Receptor Binding Properties of Monoiodotyrosyl Insulin Isomers Purified by High Performance Liquid Chromatography

BRUCE H. FRANK, DANIEL E. PEAVY, CAROL S. HOOKER, AND WILLIAM C. DUCKWORTH

SUMMARY

The four monoiodinated forms of pork insulin were prepared by lactoperoxidase catalyzed iodination followed by separation on reverse-phase high-performance liquid chromatography. The procedure was rapid and extremely reproducible, resulting in total separation of the B16, B26, and A14 monoiodinated insulins from one another and from unreacted insulin. The A19 derivative eluted closely to the unreacted insulin, requiring an additional step on ion exchange chromatography for complete purification of this derivative. The receptor binding properties of the derivatives were examined in isolated rat adipocytes and hepatocytes and in cultured human IM-9 lymphocytes. The relative binding affinities of the derivatives varied in the different cell types. At tracer concentrations, the B26 derivative had the highest apparent affinity in IM-9 lymphocytes and in adipocytes, but the A14 had the greatest affinity in hepatocytes. At equivalent concentration of each tracer, IM-9 lymphocytes bound the B26 isomer 144 ± 9% ($P < 0.05$) and the B16 isomer 118 ± 3% ($P < 0.05$) as well as the ^{125}I (A14) insulin. Isolated rat adipocytes also bound the B26 to a greater extent at tracer concentrations (117 ± 6%; $P < 0.01$) compared with A14, but bound B16 less than A14 (91 ± 2%; $P < 0.01$). Isolated rat hepatocytes bound more of the A14 derivative than either of the B-chain labels. The B26 bound 72 ± 2% ($P < 0.01$) and the B16 90 ± 1% ($P < 0.01$) as well as the A14 derivative to isolated hepatocytes.

In contrast to the results obtained at tracer concentrations of the isomers, higher concentrations of native insulin were required for 50% competitive displacement (ED_{50}) of the B26 derivative as compared with the other two derivatives in all three cell types examined. These results indicate a higher average affinity for the B26 isomer in the three tissues, and suggest that results obtained from tracer binding alone may not provide an accurate reflection of receptor affinity for the monoiodoinsulin isomers.

Competitive displacement of the ^{125}I-labeled derivatives by ^{127}I (nonradioactive) labeled homologues in the IM-9 lymphocytes also showed the B26 derivative to have the highest affinity.

The present study demonstrates that monoiodoinsulin selectively labeled on each of the tyrosines can be prepared and separated on HPLC and used to examine insulin receptor binding in different cell types. Furthermore, the data demonstrate different apparent affinities of the selectively-labeled monoiodoinsulin isomers in different cell types, suggesting variability in insulin binding properties in different tissues. DIABETES 32:705–711, August 1983.

From the Veterans Administration Medical Center, the Departments of Medicine and Physiology, Indiana University School of Medicine, and the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana.

Address reprint requests to Dr. Bruce H. Frank, Biochemistry Department, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285.

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Most studies using iodinated insulin for the examination of various properties of insulin have emphasized the importance of using "monoiodinated" hormone since more heavily iodinated materials appear to have altered activity.[1] However, since insulin contains four tyrosine residues, even monoiodinated insulin preparations can be heterogeneous mixtures, and it is now quite clear that the location of the iodine affects the properties of the molecule.[2–5] In spite of this, extensive characterization of the individual single-site-iodinated insulins has not been reported, partly because of the difficulty of obtaining each isomer in a rapid and selective manner.

Earlier investigators[2–4] described a multistep procedure for isolating the four monoiodinated tyrosine forms (A14, A19, B16, and B26) of insulin and reported a limited evaluation of the binding and biologic activity of these materials. Their isolation procedure consisted of a laborious combination of ion exchange chromatography and polyacrylamide gel electrophoresis. Our laboratory has been evaluating the potential of reverse phase high performance liquid chromatography (HPLC) as a more rapid and generally applicable method

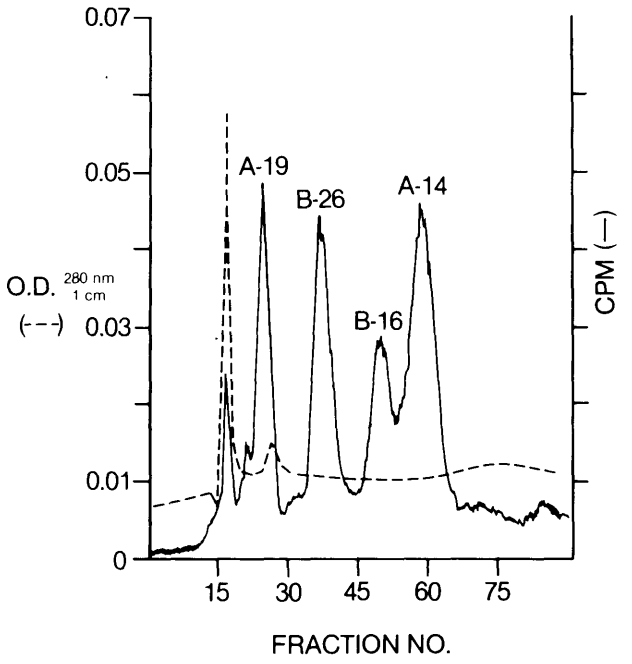

FIGURE 1. The HPLC elution of an iodination mixture of porcine insulin (see text for details of reaction and chromatography conditions). The identity of each of the monoiodinated insulin forms is noted. Unlabeled insulin elutes over fractions 24–31 as shown in the O.D. profile. The O.D. recorder is initially and deliberately offset 0.01 O.D. units for illustrative purposes and ease of chart reading.

