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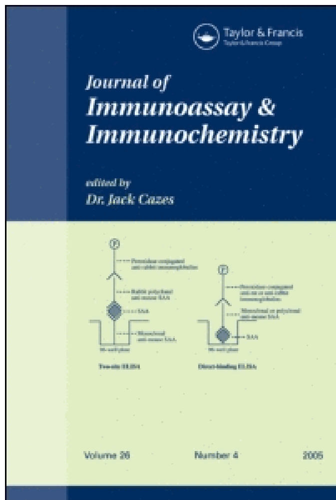

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HUMAN PARATHYROID HORMONE: ANTIBODY CHARACTERIZATION

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ABSTRACT

PTH antibodies were raised in two sheep (S 469 and S 478) by immunizing with porcine and bovine parathyroid extracts. Both antisera were characterized with various PTH preparations and fragments. Both antisera cross react with human, bovine and porcine PTH, one antiserum also binds rat PTH. Region specificity could be attributed to the mid region of the PTH molecule with particularly high affinities of both antisera for the fragment 44-68 hPTH. S 478 has smilarly high affinity for intact hormone (affinity constants 0.6 x 10^{13} l/mol), while S 469 has much higher affinity for the 44-68 fragment (affinity constant 0.84 x 10^{13} l/mol) than for intact hormone. The antibodies are useful not only for clinical radioimmunoassay, but also for experimental work. They have been distributed to many laboratories.

INTRODUCTION

The radioimmunochemical measurement of human parathyroid hormone (hPTH) in plasma is complicated by the heterogeneity of circulating PTH peptides. Intact 1-84 hPTH (27) is secreted by the parathyroid glands and also, under certain conditions, PTH fragments (16). Pro-PTH and Pre-Pro-PTH (20) do not seem to

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enter the circulation to any significant extent. Glandular and secreted molecular species of PTH are therefore quite different (2). Circulating intact PTH and PTH fragments are cleaved, degraded and partially cleaved in peripheral organs (18,19,35). Kidney (25,29,30) and liver (9,28) are the most important organs giving rise to a large variety of PTH fragments, which finally account for the heterogeneity of circulating PTH in plasma (3,4,7,41). Figure 1 gives a schematical survey of the possible types of PTH fragments.

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This heterogeneity exists with respect to molecular form and size, to biological activity, to immunochemical reactivity and to the half life of different fragments in the plasma. For biological activity the amino (N-)terminal sequence 1-34 seems to be required as a minimum, so that intact 1-84 PTH, 1-34 PTH

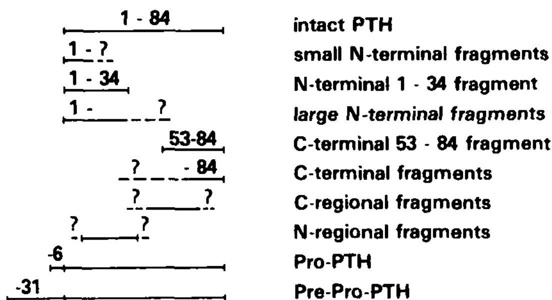

Figure 1:

Schematic and theoretical survey of PTH peptides, precursors and fragments, which play a role during synthesis, secretion and metabolism of the hormone.

and larger N-terminal fragments are biologically active (43). Carboxy (C-) terminal fragments are biologically inert (38); most likely this is also true for N- and C-regional fragments, since the positions 1 and 2 of the PTH molecule seems to be essential for biological activity (43).

The variety of plasma half lives of different PTH fragments will strongly influence measurements of circulating PTH immunoreactivity, since PTH peptides with short half lives in the range of several minutes, intact PTH and N-terminal fragments, (19,16) will account for only a small fraction of total circulating PTH immunoreactivity (34), whereas carboxyl-terminal (7,41) and carboxyl-regional (24) fragments with longer half lives in the range of 20 minutes to hours are circulating at much higher concentrations, 2-10 fold as compared to N-terminal fragments (16). Half lives of PTH peptides are prolonged in renal failure (23,24).

On the basis of these considerations it becomes evident that the measurement of PTH immunoreactivity in the plasma of patients can lead to interpretations with clinical relevance only if those PTH measurements are carried out with PTH antibodies that have been characterized with regard to their region specificity and region specific affinity (31). With the availability of synthetic 1-34 N-terminal and 53-84 C-terminal

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PTH, most antisera could be defined as cross reacting with N-terminal fragments (15): "Brewer-Sequence" (6,45) or "Niall-Sequence" (33) or with C-terminal fragments (38).

