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Research Article

Radioreceptor Assay for Insulin Formulations

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A sensitive radioreceptor assay was developed for pharmaceutical insulin formulations with the use of rat liver membranes and ^{125}I-insulin. The addition of unlabeled insulin or insulin analogues inhibited the binding of ^{125}I-insulin to the receptors in a concentration-dependent way. Insulin concentrations between 3×10^{-9} and 3×10^{-8} *M* were used for a three-point assay which fulfilled pharmacopoeial statistical requirements for validity of the assay. The coefficient of variation for five assays was 4%. Potency estimates of insulin preparations obtained with the radioreceptor assay correlated well with corresponding estimates from *in vivo* assays. Both assays also gave similar potency readings for insulin preparations that were partly degraded during storage at elevated temperatures, but the radioreceptor method was more precise.

KEY WORDS: insulin; mouse bioassay; rat liver membranes; radioreceptor assay.

INTRODUCTION

Most pharmacopoeias require *in vivo* bioassays for quantitation of insulin in pharmaceutical preparations. The *European Pharmacopeia* (1) accepts the mouse (2) or rabbit blood glucose as well as the mouse convulsion assay. These methods use large number of animals and are time-consuming for attending technicians. Thus, they are not well suited for screening the insulin contents of a great number of samples in a laboratory with limited resources for *in vivo* experiments.

This paper describes a radioreceptor assay for insulin using rat liver membranes as receptor preparation. The method is less resource-demanding than the *in vivo* assay and has a higher degree of precision. It has been designed as a three-dose assay in which the results are subjected to normal pharmacopeial statistical tests for validity of the assay (3). The present method represents a development of an earlier assay described by us (4). The precision has been improved, and extensive validation of the method including comparisons of the results with those of an *in vivo* assay has been undertaken.

MATERIALS AND METHODS

Chemicals and Equipment. Monoiodinated porcine ^{125}I-insulin (sp act approx $2.2 \cdot 10^{13}$ Bq · mmol^{-1}, 600 Ci · mmol^{-1}) was purchased from New England Nuclear Corp., Boston, Mass. This tracer could be used for 2 months after iodination without a marked reduction of specific binding to liver membranes. The international standards for insulin, corticotropin, and vasopressin (the fourth international standard for insulin, the third international standard for corticotropin, and the first international standard for arginine vasopressin) (5) and preparations of candidate international standards for bovine, human, and porcine insulin were from the WHO International Laboratory for Biological Standards, London. The commercial insulin preparations were from KabiVitrum, Stockholm, Sweden, Nordisk Gentofte, and Novo, Bagsvaerd, Denmark. Bovine and porcine proinsulin, porcine C-connecting peptide, and porcine glucagon were obtained from Novo, and chicken insulin and porcine monoarginine insulin from Dr. Ronald Chance, Eli Lilly Co, Indianapolis, Ind. Synthetic bombesin, cholecystokinin-8, and substance P were for Peninsula Laboratories, Belmont, Calif. Synthetic tetracosactrin was from Ciba–Geigy Ltd., Basle, Switzerland. Natural porcine gastric inhibitory polypeptide (GIP), secretin, and vasoactive intestinal polypeptide (VIP) were gifts from Professor Viktor Mutt, Karolinska Institutet, Stockholm, Sweden. Purified soybean trypsin inhibitor was purchased from Sigma Chemical Co., St Louis, Mo., and bovine serum albumin from Miles, Elkhart, Ind. All other chemicals were of the highest grade commercially available.

Incubated membrane samples were centrifuged in a Beckman model B microcentrifuge (Beckman Instruments, Palo Alto, Calif.).

Radioactivity was counted in a Packard 5160 or 800 C gamma counter (Packard Instrument Co., Downers Grove, Ill.)

Animals. Male Sprague–Dawley rats (100g) were obtained from Alab, Sollentuna, Sweden.

