Analogues of Parathyroid Hormone Containing D-Amino Acids: Evaluation of Biological Activity and Stability†

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ABSTRACT: Four analogues of parathyroid hormone (PTH) containing D-amino acids were synthesized. Substitutions were made within the fully biologically active fragment of parathyroid hormone in the amino-terminal region, at position 2, and at the carboxyl terminus, at position 34. The carboxyl-terminal region contains structural determinants important to receptor binding. The amino-terminal region plays a critical role in the hormone-stimulated activation of adenylate cyclase in vitro and the expression of hormonal activity in vivo. Placement of a D-amino acid at the carboxyl terminus yielded an analogue, [D-Tyr^{34}]bPTH-(1–34)-amide, 270% as active in the in vitro renal adenylate cyclase assay as unsubstituted bPTH-(1–34). In contrast, placement of a D-amino acid in the amino-terminal region, as in analogues [D-Val^{2},D-Tyr^{34}]-bPTH-(1–34)-amide and [D-Val^{2},D-Tyr^{34}]bPTH-(2–34)-amide, resulted in nearly a complete loss of in vitro biological activity. Deletion of a single residue at the amino terminus, as in the analogue [D-Tyr^{34}]bPTH-(2–34)-amide, also caused nearly total loss of biopotency. These marked declines in potency occurred despite the presence of activity-enhancing modifications at the carboxyl terminus of the latter three analogues. The most potent of the analogues, [D-Tyr^{34}]-bPTH-(1–34)-amide, sustained an apparently spontaneous and complete loss of biopotency over a period of several weeks. Detailed studies of the mechanism of inactivation revealed an unusual lability of methionine residues to oxidation. Reduction under controlled conditions restored nearly completely both the methionine content and biological activity. These studies indicate that placement of D-amino acids in the parathyroid hormone sequence markedly alters hormonal activity: the change in bioactivity depends critically on the position selected for substitution. The observed instability of bioactivity also illustrates the need to either monitor biopotency of parathyroid hormone and its analogues or utilize oxidation-stable hormone analogues in investigations of parathyroid hormone-mediated biological effects.

Substitution of D-amino acid enantiomers for naturally occurring amino acids has generated analogues of several peptide hormones or other biologically active peptides that are considerably more potent than their native counterparts (Ferland et al., 1976; Happ et al., 1978; Kastin et al., 1974; Vale et al., 1977; Nillius et al., 1978; Casper & Yen, 1979; Manning et al., 1977; Vávra et al., 1968; Sawyer et al., 1974a,b; Cobb et al., 1978; Rudinger, 1971; Marshall, 1976; Roemer et al., 1977; Cusan et al., 1977; Malfroy et al., 1978; Smith & Walter, 1979; Veber, 1980; Rivier et al., 1975; Ondetti et al., 1977). The enhanced bioactivity observed for hormone analogues containing D-amino acids may be the result of one or more mechanisms. Changes in hormone conformation produced 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 its biological survival and availability (Roemer et al., 1977; Cusan et al., 1977; Malfroy et al., 1978; Ondetti et al., 1977).

For parathyroid hormone (PTH),[1] previous structure–activity studies (Tregear & Potts, 1975; Tregear et al., 1973; Goltzman et al., 1975; Herrmann-Erlee et al., 1976; Mahaffey et al., 1979; Rosenblatt et al., 1976, 1977a; Rosenblatt & Potts, 1977; Parsons et al., 1975) directed attention to the amino- and carboxyl-terminal regions of the fully biologically active fragment of PTH, the sequence 1–34 (Potts et al., 1971; Tregear et al., 1974), because of the marked alteration in bioactivity that results when structures in these regions are modified. In particular, certain modifications of either position 1 or position 34 can produce up to a threefold increase in biological activity both in vitro and in vivo (Parsons et al., 1975; Rosenblatt & Potts, 1977).

Consequently, we undertook examination of the effects on biological activity resulting from placement of D-amino acids in either the amino- or carboxyl-terminal region of PTH-(1–34). Four D-amino acid-containing hormone analogues were synthesized by the Merrifield solid-phase method (Merrifield, 1962, 1963, 1969): [D-Tyr^{34}]bPTH-(1–34)-amide, [D-Val^{2},D-Tyr^{34}]bPTH-(1–34)-amide, [D-Tyr^{34}]bPTH-(2–34)-amide, and [D-Val^{2},D-Tyr^{34}]bPTH-(2–34)-amide. A single synthesis was branched to obtain all four peptides, to permit the most valid comparison of the biological activity of these analogues. The biological activity of these analogues was evaluated in the in vitro rat renal adenylate cyclase assay (Krishna et al., 1968; Marcus & Aurbach, 1969, 1971).

