LABELLED ANTIBODY MEMBRANE ASSAY FOR PARATHYROID HORMONE A NEW APPROACH TO THE MEASUREMENT OF RECEPTOR BOUND HORMONE

C.H.S. M^{c}Intosh, and R.-D. Hesch
Department of Medicine
Endocrinology Section
University of Göttingen
Germany

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SUMMARY: A new method is described for the measurement of hormone bound to membrane receptors. Antibodies specific for the C-terminal and N-terminal regions of parathyroid hormone were labelled with ^{125}I and incubated with renal membranes which had been previously incubated with unlabelled hormone. The uptake of hormone demonstrated pH and time dependence and was a saturable process. Treatment of the membranes with acid or heating to 100^{o}C, or inactivation of the hormone with hydrogen peroxide, completely abolished detectable hormone uptake to the membranes.

INTRODUCTION: The action of polypeptide hormones is mediated by interaction with specific cell membrane receptors and this has enabled the development of a number of radio-receptor assays with high sensitivity and specificity (1-4). A suitable receptor preparation and labelled hormone of high specific radioactivity are required for these studies, and sophisticated methods for the production of biologically active, monoiodinated hormone have been developed (5-7). However, for some hormones it has been difficult to retain biological activity during iodination (8-9) and it is probable that during the chemical process of iodination some form of conformational change will be induced even under relatively mild conditions. We have therefore devised a new assay system in which the receptor bound hormone is detected by labelled antibodies, thus avoiding any deleterious effects that labelling may exert on the hormone. The system has been applied to the binding of bovine parathyroid hormone, human parathyroid hormone and the synthetic human 1-34 fragment to bovine, porcine and chick renal membranes.

MATERIALS AND METHODS: Parathyroid hormone standard preparations - Bovine parathyroid hormone (71/324) and human parathyroid hormone (approximately 4% pure) were generously provided by the MRC (Mill Hill, London) and Prof. J.L.M. O'Riordan (Middlesex Hospital Medical School, London) respectively. Synthetic human 1-34 parathyroid hormone (Lot No. 14014) was obtained from Beckman, Palo Alto.
Antibodies - Anti-BPTH (AS 262) and anti-BPTH cross reacting with human PTH (AS 201), both reacting primarily with the C-terminal region of PTH, were obtained from the Medical Research Council (Mill Hill, London). Antibody A-VII was kindly supplied by Dr. R. Bouillon, Rega Institut, Leuven, Belgium. Anti-Synthetic 1-34 HPTH was a generous gift of Prof. F.H. Dietrich, CIBA-Geigy, Basle and Dr. J. Fischer, Orthop. Klinik, Balgrist, Zurich.
The preparation of immunoadsorbent of BPTH (Wilson Laboratories, Lot No. 15045) and 1-34 HPTH was carried out by the procedure of Miles and Hales (10). After extraction of the antibody on the respective immunoadsorbent, iodination was performed by the method of Hunter and Greenwood (11) and further purification of ^{125}I-labelled antibodies by the technique of Addison et al (12). Partially purified plasma membranes were prepared from renal cortex by the method of Fitzpatrick et al (13). The biological potency of standard parathyroid hormone preparations was checked by measuring the stimulation of adenyl cyclase activity of porcine plasma membranes using the method of Albano et al (14). Further experimental details are given in the texts or legends.

RESULTS AND DISCUSSION: An outline of the protocol for the labelled antibody membrane assay (LAMA) is given in Fig. 1. Parathyroid hormone (80 - 10,000 pg per tube) was incubated in 50 mM Tris HCl buffer (pH 7.4) + 2% bovine serum albumin with renal plasma membranes (100 μg/tube) in a total volume of 200 μl for 2 h. at 24^{o}C. The membranes were rapidly sedimented by centrifugation (16,000 g 20 sec), washed once by resuspension in incubation buffer and resuspended in 100 μl labelled antibody (3 - 50,000 cpm) in NIRG buffer (Veronal buffer pH 8.0, 0.05 M containing bovine serum albumin (1 mg/ml), non-immune bovine immunoglobulin (50 μg/ml), 0.9% NaCl, and NaN_3 (0.1 mg/ml)). After a further incubation period of 1 h. the membranes were sedimented, washed once with NIRG buffer, and bound radioactivity counted in an automatic gamma counter. Radioactivity due to non-specific binding of antibody to membranes and to tubes incubated in the absence of membranes, was subtracted from all reported binding data. These standard conditions were determined from a number of experiments on the characteristics