Since most antisera have been raised by injection of crude parathyroid extracts or of the "TCA supernate" generated during these extractions, containing many fragments predominantly from the carboxyl terminus (11,12,42), these antisera do not necessarily cross react with N- or C-terminal parts of the PTH molecule. Recently specific PTH fragments have been synthesized, so that characterization of antisera can be carried out with synthetic fragments representing parts of the whole molecule.

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We have produced two antisera (S 469 and S 478) in sheep (21,22) that were at first thought to be C-terminal antibodies, since they showed little cross reaction with N-terminal and N-regional fragments. Clinical application of assay results from such antibodies requires much more information about region specificity. The results presented here describe a more complete characterization of these two antisera as a basis for clinical application.

MATERIALS AND METHODS

Antibodies: The antisera S 469 and S 478 were raised in sheep by immunization with porcine and bovine parathyroid extracts.

The porcine immunogen was an approximately 2% pure parathyroid extract from 30,000 porcine glands which had been collected at the abattoir in Hamburg (23). The extraction was performed in C.D. Arnaud's laboratory in Rochester in 1975. The bovine material was partially purified bPTH (Calbiochem, San Diego, Lot No. 400 150, biopotency 457 U/mg.) The details of the immunization scheme have been described previously (21,23).

Several bleedings of both sheep at approximately two week intervals were available and coded S 469 VI-XIV and S 478 VI-XIV. The characterization reported here refers to both S 469 VI and S 478 VI; there were no major differences between different bleedings of each sheep except for the titer (see below).

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The assay procedure is a modification of the technique of Arnaud et al 1971 (1) with dextran coated charcoal to separate bound and free hormone and with ^{125}I bPTH as tracer. 10 µg of bPTH (Wilson Lot No. 156 552, purchased in 1974) were labelled with 0.5 mCi 125I by a chloramine T technique. Purification of the tracer was a two step procedure with adsorption chromatography on Whatman CF 1 cellulose followed by gel filtration on Biogel P10, (21,23). This tracer gives a nonspecific binding between 2 and 6% and is stable for more than 10 weeks if kept frozen at working dilution. The total incubation volume of 350 µl included 50 µl of PTH free plasma

(charcoal extracted), 200 µl of diluted antiserum and 100 µl of tracer. Incubation was 24 hours without tracer plus 48 hours after addition of tracer.

PTH preparations and peptides for characterization:

Bovine PTH (bPTH):

1. WHO 1st International Reference Preparation for parathyroid hormone, bovine for immunoassay (Ampoule Code 71/324) obtained from the National Institute of Biological Standards and Control (NIBSC) London.
2. 1-34 bPTH synthetic fragment obtained from Beckman, Palo Alto.
3. 28-48 bPTH synthetic fragment prepared by Drs. M. Rosenblatt and H. Keutman, Boston, and made available to us by Dr. Joan M. Zanelli, NIBSC, London.
4. 53-84 bPTH cleavage product, prepared and donated by Dr. H. Keutman, Boston.

Porcine PTH (pPTH):

5. Porcine parathyroid extract, approximately 2% pure, prepared by Prof. C.D. Arnaud, (Rochester/San Francisco).

Human PTH (hPTH):

6. NIBSC Reference Preparation (Ampoule Code 75/549) obtained from NIBSC, London.

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7. Tissue culture human PTH ("P2") prepared and donated by Drs. G. Dorn and R. Montz, Hamburg (13,14).

8-11. Synthetic fragments 1-34, 13-34, 18-34 and 23-34 hPTH (Brewer sequence (6)), donated by Dr. Rittel, Ciba-Geigy, Basel.

12. 44-68 hPTH synthetic fragment prepared and donated by Drs. M. Rosenblatt and H. Keutman, Boston.

13. 53-84 hPTH synthetic fragment prepared by Dr. Joan M. Zanelli, NIBSC, London.

14. Plasma from patients with primary hyperparathyroidism.

15. Plasma from patients with secondary (renal) hyperparathyroidism.

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Rat PTH:

16. Plasma from uraemic rats.

17. Rat PTH from tissue cultures of rat parathyroids (prepared and donated by Drs. Dorn and Dietel, Hamburg).

Affinity estimation:

Since the sensitivity of radioligand assays is limited by the affinity of the antiserum used, it is desirable to provide quantitative data on affinity. This may be derived from a Scatchard plot (17,39,47). B/F is plotted against the absolute amount of hormone bound (B) (see Fig. 4 and 8). If there is a linear relation between B/F and (B), the slope defines the affinity constant.