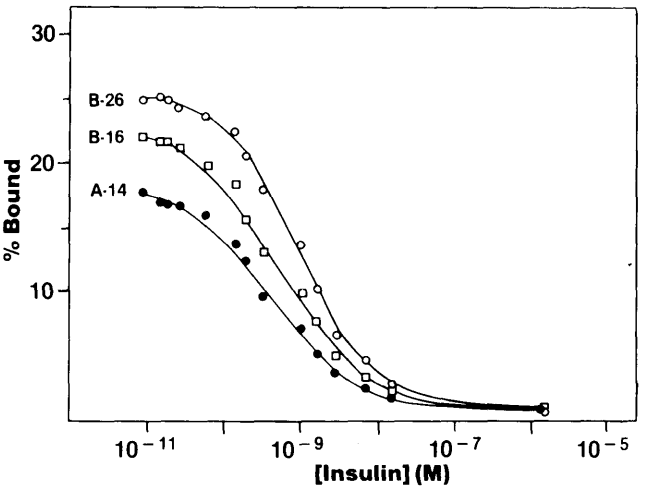

FIGURE 2. Competitive binding of the ^{125}I-monoiodo insulin isomers to IM-9 lymphocytes in the presence of increasing concentrations of native pork insulin. IM-9 lymphocytes were incubated for 2 h at 15°C with tracer concentrations (approximately 2×10^{-11} M) of the three indicated ^{125}I-labeled derivatives either in the absence or in the presence of native pork insulin at the indicated concentrations. The amount of cell-associated radioactivity was then determined as described in MATERIALS AND METHODS and is expressed as a percent of the total radioactivity present. Results shown are the mean of three experiments in which individual determinations were made in triplicate. At tracer concentrations the percent specifically bound for B26, B16, and A14 was 25.0 ± 1.6%, 20.4 ± 0.5%, and 17.3 ± 1.5%, respectively.

for isolating radiotracers of peptide hormones, including insulin.[6] The present report clearly demonstrates that the reverse phase HPLC technique can be used as a single isolation step to rapidly and selectively prepare each of the monoiodotyrosine forms of insulin. In addition, this separation can be accomplished directly from the iodination reaction mixture. The tracers are quite stable and in general are of the highest possible specific activity. The binding characteristics of these tracers have been evaluated extensively and compared with the more limited earlier results obtained on these tracers derived by the previous isolation methodology.

MATERIALS AND METHODS

The porcine insulin was kindly provided by Dr. Ronald E. Chance of the Eli Lilly Research Laboratories (Indianapolis, Indiana). The Na^{125}I (IMS 300) was purchased from Amersham. Lactoperoxidase was obtained from Calbiochem (La Jolla, California), and crystalline human serum albumin was purchased from Sigma Chemical Company (St. Louis, Missouri). All other reagents were either analytical or HPLC grade and were used without further purification.

The iodination of the insulin was performed using the following procedure. All solutions of the reagents were freshly prepared. A 100-μl aliquot of a 1 mg/ml solution of pork insulin in 0.06 M phosphate, pH 7.0 buffer was placed in a 1.5 ml snap-cap polyethylene vial. To this, 100 μl of a 7.5 M urea solution was added, followed by the addition of 4 mCi (approximately 5 μl) of Na^{125}I. Then 10 μl of a 25 U/ml solution of lactoperoxidase in 0.06 M phosphate, pH 7.0 buffer was added. To initiate the reaction, 10 μl of a fresh 1:1000 dilution of 3% H_2O_2 in 0.06 M phosphate, pH 7.0 buffer was added. The reaction was allowed to occur for 30 min at room temperature (approximately 22°C). To terminate the reaction, 700 μl of 7.0 M urea, pH 7.0 was added. This entire solution was immediately applied, via a 1.0-ml sample loop on a three-way slider injector valve, to the reverse phase HPLC column. The entire procedure, including the collection of fractions, was carried out in a radiochemical safety hood.

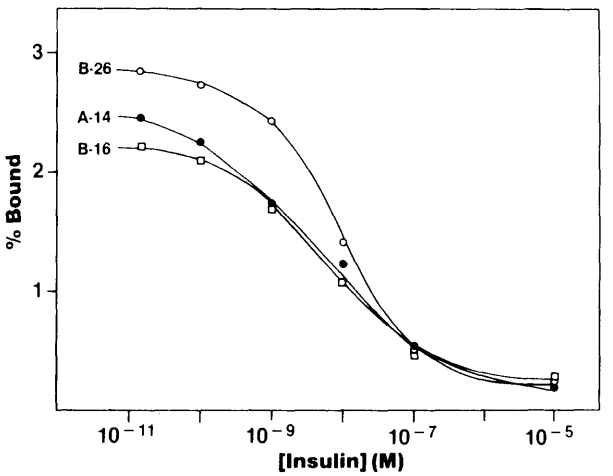

FIGURE 3. Binding of ^{125}I-monoiodo insulin isomers to isolated rat adipocytes in the presence of increasing concentrations of native pork insulin. Isolated fat cells were incubated for 2 h at 15°C with various concentrations of native pork insulin. ^{125}I-insulin binding was determined as described in MATERIALS AND METHODS. Results shown are the mean of three experiments in which individual determinations were made in triplicate. At tracer concentrations the percent specifically bound for B26, B16, and A14 was 2.46 ± 0.13%, 1.92 ± 0.05%, and 2.10 ± 0.07%, respectively.

