

# Characteristics of Human Erythrocyte Insulin Receptors

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## SUMMARY

Highly specific insulin receptors have been identified on human erythrocytes. A modification of the monocyte insulin radioreceptor technique permitted distinct separation of human erythrocytes with their bound insulin from the free insulin. When incubated with 80 pg. per milliliter of  $^{125}\text{I}$ -insulin (pH 8.0, 3.5 hours,  $15^\circ\text{C}$ .), erythrocytes from 17 normal volunteers specifically bound 10 per cent ( $\pm 1.450$  S.D.) of the total  $^{125}\text{I}$ -insulin. Less than 15 per cent of the total  $^{125}\text{I}$ -insulin bound was nonspecific. Binding of  $^{125}\text{I}$ -insulin to human erythrocytes was dependent on pH and temperature. Less than 5 per cent of the insulin available to the plasma membrane was degraded. Both calcium and magnesium enhanced  $^{125}\text{I}$ -insulin binding by 100 per cent but had no synergistic effect when mixed in a 1:1 molar ratio.

Scatchard analysis of the binding data resulted in a curvilinear plot with characteristics typical of negative cooperative interactions between receptor sites and with an unoccupied site affinity constant of  $0.1 \times 10^8 \text{ M}^{-1}$ . Human erythrocytes have 2,000 insulin binding sites per erythrocyte with 14 sites per square micrometer of surface area. The readily available human erythrocyte, thus, has both specific insulin binding sites and binding characteristics similar to other human cell types. These studies have provided the basis for further clinical investigation of polypeptide hormone receptors on human erythrocytes. *DIABETES* 27:701-08, July, 1978.

During the past seven years, radioreceptor assays have been utilized to demonstrate specific polypeptide hormone receptors on both freshly isolated and cultured circulating human cells. Monocytes<sup>1</sup> and granulocytes<sup>2</sup> have been shown to possess specific receptor sites for insulin and growth hormone.<sup>3</sup>

Freshly isolated circulating human erythrocytes have recently been shown to have highly specific insu-

lin receptors.<sup>4</sup> Initially, Gavin et al.<sup>5</sup> demonstrated insulin binding to human erythrocytes; however, they utilized a technique that suggested human erythrocytes had a high degree of nonspecific insulin binding.

Human erythrocytes possess properties that make them ideal cells for human hormonal radioreceptor studies. The membrane characteristics of these cells are similar to those of other circulating human cells.<sup>6</sup> Like other cells, specific membrane transport mechanisms are present, the membrane proteins are specifically organized, and calcium-dependent ATPase activity is evident.<sup>7</sup> Moreover, human erythrocytes are readily available in large quantities and are normally found in suspension with easily accessible free plasma membranes.

Our recent modifications of the insulin radioreceptor assay<sup>4</sup> have permitted us to further study insulin binding in human erythrocytes. The studies we now present demonstrate the similarity of human erythrocyte insulin binding to that of other human cell types.

## MATERIALS AND METHODS

*Subjects.* Erythrocytes from 17 normal volunteers, 18 to 35 years of age, were studied. After a physical examination, scanning blood chemistries, chest X-rays, and electrocardiograms, each of these volunteers was determined to be normal by a Howard University Health Service physician. All had family and personal histories negative for diabetes mellitus. Erythrocyte specimens were obtained after a 12-hour fast and three hours of morning ambulatory activity.

*Hormones.* Purified porcine insulin (lot 8GN814) was obtained from Elanco Laboratories, a division of Eli Lilly Research Laboratories, Indianapolis, and was used both for the unlabeled ligand and for iodination.

Growth hormone (GH), glucagon, and human chorionic gonadotropin (HCG) were purchased from Sigma Chemical Company. Guinea pig insulin was a gift from Dr. C. C. Yip of Toronto, Canada. Rabbit,

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bovine, and ovine insulins and bovine proinsulin were generous gifts from Dr. Ronald Chance of Eli Lilly Research Laboratories, Indianapolis.

Bovine serum albumin (BSA, fraction V) was obtained from Pentex. Carrier-free Na<sup>125</sup>I was purchased from Amersham Searle. Dibutyl phthalate (density 1.043) was obtained from Aldrich Chemical Company. Microfuge B, microfuge tubes, and other microfuge accessories were supplied by Beckman Instruments. Other chemicals were of reagent grade.

Radioiodinated insulin was prepared as previously described<sup>4</sup> and generously supplied by Dr. Jesse Roth (N.I.H.).

**Buffer G.** The following composition of buffer G was determined after a series of experiments: hepes 50 mM, tris 50 mM, MgCl<sub>2</sub> 10 mM, ethylenediamine tetra-acetic acid 2 mM, dextrose 10 mM, CaCl<sub>2</sub> 10 mM, NaCl 50 mM, KCl 5 mM, and 0.1 per cent BSA. The pH of this buffer was adjusted to 8.0 at room temperature (23 to 25° C.). This buffer has a calculated osmolarity of 286 milliosmoles per liter.

