Two fundamental problems in characterizing hormone-receptor interactions are determining the number and character of hormone binding sites present in a given situation. Inferences drawn from these findings have had profound impact on our understanding of receptor-mediated processes and certain general aspects of cellular metabolism. A few examples are cited to illustrate these points.

It is known that certain hormones occupying only a fraction of the receptor population can elicit a maximal biological response. This occurs in the cases of steroidogenesis stimulated by corticotropin binding to isolated adrenal cells (1), the elevation of cyclic adenosine monophosphate level following epinephrine association with adrenergic receptors (2), and enhanced glucose transport rates caused by insulin binding to adipocytes’ insulin receptors (3). This partial occupancy phenomenon suggests that one parameter of a cell’s responsiveness to hormonal stimuli is the level of the cognate receptor at the onset of hormone binding.

In addition to conditions such as denervation (4), differentiation (5), or autoimmune diseases (6) which may alter the level and/or location of specific receptors, and thus the sensitivity to a given level of a hormone or transmitter, the presence of the hormone may itself change these parameters (7). A specific example concerning insulin receptors is the ligand-induced receptor “down regulation” phenomenon (cf. Refs. 8 and 9) which has been ascribed to different cellular processes according to the cell type investigated. In chick hepatocytes (10), it was shown that insulin receptor was redistributed from the surface to an intracellular pool. However, using 3T3-L1 adipocytes (11), it was shown that a reduced receptor level was accounted for by an enhanced rate of surface-receptor inactivation. Both studies required measurements of hormone binding under dissimilar conditions; specifically, cell-associated receptor versus solubilized receptor. In these situations, a major concern has been defining optimal conditions for evaluating the total numbers of hormone binding sites present. This problem is analogous to quantifying the presence of an enzyme from measurements of its catalytic activity, where the experimental parameters affecting these measurements must be determined.

Optimal assay conditions often differ greatly from known physiological parameters, which raises a second basic question: What effectors of hormone binding have regulatory significance in physiological situations? The importance of obtaining this information stems from the fact that hormone receptors at the cell’s surface are mediators between extracellular stimuli and membrane and/or intracellular processes. Therefore, they are the first regulatory protein in a chain of metabolic events. Cornish-Bowden and Koshland (12) have stressed a need for the “quantitative expression of a saturation curve for a regulatory protein” in order to approach the fundamental issues mentioned above. This report describes titrations of solubilized insulin receptor with 125I-insulin, in order to characterize quantitatively that association under various conditions.

**EXPERIMENTAL PROCEDURES**

**Materials**

Bovine insulin was obtained from Eli Lilly Co. (courtesy of Dr. R. Chance). Triton X-100 was purchased from PACKARD Instruments; carrier-free sodium (125I)iodide was from Amersham Corp.; Enzymo-Bead Reagent was from Bio-Rad; bovine serum albumin (radioimmunoassay grade and Fraction V), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, ethylenediaminetetraacetic acid (disodium salt), phenylmethylsulfonyl fluoride, and fluorescamine were from Sigma; Trasylol was from FBA Pharmaceuticals; Whatman DE52 cellulose from Reeve Angel Co.; Sephadex G-50 (fine) from Pharmacia Fine Chemicals; and barbital-sodium barbital mixture (Veronal buffer, pH 8.6) was from Buchler Instruments, Inc. All other chemicals were reagent grade.
Radioiodination of Bovine Insulin—This procedure was performed using the solid phase lactoperoxidase/glucose oxidase system (Enzymobead Reagent) from Bio-Rad, essentially as described by the manufacturer. Recoveries were maintained at 4°C. The Enzymobead Reactant solution was prepared at room temperature. To the manufacturer's vial containing 5 ml of carrier-free sodium (125)I-iodide (9 or 10 µl) were added 50 µl of 0.2 M Na2HPO4 (pH 7.2), 10-30 µl of bovine insulin in H2O at pH 3.5 (30-120 µg, based on an absorbance at 278 nm of 1.06 for a solution containing 1.0 mg of insulin/ml), 25 µl of 2% glucose (w/v in water), and 50 µl of the Enzymobead Reactant solution and a crude plasma membrane pellet was generated by homogenization vessel (on ice) in a solution containing 0.1 M NaCl, 0.2 mM MgSO4, 10-30 µl of 0.2 M Na2HPO4, 0.05 M Hepes (pH 7.4). After 30 min, the reaction mixture was transferred to an Eppendorf microtube containing 600 µl of Veronal buffer and centrifuged for 2 min in a Beckman microcentrifuge. Aliquots were taken to determine the extent of incorporation (specific activity) by precipitation in 10% trichloroacetic acid, with 0.06% bovine serum albumin added as a co-precipitant. The remainder of this mixture was applied to a 2-ml column of Whatman DE52 cellulose equilibrated with the same buffer and then washed with 30 ml of Veronal buffer. The products were eluted in 0.3 M Na2HPO4 (pH 7.4) containing 2% (w/v) radiolabellum grade bovine serum albumin. Fractions included in the peak of radioactivity were pooled and aliquots were taken to determine the yield. Preparations of (125)I-insulin contained 0.2-0.3% radioactivity iodine per molecule of insulin and were at least 95% precipitable in ice-cold 10% trichloroacetic acid. Preparations were stored for up to 3 weeks in Eppendorf microcentrifuge tubes at -20°C and were thawed only once. Gel filtration was performed routinely at 4°C, using Sephadex G-50 fine, equilibrated with 0.15 M Hepes containing 0.1% Triton X-100 (w/w) at the appropriate pH. Two peaks of radioactive material were observed and material was used from the center of the peak of lower molecular weight material. This (125)I-insulin was used within 48 h.