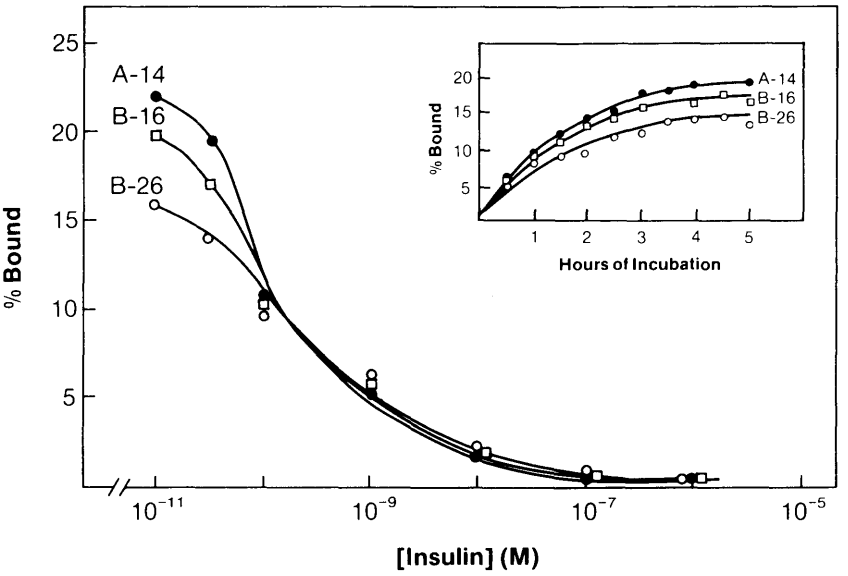

FIGURE 4. Competitive binding of ^{125}I-monoiodoinsulin isomers to isolated rat hepatocytes in the presence of increasing concentrations of native pork insulin. Isolated hepatocytes were incubated for 4 h at 15°C (pH 7.2–7.3) with each of the ^{125}I-labeled insulins and varying concentrations of native pork insulin. ^{125}I-insulin binding was determined as described in MATERIALS AND METHODS. Results shown are the mean from three experiments in which individual determinations were made in duplicate or triplicate. At tracer concentrations the percent specifically bound for B26, B16, and A14 was 15.4 ± 0.5%, 19.3 ± 0.3%, and 21.6 ± 0.7%, respectively. The time course for specific binding is shown in the inset.

The reverse phase solid support was a LP-1/C_{18} silica gel (10–20 μm) that had been prepared by reacting the silica gel with octadecyl trichlorosilane. Excess free hydroxyl groups had been capped with trimethylsilyl groups. The final carbon content of the support was 20.6%. The details of the preparation of this solid support have been described elsewhere.[7] The 1.2-×-50-cm refillable glass columns (Michel-Miller) with teflon end-fittings were purchased from the Ace Glass Company (Vineland, New Jersey). The method for packing the support into the columns was provided by the column manufacturer. The solvents were pumped through the columns using an FMI (Fluid Metering, Inc., Oyster Bay, New York) explosion-proof lab pump at back pressures of 80–120 psi and flow rates of approximately 2 ml/min. The samples were applied to the column using a 1-ml sample loop attached to a slide-injection valve (Altex Scientific, Inc., Berkeley, California). The samples were eluted isocratically using a degassed solvent of 28.0% CH_3CN in 0.2 M NH_4 OAc, pH 5.5 at room temperature. The column eluate was monitored for uv absorption at 280 nm using an ISCO UA-5 monitor, and for radioactivity using a Baird-Atomic ratemeter with a JP-200 Johnson probe. Both of these outputs were recorded simultaneously on a three-channel strip-chart recorder. The eluate was collected in 16-×-100-mm polystyrene tubes in an ISCO fraction collector (Model 328). One-minute fractions were collected. The appropriate fractions were chosen based on the uv and CPM profiles, and these fractions pooled. Total recovery of radioactivity from the column was greater than 95%. The fraction pools were each then diluted with an equal volume of a 0.1% human serum albumin in 0.1 M phosphate—0.05 M NaCl, pH 7.5 buffer, and stored at 4°C. A second dilution of the stored material with four times the volume of the albumin-PBS buffer, was usually made before use. This second dilution yielded a solution with approximately 10 μCi/ml. The trichloracetic acid precipitability of the labeled derivatives was routinely 98% or greater.

Details of the preparation of the ^{127}I analogs and the physicochemical characteristics of these compounds will be described elsewhere. In general terms, the iodination mixture was first subjected to chromatography on DEAE-Cellulose[8] to remove unreacted insulin, and the material subsequently purified on preparative reverse-phase HPLC as described above.

