Measurement of Rat Insulin

Enzyme-Linked Immunosorbent Assay With Increased Sensitivity, High Accuracy, and Greater Practicability Than Established Radioimmunoassay

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Total immunoreactive insulin (IRI) is conventionally determined by radioimmunoassays. IRI measurement in rats can be made more sensitive, accurate, and practical, as demonstrated by a new modified enzyme-linked immunosorbent assay (ELISA). It is characterized by indirect binding of an anti-insulin antibody by an antiglobulin antibody and uses the principle of competitive saturation. In this ELISA, IRI can be determined in a wide range of concentrations, corresponding to the standards. The standard curve ranges from 100 to 0.049 ng/ml IRI (1 ng/ml ~ 23.4 μU/ml ~ 172 pM rat insulin). The statistical analysis shows between- and within-assay coefficients of variation of ≤15%. *Diabetes* 37:321–26, 1988

Insulin secretion is a β-cell–specific function. Yalow and Berson (1) were the first to develop a reliable method for determining this hormone in humans. Meanwhile, their radioimmunoassay (RIA) method is widely used in various modified forms (2). For research purposes, RIAs have been adapted for application in animals, with a species-matched standard insulin preparation. In addition to in vivo experiments, insulin secretion can be evaluated in vitro with cultivated β-cells or islets of Langerhans (3). Such experiments are done preferentially in the rat model.

We describe an enzyme-linked immunosorbent assay (ELISA) for optimized detection of rat insulin (for principal considerations cf. refs. 4 and 5). The assay was developed to allow rat insulin to be determined in large numbers of specimens. We were especially interested in being able to detect extremely varied amounts of rat insulin, ranging from very small ones in sera to very high ones in culture supernatants from islets. Emphasis was placed on readily usable stable reagents. Previous work with a commercially available RIA (Novo, Bagsvaerd, Denmark) was not fully satisfactory because it was not sensitive enough when used with the serum quantities available in rats and because it depended on freshly ^{125}I-labeled reagents. Another disadvantage of the RIA was the multiple dilution steps, costing time and reagents, that were necessary to fit high insulin concentrations in culture supernatants from islets to the range of the standard curve.

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Received for publication 15 June 1987 and accepted in revised form 28 August 1987.

MATERIALS AND METHODS

The ELISA described in this paper is based on the principle of competitive saturation of an anti-insulin antibody with either unlabeled or peroxidase-labeled insulin.

Reagents. The rabbit anti-guinea pig antibody (antiglobulin antibody) used to fix the anti-insulin antibody to the microtiter plate was purchased from EY Laboratories (San Mateo, CA; cat. no. AT-2358). The antibody was obtained in an affinity-purified quality and consisted of ~1 mg of anti-guinea pig–specific antibodies per milliliter. The anti-insulin antibody was produced by Novo (antibody M 8309). This antibody was generated in guinea pigs immunized with porcine insulin. The undiluted guinea pig serum had a binding capacity of ~3 U porcine insulin per milliliter. The rat standard was also provided by Novo. All experiments described herein were performed with this Novo standard, unless otherwise mentioned. To validate the ELISA system, another standard was obtained from Lilly (Indianapolis, IN).

The peroxidase-labeled insulin (conjugate) was from Sigma (St. Louis, MO; cat. no. 1-2133). It was prepared from bovine insulin, which was coupled via an *N*-hydroxysuccinimide ester (6); 1.2 mol of horseradish peroxidase type VI (Sigma) was coupled with 1 mol of insulin. The commercially available preparation used in our assay was provided in an affinity-purified quality and had a protein concentration of 1 mg/ml. The substrate for the enzyme reaction was *o*-phenylenediamine dihydrochloride (OPD; Fluka, Buchs, Switzerland).

Specimens other than standards were sera from rats or

supernatants from islet cultures. The supernatants were harvested at the end of the incubation period by pipetting and stored in a refrigerator at −20°C. Each specimen was assayed in triplicate.

