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DETERMINATION OF THE EFFECT OF ACETYLATION OF SPECIFIC LYSINE RESIDUES IN HUMAN GROWTH HORMONE ON ITS AFFINITY FOR SOMATOGENIC RECEPTORS BY AN AFFINITY SELECTION TECHNIQUE

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A technique is described to study the effect of acetylation of individual lysine residues in peptide hormones on the affinity for their receptors, and is illustrated for the case of human growth hormone (hGH) binding to somatogenic receptors. The hGH was partially acetylated with high specific activity [^{3}H]-acetic anhydride and the product ([^{3}H]-Ac-hGH) was incubated with solubilised affinity-purified somatogenic receptors (from male rat liver) in the presence and absence of excess unlabelled hGH. The receptor-bound and unbound labelled hormone were separated by gel filtration and subjected to HPLC tryptic peptide mapping after the addition of cold carrier Ac-hGH. Peaks of [^{3}H] radioactivity were assigned to peptides corresponding to the acetylation of specific lysine residues in the hGH sequence by amino acid analysis and sequencing. Comparison of the relative intensities of corresponding [^{3}H] peaks in the peptide maps of added receptor, bound and unbound [^{3}H]-Ac-hGH, enabled the relative receptor-binding potencies of different acetylated hGH species to be determined. Acetylation of lysine 168 or 172 in hGH greatly decreases its receptor-binding affinity, acetylation of lysine 115 probably causes a minor decrease, whereas acetylation of lysines 38, 70, and the N-terminal amino group have no appreciable effect. Acetylation of lysine 140 causes a significant increase in receptor-binding affinity

Various chemical modifications of the lysine sidechains of hGH have been reported to significantly reduce its affinity for lactogenic (1) and somatogenic (2) receptors. The degree of modification in the reported experiments (1,2) was not sufficiently high to establish if modification of a specific residue has a major effect on receptor binding affinity. Experiments in this laboratory have shown that complete guanidylation of the lysine residues of hGH reduces its affinity for somatogenic receptors by a factor of about 2, whereas complete acetylation of the lysine residues reduces the receptor binding affinity by a factor of at least 100 (Huq, Surus and Chapman, unpublished observations). It may be inferred that acetylation of one or more of the nine lysine residues in the hGH sequence inhibits its binding to membrane receptors and the subsequent expression of biological activity. It does not, however, shed any light on which lysine residues are the critical ones.

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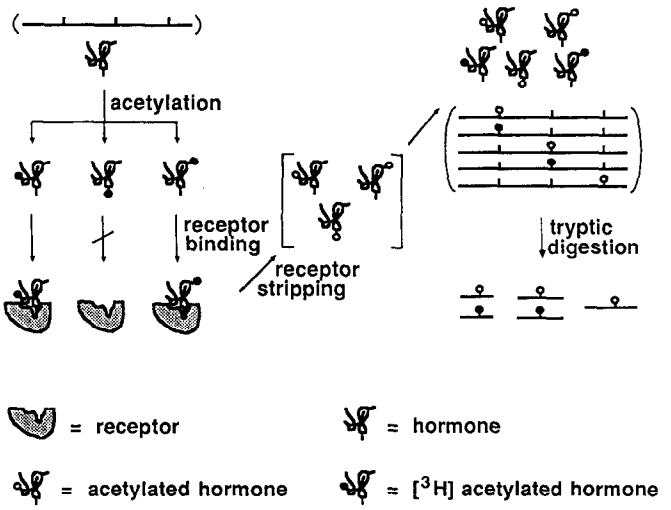

Fig.1. Schematic representation of the [^{3}H]-acetylation technique for a hypothetical folded protein hormone containing three lysine residues. Parts of the figure in round brackets are linear representations of the protein sequence.

We present here the application of a technique we have devised to enable the identification of lysine residues in peptide hormones which are involved in receptor binding, by observation of the inhibition of binding when specific lysines are acetylated. The technique involves partial acetylation of the hormone with high specific activity [^{3}H]-acetic anhydride, giving a mixture of molecular species which are coincidentally acetylated and radioactively labelled. These are incubated with receptors, and then stripped from the receptors in the presence of excess cold partially acetylated hormone as a carrier. This material is digested with trypsin and subjected to reverse phase HPLC peptide mapping. The absence of radioactivity in a peak from the stripped material which is present in digests of the added hormone corresponds to a specific lysine residue in the sequence, the acetylation of which abolishes receptor binding. The residue may be identified by amino acid analysis and/or amino acid sequencing of the peak material, with reference to the peptide hormone sequence. The principle of the technique is shown in schematic form in figure 1.