Experimental Procedures

Synthesis and Purification. Four analogues of bovine parathyroid hormone, [D-Tyr^{34}]bPTH-(1–34)-amide, [D-Val^{2},D-Tyr^{34}]bPTH-(1–34)-amide, [D-Val^{2},D-Tyr^{34}]bPTH-(2–34)-amide, and [D-Tyr^{34}]bPTH-(2–34)-amide, were prepared by a modification (Rosenblatt et al., 1976, 1977c; Erickson & Merrifield, 1976) of the solid-phase method of Merrifield (1962, 1963, 1969). 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 ($CONH_2$) carboxyl-terminal modification.

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[1] Abbreviations used: PTH, parathyroid hormone; bPTH, bovine PTH; hPTH, human PTH; MRC, Medical Research Council (United Kingdom); ATP, adenosine 5′-triphosphate; cAMP, adenosine cyclic 3′,5′-phosphate.

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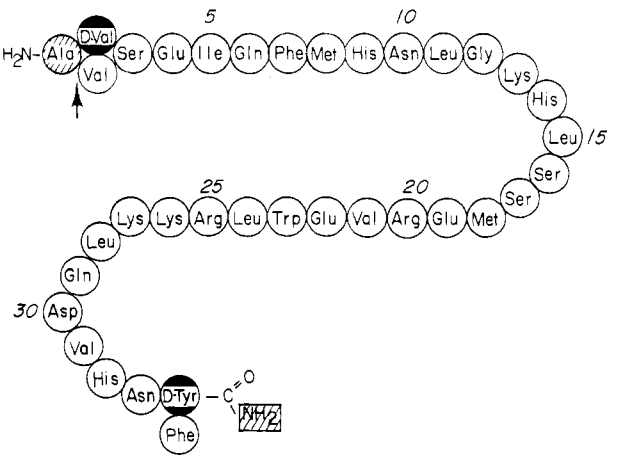

FIGURE 1: Sequence of four analogues of PTH containing D-amino acid substitutions. All four analogues contain D-tyrosine substitution for phenylalanine and a carboxylamide function at the carboxyl terminus, position 34. The valine of position 2 was replaced with D-valine in two analogues. In addition, the synthesis was terminated after incorporation of the amino acid at position 2 (→) in two analogues, thus producing the compounds [D-Tyr^{34}]bPTH-(1–34)-amide, [D-Val^{2},D-Tyr^{34}]bPTH-(1–34)-amide, [D-Tyr^{34}]bPTH-(2–34)-amide, and [D-Val^{2},D-Tyr^{34}]bPTH-(2–34)-amide.

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 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); (e) the lysine ϵ-amino group was protected by the 2-chlorocarbobenzoxy group. L-Amino acids were obtained from Beckman Instruments and Peninsula Laboratories; D-amino acids were obtained from Bachem Chemicals and Peninsula Laboratories. Details of the synthetic procedure have been provided previously (Rosenblatt et al., 1976, 1977c; Rosenblatt & Potts, 1977).

Amino acids were incorporated by using dicyclohexylcarbodiimide as the coupling agent, except 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 glutamine at 29, leucine at 28, lysine at 27, tryptophan at 23, glutamic acid at 22, arginine at 20, and methionine at 8. Triple couplings were required for leucine at 24 and arginine at 25. Purification of the peptides was performed by gel filtration, followed by ion-exchange chromatography, as previously described (Rosenblatt et al., 1976, 1977c; 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. Total enzymic digestions were performed by using papain (enzyme/substrate ratio 1:50, pH 5.4, 2 h, 37 °C), followed by aminopeptidase M (enzyme/substrate ratio 1.5:1.0, pH 8.2, 3 h, 37 °C) (Keutmann et al., 1971).

The peptides were analyzed by thin-layer chromatography and thin-layer electrophoresis as previously described (Rosenblatt et al., 1977b). 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); (B) pyridine/acetic acid/water (30:1:270). Two thin-layer electrophoretic systems using cellulose-coated plates (100 μm) and ninhydrin staining were employed: (A) pyridine/acetic acid/water (30:1:270), pH 6.5; (B) 2% formic acid and 8% acetic acid, pH 2.0. 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 with a C_{18} μBondapak column (Waters Associates), two buffers (buffer 1, 20% acetonitrile and 80% water with 0.1% trifluoroacetic acid throughout; buffer 2, 50% acetonitrile and 50% water with 0.1% trifluoroacetic acid throughout), a flow rate of 1.5 mL/min, and a linear gradient of 0–100% buffer 2 over 20 min (Bennett et al., 1980).

Bioassay. 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 Standard, lot no. MRC 72/286. Each preparation, except [D-Tyr^{34}]bPTH-(1–34)-amide, was assayed at least 3 times at multiple concentrations within 4 weeks of completion of purification. Preparations were not treated with reducing agents prior to assay, except as described below for a single preparation of [D-Tyr^{34}]bPTH-(1–34)-amide. The separate potency estimates were combined to yield the mean potency of each analogue. For [D-Tyr^{34}]bPTH-(1–34)-amide, a combined potency determination was not possible because of the observed instability of biological activity.

