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Radioreceptor Assay of Insulin using Rabbit Erythrocyte Membrane

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A simple radioreceptor assay for insulin was established with sensitivity sufficient to detect 10 μU/ml of insulin in human sera. This radioreceptor assay was carried out at 0° using rabbit erythrocyte membranes. The binding assay for the standard curve was performed in the presence of insulin-free serum, since the binding of insulin was greatly reduced by the addition of serum. Under the conditions used, 38% of the initial binding of ^{125}I-insulin was displaced by 250 μU/ml of native insulin. The sensitivity of the radioreceptor assay was dependent on the concentrations of both membrane protein and ^{125}I-insulin. The ratio of IRI to insulin values obtained by the present assay was 0.93 ±0.10 for 19 healthy and diabetic subjects.

Keywords——radioreceptor assay; serum insulin; erythrocyte membrane; insulin binding; scatchard analysis; radioimmunoassay

Introduction

The specific binding of ^{125}I-insulin to human circulating cells such as granulocytes, lymphocytes and red blood cells has been demonstrated by several authors.[2)] Recently, we showed that the binding of ^{125}I-insulin to rabbit erythrocyte membranes can be specifically displaced by native insulin and is unaffected by glucagon, adrenocorticotropic hormone and thyrotropic hormone. Scatchard analysis demonstrated that insulin receptor sites with two different affinities existed on the membranes.

The present report describes a simple, sensitive radioreceptor assay (RRA) capable of determining concentrations of insulin in human sera using rabbit erythrocyte membranes.

Materials and Methods

Reagents——Crystalline pork insulin was a gift from Shimizu Seiyaku Co., Ltd., Japan. ^{125}I-insulin (199—234 mCi/mg) was purchased from Dainabot RI Laboratory, Japan. Bovine serum albumin (BSA, fraction V) was obtained from Armour Pharmaceutical Co., U.S.A. Other reagents were from Wako Junyaku Co., Ltd., Japan and were of analytical grade.

Preparation of Rabbit Erythrocyte Membranes——The preparation of rabbit erythrocyte membranes was carried out by a modification of the method of Ginsberg *et al.*[3)] The freshly heparinized blood was collected from three to four male rabbits weighing 3—5 kg to minimize individual variation. Erythrocyte cells were prepared by centrifuging the blood at 400 g for 10 min and washing the cells with saline three times. The buffy coat was removed at the end of each centrifugation. The washed cells were hemolyzed in three volumes of cold Tris buffer (8.5 mM, pH 7.4) containing 3 mM NaCl, 1 mM glucose and 0.1 mg/ml BSA (buffer A), supplemented with 2 mM $MgCl_2$, and centrifuged at 20000 ***g*** for 10 min. The pellets were rinsed with the same buffer six to seven times. The washed pellets were further rinsed three times with buffer A supplemented with 0.2 mM $MgCl_2$ and the erythrocyte membranes obtained were frozen at −60° until use for RRA of insulin.

1) Location: *1-1, Mukogawa-cho, Nishinomiya, 663, Japan.*
2) J.R. Gavin, J. Roth, P. Jen and P. Freychet, *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 747 (1972); K.K. Gambhir, J.A. Archer, and C.J. Bradley, *Diabetes*, **27**, 701 (1978).
3) B.H. Ginsberg, C.R. Kahn, and J. Roth, *Biochim. Biophys. Acta*, **443**, 227 (1976).

Procedure for Radioreceptor Assay——Insulin-free serum used in experiments was prepared according to the dextran-charcoal method of Herbert *et al.*[4] from human sera without insulin antibody; the absence of the antibody in serum was confirmed by the polyethylene glycol method for insulin measurement.[5]