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Abbreviations: BPTH, Bovine Parathyroid Hormone; HPTH, Human Parathyroid Hormone.

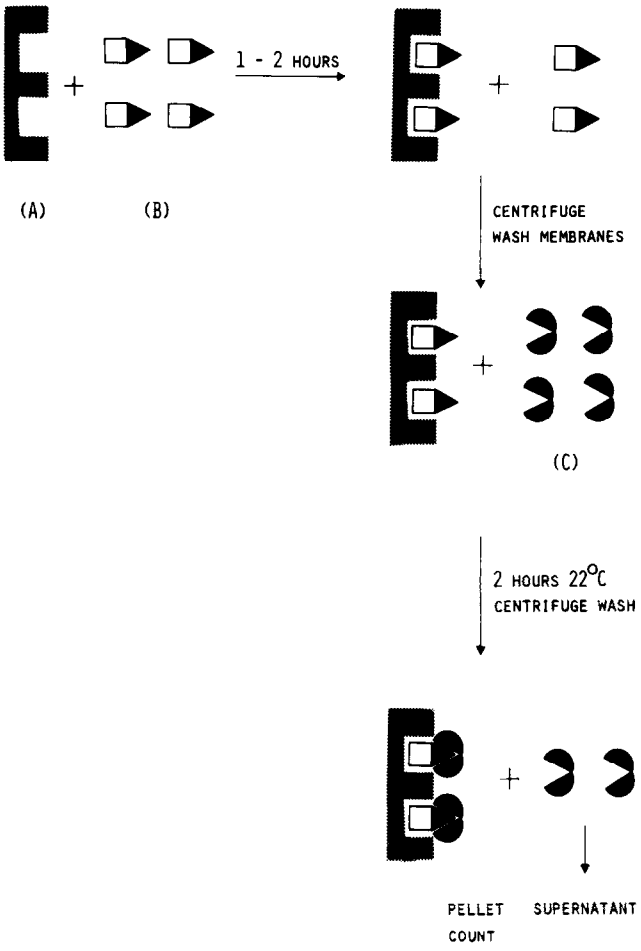

Fig. 1. General Scheme of the Labelled Antibody Membrane Assay.
(A) Membrane Receptor Preparation
(B) Hormone
(C) Labelled Antibody

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of labelled antibody binding to membranes following incubation with hormone.

Using antibody AS 262 and BPTH (10 ng/tube) maximum binding was observed with 100 - 150 /ug membrane protein (Fig. 2). The initial incubation of PTH with membranes exhibited a broad pH dependence between pH 7.0 and 8.0, with a maximum at pH 7.4. Below pH 6.0 binding was reduced to less than 30% of maximum but above pH 9.0 significant binding still existed.

