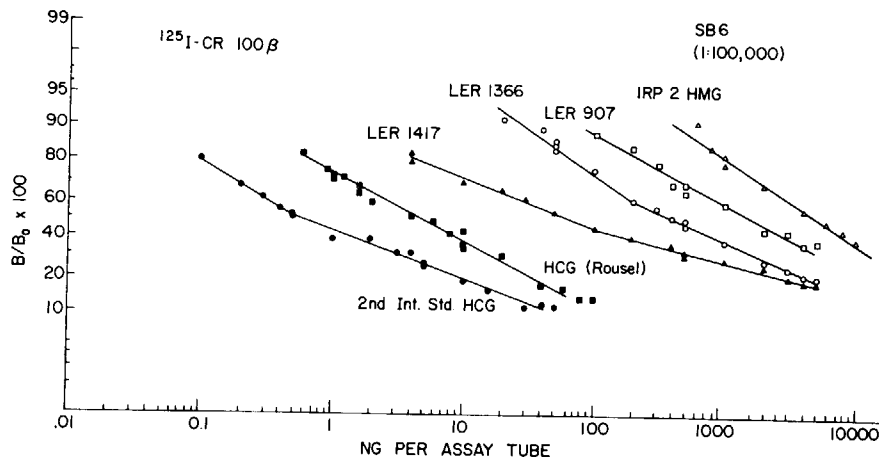


Fig. 1. Inhibition lines of Second International Standard HCG (●), HCG (Roussel) (■), HLH (LER 1417) (▲), HFSH (LER 1366) (○), HLH and HFSH (LER 907) (□), and Second International Reference Preparation HMG (Δ) in a radioimmunoassay based upon anti-HCG  $\beta$ -subunit serum and  $^{125}\text{I}$ -CR100 $\beta$  in a buffer system. The response variant is displayed as the logit transform.  $B$  = counts bound in the presence of labeled and unlabeled ligand and  $B_0$  = counts bound in the presence of labeled ligand alone.

men and women were run in both radioimmunoassay systems. Some castrated men also received exogenous HCG during the sampling period. Random samples obtained from endocrinologically normal men and women were also assayed.

Blood samples were obtained from C. P., a woman undergoing treatment for a choriocarcinoma. Twenty-four-hour urine samples were also collected and bioassayed by the mouse uterine weight (MUW) technique.<sup>9</sup>

**Results**

The highly purified HCG preparation (Roussel) used as reference preparation for all the radioimmunoassays contained a biologic activity of 12,000 I.U. per milligram by the VPW assay and 5,000 I.U. per milligram by radioimmunoassay, relative to the Second International Standard HCG.

**Specificity.** Fig. 1 shows the inhibition lines for the Second International Standard HCG, HCG (Roussel), HLH (LER 1417), HFSH (LER 1366), HLH and HFSH (LER 907), and Second International Reference Preparation HMG in the  $\beta$ -assay system without any added human plasma. HTSH (not shown in that figure) was as ineffective as HFSH in the  $\beta$ -assay system. All inhibition lines are displayed as the logit

transform of the response variant versus the log dose of antigen.<sup>10, 11</sup> None of the gonadotropin preparations tested cross-reacted at physiologic levels in the  $\beta$ -assay system.

**Nonspecific plasma protein effect.** Fig. 2 shows the inhibition lines for Second International Standard HCG and HCG (Roussel) when 200  $\mu\text{l}$  of normal human plasma was added to all assay tubes. A nonspecific plasma protein effect was found in the assay system, and that nonspecific effect was eliminated by adding an equal volume of human plasma to all assay tubes. When assays were conducted in that way, the inhibition lines for the two HCG preparations were not statistically different. Furthermore, when plasma samples obtained from pregnant women or from patients with HCG-secreting tumors were dosed out in the assay system, the inhibition lines generated were parallel to those of the reference preparation; sufficient outdated male blood bank plasma was added to the assay tubes so that all tubes contained an equal volume of plasma.

**Assay sensitivity.** Sensitivity was defined as that level in the assay at which no false positive HCG values would be encountered. Plasma samples from normally cycling women sampled serially and randomly sampled normal men and women were run in the