The buffer used consisted of 150 mM Tris, 125 mM NaCl, 100 mM glucose and 2.5 mg/ml BSA (buffer B), and was adjusted to pH 7.4 by the addition of HCl. Erythrocyte membranes were resuspended in buffer B prior to binding assay experiments. The binding assay was carried out in tubes (1.26×6.5 cm, polyethylene tube, Hitachi Koki Co., Ltd., Japan) containing 50 μl of ^{125}I-insulin (0.011 μCi/50 μl), 100 μl of membrane suspension (0.6—1.0 mg/ml of protein) 200 μl of insulin-free serum or unknown serum and 50 μl of buffer B containing native insulin (2—2000 μU/ml) or of buffer B. All the tubes were kept at 0° in an ice-bath throughout the above procedures and the incubation was continued for 20 hr at 0°. After incubation, the reaction was stopped by adding 3 ml of cold buffer A centrifuging at 50000 ***g*** for 2 min at 4°. The aqueous phase was aspirated and the radioactivity in the pellet was counted in a γ scintillation counter (Shimadzu Seisakusho Co., Ltd., Japan).

Measurement of IRI (Immunoreactive Insulin) and Membrane Protein——Insulin contents in human sera were measured by a double antibody method (Insulin-Riakit, Dainabot RI Laboratory, Japan). The concentration of membrane protein was determined according to the method of Lowry *et al.*[6]

Results

Effects of pH, Temperature and Incubation Time on the Binding of Insulin

The binding of ^{125}I-insulin to erythrocyte membranes was unaffected by pH within a range of 7.0 to 7.4 and decreased at higher pH. The binding of insulin was greatly affected by the incubation temperature, and the maximal binding was achieved by incubation at 0° for 20 hr. Thus, the following binding experiments were carried out under the following conditions: pH 7.4, 0° and 20 hr (data not shown).

Effect of Concentration of ^{125}I-Insulin on the Sensitivity of Radioreceptor Assay

Figure 1A shows that the total and nonspecific binding of ^{125}I-insulin increased in proportion to the concentration of ^{125}I-insulin. The specific binding is thus a linear function of total ^{125}I-insulin concentration. At 3.59 μU/ml of ^{125}I-insulin, the maximal sensitivity of RRA was obtained within a range of insulin concentration from 3 to 125 μU/ml, and 35% of the initial binding was displaced by 125 μU/ml of native insulin (Fig. 1B).

Effect of Membrane Protein Concentration on the Sensitivity of Radioreceptor Assay

Parallel incubations of various concentrations of membranes with 1.56 μU/ml of ^{125}I-insulin in the presence of 36 μg/ml of native insulin and in its absence demonstrated that the total and nonspecific binding increased with the concentration of membrane protein. The specific binding was a linear function of the membrane protein concentration within a range of 0.16 to 1.15 mg/ml (Fig. 2A).

The sensitivity of RRA was dependent on the concentration of membrane protein. At 0.85 mg/ml of the membrane protein, the total binding was 13.7% of total ^{125}I-insulin, and 37% of the initial binding was displaced by 250 μU/ml of native insulin (Fig. 2B).

Effect of Concentration of Serum Protein on the Binding of Insulin

Freeze-dried sera not containing insulin were diluted with distilled water to appropriate concentrations of protein. As shown in Fig. 3A, insulin binding was equally inhibited by the addition of insulin-free serum containing 1.4 to 6.0 g/dl of protein, and this inhibitory effect of serum was more marked for the total binding than for the nonspecific binding. At a protein concentration of 6.0 g/dl the total binding was inhibited by 40% of the control obtained in the absence of serum.

Figure 3B shows displacement curves of insulin binding in the presence of insulin-free serum and in its absence. Scatchard plots shown in the inset of Fig. 3B demonstrate that

4) V. Herbert, K.S. Lau, C.W. Gottlieb, and S.J. Bleicher, *J. Clin. Endocrinol. Metab.*, **25**, 1375 (1965).
5) K. Kobayashi, T. Mochizuki, T. Ichiki, M. Hata, and A. Matsuoka, *Acta Med. Hogo.*, **3**, 283 (1978).
6) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

