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The Influence of a Parotid Hypocalcemic Substance on the Levels of Calcium, Inorganic Phosphate, and Hydroxyproline in Rabbit Serum

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The effects of a bovine parotid hypocalcemic substance (pure parotin) on several components in rabbit serum were investigated after injection into the aural vein. The concentration of inorganic phosphate was significantly increased at 4–6 hr after the injection of the hypocalcemic protein, and that of serum calcium was decreased. The hypocalcemic protein also significantly reduced the concentration of hydroxyproline at 4 hr after the injection. These results suggest that the protein might act on bone tissues, directly or *via* mediators. The concentrations of sodium, potassium and magnesium ions, and of alkaline phosphatase in the serum did not change significantly after the injection.

Keywords—parotin; hypocalcemic protein; parotid gland; inorganic phosphate in serum; hydroxyproline in serum

We previously reported some properties of a hypocalcemic substance (PHCP, pure parotin) purified from a precipitate at pH 5.4 of the extract of bovine parotid gland; these included amino acid composition, terminal sequences, and circular dichroism.²⁾ We suggested, based on a chemical modification study of PHCP, that histidyl, tryptophanyl, and tyrosyl residues might play a role in the appearance of hypocalcemic activity.³⁾ PHCP also increases the populations of lymphocytes and of antibody-producing cells by stimulation of the mesenchymal system in mice.⁴⁾ We subsequently purified an active core from a digest of PHCP with chymotrypsin and studied its biochemical character.⁵⁾ In connection with calcitonin, we showed that the concentration of serum calcitonin in rabbits after administration of PHCP did not change, and that PHCP did not bind to calcitonin antibody.⁶⁾

In this work, we report the changes in the concentrations of calcium, inorganic phosphate, and hydroxyproline in rabbit serum after the administration of PHCP.

Experimental

Materials—The samples used were the pure bovine parotid hypocalcemic protein and some fractions obtained from the parotid gland extracts in the course of purification according to the method described previously.^{2a)} Sample D-III was an active fraction obtained by chromatography of an ammonium sulfate precipitate (7–15%) on DEAE-cellulose, and G-II was the main fraction obtained by gel filtration of D-III on Sepharose 6B. Pure hypocalcemic protein (PHCP) was obtained by preparative disc electrophoresis of the G-II fraction.

Bioassay—The hypocalcemic protein was intravenously injected at various effective doses selected from dose-response relationships given in the literature.^{2a,3)} Biological activity was measured by the method

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described previously,⁷⁾ using six male rabbits as one group. Serum calcium, magnesium, potassium, and sodium were determined with a Shimadzu model 610 flame atomic absorption spectrophotometer. Hydroxyproline in rabbit serum was determined by the reported method.⁸⁾ The hydrolysate of rabbit serum in 6 N HCl was neutralized with KOH, then KCl and alanine were added and the mixture was oxidized with chloramine T for 25 min. After reduction with thiosulfate, the mixture was extracted with toluene and Ehrlich's reagent was added to the toluene fraction. The absorbance of the red-colored solution was measured at 560 nm. Inorganic phosphate in rabbit serum was determined according to the method of Miyazaki and Takemura,⁹⁾ using a mixture of ammonium molybdate and ascorbic acid. The activity of alkaline phosphatase in rabbit serum was measured as follows: 0.1 ml of serum was mixed with 0.1 ml of 0.05 M *p*-nitrophenyl phosphate in 0.2 M Tris-HCl (pH 8.6). After incubation for 30 min at 37°, the mixture (0.2 ml) was diluted to 2 ml and the absorbance was immediately measured at 410 nm.