RESULTS

Titers and working dilutions of the antisera:

Titer may be defined as that dilution of antiserum which specifically binds 50% of tracer in the absence of unlabelled antigen. A working dilution of the PTH antisera is chosen to give approximately 30% of specific tracer binding. This working dilution is 1:35,000 for S 469 VI and 1:5,000 for S 478 VI. Surprisingly, titers of sequential bleeds increased from January, 1976 without further booster injections until the bleeding IX in April, 1976 and then slowly decreased. Bleeding XIV in August, 1976 still gave titers of 1:12,000 for S 469 and of 1:3,000 for S 478.

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Species cross reactivity:

As demonstrated in Fig. 2 for S 469, both antisera cross react with bovine, porcine and human PTH. S 478 additionally binds rat PTH. The affinity for pPTH is sufficiently high to measure PTH concentrations in normal and rachitic pigs (44). No international reference preparations are available to calculate affinity constants for rat and porcine PTH.

Affinity for intact PTH:

Figures 3a and b show standard curves for intact bovine (71/324) and human (75/549) PTH and for a serum dilution in chronic renal failure with a high proportion of PTH fragments.

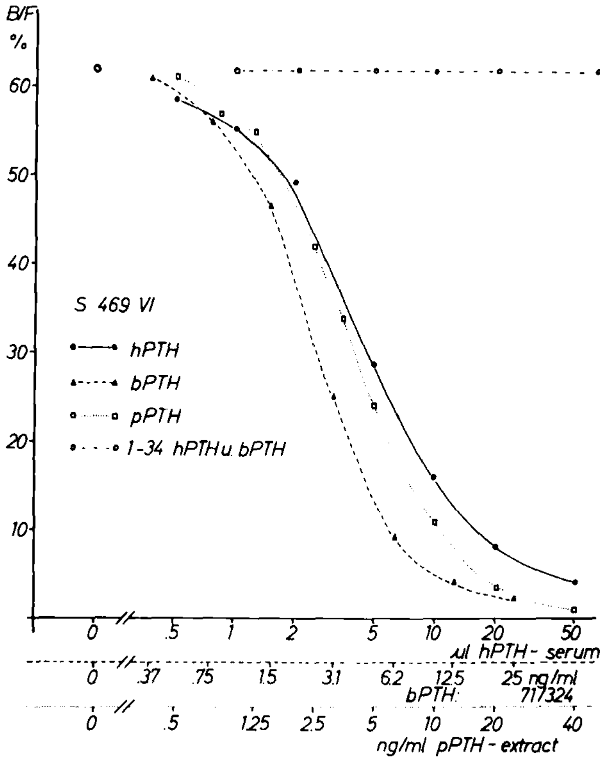

Figure 2:

Standard curves of antiserum S 469 with human PTH (serum dilution of primary hyperparathyroidism), bovine PTH (WHO first international reference preparation 71/324) porcine PTH (glandular extract) as well as for bovine and human 1-34 PTH.

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For S 469 standard curves of bovine and human intact hormone are reasonably parallel; Scatchard plot analysis, however, reveals no linearity between B/F and (B), so that affinity constants cannot be calculated from these data. For S 478 standard curves for bovine and human intact PTH are not parallel. With both antigens, Scatchard plots show linearity

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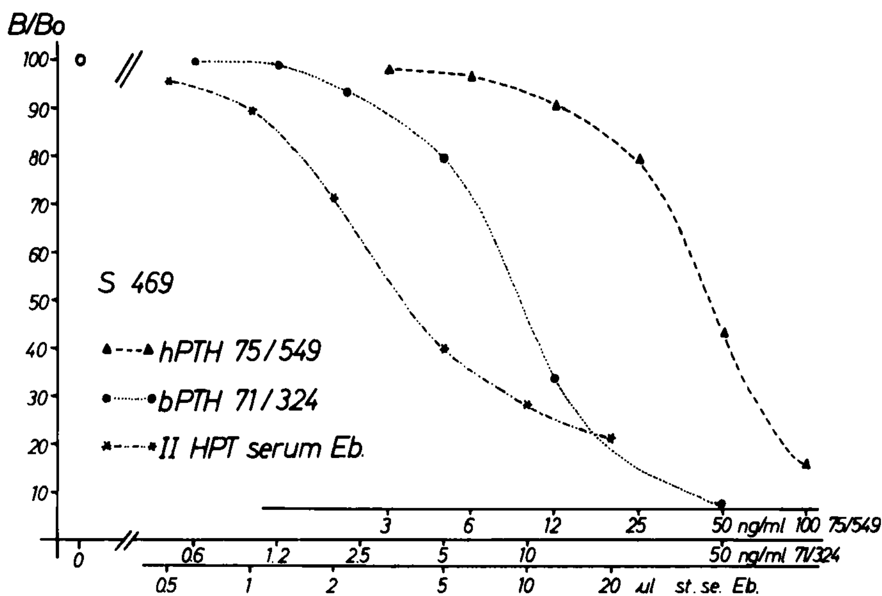