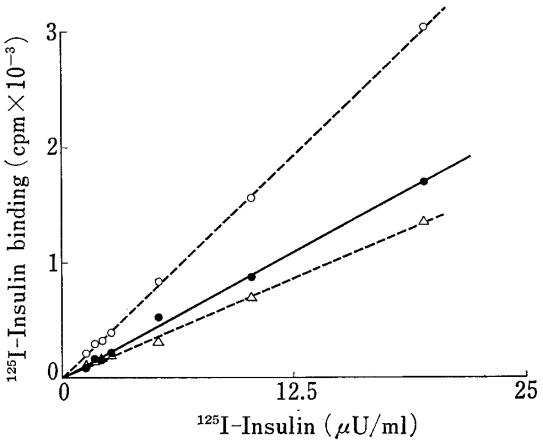

Fig. 1A. Binding of ^{125}I-Insulin to Erythrocyte Membranes as a Function of the Concentration of ^{125}I-Insulin

In the experiments shown in Fig. 1 and 2, the reaction mixture consisted of 200 μl of membrane suspension, 100 μl of the buffer containing ^{125}I-insulin and 50 μl of the buffer containing or not containing native insulin. Membrane material (0.40 mg/ml of protein) was incubated at 0° for 20 hr in 350 μl of the buffer containing various concentrations of ^{125}I-insulin (1.33—19.48 μU/ml) in the presence of 36 μg/ml of native insulin (△——△, nonspecific binding) and in its absence (○——○, total binding). The specific binding (●——●) was obtained by subtracting the nonspecific binding from the total binding. Each point represents the mean value of triplicate determinations.

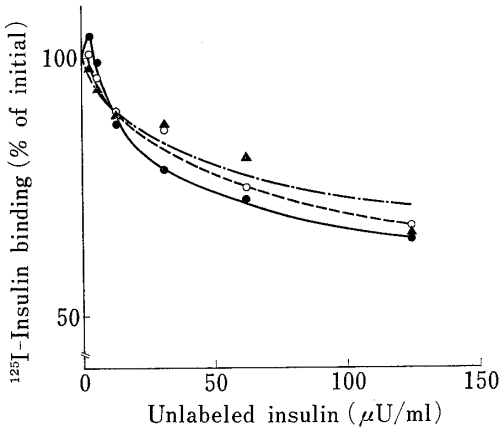

Fig. 1B. Sensitivity of the Displacement Curve to the Concentration of ^{125}I-Insulin

Membrane material (0.40 mg/ml of protein) was incubated at 0° for 20 hr in 350 μl of the buffer containing various concentrations of ^{125}I-insulin (○——○, 1.80 μU/ml; ●——●, 3.59 μU/ml; ▲——▲, 14.38 μU/ml) in the presence of native insulin ranging from 0 to 125 μU/ml. Each point represents the mean value of triplicate determinations.

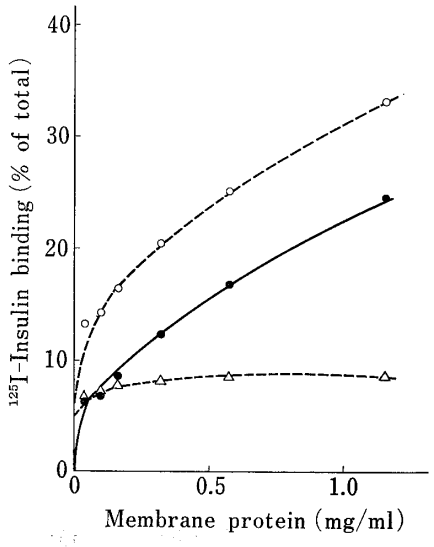

Fig. 2A. Binding of ^{125}I-Insulin to Erythrocyte Membranes as a Function of the Concentration of Membrane Protein

Incubation media contained the indicated concentration of membrane protein in 350 μl of the buffer containing 1.56 μU/ml of ^{125}I-insulin. Incubation was carried out at 0° for 20 hr in the presence of 36 μg/ml of native insulin (△——△, nonspecific binding) and in its absence (○——○, total binding). The specific binding (●——●) was obtained by subtracting the nonspecific binding from the total binding. Each point represents the mean value of triplicate determinations.

