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INTERACTIONS OF HUMAN INSULIN WITH DEAE-DEXTRAN

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ABSTRACT

In order to develope an oral drug delivery system for human insulin, interactions of human insulin with DEAE-dextran was studied under different conditions. Effects of various ionic strengths (0.02 M, 0.067 M, 0.01 M and 0.20 M), different temperatures (25°C, 37°C and 45° C) and pH's (6.9, 7.4 and 8) as well as lyophilization and the addition of urea on the interaction were performed. Bound human insulin was separated from the unbound insulin by gel chromatography. Insulin concentration was determined spectrophotometrically at 276 nm. The amount of fraction bound was calculated from area under the curve of the

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chromatographic peaks. The experiments showed that ionic strength had no effect on the binding capacity. However, fraction bound was increased with increased temperature. Under a pH range studies, maximum binding was observed at pH 7.4. The presence of 0.042 M urea seems to increase the binding of insulin to the polymer for approximately 23 %. Lyophilization appears to have no effect on insulin integrity.

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INTRODUCTION

Since the discovery of insulin, innumerable attempts have been made to find a satisfactory means of maintaining a normal blood sugar and controlling diabetes without the inconvenience of injection and complications of the treatment(1,2). These efforts include the changing of routes of administration, the modification of the hormonal drug itself, development of new drug delivery systems and the improvement of insulin treatment regimens such as the development of mechanical insulin infusion pumps (2-13).

For drug delivery systems, the drug carriers may be either homogeneous or heterogeneous systems. Heterogeneous carriers are water-soluble materials suspended in an appropriate buffer. They have included such materials as albumin microspheres (14), human erythrocytes(15), latex particles(16) and phospholipid vesicles or liposomes(17,18). Homogeneous carriers are generally water-soluble macromolecules such as nucleic acid (19,20), antibodies (21,22) or synthetic polymer(23) to which drug may be either covalently bound or noncovalently associated.

Previous works have suggested that insulin has affinity to bind to many polysaccharides such as agarose(24-26). Dextrans which are polysaccharides have been demonstrated to form many complexes with a variety of drugs(27-34). This is closely related to the basic chemical structure of dextran that provides several reactive hydroxyl groups which can form stable complexes(28).

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DEAE(diethylaminoethyl) dextran which has a positive charge on one reference unit(M.W. of 619.5) would be expected to exhibit electrostatic and/or hydrophobic affinity for insulin. It is a chemically modified dextran which has the advantage over simple dextrans since complex formation can be achieved by simple mixing of component species in aqueous solution, with no coupling or activation needed. DEAE-dextran has been found to be taken up by mammalian cells at higher rates than serum albumin(35-37). This may be due to the increased permeability of the cell membrane to DEAE-dextran and/or the enhanced pinocytic activity of cells (35,36). Moreover, the electrostatic interaction of positively charged polycations with the negatively charged cell membrane surface may be a prerequisite step for initiation and enhancement of cellular uptake(38-41). Suzuki et al. have demonstrated that insulin-dextran complex can protect the drug from degradation, thereby enhancing the drug's half life (42). Hence, insulin-DEAE dextran complex may be a better system than the unbound insulin. By the present study, the feasibility and factors affecting the interaction of human insulin and DEAE-dextran polymer was examined. Results obtained from this study will be further used for human insulin in the development of oral drug delivery system.

MATERIALS

Human insulin (Hoechst AG, D-6230 Frankfurt/M. 80), DEAE-dextran (M.W. 500,000, Pharmacia GmbH, D-7800 Freiburg) and Sepharose CL-6B (Pharmacia GmbH , D-7800 Freiburg) were used as received.

METHODS

Effect of Ionic Strength on Human Insulin-DEAE-dextran Binding

Ten milliliters of human insulin-DEAE dextran solution were

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prepared in 0.02 M, 0.067 M, 0.1 M and 0.2 M phosphate buffer at pH 7.4. The solutions were incubated in a controlled temperature water bath at 37° C for 1 hour. The separation of bound and unbound human insulin was achieved by gel chromatography.

