Sulfur-Free Parathyroid Hormone Analogues Containing D-Amino Acids: Biological Properties in Vitro and in Vivo†

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ABSTRACT: Three sulfur-free analogues of bovine parathyroid hormone (bPTH) containing D-amino acids were synthesized by the solid-phase method and their biological properties compared in an in vitro bioassay (rat renal adenylate cyclase assay), a receptor assay for parathyroid hormone (PTH) (canine renal membranes), and an in vivo bioassay (chick hypercalcemia assay). The analogue [Nle^8,Nle^{18},D-Tyr^{34}]-bPTH-(1–34)-amide, which was found to be more than 4 times as potent in vitro as unsubstituted PTH, is the most potent analogue of PTH yet synthesized. The enhanced potency was largely attributable to increased affinity for the PTH receptor. In vivo, however, this analogue was only one-third as potent as bPTH-(1–34). Cumulative evidence suggests that the nearly 15-fold decline in the relative potency when the compound was assayed in vivo is due to the substitution of norleucine for methionine. The other analogues, [D-Val^2,Nle^8,Nle^{18},D-Tyr^{34}]bPTH-(1–34)-amide and [D-Val^2,Nle^8,Nle^{18},D-Tyr^{34}]bPTH-(2–34)-amide, were only weakly active in vitro and in vivo, indicating that substitution with D-amino acids at the NH_2 terminus of PTH causes markedly diminished receptor affinity. In fact, the placement of a D-amino acid at the NH_2 terminus is more deleterious to biological activity than is omission of amino acids at positions 1 and 2.

Substitution of D-amino acid enantiomers for naturally occurring amino acids has generated analogues of parathyroid hormone (PTH)[1] (Coltrera et al., 1980), as well as of other peptide hormones (Vavra et al., 1968; Rudinger, 1972; Kastin et al., 1974; Sawyer et al., 1974a,b; Rivier et al., 1975; Ferland et al., 1976; Marshall, 1976; Cobb et al., 1978; Cusan et al., 1977; Manning et al., 1977; Ondetti et al., 1977; Roemer et al., 1977; Vale et al., 1977; Happ et al., 1978; Malfroy et al., 1978; Nillius et al., 1978; Casper & Yen, 1979; Smith & Walker, 1979; Veber, 1980), that are considerably more potent than their native counterparts. Incorporation of a D-tyrosine at the COOH-terminal position (residue 34) of an analogue of the fully active 1–34 fragment of PTH (Potts et al., 1971; Tregear et al., 1974) yielded a compound nearly 3-fold more active than bPTH-(1–34) in stimulating renal adenylate cyclase activity in vitro (Coltrera et al., 1980).

However, the D-amino acid containing analogue of enhanced biological activity was found to be unstable. Within a period of 18 weeks from the completion of synthesis, [D-Tyr^{34}]-bPTH-(1–34)-amide sustained a spontaneous and complete loss of biopotency: biological activity declined from an estimated potency of 14 500 MRC units/mg to an undetected level. Detailed chemical studies revealed the mechanism of inactivation to be an unusual lability of the two methionine residues (at positions 8 and 18) to oxidation (Coltrera et al., 1980). The instability of this analogue precluded accurate evaluation of its biological properties in vivo.

To evaluate in vivo the effects of D-amino acid substitutions on PTH biological activity, we synthesized three sulfur-free analogues of the hormone by substituting norleucine for methionine (Figure 1). Previous studies (Rosenblatt et al., 1976) showed that substitution of norleucine (a nonnatural, sulfur-free amino acid that is nearly isosteric with methionine) for methionine was well tolerated in terms of biopotency and produced oxidation-resistant PTH analogues of demonstrated long-term stability.