The effect of time and temperature on the hormone binding

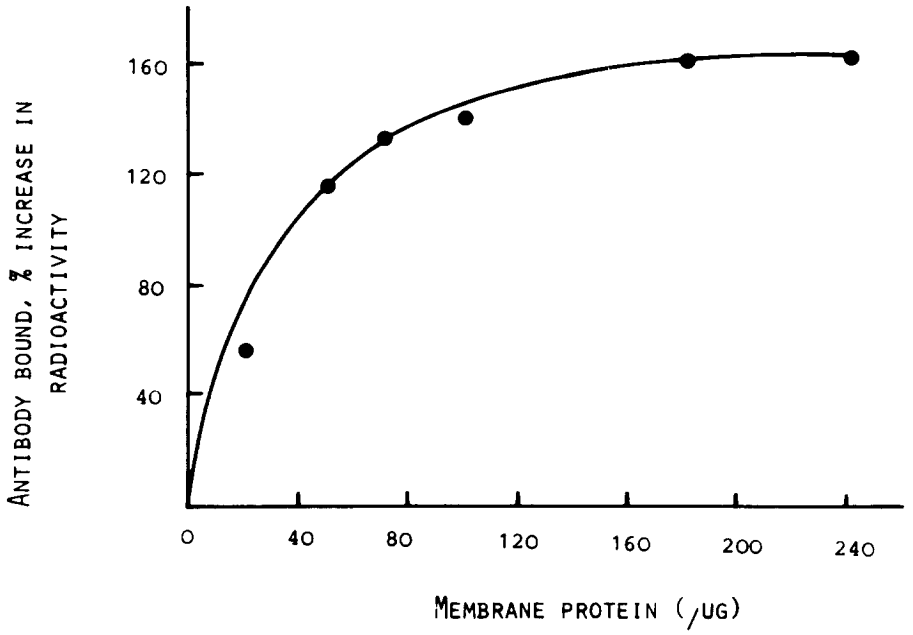

Fig. 2. Effect of increasing membrane concentration on binding of labelled antibody (AS 262) to bovine membranes after incubation with bovine PTH (10 ng).

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were investigated by keeping the time and temperature of antibody incubation constant and varying the conditions for the hormone-membrane interaction (Fig. 3). Increasing the temperature of incubation increased the binding; however, on prolonged incubation a decrease in bound hormone occurred. The rate of reaction of antibody with the membrane-hormone complex (after an initial 1 hour incubation at 24°C) also showed a decrease after 60 minutes (Fig. 4). The decreased binding observed on prolonged incubation with antibody and with increasing temperature for the first incubation may be a complex system involving dissociation of bound hormone by antibody coupled with endogenous dissociation / inactivation phenomena.

The binding curves for BPTH (Fig. 5) indicate that with the present conditions as little as 80 pg/tube produced a significant increase in the amount of antibody bound to the membranes. Almost identical binding curves were obtained with antibody A-VII. HPTH also bound to bovine membranes and could be detec-

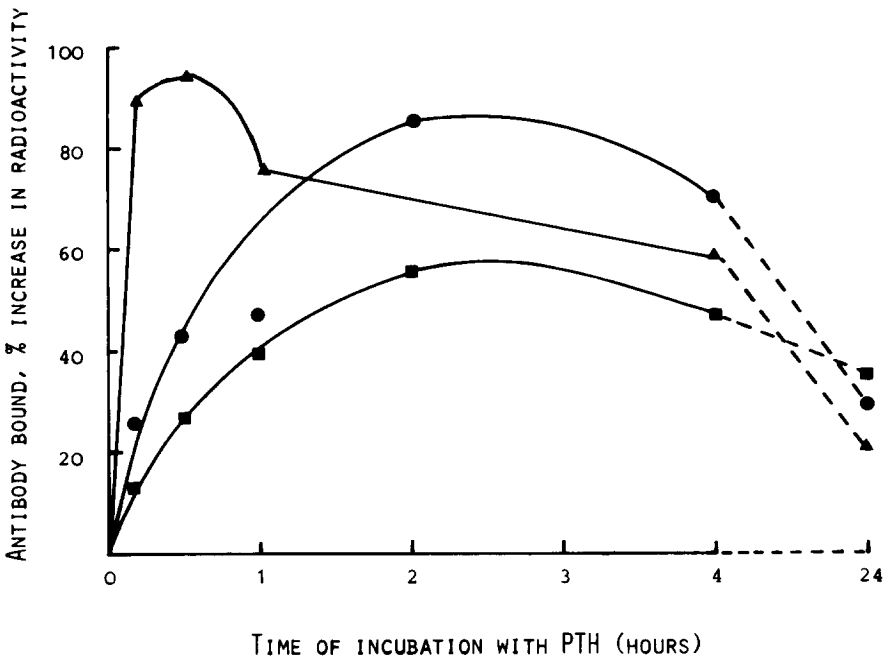

Fig. 3. Effect of incubation time and temperature on binding of bovine PTH (10 ng) to membranes. Incubation time with antibody (AS 262) = 2 h. ■ 0°C ● 20°C ▲ 37°C

