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Preparation of Iodine-125-Labeled Insulin for Radioimmunoassay: Comparison of Lactoperoxidase and Chloramine-T Iodination

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The enzymatic radioiodination of porcine insulin by a system consisting of lactoperoxidase, hydrogen peroxide, and $Na^{125}I$ was compared with a modified chloramine-T technique. Satisfactory specific activity of the labeled hormone was obtained with the enzymatic iodination, with much greater immunoreactivity and stability than after chloramine-T, besides being quite suitable for the measurement of low plasma insulin levels. There was a positive and highly significant correlation between the insulin concentrations measured with the two tracers, with the regression line defined by the equation: y (chloramine-T) = 8.34 + 0.99 x (lactoperoxidase).

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Chloramine-T is the oxidizing agent most widely used for radioiodination of insulin (*1,2*), but it can produce overoxidation and structural changes in the protein. Recently several authors have used lactoperoxidase for the iodination of polypeptide hormones (*3–5*), since in addition to oxidizing iodide to "active iodine," it has the advantage of specifically incorporating the halogen into the tyrosyl radicals of the molecule.

In this report we compare the chloramine-T and lactoperoxidase methods of iodination of insulin for the production of radioinsulin of high specific activity, adequate immunoreactivity, and low susceptibility to damage. The enzymatically labeled hormone was shown to be quite suitable for the measurement of low plasma insulin levels such as those obtained during ethanol-induced hypoglycemia or prolonged fasting.

MATERIALS AND METHODS

The following reagents were used: porcine insulin, recrystallized; human insulin, six times recrystallized; $Na^{125}I$ with specific activity of at least 200 mCi/ml, specified as carrier-free and without reducing agents; antisera to commercial porcine insulin, produced in guinea pigs*; lactoperoxidase, prepared as a stock solution at 5 μg/μl in 0.05 *M* sodium phosphate buffer (pH 7.5) and stored at −20°C; hydrogen peroxide 30%; hydrolyzed starch; BioGel P-60 (100–200 mesh); and Sephadex G-50.

· **Enzymatic radioiodination of porcine insulin.** The reaction was carried out at room temperature (25°C) in small polystyrene tubes (11 × 68 mm). The reagents were added in the following order: 1. 4 μg of porcine insulin (1 μg/μl). 2. 20 μl of acetate buffer 0.4 *M* (pH 5.6). 3. 2 μl of $Na^{125}I$ (1 mCi). 4. 5 μl of lactoperoxidase (5 μg/μl). 5. 5 μl of hydrogen peroxide (300 ng) added twice, with a 1-min interval. 6. 100 μl of enzymatic inhibitor, a solution containing 16% sucrose (w/v), 1% potassium iodide (w/v), 0.02% of sodium nitrite (w/v), and 0.1% of bovine serum albumin (BSA) (w/v).

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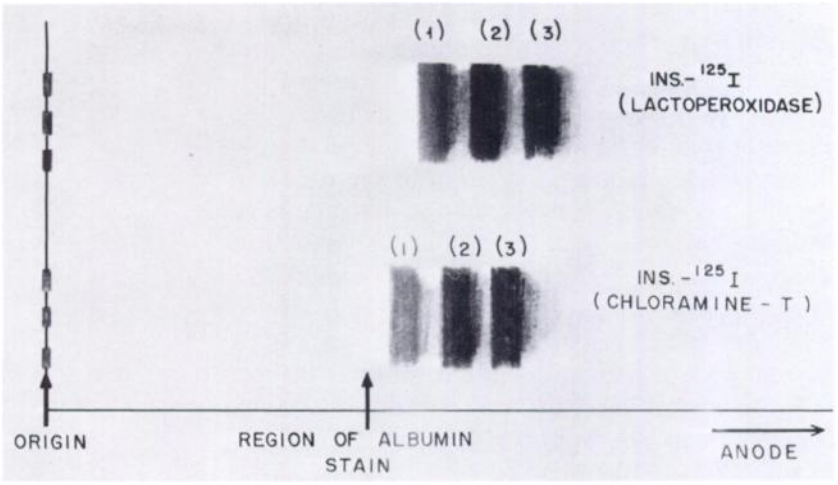

FIG. 1. Scheme of autoradiogram of starch-gel electrophoretogram of I-125 insulin immediately after iodination.

