# MONOIODOINSULIN: DEMONSTRATION OF ITS BIOLOGICAL ACTIVITY AND BINDING TO FAT CELLS AND LIVER MEMBRANES

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SUMMARY - A method is described for the preparation of monoiodoinsulin that is active biologically. $^{125}$I-insulin prepared by this method was bound to isolated fat cells and to purified plasma membranes from liver. $^{125}$I-insulin binding to liver membranes was inhibited by unlabeled insulin at physiological concentrations.

The first step in the action of insulin on its target tissues is binding of the hormone to a specific receptor on the plasma membrane of the target cell. Using iodo-ACTH and subcellular fractions from the adrenal, we have previously studied directly the interaction of ACTH with its biologically important receptors (1). In studies of this type, it is essential that the iodo-hormone retain biological activity. Because of controversy about the biological activity of iodo-insulin, we prepared pure monoiodoinsulin and demonstrated that monoiodoinsulin retains full biological activity. We also prepared iodoinsulin at high specific radioactivity that binds to isolated fat cells and to liver plasma membranes.

Purification of insulin - For iodination, porcine insulin (crystalline zinc, 24.4 U/mg, Elanco) was filtered on G-50 medium Sephadex (Pharmacia) in 1 M acetic acid to remove proinsulin-like components (2). The major peak was purified further (3) by gradient chromatography on diethylaminoethylcellulose (DEAE) (see legend to Fig. 1). Effluent fractions representing the major peak of $A_{280}$ were combined,

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dialyzed, lyophilized, and stored at -15° until use.

Iodination of Insulin - In order to minimize the introduction of more than one atom of I per insulin molecule, iodination was performed with [I]/[insulin] in molar equivalents of 1/10. To minimize the deleterious effects of the oxidizing agent, chloramine T (4), chloramine T was approximately equimolar with $I^-$. To obtain large quantities of iodoinsulin for chemical and biological analysis, milligrams of purified insulin were iodinated with $^{127}I$ to which was added a trace of $^{125}I$. After each addition of chloramine T an aliquot of the iodination solution was added to 10% trichloracetic acid (TCA) to measure the progress of iodination. At the completion of iodination, the degree of iodination was confirmed by chromatoelectrophoresis (5). Typically, the iodination mixture contained, in a total volume of 5 ml of 0.02 M Tris-HCl buffer, pH 8.6, 1.5 x $10^{-3}$M insulin, 0.18 x $10^{-3}$M KI, 5 µCi of Na $^{125}I$ (Union Carbide) and 0.08 x $10^{-3}$M chloramine T. With two further additions of chloramine T, 75% of the radioactivity was bound to protein. Thus, one would predict that 9% of the insulin molecules had been converted to monoiodoinsulin that had a specific radioactivity of 0.92 µCi/mg.

Separation of $^{127}I$-insulin from insulin - Iodoinsulin (I-insulin) was separated from uniodinated insulin by gradient chromatography on DEAE-cellulose (see Fig. 1 legend). Two major peaks were obtained (Fig. 1). Peak 1 contained 72% of the absorbance at 276 mµ ($A_{276}$) and 75% of the insulin immunoreactivity applied, but less than 3% of the protein-bound radioactivity. Peak 2 contained 75% of the protein-bound radioactivity applied but only 8% of the $A_{276}$ and 7.5% of the immunoreactivity. Thus, of the total material recovered in the two major peaks, peak 2 had 96% of the protein-bound radioactivity, 9-10% of the insulin and a specific radioactivity of 0.80 µCi/mg, in excellent agreement with theoretical expectations of 100%, 9% and 0.92 µCi/mg, respectively.

Analysis of I-insulin - Since each insulin molecule has 4 tyrosines, pure monoiodoinsulin should have 25% of its total tyrosines as monoiodotyrosine (MIT). The iodination state of the tyrosines in each of the four parts of peak 2