Effect of Temperature on Human Insulin-DEAE-dextran Binding

Ten milliliters of human insulin-DEAE dextran solution were prepared in 0.067 M phosphate buffer(pH 7.4). The solution was incubated in a controlled temperature water bath at 25°C, 37°C and 45°C for 1 hour.

Effect of pH on Human Insulin-DEAE-dextran Binding

Ten milliliters of insulin-DEAE dextran solution were prepared in 0.067 M phosphate buffer in different pH 's of 6.9, 7.4 and 8. The solutions were incubated in a controlled temperature water bath at 37° C for 1 hour.

Effect of Urea on Human Insulin-DEAE-dextran Binding

Ten milliliters of insulin-DEAE dextran solution were prepared in 0.067 M phosphate buffer in presence of 0.0042 M urea and 0.042 M urea. The solutions were incubated at 37° C for 1 hour.

Effect of lyophilization on Human Insulin-DEAE-dextran Complex

The pure human insulin-DEAE dextran complex (prepared in 0.067 M phosphate buffer pH 7.4, at 37° C and incubation for 1 hour) was obtained by gel chromatography of fractions 9 to 19 and then lyophilized (Gefriertrocknungsanlage, GT2, Leybold-Heraueus GmbH & Co. KG, D-5000 Köln) at 0.1 Torr, -30° C for 8-10 hours. The resulting lyophilized powder was reconstituted in the buffer and chromatograms of bound and unbound insulin were obtained by gel chromatography.

In all cases, a 0.5 ml sample(a 1 ml sample was used instead in lyophilization study) was loaded on the column(1.6 X 20 cm) previously packed with Sepharose CL-6B. The column was eluted with 0.067 M phosphate buffer, pH 7.4 at a flow rate of 0.6 ml/min.

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About forty fractions of 45 drops per tube were collected with an automatic fraction collector(Foxy, Lincoln, Nebraska, USA). Insulin was detected by UV at 276 nm and dextran by precipitation test using 0.1 ml aliquot of each fraction and 0.3 ml acetone. In this study, only the freshly prepared samples were used and at least three different sample preparations were run for each experimental system.

Absorbance of each fraction was plotted against the number of tubes or fractions. Fraction bound of insulin was calculated from the quotient of AUC of the bound insulin and the AUC of total insulin.

RESULTS AND DISCUSSION

The separation of human insulin-DEAE dextran complex from the unbound insulin by gel chromatography resulted, in all cases, two distinct peaks. Bound insulin was eluted first and was followed by unbound insulin as shown in Figure 1. The shade area indicated fractions which were precipitated with 3 volume of acetone and represented insulin-dextran complex whereas the second peak was not precipitated with 3 volume of acetone and corresponded to unbound insulin. The elution profile is highly reproducible. Fraction bound was calculated from AUC of these two peaks. In each experimental system, an average fraction bound was obtained from at least three different sample preparations with a standard deviation of less than 5 %.

At pH 7.4, DEAE dextran has one positively charged diethylaminoethyl group per reference unit and human insulin has a net charge of -2. One might expect that the primary interaction between them would be electrostatic involving an interaction between carboxyl groups of human insulin and the positively charged nitrogens of the polymer. However, Table 1 shows that the binding of human insulin to dextran polymer appears not to be