Reduction of Oxidized [D-*Tyr*^{34}]*bPTH-(1–34)-amide.* [D-Tyr^{34}]bPTH-(1–34)-amide (400 μg), which had declined (presumably due to oxidation) to an undetectable level of bioactivity, was reduced by treatment with 1.0 mL of 0.1 M ammonium acetate (pH 7.4) that was 2 M in mercaptoethanol for 20 h at 37 °C. After treatment, the solution was diluted with 2.0 mL of water, placed on a gel-filtration (P-2, Bio-Rad) column for desalting, then frozen and lyophilized, and immediately assayed.

Results

Analytical Data. Amino acid analysis of each of the four peptides is presented in Table I. No heterogeneity of the purified peptides was detected in the thin-layer chromatographic or electrophoretic systems employed. Analytical data for one of the four peptides obtained from the single synthesis, [D-Val^{2},D-Tyr^{34}]bPTH-(1–34)-amide, are provided. The peptide had R_f = 0.62 in TLC system A and R_f = 0.81 in system B. Electrophoretic mobility relative to leucine was 0.8 in thin-layer electrophoretic system A and 1.43 in system B. 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). A high-pressure liquid chromatographic profile of 20 μg of [D-Val^{2},D-Tyr^{34}]bPTH-(1–34)-amide is shown in Figure 2. Only a small amount of heterogeneity was revealed; contaminants were estimated to be <5%.

Bioactivity. Figure 3 depicts stimulation of rat renal cortical membranes in the adenylate cyclase assay by native bPTH-(1–84) and the four D-amino acid-containing analogues. [D-

Table I: Amino Acid Content of Synthetic Peptides[a]

residue	predicted	[D-Tyr^{34}]bPTH-(1–34)-amide obtained	[D-Val^{2},-D-Tyr^{34}]bPTH-(1–34)-amide obtained	predicted	[D-Tyr^{34}]bPTH-(2–34)-amide obtained	[D-Val^{2},-D-Tyr^{34}]bPTH-(2–34)-amide obtained
Asp	3	3.2	3.3	3	3.1	3.1
Ser[b]	3	2.8	3.1	3	2.8	2.8
Glu	5	5.3	5.4	5	5.1	5.0
Gly	1	1.1	1.2	1	1.2	1.1
Ala	1	0.8	0.9	0	<0.1	<0.1
Val	3	2.9	3.0	3	2.8	2.8
Met	2	1.9	2.0	2	1.8	2.0
Ile	1	0.9	0.9	1	0.9	0.9
Leu	4	4.2	4.4	4	4.2	4.2
Tyr	1	0.9	0.9	1	1.0	1.0
Phe	1	0.8	0.7	1	1.0	1.0
Lys	3	3.2	3.2	3	3.2	3.2
His	3	3.0	3.0	3	3.0	3.1
Arg	2	2.1	2.1	2	2.1	2.1

[a] All values represent the average of three separate aliquots of peptide after acid hydrolysis. Tryptophan content was not determined. [b] Corrected for degradative losses occurring during acid hydrolysis.

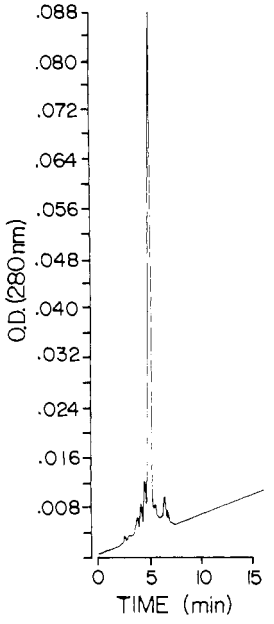

FIGURE 2: Chromatographic profile of [D-Val^{2},D-Tyr^{34}]bPTH-(1–34)-amide, one of the four analogues derived from a single synthesis, obtained by reverse-phase high-pressure liquid chromatography. Conditions employed are those of Bennett et al. (1980) and are given in the text.