Results

The concentration of inorganic phosphate in rabbit serum significantly increased at 4–6 hr after the injection of PHCP at a dose of 0.2 mg/kg of rabbit, and that of serum calcium decreased at the same time after the injection. The maximum increase ($26.3 \pm 8.8\%$) of inorganic phosphate at 5 hr was approximately three times the maximum decrease ($8.3 \pm 1.6\%$) of calcium. Table I shows the relation between the increase of inorganic phosphate and the decrease of calcium in serum, using PHCP and some crude preparations. The values in Table I are the means of the maximum values at 4, 5, and 6 hr after the injection, using six male rabbits. The difference between the experimental group and the control group was examined by means of the *t*-test. The results in Table I indicate a good correlation between the increase of phosphate and the decrease of calcium by PHCP, but it was not clear whether either of these effects was causative with respect to the other. Figure 1 shows the time course of alkaline phosphatase in the same rabbit serum in comparison with the control. The changes of alkaline phosphatase level in rabbit serum were insignificant, and the values were lower than those of inorganic phosphate. Therefore, the change of inorganic phosphate level might not be caused by alkaline phosphatase in serum but by some other factor, such as phosphatase in bone tissues.

TABLE I. Relation between the Decrease of Calcium and the Increase of Inorganic Phosphate

Fractions	Dose (mg/kg)	Mean \pm S.E. ($n=6$)	
		Percent increase of serum inorganic phosphate	Percent decrease of serum calcium
D-III ^{a)}	0.5	17.50 \pm 6.66	5.60 \pm 0.83 ^{b)}
	1.0	34.52 \pm 9.73 ^{b)}	10.65 \pm 0.69 ^{b)}
G-II ^{c)}	0.5	17.28 \pm 5.64	9.23 \pm 1.77 ^{b)}
PHCP	0.2	26.76 \pm 7.56 ^{d)}	8.90 \pm 2.06 ^{b)}
BSA	1.0	7.12 \pm 4.73	3.13 \pm 1.40
Control	—	3.01 \pm 4.88	1.57 \pm 0.46

a) the main active fraction obtained by DEAE-cellulose chromatography.

b) significantly different from saline control, $p < 0.01$.

c) the fraction obtained by gel-filtration on Sepharose 6B.

d) significantly different from saline control, $p < 0.05$.

The concentration of hydroxyproline in serum soluble collagen of rabbit is correlated with bone tissue metabolism. The time course of the change of concentration of hydroxy-

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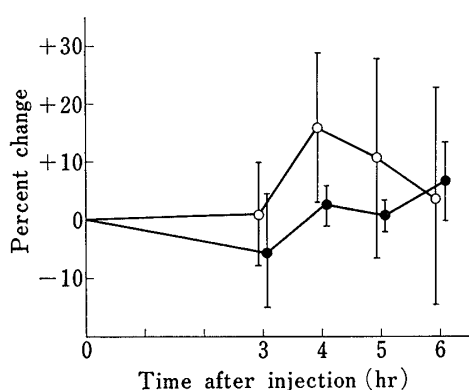


Fig. 1. Time Course of Alkaline Phosphatase in Rabbit Serum

The symbols ○ indicate the results after injection of PHCP at a dose of 0.20 mg/kg and ● indicate those of control experiments. Bars indicate S.E.

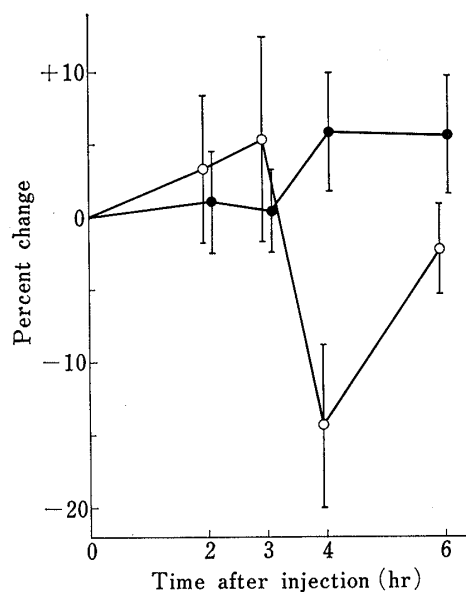


Fig. 2. Time Course of Rabbit Serum Hydroxyproline

○, PHCP 0.1 mg/kg; ●, control; $n=6$.