Preparation of Hepatic Plasma Membranes. Plasma membranes were prepared from rat livers according to the method of Neville (6) with the modification that the final sucrose gradient centrifugation was omitted. The protein content of the membrane fraction was determined according to Lowry *et al.* (7). Membranes corresponding to 20 g of liver tissue were suspended in 5 ml of Krebs–Ringer phosphate buffer (pH 7.5) and stored frozen at −70°C in portions

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of 0.25–1.00 ml. The phosphate buffer had the following composition: NaCl, 118 m*M*; KCl, 5 m*M*; $MgSO_4$, 1.2 m*M*; KH_2PO_4, 1.2 m*M*; Na_2HPO_4, 10 m*M*. Under such storage conditions, membranes could be used for at least 3 months without an appreciable loss of binding activity.

Binding Assay. Membranes were thawed and diluted with Krebs–Ringer phosphate buffer with 1% bovine serum albumin to a concentration normally corresponding to 0.5 mg of membrane protein/ml. Membranes were preincubated at specified temperatures for 30 min in a shaking water bath. Tracer was, unless otherwise specified, added at a concentration of approximately 3×10^{-11} *M* together with various concentrations of unlabeled peptide. At appropriate times after the addition of hormone, duplicate 100-μl samples were layered over 300 μl of iced incubation buffer in microcentrifuge tubes of polypropylene. Membranes were centrifuged at 9000*g* for 30 sec in a microcentrifuge. Pellets were washed a total of four times with iced buffer, after which the microfuge tubes were counted for radioactivity. In parallel, the radioactivity of duplicate 100-μl samples from the incubation mixture containing both membranes and medium was counted. Obtained values were used to calculate the binding of ^{125}I-insulin as a percentage of added insulin. Binding of tracer in the presence of 10^{-6} *M* unlabeled insulin is referred to as nonspecific binding. Nonspecific binding after 60 min of incubation at 22°C was on average 0.87 ± 0.04% (SE) of the added radioactivity, corresponding to 6.6% of the total binding.

Binding parameters for ligand–binding site data obtained during apparent equilibrium conditions were estimated using a computer program based on an algorithm for least-squares curve fitting with objective measurement of goodness of fit (8).

In assays, three serial dilutions of unlabeled insulin were chosen from the linear portion of the ^{125}I-insulin binding curve. In most cases, the concentrations ranged from 0.5 to 5 mIU/ml corresponding approximately to 3×10^{-9} to 3×10^{-8} *M*. Five hundred microliters of membrane suspension (0.5 mg membrane protein/ml) was incubated in plastic tubes (11 × 55 mm) with ^{125}I-insulin (3×10^{-11} *M*) in the absence and presence of chosen dilutions of unlabeled insulin from test and standard samples, respectively. A tube with an excess of unlabeled insulin (10^{-6} *M*) was included in each incubation series. Long-acting insulin suspensions were assayed after initial acidification of the formulations with 0.1 *M* HCl according to pharmacopeial directions (1). Dilutions were made in acidified saline (pH 2.5) with 0.1% (w/v) bovine serum albumin. Incubations were performed at 22°C for 60 min and terminated as already described.

Results from three separate series of incubations carried out in one single experiment were employed for the calculation of the relative potency of a test preparation in comparison with the standard preparation, with the concentration of unlabeled ligand as the dose and the degree of specific binding as the response. Estimated potency was calculated by analysis of variance for a three-point assay according to pharmacopeia rules (3). The statistical weight is defined as the reciprocal value of the variance of the log potency estimate (3). The index of precision is calculated by dividing the standard deviation of responses by the slope of the dose–response relationship (9).

Dissociation of Bound Insulin. ^{125}I-Insulin ($0.6–1.2 \times 10^{-10}$ *M*) was incubated with membranes (0.5 mg protein/ml) for 60 min at 22°C. After centrifugation (5 min, 2000*g*), the supernatant was discarded and the membranes were resuspended in fresh buffer with unlabeled insulin (10^{-6} *M*). Samples were taken from the incubation mixture before and at specified time intervals after resuspension. The total radioactivity in the medium immediately after resuspension was 3.9 ± 0.4% (SE) of the original activity.