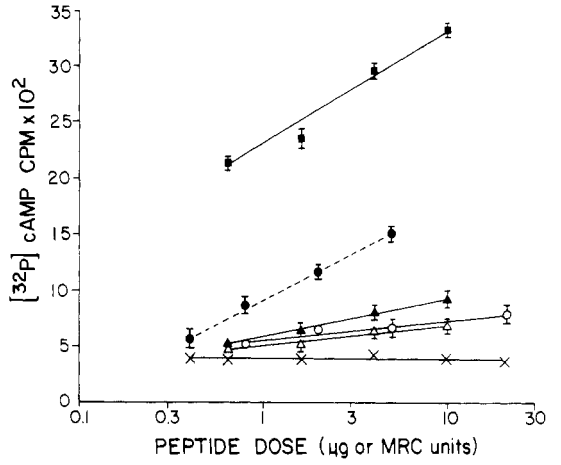

FIGURE 3: Composite of representative rat renal cortical adenylate cyclase assays of the following: [D-Tyr^{34}]bPTH-(1–34)-amide (■); native bovine hormone standard, bPTH-(1–84), 2500 MRC units/mg (●); [D-Tyr^{34}]bPTH-(2–34)-amide (▲); [D-Val^{2},D-Tyr^{34}]bPTH-(2–34)-amide (○); [D-Val^{2},D-Tyr^{34}]bPTH-(1–34)-amide (△); [D-Tyr^{34}]bPTH-(1–34)-amide (×), assayed 18 weeks after purification was completed. Each point is the mean of triplicate determinations. Peptide concentration was quantitated by amino acid analysis. Each peptide was assayed within 4 weeks of completion of purification without prior treatment with reducing agents.

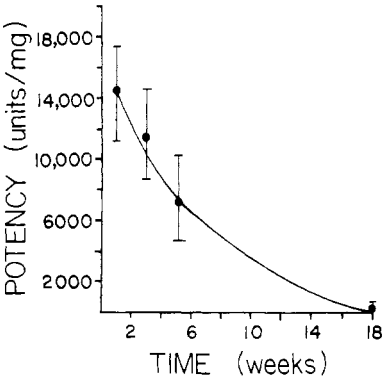

FIGURE 4: Biological activity of [D-Tyr^{34}]bPTH-(1–34)-amide vs. time elapsed from the completion of synthesis. Bioactivity was determined without prior treatment by reducing agents. Error bars depict the standard error of the mean.

Tyr^{34}]bPTH-(1–34)-amide is more potent than native bPTH-(1–84). The other analogues are only weakly biologically active, and the dose–response curves obtained for each of these analogues are nonparallel to the curve generated by the native hormone standard, indicating a qualitative difference in the nature of the observed adenylate cyclase stimulation. Mean potencies for each of the four analogues are listed and compared with the potency of unsubstituted bPTH-(1–34) and a previously synthesized (Rosenblatt & Potts, 1977) highly active PTH analogue, [Tyr^{34}]bPTH-(1–34)-amide, in Table II. Owing to instability of bioactivity of the analogue [D-Tyr^{34}]bPTH-(1–34)-amide, the potency of 14 500 MRC units/mg obtained in the first assay of this analogue, 10 days after completion of purification, may actually be an underestimate of the true potency. A potency estimate made by extrapolation back to completion of synthesis suggests that the actual potency may be 18 000 MRC units/mg or higher.

Instability of [D-*Tyr*^{34}]*bPTH-(1–34)-amide.* A progressive decline in biological activity was observed for the analogue [D-Tyr^{34}]bPTH-(1–34)-amide. The peptide had been stored in a closed glass vial at room temperature in the dark. Within 18 weeks of completion of purification, potency fell from 14 500

Table II: Biological Activity of Bovine Parathyroid Hormone Analogues in Rat Renal Adenylate Cyclase Assay

substitution	fragment length	combined potency estimate[a]	rel potency[b] (%)
none	1-34[c]	5400 (3900-8000)	100
[Tyr^{34}]	1-34 amide[c]	16000 (11000-23000)	300
[D-Tyr^{34}]	1-34 amide	14500 (11000-17000)	270
[D-Tyr^{34}]	2-34 amide	130[d] (120-150)	3
[D-Val^2, D-Tyr^{34}]	2-34 amide	90[d] (60-100)	2
[D-Val^2, D-Tyr^{34}]	1-34 amide	80[d] (60-100)	1

[a] Combined potency estimate based on three independent assays, except for the compound [D-Tyr^{34}]bPTH-(1-34)-amide. Limits in parentheses represent standard error of the mean for each of the peptides, except [D-Tyr^{34}]bPTH-(1-34)-amide, for which 95% confidence limits are provided. Peptides were assayed without prior treatment by reducing agents. [b] Relative potency calculated on the basis of the mean potency with the activity of the reference peptide, unsubstituted bPTH-(1-34), taken as 100%. [c] Potencies previously reported (Potts et al., 1971; Rosenblatt & Potts, 1977). [d] Response curve nonparallel to that of the standard. Although the potency value cannot be formally assigned, potency has been estimated by comparing the activity with that of the standard at half-maximal stimulation by the analogue.

MRC units/mg to an undetectable level. Intermediate potencies are plotted as a function of time elapsed from completion of purification (Figure 4).

Accompanying the loss of biological activity was a complete loss of methionine content as determined by amino acid analysis after enzymic hydrolysis. No methionine sulfone was detected at that time (18 weeks), indicating probable spontaneous oxidation of methionines to the intermediate oxidation state of methionine sulfoxide. Controlled reduction of the inactive material caused a 90% restoration of methionine content with a concomitant restoration of most of the bioactivity—the potency of reduced material was 11100 MRC units/mg.

