BINDING OF INSULIN AND OTHER HORMONES TO NON-RECEPTOR MATERIALS: SATURABILITY, SPECIFICITY AND APPARENT "NEGATIVE COOPERATIVITY"

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SUMMARY. Studies of interactions of ^{125}I-insulin with non-tissue materials (talc, silica, protein-agarose derivatives, glass test tubes) are presented as examples of nonreceptor ("nonspecific") interactions which share, at least superficially, the criteria (saturability, specificity, high affinity and reversibility) commonly attributed to "specific" hormone-receptor interactions. Such "specific" nonreceptor interactions, which can potentially complicate interpretations of receptor binding studies, also exhibit characteristics analogous to those observed in liver membranes where the data have been interpreted (3) to indicate "negative cooperativity" between receptors. Similar data are presented here for insulin and epidermal growth factor binding to placenta membranes, and the effects of porcine insulin are compared to those of guinea pig insulin, a derivative which dimerizes very poorly. The "negative cooperativity" observed in the nonreceptor systems is most likely attributable to insulin-insulin interactions. Wherever ligand (e.g., hormone) self-association or isomerization occurs, the binding data (e.g. Scatchard plot) may yield nonlinear relationships which can falsely be interpreted as indicating cooperative interactions between receptors, or the presence of "additional groups" of binding sites.

There are by now numerous studies of the binding of a variety of hormones to putative tissue receptors (reviewed in refs. 1-2). The binding is generally believed to reflect a "specific" (i.e., receptor) interaction if it satisfies certain criteria such as chemical and tissue specificity, saturability, high affinity and reversibility. Although these are necessary criteria for receptor identification, it is often overlooked that nonreceptor ("nonspecific") interactions may also display certain of these properties. For example, it is now almost standard practice in receptor binding assays to assume as "specific" that portion of the binding which can be "displaced" (by competition) by very high concentrations of the unlabelled hormone, since this is indicative of a "saturable" process.

In the present report we describe examples in which the binding of insulin to substances in the absence of tissue can be shown to demonstrate saturability, reversibility, relatively high affinity and some specificity. The awareness that nonspecific, nonreceptor interactions can exhibit certain of the properties expected for receptors should prove cautionary. Furthermore, the availability of simple systems for measuring such nonspecific interactions should prove useful for testing certain properties of the binding which are observed in complex biological systems and which are believed to reflect special properties of receptors. For example, certain features of insulin binding to cell membranes which have been interpreted as "negatively cooperative" interactions between receptors (3) can be reproduced in the nonreceptor systems to be

described and might therefore be ascribed to phenomena which occur independently of tissue receptors.

METHODS. The preparation of ^{125}I-labelled insulin (porcine, 24 units/mg) has been described elsewhere (4, 5). Binding was measured by Millipore filtration procedures (4, 5) as described in the legends. Human placenta membranes (microsomes) were prepared from sucrose (0.25 M) homogenates as described earlier for rat liver (6). This membrane preparation was used in the present studies because the quantity of specific binding of insulin is far greater than that seen with other preparations such as rat liver membranes (7-9 and unpublished). Dodecadiamine was coupled to Sepharose 4B by the CNBr procedure (10). Human growth hormone (peptide 1-39) was a generous gift of Dr. M. Sonenberg and guinea pig insulin of Dr. C. C. Yip. Talc tablets (25 mg) were obtained from Gold Leaf Pharmacal Co., Inc. and microfine silica, QUSO G-32, from the Philadelphia Quartz Co.

RESULTS. Artifactual "specific" binding of insulin by agitation in glass tubes. When incubation media commonly used in receptor binding assays are shaken vigorously in glass test tubes (Becton-Dickinson 12 x 75 mm RTU culture tubes), very significant "specific" binding of ^{125}I-labelled insulin can be detected despite the absence of tissue material. Such binding, defined as "specific" on the basis of competition by native insulin, is not observed if plastic tubes are used. This effect is probably not explained by the binding of insulin in a saturable manner to particulate matter dislodged from the glass surface during the agitation process, since the effect is observed only if insulin is present during the agitation procedure and not if the binding assay is performed with incubation media which have been previously shaken (Table I). This artifactual system demonstrates relatively high affinity, since significant inhibition of binding can be observed with relatively low concentrations (10 ng/ml) of native insulin (Table I). Detailed examination of the binding as a function of increas

