Comparison of an Immunoassay and the U.S.P. Bioassay for Determining Potency of Extracted Insulin

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An immunoassay procedure is described for determining the potency of extracted insulin. The method, based on salt precipitation for the separation of antibody bound and free insulins originally described by Grodsky and Forsham, was modified and simplified to obtain greater precision and reliability of results. Repetitive immunoassay results, conducted on different days with pork, beef, and mixed source zinc insulins, commercial insulin products, and insulin preparations from different stages of manufacture, were compared with the corresponding U.S.P. bioassay results. Statistical analysis of the immunoassay data revealed a confidence coefficient of ±11.2 per cent ($p = 0.95$) for a single immunoassay. This value is within the limits established by the U.S.P. for insulin potency bioassay. A desired variation of ±5–6 per cent is obtained by statistical combination of several bioassay results. Similar variation is achieved by repeating the immunoassay on 4 different days. An analysis of variance indicated that the immunoassay, under the conditions employed, is not subject to variations due to species specific insulins if the antigen used to produce antiserum is a mixed species insulin. The beef and pork zinc insulins, originally selected to serve as standards for immunoassay, were found to have a significantly different potency by immunoassay when compared to the potency established by bioassay. In order to establish an immunoassay secondary reference standard equivalent to the U.S.P. reference standard, the reaction of three lots of pooled insulin was determined repetitively at all standard curve values. Statistical analysis by least squares, of the data obtained at 20, 30, and 40 milliunits/ml. (munits/ml.) insulin concentrations, yielded a calculated common slope which lies within the computed individual slope confidence interval. These data further support the validity of the immunoassay in establishing the potency of extracted insulin and was given credence by comparing immunoassay and bioassay results obtained on large manufactured lots of crystalline zinc insulins.

FOR MORE than two decades the potency of extracted insulin has been determined by the official U.S.P. bioassay (1). Like all bioassays, the "twin crossover" rabbit blood sugar-lowering assay, used as the official method for determining insulin potency, is beset with many shortcomings. These include inherent biological variation, high cost of facilities, considerable time consumption, and lack of sensitivity. The development of specific and sensitive immunological assays (2–6) provided rapid, accurate, and economic methods for the quantitative determination of insulin. These methods are based on isotope dilution as a quantitative index for measuring the competitive reaction of labeled and unlabeled insulins for specific antibody. The separation of the antigen–antibody complex from the free antigen in the immunological reaction is achieved by different means in these reported methods. A sensitivity great enough for measuring microunit quantities of insulin found in plasma and serum is a cardinal feature of the immunoassay. However, for control of insulin development and production, milliunit sensitivity is satisfactory. In the present study, the immunoassay method of Grodsky and Forsham (3) as modified by Baum *et al.* (7) was further explored and appropriately modified to determine the best conditions for reproducible results. Immunoassays possess the potential capability of determining insulin with an accuracy equivalent to that obtained with the bioassay. Although the immunoassay and bioassay measure quite different properties of the insulin molecule, attainment of analytical equivalence makes the immunoassay attractive for establishing insulin potency followed, when indicated, by a simplified animal response as a means of confirming the hormonal activity of extracted insulin.

Experiments were designed to determine the precision of the immunoassay under specifically defined conditions, utilizing a series of insulin preparations. The results of the immunoassay were compared with the results of the U.S.P. bioassay for these same insulin samples. Statistical analysis of the results reveal that the immunoassay exhibits precision and reliability for establishing insulin potency well within the confidence limits for computed potency defined in the U.S.P.

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MATERIALS AND METHODS

Antibody Production.—Mixed source insulin (75% beef crystalline zinc insulin and 25% pork crystalline

zinc insulin, by weight) is used as the antigen in the production of antibody used in routine analysis.

The antigen is prepared as follows. To a sterilized 250-ml. (Virtis 45) homogenizer cup add 250 mg. of heat killed *Mycobacterium tuberculosis* (BP008), cells (Biological Production, Eli Lilly and Co.), 50 Gm. of a mixture of 45 Gm. mineral oil[1] plus 5 Gm. sorbitan ester,[2] and 0.23 ml. of liquefied phenol. Sterilize the mixture of mineral oil and the sorbitan ester separately and weigh directly into the homogenizing cup. Add 50 ml., 80 units/ml., of mixed source insulin solution and homogenize the mixture for 5 min. at full speed. Prepare 80 units/ml. of insulin by weighing an appropriate mixed beef-pork insulin (potency previously established by U.S.P. bioassay) and dissolve the solid in insulin diluent (1).

Use the resulting emulsion of "complete" antigen immediately for immunization. If injection delay is encountered, repeat the homogenizing step. A "modified" antigen is also prepared using 40 units/ml. of mixed source insulin in the same manner but without the heat killed *M. tuberculosis* cells. Smaller or larger quantities of the emulsion can be prepared using proportional quantities of the components in the mixture.

Inject mongrel guinea pigs (500–600 Gm. each) subcutaneously with 1.0 ml. of the "complete" antigen divided into 0.2-ml. increments at five hind quarter sites on the initial day of immunization. Repeat same treatment with "complete" antigen on the 15th day. On the 30th day of the immunization regimen and every 30 days, thereafter, further stimulate the animals with a 0.5 ml. intraperitoneal injection of the "modified" antigen. Seven days after antigenic stimulation; namely, the 37th, 67th, 97th, etc., day of the immunization regimen, recover 10 ml. of blood from each animal by cardiac puncture. Use clear antiserum, obtained in the conventional manner, immediately for determining insulin antibody titer or freeze and store at −25° for future antibody determination.

