Immunological and Biological Properties of Iodoinsulin Labeled with One or Less Atoms of Iodine per Molecule

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ABSTRACT Experiments were designed to compare the distribution of free and antibody-bound unlabeled insulin to the distribution of free and antibody-bound insulin-^{125}I. The insulin antibody was incorporated in a specific immune precipitate similar to the one used by Hales and Randle for the radioimmune assay of insulin. Insulin which was not bound by the specific immune precipitate was measured by the immune hemolysis inhibition assay. This report contains evidence that the addition of the unlabeled insulin in the radioimmune assay results in relatively more insulin-^{125}I which remains free and less bound by antibodies than is the case with the unlabeled insulin. Methods are described for the separation of an electrophoretically homogeneous iodoinsulin from samples of crude iodoinsulin with average incorporations of less than 0.2 atoms iodine per molecule. These purified iodoinsulin fractions have a markedly attenuated biological activity. Evidence is presented which supports the postulate that only a portion of the antibodies in guinea pig insulin antiserum are capable of effectively binding with purified iodoinsulin.

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INTRODUCTION

It has been reported that an average incorporation of one iodine atom into one molecule of insulin results in little, if any, loss of biological (1) or immunological (1, 2) activity.

Since it has recently been demonstrated that at least half the molecules of insulin labeled with an average of 0.8 atoms of iodine per molecule are not iodinated (3, 4), it was considered possible that the biological activity of iodoinsulin containing an average of one iodine atom per molecule (1) might be due to the presence of unlabeled insulin in the preparation used. Furthermore, although antibody binding of iodoinsulin preparations containing one to six iodine atoms per molecule has been demonstrated, this demonstration has been based solely on the distribution of radioactivity. The binding of both the unlabeled and labeled insulin by antibody has never been measured by a method that is not dependent upon the distribution of radioactivity.

These studies were designed to test whether iodoinsulin preparations labeled with an average of less than one atom of iodine per molecule retains immunological and biological activity. It has been possible to show that relatively more insulin-^{125}I remains free and less is bound by antibodies than is the case with unlabeled insulin.

In addition, electrophoretically homogeneous iodoinsulin was prepared by acrylamide block electrophoresis from insulin preparations with average incorporations of 0.2 atoms or less of

iodine per molecule. This report presents evidence that iodoinsulin prepared in this manner has a markedly attenuated biological activity in the rat epididymal fat pad, and reacts less well with guinea pig insulin antibodies than does the unlabeled insulin, as measured by immune hemolysis inhibition.

METHODS

Solutions and suspensions

Veronal-buffered saline (VBS). The diluent used for titrating complement and insulin antisera was VBS, pH 7.4 (5), containing 5×10^{-4} M $MgCl_2$ and 1.5×10^{-3} M $CaCl_2$.

VBS albumin (VBS-alb). The diluent used for radioimmune assays and immune hemolysis inhibition assays was VBS containing 1.5 g of bovine serum albumin (Armour & Co., Chicago, Ill.) per liter, which had been incubated at 80°C for 1 hr and stored at 4°C.

Buffered saline. This buffer consisted of 0.15 M NaCl with 0.01 M phosphate buffer, pH 7.4.

Insulin. Insulin was dissolved in 0.01 N NaOH equal to one-half the desired volume; an equal volume of 0.22 M phosphate buffer, pH 7.4, was added as soon as all the insulin was dissolved. Fresh insulin solutions were prepared for each experiment.

Absorbed guinea pig complement (C'a). C'a was used for immune hemolysis. The preparation and titration of C'a has been previously described (6).

Insulin-coated red cells (insulin-cells). We prepared insulin-cells by conjugating insulin to the surface of washed sheep red blood cells with bisdiazo-benzidine as previously described (7). In order to standardize the cell concentration for immune hemolysis and immune hemolysis inhibition experiments, we standardized the insulin-cells by adjusting the volume with VBS-alb so that 0.5 ml of cells diluted to 2.0 ml with distilled water gave an absorbance of 0.20 at 580 mμ in a Beckman D B Spectrophotometer.

Insulin preparations

Three preparations of insulin were used. Lilly insulin [1] (Lot 795372) was a preparation of zinc crystals of beef insulin with a biological potency of 25.6 U/mg. Novo insulin [2] (Lot 018864) was a preparation of 10× recrystallized beef insulin with a potency of 24.0 U/mg. Lilly insulin (Lot PJ4086) was a preparation of beef insulin with a potency of 23.5 U/mg.

[1] We wish to thank Dr. Otto K. Behrens and Dr. W. W. Bromer, Eli Lilly and Company, Indianapolis, Ind., for the generous supply of crystalline beef insulin.

[2] We wish to thank Dr. J. Schlichtkrull, Novo Industri A/S, Copenhagen, Denmark, for the generous supply of 10× crystalline beef insulin.

Crude insulin-^{125}I preparations

Preparation A (prep A). Abbott Laboratories, North Chicago, Ill., (Lot IN-024-1) insulin-^{125}I was used to compare the distribution of free and antibody-bound unlabeled insulin to the distribution of free and antibody-bound insulin-^{125}I. This lot of insulin-^{125}I was prepared by a modification of the McFarlane reaction (8) in which ICl is used. Abbott Laboratories estimated that this preparation contained an average of one atom ^{125}I per molecule of insulin, which had a specific activity of 13.1 mc/mg and contained 9.2 μg of insulin/ml. Contaminating materials in this preparation of insulin-^{125}I were removed by gel filtration through Sephadex G-75 with buffered saline.

Three ^{125}I-labeled peaks were obtained. The first peak was in the void volume. Although most of the radioactivity was precipitable with 10% trichloroacetic acid, much of it could not be bound by excess insulin antibodies. Approximately 80% of the radioactivity of the isolated core of the second peak was bound by *excess* insulin antibodies. Much of the radioactivity in the trailing third peak was dialyzable. After the material in the core of the second peak was dialyzed against buffered saline, 95% of the radioactivity was precipitated with excess insulin antiserum. *Consequently, 95% of the insulin-^{125}I used in these experiments was considered to be capable of binding to excess insulin antibodies.* Utilizing the immune hemolysis inhibition assay, we estimated this fraction to contain 0.3 μg of insulin/ml, and a minimum specific activity of 2.9 mc/mg. 1/10 ml of a one-twentieth dilution of this preparation was used as the labeled trace component in all the experiments comparing the distribution of free and antibody-bound unlabeled insulin to the distribution of free and antibody-bound insulin-^{125}I.

