IDENTIFICATION OF INSULIN BINDING ACTIVITY AND ISOLATION OF ENDOGENOUS INSULIN FROM RAT LIVER*

John G. Hemington** and Arnold Dunn

Department of Biological Sciences
University of Southern California
Los Angeles, California 90007

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SUMMARY: A discrete peak of immunoinsulin activity (molecular weight 300,000) has been separated by gel filtration from liver homogenates. This fraction chromatographs with insulinase activity and cyclic nucleotide phosphodiesterase. Endogenous insulin has been separated from this insulin binding activity by various methods. Our results suggest that when bound, insulin is not degraded by the insulinase activity.

Much of the insulin released by the pancreas is taken up by the liver. Although insulin has multiple effects on hepatic metabolism, its site of action and ultimate fate are unknown. These insulin effects might be mediated by reduction of intracellular cyclic 3',5' adenosine monophosphate (cyclic AMP) levels.[1,2] One mechanism suggested for this effect is insulin induced activation of cyclic nucleotide phosphodiesterase (PDE). Insulin activation of PDE has been reported in liver[3] and fat cell[4,5] preparations. Extrahepatic tissues contain two forms of cyclic AMP phosphodiesterase: a high molecular weight form (400,000) and a low molecular weight form (200,000). Previously only the high molecular weight form, which hydrolyzes both cyclic AMP and cyclic 3',5' guanosine monophosphate (cyclic GMP), has been reported in liver[6]; we have recently reported the presence, in liver, of the low molecular weight form specific for cyclic AMP hydrolysis.[7]

The experiments described in this paper indicate that insulin can be separated from liver homogenates in association with a high molecular weight fraction, presumably protein. The insulin containing fraction chromatographs (by gel filtration) with PDE and insulinase activity. Our results suggest that when insulin is associated with this protein, it cannot be degraded by the insulinase.

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** Present address: Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania 19104.

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Materials and Methods

Following sodium pentobarbital anesthesia, livers were removed from male Sprague-Dawley rats (220-300 g) and homogenized in 8 volumes (w/v) of homogenizing medium in a Sorvall Omni-Mixer. The preparation and assay of PDE were performed according to the methods of Thompson and Appleman[8], except that the homogenizing medium contained 10.9% sucrose, 36.5 mM Tris-acetate buffer, pH 6.0, and 2.82 mM 2-mercaptoethanol. In this method, approximately 7% of the total liver protein and 100% of the total liver PDE activity is applied to agarose gel columns (Biogel A-5m). Protein was measured by the Lowry method.[9] The PDE assay is a two-step enzymatic conversion of tritiated cyclic nucleotide to labeled adenine. Assay concentrations of cyclic AMP and cyclic GMP were 1.25 and 2.5 x 10^{-7} M, respectively. A unit of PDE activity equals one picomole of cyclic nucleotide hydrolyzed per minute in an initial assay volume of 0.4 ml. Results are expressed as units/ml. Fractions were concentrated using an Amicon Ultrafiltration Cell (UM20E membrane- 20,000 molecular weight "cut-off") and the pH was reduced to 3.0 with acetic acid. Insulin was extracted from these concentrated fractions by various means: ultrafiltration, acid-ethanol extraction as modified by Arquilla[10], and by chromatography on Sephadex G-75 (fine grade) with 0.167 M acetic acid. Acetic acid solutions were neutralized with 2 M Tris prior to insulin and enzyme assays.

Insulin was assayed using a radioimmunoassay "kit" (Schwarz/Mann BioResearch, Inc., Orangeburg, New York). Results are expressed as μunits/ml. Insulinase activity was measured by two different procedures. In the first method, 100 μunits of standard human insulin (Schwarz/Mann) was added to fraction aliquots and incubated overnight at 4°C. The insulin remaining at the end of the incubation was assayed immunologically as above. Results are expressed as the difference between μunits added and μunits remaining after the incubation. In the second method, ^{125}I-insulin (Schwarz/Mann) was added to aliquots and incubated for 10 min at 30°C. The reaction was stopped with 1 ml of 5% trichloroacetic acid (TCA). After centrifugation, 0.5 ml aliquots of the TCA supernatant were counted by liquid scintillation spectroscopy. Results are expressed as the percent of the initial counts per min ^{125}I (corrected for blank) not precipitated by the TCA.

