

# Defect in Cooperativity in Insulin Receptors from a Patient with a Congenital Form of Extreme Insulin Resistance

SIMEON I. TAYLOR and SHERYL LEVENTHAL, *Diabetes Branch, National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20205*

**ABSTRACT** Previously, we have described a novel qualitative defect in insulin receptors from a patient with a genetic form of extreme insulin resistance (leprechaunism). Receptors from this insulin-resistant child are characterized by two abnormalities: (a) an abnormally high binding affinity for insulin, and (b) a markedly reduced sensitivity of  $^{125}\text{I}$ -insulin binding to alterations in pH and temperature. In this paper, we have investigated the kinetic mechanism of this abnormality in steady-state binding. The increased binding affinity for  $^{125}\text{I}$ -insulin results from a decrease in the dissociation rate of the hormone-receptor complex. In addition, the cooperative interactions among insulin binding sites are defective with insulin receptors from this child with leprechaunism. With insulin receptors on cultured lymphocytes from normal subjects, both negative and positive cooperativity may be observed. Porcine insulin accelerates the dissociation of the hormone-receptor complex (negative cooperativity). In contrast, certain insulin analogs such as des-octapeptide-insulin and desalanine-desasparagine-insulin retard the dissociation of the hormone-receptor complex (positive cooperativity). With insulin receptors from the leprechaun child, positive cooperativity could not be demonstrated, although negative cooperativity appeared to be normal. It seems likely that the same genetic defect may be responsible for the abnormalities in both insulin sensitivity and positive cooperativity.

## INTRODUCTION

Inherited diseases often provide insights into normal biochemistry and physiology (1). Therefore, genetic

syndromes of extreme insulin resistance have the potential to give insight into the mechanisms of insulin action. Using cultured lymphocytes, we have characterized insulin receptors from many patients with severe insulin resistance (2, 3). One such patient with the syndrome of leprechaunism (i.e., leprechaun/Ark-1) had a unique qualitative abnormality of her insulin receptors: a markedly diminished sensitivity to alterations in pH and temperature (2). Moreover, the Scatchard plot for insulin binding to this patient's cells had an abnormal shape, suggesting that the receptor from this patient had an abnormally high affinity for insulin (2). According to one model of the insulin receptor, the curvilinear shape of the Scatchard plot results from cooperative interactions among insulin binding sites (4, 5). Consequently, we hypothesized that the cooperative interactions among insulin receptors might be abnormal in cells from leprechaun/Ark-1.

In the case of hormones that activate adenylate cyclase, the regulation of the receptor's binding affinity is intimately associated with the mechanism of hormone action (6-8). Similarly, with cells from this patient with a genetic defect causing extreme insulin resistance, we have observed a defect in the regulation of the receptor's affinity for insulin. This defect in regulation of receptor affinity is manifested as an abnormality in the cooperative interactions among insulin binding sites. It seems likely that the same genetic lesion gives rise to the defects in both insulin sensitivity and cooperative binding interactions.

## METHODS

**Patients.** Leprechaun/Ark-1 is a young girl with extreme insulin resistance originally described by Elders and her co-workers (9, 10). The other subjects were normal volunteers, with the exception of two patients with extreme insulin resistance: in one case, a patient with autoantibodies directed against the insulin receptor; in the other, a patient with lipotrophic diabetes. All studies were approved by the In-

Ms. Leventhal was supported by a grant from the Washington, DC affiliate of the American Diabetes Association.

Received for publication 20 September 1982 and in revised form 8 February 1983.

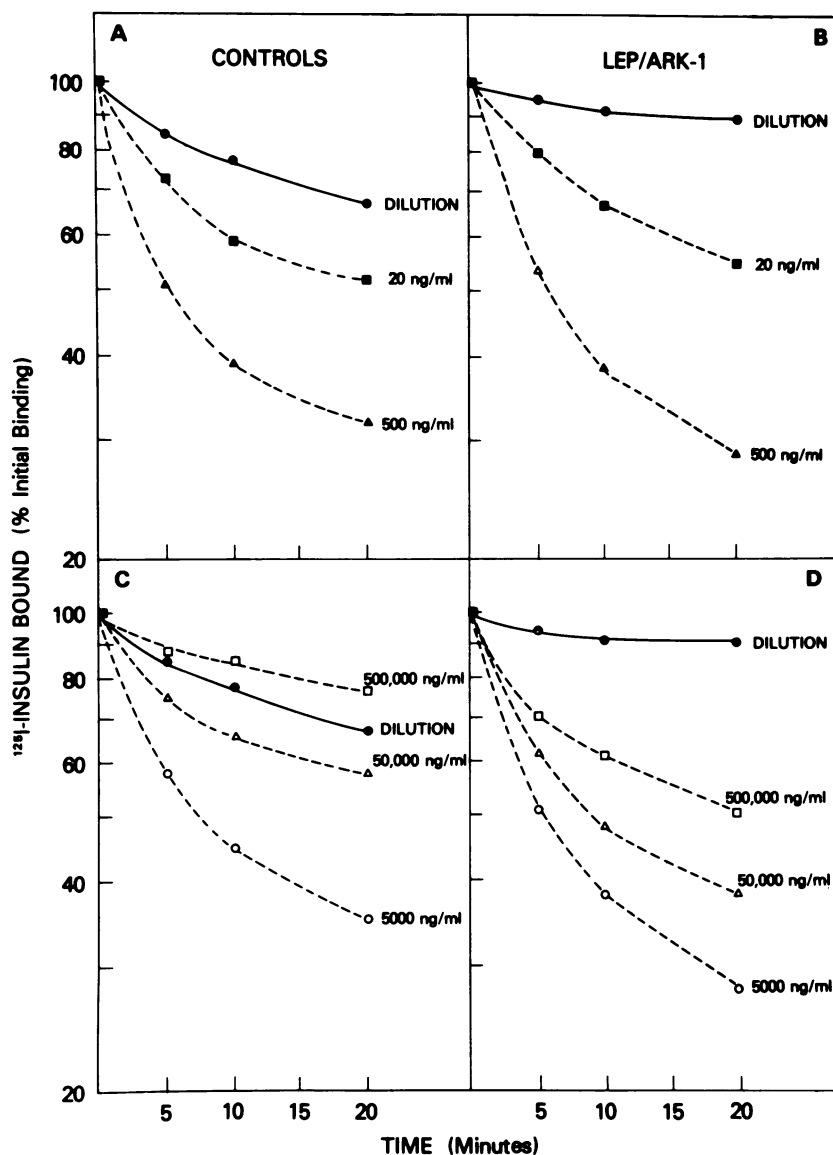


FIGURE 1 Effects of porcine insulin upon the dissociation of  $^{125}\text{I}$ -insulin ( $15^\circ\text{C}$ ). The dissociation of  $^{125}\text{I}$ -insulin from cultured lymphocytes was studied as outlined in Methods. The concentration of porcine insulin present during the dissociation phase of the experiment was varied: no added insulin ( $\bullet$ ), 20 ng/ml ( $\blacksquare$ ), 500 ng/ml ( $\blacktriangle$ ), 5,000 ng/ml ( $\circ$ ), 50,000 ng/ml ( $\Delta$ ), and 500,000 ng/ml ( $\square$ ). Data on control subjects (panels A and C) are means of five separate experiments carried out with cells obtained from two normal subjects. Data on cells from leprechaun/Ark-1 (panels B and D) are means of five separate experiments carried out on the same days as the studies with normal subjects. Under the conditions of the experiments, the  $^{125}\text{I}$ -insulin that dissociates is not significantly degraded ( $<5\%$ ), as judged by precipitability with trichloroacetic acid.

stitutional Review Board of the National Institute of Arthritis, Metabolism, and Digestive Disease. Informed consent was obtained from all subjects.

