Parathyroid hormone alters collagen synthesis and procollagen mRNA levels in fetal rat calvaria

(cell-free translation/bone/calcium metabolism)

BARBARA E. KREAM*, DAVID W. ROWE†, SUSAN C. GWOREK*, AND LAWRENCE G. RAISZ*

*Division of Endocrinology and Metabolism, Department of Medicine, and †Department of Pediatrics, University of Connecticut School of Medicine, Farmington, Connecticut 06032

ABSTRACT   Parathyroid hormone decreased the incorporation of [³H]proline into collagenase-digestible protein in cultured 21-day fetal rat calvaria but had little effect on the labeling of noncollagen protein. After 24 hr of culture, there was a 50% reduction in collagen synthesis and a 40% decrease in the level of functional procollagen mRNA as measured in a reticulocyte lysate translation assay. The effect of parathyroid hormone on both parameters was detectable after 6 hr of treatment. In these cultures, there was also a substantial degradation or release of newly synthesized collagen from the calvaria, but parathyroid hormone had little effect on the release of collagen into the medium. These results suggest that parathyroid hormone inhibits collagen synthesis primarily by decreasing the steady-state level of procollagen mRNA.

When bone is treated with parathyroid hormone (PTH), there is a substantial decline in osteoblast function and collagen synthesis (1–3). In 21-day fetal rat calvaria maintained in organ culture for 24–96 hr, PTH at 1 nM to μM decreases the incorporation of [³H]proline into collagenase-digestible protein (CDP) but has little effect on the labeling of noncollagen protein (NCP) (4). During this interval, characteristic polygonal osteoblasts are replaced by spindle-shaped cells (5), and net accumulation of bone collagen decreases (6). Similarly, PTH inhibits the synthesis of collagen in osteoblast-like cells derived from 2- to 3-day mouse calvaria by sequential enzymatic digestion (7).

The molecular mechanism by which PTH alters collagen synthesis in these bone cultures is unknown. Because procollagen mRNA levels appears to be the primary regulatory site for changes in collagen synthesis in embryonic chicken calvaria (8), a likely hypothesis is that PTH regulates collagen synthesis in fetal rat bone by altering the level of procollagen mRNA. On the other hand, PTH is a potent stimulator of bone resorption (9, 10) and could have a posttranslational effect on collagen levels by enhancing the degradation of newly synthesized collagen.

One objective of the present study was to determine whether the ability of PTH to inhibit collagen synthesis in cultures of fetal rat calvaria could be correlated with alterations in the level of functional procollagen mRNA. Another was to assess the effect of PTH on the release of newly synthesized collagen from these calvaria into the culture medium. Our results demonstrate that PTH decreases both collagen synthesis and the bone level of procollagen mRNA but has only a small effect on the release of collagen into the medium, indicating that PTH regulates collagen synthesis primarily through an effect on procollagen mRNA levels.

MATERIALS AND METHODS

Culture Method. The frontal and parietal bones were removed from 21-day fetal rats (Sprague–Dawley; Charles River Breeding Laboratories) and split along the sagittal suture. Half calvaria were cultured at 37°C under a humidified atmosphere of 5% CO_2/95% air in 25-ml erlenmeyer flasks containing 2 ml of modified BGJ medium (11) supplemented with bovine serum albumin at 1 mg/ml or with 5% human serum that had been heated at 56°C for 30 min. The concentration of unlabeled proline in the medium was 1 mM except where indicated. Half calvaria were cultured for 24 hr with continuous shaking (60 oscillations per min) at 37°C. Either natural bovine PTH 1-84 (National Institutes of Health) or the synthetic bovine 1-34 peptide (Beckman) was added to cultures in a solution containing 1 mg of bovine serum albumin per ml. During the final portion of the culture period, 5–10 μCi of [2,3-³H]proline (30 Ci/mmol; 1 Ci = 3.7 × 10¹⁰ becquerels; New England Nuclear) was added to each flask for 15–120 min. To terminate the culture each calvarium was removed, extracted with 5% trichloroacetic acid, acetone, and ether and then dried, weighed, and homogenized in 1.0 ml of 0.5 M acetic acid.

