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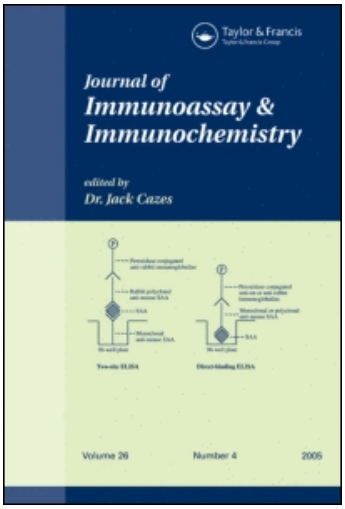

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Harald Juppner[a]; Rolf-Dieter Hesch[a]
[a] Dept. Innere Medizin Medizinische Hoohschule Hannover, Abt. fur Klinische Endokrinologie, Hannover

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PARATHORMONE RECEPTOR BINDING AND THE INFLUENCE OF MEMBRANE DEGRADATION OF THE HORMONE

Harald Juppner and Rolf-Dieter Hesch
Abt. fur Klinische Endokrinologie, Dept. Innere Medizin
Medizinische Hochschule Hannover, 3 Hannover 61, GFR

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ABSTRACT

Concentration of receptor bound parathormone was determined using a labelled-antibody-membrane-assay (LAMA). 4.2-31.2 fmol PTH per mg membrane protein (0.42-3.12 fmol/tube) were bound at apparent PTH concentrations of 15.6-500 fmol/tube. Only 0.65%-3.2% of PTH could be detected at the receptor sites. The amount of free hormone was corrected for inactivation of intact immunologically reactive PTH and of intact biologically reactive PTH. Only after the latter correction were values for binding affinity (Kd ◻ 1.1×10^{-12} mol/mg) and number of binding sites (B_{max} = 5.8×10^{-14} mol/mg) obtainable. These results indicate that allowance must be made for loss of biological activity of hormone at membrane receptor sites if binding parameters are to be defined free of artifacts. Binding data may otherwise be misinterpreted as indicating negative cooperativity, heterogeneity of binding sites or multi-step reactions.

INTRODUCTION

Most receptor assays for peptide hormones are based upon the principle of 'competitive-ligand-assay' (1,2). Binding of labelled hormone and competition by nonradioactive hormone at the receptor site is observed. It is generally assumed that data from such experiments allows one to quantify receptor occupancy, binding capacity, affinity and the receptor concentration (3). Conflicting interpretations of the

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physicochemical characteristics of the underlying reactions have been made because two prerequisites of current receptor assays are subject to possible criticism. First: labelled hormone should maintain its biological activity. Several procedures have been used to incorporate ^{125}I into the molecule but it has not always been excluded that unlabelled peptide populations may determine the resultant biological activity. It has been demonstrated that ^{125}I insulin prepared by conventional methods exhibits a loss of biological activity (4). This is also true for glucagon, FSH and especially for parathormone (5), where the N-terminal loop is destroyed during the oxidative labelling procedure (6). Even incorporation of ^{3}H into the PTH-molecule has not completely overcome this problem (7). Surprisingly, some hormones seem to exhibit higher biological activity when radioactively labelled (8). For gastrin, activity identical to native hormone after incorporation of ^{125}I has recently been claimed (9). Labelled hormones may undergo dramatic conformational changes and these have been demonstrated recently for FSH (10). This is important because such changes must be expected to influence the binding kinetics at the receptor site. Secondly: Inactivation of the ligand may occur during incubation with the receptor because most hormonal receptors are associated with a hormone inactivating site. Several chemical or immunological methods have been used to correct for this, but they do not detect changes in biological activity of the ligand (11).

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The measurement of receptor site occupation by unlabelled, structually unaltered hormone is described here using a modification of the labelled antibody technique (LAMA) (12,13) to determine receptor bound hormone and to measure hormone inactivation during the assay incubation. The resulting data is used to define receptor-ligand binding constants.