The presence of antibody in the individual guinea pig antisera is readily detected by substituting 100 μl. of 160 munits/ml. insulin standard, 20 μl. of antiserum and 1.0 ml. of immuno-diluent into the standard immunoassay procedure. An antiserum is considered to have suitable antibody concentration if 50% or more of the labeled insulin in the system is bound. Those antisera exhibiting suitable antibody concentration are pooled and freeze-dried. This freeze-dried antiserum is stored at −25° indefinitely and serves as a uniform source of antibody for extended periods of routine analysis.

Prior to use in the immunoassay, a given lot of freeze-dried antiserum is carefully titered against insulin concentrations (10–50 munits/ml. range) chosen from the standard reference curve at three selected antibody concentrations. Experience indicates that antisera, selected on the basis of the detection assay, should be diluted in a range of 1:1000–1:2000 to provide a linear relationship when per cent radioactivity remaining in the supernatant liquid is plotted as a log function of insulin concentration, as shown in Fig. 1. In this manner, a five-point reference curve is prepared for each antibody concentration. A visual inspection of the linearity of the three resulting curves permits a selection of the proper antibody concentration to be used in the immunoassay system in the range of the selected insulin concentrations.

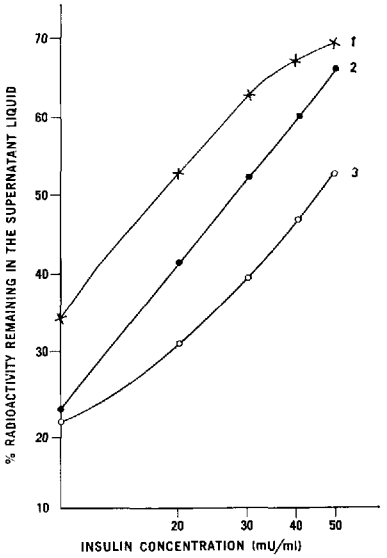

Fig. 1.—A typical insulin antibody titer determination and standard reference curve obtained with pork zinc insulin (lot PJ-5682) and guinea pig antiserum. Key: curve 1, antiserum diluted 1:1000 (1.0 μl./ml.), exhibits the effect of excess antibody; curve 2, a typical standard reference curve for insulin immunoassay is obtained with antiserum diluted 1:1500 (0.67 μl./ml.); curve 3, antiserum diluted 1:2000 (0.50 μl./ml.), shows the effect of insufficient antibody in the immunological reaction.

Due to inherent biological variation in antibody production from individual animals, the results obtained in this type antibody titer determination may indicate a repetition of the determination at higher or lower antibody concentrations. In addition to determining antibody titer, this assay adjustment system is also used to check new lots of human serum albumin, γ-globulin, or other reagents and standards.

REAGENTS

Immuno-Diluent.—*0.25% Serum Albumin-Borate Buffer.*— The immuno-diluent (pH 8.2, 0.1 ionic strength) is prepared with 6.18 Gm. of boric acid, 5.00 Gm. of sodium tetraborate, 4.62 Gm. of sodium chloride, and 10 ml. of 25% salt-poor human serum albumin (Cutter Laboratories) per liter. Stock of 10–20 L. can be prepared and stored at room temperature for 2 months.

Urea–γ-Globulin Reagent.—Urea solution (17%) containing 0.1% bovine γ-globulin (Cohn, fraction II) is prepared by dissolving 510 Gm. of urea, 3.0 Gm. of bovine γ-globulin, and diluting to 3 L. with immuno-diluent. The solution is adjusted to pH 8.2 with 5.0 *N* HCl. The reagent is stored under refrigeration (5°) when not in use and is stable for 6 weeks.

[1] Marketed as Drakseol-6VR by Pennsylvania Refining Co., Butler, Pa.
[2] Marketed as Arlacel A by Atlas Chemical Industries, Inc., Wilmington, Del.

Labeled Insulin.—Insulin labeled with ^{131}I or ^{125}I is satisfactory as a tracer in the immunoassay. ^{131}I insulin is obtained from Abbott Laboratories with a specific activity of 250–300 mc./mg. The concentrate, supplied in 1% human serum albumin with glycine buffer at pH 8.2, is diluted for use with immuno-diluent to 0.266 μc./ml. ^{125}I insulin is prepared at Eli Lilly and Co. with a specific activity of 6–12 mc./mg. and is diluted for use with immuno-diluent to 0.133 μc./ml. The diluted ^{125}I insulin is portioned into 25-ml. aliquots. A fresh aliquot of labeled insulin is used each week, the remainder is stored at −25°. A lot of ^{131}I insulin can be used for a 2-week period and then must be replaced with a freshly prepared material. On the other hand, a lot of ^{125}I insulin,[3] when frozen in aliquots as described, can be used for 3 months. The weekly supply of ^{125}I insulin is stored under refrigeration (5°) when not in use.

Insulin Standards.—Two reference standards used in the present study were selected at random; namely, pork zinc insulin, lot No. PJ-5682 (U.S.P. bioassay: 23.9 unit/mg. ±5.51%) and beef zinc insulin, lot No. 836550 (U.S.P. bioassay: 25.4 units/mg. ±5.59%). Fifty units/ml. stock solution of the reference standards are prepared in insulin diluent and carefully diluted (two steps) to 0.25 units/ml. with insulin diluent. In the final dilution of the 0.25 unit/ml. solution to 10–50 munits/ml. solutions used for the preparation of the standard reference curve, immuno-diluent is used for the dilutions. The mixed source insulin reference is prepared by mixing the 50 units/ml. stock reference standards in the ratio of 25% pork, 75% beef zinc insulin, and appropriately diluting the mixed stock for use. Fresh reference standards are prepared weekly.