EXPERIMENTAL

Preparation of hGH: The preparation of the highly purified monomeric hGH used in this study has been described previously (3).

Preparation of [^{3}H]-acetyl-hGH: [^{3}H]-acetic anhydride with specific activity of 4.36Ci/mmole was obtained from Amersham International, UK. 16mg of hGH was dissolved in 5ml of 50mM $NaHCO_3$ and added to 25mCi of [^{3}H]-acetic anhydride in a sealed vial. After 10min, the pH was adjusted to 10 by the addition of 2M NaOH and the reaction mixture was left

at room temperature for 3h, to hydrolyse acetyl tyrosine residues, this process being monitored spectrophotometrically. The protein solution was adjusted to pH 7.8 with HCl and loaded on to a high performance anion exchange Mono Q (Pharmacia Biotechnology) column (0.5 x 5cm) and eluted with a 60ml linear gradient of 0 to 500mM NaCl in 25mM Tris/HCl, pH 7.8 at a flow rate of 1ml/min, monitoring absorbance at 280nm. Appropriate fractions were pooled and stored at -20°C. Appropriate [^{3}H]-acetyl-hGH cuts from the Mono Q fractionation were diluted and rerun on the Mono Q column, eluting with a 60ml linear gradient of 0 to 500mM NaCl in 50mM ethanolamine/HCl, pH 8.7 at a flow rate of 1ml/min. Non-radioactive acetylated hGH was made in precisely the same way, using non-radioactive acetic anhydride.

Preparation of Crude Somatogenic Receptors: All procedures were conducted at 4°C. The microsomal membrane preparations were prepared from male rat liver by the procedure of Tsushima and Friesen (4). The 100,000g x 60min pellet was resuspended in 25mM Tris/HCl, 10mM $MgCl_2$, 0.1% BSA, 0.1% NaN_3, pH 7.4 at a concentration corresponding to about 15mg/ml protein and stored at -70°C until use.

Preparation of Affigel-10-hGH Column: 10ml of activated affinity agarose support, Affigel-10 (Bio-Rad), was washed with 300ml of isopropanol, followed by 300 ml of ice-cold water. 10mg of hGH (dissolved in 10ml of 0.1M $NaHCO_3$, pH 8) were added to 10ml of Affigel-10 (resuspended in water). The gel slurry was tumbled for 4h at room temperature. 1ml of 1M ethanolamine was added and the gel mixture was left at 4°C overnight. The gel was washed sequencially with 3L of 8M urea (made freshly and deionised) in 0.1M $NaHCO_3$, pH 8; 2L of 25mM Tris/HCl, 10mM $MgCl_2$, 0.1% NaN_3, pH 7.4; and 1L of Tris/HCl buffer, pH 7.4 with 0.1% (v/v) Triton-X 100, and stored at 4°C.

Preparation of Affinity-purified Receptor: 10ml of crude microsomal membrane preparation was obtained from rat liver and Triton X-100 was added to give a final concentration of 1% (v/v). The mixtures were stirred for 1h at 25°C, and then centrifuged at 100,000g for 1h at 4°C. The supernatants were diluted with an equal volume of 25mM Tris/HCl, 10mM $MgCl_2$, 0.1% BSA, 0.1% NaN_3, pH 7.4 and batch-stirred with 1ml of Affigel-10-hGH at 25°C for 4h. The gel was washed extensively with 25mM Tris/HCl, 10mM $MgCl_2$, 0.1% BSA, 0.1% NaN_3, 0.1% (v/v) Triton X-100, pH 7.4 until no more protein was eluted. The bound receptor was stripped from the column with 4ml of 5M $MgCl_2$ and the eluant diluted immediately with 8ml of 25mM Tris/HCl, 10mM $MgCl_2$, 0.1% BSA, 0.1% NaN_3, 0.1% (v/v) Triton X-100, pH 7.4. The receptor solution was dialysed by centrifugation using a centrifugal ultrafiltration unit (Centricon, Amicon) to give a concentrated receptor solution in 25mM Tris/HCl, 10mM $MgCl_2$, 0.1% BSA, 0.1% NaN_3, 0.1% Triton X-100, pH 7.4.