Experimental Design

1. Immunoadsorbed bPTH (ImAd-bPTH) linked at the N-terminus was assayed in a C-regional RIA using bPTH as standard. The ImAd-bPTH showed parallelism and was assigned an assay value in terms of C-regional standard.
2. bPTH was reacted with membranes for 2 hrs and the bound bPTH was assayed with labelled C-regional antibody (*Ab) using the ImAd-bPTH as standard.
3. The bPTH not bound after incubation with membranes for 2 hrs was assayed using fresh membranes to derive remaining biological activity. The same unbound component was assayed for immunologically reactive hormone by a immunoradiometric assay using polyethylene tubes to adsorb intact bPTH, followed by labelled C-regional antibody, with bPTH 1-84 as standard.

MATERIALS AND METHODS

The labelled-antibody-membrane-assay (LAMA) was performed as previously described (13) using chick renal plasma membranes (100 µg/tube) prepared by the technique of Fitzpatrick et al (14). Immunoadsorbent (ImAd) was prepared according to Addison

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and Hales (15) from Ultro-Gel AcA 54 (LKB, France). Purified bovine PTH was kindly provided by the Hormon-Chemie, Munchen, GFR. C-regional PTH was determined by radioimmunoassay (16) using antibody S478 X (final dilution 1:5000). To separate bound and free hormone a precipitating second antibody (Wellcome: AS 796 - donkey anti-sheep/ goat) was used in a final concentration of 1:500. The immunoradiometric assay for 1-84 bPTH was performed as described (17). For all assays we used the international bPTH standard 71/324, kindly provided by the National Institute for Biological Standards and Control (NBSB), London. All other reagents were of the highest purity available.

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Concentration of ImAd-bound-bPTH

Bovine parathormone with the N-terminus covalently bound to activated amino cellulose (ImAd) was assayed at six dilutions (1:200 - 1:6400) using a C-regional specific RIA (16). ImAd dilution ran parallel with the standard curve. 100 µl of this ImAd solution contained 10 pMoles bPTH (71/324).

Absolute Receptor Bound Hormone

For the assay of hormone bound to the receptor, a LAMA standard curve was set up using chick renal plasma membranes (100 µg/tube). Hormone concentrations ranged from 15.6 to 500.0 fmol/tube. Non-specific binding of PTH to the plastic tube was measured using the same standard concentrations incubated

without the addition of membranes. The first incubation was carried out for 2 hrs at 22°C. After washing the membrane pellet once, the labelled antibody was added and incubated for one hour. In a simultaneous assay, dilutions of ImAd (1:25600 - 1:1600) representing 0.39 - 6.25 fmol bPTH/tube were incubated with the same labelled antibody. After washing twice the membrane pellet and the ImAd-pellet and cutting off the tips of the tubes, the samples were counted in an automatic gamma counter (Berthold, Wildbad, GFR). Each sample measurement was corrected for non-specific binding of the hormone to the plastic tubes at the respective hormone concentrations (not presented). Radioactivity of the labelled antibody increased with increasing amounts of hormone bound to the receptor sites on the membranes. Thus the amount of radioactivity is directly related to the amount of receptor bound hormone (PTH_r). Radioactivity also increased when the labelled antibody was incubated with increasing amounts of ImAd. Since the solid-phase bound PTH concentration had already been determined, the respective amount of radioactivity refers to a known concentration of the hormone. By comparison of the receptor-bound radioactivity with the ImAd bound radioactivity, the hormone concentration in the membrane pellet could be read off the ImAd standard curve (Fig. 1). The two reactions compared are as follows:

i) $PTH_a + R \rightleftharpoons PTH_r$; wash; $PTH_r + *Ab \rightleftharpoons PTH_r*Ab$; wash; count PTH_r*Ab

ii) ImAd-PTH + *Ab $\rightleftharpoons$ ImAd-PTH*Ab; wash; count ImAd-PTH*Ab

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(H=hormone; R=receptor; Ab=Antibody; PTH_a=initial parathormone;
*Ab=labelled antibody; PTH_r=receptor bound parathormone)