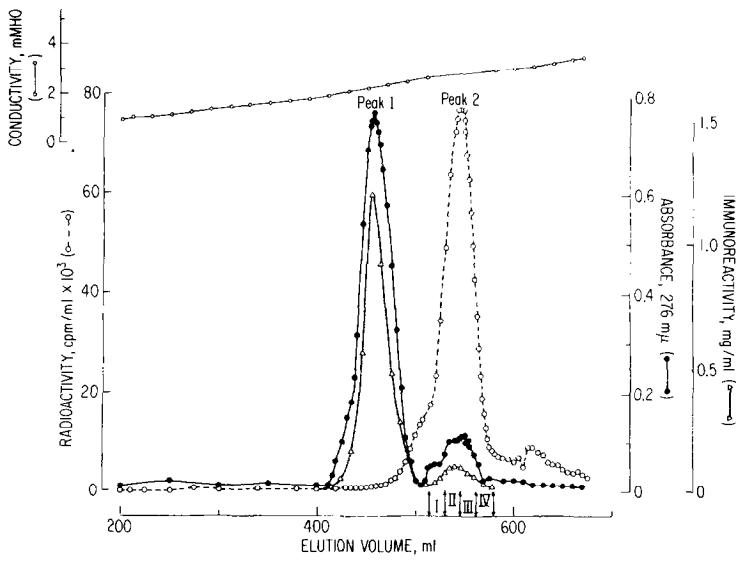


Fig. 1. Separation of $^{127}$I-insulin from uniodinated insulin. The iodination solution (see text) was applied to a 2.5 x 22 cm column of DEAE-cellulose (microgranular, Whatman DE 52, Reeve Angel) that had been equilibrated in 0.05 M Tris HCl, 7 M urea (Ultra-Pure, Mann), pH 9.4 ("starting buffer"), at 4°. After 500 ml of starting buffer had passed over the column, a gradient to 0.1 M NaCl at constant pH was applied, using a Varigrad apparatus in which successive compartments contained 100 ml of the following concentrations of NaCl in the starting buffer: starting buffer (without NaCl); 0.01 M; 0.02 M; 0.03 M; 0.04 M; 0.06 M; 0.08 M; 0.1 M; 0.1 M. Porcine $^{131}$I-insulin and guinea pig anti-porcine insulin serum (6) were used for radioimmunoassay of insulin.

Table I. SPECTRAL TITRATION OF IODOINSULIN in 7M UREA*

| Fractions (peak 2 of Fig. 1) | Δ absorbance at mμ 295 pH 9-12 | 305 8.4-10 | 325 3-9 | Percent of total tyrosines MIT | DIT |
|---|---|---|---|---|---|
| I | 0.20 | 0.07 | 0 | 17% | 0% |
| II | 0.61 | 0.29 | 0 | 22% | 0% |
| III | 0.49 | 0.19 | 0.01 | 19% | <1% |
| IV | 0.07 | 0.03 | 0.03 | 18% | 15% |

*The molar extinction coefficients used are: 2,550 for Tyrosine; 4,270 for MIT and 5,000 for DIT.

(Fractions I-IV of Fig. 1) was examined by spectral titration (7) (see Table I legend). MIT constituted 16-22% of the total tyrosines in each of the fractions and, except for fraction IV, diiodotyrosine (DIT) content was trivial (Table I).

As a whole, the tyrosines in peak 2 were 20% MIT and less than 1% DIT, confirming the conclusion, based on measurements of $A_{276}$, immunoreactivity and protein-bound radioactivity, that at least 80% of the insulin in peak 2 was iodoinsulin.

Bioactivity of monoiodoinsulin - Fraction II of peak 2, whose contamination with uniodinated insulin was 12% by spectral titration, was indistinguishable from native insulin over a 50-fold range of concentrations in its capacity to stimulate glucose oxidation by isolated fat cells (Fig. 2). To exclude the conversion of I-insulin to insulin + $I^-$ during incubation with fat cells, an aliquot of fraction II that had been incubated with fat cells was mixed with TCA;

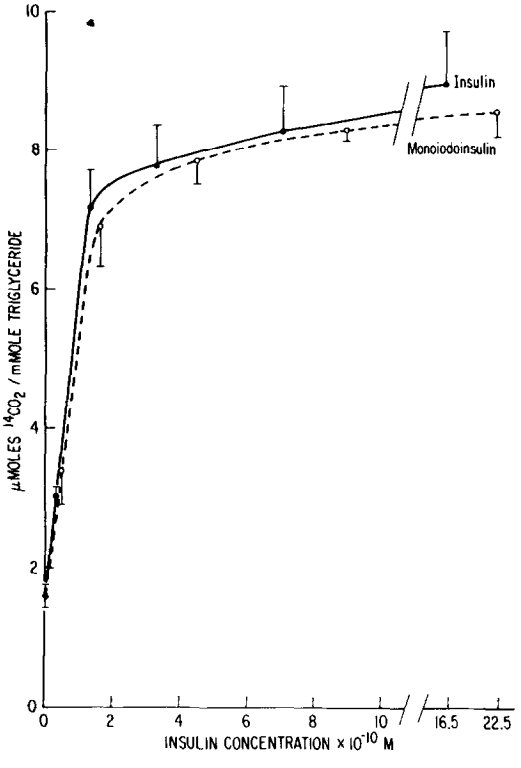


Fig. 2. Insulin bioactivity (8). Each incubation flask in a total volume of 2 ml of Krebs Ringer bicarbonate buffer adjusted to pH 7.4 under an atmosphere of 95% $O_2$ - 5% $CO_2$ contained isolated fat cells (30-40 mg), glucose (1 mM), glucose-U-$^{14}C$ (2.2 x $10^5$ dpm, New England Nuclear), and albumin (50 mg, bovine Fraction V, Armour), with and without insulin. After shaking for 2 hours at 37°, the $^{14}CO_2$ was trapped in hyamine and counted. The data are expressed as μmoles of $^{14}CO_2$ produced per mmole triglyceride (9) as a function of insulin concentration. Each point is the mean ± SEM of three determinations. The insulin was porcine insulin (Elanco). The monoiodoinsulin was Fraction II of peak 2 (Fig. 1); the insulin concentration in Fraction II was measured by immunoassay which agreed closely with data obtained by spectral analysis.