The three D-amino acid containing, sulfur-free analogues of PTH were synthesized by the Merrifield solid-phase method (Merrifield, 1963). All three analogues contained a carboxyl-terminal D-tyrosine, and two contained D-amino acid substitutions at the amino terminus: [Nle^8,Nle^{18},D-Tyr^{34}]-bPTH-(1–34)-amide, [D-Val^2,Nle^8,Nle^{18},D-Tyr^{34}]bPTH-(1–34)-amide, and [D-Val^2,Nle^8,Nle^{18},D-Tyr^{34}]bPTH-(2–34)-amide. To permit the most valid comparison of biological activity, we branched a single synthesis to generate the three peptides. Biological properties were evaluated in a renal adenylate cyclase assay in vitro (Krishna et al., 1968; Marcus & Aurbach, 1969, 1971), in a PTH-specific renal radioreceptor assay (Segre et al., 1979a), and in a chick hypercalcemia assay in vivo with intravenous administration of hormone (Parsons et al., 1973).

Experimental Procedures

Synthesis and Purification. Three analogues of bovine parathyroid hormone, [Nle^8,Nle^{18},D-Tyr^{34}]bPTH-(1–34)-amide, [D-Val^2,Nle^8,Nle^{18},D-Tyr^{34}]bPTH-(1–34)-amide, and [D-Val^2,Nle^8,Nle^{18},D-Tyr^{34}]bPTH-(2–34)-amide, were prepared by a modification (Rosenblatt et al., 1976, 1977b; Erickson & Merrifield, 1976; Barany & Merrifield, 1979) of the solid-phase method of Merrifield (1963). The primary structure of the analogues is depicted in Figure 1. Synthesis was performed manually. Benzhydrylamine hydrochloride resin (polystyrene–1% divinylbenzene, Beckman) was employed to effect the carboxyamide ($CONH_2$) COOH-terminal modification. The *tert*-butoxycarbonyl (Boc) group was used to protect α-amino groups during coupling, except for arginine, which was protected by an amyloxycarbonyl group. Amino acid side-function protection was obtained as follows: (a) the serine hydroxyl group was protected as an *O*-benzyl ether; (b) the tyrosine hydroxyl group was protected as the *O*-2,6-dichlorobenzyl ether; (c) the carboxyl group of aspartic and glutamic acids was protected as the benzyl ester; (d) the

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[1] Abbreviations used: PTH, parathyroid hormone; bPTH, bovine parathyroid hormone; Boc, *tert*-butoxycarbonyl; ATP, adenosine 5′-triphosphate; cAMP, adenosine cyclic 3′,5′-monophosphate.

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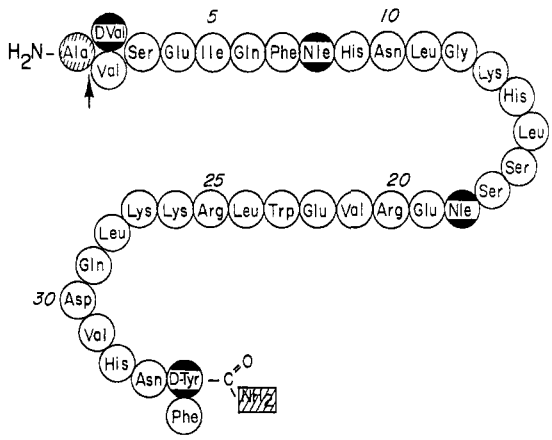

FIGURE 1: Sequence of three analogues of PTH containing D-amino acid substitutions. All analogues contain D-tyrosine substitution for phenylalanine (position 34), norleucine for each methionine (positions 8 and 18), and a carboxamide function at the COOH terminus. The valine of position 2 was replaced with D-valine in two analogues. In addition, the synthesis was terminated after incorporation of the amino acid at position 2 in one analogue, thus producing the compounds [Nle^8,Nle^{18},D-Tyr^{34}]bPTH-(1–34)-amide, [D-Val^2,Nle^8,Nle^{18},-D-Tyr^{34}]bPTH-(1–34)-amide, and [D-Val^2,Nle^8,Nle^{18},D-Tyr^{34}]-bPTH-(2–34)-amide.

histidine imidazole nitrogen and the arginine guanidine function were protected by the *p*-toluenesulfonyl group (histidine was stored as the dicyclohexylamine salt and desalted immediately before use); and (e) the lysine ϵ-amino group was protected by the 2-chlorobenzyloxycarbonyl group. Amino acids were obtained from Bachem Fine Chemicals, Beckman Instruments, and Peninsula Laboratories. Details of the synthetic procedure have been provided previously (Rosenblatt et al., 1976, 1977b; Rosenblatt & Potts, 1977).

