Immunoassay for Extracted Insulin

By W. E. BAUM*, W. F. BROWN, and R. E. CRABTREE

A rapid, accurate, and reproducible assay has been developed for commercial insulin. The assay is based on the competition between the insulin sample to be analyzed and I^{131}-labeled insulin for binding to guinea pig anti-insulin antibody. Antibody bound insulin is separated from free insulin by the salt precipitation technique of Grodsky and Forsham. This procedure is described in detail together with the adjustment of variables that must be made. Examples are given to show the influence of the purity of the samples, species differences, statistical variation, and specificity of the assay. Comparisons with the mouse convulsion and U.S.P. rabbit assays show that this immunological approach is valid and has many advantages over the *in vivo* assays.

FOR MANY YEARS a method has been sought to supplant the small animal assays used to control the development and production of insulin. The requirements for a new assay were (*a*) that it not be subject to the biological variations exhibited in live animals, (*b*) that it yield results in a few hours, (*c*) that it be relatively inexpensive to operate, and (*d*) that it be sensitive to hormonal activity in preparations having specific activities from 0.5 to 26.0 u./mg. and yet not be too sensitive to operate in areas where insulin contamination is a possibility.

The introduction of the immunochemical assay of Grodsky and Forsham (1), which uses salt precipitation of antibody bound insulin-I^{131}, provided for the first time a procedure which met these requirements.

The present study was directed at determining the capability of the assay with regard to the number of samples that could be assayed, its ability to assay low purity samples, its specificity for hormonally active protein, its statistical reproducibility, and its ability to assay insulin from various species. In the process of the investigation, data were collected on technique and the effect of possible chemical interference.

We have made several studies comparing the immunochemical assay per Grodsky (1) with the mouse convulsion assay (2) and with the "twin-crossover" rabbit blood-sugar-lowering assay (3).

Though there are other aspects to investigate, it is our belief that this flexible and reliable assay procedure should be made available to others interested in the assay of insulin.

MATERIALS AND METHODS

Insulin Antibody Production.—A modification of the procedure of Morse (4) is used to prepare insulin in adjuvant. Crystalline zinc-insulin is dissolved in 37.0 ml. of insulin diluent (3) at pH 3.2. This solution is then mixed with 25.0 Gm. of heavy liquid petrolatum and 12.5 Gm. of Aquavor (Dukes Laboratories). The mixture is homogenized in a Virtis 45 homogenizer. Enough insulin is used to make a final concentration of about 0.5 mg./ml.

Young guinea pigs (250 Gm.) of either sex are given subcutaneous injections of 0.5 ml. of insulin in adjuvant. The injections are repeated 30 and 270 days later and 10 days before each blood collection. After each injection, the animals are observed for hypoglycemic convulsions for a 7-hour period. Convulsions are treated with 1 ml. of 50% (w/v) dextrose (subcutaneous injection preferably) as soon as the hypoglycemic response is recognized. Blood is withdrawn *via* heart puncture, and after coagulation in an ice bath, is centrifuged at 15°. Serum is held at −20°.

Pooled batches of serum are evaluated in the adjustment procedure below and diluted to the titer indicated with 5% albumin stock solution. Aliquots of the diluted antibody, sufficient for each half day of operation, are placed in plastic bottles and kept frozen until needed.

Standard Reagents.—All standard insulins used were compared in the rabbit bioassay (3) against U.S.P. zinc-insulin crystals reference standard "G." The radioactive insulin was obtained from Abbott Laboratories.[1] This form of iodo-insulin had full potency when tested in the mouse-convulsion test. This labeled material is diluted in 5% (w/v) human albumin stock solution; sufficient nonradioactive insulin is added to make a concentration of 100 microunits (μu.) and 0.04 μc. in 20 μl. of the iodo-insulin stock solution. For preparation of the albumin stock solution, a 25% (w/v) commercial solution of human serum albumin (Cutter Laboratories) was diluted to 5% with a 0.9% (w/v) NaCl solution containing 0.2% (w/v) phenol. The pH was adjusted to 8.5 with 40% (w/v) NaOH. This solution was stored at 5°. The 30% (w/v) urea solution in 5% albumin, as used by Grodsky, is always made daily. An ammonia-free reagent grade glycine is required for 0.2 *M* glycine solution in 5% albumin.

Inactivated Human Plasma.—Several bags of blood bank plasma (relatively free of hemoglobin) are pooled, centrifuged in the cold to remove residual blood cells, adjusted to pH 10.5 with 40% NaOH, and stored 24 hours at 5°. Following this aging, the pH is lowered to 7.4 with 10% HCl, the pooled

Received July 29, 1963, from the Insulin Testing Department, Statistical Research Department and Chemical Research Division, Eli Lilly and Co., Indianapolis, Ind.
Accepted for publication October 22, 1963.
The authors thank Mr. J. P. Ohlrogge, Mr. R. W. Colwell, and Mr. J. G. Williams, Jr., for their technical assistance.
* Present address: Lincoln Laboratories, Inc., Decatur, Ill.

[1] Marketed as Iodo-Insulin Sterile-I^{131}.

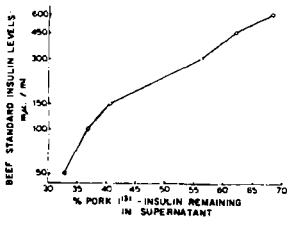

Fig. 1.—An extended standard curve to indicate the acceptable operating range, 150, 300, and 450 mu./ml., selected for one of our adjusted systems.

plasma is centrifuged in the cold, and the supernatant decanted from a slimy precipitate. After experimental determination of the dilution strength needed in the assay, the pooled plasma is divided into aliquots sufficient for each half day of operation and frozen until needed. Dilutions in this plasma are made immediately before use.