TABLE I. "Specific" Insulin Binding in the Absence of Tissue by Vigorous Mixing of Assay Mixture in Glass Tubes - Incubation media (0.2 ml) consisting of Krebs-Ringer-bicarbonate buffer containing 0.1% albumin (in 12 x 75 mm disposable glass tubes) were shaken vigorously for 50 min at 24° in the absence ("before") or presence ("during") of various concentrations of native insulin and 2.8 x 10^5 cpm ^{125}I-insulin (1.6 Ci/μmole). The samples were then assayed for binding ("during"), or native and ^{125}I-labelled insulin were added and binding was assayed after incubation (without shaking) for another 50 min period at 24° ("before"). Binding was determined as in Fig. 1. Essentially the same results were obtained using EH Millipore filters, and by performing incubations in Hank's-buffered saline, 0.1% albumin.

	^{125}I-insulin bound	
Addition	Shaking "before" assay	Shaking "during" assay
	cpm	
None	3,200	136,000
Insulin, 15 μg/ml	3,600	36,000
5 μg/ml	3,400	53,000
1 μg/ml	3,300	96,000
10 ng/ml	3,300	110,000

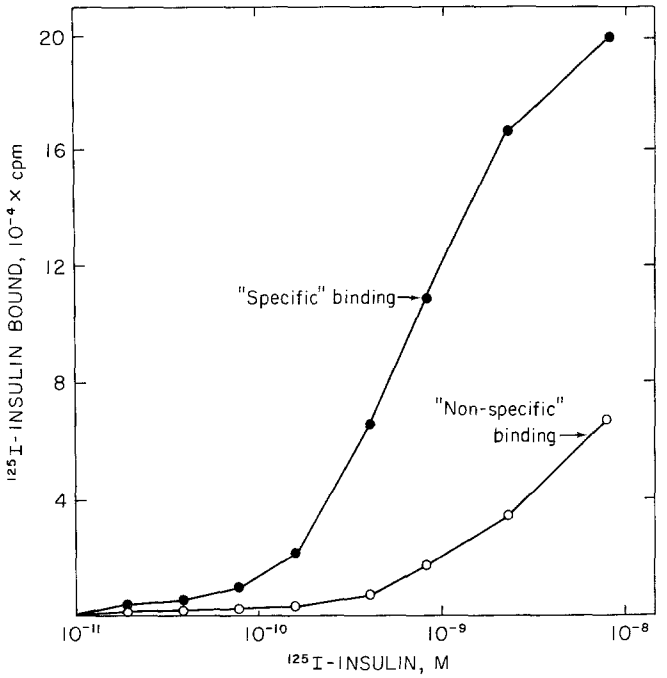

Fig. 1. Dependence on insulin concentration of "specific" binding of insulin during vigorously agitated assays in glass tubes in the absence of tissue. Incubations consisting of Hank's buffered saline containing 0.1% albumin (0.2 ml) were shaken vigorously for 50 min at 24° in the presence of varying concentrations of ^{125}I-labelled insulin (1.6 Ci per mmole) and in the presence and absence of native insulin (50 μg per ml). Binding was determined (4, 5) by filtration over EG cellulose acetate Millipore filters. "Specific" binding (●) was calculated by subtracting the binding observed in the presence of native insulin ("nonspecific" binding, 0) from the total binding (not shown) in the absence of native insulin.

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ing ^{125}I-insulin concentration demonstrates detectable "specific" binding at concentrations well below 10^{-10} M, and the "apparent" affinity is near 10^{-9} M (Fig. 1). The basis of this peculiar "binding" is not understood, although it may be related to special physical alterations or aggregation of the insulin molecules. Large, dense particulate structures are probably not involved since the ^{125}I-insulin trapped (bound) on the Millipore filters does not readily sediment (Beckman Microfuge, 10 min) through an oil mixture of dibutylphthalate (density, 1.043):dinonyl phthalate (density, 0.955), 3:1 (11).

