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# STUDY OF MODIFIED SEPHADEX-BOUND INSULIN IN ANIMAL EXPERIMENTS

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Preparations of insulin covalently coupled to Sephadex were obtained using modified aldehyde-containing Sephadex carriers. In all cases carriers used had the same structure but differed in molecular weight. Depending on the reaction conditions insulin might or might not be split from the carrier. In animal experiments it was shown that in the homogeneous state all preparations retain high biological activity independent of molecular weight of the carriers, which varied from 10,000 to 150,000. The binding of insulin with polymeric carriers prolonged the time of its action from 5–6 to 14–16 h.

## INTRODUCTION

The binding of insulin to polymer carriers is of interest from at least two aspects. First, it is desirable to prolong the insulin lifetime in an organism by, for instance, decreasing its clearance rate. This can be done by the use of a carrier of certain molecular weight which hinders its clearance. Second, coupling of insulin to carriers is of importance in understanding the mechanism of insulin effects, that is, whether the hormone must enter the cell to exert its effect.

The detailed works of Cuatrecasas and co-workers (1–5) show that insulin bound to Sepharose preserves its biological activity. However, other authors (6) state that the activity of these preparations may be explained by insulin which was split from the carrier. Similar discrepancy of opinions is also noticed in the study of other preparations of insulin bound to polymer (7–9). It is thought that, for example, the effect of insulin–Sepharose on transaminase activity is actually due to both the split and bound hormone (10). Several authors point out that there occur prolongations of the action of insulin covalently bound to polymer carrier (16,17).

In this work we have compared in experiments on dogs the biological activity and the effective time of action of insulin preparations, in which insulin is bound covalently to the same carrier, but in which certain conjugates are able to split insulin and others are not. Modified Sephadex preparations of various weights were used as carriers.

## MATERIALS AND METHODS

### *Materials*

Crystalline insulin was the product of the Institute of Hormonal Preparations and had the activity of 24 units/mg. Sephadex G-25 and G-150 were brought from Pharmacia (Sweden); periodic acid and acetone were from Chemapol (Czechoslovakia). Glucosoxidase and peroxidase were obtained from Koch-Light (England). All other chemicals were bought from Reachim (USSR). Female and male dogs weighing from 10 to 15 kg were used in the experiments.

### *Methods*

The molecular weight of fragments which had formed after solubilization of modified Sephadex preparations was determined by gel filtration on Sephadex G-25 and G-150 columns. Dextrans of known molecular weight were used as standards (see Fig. 1). The column (2.5 × 45 cm) was equilibrated with 0.1 M phosphate buffer pH 7.2, containing 0.02% sodium azide. Elution was performed with the same buffer; velocity was 0.8 ml/min.

Insulin was coupled to modified Sephadex preparations by formation of Schiff bases. This was done by adding 50 mg of insulin to a suspension of 100 mg of carrier in phosphate buffer at pH 9.4, and terminating the reaction after 20 h at 4°C. These conditions had previously been found to be optimal (1). Schiff bases in the product of the conjugation were reduced by addition of an excess of sodium borohydride. Free insulin was washed off by successive washing with buffer, 1 M NaCl, and water. The product obtained was dried with acetone. The amount of the bound hormone was determined by the Lowry method (18).

In the test of biological activity of the bound insulin, the preparations of modified insulin (1 unit/kg of animal weight) were injected intravenously into anesthetized animals. Animals were anesthetized with 10 mg of morphine and 20 mg of barbamil per 1 kg of weight. Preliminary blood sampling and regular blood sampling at 30-min intervals after adminis-

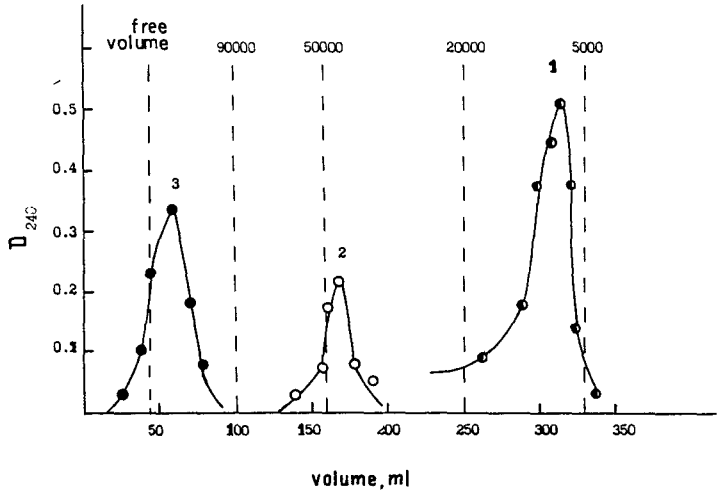


FIG. 1. Gel chromatography of aldehyde-containing Sephadex preparations after complete solubilization. Curve 1—P-1, molecular weight 10,000; curve 2—P-2, molecular weight 50,000; curve 3—P-3, molecular weight 150,000. $D_{240}$ is optical density of eluate at 240 nm. Dashed lines show elution volumes of standard dextrans of known molecular weight. For conditions see the Methods section.