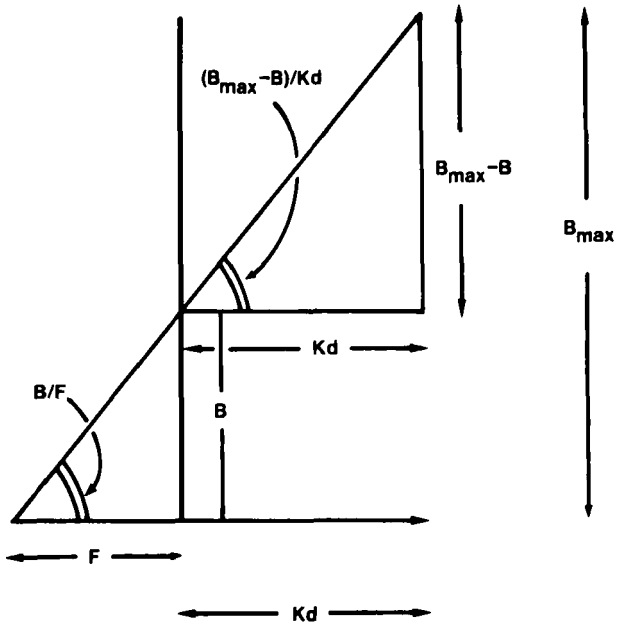

Figure 1.

Determination of receptor bound Parathormone. Increasing amounts of Parathormone were incubated with chick renal plasma membranes. PTH bound to the receptor sites was determined by a radiolabelled antibody directed against the C-terminal part of the hormone. Simultaneously, the ^{125}I antibody was incubated with a solid phase PTH (ImAd-PTH) with a previously determined hormone concentration. The concentration of PTH bound to the membranes was directy read off the ImAd standard curve.

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Determination of Hormone Inactivation

After incubation we measured the concentration of immunologically intact 1-84 PTH (PTH_i) and of biologically active 1-84 PTH (bioPTH) (biological active hormone in this context is hormone which is able to bind to membranes). Native hormone (1.56 - 50.0 ng/ml) was incubated with membranes for 2 hrs at 22°C. After centrifugation at 4°C the supernatants were aspirated and assayed for 1-84 PTH_i in a two-site sandwich assay (17) and for 1-84 bioPTH in a second LAMA. Resulting standard curves were compared to those for PTH without prior membrane exposure. Data obtained for receptor bound hormone (PTH_r) were subtracted from initial amount of PTH (PTH_a) in the incubation milieu to obtain free hormone (F_1) concentrations. The following formulas were used for calculation of the free hormone concentration:

i) without correction for inactivation:

$$F_1 = PTH_a - PTH_r \quad \text{(immunological inactivation)}$$

ii) with correction for immunological inactivation:

$$F_2 = PTH_i \quad \text{(biological inactivation)}$$

iii) with correction for biological inactivation:

$$F_3 = \text{bioPTH}$$

Mathematical Analysis

For the analysis of raw data from binding and inactivation studies we used the geometric construction described by Eisenthal and Cornish-Bowden (18). For ligand-binder