Isolated hepatocytes were prepared from male Sprague-Dawley rats (150–200 g) by a modification of the method of

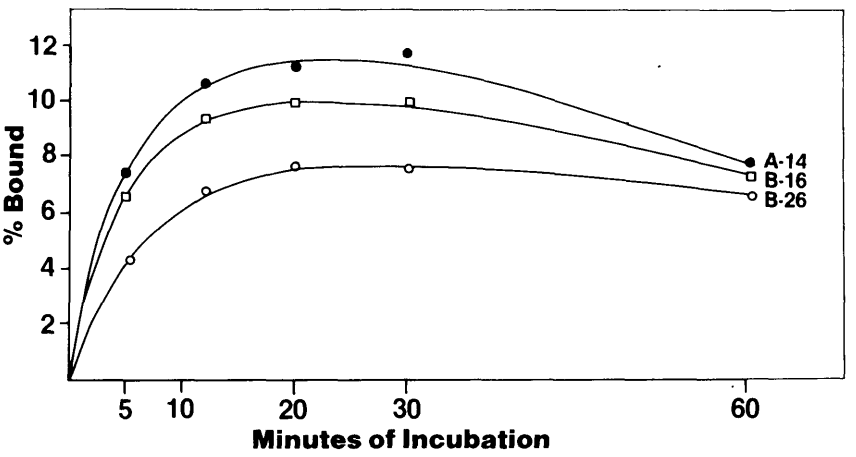

FIGURE 5. Time course of specific binding of the ^{125}I-insulin isomers to hepatocytes incubated at 37°C. Isolated hepatocytes were incubated with equal tracer concentrations (approximately 2×10^{-11} M) of each of the labeled insulin isomers and at the times indicated samples were taken to determine the amount of cell-associated radioactivity ("binding"). Results have been corrected for nonspecific binding determined in the presence of 10^{-5} M native insulin and are the mean of three experiments in which individual determinations were made in triplicate.

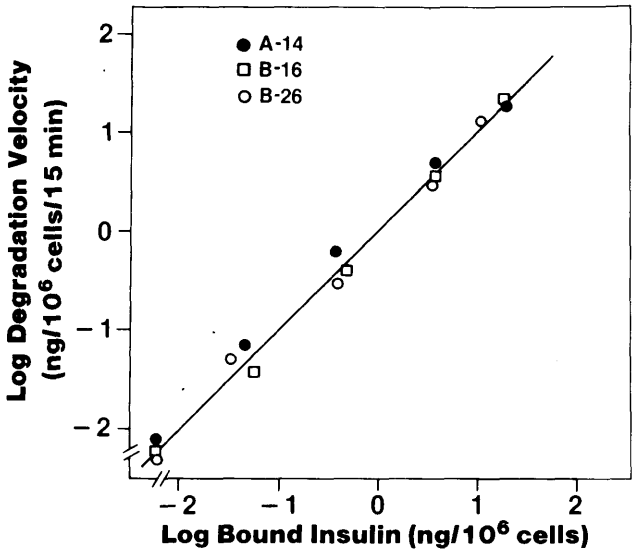

FIGURE 6. Relationship between specific binding and degradation of the ^{125}I-insulin derivatives in hepatocytes incubated at 37°C. Isolated hepatocytes were incubated for 15 min at 37°C with each of the ^{125}I-insulins and varying concentrations of native insulin. Binding and degradation were determined as described in MATERIALS AND METHODS.

Berry and Friend,[9] as described previously.[10] Approximately 10^6 cells were incubated in 1 ml of Krebs Improved Ringer II buffer containing 3% bovine serum albumin (Cohn Fraction V, Miles Laboratories, Elkhart, Indiana) and 27.7 mM glucose. Incubations were carried out in 20-ml polyethylene vials which were thoroughly flushed with 95%:5%, O_2:CO_2, sealed prior to the incubation period, and were incubated in a reciprocal shaking water bath at a rate of 90/min. After incubation with the individual ^{125}I-insulins for the times indicated in the figure legends, a sample of the cell suspension (100 μl) was taken and the cells rapidly separated from the

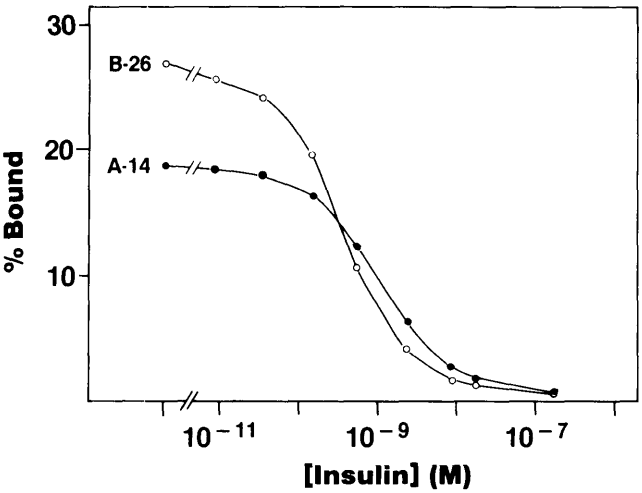

FIGURE 7. Competitive binding of ^{125}I-monoiodo insulin to IM-9 lymphocytes in the presence of increasing concentrations of the homologous ^{127}I derivative. IM-9 lymphocytes were incubated for 2 h at 15°C with tracer concentrations of each ^{125}I-insulin and increasing concentrations of the homologous ^{127}I-insulin derivative. Binding was determined as described under MATERIALS AND METHODS and the results shown are the mean of three experiments in which individual determinations were made in triplicate.

incubation media by centrifugation through gradients consisting of 14% perchloric acid (40 μl) overlaid with silicone oil (160 μl) (density 1.02) prepared in microfuge tubes.[11] After 1 min of centrifugation in a Beckman microfuge, a sample of the incubation medium was removed from the top of the gradient to determine the integrity of the ^{125}I-insulin by precipitation with 10% TCA, and the perchloric acid layer including the cell pellet, was taken for the determination of cell-associated radioactivity.