"Specific" binding of insulin to talc. Binding of ^{125}-labelled insulin in a fashion that is at least partially inhibited by native insulin (i.e., "specific binding") can be demonstrated with a variety of particulate substances (i.e., talc, microfine silica, alumina powder, thyroglobulin-agarose and fetuin-agarose) provided sufficiently small quantities of these materials are present in the binding assay. Talc was chosen for more detailed study as a possible prototype for nonreceptor, "nonspecific" insulin

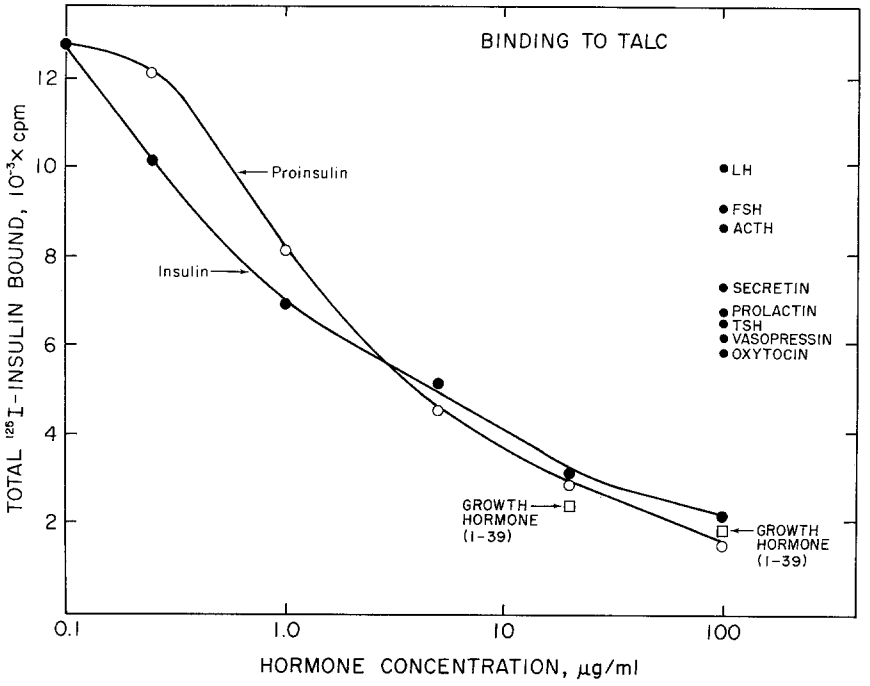

Fig. 2. Specificity of binding of insulin to talc. Samples (0.2 ml) containing 100 μg per ml talc were incubated at 24° for 30 min with 2.4 x 10^5 cpm ^{125}I-insulin (1.1 Ci per mmole) in the presence of various unlabelled hormones. The latter were added 5 min before addition of ^{125}I-insulin. Binding was determined (4, 5) by Millipore (EG, cellulose acetate) filtration.

interactions. Homogeneous, stable suspensions of talc can be prepared which are convenient to use and give highly reproducible results.

It is clear that native insulin competes for the binding of ^{125}I-insulin to talc (Fig. 2) in a way not too dissimilar to some previously described reports of insulin binding to plasma membrane preparations. Furthermore, as in plasma membrane systems (12-14), insulin inhibits more effectively than proinsulin, at least at low concentrations. A variety of other peptide hormones unrelated to insulin inhibit very poorly (Fig. 2). However, unlike the findings with biological receptors, growth hormone (peptide 1-39) competes very well for binding, and desoctapeptide insulin and reduced and carboxymethylated insulin (but not the separated chains) compete nearly as well as native insulin. Binding to talc is time and temperature dependent, requiring about 1 hour (24°) for achievement of near-steady state. Studies on the effect of increasing the concentration of ^{125}I-insulin on binding give very complex data suggestive of "positive cooperativity", and Scatchard plots (15) of these data are not readily interpretable.