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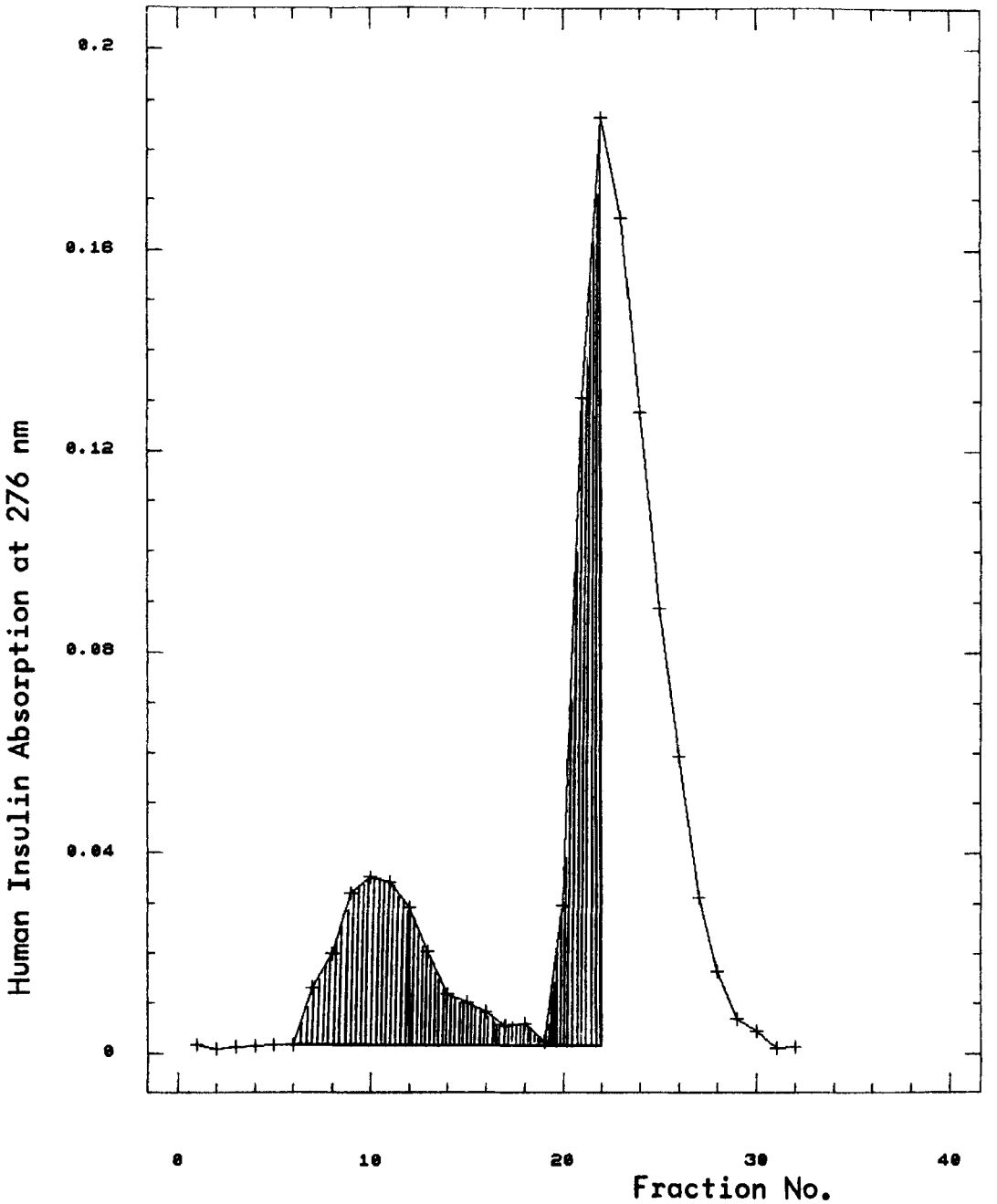

FIGURE 1

Elution Pattern of Human Insulin-DEAE Dextran Complex Solution Prepared in 0.067 M Phosphate Buffer, pH 7.4 and at 37° C.

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TABLE 1

Effect of Ionic Strength on Human Insulin-DEAE-dextran* Binding

Ionic Strength	Average Fraction Human Insulin Bound
0.020	0.1970
0.067	0.2080
0.100	0.2027
0.200	0.1907

* DEAE-dextran concentration = 16.14 X 10^{-2} M (M.W. 619.5 "Reference Unit" Basis) M.W. of Polymer Approximately 500,000 , Human Insulin Concentration = 6.87 X 10^{-4} M = 4.0 mg/ml, pH 7.4 and at 37° C.