Amino acids were incorporated by using dicyclohexylcarbodiimide as the coupling agent, except for glutamine and asparagine, which were coupled as "active" *p*-nitrophenyl esters. Couplings were monitored qualitatively for completeness by the fluorescamine test (Felix & Jimenez, 1973). Double couplings were required to obtain a negative fluorescamine test after addition of lysine at 26, leucine at 24, and isoleucine at 5. Triple couplings were required for glutamine at 29 and asparagine at 10. After double couplings of asparagine at 33, a positive fluorescamine test was obtained; hence, the peptide resin was acetylated to terminate relatively unreactive peptide chains at an early point in the synthesis. Purification of the peptides was performed by gel-filtration followed by ion-exchange chromatography, as previously described (Rosenblatt et al., 1976, 1977b; Rosenblatt & Potts, 1977).

Analytic Methods. Amino acid analyses were conducted with a Beckman Model 121 M-B automated analyzer. Acid hydrolysis was performed in 5.7 N HCl at 110 °C in an evacuated desiccator for 24 h in the presence of 1/2000 (v/v) mercaptoethanol.

The peptides were analyzed by thin-layer chromatography and thin-layer electrophoresis as previously described (Rosenblatt et al., 1977a). Two thin-layer chromatography systems using precoated cellulose plates (100 μm, Brinkmann) and ninhydrin staining were employed: (A) butanol–pyridine–acetic acid–water (15:10:3:12) and (B) pyridine–acetic acid–water (30:1:270). Two thin-layer electrophoresis systems using cellulose-coated plates (100 μm) and ninhydrin staining were employed: (A) pyridine–acetic acid–water (30:1:270), pH 6.5, and (B) pyridine–acetic acid–water (1:10:289), pH 3.5. Sequence analysis was performed to quantitate contamination by deletion-containing error peptides as well as to confirm the presence of the correct amino acid sequence (Tregear, 1975; Tregear et al., 1977). Reverse-phase high-pressure liquid chromatography was performed on a μ-Bondapak column (Waters Associates) with two buffers (buffer 1, 20% acetonitrile and 80% water containing 0.1% trifluoroacetic acid throughout; buffer 2, 90% acetonitrile and 10% water containing 0.1% trifluoroacetic acid throughout), flow rates of 1–5 mL/min, and a linear gradient of 0–100% of buffer 2 over 20 min (Bennett et al., 1980).

Bioassays. (*1*) *Rat Renal Adenylate Cyclase Assay.* Assessment of biological activity in vitro was performed by using a modification of the rat renal cortical adenylate cyclase assay (Krishna et al., 1968; Marcus & Aurbach, 1969, 1971). [^{32}P]ATP and [^{3}H]cAMP were obtained from New England Nuclear. The bPTH standard used in the assays was Medical Research Council house standard, lot MRC 72/286. Each preparation was assayed at least 3 times at multiple concentrations. The separate potency estimates were combined to yield the mean potency of each analogue.

(*2*) *Radioreceptor-Binding Assay.* The assay for the binding of PTH to a renal receptor, based on canine renal cortical membranes and a sulfur-free radioiodinated PTH analogue ligand (Segre et al., 1979a), was used to evaluate the receptor-binding properties of each analogue. The analogues were tested over a concentration range of 1×10^{-9} to 1×10^{-4} M. Each peptide was assayed at least 3 times.