**Cultured lymphocytes.** Peripheral blood lymphocytes were transformed with Epstein-Barr virus as described elsewhere (2, 3). Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Flow Laboratories, Inc., Rockville, MD).

**Materials.** Porcine insulin was purchased from Elanco

Products Co., Indianapolis, IN. Porcine DOP-insulin<sup>1</sup> was provided by the National Institute of Arthritis, Metabolism,

<sup>1</sup> *Abbreviations used in this paper:* DAA-insulin, desalanine-desasparagine insulin (i.e., insulin lacking the C-terminal amino acids of both the A- and B-chains); DOP-insulin, desoctapeptide insulin (i.e., insulin lacking the C-terminal octapeptide on the B-chain).

and Digestive Disease. Guinea pig insulin and turkey DAA-insulin were the generous gifts of Drs. Cecil Yip and Pierre De Meyts, respectively. Antiserum directed against the receptor for insulin was obtained from patient B-2, a patient with extreme insulin resistance as a result of anti-receptor antibodies (11).

**<sup>125</sup>I-insulin binding studies.** <sup>125</sup>I-insulin binding studies were carried out as described elsewhere (2, 3) in a medium containing 120 mM NaCl, 2.5 mM KCl, 15 mM sodium acetate, 10 mM glucose, 1 mM EDTA, 1.2 mM MgSO<sub>4</sub>, 50 mM Hepes (pH 7.8) plus 10 mg/ml bovine serum albumin. Cultured lymphocytes (10<sup>7</sup> cells/ml) were incubated for 3 h at 15°C in the presence of <sup>125</sup>I-insulin (100–150 Ci/g, 0.1 ng/ml) and varying amounts of unlabeled insulin derivatives. At the end of the incubation period, aliquots (0.2 ml) of cell suspensions were layered over ice-cold assay buffer (0.175 ml) in microcentrifugation tubes. The cells were separated from the medium by centrifugation for 45 s in a Beckman Microfuge (~10,000 g) and the supernatant medium was aspirated and discarded (Beckman Instruments, Inc., Fullerton, CA). The tips of the tubes, containing the cell pellet, were excised and placed in 12 × 75-mm glass test tubes for

determination of cell-associated radioactivity. Nonspecific binding was defined as <sup>125</sup>I-insulin binding in the presence of excess porcine insulin (50,000 ng/ml). Specific binding was calculated by subtraction of nonspecific binding from total binding.

In these experiments, the range of specific binding of <sup>125</sup>I-insulin (mean±SD) averaged 37±15% (normal subjects) and 23±6% (leprechaun/Ark-1) of the added <sup>125</sup>I-insulin (0.1 ng/ml) per 10<sup>7</sup> cells/ml. Nonspecific binding was 1.1±0.3% in both types of cells.

**<sup>125</sup>I-insulin dissociation kinetics.** Cultured lymphocytes (10<sup>7</sup> cells/ml) were suspended in incubation medium (10 ml) containing <sup>125</sup>I-insulin (0.1 ng/ml). After incubation at 15°C for 3 h, the cells were cooled by addition of 35 ml of ice-cold medium. Cells were separated from the medium by centrifugation (250 g, 10 min, 4°C) and were immediately resuspended in 10 ml of fresh medium at 4°C. Plastic test tubes (12 × 75 mm, Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) containing medium (4.8 ml) were pre-equilibrated at 15°C. Insulin or insulin analogs had been added to the incubation medium as indicated. The dissociation of <sup>125</sup>I-insulin was initiated by diluting aliquots (0.2 ml) of the resuspended cells into the tubes containing incubation medium (4.8 ml) at 15°C. In the experiments described in Figs. 3 and 4, the incubation temperature during the dissociation phase was 37°C; the temperature during association remained 15°C. To monitor the rate of dissociation, duplicate tubes were removed at 0, 5, 10, and 20 min and the cells were separated from the medium by centrifugation (1,000 g, 5 min, 4°C). After the supernatant medium was aspirated, the tubes were placed directly into a Searle automatic gamma system (model 1285; Searle Radiographics Inc., Des Plaines, IL) for determination of the cell-associated <sup>125</sup>I-insulin.

## RESULTS

### Effects of porcine insulin upon dissociation of <sup>125</sup>I-insulin

**Studies with receptors from control subjects.** As shown previously with normal insulin receptors (4),

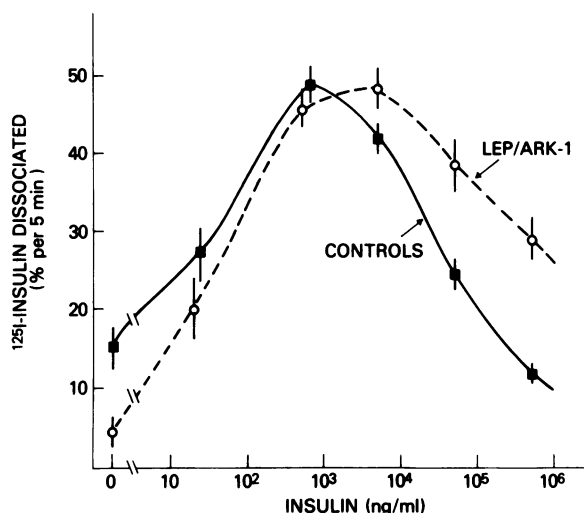


FIGURE 2 Dose-response curves for effects of porcine insulin upon the dissociation of <sup>125</sup>I-insulin (15°C). The data from Fig. 1 were analyzed as follows. The initial rates of dissociation of <sup>125</sup>I-insulin (observed during the first 5 min of dissociation) are plotted as a function of the concentration of unlabeled insulin. Data are presented as means±SEM of five experiments with cells from control subjects (■) or leprechaun/Ark-1 (○). The differences in dissociation rates were statistically significant at all concentrations of insulin, with the exception of 500 ng/ml. Values of *P*, calculated using a two-tailed *t* test for paired observations, were as follows: *P* < 0.001 (no added insulin), *P* < 0.04 (20 ng/ml), *P* = 0.23 (500 ng/ml), *P* < 0.005 (5,000 ng/ml), *P* < 0.01 (50,000 ng/ml), and *P* < 0.0005 (500,000 ng/ml). Paired *t* tests are significant because the differences were highly reproducible within each experiment. The overlap of the error bars at some concentrations derives from the fact that the interexperiment variability in dissociation rates was greater than the intraexperiment variability. Although the data presented in Fig. 2 derive only from the 5-min time points of Fig. 1, qualitatively similar results may be obtained with later time points.