Preparation B (prep B). Preliminary experiments designed to separate insulin-^{125}I from unlabeled insulin were performed with Abbott iodoinsulin (Lot IN5-027-6). This material contained an average of one atom ^{125}I per molecule insulin, and had a specific activity of 13.4 mc/mg. *Excess insulin antibodies bound 95% of the radioactivity present in this preparation.*

Preparations C and D (prep C and D). Crude iodoinsulin preparations were labeled with ICl according to the method of Springell (9) as modified by Izzo, Roncone, Izzo, and Bale (1). Since it was necessary to prepare large batches of the crude preparation for use in the acrylamide block purification procedure, it was not feasible to label the iodoinsulin in preparations C, D, and E with ^{125}I alone. Accordingly, ICl was used with a trace amount of carrier-free Na^{125}I obtained from Abbott Laboratories. In prep C the ratio of ^{127}I to ^{125}I which was substituted to insulin was 7120. In prep D, this ratio was 95. Immediately after labeling, we dialyzed each preparation against pH 3 saline (3 ml of glacial acetic acid and 9 g of NaCl/liter) at 4°C until the dialysates contained less than 500 cpm/ml. They were then precipitated isoelectrically by dialysis against 0.1 M sodium acetate buffer, pH 4.5 at 4°C, and washed twice with 40 ml of acetone. The precipitated crude iodoinsulin was dried under vacuum. Between 70 and 75% of the insulin was recovered.

Prep C and D had average incorporations of 0.22 and 0.11 atoms of iodine, respectively, per molecule of insulin.

Preparation E (prep E). Crude iodoinsulin was labeled with KI and trace amounts of carrier-free Na ^{125}I according to the method of DeZoeten and De Bruin (3). The ratio of ^{127}I to ^{125}I was 95. After iodination, this preparation was dialyzed, precipitated, dried, and stored in the same manner as described for preps C and D. Prep E had an average incorporation of 0.16 atoms iodine per molecule of insulin.

Antisera

Pooled guinea pig insulin antisera were prepared as previously described (6). Each of the insulin antiserum pools used were prepared from at least 10 but not more than 20 guinea pigs.

Guinea pig gamma G globulin (GPGG). We prepared GPGG from pooled insulin antisera by diethylaminoethyl (DEAE) column chromatography utilizing the method of Yagi, Maier, and Pressman (10). The GPGG fraction was rechromatographed, dialyzed against 0.025 M ammonium formate, and lyophilized. GPGG in excess, 15 mg/ml, gave a single precipitin arc against rabbit anti-whole guinea pig serum by immunoelectrophoresis. This material was used as the antigen for the production of rabbit antiserum against guinea pig gamma G globulin (Rab-GPGG). The rabbit antiserum induced by this antigen also gave a single precipitin arc in the gamma globulin region when tested against whole guinea pig serum.

Rabbit antiserum against guinea pig gamma globulin (Rab-GPGG). We developed antisera to purified GPGG in adult white rabbits by injecting 0.2 ml of GPGG (0.7 mg) in Freund's adjuvant into each toe pad. 3–4 wk later, 0.5 ml of GPGG (2 mg) was given as an intravenous booster, followed by another 0.5 ml within 48–72 hr. We took a sample of blood for testing, 3–4 ml, within 7–12 days after the second booster. When high antibody levels were observed, 50–70 ml of blood was taken from the marginal ear vein 1–2 days after the test sample was obtained. We considered adequate antisera which yielded precipitin lines at a dilution of 1/100 utilizing a modification (11) of the micro double diffusion method of Ouchterlony (12).

Titration of insulin antisera. Insulin antisera were titrated by the immune hemolysis technique previously described (6). The amount of hemoglobin released after the reaction of antibodies with insulin-cells in the presence of excess C'a is an accurate measure of relative antibody concentration in insulin antisera (11).

Insulin immune hemolysis inhibition insulin assay

The immune hemolysis inhibition analyses were carried out by a modification of the method previously described (13). In this test, insulin antiserum was preincubated with different concentrations of insulin and test samples. Insulin-cells and C'a were then added, a second incubation was performed and the amount of lysis determined. Appropriate controls such as the degree of immune hemolysis and immune hemolysis inhibition with a standard antisera were included in each experiment. In the absence of added insulin, the insulin antibodies combined with insulin fixed to the cells, activating the complement system which lysed the cell. In the presence of insulin, the antibodies were partially neutralized and hemolysis was inhibited.

An example of the immune hemolysis inhibition assay as used in these studies is presented in Fig. 1. Master dilutions of insulin antiserum (1/50, 1/70, 1/110, and 1/130) were prepared for each experiment in volumes sufficient to allow an aliquot (0.1 ml) of each dilution to be used for the control, standards, and test samples. Control samples, test samples, and standards were added to the antiserum dilutions in 0.5 ml volumes. These mixtures were incubated for 1 hr at 37°C in a Dubnoff metabolic shaker with gentle agitation (4 cycles/sec). ½ ml of a suspension of insulin-cells (0.4%), containing 10 U of C'a was then added rapidly with an automatic pipette to

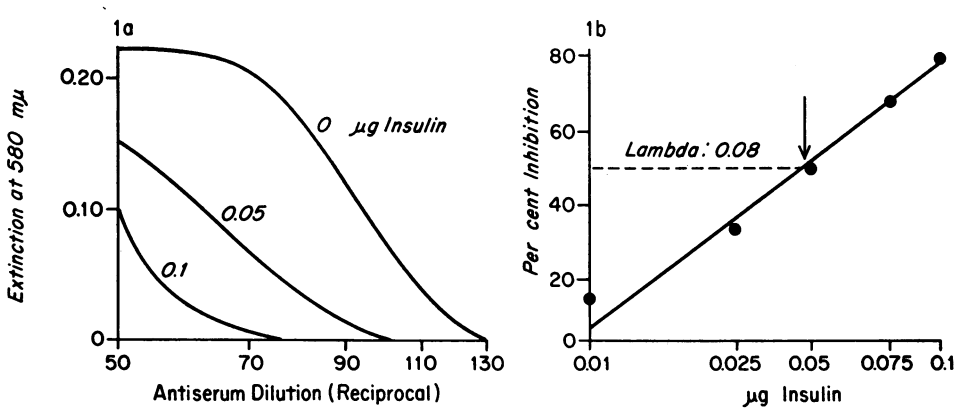

FIGURE 1 Immune hemolysis inhibition assay. *a,* Immune hemolysis titration curves for control (0 μg of insulin indicates that no insulin was added). *b,* Per cent inhibition based on decrease in area compared to control titration curve, plotted against amount of insulin added. The arrow indicates the amount of insulin necessary to inhibit 50% of the antibodies (0.05 μg).