(*3*) *Hypercalcemia Assay in the Chick in Vivo.* The intravenous hypercalcemia assay in the chick (Parsons et al., 1973) was used to assess biological properties in vivo, and, just as in the in vitro system, potencies were estimated in terms of the native bovine PTH-(1–84) (house standard 72/286). Compounds were injected intravenously for this in vivo assay.

Results

Analytic Data. Amino acid composition of each of the three peptides conformed with theoretically predicted amino acid ratios. No heterogeneity of the purified peptides was detected in the thin-layer chromatographic or electrophoretic systems employed. Analytical data obtained for one of the three peptides obtained from the single synthesis, [D-Val^2,Nle^8,Nle^{18},D-Tyr^{34}]bPTH-(1–34)-amide, are provided below. The peptide had R_f = 0.69 in thin-layer chromatographic system A and R_f = 0.83 in system B. Electrophoretic mobility relative to lysine was 0.79 in thin-layer electrophoretic system A and 0.89 in system B. The product appeared homogeneous by analytical high-pressure liquid chromatography (Figure 2). Accumulated "preview" was determined through 30 cycles of automated Edman sequence analysis; the purified peptide contained no more than 4% contamination by deletion-containing error peptides (Tregear, 1975; Tregear et al., 1977). Hence, purity was determined to be 96% or greater.

Bioactivity. Figure 3 depicts stimulation of rat renal cortical membranes in the adenylate cyclase assay by native bPTH-(1–84) (used as the assay standard) and the three analogues. [Nle^8,Nle^{18},D-Tyr^{34}]bPTH-(1–34)-amide is more potent than native bPTH-(1–84). The weighted mean potency of 24 000 MRC units/mg ranks this peptide as the most potent cyclase-stimulating PTH analogue synthesized thus far. The other analogues are only weakly biologically active, and the log dose–response regressions obtained for each of these analogues are not parallel to the regression generated by the native hormone standard, indicating a qualitative difference in the nature of the observed adenylate cyclase stimulation. With this restriction, approximate potencies were calculated at the level of half-maximal stimulation and compared with the potency of unsubstituted bPTH-(1–34) (Table I).

Table I: Biological Activity of Bovine Parathyroid Hormone Analogues

analogue	in vitro rat renal adenylate cyclase assay		in vivo chick hypercalcemia assay	
	potency[a]	rel potency (%)[b]	potency[a]	rel potency (%)[b]
bPTH-(1-34)	5400 (3900-8000)	100	7700 (5300-11300)	100
[D-Tyr^{34}]bPTH-(1-34)-amide[c]	14500 (11000-17000)	270	not tested	
[Nle^{8},Nle^{18},D-Tyr^{34}]bPTH-(1-34)-amide	24000 (21000-28000)	440	2300 (1700-3000)	30
[D-Val^{2},Nle^{8},Nle^{18},D-Tyr^{34}]bPTH-(1-34)-amide[d]	50	2	<100	<1
[D-Val^{2},Nle^{8},Nle^{18},D-Tyr^{34}]bPTH-(2-34)-amide[d]	30	1	<170	2

[a] Combined potency estimate based on at least two independently valid and statistically homogeneous assays. Limits in parentheses represent standard error for each of the peptides. [b] Relative molar potency calculated on the basis of mean potency with activity of the reference peptide, unsubstituted bPTH-(1-34), taken as 100%. [c] Potencies previously reported (Coltrera et al., 1980). Instability of this analogue precluded evaluation of in vivo biopotency; in vitro potency was determined by extrapolation back to the date of completion of synthesis. [d] Response curve nonparallel to the standard. Although potency value cannot be formally assigned, potency has been estimated by comparing activity with that of the standard at half-maximal stimulation by the analogue.

