Effect of Parathyroid Hormone on Mucopolysaccharide Synthesis in Rachitic Rat Cartilage *in vitro*.* (29349)

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Several recent investigations have suggested that parathyroid hormone (PTH) has a direct effect on bone matrix metabolism *in vitro* and *in vivo*. Gaillard, using tissue culture technique, found that parathyroid explants caused disappearance of osteoblasts and cessation of bone matrix deposition in limb rudiment preparations in association with significant alterations in fibroblasts and cartilage(1). Addition of both commercial and purified parathyroid hormone preparations produced similar changes. Vaes and Nichols observed a significant decrease in incorporation of glycine-1-C^{14} into the matrix of bone fragments from parathyroid treated rats *in vitro*(2). Studies on bone matrix biosynthesis by Johnston and Deiss suggested that parathyroid hormone increased the rate of matrix hexosamine synthesis while the turnover of hydroxyproline was decreased(3).

There have been relatively few studies on the effect of parathyroid hormone on the synthesis of matrix mucopolysaccharides in an isolated system, even though alteration in both tissue and serum mucopolysaccharides have been observed or suggested(4-7). Previous studies by one of us showed that after *in vivo* administration of parathyroid hormone the incorporation and release of sulfate-S^{35} in rachitic rat epiphyseal mucopolysaccharides was altered(5). In addition, rachitic rat cartilage was found to be a suitable tissue preparation for studying mucopolysaccharide synthesis *in vitro*(10).

The present investigation was designed to delineate the effect of physiological doses of parathyroid hormone on mucopolysaccharide synthesis *in vitro*. The parathyroid effect was rendered potentially more specific and precise by adding parathyroid hormone *in vitro* since *in vivo* administration results in sequential tissue changes and hypercalcemia, a potent metabolic inhibitor.

Experimental. Male weanling rats of the Sprague-Dawley strain were maintained on a rachitogenic diet (Nutritional Biochemicals Diet #2, U.S.P.) for 3 to 4 weeks until they were grossly rachitic. The animals were anesthetized with ether and killed by a blow on the head. Both femoral and tibial epiphyses were dissected clean of adherent tissue and sliced longitudinally with a sharp blade by hand into 3-5 mm segments. Initially, the pooled slices were washed quickly in cold modified Krebs-Ringer bicarbonate solution (*vide infra*) and aliquoted into 25 ml flasks so that each flask contained the epiphyses from a total of one rat. Eventually, the epiphyses from one leg of each of 2 paired rats were placed in one of 2 paired flasks; one flask served as control and the other as the parathyroid containing flask.

Each 25 ml flask contained 2.5 or 3.0 cc of incubation media consisting basically of Krebs-Ringer bicarbonate solution modified to contain 1.25 mM calcium; 200 mg% gelatin; 100 mg% glucose; 10 mg% cysteine; 1× Eagles amino acid mixture; and 1.0 μc of $Na_2S^{35}O_4$ per flask.

Commercial parathyroid hormone (Eli Lilly Co., Indianapolis, Ind.) was lyophilized, redissolved in dilute HCl (0.001 N) and then serially diluted in modified Krebs-Ringer bicarbonate solution containing 200 mg% gelatin to the specified concentrations. Purified parathyroid hormone,† which had been assayed at 9000 units/mg protein nitrogen, was dissolved in dilute acid (0.01 N HCl) and then serially diluted in the same buffer as described above.

Incubations were carried out for 3 hours in a Dubnoff metabolic shaker (72 oscillations/min) at 37° after 10 minutes prelim-

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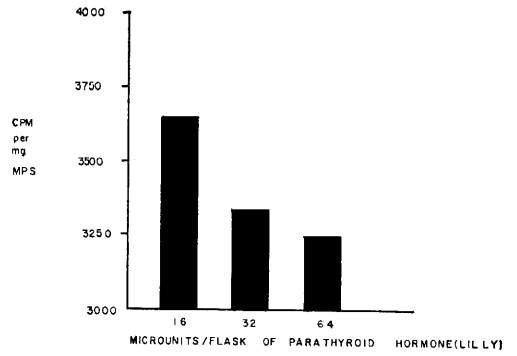

FIG. 1. The effect of variable concentrations of parathyroid hormone (Lilly) on S^{35} incorporation into rachitic rat cartilage mucopolysaccharide.

inary aeration with 95% oxygen-5% CO_2.