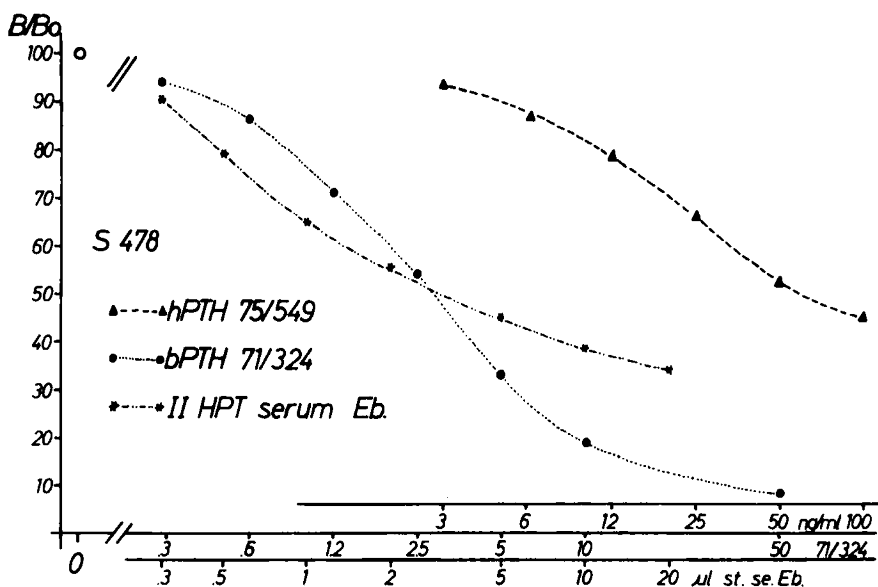

Figure 3 (a and b):

Standard curves of antisera S 469 (a) and S 478 (b) with bPTH (WHO first international reference preparation 71/324) and hPTH (NIBSC reference preparation 75/549) compared to a dilution curve of a patients serum with renal insufficiency and secondary hyperparathyroidism containing large amounts of PTH fragments.

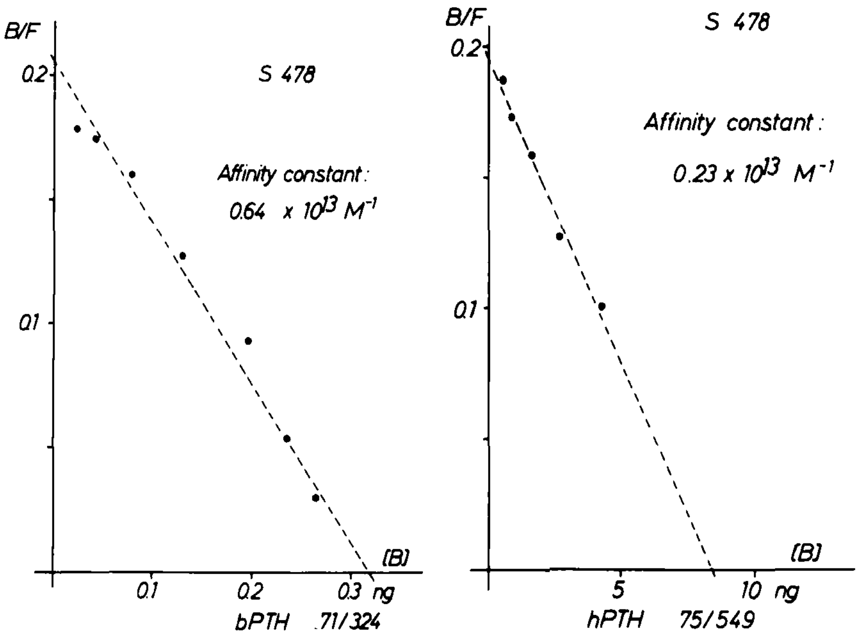

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Figure 4 (a and b):