**Preparation of erythrocytes for binding studies.** Human blood, 10 ml., was obtained in a vacutainer tube containing 143 U.S.P. units of sodium heparin per tube (Becton-Dickinson Co.). Following centrifugation (10 minutes, 400 × g, 20° C.), the plasma was aspirated. The cell pellet was mixed with one part of physiologic saline and then layered on 3 ml. of Hypaque (33.9 per cent)-Ficoll (9 per cent) mixture (1:2.4 volume/volume) in a glass tube as described by Boyum.<sup>8</sup> After centrifuging at 400 × g for 20 minutes at 20° C., the saline, monocyte, Hypaque-Ficoll, granulocyte phases, and the upper layer of the erythrocyte phase were aspirated. The cell pellet was then suspended in one part saline, and the above procedure was repeated. The resulting erythrocyte pellet was then resuspended in two parts of buffer G to equilibrate the cells. After centrifugation of the cell suspension (10 minutes, 400 × g, 4° C.), the buffer was aspirated and the cell pellet resuspended with a volume of buffer G that resulted in a suspension containing 4.4 × 10<sup>9</sup> cells per milliliter. The mean hematocrit and hemoglobin for this suspension were 41.1 per cent (± 4.4 S.D.) and 13.6 gm. (± 1 S.D.), respectively; the mean erythrocytic indexes were mean corpuscular volume of 88.8 μ<sup>3</sup> (± 9.1 S.D.), mean corpuscular hemoglobin of 30.2 pg. (± 3.5 S.D.), and mean corpuscular hemoglobin concentration of 32.9 per cent (± 2.9 S.D.). More than 95 per cent of the cells were viable, as determined by the trypan blue dye exclusion technique.<sup>9</sup> Total RBC counts, hematocrit, hemo-

globin, and erythrocyte indexes were determined by a Coulter counter (model S Senior) that was standardized three times before counting each sample.

**Calculations.** The average number of insulin binding sites per human erythrocyte was obtained by Scatchard analysis<sup>10</sup> of <sup>125</sup>I-insulin binding data. Using the amount of the insulin bound (B) and total insulin concentration minus B as the free insulin concentration (F), a plot of B/F to B can be derived. Since this plot was curvilinear, a constant slope was not possible and, thus, estimation of the exact affinity constant could not be obtained. The average number of estimated sites per cell was calculated using the following expression:

$$\text{Sites per cell} = \frac{\text{moles of insulin bound per liter}}{\text{cell concentration per liter}} \times 6.03 \times 10^{23} \text{ (Avogadro's number).}$$

The maximal amount of insulin bound (moles of insulin bound per liter) is the abscissa intercept of the curve.

The average number of receptor sites per unit surface area (square micrometer) was calculated from the following formula:

$$\text{Sites per square micrometer} = \frac{\text{number of sites per cell}}{\text{surface area (140 } \mu\text{m.}^2\text{)}}.$$

The affinity constant of the empty sites ( $\bar{K}_e$ ) was obtained from the negative slope of a line joining the initial B/F ratio to the abscissa intercept of the curve.<sup>12</sup>

**Binding studies.** Binding of <sup>125</sup>I-insulin to human erythrocytes was determined by incubating a 400 μl. cell suspension (1.76 × 10<sup>9</sup> cells in buffer G), 40 pg. of <sup>125</sup>I-insulin (in 25 μl. of buffer), and concentrations of unlabeled insulin (0 to 0.5 × 10<sup>5</sup> ng.) and buffer G to constitute a volume of 0.5 ml. After incubating at 15° for 3.5 hours, 200 μl. of the incubated suspension was aliquoted into prechilled microfuge tubes containing 200 μl. of buffer G and 200 μl. of dibutyl phthalate. These tubes were then centrifuged in a Beckman microfuge B (kept in a 4° C. cold room) for 2.5 minutes. The buffer and dibutyl phthalate layers were aspirated with a Pasteur pipet, leaving about 1/10 of the total dibutyl phthalate on the pellet.

During aspiration, formation of bubbles containing trapped free <sup>125</sup>I-insulin in the dibutyl phthalate phase can be avoided by aspirating with a Pasteur pipet that has an uneven tip. Removal of these trapped bubbles prevents a false positive binding of <sup>125</sup>I-insulin. To determine the bound radioactivity, the tip of the microfuge tube containing the cell pellet was cut with a heated scalpel and counted in a gamma

counter (Searle model 1185). The radioactivity bound to the cells was determined by the following:

$$\text{Per cent radioactivity bound} = \frac{\text{erythrocyte pellet radioactivity}}{\text{total radioactivity (in 200 } \mu\text{l. of the incubated cell suspension)}} \times 100.$$

The percentage of specific insulin bound<sup>11</sup> at each concentration of unlabeled insulin was determined by subtracting the percentage of <sup>125</sup>I-insulin bound at  $1 \times 10^5$  ng. per milliliter of unlabeled insulin from the total percentage of <sup>125</sup>I-insulin bound at each concentration of unlabeled insulin.