Human Placental Plasma Membrane Preparations—Placentas were obtained within 10 min of normal full term delivery, the blood was drained, and the villous tissue was scraped and placed into ice-cold phosphate-buffered saline. All subsequent steps were performed at 4°C. This tissue was drained of liquid and homogenized for 30 s using a Sorvall Omni-Mixer in 100 ml (per 50 g of tissue) of 0.25 M sucrose, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, Trasylol (1 unit/ml), and 50 mM Hepes (pH 7.4). Gross debris was removed by centrifugation at 600 × g for 10 min followed by recentrifugation of the supernatant at 600 × g for 10 min. The second supernatant was centrifuged twice at 12,000 × g for 50 min each yielding a "postnuclear" supernatant. This supernatant was adjusted to 0.1 M NaCl and 0.2 mM MgSO4 by adding 1/4 volume of a 20x-concentrated salt solution and a crude plasma membrane pellet was generated by centrifugation at 40,000 × g for 50 min. In each subsequent step, the resuspended pellets were rehomogenized once; only once. Gel filtration was performed routinely at 4°C, using Sephadex G-50 fine, equilibrated with 0.15 M Hepes containing 0.1% Triton X-100 (w/w) at the appropriate pH. Two peaks of radioactive material were observed and material was used from the center of the peak of lower molecular weight material. This (125)I-insulin was used within 48 h.

Methods

125I-Insulin Binding Assays—All steps were performed at 4°C. Incubations were done in polystyrene tubes (12 × 75 mm) (Lancer) at a final volume of 300 µl (see below). Total (125)I-insulin binding was determined by co-incubation of solubilized insulin receptor with radiolabeled (125)I-insulin and Tris (125)Hepes prepared in 0.15 M Hepes, 0.1% Triton X-100 (w/w) at pH 7.4 for indicated for each experiment. Nonspecific binding was determined using unlabeled insulin at final concentrations of 0.4 or 0.5 µCi/ml, respectively, the unlabeled and solubilized insulin mixture were incubated for 3-5 h prior to forming the final assay-incubation. The initial concentration of unlabeled insulin in the preincubation was 2.0 or 2.5 µM in order to achieve the respective final concentrations following the addition of 0.6 µl of solubilized insulin receptor to 240 µl of radiolabeled insulin. Mock incubations were prepared with only unlabeled insulin to measure the pH of each mixture at 4°C. Bound radioligand was precipitated by the addition of 0.5 ml of 29.8% polyethylene glycol (w/w in water) and 0.5 ml of 0.15% bovine γ-globulins (w/w in 5 mM Hepes, pH 7.6) essentially according to the procedure of Cuatrecasas (18). Twenty min after vigorous mixing, the insoluble material was collected by centrifugation at 6000 rpm in a Sorvall HS-4 rotor. After standing at room temperature for 10 min, the supernatant was removed by aspiration and radioactivity in the pellet was measured in a Packard Auto-Gamma Scintillation Spectrometer Model 5266.

To determine the time course of (125)I-insulin binding to the solubilized insulin receptor, initial binding experiments were performed at final volumes of 8.2 ml and aliquots of 300 µl each were removed at timed intervals. These were added to tubes containing polyethylene glycol and bovine γ-globulin (unmixed) as indicated above and the protocol continued as described previously. Unlabeled insulin and solubilized plasma membrane protein were incubated for 4 h prior to the determination of specific binding.

Further details of specific incubation conditions are given in the figure legends.

Data Analysis—This section describes the pertinent equations used in analyzing the data obtained from (125)I-insulin titrations of solubilized insulin receptor.

