Glucagon Immunoassay by Tracer Displacement*

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ABSTRACT: Nonprecipitating antibody to pork–beef glucagon is readily produced in rabbits and guinea pigs with alum-precipitated glucagon in Freund's complete adjuvant. The immunoassay for glucagon is based on the formation and isolation of a [^{131}I]glucagon–antibody complex, the subsequent displacement of the label with unlabeled glucagon, and separation of the soluble mixture of antibody bound and free glucagon by salt precipitation. The amount of [^{131}I]glucagon displaced from the antibody complex is proportional to the log of the glucagon concentration. The evidence demonstrates that labeled glucagon is stabilized in the isolated [^{131}I]-glucagon–antibody complex. Complex formation eliminates nonspecific radioactivity and provides a consistent reagent for routine analysis. Characteristics of the standard glucagon–antibody complex prevents secondary reactions in which [^{131}I]glucagon is bound irreversibly. Insulin interferes with the immunoassay in concentrations >1%, limiting the detection level of glucagon to 0.0025%. The glucagon assay by tracer displacement, with a sensitivity range 6.25–100 mμg/replicate sample, provides a rapid, accurate, and economic method, sufficiently sensitive for controlling glucagon development and manufacturing processes and with further development, the specific measurement of glucagon in biological samples.

Glucagon is isolated from a side fraction of the commercial insulin process. In order to facilitate efficient processing for optimum yields of glucagon, a reliable potency determination of glucagon in source material is essential. The bioassay, based on the hyperglycemic response in cats (Staub and Behrens, 1954; Bromer and Behrens, 1962), is limited by inherent biological variation, cost of facilities, time consumption, and lack of sensitivity. Insulin in pancreatic fractions must be destroyed with cysteine (Staub and Behrens, 1954) before estimating glucagon activity. Other pancreatic proteins can interfere with the hyperglycemic response.

The immunoassays reported for insulin (Yalow and Berson, 1960, 1961; Grodsky and Forsham, 1960; Morgan and Lazarow, 1962; Hales and Randle, 1963) and glucagon (Unger *et al.*, 1961; Grodsky *et al.*, 1961) offer methods to circumvent many bioassay shortcomings. In preliminary studies an immunoassay based on direct competitive isotope dilution procedure (Grodsky *et al.*, 1961), as modified by Baum *et al.* (1964), was investigated. The standard response curve of glucagon showed a continuous characteristic change over a period of 2 weeks indicative of a progressive loss in the binding capacity of [^{131}I]glucagon with the antibody. This apparent spontaneous change in binding capacity of [^{131}I]glucagon results in an erratic and insensitive assay. Damage to labeled antigen has been recognized as contributing to immunoassay variation (Berson and Yalow, 1957; Unger *et al.*, 1961). [^{131}I]Glucagon in the frozen state or diluted for use and stored at 5° appears to be particularly susceptible to spontaneous modification.

This difficulty is overcome by forming and isolating [^{131}I]glucagon–antibody complex which eliminates nonreactive radioactivity from source labeled glucagon and stabilizes bound [^{131}I]glucagon from further change. The displacement of [^{131}I]glucagon in the soluble purified antibody complex with varying concentrations of unlabeled glucagon provides a sensitive measurable index of glucagon concentration.

Materials and Methods

Immunization. Preliminary immunization studies indicated that glucagon antibody was most effectively produced in New Zealand white males and both sexes of Dutch rabbits. Both rabbits and guinea pigs exhibited similar patterns of antibody production with maximal titers occurring 70–100 days after initial immunization. Thereafter, antibody titers gradually decreased, essentially disappearing after 190 days, even though glucagon stimulation was continued at 30-day intervals. The subcutaneous administration of alum-precipitated glucagon in complete Freund's adjuvant 190 days after initial immunization stimulated a temporary rise in rabbit antibody titer, but failed to restore antibody titers in guinea pigs. The antigen preparation and immunization regimen which yields glucagon antibody in 80% of the animals (New Zealand white male rabbits and mongrel guinea pigs) are described.