## RESULTS

When specific insulin binding to  $3.52 \times 10^9$  erythrocytes per milliliter from 17 normal human volunteers was studied, a maximum of 10 per cent ( $\pm 1.4$  S.D.) of the <sup>125</sup>I-insulin was specifically bound at 80 pg. per milliliter of <sup>125</sup>I-insulin (figure 1). Nonspecific <sup>125</sup>I-insulin binding at  $10^5$  ng. per milliliter was 12.8 per cent ( $\pm 4.2$  S.D.) of the total insulin bound. Inhibition of <sup>125</sup>I-insulin by the unlabeled ligand was linear over the range of insulin

concentrations from 2 to 15 ng. per milliliter. Greater than 50 per cent of the <sup>125</sup>I-insulin binding was inhibited in the physiologic range of insulin concentrations.

*Effects of  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$ ,  $\text{K}^+$ , and  $\text{Na}^+$ .* To determine the effect of various cations on the binding of insulin, the erythrocytes were incubated in a control buffer of 70 mM tris, 10 mM glucose, 0.23 M sucrose, and 0.1 per cent bovine serum albumin and insulin binding was compared with that of erythrocytes incubated in this buffer plus each of the following cations: sodium, potassium, magnesium, and calcium. Both sodium and potassium (20 mM each) decreased the binding of <sup>125</sup>I-insulin by 13 and 30.6 per cent, respectively. When these two cations were mixed in a 1:1 molar ratio (10 mM each), the binding was decreased by 20 per cent. Magnesium and calcium at 20 mM each resulted in a 100 per cent increase of <sup>125</sup>I-insulin binding. When mixed in a 1:1 molar ratio (10 mM each), a 93 per cent increase in binding was observed. Calcium and sodium in a 1:1 molar ratio (10 mM each) increased the binding by 58 per cent, whereas magnesium and sodium increased the binding by 38 per cent.

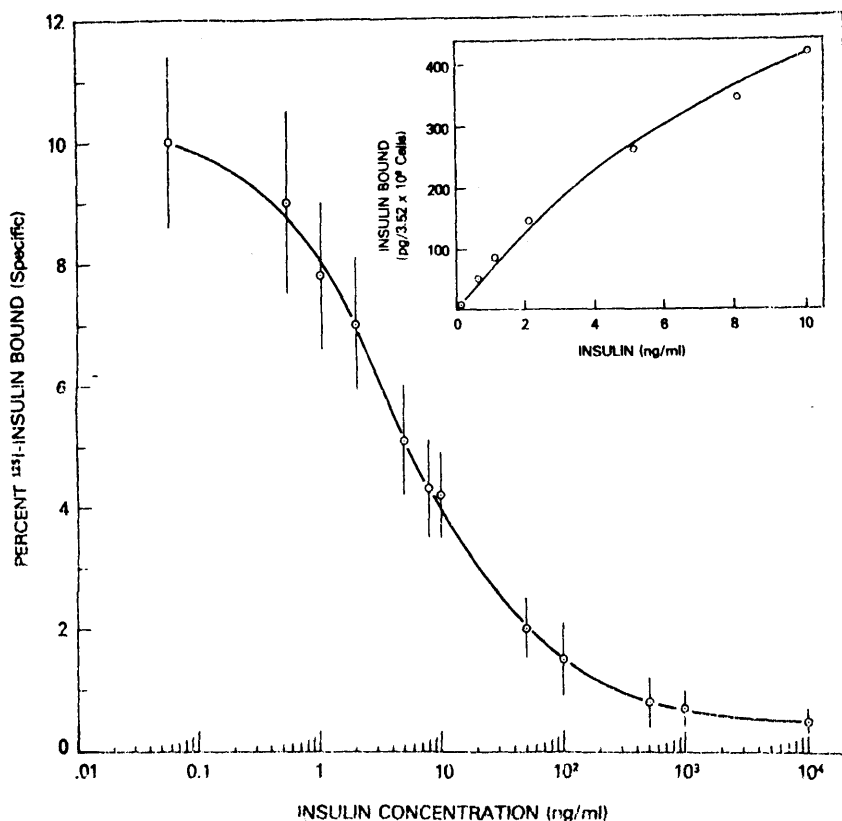


FIGURE 1

Per cent of <sup>125</sup>I-insulin bound to human erythrocytes. Human erythrocytes were isolated, washed, and resuspended in buffer G, pH 8.0, to constitute a suspension with  $4.4 \times 10^9$  erythrocytes per milliliter. Then 400  $\mu$ l. ( $1.76 \times 10^9$  erythrocytes) of this cell suspension was incubated with buffer G, unlabeled insulin (0 to  $0.5 \times 10^5$  ng.), and 40 pg. of <sup>125</sup>I-insulin for 3.5 hours at 15° C. (total volume 0.5 ml.). After termination of the incubation, duplicate aliquots (200  $\mu$ l.) of each cell suspension were centrifuged through a cold buffer G and dibutyl phthalate gradient. To determine the amount of <sup>125</sup>I-insulin bound, the <sup>125</sup>I-insulin in the cell pellet was counted. The data expressed are the means  $\pm 1$  S.D. for 17 normal volunteers, ages 18 to 35 years. The inset shows the amount of insulin bound (ordinate) at each insulin concentration in the normal physiologic range (abscissa).