Assay System Adjustment.—Inactivated plasma and the antibody serum are diluted to concentrations of 1:3, 1:5, and 1:8 with 5% albumin stock solution. Beef insulin in insulin diluent (3) is diluted with 5% human albumin stock solution to 50, 100, and 150 milliunits (mu.)/ml. concentrations for use as a reference standard. A 27.32% (w/v) sodium sulfite solution in H_2O is prepared which will give an 18.5% (w/v) salt concentration after addition to the reaction mixture.

Four standard levels (one tube each of 0, 50, 100, and 150 mu.) are run with each possible combination of the serum and plasma dilutions. The set of conditions which gives the largest difference in supernatant radioactivity between the 50- and 150-mu. levels, while maintaining a low value for the 0-mu. level, is selected. In our hands, this difference is about 19%, and the low value for 0 mu. is about 20% of the initial radioactivity in the supernatant. A curve, as shown in Fig. 1, is plotted to show the linearity and the slope at these standard points.

With some antibody pools, pork and human insulins require 75, 150, and 225 mu./ml. standard levels to duplicate the competitive binding affinity of beef insulin for the same antibody. Assay system adjustment is usually required each time the albumin, serum, or plasma is changed.

Sample Preparation.—Micro dilutions of highly

TABLE I.—STATISTICAL DESIGN AND GENERALIZED FORMULAS FOR AN n POINT STANDARD CURVE AND m UNKNOWNS WITH K REPLICATION IN THE INSULIN IMMUNOASSAY

	Standards						Unknowns					
Concn.	Recovery						Potency	Recovery				
c_1	$S_{1,1}$	$S_{1,2}$	...	$S_{1,k}$	...	$S_{1,K}$	p_1	$U_{1,1}$	$U_{1,2}$	...	$U_{1,k}$	... $U_{1,K}$
.	.	.	.	.	.	.	.	.	.	.	.	. .
.	.	.	.	.	.	.	.	.	.	.	.	. .
.	.	.	.	.	.	.	.	.	.	.	.	. .
c_i	$S_{i,1}$	$S_{i,2}$	...	$S_{i,k}$	...	$S_{i,K}$	.	.	.	.	.	. .
							.	.	.	.	.	. .
							.	.	.	.	.	. .
c_{i+1}	$S_{i+1,1}$	$S_{i+1,2}$	...	$S_{i+1,k}$	...	$S_{i+1,K}$	.	.	.	.	.	. .
							.	.	.	.	.	. .
.	.	.	.	.	.	.	.	.	.	.	.	. .
.	.	.	.	.	.	.	p_j	$U_{j,1}$	$U_{j,2}$	...	$U_{j,k}$	... $U_{j,K}$
.	.	.	.	.	.	.	.	.	.	.	.	. .
c_n	$S_{n,1}$	$S_{n,2}$	...	$S_{n,k}$	...	$S_{n,K}$	.	.	.	.	.	. .
							.	.	.	.	.	. .
							p_m	$U_{m,1}$	$U_{m,2}$	...	$U_{m,K}$	... $U_{m,K}$

Situation	Formulae
$\bar{S}_{i-1} < \bar{U}_j < \bar{S}_i$	$\ln p_j = \ln c_i + \left[\frac{\bar{S}_i - \bar{U}_j}{\bar{S}_i - \bar{S}_{i-1}}\right] \ln (c_{i-1}/c_i)$
when $(1 < i \leq n)$	$\text{var}(\ln p_j) = \frac{[\ln (c_{i-1}/c_i)]^2}{(\bar{S}_i - \bar{S}_{i-1})^4} \{(\bar{S}_i - \bar{U}_j)^2 \text{ var } \bar{S}_{i-1} + (\bar{U}_j - \bar{S}_{i-1})^2 \text{ var } \bar{S}_i + (\bar{S}_i - \bar{S}_{i-1})^2 \text{ var } \bar{U}_j\}$
$\bar{U}_j = \bar{S}_i$	$\ln p_j = \ln c_i$
when $(1 < i < n)$	$\text{var}(\ln p_j) = [\ln (c_{i-1}/c_i)]^2 \left[\frac{1}{(\bar{S}_i - \bar{S}_{i-1})^2} + \frac{1}{(\bar{S}_{i+1} - \bar{S}_i)^2}\right] \text{var } \bar{S}_i$

where:

$$\bar{S}_i = \frac{1}{K} \sum_{k=1}^{K} S_{i,k} \qquad \bar{U}_j = \frac{1}{K} \sum_{k=1}^{K} U_{j,k}$$

$$\text{var } \bar{S}_i = \frac{K \sum_{k=1}^{K} (S_{i,k})^2 - \left(\sum_{k=1}^{K} S_{i,k}\right)^2}{K^2(K-1)}$$

$$\text{var } \bar{U}_j = \frac{K \sum_{k=1}^{K} (\bar{U}_{j,k})^2 - \left(\sum_{k=1}^{K} U_{i,k}\right)^2}{K^2(K-1)}$$

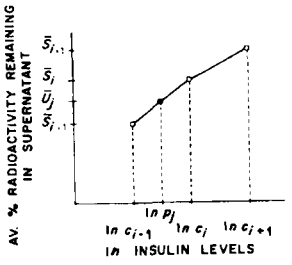

Fig. 2.—A sample graph to illustrate calculation of immunoassays for extracted insulin. Key: $\bar{U}_j$ = average response of the jth sample; $\bar{S}_i$ = average standard response at the ith dilution; p_j = insulin concentration of the jth sample; c_i = insulin concentration at the ith dilution.

concentrated insulin solutions are made with insulin diluent to about 50 u./ml., and the final dilution step is always made using 5% albumin stock solution. After final dilution, the samples are allowed to stand overnight at 5°.