Isolated fat cells were prepared by a modification[12] of the method of Rodbell.[13] All incubations were in Krebs-Ringer-Hepes (KRH) buffer, pH 7.4, with 4% BSA in a total volume of 2 ml. The fat cells were incubated with ^{125}I-labeled insulin ($1–2 \times 10^{-11}$ M) at 15°C for 2 h. At the indicated times triplicate 300-μl aliquots were removed and added to microfuge tubes (Beckman Instruments, Palo Alto, California) containing 100 μl dinonyl phthalate.[14] After centrifugation for 1 min in a Beckman microfuge, the tubes were cut through the oil layer and the cell pellet counted in a gamma counter for determination of binding. Degradation of the ^{125}I-labeled insulin was determined by adding the buffer layer from the microfuge tube to ice-cold KRH buffer followed immediately by sufficient trichloracetic acid to give a final concentration of 5%.[15]

IM-9 cells were grown in RPMI media containing 2 mM glutamine, 25 mM HEPES, and 10% fetal bovine serum. Cells were harvested by centrifugation, washed, and resuspended in HEPES assay buffer, pH 7.6.[16] Cell viability, determined by exclusion of trypan blue, was greater than 90% in each experiment. Triplicate tubes were prepared containing 100 μl assay buffer, standard, or analog, 200 μl ^{125}I-insulin, (final concentration $1–2 \times 10^{-11}$ M), and 200 μl cells (500,000 cells). Incubations were carried out in 1.5 ml microfuge tubes at 15°C for 2 h. Concentrations of ^{127}I-insulin stock solutions used in the binding studies were established by amino acid analysis and by their absorbance at 276 nm. The cells were resuspended during the assay every 30 min by inverting the tubes several times. At the end of the incubation, the tubes were centrifuged for 1 min in a Beckman Microfuge, the supernatant aspirated, the tips of the tubes containing the cell pellet were excised, and the radioactivity measured.

ED_{50} values, maximal binding, and minimal (nonspecific) binding were determined using the PREFIT and ALLFIT programs[17] based on the four-parameter logistic model. Specific binding was determined by subtracting nonspecific from total binding. A one-way analysis of variance followed by the Student-Newman-Keuls multiple range test was used for comparison of sample means, which are presented as the mean ± SE.

RESULTS

The four monoiodinated forms of pork insulin were prepared by lactoperoxidase catalyzed iodination followed by chromatography using reverse phase HPLC (Figure 1). This procedure was rapid and extremely reproducible. The identity of each of the peaks as the monoiodinated insulin derivatives (either on A14, A19, B16, or B26 tyrosine) was established by performing amino acid sequence analysis[18] on each peak. The separation of the B16, B26, and A14 monoiodinated insulins from one another and from unreacted insulin was accomplished very efficiently in our HPLC system. The A19

derivative unfortunately eluted very close to the unreacted insulin. This fact made the absolute purity of the A19, ^{125}I tracer questionable. However, since earlier studies[2,19] had demonstrated that this derivative was quite different from native insulin, extensive studies were not performed with this particular tracer preparation. However, when either pure ^{125}I-Tyr A19-insulin or ^{127}I-Tyr A19-insulin were desired, we first subjected the iodination mixture to DEAE-Cellulose chromatography[8] to remove the unreacted insulin, and then performed a final purification by reverse-phase HPLC. Polyacrylamide gel electrophoresis of each of the monoiodinated insulin forms demonstrated the absence of unreacted insulin (sensitivity of procedure would have detected 0.5–1.0% unreacted insulin).

The presence of the urea in the reaction mixture should be noted since this resulted in greater labeling of the B-chain tyrosine residues relative to the A-chain tyrosine residues.[3] For example, in the absence of the urea, about 10–15% of the iodine was incorporated into the B-chain with the remainder of the iodine in the A-chain. In the presence of urea, approximately 40–45% of the iodine was incorporated into the B-chain residues with the remainder in the A-chain. Usually, greater than 90% of the iodine introduced into the reaction mixture was incorporated into the insulin. This occurred primarily because of the excess of insulin used. In addition, very little diiodinated insulin was observed under our reaction conditions. Finally, any unreacted iodine was separated on the HPLC column—the iodine eluted at the break through volume of the column (see peak at approximately fraction 17 in Figure 1).