*pH Dependence of insulin binding.* To determine the optimal pH of  $^{125}\text{I}$ -insulin binding to human erythrocytes, binding was studied over a pH range from 6.0 to 10.0 with  $3.52 \times 10^9$  cells per milliliter incubated for 2.5 hours. At a pH of 6.0, 0.7 per cent of  $^{125}\text{I}$ -insulin was bound as compared with 6.1 per cent bound at a pH of 8.0 and 3.2 per cent at a pH of 10.0 (figure 2).

*Effect of temperature on insulin binding.* At  $4^\circ\text{C}$ ., slowly progressive association of  $^{125}\text{I}$ -insulin binding

to human erythrocytes was observed for seven hours (figure 3). By the end of 30 minutes 1.6 per cent of the total  $^{125}\text{I}$ -insulin was bound; by one hour 2.9 per cent was bound, and the binding then slowly increased to 7.9 per cent at the end of seven hours. At  $15^\circ\text{C}$ ., association is rapid and progressive during the first hour; however, an equilibrium was obtained between 3 and 3.5 hours and was maintained thereafter for two hours. At  $30^\circ\text{C}$ ., a progressive increase of binding was evident during the five hours of incubation. No decrease of binding was observed and no equilibrium was evident at  $30^\circ\text{C}$ . during the time of the incubation. This increase of binding at  $30^\circ\text{C}$ . occurred in three successive studies.

*Dissociation.* The rate of dissociation of  $^{125}\text{I}$ -insulin from human erythrocyte insulin receptors was determined after  $3.52 \times 10^9$  erythrocytes per milliliter were incubated at  $15^\circ\text{C}$ ., pH 8.0, for 3.5 hours. Duplicate aliquots (200  $\mu\text{l}$ .) were removed to determine the binding of insulin, then excess cold insulin (100,000 ng. per milliliter) was added. At various time intervals, aliquots were removed to determine the amount of  $^{125}\text{I}$ -labeled insulin still bound after dissociation (figure 4). Initially there was a rapid rate of dissociation, with 33 per cent of the bound activity dissociated in 30 minutes. At one hour, 38 per cent was dissociated, whereas after two and three hours of excess insulin addition only 41 and 42 per cent, respectively, had dissociated.

*Degradation.* Five per cent or less of the total insulin concentration was degraded when insulin degradation was determined by either TCA precipitation or re-binding experiments utilizing supernatants obtained at the end of a receptor assay.

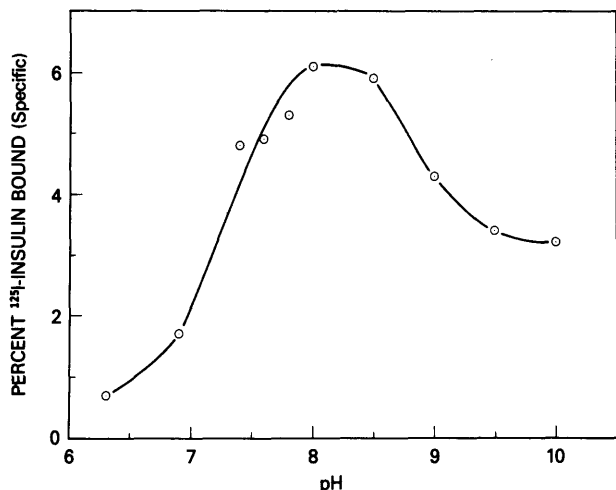


FIG. 2. Effect of pH on  $^{125}\text{I}$ -insulin binding to human erythrocytes.  $1.76 \times 10^9$  isolated human erythrocytes,  $^{125}\text{I}$ -insulin (40 pg.), and unlabeled insulin were each suspended in buffer G with pH ranging from 6 to 10. The pH of the buffer was adjusted at  $23^\circ\text{C}$ . Then these were incubated for 2.5 hours, with a similar pH range, in 0.5 ml. of buffer G (abscissa) as described in figure 1. After incubation and removal of duplicate aliquots (200  $\mu\text{l}$ .), the specific per cent of insulin bound (ordinate) was determined.