TABLE I  
Potencies of Insulin Analogs in Inhibiting <sup>125</sup>I-Insulin Binding to Cultured Lymphocytes from Normal Subjects and Leprechaun/Ark-1

Insulin analog	I <sub>50</sub>		I <sub>50</sub> Ratio*
	Controls	Lep/Ark-1	
ng/ml			
Porcine (7)	4.0±0.4	1.3±0.2	3.1
Turkey DAA (2)	225±75	38±12	5.9
Guinea pig (6)	270±80	115±15	2.4
Porcine DOP (2)	1250±250	250±0	5.0

The data summarized in Fig. 3 were analyzed to estimate the concentration (I<sub>50</sub>) of insulin analog which displaced 50% of <sup>125</sup>I-insulin binding. Values of I<sub>50</sub> are presented as means±SEM of *n* experiments. (The value of *n* is given in parentheses after the name of the insulin analog.)

\* Control: Lep/Ark-1.

the effects of porcine insulin upon the dissociation of  $^{125}\text{I}$ -insulin at  $15^\circ\text{C}$  are complex (Figs. 1, 2):

Phase 1: Addition of moderate concentrations of insulin ( $\leq 500$  ng/ml) causes a progressive acceleration of dissociation of  $^{125}\text{I}$ -insulin (Figs. 1A, 2).

Phase 2: Higher concentrations of unlabeled insulin (5,000–50,000 ng/ml) begin to inhibit the acceleration of the dissociation of  $^{125}\text{I}$ -insulin (Figs. 1C, 2).

Phase 3: The highest concentrations of porcine insulin (500,000 ng/ml) retard the dissociation of  $^{125}\text{I}$ -insulin (Figs. 1C, 2).

*Studies with receptors from leprechaun/Ark-1.* With cells from leprechaun/Ark-1, we observed several differences in the dissociation kinetics (Figs. 1B, 1D, 2) at  $15^\circ\text{C}$ :

(a) The spontaneous rate of dissociation of  $^{125}\text{I}$ -insulin was markedly retarded as compared to the dis-

sociation rate with cells from control subjects (Figs. 1, 2). This slower dissociation rate (approximately one-third as fast as with normal subjects) may account for the threefold increase in apparent binding affinity of insulin receptors from cells of leprechaun/Ark-1 (Table I and ref. 2).

(b) Maximal acceleration of  $^{125}\text{I}$ -insulin dissociation required a higher concentration (5,000 vs. 500 ng/ml) of unlabeled porcine insulin (Figs. 1B, 1D, 2).

(c) Phase 2 was abnormal in that the inhibition of the acceleration of dissociation required tenfold higher concentrations of unlabeled insulin (50,000 vs. 5,000 ng/ml).

(d) The most striking abnormality was the failure to observe phase 3 (i.e., retardation of dissociation) in cells from leprechaun/Ark-1 (Fig. 1D).

Because the temperature sensitivity of the insulin receptor of leprechaun/Ark-1 is abnormal, we also studied the dissociation kinetics at physiological temperature (i.e.,  $37^\circ\text{C}$ ). Qualitatively similar results were observed (Figs. 3, 4). However, dissociation proceeds more rapidly at  $37^\circ\text{C}$  under all conditions. Nevertheless, the dissociation kinetics observed with insulin receptors from leprechaun/Ark-1 are strikingly abnormal at  $37^\circ\text{C}$ . In fact, the differences in the physiological range of insulin concentrations are more obvious at  $37^\circ\text{C}$  than at  $15^\circ\text{C}$ .

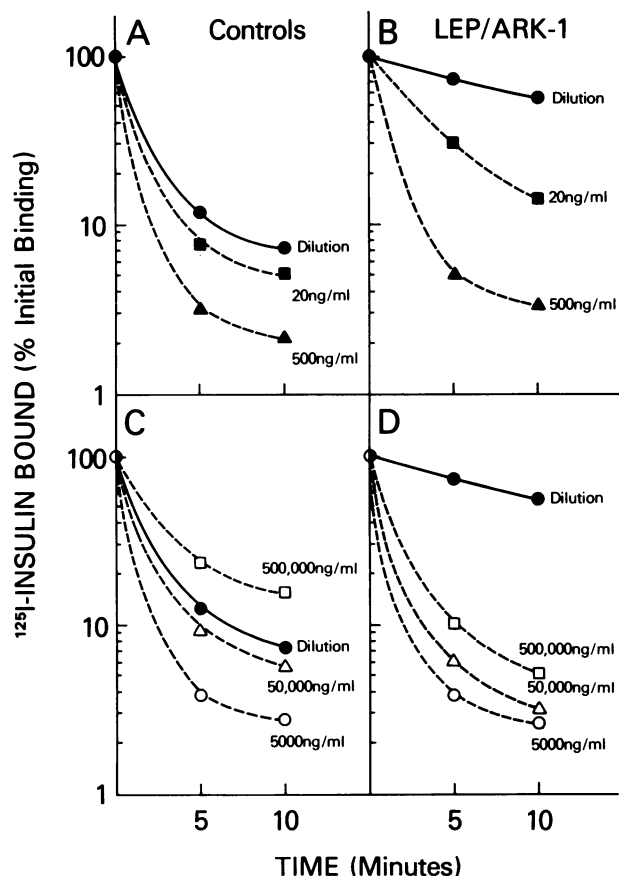


FIGURE 3 Effects of porcine insulin upon the dissociation of  $^{125}\text{I}$ -insulin ( $37^\circ\text{C}$ ). These studies were identical to those described in the legend to Fig. 1, with the exception that the dissociation was carried out at  $37^\circ\text{C}$ . The symbols are the same as outlined in Fig. 1. Data are means of two separate experiments.