The incorporation of [³H]proline into CDP and NCP was determined by using repurified bacterial collagenase (Worthington) according to the method of Peterkofsky and Diegelmann (12) and was linear for at least 120 min in both control and PTH-treated calvaria (Fig. 1). The percentage collagen synthesized by the bones was corrected for the relative abundance of proline residues in collagen compared to NCP (13).

Analysis of [³H]Hydroxyproline. To assess the recovery of [³H]hydroxyproline, [2-¹⁴C] hydroxyproline (Amersham) was added to portions of the bone homogenates and culture medium. After adsorption to an acidic sulfonated ion exchange resin (Organon) each sample was hydrolyzed at 104°C for 18–24 hr. Hydrolysates were lyophilized, redissolved in 0.1 M citrate buffer (0.2 M in Na^+) at pH 2.90, and chromatographed on a 1.5 × 42 cm or 1.6 × 86 cm column (for bone or medium hydrolysate, respectively) of AG-50W-X8 resin in the Na^+ form (Bio-Rad); the column was equilibrated and developed with the above buffer (14). Aliquots of column fractions were assayed in 10 ml of ACS scintillation fluid (Amersham) and cpm of ³H were corrected for the spillover of ¹⁴C. [³H]Hydroxyproline was identified as the peak of radioactivity comigrating with authentic [¹⁴C]hydroxyproline. Recovery of [³H]hydroxyproline after hydrolysis and chromatography was 60–80%.

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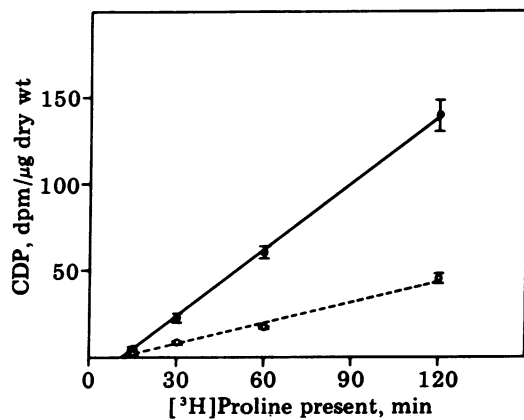
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FIG. 1. Incorporation of [3H]proline into CDP of control and PTH-treated fetal rat calvaria. Calvaria were cultured for 24 hr; between 15 min and 2 hr before the end of culture, 20 μCi of [3H]proline was added. The incorporation of [3H]proline into CDP was determined. Data are mean ± SEM. ●, Control; O, with PTH.

Extraction and Translation of Procollagen mRNA. Methods previously developed for embryonic chicken calvaria and fibroblasts were used to extract RNA from rat calvaria quantitatively (8, 15). Calvaria were cultured as described but were not given [3H]proline. After culture, these calvaria were frozen in liquid N_2 and stored at $-80°C$. Eight to 10 half calvaria were disrupted with a Polytron (Brinkman) and then homogenized by using several strokes of a tight-fitting Dounce homogenizer in 5 ml of SET buffer containing proteinase K (E. Merck Darmstadt, Germany) at 50 μg/ml. After 1 hr at 37°C the homogenate was extracted with 8 ml of phenol (saturated with 0.5 M Tris·HCl at pH 7.5)/chloroform, 1:1 (vol/vol). The aqueous phase was adjusted to 0.1 M in NaCl, and total nucleic acids were precipitated overnight by addition of 2 vol of absolute ethanol. The precipitate was collected by centrifugation at 19,000 × g for 10 min, washed once with 66% ethanol/0.1 M NaCl, and lyophilized. Total nucleic acids were dissolved in 0.4 ml of 6 M guanidine·HCl at pH 7.0 and heated to 60°C for 5 min. DNA was quantitated by using a fluorometric assay (16). RNA was separated from DNA by precipitation with 0.2 ml of absolute ethanol after overnight storage at $-20°C$. The precipitate was collected by centrifugation at 19,000 × g for 10 min, washed once with 4 M guanidine·HCl/33% ethanol and twice with 60% ethanol/0.1 M NaCl, drained, and lyophilized. The residue (total RNA) was redissolved in 0.1–0.15 ml of 10 mM Hepes (pH 7.4), heated at 60°C for 5 min, and stored at $-70°C$. In some experiments, the RNA was dissolved in 200 μl of SET buffer, heated at 60°C for 5 min, cooled, and centrifuged through a 5–20% sucrose gradient in SET buffer (4.5 hr; 20°C; 285,000 × g; Beckman SW 41 Ti rotor). Fractions were obtained by using an Isco model 184 sucrose gradient fractionator. The 28S ribosomal RNA peak was collected and precipitated with 2 vol of 60% ethanol containing 0.1 M NaCl. The yields of extractable DNA or RNA were comparable from the control and PTH-treated calvaria: eight half calvaria (8–10 mg wet weight) contained 50–80 μg of DNA, 25–50 μg of total RNA, and 10–25 μg of 28S RNA. The 260/280 absorbance ratio of the 28S RNA was 2.02–2.11, and the 28S/18S RNA ratio as assessed on the SET/sucrose gradients was 1.2–1.6, indicating little degradation of the 28S ribosomal RNA.