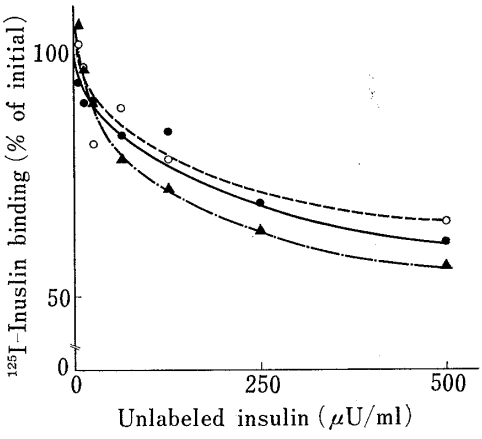

Fig. 2B. Sensitivity of the Displacement Curve to the Concentration of Membrane Protein

Incubation media contained various concentrations of membrane protein (○——○, 0.21 mg/ml; ●——●, 0.43 mg/ml; ▲——▲, 0.85 mg/ml) in 350 μl of the buffer containing 3.63 μU/ml of ^{125}I-insulin. Incubations was carried out at 0 ° for 20 hr. Each point represents the mean value of triplicate determinations.

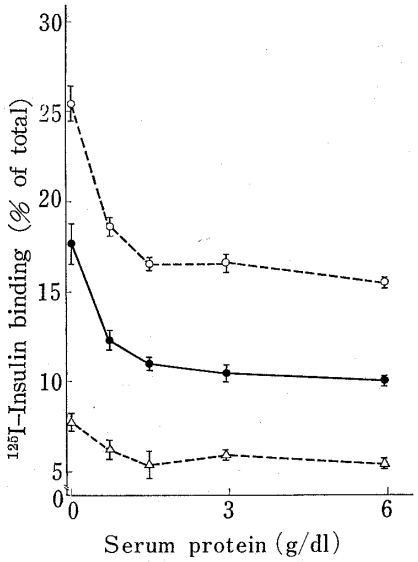

Fig. 3A. Inhibitory Effect of Serum Protein on the Binding of Insulin to Erythrocyte Membranes

Incubation media contained the indicated concentration of serum protein in 400 μl of the buffer containing 3.13 μU/ml of ^{125}I-insulin. Incubation was carried out at 0° for 20 hr in the presence of 36 μg/ml of native insulin (△——△, nonspecific binding) and in its absence (○——○, total binding). The specific binding (●——●) was obtained by subtracting the nonspecific binding from the total binding. Each point represents the mean (±SEM) value of triplicate determinations.

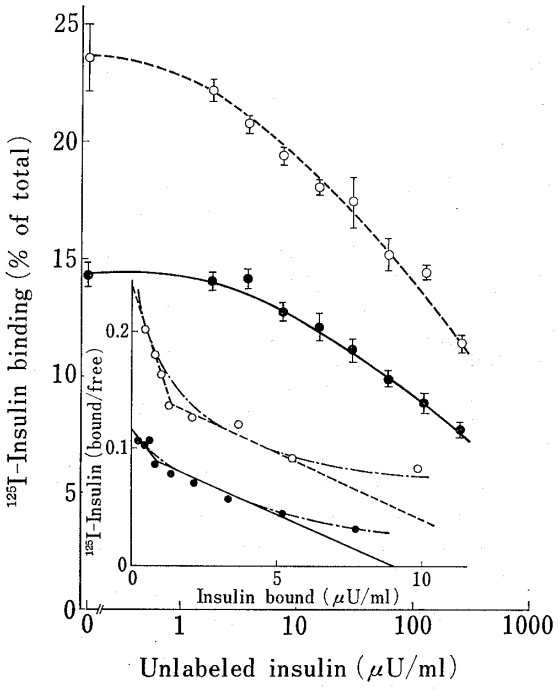

Fig. 3B. Displacement Curves of ^{125}I-Insulin by Native Insulin

Membrane material (0.60 mg/ml of protein) was incubated at 0° for 20 hr in 400 μl of the buffer containing 3.20 μU/ml of ^{125}I-insulin and various concentrations of native insulin in the presence of insulin-free serum (●——●) and in its absence (○——○). The inset shows Scatchard plots. Each point represents the mean±SEM or the mean value of triplicate determinations.