Apparent "negative cooperativity" of insulin binding to talc and to cell membranes. The existence of negatively cooperative interactions between insulin receptors in live

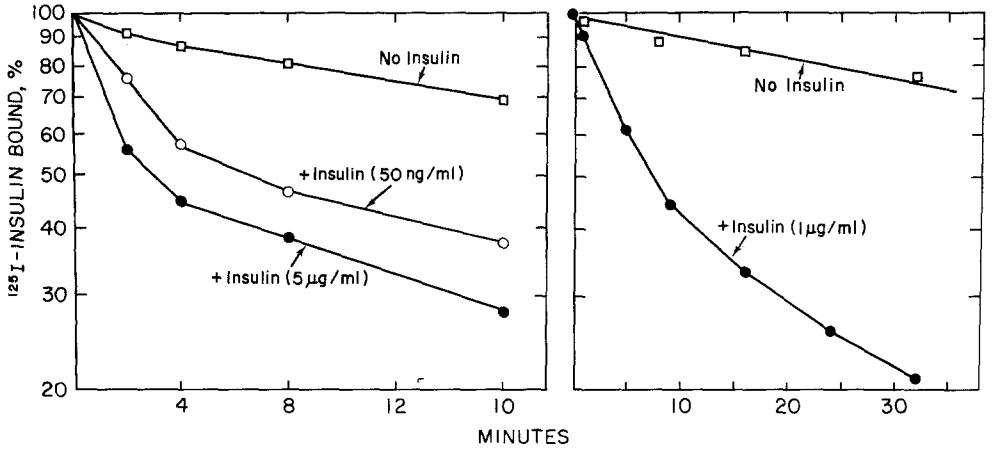

Fig. 3. Native insulin enhances the rate of dissociation of the ^{125}I-insulin-placenta membrane complex, as measured by two very different methods (left and right). Left: Nine ml of Krebs-Ringer-bicarbonate buffer containing 0.1% albumin and 2.5 mg of placenta membrane protein were incubated for 30 min at 24° with ^{125}I-insulin (8 x 10^4 cpm per ml, 1.4 Ci per mmole). The suspension was cooled in ice, centrifuged at 40,000 rpm, and the pellet was washed three times with ice-cold buffer. The pellet was suspended in 8 ml of ice-cold buffer and divided in 0.4 ml portions for assay. Native insulin (50 ng or 5 μg per ml) was added to some samples, and some were immediately (within 2 min) filtered on EG Millipore filters for determination of the zero time values. The samples were then placed in a 24° water bath and assayed for binding at various times. The zero time values (100%) correspond to about 22,000 cpm. Right: Spontaneous dissociation of ^{125}I-insulin in the absence of native insulin was determined after first collecting and washing the samples on filters (EGWP) with buffer at 4° and then percolating buffer at 24° for timed intervals (4). Results are corrected for nonspecific binding (1 μg per ml of native insulin added before ^{125}I-insulin). The 100% value corresponds to about 35,000 cpm.

membranes has been proposed (3) on the basis of the fact that the spontaneous rate of dissociation of the ^{125}I-insulin-membrane complex is accelerated by the addition of native insulin to the incubation medium. Using human placenta membranes, which bind much greater quantities of insulin and thus greatly facilitate accurate measurements, we have confirmed these observations using two entirely different assay procedures (Fig 3). The major effect of adding native insulin occurs very rapidly (first few minutes), depending on the concentration of native insulin which is used. On the basis of at least six experiments, the $t_{1/2}$ for dissociation in the absence of added insulin was about 50 to 60 min (at 24°). With native insulin, a rapid initial phase with a $t_{1/2}$ of 4 to 8 min is observed using 1 to 5 μg per ml of insulin; if the subsequent course of dissociation is corrected for this initial, rapid phase (assuming a $t_{1/2}$ of 4 to 9 min for the rapid phase), the remaining bound insulin dissociates with a $t_{1/2}$ of about 30 to 40 minutes. If very early times in the course of dissociation are examined, significant differences can be seen between various concentrations of insulin which are all supersaturating with respect to receptor binding (Fig. 3 and Table II).

TABLE II. Effect of insulin concentration on the extent of dissociation of the insulin-placenta membrane complex - The experiments were performed at 24° as described in Figure 3 (left).