Translation of total RNA was performed by using a micrococcal nuclease-treated reticulocyte lysate, filtered on Sephadex G-50, prepared in this laboratory as described (15). The 50-μl reaction mixture contained 10 μl of the treated lysate, 5–40 μg of RNA per ml, 1 mM ATP, 0.2 mM GTP, 15 mM creatinine

phosphate, 25 μg creatine kinase per ml, 60 mM KCl, 30 mM NaCl, 4 mM KH_2PO_4 (pH 7.5), 1.75 mM $MgCl_2$, 0.3 mM spermidine, 3 mM fructose 6-phosphate, and 100 μCi [5-3H]-proline (50 Ci/mmol; ICN, Irvine, CA) per ml. After 2 hr at 26°C, 20 μl of 5% Triton X-100/1 mM proline/0.1 mM cycloheximide containing 2 mg of bovine serum albumin per ml was added to the reaction mixture with a 1:25 dilution of rat antiprocollagen antiserum. The antiprocollagen antibody, raised in rabbits inoculated with purified type I rat skin procollagen, was the generous gift of Paul Bornstein. After 1 hr at room temperature, 40 μg of goat anti-rabbit Ig antiserum (15) was added and the resulting precipitate was collected by centrifugation through a sucrose/detergent solution as described (17).

Background radioactivity nonspecifically trapped by the immunoprecipitate was estimated by determining the cpm in an albumin–antialbumin precipitate of size similar to the antiprocollagen precipitate. Radioactivity in the immunoprecipitate was solubilized in TS-1 (Research Products International, Elk Grove Village, IL) and assayed in 4 ml of toluene scintillation fluid. Procollagen mRNA is expressed as cpm of immunoprecipitable collagen translated per μg of total RNA per ml.

The products made during *in vitro* translation were analyzed on 5% acrylamide slab gels (18) and identified by fluorography (19). The immunoprecipitate was incubated for 1 hr in 50 μl of 20 mM Hepes, pH 7.2/0.5 mM $CaCl_2$/2.5 mM N-ethylmaleimide in the presence or absence of purified bacterial collagenase (Advance Biofactures, Lynnbrook, NY). The reaction was terminated by the addition of an equal volume of the gel sample buffer and heating at 100°C. The solution was applied to the acrylamide gel. For comparison, procollagen synthesized by control or PTH-treated calvaria was prepared by labeling calvaria maintained in culture with [2,3-3H]proline (30 Ci/mmol) for 15 min. The calvaria were extracted in 5 vol of 0.15 M NaCl/0.05 M Tris·HCl, pH 7.5/25 mM EDTA/0.2 mM phenylmethylsulfonyl fluoride/6 mM N-ethylmaleimide. Procollagen was precipitated by adjusting the solution to 30% ammonium sulfate and collected by centrifugation at 17,000 × g for 10 min. The precipitate was washed with 18% ethanol, lyophilized, dissolved in gel buffer, and applied to the acrylamide gel.