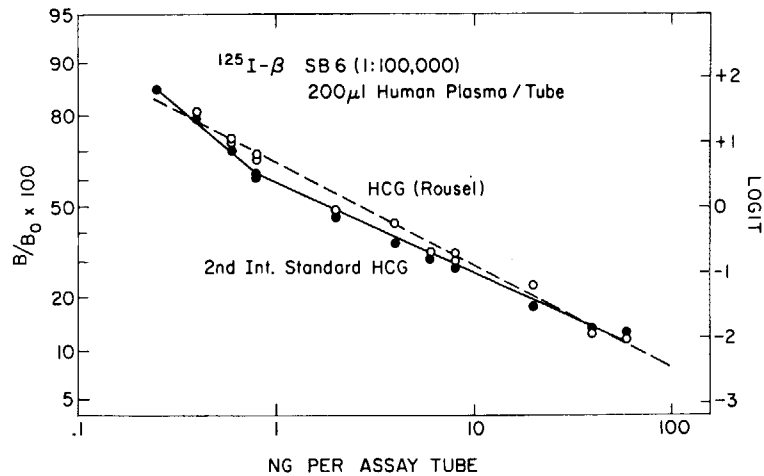


Fig. 2. Inhibition lines for Second International Standard HCG (●) and HCG (○) Rousell) with 200  $\mu$ l of normal male plasma added to all assay tubes.

Table I. Frequency distribution of precipitation by plasma samples in the specific HCG radioimmunoassay

	$B/B_0 \times 100$				
	75-80	81-88	86-90	91-95	96-100
Castrated women	5 (5)*	11 (11)	11 (11)	30 (30)	45 (45)
Women with cycles	2 (0.6)	9 (3)	27 (9)	55 (18)	207 (70)

\*Figures in parentheses are per cent frequencies.

assay system. The  $B/B_0 \times 100$  (where  $B$  = counts bound in the presence of labeled and unlabeled ligand and  $B_0$  = counts bound in the presence of labeled ligand alone) of the samples were grouped as a frequency distribution (Table I). None of the plasma samples had a  $B/B_0 \times 100$  of less than 75 (75 per cent) which corresponded to 0.2 ng. of HCG on most assays. Therefore, 75 per cent was used as the cutoff point on most assays, and that point corresponded to 5 mIU. per milliliter for a 200  $\mu$ l plasma or serum sample.

**Within- and between-assay variation.** All radioimmunoassays were initially calculated by high-speed digital computers with programs designed by Rodbard and Lewald.<sup>10</sup> The within- and between-assay variation of potency estimates on replicate samples run on the assays was computed as described by Rodbard.<sup>11</sup> The within-assay coefficient of variation was 15 per cent, and that for between-assay was 27 per cent for 4 replicate

samples ranging from 7.3 to 19.7 ng. per milliliter.

**Clinical samples.** Fig. 3 shows the HLH and HCG values from plasma samples obtained from a woman with presumptively ovulatory menstrual cycles. The high mid-cycle HLH values did not interfere with the specific HCG radioimmunoassay. Tonicly elevated HLH levels of plasma samples obtained from a castrated man resulted in no significant inhibition in the specific HCG assay (Fig. 4). Another castrated man with tonically elevated HLH levels was given exogenous HCG. Fig. 5 shows the HLH and HCG levels in his plasma samples over his course of observation. It should be recalled that the HLH values were ascertained in an assay system which measured both HLH and HCG in a given plasma sample.

Fig. 6 shows the disappearance of plasma and urinary HCG activity in C. P., a patient undergoing therapy for a gestational choriocarcinoma. Plasma HCG immunoreactivity

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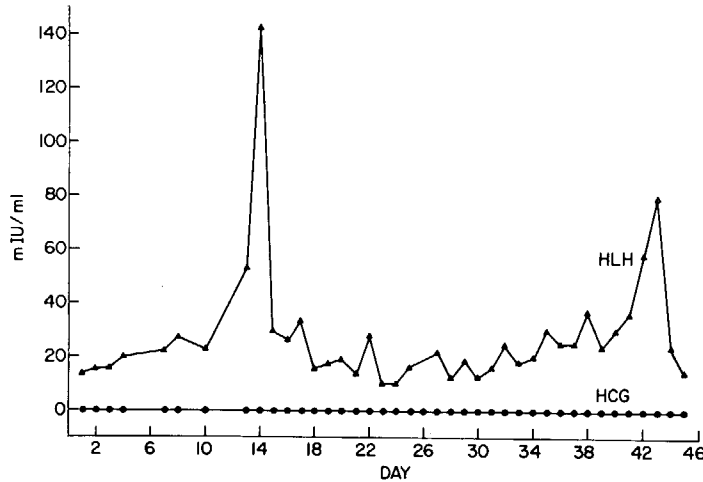


Fig. 3. Plasma HLH (▲) values determined by a radioimmunoassay which could not discriminate between HCG and HLH and plasma HCG (●) values obtained by our specific HCG radioimmunoassay. Plasma samples were obtained from a woman with presumptively ovulatory cycles.