tration of the preparation were conducted during 8–15 h. The time of the experiment was no more than 16 h because of changes in hemodynamic parameters (increase in the volume of circulating blood, hypotension, decrease in $O_2$ concentration) which, in turn, affected the glucose level in the blood. Blood glucose was determined enzymatically by the glucosoxidase technique of Gorodetsky (19). In each experiment the insulin preparation was solubilized completely before administration in the same amount of 0.05 M phosphate buffer.

Separate experiments were performed with the administration of suspensions of nonsolubilized modified insulin preparations bound to aldehyde-containing Sephadex preparations. In each series of experiments four animals were used.

## RESULT AND DISCUSSION

The method of immobilization on Sephadex preparations and water-soluble dextrans, modified by oxidative administration of aldehyde groups, was described earlier (12–15). The binding of protein to carrier occurs

owing to the formation of Schiff bases between aldehyde groups of a carrier and $\varepsilon$-amino groups of lysine residues of the protein. It was demonstrated that after this treatment Sephadex preparations become soluble in water. The molecular weight of the fragment which is split from the granule depends on the degree of oxidation and may vary rather widely. Hydrolysis of Schiff bases, which occurs in physiological conditions, can be completely stopped by reduction of the Schiff bases with sodium borohydride.

As carriers aldehyde-containing Sephadex preparations were made which form fragments with average molecular weights of 10,000, 50,000, and 150,000 (P-1, P-2, P-3, respectively) after their complete solubilization (Fig. 1).

In all cases in the experiments on animals preparations containing the maximal amount of bound insulin were used: 62 mg/g of carrier for P-1, 40 mg/g for P-2, and 30 mg/g for P-3.

Gel chromatography of the bound insulin samples which were stored after complete solubilization for 24 h at 40°C in 0.05 M phosphate buffer, pH 7.4, showed no free insulin in the mixture when insulin–polymer linkage was reduced, and up to 30% of split free insulin in the mixture when no reduction of Schiff bases was made (Fig. 2). Even under conditions that were harsher than those in the living organism (0.5 M phosphate buffer, pH 8.0, 45°C), we had no free hormone after storage of solubilized preparation during 24 h (see Fig. 2).

To study the effect of insulin preparations we administered intravenous injections to anesthetized animals and then determined their glucose levels in the blood for prolonged periods (Fig. 3).

It is seen that native hormone causes a decrease in the glucose level, which persists about 6 h (Fig. 3a). At the same time the insulin bound to carrier has a prolonged effect, probably due to the decrease in the rate of its clearance (Figs. 3b–3d and Table 1).

The study of preparations in which hormone is able to split from its matrix (Fig. 3b) showed that the time of insulin effect for this preparation is less than that for the preparations with firmly bound hormone. This can be explained by the fast inactivation or clearance of the split free insulin.

In all cases not depending on the molecular weight of the carrier the solubilized preparation of bound insulin causes a drop in glucose level to that caused by native insulin. The time of onset of hormone effect also remains constant. This fact demonstrates that insulin need not enter the cell to exert its effect but can interact with receptors on the cell membrane surface, since if it must enter the cell, the increase in the molecular weight of the carrier from 10,000 to 150,000 would inevitably cause a decrease in the activity of the bound hormone due to a decrease in the rate of diffusion of the preparation through the cell membrane (see Table 1). As we have

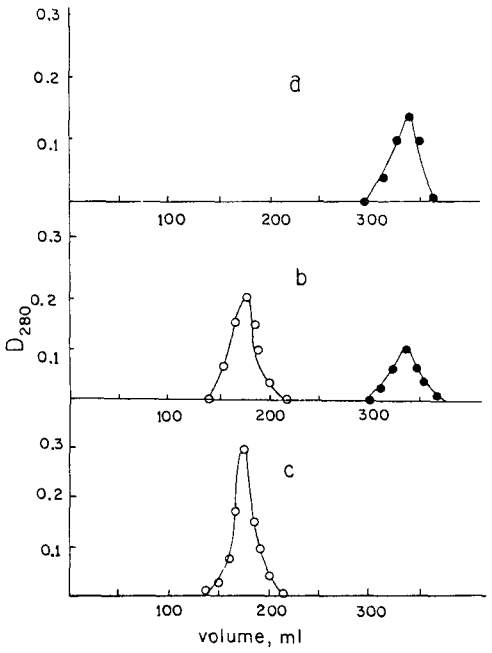


FIG. 2. Gel chromatography of native and bound insulin on Sephadex G-150 column. (a) Native insulin. (b) Bound insulin (preparation P-2) after complete solubilization and after storage for 24 h in 0.05 M phosphate buffer, pH 7.4, at 36°C. No Schiff base reduction. (c) The same experiment as in (b), but Schiff bases were initially reduced. For conditions see the Methods section.

already mentioned, bound and native insulin cause a similar drop in glucose level, but in order to answer the question, does the solubilized Sephadex-bound insulin retain its full biological activity, additional experiments are necessary.