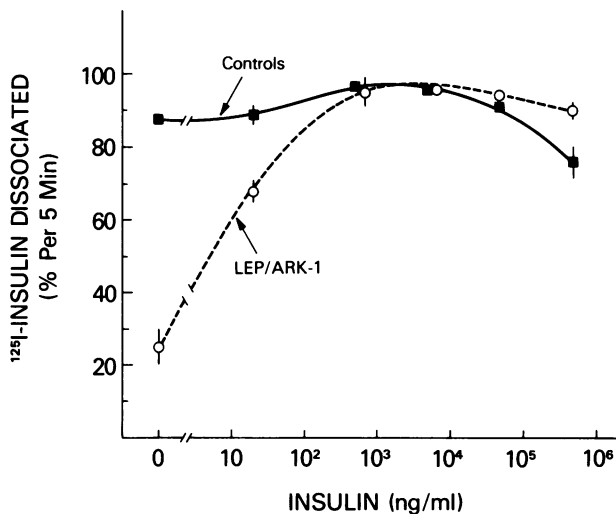
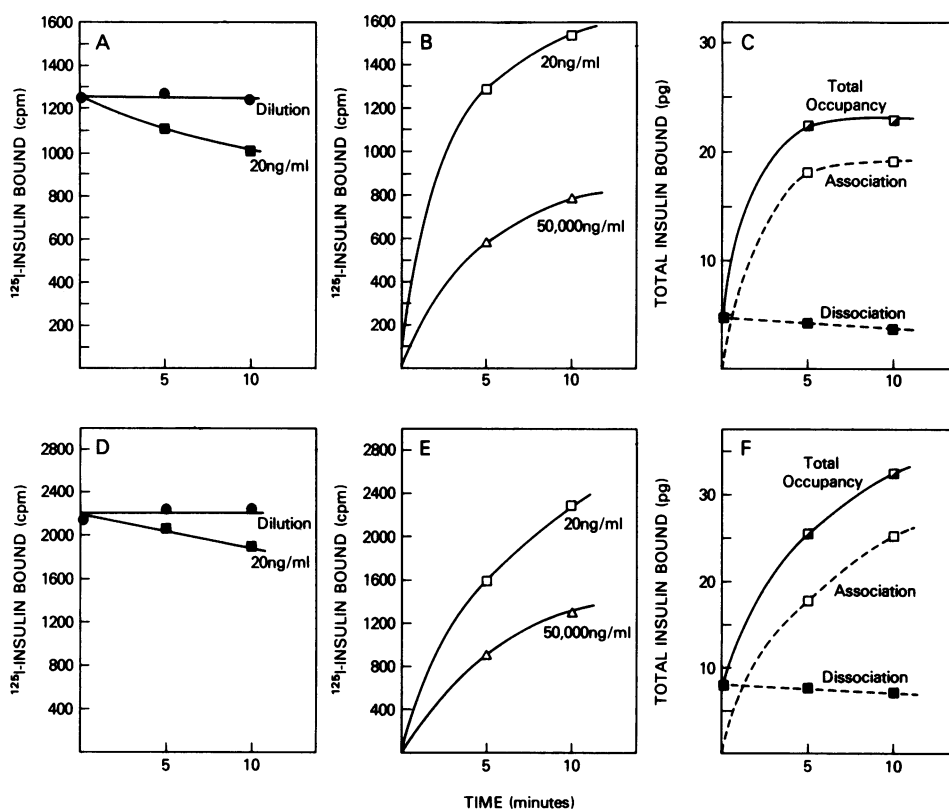


FIGURE 4 Dose-response curves for effects of porcine insulins upon the dissociation of  $^{125}\text{I}$ -insulin ( $37^\circ\text{C}$ ). The data of Fig. 3 were analyzed as described in the legend to Fig. 2. The differences in dissociation rate were statistically significant at all concentrations of insulin with the exception of 500 and 5,000 ng/ml. Values of  $P$ , calculated using a one-tailed  $t$  test for paired observations were as follows:  $P < 0.02$  (no added insulin),  $P < 0.01$  (20 ng/ml),  $P = 0.18$  (500 ng/ml),  $P = 0.5$  (5,000 ng/ml),  $P \leq 0.04$  (50,000 ng/ml),  $P \leq 0.04$  (500,000 ng/ml).

The effect of insulin to accelerate dissociation of  $^{125}\text{I}$ -insulin is very rapid—with onset in less than 5 min. If one proposes that this acceleration results from an increase in receptor occupancy (4, 5), it is important to document that the increase in receptor occupancy also has rapid onset. When insulin (20 ng/ml) is included in the medium during the dissociation of  $^{125}\text{I}$ -insulin, receptor occupancy increases by 200–400% within 5 min (Fig. 5). Thus, it is clear that the time-

course of the increase in receptor occupancy is sufficiently rapid so that it might be a cause of the accelerated rate of dissociation of  $^{125}\text{I}$ -insulin (Figs. 1–4).

**Retardation of the dissociation of  $^{125}\text{I}$ -insulin by DOP- and DAA-insulins.** We chose to investigate further the possibility that there is a defect in phase 3 (i.e., the retardation of  $^{125}\text{I}$ -insulin dissociation by very high concentrations of porcine insulin). For this purpose, to simplify the interpretation of experiments,



**FIGURE 5** Receptor occupancy during dissociation experiments. Panels A and D: Cultured lymphocytes ( $12 \times 10^6$  cells/ml) from either a normal subject (panels A–C) or leprechaun/Ark-1 (panels D–F) were incubated with  $^{125}\text{I}$ -insulin (0.1 ng/ml; 150 Ci/g) for 3 h at  $15^\circ\text{C}$ . After addition of 20 ml of ice-cold buffer, the cells were sedimented by centrifugation at  $4^\circ\text{C}$ . The cells were resuspended in the original volume of ice-cold buffer. Thereafter, aliquots (0.8 ml) of resuspended cells were added to tubes containing buffer (0.2 ml) in the presence or absence of unlabeled insulin (20 ng). The cells were incubated at  $15^\circ\text{C}$  and duplicate aliquots (0.2 ml) were taken after 5 and 10 min for determination of cell-associated  $^{125}\text{I}$ -insulin. The dissociation of  $^{125}\text{I}$ -insulin (cpm per  $2 \times 10^5$  cells) in the presence (■) or absence (●) of unlabeled insulin (20 ng/ml) is plotted as a function of time. Panels B and E: Cultured lymphocytes were preincubated exactly as above, with the exception that unlabeled insulin (0.1 ng/ml) was substituted for  $^{125}\text{I}$ -insulin. In the second incubation, the unlabeled insulin concentration was either 20 ng/ml (□) or 50,000 ng/ml (Δ). In addition,  $^{125}\text{I}$ -insulin (788,000 cpm) was added to each of the tubes during the second incubation. Panels C and F: These panels integrate the observations in panels A–B and D–E, respectively. The rate of dissociation of pre-bound  $^{125}\text{I}$ -insulin (panels A and D) in the presence of unlabeled insulin (20 ng/ml) is plotted in pg- $^{125}\text{I}$ -insulin per  $2 \times 10^5$  cells (■). The rate of association of specifically bound  $^{125}\text{I}$ -insulin (20 ng/ml) is plotted in pg of insulin per  $2 \times 10^5$  cells (□). (To calculate the specifically bound  $^{125}\text{I}$ -insulin, cpm bound in the presence of 50,000 ng/ml insulin was subtracted from cpm bound in the presence of 20 ng/ml insulin [panels B and E].) Total occupancy of insulin receptors (■) was calculated by adding the dissociation (■) and association (□) curves.

