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Mouse Bone Collagenase

The Effect of Heparin on the Amount of Enzyme Released in Tissue Culture and on the Activity of the Enzyme

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The amount of mouse bone collagenase recovered in the tissue culture medium of bone cultured *in vitro* was increased by the addition of heparin at an optimal concentration of approximately 50 units/ml of tissue culture medium. Dextran sulfate and Treburon (a synthetic polysaccharide-sulfuric ester) which are structurally and chemically related to heparin were as effective as heparin in increasing the amount of mouse bone collagenase recovered in the tissue culture medium. In addition to stimulating the synthesis and/or release of mouse bone collagenase, heparin was also found to increase the specific activity of both crude and purified preparations of the enzyme when assayed using collagen in the solid state as the substrate, but showed no enhancement of enzyme activity when assayed using collagen in solution as the substrate. Dextran sulfate was as effective as heparin in increasing the activity of the enzyme using collagen in the solid state as a substrate. Neither heparin or dextran sulfate enhanced the activity of *Clostridium histolyticum* collagenase. For the first time, a purified tissue collagenase has been shown to both degrade and solubilize undenatured, insoluble tissue collagen at 37°. Moreover, since this action was markedly enhanced by the addition of heparin, it suggests that heparin and similar substances may play an important role in the regulation of collagen degradation during the remodeling of collagenous tissues *in vivo*.

Key words: Bone — Collagenase — Heparin — Collagen.

La quantité de collagénase d'os de souris, obtenue dans un milieu de culture d'os, cultivé *in vitro*, est augmentée pas l'addition d'héparine à une concentration optimale d'environ 50 unités/ml de milieu de culture. Le sulfate de dextrane et le Treburon (un ester polysaccharide-sulfurique synthétique), qui sont voisins au point de vue chimique et structural à l'héparine, sont aussi efficaces que l'héparine pour augmenter la quantité de collagénase de l'os de souris, récupérée dans le milieu de culture. L'héparine, outre son action de stimulation de synthèse et/ou de libération de callogénase d'os de souris, augmente aussi l'activeté spicifique des préparations globales et purifiées de l'enzyme, dont l'activité est testée sur du collagène à l'état solide comme substrat. Aucune augmentation d'activité enzymatique n'est notée lorsque du collagène en solution est utilisé comme substrat. Le sulfate de dextrane est aussi effectif que l'héparine pour obtenir une augmentation de l'activité enzymatique, en utilisant le collagène solide comme substrat. Ni l'héparine ou le sulfate de dextrane augmente l'activité de la collagénase de *Clostridium histolyticum*. Pour la première fois, une collagénase tissulaire purifiée dégrade et solubilise du collagène tissulaire non dénaturé et insoluble à 37° C. De plus, étant donné que cette action est nettement augmentée par l'addition d'héparine, il semble que cette dernière et des substances similaires peuvent jouer un rôle important dans la régulation de la dégradation collagénique pendant le remaniement des tissus collagéniques *in vivo*.

Die Menge von Mäuseknochen-Kollagenase, die sich im Gewebezucht-Medium von *in vitro* gezüchteten Knochen wiederfindet, konnte durch Zusatz von Heparinat in einer optimalen