Discussion

Structure–activity studies for parathyroid hormone have thus far followed two general directions. The first has been synthesis of fragments of the fully biologically active segment of the molecule [the amino-terminal region (1–34)] to ascertain the role of structural determinants in the expression of parathyroid hormone action. From these studies the minimum sequence necessary for biological activity both in vitro and in vivo was determined (Tregear et al., 1973; Parsons et al., 1975). In addition, the fully biologically active fragment was found to contain a small region, the two amino-terminal amino acids (positions 1 and 2), critical for the activation of adenylate cyclase once receptor binding has occurred. This region is distinct from the region 3–34, which is largely responsible for binding to presumed parathyroid hormone receptors (Goltzman et al., 1975; Rosenblatt et al., 1977a). Further structure–activity studies (Mahaffey et al., 1979) of the binding region using a renal radioreceptor assay (Segre et al., 1979) led to the identification of a minimum binding sequence that is greater than seven but less than or equal to ten amino acids in length at the carboxyl terminus of the active segment (region 25–34) (Rosenblatt et al., 1980).

The second major direction pursued has been the substitution of naturally occurring amino acids at selected positions along the native sequence of parathyroid hormone. Substitutions at position 1 were extensively explored by Tregear & Potts (1975). Even subtle structural modifications were found to confer large changes in biopotency of PTH. Similarly, modification of the carboxyl-terminal residue, phenylalanine at position 34, yields analogues of enhanced activity. Substitution of tyrosine for phenylalanine, i.e., the addition of a single hydroxyl function, results in an analogue 140% as potent as unsubstituted bPTH-(1–34) (Rosenblatt et al., 1976). Conversion of the carboxyl-terminal carboxylic acid to a carboxyamide produces a compound 270% as active as bPTH-(1–34) (Parsons et al., 1975), and combining both of these modifications yields the analogue [Tyr^{34}]bPTH-(1–34)-amide, which is nearly 300% as potent as bPTH-(1–34) (Rosenblatt & Potts, 1977).

Because these earlier studies suggested that large changes in bioactivity occur with alterations or substitutions at the amino and carboxyl termini (the activation and binding regions, respectively, of the active fragment of PTH), a third direction of analogue design, namely, the use of nonnatural amino acids, was pursued. Placement of a D-amino acid at the carboxyl terminus of the active fragment of PTH is well tolerated in terms of in vitro bioactivity. The analogue [D-Tyr^{34}]bPTH-(1–34)-amide is nearly equal in potency to the most active analogue of PTH yet synthesized, [Tyr^{34}]-bPTH-(1–34)-amide (Table II). Hence, future incorporation of D-amino acids at the carboxyl terminus of PTH analogues may generate both agonists and antagonists of enhanced activity which may prove to be more active in vivo than in vitro because D-amino acids confer resistance to enzymatic degradation. In addition, such analogues would be ideally suited to the investigation of the physiological role of the cleavage (which occurs between positions 33 and 34) of PTH observed in vivo (Rosenblatt et al., 1977c; Martin et al., 1978, 1979).

Structural alteration of the amino-terminal activation region of the molecule is poorly tolerated in terms of biopotency. Substitution of D-valine for the naturally occurring L-valine at position 2 causes a nearly complete loss of biological activity, as determined by adenylate cyclase assay of the analogues [D-Val^2,D-Tyr^{34}]bPTH-(1–34)-amide and [D-Val^2,D-Tyr^{34}]-bPTH-(2–34)-amide. Similarly, deletion of position 1 in the analogue [D-Tyr^{34}]bPTH-(2–34)-amide causes a nearly complete loss of bioactivity (Table II). In all three of these analogues, the marked decline in potency [potencies <3% that of unsubstituted bPTH-(1–34)] resulting from amino-terminal structural modification occurs despite the presence of activity-enhancing modifications at the carboxyl terminus of the molecule.

Evaluation of the biological activity in vivo of the most potent of the PTH analogues was precluded because of chemical instability of the compound. The total loss of biological activity in vitro of the analogue [D-Tyr^{34}]-bPTH-(1–34)-amide is attributable to the spontaneous oxidation, over a period of 18 weeks, of both of the methionine residues (present at positions 8 and 18) in PTH. Oxidation of methionine was previously demonstrated to result in the complete loss of PTH bioactivity (Rasmussen & Craig, 1962; Tashjian et al., 1964; Potts et al., 1966; O'Riordan et al., 1974). This occurred despite the storage of the peptide as a lyophilized powder in a closed vial in the dark.