When the suspension of nonsolubilized bound insulin is used the time of onset of the maximal effect is much later (Fig. 3d). It is thought that the hormone in heterogeneous phase is not able to interact with receptor due to steric hindrances; in other words, insoluble insulin–Sephadex did not work immediately because it did not cross the vascular barrier. The hormone begins to exert its effect only as it solubilizes into fragments of the carrier. In this case the time of onset corresponds to the time of solubilization of this preparation *in vitro* (modified Sephadex with the complete solubilization time in 0.1 M phosphate buffer, pH 7.4 at 37°C for about 5 h

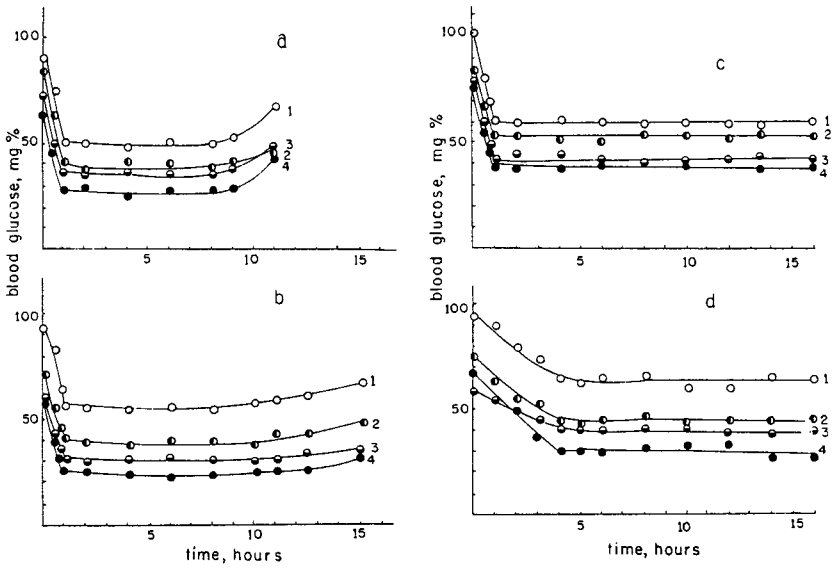


FIG. 3. Blood glucose levels after administration of insulin preparations. (a) Native insulin; (b) solubilized preparation, unreduced (P-2 was a carrier); (c) solubilized preparation, reduced (P-2 was a carrier); (d) insulin–Sephadex (reduced) in suspension (P-11 was a carrier).

TABLE 1. **Properties of Different Insulin Preparations**[a]

| Preparation | Decrease in glucose level, % from initial ($M \pm m$) | The time of onset of hormone effect (h) | The time of active life of preparation (h) |
|---|---|---|---|
| Native insulin | 50±3 | ~1 | ~8 |
| Solubilized Sephadex–insulin | | | |
| Unreduced | 54±5 | ~1 | ~10 |
| Reduced, average mol. w. of carrier fragments ~10,000 | 55±3 | ~1 | >15 |
| Reduced, average mol. w. of carrier fragments ~150,000 | 56±4 | ~1 | >15 |
| Suspension of Sephadex–insulin (time of solubilization ~5 h) | 63±6 | ~4 | >15 |

[a] In each group $n = 4$. For conditions see the Methods section.

was used as a carrier). This also proves our point that insulin does not split off the carrier *in vivo*.

In conclusion it can be stated that one of the possible ways to prolong the effect of insulin may be its binding with the inert polymer carriers. It should be emphasized that up to 150,000 molecular weight the size of the carrier molecule does not influence noticeably the behavior of bound hormone. This permits the suggestion that the hormone interacts with receptors which are located on the surface of the cell membrane. In the case of heterogeneous carrier sterical difficulties due to the large matrix of the carrier molecule may interfere with hormonal action.[1]

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[1] In preparing this paper we became aquainted with the work of Y. Sakamoto, Y. Akanuma, K. Kosaka, and B. Jeanrenaud, "Comparative effects of native insulin and insulin–dextran complexes on the metabolism of adipose," in Biochimica et Biophysica Acta, 498 (1977) 102–113, which, in our opinion, conforms well and is complementary to our data.