93% of the radioactivity was precipitated compared to 96% of fraction II incubated in the absence of fat cells. Thus, monoiodoinsulin retained bioactivity unaccounted for by its small content of uniodinated insulin.

Preparation of I-insulin of high specific radioactivity - $^{125}$I-insulin was prepared by the same methods as described above for mono$^{127}$I-insulin. Typically the iodination mixture contained, in a total volume of 130 µl, 5 x $10^{-4}$M insulin and 0.3 x $10^{-4}$M $^{125}$I; with one addition of 0.4 x $10^{-4}$M chloramine T, about 40% of the radioactivity was bound to insulin. On chromatography (Fig. 3) we observed

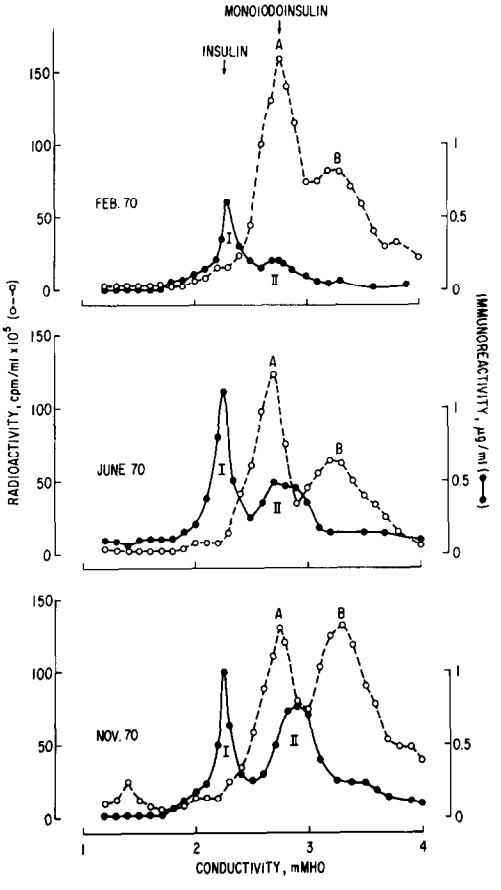


Fig. 3. Chromatography of $^{125}$I-insulin. The iodination solutions (see text) were applied to DEAE-cellulose columns. Methods were identical to those in Fig. 1, except that the columns were 0.9 x 20 cm and each Varigrad chamber contained 70 ml. The insulin used for these iodinations had been purified on DEAE-cellulose in January 1970. The two arrows at the top refer to the location of uniodinated insulin and monoiodoinsulin (peaks 1 and 2) observed in Fig. 1.

two major peaks of immunoreactivity (I and II) and two of radioactivity (A and B). Peak I and Peak A were eluted at the same ionic strength as uniodinated insulin and mono$^{127}$I-insulin, respectively (Fig. 3). Peak II and B are thought to be desamido- and iodinated desamidoinsulin, respectively; both increased with the duration of storage of the insulin prior to iodination and, in all cases, the immunoreactivity in peak I relative to that in peak II was equal to the radioactivity in peak A relative to that in peak B (Fig. 3). TCA precipitated 95% and insulin antibody bound 93% of the radioactivity in both peaks A and B. The chromatography on DEAE-cellulose increased the specific radioactivity by 3-12 fold to yield labeled hormone at 25-100 μCi/μg, which is considerably less than the 360* μCi/μg expected for mono$^{125}$I-insulin, probably because the peak II of immunoreactivity contaminated both peaks A and B (Fig. 3). Presumably, purification of the insulin immediately prior to its iodination will yield $^{125}$I-insulin at higher specific activities.

Binding to fat cells and liver membranes - Using $^{125}$I-insulin and isolated fat cells with $^{125}$I-albumin to assess trapped and nonspecifically adsorbed radioactivity, we found, with insulin at about 1 ng/ml, that approximately 100 pg of insulin were bound per 100 mg of fat cells (Table II) which is in agreement with data of Crofford who, under similar conditions, measured insulin uptake by fat cells as the rapid disappearance of immunoassayable insulin from the medium in the presence of fat cells (10). In the presence of unlabeled insulin at 4 μg/ml, the uptake of insulin radioactivity was reduced to that observed with radioactive albumin (Table II), suggesting that insulin receptors were virtually saturated by insulin at this concentration. The $^{125}$I-albumin content of fat cells was unaffected by the addition of excess insulin (Table II).