The spontaneous loss in bioactivity of the analogue [D-Tyr^{34}]bPTH-(1–34)-amide seems to be much more rapid than the spontaneous decline in biopotency of the larger molecule, native bPTH-(1–84), which occurs over a period of months to years when it has been observed in our laboratory. We have observed similar, although less marked, losses of bioactivity of other potent analogues or fragments of PTH, such as bPTH-(1–34)-amide, [Tyr^{34}]bPTH-(1–34)-amide, and

hPTH-(1–34) (unpublished experiments). Our findings indicate that synthetic fragments of the hormone may be more susceptible to spontaneous oxidation than is the native hormone, perhaps because the shorter, synthetic peptides assume conformations that render methionines more exposed than they are in the native molecule. The instability in biological activity of a PTH analogue emphasizes the need to monitor PTH analogue biopotency during any course of experiments lasting several weeks or longer. Specialized storage conditions for PTH or PTH analogues in a reducing atmosphere or in the presence of reducing agents need to be developed and systematically evaluated for effectiveness in preserving hormonal activity over long periods.

Alternatively, evaluation of the promising effects of D-amino acid substitutions on bioactivity in vivo may require an additional approach, namely, the synthesis of a group of analogues incorporating further structural modifications. In addition to D-amino acids, oxidation-stable, sulfur-free analogues containing norleucine instead of methionine, a substitution previously shown to be well tolerated in terms of biopotency (Rosenblatt et al., 1976, 1977a; Rosenblatt & Potts, 1977), may have to be prepared to assure long-term stability of biopotency. The PTH analogue [D-Tyr^{34}]bPTH-(1–34)-amide provides a particularly important example of the need to develop approaches that preserve the bioactivity of PTH analogues. The initial biopotency of this analogue may be considerably greater than that obtained on the first assay performed 10 days after purification. Hence, the actual value of certain structural modifications in enhancing bioactivity could go unrecognized unless appropriate methods for stabilizing bioactivity are found.

Acknowledgments

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References

Bennett, H. P. F., Browne, C. A., Goltzman, D., & Solomon, S. (1980) in *Peptides: Structure and Biological Function* (Gross, E., & Meienhofer, Eds.) pp 121–124, Pierce Chemical Co., Rockford, IL.

Casper, R. F., & Yen, S. S. C. (1979) *Science (Washington, D.C.) 205*, 408.

Cobb, W. E., Spare, S., & Reichlin, S. (1978) *Ann. Intern. Med. 88*, 183.

Cusan, L., Dupont, A., Kledzik, G. S., Labrie, F., Coy, D. H., & Schally, A. V. (1977) *Nature (London) 268*, 544.

Erickson, B. W., & Merrifield, R. B. (1976) *Proteins 2*, 255–527.

Felix, A. M., & Jimenez, M. H. (1973) *Anal. Biochem. 52*, 377.

Ferland, L., Labrie, F., Savary, M., Beaulieu, M., Coy, D. H., Coy, E. J., & Schally, A. V. (1976) *Clin. Endocrinol. (Oxford) 5*, 279s.

Goltzman, D., Peytremann, A., Callahan, E., Tregear, G. W., & Potts, J. T., Jr. (1975) *J. Biol. Chem. 250*, 3199.

Happ, J., Weber, T., Callensee, W., Ermert, J. A., Eshkol, A., & Beyer, J. (1978) *Fertil. Steril. 29*, 552.

Herrmann-Erlee, M. R. M., Heersche, J. N. M., Hekkelman, J. W., Gaillard, P. J., Tregear, G. W., Parsons, J. A., & Potts, J. T., Jr. (1976) *Endocr. Res. Commun. 3*, 21.

Kastin, A. J., Schally, A. V., Gonzales-Barcena, D., Coy, D. H., Miller, M. C., Porias, H., & Schalch, D. S. (1974) *J. Clin. Endocrinol. Metab. 38*, 801.

Keutmann, H. T., Aurbach, G. D., Dawson, B. F., Niall, H. D., Deftos, L. J., & Potts, J. T., Jr. (1971) *Biochemistry 10*, 2779.

Krishna, G., Weiss, B., & Brodie, B. B. (1968) *J. Pharmacol. Exp. Ther. 163*, 379.

Mahaffey, J. E., Rosenblatt, M., Shepard, G. L., & Potts, J. T., Jr. (1979) *J. Biol. Chem. 254*, 6496.

Malfroy, B., Swerts, J. P., Guyon, A., Roques, B. P., & Schwartz, J. C. (1978) *Nature (London) 276*, 523.

Manning, M., Lowbridge, J., Haldar, J., & Sawyer, W. H. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36*, 1848.

Marcus, R., & Aurbach, G. D. (1969) *Endocrinology 85*, 801.

Marcus, R., & Aurbach, G. D. (1971) *Biochim. Biophys. Acta 242*, 410.

Marshall, G. R. (1976) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35*, 2494.

Martin, K. J., Freitag, J. J., Conrades, M. B., Hruska, K. A., Klahr, S., & Slatopolsky, E. (1978) *J. Clin. Invest. 62*, 256.

