# HYPOGLYCEMIC EFFECTS OF INSULIN COVALENTLY LINKED TO POLYACRYLATE

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## 1. Introduction

Considerable research work has been done to improve insulin preparations in terms of controlled onset and duration of action [1–5]. However, despite the various types of insulin preparations available for therapy, it may still be of some interest to follow new ideas in this field.

Thus, based on Katchalski's work [6] and our own observations with enzymes and other biological active peptides covalently linked to synthetic polymers [7], we prepared a water soluble insulin-polyacrylate compound (I–PA).

## 2. Methods and materials

### 2.1. *Polyacrylic anhydride*

(PAA) was prepared from its monomer (acrylic anhydride, 10% in water-free benzene) in 60% yield as described in [8]. The weight average molecular weight of our PAA measured in a light scattering photometer (Sofica, PGD 42000) and calculated from a Zimm plot was 140,000. For preparation of I–PA, PAA was dissolved in water-free dimethylformamide at a concentration of 33 mg/ml. Since prior to solution PAA forms an invisible gel, this system has to be shaken overnight.

### 2.2. *Insulin–polyacrylate (I–PA)*

100 mg bovine insulin (Serva, Feinbiochemica-Heidelberg, 23 international units per mg, 0.4% zinc) was dissolved in 15 ml 0.2 M phosphate buffer, pH 7.5, containing 80 mg EDTA (tetra sodium salt) at room temp. The solution was cooled in an ice bath and under rapid stirring 3 ml of dimethylformamide containing 99 mg PAA was added over a period of 1–2 min. The mixture was stirred for 6 hr at 3°, allowed to stand overnight at 8°, and lyophylized. The dry material was dissolved in 10 ml $H_2O$ and submitted to gel chromatography (Sephadex G-100 fine, 3 × 40 cm, $H_2O$).

Fractions containing 10 ml were collected and lyophylized. From each fraction a UV-spectrum at a standard concentration of 1 mg/ml 0.05 M phosphate buffer was recorded, from which the respective insulin content was calculated. This procedure of preparation of I–PA was repeated five times with essentially the same result.

### 2.3. *I–PA-(insulin-$^{125}I$)*

Insulin-$^{125}$I was prepared according to Greenwood and Hunter's method [9] using 2 mCi sodium iodide-$^{125}$I, type IMS 30, from Radiochemical Center, Amersham. The procedure was slightly modified in the following way: a) for iodination 50 μg insulin (Serva) was added, b) after iodination, 0.8 ml phosphate buffer (0.05 M, pH 7.5) containing 1000 μg insulin (Serva) was added, c) gel chromatography was carried out using a 1.5 × 25 cm Sephadex G-50 column, 0.05 M phosphate buffer pH 7.5, 2 ml fraction was determined after appropriate dilution ($H_2O$) in a liquid scintillation spectrometer, Packard TriCarb, model 544, using NEN aquasol as scintillation liquid.

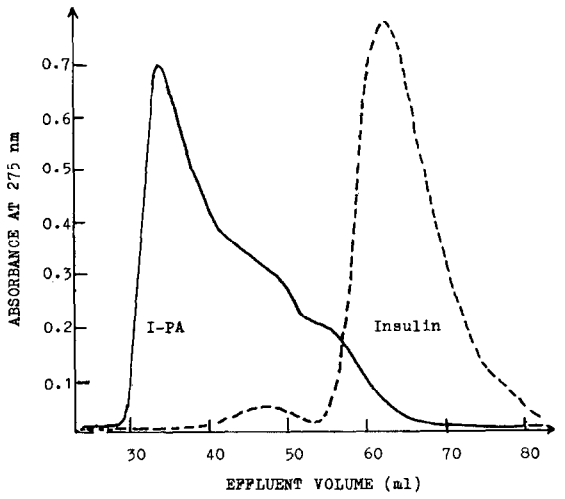


Fig. 1. Gel chromatogram of I–PA. a) Solid line: 1.8 ml of the reaction mixture of I–PA was Sephadexed on G-100 fine (1.5 × 40 cm column, $H_2O$, for further details see Methods and materials). b) Broken line: 1.8 ml of solvent containing 1.5 ml 0.2 M phosphate buffer, pH 7.5, 0.3 ml dimethylformamide, 10 mg insulin and 8 mg EDTA (tetrasodium salt) was submitted to gel chromatography as described in a.