The Adair Equation (16) was used in analyzing the binding isotherms. Allowing two classes of sites

\[ N_s = \frac{K_s (X) + 2K_s K_B (X)^2}{1 + K_s (X) + K_B (X)^2} \]  

(1)

where \( N_s \) = number of moles of (125)I-insulin bound/mol of receptor (partial occupancy), \( X \) = the equilibrium (free) concentration of (125)I-insulin, and \( K_s \) and \( K_B \) are the first and second stoichiometric (macromolecular) association constants. Since the stoichiometry of moles of (125)I-insulin bound/mol of receptor was unknown, we determined the stoichiometry of the Adair equation for the total available insulin binding sites; total binding sites were estimated graphically from midpoints of the binding isotherms. This was done prior to the fitting procedure described below. Thus, \( N_s = n \) (concentration of bound ligand)/(concentration of binding sites), where \( n = 1 \) or 2. Allowing two classes of sites requires a minimum of two binding sites per receptor holomer causing \( N_s \) to vary between 0 and 2. Alternatively, the simplest model for a single class of sites requires a stoichiometry of 1, restricting \( N_s \) to values between 0 and 1. In the latter case, \( K_B = 0 \) and the equation is simplified accordingly. If one assumes a stoichiometry of 2 and a single class of sites (0 ≤ \( N_s \) ≤ 2), then \( K_B = 4K_s \) (see "Discussion").

An iterative fitting procedure was devised for each case, following the general method suggested by Simon (17), where the parameters to be fitted were assigned the relations \( \psi_1 = 1/K_s \) and \( \psi_2 = \psi_1/K_B \) according to Cornish-Bowden and Koshland (12). The "best" fit was obtained when a minimum was reached in the sum of squares of the deviations in experimental and theoretical values of \( N_s \) for all experimental values of \( X \) (the error function described by Equation 5 in "Methods"). These parameters were reestimated by starting the fitting program with initial estimates of \( K_s \) and \( K_B \) above and below each fitted parameter. In this somewhat laborious fashion, local minima far from the true minimum in the error function were avoided.

Scatchard plots (18) employed the formulation (for a single class of sites)

\[ B/F = n \times K_s - K_s B \]  

(2)

where \( K_s \) is the association constant, \( B \) is the concentration of specifically bound (125)I-insulin, and \( n \) is the concentration of insulin binding sites. Properties of this function describing the equilibrium

1. The abbreviation used is: Hepes, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid.
concentrations of associating species have been discussed by Klotz (19, 20) and others (21).

Hill plots (22) were generated from binding isotherms according to the equation

$$\log [y/(n - y)] = \log K_0 + nH \log (X)^n$$

where y is the partial occupancy (equivalent to $N_c$ above), $K_0$ is the association constant, n is the binding stoichiometry (number of sites/bound insulin, and $H$ is the Hill coefficient which is always less than the value of n (23). In the case where $n_H = 1$, this equation is exact (24).

Treatment of the pH dependence for insulin binding included the following equations which are described in more detail under "Appendix":

$$K_{app} = B/L - B(R_0 + B)$$

$$K' = K_{app} (1 + (\frac{pK_1}{-pK_2}) (1 + (\frac{pK_3}{-pK_5}))$$

$K_{app}$ is the association constant determined at a given pH. $K'$ is the pH-independent (true) association constant, $L$ and $R_0$ are total 128-1-insulin and insulin receptor (binding site) concentrations, respectively, and $pK_1$ and $pK_2$ are the logarithms of the proton dissociation constants of the insulin receptor. Thus, constant $pK_3$ describes the insulin dissociation and $pK_4$ the insulin-protein association in forming species that are competent for binding (see "Appendix," Scheme II). This expression differs slightly from that employed by Waelbroeck and colleagues (25, 26).

RESULTS

Parameters of Nonspecific Binding—Specific 128-1-insulin binding was calculated as the difference between total radioligand precipitated in the absence of unlabeled insulin and the amount precipitated following incubation with excess unlabeled insulin, where the latter is considered a measure of "nonspecific binding." The results given here delimit the usable concentration range of unlabeled ligand for determining nonspecific binding by the polyethylene glycol precipitation technique. We describe several aspects of the method which influence this determination, as shown in Fig. 1.

First, for a fixed quantity of iodinated insulin and in the absence of added membrane protein, the precipitation of radiolabeled hormone was independent of the native hormone concentration only below a limiting value that was less than 1 pM (Fig. 1A). Above this concentration of unlabeled insulin, there was a definite increase in the amount of 128-1-insulin appearing in the pellet and this effect was not relieved by increasing the precipitation volume from 1.3 ml to 3.0 ml.

A second factor, the effect of added solubilized membrane protein on the precipitation of 128-1-insulin, was examined to find optimal conditions for measuring nonspecific binding. Solubilized membrane protein at a final concentration of 290, 145, or 73 $\mu$g/ml was incubated with 5 nM radiolabeled insulin and unlabeled insulin (0-5 pM) for 10 h prior to precipitation. The following expectations were met, as illustrated in Fig. 1.

1) As the amount of solubilized membrane protein added approached zero, the profile of nonspecific binding versus native insulin concentration became more similar to the control (no added protein). 2) In every case, there was a minimum value of 128-1-insulin precipitated and this minimum occurred at the same concentration of native insulin irrespective of the amount of solubilized membrane protein added (Fig. 1A). 3) The nonspecific binding at this minimum was proportional to the amount of added membrane protein, but showed nonlinear behavior at concentrations of unlabeled insulin above and below that generating the minimum (Fig. 1B). Using these optimum values for nonspecific binding, the specific binding of 128-1-insulin was proportional to the concentration of solubilized membrane protein present in the incubation, between 0 and 300 $\mu$g/ml.