The purity of the tracer materials was verified by two procedures in addition to the polyacrylamide gel method described above. First, each isolated peak was subjected to rechromatography on analytical HPLC and each yielded a single symmetrical peak of radioactivity that eluted at the appropriate volume and no O.D. (220 nm) at the elution position of insulin was observed.* Second, the binding characteristics of each peak were compared both before and after rechromatography on the glass column and no change in the results was observed. Based on these data, we concluded that these tracers were free from contamination with unlabeled insulin and that each represented a single-site monoiodinated form of insulin. The specific activity of these tracers was usually 300–340 μCi/μg of insulin. Thus they provided us with essentially pure tracers to undertake the studies described below. Four separate preparations of ^{125}I-labeled derivatives were used for these studies.

Binding of the ^{125}I-labeled isomers to IM-9 lymphocytes and the competitive displacement of each isomer by native pork insulin are shown in Figure 2. Relative to binding of the A14 isomer, both B-chain-labeled insulins were bound to a significantly greater extent. At equivalent concentrations of each tracer, the B26 isomer-bound 144 ± 9% ($P < 0.05$) and the B16 isomer 118 ± 3% ($P < 0.05$) as well as the ^{125}I (A14)-insulin. Relative apparent binding affinities, based on the concentration of native insulin required to prevent 50% of the tracer binding (ED_{50}) also indicated higher average relative affinities of the B-chain-labeled derivatives as compared with the A14 isomer. The ED_{50} for the A14 label was 6.3×10^{-10} M. Relative to A14-labeled material values of 203 ± 30% ($P < 0.05$) and 126 ± 20% (P = NS) were obtained for the B26 and B16 isomers, respectively.

Similar experiments were also conducted using isolated rat adipocytes (Figure 3). Again, at tracer concentrations, a significantly greater percent of ^{125}I (B26)-insulin (117 ± 6%; $P < 0.01$) was bound as compared with A14 and a higher average affinity (114% compared with A14) was indicated by the ED_{50} although this did not reach statistical significance. The ED_{50} value for A14 was 2.9×10^{-9} M. Tracer binding of the B16 isomer was less (91 ± 2%; $P < 0.01$) than A14 tracer binding and the relative affinity was similarly reduced (91%).

In contrast with the other two cell types, isolated rat hepatocytes bound significantly more of the A14 derivative at tracer concentrations than either of the two B-chain-labeled insulins (Figure 4). The B26-labeled material bound only 72 ± 2% ($P < 0.01$) as well, and the B16 isomer 90 ± 1% ($P < 0.01$) as well as the A14 derivative. In contrast, however, the competition curves indicated that a higher native insulin concentration was required to displace 50% of the B-chain-labeled materials as compared with the A14 isomer. The ED_{50} for A14 was $1.08 \pm 0.16 \times 10^{-10}$ M, for B16 was $1.52 \pm 0.22 \times 10^{-10}$ M, and for B26 was $3.95 \pm 0.65 \times 10^{-10}$ M ($P < 0.01$ for B26 versus either A14 or B16; P = NS for A14 versus B16).

Since hepatocytes are active in the uptake and degradation of insulin, experiments also were conducted at 37°C to examine these processes. Figure 5 shows the time course of binding of tracer concentrations of the isomers at 37°C. At all time points, the amount of cell-associated radioactivity was greatest with the A14-labeled material and somewhat less with the B16 and B26 isomers. Degradation of the isomers was also measured and the degradation closely paralleled the binding. After an initial 5-min lag phase, degradation of each of the isomers was linear and Figure 6 shows the relationship between binding and degradation over a wide concentration range, demonstrating a linear relationship between degradation velocity and cell-associated radioactivity for each of the ^{125}I-isomers.

To obtain more information about the binding characteristics of the isomers, nonradioactive ^{127}I-labeled insulin was prepared and the specific monoiodo isomers were isolated. Figure 7 shows the binding of the ^{125}I-labeled tracers and displacement by the homologous ^{127}I-labeled isomer, using the IM-9 lymphocyte. Consistent with the experiments shown in Figure 2, again at tracer concentrations the B26-labeled (148%) and the B16-labeled (125%) materials bound better than the A14. The ED_{50} for the ^{127}I (A14) analogue competitively displacing ^{125}I (A14) iodoinsulin was $0.95 \pm 0.08 \times 10^{-9}$ M, and for ^{127}I (B26) displacing ^{125}I (B26) iodoinsulin was $0.32 \pm 0.02 \times 10^{-9}$ M. The difference was significant at the 0.01 level.

DISCUSSION

With the procedure described here, monoiodinated insulin can be prepared and separated into the four isomers by a single-step isolation procedure. Three of the isomers can be obtained with no contamination with unlabeled insulin or with other iodinated species. The A19 isomer eluted from the

*The commercial analytical HPLC system used for these analyses is described in reference 30.

HPLC column too close to native insulin for complete separation, but with a preliminary step of ion exchange chromatography this isomer could also be obtained in pure form. Since the A19 isomer has been shown to exhibit altered properties, with decreased binding and biologic activity,[2,19] we have not done extensive studies with this isomer, but have concentrated on the A14 and the two B-chain-labeled materials and have examined binding to various tissues. In studies with the A19 isomer, however, we consistently found a binding potency of 50–60% of that of the A14 derivative in all cell systems examined (data not shown).