Insulin concentration	^{125}I-insulin bound (%)			
	Assay at 24°		Assay at 4°	
	2 min	4 min	2 min	4 min
No insulin	100	100	100	100
Insulin, 10 ng/ml	90	83	98	97
40 ng/ml	84	73	93	85
0.2 μg/ml	77	70	87	80
1 μg/ml	72	70	81	76
5 μg/ml	67	68	73	72

Effects similar to those described above for the enhancement by native insulin of the dissociation of the insulin-placenta membrane complex can be reproduced with insulin talc complexes (Fig. 4). Although in this system the spontaneous rate of dissociation is slower ($t_{1/2}$ varies between 120 to 150 min at 37°), profound effects are observed with concentrations of native insulin comparable to those used with the placenta membranes. With ^{125}I-insulin-talc complexes, the major effect of native insulin also occurs very rapidly; with 5 to 50 μg of insulin per ml, the $t_{1/2}$ (37°) of the early phase varies between 10 to 20 minutes. The ability of relatively high concentrations (above 0.05 μg per ml) of native insulin to accelerate the spontaneous rate of dissociation has also been observed with other kinds of nonreceptor insulin complexes (e.g., binding to dodecadiamine-agarose).

The insulin-induced enhancement of dissociation of the ^{125}I-insulin-placenta membrane complex was studied further using guinea pig insulin since this derivative reportedly (16) does not dimerize. At sufficiently high concentrations both pork and guinea pig insulins can decrease membrane-bound ^{125}I-labelled pork insulin to the same extent. However, when the concentration dependence of both insulins is studied at early times (5 or 10 min) and in the range where only about 30% of the maximal effect is evident, it can be demonstrated that pork insulin is 60- to 100-times more effective than guinea pig insulin (six experiments). When the rate of dissociation of ^{125}I-labelled guinea pig insulin is studied, higher concentrations of native pork insulin are required to affect dissociation than when ^{125}I-pork insulin is used, the maximal effects are less pronounced, and pork insulin is only 10- to 20-times more effective than guinea pig insulin. These effects obtain even though the apparent affinity of guinea pig insulin for placenta membranes is between 6-fold (by ratio of rate constants or 20-fold (by competition curves) lower than that of pork insulin. These results are

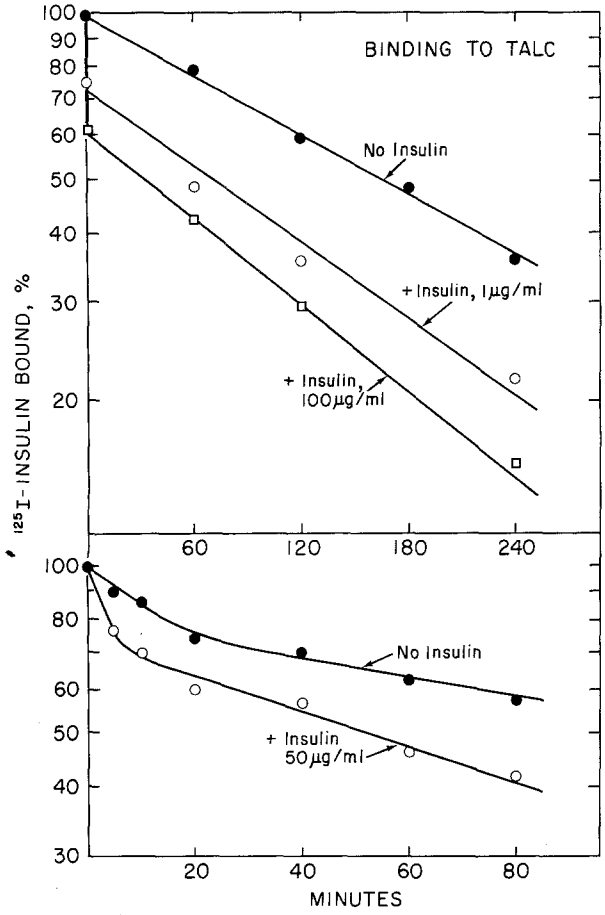

Fig. 4. Enhancement of dissociation of ^{125}I-labelled insulin-talc complex by native insulin. A suspension of talc (0.25 mg per ml) in Hank's buffer - 0.1% albumin was incubated for 40 min at 37° with ^{125}I-insulin (2.5 x 10^5 cpm per ml, 1.7 Ci per mmole). The suspension was cooled in ice and washed two times with ice-cold buffer to remove all the unbound ^{125}I-insulin. The talc was suspended (30 μg of talc per ml) in ice-cold buffer and native insulin was added to some samples. Samples without (100% value) and with insulin were filtered at once (EG Millipore filters) to determine zero time values. The suspensions were then incubated at 37° and samples were filtered periodically to determine insulin binding.