Antigen Preparation. Alum-precipitated glucagon in complete Freund's adjuvant–crystalline egg albumin (800 mg) (Nutritional Biochemicals Corp.) was dissolved in 12.5 ml of isotonic saline in a 250-ml centrifuge bottle. Pork–beef glucagon (150 mg) (Lot No. 258-234B-167-1) was dissolved in 30 ml of distilled water

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by the addition of 4–5 drops of 1 N sodium hydroxide. The glucagon solution was added to the centrifuge bottle, followed by 45 ml of 10% alum [$AlK(SO_4)_2 \cdot 12H_2O$] by weight in distilled water. The mixture was adjusted to pH 6.5 with 5 N sodium hydroxide and an additional 4-ml excess of alkali was added. The mixture was centrifuged at 2500 rpm at 5° for 5 min. The recovered precipitate was washed with two 50-ml portions of 4.0×10^{-4} M sodium borate and the centrifugation steps repeated. The resulting precipitate was suspended in 50 ml of isotonic saline. An emulsion was prepared in a Virtis homogenizer using 50 ml of alum-precipitated glucagon, 45 g of mineral oil (Drakeol 6-VR, Pennsylvania Refining Co.), 5 g of Arlacel A (Atlas Chemical Industries), and 250 mg of heat-killed *Mycobacterium tuberculosis* (BP 088) cells.

Glucagon in incomplete Freund's adjuvant–modified antigen was prepared by emulsifying 50 ml of aqueous 5.0 mg/ml glucagon solution with 45 g of Drakeol 6-VR and 5 g of Arlacel A. All vessels, water, isotonic saline, Drakeol 6-VR, and Arlacel A were sterilized prior to use in preparation of the antigens.

Immunization Regimen. Twelve New Zealand white male rabbits, weighing 2–3 kg each, were initially immunized with 2.0 ml of complete emulsion injected subcutaneously using 0.2-ml increments in nine hindquarter sites and one 0.2-ml injection in a rear foot pad. On the 15th day after the initial treatment, 1 ml of complete emulsion was injected subcutaneously in four divided sites in the scapular region. All animals were subsequently stimulated on the 30th day after the initial treatment and every 30 days thereafter with 0.5 ml of modified antigen injected subcutaneously in the right thorax. The animals were ear bled 7 days post stimulation, and glucagon antibody titer was determined. In those animals exhibiting serum antibody, 50 ml of blood was recovered by cardiac puncture.

Six mongrel guinea pigs (500–600 g each) were injected subcutaneously with 1.0 ml of the complete emulsion using 0.2-ml increments at five hindquarter sites. The animals were further stimulated at 30-day intervals with a 1.0-ml intraperitoneal injection of the modified antigen. At 7 days post stimulation, 10 ml of blood was recovered from each animal by cardiac puncture, and the glucagon antibody titer was determined.

Glucagon Standards. Glucagon Lot No. 258-234B-167-1, dissolved in insulin diluent (United States Pharmacopeia, 1960), was used as a reference standard. Stored at 5°, the solution is stable for 30 days. Reference solutions for preparation of the standard reference curve were prepared weekly in 1% human serum albumin in borate buffer. The serum albumin–borate buffer (pH 8.2, 0.1 ionic strength) was prepared using 6.184 g of boric acid, 5.004 g of sodium tetraborate, 4.628 g of sodium chloride, and 40 ml of 25% salt-poor human serum albumin (Cutter Laboratories)/l.