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ted with the cross reacting antibody AS 201. However, no binding of either hormone could be detected with acid treated (0.1 N HCl) or boiled membranes (100°C 5 min), or with a mitochondrial fraction from bovine kidney. The binding to rat membranes was extremely low and exhibited the characteristics of non-specific binding. This is probably due to the rapid metabolism of PTH (9). Binding of 1-34 HPTH could not be detected in any of the systems examined, with either antibody 201 or anti-1-34 HPTH. Since this fragment stimulated the adenyl cyclase of rat and porcine membranes it is possible that the lack of detection is due to non-accessibility of the antibody to the receptor bound fragment. Alternatively rapid metabolism or displacement by antibody could explain this result.

Preliminary experiments on the binding of BPTH to chick renal membranes have demonstrated saturation of binding at 2.5 - 5.0 ng BPTH/100 /ug membrane protein. It therefore seems probable that hormone degradation, although low in the case of

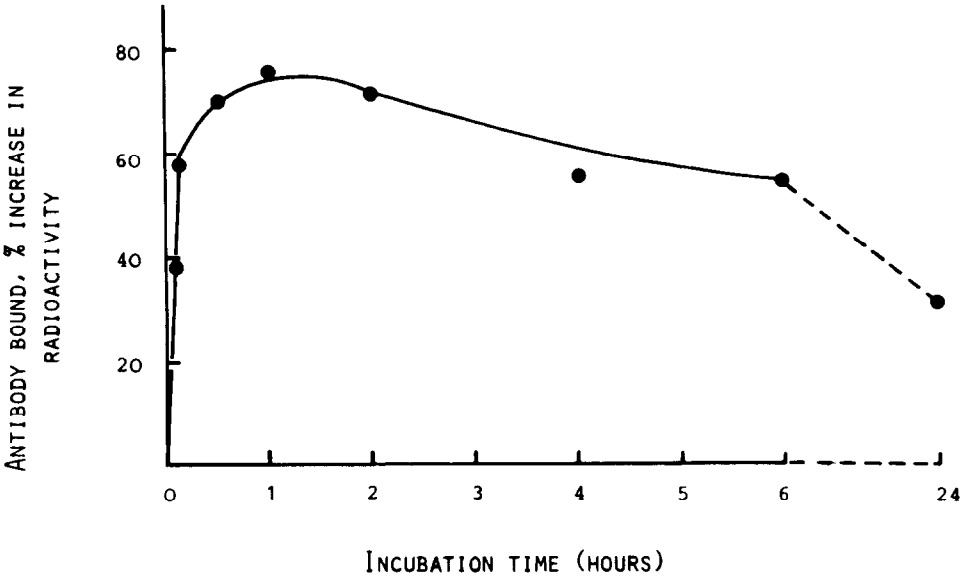

Fig. 4. Effect of incubation time on binding of antibody (AS 262) to bovine membranes. Incubation with bovine PTH = 2 h 22°C.

porcine and bovine membranes, occurs at a sufficient rate to prohibit detection of saturation of membrane receptors. In addition, BPTH oxidised with H_2O_2 (17) exhibited undetectable binding in this system whereas hormone treated in an identical manner but in the absence of H_2O_2 showed no loss of binding. This clearly demonstrates that specific hormone-receptor binding is being studied.

Previous attempts to study PTH-receptor interaction using iodinated (16) or tritiated (17) hormone have demonstrated tracer binding, but displacement with unlabelled PTH has only been obtained at unphysiological levels of hormone. In addition, there is still considerable confusion over the in vivo biologically active species of PTH and the possibility exists that, prior to receptor binding the 1-84 secreted hormone must be metabolized to a smaller N-terminal fragment. The reported results clearly do not support this proposal although the question as to whether both intact and N-terminal fragments are equally active in vivo clearly cannot be answered. Additional experiments are at present in progress to further elucidate this process.