Receptor-binding Studies: 50,000dpm of [^{3}H]-diacetyl hGH and 50KIU of basic pancreatic trypsin inhibitior (Trasylol, Bayer) were added to 100µl of concentrated affinity-purified rat liver receptor and incubated in a total volume of 500µl of 25mM Tris/ HCl, 10mM $MgCl_2$, 0.1% BSA, 0.1% NaN_3, 0.1% (v/v) Triton X-100, pH 7.6, for 16h at 4°C, in the presence and absence of 50µg of hGH. The receptor-bound [^{3}H]-di-acetyl hGH was separated from the unbound by high performance gel filtration using Superose 12 (Pharmacia) column (1 x 30cm) and eluted with 25mM Tris/HCl, 10mM $MgCl_2$, 0.1% BSA, 0.1% NaN_3, 0.1% (v/v) Triton X-100, pH 7.6 at 0.3ml/min. Fractions containing specific displaceable radioactivity were pooled, 0.3mg of cold diacetyl-hGH was added, and the solution was lyophilised.

Trypsin Digestion: Lyophilised protein samples were dissolved in 50mM Tris/HCl, 10mM $CaCl_2$ to a concentration of 5mg/ml and a 1mg/ml solution of bovine trypsin (Worthington) in 2mM HCl was added to give a substrate:enzyme ratio of 50:1. Incubation was at 37°C for 6h. The control digest was 1.2mg of non-radioactive diacetyl-hGH with 10Kdpm of [^{3}H]-diacetyl-hGH added.

HPLC Peptide Mapping: The digested samples were acidified to pH 2 with glacial acetic acid and loaded onto a Waters Associates HPLC system, using a Radial-Pak µNovapak C_{18} radial compression cartridge (5µm, 0.8 x 10cm). Chromatograms were developed with an 80ml linear gradient of 0.1% v/v aqueous trifluoracetic acid (solvent A) to 0.1% v/v trifluoracetic acid in 80% acetonitrile/20% water v/v (solvent B) at a flow rate of 1.5ml/min. The system effluent was monitored by absorption at 214nm. Fractions were collected where appropriate, for lyophilisation followed by amino acid sequencing and acid hydrolysis/amino acid analysis.

Amino Acid Analysis: Samples were hydrolysed in 6M HCl with a crystal of phenol added to each sample, at 110°C for 24h under vacuum, dried under vacuum, then run on a Waters Associates ion exchange amino acid analysis system with ninhydrin detection.

Amino Acid Sequencing: Peptides were sequenced on an Applied Biosystems Model 470A gas phase sequencer, according to published methods (5).

RESULTS

The anion exchange chromatography profile of the [^{3}H]-acetylated hGH is shown in figures 2a and 2b. The specific activities of the first two peaks following the hGH parent peak in figure 2a are consistent with their having approximately 1 and 2 [^{3}H]-acetyl groups per hGH molecule respectively. The positions of these peaks are consistent with an increased net negative charge due to the neutralisation of the positive charges of the the lysine sidechains. Material from the shaded area indicated in figure 2b was used in the receptor binding studies.

The gel filtration [^{3}H] radioactivity profiles of the [^{3}H]-diacetyl-hGH/receptor incubations are shown in figure 2c. The first major peak in the profile of the incubation in the