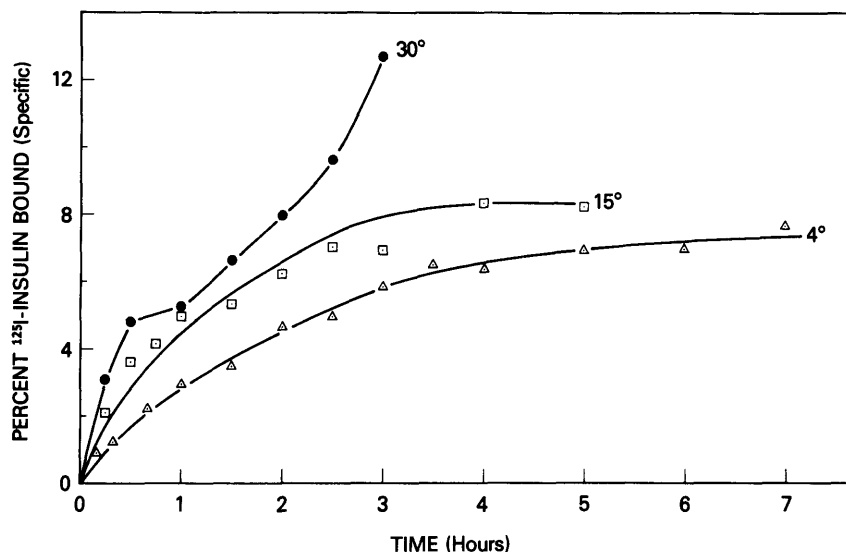


FIGURE 3

Temperature effect on  $^{125}\text{I}$ -insulin binding to human erythrocytes. Erythrocytes ( $14.08 \times 10^9$ ) were incubated with 320 pg. of  $^{125}\text{I}$ -insulin with and without unlabeled insulin (400,000 ng.) in buffer G, pH 8.0, at a total volume of 4 ml. at 4, 15, and  $30^\circ\text{C}$ . Duplicate aliquots (200  $\mu\text{l}$ .) were removed at the indicated time periods and the specific per cent of  $^{125}\text{I}$ -insulin bound determined as described in figure 1.

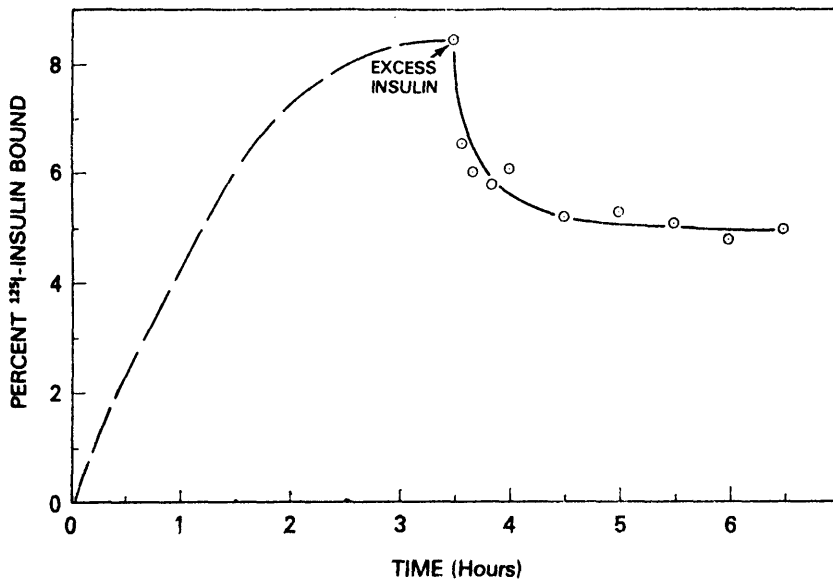


FIGURE 4

Effect of excess insulin on the dissociation rate. Erythrocytes were incubated as described in figure 1. At the end of 3.5 hours,  $10^5$  ng. of unlabeled insulin per milliliter of incubation volume were added. Duplicate aliquots (200  $\mu$ l.) were removed at intervals of 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 hours. Bound  $^{125}$ I-insulin was determined as described in figure 1.

When compared with preincubated ( $4^\circ$  C., pH 8.0, 90 minutes) control cells, erythrocytes preincubated at  $25^\circ$  C., pH 8.0, for 90 minutes had no decrease of receptor sites. No degradation or solubilization of erythrocyte receptor sites was thus evident under these conditions.