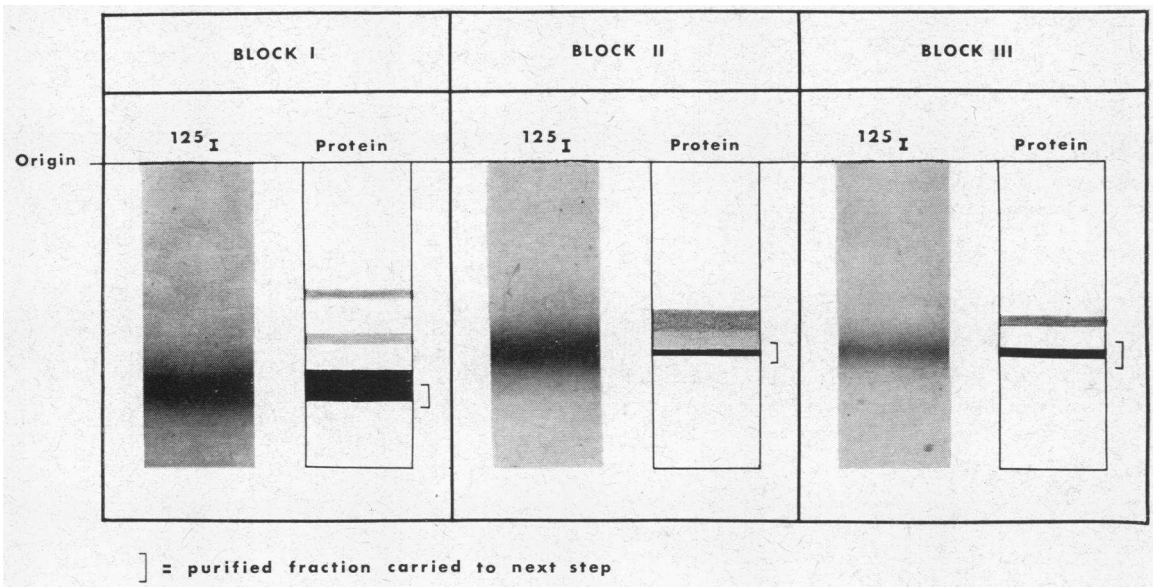

FIGURE 2 Purification of iodoinsulin by repeated acrylamide block gel electrophoresis. ^{125}I, photograph of a portion of the radioautograph of the acrylamide block. Protein, schematic representation of the protein–stained vertical strip of the acrylamide block. Migration from the origin is toward the anode at bottom of the figure. (*1*) Block I contains the crude iodo preparation. (*2*) Block II contains the material electrophoresed from Block I indicated by the brackets on the protein-stained strip. (*3*) Block III contains the material electrophoresed from Block II indicated by the brackets on the protein-stained strip.

each test tube and incubation at 37°C was continued for 30 min with vigorous shaking (9 cycle/sec). The unhemolyzed insulin-cells were separataed by centrifugation at 800 *g*. The supernates were immediately decanted and the hemoglobin released was measured by absorbances at 580 mμ.

Hemolytic titration curves were plotted for controls, insulin standards, and test samples (Fig. 1 *a*). The areas of these curves were determined by planimetry. The area of the control (zero insulin) immune hemolysis curve was considered as 100% lysis. The decrease in area of the immune hemolysis titration curves obtained with given amounts of insulin was used to calculate the per cent inhibition. We obtained a linear dose-response by plotting the per cent inhibition against added insulin (Fig. 1 *b*). Insulin in test samples was calculated from the standard curve, which ranged between 0.01 and 0.1 μg of insulin and had a lambda of 0.08.

Preparation of electrophoretically homogeneous iodoinsulin

Electrophoretically homogeneous iodoinsulin was isolated by sequential electrophoresis in polyacrylamide gel (Fig. 2). Polyacrylamide gel was prepared by the method of Davis (14) as modified by Hokama and Riley (15). A lucite cell, 78 mm high with an internal opening 205 × 6 mm was temporarily sealed at the bottom by coating the edges with vaseline and inserting it into a tightly fitting Silastic (Dow Corning, Midland, Mich.) rubber holder. Approximately 80 ml of a 10 or 15% acrylamide gel was poured into the cell and allowed to polymerize. 10 ml of large pore upper gel was layered over it for a spacer; this was also allowed to polymerize. Tris-glycine buffer, pH 8.3, was layered over the top of the cell. 3–5 ml of labeled insulin containing approximately 20 mg of material was mixed with an equal volume of 50% dextrose and carefully pipetted under the buffer on top of the spacer gel. The cell was then inserted into the buffer of the anode vessel. The top of the cell was connected to a vessel containing Tris-glycine buffer which was connected with a saturated flannel wick to the cathode. Optimal separation was obtained in 3 hr with a current of 6 ma/cm^2. Vertical strips were taken from the edge of the block and stained for protein bands with aniline black. The remainder of the block was frozen and stored at −80°C. A radioautograph with Eastman Kodak Industrial type KK X-ray film was obtained in order to locate the iodoinsulin band (Fig. 2).

The protein-stained bands which were not radioactive by radioautography were located from the vertical strips. The fractions separated from these segments will be referred to as "unlabeled insulin." The iodoinsulin band was fragmented and mixed with upper gel and reelectrophoresed. In all cases the third and final electrophoresis was in 15% acrylamide gel.

Iodoinsulins and unlabeled insulins were separated from the gel by electrophoresis. The gels containing the respective samples were fragmented and suspended on a filter in a Buchner funnel to which dialysis tubing was attached. The whole system was filled with VBS and all of the air expressed from it. The contents in the funnel

were attached to the cathode with a flannel wick and the dialysis tubing was immersed into the solution at the anode. A current of 60 ma for 2 hr was sufficient to elute 95% of the radioactivity from those gels containing the iodoinsulin. The material in the dialysis sack was then passed through a G-25 Sephadex column with saline, precipitated isoelectrically in 0.1 M acetatae, pH 4.5, and dried under vacuum, as were the crude preparations.