Scatchard plots of S 478 for intact bPTH (a) and hPTH (b) standards. Affinity constants were calculated assuming, that 71/324 was 100% and 75/549 was approximately 10% pure.

between B/F and (B) (Fig. 4a and b) and affinity constants can be calculated to be 0.6 x 10^{13} l/mol for bPTH (71/324) and 0.2 x 10^{13} l/mol for hPTH (75/549), provided that 75/549 is approximately 10% pure (46). By comparison of standard curves of both antisera with intact hormone preparations, the affinity of S 469 for 1-84 hPTH can be estimated to be lower by a factor of approximately 2.

Another PTH preparation which consists predominately of intact

1-84 hPTH is the Peak 2 ("P2") material from Hamburg (13,14), extracted and purified from tissue culture media, in which human parathyroid adenoma have been maintained. The material is biologically active in stimulating adenylate cyclase of rat, bovine and human cortical tubular membranes. (Mohr et al unpublished data, (14)). It is also active in the cytochemical PTH bioassay (8). Standard curves with this material show a two times higher affinity of S 478 for intact hPTH as compared to S 469 (see Fig. 6 and 7).

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Affinity for N-terminal and N-regional PTH peptides:

Under routine assay conditions with ^{125}I bPTH as tracer both antisera do not bind 1-34 bPTH, or 1-34 hPTH (Brewer Sequence (6)) (see Fig. 2), and there is no cross reaction with the synthetic N-regional fragments 13-34, 18-34 and 23-34 hPTH up to concentrations of 2 μg/ml.

However, Dr. J.M. Zanelli in the laboratories of the NIBSC, London, provided data (Fig. 5) that S 478 is a high affinity antibody for 1-34 hPTH (Neall Sequence (33)) if ^{125}I 1-34 hPTH is used as a tracer. The affinity is similar to an antiserum of Dr. Deslan, Paris (10) produced with 1-34 hPTH as the immunogen. Less than 100 pg/ml or 5 pg/tube of 1-34 hPTH can be detected in this assay system (Fig. 5).

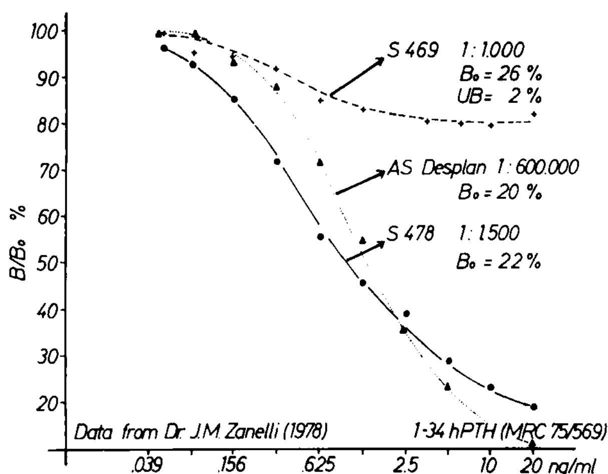

Figure 5:

Standard curves of S 469 and S 478 in a homologous 1-34 hPTH assay. At a dilution of 1:1500, S 478 contains anti-N-terminal populations, which have similar affinity as Dr. Desplans (Paris) anti-N-antiserum.

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Affinity for C-terminal PTH peptides:

Both antisera reveal very poor cross reactivity with bovine and human 53-84 PTH. Only at very high concentrations (>100 ng/ml) displacement of ^{125}I bPTH tracer can be detected (see Fig. 6 and 7).