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Konzentration von ungefähr 50 E/ml Medium erhöht werden. Dextransulfat und Treburon (ein synthetischer Polysaccharid-Sulfatester), welche strukturmäßig und chemisch dem Heparin nahestehen, wirkten sich auf die Erhöhung der im Gewebezucht-Medium zurückgewonnenen Mäuseknochen-Kollagenase im gleichen Maße aus wie Heparin. Nebst der stimulierenden Wirkung auf die Synthese und/oder die Freisetzung von Mäuseknochen-Kollagenase vermochte Heparin auch die spezifische Aktivität von ungereinigten und von gereinigten Enzympräparaten zu erhöhen, wenn für den Versuch Kollagen in fester Form als Substrat verwendet wurde. Mit gelöstem Kollagen als Substrat trat diese Wirkung dagegen nicht ein. Dextransulfat zeigte die gleiche Wirksamkeit wie Heparin, indem es die Enzymaktivität zu erhöhen vermochte, wenn Kollagen in fester Form als Substrat vorlag. Weder Heparin noch Dextransulfat erhöhten die Aktivität der Kollagenase aus *Clostridium histolyticum*. Erstmals konnte gezeigt werden, daß eine gereinigte Gewebe-Kollagenase in der Lage ist, nicht-denaturiertes, unlösliches Gewebekollagen bei 37° sowohl abzubauen als auch aufzulösen. Da diese Wirkung durch Zusatz von Heparin noch deutlich erhöht werden konnte, läßt sich überdies vermuten, daß Heparin und heparinähnlichen Substanzen bei der Regulierung des Kollagen-Abbaues während der Umgestaltung von Kollagengewebe *in vivo* eine wichtige Rolle zufällt.

Introduction

It has been previously found that the addition of small amounts of heparin to the tissue culture medium of bone explants enhanced the bone resorption produced by suboptimal concentrations of parathyroid hormone extract and other substances [2], and that this was accompanied by an increase in the collagenolytic activity released from the explants [6]. The role of heparin as a "cofactor" enhancing bone resorption was also suggested by the studies of Griffith *et al.* (1965) and Jaffe and Wilson (1965) who showed that osteoporosis developed in patients given large amounts of heparin in the treatment of clotting disorders. More recently, Jowsey *et al.* (1970) demonstrated that both normal and thyroparathyroidectomized animals showed a significant rise in their serum calcium levels after heparin administration, suggesting that the mobilization of calcium from the skeleton by heparin was a direct effect and was not secondary to increased thyroid or parathyroid gland activity.

Unpublished observations of M. Shimizu in our laboratories which showed that the addition of small amounts of heparin to collagen gels on which mouse bone or skin were cultured *in vitro* caused an increase of gel lysis, led to the finding that increased amounts of mouse bone collagenase could be recovered from the tissue culture media of bone explants when heparin was added to the medium during culture [15, 14].

In the present study the effects of heparin and structurally and chemically related compounds in enhancing the synthesis and/or release of mouse bone collagenase by bone explants in tissue culture and its direct effect on the enzyme activity of crude and purified preparations of mouse bone collagenase are presented.

Materials and Methods

Tissue Culture of Bone

The tibiae of 5-day-old Swiss albino mice of the Webster strain were cultured in mammalian Tyrode solution containing amino acids, vitamins, L-glutamine, penicillin and streptomycin in a roller tube as previously described. Commercial sodium heparin solution (Eli Lilly & Co., Indianapolis, Indiana) was diluted with Tyrode solution and added to the culture. Heparin

sodium salt (Fisher Scientific Co., Fair Lawn, New Jersey) was dissolved in water and extensively dialyzed against 1 M $CaCl_2$ and then against water. The heparin solutions were diluted with Tyrode solution, millipore-filtered and added to the tissue culture medium. Treburon (a synthetic polysaccharide-sulfuric ester) was kindly provided by Hoffman-La Roche, Inc., Nutley, New Jersey. Polyethylenesulfonic acid was obtained from the Upjohn Co., Kalamazoo, Michigan; chondroitin sulfate (bovine nasal septa) from Mann Research Laboratories, New York, New York; hyaluronic acid (bovine vitreous humor) from Worthington Biochemicals Corp., Freehold, New Jersey; dextran sulfate (molecular weight 16200) and dextran (molecular weight 40000) from Nutritional Biochemicals Corp., Cleveland, Ohio. These substances were dissolved in water at a concentration of 30 mg/ml and diluted with Tyrode solution, millipore-filtered and added to the tissue culture medium or to the solution used in the assay of enzyme activity. The incubation was carried out at 37° in an atmosphere of 95% O_2 and 5% CO_2 for 6 days. After the period of tissue culture, the media were pooled and dialyzed at 4° against distilled water.