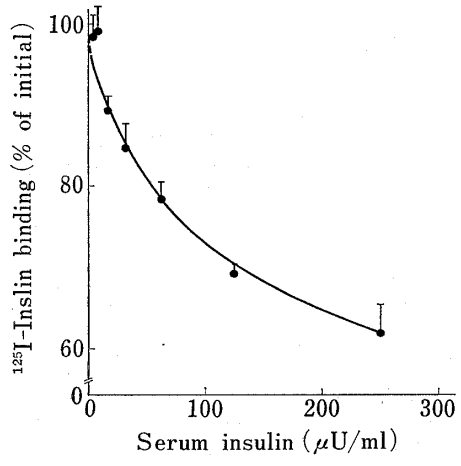

Fig. 4A. Standard Curve for the Measurement of Insulin in Serum

The standard curve was derived from a displacement curve shown in Fig. 3B and is shown in terms of percentages of the initial binding. Each point represents the mean (±SEM) value of triplicate determinations.

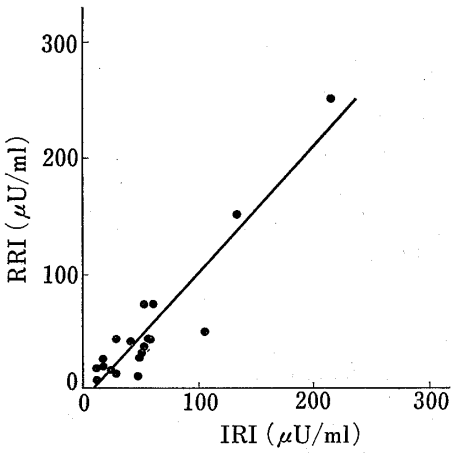

Fig. 4B. Correlation between Values of Serum Insulin obtained by the Present Radioreceptor Assay and by Radioimmunoassay

Each plot represents the mean of triplicate receptor assays and duplicate radioimmunoassays.

$n=19$
$y=1.10\,x-10.03$
$r=0.9323$

the addition of serum reduced the number of binding sites, but the affinity for insulin binding was not significantly affected.

Insulin Values in Human Sera measured by the Present Assay and by Radioimmunoassay

The standard curve for the measurement of insulin is given as percentages of the initial binding of ^{125}I-insulin, and was derived from a displacement curve shown in Fig. 3B (Fig. 4A).

Insulin values measured by the present assay were in close agreement with those measured by radioimmunoassay (Fig. 4B).

Discussion

The radioimmunoassay of insulin has been widely used in fundamental and clinical studies. It has been reported that values of serum insulin determined by bioassay are much higher than immunoreactive insulin values. The radioreceptor assay, which can measure molecules, or at least the portions of them that bind to receptors, is more significant in a biological sense than radioimmunoassay. In addition, the former is as easy to carry out as the latter.

In the present study, we demonstrated that insulin values in human sera measured by the radioreceptor assay are consistent with immunoreactive insulin values. Freychet *et al.*[7] showed that insulin values obtained by radioreceptor assay correlate with those measured by bioassay. Recently, Kabuto *et al.*[8] observed an excellent correlation of insulin values determined by radioreceptor assay using rat liver membranes and by radioimmunoassay.

The present radioreceptor assay using rabbit erythrocyte membranes was proved to be a simple and effective tool for detecting serum insulin. In addition, the erythrocyte membranes are convenient as a source of receptor sites, since they are readily available in large quantities and are rather homogeneous. The present assay system was capable of measuring human serum insulin ranging from 10 to 250 μU/ml. The within-assay precision of insulin values expressed as a coefficient of variation was relatively high, except in the cases of two samples (Table I).

Table I. Within-assay Variation of Insulin Values measured by the Present Radioreceptor Assay

Sample	Insulin measured (μU/ml)		Sample	Insulin measured (μU/ml)	
	Mean±S.D.	C.V.[a]		Mean±S.D.	C.V.[a]
1	74.0± 1.4	1.9	11	50.3± 8.4	16.7
2	15.0± 2.8	18.7	12	44.3±10.7	24.1
3	27.5± 0.7	2.6	13	7.0± 0.0	0.0
4	44.0±14.1	32.1	14	152.0±23.1	15.2
5	30.3± 7.5	24.8	15	251.0±13.5	5.4
6	14.3±12.1	84.4	16	11.0± 3.6	32.8
7	15.7± 1.5	9.8	17	32.5± 3.5	10.8
8	28.0± 8.5	30.3	18	43.0±11.0	25.6
9	41.0± 1.4	3.4	19	18.5± 5.0	26.8
10	73.3±42.0	57.3			

Samples were sera of 19 healthy and diabetic subjects. Each value represents the mean (±S.D.) of triplicate determinations and is plotted in Fig. 4B.
a) Coefficient of variation.

