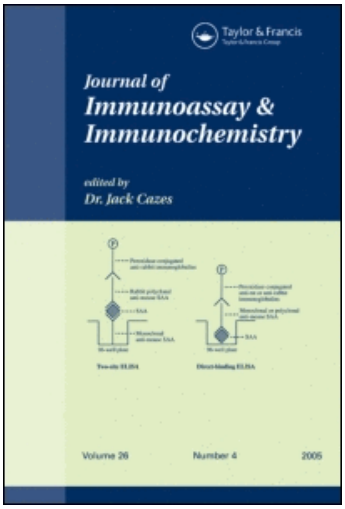

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The Cytochemical Bioassay of Parathyroid Hormone: Further Experience

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THE CYTOCHEMICAL BIOASSAY OF PARATHYROID HORMONE: FURTHER EXPERIENCE

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ABSTRACT

The procedure for the cytochemical bioassay of parathyroid hormone has been examined to improve the reliability and robustness of the assay. It has been shown that the control of the pH, at all stages of the assay, is essential. The use of the assay confirmed that the circulating level of the biologically active form of this hormone is less than one tenth of the values found by the more recent immunoassays, and is in good agreement with previously calculated values. The assay clearly distinguished between normal, hypoparathyroid and hyperparathyroid levels and allowed relative potencies to be ascribed to preparations of the hormone obtained from human, bovine and porcine sources.

INTRODUCTION

In the past few years cytochemical techniques have been shown to be capable of measuring sub-physiological concentrations of several polypeptide hormones (1). Guinea-pig kidney has been found to be a suitable target tissue for the measurement of the

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biological activity of parathyroid hormone (PTH); the specificity of the response of glucose 6-phosphate dehydrogenase (G6PD) activity in distal convoluted tubules in this tissue to concentrations of PTH within and below the physiological range has been established (2,3).

Where cytochemical bioassays have been used to define the normal circulating concentrations of biologically active hormones, the results have, in general, been in good agreement with the results of radioimmunoassay. However, PTH is a special case in that the circulating level of the biologically active form of the hormone, which has been calculated to be about 10pg/ml(4), represents no more than 5% of the immunoreactive hormone. This discrepancy between bioassay and immunoassay has been attributed to the fact that PTH in the circulation is heterogeneous (5) and consists mainly of biologically inactive fragments, mostly derived from the biologically inactive carboxy-terminus (C-terminus), which are measured by immunoassay. Nevertheless, even those assays which measure the N-terminus exclusively (6,7) have still demonstrated molar concentrations of immunoassayable hormone thirty to fifty times greater than those which have been reported for bioassayable PTH.

For these reasons it is of particular importance to be able to determine the circulating levels of bioassayable PTH. Although recent reports of a bioassay for PTH based upon the stimulation of adenylate cyclase activity in renal cortical membranes have shown that that assay is capable of measuring

physiological concentrations of PTH (8), the cytochemical bioassay is currently the only method available which can readily measure both physiological and sub-physiological levels. Studies were therefore made to try to ensure its ready reproducibility and to make it more robust so that it can be used more widely.

METHODS

Cytochemical bioassay

The procedure was similar to that used previously by Chambers et al. (2) and by Goltzman et al. (9). Female guinea-pigs of the Dunkin-Hartley strain weighing 400-550g were fed on 350 Coney bran pellets (BOCM Silcock, Basingstoke, Hampshire) with added fresh cabbage. Adexoline (Farley Health Products, Plymouth, Devon, England) 0.6ml/day (containing 400U Vitamin D_2, 45µg Vitamin A and 3mg Vitamin C per ml) was added to their drinking water for at least one week before they were used in the assay. The animals were killed by asphyxiation in nitrogen and the kidneys removed and decapsulated. Each kidney was then cut longitudinally through the pelvis and the poles were cut into equal sized pyramid-shaped segments less than 5mm in greatest dimension, yielding a maximum of sixteen segments from each animal. Each segment was then placed on defatted lens tissue covering a stainless steel wire mesh table which stood in a vitreosil dish. Non-proliferative culture medium (Trowell's T8, Gibco Biocult, Paisley, Scotland) was added up to the level of