TABLE 2

Effect of Temperature on Human Insulin-DEAE-dextran* Binding

Temperature	Average Fraction Human Insulin Bound
25° C	0.1492
37° C	0.1952
45° C	0.2593

* Same as Table 1 and in 0.067 M Phosphate Buffer pH 7.4.

affected by ionic strength. This suggests that the interaction should not be mainly caused by electrostatic binding but probobly also via hydrophobic interaction. This may be explained by the consideration of human insulin as a polymer of amino acids. The tertiary and quaternary structures of both polymers, DEAE-dextran and human insulin, may prevent the approaching of the charged groups which are only small parts comparing to the uncharged or hydrophobic parts of the molecules. Hydrophobic interaction seems therefore to be more possible. The average fraction bound of all cases is quite a small number of not more than 0.30. This may be also due to steric hindrance effect of the two polymers.

The binding of human insulin to DEAE dextran appears to increase with temperature as shown in Table 2. The results also gave a linear relationship between average fraction bound and

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TABLE 3

Effect of pH on Human Insulin-DEAE-dextran* Binding

pH	Average Fraction Human Insulin Bound
6.9	0.1792
7.4	0.2020
8.0	0.1655

* Same as Table 1 and in 0.067 M Phosphate Buffer at 37° C

TABLE 4

Effect of Urea on Human Insulin-DEAE-dextran* Binding

Urea Concentration	Average Fraction Human Insulin Bound
0.0000 M	0.2045
0.0042 M	0.2064
0.0420 M	0.2518

* Same as Table 1 and in 0.067 M Phosphate Buffer pH 7.4 at 37° C.

temperature(° C) with the relation coefficient of 0.98. The increase of kinetic energy of the system by increasing temperature may provide more molecules to come close together and have the interaction. The linear relationship probably indicates a linear relation between kinetic energy and numbers of interacting molecules.

The binding of human insulin to DEAE dextran appears to be affected by pH and shows the maximum binding of 0.2020 at pH 7.4 as shown in Table 3. This suggests that hydrophobic interaction was not the only interaction that could occur but also electrostatic interaction as well, since charge dissociation is pH dependent. At pH 7.4, maximum solubility of human insulin and charge dissociation combination giving maximum binding of the two polymers may occur.

The presence of 0.0042 M urea, concentration in normal human blood, had no effect on the binding of insulin to dextran as shown in Table 4. However, when urea concentration was increased to 0.042 M, the binding was increased to about 23 %. At this urea