we used insulin analogs that do not accelerate the dissociation of  $^{125}\text{I}$ -insulin (4, 12–13). Two such analogs, porcine DOP- and turkey DAA-insulins, actually retard the rate of dissociation of  $^{125}\text{I}$ -insulin from cells derived from normal subjects (Figs. 6A, 6C). Thus, these analogs retard the dissociation of  $^{125}\text{I}$ -insulin without duplicating the effect of monomeric insulin to accelerate dissociation. Interestingly, porcine DOP-

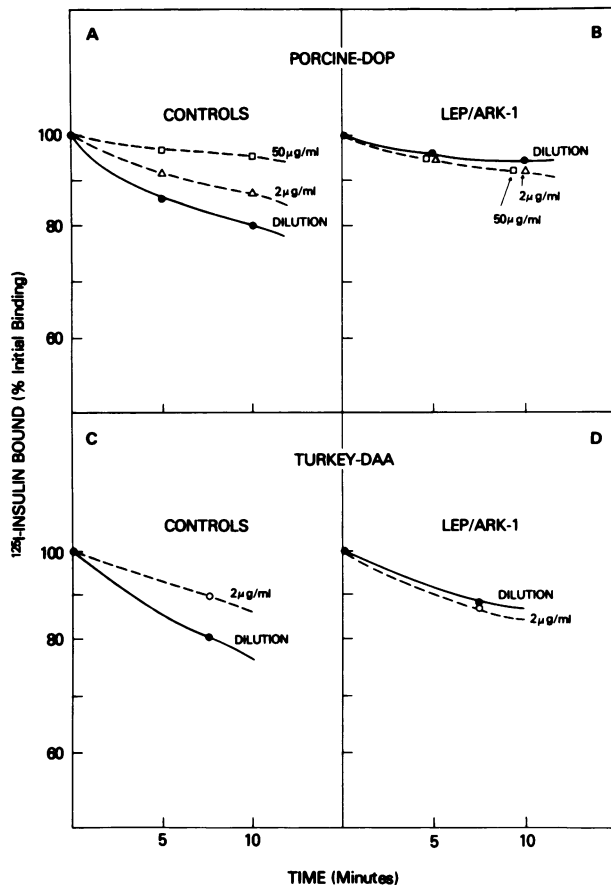


FIGURE 6 Effects of porcine DOP- and turkey DAA-insulins upon the dissociation of  $^{125}\text{I}$ -insulin. The dissociation of  $^{125}\text{I}$ -insulin from cultured lymphocytes was studied as outlined in Methods. The concentration of porcine DOP-insulin (panels A and B) and turkey DAA-insulin (panels C and D) present during the dissociation phase of the experiment was as follows: no added insulin (●); porcine DOP-insulin, 2  $\mu\text{g}/\text{ml}$  ( $\Delta$ ); porcine DOP-insulin 50  $\mu\text{g}/\text{ml}$  ( $\square$ ); turkey DAA-insulin, 2  $\mu\text{g}/\text{ml}$  ( $\circ$ ). Data with porcine DOP-insulin are means of two separate experiments; data with turkey DAA-insulin are results of duplicate determinations in a single experiment. Studies using cells from control subjects (panels A and C) were carried out simultaneously with studies using cells from lepchaun/Ark-1 (panels B and D). Effects of porcine DOP-insulin and turkey DAA-insulin to retard dissociation were significant ( $P < 0.05$  for both analogs at 2  $\mu\text{g}/\text{ml}$ ;  $P < 0.01$  for 50  $\mu\text{g}/\text{ml}$ ) in the cells from control subjects. However, neither analog had a significant effect upon the dissociation rate with cells from lepchaun/Ark-1.

and turkey DAA-insulins do not affect the rate of dissociation of  $^{125}\text{I}$ -insulin from cells of lepchaun/Ark-1 (Figs. 6B, 6D). This is compatible with the possibility that receptors from lepchaun/Ark-1 exhibit a defect in phase 3.

**Steady-state  $^{125}\text{I}$ -insulin binding.** In view of the failure of porcine DOP- and turkey DAA-insulins to retard dissociation of  $^{125}\text{I}$ -insulin with cells from lepchaun/Ark-1 (Figs. 6B, 6D), it is important to document that these insulin analogs are able to bind to insulin receptors from lepchaun/Ark-1. In fact, these analogs bind quite well to cells from lepchaun/Ark-1 (Fig. 7)—even more tightly than to cells from control subjects. The binding-competition curves for turkey DAA- and porcine DOP-insulins are shifted five- to sixfold to the left as compared to the curves with cells from control subjects (Figs. 7C, 7D, Table I).

**Effects of anti-receptor antibodies on  $^{125}\text{I}$ -insulin binding.** We inquired whether the leftward shift of the binding-competition curves is specific for insulin and insulin analogs. In the case of antibodies to the insulin receptor, another ligand for the receptor (14), the leftward shift of the binding curve was not observed. Anti-receptor antibodies appeared to recognize insulin receptors equally well from all cell types studied (Fig. 8).

## DISCUSSION

In lepchaun/Ark-1, the genetic defect in the insulin receptor results in an abnormally high binding affinity for insulin under physiological conditions (i.e., 37°C and pH 7.4) as well as a marked insensitivity of binding to alterations of pH and temperature (2). Paradoxically, the increased occupancy of receptors by insulin under physiological conditions is associated with extreme insulin resistance (2, 9, 10, 15). Unraveling this paradox may provide insight into the mechanism of coupling hormone binding to hormone action.

**Kinetic model for the binding properties of the insulin receptor.** The complex dissociation kinetics observed with the insulin receptor may be analyzed more simply by considering three limiting conditions, characterized by different dissociation rates (Fig. 9A):

(a) Spontaneous dissociation rate (curve E): In the absence of unlabeled insulin,  $^{125}\text{I}$ -insulin dissociates relatively slowly (Figs. 1A, 6A, 6C, 9A).

(b) Maximally accelerated dissociation rate (curve F): Moderate concentrations of porcine insulin ( $\sim 500$  ng/ml) lead to maximal acceleration of the dissociation of  $^{125}\text{I}$ -insulin (Figs. 1A, 9A).

(c) Maximally retarded dissociation rate (curve G): Insulin analogs (e.g., porcine DOP- and turkey DAA-insulins), as well as covalent dimers of insulin (12, 13, 19), retard the rate of dissociation of  $^{125}\text{I}$ -insulin (Figs.