Functional Alteration of Insulin (Reduced Binding) Following Incubation with Liver Membranes. ^{125}I-insulin (3×10^{-11} *M*) was incubated with liver membranes (0.5 mg protein/ml) for 60 min at 22°C. After centrifugation (5 min, 2000*g*), supernatant was added to fresh membranes in such volumes that the protein concentration of the incubation mixture remained approximately unchanged. Binding of labeled insulin to membranes at 22°C was then assessed as described under Binding Assay after various incubation times. Fresh tracer was added to membranes in control incubations.

Chemical Alteration (TCA Precipitability) of Insulin Following Incubation with Membranes. Membranes (0.5 mg protein/ml) were incubated with ^{125}I-insulin (3×10^{-11} *M*). Duplicate 200-μl samples of the incubation mixture were withdrawn at specified times and layered over 500 μl of iced Krebs–Ringer phosphate buffer in microcentrifuge tubes. The tubes were centrifuged for 60 sec at 9000*g*. Samples (2 × 100 μl) were taken from the supernatant and added to tubes containing 300 μl ice-cold 20% trichloroacetic acid (TCA) and spun for 60 sec. After four washes with 20% TCA, the pellets were counted together with samples from the supernatant after the initial centrifugation.

In Vivo *Assay of Insulin.* Insulin activity of various commercial insulin preparations as well as candidate materials for new international standards was determined against the fourth international standard for insulin using the mouse blood glucose method (1,2).

RESULTS

Time Course of Specific Binding of Insulin to Liver Membranes. Specific binding of ^{125}I-insulin to liver membranes increased rapidly at 22 and 12°C (Fig. 1), reaching a steady state after 45 to 60 min. Incubation at 4°C slowed down the binding process somewhat, while an incubation temperature of 37°C resulted in reduced maximal binding (Fig. 1). Nonspecific binding increased only slightly over time and reached an average value of 0.87 ± 0.04% (SE) of the added radioactivity after 60 min of incubation at 22°C.

Binding of insulin at apparent equilibrium was increased in an approximately linear fashion when the concentration of membrane protein was raised from 0.125 to 0.5 mg/ml (data not shown). At 1 mg/ml binding was further increased. In most experiments, 0.5 mg/ml was chosen.

Time Course of Dissociation of Bound Insulin. The dissociation rate for membrane-bound insulin was markedly temperature dependent. The mean dissociation rate for the first 15-min period was 12.3% min^{-1} at 37°C, 2.2% min^{-1} at 22°C, and 0.7% min^{-1} at 12°C. After 90 min of dissociation 5, 27, and 73% of the initially bound radioactivity remained

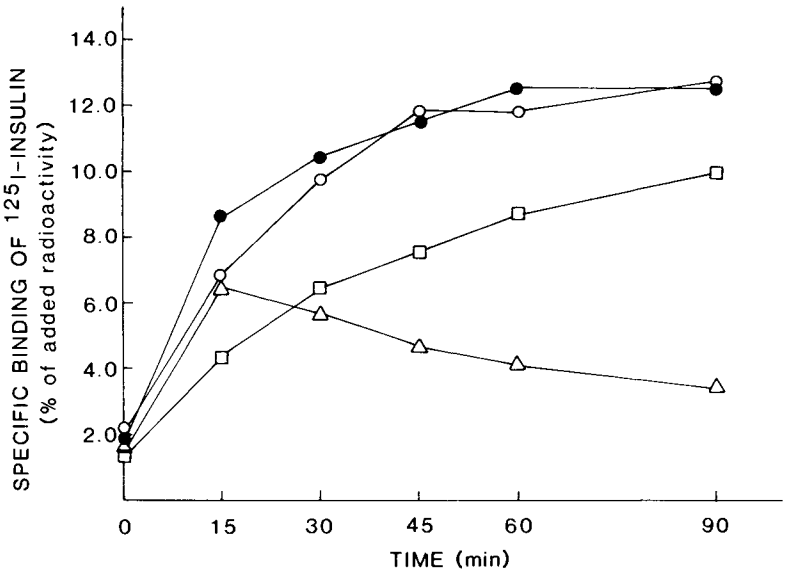

Fig. 1. Time course of specific binding of ^{125}I-insulin to rat liver membranes at indicated temperatures. Membranes (0.5 mg/ml) were incubated with 3×10^{-11} *M* of ^{125}I-insulin in Krebs–Ringer phosphate buffer at pH 7.5. Each point corresponds to the mean of two to six experiments. Incubation temperatures were 37 (open triangles), 22 (filled circles), 12 (open circles), and 4°C (open squares).