Comparison of the results using solubilized membrane protein, with the "control" (dashed line, Fig. 1A), showed that the pronounced increase in precipitated radioligand did not occur except where low levels of protein were present. It was the case, however, that "excess" unlabeled insulin, as described above, produced artifactually high values of "nonspecific binding" under all conditions tested. This analysis, therefore, defined the optimum concentration of unlabeled insulin usable to determine nonspecific binding (0.4-0.5 pM) and imposed an upper limit on the concentration of radiolabeled insulin used in subsequent titrations. The latter condition is illustrated in Fig. 1C, where it is clear that nonspecific binding was linear with respect to the concentration of 128-1-insulin. This linearity was pH-independent, although a slight deviation was noted between 6 and 8 $\mu$g/ml at pH 9.6.

Fig. 1C shows the influence of pH on the net precipitation of 128-1-insulin in the presence of 0.4 $\mu$g/ml unlabeled insulin, a
third factor affecting nonspecific $^{125}\text{I}$-insulin binding. The effect of decreasing the pH was an increase in the precipitation of radiolabeled insulin. These observations were qualitatively uniform for $^{125}\text{I}$-insulin varied between 1 and 30 nM and quantitatively identical in that $1 \mu\text{M}$ unlabeled insulin was the lower limit for the concentration of hormone including artifactual $^{125}\text{I}$-insulin precipitation. This was true for all combinations of unlabeled insulin (0.1-10 nM) in the presence or absence of solubilized membrane protein (data not presented).

The Equilibrium Condition of $^{125}\text{I}$-Insulin Binding—The theories underlying all data analysis in this communication rest upon the assumption that the associating species have attained thermodynamic equilibrium. It was therefore necessary to establish that, for a given experimental situation, equilibrium was reached in a path-independent manner. A simple demonstration that this criterion was met is shown in Fig. 2, which indicates the equilibrium position as a function of pH and radioligand concentration. Three initial conditions and one final condition were employed. At pH 8.2 and pH 6.8, the initial $^{125}\text{I}$-insulin concentration was 1 nM and at pH 7.5 this concentration was 0.5 nM. The amount of solubilized insulin receptor was constant throughout the experiment. At 9.7 h elapsed time, one-half of each incubation at pH 8.2 and pH 6.8 was adjusted to 0.5 nM $^{125}$I-insulin and pH 7.5 by mixing with an equal volume of the appropriate buffer, thereby yielding a uniform final condition. The initial rapid drop at 9.7 h was due to the 2-fold dilution required to achieve the perturbation. The “zero time” point for the pH 6.8 to pH 7.5 transition was greater than 2.8 fmol bound because, at pH 7.5, about 0.5 fmol of $^{125}$I-insulin was bound before or during the precipitation.

It was clear that without further manipulations the equilibrium position of $^{125}$I-insulin binding remained unchanged and the perturbations described resulted in the new equilibrium “predicted” by the intermediate condition. Importantly, it was also concluded that prolonged incubation under these acidic and basic conditions did not alter the association properties of either the solubilized insulin receptor or the radiolabeled hormone.

$^{125}$I-Insulin Binding at Different Concentrations of Solubilized Receptor—The binding of radiolabeled insulin to solubilized insulin receptor was studied at three different concentrations of plasma membrane protein at pH 8.2. The binding isotherms shown in Fig. 3A were analyzed independently according to the Adair equation and gave an average association constant of $1.35 \pm 0.22 \times 10^8 \text{M}^{-1}$. Linear regression analysis of Scatchard plots for these data, shown in Fig. 3B, gave nearly parallel lines and an average association constant of $1.41 \pm 0.12 \times 10^8 \text{M}^{-1}$, in good agreement with the above value. An aggregate Hill plot (Fig. 3C) further substantiated the conclusions that: 1) under these experimental conditions, the parameters of $^{125}$I-insulin binding to solubilized receptor were independent of receptor concentration and 2) since the

![Fig. 2. Time course of specific $^{125}$I-insulin binding at 4 °C.](image1)

Two initial incubations were prepared with 1 nM radiolabeled insulin at pH 8.2 (●) and at pH 6.8 (▲) and one containing 0.5 nM radioligand at pH 7.5 (■). Each incubation contained 44 μg/ml of solubilized plasma membrane protein. After 9.7 h (arrows), one-half of the remainder from each of the first two incubations was adjusted to 0.5 nM $^{125}$I-insulin and pH 7.5 by mixing with buffer of the appropriate pH containing the same initial concentration of receptor protein. After 9.7 h elapsed time, one-half of each incubation at pH 8.2 and pH 6.8 were independent of receptor concentration and 2) since the

![Fig. 3. $^{125}$I-insulin binding at three receptor concentrations.](image2)