The mucopolysaccharide was isolated from the incubated cartilage by methods previously described(5). The lyophilized mucopolysaccharide was weighed and dissolved in distilled water. Suitable aliquots were separated for determination of radioactivity in a liquid scintillation system. In later experiments, it was found to be more convenient to determine the amount of mucopolysaccharide by the carbazole method of Dische employing chondroitin sulfate as a standard (8). The range of mucopolysaccharide isolated from individual flasks was 500-700 μg.

Results. Initial experiments demonstrated increased labeling of isolated mucopolysaccharide with the addition of parathyroid hormone (Lilly) in micro-amounts. Gelatin was added empirically to prevent the parathyroid hormone from adhering to the glass flask. Superficial observation also revealed that the addition of cyteine appeared to insure a response, perhaps by preventing the oxidation of parathyroid hormone and thereby maintaining its biologic activity during the period of incubation(9).

Fig. 1 illustrates the effect of variable concentrations of parathyroid hormone (Lilly) on the S^{35} labeling of mucopolysaccharide. Within the range of 16 to 64 microunits/flask, there appeared to be a significant increase in the labeling of the mucopolysaccharide over control values which approached a peak at 16 microunits. At concentrations above 64 microunits/flask there was a tendency to depress S^{35} incorporation. This increase in

TABLE I. Effect of Parathyroid Hormone (Lilly) on Incorporation of Sulfate-35 into Rachitic Rat Cartilage Mucopolysaccharide *in vitro*.

Exp No.	No. of observations	Mucopolysaccharide radioactivity (epm/mg) Control flasks	Parathyroid flasks	Ratio PTH/control
1	2	2030 ± 130	2840 ± 120	1.39
2	2	5970 ± 700	6280 ± 680	1.05
3	1	4320 ±	6110 ±	1.41
4	2	8200 ± 210	9560 ± 420	1.17
5	2	1300 ± 270	1360 ± 270	1.05
6	1	4350 ±	5040 ±	1.17
7	6	8750 ± 500	9510 ± 720	1.09
			Mean	1.17
			Significance	$P < .05$

200-333 mg of pooled rachitic rat epiphyses were placed into each of 2 sets of 25 cc flasks containing 3.0 cc of incubation media. Parathyroid flasks contained 16 microunits of commercial parathyroid (Lilly). Radioactivity expressed as cpm per mg of isolated mucopolysaccharide.

labeling was repeatedly observed in 7 consecutive experiments (Table I).

Since S^{35} incorporation into mucopolysaccharide may represent an exchange process rather than an increased biosynthesis of mucopolysaccharide, three experiments were performed using glucose-C^{14} (uniformly labeled) in an attempt to demonstrate that parathyroid hormone stimulated glucose incorporation into mucopolysaccharide *in vitro*, thereby suggesting overall net increase in mucopolysaccharide synthesis *in vitro*. Our results (Table II) appear to confirm this hypothesis.

These initial results, which suggested that parathyroid hormone stimulated mucopolysaccharide synthesis *in vitro*, were obtained with the commercial parathyroid preparation (Eli Lilly Co.). Because of the crude nature of commercial parathyroid hormone, the possibility existed that this effect might be mediated by a contaminant of this type of preparation. To eliminate this remote possibility, purified parathyroid hormone (9000 units/mg protein nitrogen) was employed and as Table III demonstrates, this also increased the S^{35} labeling of mucopolysaccharide in rachitic rat cartilage at similar concentrations. Higher concentrations of purified parathyroid hormone were also found to decrease S^{35} uptake into mucopolysaccharide in a

manner similar to the commercial preparation.

