The Effects of Parathyroid Extract Upon Incorporation of 1-^{14}C-Glucosamine Into Bone (36139)

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The mechanism of action of parathyroid hormone is not known; however, a number of effects have been reported. These include effects on serum calcium levels, cyclic AMP, nucleotide pools, protein biosynthesis and glycoprotein biosynthesis. Johnston, Deiss and Miner (1) reported an increased *in vitro* synthesis of material containing ^{14}C-labeled hexosamine when incubating 1-^{14}C-glucose with bone from rats previously treated with parathyroid extract. Owen and Shetlar(2) reported an early labeling of the osteoclasts of young rabbits given tritiated glucosamine. Bingham, Brazell and Owen (3) later showed that the uptake of ^{3}H-glucosamine into bone is increased by the administration of parathyroid extract. As glucosamine is efficiently incorporated into glycoproteins and mucopolysaccharides, it has been assumed that the radioactive material in bone represents glycoprotein, mucopolysaccharides, or precursors of these compounds. A glycoprotein with a high content of sialic acid and chondroprotein has been isolated from bovine bone by Herring and Kent (4). However, previous to this paper, labeled glycoprotein and chondroprotein have not been isolated from bone following the administration of labeled glucosamine. It is the purpose of this paper to report the isolation of glycoprotein and chondroprotein from the bone of rats treated with parathyroid extract and then given ^{14}C-glucosamine. The influence of parathyroid extract upon the incorporation of glucosamine into uncalcified connective tissue and into plasma proteins has also been investigated.

Materials and Methods. Adult male rats weighing between 332 and 423 g were implanted subcutaneously with 3 stainless steel cylinders as previously described for studies of non-calcified connective tissue (5). These cylinders were left in the rats for 2 weeks before killing the animals as described below. The animals were divided into 6 groups. Three groups were given parathyroid extract (Lilly); the other 3 groups were given injections of the vehicle solution (0.2% phenol, 1.5% glycerol in deionized water). Injections were administered subcutaneously in 4 doses of 50 units per day. One group received extract for 1 day, a second for 2 days and the third group 3 days. In an experiment to study long term effects of the extract, rats were given 3 doses daily of a less potent extract for 14 days. Control rats were injected similarly with the vehicle solution. Six hours before rats were sacrificed, each animal was given a single intraperitoneal injection of 20 μCi of 1-^{14}C-glucosamine in saline. At this time, blood, kidney, liver, femurs and the connective tissue samples were taken. A section of kidney was placed in formalin solution for histological examination. Liver was perfused *in situ* to remove blood. All uncalcified tissues were then washed with tap water, blotted dry and freeze-dried. The freeze-dried samples were ground in a Wiley mill to pass through a 40-mesh screen. The femurs were cleaned of adhering tissue, split longitudinally, the marrow removed, and the bone rinsed thoroughly with cold 0:9% saline solution, followed by cold water. They were then freeze-dried and ground in the Wiley mill.

For the determination of total bound radioactivity, uncalcified tissue samples were weighed and homogenized in water, placed in dialysis bags and dialyzed against deionized water. Bone samples were demineralized by suspended in 10% EDTA solution and dialyzing first against EDTA solution and then against deionized water. Aliquots were taken for counting in the liquid scintillation counter. NCS solubilizer was used with a toluene

counting cocktail (4 g PPO and 50 mg POPOP in 1 liter of toluene). Counting was done in a Beckman Model LS200 liquid scintillation counter. All counts were corrected for quenching. Results are summarized in Table I.

In order to see if the radioactivity was present in bone as hexosamine, the hexosamines were isolated by hydrolyzing the demineralized bone with 4.0 *N* HCl for 6 hr at 100°, taking the hydrolysate to dryness, dissolving in water and chromatographing on Dowex-50 resin. The radioactivity on this isolated fraction was then determined in the scintillation counter. Results are shown in Table II.

In order to isolate sialoprotein and chondroprotein, the demineralized bone was digested with collagenase (clostridiopeptidase A EC 3.4.4.19, Worthington Code CLS, 125-200 units/mg), 5 μg enzyme/mg of bone in 0.67 *M* phosphate-0.45% NaCl

TABLE I. Total Bound Radioactivity Found in Various Tissues after Treatment of Rats with Parathyroid Extract and Administration of 1-^{14}C-Glucosamine.

Values given are the means of results from 8 rats treated with parathyroid extract (PTE) and 9 control rats (± the standard error of the mean).