Determinations were made of the amounts of radiolabeled insulin bound specifically at equilibrium to solubilized insulin receptor present at final binding site concentrations of 0.396 nM (□), 0.175 nM (○), 0.096 nM (△), as determined from the inflection point of each binding isotherm. Each point represents the average of quadruplicate determinations of total binding and of nonspecific binding at 0.5 μM unlabeled insulin. A, binding isotherms. The solid lines show the best theoretical fit according to the Adair equation for a single class of sites. B, Scatchard plots. Data from A were replotted and analyzed by linear regression (solid lines) to obtain the corresponding slopes and intercepts. C, Hill plot. Data from A were replotted according to Equation 3 (see “Experimental Procedures”) and the Hill coefficient determined by linear regression analysis (solid line). The Hill coefficient was found to be uniform by examining the slopes of the lower, middle, and upper third of each data set.
Hill coefficient for each data set was 1, there were no detectable cooperative interactions in the binding of 125I-insulin to its receptor over the concentration range 0.01-8.0 nM radioligand.

The pH Dependence of Binding at a Single 125I-Insulin Concentration—The binding of 125I-insulin to a fixed amount of solubilized insulin receptor was studied at 1 nM radioligand over the pH range 6.5-9.4. Consistent with earlier observations, the nonspecific binding decreased monotonically with increasing pH (results are not shown for simplicity). The semilogarithmic plot shown in Fig. 4 depicts the pH dependence of 125I-insulin binding, where the optimum association occurred between pH 8.2 and 8.7. By analogy with the behavior of Michaelis pH functions for enzyme kinetics, interpolation from the linear ascending and descending wings of the profile yielded estimates of the proton dissociation constants as \( pK_1 = 8.0 \) and \( pK_2 = 8.8 \) (indicated by the arrows). Although these two constants were sufficient to account for the findings described below, it must be emphasized that there was neither enough data not sufficient precision to rule out three or more significant \( pK \) values, which would in fact be consistent with the asymmetry of this profile (see “Appendix”). Furthermore, the value for \( pK_2 \) is probably somewhat inaccurate since the linear extrapolation was based only upon four points obtained at high pH. In the absence of independent chemical evidence, it is not possible to assign these \( pK \) values to particular functional groups on either (or both) associating species.

The pH Dependence of 125I-Insulin Binding Parameters—Binding of 125I-insulin to a fixed amount of solubilized insulin receptor was studied between pH 6.8 and pH 9.6, for a concentration range of radioligand from 0.01-8.0 nM. Fig. 5A shows the binding isotherms derived from this study; it is apparent that the level of saturation attained increased with increasing pH between pH 6.8 and pH 8.3. In order to employ the Adair equation for data analysis, it was necessary to have an estimate of the total number of 125I-insulin binding sites present (see above). The inflection point, representing half-saturation of the receptor, was estimated graphically from the binding isotherms obtained at the three highest pH values since saturation was approached more closely under those conditions. The average value (358 pm sites) was taken to be the same for each titration and was not a variable in the curve-fitting procedures. The behavior of the nonspecific binding was discussed previously in connection with Fig. 1C.

Replotting these data according to the algorithm proposed by Scatchard gave the results shown in Fig. 5B. Linear regression analysis was employed to determine the association constants (the negative value of each slope) and the total number of specific ligand binding sites (the intercept on the “Bound” axis). The average number of sites according to this method was 351 pm, in agreement with the previous estimate. The contention that these data represent a single straight line includes the assumption of a single class of binding sites (see above).

Fig. 4. The pH dependence of specific binding at 1 nM 125I-insulin. Equilibrium binding of radioligand as a function of pH, measured at 4 °C, was determined in the presence or absence of 0.5 μM unlabeled insulin and insulin receptor at 0.20 nm binding sites. Each point represents the average of triplicate determinations. The arrows indicate values for \( pK_1 = 8.0 \) and \( pK_2 = 8.8 \), as described in the text.

Fig. 5. 125I-insulin binding between pH 6.8 and pH 9.6. Specific binding of radioligand (0.01-8.0 nM) was measured following 12-16-h incubations at pH 6.8 (●), pH 7.2 (○), pH 7.5 (△), pH 7.9 (□), pH 8.3 (×), and pH 9.6 (○). Nonspecific binding was determined in the presence of 0.4 μM unlabeled insulin (see Fig. 1C). Total insulin binding sites present was 356 pm (19 μg of solubilized membrane protein per incubation of 300 μl) based upon the average of the inflection points of the binding isotherms. Each point represents the average of quadruplicate determinations of total binding and of nonspecific binding. A, binding isotherms. The solid lines show the best theoretical fit to the Adair equation, assuming a single class of binding sites. B, Scatchard plots. Data from A were replotted and fit by linear regression analysis (solid lines). Results obtained at pH 6.8 and at pH 9.6 are not shown (see “Discussion”). C, Hill plots. These were derived from data for the binding isotherms. The solid lines were generated by linear regression analysis, giving Hill coefficients that were uniform for each data set, as described for Fig. 3C.
There is no consensus regarding the nature of insulin binding to the insulin receptor. Studies of this phenomenon have utilized receptor from numerous species and tissues in cell-associated, membrane-bound, and nonionic detergent-solubilized states, covering the effects of pH, ions, and temperature. Most investigators have relied upon Scatchard plots as the primary graphical method of data analysis, even in situations where the binding process did not reach equilibrium. Many reports have addressed the kinetics of insulin association and dissociation in attempts to demonstrate a homogeneous class of binding sites consistent with a linear Scatchard plot. Others have tried to distinguish between site heterogeneity and negative cooperativity, both of which are consistent with "concave upward" Scatchard plots.