Assay Procedure.—Six replicate tubes are run on each sample. Two patterns are used: eight samples per day in triplicate with two sets of 36 tubes. Fourteen samples per day can be run in duplicate with three sets of 36 tubes (only one "zero" tube is used with this pattern).

All glassware is cleaned with detergent, rinsed in 2% (w/v) HCl, water-rinsed, and oven-dried before use. A primary solution containing 38.0 ml. of 30% urea and 38.0 ml. of 0.2 *M* glycine solution is prepared for a set of 36 tubes. After removal of 2.04 ml. of the primary solution for a blank tube, the diluted antibody serum is added. The pH of this primary solution is adjusted to 8.5 with 10% NaOH, and the solution is stirred until it reaches room temperature. Twenty microliter-volumes of the diluted I^{131} insulin are measured into glass test tubes using a $^1/_4$-ml. syringe microburet (Micrometric Instruments Co.). The microburet, with a syringe for each dilution, is then used to measure 20 µl. aliquots of each of the standard dilutions and of the samples into their appropriate test tubes. Two zero tubes (no unlabeled insulin added) and one blank tube (no unlabeled insulin or antibody added) are included in each set with the eight-sample-per-day pattern. One control tube is made containing 20 µl. of the radioactive insulin stock solution. The volume differentials in the blank, zero, and control tubes are corrected with 5% albumin stock solution. Next, 2.04 ml. of the primary solution with antibody is rapidly injected sequentially into all the tubes except the blank. Glass rods are inserted in the tubes, and the set of tubes is placed on a rotating shaker (A. H. Thomas No. 3623) for 1 hour of gentle agitation at 24°. At the end of 1 hour, 0.8 ml. of the diluted inactivated plasma is injected sequentially into each of the tubes. Following the addition of plasma and a 15-minute agitation period on the rotating shaker, 6.0 ml. of 27.32% Na_2SO_3 is added to each of the tubes sequentially. This mixture is agitated on the rotary shaker for 1 hour at 24°. After the glass rods are removed, the tubes are centrifuged in a refrigerated

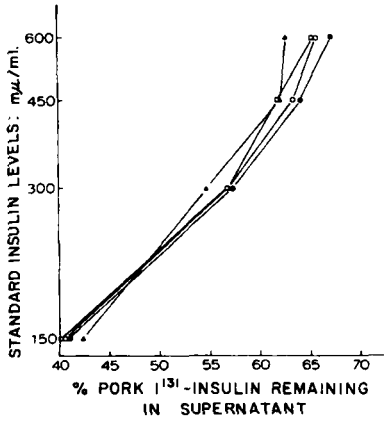

Fig. 3.—Responses of an antibody pool to insulins from different species. Rabbit assays (P = 0.95) on the insulins used are: W-3776, solution of U.S.P. reference standard at 24.0 u./mg.; 493-10GP-182—20.80 (22.63 to 19.12) u./mg.; ML 26—24.78 (26.01 to 23.61) u./mg.; PJ-5158—22.84 (24.75 to 21.07) u./mg. Key: ●—●, U.S.P. insulin, W3776; ▲—▲, human insulin, 493-10GP-182; ○—○, beef insulin, ML 26; and □—□, pork insulin, PJ5158.

TABLE II.—A SCHEMATIC INSULIN PURIFICATION PROCEDURE WITH THE IMMUNOASSAY RESULTS ON ONE LOT EXPRESSED AS A PER CENT OF TOTAL INITIAL PANCREAS EXTRACT ACTIVITY AT EACH ASSAY POINT

Active Portion	Discarded Portion
Pancreas Extract 100%	Meat Residue
↓	
Salt Precipitate 97.3%	Salt Filtrate 1.96%
↓	
1st Isoelectric Point Precipitate 82.8%	1st Isoelectric Point Filtrate 1.19%
↓	
2nd Isoelectric Point Precipitate 102.5%	2nd Isoelectric Point Filtrate 2.52%
↓	
3rd Isoelectric Point Precipitate 98.9%	3rd Isoelectric Point Filtrate (not assayed)
↓	
Zinc-Insulin Crystals 92.58%	
Amorphous Fraction 5.53%	
Soluble Insulin 2.03%	

TABLE III.—EFFECT OF PH AND VARIOUS IONS USED IN INSULIN PURIFICATION ON IMMUNOASSAY RESULTS[a]