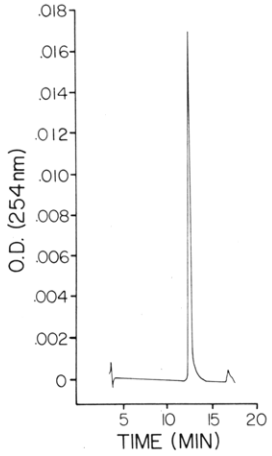

FIGURE 2: Chromatographic profile of [D-Val^{2},Nle^{8},Nle^{18},D-Tyr^{34}]-bPTH-(1-34)-amide, one of the three analogues derived from a single synthesis, obtained by reverse-phase high-pressure liquid chromatography. Conditions employed are those of Bennett et al. (1980) and are given in the text.

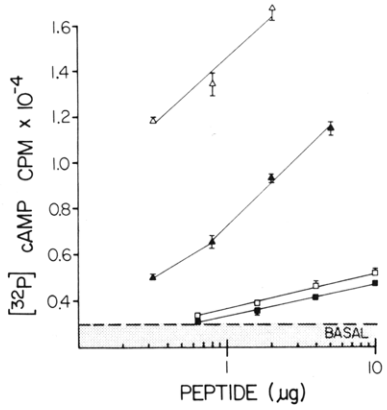

FIGURE 3: Plot of data from a rat renal cortical adenylate cyclase assay of [Nle^{8},Nle^{18},D-Tyr^{34}]bPTH-(1-34)-amide (△), native bovine hormone standard, bPTH-(1-84), 2500 MRC units/mg (▲), [D-Val^{2},Nle^{8},Nle^{18},D-Tyr^{34}]bPTH-(1-34)-amide (□), and [D-Val^{2},Nle^{8},Nle^{18},D-Tyr^{34}]bPTH-(2-34)-amide (■). Each point is the mean of triplicate determinations. Error bars depict standard error. Peptide concentration was quantitated by amino acid analysis.

In vivo biopotencies determined in the chick hypercalcemia assay are also listed in Table I. For the analogue [Nle^{8},Nle^{18},D-Tyr^{34}]bPTH-(1-34)-amide, there is a marked disparity in biopotency in vitro and in vivo relative to unsubstituted bPTH-(1-34). Although this analogue has enhanced potency in vitro, being approximately 440% as active as bPTH-(1-34), the compound is only 30% as active as bPTH-(1-34) in vivo. This represents more than a 90% decline

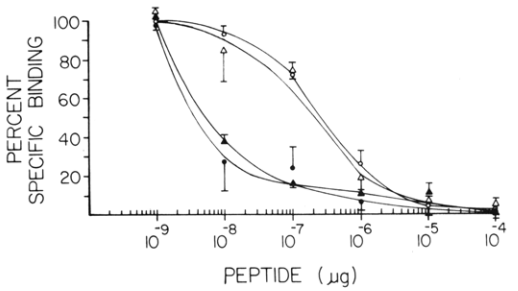

FIGURE 4: Binding properties of three analogues of PTH in a canine renal radioreceptor assay for PTH (Segre et al., 1979a). ^{125}I-Labeled [Nle^{8},Nle^{18},D-Tyr^{34}]bPTH-(1-34)-amide was used as the radioligand. Inhibition of radioligand-specific binding is shown by ● for [Nle^{8},Nle^{18},D-Tyr^{34}]bPTH-(1-34)-amide, by △ for [D-Val^{2},Nle^{8},Nle^{18},D-Tyr^{34}]bPTH-(1-34)-amide, and by ○ for [D-Val^{2},Nle^{8},Nle^{18},D-Tyr^{34}]bPTH-(2-34)-amide. [Nle^{8},Nle^{18},D-Tyr^{34}]bPTH-(1-34)-amide, shown by ▲, served as the standard in this assay. Each point is the mean of triplicate determinations. Error bars depict standard error.

in relative biopotency in the transition from in vitro to vivo bioassay systems.

The remaining two analogues, [D-Val^{2},Nle^{8},Nle^{18},D-Tyr^{34}]bPTH-(1-34)-amide and [D-Val^{2},Nle^{8},Nle^{18},D-Tyr^{34}]-bPTH-(2-34)-amide, are correspondingly weak in bioactivity in vitro and in vivo. The weak potency of these compounds can be directly attributed to a decreased affinity for the PTH receptor, as demonstrated in the renal-receptor binding assay (Figure 4).