the top of the table, and the dish was placed in a culture chamber. The segments were maintained for 5 hours at 37°C in an atmosphere of 5%CO_2/95%O_2 which buffered the bicarbonate in the medium to achieve a pH of 7.56. At the end of this time, the medium was removed and replaced with fresh medium which was poured over the segments and left for eight minutes. This was then removed and to each segment was added either one of several graded doses of a standard preparation of PTH (1.0 to 0.001pg/ml) or dilutions of plasma (1:100 to 1:10 000), all diluted in T8 medium. To the highest concentration of the standard preparation was added 1% hypoparathyroid plasma, this being diluted serially with the standard preparation.

After exposure to the hormone, the segments were chilled rapidly to -70°C in n-hexane (low in aromatic hydrocarbons, boiling range 67-70°C) for 30-45 seconds and stored at -70°C in corked dry tubes. Within 72 hours they were mounted on a platform of ice on a microtome chuck and sections, 16μm thick, were cut using a Bright's cryostat (Bright Instrument Company, Huntingdon, England) at a cabinet temperature of -25°C with the knife cooled to -70°C.

As soon as possible after cutting, unfixed sections were reacted for G6PD activity. The reaction medium contained 5mmol/l glucose 6-phosphate (G6P), 3mmol/l NADP, 5mmol/l neotetrazolium, 10mmol/l KCN, 0.67mmol/l phenazine methosulphate and 12% polyvinyl alcohol (G18/140 Wacker Chemicals Ltd., Walton-on-Thames, Surrey, England) in glycyl glycine buffer, 50mmol/l, adjusted to pH 8.0.

The sections were exposed to the reaction medium for the same time, usually 5-7 minutes depending on the intensity of the reaction. Under these conditions neotetrazolium is reduced to two intensely coloured insoluble formazans which were measured in cells of individual distal convoluted tubules by means of a Vickers M85 scanning and integrating microdensitometer (Vickers Instruments, York, England) at 585nm, the isobestic point of the two formazans. A x40 objective was used; the scanning spot size was 0.5μm in diameter in the plane of the section, and the diameter of the mask was 17μm. Ten separate tubules in duplicate sections from each segment of kidney were measured, and the activity of the enzyme was expressed as mean integrated extinction (MIE). The mean of these readings (mean ± SEM of twenty readings) was plotted against the concentration of the standard preparation of the hormone or the dilution of the plasma. The concentration of hormone in the plasma, the 95% confidence limits and the indices of precision for each sample were then calculated according to the method of Gaddum (10) as modified by Borth (11) for multiple bioassays.

Preparation of Medium for Hormone Dilution

To ensure a standard pH, all the solutions that were applied to segments had 5%CO_2/95%air passed through them for 10-15 minutes before they were used. The passage of gas was continued while the medium was pipetted into the tubes used for dilution; the tubes were immediately stoppered to ensure that the pH of the medium remained stable.

For the study of the effect of pH on the time required to evoke the maximum response in the tissue, the pH of the medium was adjusted to one of two different pH values. The higher pH of 7.56 was that usually used; this was achieved by bubbling the medium with 5%CO_2/95%air. The lower pH, namely 7.26, was achieved by the addition of 1N HCl (2ml to 100ml of medium) to the medium before bubbling with the gas mixture.

Preparation of Hormone Standard and Plasma Samples

Standard preparations of hormone were prepared by initially dissolving the contents of an ampoule of the lyophilized standard reference preparation in 0.5ml of 0.1M acetic acid and then diluting 1:10 in either reconstituted freeze-dried or fresh normal plasma; aliquots (about 150µl) were snap frozen and stored at -70°C. Samples of plasma were taken by venepuncture into cool plastic tubes containing lithium heparin (2mg/tube) and quickly centrifuged at 4°C, snap frozen in aliquots (about 150µl) and stored at -70°C. For each assay 100µl of a fresh aliquot of the reference preparation or of plasma was used.