Estimation of biological activity

Biological activity was estimated with the rat epididymal fat pad assay of Ball and Merrill (16). Insulin added to the epididymal fat pad of the rat results in a proportionate liberation of carbon dioxide which can be measured manometrically. The results were expressed as per cent of maximal activity noted with 100 mU of insulin.

RESULTS

Distribution of free and antibody-bound insulin-^{125}I, compared to free and antibody-bound unlabeled insulin

If antibodies can combine with both insulin-^{125}I and crystalline insulin to the same degree, then the free and bound insulin assayed radioimmunologically should equal the free and bound insulin assayed by immune hemolysis inhibition. If, however, binding of antibodies with insulin-^{125}I is impaired by the iodine, then the radioimmune assay should show more insulin free and less bound to antibodies than noted by the immune hemolysis inhibition assay.

In order to test these two possibilities, we prepared a specific immune precipitate (Table I) similar to the one used by Hales and Randle (17) by adding Rab-GPGG to pooled guinea pig insulin antiserum. Variable amounts of unlabeled insulin with a constant trace of insulin-^{125}I were then added to the specific immune precipitate. The distribution of free and antibody-bound insulin measured by radioactivity was compared to the distribution measured by immune hemolysis inhibition.

It was necessary to make a specific immune precipitate capable of binding amounts of insulin which could be measured by immune hemolysis inhibition. The detailed procedure for this method is presented in Table I.

The radioimmune assay was based on the amount of insulin-^{125}I displaced by added insulin. The amount of trace insulin-^{125}I bound when no insulin was added was assumed to be 100% binding by antibody. The mean per cent of insulin-^{125}I displaced, plotted against insulin added, resulted in a linear dose-response with a lambda of 0.05 (11). This method and index of precision are similar to that previously reported by Hales and Randle (17). The cpm in the wash of the specific immune precipitate when added to the cpm of either the supernate or precipitate had no significant effect on the curve obtained by plotting bound/free (B/F) insulin ratios against added insulin (Fig. 3). There was a residual amount of supernate adherant to the vials within which the binding reactions were performed. It was therefore considered more valid to include the cpm of the wash with the supernate, rather than with the precipitate.

We calculated the amount of unlabeled insulin bound by the specific immune precipitate by subtracting the amount of free insulin in the super-

TABLE I

Experimental Design: Distribution of Free and Antibody-Bound Insulin-^{125}I Compared to Free and Antibody-Bound Unlabeled Insulin

I Preparation of specific immune precipitate.
- A. Add 0.5 ml of insulin antiserum to 0.2 ml of Rab-GPGG.
  1. Incubate, 4°C, 2 hr.
  2. Centrifuge, 800 *g*, 4°C, 10 min.
  3. Wash with 1 ml of VBS-alb.

II Insulin-binding reaction.
- A. Add to precipitate varying amounts of insulin (0–6 μg/ml) and constant trace of insulin-^{125}I in 0.1 ml. Total volume 1.3 ml.
  1. Incubate, 4°C, 2 hr.
  2. Centrifuge, 800 *g*, 4°C, 10 min.

III Analysis of free and bound insulin-^{125}I and unlabeled insulin.
- A. Precipitate.
  1. Wash with 1 ml of VBS-alb.
  2. Measure cpm in precipitate for assay of antibody-bound insulin-^{125}I.
- B. Supernate.
  1. Add cpm in wash to cpm in supernate for assay of free insulin -^{125}I.
  2. Take 0.5 ml of aliquot of supernate for assay of free unlabeled insulin by immune hemolysis inhibition.
  3. Determine amount of antibody-bound unlabeled insulin by subtraction from amount of insulin added.

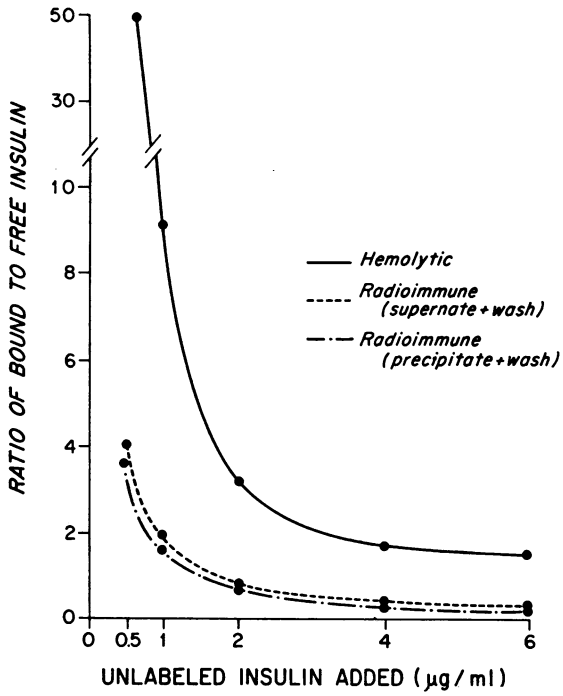

FIGURE 3 Antibody binding with insulin-^{125}I compared to binding with unlabeled insulin. ————, Ratio of bound to free unlabeled insulin as calculated from the amount of insulin in the supernate, measured by immune hemolysis inhibition. Approximately 100% of added insulin was recovered in the supernate when normal serum was used instead of insulin antiserum. – – – – –, Ratio of bound to free insulin-^{125}I as measured by distribution of radioactivity, adding cpm in wash to cpm in supernate. —— · ——, Ratio of bound to free insulin-^{125}I as measured by distribution of radioactivity, adding cpm in wash to cpm in precipitate.

nate (measured by immune hemolysis inhibition) from the amount of added insulin. The free and bound insulin estimated by the radioimmune assay was compared to the free and bound insulin estimated by immune hemolysis inhibition.

In Table II, the ratios of B/F insulin measured by radioimmune and immune hemolysis inhibition assays are tabulated for each of three experiments. In all experiments and at each level of added insulin, less insulin was free and more was bound to antibody as measured by immune hemolysis inhibition than as measured by the radioimmune assay. Analysis of variance of the data showed that the distributions of free and antibody-bound unlabeled insulin were significantly different ($P < 0.00001$) than the distributions of free and antibody-bound insulin ^{125}I.