Discussion

Changes in hormone conformation induced by substitution with a D-amino acid may alter the interaction between hormone and receptor (Smith & Walter, 1979; Veber, 1980). Alternatively, D-amino acids may confer on a hormone analogue resistance to enzymatic degradation, thus prolonging the analogue's biological survival and availability (Cusan et al., 1977; Ondetti et al., 1977; Roemer et al., 1977; Malfroy et al., 1978). Previous structure–activity studies (Coltrera et al., 1980) demonstrated that incorporation of D-amino acid substitutions in analogues of PTH could enhance biological activity in vitro. For these reasons, incorporation of D-amino acid substitutions was thought to be a promising approach for the design of PTH analogues of enhanced biological activity in vivo.

PTH analogues containing either COOH-terminal modifications alone or combined COOH- and NH_2-terminal modifications were synthesized previously, and their biological activity was evaluated in vitro (Parsons et al., 1975; Tregear & Potts, 1975; Rosenblatt & Potts, 1977). However, marked

instability of biological activity was observed for the most potent of these compounds, [D-Tyr^{34}]bPTH-(1–34)-amide, due to spontaneous oxidation of methionine (Coltrera et al., 1980). This instability precluded a program of biological evaluation in vivo. To obtain comparable but stable analogues for studies in vivo, we synthesized sulfur-free analogues of PTH corresponding to the methionine-containing analogues previously prepared (Coltrera et al., 1980).

Biological evaluation of these analogues was undertaken in three different assay systems to probe several issues. The rat renal adenylate cyclase assay was utilized to determine biological activity in vitro and to provide direct comparison of the potency of the sulfur-free analogues with the series of D-amino acid containing analogues synthesized and evaluated previously (Coltrera et al., 1980). In addition, the rat renal adenylate cyclase assay utilizes a relatively unpurified renal membrane preparation that is rich in enzymes that rapidly degrade parathyroid hormone. Hence, this system serves as an important screening assay for compounds that might possess enhanced biological activity as a result of design features that provide resistance to enzymatic degradation.

The renal radioreceptor-binding assay, based on highly purified canine renal membranes, is relatively devoid of PTH-degrading enzymatic activity. This assay provides a measure of the affinity of interaction of analogues with the PTH-specific renal receptor (Segre et al., 1979a).

The chick hypercalcemia assay was used to evaluate biological properties in vivo and the biological action of PTH on bone. Radiocalcium studies show that the hypercalcemic response observed in this assay requires 1 h or more to develop and reflects calcium mobilization from bone (Parsons et al., 1973). However, activation of receptor takes place within 4 min (Parsons & Potts, 1972). Hence, rapid destruction or clearance of peptide is unlikely to account entirely for differences in biological properties displayed in this assay vs. assays in vitro. In general, there has been a very close correlation between biological properties of analogues observed in vitro and in vivo (Tregear et al., 1973; Parsons et al., 1975).

The analogue [Nle^{8},Nle^{18},D-Tyr^{34}]bPTH-(1–34)-amide, which was more than 4-fold as active as unsubstituted bPTH-(1–34) in vitro, ranks as the most potent PTH analogue yet synthesized. This increased bioactivity correlated only partially with increased affinity for PTH-specific renal binding sites. However, this analogue appeared to have an equal or slightly increased affinity for PTH receptors relative to [Nle^{8},Nle^{18},Tyr^{34}]bPTH-(1–34)-amide, as determined in the renal-receptor binding assay. The variability and lower degree of precision of the radioligand assay compared to the adenylate cyclase assay do not preclude the possibility that the analogue has increased avidity for receptors. Alternatively, for this analogue increased potency in the cyclase assay may result from enhanced efficacy of cyclase stimulation once receptor binding has occurred and not from increased receptor avidity per se.