Protein Precipitant.—A 1.25 *M* sodium citrate dihydrate solution is prepared by dissolving 367.6 Gm. in distilled water and diluting to 1 L. It is essential that analytical reagent grade sodium citrate be used in the preparation of this salt solution.

INSULIN IMMUNOASSAY METHOD

Sample Preparation.—In order to minimize volumetric error, samples are carefully diluted with conventional laboratory pipets and volumetric flasks; or, micro dilutions are prepared by employing a syringe microburet (Micro-metric Instruments Co.). If micro dilutions are utilized in sample preparation, volumetric error is avoided if the delivery volume from the syringe microburet is not less than 50 μl. with highly concentrated insulin solutions. The first step dilution is performed to about 50 units/ml. using insulin diluent. The final dilutions are made with immuno-diluent. After the final dilution, the samples are conveniently stored overnight at 5°.

Assay Procedure.—Clean all glassware with detergent (Alconox), rinse with 2% (w/v) HCl, then deionized water, and dry in an oven before use. Number a set of seventy-two 10-ml. conical flasks, 12-ml. double strength centrifuge tubes, and plastic counting tubes in sequence. Add 100 μl. of 0.133 μc./ml. ^{125}I insulin (by syringe microburet) and 1.8 ml. urea–γ-globulin reagent to all flasks. Flasks 1 and 2 serve as control blanks for labeled insulin; add 1.10 ml. immuno-diluent to each in place of the sample and antibody. Flask 3 (reference zero) serves as a guide in measuring the binding capacity of the antibody with the labeled insulin. It contains 100 μl. of immuno-diluent in place of the sample. The insulin reference standard (flasks 4–18) and the samples (flasks 19–72) are run in triplicate. Using the syringe microburet, deliver 100-μl. aliquots of each of the insulin reference standards (10, 20, 30, 40, and 50 munits/ml., respectively) and aliquots of the unknown samples into the appropriate flask. Initiate the immunological reaction by the sequential addition of 1.0 ml. of antibody solution (flasks 3–72). Momentarily agitate the mixture by hand and continue gentle agitation on a rotary shaker (A. H. Thomas No. 3623) for 15 min. at room temperature. Commence all timing sequences after additions are made to the last flask. Precipitate the antigen–antibody complex (bound form) by adding 6.0 ml. of 1.25 *M* sodium citrate into each flask. Again agitate this mixture on the rotary shaker for 15 min., then transfer the contents to the appropriate numbered centrifuge tube, and centrifuge in a refrigerated angle head centrifuge at 2400 × g for 45 min. at 15°.

Following centrifugation, carefully remove 5 ml. of the supernatant liquid and transfer the liquid to the appropriately numbered plastic tube.

Radioactivity Measurement.—Measure the radioactivity of the sample in the plastic tube in an automatic gamma spectrometer (model 410A, Packard Instrument Co.) precalibrated for a peak emission of ^{125}I or ^{131}I. The counting efficiency of the instrument is measured with a γ-ray emission reference source (^{133}Ba) lot No. B-508 (Abbott Laboratories).

Prepare a radioisotope control tube for monitoring a given lot of labeled insulin during the entire period of time that the particular lot is used for routine assay. Dilute a 100-μl. aliquot of diluted labeled insulin (0.133 μc./ml.) with 8.9 ml. of immuno-diluent and transfer a 5-ml. aliquot of this mixture to a plastic tube. Tightly seal the tube with a rubber stopper and tape.

The above control tube is followed by an empty plastic tube used to measure background radiation. Counting time is usually 5 min./tube. A radioactivity concentration is selected such that the 5-min. count of the "reference zero" tube is never less than 5000.

Calculation of Potency.—By convention, correct the individual tube counts for background and decay. Calculate the amount of radioactivity remaining in the supernatant liquid and express as per cent (average corrected sample count × 100 divided by average corrected blank count). Plot a five-point curve of the standard reference insulin concentration, 10, 20, 30, 40, and 50 munits/ml., respectively, on semilog graph paper. Curve 2, Fig. 1, represents a typical standard response curve exhibited by a plot of these concentrations. Sample concentration is estimated from this graph or calculated on a point-to-point linear assumption as a logarithmic function of the insulin concentration. For greater reliability, the calculation is limited to three center standard reference points; namely, 20, 30, and 40 munits/ml. of insulin. For sample values (per cent radioactivity in the supernatant) equal to

[3] ^{125}I insulin was prepared by Dr. R. E. Crabtree, Analytical Research, Eli Lilly and Co., Indianapolis, Ind.

or less than the midpoint of the standard curve, the following equations are used to calculate insulin concentration in the sample. (Assuming 20, 30, and 40 munits/ml. = standard curve.)

$$\text{slope} = \log 30 - \log 20/(\%\text{ at } 30) - (\%\text{ at } 20)$$
$$\log \text{sample} = \log 30 - (\text{slope})(\%\text{ at } 30 - \%\text{ sample})$$
$$\text{antilog sample} = \text{sample concentration}$$
$$\%\text{ estimate} = (\text{sample concentration/midpoint standard concentration}) \times 100$$
$$(\%\text{ estimate})(\text{estimate}) = \text{unknown insulin concentration}$$

For sample values (per cent radioactivity in the supernatant) equal to or greater than the midpoint of the standard curve, the equations are the same as above except for calculation of the slope, which is:

$$\text{slope} = \log 40 - \log 30/(\%\text{ at } 40) - (\%\text{ at } 30)$$

Immunoassay results are reported with their 95% confidence interval values. The variance of the indicated sample potency is calculated as described by Baum *et al.* (7). For routine immunoassay of insulin, a digital computer (IBM 360) is programed to perform the calculations.