$^{125}$I-insulin bound to purified plasma membranes (11) from rat hepatocytes (Table III). Twenty percent of the $^{125}$I-insulin binding was inhibited by unlabeled insulin at $10^{-9}$M which is in the range of physiological hormone

*Specific activity of carrier-free $^{125}$I = 17.4 mCi/μg; insulin mol. wt. ∿ 6000.

TABLE II. UPTAKE OF $^{125}$I-INSULIN BY ISOLATED FAT CELLS

| $^{125}$I-protein | Insulin content | | | Uptake of radioactivity | | Specific insulin uptake | |
|---|---|---|---|---|---|---|---|
| | Radio-activity nc/ml | Immuno-activity ng/ml | Bio-activity μU/ml | No unlabeled insulin | With unlabeled insulin | ng of immuno-reactivity | μU of bio-activity |
| Insulin "A" | 30 | 1.2 | 19 | 36% | 25% | 0.12 | 1.9 |
| Insulin "B" | 74 | 1.3 | 12 | 31% | 26% | 0.06 | 0.6 |
| Albumin | 18 | - | - | 26% | 26% | - | - |

Isolated fat cells (approximately 100 mg/ml) were incubated in plastic syringes with $^{125}$I-insulin or $^{125}$I-albumin in the absence and in the presence of unlabeled insulin (4 μg/ml) in 3 ml of Krebs Ringer bicarbonate buffer with bovine albumin (20 mg/ml), pH 7.4. After 15 minutes at 24°, the cells were floated by centrifugation, separated from the infranatant, and counted for their radioactivity. The $^{125}$I-insulin used in this experiment ("A" and "B") represent fractions at the apex of peaks A and B in Fig. 3 (Nov. 70). The radioactivity and content of insulin immunoreactivity (6) and bioactivity (8) were measured in both fractions. The specific insulin uptake = (% uptake of $^{125}$I-insulin - % uptake of $^{125}$I-albumin) x insulin concentration.

TABLE III. INHIBITION BY UNLABELED INSULIN OF $^{125}$I-INSULIN BINDING TO LIVER MEMBRANES

| Conc. of unlabeled insulin (M/L) | 0 | $10^{-9}$ | $10^{-8}$ | $10^{-7}$ | $10^{-6}$ |
|---|---|---|---|---|---|
| $^{125}$I-insulin bound (% of total) | 26 | 21 | 12 | 6 | 4 |

Purified plasma membranes (step 15, ref. 11) were kept at -70° until use. Immediately after thawing, 160 μg of membrane protein (12) were diluted to 100 μl with 1 mM KH $CO_3$ and unlabeled insulin was added in a volume of 10 μl. After 20 minutes incubation at 30°, 50 μl of Krebs Ringer phosphate ($Ca^{2+}$-free) buffer, pH 7.5, was added that contained bovine albumin (1.5 mg, fraction V) and $^{125}$I-insulin (12,000 cpm of peak A, Fig. 3, Nov. 70) to give a final concentration of $^{125}$I-insulin at 7 x $10^{-10}$M. After 40 minutes further incubation, 60 μl aliquots of each tube were layered onto 250 μl of Krebs Ringer phosphate buffer, pH 7.4, with albumin 10 mg/ml, in plastic microtubes. After centrifugation, the supernatants were discarded and the radioactivities in the pellets were counted. This method of separation is that described by Rodbell et al. (13) for measurement of $^{125}$I-glucagon binding to liver membranes.

concentrations in hepatic portal blood. With insulin at $10^{-7}$M, 80% of the $^{125}$I-insulin binding was inhibited (Table III).

Discussion - The differences in the methods of preparing iodoinsulin probably account for the conflicting conclusions about the bioactivity of iodoinsulin (14-21). We showed with $^{127}$I-insulin that monoiodoinsulin had biological activity far beyond that which could be accounted for by any contamination with uniodinated hormone. We have no conclusive data on the bioactivity of the $^{125}$I-insulin since the radioactive insulin represented only a minority of the total insulin content and was therefore not accurately reflected by our bioassays of these preparations. We do feel confident, from the method of iodination and the chromatography data, that the radioactive insulins in these preparations are almost exclusively monoiodinated species and that peak A represents mono $^{125}$I-insulin.

We have confirmed the specificity of the $^{125}$I-insulin binding reported here by further studies reported in detail elsewhere (22). Binding of $^{125}$I-insulin to liver plasma membranes has also been reported recently by others (23). These studies extend to insulin the methods developed for the direct study of binding of polypeptide hormones to target tissues: ACTH to adrenals (1), angiotensin to various tissues (24), and glucagon to liver (13, 25).

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