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# HUMAN INSULIN BINDING TO ERYTHROCYTE-MEMBRANE

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## ABSTRACT

The binding of human insulin to erythrocyte membrane in the form of ghosts, vesicles (ultrasonicated ghosts), lipid-coated-ghosts or lipid-coated-vesicles was the subject of this study. Insulin was found to associate with ghosts in two mechanisms, by encapsulation and adsorption on the surface. Insulin binding reached equilibrium with a much faster rate with ghosts than the other carriers, due to these two mechanisms. The findings from this study suggest that the use of these carrier-insulin systems may be of value in the delivery of insulin in the treatment of diabetes.

## INTRODUCTION

Diabetes mellitus in man is a well documented disease. With the discovery of insulin during the first half of this century, clinicians thought that diabetes would be well controlled. Until recently porcine or bovine insulins were the primary insulins used in this treatment. However, advances in biotechnology in the field of recombinant DNA have allowed the synthesis of human insulin using microorganisms. This synthetic human

insulin differs from insulins of animal origin in that it is absorbed much faster from subcutaneous injections and therefore it reduces the blood glucose level at a much more rapid rate.[1] The usual route of insulin administration is subcutaneous which offers easy vascular access but frequently results in a slow and variable insulin absorption. The intravenous route results in a rapid onset of insulin effect, but this is accompanied by a short duration of action and peripheral hyperinsulinemia.[2] Despite the therapeutic efficacy of parenterally administered insulin, significant inconveniences of these routes have prompted a search for alternate means of administration. Consequently, new drug delivery systems to permit administration of insulin by other routes have become the target for a new era of insulin research.

Insulin absorption from lung tissue has been accomplished by administering insulin in an aerosol dosage form.[3] Some success has been achieved in the preparation of an intranasal dosage form for insulin.[4,5,6,7,8] An oral dosage form, however, would offer still greater ease of administration. Development of a useful oral dosage form has been complicated by the fact that enzymes in the digestive juices destroy substances such as orally administered peptides. Thus, orally administered insulin, if it is to be useful, must be protected from these enzymes. Recent work suggested that insulin absorption from the intestine, although low, occurred largely in the jejunum.[9] Although the use of liposomes has enhanced the absorption of insulin from the intestine, the insulin that was absorbed was partially degraded yielding no hypoglycemic effect.[10,11] Polyalkylcyanoacrylate nanoparticles were used to entrap

insulin and have also been examined as an oral delivery system.[12]

The use of intact erythrocytes[7,13,14] erythrocyte-ghosts[15] and liposomes-incorporating-erythrocyte-ghosts[14] as carriers for insulin have not been fully assessed. In this report, the mode of interaction of human insulin with erythrocyte membrane (i.e, erythrocyte-ghosts and -vesicles) and lipoid-coated erythrocyte membrane is described.

## MATERIALS AND METHODS

### Materials

Human insulin (Humulin R, Eli Lilly) was purchased from N.C. Mutual, North Carolina. Human Erythrocytes were obtained from the American Red Cross, North carolina. Cholesterol and L-α-phosphatidyl-choline (type XI-E, from fresh egg yolk) were purchased from Sigma Chemical Company, St. Louis, Missouri. All chemicals were of analytical grade except those that were used in the analysis of insulin, which were of HPLC grade.

### Preparation of Erythrocyte-Ghosts

Erythrocyte-ghosts were prepared according to the general method previously described.[16] Briefly, a volume of human red blood cells was washed with an isotonic phosphate buffer solution. The suspension of erythrocytes was centrifuged at 23,500 x g for 20 min. The supernatant was aspirated and discarded. The sedimented erythrocytes were treated with a hypotonic buffer solution. The mixture was then centrifuged for 20 min at 23,500 x g. The supernatant was aspirated and discarded. The addition of the hypotonic buffer solution was repeated several times until the supernatant became colorless. The erythrocyte-ghosts were stored in the

refrigerator (4°C) for further use. The preparation yields, on average, 4 x $10^6$ ghosts/ml.[16]

**Preparation of Erythrocyte-Vesicles**

Erythrocyte-vesicles were prepared using the method previously described.[16] Five ml of erythrocyte-ghosts suspension were sonicated using a sonic dismembrator (Tekmar sonic disrupter, 50-watt model, Tekmar Company, Cincinnati, Ohio) set at an energy level of 50.