Rats were made diabetic by intraperitoneal injections (50 or 100 mg/kg body weight) of streptozotocin.[11] They were maintained on 0.45% saline and Purina Lab Chow *ad libitum*. All diabetic animals tested positively for urinary glucose, had plasma glucose concentrations greater than 400 mg %, exhibited weight loss (50-60 g) and reduced plasma insulin levels. The animals were used 3 weeks after onset of diabetes.

Results

Figure 1 compares column profiles (Biogel A-5m) obtained from normal (Fig. 1A) and diabetic (Fig. 1B) livers. Similar results were obtained in 8 normal and 4 diabetic rats. All preparative steps and assays were performed at the same time; equal amounts of protein (150 mg) were applied to the columns. The only apparent difference between normal and diabetic livers is the marked reduction in immunoinsulin content of the diabetic profile. Insulinase activity, as measured by the disappearance of added standard insulin, was not affected by streptozotocin induced diabetes. In both normal and diabetic profiles, in-

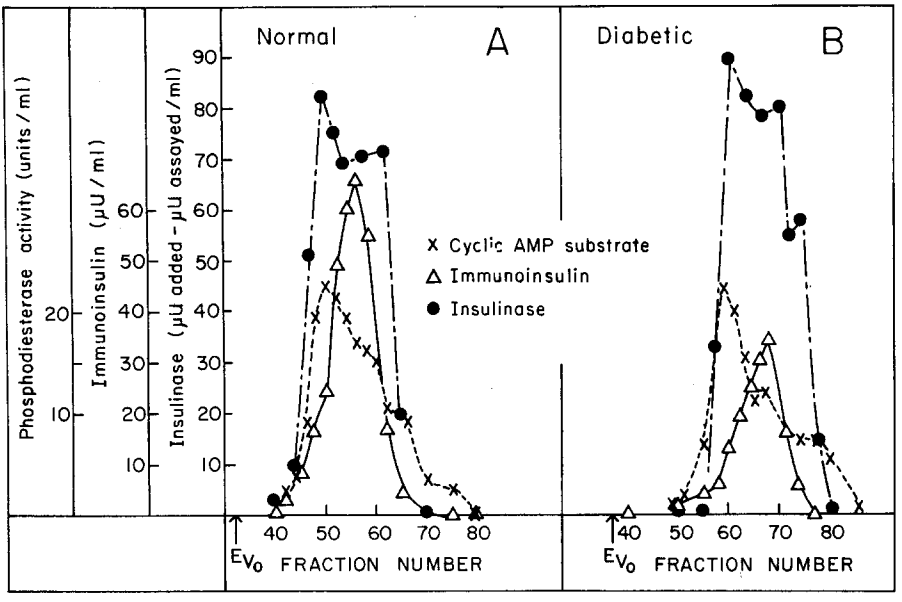

Figure 1. Chromatographic profiles of cyclic AMP phosphodiesterase, immunoinsulin and insulinase activity measured immunologically on agarose gel (Biogel A-5m) from normal (A) and diabetic (B) rat livers. Equal amounts of protein (150 mg) applied to columns. Buffer is 50 mM Tris-acetate, pH 6.0, 3.75 mM 2-mercaptoethanol.

sulinase activity does not appear as a single peak as do PDE and immunoinsulin activities. In the preparation from normal rat liver, insulinase activity peaks in fraction 50, decreases until fraction 54 and then increases again to a second peak in fraction 64. This "notch" pattern is characteristic of insulinase activity when measured by this immunological method, and occurs in both normal and diabetic preparations. As can be seen in Figures 1 and 2 (B and C), the "notch" in insulinase activity is coincident with the rise in immunoinsulin. The immunoinsulin fraction in normal rat liver in Figure 2 peaks at 37 μU/ml, apparently similar to the amount shown for the diabetic liver in Figure 1B. This is due to different amounts of protein placed on the column