These distributions were much more distorted at the low levels of added insulin than at the higher levels. This is emphasized in Fig 3, where the data obtained in Experiment 2 (column 2, Table II) are presented by plotting B/F insulin ratios against added insulin.

The disparity in distributions of antibody-bound and free insulin-^{125}I is probably best explained by the presence of insulin antibodies in the antiserum that have combining site configurations directed to portions of the insulin molecule to which the iodine is attached. The capacity of such antibodies to combine with insulin-^{125}I would be significantly reduced. Such antibodies would, however, still be capable of combining with unlabeled insulin. Consequently, in the region of antibody excess, such antibodies would combine with unlabeled insulin much more efficiently than with insulin-^{125}I. In each of three experiments the disparity in distribution was more marked in the region of antibody excess (Table II and Fig. 3).

Biological and immunological characteristics of partially purified fractions from crude insulin-^{125}I, separated by acrylamide gel electrophoresis

Since significant amounts of unlabeled insulin are present in insulin-^{125}I preparations labeled with an average of one atom or less of ^{125}I per molecule, we feel that it is difficult to evaluate the biological and, to a degree, the immunological characteristics of such insulin-^{125}I preparations unless the unlabeled insulin is separated from the insulin-^{125}I.

Approximately 75 μc of crude insulin-^{125}I (1 μg of prep B) was applied to each of six acrylamide (10%) gel columns (0.6 × 6.7 cm) and subjected to electrophoresis. Five of the columns were frozen and stored at −80°C. The sixth column was split longitudinally; one half was stained for protein, the other half was radioautographed. Three distinct protein bands could be identified: two light-staining bands and one more intense-staining and less electronegative band. The most intense exposure of the radioautograph corresponded to the most electronegative protein component (Fig. 4), which by inspection, accounted for less than half of the protein stained with aniline black. Therefore, by radioautography, it appears that most of the radioactivity was attached to less

TABLE II

*Distribution of Insulin Bound/Free (B/F) Determined by Radioimmune and Immune Hemolysis Inhibition Assays**

Insulin added		Experiment 1		Experiment 2		Experiment 3	
		Radioimmune assay	Hemolytic assay‡	Radioimmune assay	Hemolytic assay	Radioimmune assay	Hemolytic assay
0.5	B/F§			0.4/0.1	0.49/0.01	0.4/0.1	0.45/0.05
1	B/F	0.7/0.3	0.98/0.02	0.6/0.4	0.9/0.1	0.5/0.5	0.6/0.4
2	B/F	0.9/1.1	1.5/0.5	0.8/1.2	1.5/0.5	0.8/1.2	1.4/0.6
4	B/F	1.3/2.7	1.8/2.2	1.0/3.0	2.5/1.5	1.0/3.0	2.0/2.0
6	B/F	1.2/4.8	2.8/3.2	1.2/4.8	3.6/2.4	1.2/4.8	3.0/3.0

* Insulin concentrations in all cases are in μg/ml.

‡ Calculated from the amount of insulin in the supernate, measured by immune hemolysis inhibition. Approximately 100% of added insulin was recovered in the supernate when normal serum was used instead of insulin antiserum.

§ Ratio of insulin bound by specific immune precipitate over insulin in supernate.

than one half the insulin in the crude insulin-^{125}I (prep B) labeled with an average of one atom of ^{125}I per molecule.

A 2 mm section corresponding to the core of the most heavily exposed area on the radioautograph was cut from each of the five remaining gel columns, counted, and then pooled as Fraction 2 (Table III, Fig. 4). 1 cm sections from each acrylamide gel column cathodal from the core were counted and then pooled as Fraction 1. 1 cm sections anodal from the core were counted and then pooled as Fraction 3. The pooled acrylamide discs corresponding to Fractions 1, 2, and 3 were crushed with glass wool, extracted at room temperature with 6 ml of VBS, centrifuged at low speeds, and the supernates decanted and stored at 4°C. Analyses and tests with these fractions were performed within 1 wk after the extraction.

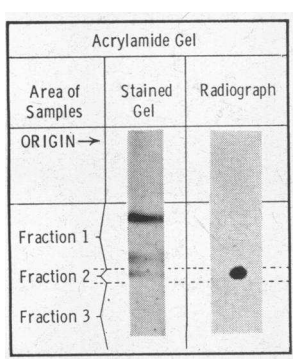

FIGURE 4 Disc electrophoresis of insulin-^{125}I in acrylamide gel. Fraction 1 comprised a 1 cm segment from the acrylamide gel column cathodal from the radioactive core. Fraction 2 comprised a 2 mm segment of the acrylamide gel column from the core of the radioactive band. Fraction 3 comprised a 1 cm segment from the acrylamide gel column anodal from the radioactive core.

Aliquots of each fraction were diluted with VBS-alb until their cpm/ml was comparable (± 2%) to that of crude insulin-^{125}I which had a concentration of 0.025 μg/ml as measured by immune hemolysis inhibition. Fractions 1, 2, 3, and an aliquot of crude insulin-^{125}I were then assayed by immune hemolysis inhibition. Fraction 1 contained approximately twice the insulin, Fraction 2 about three-fifths the insulin, and Fraction 3 equal amounts of insulin as measured by immune hemolysis inhibition compared to the crude prep B from which they were separated (Table IV).

Compared to Fractions 1 and 3, there was a concentration of ^{125}I in Fraction 2 (Table III). However, equivalent amounts of Fraction 2 (cpm/ml) neutralized fewer insulin antibodies in

TABLE III

Distribution of Radioactivity in Acrylamide Gel after Electrophoresis of Crude Insulin-^{125}I

Samples*	cpm/mm gel‡
	×10^{-3}
Fraction 1	9.95
Fraction 2	158.73
Fraction 3	11.97

* Five acrylamide columns from the same run were each subdivided into Fractions 1, 2, 3 according to Fig. 4. The segments corresponding to each fraction were counted individually and then pooled.

‡ The cpm/mm of gel was calculated from the average cpm of five segments divided by the mm of gel column from which the fractions were obtained (Fig. 4).

the immune hemolysis inhibition assay than did Fraction 1 or 3 (Table IV). It appears likely therefore, that insulin-^{125}I in preparations labeled with an average incorporation of one atom ^{125}I per molecule reacts less well with insulin antibodies than does the "unlabeled insulin" in such preparations.