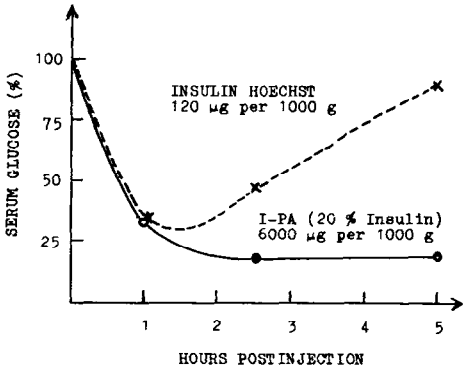


Fig. 2. Depression of serum glucose level in rats after subcutaneous injection of I–PA. Ordinate: serum glucose level in percent of initial (normal) concentration. I–PA (solid line) contains 20% bound insulin, and the dose corresponds therefore to 1200 µg bound insulin/1000 g rat.

The yield of $^{125}$iodine bound to insulin was 90%. The insulin containing fractions were pooled and divided into two aliquots, which were lyophylized.

For preparation of I–PA-(insulin-$^{125}$I) one aliquot was dissolved in 3.2 ml phosphate buffer, 0.1 M, pH 7.5, containing 19 mg insulin (Serva) and 12 mg EDTA, and, under cooling and rapid stirring, 0.6 ml dimethylformamide containing 20 mg PAA was added. Isolation and purification procedures were carried out as described above for the preparation of unlabelled I–PA. The final product was separated on Sephadex G-100 fine, 1.5 × 40 cm. $H_2O$ was taken for elution and 3 ml fractions were collected. Each fraction was measured for radioactivity as described above. According to the data obtained, more than 98% of the insulin had reacted with PAA.

To assure the covalent nature of the binding of insulin in I–PA, an aliquot equivalent to 120 µg I–PA-(insulin-$^{125}$I) was taken from the peak fraction of the gel chromatogram and 5000 µg non-radioactive insulin dissolved in 1.0 ml 0.05 M phosphate buffer was added. The solution was allowed to stand overnight at 15° and was submitted to gel chromatography as described above. The absorbance at 275 nm and the radioactivity of each fraction was measured.

### 2.4. *Hypoglycemic effect of I–PA*

The biological activity of I–PA was measured according to the directions for insulin assay issued by the United States Pharmacopeia (U.S.P. XVIII, page 883, Blood-Sugar Method). Female Sprague Dawley rats (220–240 g), and female NZW rabbits (2300–2700 g) were used.

Serum glucose levels were determined in a Technikon-Autoanalyzer according to Brown's method [10].

## 3. Results and discussion

As is evident from fig. 1, the reaction system used allows almost quantitative binding of insulin to polyacrylate. In the control experiment, when insulin was submitted to gel chromatography in the presence of polyacrylate under exactly the same conditions of pH and concentration of buffer and EDTA (as employed for I–PA gel chromatography), no insulin was found in the polymer peak at the exclusion volume (fig. 1).

Moreover, using I–PA which contained $^{125}$I-labelled insulin, no dissociation of the I–PA molecule after addition of a hundred-fold excess of nonradioactive insulin was observed by gel chromatography.