Hormone Preparations

Four preparations of hormone were used. A purified standard preparation of bovine PTH (NIBSC 77/533) containing 100µg PTH/ampoule and equivalent to 200 U PTH was most frequently used; all results have been expressed as pg/ml of this standard. A second standard preparation of bovine PTH (NIBSC 72/286) containing 200µg PTH/ampoule and equivalent to 520U was also used. Both of these

preparations were obtained from the National Institute of Biological Standards and Control, Hampstead, England. Human hormone had been prepared by trichloroacetic acid extraction of human parathyroid tumours; each ampoule contained 100ng of PTH material and was thought to be about 10% pure (R. Manning, personal communication). A purified preparation of porcine hormone was also studied. After the porcine glands had been extracted with trichloroacetic acid, the extract had been purified by chromatography on carboxymethyl cellulose; it was designated CMC-PPTH (12).

Subjects

The ability of the assay to discriminate between normal subjects and patients with either primary hyperparathyroidism or hypoparathyroidism was studied in twelve normal subjects (three females and nine males) aged between 23 and 35 years; seven patients aged between 13 and 67 years with hypercalcaemia due to primary hyperparathyroidism proven at operation; and nine patients aged between 8 and 61 years with hypoparathyroidism of thalassaemic, idiopathic or postsurgical origin.

RESULTS

1. Dose Response Curve of Concentrations of a Standard Preparation of the Hormone

In guinea-pig kidney distal convoluted tubules PTH (0.001 to 1.0pg/ml) increased G6PD activity linearly (Fig. 1). Two dilutions

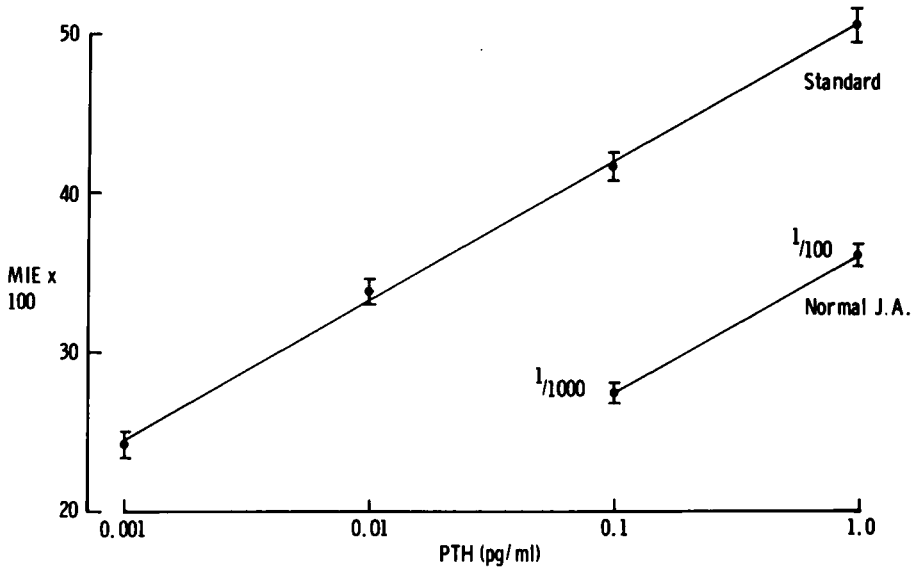

Figure 1. Regression line of standard preparation of bovine PTH (77/533) over four serial 1:10 dilutions. A plasma sample in two serial 1:10 dilutions gave a parallel response and a value of 2.4 pg/ml. Responses shown as mean ± SEM.

of plasma from a normal subject gave responses that were parallel to that of the standard regression line and, when corrected for dilution, gave a result of 2.4pg/ml with 95% confidence limits of 76-138%. The index of precision for this assay was 0.09.

However, a linear response was not always obtained over all four graded concentrations of the standard preparation of the hormone. Frequently a linear response was elicited only over three concentrations and the standard graph showed either low activity in the segments exposed to the highest concentration of hormone (as in Fig. 5) or aberrantly high activity in the tissue exposed

to the lowest concentration. Dilutions of plasma gave parallel responses when the activities induced by them fell on the linear part of the standard graph. To ensure that this occurred, plasma samples were usually assayed in at least three, or sometimes four, dilutions, particularly if they were expected to contain a high concentration of hormone. The parallel sections of the graphs of the standard and the unknown were then compared when calculating the concentration of the hormone in the unknown.