Insulin monoiodinated on either of the tyrosines of the B chain bound to the insulin receptor in spite of the fact that these residues are believed to be close to or within the binding site.[20] In fact, the B26-labeled isomer bound better to receptors of IM-9 lymphocytes and of rat adipocytes than did the A14 isomer, which has been reported previously to have the same binding potency and biological activity as native insulin in adipocytes.[19] This would suggest that the B26 isomer has a higher affinity for the insulin receptor than does native insulin. Since the B26 residue is in the region of the molecule which is thought to be involved in both receptor binding and cooperative interactions,[20,21] alterations in either or both of these properties might explain the apparent increased affinity for binding.

Of considerable interest was the fact that hepatocytes bound the isomers with a different order from other cell types. In both lymphocytes and adipocytes the B26 isomer bound better at tracer concentrations than did the A14 and higher concentrations of native insulin were required for competitive displacement of the B26 than of the A14. Thus, under both conditions the B26 showed a higher affinity for the receptor than the A14. In hepatocytes, however, at tracer concentration the A14 consistently bound better than the B26, suggesting a higher affinity of the A14 for the receptor.[3] In contrast, the amount of native insulin required for 50% displacement of the tracer was higher for B26 than for A14. Examination of Figure 4 shows that the competitive displacement curves in the hepatocytes are not parallel but rather the A14 curve actually crosses over the curves for the B-chain-labeled isomers thus supporting the concept that insulin receptor interaction cannot be described by a simple competitive model.

The explanation for the differences between hepatocytes and the other cell types is not immediately apparent. Several possibilities exist. One possibility is that the hepatocyte receptor has different properties from receptors in adipocytes or cultured lymphocytes. The difference could be due to an intrinsic difference in the receptor, e.g., variations in structure, or to extrinsic influences, e.g., lipid environment of the membrane.[22] Differences between hepatocytes and adipocytes in the structural organization of the receptor have been reported previously.[23]

Another possibility is that more than one type of insulin receptor exists, as has been suggested by several studies.[24–26] If this is the case, the relative proportions of receptor types could vary from tissue to tissue. If the iodinated analogues had differential affinities for different types of receptors, it could explain the results obtained in these experiments. This seems to be the most likely explanation for the observed differences between tracer binding and competition curves for A14 and B26 in hepatocytes. This interpretation would imply a high affinity, low-capacity site which preferentially binds A14 at tracer concentrations and a lower affinity, higher capacity site with which the B26 is interacting at higher concentrations. Differences in binding of A14-labeled insulin to adipocytes and hepatocytes have been reported previously.[3] In a study using tracer concentrations only, Linde et al. found that B26, relative to A14, bound better to adipocytes than to hepatocytes. Competition curves were not reported.

Since liver has a major function of clearing and degrading insulin as well as responding to the hormone,[27] the observed differences between tissues could be related to this function in some manner. Our binding studies were done at low temperature to minimize internalization and degradation and thus differences in binding potencies were presumably not due to these processes. When examined at 37°C, the relative "binding" potency (actually cell-associated radioactivity) remained the same in the hepatocyte with A14 bound the most at tracer concentrations. Relative degradation rates were consistent with the binding potency, as would be expected since degradation is a receptor-linked process,[28] and degradation velocity was linearly related to binding over a wide range of concentrations. The B-chain-labeled isomers will therefore be of use in examining cellular processing and degradation of insulin and in following the fate of the B chain during cellular metabolism of the hormone.

The biologic activity of the iodinated isomers is of obvious interest. In preliminary studies the biologic activity in the adipocyte correlated well with receptor binding, with the B26 exhibiting the highest biologic activity (data not shown). Additional studies of the other isomers and comparisons with activity in the hepatocyte are in progress.

In conclusion, we have demonstrated that our HPLC isolation method will yield, in a rapid, reproducible and selective manner, single-site carrier-free tracers whose binding and biologic properties can be readily evaluated. Moreover, studies involving the evaluation of the binding characteristics of insulin analogs can be performed using the same single-site tracer of both the analog and insulin, thereby yielding a more cogent comparison of results.[29]

ACKNOWLEDGMENTS

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REFERENCES

[1] Sodoyez, J. C., Sodoyez-Goffaux, F., Goff, M. M., Zimmerman, A. E., and Arquilla, E. F.: [^{127}I] or carrier-free [^{125}I] monoiodoinsulin. Preparation, physical, immunological, and biological properties, and susceptibility to "insulinase" degradation. J. Biol. Chem. 1975; 250:4268–77.

[2] Linde, S., and Hansen B.: Monoiodoinsulin specifically substituted in Tyr A14 or Tyr 19. Int. J. Peptide Protein Res. 1980; 15:495–502.

[3] Linde, S., Sonne, O., Hansen, B., and Gliemann, J.: Monoiodoinsulin labelled in tyrosine residue 16 or 26 of the insulin-B-Chain. Hoppe-Seyler's Z. Physiol. Chem. 1981; 362:573–79.

[4] Linde, S., Hansen, B., Sonne, O., Holst, J. J., and Gliemann, J.: Tyrosine A14 [^{125}I] monoiodoinsulin preparation, biologic properties, and long-term stability. Diabetes 1981; 30:1–8.

[5] Peavy, D. E., Hooker, C., Frank, B. H., and Duckworth, W. C.: Receptor binding of mono-iodo 125-I-Insulin specifically labeled at each of the tyrosine residues in insulin. Fed. Proc. 1982; 41:1087A.