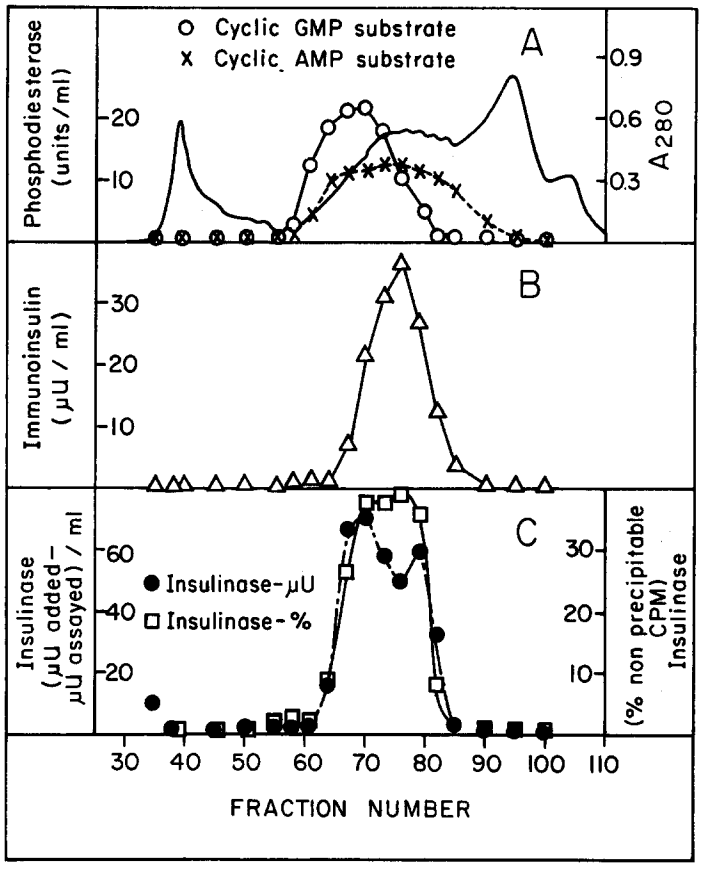

Figure 2. Chromatographic profiles of protein (A_{280}), cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase (A), immunoinsulin (B) and insulinase measured both immunologically and by % non-precipitable CPM (C) from the same normal rat liver preparation. 70 mg of protein applied to column (Biogel A-5m). Buffer is 50 mM Tris-acetate, pH 6.0, 3.25 mM 2-mercaptoethanol.

(70 mg from normal liver vs. 150 mg from diabetic liver). In most cases, the immunoinsulin peak occurs in the same fraction as the lowest point of the "notch". When insulinase is determined by measuring the ^{125}I remaining in the TCA supernatant, the "notch" is absent. With the exception of this "notch", insulinase activity profiles, measured by both methods can be superimposed (Fig. 2C). Also in Figure 2A, the low molecular weight form of PDE activity (cyclic AMP hydrolysis only) appears as a shoulder of the total PDE activity between fractions 76 and 95. Both insulinase and immunoinsulin appear as discrete peaks of activity between the two forms of PDE and are not coincident with either form. The estimated molecular weight of these peaks is 300,000; presumably, the insulin is bound to a protein.