The first report by Cuatrecasas (27) on the properties of insulin receptor solubilized from rat hepatocyte and adipocyte membranes indicated a single class of binding sites (see Fig. 5 in Ref. 27). The concentration range of 125I-insulin employed for these equilibrium binding studies was 10–100 pM, yielding dissociation constants in the range of 1.3 × 10–10 M to 1.8 × 10–10 M, at pH 7.4 and 25°C; results from kinetic experiments were in good agreement for the respective dissociation constants for adipocyte and hepatocyte insulin receptors. Reports dealing with the properties of solubilized avian insulin receptor (28, 29), however, presented evidence for binding site heterogeneity, interpreted as a display of negative cooperativity, for which a molecular model was proposed (albeit not confirmed to date).

Further justification for analyzing the data according to models using a single class of binding sites came from the Hill plots shown in Fig. 5C. The slopes (Hill coefficients) generated by linear regression analysis were found uniformly to be equal to 1, for radioligand concentrations varied over nearly three orders of magnitudes. The coefficient of correlation for each of these lines was greater than 0.992. These results are summarized in Table I.

The association constants derived from the binding isotherms according to the Adair equation or from Scatchard plots using a linear regression analysis show roughly the same pH dependence. A theoretical prediction of this behavior was made using equation 5 (see "Methods"), values of pK1 = 8.8 obtained from Fig. 4, and an estimate of the pH-independent association constant, K′d = 2.9 × 10^9 M–1, derived also from the data in Fig. 4. Fig. 6 shows this result and the association constants from the Adair equation as listed in Table I. Complex pH dependence schemes involving more than two pK values are considered under "Appendix."

**DISCUSSION**

The sites were taken from the stipulated assay conditions and the inset of Fig. 4, Ref. 30.

Further work characterizing this kinetic behavior (37) led to a model involving oligomeric insulin receptor structures and was consistent with solution studies (28) which indicated
Characteristics of Insulin Binding to Solubilized Receptor

Insulin-dependent changes in receptor subunit "aggregation." It must be pointed out, however, that no reports have appeared quantifying the site-site interaction parameters for that tetrameric model in a manner such as that described by Koshland (50).

Kahn et al. (39) reported two classes of specific binding sites and one class of nonspecific insulin binding sites using rat liver plasma membranes. They noted a discrepancy between the apparent kinetic behavior of insulin dissociation and the numbers of sites derived from equilibrium analyses, even when the latter were corrected for hormone degradation. In a similar system (40), Donner reported that correcting for the effects of insulin degradation "linearized" the Scatchard plot and concluded that the insulin receptor had a homogeneous population of binding sites for insulin. This correction had an impact on determining both the number of sites and the affinity constant. Gameltoft et al. (41) showed a single class of 125I-insulin binding sites by kinetic and steady state analyses using rat hepatocytes under conditions which minimized hormone degradation, showing a $K_d = 0.52$ nM ($37 \text{ C}$, pH 7.4). It was also shown that the slowly dissociating species was accounted for by cellulyarly processed radioligand; similar findings have been reported in other systems where the binding phenomenon was considered dependent upon a single class of sites (42, 43). Further arguments against negative cooperativity were presented by Pollet et al. (44) who maintained that there were two classes of noninteracting sites and by Corin and Donner (45) who argued for a single class of site. Both groups showed that enhanced dissociation rates induced by the presence of native hormone occurred even with decreased binding site occupancy. In fact, the latter group proposed a model involving hysteresis, suggesting a ligand-induced conversion of the membranous receptor to a higher affinity state. A recent review by Levitski discusses briefly these and other reservations concerning the negative cooperativity model for insulin receptor-insulin association (46).

Included in many investigations are reports of pH optima for 125I-insulin binding to insulin receptor in various states and under numerous conditions. Irrespective of the cellular source, state of the receptor, or conditions of the assay it was generally found that the optimal pH for binding was near pH 7.5, although relatively broad optima in the pH range 7.5-8.4 have been reported (47, 48). A more rigorous analysis of this phenomenon was given recently by Waelbroeck (26), showing a broad optimum between pH 7.6 and pH 8.0 for 125I-insulin binding to IM-9 lymphocytes but an upward shift in this range to pH 8.0-8.7 for the N\textsuperscript{4}-modified trifluoroacetyl insulin derivative. Two significant proton dissociations were noted having a $pK_a = 7.5$ and a $pK_b = 8.0$ ($25 \text{ C}$); the latter was ascribed to protonation of the amino group of the insulin A-chain based upon the binding characteristics of the derivative. In addition to mechanistic implications of this pH dependence, the metabolic significance has been underscored in studies of diabetic ketoacidosis (49, 50), where the extracellular pH was lowered due to the diseased state, although the pH optimum for binding was unchanged.