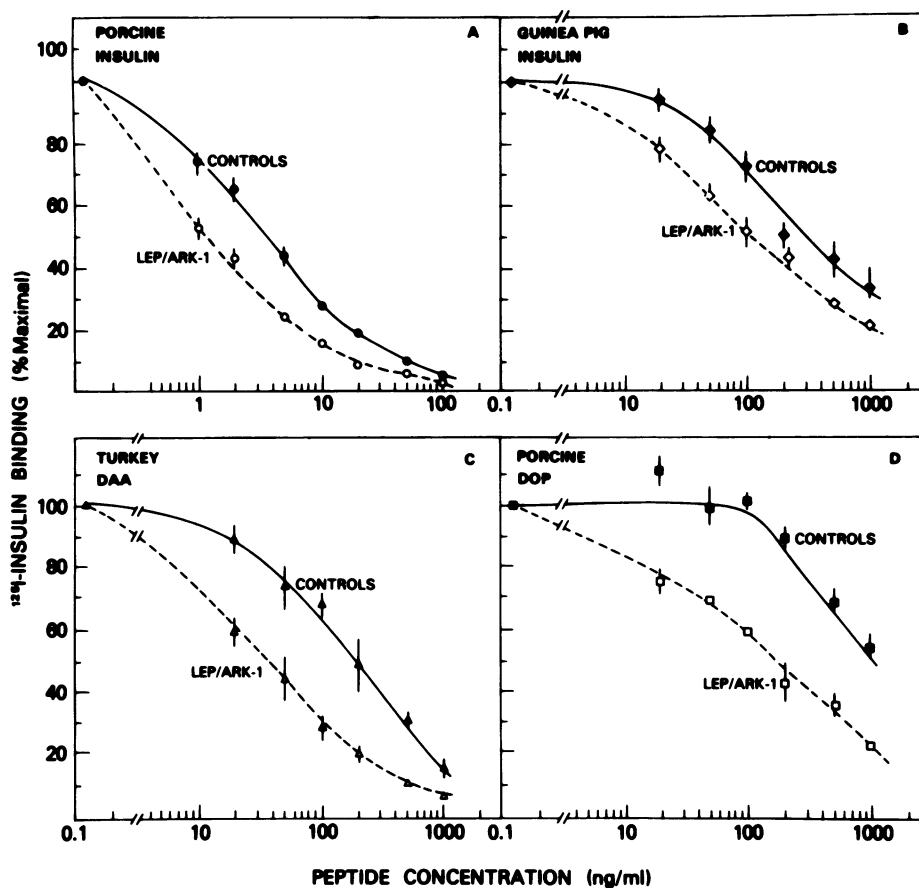


FIGURE 7 Binding-competition curves with insulin analogs.  $^{125}\text{I}$ -insulin binding studies were carried out at  $15^\circ\text{C}$  with cultured lymphocytes from leprechaun/Ark-1 as well as three control subjects (two normal volunteers and a patient with anti-receptor antibodies). Results are expressed as a percentage of the specific binding of  $^{125}\text{I}$ -insulin ( $0.1\text{ ng/ml}$ ) observed in the absence of unlabeled insulin. The data are means  $\pm$  SEM of seven (porcine insulin, panel A), six (guinea pig insulin, panel B), or two (turkey DAA-insulin, panel C; porcine DOP-insulin, panel D) separate experiments. Data with cells from control subjects are represented with closed symbols and solid lines; data with cells from leprechaun/Ark-1 are represented with open symbols and broken lines.

6A, 6C). Presumably because of the tendency of insulin to dimerize when present in concentrated solutions, high concentrations of insulin (e.g.,  $500,000\text{ ng/ml}$ ) similarly retard the dissociation rate (Fig. 1C). Thus, the effects of any concentration of porcine insulin depends upon a balance between the effects of that fraction of insulin that exists as monomer and that fraction that exists as dimer. At concentrations of porcine insulin  $\leq 500\text{ ng/ml}$ , the predominant species is monomeric insulin, which accelerates the dissociation of  $^{125}\text{I}$ -insulin. At concentrations of porcine insulin  $\geq 5,000\text{ ng/ml}$ , the effects of dimeric insulin to inhibit monomeric insulin binding as well as to retard  $^{125}\text{I}$ -insulin dissociation begin to be observed. It should be emphasized that insulin concentrations in excess of  $10\text{ ng/ml}$  are almost certainly supraphysiologic. These high

concentrations are being exploited as an experimental tool to probe receptor function. Moreover, we do not wish to imply that there is a significant concentration of dimeric insulin in the circulation *in vivo*.

Despite the general agreement about the phenomenology of the kinetics of insulin binding, considerable controversy surrounds the question of which kinetic model correctly explains the experimental observations (4, 20–25). Although it is beyond the ambition of the present work to provide final resolution to the controversy, we have chosen to rationalize our observations in terms of a model that assumes cooperative interactions among insulin binding sites (4, 5). We believe that this model provides the simplest explanation of our data. According to this model, the insulin receptor may exist in three conformations: E, F, and

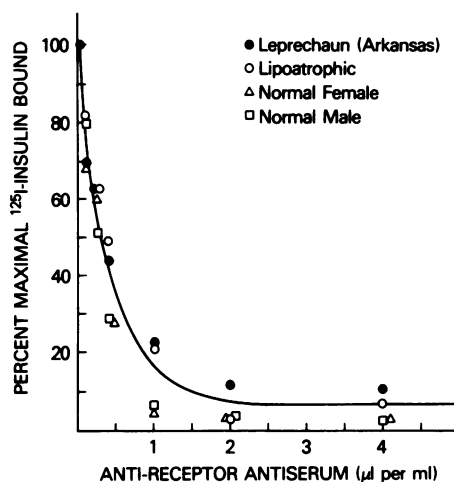


FIGURE 8 Inhibition of  $^{125}\text{I}$ -insulin binding by anti-receptor antiserum. Cultured lymphocytes ( $10^7$  cells/ml) were suspended in the usual binding buffer (0.5 ml) containing anti-receptor antiserum at a dilution of 1:10,000 to 1:250. After incubation at  $21^\circ\text{C}$  for 90 min, the cells were sedimented by centrifugation (250 g for 8 min) and the medium was aspirated. Cells were resuspended in fresh medium containing  $^{125}\text{I}$ -insulin (0.1 ng/ml) in the presence or absence of unlabeled insulin (0.05 mg/ml).  $^{125}\text{I}$ -insulin binding was assayed as outlined in Methods. Data are presented as the percentage of the maximal specific binding observed in the absence of anti-receptor antiserum. For these studies, cultured lymphocytes were obtained from leprechaun/Ark-1 (●), a male with lipoatrophic diabetes (○), a normal female (△), and a normal male (□).