Buffers. The coating buffer (0.05 M carbonate bicarbonate buffer, pH 9.6) used to attach the rabbit anti-guinea pig antibody to the polystyrene microtiter plates was made by dissolving 1.59 g of Na_2CO_3, 2.93 g of $NaHCO_3$, and 0.20 g of NaN_3 in 1 L of distilled water. The incubation buffer (0.04 M phosphate buffer, pH 7.4) used to couple the anti-insulin antibody to the anti-guinea pig antibody was prepared by dissolving 5.77 g of $Na_2HPO_4/2H_2O$, 1.05 g of NaH_2PO_4/H_2O, 1.00 g of bovine serum albumin (BSA) (RIA grade, Sigma), and 0.24 g of sodium merthiolate (Sigma, cat. no. T5125) in 1 L of distilled water. The incubation buffer for the rat insulin standards or samples differed from the previous one in that 0.6 g of NaCl and 5.9 g of BSA were added per 100 ml of the phosphate buffer. Thus, the buffer was isotonic to serum, the high albumin concentration mimicking the protein concentration of the serum (2). The washing buffer (0.15 M phosphate-buffered saline, pH 7.2) contained 8.0 g of NaCl, 0.2 g of KCl, 1.15 g of Na_2HPO_4, 0.2 g of KH_2PO_4, and 0.5 ml of Tween 20 (Merck, Darmstadt, FRG) in 1 L of distilled water. The substrate solution consisted of 20 mg of OPD and 0.15% H_2O_2 dissolved in enzyme buffer. This buffer (0.05 M) was prepared by adjusting a sodium dihydrogen phosphate solution (2.07 g of NaH_2PO_4 in 300 ml of H_2O) to pH 5.6 by adding appropriate amounts of a disodium hydrogen phosphate solution (2.67 g of $Na_2HPO_4/2H_2O$ in 300 ml of H_2O).

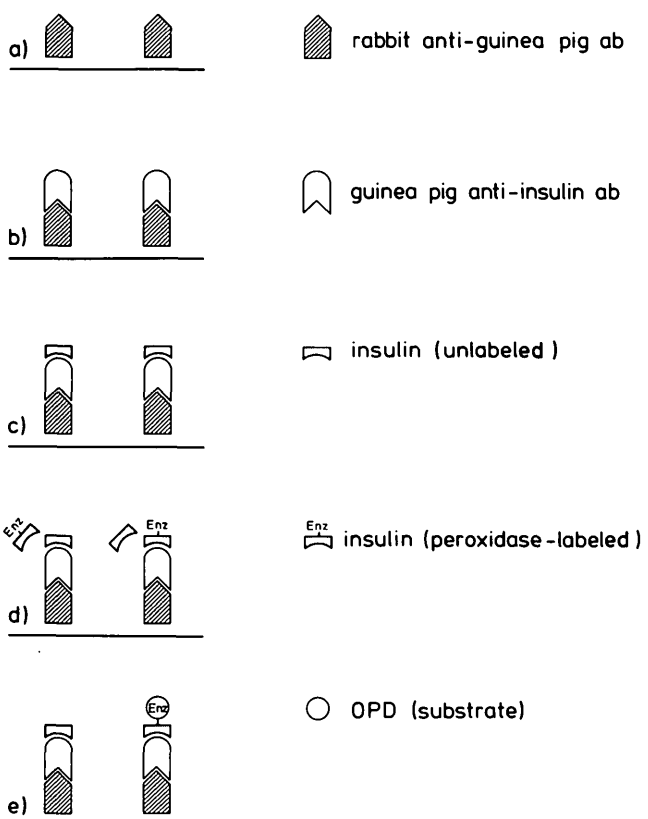

FIG. 1. Determination of rat insulin by ELISA. *a*: Coating of microtiter plate with antiglobulin antibody. *b*: Anti-insulin antibody is added and bound by antiglobulin antibody. *c*: Insulin (from sera, culture supernatants, or standard) is bound by anti-insulin antibody. *d*: Competitive saturation of anti-insulin antibody with either unlabeled or enzyme-labeled insulin. *e*: Measurement of substrate degradation after removal of all unbound insulin (labeled/unlabeled). For detailed procedure see MATERIALS AND METHODS.