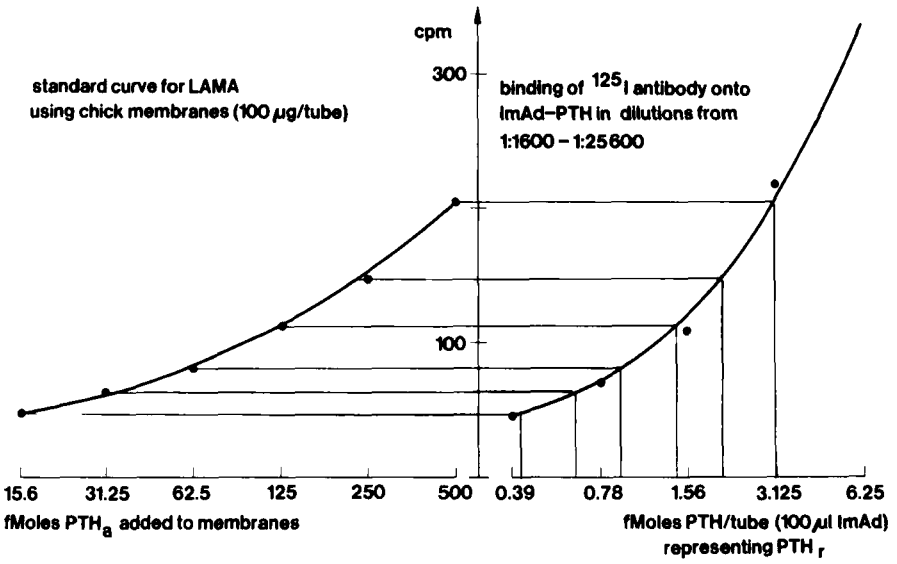

Figure 2.

The mass action relationship $B/F = (B_{max} - B)/Kd$ (B ▫ bound ligand concentration; F = free ligand concentration; B_{max} = maximal receptor binding capacity; Kd = binding affinity) is used to generate two similar right angle triangles; base F and height B, base Kd and height $(B_{max} - B)$ respectively. The first is constructed for each pair of data points. Extrapolation of each hypotenuse will yield a common intersection which defines the second triangle and hence B_{max} and Kd.

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interaction involving a homogeneous single species of binding sites, right triangles with base F, height B and a common right angle will have hypotenuses which intersect at a point which defines K_d and B_{max} (Fig. 2).

RESULTS

The concentration of receptor bound PTH (PTH_r) ranged from 4.2 - 31.2 fmol/mg membrane protein (Fig. 3). With concentrations of PTH_a added to the membranes increasing from 15.6 to 500 fmol/tube, the percentage of PTH bound to the receptor-sites decreased from 3.2 - 0.65%. As measured in the two-site assay only 42 - 50% of PTH_a at different concentrations retained immunoreactivity after 2 hrs of incubation with membranes. The concentration of remaining biologically active PTH (bioPTH) as determined in a second receptor-assay (LAMA) was also reduced to 34% - 54% (Fig. 3). All data for PTH_r and free hormone concentrations, with and without correction for inactivation, are summarized in Table 1. Figure 4i illustrates that plotting PTH_r against F_1 yielded a variety of possible values for Kd ranging from 4.0×10^{-14} to $45,0 \times 10^{-14}$ mol/100 µg, and for B_{max} from 1.7×10^{-15} to 6.3×10^{-15} mol/100 µg membrane protein. This range of values could be diminished by correcting for immunological inactivation (Fig. 4ii); but only after correcting F_1 for biological inactivation (Fig. 4iii) was one single intersection point achieved. Values of 11.2×10^{-14} mol/100 µg for Kd and $5.8 \times$