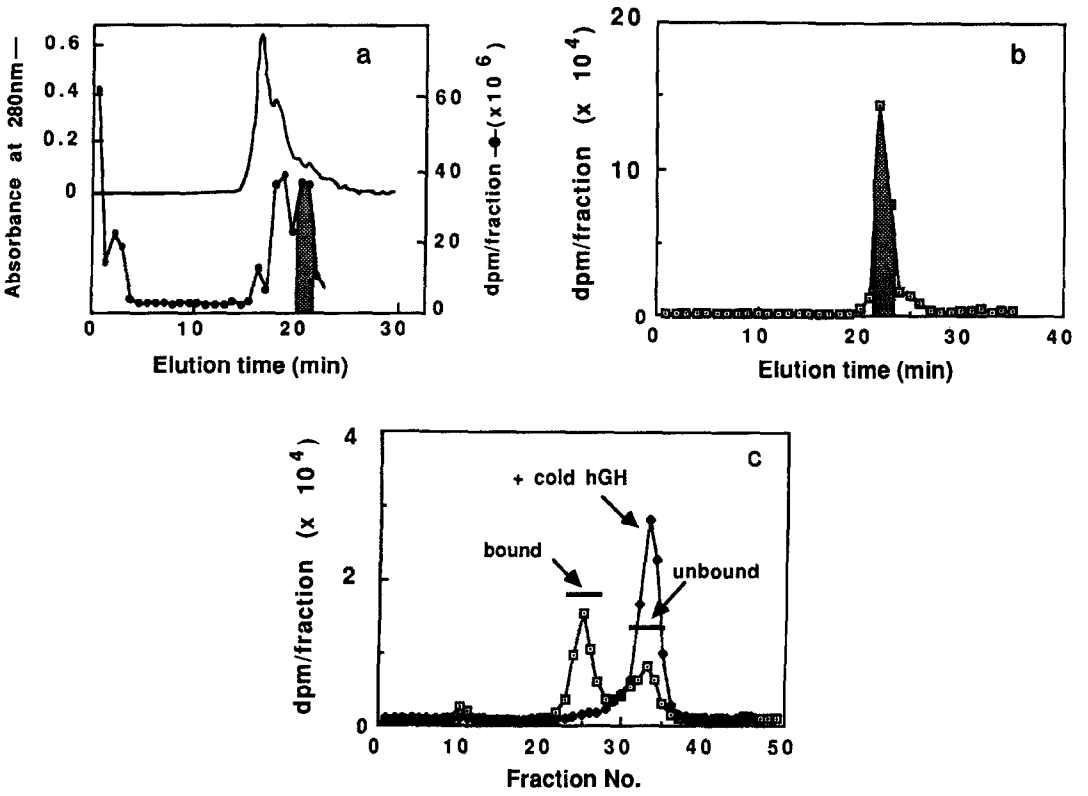

Fig.2. (a) Ion exchange chromatography of the [^{3}H]-acetylated hGH mixture on a Mono Q column (0.5 x 5cm), eluting with a linear gradient of NaCl in 25mM Tris/HCl, pH 7.8. (b) Rechromatography of material from the shaded area of (a) on Mono Q, eluting with a linear gradient of NaCl in 50mM ethanolamine HCl, pH 8.7. Material from the shaded area of (b) was used for receptor-binding studies. For full details, see Experimental Section (c) Gel filtration [^{3}H] profiles of [^{3}H]-diacetyl-hGH/receptor incubations on a Superose 12 column (1 x 30cm), eluting with 25mM Tris/HCl, 10mM $MgCl_2$, 0.1% BSA, 0.1% NaN_3, 0.1% (v/v) Triton X-100, pH 7.6 at 0.4ml/min. 0.4ml fraction size. Incubations in the absence and presence of cold hGH are shown as open and closed symbols respectively.