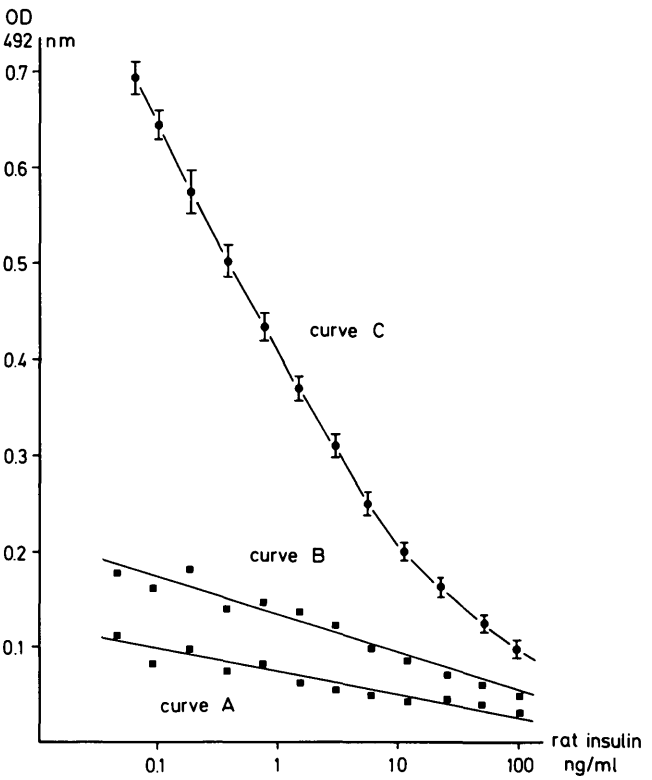

FIG. 2. Typical standard curves. *Curves A* and *B*: anti-insulin antibody is coated directly onto microtiter plate without using antiglobulin antibody (dilution of anti-insulin antibody is 1:18,000 for *curve B* and 1:72,000 for *curve A*). *Curve C*: optimization of standard curve by fixing anti-insulin antibody (dilution 1:72,000) with antiglobulin antibody. Optical density (OD) is expressed as mean ±SD of triplicate samples.

ELISA. Figure 1 illustrates the individual steps that were performed. Microtiter plates with 96 round-bottomed wells (Nunc, Roskilde, Denmark) were coated by a sandwich principle. First, the plates were coated with the rabbit anti-guinea pig antibody (antiglobulin antibody) at a pH of 9.6 in a 2-h incubation period at 37°C (150 μl/well) (*a*). This antibody was used in a dilution of 1:1000. The plates were washed and then incubated with the 1:72,000 diluted anti-insulin an-

TABLE 1
Effect of various glucose concentrations on insulin secretion of rat islets cultured in CMRL medium with 10% fetal calf serum

Islets (*n*)	Glucose (mM)	Insulin secretion: Total (ng · ml^{-1} · 24 h^{-1})	Insulin secretion: Per islet (ng · ml^{-1} · 24 h^{-1})
8	5	397	50
4	5	620	155
8	5	496	62
7	20	4365	623
9	20	4558	506
4	20	2819	705

CMRL medium was from Gibco (Grand Island, NY; cat. no. 041-1530); fetal calf serum was from Biochrom (West Berlin).

