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# The Effect of Proteolytic Enzymes and Hyaluronidase *in Vitro* on the Calcification Mechanism of Epiphyseal Cartilage*

(33394)

ALBERT HIRSCHMAN AND DAVID SILVERSTEIN
(Introduced by R. S. Speirs)
*Department of Anatomy, Downstate Medical Center, State University of New York, Brooklyn, New York 11203*

Alteration or breakdown of the glycosaminoglycan-proteins (proteinpolysaccharides) in the hypertrophic zone of epiphyseal cartilage matrix by proteolytic enzymes has been proposed as an important phase of the calcifying mechanism (1–4). It has been suggested by Jibril (4), that the resulting removal of part of the ground substance would permit more rapid mobility of water and ions to the site of mineralization and thereby facilitate calcification. Weinstein *et al.* (1) demonstrated that the precipitation of calcium phosphate *in vitro* can be retarded or prevented by the presence of the high molecular weight proteinpolysaccharides, whereas trypsin- or hyaluronidase-treated proteinpolysaccharide, or chondroitin sulfate per se, has little or no effect. This evidence suggests that the role of intact proteinpolysaccharides in cartilage is to inhibit calcification.

On the other hand, it may also be hypothesized that the cartilage protease acts on the glycosaminoglycan-protein to produce a substance which is (or subsequently becomes) a component of the system which brings about calcification. Part of the product remaining after the *in vivo* degradation of glycosaminoglycan-protein by cartilage protease does remain *in situ* as indicated by the intense staining with toluidine blue usually seen in the matrix of the calcifying zone of epiphyseal cartilage and in the cartilage cores of the metaphyseal trabeculae.

In the present investigation, the technique of *in vitro* calcification of rachitic epiphyseal cartilage of rats was used as a means of studying the effect of proteolytic enzymes *in vitro* on the calcifiability of cartilage matrix. Inasmuch as hyaluronidase also degrades glycosaminoglycan-protein, the effect of this en-

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zyme on calcifiability was also studied.

*Materials and Methods.* Albino rats, 21 days of age, were maintained on a rachitogenic diet[1] for 3–4 weeks, at which time they were sacrificed and the tibias were removed. Four to five sections were cut free-hand in a frontal plane from the proximal end of each tibia. Each section was treated by one of the following procedures:

(a) Untreated section was immersed in 2% silver nitrate for a few minutes, rinsed, and exposed to light to demonstrate the initial state of calcification at the time of sacrifice.

(b) Control section was kept for 2 hr at 37°, then incubated overnight in calcifying solution and stained with silver nitrate to demonstrate normal calcifiability under the conditions used.

(c) Enzyme-treated sections, incubated for 2 hr at 37° in one of the following: trypsin[2] (0.046 *M* Tris, 0.0115 *M* $CaCl_2$, pH 7.8), chymotrypsin[2] (0.08 *M* Tris, 0.1 *M* $CaCl_2$, pH 7.8), pronase[3] (0.05 *M* Tris, 0.0115 *M* $CaCl_2$, pH 7.8), or hyaluronidase[4] (0.2 *M* acetate buffer, pH 6, 0.025 *M* $CaCl_2$). The concentration of the enzymes was 5 mg/ml, except for pronase, which was used at 2.5 mg/ml. After incubation in the hydrolytic enzymes, the sections were rinsed briefly in distilled water, incubated overnight in a calcifying solution, and stained with silver. The composition and preparation of the calcifying solutions are described by Hirschman *et al.* (5). The solutions used were buffered with bicarbonate and contained 7 mg/100 ml of Ca and 4 mg/100 ml of P, 9 mg/100 ml of Ca and 5 mg/100 ml of P, or 10 mg/100 ml of Ca and 6 mg/100 ml of P.

(d) Control buffer-treated sections were simultaneously incubated in the various buffers used, and otherwise handled like the enzyme treated specimens.

(e) Untreated section, tested to demonstrate the presence of nuclei of crystallization or incipient calcification (6).

After staining with silver, the specimens were rinsed thoroughly to remove excess silver nitrate, placed in a drop of glycerine, and examined.