Salt Soln.	Initial Biological Activity, %			
	pH 6.5 Soln.		pH 3.0 Soln.	
	Pork Insulin[b]	Beef Insulin[c]	Pork Insulin[b]	Beef Insulin[c]
0.5 *M* NH_4Cl	53.48(54.94–52.06)	38.80[e]	99.65(109.68–90.52)	114.41(118.68–110.28)
0.5 *M* $NH_4C_2H_3O_2$[d]	51.94(56.30–47.90)	41.40[e]	101.92(111.06–93.52)	113.21(122.60–104.52)
0.5 *M* $NaC_2H_3O_2$[d]	54.69(59.68–50.10)	40.60[e]	91.27(97.46–85.46)	110.43(117.94–103.38)
0.02 *M* Na_2HPO_4	56.66(58.98–54.42)	45.70[e]	107.41(112.76–103.02)	107.71(112.28–103.32)
0.05 *M* $Na_3C_6H_5O_7$	59.37(63.02–55.92)	48.90[e]	99.61(104.38–95.04)	104.60(115.44–94.76)
0.05 *M* $(NH_4)_2HC_6H_5O_7$	66.93(70.62–63.42)	74.46(78.70–70.44)	105.66(118.92–93.88)	110.94(120.94–101.76)
0.02 m*M* $ZnCl_2$	93.52(101.30–86.34)	95.38(102.88–88.42)	96.10(102.68–89.92)	101.04(109.04–93.62)
0.3 *M* NaCl	46.00[e]	85.07(88.96–81.34)	101.31(114.20–89.86)	106.13(112.50–100.10)

[a] Standard insulin solutions, 50 u./ml., were diluted to 5 u./ml. with the salt solution, followed by pH adjustment prior to final dilution with albumin stock solution. [b] Pork insulin lot was PJ-5158 (C-2598-9) which assayed 26.99 u./mg. (±7.87% $p = 0.95$) in 96 rabbits. [c] Beef insulin was ML 26 which assayed 24.8 u./mg. (±4.85% $p = 0.95$) in 192 rabbits. [d] pH adjustment was with the respective acid or base in all solutions, except those where H_2SO_4 was used. [e] Statistics used do not provide limits on values obtained by extrapolation from the standard curve which only extends from 50 to 150% of estimate.

angle head centrifuge at 15° at a relative centrifugal force of 2400 × g. for 45 minutes. Following centrifugation, 5 ml. of supernatant is carefully removed from each tube and placed in a plastic tube for counting.

Radioactivity Measurement.—A control tube is made up for counting with each set of samples containing 5 ml. of the diluted iodo-insulin solution (20 μl. diluted to 8.86 ml. with 5% albumin stock solution). Due to the short half-life of I^{131} the control count must be corrected for decay when calculating the result on each sample tube. Sets of samples are conveniently counted overnight in an automatic gamma spectrometer (Packard Instruments Co.) for the peak emission of I^{131}. A thallium activated sodium iodide crystal is used in the spectrometer. The usual counting time is 5 minutes per sample.

Calculation of Potency.—The statistical design of the assay procedure is presented in Table I. Total counts are corrected for backgrounds and reduced to counts per minute. The tube count is then divided by the control count (corrected for decay during counting) to obtain the per cent radioactivity remaining in the supernatant, S_i or U_j. A three point standard curve may be plotted on semilog graph paper as shown in Fig. 2. Sample potency can be estimated from this graph or calculated using Eqs. 1 or 2. With a point-to-point linear assumption (when $S_1 < \ldots < S_i < \ldots < S_n$), any one of $2n - 1$ situations may be encountered. For an n point standard curve with K sample replication, the generalized formulas are: when $\bar{S}_{i-1} < \bar{U}_j < \bar{S}_i \; (1 < i \leq n)$

$$\ln p_j = \ln c_i + \left[\frac{\bar{S}_i - \bar{U}_j}{\bar{S}_i - \bar{S}_{i-1}}\right] \ln (c_{i-1}/c_i) \quad \text{(Eq. 1)}$$

where

$$\bar{S}_i = 1/K \sum_{k=1}^{K} S_{ik} \text{ and } \bar{U}_j = 1/K \sum_{k=1}^{K} U_{jk}.$$

When $\bar{U}_j = \bar{S}_i \; (1 \leq i \leq n)$,

$$\ln p_j = \ln c_i \quad \text{(Eq. 2)}$$

Variance of Sample Potency.—The variance of the ln p_j may be estimated by the formula for the propagation of error (5). This solution becomes asymptotic as the value of K increases. For the situation $\bar{S}_{i-1} < \bar{U}_j < \bar{S}_i \; (1 < i \leq n)$

$$\text{var } (\ln p_j) = [\ln (c_{i-1}/c_i)]^2 \text{ var } \left[\frac{\bar{S}_i - \bar{U}_j}{\bar{S}_i - \bar{S}_{i-1}}\right]$$

Let

$$y = \frac{\bar{S}_i - \bar{U}_j}{\bar{S}_i - \bar{S}_{i-1}}$$

then

$$\text{var } (\ln p_j) = [\ln (c_{i-1}/c_i)]^2 \left\{\left(\frac{\partial y}{\partial \bar{S}_{i-1}}\right)^2 \text{var } \bar{S}_{i-1} + \left(\frac{\partial y}{\partial \bar{S}_i}\right)^2 \text{var } \bar{S}_i + \left(\frac{\partial y}{\partial \bar{U}_j}\right)^2 \text{var } \bar{U}_j\right\}$$

By the usual methods of partial differentiation and variance determination of an independent variable

$$\text{var } \bar{S}_i = \frac{K \sum_{k=1}^{K} (S_{ik})^2 - \left(\sum_{k=1}^{K} S_{ik}\right)^2}{K^2(K-1)}$$