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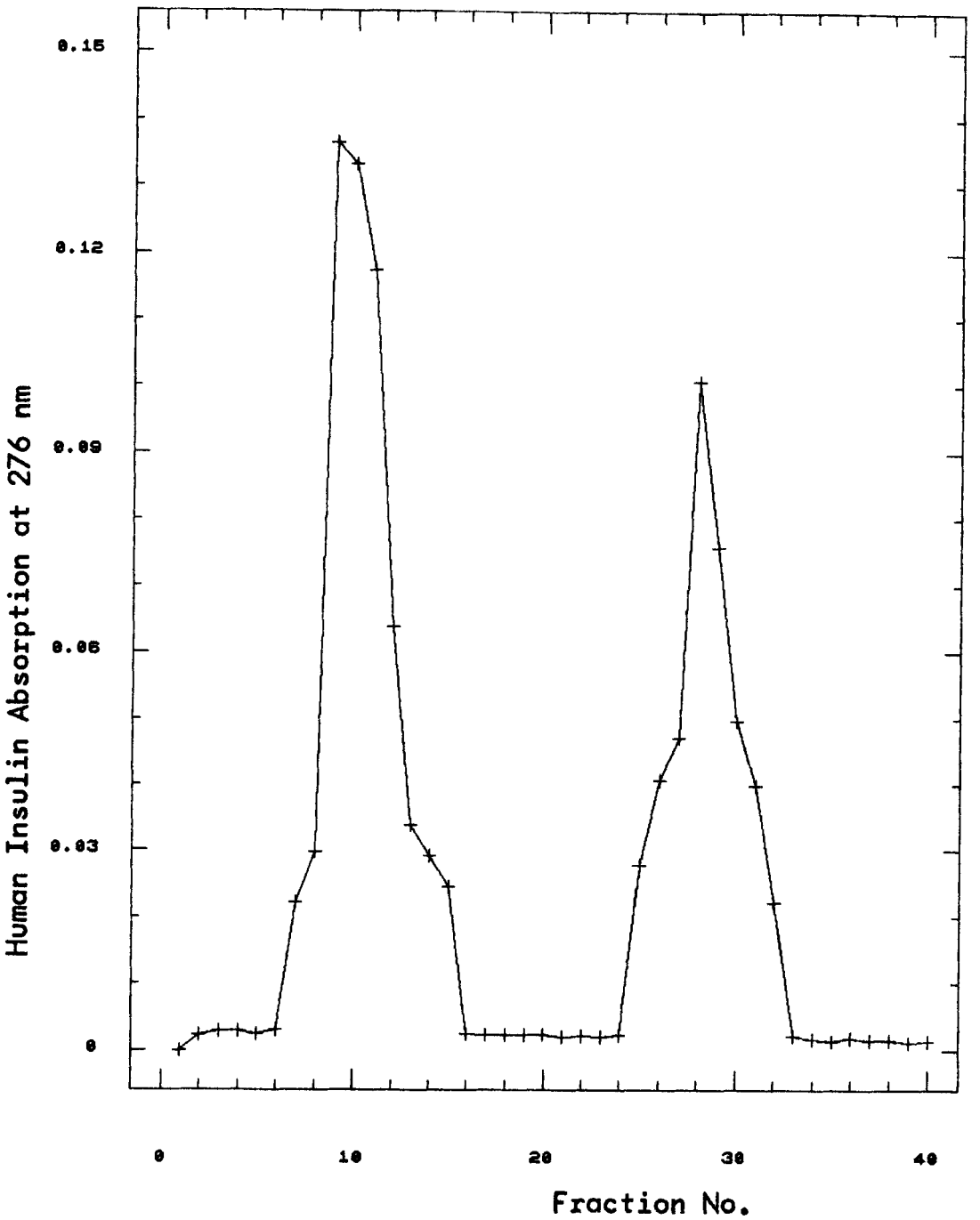

FIGURE 2

Elution Pattern of Reconstituted Pure Human Insulin-DEAE Dextran Complex Prepared in 0.067 M Phosphate Buffer with 0.042 M Urea, pH 7.4 at 37° C, Insulin Concentration 0.6 mg/ml.