The supernatant solutions from the enzyme-treated and buffer control-treated sections, were treated with 1 ml of 10% trichloroacetic acid and centrifuged. Increased absorbance at 280 mμ of the supernatant from the enzyme treated sections indicated that breakdown products from the sections were released into the solution. Positive results were obtained with all enzymes.

In order to ensure the validity of the results, the bones from an animal were rejected if: (i) an untreated section showed signs of healing rickets, as indicated in (a) or in a test for nucleation (e), or (ii) if untreated sections did not calcify well in (b).

The extent of calcification of each section was assessed by microscopic evaluation of the silver-stained specimens.

The calcifiability of an enzyme-treated section (c) is expressed as the ratio of the extent of its calcification to that of the corresponding buffer control (d). Thus, a value of 1 was assigned if the calcification of an enzyme-treated section equaled that of its buffer control, and lower values down to 0 for decreasing ratios.

*Results.* Our microscopic observations indicated that incubation in chymotrypsin, trypsin, pronase, and hyaluronidase did not promote calcifiability in areas of epiphyseal and articular cartilage which do not normally calcify. In fact, incubation in these enzymes decreased or destroyed the normal calcifiability of the hypertrophic zone of the epiphyseal cartilage.

The results are summarized in Table I. The data from the different calcifying solutions were pooled, because the loss of calcifiability of the enzyme-treated specimens was not affected by differences in the calcium and

[1] The rachitogenic diet consisted of: wheat gluten, 10%; vitamin free casein, 15%; corn starch, 50%; sucrose, 15%; calcium carbonate, 4%; brewer's yeast, 3%; sodium chloride, 1%; vegetable oil (vitamin D-free), 2%. It was compounded to order by Nutritional Biochemicals Corporation, Cleveland, Ohio.

[2] Purchased from Worthington Biochemical Corporation, Freehold, New Jersey.

[3] Purchased from Calbiochem, Los Angeles, California.

[4] Bovine testicular hyaluronidase containing 1380 USP units/mg, donated by the Wyeth Institute for Medical Research.

phosphorus levels, even at calcium and phosphorus concentrations as high as 10 and 6 mg/100 ml, respectively. At higher calcium and phosphorus levels, there is increased likelihood for spontaneous precipitation to occur, which would render the experiment invalid.

*Discussion.* Our results show that following incubation *in vitro* in trypsin, chymotrypsin, pronase, and hyaluronidase, there is a marked decrease in the calcifiability of rachitic rat epiphyseal cartilage, under the conditions used. In preliminary experiments papain also appeared to decrease calcifiability. This suggests that a protein or peptide combined with or associated with glycosaminoglycan is a necessary part of the calcifying mechanism.

It is to be noted that the proteolytic enzymes used in this study are not lysosomal enzymes, and are active at alkaline pH whereas lysosomal enzymes act in the acid range.

It is generally known that most proteolytic enzymes act at specific sites on the protein that they are cleaving. It has been demonstrated (7–9) that different proteolytic enzymes have different effects on glycosaminoglycan-protein. Immunochemical tests (10) showed that trypsin and chymotrypsin produce immunologically different products from glycosaminoglycan. Thus, we must consider the possibility that the presumably lysosomal protease normally present in calcifying cartilage may produce from the glycosaminoglycan-protein a specific product which is an important part of the calcifying mechanism, and which differs from any of the products formed by the enzymes used in this study.

In support of the concept that degraded glycosaminoglycan-protein forms part of the calcifying mechanism, Lipp (11) demonstrated the uptake of labeled serum proteins at sites of calcification and proposed that the product of the degradation of glycosaminoglycan-protein by cartilage protease combined with some of the serum proteins to form part of the calcifying mechanism.