specifically bound to membranes incubated at 37, 22, and 12°C, respectively (data not shown).

Functional Alteration (Deteriorated Binding of Insulin) Following Incubation with Liver Membranes. After preincubation of ^{125}I-insulin with liver membranes for 60 min at 22°C, the mean specific binding of ^{125}I-insulin (60 min, 22°C) was 87% of the control value with fresh tracer (data not shown).

Chemical Alteration (TCA Precipitability) of Insulin Following Incubation with Membranes. TCA precipitability decreased from 96% immediately after the start of incubation to 90% after 60 min at 22°C (data not shown).

Inhibition of Binding of ^{125}I-Insulin by Unlabeled Peptides. Binding of ^{125}I-insulin to membranes was inhibited in a dose-related way by unlabeled porcine insulin (Fig. 2) as well as bovine and human insulin (data not shown). Unla-

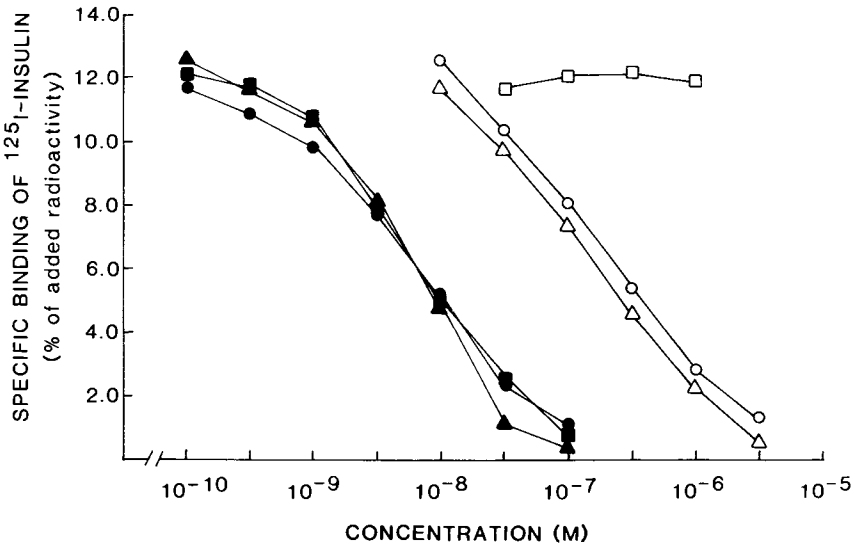

Fig. 2. Inhibition of specific binding of ^{125}I-insulin (3×10^{-11} *M*) by the addition of increasing concentrations of porcine insulin (filled circles) or the following insulin related peptides: chicken insulin (filled triangles), monoarginine bovine insulin (filled squares), porcine proinsulin (open circles), bovine proinsulin (open triangles), and porcine c-connecting peptide (open squares). Incubations with membranes (0.5 mg/ml) at 22°C for 60 min. Each point represents the mean of at least three separate incubations.

beled insulin (5×10^{-9} *M*) caused an approximately 50% inhibition of specific binding. Proinsulin was about 40 times less potent (Fig. 2). Chicken insulin and monoarginine insulin had affinities similar to those of bovine, porcine, and human insulin (Fig. 2), while the C peptide was devoid of affinity for insulin receptors (Fig. 2).

Bombesin, cholecystokinin-8, caerulein, corticotropin, gastric inhibitory polypeptide, glucagon, substance P, tetracosactrin, vasoactive intestinal peptide, and vasopressin at concentrations of 1 or 10 μM did not affect the binding of ^{125}I-insulin to liver membranes (data not shown).