Radioiodination of porcine insulin by chloramine-T method. The standardized method using chloramine-T (*6*), was modified in our laboratory, with reduction in the amount of the oxidative agent to 1 μg of chloramine-T per μg of hormone, to minimize over-oxidation and iodination. The reagents were added as follows: 1. 4 μg of porcine insulin (1 μg/μl). 2. 20 μl of 0.2 *M* sodium phosphate buffer, pH 7.5. 3. 2 μl of $Na^{125}I$ (1 mCi). 4. 4 μg of chloramine-T (26.5 mg/100 ml phosphate buffer). 5. 25 μg of sodium metabisulfite (52.5 mg/100 ml phosphate buffer). 6. 40 μl of human plasma stained with bromophenol blue ("blue plasma").

The reaction was carried out at 4°C in small glass tubes (11 × 68 mm) and a few seconds elapsed before the addition of each new reagent.

A small aliquot (2–5 μl) of the iodination mixture, obtained by each method, was removed for the assessment of the efficiency of the labeling by its adsorption to dextran-coated charcoal, a method that compares favorably with the standard paper chromatoelectrophoresis (*7*). The efficiency is expressed as percentage of the total radioactivity incorporated into the intact hormone.

Purification of the labeled hormone. To purify the labeled hormone preparation, freeing it from unreacted iodine and damaged components, the following methods were employed:

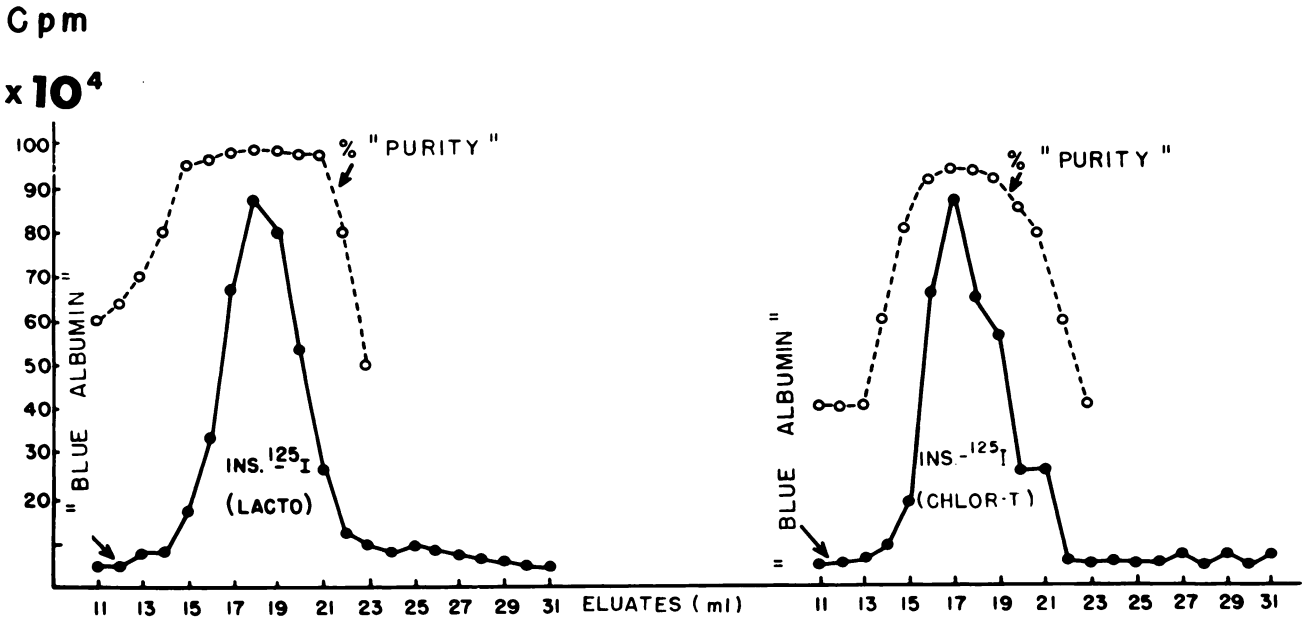

FIG. 2. Gel filtration on Sephadex G-50 of I-125 insulin labeled with lactoperoxidase or chloramine-T, after previous purification on starch-gel electrophoresis (fraction 2).