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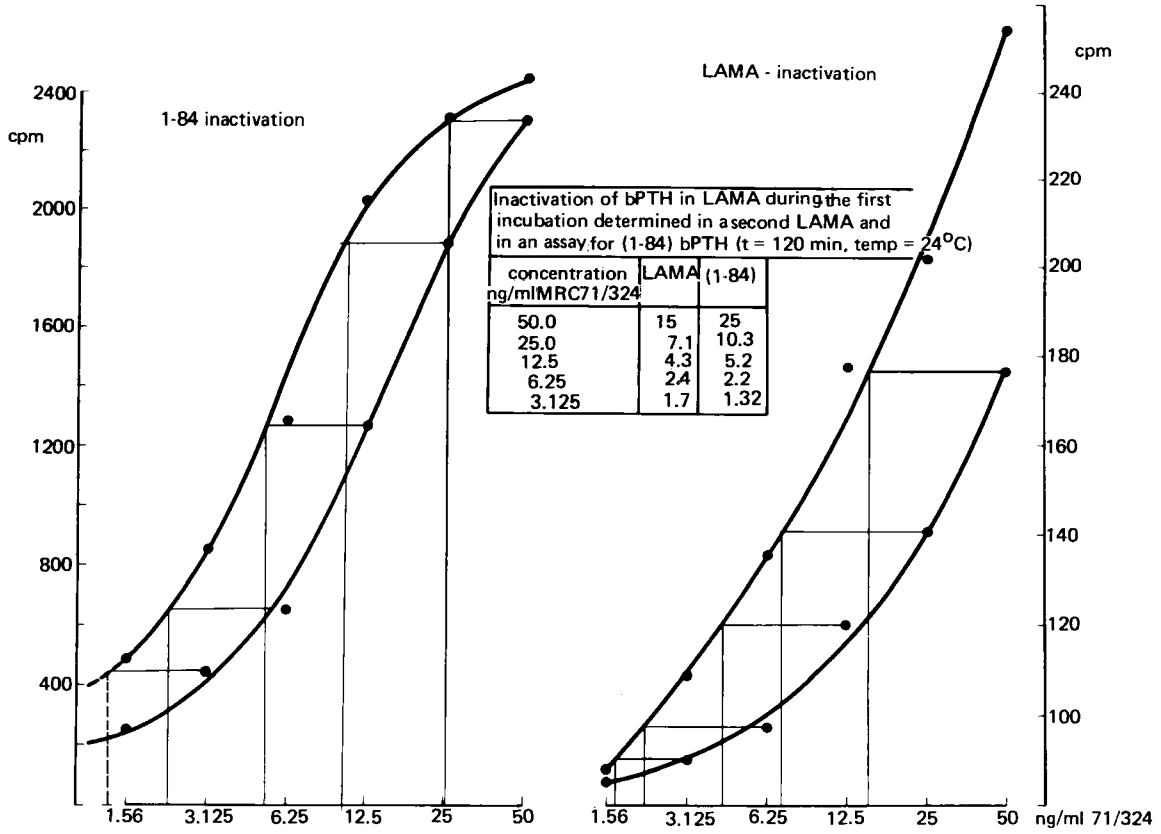

Figure 3: Inactivation of PTH_i and bioPTH. Parathormone (1.56 - 50.0 ng/ml) was incubated with chick renal plasma membranes for 2 hrs at 22°C. After centrifugation PTH was determined and compared to PTH without prior membrane exposure: i) in a two-site assay for PTH, detecting immunological intact parathormone (PTH_i) and ii) in a second receptor assay (LAMA) detecting biologically intact PTH (bioPTH).

TABLE 1

B (fMoles/tube)	F_1	F_2 (fMoles/tube)	F_3
3.33	500.0	250.0	150.0
2.27	250.0	103.0	71.0
1.64	125.0	52.0	43.0
1.07	62.5	22.0	24.0
0.78	31.3	13.2	17.0
0.49	15.6	n.d.	n.d.

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10^{-15} mol/100 µg membrane protein for B_{max} resulted. Other graphical designs were ambiguous in their interpretation, if the free hormone concentrations were not corrected for inactivation. For example, without accounting for hormone inactivation, the Scatchard plot seemed to indicate heterogeneity of binding sites, negative cooperativity or a two-step reaction.