2. Effect of pH on the Response of Tissues

The effect of the pH of the medium used both for the post-maintenance wash and for exposing the tissue to the hormone, was studied by determining the time required to produce a maximal response. The tissue segments were maintained under the normal conditions of culture at pH 7.56, subjected to a post-maintenance wash at the pH to be used for subsequent incubation with standard, and then exposed for different periods of time, from four to fourteen minutes, to 0.1pg/ml of bovine PTH (NIBSC 77/533) dissolved in culture medium that had been adjusted to either pH 7.56 or 7.26.

At pH 7.56 a peak response was obtained six minutes after the hormone was poured on while at pH 7.26 the peak response did not occur until twelve minutes (Fig. 2).

3. Effect of Hormone Concentration on the Response of the Tissue

A study was made to define how the concentration of the hormone, applied to the tissue, influenced the time of maximal

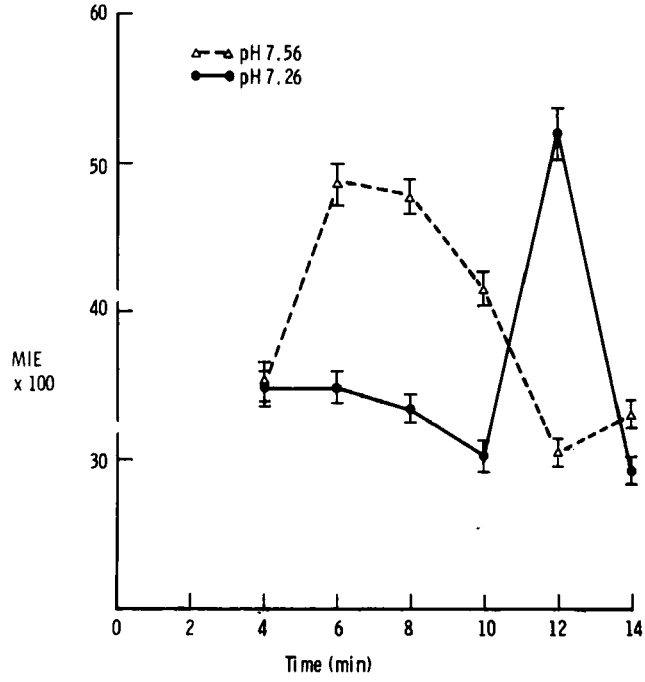

Figure 2. Effect of variation of pH of culture medium on time of peak response using a hormone concentration of 0.1 pg/ml. At pH 7.56 (Δ --Δ) a peak response was present after six minutes exposure to hormone; at pH 7.26 (●—●) twelve minutes were required for a peak response to occur. Responses shown as mean ± SEM.

response. Two concentrations of the standard preparation (NIBSC 77/533) of the hormone were used.

At a pH of 7.56 and a concentration of 1.0pg/ml, the peak response time (four minutes) occurred earlier than with 0.01pg/ml (six minutes), by which time the G6PD activity in the segments exposed to the higher concentration had fallen to a level below that resulting from exposure to the lower concentration (Fig. 3). This would explain the finding that this higher concentration of