TABLE IV.—REPLICATE IMMUNOASSAYS ON DIFFERENT DAYS ON SAMPLES OF DIFFERENT SPECIFIC ACTIVITIES

Lot No.	Species	Specific Activity, u./mg.	Anticipated Potency Found ($p = 0.95$), % Day 1	Day 2	Day 3	Day 4	Day 5	Weighted Mean
813527-31	Beef	0.16	139.6(147.8–128.3)[a]	142.9(191.3–104.3)[b]	139.6(158.7–121.7)[a]	140.8(147.83–132.61)	129.1(137.0–120.0)	137.3
813527-31	Beef	11.47	110.1(121.1–100.1)	107.4(116.4–99.1)	103.0(107.9–97.4)	106.6(113.83–99.87)	101.0(105.8–96.6)	105.6
813527-31	Beef	24.22	115.1(127.4–104.3)	96.8(104.6–89.7)	105.4(113.2–98.3)	110.0(118.5–102.2)	95.0(101.6–88.9)	104.2
815271-5	Pork	0.08	93.76(98.7–88.3)	102.6(113.3–90.0)	97.5(110.0–83.3)[a]	87.0(92.7–81.3)	93.1(101.0–85.3)	94.6
815271-5	Pork	0.92	86.4(106.0–70.0)	75.2(80.4–70.3)[a]	70.5(77.6–63.9)[a]	75.2(83.2–67.9)	69.5(75.5–64.1)	75.1
815271-5	Pork	8.02	92.6(98.0–87.5)	99.9(107.6–92.8)	103.7(111.9–96.2)	98.0(106.8–90.0)	92.1(101.4–83.6)[a]	97.2
815271-5	Pork	15.26	99.1(106.3–92.4)	113.8(122.1–106.1)	99.7(110.6–90.1)	102.4(108.7–96.5)	94.5(100.7–88.6)	101.7
815271-5	Pork	17.55	105.8(115.7–96.8)	103.3(115.3–92.6)	94.0(101.6–87.5)	98.9(107.8–90.8)	93.8(100.5–87.5)	99.0
815271-5	Pork	20.42	106.2(113.0–99.9)	99.5(108.3–91.4)	94.3(104.5–85.3)	101.2(107.4–95.4)	92.7(96.6–88.9)	98.7

[a] Two of the six-tube results rejected as aberrant values. [b] Four of the six-tube results rejected as aberrant values.

$$\text{var } \bar{U}_j = \frac{K \sum_{k=1}^{K} (\bar{U}_{jk})^2 - \left(\sum_{k=1}^{K} \bar{U}_{jk}\right)^2}{K^2(K-1)}$$

the variance of the ln pj may be written as

$$\text{var } (\ln p_j) = \frac{[\ln (c_{i-1}/c_i)]^2}{(\bar{S}_i - \bar{S}_{i-1})^4} \{(\bar{S}_i - \bar{U}_j)^2 \text{ var } \bar{S}_{i-1} + (\bar{U}_j - \bar{S}_{i-1})^2 \text{ var } \bar{S}_i + (\bar{S}_i - \bar{S}_{i-1})^2 \text{ var } \bar{U}_j\} \quad \text{(Eq. 3)}$$

The variance, when ln p_j = ln c_i, must be estimated from the limits of Eq. 3 as $\bar{U}_j$ approaches $\bar{S}_i$ from both a positive and negative direction. By averaging (when $1 \leq i \leq n$)

$$\text{var } (\ln p_j) = [\ln (c_{i-1}/c_i)]^2 \left[\frac{1}{(\bar{S}_i - \bar{S}_{i-1})^2} + \frac{1}{(\bar{S}_{i+1} - \bar{S}_i)^2}\right] \text{var } \bar{S}_i \quad \text{(Eq. 4)}$$

Reporting of Assay Results.—The logs of the results from each set of tubes are averaged. The antilog of this average coupled with the 95% confidence interval values is reported as the assayed potency of the sample dilution. A digital computer (IBM 1620) program is available to perform the necessary calculations.

RESULTS

Species Similarities in Competitive Insulin Binding Situations.—The data in Fig. 3 demonstrate the similarity in binding affinity for human, beef, and pork insulins, and U.S.P. reference standard in-

TABLE V.—COMPARISON OF PORK AND BEEF IODO-INSULIN IN LIKE ASSAY SYSTEMS[a]

Tube Description	I^{131} Remaining in the Supernatant, % Beef Iodo-Insulin A.M.	Beef Iodo-Insulin P.M.	Pork Iodo-Insulin A.M.	Pork Iodo-Insulin P.M.
Blank	82.53	85.12	84.10	78.86
Zero	22.35	21.71	21.90	19.15
50 mu./ml. W-3743 beef[b] standard	32.53	33.60	39.45	38.80
100 mu./ml. W-3743 beef[b] standard	49.13	49.78	55.98	54.38
150 mu./ml. W-3743 beef[b] standard	56.07	55.92	60.62	60.01
50 mu./ml. U.S.P. standard	29.87	31.05	38.21	37.29
100 mu./ml. U.S.P. standard	45.89	45.74	55.76	52.86
150 mu./ml. U.S.P. standard	51.71	50.66	57.50	58.95
100 mu./ml. W-3752 acidified U.S.P. protamine zinc insulin	44.97	45.40	51.72	52.73
100 mu./ml. W-3751 acidified beef NPH insulin	49.25	48.96	55.03	55.56
100 mu./ml. W-3750 acidified beef protamine zinc insulin	48.64	49.64	54.60	54.82
100 mu./ml. W-3751 supernatant	21.23	21.16	20.08	19.39
100 mu./ml. W-3750 supernatant	21.07	20.62	19.65	19.40

[a] Three-tube averages except on blank and zero. [b] Beef insulin used in W-3743, W-3750, and W-3751 was from ML 795372 which assayed 25.54 u./mg. in the U.S.P. rabbit test for insulin (368 rabbits).