Purification of the Mouse Bone Collagenase

The dialyzed culture medium was buffered by the addition of 0.2 M tris-HCl buffer, pH 7.6, to a final concentration of 0.05 M tris. Solid $(NH_4)_2SO_4$ was added and the fraction precipitated out between 20% and 50% $(NH_4)_2SO_2$ saturation collected. The precipitate was dissolved in a small volume 50 mM tris-HCl buffer, pH 7.6, containing 5 mM $CaCl_2$ and dialyzed against a large volume of the same solution.

The dialyzed, partially purified mouse bone collagenase from each group of cultures was assayed. The similar, partially-purified mouse bone collagenase obtained by ammonium sulfate fractionation from larger culture preparations was used for most of the experiments reported in this study. For some experiments, especially those involving the insoluble tissue collagens (*vide infra*), highly purified mouse bone collagenase was prepared by gel filtration of partially purified enzyme on Bio-Gel P-150 resin columns [15]. The heparin added to the tissue culture medium was removed by the ammonium sulfate precipitation. No free heparin was detected in the enzyme preparations. This partial purification step was necessary in order to determine mouse bone collagenase activity in each of the culture groups studied.

Collagenase Assays

The following four assay methods for collagenase activity were utilized in this study.

Radioactively Labeled, Reconstituted Collagen Fibrils as the Substrate [7]. Reconstituted collagen fibrils were prepared in plastic centrifuge tubes (11 × 70 mm) by preincubation of 50 µl of 0.2% rat skin neutral soluble collagen in 0.4 M NaCl, labeled with ^{3}H-proline and ^{3}H-hydroxyproline (5×10^4 dpm/mg collagen), prepared as described [9]. The reaction mixture consisted of the collagen gel containing 3000 cpm, 25 to 50 µl of enzyme solution, and other substances as noted in the individual experiments. The mixture was made up to a total volume of 300 µl with 50 mM tris-HCl buffer, pH 7.6, containing 5 mM $CaCl_2$. The incubation was carried out for 2 or 4 h at 37° on a shaker. Crystalline trypsin (Worthington Biochemical Corp., 25 µg) was incubated with collagen gels as a control. After incubation the tubes were centrifuged at 15000 rpm for 20 min at room temperature and the radioactivity was measured in 200 µl samples of the supernatant solution dissolved in toluene scintillator fluid with 5% (v/v) Bio-Solv-3 (Beckman Instruments, Inc., Fullterton, California) in a liquid scintillation counter.

Radioactively Labeled Collagen in Solution. This assay method has been recently developed in our laboratory [12–14]. The reaction mixture consisted of 50 µl of an 0.2% radioactively-labeled collagen solution in 0.4 M NaCl, 20 to 25 µl of enzyme solution and other substances as noted in the individual experiments. The mixture was made up to a total volume of 250 µl with 50 mM tris-HCl buffer, pH 7.6, containing 5 mM $CaCl_2$ and 0.2 M NaCl. The incubation was carried out for 2 h at 25°. Crystalline trypsin (25 µg) was incubated with collagen solution as a control. After incubation, 50 µl of an 0.2 M EDTA solution, pH 7.6, was added to each tube to stop the reaction. Fifty microliters of an 0.2 M EDTA solution containing 50 µg of crystalline soya bean trypsin inhibitor (Nutritional Biochemicals Corp., Cleveland, Ohio) was added to the trypsin blank. The tubes were then further incubated at 37° for 12 h to allow the precipitation

of intact collagen molecules as fibrils. The radioactivity remaining in solution was measured as described. Some of the polysaccharides, when used at high concentrations, inhibited collagen gel formation. Therefore, the corresponding blank (containing the same amount of the polysaccharide and 25 μg of crystalline trypsin without mouse bone collagenase), was prepared for each assay.