In this context, the goals of the present study were the following: 1) A quantitative description of the insulin binding isotherm, leading to an evaluation of the site binding constants and site-site interaction parameters, in the event that cooperative behavior could be established. 2) Investigation of the effects of varying assay conditions on the parameters of insulin binding, given known physiological situations. 3) Determination of the optimal conditions for measuring the total number of binding sites present. We chose to use solubilized insulin receptor from human placental plasma membranes, a rich source of this binding protein, because in the solubilized state the receptor was presumably freed from potential interactions with other membrane proteins and lipids, and from the attendant temperature dependence of membrane-associated transitions (e.g. phase changes or aggregation). Reports by other investigators treating these problems have been presented for which there are conflicting interpretations (compare Refs. 55 and 54 with Ref. 55). In addition, many of the published methods used to enhance the specific activity of the solubilized receptor are known to alter its binding characteristics (50, 31, 56-58) and have therefore been avoided for the purpose of this characterization. To facilitate subsequent studies on the effects of various physiological agents (Mg\textsuperscript{2+}, Ca\textsuperscript{2+}, etc.), we performed these experiments in a minimal assay buffer; a buffering component (Hepes) and nonionic detergent (Triton X-100).

Determination of the binding parameters for hormone association with the solubilized insulin receptor by direct titration with 125I-insulin, rather than by the more commonly used "competition" assay, required that artifacts be excluded from the measurement of nonspecific binding. We have demonstrated that this criterion was met for the three major parameters of the experiments: unlabeled insulin concentration, radiolabeled insulin concentration, and the level of solubilized plasma membrane protein (Fig. 1). We have also shown that the equilibrium condition was reached in the time allowed for co-incubation of insulin receptor and 125I-insulin (Fig. 2). This was critical since the equations and graphical forms used to analyze the data were predicated upon the equilibration of all binding species. Equally important was the demonstration that the physical stabilities of the associating species were unaffected by pH, within the range studied, as shown by the attainment of a common equilibrium state following changes in pH (Fig. 2). Therefore, although there were differences in nonspecific binding as a function of pH or solubilized protein concentrations (Fig. 1), these differences did not account for the observed changes in the pH-dependent association constant ($K_d^{app}$, Table I).

By studying the binding at a single concentration of 125I-insulin over the pH range 6.5-9.5 (Fig. 4), we were able to discern one significant proton dissociation and one proton association and to calculate the pH-independent association constant, $K_a = 2.9 \times 10^9$ M$^{-1}$ ($K_d \approx 0.3$ nM). The latter value reflects the "true" equilibrium between states of the associating species that are competent for binding; its relationship to the apparent association constant is a function of the distribution between protonated and deprotonated species at a given pH (Equation 5). The proton dissociation constants define that distribution (Fig. 6), but as a basis for understanding the binding process in molecular terms, it is noteworthy that the pH profile shown in Fig. 4 is asymmetric, suggesting the occurrence of a pK less than 8.0. Attempts to fit data for log($K_d^{app}$) versus pH required at least 3 pK values, generally in the ranges $7.0-7.5$, $7.9-8.1$, and $8.8-9.3$. Unfortunately, any fit of the data between pH 6.4 and pH 6.9 could not be reconciled with a good fit to the data obtained over the pH range $7.0-7.7$. Without further refinement of the data, we cannot justify using a model with 3 or more pK values. Thus, we cannot rule out nor identify proton dissociations in the physiologically relevant range of pH.

The results in Fig. 6 indicate that the greatest alteration in apparent affinity as a function of pH occurs between pH 7.5 and pH 8.0. Two potentially significant factors in short term

4 Reports have appeared suggesting the existence of a membrane-associated protein, distinct from the insulin-binding moiety, that may be an effector of insulin binding (51, 52).

5 Jacobs et al. (59) have discussed several limitations of this method.
cellular responsiveness to and processing of insulin are: 1) transient changes in extracellular pH accompanying alterations in the metabolic state and 2) intracellular compartmentation, either achieving local pH differences which could alter the extent of hormone-receptor association. Especially in relation to cellular responsiveness, it was expected that the greatest affinity changes would occur between pH 7.0 and pH 7.4, the normal range of blood plasma pH. It must be noted that we have excluded from our assay system components such as monovalent and divalent cations which are known to alter the apparent affinity (48, 60, 61). Work in progress suggests that at physiologically significant concentrations these constituents shift the ascending wing of the pH dependence profile toward a lower pH values; these findings differ from those reported by Harrison et al. (62). From a practical viewpoint, however, assessing the total binding capacity of a preparation of solubilized insulin receptor would best be accomplished near the optimal pH of 8.4, since saturation was more closely approached at usable 125I-insulin concentrations.