**Preparation of Liposomes-Incorporated Erythrocyte-Ghosts or Erythrocyte-Vesicles**

These two dosage forms were prepared by a method previously described[15] with minor modifications. A swelling solution for the lipid phase that contained 0.0725 M each of NaCl, KCl, and CsCl was used. The lipid phase consisted of 0.375 g L-α-phosphatidyl-choline (type XI-E from fresh egg yolk) and 0.05 g cholesterol. The lipid phase was first dissolved in 50 ml of chloroform in a round-bottom glass container (capacity 350 ml) and was evaporated to dryness under vacuum using a rotary evaporator set at a speed of 35 r.p.m. Then, 5 ml of a liquid mixture (50 % (v/v) of the swelling solution and 50 % (v/v) of either erythrocyte-ghosts suspension or erythrocyte-vesicles suspension) were added to the dry lipids. The flask was shaken gently for one hour at room temperature, and allowed to stand for two hours at room temperature or overnight in the refrigerator (4°C). The mixture was then centrifuged at 23,500 x g for 20 min. The supernatant was aspirated and discarded. Five ml of the swelling solution were added to the sediment and the mixture was vortexed. The resulting suspension was stored in the refrigerator for further use.

**Incubation of Human Insulin with the Carriers**

Human insulin was incubated with either one of the four preparations mentioned above under the following conditions: one ml of the insulin solution with various concentrations (10 - 100 U/ml) was mixed with one ml of carrier. The incubation time was 24 hours, at a temperature of 37°C. Following incubation, the suspension was centrifuged at 23,500 x g for 20 min. The amount of insulin that was associated with the carrier was determined from the difference between the initial amount of insulin added and the amount that was found in the supernatant. To correct for the possibility of insulin binding to the surface of glass or other materials during processing, a blank solution that contained insulin solution with no carrier was also tested. To test for the effect of incubation time on the amount of insulin associated with carrier, 1 ml of insulin solution (100 U/ml) was incubated with 1 ml of carrier for either 1 or 6 hours.

**Mode of Binding of Human Insulin to Erythrocyte-Ghosts**

Ghosts (1 ml) were mixed with 1 ml of insulin solution of different concentrations (10 - 100 U/ml). The samples were incubated for 24 h at 37°C in a shaking water bath. Following the incubation, the samples were centrifuged for 20 min at 23,500 x g. An aliquot (100 μl) taken from the supernatant was tested for insulin content. The samples were then vortexed and ultrasonicated for 3 min at an energy level of 50 to disintegrate the cellular membrane of the ghosts. The portion of insulin entrapped inside the cells, if any, was released upon ultrasonication. The samples were centrifuged for 20 min at 23,500 x g and another aliquot

(100 μl) was taken from the supernatant to be tested for insulin.

### High Performance Liquid Chromatography Assay for Human Insulin

The HPLC system used in this study had the following specifications: U6K injector, Lambda-Max model 481 LC spectrophotometer, Waters 740 data module (Millipore/Waters, Milford, MA), Perkin-Elmer series 3 solvent delivery system, and Dupont Instruments C-18 reverse phase column. The mobile phase was composed of acetonitrile:water:trifluoroacetic acid:hexanesulfonic acid 35:65:0.1:0.1. The solvent flow was set at 1 ml/min. Measurements were carried out at a wavelength of 215 nm.