[^{131}I]Glucagon. Labeled glucagon, with a specific activity of 180–300 mc/mg, was obtained from Abbott Laboratories. It was prepared by a modification of the method described by Unger *et al.* (1961) as follows: to a solution of carrier-free $Na^{131}I$ sufficient sodium nitrite and HCl were added to produce nitrous acid to oxidize the iodide to elemental iodine or NaI_3. The glucagon, dissolved in glycine buffer, pH 11.0, is mixed with the iodinating solution. Glycine buffer is added to pH 10. The resulting mixture is passed over an anion exchange resin to remove unreacted iodine compounds (B. J. Green, Abbott Laboratories, personal communications). The concentrate, supplied in 1.0% serum albumin, was diluted to 1.0 μc/ml in 1.0% human serum albumin–borate buffer for use in preparing the [^{131}I]-glucagon–antibody complex. The [^{131}I]glucagon used in determining antiserum titer was diluted to 0.216 μc/ml. Labeled glucagon was stored in the frozen state in the concentrated form and at 5° in the diluted form.

Determination of Antibody Titer. Glucagon antibody content was determined by a modification of the salt precipitation method described by Grodsky *et al.* (1961) A reaction mixture in a 10-ml Erlenmeyer flask was prepared with 100 μl of 0.5 μg/ml standard glucagon, 100 μl of 0.216 μc/ml [^{131}I]glucagon, 1.0 ml 1% human serum albumin–borate buffer, and 20 μl of antiserum at room temperature. After a 15-min reaction period, 1.8 ml of diluted human plasma, prepared according to the method of Grodsky *et al.* (1961), and 6.0 ml of 4.0 M ammonium sulfate were added to the flask in sequence. The mixture was agitated on a rotary shaker for 30 min, transferred to centrifuge tubes, and centrifuged at 2400*g* for 45 min at 15°. Following centrifugation, 5.0 ml of supernatant liquid was transferred to a plastic tube for counting. Total counts (5 min) were corrected for background and decay. The average count of triplicate tubes (times 100) was divided by the average blank count (the blank contains 20 μl of serum from nonimmunized animals) to obtain the per cent radioactivity remaining in the supernatant liquid. Serums were considered to have a suitable antibody level if 50% or more of the label was bound. On the basis of this direct assay, the antisera were pooled and stored in 1-ml aliquots at −25°. A given pool of antisera was systematically titrated *vs.* the glucagon reference standard to determine the proper dilution of antiserum required in conduct of the glucagon displacement immunoassay.

Immunoassay of Glucagon

Preparation of the [^{131}I]Glucagon–Antibody Complex. A reaction mixture, consisting of 1.0 ml of [^{131}I]glucagon (1.0 μc/ml) and 4.0 ml of diluted antiserum, was introduced into a 15-ml centrifuge tube and was gently agitated for 30 min at room temperature. (The antiserum used in this study was diluted 1:10.) The complex was precipitated by the addition of 5.0 ml of 4.0 M ammonium sulfate. After allowing 30 min for the precipitate to aggregate, the mixture was centrifuged at 2400*g* for 20 min and the supernatant liquid was discarded. The precipitate was washed with 5.0 M ammonium sulfate and was recovered as before. The supernatant liquid was carefully drained from the residue. The resulting complex was dissolved in 70 ml of 1% human serum albumin–borate buffer. For routine analysis, utilizing a standard reference curve, the complex is prepared fresh daily.