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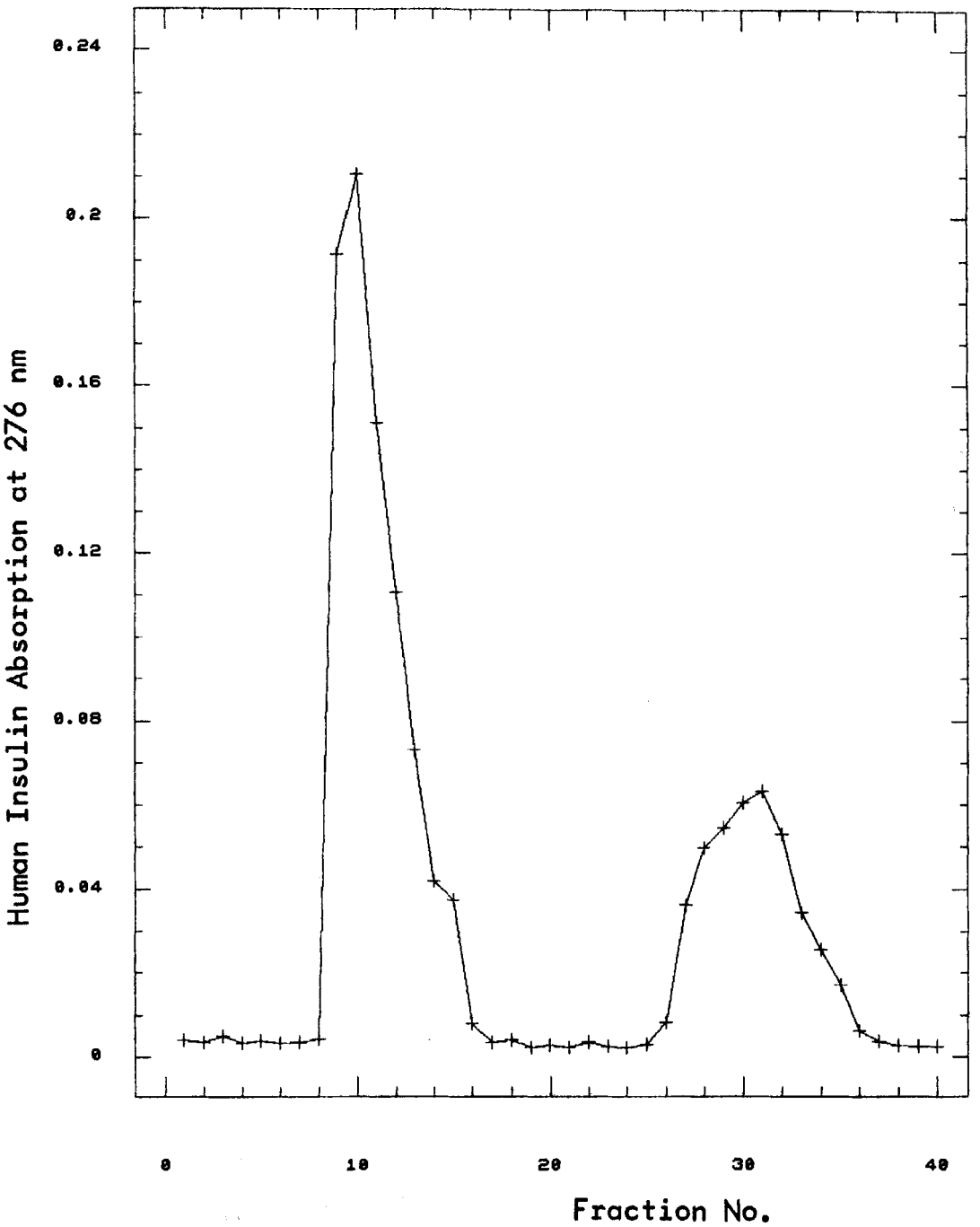

FIGURE 3

Elution Pattern of Reconstituted Pure Human Insulin-DEAE Dextran Complex Prepared in 0.067 M Phosphate Buffer without Urea, pH 7.4 at 37° C, Insulin Concentration 0.6 mg/ml.

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concentration level, urea may unfold the protein chain resulting in the reduction of steric hindrance effect of the two polymers, thereby facilitating the binding of insulin to DEAE-dextran polymer.

Figure 2 and 3 show the elution pattern of the reconstituted pure human insulin-DEAE dextran complex after undergone lyophilization with and without 0.042 M urea respectively. From the chromatograms, lyophilization appears not to change the integrity of bound and unbound human insulin. However, dissociation of the reconstituted complex insulin with urea was more than that without urea of about 27 %. Again, urea may unfold insulin chain and facilitate complex dissociation when reconstituted. Thus, presence of urea may be disadvantageous since urea facilitated complex dissociation more than complex binding.

From an analysis of the above data, it would appear to suggest that the proper conditions in preparing a human insulin - DEAE dextran complex solution is in 0.067 M phosphate buffer, pH 7.4 at 37° C and with no presence of urea. The complex could be stored after lyophilization. An application of these results together with the study of the development of human insulin as oral drug as well will be further reported in a separate communication.

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