TABLE VI.—ASSAYS OF PROTAMINE-INSULIN MIXTURES[a]

Insulin Soln.	Estimated Potency, u./ml.	Estimated Potency Found, % Iodo-Insulin Beef	Iodo-Insulin Pork
W-3752, U.S.P. protamine zinc insulin[b]	40.0	83.46(87.05–80.00)[d]	88.17(92.05–84.43)[d]
W-3751, Beef NPH insulin[b]	40.0	98.60(106.28–91.48)	102.31(108.48–96.48)
W-3750, Beef protamine zinc insulin[b]	40.0	98.68(103.23–94.33)	98.71(104.95–92.83)
W-3751, NPH supernatant[b]	1.0	29.60[c]	21.60[c]
W-3750, P.Z.I. supernatant[e]	1.0	29.80[c]	21.80[c]

[a] Results are calculated against the recoveries shown in Table V for beef standards. [b] Acidified with 30 μl. concentrated HCl/10 ml. of mixture for solution prior to dilution with albumin stock solution. [c] Values were extrapolated from standard curve; confidence limits not calculated. [d] Antibody pool used was demonstrating species specific properties; thus the pork insulin content of the U.S.P. reference standard insulin causes low results. [e] The result on this sample from a 24-rabbit U.S.P. assay was 0.09 (0.195–0.013) u./ml.

sulin at various dilution levels. Each of these samples has been carefully tested on the U.S.P. assay for insulin in rabbits (3). The actual rabbit assay results are given in the caption under Fig. 3. When antibody pools demonstrate differential competitive binding, it can be circumvented by either using the same species on both the standard and sample or by appropriate mixing of the standards from different species. Thus, reliable assay results can be obtained for an insulin solution of known species composition.

Total Competitive Binding Curve.—Figure 1 gives a typical total competitive binding curve which is shown to illustrate the portion selected for the performing of assays.

Assay of Low Purity Samples.—Low purity samples from the manufacturing process are normally not tested in rabbits because of the toxic effects caused by the impurities present. Table II illustrates an experiment to show that immunoassay results on low purity samples are within the range of expectation. Both the beef and pork insulin manufacturing processes were sampled at various stages of purification and material balance figures computed from the immunoassay results.

Acidification Before Dilution.—In an experiment to test for ionic interference with the immunoassay results, the apparent interference caused by some ions could be nullified by acidification of the sample prior to dilution in the 5% albumin stock solution. Table III illustrates the differences found with pH 6.5 and pH 3.0 solutions in the presence of various ions.

Day-to-Day Replications.—Replicate samples of two different production lots of insulin were prepared and frozen. A fresh sample was removed and assayed each day for a period of 5 days. The data in Table IV show the characteristic variations in results.

TABLE VII.—SAMPLES OF CRYSTALLINE INSULIN DILUTED TO ABOUT 80 u./ml. IN INSULIN DILUENT AT pH 3.0[a]

Boiling Time, Hr.	Immunoassay Results, u./ml. Beef Insulin	Pork Insulin
0	77.9 (87.4–69.3)[b]	75.2 (94.5–59.9)[c]
1	62.2 (71.8–53.9)	47.1 (56.5–39.3)
2	48.0 (52.2–44.1)	48.8 (59.5–40.0)
3	46.5 (53.6–40.4)	39.8 (47.5–33.4)
4	41.6 (46.7–37.0)	35.2 (42.1–29.4)
5	37.8 (42.4–33.7)	29.5 (37.2–23.5)
6	32.2 (35.7–29.1)[d]	32.5 (38.7–27.3)[e]

[a] Solutions gently refluxed at 100°C. [b] U.S.P. rabbit assay in 48 rabbits on pooled lots C2730, 1, 3, and 4 showed this to be 76.58 (93.89 to 62.46) u./ml. [c] U.S.P. rabbit assay in 240 rabbits on lot C2732X showed this to be 76.48 (80.80 to 72.38) u./ml. [d] U.S.P. rabbit assay in 96 rabbits showed this to be 23.11 (25.60 to 20.87) u./ml. [e] U.S.P. rabbit assay in 96 rabbits showed this to be 27.48 (30.38 to 24.86) u./ml.

Comparison of Pork and Beef I^{131} Labeled Insulin.—Duplicate assays on known standard material were run first using beef I^{131} insulin and then pork I^{131} insulin. The comparative results are shown in Table V.

Specificity for Biologically Active Insulin.—To demonstrate that the antibodies were specific for biologically active insulin and also to demonstrate that the assay could be used with long-acting forms of insulin, several experiments were seen to show this selective action. Table VI shows the final results obtained with acidified NPH and protamine zinc insulins run against their respective solutions of insulin crystals in insulin diluent (computed from the data in Table V). Table VII gives the results when solutions of insulin (either beef or pork) are boiled in acid solution. The data in Table VIII show a comparison between mouse, rabbit, and immunoassay. These data also illustrate the sensitivity of the immunoassay for that insulin which has not been attacked by trypsin. The mouse and rabbit data were previously published in Table I of (6).

Comparative Assay Results.—Table IX shows a collection of routine assays performed on the samples with both the U.S.P. rabbit assay (3) and this immunological technique.

Influence of Ethanol.—Table X shows how small amounts of ethanol present before dilution with albumin diluent affect the antibody.