The considerable decrease in the binding of insulin upon addition of serum is a defect of the present assay. A similar effect was observed upon adding bovine serum albumin,

7) P. Freychet, J. Roth, and D.M. Nevill, Jr., *Proc. Natl. Acad. Sci. U.S.A.*, **68**, 1833 (1971).
8) M. Kabuto, K. Suzuki, N. Ohsawa, and K. Kosaka, *Endocrinol. Japon.*, **24**, 173 (1977).

but washing out the serum or bovine serum albumin removed the inhibition of insulin binding. Therefore, the inhibitory effect of serum on insulin binding is not due to the degradation of insulin receptors but to some other unknown factor (s).

In summary, erythrocyte membranes serve as receptor sites for the radioreceptor assay; a more sensitive assay should be possible simply by using solubilized erythrocyte membranes or larger amounts of membranes.

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Synthesis of Bradykinin Fragments and Their Analogs modified at a Phenylalanine Residue[1,2]

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Bradykinin fragments and their analogs, Gly–X–Ser–Pro (X: L-Phe I, D-Phe II, L-Ala III, D-Ala IV, β-Ala V and Gly VI) and X–Ser–Pro (X: L-Phe VII and D-Phe VIII) were synthesized. Compounds I, II, V, VII and VIII prolonged the pentobarbital-induced sleeping time in mice at the dosage of 2.5 nmol per mouse.

Keywords——bradykinin fragments; analogs; chemical synthesis; AP–M digest; effects on central nervous systems

Bradykinin injected intracerebrally into experimental animals produces a short-lasting phase of behavioral excitation, followed by sedation.[4] Iwata *et al.*, suggested that the excited state might be elicited by bradykinin, and that its metabolites might cause the depression.[5]

Synthesis of bradykinin fragments and their analogs is under way in our laboratory to investigate their action on the central nervous system by assessing their effects on pentobarbital-induced sleeping time in mice as an index. In a preliminary report,[1] we stated that some synthetic bradykinin fragments, Gly–Phe–Ser–Pro (I), Phe–Ser–Pro (VII) and Ser–Pro (IX) (Fig. 1) prolonged the pentobarbital-induced sleeping time in mice, and it was shown that the Ser–Pro moiety at the C-terminus of the peptides was required for effectiveness in this action. In this paper, we describe the synthesis of Gly–X–Ser–Pro (X: L-Phe I, D-Phe II, L-Ala III, D-Ala IV, β-Ala V, and Gly VI) and X–Ser–Pro (X: L-Phe VII, D-Phe VIII), as shown in Fig. 2, to study their effects on pentobarbital-induced sleeping time in mice.

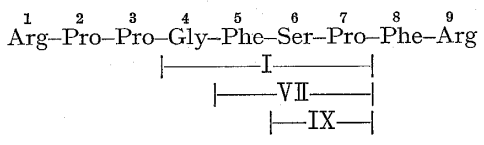

Fig. 1. Structure of Bradykinin and Its Fragments

1) Previous paper of this series, Y. Okada, Y. Tsuchiya, M. Yagyu, S. Kozawa, and K. Kariya, *Neuropharmacology*, **16**, 381 (1977).
2) Abbreviations used are those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 3485 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1972). Z=benzyloxycarbonyl, DCC=N,N′-dicyclohexylcarbodiimide.
3) Location: *Ikawadani-machi, Tarumi-ku, Kobe, 673, Japan.*
4) F.G. Graeff, R.I. Pela, and M. Rocha E. Silva, *J. Pharmacol.*, **37**, 723 (1969).
5) H. Iwata, T. Shikimi, M. Iida, and H. Miichi, *Jap. J. Pharmacol.*, **20**, 80 (1970).