RESULTS

In this organ culture system, both the natural bovine PTH 1-84 and the synthetic bovine 1-34 peptide were equipotent in inhibiting the incorporation of [3H]proline into CDP and the percentage collagen synthesized by 21-day fetal rat calvaria (Table 1). The natural hormone had a small inhibitory effect on the labeling of NCP.

Standard curves were constructed for the translation of total

Table 1. Effect of 10 nM PTH on labeling of CDP and NCP in 21-day fetal rat calvaria

| | n | dpm/μg dry wt | | % collagen synthesized |
		CDP	NCP	
Control	25	34 ± 3	42 ± 2	12.6 ± 0.4
PTH 1-84	10	13 ± 1*	34 ± 1†	6.7 ± 0.3*
PTH 1-34	16	17 ± 1*	40 ± 2	7.5 ± 0.3*

Calvaria were cultured individually in BGJ medium supplemented with 5% heated human serum for 24 hr. All samples were exposed to 10 μCi of [3H]proline for 2 hr. The bones were extracted, homogenized in 0.5 M acetic acid, and analyzed for incorporation of [3H]proline into CDP and NCP. Data are shown as mean ± SEM.
* For difference from control, P <0.01.
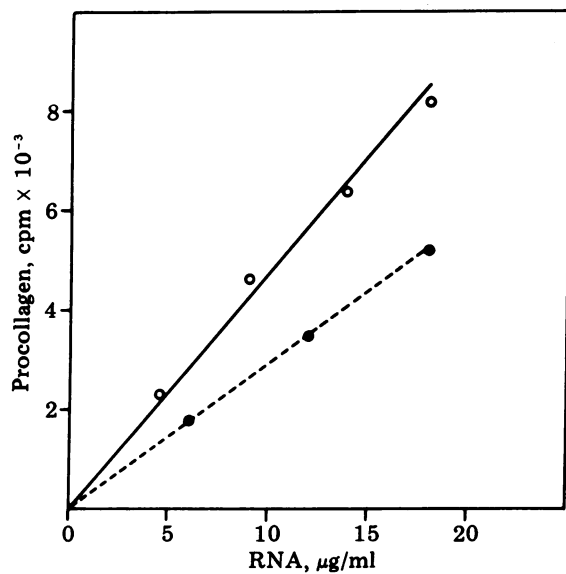† For difference from control, P <0.05.

FIG. 2. Procollagen mRNA activity in reticulocyte lysate with RNA extracted from control (O) or PTH-treated (●) 21-day fetal rat calvaria. Calvaria were cultured for 24 hr in BGJ medium containing 1 mg of bovine serum albumin per ml. RNA was extracted, and translated procollagen was identified by immunoprecipitation.

RNA in the reticulocyte lysate (Fig. 2). Over the range 5–20 μg of total RNA per ml, the curve for translation of RNA extracted either from control or PTH-treated calvaria was linear. Furthermore, RNA extracted from PTH-treated calvaria had less activity than did RNA extracted from control calvaria. Analysis of the translation products on 5% acrylamide slab gels (Fig. 3) indicated that the immunoprecipitate contained procollagen α_1 and procollagen α_2 chains plus smaller products. All translation products were collagenase-sensitive. The intensity of the bands representing procollagen synthesized by the calvaria in organ culture or in the cell-free translation system was decreased by PTH.

Results of a typical experiment in which calvaria were analyzed for protein synthesis and procollagen mRNA levels are
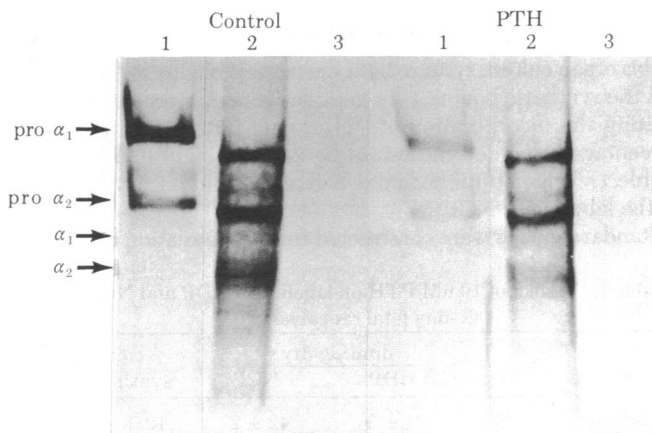