[6] Frank, B. H.: HPLC preparation of high specific activity ^{125}I-labels of insulin, proinsulin and other polypeptide hormones. Diabetes 1980; 29 (Suppl. 2):106A.

[7] Gesellchen, P. D., Tafur, S., and Shields, J. E.: Peptides, structure and biological function. Proc. 6th Amer. Peptide Symp. Gross, E., and Meienhofer, J., Eds. Rockford, Illinois, Pierce Chemical Co., 1979:117–20.

[8] Chance, R. E.: Amino acid sequences of proinsulins and intermediates. Diabetes 1972; 21 (Suppl. 2):1461–67.

[9] Berry, M. N., and Friend, D. S.: High-yield preparation of isolated rat liver parenchymal cells. A biochemical and fine structural study. J. Cell Biol. 1969; 43:506–20.

[10] Peavy, D. E., Taylor, J. M., and Jefferson, L. S.: Alteration in albumin secretion and total protein synthesis in livers of thyroidectomized rats. Biochem. J. 1981; 198:289–99.

[11] Hoek, J. B., Nicholls, D. G., and Williamson, J. R.: Determination of the mitochondrial proton motive force in isolated hepatocytes. J. Biol. Chem. 1980; 255:1458–64.

[12] Huber, C. T., Solomon, S. S., and Duckworth, W. C.: The time course of insulin degradation in the perifused isolated fat cell. J. Clin Invest. 1980; 65:461–68.

[13] Rodbell, M.: Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. J. Biol. Chem. 1964; 239:375–80.

[14] Gliemann, J., Osterlind, K., Vinten, J., and Gammeltoft, S.: A procedure for measurement of distribution spaces in isolated fat cells. Biochem. Biophys. Acta 1972; 286:1–9.

[15] Duckworth, W. C.: Insulin degradation by liver cell membranes. Endocrinology 1979; 104:1758–63.

[16] DeMeyts, P.: Insulin and growth hormone receptors in human cultured lymphocytes and peripheral monocytes. Blecher, M., Ed. New York, Marcel Dekker, Inc., 1976:301–30.

[17] De Lean, A., Munson, P. J., and Rodbard, D.: Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. Am. J. Physiol. 1978; 235:E97–102.

[18] Chan, S. J., Weiss, J., Konrad, M., White, T., Bahl, C., Yu, S.-D., Marks, D., and Steiner, D. F.: Biosynthesis and periplasmic segregation of human proinsulin in Escherichia coli. Proc. Natl. Acad. Sci. USA 1981; 78:5401–5405.

[19] Gliemann, J., Sonne, O., Linde, S., and Hansen, B.: Biological potency and binding affinity of monoiodoinsulin with iodine in tyrosine A14 or tyrosine A19. Biochem. Biophys. Res. Commun. 1979; 87:1183–90.

[20] Pullen, R. A., Lindsay, D. G., Wood, S. P., Tickle, I. J., Blundell, T. L., Wollmer, A., Krail, G., Brandenburg, D., Zahn, H., Gliemann, J., and Gammeltoft, S.: On the receptor binding region of insulin. Nature 1976; 259:369–73.

[21] DeMeyts, P., Van Obberghen, E., Roth, J., Woomer, A., and Brandenburg, D.: Mapping of the residues responsible for the negative cooperativity of the receptor binding region of insulin. Nature 1978; 273:504–509.

[22] Ginsberg, B. H., Brown, T. J., Simon, I., and Spector, A. A.: Effect of the membrane lipid environment on the properties of insulin receptor. Diabetes 1981; 30:773–80.

[23] Jarett, L., Schweitzer, J. B., and Smith, R. M.: Insulin receptor differences in structural organization on adipocyte and liver plasma membranes. Science 1980; 210:1127–28.

[24] Pollet, R. J., Standaert, M. L., and Haase, B. A.: Insulin binding to the human lymphocyte receptor. Evaluation of the negative cooperativity model. J. Biol. Chem. 1977; 252:5828–34.

[25] Olefsky, J. M., and Chang, M. S.: Insulin binding to adipocytes, evidence for functionally distinct receptors. Diabetes 1978; 27:946–48.

[26] Wheeler, F. B., Santora, A. C., and Elsas, L. J.: Evidence supporting a two receptor model for insulin binding by cultured embryonic heart cells. Endocrinology 1980; 107:195–207.

[27] Duckworth, W. C., and Kitabchi, A. E.: Insulin metabolism and degradation. Endocr. Rev. 1981; 2:210–33.

[28] Terris, S., and Steiner, D. F.: Binding and degradation of ^{125}I-insulin by rat hepatocytes. J. Biol. Chem. 1975; 250:8389–98.

[29] Green, A., Frank, B. H., Tager, H., and Olefsky, J. M.: Characteristics of binding of a low affinity non-cooperative insulin (Leu^{B25} Insulin) to IM-9 cultured lymphocytes. The Endocrine Society, 64th Annual Meeting. 1982:1020A.

[30] Markussen, J., and Larsen, U. D.: The application of HPLC to the analysis of radioiodinated tracers of glucagon and insulin. *In* Insulin, Chemistry, Structure and Function of Insulin and Related Hormones. Brandenburg, D., and Wollmer, A., Eds. New York, Walter de Gruyter, 1980:161–68.