Viscometry. The reaction mixture consisted of 0.5 ml of an 0.2% solution of acid soluble rat skin collagen [1], in 0.4 M NaCl, 0.1 ml of mouse bone collagenase solution, 0.3 ml of 0.2 M tris-HCl buffer, containing 5 mM $CaCl_4$ and 0.1 ml of heparin solution or tris buffer. The mixture was incubated at 25° in a semimicro Cannon Manning viscosimeter (Cannon Instrument Co., State College, Pennsylvania) for 37 h.

Collagenase Assay Using Insoluble Collagen Fibers. Collagenase was also assayed by the release of soluble hydroxyproline from insoluble tissue collagen. The following two insoluble tissue collagens were used in this study:

1. A commercial preparation of purified bovine Achilles tendon collagen (Sigma Chemical Co., St. Louis, Mo.) was used. This insoluble collagen was suspended in 50 mM Tris-HCl buffer, pH 7.6, and finely divided by homogenization using Vir-Tis-45 homogenizer at top speed for 20 min in the cold. The homogenate was diluted to give a concentration of approximately 20 mg/ml with the same buffer. Samples (400 μl) of homogenized insoluble collagens were mixed either with 50 μl of mouse bone collagenase solution, 50 μl of heparin solution, or 50 μl of trypsin solution as noted in the individual experiments in small plastic centrifuge tubes. The mixture was made up to a total volume of 500 μl with 50 mM Tris-HCl buffer, pH 7.6, containing 5 mM $CaCl_2$ with NaN_3 (1 mg/ml).

2. The insoluble collagen of mouse bone calvaria was prepared from 10 young adult female mice. Freshly-dissected calvaria, freed from any adhering soft tissues and cartilage were extensively rinsed with several changes of cold 0.15 M NaCl. The washed calvaria were then cut into pieces approximately 1 × 1 mm by sharp scissors. The pieces were then demineralized in 0.5 M EDTA, pH 8.3, for 2 days with 4 changes of the EDTA solution. The demineralized pieces of calvaria were then extensively washed with cold water and then with 50 mM tris buffer, pH 7.6. This demineralized bone collagen preparation contained 114 μg of hydroxyproline per mg of sample. Aliquots of the decalcified collagen of mouse bone calvaria (approximately 450 μg as hydroxyproline) were placed in a reaction tube with 0.05 M tris buffer. Fifty microliters of trypsin solution, 50 μl of mouse bone collagenase solution with 50 μl of heparin solution, or 50 μl of buffer were added to each tube as noted in the individual experiments. The mixture was made up to a total volume of 500 μl with buffer and NaN_3 (1 mg/ml).

The incubations were carried out for 16 to 24 h at 37° with vigorous shaking. At the end of the incubation, the tubes were centrifuged at 15000 rpm for 20 min in the cold and the supernatant separated from the precipitate. Both fractions were hydrolyzed in sealed tubes in 6 N HCl for 20 h at 105° and the hydroxyproline content determined by the method of Woessner (1961). In some experiments, aliquots of the supernatant were dialyzed against water and the hydroxyproline in both the non-dialyzable and dialyzable fractions determined.

Protein Determination

The protein concentration of mouse bone collagenase samples was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as a standard. Collagen concentration was calculated from the hydroxyproline content measured by the method of Woessner.

Results

Effect of Heparin on the Amount of Mouse Bone Collagenase Released into the Culture Medium

The addition of varying amounts of commercial heparin solution to the culture medium increased the amount of mouse bone collagenase released into the tissue culture medium. The increase was roughly proportional to the amount of heparin added up to a concentration of 50 units of heparin/ml, and then leveled off (Table 1).