FIG. 3. Fluorogram of slab gel after electrophoresis of rat calvarial procollagen and immunoprecipitable products from the translation of calvarial RNA in a reticulocyte lysate. Rat calvaria were maintained in organ culture for 24 hr in the absence (control) or presence (PTH) of 25 nM PTH. Lanes: 1, procollagen labeled in calvaria during a 15-min incubation with [2,3-^{3}H]proline; 2, immunoprecipitable products of the cell-free translation with RNA extracted from rat calvaria; 3, sample identical to that in lane 2 but treated with bacterial collagenase for 1 hr at 37°C.

Table 2. Effect of 4 nM PTH on procollagen mRNA levels and protein synthesis in 21-day fetal rat calvaria

| | mRNA* | dpm/μg dry wt | | % collagen synthesized |
		CDP	NCP	
Control	2953	20 ± 1	27 ± 1	12.2 ± 0.3
PTH	1867	10 ± 1†	26 ± 2	6.4 ± 0.3†

Calvaria were cultured in BGJ medium containing 5% heated human serum. Bones used for the analysis of the labeling of CDP and NCP were exposed to 10 μCi of [^{3}H]proline for 1 hr at the end of culture. For analysis of procollagen mRNA, some bones were not exposed to [^{3}H]proline but were frozen in liquid N_2 and then extracted and assayed. Data are shown as mean ± SEM.
* cpm of immunoprecipitable procollagen/μg of total RNA per ml.
† For difference from control, $P < 0.01$.

shown in Table 2. After 24 hr of exposure to PTH, bone procollagen mRNA was decreased by nearly 40% and the ability to incorporate [^{3}H]proline into CDP and the percentage collagen being synthesized by the calvaria were decreased about 50%. In five separate experiments, a 24-hr incubation with PTH consistently decreased the steady-state level of procollagen mRNA by 50 ± 6% (SEM) and the labeling of CDP by 58 ± 3% (SEM), demonstrating that the decline in collagen synthesis is associated with a decrease in the level of functional procollagen mRNA. PTH specifically decreased functional procollagen mRNA because total protein synthesis in the reticulocyte lysate was equal with RNA extracted from control or PTH-treated calvaria.

To determine the time course of action of PTH in inhibiting collagen synthesis and procollagen mRNA accumulation, calvaria were put into cultures supplemented with PTH at various times before the end of the 24-hr incubation period. Procollagen mRNA levels were unaffected when calvaria were incubated with PTH for 3 hr. Bone procollagen mRNA steadily decreased after longer exposure to the hormone and was 60% of the control at 24 hr. Similarly, the labeling of CDP declined in the presence of PTH (Fig. 4). However, at all time points examined after 3 hr, the decrease in the labeling of CDP was greater than the decrease in procollagen mRNA levels.

One explanation for the observation that PTH inhibits collagen synthesis to a greater extent than it inhibits procollagen mRNA accumulation is that PTH also promotes the degradation of newly synthesized collagen or impairs its incorporation into bone matrix. If this were the case, the medium from PTH-treated cultures should be enriched in [^{3}H]hydroxyproline compared to medium from control cultures. To investigate this possibility the distribution of [^{3}H]hydroxyproline between bone

Table 3. [^{3}H]Hydroxyproline in control and PTH-treated cultures of 21-day fetal rat calvaria

| | [^{3}H]Hydroxyproline, dpm | |
	Control	100 nM PTH
Bone	174,000	84,000
Medium	213,600	135,600
Total	387,600	219,600
% in medium	55	62

Calvaria were cultured individually in BGJ medium containing 5% heated human serum and no added unlabeled proline. After 24 hr the bones were transferred to fresh medium so that there were four bones per flask and three flasks per group. To each flask was added 2 μCi of [^{3}H]proline, incubation was continued for 24 hr. All bones in each treatment group were pooled, rinsed twice with saline, and homogenized in 6 ml of 0.5 M acetic acid. Culture medium from each group was pooled. After hydrolysis at 104°C, [^{3}H]hydroxyproline in bones and medium was measured.