DISCUSSION

We have determined the amount of hormone bound to plasma membranes using non-labelled native PTH for binding to the receptor sites and a radiolabelled antibody directed against the C-region of the hormone. This method avoids the use of ^{125}I-labelled hormone and may more appropriately reflect the

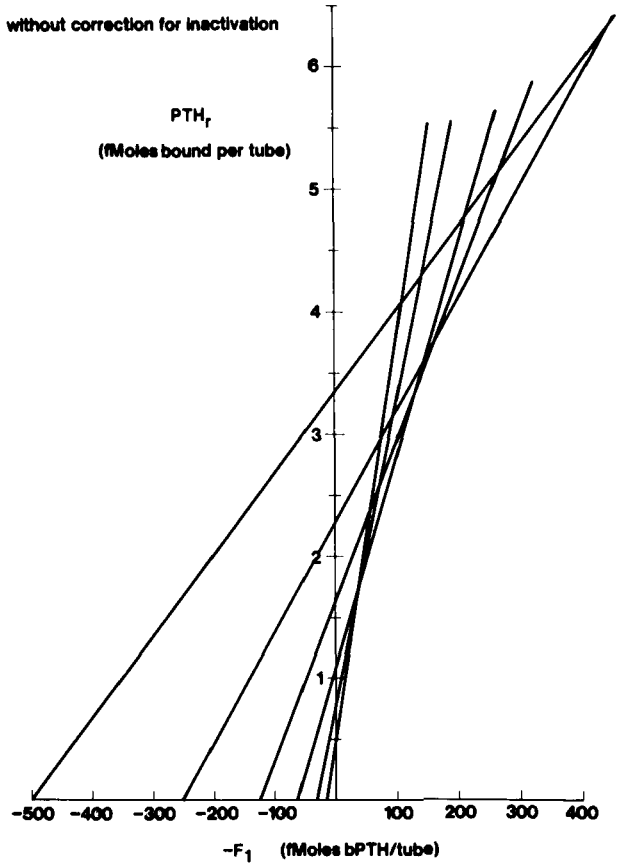

Figure 4. i

physiological circumstances of N-regional hormone-receptor interaction, since labelled PTH is probably conformationally altered and exhibits diminished biological activity (19). Parallelism of ImAd-PTH with receptor bound PTH suggests that the conformation of the hormone is not altered after binding of the C-regional antibody. This phenomenon is regularly observed and also reproduced using lymphocytes as receptor targets (20).

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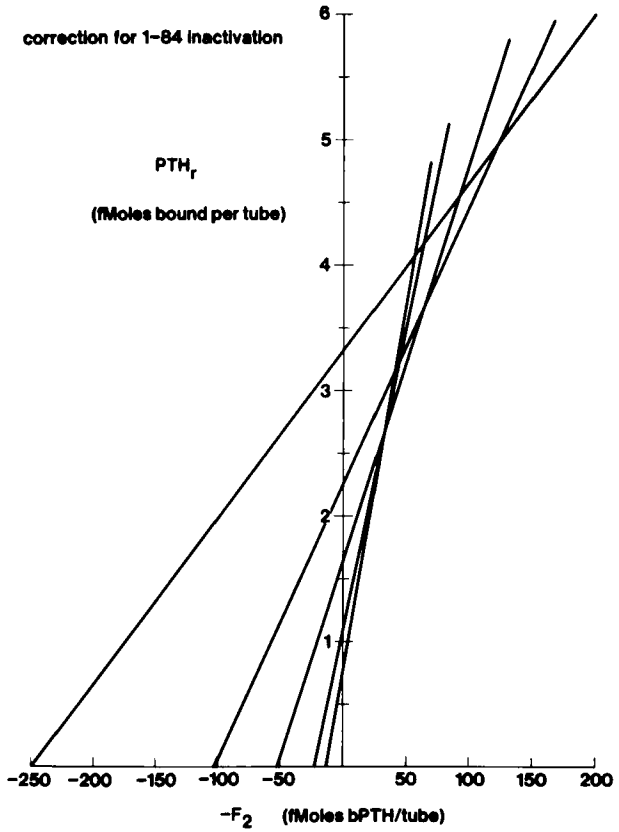