Table 1. The effect of heparin on the release of mouse bone collagenase by bone cultured *in vitro*

Culture	Assayed by the method utilizing radioactively labeled reconstituted collagen fibrils as the substrate		Assayed by the method utilizing radioactively labeled collagen in solution as the substrate	
	Activity (cpm)	Ratio of collagenase activity of test sample/ control	Activity (cpm)	Ratio of collagenase activity of test sample/ control
Control (no heparin added to culture)	451	1.00	355	1.00
5 units/ml heparin added to culture	1210	2.57	816	2.30
50 units/ml heparin added to culture	1488	3.16	1136	3.20
500 units/ml heparin added to culture	1492	3.17	1016	2.86

Each culture group consisted of 5 tubes, the media of which were pooled. The partially purified enzyme (mouse bone collagenase) was obtained from the pooled media by $(NH_4)_2SO_4$ precipitation (20–50% saturation) and dissolved in 0.5 µl of tris buffer. After dialysis against the same buffer, 50 ml of each sample was assayed using radioactively labeled reconstituted collagen fibrils as the substrate [10] and by the radioactive collagen solution method. The incubation period for the assay was 4 h. See text for further details.

For practical purposes, the maximum increase in the amount of collagenase released occurred at about 50 units of heparin/ml and this concentration was therefore used in the remaining experiments.

Further evidence for the enhancement of bone collagenase activity in the presence of heparin was the finding that the amount of hydroxyproline released into the tissue culture medium was increased by 53% with a concomitant decrease of 14% in the dry weight of the explant, as compared with control samples containing no heparin in the tissue culture medium.

The amount of mouse bone collagenase released into the culture medium was also increased by the addition of several substances chemically and structurally related to heparin (Table 2). Although the concentration of these substances which produced the maximum effect has not been determined, dextran sulfate, at the concentration used, was as effective as heparin used at its optimal concentration and Treburon was only slightly less effective. The addition of chondroitin sulfate and dextran also increased the amount of enzyme released. The differences in the effectiveness of these substances may be partly due to the rate at which they are metabolized in tissue culture, as well as to differences in their chemistry and structure.

Table 2. The effect of heparin and several related compounds on the release of mouse bone collagenase in tissue culture

Compound added to tissue culture	Collagenase activity (cpm)	Ratio of collagenase activity of test sample/control
None	490	1.00
Heparin, 50 units/ml	1472	3.00
Treburon (synthetic polysaccharide-sulfuric ester), 1.5 mg/ml	1156	2.36
Chondroitin sulfate, 1.5 mg/ml	764	1.56
Dextran sulfate, 1.5 mg/ml	1556	3.18
Dextran, 1.5 mg/ml	864	1.76

The cultures and the enzyme samples were prepared in the same fashion as those described in Table 1. Each sample was assayed using radioactive reconstituted collagen fibrils as the substrate [10]. The incubation period for the assay was 4 h. See text for further details.

Table 3. The effect of heparin on the activity of mouse bone collagenase assayed using radioactively labeled reconstituted collagen fibrils

Assay System	Activity (cpm)	Ratio test/control
Experiment 1		
Partially purified mouse bone collagenase		
enzyme (25 μg) without heparin (control)	762	1.00
enzyme (25 μg) + heparin (1 μg)	837	1.10
enzyme (25 μg) + heparin (10 μg)	978	1.28
enzyme (25 μg) + heparin (100 μg)	1298	1.70
enzyme (25 μg) + heparin (1000 μg)	1311	1.72
Experiment 2		
Highly purified mouse bone collagenase		
enzyme (5 μg) without heparin (control)	342	1.00
enzyme (5 μg) + heparin (10 μg)	715	2.09
enzyme (5 μg) + heparin (1000 μg)	918	2.68

The incubation period for the assay was 2 h in all cases.

Direct Effect of Heparin on the Activity of Mouse Bone Collagenase Assayed by the Method Utilizing Radioactively Labeled, Reconstituted Collagen Fibrils

Surprisingly, the addition of heparin to the incubation mixture significantly increased the enzyme activity of partially and highly purified mouse bone collagenase when assayed using radioactively labeled reconstituted collagen fibrils as the substrate (Table 3). Moreover, the enhancement was more marked in the case of the more highly purified enzyme preparations (Experiment 2, Table 3).