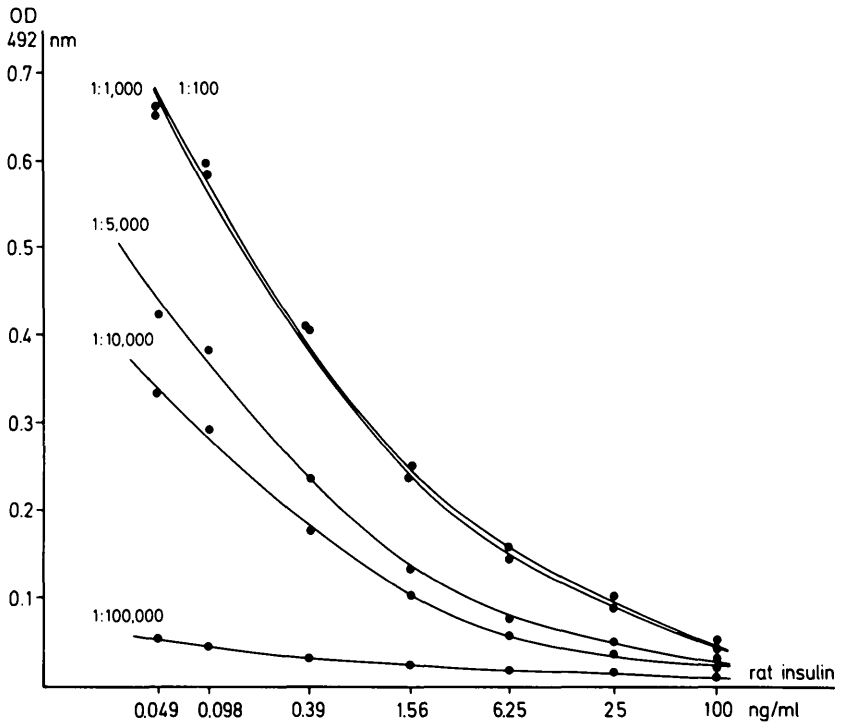

FIG. 3. Evaluation of optimum antiglobulin antibody dilution for assay. Optimum standard curve is achieved with antiglobulin antibody dilution of 1:1000. Higher dilutions result in lower optical density (OD) values, indicating incomplete binding of anti-insulin antibody and, consequently, less binding of enzyme-labeled insulin.

tibody at a pH of 7.4 with the phosphate buffer containing 1‰ BSA (100 μl/well; *b*). This incubation step again lasted 2 h at 37°C.

Appropriate standards were prepared, e.g., starting with 100.0 ng/ml. The standards or the test samples (rat sera or the supernatants from cultivated rat islets) were all diluted appropriately in phosphate buffer containing 6% BSA and used in triplicate (100 μl each/well). One hundred microliters of the standards or the test samples were added in triplicate to the plates after free antibodies had been removed by repeated washings (Fig. 1*c*). The plates were allowed to incubate overnight at 4°C. The next day, 100 μl of a 1:10,000 dilution of the insulin peroxidase conjugate was added to each well without washing the plates (Fig. 1*d*). The buffer used for dilution of the peroxidase-labeled insulin was identical to that used for diluting the standards. The incubation lasted for 4 h at 4°C. Then the plates were washed, and 100 μl/well OPD solution was added (Fig. 1*e*). The enzyme re-