Use of norleucine as a methionine substitute, although affording resistance to oxidation, is not responsible for enhancement of bioactivity. In fact, this modification results in diminished biopotency. In the rat renal adenylate cyclase assay, norleucine substitution for methionine causes a 50–60% decline in biopotency (Rosenblatt et al., 1976). In the chick hypercalcemia assay in vivo, the analogue [Nle^{8},Nle^{18}]-bPTH-(1–34) is only 25% as active (potency = 1800 MRC units/mg) as unsubstituted bPTH-(1–34) (J. A. Parsons, G. W. Tregear, and M. Rosenblatt, unpublished data). Similar findings were obtained for the analogue [Nle^{8},Nle^{18},Tyr^{34}]-bPTH-(1–34)-amide, which is almost 200% as active as bPTH-(1–34) in vitro but only 33% as active (potency = 2500 MRC units/mg) in vivo (J. A. Parsons, G. W. Tregear, and M. Rosenblatt, unpublished data). Taken together with the present studies, the cumulative evidence indicates that the norleucine substitution is responsible for the nearly 15-fold decline in biopotency in vivo observed for the analogue [Nle^{8},Nle^{18},D-Tyr^{34}]bPTH-(1–34)-amide.

This evidence concerning a deleterious effect of norleucine on biological activity of PTH analogues, especially in vivo, has important implications for the design of PTH antagonists. Recently, a potent competitive inhibitor of PTH action in vitro, [Nle^{8},Nle^{18},Tyr^{34}]bPTH-(3–34)-amide, which inhibits by 50% PTH-stimulated levels of cAMP when present at concentrations equimolar to PTH, was found to be devoid of either agonist or antagonist properties in vivo in the rat and the chick (R. W. Stevenson and J. A. Parsons, personal communication) and to possess weak, but definite, agonist and perhaps weak antagonist properties in vivo in the dog (Segre et al., 1979b). In view of the present findings, it is quite possible that some of the loss of inhibitory action evident in vitro when the analogue [Nle^{8},Nle^{18},Tyr^{34}]bPTH-(3–34)-amide was tested in vivo is attributable to diminished receptor interaction as a result of the presence of norleucine instead of methionine.

Further decline in potency in vivo occurred when a D-amino acid was placed at position 2. The analogues [D-Val^{2},Nle^{8},Nle^{18},D-Tyr^{34}]bPTH-(1–34)-amide and [D-Val^{2},Nle^{8},Nle^{18},D-Tyr^{34}]bPTH-(2–34)-amide were less than 4% as active as bPTH-(1–34) in vivo. An altered and less favored (by the PTH receptor) conformation of the molecule resulting from incorporation of a D-amino acid appears to be responsible for the decline in biopotency. Our studies in vitro in the radioreceptor assay show that placement of a D-amino acid at the COOH terminus tends to enhance affinity, but placement of a D-amino acid at the NH_2-terminal region is so poorly tolerated that the net affinity for the receptor falls by a factor of approximately 30, despite the simultaneous presence of the affinity-enhancing modifications at the COOH terminus. In fact, placement of a D-amino acid in the NH_2-terminal region is more deleterious to biological activity than is the complete omission of the NH_2 terminus: the compound [Nle^{8},Nle^{18},Tyr^{34}]bPTH-(3–34)-amide, which is lacking residues at positions 1 and 2, is equal in binding affinity to [Nle^{8},Nle^{18},Tyr^{34}]bPTH-(1–34)-amide (Segre et al., 1979a,b; Nussbaum et al., 1980), and in vivo bPTH-(2–34) is approximately 50% as potent as bPTH-(1–34) (Tregear et al., 1973; Parsons et al., 1975). Although the effects, on in vivo bioactivity, of placing of D-amino acids in the COOH-terminal region (and perhaps in other regions away from the NH_2 terminus) appear promising, a more extensive evaluation of their actual value must await either the synthesis of oxidation-stable analogues of PTH that do not contain norleucine or the development of storage conditions that preserve the biological activity of potent methionine-containing analogues of the hormone.

Acknowledgments

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