DEXTRAN-LINKED INSULIN: A SOLUBLE HIGH MOLECULAR WEIGHT DERIVATIVE WITH BIOLOGICAL ACTIVITY *IN VIVO* AND *IN VITRO*[*]

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SUMMARY

Insulin has been covalently linked to dextran, $\overline{MW}$ 2,000,000. The soluble derivative has intrinsic biological activity in lowering blood glucose levels *in vivo* and in glucose uptake in isolated fat cells *in vitro*.

Insulin linked to insoluble bead supports such as agarose or polyacrylamide has been used to study insulin interactions with membrane receptors of fat cells and liver cells (1,2). We have recently synthesized a new type of hormone derivative with thyroxine linked to dextran which is soluble and retains full biological activity (3). In this paper we describe the preparation of a similar derivative of insulin, which is also soluble and has intrinsic biological activity both *in vivo* and *in vitro*.

Dextran of high molecular weight was chosen as the matrix to which the hormone molecules were to be covalently linked. It has a high degree of solubility and its relative lack of toxicity has been demonstrated by extensive clinical use as a blood plasma expander. Dextran-linked hormone derivatives provide several advantages over insoluble bead preparations in studies of hormone receptor interactions. The

[*] Some of the results presented here were discussed at the Symposium on Insulin Action, Toronto, October 25, 1971.

dextran-hormone derivative is essentially linear; each hormone molecule linked to the polymer has potential access to the membrane surface of the cell. With bead preparations, it is difficult to obtain an accurate estimate of the number of bound hormone molecules on the surface of the sphere, and which are available to receptor sites on the cell membrane at the point of contact with the bead. A soluble derivative covalently linked to a stable non-toxic support such as dextran affords the additional important advantage of permitting whole animal studies.

MATERIALS

Dextran 2000, average molecular weight 2,000,000, is a product of Pharmacia. Amorphous beef insulin, Lot No. PJ-7230, and beef zinc insulin crystals, Lot No. PJ-4609, were gifts from Eli Lilly and Company. ^{125}I-Insulin was obtained from Amersham Searle; cyanogen bromide from Eastman Organic Chemicals. Bovine albumin fraction V powder, "Pentex", from Miles Laboratories, was dialyzed overnight against buffer before using. Epinephrine was obtained from Sigma. Rats used were male animals from Holtzman. Fat cells were incubated in plastic vials. Carbon dioxide was collected in polypropylene center wells containing Hyamine hydroxide after the incubation had been stopped and 0.2 ml of 10 N H_2SO_4 had been added.

METHODS

Preparation of Dextran-Linked Insulin

Dextran 2000 (500 mg) in 12 ml of water was added to a well-stirred solution of cyanogen bromide (100 mg) in 50 ml, in a Radiometer pH-stat with the pH maintained at 11 by the addition of 2 N NaOH. Activation was allowed to proceed for about eight minutes. Then 10 mg insulin (containing a tracer amount of ^{125}I-insulin) in 10 ml of 1 M sodium bicarbonate were quickly added, bringing the pH of the reaction solution to 9.4. The solution was stirred overnight in the cold in an Amicon

ultrafiltration apparatus, fitted with an XM-50 membrane, then concentrated and washed with 6 molal guanidine hydrochloride. When no further radioactivity was detected coming through the membrane, the product was thoroughly washed with water and concentrated to a final volume of 30-40 ml. Insulin was coupled to dextran with a yield of 23%.

Preparation of Fat Cells

Fat cells were prepared essentially according to the procedure described by Rodbell (4) with 3.5% bovine serum albumin Krebs Ringer bicarbonate buffer equilibrated with 95% CO_2-5% O_2 and containing 1.3 mM Ca^{++}.

Rat Diaphragm Muscle

The procedures employed were essentially those previously described (5). Intact rib cages and diaphragm muscle removed from male rats weighing approximately 100 g were given a 10 minute preincubation wash in 20 ml of Krebs Ringer phosphate buffer at 37°. These were then transferred to 15 ml of buffer containing either ^{14}C-α-aminoisobutyric acid or ^{14}C-glucose and the appropriate hormone and incubated with gentle shaking for two hours at 37°. After this time the diaphragm muscle was cut out, weighed and homogenized with four times its own weight of 0.008 N acetic acid. Each homogenate was placed in boiling water for five minutes, cooled and centrifuged and an aliquot of the supernatant removed and counted in 10 ml of Diatol in the liquid scintillation counter.

RESULTS

The attachment of the insulin molecule to the dextran polymer is presumed to be via the epsilon amino group of the lysine residue at position 29 in the B chain, since coupling of activated dextran to the hormone was carried out at pH 9.4 (1). It is of interest that Katsoyannis has recently shown that insulin lacking both lysine at position 29 and the C-terminal alanine at position 30 has full biological activity (6). Thus, the region where the linkage to the polymer occurs is not required for receptor site interaction.

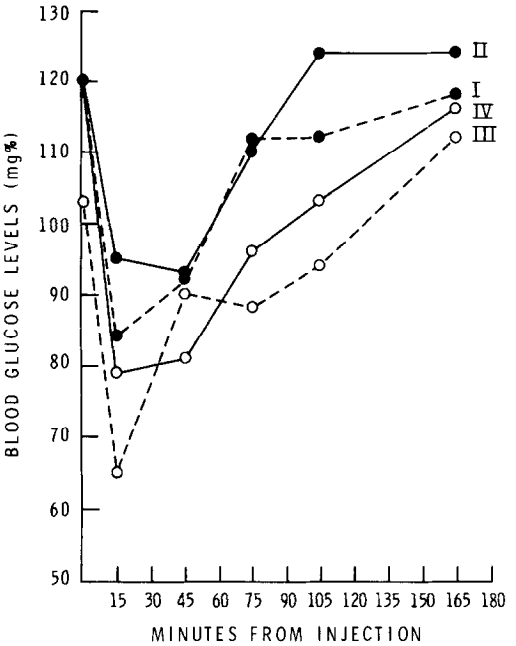

FIG. 1 - Dextran-linked insulin (I and II) or free insulin (III or IV) was injected intravenously at a dose of 0.5 units/kg. Blood samples were taken at the time intervals shown, and the plasma analyzed for glucose with a Beckman glucose analyzer.