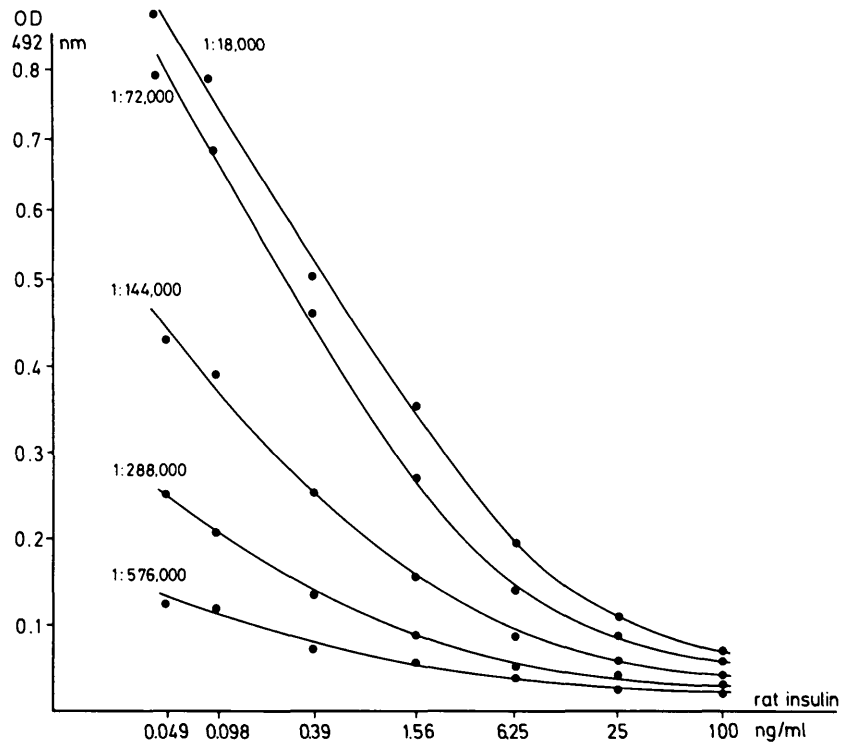

FIG. 4. Evaluation of optimum anti-insulin antibody dilution for assay. High anti-insulin antibody concentrations result in higher optical density (OD) values, indicating increased binding of enzyme-labeled insulin. Acceptable standard curve is achieved by anti-insulin antibody dilution of 1:72,000.

TABLE 2
Effect of administration of glucose (1 g/kg body wt, i.v.) on insulin secretion in Lewis rats

	Time (min)									
Experiment no.	−10	+1	+2	+3	+4	+5	+10	+20	+30	+40
1*	1.6	4.7	4.5	6.0	4.3	3.3	0.9	0.7	0.7	0.6
2*	0.7	7.8	10.3	6.4	5.6	4.4	2.3	1.9	1.6	0.8
3†	0.7	12.0	6.0	1.7	1.3	0.8	0.5	0.5	1.2	1.9
4†	5.6	34.1	11.7	6.8	5.3	9.7	11.7	7.2	11.8	3.5

Values are insulin secretion in nanograms per milliliter. Specimens, either posthepatic (vena cava*) or prehepatic (vena portae†), were taken after various time intervals (min).

action was stopped after 30 min by adding 100 μl of 0.5 M H_2SO_4 per well, and the optical density (OD) was measured at 492 nm. The mathematical computations for the calculation of the specimens were done by infinitesimal approximation. All washings were done with phosphate-buffered saline, pH 7.2. The plates were washed four times each with 250 μl of buffer/well. All steps of this assay were performed with an ELISA processor (Behring, Marburg, FRG) to guarantee reproducible assay conditions.

To study the influence of the coating procedure, in one series the antiglobulin antibody (final dilutions 1:18,000 and 1:72,000) was coated directly onto the microtiter plate at a pH of 9.6 in a 2-h incubation period at 37°C. In other words, step *a* was carried out with the anti-insulin antibody instead of the antiglobulin antibody, and step *b* was omitted.

RESULTS

For a high-quality ELISA with an anti-insulin antibody fixed to a solid phase and the principle of competitive saturation, optimum insulin binding to the anti-insulin antibody is essential. In our assay the insulin-binding properties were improved by fixing the anti-insulin antibody with an antiglobulin antibody. Fig. 2, *curve C*, illustrates a typical dose-response curve starting at 100 ng/ml rat insulin. The standard could be diluted down to a concentration of 0.049 ng/ml rat insulin, and the curves were clearly dose dependent. When only buffer was added instead of insulin standard, the OD was ≥.804. The blanks (OD values when substrate or conjugate was omitted to prevent degradation of the substrate by the enzyme) were no higher than .007. This was also true of a standard curve for rat insulin omitting the anti-insulin antibody in the coating procedure. To demonstrate the efficiency of the indirect coating method, Fig. 2, *curves A* and *B*, show the standard curves that resulted when the anti-insulin antibody was coated directly onto the microtiter plates without using the antiglobulin antibody. These standard curves express only a poor increase in the OD in relation to the amount of insulin. Even a fourfold increase in concentration of the anti-insulin antibody (Fig. 2, *curve B*) did not result in a standard curve like that seen with the indirect coating procedure (Fig. 2, *curve C*).