Displacement of [^{131}I]Glucagon from the Antibody Complex. The glucagon displacement immunoassay was performed in triplicate. A five-point standard reference curve was prepared with 0.0625–1.00 μg/ml of glucagon standard solutions. Appropriate controls were prepared; the blank consists of 1.0 ml of antibody complex and 8.0 ml of 1% human serum albumin–borate buffer. The antibody complex control was prepared identical with to the sample replicate, but 100 μl of buffer was substituted for the sample. Unknown glucagon samples were diluted with 1% human serum albumin–borate buffer to an estimated 0.25 μg/ml of glucagon, the midpoint of the standard reference curve. The individual assay replicates were prepared as follows: Into a 10-ml Erlenmeyer flask was delivered 100 μl of glucagon reference standard or glucagon unknown sample and 1.0 ml of [^{131}I]glucagon–antibody complex. The mixture was gently agitated on a rotary shaker for 16 hr at room temperature. After completion of the displacement reaction, 1.9 ml of human plasma, diluted 1:100 in 1% human serum albumin–borate buffer, was added to the reaction mixture. Human plasma served as a protein carrier for the precipitation of the soluble antigen–antibody complex. The antibody complex was then precipitated with 6 ml of 2.75 M ammonium sulfate. The resulting suspension was transferred to a centrifuge tube, allowed to stand 15 min, and then was centrifuged for 45 min at 2400*g*. A 5.0-ml aliquot of the supernatant liquid was transferred to a plastic tube and counted in an automatic γ-spectrometer (Packard Instrument Co.) Tube counts were corrected for background and decay. The amount of radioactivity displaced into the supernatant liquid, expressed as per cent (average corrected sample count times 100 divided by average corrected blank count) was calculated. A five-point curve of the standard reference glucagon concentrations, 0.063, 0.125, 0.25, 0.50, and 1.00 μg/ml, respectively, was plotted on semilog graph paper. Curves 2 of Figure 2 represent typical standard response curves exhibited by a plot of these concentrations over the useful life of the isotope. Sample concentration can be estimated from this graph or calculated on a point-to-point linear assumption as a logarithmic function of the glucagon concentration. For greater reliability, the calculation is limited to three standard reference points, namely, 0.125, 0.25, and 0.5 μg/ml glucagon, respectively. For routine immunoassay of glucagon, a digital computer (IBM 1620) program performed the calculations.

Results and Discussion

Stabilization of [^{131}I]Glucagon as an Antibody Complex. Originally an attempt was made to design an immunoassay for glucagon in accordance with the salt precipitation method of Grodsky *et al.* (1961) as modified by Baum *et al.* (1964) for insulin, in which labeled and unlabeled antigen compete for antibody. Initial studies indicated a progressive change in the characteristics of the [^{131}I]glucagon if the labeled material was stored in the frozen state or at 5°. This change in the characteristics of the labeled glucagon is reflected by the amount of label that fails to bind with the antibody. As shown in Table I, *ca.* 45% of the radioactivity in day-old [^{131}I]glucagon remains in the supernatant liquid and gradually increases to more than 60% in 14 days. This apparent spontaneous change in labeled glucagon results in an insensitive and unreliable immunoassay as measured by direct competitive isotope dilution. In contrast, the formation and isolation of the [^{131}I]glucagon–antibody complex eliminate nonreactive radioactivity from the labeled product and tend to stabilize the bound [^{131}I]glucagon. Table I compares the stability of the labeled glucagon stored at 5° for direct competitive isotope dilution immunoassay with labeled glucagon–antibody complex prepared fresh daily or antibody complex prepared and stored at 5° over the useful life of the isotope. Although the antibody complex prepared fresh daily reflects the change in the [^{131}I]-glucagon binding capacity for antibody, the elimination of nonreactive ^{131}I in preparing the complex yields a product suitable for immunoassay. On the other hand, the antibody complex prepared and stored at 5° shows little change with time, indicating a stabilizing effect by antibody complex formation.

TABLE I: Improved Stability of [^{131}I]Glucagon Resulting from Antibody Complex Formation Determined over a 14-Day Period.

	^{131}I Remaining in the Supernatant Liquid (%)		
		[^{131}I]Glucagon–Antibody Complex	
[^{131}I]Glucagon Age in Days	Direct Competitive Isotope Dilution[a]	Prepared Fresh Daily[b]	Prepared and Stored at 5°[c]
1	46.5	14.6	...
2	...	...	7.2
4	50.7	18.9	7.7
6	53.2	19.2	8.2
10	58.0	21.0	10.0
14	62.1	24.2	11.9

[a] Abbott Laboratories, Lot E-1017-60-1 (specific activity 306 μc/μg). The amount of ^{131}I which failed to bind with antibody by direct competitive isotope dilution was determined in a 15-min reaction of replicates containing 6.7×10^{-3} μc of [^{131}I]glucagon/μl of antiserum. [b] Abbott Laboratories, Lot E-1017-62-1 (specific activity 182 μc/μg). The amount of ^{131}I liberated from [^{131}I]glucagon–antibody complex prepared fresh daily containing 1.87×10^{-2} μc of [^{131}I]-glucagon/μl of antiserum in 1 ml of diluted antibody complex. [c] Same as ref *b* but represents an antibody complex prepared and stored at 5° containing 1.85×10^{-2} μc of [^{131}I]glucagon/μl of antiserum in 1 ml of diluted antibody complex.