Insulin Bioassay Method.—The potencies of all standard insulins and insulin samples used in this study were established in accordance with the official U.S.P. bioassay (1) utilizing the U.S.P. zinc insulin reference standard for comparison. Sufficient individual bioassay results were combined to yield 95% fiducial limit in the range of 5–8%.

Insulin Testing Samples.—Triplicate sample of regular pork, beef, mixed source master lots of crystalline zinc insulin, NPH (isophane) insulin, and protamine zinc insulin were selected at random for establishing assay precision. The samples, to be subjected to repetitive immunoassay, were the same trial dilutions of the respective insulin master lots used previously in establishing their insulin potency by the official U.S.P. bioassay. In addition to the commercial insulin products, several process samples from insulin manufacture were obtained and assayed with both beef and pork insulin standards. Two of these samples, 174-A and 9CR40 + 41-A, were subjected to U.S.P. bioassay to establish the biological potency for comparison with the immunoassay results.

The initial aspects of the study revealed the desirability of having a secondary reference standard which is equivalent to the U.S.P. standard. For this purpose three pooled lots of pork, beef, and mixed source insulin were carefully selected from a number of lots which had previously been thoroughly tested by U.S.P. bioassay. Lot 14GP-257, pork zinc insulin crystals, was obtained from two pooled lots of pork insulin, and the average biopotency value was used as a basis of preparing a solution of this pork zinc insulin at 40 units/ml. Similarly, lot 14GP-258, beef zinc insulin crystals, was obtained from six different beef lots of established potency and lot 14GP-259, a mixed source zinc insulin, was a pool of 32 lots of mixed pork and beef zinc insulin crystals with potency also established by bioassay. The average potency value of these pooled lots of insulin should be closely related to the potency of the U.S.P. reference standard. Therefore, the lot, which under conditions of the immunoassay, gave results equivalent to the U.S.P. reference standard, would provide an insulin standard that permits immunoassay results to be expressed in terms of the U.S.P. standard.

Requirements for Immunoassay Precision.—The experimental plan was designed to yield results which were considered as "maximum capability" of the immunoassay under presently recognized conditions. "Maximum capability" is defined as those conditions of procedural operations which would yield the smallest possible variation from one daily assay to another. These conditions place special demand on the technical operators which would not be imposed on the regular daily "routine" testing.

Samples were prepared for each day's assay by a two-step dilution from the concentrated sample. The same pipet was used with a given sample each time that sample was diluted. In order to achieve more accuracy throughout, each technical operator used only one syringe for the ^{125}I insulin and only one syringe for the sample in the micrometric delivery. The sample syringe was carefully rinsed each time with the sample to be delivered into the reaction mixture. Every effort was made to keep dilutions and volumetric errors to a minimum. All reagents utilized throughout the testing sequence were prepared, as necessary, from the same lots of antibody, γ-globulin, human serum albumin, urea, sodium citrate, and borate buffer.

In all experimental testing, each sample was evaluated 10 times on different days by each of two technical operators. The experiments in each sequence were carried out over a period of a month with daily runs scheduled not to interfere with the routine insulin immunoassays also being performed. The tabulated results were submitted for statistical calculation and an analysis of variance.

RESULTS AND DISCUSSION

Comparative Assay Results.—*Regular Insulin.*—Table I shows the comparative results of the U.S.P. bioassay and the immunoassay of three lots each of pork, beef, and mixed zinc insulin. Both the bioassay and the immunoassay were conducted on a 40 units/ml. sample solution of each lot of insulin. The results were calculated in terms of units/mg. so that both assay systems could be directly compared. The 95% confidence limits of the immunoassay results are expressed for 4 and 10 daily repetitive tests. The insulin test samples were measured against the randomly selected pork standard, beef standard, and the mixed standard to determine the degree, if any, of species specificity affecting the immunoassay. The results reveal that the deviation of the immunoassay values for 4 repetitive tests is generally less than that of the corresponding bioassay and is always less in the case of 10 repetitive immunoassays. The test sample potency values obtained with the immunological assay using the pork insulin reference standard closely coincides with the bioassay results which were established with the U.S.P. insulin standard. On the other hand, the immunological data obtained with the beef insulin reference standard are consistently higher than the corresponding bioassay results. The statistical analysis indicates a significant difference in the assigned bioassay potencies of the two randomly selected reference standards. The overall results revealed that the beef zinc insulin refer-

TABLE I.—COMPARATIVE RESULTS OF THE U.S.P. BIOASSAY AND THE IMMUNOASSAY

U.S.P. Bioassay			Immunoassay								
				Pork Std.			Beef Std.			Mixed Std.	
Sample	units/mg.	$\%2\sigma$	Animals No.	units/mg.	$\%2\sigma_4$[a]	$\%2\sigma_{10}$[a]	units/mg.	$\%2\sigma_4$[a]	$\%2\sigma_{10}$[a]	units/mg.	$\%2\sigma_4$[a]	$\%2\sigma_{10}$[a]
Regular Pork Zinc Insulin												
PJ5682	23.9	±5.51	240	27.56	±3.99	±2.55	30.25	±4.85	±3.07	30.18	±3.11	±1.97
W-3789	25.6	±6.59	216	25.02	±3.89	±2.46	28.23	±3.95	±2.50	26.62	±2.47	±1.56
W-3864	25.2	±5.46	144	25.26	±3.99	±2.52	27.04	±4.71	±2.98	26.46	±4.65	±2.94
Regular Beef Zinc Insulin												
W-3879	24.64	±7.06	192	24.71	±4.61	±2.92	26.78	±2.00	±1.27	25.65	±4.57	±2.89
W-3912	24.60	±5.11	192	23.69	±4.10	±2.59	26.65	±6.31	±3.99	25.57	±5.67	±3.59
W-3905	25.47	±5.77	168	23.95	±4.43	±2.80	26.13	±6.37	±4.03	25.58	±5.74	±3.63
Regular Mixed Zinc Insulin												
W-3885	25.69	±5.43	240	24.78	±5.17	±3.27	26.82	±2.80	±1.77	25.45	±6.33	±4.00
W-3867	24.80	±5.92	192	25.45	±6.43	±4.07	27.66	±7.80	±4.93	25.76	±5.35	±3.38
W-3846	24.72	±5.18	192	24.07	±5.68	±3.59	26.54	±5.42	±3.42	25.96	±6.65	±4.21