Table 1
Insulin content of I–PA.

| Fraction no. | Weight (mg) | Absorbance at 275 nm for 1 mg/ml* | Insulin in fraction (%) | Remarks |
|---|---|---|---|---|
| 14 | 6 | 0.092 | 10.5 | – |
| 15 | 14 | 0.168 | 19.3 | For biol. test |
| 16 | 18 | 0.250 | 28.6 | – |
| 17 (peak) | 34 | 0.358 | 40.6 | For biol. test |
| 18 (peak) | 38 | 0.372 | 42.5 | – |
| 19 | 42 | 0.312 | (35.5) | Frac. 19 and 20 contain phosphate from the reaction |
| 20 | 42 | 0.257 | (29.0) | medium |

For details see Methods and materials.
* 1140 μg insulin (Serva)/ml 0.05 M phosphate buffer shows an absorbance of 1.00 (1 cm cuvette).

Therefore, complexes of insulin with polyacrylates, as described in [11], do not occur in our system, nor was aggregation of insulin to high molecular forms [12] observed under our conditions. Thus in I–PA, insulin is covalently attached to polyacrylate, presumably by its lysylamino or its N-terminal amino groups via amide bonds. In the reaction mixture, there are about 24 insulin molecules (72 amino groups) per 1 PAA molecule (1100 anhydride groups). Thus at the best, only 3.3% of the total carboxylic groups in the synthetic polymer are engaged in binding of insulin.

However, the I–PA preparation is not homogeneous in terms of insulin content. The I–PA fraction with the highest molecular weight contains much less insulin than those fractions which are close to the weight average molecular weight of the product (table 1).

For biological tests we used I–PA preparations containing 20% or 40% insulin (table 1).

Subcutaneously injected, I–PA lowers the serum glucose level of rats in a pronounced way (fig. 2). Taking into account that the dose applied contains 20% bound insulin, one finds that about 10 times as much bound insulin is needed to evoke the same onset in glucose depression as one observes with free insulin of the fast acting type. However, while free insulin is rapidly inactivated (fig. 2), I–PA remains fully active for the whole period of observation (5 hr).

Essentially the same findings were obtained with rabbits, where the action of subcutaneously injected I–PA persisted for at least 12 hr, when a dose of 1000 μg I–PA per 1000 g rabbit corresponding to 200 μg bound insulin was administered.

Upon intraveneous application the hypoglycemic effect of I–PA is comparable to "Actrapid" insulin (Novo) in terms of rapid onset. With respect to its insulin content, intraveneous I–PA is about 6 times less active than free insulin in the initial phase, however, it is only 3 times less active as far as its overall efficiency is concerned.

From all these observations, the following conclusions can be drawn: a) due to its solubility and due to its polymeric nature subcutaneously administered I–PA is released into the bloodstream in a rather constant way over an extended period, thus representing an insulin preparation of the long acting type with a rapid onset, b) the covalent binding to the polyanion does not prevent insulin to be inactivated, presumably in the same way as insulin is degraded in the organism [13–16], however some delay in inactivation is observed. According to Merigan's findings with synthetic polyanions of the maleic acid-divinylether type [17], I–PA is likely to be deposited and eventually its insulin moiety catabolized in the cells of the reticulo-endothelial system [16].

With respect to Cuatrecasas' findings of biological activity of insulin bound to agarose particles [20] and our own data about the rapid onset of subcutaneously injected I–PA, it appears to be more likely that the whole, intact I–PA molecule is biologically active.

It is scarcely conceivable that there is an enzymatic *in vivo* mechanism which would split specifically the amide bonds between insulin and PAA, and would release the intact insulin molecule, and even if so, such a mechanism would have to be more effective or faster than the mechanism of insulin degradation which is unlikely [13–16].

This concept is also supported by recent findings of Cuatrecasas who demonstrated that the significant primary event of its biological action involves an interaction of the intact insulin molecule with a highly specific receptor located in the cell membrane, while only insignificant amounts of insulin are found in other cell compartments [20]. Since I–PA, with its negative charges and its high molecular weight, should penetrate into cells much slower than insulin, the rapid onset of glucose depression upon intraveneous injection of I–PA can be interpreted in terms of a direct interaction of I–PA with Cuatrecasas receptor site in the cell membrane.

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