When aliquots of crude prep B and Fractions 1, 2, and 3 were each diluted so that they contained approximately 0.01 μg of insulin/0.2 ml (250 μU) by immune hemolysis inhibition and tested for biological activity, crude prep B gave a 100% response (Table V). Fraction 1 gave a 100% response and Fraction 3 gave a 62% response. Fraction 2, however, gave a very markedly attenuated response (10%) (Table V).

These results provide evidence that the insulin-^{125}I in a preparation with an average incorporation of one atom per molecule has a markedly attenuated biological activity. They suggest that most of the biological activity can be attributed to the unlabeled insulin present in such preparations.

Immunological characteristics of lightly labeled, purified iodoinsulins

The results obtained with disc electrophoresis using prep B suggested that much of the insulin with attached iodine reacted less well immunologically and biologically than the "unlabeled insulin" in the preparation. The possibility existed that more lightly labeled iodoinsulin preparations contained biologically and immunologically more active forms of iodoinsulin. To test this, insulin was labeled at 0.2 or less atoms per molecule and the iodoinsulin and "unlabeled insulin" were separated by repetitive acrylamide gel electrophoresis.

TABLE IV

Insulin Assay with Immune Hemolysis Inhibition of Fractions from Crude Insulin-^{125}I Separated by Acrylamide Gel Electrophoresis

Sample	cpm/ml*	Immune hemolysis
	$\times 10^{-5}$	*μg of insulin/ml*
Crude insulin-^{125}I	1.43	0.025
Fraction 1‡	1.43	0.056
Fraction 2‡	1.43	0.015
Fraction 3‡	1.43	0.024

* Each of the samples assayed by immune hemolysis inhibition was diluted with VBS-alb until cpm/ml were within 2% of 0.025 μg/ml of crude insulin-^{125}I (prep B).
‡ Fraction 1, 2, and 3 (Fig. 4) are VBS-alb extracts of pooled acrylamide gel segments from five columns.

TABLE V

Biological Activity of Insulin-^{125}I Fractions Separated by Acrylamide Gel Electrophoresis Measured by Rat Epididymal Fat Pad Assay

Sample*	cpm/0.2 ml	μg insulin in 0.2 ml by immune hemolysis‡	Biologic activity§
	$\times 10^{-5}$		%
Crude insulin-^{125}I	1.14	0.01	100
Fraction 1	0.56	0.01	100
Fraction 2	1.90	0.01	10
Fraction 3	1.14	0.01	62

* All samples were diluted with VBS-alb to 0.01 μg insulin/0.2 ml based upon assay by immune hemolysis inhibition.
‡ 0.01 μg is equivalent to 250 μU.
§ Biological activity as a percentage of the activity of maximal stimulation with 4 μg (100 mU) insulin according to the method of Ball and Merril (16).

The amount of insulin as measured by immune hemolysis inhibition agreed with the amount of protein measured by the Lowry, Rosebrough, Farr, and Randall determination (18) for each of the "unlabeled insulin" fractions (Table VI). Therefore, the methods used to prepare these fractions had very little effect on the immunological properties of insulin. Furthermore, the "unlabeled" fractions contained no detectable contaminating materials which reacted in the protein determination. Thus the samples of "unlabeled insulin" contained only trace amounts of iodoinsulin. On the other hand much more iodoinsulin was needed to neutralize antibodies in the immune hemolysis inhibition system than was measured by the Lowry determination. Therefore, relatively more of the purified iodoinsulin from preps C, D, and E was needed to neutralize antibodies in the immune hemolysis inhibition system compared to the amount of "unlabeled insulins."

To evaluate more precisely the immunological competence of iodoinsulin purified by electrophoresis in acrylamide gel, we designed immune hemolysis inhibition experiments in which the quantities of "unlabeled insulin" from preparations D and E were compared to the respective quanti-

TABLE VI

Attenuated Immunological Reactivity of Purified Iodoinsulins Separated by Acrylamide Gel Block Electrophoresis

Crude preparations		Purified fractions		
Preparation	Atoms iodine/ molecule insulin	Fractions	Protein*	Insulin‡
			cpm/mg × 10^{-3}	*cpm/mg* × 10^{-3}
C	0.22	Unlabeled§	2.9	2.9
		Iodoinsulin	20.0	270.0
D	0.11	Unlabeled	0.2	0.2
		Iodoinsulin	7.0	805.0
E	0.16	Unlabeled	1.1	1.1
		Iodoinsulin	31.0	2485

* Protein concentration was determined by the Lowry method (18).

‡ Insulin concentration was determined by immune hemolysis inhibition.

§ Unlabeled fraction refers to the fraction eluted from the segments of the acrylamide block which had no radioactivity by radioautography but which contained protein-staining material.

ties of purified iodoinsulin required to inhibit 50% of the antibodies (Fig. 1). The "unlabeled insulin" from Fraction D (Table VII) was 140 times more effective in neutralizing one-half of the antibodies than was the purified iodoinsulin from the same preparation. With preparation E, 80 times more insulin-^{125}I than "unlabeled insulin" was needed.

These results indicate that the purified iodoinsulin from the crude preparations D and E, labeled with an average incorporation of 0.11 and 0.16 atoms of iodine per molecule, respectively, had markedly attenuated abilities to combine with antibodies compared to the "unlabeled insulin" from the same fractions. Whether the significantly attenuated immunological characteristics of the purified iodoinsulin were due to radioactive degradation products which allowed only a portion of the antibodies in a given antiserum to combine with the labeled insulin was not known. In order to test this possibility we subjected iodoinsulin at a concentration of 1 μg/ml by immune hemolysis inhibition to the Hales-Randle double antibody technique (17), using increasing amounts of the pooled insulin antiserum. In this way it was possible to estimate whether or not all of the iodoinsulin was sufficiently intact to be bound by antibodies (Table VIII). If degradation products existed in the purified insulin-^{125}I fraction, then a significant proportion of radioactivity would not be bound to antibodies regardless of how great an excess of antiserum was employed. The amounts of pooled antiserum required to precipitate 95% of the radioactivity in one μg of purified iodoinsulin from preps D and E were 0.7 and 0.63 ml of insulin antiserum, respectively. One-tenth this amount of antiserum was needed to precipitate 95% of the radioactivity in a comparable amount of prep C.