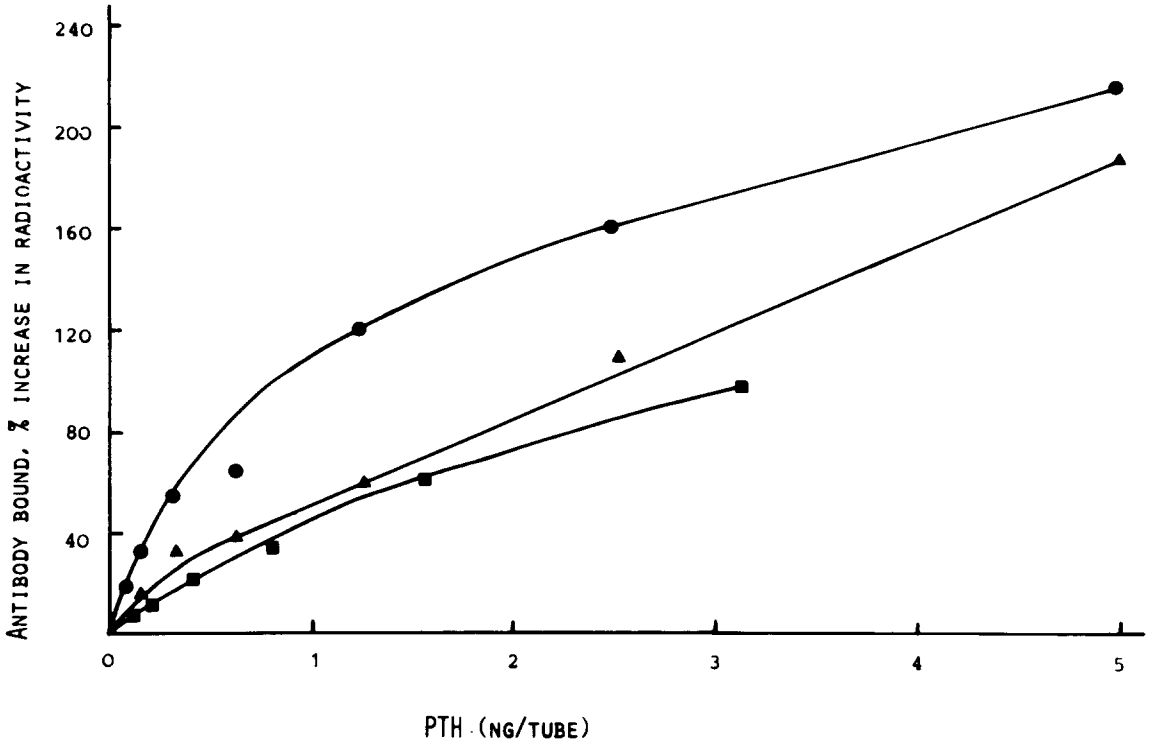

Fig. 5. Standard curves for binding of PTH to renal membranes:
● Pig Membranes BPTH Antibody AS 262 (Antibody Bouillon A-VII gave almost identical binding curves).
▲ Bovine Membranes BPTH Antibody AS 262
■ Bovine Membranes HPTH Antibody AS 201
Incubation with parathyroid hormone 2 h. 22°C followed by 1 h. 22°C incubation with antibody.

In conclusion, the described system is both a useful adjunct to existing radioreceptor assays and an alternative method for studying receptor interaction of hormones which cannot be readily iodinated (e.g. CCK, secretin). By combined studies of both inhibition of binding by hormone fragments and the use of region specific antibodies it is evident that a greater knowledge of the biologically significant region of a hormone may be obtained. In addition, by increasing the sensitivity of the assay the measurement of biologically active PTH in human serum seems possible.

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