Our results appear to be at variance with the work of Hjertquist and Westerborn (12), Howell and Carlson (13), and Murray (14) in which it was shown that after administration of papain *in vivo*, epiphyseal cartilage retained the ability to calcify *in vivo* although there was a decrease in the sulfated glycosaminoglycans present. This discrepancy may be due to the fact that we used different proteolytic enzymes although even papain appeared to inhibit in our preliminary experiments. In addition, the concentrations of enzymes that we used were at least 50 times the concentration of papain probably attained in the *in vivo* experiments of the other investigators. Thus, in our experiments, we may have broken down, in addition to glycosaminoglycan-protein, other proteinaceous components of the matrix necessary for calcification. It is also possible that a calcifying factor rendered diffusible by the *in vitro* enzyme incubations was lost in the supernatant fluid, whereas in the *in vivo* work this factor remains *in situ* or is replenished as it is lost.

TABLE I. *In Vitro* Calcifiability of Rachitic Rat Epiphyseal Cartilage after Treatment with Proteolytic Enzymes and Hyaluronidase.[a]

| Enzyme | Av calcifiability |
|---|---|
| Trypsin | 0.4 (11) |
| Chymotrypsin | 0.5 (10) |
| Pronase | 0.1 (11) |
| Hyaluronidase | 0.3 (6) |

[a] The values give the relative extent of calcification, as compared with the corresponding buffer-incubated sections. A value of 1.0 would indicate no loss of calcifiability. Average values are given; numbers in parentheses refer to the number of sections observed.

*Summary.* Incubation of slices of rachitic epiphyseal cartilage of rats in trypsin, chymotrypsin, pronase, and hyaluronidase resulted in a loss of calcifiability, as determined by subsequent incubation in calcifying solutions. It is concluded that breakdown of glycosaminoglycan-protein, per se, in cartilage is not a sufficient condition to make the tissue calcifiable.

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## Influenza Infections of Mice. I. Curative Activity of Amantadine HCl (33395)

J. W. McGAHEN AND C. E. HOFFMANN
(Introduced by J. A. Schneider)
*Pharmaceuticals Division, Industrial and Biochemicals Department, E. I. du Pont de Nemours and Co., Wilmington, Delaware 19898*

The reports of anti-influenza activity of amantadine HCl[1] (Symmetrel) have been concerned chiefly with the prophylactic action of this compound. However, Davies *et al.* (1) and Grunert *et al.* (2) reported that treatment with amantadine HCl as late as 72 hr, but not 96 hr, after infection time reduced mortality and increased survival time of mice infected with influenza A2/AA/2/60. Similarly, Solovyov and Tolmacheva (3, 4) showed that amantadine HCl was effective when given to mice 24 hr after infection with influenza A2/Frunze and with influenza A2/Lvov. Floor-Wieringa *et al.* (5) indicated that amantadine HCl reduced disease duration and some symptoms in some, but not all, human therapeutic trials during an influenza A2 epidemic in the Netherlands.

Although the mouse studies showed that postinfection treatment with amantadine HCl was protective, no curative action could be claimed since it was not shown that dosing began after the appearance of influenza A2 disease symptoms. Our studies of influenza A strains in mice have shown that a decrease of water consumption is the earliest observable disease symptom or sign. The present studies were undertaken to determine the effects of amantadine HCl on influenza A2 mouse infections when dosing was begun after the appearnce of this symptom.

*Materials and Methods.* Female white mice 28 to 30 days old (t. 18 ± 2 g) which had been caged on wire in continuous light 5 days before use were used throughout. Such mice, under light ether anesthesia, were infected intranasally with 0.05 ml of a mouse lung preparation of either influenza A2/Bethesda/10/63 or influenza A2/AA/2/60 diluted to a concentration calculated to cause 85% mortality on the tenth day after infection (3–5 $LD_{50}$).

The water consumption of virus-infected and sham-infected mice was measured to the nearest 1 ml at 4-hr intervals beginning 4 hr after infection. The data were analyzed as the cumulative milliliters of water consumed by groups of 60–80 mice.

Amantadine HCl was administered 12 hr after the first measurement period of significant water consumption difference. With influenza A2/Bethesda/10/63, amantadine HCl was dosed by two methods, singly and in combination: *ad libitum* in the drinking water as a 0.5 mg/ml solution from 48 hr

[1] 1-Adamantanamine HCl, EXP 105-1.