The effect of a number of compounds which are chemically and structurally related to heparin in collagenase activity is shown in Table 4. Although most of the

Table 4. The effect of heparin and other related compounds on the activity of mouse bone collagenase assayed using radioactively labeled reconstituted collagen fibrils

Assay System	Activity (cpm)	Ratio test/control
Partially purified mouse bone collagenase (25 μg) without heparin (control)	761	1.00
+ heparin (10 μg)	1030	1.35
+ heparin (1000 μg)	1324	1.74
+ chondroitin sulfate (7.5 μg)	808	1.06
+ chondroitin sulfate (750 μg)	1152	1.51
+ dextran sulfate (7.5 μg)	1030	1.35
+ dextran sulfate (750 μg)	1396	1.83
+ polyethylene sulfonic acid (7.5 μg)	943	1.37
+ polyethylene sulfonic acid (750 μg)	1439	1.89
+ dextran (7.5 μg)	793	1.04
+ dextran (750 μg)	764	1.00
+ Treburon (7.5 μg)	533	0.70
+ Treburon (750 μg)	495	0.65
+ hyaluronic acid (7.5 μg)	616	0.81
+ hyaluronic acid (750 μg)	228	0.30

The incubation period for the assay was 2 h in all cases.

Table 5. The failure of heparin and other related compounds to enhance the activity of mouse bone collagenase activity assayed using radioactively labeled collagen in solution as the substrate

Assay System	Activity (cpm)	Ratio test/control
Partially purified muose bone collagenase (25 μg)		
without heparin (control)	984	1.00
+ heparin (10 μ)	994	1.01
+ heparin (1000 μg)	1013	1.03
+ chondroitin sulfate (750 μg)	863	0.88
+ dextran sulfate (750 μg)	919	0.93
+ dextran (750 μg)	935	0.95
+ polyethylenesulfonic acid (750 μg)	895	0.91
+ Treburon (750 μg)	679	0.69
+ hyaluronic acid (750 μg)	472	0.48

The incubation period for the assay was 2 h in all cases.

compounds also showed an enhancement of enzyme activity, Treburon, which increased the release of mouse bone collagenase, inhibited enzymatic activity. Hyaluronic acid likewise inhibited enzyme activity.

The Absence of Heparin Enhancement on the Activity of Mouse Bone Collagenase Assayed Using Collagen in Solution as the Substrate

The direct enhancing effect of heparin and several substances related to heparin on the activity of mouse bone collagenase when assayed using collagen in the solid

Table 6. The failure of heparin to enhance the activity of mouse bone collagenase using collagen in solution as the substrate and assayed by viscometry

	Time elapsed in min						
	0	15	30	95	1150	1335	2215
Experiment 1: Control—Enzyme + Substrate Only							
Specific viscosity	4.99	4.70	4.51	4.07	2.35	2.15	1.63
% specific viscosity	100	94.1	90.4	81.6	47.1	43.1	32.7
Experiment 2: Enzyme + Substrate + Heparin (30 μg)							
Specific viscosity	4.86	4.61	4.45	4.05	2.23	2.03	1.55
% specific viscosity	100	94.8	91.6	83.3	45.9	41.8	31.9
Experiment 3: Enzyme + Substrate + Heparin (3 mg)							
Specific viscosity	4.80	4.51	4.28	3.80	2.09	1.91	1.50
% specific viscosity	100	94.0	89.2	79.2	43.5	39.8	31.3

Partially purified mouse bone collagenase, 50 μg, was used for each assay.