Affinity for mid regional PTH peptides:

As shown in Fig. 6 and 7, very poor cross reactivity of both antisera was found with the bovine synthetic fragment 28-48. However, for the synthetic human fragment 44-68 extremely

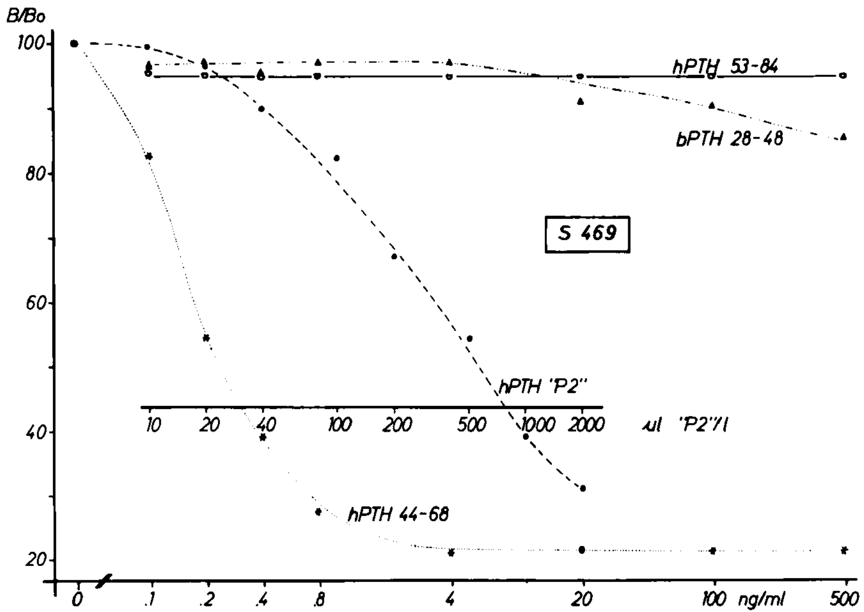

Figure 6:

Standard curves of S 469 with bPTH 28-48 and hPTH 53-84 and 44-68 and with the human tissue culture PTH preparation "P2" (Hamburg). There is only little cross reaction with 53-84 PTH, but very high affinity for the mid-region fragment 44-68 (less than 100 pg/ml or 5 pg/tube can be measured).

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sensitive standard curves could be obtained. Both antisera detect much less than 100 pg/ml or 5 pg/tube, the standard curve of S 469 (Fig. 6) being even more sensitive than for S 478 (see Fig. 7). Affinity constants calculated from Scatchard plots (Fig. 8) were 0.84 x 10^{13} l/mol (for S 469) and 0.62 x 10^{13} l/mol (for S 478).

The affinity of S 469 for this particular mid-C-regional

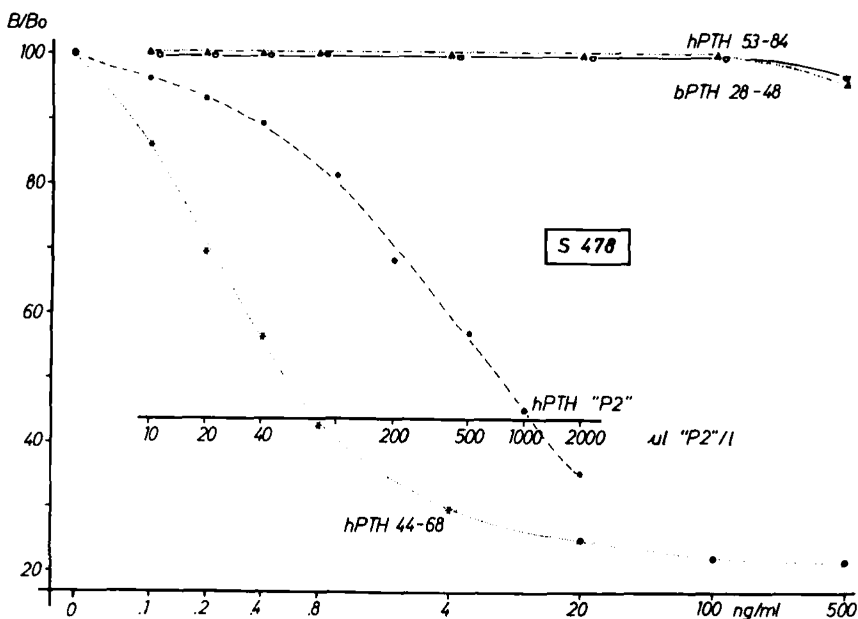

Figure 7:

Standard curves of S 478 with the same PTH fragments as in Fig. 6 Similar to the antiserum S 469 there is little cross reaction with 53-84 and 28-48 PTH and again high affinity for the mid-region fragment 44-68.

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peptide 44-68 is much higher than its affinity for intact hormone, while for S 478 the high affinity for intact 1-84 hPTH and bPTH is identical with the affinity for this fragment.