Equilibrium constants for binding of porcine insulin to liver membranes are presented in Table I. A two-site model gave a statistically significantly better fit than a one-site model. However, the current data are also compatible with negative cooperativity between insulin–receptor complexes (10).

Radioreceptor Assay of Insulin Preparations. Using concentrations of unlabeled insulin, producing a linearly dose-related inhibition of tracer binding, results as in Fig. 3 were obtained when a commercial preparation was assayed against the international standard. Using analysis of variance for a three-point assay, the commercial preparation in Fig. 3 was found to have 110% of the affinity of the international standard for the receptors. The fiducial limits (P = 0.95) were 89–113%. The assay was repeated four times. The weighted mean potency was found to be 105% of the potency of the international standard, with a coefficient of variation of 4%.

In single experiments, the results deviated slightly from strictly linear log dose–response relationships or from parallellism between the test and the standard preparation. Since the variation in binding results was slight, such deviations sometime reached statistical significance.

In a series of experiments, various dilutions of a test preparation were assayed against the same preparation at a constant concentration (Table II). The estimated potencies were not significantly different from the nominal potencies.

Seventeen batches of 15 different commercial preparations of insulin (Table III) were assayed against the international standard with both the radioreceptor assay and the mouse blood glucose assay (1,2). Similar results were obtained with the two methods and there was no consistent tendency toward higher or lower activities with the receptor assay (Table IV). The index of precision was significantly lower with the ligand method, indicating a higher precision in comparison with the *in vivo* method (9).

In further experiments, candidate international standard preparations of bovine, porcine, and human insulin were assayed against each other (Table V). In these experiments, human insulin tended to have a slightly higher activity than the other forms of insulin.

Finally, three samples of insulin which had been stored at 56°C for more than 120 days were assayed with both the receptor and the mouse blood glucose method (Table VI). The activity was similar when measured with the radioreceptor and with the *in vivo* assay.

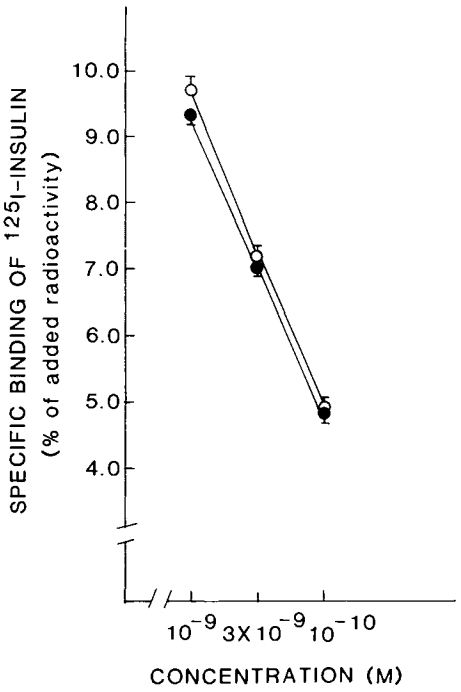

Fig. 3. Radioreceptor assay of a commercial preparation of soluble porcine insulin (filled circles) against the fourth international standard of insulin (open circles). ^{125}I-Insulin (3×10^{-11} *M*) and unlabeled insulin were incubated with membranes (0.5 mg/ml) at 22°C for 60 min. Each point represents a mean of three separate incubations. Vertical bars denote SE. Estimated potency of test preparation was 110% of the standard with finducial limits (P = 0.95) of 89–113%.

DISCUSSION

Several radioreceptor assays for insulin have been described by us (4) and others (11–13). Only two have, to our

Table I. Characteristics of Binding Sites for ^{125}I-Insulin on Rat Liver Membranes[a]

Sites	Dissociation constant (*M*)	Binding capacity (pmol/mg)
High affinity	$9.37 \times 10^{-10} \pm 4.80 \times 10^{-10}$	0.15 ± 0.10
Low affinity	$1.28 \times 10^{-8} \pm 2.56 \times 10^{-9}$	2.28 ± 0.14

[a] Incubation of ^{125}I-insulin (3×10^{-11} *M*) with membranes (0.5 mg protein/ml) at 22°C for 60 min. Data from eight separate experiments. Mean ± SE.