G, corresponding to the dissociation curves depicted in Fig. 9. Each conformation has a characteristic affinity for insulin:  $K_e$ ,  $K_f$ , and  $K_g$ , respectively. When unoccupied by insulin, the insulin receptor from normal subjects is ordinarily in the E-conformation. Monomeric porcine insulin promotes a transition to the F-conformation (negative cooperativity). In contrast, porcine DOP- and turkey DAA-insulins promote a transition to the G-conformation (positive cooperativity).<sup>2</sup>

**Abnormal dissociation kinetics with receptors from leprechaun/Ark-1.** Cultured lymphocytes from leprechaun/Ark-1 revealed unique abnormalities in the kinetics of dissociation of  $^{125}\text{I}$ -insulin from its receptor:

(a) The spontaneous dissociation rate observed in the absence of unlabeled insulin was already as slow

<sup>2</sup> De Meyts has previously reported that turkey DAA-insulin gives rise to a linear Scatchard plot when binding studies to cultured lymphocytes are analyzed (26). However, on theoretical grounds, the positive cooperativity observed with turkey DAA-insulin suggests that the Scatchard plot should be curvilinear (i.e., concave downward). We do not understand this apparent inconsistency. Perhaps the curvature of the Scatchard plot is so slight that the curve is well approximated by a straight line.

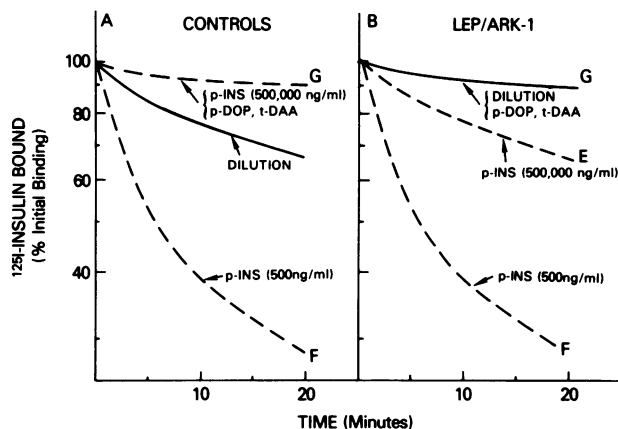


FIGURE 9 Schematic summary of studies on kinetics of dissociation of  $^{125}\text{I}$ -insulin. The data from Figs. 1 and 6 are partially summarized here. We have interpreted the data in terms of three limiting dissociation curves: curve E, slow dissociation rate; curve F, rapid dissociation rate; and curve G, ultraslow dissociation rate. For each cell type (panel A, control subjects; panel B, leprechaun/Ark-1), the curve describing the spontaneous dissociation rate is denoted with a solid line. The other two dissociation curves are represented with dotted lines. According to the conventional nomenclature of the negative cooperativity model (4), curves E and F may be correlated with the  $K_e$  and  $K_f$  conformations of the insulin receptor. While others (16–18) have suggested various forms of ultra slowly dissociating forms of the insulin receptor, we have not adopted their nomenclature because it is not certain that all of the groups are describing the same phenomena.

as the maximally retarded rate in normal cells (Fig. 9B, curve G).

(b) Positive cooperativity (i.e., retardation of the dissociation rate) was not observed with leprechaun/Ark-1. Those analogs (i.e., porcine DOP- and turkey DAA-insulins) which ordinarily retard dissociation of  $^{125}\text{I}$ -insulin had no effect upon the dissociation rate with cells from leprechaun/Ark-1 (Figs. 6B, 6D, 9B).

(c) High concentrations of porcine insulin (i.e., 500,000 ng/ml) led to acceleration rather than retardation of dissociation (Figs. 1D, 9B). In contrast, acceleration of the dissociation rate by moderate concentrations of insulin was similar in both cell types (Figs. 1A, 1B, 9A, 9B). It seems likely that the leprechaun/Ark-1 insulin receptor exists in the G-conformation rather than the E-conformation, even when the receptor is unoccupied by hormone (Fig. 9B). This accounts for the abnormally slow spontaneous dissociation rate. Moreover, positive cooperativity is not observed with receptors from leprechaun/Ark-1, because the receptor already exists in the G-conformation, even in the absence of porcine DOP- or turkey DAA-insulins.

**Biological significance of the defect in positive cooperativity.** The receptor from leprechaun/Ark-1 would be predicted to bind abnormally large amounts



of insulin under physiological conditions of temperature and pH (2). Nevertheless, this insulin binding appears not to be coupled efficiently to the induction of biological activity (9). Consequently, it seems likely that the G-conformation of the receptor, despite its ultrahigh affinity for insulin, may be compromised in its ability to mediate the early events in insulin action. This suggests that the same molecular defect in the receptor simultaneously interferes with positive cooperativity and insulin action, possibly because the two phenomena share common mechanisms.

While the detailed mechanism for the uncoupling of the insulin receptor is not certain, these observations closely resemble the situation with the UNC mutation in S49 lymphoma cells (6, 7). Normally, the guanine nucleotide binding subunit has at least two functions: (a) to couple hormone binding to activation of adenylate cyclase, and (b) to regulate the affinity of receptor for agonistic ligands. In the case of the UNC mutation, the primary genetic defect appears to affect the guanine nucleotide binding subunit. As a result of the UNC mutation, the defective guanine nucleotide binding subunit does not interact normally with hormone receptors. Just as with leprechaun/Ark-1, the UNC mutation is associated not only with hormone resistance, but with defective regulation of hormone receptor affinity as well. We have previously proposed (15) that the mutation in leprechaun/Ark-1 may be a structural defect in the insulin receptor such that the receptor does not interact normally with the "affinity regulator" (27). The fact that this defect gives rise to insulin resistance suggests that the affinity regulator may serve to couple insulin binding to bioactivity, a function similar to that of the guanine nucleotide binding subunit in the adenylate cyclase system. We may now extend this model to propose that the ultraslowly dissociating form of the insulin receptor (i.e., the G-state) may be a form which is uncoupled from the "affinity regulator." At present, this model remains highly speculative. Nevertheless, it seems likely that elucidation of the binding abnormalities in receptors from leprechaun/Ark-1 may give insight into the molecular details of the mechanism of insulin action just as mutations affecting the guanine nucleotide binding subunit of adenylate cyclase (e.g., UNC, CYC<sup>-</sup>, and H21a) have contributed to our understanding of the mechanism of action of catecholamines (6, 7).

#### ACKNOWLEDGMENTS

We gratefully acknowledge Dr. Jesse Roth for his support and encouragement as well as his critical review of this manuscript. In addition, we thank Mrs. Bernice Samuels for assistance in growing the cells and Ms. Laurie Tuchman for assistance in preparation of the manuscript. Dr. Taylor is indebted to the American Diabetes Association for partial research support (Roger Staubach Feasibility Grant). Fi-

nally, we are grateful to Dr. M. Joycelyn Elders for referral of this patient.