Martin, K. J., Hruska, K. A., Freitag, J. J., Klahr, S., & Slatopolsky, E. (1979) *N. Engl. J. Med. 301*, 1092.

Merrifield, R. B. (1962) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 21*, 412.

Merrifield, R. B. (1963) *J. Am. Chem. Soc. 85*, 2149.

Merrifield, R. B. (1969) *Adv. Enzymol. Relat. Areas Mol. Biol. 32*, 221.

Nillius, S. J., Bergquist, C., & Weid, L. (1978) *Contraception 17*, 537.

Ondetti, M. A., Rubin, B., & Cushman, D. W. (1977) *Science (Washington, D. C.) 196*, 441.

O'Riordan, J. L. H., Woodhead, J. S., Hendy, G. N., Parsons, J. A., Robinson, C. J., Keutmann, H. T., Dawson, B. F., & Potts, J. T., Jr. (1974) *J. Endocrinol. 63*, 117.

Parsons, J. A., Rafferty, B., Gray, D., Zanelli, J. M., Keutmann, H. T., Tregear, G. W., Callahan, E. N., & Potts, J. T., Jr. (1975) *Int. Congr. Ser.—Excerpta Med. No. 346*, 33–39.

Potts, J. T., Jr., Sherwood, L. M., & Aurbach, G. D. (1966) *Recent Prog. Horm. Res. 22*, 101.

Potts, J. T., Jr., Tregear, G. W., Keutmann, H. T., Niall, H. D., Sauer, R., Deftos, L. J., Dawson, B. F., Hogan, M. L., & Aurbach, G. D. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 63.

Rasmussen, H., & Craig, L. C. (1962) *Recent Prog. Horm. Res. 18*, 269.

Rivier, J., Brown, M., & Vale, W. (1975) *Biochem. Biophys. Res. Commun. 65*, 746.

Roemer, D., Buescher, H. H., Hill, R. C., Pless, J., Bauer, W., Cardinaux, F., Closse, A., Hauser, D., & Huguenin, R. (1977) *Nature (London) 268*, 547.

Rosenblatt, M., & Potts, J. T., Jr. (1977) *Endocr. Res. Commun. 4*, 115.

Rosenblatt, M., Goltzman, D., Keutmann, H. T., Tregear, G. W., & Potts, J. T., Jr. (1976) *J. Biol. Chem. 251*, 159.

Rosenblatt, M., Callahan, E. N., Mahaffey, J. E., Pont, A., & Potts, J. T., Jr. (1977a) *J. Biol. Chem. 252*, 5847.

Rosenblatt, M., Keutmann, H. T., Tregear, G. W., & Potts, J. T., Jr. (1977b) *J. Med. Chem. 20*, 1452.

Rosenblatt, M., Segre, G. V., & Potts, J. T., Jr. (1977c) *Biochemistry 16*, 2811.

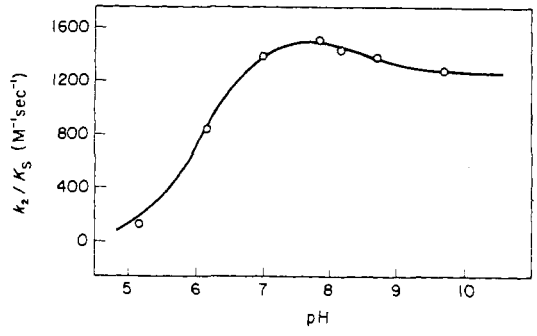
Rosenblatt, M., Serge, G. V., Tyler, G. A., Shepard, G. L., Nussbaum, S. R., & Potts, J. T., Jr. (1980) *Endocrinology* (in press).

Rudinger, J. (1972) *Med. Chem. (Academic) 11 (2)*, 319–419.
Sawyer, W. H., Acosta, M., Balaspiri, L., Judd, J., & Manning, M. (1974a) *Endocrinology 94*, 1106.
Sawyer, W. H., Acosta, M., & Manning, M. (1974b) *Endocrinology 95*, 140.
Segre, G. V., Rosenblatt, M., Reiner, B. L., Mahaffey, J. E., & Potts, J. T., Jr. (1979) *J. Biol. Chem. 254*, 6980.
Smith, C. W., & Walter, R. (1979) *Science (Washington, D.C.) 199*, 297.
Tashjian, A. H., Jr., Ontjes, D. A., & Munson, P. L. (1964) *Biochemistry 3*, 1175.
Tregear, G. W. (1975) *Pept., Proc. Eur. Pept. Symp., 13th, 1974*, 177–189.
Tregear, G. W., & Potts, J. T., Jr. (1975) *Endocr. Res. Commun. 2*, 561.
Tregear, G. W., van Rietschoten, J., Greene, E., Keutmann, H. T., Niall, H. D., Reit, B., Parsons, J. A., & Potts, J. T., Jr. (1973) *Endocrinology 93*, 1349.
Tregear, G. W., van Rietschoten, J., Greene, E., Niall, H. D., Keutmann, H. T., Parsons, J. A., O'Riordan, J. L. H., & Potts, J. T., Jr. (1974) *Hoppe-Seyler's Z. Physiol. Chem. 355*, 415.
Tregear, G. W., van Rietschoten, J., Sauer, R., Niall, H. D., Keutmann, H. T., & Potts, J. T., Jr. (1977) *Biochemistry 16*, 2817.
Vale, W., Rivier, C., & Brown, M. (1977) *Annu. Rev. Physiol. 39*, 473.
Vávra, I., Machova, A., Holecek, V., Cort, J. G., Zaoral, M., & Sorm, F. (1968) *Lancet i*, 948.
Veber, D. (1980) in *Peptides: Structure and Biological Function* (Gross, E., & Meienhofer, J., Eds.) pp 409–419, Pierce Chemical Co., Rockford, IL.