[a] The symbols indicated as $2\sigma_4$ and $2\sigma_{10}$ represent the 95% confidence limit of the mean of 4 and 10 daily immunoassays respectively.

ence standard (lot No. 836550) gave consistent values which were higher than expected in the immunoassay when compared to the bioassay. This suggests that the original bioassay of this lot of beef zinc insulin crystals, although within the bioassay fiducial limit, was unrealistic as an estimate of the true potency; hence, in preparing the beef reference standard based on the mean biopotency, more insulin was introduced into the standard than would normally be expected. This was further supported by the analysis of variance and cross checking the reference standards in the immunoassay. Of the two immunoassay reference standards, randomly chosen, the pork zinc insulin (PJ-5682) compares most favorably with the U.S.P. reference standard.

The effect of species specificity on immunoassay reliability could be determined by an analysis of variance of the data obtained in Table I. The analysis of variance revealed no interaction of the groups studied under the conditions employed in the immunoanalysis.

Therefore, immunoassay, performed with antiserum obtained from guinea pigs immunized with mixed pork and beef insulin as the antigen, can be used with either pork or beef insulin as a standard in the immunoassay of beef, pork, or mixed source insulin samples. The fact that species specificity has no effect upon the method employed in this study is important for a satisfactory method for control of insulin development and manufacture.

Immunoassay precision is influenced from two principal sources: the variation encountered within a single day's testing and the variation experienced between days. The within-day assay variation is largely dependent upon sample replication, instrumentation, etc. Differences between days which exceed the within-day variation, are less amenable. The result of these two types of variation often produced deviations as large as ±15–25% in the immunoassays obtained by the salt precipitation procedure as described by Baum *et al.* (7). By carefully modifying the procedure, standardizing reagents, practicing rigid analytical techniques, and strict attention to details, the assay variation was reduced to ±11.2% when expressed as a 95% con-

TABLE II.—COMPARISON OF CONFIDENCE INTERVALS OF THE INSULIN IMMUNOASSAY CONDUCTED BY "MAXIMUM" AND "ROUTINE" CAPABILITY IMMUNOASSAY WITH PORK INSULIN STANDARD

	Max. Capability			Routine Capability	
Sample	units/mg.	$\%2\sigma_4$[a]	$\%2\sigma_{10}$[a]	$\%2\sigma_4$[a]	$\%2\sigma_{10}$[a]
Regular Pork Zinc Insulin					
PJ-5682	27.56	±3.99	±2.52	±4.90	±3.10
W-3789	25.02	±3.89	±2.46	±1.98	±1.25
W-3864	25.26	±3.99	±2.52	±1.90	±1.20
Regular Beef Zinc Insulin					
W-3879	24.71	±4.61	±2.92	±5.49	±3.47
W-3912	23.69	±4.10	±2.59	±1.76	±1.11
W-3905	23.95	±4.43	±2.80	±8.12	±5.14
Regular Mixed Zinc Insulin					
W-3885	24.78	±5.17	±3.27	±6.10	±3.86
W-3867	25.45	±6.43	±4.07	±6.31	±3.99
W-3846	24.07	±5.68	±3.59	±8.88	±5.62

[a] The symbols indicated as $2\sigma_4$ and $2\sigma_{10}$ represent the 95% confidence limit of the mean of 4 and 10 daily immunoassays, respectively.

TABLE III.—COMPARISON OF CONFIDENCE INTERVALS OF THE IMMUNOASSAY OF NPH AND PZ INSULIN BY "MAXIMUM" AND "ROUTINE" CAPABILITY

				Immunoassay					
				Max. Capability			Routine Capability		
	U.S.P. Bioassay			Mixed Insulin Std.			Pork Insulin Std.		
Sample	Type	units/mg.[b]	$\%2\sigma$	units/ml.	$\%2\sigma_4$[a]	$\%2\sigma_{10}$[a]	units/ml.	$\%2\sigma_4$[a]	$\%2\sigma_{10}$[a]
W-3891[c]	NPH	25.4	±5.43	41.92	±9.45	±5.98	38.24	±6.08	±3.85
W-3871[c]	NPH	24.8	±5.92	41.23	±4.24	±2.68	37.00	±5.50	±3.48
W-3858[c]	NPH	24.9	±5.18	41.47	±6.02	±3.81	37.40	±6.52	±4.12
W-3857[c]	PZI	24.9	±5.18	44.91	±6.35	±2.01	39.56	±6.35	±2.01
W-3870[c]	PZI	24.8	±5.92	42.19	±6.62	±2.09	40.16	±5.56	±3.54
W-3892[c]	PZI	25.4	±5.43	42.81	±4.65	±1.47	39.10	±5.65	±3.57

[a] The symbols indicated as $2\sigma_4$ and $2\sigma_{10}$ represent the 95% confidence limit of the mean of 4 and 10 daily immunoassays, respectively. [b] All samples were prepared at 40 units/ml. based on the insulin potency established by bioassay. [c] Acidified with 30 µl. concentrated HCl/10 ml. of mixture for solution prior to dilution with immuno-diluent.

fidence limit for a single assay. The data in Table I were used to calculate such a generalized confidence interval of 88.8–111.2%.