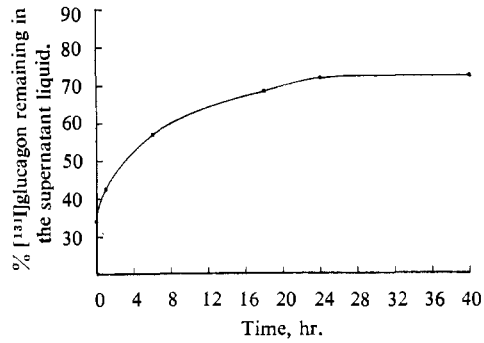

FIGURE 1: The displacement reaction of the [^{131}I]glucagon–antibody complex by unlabeled glucagon approached equilibrium after 24 hr with all concentrations of glucagon employed in the standard reference curve. The above curve was obtained with 0.5 μg/ml standard glucagon preparation.

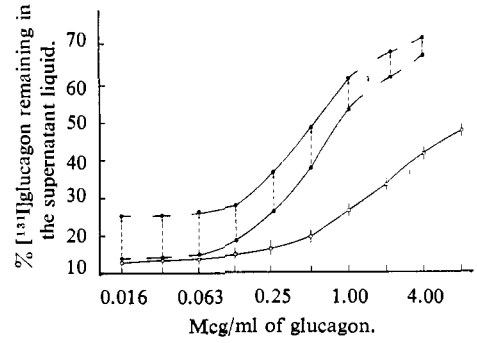

FIGURE 2: The different characteristics of standard reference curves obtained by the displacement of [^{131}I]glucagon from the labeled antibody complex with unlabeled glucagon are shown. Curve 1 (□) represents the mean values obtained by the displacement of labeled glucagon from a complex prepared and stored (5°) for 24 hr, then used over a 14-day period. Curves represented by 2 (○) shows the standard displacement reference curve obtained from an antibody complex prepared fresh daily. The gradual shift in the reference curve over a 2-week period is indicated by the arrows.

If the isolation yields only a specific [^{131}I]glucagon–antibody complex, the amount of free radioactivity released from the complex in the absence of unlabeled glucagon should be 0. In application, 14–24% of the radioactivity in the antibody complex, diluted for use in routine immunoassay, remains in the supernatant liquid as shown in Table I, column 2. This amount of the free radioactivity is probably due to dissociation of the antibody complex or incomplete precipitation of the complex with ammonium sulfate (Table I, columns 2 and 3). The [^{131}I]glucagon–antibody complex serves as a reagent in routine analysis and provides a means of delivering both the labeled antigen and antibody into the assay system together, eliminating a possible source of volumetric error.

Displacement of [^{131}I]Glucagon from Its Antibody Complex. The reaction of [^{131}I]glucagon with antiserum followed by simple ammonium sulfate fractionation distributes the radioactivity *ca.* 75% in the precipitated antibody complex and 25% in the supernatant liquid. A single precipitation and wash of the precipitated [^{131}I]-glucagon–antibody complex suffices for the preparation of a stable product for displacement studies. Although the distribution of radioactivity between the antibody complex and its corresponding supernatant liquid varies from lot to lot of [^{131}I]glucagon, the amount in the supernatant liquid always increases with the age of the labeled glucagon. This progressive loss of label reactivity reflects some modification of the [^{131}I]glucagon.

Figure 1 shows the displacement of [^{131}I]glucagon from the antibody complex with 0.5 μg/ml of glucagon as a function of time. The displacement reaction approaches equilibrium in 24 hr at room temperature. A 16-hr displacement time yields reproducible results for immunoassay and is used for convenience.