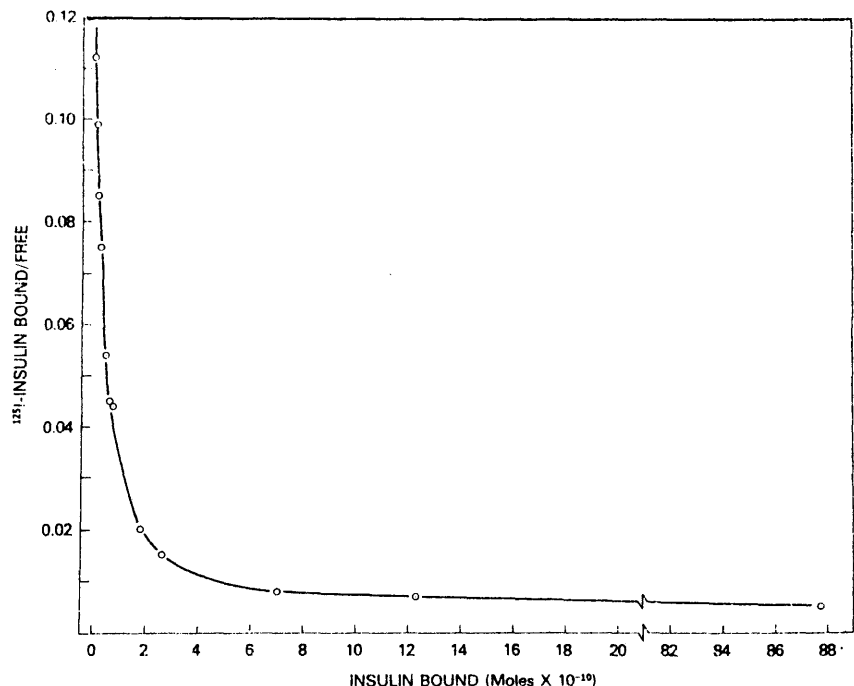
*Scatchard analysis.* Analysis of the insulin binding data according to Scatchard<sup>10</sup> resulted in a curve with an upward concave configuration (figure 5). When this curve is extrapolated to its intersecting point on the abscissa,  $3.52 \times 10^9$  cells were determined to

have bound  $115.5 \times 10^{-10}$  moles of insulin. On the basis of this determination, each erythrocyte had  $\sim 2,000$  receptor sites.

To demonstrate the presence of negative cooperativity as described by DeMeys et al.,<sup>12</sup> the effect of dilution and of dilution plus excessive concentration of unlabeled insulin on the rate of dissociation was studied (figure 6). Addition of excess insulin (60 ng. per milliliter or  $10^{-8}$  M) to obtain a 1:100 dilution increased the dissociation by 15, 16, and 14 per cent at 15, 60, and 120 minutes, respectively.

FIGURE 5

Scatchard analysis. Data from insulin radioreceptor assay ( $15^\circ$  C., 3.5 hours, pH 8.0, as in figure 1) for the 17 normal volunteers were analyzed. On the abscissa is the amount of insulin bound calculated by multiplying the per cent of  $^{125}$ I-insulin bound by the total insulin concentration of the incubation medium. The ratio of bound insulin to free insulin is plotted on the ordinate.



Further, the above Scatchard plot was analyzed for the affinity profile according to DeMeys et al.<sup>12</sup> The average affinity profile expresses the relationship between the average affinity for insulin ( $\bar{K}$ ) and the receptor occupancy ( $\bar{Y}$ ). At any point *i* on the Scatchard curve, the average affinity  $\bar{K}_i$  equals  $(B/F)_i/R_o - B_i$

where  $B_i$  = the concentration of the bound hormone and  $(B/F)_i$  = the bound/free hormone at that point. The affinity ( $\bar{K}$ ) plotted versus the log of the fractional occupancy is referred to as the "average affinity profile."<sup>12</sup> In the erythrocytes studied, the highest or "empty sites" affinity,  $\bar{K}_e$ , was  $0.01 \text{ nM}^{-1}$  and begins to decrease when only 0.1 per cent of the total sites are occupied. With the increasing occupancy of the receptors by insulin, apparent  $\bar{K}$  progressively decreases until  $\bar{K}_f$  is reached.  $\bar{K}_f$  ranges from 0.001 to  $0.002 \text{ nM}^{-1}$  and is reached when only 5 to 10 per cent of the available receptor sites are occupied (figure 7).

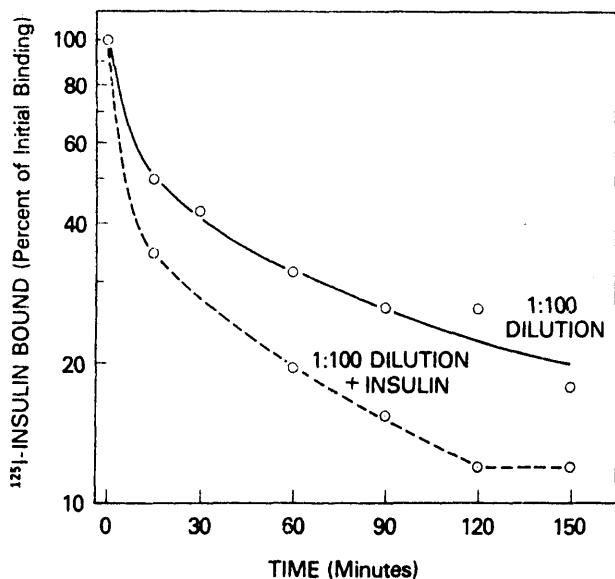


FIG. 6. Rate of dissociation of total bound <sup>125</sup>I-insulin from human erythrocytes. Labeled insulin (240 pg.) was incubated with human erythrocytes  $10.56 \times 10^9$  in a total volume of 3 ml. for 3.5 hours at 15° C. Duplicate aliquots of 100  $\mu$ l. each were diluted with 10 ml. of buffer G or with buffer G containing 60 ng. per milliliter unlabeled insulin. These dilutions were then incubated at 15° C. for 15, 30, 60, 90, 120, and 150 minutes. After each incubation, the cells were centrifuged at  $1,500 \times g$  for 15 minutes after the addition of 200  $\mu$ l. of dibutyl phthalate. The cell pellets were then counted for residual bound activity, and the percentage of the total <sup>125</sup>I-insulin bound was calculated.