## RESULTS AND DISCUSSION

The present report addresses the interaction of human insulin with erythrocyte-membrane and lipid-coated-membrane. These forms of the membrane may potentially be used as carrier systems for insulin *in vivo*. The association of insulin with carriers was determined at 37°C; furthermore, the effect of incubation time on the amount associated was also studied. Insulin binding to ghosts appeared to be nearly complete at 1 h, yet binding to other carriers increased over the 24 h incubation period (Fig. 1). This suggests that the association of insulin with ghosts can be of two types: i) adsorption on the surface of the cytoplasmic membrane and/or ii) diffusion through the membrane and establishing an equilibrium with the outside concentration of the drug. This was evident when the ghosts were incubated with insulin and then ultrasonicated to release any entrapped insulin. The

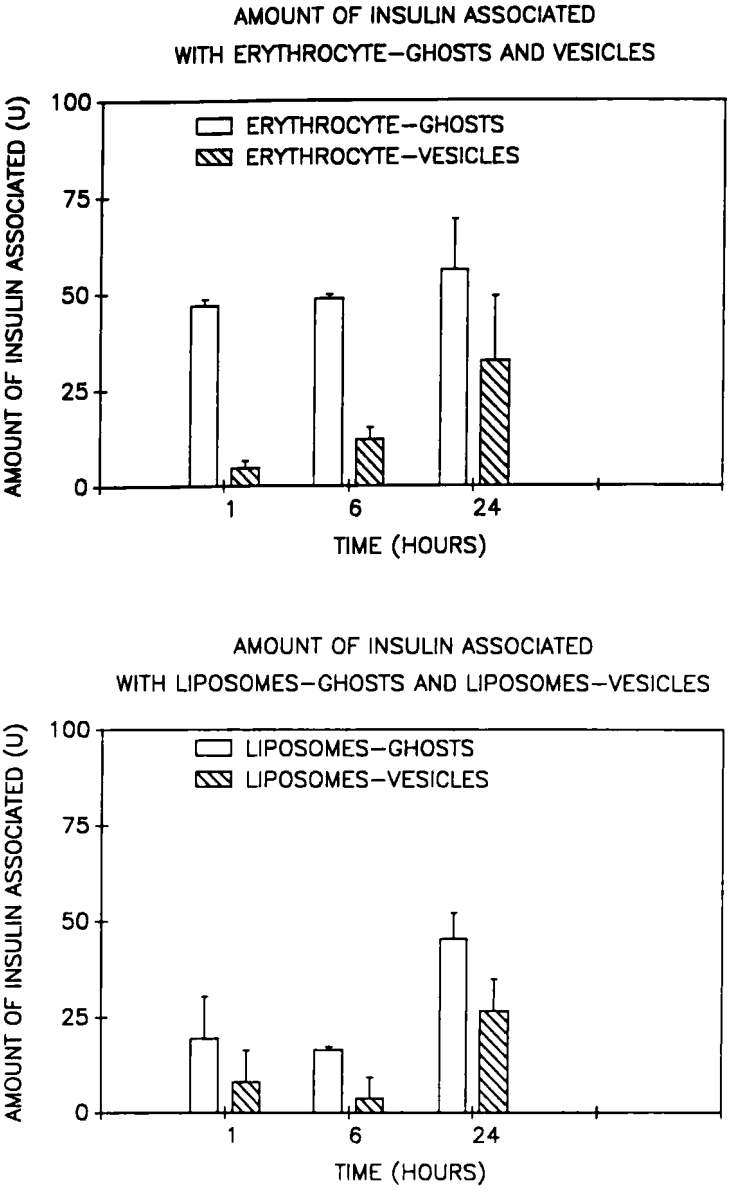


FIGURE 1.

Fig. 1. The amount of insulin bound to carrier systems at 1, 6, and 24 h. Insulin equilibrated rapidly with erythrocyte-ghosts; however, with the other carriers there was an increase in the amount bound as the incubation time increased. Data points represent mean ± s.d., n = 3-6. Initial amount of insulin added was 100 U for all systems.