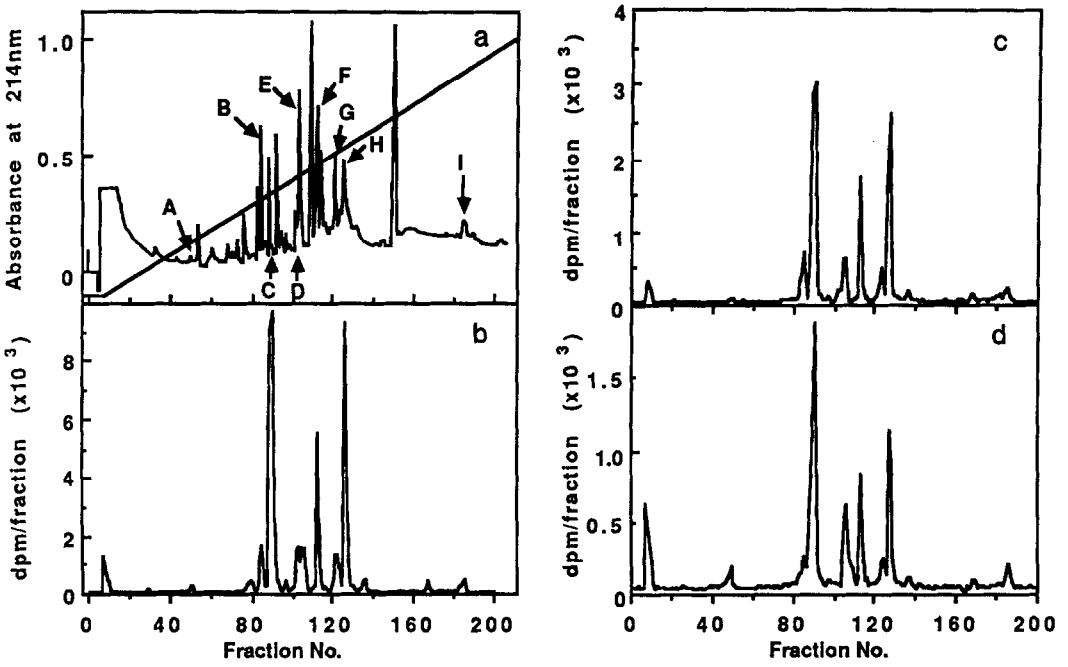

Fig.3. (a) HPLC peptide map of a tryptic digest of [^{3}H]-diacetyl-hGH on a Radial-Pak μNovapak C_{18} radial compression cartridge (5μm, 0.8 x 10cm), using a linear gradient of acetonitrile in 0.1% trifluoracetic acid, and (b) its corresponding [^{3}H] radioactivity profile, counting 15s fractions. Full details are in the Experimental Section. Sequence assignments of peptides containing [^{3}H]-acetyl lysine derived from amino acid analysis and peptide sequencing, are as follows: A=[acetyl-lysine 168]-(168-172), B=[acetyl-lysine 140]-(135-145), C=[acetyl-lysine 70]-(65-77), D=[acetyl-lysine 172]-(169-178), E=[N-acetyl]-(1-8), F=[acetyl-lysine 38]-(20-41), G=[acetyl-lysine 41]-(39-64/159-167), H=[acetyl-lysine 41,158]-(39-64/146-167) and I=[acetyl-lysine 115]-(95-127). (c) [^{3}H] profile of the HPLC tryptic peptide map of receptor-bound [^{3}H]-diacetyl-hGH. (d) [^{3}H] profile of the HPLC tryptic peptide map of unbound [^{3}H]-diacetyl-hGH.

presence of [^{3}H]-diacetyl-hGH alone is absent in the profile of the incubation performed in the presence of 50μg hGH. This peak thus represents the [^{3}H]-diacetyl-hGH/receptor complex.

The HPLC tryptic peptide map of the [^{3}H]-diacetyl-hGH taken from the gel filtration cut in the presence of 10 μg/ml hGH is shown in figure 3a, with the corresponding [^{3}H] radioactivity profile in figure 3b. Peaks containing [^{3}H] radioactivity were assigned to hGH peptides corresponding to acetylation of eight of the nine lysine residues and the N-terminal amino group of hGH, by amino acid analysis, with reference to the hGH sequence (6). The radioactivity yields of the peptides vary considerably, indicating either (i) that the rate of acetylation is widely different for specific lysine residues, or (ii) that acetylation of specific lysine residues affects the anion exchange chromatographic properties of the resulting acetyl-hGH species to different degrees. The largest single peak of activity is assigned to hGH peptide [ε-acetyl-lysine70]-(65-77).