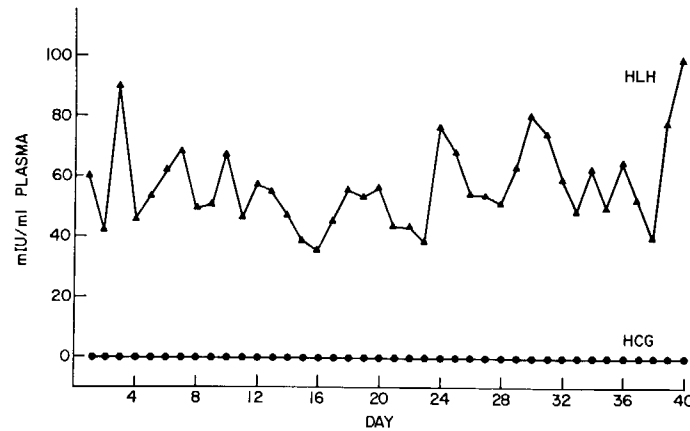


Fig. 4. Plasma HLH (▲) and HCG (●) levels in a castrated man. HLH values were determined in a radioimmunoassay system which could not distinguish between HLH and HCG. HCG values were determined by our specific HCG radioimmunoassay system.

remained detectable even after several 24 hour urine collections contained biologic activity within the normal range for cycling women.<sup>12</sup>

**Comment**

The human glycoprotein hormones are composed of 2 dissimilar non-covalently linked subunits.<sup>4, 12-15</sup> It would appear that the  $\alpha$ -subunit is a common subunit among the hormones. In contrast to  $\alpha$ -subunits, the amino acid composition of the  $\beta$ -subunits of the human glycoprotein hormones differs considerably.<sup>4, 12-13</sup> Biologic and immuno-

logic studies with the subunits of the gonadotropins are consistent with the  $\beta$ -subunit's conferring the unique biologic and immunologic activities of a given hormone.<sup>1, 2, 13, 15</sup>

All antisera generated to the  $\beta$ -subunit of HCG discriminated between HCG and HLH.<sup>1-3</sup> Those antisera enabled us to develop a radioimmunoassay, specific for HCG, in which highly purified human pituitary FSH, LH, and TSH did not significantly cross-react with HCG in the range in which those pituitary hormones are present in human serum under physiologic conditions. The latter observation permitted us to de-

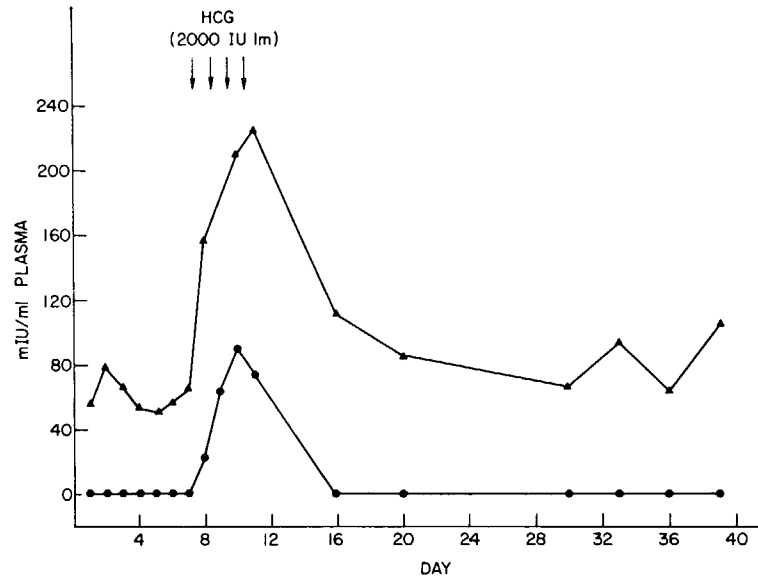


Fig. 5. Plasma HLH (▲) and HCG (●) levels in a castrated man before, during, and after administration of exogenous HCG. HLH levels were determined in an assay which could not discriminate between HCG and HLH.