The U.S.P. regulates the potency of insulin with a 95% fiducial limit of 87–115%. However, specifications for establishing insulin potency at Eli Lilly and Co. require a 95% fiducial limit of ±5–6% for the U.S.P. insulin bioassay. The number of daily immunoassays which give equivalent results is predicted from the average variation observed in the data summarized in Table I. Under the assumption that the daily immunoassays of a single sample are normally distributed, the required sample size (repetitive immunoassays conducted on different days, n) is calculated as:

$$\pm 5.5 = \pm 11.2\sqrt{\frac{}{n}}$$

$$n = 4.15$$

Hence, four daily immunoassays must be conducted to be within the Eli Lilly and Co. specifications.

The coefficient of variation (±5.6%) established from the data in Table I was obtained under conditions of "maximum capability." These rigid analytical techniques are impractical in the routine immunoassay for insulin. Therefore, the same series of insulin samples was re-evaluated under conditions defined as "routine capability." "Routine capability" permits the technician to utilize different syringes and pipets for all analytical measurements. Table II shows a comparison of the confidence limits of the U.S.P. bioassay and the insulin immunoassay conducted under conditions of "maximum" and "routine" capability. In this series of immunoassays, only the pork insulin standard was used since it has a potency very similar to that of the U.S.P. reference standard. Visual inspection of the "routine capability" results reveals little difference from the results of "maximum capability." The results further support the validity of the calculated coefficient of variation (±5.6%) as originally determined and indicate that the between-days variation encountered in the immunoassay is not due to operator error or analytical measurement.

Immunoassay of Some Commercial Insulin Products and Manufacturing Intermediates.—The reliability of the immunoassay for determining insulin concentrations in commercial products containing protamine is shown in Table III. NPH (isophane) insulin and protamine zinc insulin were from lots with potency established previously by bioassay. The immunoassay results under "maxi-

TABLE IV.—IMMUNOASSAY OF SAMPLES FROM DIFFERENT STAGES OF INSULIN MANUFACTURE

						Immunoassay					
	U.S.P. Bioassay				Animals,	Pork Std.			Beef Std.		
Sample	Species	Type	units/ml.	$\%2\sigma$	No.	units/ml.	$\%2\sigma_4$[a]	$\%2\sigma_{10}$[a]	units/ml.	$\%2\sigma_4$[a]	$\%2\sigma_{10}$[a]
9CR-48 2nd Ch.	Beef	Cr. ext.	Not possible	...	...	6.23	±1.30	±0.83	6.68	±2.58	±1.63
9CR-48 3rd Ch.	Beef	Cr. ext.	Not possible	...	...	6.39	±1.65	±1.04	6.72	±2.10	±1.33
9CR-48 4th Ch.	Beef	Cr. ext.	Not possible	...	...	6.97	±3.90	±2.47	7.60	±5.11	±3.23
9CR40-41A	Pork	pH 5.6 ppt.	363.4 ± 6.92		144	387.4	±4.66	±2.95	397.0	±8.60	±5.44
9CR40-41B	Pork	pH 5.6 ppt.	...	..	...	405.0	±2.27	±1.44	422.9	±2.48	±1.57
9CR40-41C	Pork	pH 5.6 ppt.	...	..	...	404.5	±3.65	±2.31	...	...	...
L-174A	Pork	1st iso.	667.3 ± 7.87		144	666.5	±1.61	±1.02	713.0	±6.10	±3.86
L-174B	Pork	1st iso.	...	...	...	658.4	±2.88	±1.82	703.1	±2.09	±2.32
L-174C	Pork	1st iso.	...	...	...	660.0	±6.03	±3.81	692.8	±7.54	±4.77

[a] The symbols indicated as $2\sigma_4$ and $2\sigma_{10}$ represent the 95% confidence limit of the mean of 4 and 10 daily immunoassays, respectively.