The data as presented show that parathyroid hormone has an effect on the synthetic pathway of mucopolysaccharide in rachitic rat cartilage *in vitro*. Perhaps even more exciting is the fact that this effect has been shown in dose ranges which are probably physiological and not pharmacological.

The mode of action of parathyroid hormone in the system described is still unclear. Since it has been shown that mucopolysaccharide is produced in the cytoplasm of the osteoblast or chondroblast(11), it is conceivable that parathyroid hormone has an intracellular effect. Recent experiments by DeLuca *et al* on mitochondrial effects of parathyroid hormone *in vitro* lend some credence to this concept(12). The exact locus of action of parathyroid hormone has still not been defined, however. The evidence presented here is in agreement with the observations that parathyroid hormone stimulates chondroblasts and fibroblasts *in vitro*.

Previous experiments utilizing rachitic rat cartilage by one of us have shown that other polypeptide hormones (insulin, growth hormone) effect glucose uptake into mucopolysaccharide *in vitro*(10). Since increased osteoid production is seen clinically in hyperparathyroidism as well as in states of growth hormone excess, the increased synthesis stimulated by parathyroid hormone *in vitro* is not unexpected.

TABLE II. Effect of Parathyroid Hormone (Lilly) on Incorporation of Glucose-C^{14} into Rachitic Rat Cartilage Mucopolysaccharide *in vitro*.

Exp No.	No. of rat cartilage/flask	Flask content	Mucopolysaccharide radioactivity, cpm/μg	Ratio PTH/control
1	3	Control	24.4	
	3	Parathyroid	51.1	2.05
2	3	Control	36.5	
	3	Parathyroid	60.0	1.65
3	4	Control	28.3	
	4	Parathyroid	42.7	1.51
		Mean		1.76
		Significance		$P < .05$

Rachitic rat epiphyses from 6-8 animals were divided into 2 pools and placed into individual 50 cc flasks containing 6.0 cc of incubation media; one serving as the control and the other as the treated flask containing 25 microunits of parathyroid hormone (Lilly). Each flask contained 5.0 μcuries of uniformly labeled glucose-C^{14}. The radioactivity is expressed as cpm per μg of isolated mucopolysaccharide.

TABLE III. Effect of Purified Parathyroid Hormone on Incorporation of Sulfate-35 into Rachitic Rat Cartilage Mucopolysaccharide *in vitro*.

No. of paired flasks	Mucopolysaccharide radioactivity (cpm/μg) Control flasks	Parathyroid flasks	Ratio PTH/control
1	8.91	12.30	1.38
2	8.79	12.88	1.45
3	8.85	10.31	1.16
4	7.32	6.44	.87
5	3.62	6.68	1.85
6	5.53	6.45	1.17
7	5.51	5.82	1.06
8	3.47	3.47	1.00
9	3.98	4.28	1.07
10	5.79	5.81	1.00
11	5.65	6.42	1.14
12	4.76	7.00	1.47
13	6.60	5.78	.88
14	5.66	7.50	1.33
15	4.09	4.54	1.11
		Mean	1.19
		Significance	$P < .01$

Epiphyses from one leg of each of 2 paired rachitic rats were placed in each of 2 paired 25 cc flasks containing 2.5 cc of incubation media; one flask serving as control and the other as the parathyroid flask containing 12.5 microunits of purified parathyroid hormone. Radioactivity is expressed as cpm per μg mucopolysaccharide determined by the carbazole method using chondroitin sulfate as a standard.

Summary. 1. Both commercial and purified parathyroid hormone increase the uptake of S^{35} and glucose-C^{14} into rachitic rat cartilage mucopolysaccharide *in vitro*. 2. The dose range employed is probably physiological and ranges from 3 to 10 microunits/cc of incubating media.