The [^{3}H] radioactivity profile of the reverse phase HPLC tryptic peptide map of the receptor-bound and unbound [^{3}H]-diacetyl-hGH is shown in figure 3c and 3d respectively. Comparison of the [^{3}H] profiles of the digests of the added, receptor-bound and unbound [^{3}H]-diacetyl-hGH reveals that the peaks assigned by amino acid analysis to hGH peptides 168-172 and 169-178 (corresponding to acetylation of lysine 168 and lysine 172 respectively) are virtually absent in the receptor-bound fraction, and correspondingly increased in intensity in the unbound fraction relative to the added fraction. The assignment of these peaks were as confirmed by gas phase sequencing of the corresponding peaks from the HPLC tryptic peptide maps of the [^{3}H]-diacetyl-hGH. Firstly, for peptide 168-172, amino acid analysis gave the composition Asp_2,Lys_2,Met. The derived sequence was X-Asp-Met-Asp-Lys, where X was unidentified (assumed to be ε-acetyl-lysine). This peptide thus derives from tryptic cleavage of hGH acetylated at lysine 168, as follows:-

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167 (Ac)                    173
-Arg-Lys-Asp-Met-Asp-Lys-Val-
     ↑                   ↑
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Secondly, for peptide 169-178, the amino acid analysis gave the composition Asp_2,Thr,Glu, Val,Met,Leu,Phe,Lys,Arg. The derived sequence was Asp-Met-Asp-X-Val-Glu-Thr-. This peptide thus derives from tryptic cleavage of hGH acetylated at lysine 172, as follows:-

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168              (Ac)                          179
Lys-Asp-Met-Asp-Lys-Val-Glu-Thr-Phe-Leu-Arg-Ile-
 ↑                                         ↑
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It is concluded that these peptides do not occur in the receptor-bound component of the [^{3}H]-diacetyl-hGH because acetylation of either lysine 168 or lysine 172 in hGH inhibits its binding to somatogenic receptors.

Comparison of the [^{3}H] profiles of the peptides maps of the receptor-bound, unbound and added [^{3}H]-diacetyl-hGH fractions reveals two further features. Peptide 135-145 (containing acetyl-lysine 140) has a higher relative intensity in the receptor-bound fraction than in the unbound and added fractions. It is thus inferred that acetylation of lysine 140 significantly increases the affinity of hGH for somatogenic receptors. Peptide 95-127 (containing acetyl-lysine 115) has a significantly lower intensity in the receptor-bound fraction than in the unbound and added fractions. It is thus probable that aceylation of lysine 115 decreases the affinity of hGH for somatogenic receptors to a small degree, though the data do not justify a definite conclusion.

DISCUSSION

The technique described here has enabled us to test the effect of acetylation of eight of the nine lysine residues of hGH (residues 38, 41, 70, 115, 140, 158, 168 and 172) and the N-terminal amino group on its binding to somatogenic receptors. Acetylation of either lysine 168 or lysine 172 greatly decreases the affinity of hGH for the receptors, whereas acetylation of lysine 38, lysine 70 or the N-terminal amino group has no effect on receptor binding. Acetylation of lysine 140 significantly increases the affinity of hGH for its receptors whereas acetylation of lysine 115 probably decreases its affinity for the receptors to some degree. It is not possible from these experiments to say if the acetylation of lysine 41 and/or lysine 158 has any effect on receptor binding.

Lysine 168 is conserved in growth hormones from homeothermic species (7). The corresponding residue in prolactins, when the sequences are aligned for maximum homology with growth hormones is arginine (7). Lysine 172 is totally conserved in growth hormones and prolactins (7). It is noteworthy that secondary structure predictions on the hGH sequence predict that residues 168-174 form an α-helix (7), in which case residues 168 and 172 are in fairly close proximity, one turn removed in the helix.

Successful application of the [^{3}H]-acetylation technique has been demonstrated in these experiments. We note that a similar technique has recently been employed by Manalan and Klee (termed by them "affinity selection") in a study of the interaction of calmodulin and calcineurin (8), though in that study, no specific lysine residue was implicated in the interaction. The technique has potential for the investigation of the role of lysine residues in protein-ligand reactions generally. The only requirement is a knowledge of the primary structure of the protein and a means for separating the protein-ligand complex from the free protein. The technique may be extended to other specific protein chemical modifications where the chemical modification reagent is available labelled to high specific activity with a radioactive isotope, for example the S-methylation of methionine residues with high specific activity [^{3}H]-methyl iodide, the first part of a reaction scheme which has been applied to the exchange methylation [^{3}H]-labelling of parathyroid hormone (9).

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