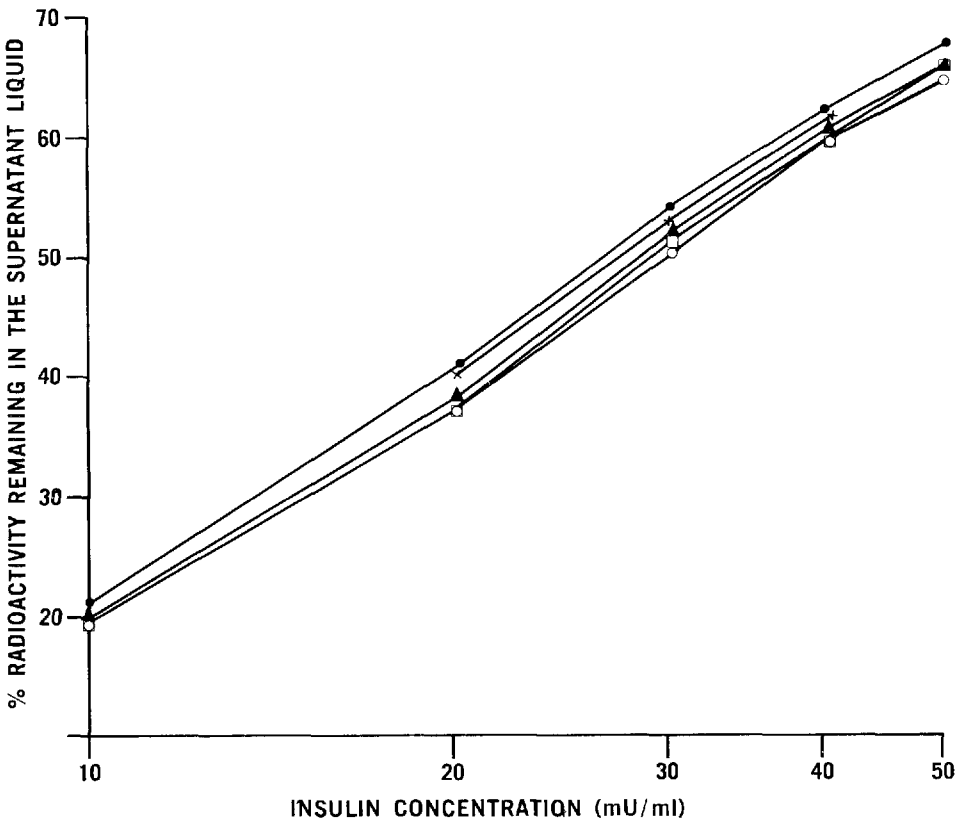

Fig. 2.—Full-scale response curves of the mean value of 10 immunoassays conducted on different days. Key: ●, pork zinc insulin, lot PJ-5682 (served as the reference standard); ○, pooled samples of pork insulin, 14GP-257; □, beef insulin, 14GP-258; ▲, mixed pork and beef insulins, 14GP-259, diluted based on the calculated mean of the individual bioassay result; ×, U.S.P. reference standard, W-3930, diluted based on the established biopotency of 24 units/mg. and determined at three of the five standard concentrations.

mum capability" conditions were obtained with a mixed source insulin reference standard having the same ratio of pork to beef insulin as that of the sample. The immunoassay values are slightly high compared to the bioassay figures. This is accounted for by the fact that the beef reference standard, lot 836550, which constitutes 75% of the mixed source reference standard, was found to give consistently high results, as previously noted. The unrealistic high results obtained with the mixed standard and the similarity of the pork insulin standard with the U.S.P. reference standard suggested that pork insulin standard should be used in the series conducted under "routine capability." The results readily reflect the difference in the absolute potency of these two randomly selected reference standards. The confidence limits, expressed for 4 and 10 repetitive tests, are slightly greater than the values presented in Tables I and II, but are within the expected range of the coefficient of variation. The results in Table III support the view that commercial insulin products can be analyzed effectively with this immunoassay.

Table IV provides evidence that the immunoassay can be used in monitoring insulin manufacture. Immunoassay results on samples selected at different stages of the insulin process show a variation that is within the limits of the coefficient of variation and agree satisfactorily with the U.S.P. bioassay. The immunoassay possesses a distinct advantage over the U.S.P. bioassay in its ability to determine insulin concentration in crude samples that cannot be satisfactorily handled under the conditions of the bioassay system.

The pooling of many lots of crystalline zinc insulin of established biological potency would seem to provide a material with a mean biopotency which would closely correspond to the U.S.P. reference standard. Three types of pooled samples were prepared by mixing aliquots of zinc insulin crystals from lots that were thoroughly assayed in the U.S.P. method; namely, 14GP-257 from two lots of pork zinc insulin crystals with an average potency of 25.45 units/mg., 14GP-258 from six lots of beef zinc insulin crystals with an average potency of 25.50 units/mg., and 14GP-259 from 32 lots of mixed source zinc insulin crystals with an average potency of 24.79 units/mg. The average potency value was used to prepare 40 units/ml. insulin solutions of each for 10 repetitive immunoassays on different days. To further substantiate and extend the findings of the initial study, these samples were tested at all concentrations used

TABLE V.—SIMILARITY OF THE INDIVIDUAL COMPUTED OF POOLED ZINC INSULIN SAMPLES

Sample	Type	Range, munits/ml.	Slope[a]	95% Confidence Interval of the Slope
PJ5682	Pork	20–40	69.57	66.96–72.18
14GP-257	Pork	20–40	70.05	67.44–72.66
14GP-258	Beef	20–40	73.60	70.99–76.21
14GP-259	Mixed	20–40	73.36	70.75–75.98
W-3930	U.S.P.	20–40	69.93	67.32–72.54
Common slope—all species from statistical analysis.		20–40	71.30[b]	

[a] Individual slopes by least squares. [b] Least squares common slope lies within each of the computed individual slope confidence intervals.

in the standard reference curve. According to the procedure described, a sample for immunoassay is diluted to a concentration which is estimated to be equivalent to that of the midpoint standard (30 munits/ml.). The statistical analysis and the interpretation of results are limited to this single point. On the other hand, a comparison of the slopes of the curves obtained from determining these pooled samples at five different concentrations would avoid this limitation. If the slopes of the curves obtained from the pooled samples are identical, within the experimental limits, with that of the randomly chosen standard (in this case pork zinc insulin PJ-5682), the original interpretation would be confirmed.

Figure 2 shows a plot of the mean values of 10 repetitive immunoassays of the pooled samples, standard PJ-5682, and three concentrations of the U.S.P. reference standard. Visual inspection indicates the curves to have similar slopes in the range of 20–40 munits/ml. insulin concentrations, respectively. In view of the fact that this immunoassay has been limited to the determination of potency of extracted insulin by definition, only the most sensitive portion of the standard curve was subjected to statistical analysis to substantiate the use of one sample concentration for routine analysis.