Optimum dilutions for the antiglobulin antibody and the anti-insulin antibody were established separately. Figure 3 demonstrates that a dilution of 1:1000 of the antiglobulin antibody resulted in sufficient binding of the anti-insulin antibody. Higher concentrations of the antiglobulin antibody did not lead to a significant increase in the OD values, indicating that in a 1:1000 dilution the antiglobulin antibody fixes the anti-insulin antibody almost completely. When the concentrations of the anti-insulin antibody were changed, higher anti-insulin antibody concentrations resulted in higher OD values (Fig. 4). This is due to increased binding of peroxidase-labeled insulin to unsaturated anti-insulin antibodies. Dilutions of <1:72,000 did not show any considerable

TABLE 3
Accuracy of immunoreactive insulin measurement as shown by recovery study

Set no.	Background	Standard added	Expected value	Measured value	Recovery (%)
1	5.7	10	15.7	13.7	80
	5.7	2.5	8.2	8.3	104
	5.7	0.6	6.3	6.5	133
2	39.7	10	49.7	48.1	84
	39.7	2.5	42.2	43.8	164
3	1.9	10	11.9	12.0	101
	1.9	2.5	4.4	4.8	116
	1.9	0.6	2.5	2.8	150
4	2.4	10	12.4	14.6	122
	2.4	2.5	4.9	5.8	136
	2.4	0.6	3.0	3.4	166

Values are nanograms of rat insulin per milliliter in a 1:50 dilution (sets 1 and 2 of culture supernatants) or in a 1:5 dilution (sets 3 and 4 of rat sera) of specimen. Relatively high serum levels were obtained from glucose-stimulated rats. Recovery = (measured value − background)/standard added.

TABLE 4
Variation within assays

	Detected (ng/ml)			
Expected (ng/ml)	Mean	Range	SD	C.V. (%)
50	47.8	44.5–54.0	3.7	7.7
25	26.9	24.2–29.5	2.5	9.5
12.5	12.0	11.2–14.5	1.4	11.7
6.25	5.3	5.0–6.2	0.5	9.4
3.12	2.8	2.2–3.2	0.4	14.3
1.56	1.4	1.3–1.6	0.14	10.0
0.78	0.71	0.63–0.84	0.078	11.0
0.39	0.33	0.29–0.41	0.044	13.3
0.19	0.16	0.14–0.20	0.025	15.6
0.098	0.096	0.084–0.121	0.010	10.4

Values are results of 5 determinations done in triplicate in same run (identical samples were prepared from standard by serial dilutions in phosphate buffer containing 6% BSA). C.V., coefficient of variation.

TABLE 5
Variation between assays

Expected (ng/ml)	Detected (ng/ml) Mean	Range	SD	C.V. (%)
50	48.5	41.5–52.0	4.4	9.0
25	25.7	22.7–27.0	1.8	7.0
12.5	10.9	8.7–13.2	1.7	15.6
6.25	5.5	4.7–6.0	0.5	9.1
3.12	3.1	2.5–3.7	0.4	12.9
1.56	1.4	1.3–1.6	0.15	10.7
0.78	0.72	0.65–0.85	0.076	10.6
0.39	0.32	0.29–0.42	0.050	15.6
0.19	0.15	0.11–0.18	0.023	15.3
0.098	0.099	0.085–0.121	0.015	15.2

Values are results of 5 determinations done in triplicate in 5 different experiments (identical samples were prepared from standard by serial dilutions in phosphate buffer containing 6% BSA). C.V., coefficient of variation.

increase in the OD values, however. This indicates that nearly all peroxidase-labeled insulin had been bound to the anti-insulin antibody when the standard concentrations were low. For reasons of economy and precision, we preferred to dilute the anti-insulin antibody 1:72,000.

For routine measuring of supernatants from cultivated rat islets, we used a standard curve ranging from 100 to 0.78 ng/ml rat insulin. With a 1:50 dilution for the culture supernatants, it was possible to detect from 39.0 to 5000 ng/ml of insulin in one run. Table 1 shows an example of an in vitro experiment in which islets of Langerhans were stimulated for 24 h. Depending on the glucose concentration of the culture medium, the values for the rat insulin in the supernatants ranged from 397 to 4558 ng/ml. For detecting insulin in rat sera, we preferred a standard curve ranging from 12.5 to 0.049 ng/ml rat insulin. This means that for sera diluted 1:5,