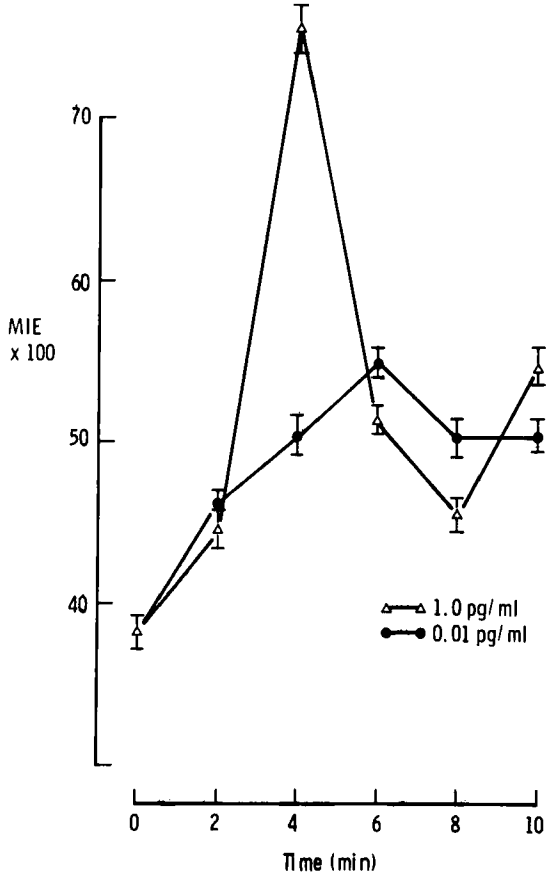

Figure 3. Effect of varying the hormone concentration on the time of peak response. At a concentration of 1 pg/ml (Δ —Δ) the peak response occurred after four minutes exposure to hormone; at a concentration of 0.01 pg/ml (●—●) the peak response occurred after six minutes and was significantly greater ($p<0.01$) than at four and eight minutes. Responses shown as mean ± SEM.

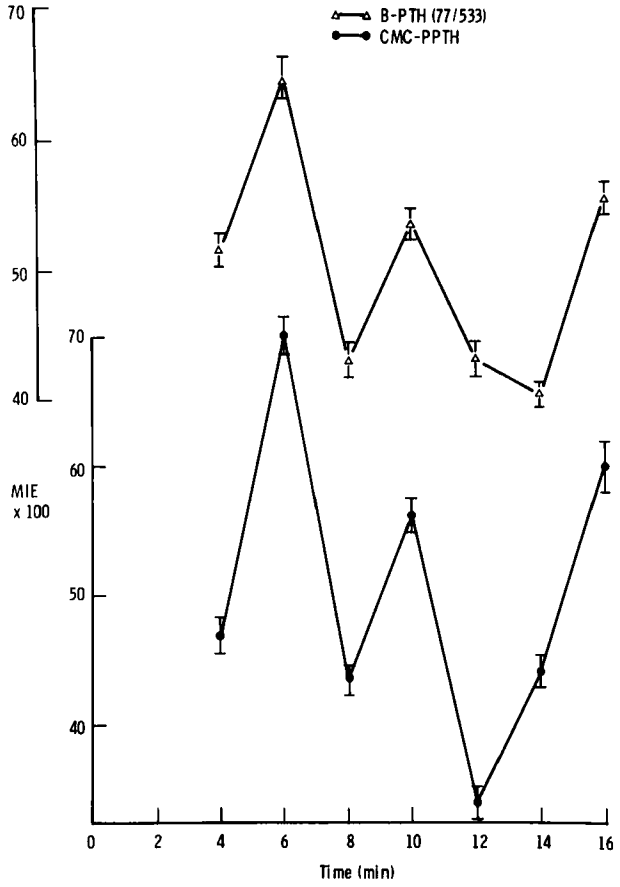

Figure 4. Response with time of glucose 6-phosphate dehydrogenase activity in the guinea-pig kidney after exposure to bovine PTH (△—△) and porcine PTH (●—●). The response to both hormone preparations was similar. Responses shown as mean ± SEM.

PTH gave anomalously low values in some standard calibration graphs.

4. Comparison of Different Hormones

i) Time courses were measured for the response of tissue from the same guinea-pig to approximately equivalent concentrations