Table II. Potency of an Insulin Preparation Diluted to Various Nominal Concentrations[a]

Potency		Fiducial limits (%)	Statistical weight	Number of expts.
Nominal	Estimated			
90.0	92.6	81–124	575	1
80.0	80.2	92–108	4418	2
60.0	60.6	93–107	4841	3

[a] Porcine insulin was diluted to 90, 80, and 60% concentrations of a standard preparation. The different dilutions were assayed under conditions similar to those in Fig. 3.

Table III. Type of Commercial Insulin Preparation Assayed Against the Fourth International Standard

Sample No.	Type of preparation	Species
1, 2	Neutral solution	Porcine
3	Neutral solution, RI[a]	Porcine
4	Neutral solution, MC[a]	Porcine
5	Neutral solution	Bovine
6, 7	Neutral solution, MC	Human, enzymatically modified
8	Neutral solution	Human, biosynthetic
9	Isophane protamine insulin, RI	Porcine
10	Isophane protamine insulin	Human, biosynthetic
11	Neutral solution, 20 IU,[a] and isophane protamine insulin, 20 IU, RI	Porcine
12	Neutral solution, 12 IU, and isophane protamine insulin, 28 IU, RI	Porcine
13	Neutral solution, 10 IU, and crystalline insulin zinc, 30 IU, MC	Porcine, bovine
14	Amorphous insulin zinc, 12 IU, and crystalline insulin zinc, 28 IU, MC	Porcine
15	Amorphous insulin zinc, 12 IU, and crystalline insulin zinc, 28 IU, MC	Porcine, bovine
16	Amorphous insulin zinc, 12 IU, and crystalline insulin zinc, 28 IU, MC	Human, enzymatically modified
17	Crystalline insulin zinc, MC	Bovine

[a] RI denotes rare immunogen, and MC monocomponent quality. Number of units are given per/ml. All preparations contained a total of 40 IU/ml.

Table IV. Comparisons of Insulin Activity in Commercial Preparations as Determined with the Radioreceptor Assay and the Mouse Blood Glucose Method

	Radioreceptor assay					Mouse blood glucose assay				
Sample No.	Potency (% of standard)	Fiducial limits (%)	Statistical weight	Index of precision	No. of expts.	Potency (% of standard)	Fiducial limits (%)	Statistical weight	Index of precision	No. of expts.
1	105	95–105	9243		5	97	92–108	3388		2
2	110	91–110	3123	0.038	1	95	92–108	3225		3
3	108	92–109	3344	0.037	1	112	89–112	1578		2
4	107	93–108	4396	0.032	1	114	90–111	1941		2
5	105	89–113	1888	0.049	1	110	92–109	2868	0.195	1
6	102	93–108	5008	0.030	1	107	86–116	940		2
7	99	88–113	1663	0.050	1	109	91–110	2161		2
8	103	91–110	3107	0.038	1	91	86–117	578		2
9	106	93–108	4895	0.030	1	110	81–124	470	0.215	1
10	100	94–107	6035	0.027	1	96	89–113	1537	0.123	1
11	103	88–114	1643	0.353	1	103	89–113	1483	0.126	1
12	110	91–110	2793	0.040	1	103	92–109	2711		3
13	108	93–107	5941	0.029	1	104	80–125	420	0.227	1
14	105	87–114	1000	0.040	1	97	83–120	629	0.189	1
15	102	87–116	1267	0.060	1	91	85–117	854	0.161	1
16	101	94–106	7491	0.025	1	110	85–117	847	0.112	1
17	100	85–118	974	0.068	1	99	88–114	1345	0.132	1

Table V. Comparisons of Relative Affinity for Receptors and Biological Activity of Bovine, Human, and Porcine Insulins