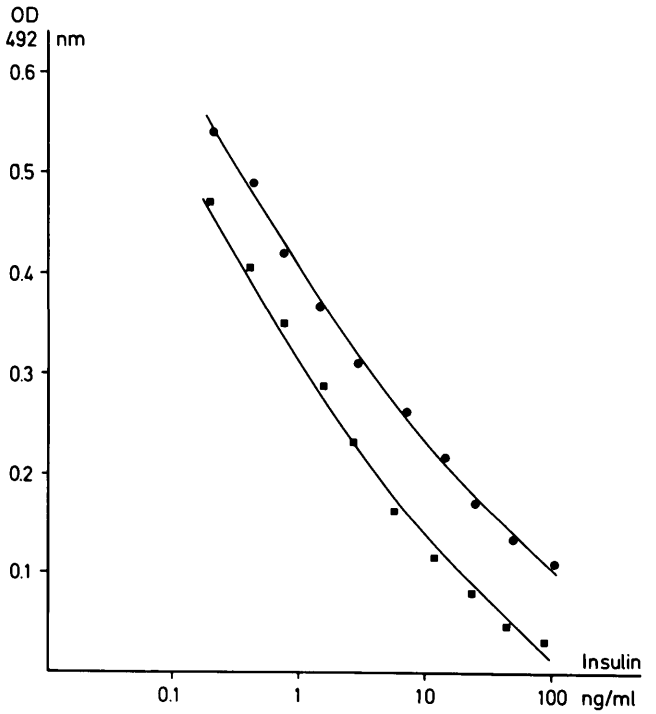

FIG. 6. Comparison of standard curves for insulin from different species: rat insulin (●, Novo) and bovine insulin (■, Novo). OD, optical density.

0.24–60.0 ng/ml of rat insulin could be detected. This was an appropriate approach for studying insulin secretion after glucose challenge in small-volume specimens. Data from a typical experiment are shown in Table 2. Dependent on the initial insulin levels and the veins from which the specimens were taken (pre- or posthepatic), varying insulin concentra-

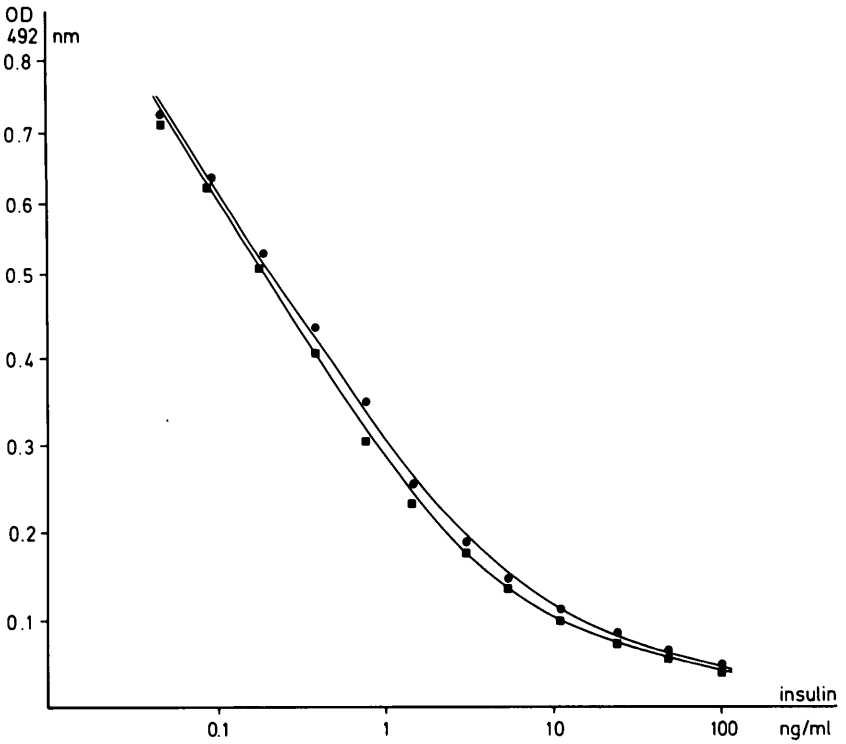

FIG. 5. Comparison of standard curves from different rat standard preparations: Novo (●) and Lilly (■). OD, optical density.

tions could be detected, reaching a peak 1–3 min after glucose administration.