1. *Starch-Gell electrophoresis followed by Sephadex-Gel (G-50) filtration.* The starch gel was prepared by the method of Smithies (*8,9*) using Connaught hydrolyzed starch. As shown in Fig. 1, the autoradiogram of I-125 insulin on starch gel showed three components migrating ahead of the region of the albumin stain, the most cathodal fraction being designated as Zone 1 and the most anodal as Zone 3. This purification separates I-125 insulin components by their degree of iodination: the most highly labeled molecules containing diiodotyrosyl residues (DIT) migrate preferentially into Zone 3, whereas the less fully iodinated insulins, with relatively greater amounts of monoiodotryrosyl residues (MIT), are found in Zone 1, the same region as unlabeled insulin. Figure 1 shows the autoradiograms on starch gel of I-125 insulin labeled with lactoperoxidase and chloramine-T. The Zone-2 fraction of the labeled hormone—preferred because of its small proportions of unlabeled hormone and DIT—was extracted from the starch gel and purified on a Sephadex G-50 (1 × 50 cm) column equilibrated with 0.02 *M* barbital buffer (pH 8.6). When packed, the column was washed with "barbital-albumin" (0.02 *M* barbital buffer containing 2.5% human serum albumin).

The flow rate was adjusted to 3 ml/hour and 1-ml eluates were collected. In all samples applied to the column, human serum albumin, labeled with bromophenol blue, was used as an internal standard emerging near the void volume, and I-125 to mark the salt peak. Purified labeled hormone emerged at about 50% of the elution volume, between "blue albumin," containing the damaged hormone, and free unreacted I-125 (Fig. 2).

2. *Purification by BioGel P-60 filtration.* BioGel P-60 was allowed to swell in water for 72 hr, packed in a 20- × 1-cm column, and washed with "barbital-albumin." An aliquot of the enzymatic iodination mixture was put directly on the gel and eluted. The rate of flow from the column was kept at 3 ml/hr, and 1-ml fractions were collected. Figure 3 shows the elution profile of I-125 insulin from BioGel P-60. Three radioactive peaks emerge, corresponding to (a) the damaged components that are eluted with the plasma proteins (eluates 4–8); (b) undamaged radioinsulin with a high degree of "purity" (eluates 9–11); and (c) I-125 (eluates 12–16).

Stability and immunoreactivity of the labeled insulin. Insulins labeled via chloramine-T and lactoperoxidase were incubated with the specific antiserum in dilutions of $1:1 \times 10^5$, $1:2 \times 10^5$ and $1:4 \times 10^5$. The incubation period lasted 3 days at 4°C, with daily checks for control of the physicochemical and immunologic behavior of the iodoinsulins.

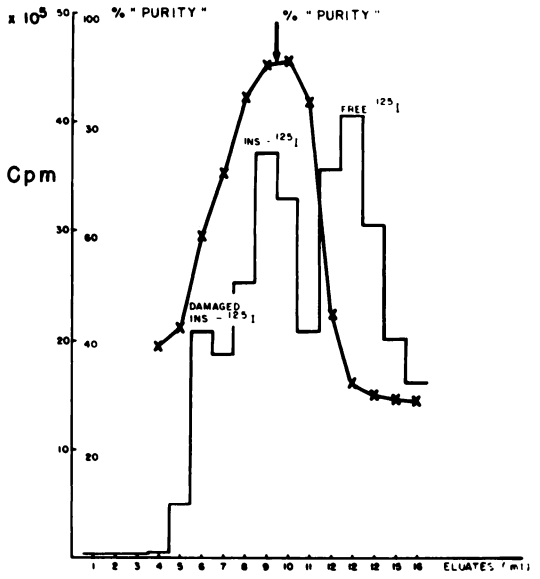

FIG. 3. BioGel P-60 chromatography of I-125 insulin labeled with lactoperoxidase.

The protocol for insulin radioimmunoassay (RIA) is given in Table 1.