Careful analysis of the equilibrium binding data revealed that the binding isoformers could be fit to the Adair equation (Equation 1) assuming a single class of sites. Attempts to fit these data, assuming two classes of sites, using the general method of Cornish-Bowden and Koshland (12), which does not require assumptions concerning site-site interactions, yielded stoichiometric association constants in the ratio K2/K1 = 4. It has been established by others (19, 63) that this ratio indicates the presence of a single class of noninteracting binding sites, where the intrinsic (site) binding constant is equal to one-half of K1. However, in the absence of direct physical data, we could not draw any conclusions concerning the stoichiometry of insulin bound per receptor “holomer.” The finding that insulin binding to its solubilized human placental receptor accounted for adequately by a single class of sites was substantiated by the Hill plots (Figs. 3C and 5C) where a single Hill coefficient, uniformly equal to 1, covered the data over the entire concentration range of 125I-insulin. This feature of insulin receptor-insulin association was independent of pH between pH 7.5 and 9.6 (Table I). It should be noted that the binding isotherm generated at pH 6.8 (Fig. 5A) did not show a sufficient degree of saturation to allow confidence in the analysis (cf. Ref. 20) and was subject to the greatest experimental error because the nonspecific binding comprised a major fraction of the total 125I-insulin precipitated. Furthermore, the Scatchard plot (not shown) from data obtained at pH 9.6 was concave downward and therefore indicative of positive or mixed positive and negative cooperativity (64). This conclusion was inconsistent with the fit of the binding isotherm to the Adair equation (Fig. 5A) or with the uniformity of the Hill plot (Fig. 5C). We therefore relied upon iterative fitting procedures using the Adair equation to describe the binding isoformers in cases where >50% of the saturation curve was covered experimentally (e.g. 20%–70% saturation, as judged by the position of the inflection point). These results gave uniformly better fittings of theoretical and experimental values than attempts to fit the binding isoformers with parameters derived from the Scatchard plots (Table I).

We have shown by measurements made at equilibrium that, in the incubation system described, only a single class of noninteracting insulin binding sites exists on solubilized insulin receptor from human placenta plasma membrane and we have not ruled out the possibility of more complex association phenomena under other circumstances.

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Characteristics of Insulin Binding to Solubilized Receptor

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Equation 9.2 may be written accordingly

\[
K_{b}^{R} = K_{b}^{P} + 1 + \frac{K_{b}^{P}K_{b}^{R}}{[Pb]_{b}}
\]

This is not formally different from allowing only pK\(_{b}^{P}\) and pK\(_{b}^{R}\) in Scheme I, i.e., one cannot assign a significant proton association or dissociation to one species or the other. However, allowing both protonations to occur on only one species alters the resulting equation. For example,

\[
\text{Scheme III}
\]

where \(K_{b}^{P}\) and \(K_{b}^{R}\) are defined giving the following equation for the pH-dependence:

\[
K_{b}^{P} + K_{b}^{R} = K_{b}^{P} + 1 + \frac{K_{b}^{P}K_{b}^{R}}{[Pb]_{b}}
\]

Equation 13 differs from equation 12 by the absence of the term \(pK_{b}^{P}\). (compare the expression of equation 12) where, as noted previously, \(pK_{b}^{P} < pK_{b}^{R}\), making this term small. For Scheme II and III, if \(pK_{b}^{P}\) is close to \(pK_{b}^{R}\), then for values of pH = 1/2 \((pK_{b}^{P} = pK_{b}^{R})\), \(K_{b}^{P} = K_{b}^{R}\). Clearly, if there are no protonations of functionally significant groups, then \(K_{b}^{P} = K_{b}^{R}\) at every pH where both hormones and receptor are stable.

\[
\text{Scheme III}
\]

where \(K_{b}^{P}\) and \(K_{b}^{R}\) are related giving the following equation for the pH-dependence:

\[
K_{b}^{P} + K_{b}^{R} = K_{b}^{P} + 1 + \frac{K_{b}^{P}K_{b}^{R}}{[Pb]_{b}}
\]

Equation 13 differs from equation 12 by the absence of the term \(pK_{b}^{R}\). (compare the expression of equation 12) where, as noted previously, \(pK_{b}^{R} < pK_{b}^{R}\), making this term small. For Scheme II and III, if \(pK_{b}^{P}\) is close to \(pK_{b}^{R}\), then for values of pH = 1/2 \((pK_{b}^{P} = pK_{b}^{R})\), \(K_{b}^{P} = K_{b}^{R}\). Clearly, if there are no protonations of functionally significant groups, then \(K_{b}^{P} = K_{b}^{R}\) at every pH where both hormones and receptor are stable.
Binding of insulin to solubilized insulin receptor from human placenta. Evidence for a single class of noninteracting binding sites.

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