Table 7. The failure of heparin to increase the activity of *Clostridium histolyticum* collagenase: assayed using radioactively labeled reconstituted collagen fibrils

Assay System	Activity (cpm)	Ratio test/control
Experiment 1: Crude *Clostridium histolyticum* collagenase, Sigma Type I (25 μg)		
without heparin (control)	1051	1.00
+ heparin (10 μg)	1062	1.01
+ heparin (1000 μg)	1075	1.02
Experiment 2: Chromatographically purified *Clostridium histolyticum* collagenase, Sigma Type III (2.5 μg)		
without heparin (control)	847	1.00
+ heparin (10 μg)	783	0.92
+ heparin (1000 μg)	850	1.00

The incubation period for the assay in Experiment 1 was 15 min, and for the assay in Experiment 2, 30 min.

state (fibrils) as substrate was not observed when the assay was carried out using collagen in solution as the substrate (Table 5). Similar to the results obtained with collagen in the solid state as the substrate for the assay, both Treburon and hyaluronic acid inhibited enzyme activity. The failure of heparin to enhance mouse bone collagenase activity when collagen in solution is used as the substrate was confirmed by assaying the enzyme activity by viscometry. Table 6 shows the typical time course of the changes in specific viscosity after the addition of 50 μg of partially purified enzyme. It is clear that heparin did not significantly affect the time course of viscometry changes compared with controls containing only the enzyme.

Table 8. The effect of heparin on the activity of mouse bone collagenase on the insoluble collagen of bovine Achilles tendon

Experiment	Collagen released into the supernatant (%)
Collagen alone	2.9
Collagen + heparin (2 mg)	3.9
Collagen + trypsin (50 μg)	8.4
Collagen + trypsin (50 μg) and heparin (2 mg)	8.8
Collagen + mouse bone collagenase (25 μg)	14.1
Collagen + mouse bone collagenase (25 μg) and heparin (2 mg)	28.5

Each reaction tube contained 0.4 ml of insoluble collagen homogenate containing approximately 1110 μg of hydroxyproline. The amount of collagen released into the supernatant was calculated from the amount of hydroxyproline recovered in the supernatant and that remaining in the residue.

Table 9. The effect of heparin on the activity of mouse bone collagenase on the insoluble collagen of mouse bone calvarium

Experiment	Collagen released into the supernatant (%)
Collagen alone	5.1
Collagen + heparin (2 mg)	8.3
Collagen + trypsin (50 μg)	9.6
Collagen + trypsin (50 μg) and heparin (2 mg)	10.6
Collagen + purified mouse bone collagenase (25 μg)	29.9
Collagen + purified mouse bone collagenase (25 μg) and heparin (2 mg)	94.6

The incubation period for the reaction was 16 h at 37°.

The Failure of Heparin to Affect the Activity of Clostridium histolyticum *Collagenase.* Addition of heparin did not increase the enzyme activity of either crude (Type I) or chromatographically purified (Type III) *Clostridium histolyticum* collagenase preparations (Sigma Chemical Co., St. Louis, Mo.) analyzed using radioactively labeled reconstituted collagen fibrils as the substrate (Table 7).

The Effect of Heparin on the Activity of Mouse Bone Collagenase on Insoluble Collagens

At 37°, purified mouse bone collagenase releases significantly more insoluble tendon collagen into the supernatant than does trypsin. This digestion of the insoluble tendon collagen is considerably enhanced by the addition of heparin which has no effect itself on the amount of collagen released into the supernatant, or on the amount dissolved by trypsin (Table 8). Even more striking, however, is the effect of heparin on the amount of collagen dissolved by the activity of mouse bone collagenase on the insoluble collagen of mouse calvaria (Table 9), where virtually the entire insoluble solid phase of bone collagen is dissolved when heparin is added to the incubation mixture.

Analyses of the supernatants after digestion of insoluble bone collagenase showed that 42% of the hydroxyproline was dialyzable and 56% nondialyzable, whereas after digestion of the insoluble collagen by collagenase in the presence of heparin, 82% of the hydroxyproline was found to be nondialyzable and only 18% dialyzable.

Discussion

Heparin has been shown to have two distinct effects with respect to mouse bone collagenase: (1), it increases the amount of the enzyme raleased into the tissue culture medium during the culture of bone, *in vitro* and (2), it greatly enhances the activity of the enzyme (especially if the enzyme is highly purified) when assayed using collagen in the solid state as the substrate. When the assay for enzyme activity is carried out using collagen in solution as the substrate, heparin does not enhance the activity of the enzyme.