DISCUSSION

Some of our antibody pools have demonstrated a species specificity such as indicated by Berson (7). Carefully standardized lots of different insulin would give curves, plotted as in Fig. 3, having different slopes. When differential binding is

TABLE VIII.—TRYPSIN HYDROLYZED BEEF INSULIN SAMPLES[a]

Hydrolysis Time, Min.	Biological Activity, u./mg. Immunoassay[b]	Mouse	Rabbit
0	22	23	25
5	17	17	18
11	15	14	...
19.5	12	9	10
40	4.6	3.4	...
80	1.8	0.7	...
160	0.8	...	0.3

[a] All hydrolyzed samples are presumed to contain insulin, desoctapeptide insulin, alanine, heptapeptide, trypsin, and soybean trypsin inhibitor. [b] These were three-tube assays.

TABLE IX.—IMMUNOASSAYS AND U.S.P. RABBIT ASSAYS ON SOME UNUSUAL AND ROUTINE SAMPLES

Lot and Description	Immunoassay Potency, u./ml.	Immunoassay Confidence Limits (p = 0.95), u./ml.	U.S.P. Rabbit Assay Potency, u./ml.	U.S.P. Rabbit Assay Confidence Limits (p = 0.95), u./ml.	No. Rabbits
Dealaninated[a] pork insulin CT-1530A	118.5	(128.4–109.4)	107.38	(116.50–98.97)	96
Human insulin 493-10GP-182	155.7	(162.9–148.7)	123.10	(133.93–113.16)	72
Acidified beef protamine zinc insulin					
190-14GP-21A	18.1	(19.3–17.1)	20.50	(25.02–17.12)	24
190-14GP-21B	9.2	(11.3–7.5)	11.18	(13.57–9.46)	24
Pork and beef insulin					
W-3775	37.9	(40.6–35.3)	40.69	(43.15–38.37)	240
W-3786	41.0	(45.1–37.2)	40.07	(42.41–37.86)	192
Beef insulin					
M.L. 31	36.0	(36.7–35.3)	39.47	(42.72–36.47)	96
M.L. 32	38.6	(39.5–37.6)	40.96	(44.43–37.76)	96
C-2695-96	41.7	(42.5–40.8)	36.72	(42.12–32.01)	48
C-2701 + 2706	38.2	(41.7–35.1)	41.79	(50.33–34.70)	48
C-2710-11 + 13-14	39.6	(43.3–36.2)	41.09	(46.06–36.65)	48
PJ-5504	41.0	(42.4–39.7)	42.82	(49.56–37.00)	96
Pork insulin					
C-2712 + 2715	39.1	(41.3–37.1)	37.70	(43.04–33.02)	48
C-2716-17	39.1	(41.2–37.0)	36.84	(40.54–33.48)	48
C-2732X	40.1	(43.3–37.2)	37.48	(39.60–35.47)	240
C-2730-31-33-34	40.2	(41.7–38.7)	36.87	(40.60–33.48)	96

[a] Dealaninated insulin, porcine insulin less the C-terminal alanine on the B-chain.

TABLE X.—IMMUNOASSAY TOLERANCE FOR ETHANOL IN SAMPLES FOR ASSAY[a]

Potency, u./ml.	Original Ethanol, %	Anticipated Activity Found, %
3	20	102.4
2	40	108.9
1	60	107.5

[a] Test solutions were made from M.L. 26 beef insulin.

observed, beef insulin is the most effective competitor for binding sites, followed in decreasing order by U.S.P. reference standard (beef and pork), pork, and human insulins. Errors in potency estimation show up as parallel lines in plots such as those shown in Fig. 3. A change in slope represents a proportionate difference and species specificity.

One of the questions which must be answered about any *in vitro* hormone assay is the specificity of the assay for the active hormone. We have attempted to supplement the work of Grodsky (8) by indirect activity correlation and some interference testing. Table II is an indirect correlation of crude extract assays and intermediate purity assays with final crystal results. One of the problems with prior *in vitro* or chemical techniques is an inability to assay insulin quantitatively in mixtures having a specific activity of less than 16 u./mg. We believe that this test gives adequate results down to 0.05 u./mg. specific activity.

The data in Table III resulted from an experiment to discover if the ions used in processing insulin would interfere with the assay. This is the first report showing that the pH of the sample must be below the isoelectric point of insulin prior to dilution with pH 8.5 albumin stock solution. This pH requirement holds for both beef and pork insulins. There are at least two explanations for the apparent loss of activity in alkaline solutions. First, insulin is lost through adsorption on glassware at higher pH's; or second, the insulin molecule is modified so that the antibody cannot bind it. We prefer the latter explanation since we found that acidification was necessary to give reliable results on NPH and PZI insulins which are originally nearly neutral. In Table IV the assay shows normal variation about a weighted mean for both beef and pork samples.

The experiments described in Tables V and VI were designed to show several points about and uses for the assay. Either the beef or pork section of Table V illustrates a complete set of converted data from an eight-sample assay pattern. Subset replication is indicated. The use of the assay with protamine containing samples both for acidified total potency and centrifuged supernatant assays, and the two forms of commercially available iodo-insulin were compared. We have concluded from the completed calculations in Table VI that there was no appreciable difference in the two tests due to changes in the species of iodo-insulin used.

The experiments described in Tables VII and VIII illustrate that the assay discriminates between natural and denatured forms of insulin. Neither heat nor enzyme denatured insulin are effective in the assay.

In Table IX we reported routine instances of direct comparison with some fairly precise U.S.P. rabbit assays. The high immunoassay results on the human insulin sample are possibly due to a low response on the standard (493-10GP-90) which could in turn be due to poor stability in albumin stock solution, statistical variation due to the relatively small samplings of the rabbit population when its potency was established in the U.S.P. test, or some other unrecognized variable.