Table 2 shows the results of the daily checks for the degradation of the tracer during incubation ("C" in Table 2, explained in footnote) and for its immunoreactivity (similarly see "B/F" in Table 2), the latter being measured by the maximal binding to the antibody in the absence of stable insulin. These controls were obtained by adding 0.2 ml of dextran-coated charcoal (prepared as indicated in footnote

TABLE 1. PROTOCOL FOR INSULIN RADIOIMMUNOASSAY (RIA)

1. 0.1 ml of plasma or standard sample of human insulin.
2. 2.4 ml of standard diluent* containing I-125 insulin (1,000 cpm/ml or 10 pg) and antiserum to porcine insulin in the appropriate dilution.
3. 72-hr incubation at 4°C. Daily checks of damage of labeled hormone and its binding to the antibody.
4. 0.2 ml of dextran-coated charcoal†.
5. Centrifugation for 30 min at 1,500 g and 4°C.
6. Daily checks for "incubation damage" (C) and immunoreactivity (B/F*). Incubation stopped when C ≧ 90% and B/F* ≧ 0.8–1.2.
7. Separation of the supernatant (antibody-bound radioinsulin + free iodine) from the precipitate (free I-125 insulin); both are counted in a well scintillation counter. Calculation of bound/free ratio, corrected for damaged hormone and free iodine.

* Standard diluent: 25 ml of 1% human serum albumin + 10 ml guinea pig serum + barbital buffer, 0.02 *M*, pH 8.6, to a final volume of 1 l.

† Dextran-coated charcoal: 20 g of activated charcoal/100 ml barbital buffer + 2 g of dextran-70 per 100 ml of barbital buffer. The two components are mixed at the time of use.

TABLE 2. COMPARISON OF THE DEGRADATION AND IMMUNOREACTIVITY OF LABELED INSULINS DURING INCUBATION FOR THE RIA

Time of incubation (days)	Test	Antiserum dilution	[^{125}I] Ins. Lacto Purif. starch + Sephadex	[^{125}I] Ins. Lacto Purif. BioGel	[^{125}I] Ins. Chlor.-T Purif. starch + Sephadex
1	C†	—	96%	82%	91%
	B/F*	1:1 × 10^5	2.81	1.13	1.35
	B/F*	1:2 × 10^5	1.68	0.55	0.91
	B/F*	1:4 × 10^5	1.00	0.45	0.67
2	C†	—	95%	82%	90%
	B/F*	1:1 × 10^5	3.44	1.24	1.44
	B/F*	1:2 × 10^5	2.53	0.77	1.33
	B/F*	1:4 × 10^5	1.62	0.63	0.78
3	C†	—	95%	82%	89%
	B/F*	1:1 × 10^5	3.84	1.65	2.58
	B/F*	1:2 × 10^5	3.06	1.28	2.00
	B/F*	1:4 × 10^5	1.86	0.83	1.40

* B/F = antibody-bound radioinsulin ÷ free radioinsulin, the former corrected for free I-125 and for percentage of damaged hormone.

† C tests the fraction of intact labeled hormone, measured by the percentage of total radioactivity bound to dextran-coated charcoal. The remainder represents "damaged" insulin.

to Table 1) to 2.4 ml of standard diluent containing the tracer and both the labeled insulin and the antibody, each in the appropriate dilution.

Standard curves for the assay of insulin. Three standard curves were run, spanning the range of human insulin concentration from 0.01 to 1.0 ng/ml, with constant antiserum dilution (1:400,000) and incubation time (3 days at 4°C). The only variable was the radioactive insulin, labeled by either chloramine-T or lactoperoxidase and purified by the two methods above described, with a tracer concentration of 10 pg/ml, corresponding to 1,000 cpm/ml. The specific activity of the labeled antigen was estimated by the self-displacement method.

For the determination of low insulin levels, the assay system was set up to increase the sensitivity of the reaction, further diluting the antiserum to 1:2 × 10^6 and reducing the tracer concentration to 5 pg/ml, corresponding to 500 cpm/ml.

RESULTS

Radioiodination. The efficiency of six labeling procedures, expressed as the percentage of the total radioactivity incorporated into the intact radioiodinated porcine insulin, averaged 51% (range, 48–53%) with chloramine-T and 52% (range, 47–60%) with lactoperoxidase.

Purification of the labeled hormone. When the labeled radioinsulins were both purified on starch gel, followed by Sephadex G-50 filtration, the lactoperoxidase preparation gave a greater number of eluates containing more undamaged hormone than did the chloramine-T product (Fig. 2). On the other hand, insulin labeled with lactoperoxidase and purified on a BioGel P-60 column (Fig. 3) yielded a lower number of eluates with a satisfactory proportion of undamaged hormone. The product was also slightly less pure than when the two purification systems were used.