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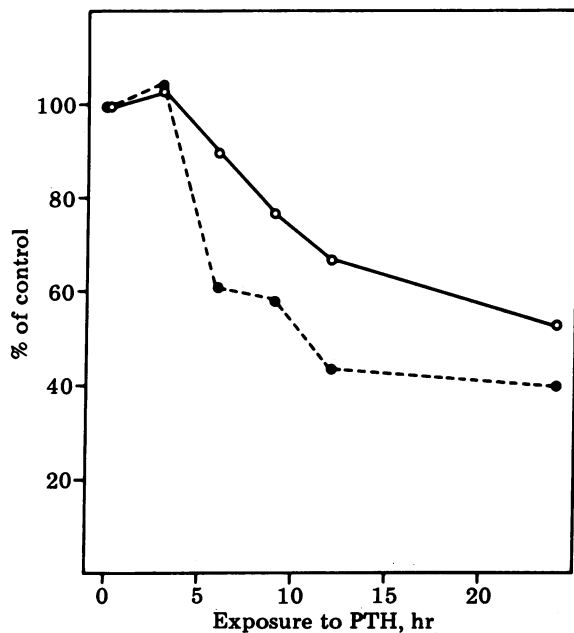
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FIG. 4. Time course of PTH inhibition of labeling of CDP (●) and the accumulation of procollagen mRNA (○) in 21-day fetal rat calvaria. Calvaria were cultured in BGJ medium containing 1 mg of bovine serum albumin per ml. At various times before the end of culture, flasks were supplemented with 25 nM PTH dissolved in albumin solution (1 mg/ml). Two hours before the end of culture, bones used for the analysis of CDP were pulsed with 10 μCi of [^{3}H]proline; then these bones were extracted and assayed. Bones used for the determination of procollagen mRNA were frozen in liquid N_2. RNA was extracted and translated in the reticulocyte lysate as described. At each time point, the experimental value is expressed as a percent control. Each point represents the average of measurements derived from bones cultured in two different experiments.

and medium in control and PTH-treated cultures was measured (Table 3). Overall, the PTH-treated cultures (bone plus medium) contained 168,000 dpm less [^{3}H]hydroxyproline than did the control cultures. The [^{3}H]hydroxyproline detected in the medium from control or PTH-treated cultures was proportional to the levels found in the bones and represented 55% or 62%, respectively, of the total [^{3}H]hydroxyproline in the cultures. Therefore, the decrease in labeled collagen in the PTH-treated calvaria was not due entirely to impaired incorporation of newly synthesized collagen into bone matrix because sufficient [^{3}H]hydroxyproline was not found in the medium from PTH-treated bones to support this hypothesis.

A PTH-induced decrease in hydroxylation of proline incorporated into collagen would invalidate the above experiment because one would underestimate medium CDP by using [^{3}H]hydroxyproline as a marker if underhydroxylated collagen peptides were degraded and released during culture. To assess the extent of hydroxylation of proline incorporated into bone collagen, calvaria were cultured in the presence of [^{3}H]proline, extracted, and homogenized in acetic acid. One portion of the homogenate was analyzed for [^{3}H]hydroxyproline by ion exchange chromatography and an equivalent portion was digested with bacterial collagenase to determine CDP radioactivity (proline plus hydroxyproline). Tritiated [^{3}H]hydroxyproline accounted for 46 ± 1% (SEM) and 49 ± 2% (SEM) of the CDP radioactivity in PTH and control cultures, respectively, indicating that PTH had no effect on the normal hydroxylation of bone collagen.