In accordance with the proposed application of the ELISA, we performed a recovery study to validate the assay. The first part of the study (details in Table 3) consisted of experiments (sets 1 and 2) in which different amounts of rat standard insulin were added to culture supernatants from rat islets diluted 1:50. The second part of the study (sets 3 and 4) was carried out with rat sera diluted 1:5, containing 1.9 or 2.4 ng/ml rat insulin. When we compared the expected values with the ones we measured, the recovery ranged from 80 to 166%. Further statistical analysis showed that the degree of precision was acceptable, with the between- and within-assay coefficients of variation ≤15% (Tables 4 and 5). In a study on the effect of serial dilution of sera or culture supernatants on the measured concentration of insulin, neither sera nor culture supernatants had an inhibitory or augmentative effect. For sera run in a dilution from 1:8 to 1:1, the maximum coefficient of variation was 12.9%; culture supernatants had a maximum coefficient of variation of 14% in serial dilutions running from 1:100 to 1:800.

To further validate the ELISA, we compared two different rat standard preparations. Figure 5 shows the standard curves of a serial dilution starting from 100 ng/ml and ending with 0.049 ng/ml for each standard preparation. The data in Fig. 5 were obtained from one run of a microtiter plate, each standard preparation covering one-half of the plate. The comparison of the OD values showed a high degree of correspondence between the standard from Novo and that from Eli Lilly. The discrepancies may not have any real significance for the practical application of the ELISA with regard to possible variations caused, for instance, by the dilution of the standard preparations. In contrast to this experiment, Fig. 6 compares standard curves obtained with insulin from different species, in this case rat and bovine insulin. As expected from the origin of the anti-insulin antibody and the strong structural similarity between bovine and porcine insulin, the OD values obtained in the bovine insulin assay were lower than those obtained in the rat insulin assay.

DISCUSSION

We present an immunoenzyme method for measuring immunoreactive insulin in both rat sera and culture supernatants from rat islets. The assay is based on a solid-phase immunoenzyme technique and the principle of competitive saturation of an anti-insulin antibody with either unlabeled or peroxidase-labeled insulin. This technique presents an alternative to previously reported ELISA procedures for measuring human insulin with two different anti-insulin antibodies, one of them enzyme labeled (7–11). In such techniques, unspecific binding of the enzyme-labeled antibody to the unlabeled antibody can be a problem.

A major difficulty, i.e., that of working in a solid phase, was overcome by fixing the anti-insulin antibody with a second antibody. This considerably increased the capacity to bind insulin, as shown in Fig. 2. The implication is that the anti-insulin antibody appears to lose some of its structural integrity when bound directly to the solid phase at a pH of 9.6. Other groups have used various kinds of beads to immobilize the anti-insulin antibody (7–11). We prefer working with microtiter plates because they can be coated easily. In addition, fast processing and automatic pipetting, washing, and photometry are feasible.

Concerning the quality of the ELISA, the variation within and between tests and the results of the experiments to demonstrate accuracy achieve a standard that conforms to commercially available RIAs. Our technique employed in the rat system has the advantage of generating a standard curve with a broad range, which is hardly achieved by RIA. This means fewer errors due to frequent dilutions and greater practicability.

Most important, the high degree of sensitivity allows more detailed time-kinetic studies of rat insulin in animals and corresponding in vitro experiments, because satisfactory measurements are now possible with small quantities of serum or supernatant. With the specimens being handled in the microtiter plate system and the lack of dependence on fresh radionucleotides, larger numbers of specimens can be processed at any given time.

Commercially available reagents can be used throughout. The use of peroxidase-labeled insulin instead of ^{125}I-labeled insulin is less expensive, and the laboratory equipment needed is less sophisticated. Therefore, with this assay, it is easier to perform detailed studies of β-cell function in vivo and in vitro in the fields of diabetes and pancreas transplantation research.

ACKNOWLEDGMENTS

We thank Drs. G. Schubert and A. Thiede, Department of Surgery, University of Kiel, who were responsible for the animal experiments.

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