	Disintegrations per minute per mg dry tissue		
	Whole tissue	Not dialyzable	Dialyzable
Bone, PTE	46.9[a] ± 2.1	33.4[a] ± 0.8	13.4[b] ± 1.8
Bone, control	23.6 ± 1.5	14.5 ± 0.6	7.9 ± 0.6
Connective tissue, PTE	9628 ± 1269	6400 ± 881	3528 ± 443
Connective tissue, control	9288 ± 1192	6275 ± 766	3012 ± 1151
Kidneys, PTE	2348[a] ± 111	1707[a] ± 111	708[a] ± 36
Kidneys, control	1567 ± 50	1215 ± 43	353 ± 30
Liver, PTE	1210 ± 82	885 ± 65	326 ± 21
Liver, control	1061 ± 34	785 ± 24	276 ± 18
Serum,[c] PTE	18,220[a] ± 1250	16,030[a] ± 1020	2193[a] ± 198
Serum, control	13,470 ± 550	12,040 ± 520	1390 ± 129

[a] Significantly higher than the control value at the 1% level of probability.
[b] Significantly higher than the control value at the 5% level of probability.
[c] Serum counts are expressed as dpm/mg protein.

TABLE II. Radioactivity in the Hexosamine Fraction of Whole Bone and in the Sialoprotein and Chondroprotein Isolated from Bone of Adult Rats Given Parathyroid Extract and 1-^{14}C-Glucosamine.

Days treated with PTE	Original whole bone (dpm/μmole hexosamine)	Sialoprotein (dpm/μmole sialic acid)	Chondroprotein (dpm/μmole uronic acid)
1	3510	2313	617
2	3503	2278	390
3	3699	2710	954
Avg (1–3 days)	3571[a] ± 54	2434[a] ± 198	663[b] ± 142
Controls	1739 ± 56	1078 ± 37	220 ± 20
14	3345[a] ± 261	2593[a] ± 21	—
Controls	1433 ± 96	1558 ± 102	—

[a] Significantly higher than the controls at the 1% level of probability.
[b] Significantly higher than the controls at the 2% level of probability.

buffer, pH 7.4; a crystal of thymol was added to prevent bacterial action. The samples were incubated at 37° for 2 days. The samples were centrifuged, the supernatants poured off, and the residue was suspended in buffer and incubated with additional collagenase for another 2 days. The supernatants were combined, filtered through Whatman No. 1 filter paper, and dialyzed against deionized water for 3 days at 4° in a rotating dialyzer. The resulting solutions were freeze-dried and then dissolved in water. The samples were placed on an Amberlite CG-50 column to separate chondroprotein and sialoprotein from other proteins. The resin was prepared by batchwise washing with 2*N* NaOH, H_2O, 2*N* HCl and final washing with water until a negative test for chloride ion was obtained. The resin was then stirred with 0.2 *M* sodium phosphate buffer (pH 5.2) for 2 days and placed in a 1.2 × 16 cm column. The samples were eluted from the column with about 70 ml of the buffer. The eluates were dialyzed, freeze-dried and dissolved in 2 ml of water.

The samples were then applied to a Dowex-1 resin (BioRad AG 1 × 2, 200–400 mesh, acetate form) column, 1.2 × 16 cm. The sialoprotein was eluted from the column with 210 ml of a 1 *M* sodium acetate–acetic acid (pH 4.6) buffer. The chondroprotein was then eluted from the column with 250 ml of 2 *M* NaCl. The sialoprotein was finally precipitated with ethanol containing 5 mg% zinc acetate and acetic acid to pH 5. The chondroprotein was precipitated with ethanol containing 5% sodium acetate and 0.5 *N* acetic acid. The precipitated fractions were dissolved in deionized water. Aliquots were used for determination of radioactivity by scintillation counting, and for determinations of salic acid (6) and uronic acid (7). Radioactivities found in bone fractions are summarized in Table II. Another aliquot was used for cellulose acetate electrophoresis in pyridine–acetic acid water buffer (1:10:89, pH 3.5). Bovine sialoprotein and chondroprotein were used as standards for the electrophoresis and the patterns were stained with Alcian Blue. The mobilities of the rat sialoprotein preparations were identical to that of the bovine standard and those of the rat chondroprotein were identical to the bovine chondroprotein.