Figure 4. ii

It should, however, be mentioned that such parallelism is only achieved after correction of PTH inactivation by the receptor protein. For definition of binding data it was also essential to allow for the possibility of hormonal degradation during membrane exposure. The degradation involves a loss of biological reactivity followed by a loss of immunological reactivity as measured by the two-site assay, which detects

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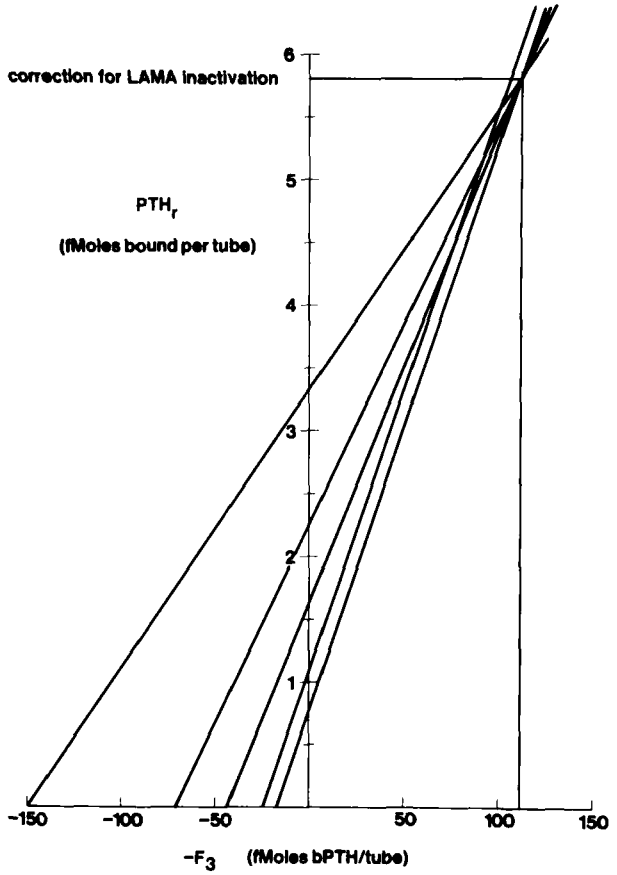

Figure 4. iii

Figure 4:

Calculation of binding affinity and capacity.

i) without correcting the apparent free concentration (F_1) for inactivation during membrane exposure a variety of possible values for B_{max} and Kd was observed.

ii) after correction of free for immunological inactivation (F_2) this widespread range could be diminished.

iii) after correction of free for biological inactivation (F_3) a single intersection results yielding a unique value for binding affinity and capacity.

almost intact PTH, bearing the N- and C-regional regions of the peptide. Synthetic peptide fragments (1-34, 24-48, 44-68, 53-84) do not react in this assay unless present in a molar excess of 1000:1 (17).

The PTH-binding site seems to be located near an inactivating enzyme which is separate from the PTH binding site and the PTH-specific adenylate cyclase. We believe, therefore, that the PTH-"receptor" is composed of three subunits whose physiological interaction in the membrane is not yet fully understood (21). The mathematical analysis of the PTH-receptor interactions suggests that allowance must be made for loss of biological reactivity in deriving the free hormone concentration. Binding data otherwise may be misinterpretated as showing either negative cooperativity, a two-step reaction or heterogeneity of binding sites. Such features are already described for several hormones (22,23).

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The values for the affinity constant (Kd) and the receptor binding capacity (B_{max}), calculated as being 1.1×10^{-12} mol/mg and 5.8×10^{-14} mol/mg, respectively, are in the range of reported concentrations of circulating biological active parathormone. Whether hormonal N- and C-regional PTH fragments generated during membrane exposure have any biological function is still open to discussion. Preliminary results suggest that synthetic PTH fragments may cause either desensitization or "oversensitization" of the receptor (24). This would indicate

a self-regulatory capacity located in the subunits of membrane receptors.

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