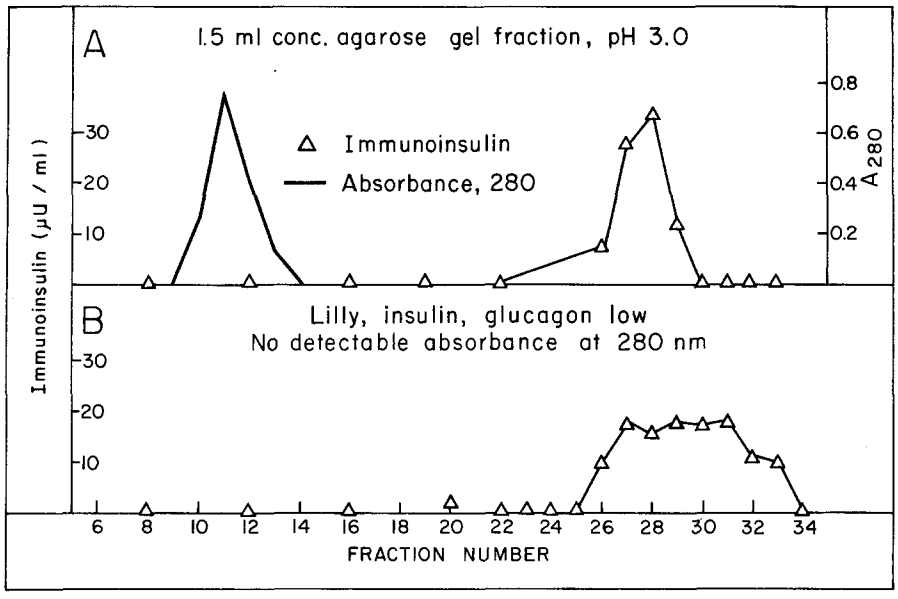

Figure 3. Camparison of chromatographic properties on Sephadex G-75 of immunoinsulin activity between concentrated agarose gel fractions (A) and standard insulin (Lilly, glucagon low) (B). Medium is 0.167 M acetic acid.

Figure 3 compares the results of chromatography on Sephadex G-75 of a standard insulin (Lilly, glucagon low) (Fig. 3B) with the concentrated agarose gel fractions (Fig. 3A). These samples were chromatographed on the same column with the same flow rate. Both insulin peaks were detected immunologically and have similar chromatographic properties. The high molecular weight protein fractions (200,000 - 400,000) are excluded from the Sephadex gel. Enzymatic activity (PDE and insulinase) is not detectable after adjusting the pH to 3.0, followed by neutralization. The immunoinsulin peak in Figure 3A does not have insulinase activity. In addition, after acidification immunoinsulin and not insulinase activity or other larger proteins pass through the UM20E membrane. Similarly, after acid-ethanol extraction only insulin is detectable. The possibility of contamination of liver preparations by circulating insulin seems remote since plasma or red blood cells prepared in a similar manner do not have comparable activity. Also, livers perfused in situ to remove blood still gave comparable results. Furthermore, the insulin appears first with the 300,000 molecular weight fraction.

Discussion

Since both insulin and insulinase occur in the same agarose gel column fractions, the insulinase activity could give a false positive immunoassay for insulin by degrading added iodinated insulin. The comparison of insulin and

insulinase content of normal and diabetic liver fractions indicates, however, that such interference may not occur. Although the insulinase activity of normal and diabetic liver fractions is the same, the amount of immunoinsulin in the diabetic fraction is reduced, as expected. The estimated molecular weight of the immunoinsulin fraction is 300,000 indicating that the endogenous insulin is probably bound to a high molecular weight fraction, probably a protein. In addition, insulin has been dissociated from the binding protein and from insulinase activity by acidic conditions and then separated on Sephadex G-75 (Fig. 3), acid-ethanol extraction and ultrafiltration. We propose that the endogenous insulin, when bound in this form, is protected from the insulinase activity present in the same fractions, but can still be recognized by the insulin antibody. The different results obtained by the two insulinase methods support this hypothesis. Insulinase, when measured by degradation of exogenous iodinated insulin, has a single peak of activity. Insulinase measured by the disappearance of exogenous human insulin, detected immunologically, should have the same result; the "notch" is interpreted as bound insulin which the insulinase could not degrade.

Since no attempt was made to separate sub-cellular components, we cannot assign a cellular locus of the insulin binding protein. It is possible that this might be a membrane insulin receptor, as reported for fat cells.[12,13] The nucleus might be a second possible site since insulin has been extracted from isolated rat liver nuclei.[10] The insulin binding protein and insulinase are discrete peaks and represent only a small portion of the total protein present in the homogenate. Furthermore, we find that these proteins are either absent or present in only small amounts in tissues known to be insensitive to insulin (brain, kidney, plasma, and red blood cells). These findings suggest that the insulin binding protein and insulinase might be involved with the physiological action of insulin in the liver.

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