1. Gaillard, P. J., *The Parathyroids*, 1961, p20.

2. Vaes, G. M., Nichols, G., Jr., *Endocrinology*, 1962, v70, 546.

3. Johnston, C. C., Jr., Deiss, W. P., Miner, E. B., *J. Biol. Chem.*, 1962, v237, 3561.

4. Bradford, R. H., Howard, R. P., Joel, W., Shetlar, M. R., *Biochem. and Biophys. Res. Communications*, 1959, v1, 350.

5. Bernstein, D. S., Handler, P., PROC. SOC. EXP. BIOL. AND MED., 1958, v90, 339.

6. Bronner, F., *Trans. N. Y. Acad. Sci.*, 1962, v24, 265.

7. Johnston, C. C., Jr., Deiss, W. P., Holmes, L. B., *Endocrinology*, 1961, v68, 484.

8. Dische, Z., *J. Biol. Chem.*, 1950, v183, 489.

9. Tashjian, A. H., Jr., Munson, P. L., *J. Chron. Dis.*, 1963, v16, 269.

10. Bernstein, D. S., LeBoeuf, B., Cahill, G. F., Jr., PROC. SOC. EXP. BIOL. AND MED., 1961, v107, 458.

11. Dziewiatkowski, D. D., *J. Cell. Biol.*, 1962, v13, 359.

12. DeLuca, H. F., Engstrom, G. W., Rasmussen, H., *Proc. Nat. Acad. Sci.*, 1962, v48, 1604.

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Drug Effects on Epineprine Release from Rat Adrenal Medulla Homogenates and Granule Preparations.* (29350)

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We previously(1) characterized the *in vitro* release of the catecholamines from the chromaffin granules of the adrenal medulla of the rat. The initial release and the absolute release of the catecholamines were increased in electrolyte media as compared with equiosmolar sucrose and at 20°C and 37°C as compared with the release at 0°C.

We will report here the presence or absence of a releasing effect by 35 different drugs upon homogenates of the adrenal medulla of the rat and upon the partially isolated chromaffin granules separated from the homogenates. Although there are many *in vivo* studies of the effects of drugs on the catecholamine content of the adrenal medulla of the rat, we are not aware of any previously reported *in vitro* studies. It seemed pertinent, therefore, to evaluate a given drug in *in vitro* preparations from the same species in which the *in vivo* studies were made. Many of these agents were ineffective or quantitatively less effective on rat medullary granules as compared with the reported data on bovine adrenal medullary and adrenergic nerve granules. Their effects on rat medullary granules are more comparable with those recently reported on rabbit medullary granules(2) except that: a) d-lysergic acid diethylamide (LSD) and mescaline were effective on rat granules and ineffective on rabbit granules; b) reserpine, at high concentrations, was effective (as a releaser) on rabbit granules and ineffective on rat granules; c) at low concentrations (10^{-5}-10^{-7} M) reserpine and chlorpromazine inhibited release of adrenaline from rabbit granules while they were ineffective at similar concentrations on the rat granules.

Methods. The preparations of the homogenates and the fractionated suspensions of the chromaffin granules of the adrenal glands of rats were previously described(1). Briefly, the medullae were mechanically expressed, gently homogenized (0°C) either in sucrose (0.3 M), NaCl (0.155 M) in Tris buffer (0.02 M) adjusted to pH 7.45, or phosphate buffer adjusted to pH 6.5 (0.125 M KCl, .005 M KH_2PO_4, .006 M $MgCl_2$), in the proportion of 3 medullae/0.5 cc, and incubated together with equal volumes of the various drugs made up in the same suspending medium. After various incubations (50 minutes, 0°C; 15 minutes, 20°C; 15 minutes, 37°C) the mixture was layered over 0.5 M sucrose and centrifuged at 20,000 *g* (Servall-ss-34 head) for 20 minutes at 0°C to bring down the granules. The catecholamine content of the supernatant represented the portion released while the catecholamine content of the pellet was presumably contributed almost entirely by the granules.

Alternatively the chromaffin granules were in part fractionated from the homogenate by

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