These results suggest that the attenuated immunological characteristics of the purified iodoinsulins were due to distortions in conformations of antigenic determinants on the intact insulin molecules and not due to degradation products

TABLE VII

Immunological Activity by Immune Hemolysis of Purified Iodoinsulin and Unlabeled Insulin*

Preparation	Sample	μg for 50% inhibition	Ratio of iodoinsulin to unlabeled insulin
C	Unlabeled		
	Iodoinsulin	0.50	
D	Unlabeled	0.11	140
	Iodoinsulin	15.00	
E	Unlabeled	0.10	80
	Iodoinsulin	8.00	

* Unlabeled refers to the fraction eluted from the segments of the acrylamide block which had no radioactivity by radioautography but which contained protein-staining material.

incapable of combining with any of the antibodies in guinea pig antiserum. This is additional evidence that pooled insulin antisera contain significant amounts of antibodies which do not effectively bind iodoinsulin as measured by the double antibody method. The fact that such a large amount of antiserum was required to combine with 95% of 1 μg of purified iodoinsulin suggests that only a small proportion of antibodies in the pooled guinea pig antiserum were capable of effectively binding with iodoinsulin.

Biological activity of purified fractions from crude iodoinsulin preparations with average incorporation of 0.11 and 0.16 atoms of iodine per molecule

The biological activities of the "unlabeled insulin" fractions from preparations C and E agreed very well with those of the crystalline insulin used to make the crude iodoinsulin preparations (Table IX). Therefore, the methods of purification produced no detectable alterations of the biological properties of the "unlabeled insulin." However, a comparison of the biological activity of the purified iodoinsulin in preparations C and E to the biological activity of the "unlabeled insulins" from the respective fractions shows that the response of the purified iodoinsulin was less than 5% of that noted with the "unlabeled insulin." The levels of purified iodoinsulin from preparations C and E had no significant biological effect, whereas one-twentieth the amount of the "unlabeled fractions" from each of these preparations had a significant biological activity. Therefore, as measured by the rat epididymal fat pad, the biological activity of the purified iodoinsulin from preparations with an average incorporation of 0.11 and 0.16 atoms iodine per molecule was very markedly diminished.

TABLE VIII

Immunological Competency of Purified Iodoinsulins: Amount of Pooled Guinea Pig Antiserum Required to Precipitate 95% of the Radioacticity of Purified Iodoinsulins

Preparation	ml of insulin antiserum to precipitate 95% of radioactivity	Protein*	Insulin‡
		μg/ml	μg/ml
C	0.064	13.5	1
D	0.63	115	1
E	0.70	80	1

* Protein concentration determined by Lowry method (18).
‡ Insulin concentration determined by immune hemolysis inhibition.

TABLE IX

Biological Activity of Purified Fractions by Rat Epididymal Fat Pad Assay

Preparation	Fraction	Protein concentration*	Insulin concentration‡	No.§	Biological activity
		μg/ml	μg/ml		%
C	Unlabeled‖	0.01	0.01	4	42
	Iodoinsulin	1.0	0.04	4	7
	Iodoinsulin	5.0	0.20	4	15
E	Unlabeled	0.01	0.01	4	47
	Iodoinsulin	3.2	0.04	8	11
	Iodoinsulin	16.0	0.20	4	11

* Protein concentration determined by Lowry method (18).
‡ Insulin concentration determined by immune hemolysis inhibition.
§ No. of determinations.
‖ Unlabeled fraction refers to the fraction eluted from the segments of the acrylamide block which had no radioactivity by radioautography but which contained protein-staining material. The response obtained with the unlabeled insulin from both prep C and E was not significantly different from that observed with 250 μU (0.01 μg) of crystalline insulin.

Distribution of radioactive iodine between A and B chains of crude and purified iodoinsulin

It is possible that the methods of separation employed in these studies were in essence selecting those molecules of iodoinsulin which were markedly distorted while the molecules of iodoinsulin minimally distorted may have migrated with the "unlabeled insulin." In order to test this possibility, we subjected the crude preparation E to oxidative sulfatolysis (19) and compared the amounts of radioactivity attached to the A and B chain to the radioactivity in the A and B chain of the purified iodoinsulin separated from prep E.

It was assumed that, if the distribution of the radioactivity between A and B chain in the crude prep E was similar to the distribution of radioactivity between A and B chain of the purified iodoinsulin from prep E, little, if any, selection of the various forms of iodoinsulin had taken place

TABLE X

Distribution of Radioactivity Between A and B Chains of Crude Prep E and Purified Iodoinsulin

	% Radioactivity on B chain	% Radioactivity on A chain
Crude prep E	10	90
Purified iodoinsulin	9	91

Iodoinsulin preparations were subjected to oxidative sulfatolysis and immediately followed by cellulose-acetate electrophoresis in 8 M urea–acetic acid buffer (pH 3.2) for 90 min.

by the method of purification. After oxidative sulfatolysis of the crude preparation, the iodine was found to be attached primarily to the A chain (Table X). Likewise, after oxidative sulfatolysis of the purified iodoinsulin, a comparable amount of radioactivity was found attached to the A chain. Consequently, very little selection took place by the method of separation.

All of the above evidence is therefore submitted as proof that iodoinsulin, even when very lightly labeled, has a markedly attenuated biological activity and that only a portion of the antibodies in guinea pig insulin antiserum are capable of effectively binding with it.

DISCUSSION

The disparity in distribution of antibody-bound and free insulin-^{125}I, compared to unlabeled insulin, is probably best explained by the presence of insulin antibodies in antiserum which have combining site configurations directed to portions of the insulin molecule to which the iodine is attached. The capacity of such antibodies to combine with iodoinsulin would be reduced. Such antibodies would, however, still combine with unlabeled insulin. The difference in the distributions of free and antibody-bound insulin-^{125}I and free and antibody-bound unlabeled insulin in these experiments were measured at levels of added insulin which are three orders of magnitude greater than circulating insulin concentrations as measured by the radioimmune assays. Experiments comparing the distribution of antibody-bound and free unlabeled insulin to antibody-bound and free insulin-^{125}I at lower levels of added insulin (250–1000 μU) have also been performed. A second radioimmune assay using insulin-^{131}I to measure the unlabeled insulin not bound to the specific immune precipitate was used in these experiments. There was less insulin free and more bound by antibody as measured by the second radioimmune assay than could be expected from the distribution of ^{125}I (20). It is therefore reasonable to propose that the binding of iodoinsulin by antibodies is also impaired at the low levels of insulin in blood as measured by radioimmune assays.