Material		Radioreceptor assay				Mouse blood glucose assay			
Test	Standard	Potency (% of standard)	Fiducial limits (%)	Statistical weight	No. of expts.	Potency (% of standard)	Fiducial limits (%)	Statistical weight	No. of expts.
Bovine insulin	Porcine insulin	100.3	95–106	7268	4	107.1 (97.2)[a]	94–107	4754	4
Bovine insulin	Human insulin	95.8	85–118	848	2	105.1 (98.3)	91–110	2539	2
Human insulin	Porcine insulin	104.2	85–117	1046	1	99.3 (102.3)	94–106	6200	4

[a] Figures within parentheses denote an overall estimate of relative potency of the present preparations based on mouse convulsion, mouse blood glucose, and rabbit blood glucose assays presented in Ref. 16.

Table VI. Potency of Bovine, Human, and Porcine Insulins Stored at 56°C for 122–140 Days in Terms of the Same Material Stored Frozen

Preparation	Radioreceptor assay		Mouse blood glucose assay	
	Potency (% of standard)	Fiducial limits (%)	Potency (% of standard)	Fiducial limits (%)
Bovine insulin	84	88–114	81 (81)[a]	87–115
Human insulin	77	89–113	66 (73)	90–111
Porcine insulin	75	85–118	64 (64)	84–114

[a] Figures within parentheses denote an overall estimate of relative potency of the present preparations based on mouse convulsion, mouse blood glucose, and rabbit blood glucose assays presented in Ref. 16.

knowledge, been adopted for quantitation of pharmaceutical insulin preparations (4,12). The present study represents an improvement of our earlier method (4) and a more extensive validation of the receptor assay including numerous parallel comparisons with the mouse blood glucose assay (2). The improvement is due mainly to optimizing the conditions for preparation of liver membranes, giving substantially enhanced specific binding of insulin resulting in an increased precision of the radioreceptor assay.

The current results show that the present radioreceptor assay give results that are similar to those obtained by the mouse blood glucose assay, irrespective of whether preparations of insulin solutions or various long-acting insulin suspensions are tested. Normally, more narrow fiducial limits and a lower index of precision, indicating more precise results, are obtained with the *in vitro* than the *in vivo* method. In contrast to an earlier study (11), in which insulin formulations containing protamine occasionally gave anomalous potency readings, the present results indicate a close correlation between the current radioreceptor assay and the mouse blood glucose assay also for protamine containing samples.

Beside precision, the radioreceptor assay has other advantages compared to the mouse *in vivo* assay. A single technician can assay two or more preparations in 1 day with the receptor method, while a single mouse blood glucose assay requires two technicians working a full day. Another advantage with the receptor method is that it needs only a small amount of liver tissue, while the *in vivo* assays require 48 or more mice or 16 or more rabbits (1). This means that a radioreceptor assay could markedly increase the assay capacity of a quality-control laboratory. A similar gain could also be obtained by a radioimmunoassay. However, earlier findings indicate that insulin immunoassays less readily reflect the biological activity of insulin than a radioreceptor assay (14). Insulin could also be quantitated by reverse-phase high-pressure liquid chromatography (15). Under optimal conditions, this technique is powerful for separation of various insulin derivatives. However, changes in the insulin component of the chromatogram and changes in biological activity may not be parallel (16). Secondary peaks may or may not represent active material.

The relative biological activity of analogues to insulin may differ *in vitro* and in vivo. It has been reported that some cross-linked insulin derivatives have full biologic activity *in vivo,* while they display only limited receptor affinity and biologic activity *in vitro* (17). Similar findings have been reported for proinsulin (11). This may be related to a breakdown into active constitutents in the circulation as well as to differences in inactivation rates *in vivo* between such derivatives and native insulin. However, the present results, with a close agreement between corresponding results from radioreceptor and mouse blood glucose assays, do not indicate a significant presence of substances with such deviating characteristics in available pharmaceutical preparations of insulin. The concordance between the assay results obtained with the two methods indicates that the radioreceptor assay is a valuable technique for screening the potency of pharmaceutical insulin preparations.

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