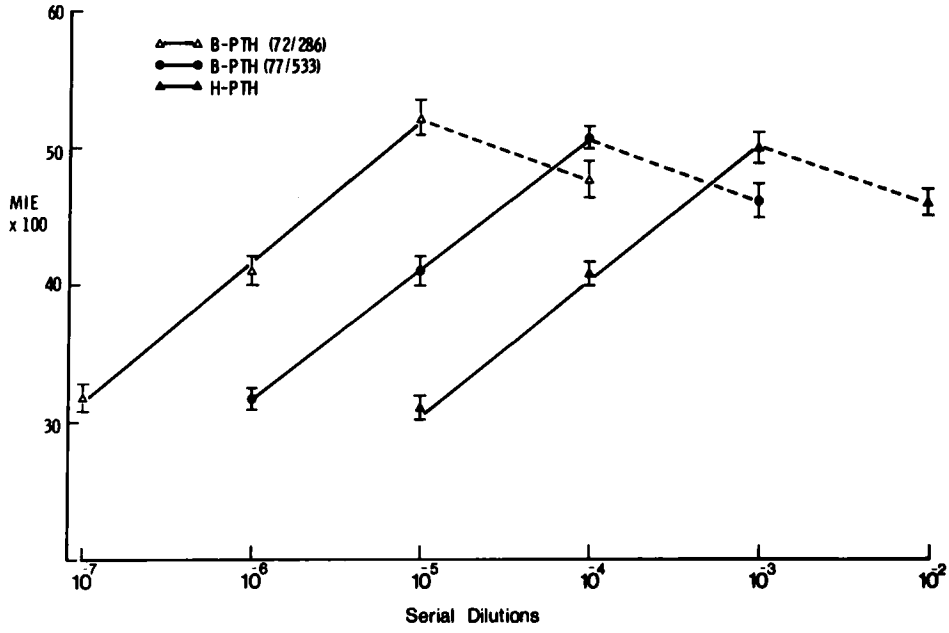

Figure 5. Assay of purified standard preparation of bovine PTH (72/286 NIBSC Δ—Δ) and partially purified human PTH (▲—▲) against purified standard preparation of bovine PTH (77/533 NIBSC o—o). In each case the highest concentration of hormone gave inappropriately low activity in the tissue. Serial 1:10 dilutions of each preparation were used. Time of exposure was six minutes. Responses shown as mean ± SEM.

of two hormone preparations (NIBSC 77/533 and CMC-PPTH). Both demonstrated an initial peak of activity after six minutes exposure to 0.1pg/ml of hormone at pH 7.56 (Fig. 4).

ii) In an intra-animal assay, a standard preparation of purified bovine hormone (NIBSC 77/533) was compared with a similar purified bovine standard (NIBSC 72/286) and with the partially purified preparation of human hormone (Fig. 5). In a second intra-animal assay, the bovine hormone (NIBSC 77/533)

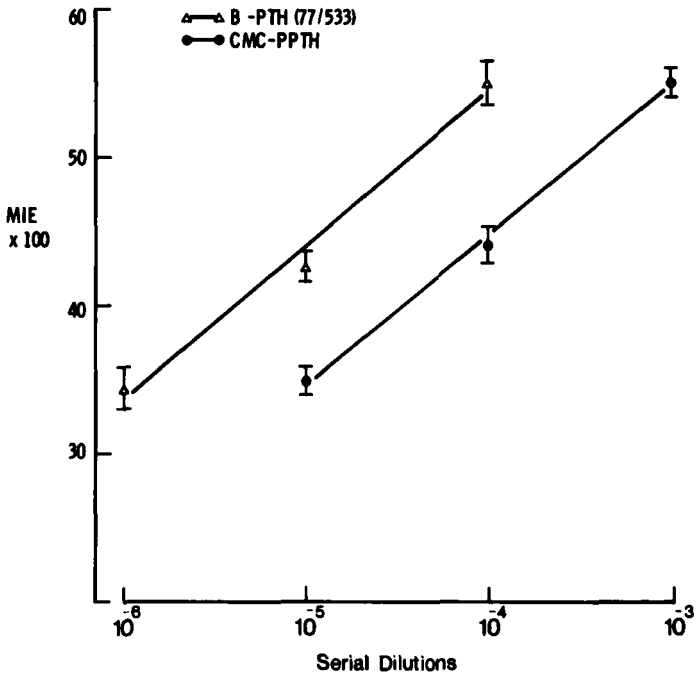

Figure 6. Assay of purified preparations of porcine PTH (CMC-PPTH o—o) against purified standard preparation of bovine PTH (77/533 NIBSC Δ—Δ). Serial 1:10 dilutions of both preparations were used. Responses shown as mean ± SEM. Time of exposure was six minutes.

was compared with the purified preparation of the porcine hormone (CMC-PPTH) (Fig. 6).