consistent with a 60- to 100-fold lower association constant for dimerization of guinea pig insulin, and with the view that this dimerization is even further compromised when the guinea pig insulin monomer is bound to the receptor.

Studies performed by procedures identical to those described in Fig. 3 (left) on the binding of ^{125}I-labelled glucagon to liver membranes (17) demonstrates no effect of a large excess of native glucagon (1 μg/ml) on the rate of dissociation of the membrane-^{125}I-glucagon complex. Epidermal growth factor (EGF) (18), however, can readily accelerate (at 1 μg/ml) the rate of dissociation of the ^{125}I-hormone-placenta membrane

complex in a manner similar to that described for insulin.[1] It is also possible to demonstrate that native EGF (2 μg/ml) can enhance dissociation of ^{125}I-EGF from talc at 4° and at 45°.

DISCUSSION. The present studies demonstrate that the basic properties (or criteria) of ligand binding by biological receptors can also be exhibited, at least in part, by nonreceptor interactions. Saturability by itself simply implies a finite or limited number of "acceptor" molecules, assuming that true steady state binding is achieved. Furthermore, it is not surprising that nonspecific adsorptive interactions should exhibit some degree of chemical or steric specificity, especially under conditions which display very high affinity and must therefore involve the simultaneous formation of several bonds to achieve the necessary energies of interaction. Other examples of nonreceptor interactions exist which at least superficially resemble those expected for true biological receptors: ^{125}I-glucagon can bind "specifically" to certain Millipore filters (19), ^{3}H-naloxone binds stereospecifically to glass filters (20), and opiate drugs bind to cerebroside sulfate stereospecifically and with a relative affinity reflecting potencies observed *in vivo* (21). Numerous "foreign" drugs interact highly specifically with naturally occurring macromolecules (22).

It is, however, important to emphasize that bringing attention to the above data is not intended to discourage or mitigate the use of binding methodology to identify and study true biological receptors. In the cases described above, as well as others that we have studied, the properties of the nonreceptor systems when studied in sufficient detail can be clearly and readily distinguished from those of true receptors. A number of receptor interactions have now been studied which satisfy relatively convincingly the necessary criteria for true or biologically significant receptors (reviewed in ref. 1). However, since biological tissues will surely also exhibit some of the "specific" types of interactions described here for simple nonreceptor systems, awareness of such interactions should encourage greater scrutiny, particularly for those interactions observed with high concentrations of hormones, where the results are frequently interpreted to indicate "second" or "multiple" classes of receptors.

The results described in this report indicate that previously presented data (3) taken as evidence for "negative cooperativity" between insulin receptors may possibly be interpreted differently, since similarly "negatively cooperative" effects are also observed in nonreceptor systems (Figs. 4 and 5). Furthermore, since in these nonreceptor systems (talc, dodecadiamine-agarose) there is no possibility for interactions between binding sites, the observed effects are most likely the result of interactions

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1. Previous measurements (18) of the rate (about 6×10^{-4} sec^{-1}) of EGF-receptor dissociation (k_{-1}) were performed in the presence of native EGF. The k_{-1} value measured (at 24°) in the absence of EGF is about 7×10^{-5} sec^{-1}. The dissociation constant, estimated on the basis of rate constants (k_{-1}/k_1), should therefore be closer to 10^{-10} M rather than 6×10^{-10} M, as reported previously (18).

between the insulin molecules. It is well known that insulin forms dimers and higher aggregates (23-27). The dissociation constant for dimer formation (at neutral pH) has been reported to be 7×10^{-7} M (26). The ability of native nerve growth factor (NGF) to enhance the rate of dissociation of the ^{125}I-NGF-membrane complex, which has been interpreted as evidence for "negative cooperativity" (28), may also be a consequence of hormone self-aggregation, as suggested for insulin, especially since it is known[2] that NGF tends to aggregate at neutral pH.