proline in rabbit serum after administration of PHCP at a dose of 0.1 mg/kg of rabbit is shown in Fig. 2 together with that of the control. The results show that the concentration of hydroxyproline significantly decreased at 4 hr after the injection of PHCP. The decreases of hydroxyproline at a dose of 0.05 mg/kg and 0.03 mg/kg after the injection were significant ($8.27 \pm 1.75\%$) and insignificant ($3.40 \pm 2.05\%$), respectively. The decrease in the concentration of serum hydroxyproline after the injection of PHCP was coincident with the decrease of concentration of calcium and the increase of inorganic phosphate. Thus, it appears that PHCP decreased the serum hydroxyproline concentration in rabbit; this might be caused by a direct or mediated inhibition of bone resorption, with a decreased breakdown of collagen as well as a decreased release of calcium.

Potassium and sodium levels did not change upon injection of PHCP at a dose of 0.2 mg/kg of rabbit. The concentration of magnesium ions in serum was reduced by the injection of PHCP at the same dose but the extent ($7.46 \pm 3.61\%$) in the case of PHCP was similar to that of the control ($7.02 \pm 2.32\%$).

Discussion

A combined decrease of serum calcium and increase of serum inorganic phosphate was reported using a partially purified preparation of parotid hypocalcemic protein.¹⁰⁾ In this report, we confirmed that the purified parotid hypocalcemic protein, PHCP, possessed both activities and found that PHCP reduced the concentration of soluble hydroxyproline in rabbit serum. The magnitudes and the time courses of the changes of serum calcium and hydroxyproline indicated that the entire process of bone catabolism was inhibited, though there is no definitive evidence that PHCP increases bone formation. However, it is well known that inhibition of bone resorption is induced by PTH or vitamin D, while calcitonin shows stimulatory activity.¹¹⁾ There is the general relation $[Ca]^3[P]^2=L$ between the concentrations

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of calcium and inorganic phosphate in rabbit serum. Our results are coincident with this equation (the data on calcium and inorganic phosphate levels produced by PHCP are shown in Table I).

The increases of phosphate were about three times those of calcium, but the standard errors were large in the case of inorganic phosphate, and the number of significant differences for phosphate in Table I is less than that for calcium. The concentration of inorganic phosphate in serum may be affected by more factors than that of calcium. The values of the standard errors of hydroxyproline are also larger than those of calcium. In conclusion, parotid hypocalcemic protein significantly affected the levels of calcium, inorganic phosphate, and hydroxyproline in rabbit serum.

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Crystalline Salts of Sucrose Octasulfate

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Sulfation of sucrose with pyridine-sulfur trioxide was carried out in dimethylformamide and pyridine, and the degree of sulfation of the sodium salt of sucrose sulfate thus obtained was estimated from the ratio of sulfur to carbon content (*S/C*). The sulfates prepared with 9—15 molar equivalents of pyridine-sulfur trioxide in dimethylformamide and those prepared with 5 and 9 equivalents in pyridine were identified as sucrose octasulfate. Potassium, cesium, rubidium, and ammonium salts of sucrose octasulfate were obtained as crystals.

Keywords—sulfation; pyridine-sulfur trioxide; sucrose; crystalline sucrose octasulfate; sucrose sulfate

Namekata and his co-workers^{2,3)} prepared sodium salts of disaccharide sulfates having strong anti-pepsin and anti-ulcer activities, and a basic aluminium salt of sucrose sulfate³⁾ having more potent activities.

In their previous work,^{2,3)} the amorphous sodium salt of sucrose sulfate obtained by sulfation of sucrose using pyridine-sulfur trioxide complex⁴⁾ in pyridine was analyzed only for sulfur content, giving a rather inappropriate value due to the presence of bound water, and the structures of the prepared sulfates were not satisfactorily confirmed.

Although Takiura and his co-workers⁵⁾ reported successful separation of sucrose di- and tri-sulfates from the products obtained by sulfation of sucrose using the same agent in dimethylformamide (DMF), no other components were examined. We prepared the salts of sucrose sulfate by using various amounts of pyridine-SO₃ and attempted to estimate their degrees of sulfation.

This paper describes a method for estimating the degree of sulfation in the sucrose molecule and reports the preparation of crystalline salts of sucrose octasulfate.

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