The two protamine zinc insulin samples were of interest as 20-year-old room temperature samples which are still active (though weaker than they were at time of manufacture). Thus, a supposedly mildly inactivated portion of these samples does not affect the immunoassay.

Ethanol in samples, such as crude extracts, apparently does not inhibit the antibody as indicated in Table X.

Output quantity is a function of manpower, equipment available, and the specialization possible with larger numbers of samples. The following

sample patterns 8, 14, and 22, respectively, require about 1.5, 2.0, and 2.5 men per day. The assay takes a little longer than a mouse convulsion test, but is many times more accurate.

Since we were not assaying serum insulin we were able to increase volumes, standard concentrations, and I^{131}-insulin concentration to provide adequate counting quantities when low specific activity commercially available iodo-insulin was used. If high specific activity iodo-insulin is used, the sensitivity of the test could be increased by reverting to the original volumes used by Grodsky (1).

As suggested by Grodsky (1), we started using Na_2SO_3 in the salt-out step but changed to Na_2SO_4 for operation at 24°. Frictional heat arising during centrifugation led us to the use of a refrigerated centrifuge. However, since Na_2SO_4 crystallizes at 5–15°, we returned to Na_2SO_3 and 15° as our operating temperature.

We have found this test to be accurate, fast, inexpensive, and specific for natural insulin.

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Gas-Liquid Chromatography of Local Anesthetics and Related Compounds

By HENRY M. KOEHLER and JOHN J. HEFFERREN

Conditions for the gas chromatography of local anesthetics and related materials were investigated. Flexol on Chromosorb W and SE 30 on glass bead columns were preferred for the low and high melting compounds, respectively.

ANALYSES OF local anesthetic drugs in various dosage forms and tissue preparations have been carried out using ultraviolet, visible, and infrared spectrophotometric and titrimetric and gravimetric methods. The use of chromatographic methods for separation, identification, and quantitation of components of mixtures is well established in pharmaceutical analysis. Gas-liquid chromatography offers the advantage of rapidity combined with the sensitivity and versatility of other chromatographic techniques (1). This technique was applied to the detection and quantitation of local anesthetic bases, hydrochloride salts and degradation products. Kirk, describing the application of gas-liquid chromatography to criminalistic problems, included procaine hydrochloride among the drugs which could be chromatographed (2).

APPARATUS

The instrument used in this work was the Aerograph Hy-Fi model 600 fitted with a gold-plated hydrogen flame ionization detector and a Sargent model SR recorder with an input filter and a Disc integrator. Hydrogen, at a flow rate of 25 ml./minute, was provided for the flame ionization detector by the Aerograph hydrogen generator, model 650. Dry nitrogen was used as carrier gas at a flow rate of 30 ml./minute. Liquid phases used included Carbowax 400, Apiezon L, Ucon Polar, Flexol plasticizer 8N8, and silicone rubber SE 30. Solid supports used included 60–80 mesh firebrick, 100–120 mesh siliconized Chromosorb W, and 100–120 mesh siliconized glass beads. Five-foot columns of $^1/_8$ inch O.D. copper, aluminum, or stainless steel tubing were used.

The liquid phases were coated on the solid supports by evaporating with a Rinco rotary evaporator a slurry of the solid support and a dichloromethane solution of the liquid phase. The columns were packed with a vibrator and shaped into $2^1/_2$ inch diam. coils which were conditioned overnight in a slow stream of nitrogen at the expected operating temperature or at 200°, whichever was lower.

RESULTS AND DISCUSSION

The structural relationships of the local anesthetic agents used in this study are summarized in Table

Received August 26, 1963, from the Division of Chemistry, American Dental Association, Chicago 11, Ill.
Accepted for publication October 4, 1963.
Samples of the local anesthetics and their component alcohols were generously furnished by their manufacturers: butacaine sulfate, benzocaine, procaine HCl, and 3-dibutylaminopropanol by Abbott Laboratories; lidocaine and *N,N*-diethylaminoacetic acid by Astra Pharmaceutical Products, Inc.; mepivacaine HCl, propoxycaine HCl and tetracaine HCl by Cook-Waite Laboratories, Inc.; diethylaminoethanol, dimethylaminoethanol, and 2,6-xylidene by Sterling Winthrop Research Institute, division of Sterling Drug; benoxinate HCl by Dorsey Laboratories, division of the Wander Co.; pyrrocaine HCl by Graham Chemical Co.; chloroprocaine HCl by Lederle Laboratories, division of American Cyanamid Co.; piperocaine HCl and *N*-(3-hydroxypropyl)-α-pipecoline by Eli Lilly and Co.; isobucaine HCl, meprylcaine HCl, 2-propylamino-2-methylpropanol, and 2-isobutylamino-2-methylpropanol by Mizzy, Inc.; butethamine HCl, metabutethamine HCl, metabutoxycaine HCl, naepaine HCl, isobutylaminoethanol, and amylaminoethanol by Novocol Chemical Manufacturing Co.; dyclonine HCl by Pitman-Moore Co., division of Dow Chemical Co.; and parethoxycaine HCl by E. R. Squibb and Sons, division of Olin Mathieson Chemical Corp.
Supported in part by USPHS Grant D-533.
Analysis of Local Anesthetics IX. A portion of this paper was presented at the 41st general meeting of the International Association for Dental Research, Pittsburgh, Pa., March 1963.