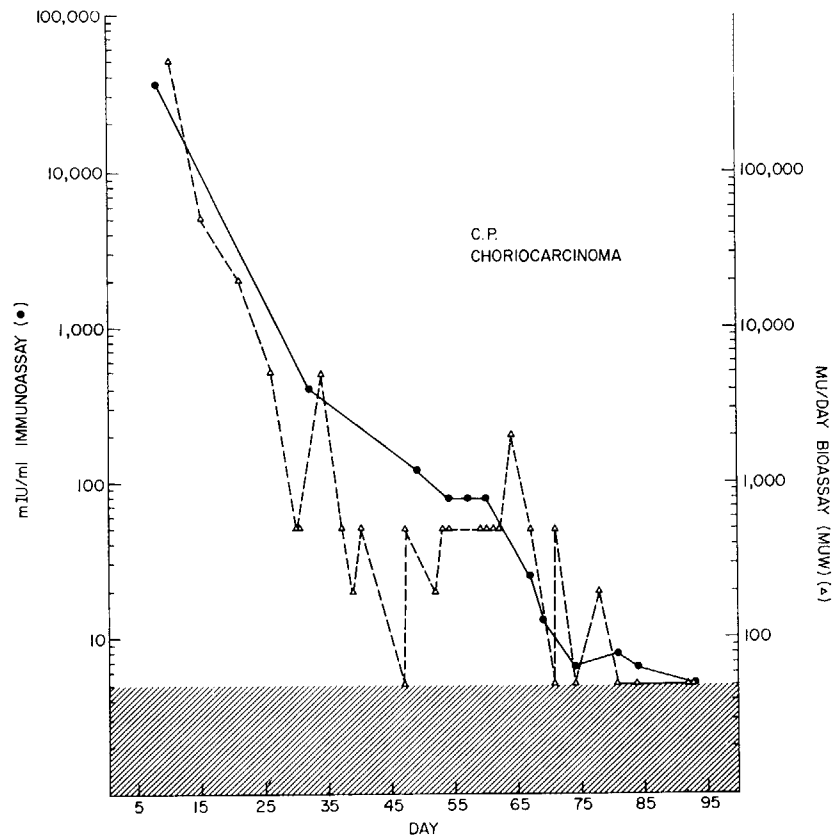


Fig. 6. Plasma HCG (●) levels and daily gonadotropin excretion (Δ) in a patient undergoing therapy for a gestational choriocarcinoma. Shaded area represents level below lower limit of HCG radioimmunoassay.

velop an assay system which selectively measured HCG when both HCG and HLH were present in a given plasma or serum sample.

A nonspecific plasma protein effect was noted with the specific HCG radioimmunoassay system. When inhibition lines for the Second International Standard HCG and highly purified HCG were generated in a buffer system, the Second International Standard HCG had greater immunoreactivity (Fig. 1) than the highly purified HCG preparation; however, when those same preparations were run in an assay system containing 200  $\mu$ l of outdated human male plasma in all assay tubes, the two inhibition lines were superimposable (Fig. 2), probably reflecting nonspecific interference by material present in the cruder Second International Standard for HCG in the assay system not containing human plasma. This nonspecific plasma protein effect has been observed in other assay systems.<sup>16, 17</sup>

Plasma samples obtained from women throughout presumptively ovulatory cycles and samples obtained from castrated men with tonically elevated HLH levels showed that high plasma HLH levels did not result in significant inhibition in the specific HCG assay system. If one examines the inhibition lines for highly purified pituitary HLH and Second International Reference Preparation HMG, a relatively crude gonadotropin extract prepared from postmenopausal urine, then one can see that the immunologic behavior of circulating HLH was intermediate between highly purified pituitary HLH and relatively crude urinary gonadotropin preparation containing HLH (Fig. 1). If circulating HLH behaved immunologically as Second International Reference Preparation HMG, then one would have expected to find significant inhibition by plasma samples ob-

tained from castrated patients or from women at their midcycle HLH peaks in the specific HCG assay. On the other hand, if circulating HLH behaved as highly purified pituitary HLH, then no samples would have caused the degree of inhibition summarized in Table I. In short, then, circulating HLH is an immunologic species different from pituitary or urinary HLH.

With administration of exogenous HCG to a castrated man, serum HCG levels became detectable at a level indistinguishable from castrated HLH levels as determined by an assay system which could not discriminate between HCG and HLH in the same plasma sample (Fig. 5). Furthermore, the specific HCG assay was sufficiently sensitive to measure plasma HCG levels when those levels fell within the range of serum HLH levels observed in regularly cycling women. In plasma samples obtained from a patient undergoing chemotherapy for a choriocarcinoma, HCG remained detectable at a time when urinary gonadotropin excretion determined by bioassay (MUW) was within the range for women with normal cycles, a point at which chemotherapy has usually been stopped.<sup>18, 19</sup>

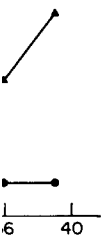
In short, a specific radioimmunoassay has been developed which specifically and selectively measures HCG in plasma or serum samples containing both HLH and HCG. The sensitivity of the assay is sufficient for distinguishing HCG from follicular or luteal phase HLH levels. The specificity and sensitivity of the assay are ideal both for diagnosis and for following the course of the disease among patients undergoing chemotherapy for HCG-secreting tumors and for following serum HCG levels in patients after termination of molar pregnancies. The entire assay can be carried out within 36 hours.

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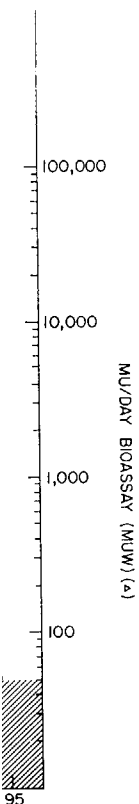
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