DISCUSSION

Human erythrocytes have highly specific insulin receptors. Insulin binding to human erythrocytes has characteristics similar to that of insulin binding previously demonstrated in other human cells such as monocytes,<sup>1</sup> granulocytes,<sup>2</sup> and adipocytes.<sup>13</sup> In this study, we have demonstrated that this insulin binding is also similar to that of rat hepatocytes,<sup>14</sup> myocytes,<sup>15</sup> adipocytes,<sup>16</sup> and turkey erythrocytes.<sup>17</sup> Since the phylogenic span between man and birds is considerable, it is remarkable to note that the insulin binding characteristics of erythrocytes from man and the turkey are similar.

As in the other cell types, the physiologic concentration of unlabeled porcine insulin bound to human erythrocytes inhibited greater than 50 per cent of the <sup>125</sup>I-labeled insulin binding. In an earlier study,<sup>4</sup> we demonstrated that the amount of insulin binding to human erythrocytes was directly proportional to the concentration of the viable cells, i.e., receptor sites per cell. When the erythrocyte concentration ranged

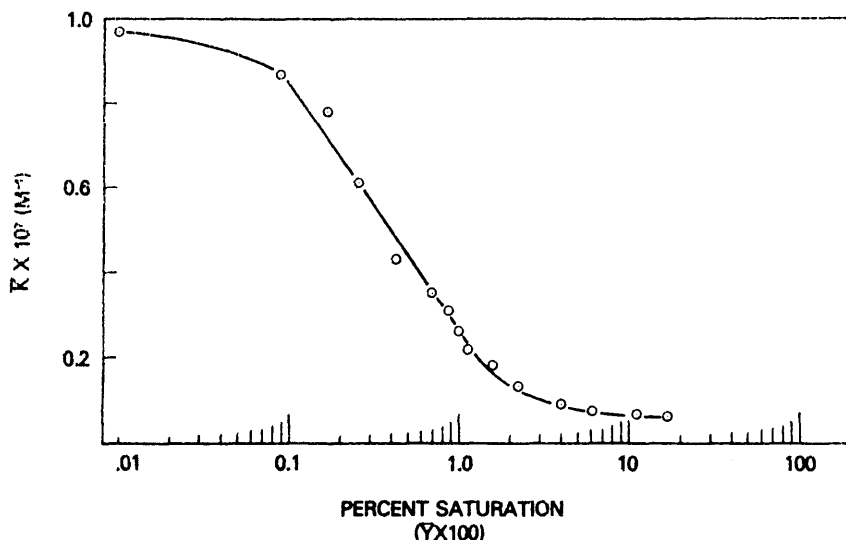


FIGURE 7

Average affinity profile of <sup>125</sup>I-labeled insulin binding data of normal human erythrocytes shown in figure 6. The average affinity constant,  $\bar{K}$ ,  $(B/F/R_o - B)$ , is plotted versus the log of the percentage of occupancy of receptor sites ( $\bar{Y} \times 100$ ), calculated by  $B/R_o \times 100$ . *B* is the amount of insulin bound, *R<sub>o</sub>* is the receptor concentration, and  $\bar{Y}$  is the fraction of receptor occupancy. In order to plot the range of receptor site occupancy, the points on the curve were derived from the Scatchard plot (figure 6).

from 0.72 to  $7.2 \times 10^9$  viable cells per milliliter, a range of from 1.35 to 13.55 per cent, respectively, of  $^{125}\text{I}$ -insulin was specifically bound. These erythrocytes have receptor sites that are highly specific for porcine insulin. When the cells were incubated with 100 ng. per milliliter of ovine, rabbit, bovine, and guinea pig insulins, bovine proinsulin, glucagon, desalanine-desasparagine insulin, GH, and 10 I.U. of HCG, less inhibition of  $^{125}\text{I}$ -insulin binding was evident when compared with the inhibition of binding by 100 ng. per milliliter of porcine insulin. All the insulins, except desalanine-desasparagine insulin, demonstrated greater than 50 per cent inhibition. However, glucagon had only 11 per cent and GH 1.3 per cent inhibition, whereas desalanine-desasparagine insulin and HCG demonstrated no inhibition of  $^{125}\text{I}$ -porcine insulin binding. In contrast to pork insulin, unlabeled insulins from other species and other hormones had less or no effect on  $^{125}\text{I}$ -labeled insulin binding.