All of these hormone preparations showed parallelism with NIBSC 77/533 over the three dilutions. Relative to this standard, the bovine standard NIBSC 72/286 had a potency of 1.12 with 95% confidence limits of 77-130%; this compared with a relative potency of 1.3 between these standards ascertained by NIBSC. Assuming 10% purity, the human preparation had a relative potency of 0.87 (76-131%), and the CMC-PPTH had a potency of 1.16

(66-154%). The indices of precision for these two assays were respectively 0.093 and 0.15. Subsequently all results have been expressed as pg/ml of bovine standard NIBSC 77/533 since this was the most readily available.

5. Assay of Plasma Samples

The twelve normal subjects had values ranging from 1.2 to 5.9 pg/ml and the seven patients with primary hyperparathyroidism from 16.4 to 406pg/ml. The two patients with the highest concentrations of hormone (406 and 328pg/ml) had the most florid hyperparathyroid bone disease. The nine patients with hypoparathyroidism had concentrations below 0.55pg/ml.

The mean index of precision for fourteen assays was 0.13 ± 0.01 (mean ± SEM) and the 95% confidence limits for 38 samples in these assays varied from 47-241% up to 77-130% with most being around 66-159%. In two assays in which two series of dilutions of the same standard preparation of hormone were assayed against each other, the coefficient of variation within assays was respectively 7.2 and 6.1%. The coefficient of variation between assays calculated for ten samples which were assayed on more than one occasion was 27%.

DISCUSSION

Our experience with the cytochemical bioassay for PTH has confirmed the findings of others (2,3,9) that the normal circulating concentration of biologically active PTH is

considerably lower than that reported for immunoassayable hormone. Furthermore, it has also been shown that it is possible to measure concentrations of hormone below the normal range. Patients with primary hyperparathyroidism had concentrations up to fifty times greater than normal and, as with the N-terminal specific immunoradiometric assay (6), the highest concentrations were associated with the most florid radiologically apparent bone disease.

However, the assay system appears to be very sensitive to changes in the conditions under which it is performed. The pH of the medium used for treating the segments with the hormone seems to be of particular importance in this respect. Most of the time course studies, undertaken under the conditions specified, demonstrated an initial peak time of response at six minutes. This is the same as that found by Goltzman et al. (9) although slightly shorter than that used by Chambers et al. (2); both of these groups used the same medium but without equilibrating it with 5%CO_2. When the pH of the medium was lowered, the initial peak response time increased to twelve minutes. The possibility that the lower pH resulted in suppression of the first peak with preservation of the second peak cannot be ruled out, but Fenton et al. (3)demonstrated an initial peak at twelve minutes using a different medium (Eagles MEM) which was presumably at a lower pH.

This variation of response with pH means that the conditions must be carefully controlled if maximum value is to be obtained

from the assay. Trowell's T8 medium presents certain difficulties in this respect since its pH is maintained in culture by equilibration with 5%CO_2. The pH of commercial preparations of the medium varies from batch to batch,but by first equilibrating with 5%CO_2 and stoppering the tubes immediately after pipetting,changes in pH that may occur during each assay are limited.

The finding of an earlier time of maximal response of tissue to 1.0pg/ml than to 0.1 or 0.01pg/ml adds a further complication to the assay system. It is consistent with the finding (13) that the response of thyroid tissue to TSH occurs earlier when exposed to 10μU/ml of hormone than when exposed to 1μU/ml. The reasons for this phenomenon are not entirely clear but the fact that it does not occur every time that PTH is assayed may be due to inter-animal variation.

Despite slow through-put, the assay has proved a useful method of assaying biological activity of PTH. It confirms the previously calculated levels of the biologically active hormone and clearly distinguishes between normal, hypoparathyroid and hyperparathyroid subjects. In addition it has proved possible to demonstrate similarities in the response of guinea-pig kidney to PTH extracted from bovine, porcine and human species and to assess the relative potencies of these preparations.

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Requests for reprints should be addressed to J.A.

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