Stability and immunoreactivity of the tracer. When the two labeled preparations, both purified on starch gel and Sephadex G-50, were tested for integrity and immunoreactivity during incubation for RIA, the lactoperoxidase radioinsulin showed greater stability than the chloramine-T product: 95% as compared with 89% "purity" on the third day (Table 2). On the other hand, the former lost 18% of its integrity after the 3 days when it had been purified only on BioGel P-60. Moreover, its immunoreactivity was always better when it was purified in the two systems.

Standard curves for radioimmunoassay. Figure 4 shows that the radioinsulins, prepared by different techniques and purified in one system or two, produced different sensitivities in the RIA as defined by the initial slope of the dose-response standard curve. It is evident that the radioinsulin prepared with lactoperoxidase but purified only on BioGel P-60 caused lower sensitivity and precision in the assay than did the same preparation purified in starch + Sephadex. The former product therefore provides poorer performance in the assay of plasma insulin.

However, the chloramine-T and lactoperoxidase preparations were almost equally effective when both were purified in the two systems indicated. The only difference, in favor of the latter, was the slightly steeper slope of the dose-response curve, related to

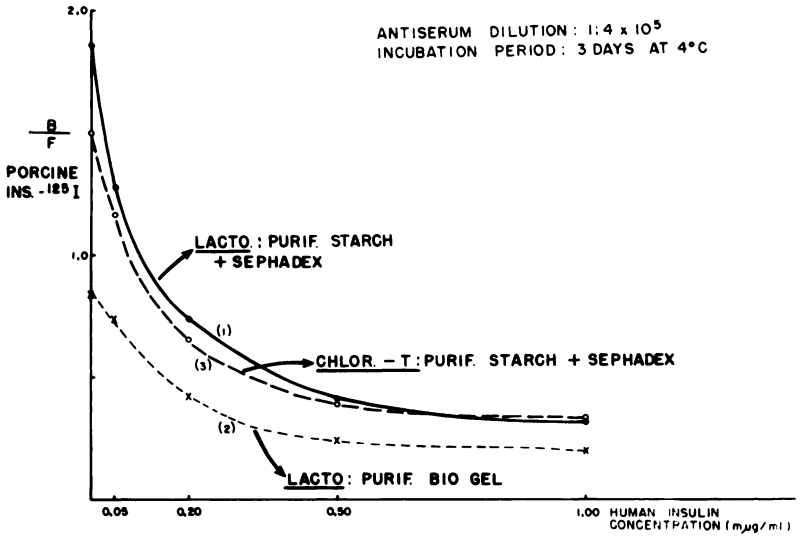

FIG. 4. Standard curves for RIA of human insulin. Comparison of radioinsulins labeled with lactoperoxidase vs chloramineT.

the initial B/F ratio, in the absence of the standard: 1.86_{lacto} against $1.50_{chlor\text{-}T}$. Accordingly, the specific activity of the labeled insulin is estimated to be greater with the lactoperoxidase preparation (300 μCi/μg) than with the chloramine-T (220 μCi/μg).

In Figs. 5 and 6, the steepness of the standard curves at very low insulin levels indicate that the tracers obtained by enzymatic iodination and two-system purification succeed in reducing the minimum detectable quantity of the hormone, operating in the range up to 0.04 ng/ml.

Immunoassay of plasma insulin. Twelve plasma samples from three normal subjects, withdrawn at different times before and after a 30-min arginine infusion, were simultaneously analyzed, in quadruplicate, in two similar series of assays whose only difference lay in the type of radioinsulin used: prepared with chloramine-T in one case, and (concurrently) with lactoperoxidase in the other. There was a positive and highly significant correlation between the insulin concentrations measured with the two tracers ($r = 0.99$; $p < 0.001$), in the range 3–180 μU/ml. The regression line is defined by the equation $y = 8.34 + 0.99\ x$, y and x being insulin concentrations in μU/ml, y derived from the chloramine-T standard curve, and x from the lactoperoxidase curve.