Variations Associated with the Displacement Reaction. The [^{131}I]glucagon–antibody complex can either be prepared and used immediately or stored and used as needed for the isotopic dating period. Standard response curves of [^{131}I]glucagon–antibody complex of both type preparations are shown in Figure 2. Curve 1 results from the use of [^{131}I]glucagon–antibody complex which has been stored at 5° for 24 hr or longer. Curve 2 represents the responses with antibody complex prepared and used immediately from [^{131}I]glucagon aged through a period of 1–14 days, respectively. These curves differ in slope and sensitivity, but either can be used satisfactorily in the conduct of the assay. Even though the standard curves obtained from freshly prepared complex, represented by curve 2, shift throughout a 14-day period, this is the preferred method for conducting routine glucagon immunoassays. The slope of the standard curve under these conditions approaches the ideal value of one providing the best sensitivity with maximum accuracy.

Curve 1 is reproducible over the useful life of the isotope revealing the stabilizing effect of antibody complex formation on labeled glucagon. If the [^{131}I]glucagon–antibody complex stored at 5° for 24 hr or more is treated with a saturating quantity of unlabeled glucagon (100 μg), only 55–60% of the radioactivity is displaced from the complex. About 30% of the [^{131}I]glucagon is irreversibly bound to the antibody. Under similar conditions, 85–90% of the radioactivity is displaced from freshly prepared [^{131}I]glucagon–antibody complex. These observations suggest that glucagon antiserum from rabbits contains antibodies having different affinity for glucagon which results in a new equilibrium mixture within 24 hr after preparation of the antibody complex. Similar characteristics of insulin antiserum from guinea pigs has been observed (Arquilla and Finn, 1963; Yagi *et al.*, 1962; Berson and Yalow, 1959). According to Merimee and Prout (1964), rabbit antiserum to beef glucagon contains two types of antibody, a γ globulin and a β-2A or β-2M globulin. The nature of

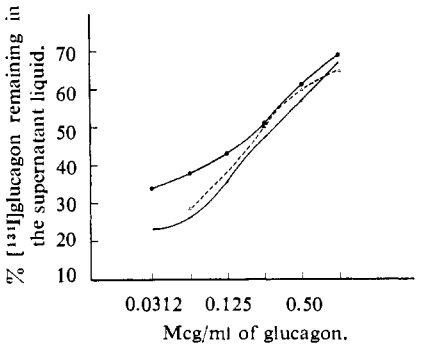

FIGURE 3: Antiserums obtained from rabbits (○) and guinea pigs (●) are compared by their respective standard curves. The concentration response curve (Δ) of glucagon source material is coincidental with that of crystalline reference glucagon determined with rabbit antiserum.

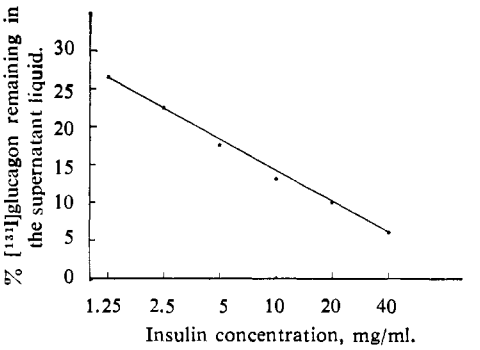

FIGURE 4: The effect of increasing insulin concentration on the [131I]glucagon–antibody complex prior to the displacement reaction indicates linear adsorption. Each replicate contained a 0.1 ml of insulin sample, 0.1 mg of unlabeled glucagon (0.25 μg/ml), 1.0 ml of [131I]glucagon–antibody complex, and 1.8 ml of diluted plasma as described. After combination, the mixture was immediately precipitated with 6.0 ml of 2.75 M $(NH_4)_2SO_4$.

the antiserum used in this study has not been established.