TABLE 1
The Increase in the Amount of Insulin Found in the Supernatant Following Ultrasonication of Ghosts-Insulin Suspension.

| | Amount of Insulin (U) (Ave. ± S.D.) | |
|---|---|---|
| Initial[a](U) | Before Ultrasonication[b] | After Ultrasonication[b] |
| 100 | 46.43 ± 3.90 | 84.62 ± 5.52 |
| 80 | 38.02 ± 2.87 | 70.00 ± 8.67 |
| 60 | 23.21 ± 2.27 | 30.36 ± 4.48 |
| 50 | 8.83 ± 1.40 | 21.72 ± 5.53 |
| 30 | 9.48 ± 1.29 | 16.54 ± 1.27 |
| 10 | 1.34 ± 0.39 | 2.16 ± 1.14 |

a. Initial Amount of Insulin Incubated with Ghosts.
b. Average of 3 Samples.



increase in the amount of insulin found in the supernatant following ultrasonication indicated that insulin was both encapsulated by the ghosts and bound to their membrane (Table 1).

The amount of insulin bound to vesicles or lipid-coated-vesicles was found to be lower than that of ghosts or lipid-coated-ghosts (Fig. 2). This can be explained by the fact that vesicles lack the internal space that the ghosts have.[16] Also, ultrasonicating the ghosts membrane may have resulted in physical deformation of the adsorption sites. Over all the carriers studied, the percent of insulin bound decreased as the amount added increased (Fig. 3). Another way to state this is that the amount of insulin bound appears to approach a maximum over the amounts studied (compare with Fig. 2). The mechanism for this observation is unclear at this time from the data collected. Further work will be needed to clarify this mechanism.

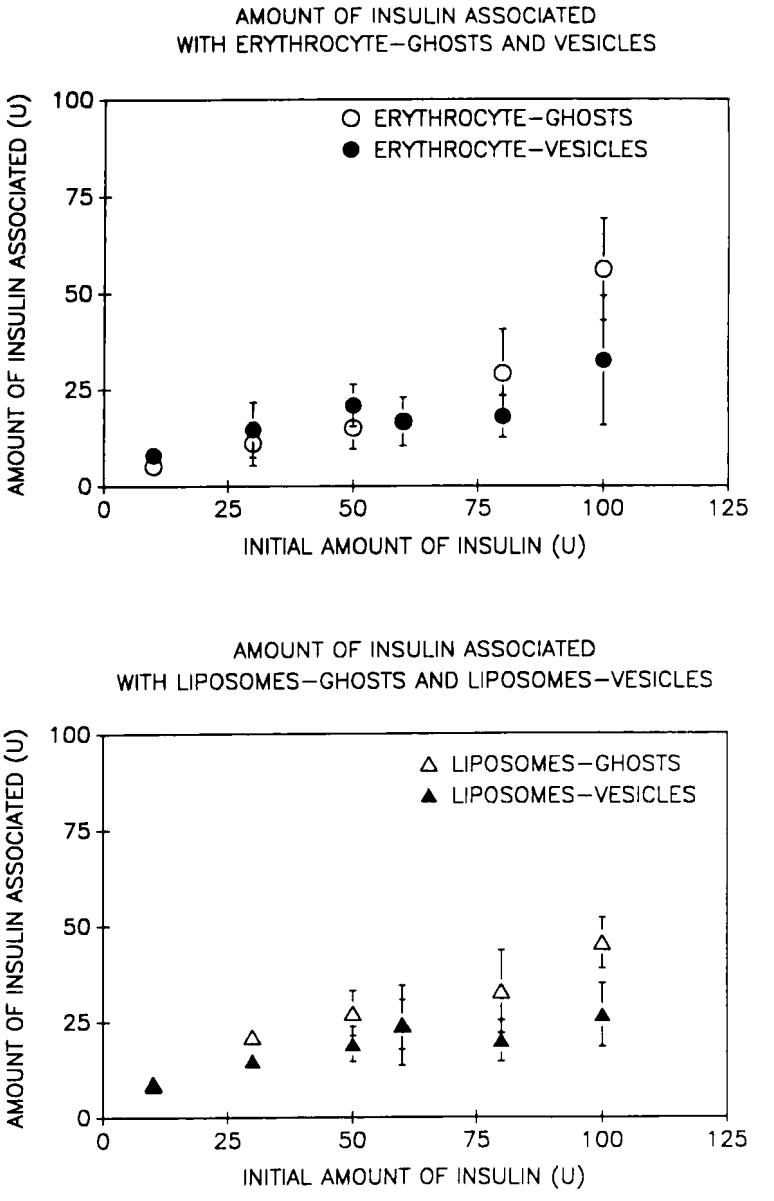


FIGURE 2.