Results and Discussion. The administration of the parathyroid extract resulted in elevated serum glycoproteins in the experimental rats (hexose contents of 2.68–3.79 g/100 g of protein for the treated animals compared with 2.07–2.28 for the control animals); these changes are similar to those previously reported for rats treated with parathyroid extract (8). Histological examination of the kidneys from the rats given parathyroid extract for 1 to 3 days showed slight calcification after the second day with increased calcification on the third day. Kidneys from the rats given the less potent parathyroid extract for 14 days showed slight calcification. The specific radioactivity of the serum proteins was increased under the influence of parathyroid extract. This indicates that the biosynthesis of serum glycoprotein is increased under these conditions. The dialyzable radioactivity of serum is also increased. The reason for this increase may be due either to increased biosynthesis or to increased metabolic products. The increases of radioactivity in the kidney may represent increased biosynthesis of serum glycoproteins in the kidney or deposition of material from the serum in the kidney. Previous work has reported early lesions in the kidney resulting from treatment with parathyroid extract; these lesions contain polysaccharides or glycoproteins and appear before calcification can be detected (10). Rapidly growing connective tissue was not significantly affected by parathyroid extract (Table II.).

The increase of radioactivity in bone after administration of parathyroid extract is in agreement with results reported by Johnston *et al.* (1), who used ^{14}C-glucose as a tool to study hexosamine metabolism. The cause of this increase in specific radioactivity may be either an increased biosynthesis of hexosamine-containing compounds or an increased breakdown of these compounds. The latter situation would result in a smaller pool of these complexes which would lead to a higher specific radioactivity. Total hexosamine content of the bones from the parathyroid

treated and control rats were essentially the same (averages 1.45 mg/g treated, 1.44, controls) indicating that the general pool of these compounds is not noticeably changed. This leads to the conclusion that an increase in biosynthesis of glycoproteins or mucopolysaccharides occurs after parathyroid extract administration. There are indications from other types of studies (3, 10) that parathyroid extract affects glycoprotein biosynthesis in bone.

When the two known hexosamine-containing components of bone were isolated, parathyroid extract was found to increase the specific radioactivity in both sialoprotein and chondroprotein. The effect of the extract was noted after 1 day's treatment and was not noticeably changed by prolonged treatment. The significance of the changes noted in regard to the overall action of parathyroid hormone on bone is not clear. Participation of mucopolysaccharide and glycoprotein complexes in the calcification process has been suggested a number of times, but there is less evidence that these compounds play a role in decalcification. Owen and Shetlar (2) have indicated the osteoclasts of young rabbits utilize ^{3}H-glucosamine for the formation of a glycoprotein which later is extruded upon the resorbing surface of the bone by the osteoclasts; later work (Bingham, *et al.*, 3) has shown that this utilization of ^{3}H-glucosamine is increased by the prior administration of parathyroid extract. Parathyroid extract appears to depress protein biosynthesis by the osteoblast (Young, 11; Goldhaber *et al.*, 12). Consequently, it would appear that increased biosynthesis of bone following parathyroid extract occurs largely in the osteoclast. The results (Table II) indicate that both sialoprotein and chondroprotein are labeled after the administration of 1-^{14}C-glucosamine. The specific activity of chondroprotein, however, is much less than that of sialoprotein. The radioactivity associated with chondroprotein may be present either as galactosamine in chondroitin sulfate or as sialic acid, glucosamine or galactosamine in the protein portion of chondroprotein (4). To answer this question the glucosamine and galactosamine were isolated by column chromatography from several of the bone samples from animals treated with parathyroid extract. Approximately 90% of the total hexosamine count was found to be associated with glucosamine; the amounts of glucosamine and galactosamine were approximately equal, consequently, the increased radioactivity in both sialoprotein and chondroprotein which occurs after parathyroid extract administration appears to be due largely to increased biosynthesis of glycoprotein. From the studies of bone cells cited above, it would appear that this synthesis may largely occur in the osteoclast. It has been suggested that parathyroid hormone exerts its action by affecting membrane permeability (13). Since sialic acid rich glycoproteins have been shown to be present in membranes, it may well be that the initial action of the hormone may be on these proteins thus altering the ion permeability of the membrane.

Summary. The radioactivity in bone, kidney, liver, serum protein and uncalcified connective tissue was determined after administration of 1-^{14}C-glucosamine to adult rats. The experimental animals were pretreated 1–3 days with parathyroid extract. Significant increases in specific radioactivity were noted in bone, kidney and serum protein of the rats treated with parathyroid extract. Radioactivity of liver and uncalcified connective tissue was not significantly changed by parathyroid extract. Sialoprotein and chondroprotein isolated from bone were both radioactive. The specific radioactivity of both fractions increased in the animals treated with parathyroid extract; however, as little radioactivity was found in galactosamine, it may be concluded that the increase is due largely to glycoprotein biosynthesis and not to increased synthesis of mucopolysaccharide.

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