A Comparison of Glucagon Antisera from Rabbits and Guinea Pigs. Glucagon antiserum obtained from rabbits and guinea pigs exhibits similar displacement reactions as indicated in Figure 3. The affinity of glucagon for glucagon antibody permits the use of antiserum from either specie in the conduct of the [131I]glucagon displacement immunoassay.

The Effect of Interfering Substances on the Immunoassay. Interfering substances in some unknown samples markedly limits assay reliability as shown by insulin in sample concentrations above 1% (w/v). On the other hand, crude glucagon source material, obtained from a side fraction of the commercial insulin process, exhibits an identical concentration response curve with that of the crystalline glucagon standard when measured with rabbit antibody (Figure 3). The symmetry of the curves indicates the amount of interfering substance in glucagon source material is negligible.

In applying the glucagon displacement immunoassay to samples containing a variety of substances, which may or may not interfere with the immuno reaction, a two-point assay is a simple and useful means of recognizing interference. An unknown sample is appropriately diluted to two different concentrations within the range of the standard reference curve, *e.g.*, 0.25 and 0.50 μg/ml, respectively, and a plot of the two experimental values is compared to the standard reference curve. Valid results are obtained if the two assay values produce a slope which parallels the standard curve. In contrast, a sample yielding a two-point slope which deviates from the standard reference slope denotes interference and unreliable immunoassay results. Assay interference can be compensated within defined limits by appropriate controls.

In determining the glucagon content of crystalline zinc–insulin lots, assay interference is readily detected by the deviation of the slope produced by the two assay values from the slope produced with the reference standards. This interference is partially due to the coprecipitation of [131I]glucagon–antibody complex with increasing quantities of insulin as shown in Figure 4. The linear adsorption of radioactivity as a function of increasing insulin concentration suggests this system may serve as a control for correcting assay interference due to insulin.

The effect of increasing insulin concentration on the immunoassay of a constant concentration of glucagon (0.025 μg/replicate) is shown in Figure 5, curve 1. These data reveal that insulin interference is not a linear function of concentration under the conditions employed in the conduct of the immunoassay. The adsorption data (Figure 4) were applied as a corrective control to the data of curve 1, Figure 5, to compensate for insulin interference in the precipitation step. The corrected results are plotted in curve 2, Figure 5. The portion of curve 2 which exhibits a positive slope reflects the glucagon content of the insulin. The portion of the curve exhibiting a negative slope suggests that an independent interaction occurred during the displacement reaction. This apparent insulin–glucagon interaction competes with the immunological displacement reaction. By preventing unlabeled glucagon from displacing [131I]glucagon from the antibody complex, a type of interference is encountered which cannot be readily controlled. Extrapolation of the two slopes to an intersecting point reveals the maximum concentration of insulin (9 mg/ml) in a sample which can be corrected by the control. Therefore, the sensitivity for determining glucagon content of insulin is limited to 0.0025% by weight. The determination of trace amounts of glucagon in protein mixtures, such as plasma, serum, or in aqueous samples containing insulin above 9 mg/ml, requires special modifications in methodology not described in this report.

TABLE II: Comparison of Results by Bioassay and Immunoassay for Glucagon.

Sample Description	Bioassay[a] % Glucagon (w/w)	Test Sample Concn (mg/ml)	Immunoassay: Mean Result % w/w	Immunoassay: Mean Result μg/ml	Immunoassay: 95% Confidence Std Dev	Immunoassay: 95% Confidence Interval Range	Immunoassay: No. Assays
258-234B-167-1 cryst glucagon std	(P = 0.95) 100 by definition	1.0	114.2	1141.57	135.14	1055.70–1227.44	12
208-158B-197 50% glucagon prepn	32–48	2.0	54.0	1080.17	106.07	1012.77–1147.57	12
PJ-6289-RC cryst beef glucagon	Not fully tested (80–120)	1.0	100.7	1006.73	53.67	950.41–1063.05	6
PJ-6418 cryst pork glucagon	Not fully tested (100)	1.0	104.0	1039.72	102.55	932.11–1147.33	6
499667 cryst pork[b] zinc-insulin	0.002–0.0075	10.0	0.0034	0.343	0.0734	0.2905–0.3955	10
W-3255 amorphous beef insulin	2.0–3.26	10.0	2.24	224.08	11.13	216.12–232.04	10
723603 cryst pork zinc-insulin	0.33–0.60	10.0	0.25	25.06	3.02	22.9–27.22	10
Glucagon source Material	Bioassay not possible	34.6	0.61	210.47	28.07	192.64–228.30	12