Fig. 2. Amount of insulin associated with carriers after 24h incubation at 37°C. Data points represent mean ± s.d., n = 6.

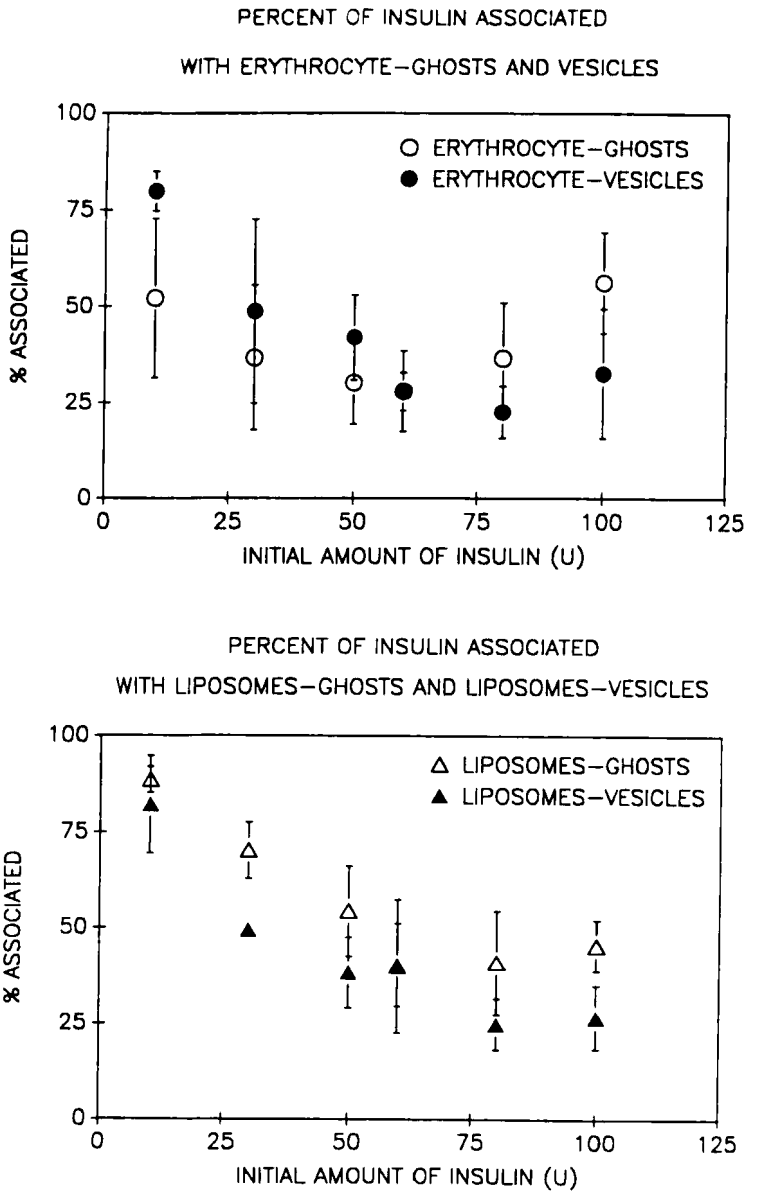


FIGURE 3.

Fig. 3. The relationship between the percent of insulin associated with the carrier systems and the initial amount of insulin incubated with the carrier. Overall, as the initial amount increased the percent decreased. Data points represent mean ± s.d., n = 6.

In conclusion, the ability of human insulin to associate with different carrier systems, and the possible influence of these carriers of changing the disposition profile of the drug may be of significant value in modifying the delivery of insulin to diabetes patients. Further work is in progress in our laboratories to test these systems *in vivo* as oral dosage forms.

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