The reasons for the increase in the amount of collagenase released into the tissue culture medium when bone is cultured *in vitro* in the presence of heparin is unknown. The increase could result from an increased rate of enzyme synthesis or an increase in the rate at which the enzyme is released from the cell, or both. Since extracellular collagenase is presumably bound to collagen and is released after digestion of the substrate, the enhancement of this process by heparin may also contribute to the increased amounts of collagenase found in the tissue culture medium.

On the other hand, it is possible that heparin and certain of its analogues may increase the amounts of collagenase recovered in the tissue culture medium by virtue of their protective action. For example, they might prevent the destruction of the collagenase either by stabilizing the molecular configuration of the enzyme so as to make it less susceptible to other specific or nonspecific degradative enzymes, or by binding or otherwise directly or indirectly inhibiting these degrative enzymes.

Since heparin does increase the enzyme activity of mouse bone collagenase, it is possible that the increased amount of enzyme activity noted to occur in the presence of heparin was merely apparent and represented increased activity and not increased amounts of the enzyme. This is ruled out, however, by the finding that an increase in the amount of enzyme was also found when the assay for enzyme activity was carried out using collagen in solution as the substrate, under which circumstances no enhancement of enzyme activity by heparin occurs. Secondly, in other experiments, when the same concentrations of heparin were added to the tissue culture medium after *in vitro* incubations, no significant increase from control values was noted.

It is quite interesting that a number of compounds which are chemically and structurally related to heparin also increase the amount of enzyme released into the tissue culture medium. Of those compounds which increase the amount of enzyme released into the tissue culture media, Treburon, a synthetic polysaccharide-sulfuric ester, and the non-sulfated compounds Dextran and hyaluronic acid, inhibited rather than enhanced enzyme activity.

The stimulation of mouse bone collagenase production and/or release and the enhancement of the enzyme activity by heparin are similar to those reported for

heparin and lipoprotein lipase (glycerol-ester hydrolase, EC., 3.1.1.3 [10, 11, 20]). The mechanism whereby heparin and the other compounds increases the activity of mouse bone collagenase is unclear. It does not act by increasing the solubility of collagen in the solid state, either as reconstituted fibrils or as the native, insoluble fibrils of bone or tendon. However, the strongly negatively-charged heparin molecule may, by its interaction with collagen, change the local charge distribution making sites for the enzyme more readily available.

With regard to experiments on the purification and mode of action of mouse bone collagenase (S. Sakamoto *et al.*, unpublished observations), it has been noted that the purer preparations of the enzyme attacked collagen in solution much more readily than they did collagen in the solid state as fibrils. This is similar to the results reported for the collagenase obtained from human granulocytes [8]. These results, combined with the marked enhancement by heparin of the activity of the purified enzyme when tested with collagen fibrils as the substrate, suggest that an anionic polysaccharide or other macromolecule is necessary for its function and that such compounds may be removed during purification of the enzyme. With regard to the effect of heparin, it is interesting that Taylor [10. 16] has observed morphologically a more rapid and complete lysis of collagen in the vicinity of mast cells, one of whose functions is the synthesis of heparin.

An important observation of this study was that purified mouse bone collagenase containing no detectable proteinase activity could degrade insoluble tissue collagens at 37°, particularly bone collagen, to a significant degree, so that solubilization of the collagen occurred. It suggests that the purified enzyme can further degrade collagen after the initial scission of the macromolecule, presumably due to the fact that the denaturation temperature of the collagen is reduced and partial gelatinization occurs after the initial scission, thus allowing the collagenase to further degrade the peptide chains [16]. The considerable enhancement of this process by heparin and the findings described earlier by Taylor [18, 19] suggest that heparin or other negatively charged macromolecules are important in regulating the rate at which collagenous tissues are remodelled *in vivo*.

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