DISCUSSION

The more highly iodinated insulin preparations usually obtained with chloramine-T are less satisfac-

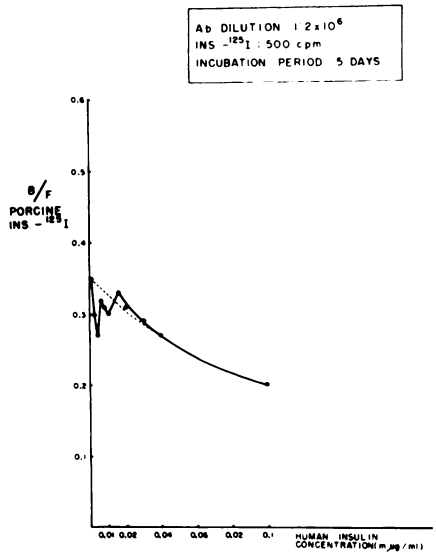

FIG. 5. Standard curve for RIA of low concentrations of human insulin. I-125 insulin labeled with chloramine-T.

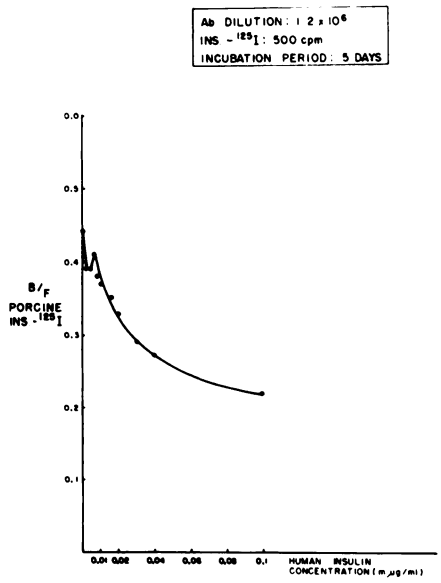

FIG. 6. Standard curve for RIA of low concentrations of human insulin. I-125 insulin labeled with lactoperoxidase.

tory when used as a tracer in RIA procedures, because of lower immunoreactivity and greater susceptibility to damage.

The purpose of this paper was to devise a method for radioiodination of insulin that would give specific activities high enough for radioimmunoassay but without loss of immunoreactivity. Insulin is favorable for such a study because of its great molecular stability (*10*).

Initially, we tried to adapt the chloramine-T method (*1,6*) to the iodination of insulin, making it suitable for our purpose by reducing the amount of the oxidizing agent from 10 $\mu g/\mu g$ of hormone to 1 $\mu g/\mu g$ of insulin. The reactions were carried out at 4°C, like those described previously for the labeling of the follicle-stimulating hormone of the human pituitary (hFSH) (*5*). The specific activity of the radioiodinated insulin varied from 200 to 300 $\mu Ci/\mu g$.

Despite the purification of the radioinsulin in two systems—starch-gel electrophoresis followed by Sephadex gel filtration—the results were uniform: excellent incorporation of the label into protein but lower immunoreactivity and greater damage on incubation during RIA, when compared with the iodoinsulin prepared by the enzymatic method and purified by the same two techniques (Table 2, Figs. 4–6).

As for the purification procedure, contrary to what we found for hFSH (*5*), our enzymatically labeled insulin, purified in one system (BioGel P-60), did not present the same immunoreactivity and stability, on incubation, as it did after two successive purifications (Fig. 4, Table 2).

The RIA standard curves obtained with I-125 insulin labeled with lactoperoxidase and purified in two systems, gave more sensitive assays (Fig. 4) due to the greater incorporation of the radioiodine into the molecule (specific activity) for the same level of total radioactivity used (1,000 cpm/ml). It can be stated, therefore, that the enzymatic iodination is more specific in relation to the substitution reaction for the incorporation of iodine atoms into insulin, while avoiding the problems resulting from the use of a powerful oxidizing agent such as chloramine-T.

Parallel assays of human plasma insulin, using the two labeled preparations, gave an excellent correlation ($r = 0.99$), the results being higher when the chloramine-T preparation was used, as expected from the previous considerations.

Likewise for the measurement of low levels of plasma insulin, when the standard samples are ten times more dilute than for the usual RIA, it is preferable to use enzymatically labeled iodoinsulin (purified in two systems), to obtain optimal sensitivity of the dose-response relationship (Figs. 5 and 6).

FOOTNOTE

* Gift from Rosalyn S. Yalow, Ph.D., Veterans Administration Hospital, Bronx, N.Y.

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