The effect of various cation concentrations on insulin binding in human erythrocytes is similar to results obtained with both rat hepatocytes<sup>18</sup> and turkey erythrocytes. Both magnesium and calcium (20 mM each) enhanced binding by 100 per cent in human erythrocytes. However, these cations in a 1:1 molar ratio increased the binding by 93 per cent, thus no synergistic effect was evident. Sodium and potassium (20 mM each) decreased the binding of  $^{125}\text{I}$ -insulin by 13 and 30.6 per cent, respectively; however, when mixed in a 1:1 molar ratio (10 mM each) the binding decreased to 20 per cent. Calcium and sodium (10 mM each) in a 1:1 molar ratio increased the binding by 58 per cent, whereas magnesium and sodium increased the binding by only 38 per cent. Gavin et al.<sup>19</sup> noted little effect from calcium and magnesium, separately or in a mixture, on cultured lymphocytes' insulin-binding. However, Podskalny<sup>20</sup> found inhibition of insulin binding to cultured placental cells with similar cation variations. Since the mature circulating human erythrocyte, unlike other cells, has no nucleus, this alteration in the erythrocytes of available receptor concentration with ionic changes surrounding the plasma membrane appears unrelated to nuclear activity.

Insulin binding in human erythrocytes has a pH optimum, 7.8 to 8.0, similar to that for other human and animal cells. That this pH optimum is also present in an un-nucleated cell strongly suggests that the insulin binding and insulin receptor characteristics of the human and animal cells studied are independent membrane properties with similar biophysical com-

position and do not require constant nuclear activity for regulation.

Association of insulin with its receptor increases in the human erythrocyte as the temperature increases. Unlike the human monocyte<sup>21</sup> and placental cells<sup>20</sup> or the rat hepatocytes,<sup>18</sup> more insulin is bound to the human erythrocyte as the temperature increases. This increase of insulin binding with the increase in temperature may reflect the minimal degradation of either the receptors or the insulin. In view of the finding of Goldfine et al.,<sup>22</sup> who reported intracellular insulin binding sites, experiments are planned to determine if the increased binding observed in three studies at 30° C. represents intracellular insulin.

In contrast to the lack of cooperative effects found with growth hormone receptors on cultured human lymphocytes,<sup>23</sup> insulin receptors of human erythrocytes, cultured placental cells,<sup>20</sup> the mouse liver membranes,<sup>24</sup> turkey erythrocytes,<sup>16</sup> mammary tumor cells,<sup>25</sup> circulating monocytes,<sup>21</sup> and cultured human lymphocytes<sup>26</sup> do show receptor site interactions of the negative cooperative type. Moreover, dependence of cooperativity on insulin concentration was similar for all these insulin receptors.

The number of insulin receptor sites per cell varies from tissue to tissue and from species to species. For the turkey erythrocyte, rat hepatocyte, cultured human lymphocyte, and human erythrocyte, the receptor concentrations per unit surface area are 29, 63, 24, and 14 sites per square micrometer, respectively. Comparatively, the turkey erythrocyte has 62 per cent, the cultured lymphocyte has 78 per cent, and the rat hepatocyte has 42 per cent more insulin receptors per unit of surface area than the human erythrocyte.

Since prior studies have not clearly defined the physiologic role of insulin for the mature circulating human erythrocyte, the significance of finding highly specific insulin receptors on these cells remains unclear. While studying the effect of insulin on rat erythrocytes, Lal and Agarwal<sup>27</sup> demonstrated a decrease of plasma free  $\alpha$ -amino nitrogen under the influence of insulin. With human erythrocytes, Zipper and Mawe<sup>28</sup> reported an increase of glucose efflux in the presence of insulin. In variance to a positive insulin effect, Wilbrandt,<sup>29</sup> however, noted inhibition of glucose transport in erythrocytes incubated with insulin. Insulin binding to human erythrocytes from a normal adult male during a glucose tolerance test demonstrated an initial increase of insulin binding followed by a decrease at three hours and a return toward baseline values at five hours.<sup>30</sup> This prelimi-

nary study suggests that erythrocyte insulin receptors in normal man vary in response to insulin concentrations subsequent to glucose stimulation. More definitive studies of the effect of physiologic alterations in human erythrocyte insulin receptors are in progress.

The circulating human erythrocyte, a mature cell, is an ideal cell for clinical evaluation of polypeptide hormone receptors. It is the most abundant circulating cell. Not only is the human erythrocyte easily isolated, but under physiologic conditions, it is also viable for extended periods. Utilizing a technique that permitted distinct separation of erythrocytes with their bound insulin from free insulin,<sup>4</sup> we have presented data that indicate the human erythrocyte has highly specific insulin receptors with characteristics similar to insulin receptors of other cell types.

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