CORRECTIONS

Comparison of the Kinetic Specificity of Subtilisin and Thiolsubtilisin toward *n*-Alkyl *p*-Nitrophenyl Esters, by Manfred Philipp, Inn-Ho Tsai, and Myron L. Bender,* Volume 18, Number 17, August 21, 1979, pages 3769–3773.

Page 3771. The following is the correct Figure 5. The data in the original caption to Figure 5 are correct and apply to this curve. This correction does not change any conclusions of the original article.



Electronic Transitions in the Isoalloxazine Ring and Orientation of Flavins in Model Membranes Studied by Polarized Light Spectroscopy, by Lennart B.-Å. Johansson, Åke Davidsson,* Göran Lindblom, and K. Razi Naqvi, Volume 18, Number 19, September 18, 1979, pages 4249–4253.

Page 4252. In column 2, the tenth line from the bottom should read as follows: One may therefore conclude that $p_i(450)$ for riboflavin must also be equated to 0.5; our results for glycerol solutions of riboflavin, FMN, fluorescein, rhodamine B, and rhodamine 6G are similar to those published by Chen & Bowman (1965)—in all cases, $p(\lambda_1)$ was close to 0.45; for lumiflavin, $p(450)$ was 0.42.

Light-Induced Permeability Changes in Sonicated Bovine Disks: Arsenazo III and Flow System Measurements, by H. Gilbert Smith, Jr., and Paul J. Bauer,* Volume 18, Number 23, November 13, 1979, pages 5067–5073.

Page 5069. In Table I, footnote *b* should read as follows: The wash buffer also contained 0.1 M imidazole–chloride buffer for all of these experiments.

Page 5071. In Figure 6, line 11 under sonication conditions, for 15 mM ^{3}H-sucrose, read 100 mM ^{3}H-sucrose.

Direct Iodination of Specific Residues in Crystals of Yeast Formylatable Methionine-Accepting Transfer Ribonucleic Acid, by James Tropp and Paul B. Sigler,* Volume 18, Number 24, November 27, 1979, pages 5489–5495.

Page 5492. The cross-hatched histograms in Figure 3 failed to reproduce clearly. The following histogram peaks should be cross-hatched: Figure 3b, peaks 1, 2, and 10; Figure 3c, peaks 1, 2, and the right-most segments in peak 10; Figure 3d, peaks 1, 2, and 10.

Page 5495. The Schevitz et al. (1979) reference should read as follows: Schevitz, R. W., Podjarny, A. D., Krishnamachari, N., Hughes, J. J., Sigler, P. B., & Sussman, J. L. (1979) *Nature (London) 278*, 188.

Binding of Platinum(II) Intercalation Reagents to Deoxyribonucleic Acid. Dependence on Base-Pair Composition, Nature of the Intercalator, and Ionic Strength, by Mary Howe-Grant and Stephen J. Lippard,* Volume 18, Number 26, December 25, 1979, pages 5762–5769.

Page 5767. In column 2, line 21, for closest, read close; in line 22, end the sentence after the word "purine" and delete the subsequent phrase; in line 40, end the sentence after the word "observed" and delete the subsequent phrase. In Table V, reverse the direction of all arrows except those in footnote *b*. In Table VI, column 1 (site type), interchange lines 3 with 4 and 5 with 6.

Mechanism of the Spontaneous Transfer of Phospholipids between Bilayers, by M. A. Roseman and T. E. Thompson,* Volume 19, Number 3, February 5, 1980, pages 439–444.

Page 443. Equation 11 should read

$$\frac{C}{M} = \frac{C}{LK_2C_h\eta_{M_{max}}} + \frac{1}{LK_2\eta_{M_{max}}}$$

Then C_h = (intercept)/(slope) = 0.0125, E_{max}/M_{max} = 0.137, and the half-time for transfer of pyrene-PC between vesicles of dimyristoylphosphatidylcholine at 36 °C should be 27 h if flip-flop is negligible or 43 h if flip-flop is faster than intervesicle exchange.