[a] Bioassay data presented by courtesy of Dr. W. W. Bromer. [b] Pork insulin trypsin treated to reduce glucagon content.

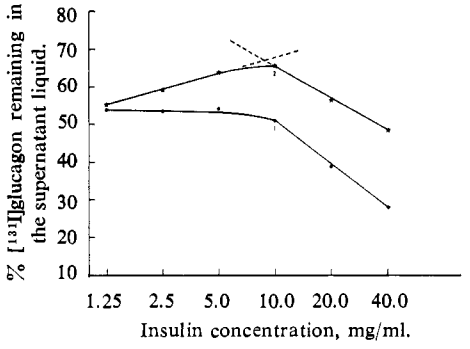

FIGURE 5: The effect of increasing insulin concentration on the precipitation of the [^{131}I]glucagon–antibody complex after the displacement reaction is shown in curve 1. Each replicate was prepared identically as described in Figure 4, then permitted to displace 16 hr prior to precipitation. The result obtained by correcting curve 1 with data from Figure 5 is shown in curve 2.

At concentrations of 1 mg/ml of sample, ACTH,[1] growth hormone, thyroid-stimulating hormone, parathyroid extract, and nonhormonal heparin failed to alter the standard glucagon response curve and confirms similar observations by Unger *et al.* (1961).

Comparative Assay Results. A series of glucagon samples of different purity and crystalline zinc-insulin samples containing variable amounts of glucagon were selected for comparing glucagon content by cat bioassay and [^{131}I]glucagon displacement immunoassay. A two-point assay system was employed and insulin interference was compensated by appropriate blanks as necessary. Table II provides evidence that the immunoassay for glucagon compares favorably with bioassay results. As a final criterion of potency, the biological response of the hormone is requisite, but the glucagon displacement immunoassay adequately fulfills the requirements for routine control of development and manufacturing processes.

This report provides a functional method for glucagon immunoassay based on the formation of a [^{131}I]glucagon–antibody complex and subsequent displacement of the label as an index of glucagon concentration. Immunoassay by direct competitive isotope dilution methods, commonly used for other protein and peptide hormones, was unsatisfactory for glucagon analysis. Disk electrophoresis studies reveal the glucagon immunoassay displacement system to have certain unique properties which will be reported separately. These new studies show that all lots of labeled glucagon contain several labeled components, of which three react with the antiserum. Successful conduct of the immunoassay is dependent on obtaining labeled glucagon(s) which suitably bind to the antibody. Forming the labeled antibody complex takes advantage of immunological

[1] Abbreviations used: ACTH, adrenocorticotropic hormone.

specificity in selecting the proper binding components from the mixed labeled antigen. Specifications for labeled glucagon based on the disk electrophoretic pattern have been established. [^{131}I]Glucagon supplied by Abbott Laboratories consistently meets the desired specifications. In addition to a multiple component labeled antigen, the antiserum contains two antibodies which show different avidity for the antigen. Hence, the displacement reaction is more involved than that which would be predicted by simple mass action calculation. This does not invalidate the usefulness of the method for the quantitative determination of glucagon within the limits defined. The principle of the displacement immunoassay offers application in the immunochemical manipulation and measurement of enzymes, other proteins, and polypeptides of biological importance. Isotopically labeled antigens can be selectively isolated from reaction mixtures and stabilized by antibody complex formation. The latter can then serve as a useful reagent for analysis under a variety of circumstances.

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