DISCUSSION

Several radioimmunoassay systems for hPTH have proved to be of considerable clinical and diagnostic value. For routine PTH determinations in peripheral plasma or serum of patients

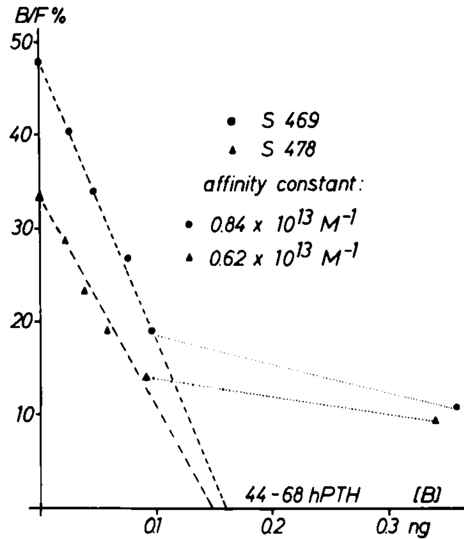

Figure 8:

Scatchard plots for both antisera S 469 and S 478 with the human PTH fragment 44-68; the calculated affinity constants are indicated and show the high affinity of both antisera for this mid-region fragment.

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with disorders of calcium metabolism and metabolic bone disease, the results obtained and their clinical relevance depend largely on the region of the peptide against which the antibody is directed. When 1-34 synthetic PTH peptides and the tryptic fragment 53-84 (32) became available, it was possible to characterize antisera with regard to their cross reactivity with these N-terminal and C-terminal fragments. On this basis PTH assays have been defined to be N-terminal or C-terminal specific. At that stage we believed that our antisera S 469 and S 478 were C-terminal antibodies, since they did not detect

N-terminal and N-regional peptides (21). In the meantime extensive analytical and synthetic work in PTH biochemistry yielded fragments along the whole 1-84 PTH molecule (26,27,32,36,37). As a result we were able to characterize our antibodies more extensively. The materials included the mid region fragments 28-48 bPTH and 44-68 hPTH. Under routine conditions both antisera S 469 and S 478 proved to be neither N-terminal nor C-terminal, although S 478 contains high affinity anti-N-populations, if ^{125}I 1-34 hPTH is used as tracer.

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Both antisera have very high affinities for the mid-C-region fragment 44-68 hPTH. For S 478 the high affinity for intact bPTH and hPTH can be explained by this mid region specificity, since affinity constants are similar for fragment and intact hormone.

In the case of S 469 the affinity for the fragment 44-68 is much higher than for the intact hormone.

It has not yet been possible to test cross reactivity with the fragment 1-64, that was used for characterization by Blum et al 1979 (5). A very pure preparation of intact hPTH 1-84 (supplied by C.D. Arnaud) will be included in the next interlaboratory comparison organized by WHO/NIBSC 1979/80.

The synthetic fragments available may not be identical with those cleavage products present in the circulation and thus may not have the same conformation and antibody interaction. However, even antiserum S 469 with comparably lower affinity for intact hormone does detect PTH in more than 95% of normal sera and is able to discriminate conditions associated with chronic hypersecretion of the hormone. This may indicate that it is not intact hormone, but a variety of PTH fragments with long half lives that is being measured in normal persons. Among more than 60 patients with proven primary hyperparathyroidism only three had borderline PTH concentrations with S 469, while measurements with S 478 showed clearly elevated levels in all patients. Possibly these three patients secrete a major proportion of intact hormone, which is adequately measured by S 478 but only with lower affinity by S 469. With regard to the half life of the mid region fragments, which both antisera detect with high affinity, we could demonstrate that with intact kidney function half life was between 20 and 50 minutes; in chronic renal failure the disappearance of these peptides was prolonged 5-10 fold (24). Similar half lives have been described for C-terminal fragments.

The clinical usefulness of such "anti-C-assays" are attributed to the relatively long persistence and the resulting higher

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concentrations of carboxyl terminal fragments in peripheral plasma of patients (3,16,40).

For the PTH radioimmunoassays with the antisera S 469 and S 478 as characterized here, clinical usefulness and adequate quality control criteria have been demonstrated (23). Since the antibodies had acceptable titers and were raised in sheep, they are available in sufficient quantities for distribution. Consequently more than 30 groups in Europe, USA and Canada have been supplied with these materials. By their cross reactivity with human, bovine, porcine and rat PTH, the antisera are not only useful for clinical, but also for experimental work.

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