Statistical analysis, by least squares, of the data obtained at 20, 30, and 40 munits/ml. insulin concentrations gave 95% confidence limits for the individual slopes as shown in Table V. The value of the calculated common slope, 71.30, lies within the limits of the individual slopes. Furthermore, by a more refined technique of regression analysis, no significant differences in slopes were detected. Figure 3 shows the dose response curves of these

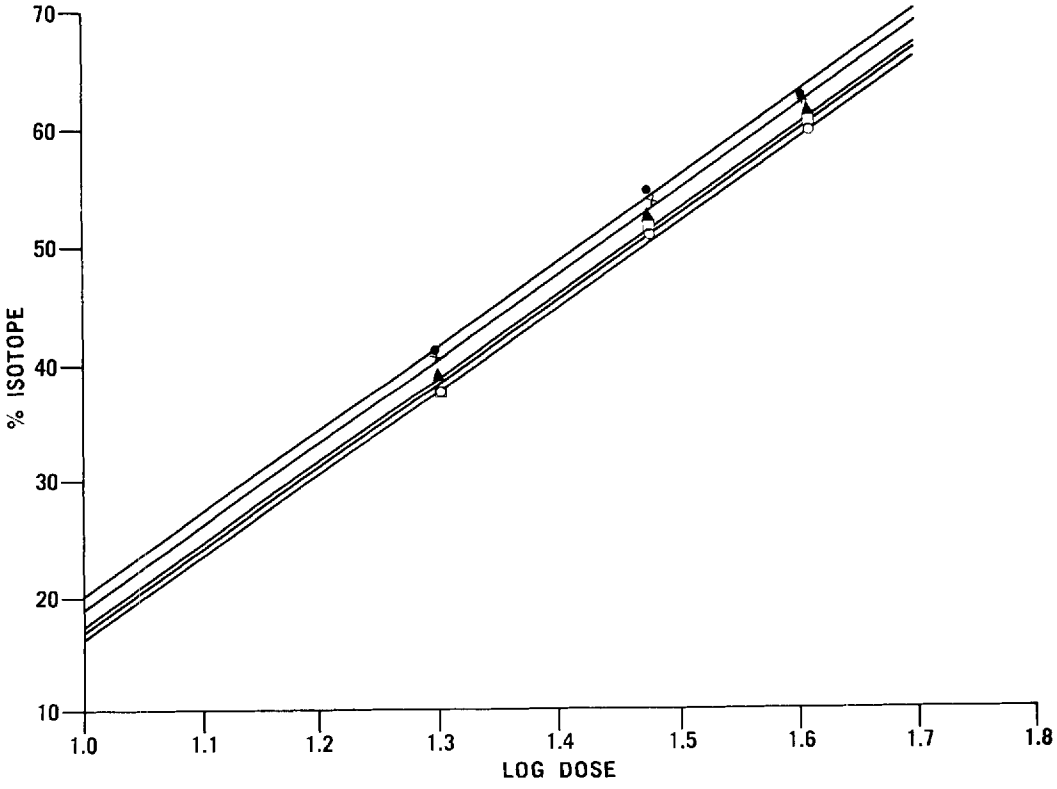

Fig. 3.—Dose response curves based on the common slope calculated by least squares for the selected concentrations at 20, 30, and 40 munits/ml. Key: ●, PJ-5682, ○, 14GP-257; □, 14GP-258; ▲, 14GP-259; ×, U.S.P. reference standard W-3930 as in Fig. 2.

TABLE VI.—COMPARATIVE POTENCY RESULTS OF INSULIN MASTER LOTS DETERMINED BY U.S.P. BIOASSAY AND IMMUNOASSAY

Sample	Estimated Potency, units/mg.	% Estimate	Potency, units/mg.	Variation, %	Rabbits, No.	Estimate, %	Potency, units/mg.	% S. D.	Replications
M.L. 47 (W-3927) Beef	25.0	99.06	24.76	5.71	192	98.75	24.69	6.3	5
M.L. 48 (W-3934) Beef	25.0	100.41	25.10	5.94	192	102.00	25.50	11.4	5
M.L. 9BV49 (W-3929) Mixed	24.5	102.86	25.20	5.46	240	102.67	25.15	4.5	5
M.L. 9GZ30 (W-3940) Mixed	25.0	101.28	25.32	5.90	192	101.44	25.36	5.6	4

[a] Pork zinc insulin (lot PJ-5682) used as the immunoassay reference standard.

insulin samples plotted with the common slope. Consequently, species specificity does not affect the validity of the immunoassay result if mixed antigen is used in production of antibody in guinea pigs. The individual regression lines for the different samples, although parallel, are not superimposable, thus revealing slight errors in the assigned potency of these samples. In order to express insulin immunoassay results in terms of the U.S.P. reference potency, a secondary reference standard would need to be experimentally adjusted to the potency of the U.S.P. reference standard. Any of these pooled insulin samples can serve as a secondary reference after appropriate matching with the U.S.P. reference by repetitive immunoassay.

Application of the Immunoassay for Establishing the Potency of Manufactured Insulin.—The information obtained in this investigation was applied to the determination of potency of several lots of manufactured insulin. Table VI compares the U.S.P. bioassay and the immunoassay of four lots of zinc insulin crystals. Instead of four repetitive immunoassays on different days as required under "maximum capability" conditions, five immunoassays were conducted (except on ML-9GZ30) under routine conditions. The mean value of insulin potency established by immunoassay compares favorably with the bioassay result being well within the limits defined in the U.S.P. (1). However, the standard deviation of the mean of the individual values, particularly with ML 48, varied somewhat more than would be predicted. The cause of this excessive variation could not be readily determined, but suggests the necessity of vigilant attention to detail in immunoassay conduct.

The results of this investigation indicate that the insulin immunoassay, in accordance with the described procedure, is a rapid and economic method, with satisfactory precision and sensitivity for establishing the potency of extracted insulin. The use of this immunoassay for extracted insulin fills an important need in monitoring the manufacture of insulin and assessing insulin concentration in commercial insulin products. The information provided in this investigation can serve to promote interest and support in establishing the immunoassay as a U.S.P. method for determining insulin potency.

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