DISCUSSION

In agreement with previous studies (4), 10–40 μM PTH decreased the labeling of CDP by 50% but had little effect on the labeling of NCP after 24 hr of culture. The important finding in this study is that the decrease in collagen synthesis in PTH-treated calvaria is accompanied by a decrease in the bone level of functional procollagen mRNA, suggesting that PTH regulates collagen synthesis by altering the level of procollagen mRNA. These observations are consistent with the autoradiographic results of Bingham *et al.* (20) which showed a decrease in the incorporation of [^{3}H]uridine into osteoblasts after administration of parathyroid extract to rabbits.

Procollagen mRNA was quantitated in a reticulocyte lysate translation assay which measures only functional procollagen mRNA. The assay would not detect procollagen mRNA molecules altered by posttranscriptional processing events (removal of introns, capping, methylation, or polyadenylation) or by covalent attachment of factors that convert the mRNA into a nontranslatable form. However quantitating procollagen mRNA by hybridization using a cDNA probe gives an estimate of total mRNA sequences irrespective of posttranscriptional modifications. In the developing chicken calvarium, collagen synthesis is proportional to collagen mRNA levels measured by either translation or hybridization (8).

Procollagen synthesized *in vitro* by cell-free translation or by calvaria maintained in organ culture was identified by electrophoresis and fluorography. In the cell-free translation, the major bands precipitated by the antiprocollagen antibody migrated slightly faster than the pro α chains made in intact calvaria, probably due to the absence of prolyl hydroxylase in the lysate (15). In the procollagen synthesized in control or PTH-heated rat calvaria, the ratio of pro α_1 to pro α_2 chains was 2:1; however, the concentrations pro α_1 and pro α_2 chains in the immunoprecipitates of the cell-free translations were equivalent. This was not due to the antibody used for precipitation because pro α_1 and pro α_2 were present in equal amounts in the translated mixture before precipitation (data not shown). The reason for this discrepancy is unclear.

As assessed by the distribution of [^{3}H]hydroxyproline between bones and culture medium, the observed decrease in [^{3}H]collagen levels in PTH-treated calvaria was not due to enhanced release of collagen into the culture medium. What is striking is that in both control and PTH-treated cultures, 55–62% of the total hydroxyproline synthesized was released into the culture medium during a 24-hr labeling period. However, such an extensive loss of newly synthesized collagen is not unexpected; Bienkowski *et al.* (21) have shown that up to 40% of the newly synthesized collagen in rabbit lung explants is degraded within minutes of its synthesis. In our results, the molecular sizes of the unincorporated collagen peptides have not yet been determined. However, at least 50% of the hydroxyproline in the medium is dialyzable, indicating extensive degradation of newly synthesized collagen (unpublished data).

The relevance of the effect of PTH on collagen synthesis in this tissue culture system to the action of PTH in the intact animal is controversial. When administered *in vivo*, intermittent low doses of PTH and parathyroid extract stimulate osteoblast proliferation and, presumably, bone formation (22–24). However, Flanagan and Nichols (2) demonstrated that acute administration of parathyroid extract to rats 6–18 hr before sacrifice decreased the incorporation of radioactive proline into collagen of excised bone fragments. In the present studies, the decline in procollagen mRNA and collagen synthesis *in vitro* occurred over a similar time interval. Johnston and coworkers (3) showed that the labeling of bone collagen *in vitro* was de-

pressed 3–15 hr after administration parathyroid extract *in vivo* but was increased above control at 36–74 hr. A likely explanation for the stimulatory effect of PTH on osteoblast proliferation and collagen synthesis *in vivo* is that these responses are indirect and are mediated by other hormones or factors. Another explanation is that osteoblast number is increased in response to enhanced bone resorption.* The reduction in procollagen mRNA induced by PTH in fetal rat calvaria could result from an alteration in the rate of transcription of the procollagen genes or a change in the metabolism of procollagen mRNA. In mammary gland organ culture, the increase in accumulation of casein mRNA caused by prolactin (25) can be explained only in part by an increased rate of gene transcription (26). Rather, the regulation of casein production by prolactin involves a coordinate response at the level of transcription, posttranscription, and other sites. It is possible that a similar pattern of coordinate regulation exists for the control of collagen synthesis by PTH. Bone organ culture will provide an excellent system for studying the effect of PTH on the synthesis and metabolism of procollagen mRNA.

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