Dimer formation can account for the enhanced "dissociation" of the labelled, receptor-bound hormone, provided: (1) that receptor-bound monomer can still participate in dimerization and (2) that dimerization alters the ligand conformation such that its affinity for the receptor is reduced. Even with relatively low stability constants of dimerization, very low concentrations of the unlabelled ligand can promote rapid "exchange" or "dissociation" of the ^{125}I-labelled receptor-bound material, since the rates of hormone diffusion and dimer formation are extremely fast. Thus, provided the total quantity of unlabelled ligand in the medium greatly exceeds that of the labelled, extensive and rapid exchange can occur even when the proportion of the total ligand present as dimer is negligibly small.

Although it had been predicted (16, 29) that guinea pig insulin would dimerize on the basis that all of the residues of the hydrophobic core of porcine insulin dimer are retained, recent physical studies (16) have failed to demonstrate dimerization. The present studies, however, would indicate that dimerization of guinea pig insulin may occur with an association constant 60- to 100-fold lower than for porcine insulin. Such weak associations may not be readily detected by ultraviolet spectroscopy or sedimentation equilibrium at low concentrations of the hormone. For example, if the dimerization constant for guinea pig insulin is about 80-fold lower (i.e., about 10^4 M^{-1}), less than 10% will be in the dimer state at concentrations (16) of 0.3 mg/ml. Similarly, although self-aggregation has not been described for epidermal growth factor, the present study suggests that n-merization may be detected by physical methods if high concentrations of peptide are used.

Self-aggregation[3] of the kind suggested here will also introduce curvatures in Scatchard plots (15) of binding data, especially if data are obtained from competition-displacement curves measured over a wide range of native hormone concentration. Self-aggregation or isomerization has been well documented for several kinds of ligands, including proteins (31), nucleosides (32), chlorophylls (33), pyrimidines (34), purines (35), cholesterol (36) and organic dyes (37). The analysis of competition-displacement

2. E. Shooter, manuscript in preparation.

3. The possible effect of ligand self-aggregation in the production of sigmoidal binding curves and nonlinear Scatchard plots, as discussed by Nichol et al. in 1969 (30), has seldom been considered in the analysis of hormone-receptor binding studies.

curves for the binding of such molecules to biological membranes will be complicated by self-aggregation phenomena. It is notable that in several studies (3, 38-43) where Scatchard analysis and kinetic interpretations have been made for competition-displacement curves using unlabelled insulin, the plots have been nonlinear and suggestive of additional "low affinity, high capacity" binding sites. Such data could be, at least in part, a consequence of the aggregation effects discussed here.

The presence of ligand-ligand interactions may thus necessitate a re-examination, not only of dissociation rate constants estimated in the presence of high concentrations of unlabelled ligand[1], but also of equilibrium estimates of affinity constants obtained by competition-displacement measurements. For example, it may be pertinent that the insulin-receptor dissociation constant estimated from measurements of rate data ($K_D = k_{-1}/k_1$) obtained in the absence of unlabelled insulin is lower (about 10^{-10} M; 4, 5, 17, 44)[4] than that estimated (K_D about 10^{-9} M; 8, 13, 14, 38-43) from native insulin competition curves. Several independent approaches to the measurement of hormone-receptor interactions may be necessary to rule out contributions from artifactual nonreceptor interactions or ligand polymerization.

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4. In the instance studied here, the ^{125}I-insulin-placenta membrane interaction, k_{-1} at 24° is about 2 x 10^{-4} sec^{-1}. Using k_1 values for these membranes (about 4 x 10^6 $mole^{-1}$ sec^{-1}), or values reported (4, 5, 17, 44) for various other tissues (range about 3 to 9 x 10^6 $mole^{-1}$ sec^{-1}), the apparent K_D ranges from 2 to 6 x 10^{-11} M.

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