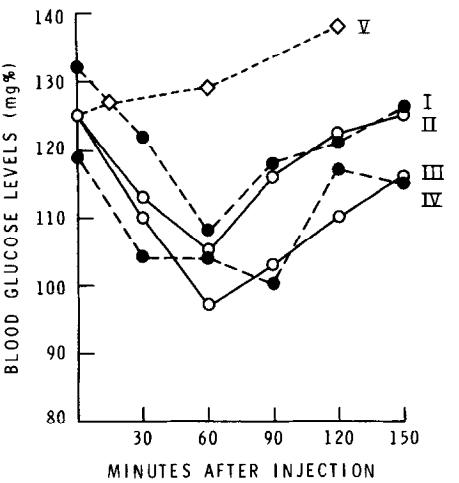

FIG. 2 - Subcutaneous injections of either dextran-linked insulin (I and IV) or free insulin (II and III) were given to rabbits weighing 1.5-2.0 kg at a dose of 0.5 units/kg. V is a dextran control. Glucose levels were determined as in Fig. 1 at the time intervals shown.

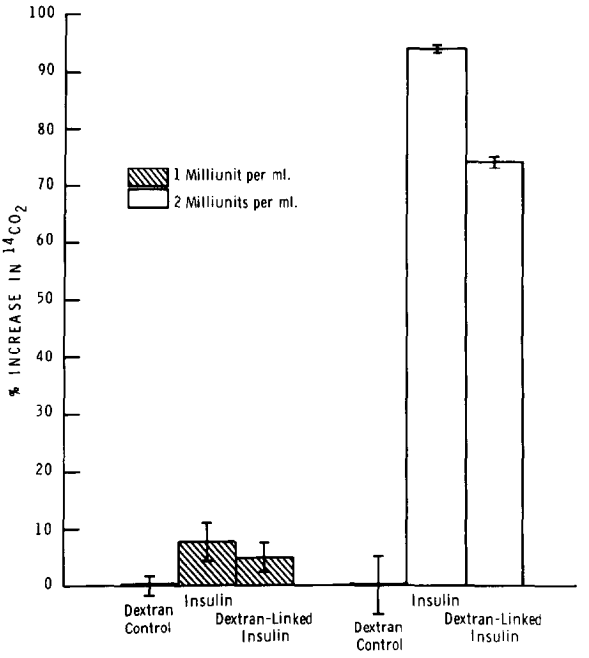

FIG. 3 - Fat cells were incubated for two hours at 37° in 3 ml of Krebs Ringer bicarbonate buffer containing 3.5% bovine serum albumin and ^{14}C-glucose at 3 μmoles/ml. Dextran-linked insulin or insulin was added to give a final concentration of 1 or 2 milliunits/ml. Controls contained equivalent amounts of dextran. The incubations at 1 milliunit/ml contained 1.01 x 10^6 cells/ml and at 2 milliunits/ml 1.9 x 10^6 cells/ml.

As can be seen in Figures 1-3, the dextran derivative has biological activity both in the whole animal and also in vitro. In order to eliminate the possibility of adsorption of free insulin to the dextran preparation, ^{125}I-insulin was added during derivatization and the reaction mixture finally washed by ultrafiltration with 6 molal guanidine-HCl under conditions which are known to dissociate insulin into monomer form (7). Tests with aliquots of this preparation under these same conditions in the presence of excess free insulin failed to displace any radioisotope. Thus, it can be reasonably concluded that all the insulin in the preparation was covalently linked to dextran.

It had been previously shown that the linkage between the hormone and the dextran polymer is very stable (3). It was also considered very unlikely

that non-specific proteolytic activity could result in the cleavage of enough active hormone from the polymer to account for the observed biological responses. Nevertheless, a direct test was suggested by studies showing a lack of penetration of dextran through the peritoneal membrane (8). In rat diaphragm preparations (5,9) in which the rib cage is intact and where the dextran-linked hormone derivative should not be able to penetrate to the surface of the muscle cells, we were able to observe biological activity only with free insulin (Table I). Thus, the intact nature of the dextran-insulin is apparent.

TABLE I

Amino Acid and Glucose Transport into Rat Diaphragm Muscle

Rat diaphragms were incubated with ^{14}C-α-aminoisobutyric acid (0.115 mM) and insulin added to give a concentration of 0.4 units/ml. Where ^{14}C-glucose (3 mM) was used, insulin was present at 1 milliunit/ml. Mean values for the ratio of isotope in 1 ml of tissue water to 1 ml of incubation medium are shown ± S.E.M.

	Dextran Control	Insulin	Dextran-Linked Insulin
α-amino-isobutyric acid	0.764 ± 0.025	1.215 ± 0.027	0.681 ± 0.012
glucose	0.633 ± 0.003	1.272 ± 0.039	0.864 ± 0.034

Dextran-linked insulin offers a number of interesting possibilities in studying insulin-receptor interactions and it also has potential clinical usefulness.

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