It is interesting that radioimmune assays do *not* give linear dose-response curves when sufficient antisera is used to bind all of the trace-labeled insulin. Linear dose-response curves are obtained only when excess labeled insulin is used, i.e., when antibody concentration is decreased to the point where only a fraction (70% or less) of the labeled insulin is bound (17, 21–23). We postulate that a linear dose-response radioimmune assay cannot be obtained unless unlabeled insulin is present in sufficient amounts to saturate those antibodies which cannot bind effectively with iodinated insulin. This is supported by the parallelism in the B/F ratios between the radioimmune and immune hemolysis inhibition assays when 4 and 6 μg of insulin were used (Fig. 3).

On this basis antisera that contain relatively large quantities of antibodies capable of reacting with the labeled hormone should be selected in order to obtain sensitive radioimmune assay systems. We are currently isolating antibody fractions from pooled inbred guinea pig insulin antisera which will presumably contain high concentrations of antibodies which will more effectively bind iodinated insulin. Preliminary results indicate that antisera from strain 2 guinea pigs may have relatively more antibodies capable of reacting with iodinated antigens than antisera from strain 13 guinea pigs. The selection of insulin antibody preparations for radioimmune insulin assays is empirical, frequently requiring that several different antisera be tested. It might be useful to use certain purified immunoglobulin fractions from strain 2 guinea pigs to consistently prepare more sensitive radioimmune insulin assays.

The immunological and biological properties of the partially purified insulin-^{125}I from prep B and purified iodoinsulin from prep C were different when compared to the purified iodoinsulins from preps D and E. Softer gel (10% acrylamide) was

used to prepare iodoinsulin from preparations B and C compared to hard gel (15% acrylamide) used to prepare iodoinsulin from D and E. Consequently, it is probable that the purified iodoinsulin from preps B and C were contaminated to a greater degree with unlabeled insulin.

There was a disproportionate loss of immunological activity relative to loss of biological activity of iodoinsulin from preps D and E compared to iodoinsulin from prep C. According to biological activity, a maximum of 5% contamination of purified iodoinsulin from prep C with unlabeled insulin could be expected (Table IX). Prep C iodoinsulin, however, was 30 and 16 times more effective in neutralizing 50% of the antibodies by immune hemolysis inhibition than were the iodoinsulins from preps D and E, respectively (Table VII). Similar results were observed with the double antibody method where 8.5 and 6 times as much antibody was needed to precipitate 95% of preps D and E, respectively, compared to the amount required to precipitate prep C (Table VIII).

The possibility exists therefore, that iodoinsulin in the presence of unlabeled insulin binds with proportionately more antibodies in antiserum than does iodoinsulin alone. Iodoinsulin may aggregate with unlabeled insulin and antibodies may be present in insulin antisera which bind with aggregated forms of insulin more effectively than with monomers. Antibodies to aggregated forms of antigen which do not react with the respective monomers have been described (24, 25). In this regard, the presence of unlabeled insulin in the trace used for radioimmune assays may be very important for maximum sensitivity.

The use of acrylamide gel to prepare highly purified fractions of iodoinsulin is being extended in our laboratory. Preliminary results indicate that, by increasing the percentage of the acrylamide above 15% to 20% in gels, we can discern subfractions of iodoinsulins in the block. Consequently, it may be possible to obtain subspecies of iodoinsulin preparations from lightly labeled crude preparations. We hope to obtain by this method a fraction which is biologically and immunologically similar to unlabeled insulin.

It is not surprising that by recent estimates (26) the half life of unlabeled insulin in man is shorter than by previous estimates with insulin-^{131}I (27, 28). Of particular concern are the studies based on the distribution of insulin-^{131}I in animals (29, 30) and tissue (31) which related localization of radioactive insulin to the site at which insulin induces its biological effect. In this regard, De Zoeten and Van Strik (32) have studied the effect of the incorporation of one or more atoms of iodine on the biological activity of insulin. They concluded that it was advisable to use iodinated insulin with an average incorporation of no more than 0.2 iodine atoms per molecule of insulin for the investigation of physiological properties. Our results show that the iodinated insulin separated from preparations labeled with average incorporations of as little as 0.11 atoms of iodine per molecule has no significant biological activity in the rat fat pad assay. The possibility exists that purified iodoinsulin may have biological activity when other bioassay methods are employed. Experiments are currently in progress to test this possibility.

In view of the results obtained by De Zoeten and De Bruin regarding the distribution of iodine on the various tyrosines in the insulin molecule (3), it appears probable that the iodination of insulin is not random. Insulin which has one iodine substituted probably becomes more susceptible to substitution by a second iodine, because of distortions imposed by the initial substitution. Evidence that some altered forms of insulin do not aggregate has been obtained by Mercola, Morris, Arquilla, and Bromer (33). The iodine substitution onto the A chain results in a significant distortion of surface conformation of iodoinsulin so that many antibodies cannot effectively bind with it. Although iodoinsulin probably aggregates with unlabeled insulin in solution, it is reasonable to assume that unlabeled insulin will associate to a much greater degree. Consequently, as insulin becomes iodinated, more of the iodinated forms will be present as monomers and dimers and therefore subject to further iodination. Results consistent with the postulate that the A-19 tyrosine is labeled first and then the A-14 is preferentially iodinated have been presented recently by Rosa, Massaglia, Pennisi, Cozzani, and Rossi (34).

The observations presented here suggest that the integrity of the C terminal portion of the A chain is necessary for biological activity. Using other derivatives of insulin with known alterations, we

have been able to show that the C terminal A chain is in close proximity to the C terminal B chain (35), thus forming a core containing six of the seven aromatic residues present in insulin. Alterations of the molecule affecting this core result in profound loss of biological activity and marked conformational changes (33).

In view of these findings, it would be surprising if any iodinated form of insulin made by iodine substitution on the tyrosine residues would be biologically active. In this regard, it might be more advantageous to prepare labeled insulins with substitutions to the N terminal phenylalanine. It has for example, been possible to prepare homogeneous insulin derivatives of 50% biological activity by substituting fluorescein isothiocyanate to the B-1 phenylalanine (36).

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