#### REFERENCES

1. Goldstein, J. L., and M. S. Brown. 1979. The LDL receptor locus and the genetics of familial hypercholesterolemia. *Annu. Rev. Genet.* 13: 259-289.
2. Taylor, S. I., J. Roth, R. M. Blizzard, and M. J. Elders. 1981. Qualitative abnormalities in insulin binding in a patient with extreme insulin resistance: decreased sensitivity to alterations in temperature and pH. *Proc. Natl. Acad. Sci. USA.* 78: 7157-7161.
3. Taylor, S. I., B. Samuels, J. Roth, M. Kasuga, J. A. Hedo, P. Gorden, D. E. Brasel, T. Pokora, and R. R. Engel. 1982. Decreased insulin binding in cultured lymphocytes from two patients with extreme insulin resistance. *J. Clin. Endocrinol. Metab.* 54: 919-930.
4. De Meyts, P., A. R. Bianco, and J. Roth. 1976. Site-site interactions among insulin receptors. Characterization of the negative cooperativity. *J. Biol. Chem.* 251: 1877-1888.
5. De Meyts, P., and J. Roth. 1975. Cooperativity in ligand binding: a new graphic analysis. *Biochem. Biophys. Res. Commun.* 66: 1118-1126.
6. Ross, E. M., and A. G. Gilman. 1980. Biochemical properties of hormone-sensitive adenylate cyclase. *Annu. Rev. Biochem.* 49: 533-564.
7. Johnson, G. L., H. R. Kaslow, Z. Farfel, and H. R. Bourne. 1980. Genetic analysis of hormone-sensitive adenylate cyclase. *Adv. Cyclic Nucleotide Res.* 13: 1-37.
8. Limbird, L., and R. J. Lefkowitz. 1978. Agonist-induced increase in apparent  $\beta$ -adrenergic receptor size. *Proc. Natl. Acad. Sci. USA.* 75: 228-232.
9. Kobayashi, M., J. M. Olefsky, J. Elders, M. E. Mako, B. D. Given, H. K. Schedewie, R. H. Fiser, R. L. Hintz, J. A. Horner, and A. H. Rubenstein. 1978. Insulin resistance due to a defect distal to the insulin receptor: demonstration in a patient with leprechaunism. *Proc. Natl. Acad. Sci. USA.* 75: 3469-3473.
10. Bier, D. M., J. Schedewie, J. Larner, J. Olefsky, A. Rubenstein, R. H. Fiser, J. W. Craig, and M. J. Elders. 1980. Glucose kinetics in leprechaunism: accelerated fasting due to insulin resistance. *J. Clin. Endocrinol. Metab.* 51: 988-994.
11. Flier, J. S., C. R. Kahn, D. B. Jarrett, and J. Roth. 1976. Characterization of antibodies to the insulin receptor: a cause of insulin-resistant diabetes in man. *J. Clin. Invest.* 58: 1442-1449.
12. Gu, J. L., P. De Meyts, L. M. Keefer, S. C. Piron, C. C. Wang, H. Gattner, and D. Brandenburg. 1981. Refined mapping of the cooperative site of the insulin molecule: crucial role of B25 Phe in negative cooperativity. *Endocrinology.* 108: 146. (Abstr. 253)
13. Keefer, L. M., J. L. Gu, M. A. Piron, and P. De Meyts. 1981. The negative cooperativity of the insulin receptor: its antagonism by non-cooperative analogues provides strong evidence for sequential, ligand-induced conformational changes. *Endocrinology.* 108: 314. (Abstr. 927)
14. Jarrett, D. B., J. Roth, C. R. Kahn, and J. S. Flier. 1976. Direct method for detection and characterization of cell surface receptors for insulin by means of <sup>125</sup>I-labeled autoantibodies against the insulin receptor. *Proc. Natl. Acad. Sci. USA.* 73: 4115-4119.
15. Taylor, S. I., J. A. Hedo, L. H. Underhill, M. Kasuga, M. J. Elders, and J. Roth. 1982. Extreme insulin resistance in association with abnormally high binding affinity of insulin receptors from a patient with lepre-

- chaunism: evidence for a defect intrinsic to the receptor. *J. Clin. Endocrinol. Metab.* **55**: 1108–1113.
16. Keefer, L. M., J. L. Gu, P. De Meyts, and J. M. Ketelslegers. 1981. Fast and slow transitions in the insulin receptor. *Diabetes*. **30**: 113A.
  17. Corin, R. E., and D. B. Donner. 1982. Insulin receptors convert to a higher affinity state subsequent to hormone binding. A two-state model for the insulin receptor. *J. Biol. Chem.* **257**: 104–110.
  18. Saviolakis, G. A., L. C. Harrison, and J. Roth. 1981. The binding of <sup>125</sup>I-insulin to specific receptors in IM-9 human lymphocytes. Detection of radioactivity covalently linked to receptors. *J. Biol. Chem.* **256**: 4924–4928.
  19. De Meyts, P., M. Michiels-Place, A. Schuttler, and D. Brandenburg. 1980. Cross-linked insulin dimers bind non-cooperatively to the insulin receptor and antagonize the negative cooperativity induced by monomeric insulin. *Endocrinology*. **106**: 112. (Abstr. 149)
  20. Pollet, R. J., M. L. Standaert, and B. A. Haase. 1977. Insulin binding to the human lymphocyte receptor. Evaluation of the negative cooperativity model. *J. Biol. Chem.* **252**: 5828–5834.
  21. Olefsky, J. M., and H. Chang. 1979. Further evidence for functional heterogeneity of adipocyte insulin receptors. *Endocrinology*. **104**: 462–466.
  22. Donner, D. B. 1980. Regulation of insulin binding to isolated hepatocytes: Correction for bound hormone fragments linearizes Scatchard plots. *Proc. Natl. Acad. Sci. USA*. **77**: 3176–3180.
  23. Caro, J. F., and J. M. Amatruda. 1980. Functional relationships between insulin binding, action, and degradation. *J. Biol. Chem.* **255**: 10052–10055.
  24. Taylor, S. I. 1975. Binding of hormones to receptors. An alternative explanation of non-linear Scatchard plots. *Biochemistry*. **14**: 2357–2361.
  25. Rodbard, D. 1979. Negative cooperativity: positive finding? *Am. J. Physiol.* **237**: E203–E205.
  26. Keefer, L. M., M. Michiels-Place, and P. De Meyts. 1980. A new method for the accurate determination of insulin receptor affinity and the concentration using a double-ligand technique (native insulin and a non-cooperative analogue). *Endocrinology*. **106**: 112. (Abstr. 151)
  27. Harmon, J. T., C. R. Kahn, E. S. Kempner, and W. Schlegel. 1980. Characterization of the insulin receptor in its membrane environment by radiation inactivation. *J. Biol. Chem.* **255**: 3412–3419.