Rat Epiphyseal Cartilage: IV. Effect of Papain on Proteinpolysaccharide Metabolism and S^{35} Incorporation, *in vitro* and *in vivo*.* (31746)

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Papain has been observed to produce profound pathologic changes in cartilage(1,2). While most studies have dealt with the cartilagenous tissue of the rabbit ear and the characteristic "donkey phenomenon," a similar destructive sequence of events has been demonstrated in various cartilagenous organs including epiphyseal tissue(3). The ability of this enzyme to liberate mucopolysaccharide (MPS) complexes from their protein residues has been shown both *in vivo* and *in vitro* (4,5). In association with this effect it has been suggested that a reciprocal increase in MPS synthesis occurs(6).

The effect of papain on cartilage is of interest since its ability to evoke matrix loss provides a tool by which the mechanisms underlying the loss of cartilage matrix and its subsequent repair as well as the effect of matrix alteration on cellular metabolism can be investigated. In addition, endogenous proteases of lysosomal origin have been implicated in the changes observed in diseased cartilage and on the processes involved in endochondral bone formation and growth(7,8). The changes induced by papain, an exogenous protease, might simulate the chemical findings one might observe if the presence of these hydrolytic substances is real and relevant.

To explore some of these possibilities, the effect of papain administration *in vivo,* and of papain added *in vitro,* on the metabolism of rat epiphyseal cartilage (REC) *in vitro,* has been evaluated and is reported here.

Materials and methods. Preparation of animals and tissue. Male Sprague-Dawley rats ranging from 3 to 5 weeks of age (60-100 g) were employed. The isolation of tissue and incubation technique were similar to our previous description with minor exceptions(9, 10). Both chemical analysis and incubation studies were performed on the epiphyses obtained from one limb of each animal (one distal femoral and one proximal tibial epiphysis). In the experiments testing the addition of papain *in vitro,* the isolated epiphyses were randomly divided into paired flasks. To evaluate the effect of papain administered *in vivo,* the animals were paired according to closest body weight and injected intraperitoneally with either a high dose (10.0 mg/0.1 kg body weight) or low dose (2.0 mg/0.1 kg body weight) of a papain or a control solution (albumin). Papain (2 × crystallized-Sigma) was prepared in a 0.03 M cysteine solution at protein concentrations of 2.0 or 10.0 mg/cc while in the control solution albumin was substituted for papain. The animals were sacrificed at appropriate time intervals and the tissue isolated. Incubations were performed on the epiphyses of one limb of the control or the papain treated animals while in some experiments the opposite limb epiphyses were used for chemical analysis unincubated.

Preparation of media and incubation. The medium employed in these experiments was modified Krebs-Ringer bicarbonate containing one-half the prescribed calcium and magnesium concentration and fortified with glucose (11.1 mM). Each flask contained 3.0 cc of incubation medium to which sodium sulfate-S^{35} (New England Nuclear) was added to a final concentration of 6×10^5 CPM/flask. To evaluate the effect of papain, *in vitro,* the incubation media were adjusted to contain cysteine at a concentration of 3.0 mM while concentrated papain solutions were added in 0.04 cc volumes so that the final concentration in each flask ranged from 10 to 40 μg. Distilled water was added in the control flasks. The method of incubation otherwise

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TABLE I

	Glucose consumption, μM/3 hr*	Lactate production, μM/3 hr*	Media polysaccharides (uronic acid), μg/3 hr*	S^{35} incorporation into: Protein-polysaccharide CPM/3 hr*	S^{35} incorporation into: Cartilage residual polysaccharides CPM/3 hr*
Control	2.64 ± .15	5.54 ± .38	38.2 ± 5.8	3027 ± 190	981 ± 83
Papain added (*in vitro*)	2.90 ± .10	6.24 ± .49	95.2 ± 18.8	2787 ± 236	1032 ± 68
p	N.S.	N.S.	<.001	N.S.	N.S.

* Expressed per 100 mg wet cartilage incubated.

REC was isolated as described and placed in individual flasks containing 3.0 cc of incubation media without (control) or with added papain (10 μg/flask) followed by incubation for 3 hr. The data presented represent the mean plus I S.D. observed in 2 consecutive experiments containing 3 and 4 flasks for each group.

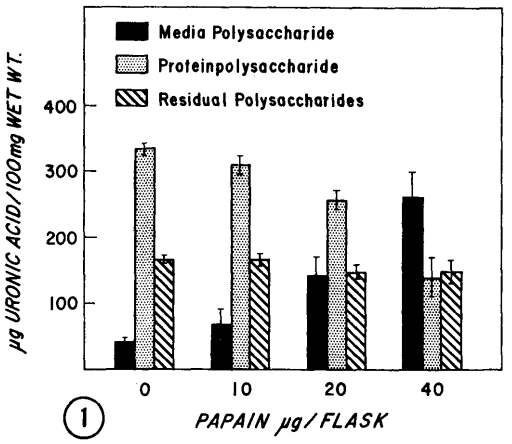
has been described previously(10).

Analytic methods. After 3 hours of incubation the media and tissue were transferred to a centrifuge tube, centrifuged for 5 minutes, and the supernatant decanted from the cartilage fragments. Glucose and lactate were determined on centrifuged media. The media (2.0 cc) was adjusted to 80% ethanol (v/v) and 2.0% potassium acetate and media MPS were allowed to precipitate overnight at 4°C. A fine ppt. was isolated by centrifugation and after reprecipitation 3 times the final ppt. was dissolved in water and a fraction removed for uronic acid determination. The cartilage fragments remaining after centrifugation were washed quickly with ice cold saline (0.9%) containing iodoacetate (10^{-3} M) and quickly frozen or dried in acetone and stored at —20°C.

Isolation of tissue MPS fractions. The tissue was suspended in 12.5 cc of distilled water and homogenized for 30 minutes at high speed in a Virtis 45 apparatus. The fine homogenate was centrifuged at 34,800 *g* for 10 minutes and the supernatant decanted. Proteinpolysaccharide (PP) was isolated from the supernatant as previously described. The residue remaining after centrifugation at 34,800 *g* was suspended in 3.0 cc of 0.6 M phosphate buffer (pH 6.5) containing EDTA (.02 M), cysteine (.01 M) and papain 2.0 mg, and digested for 16 hours at 60°C. The fine digest was adjusted to 0.2 M sodium hydroxide and, after 16 hours at 4°C, the solution was neutralized and centrifuged. The supernatant was decanted and the residue was washed with 1.0 cc of distilled water. The initial supernatant and wash were pooled and the residual tissue MPS was isolated with ethanol and potassium acetate in a similar manner as that employed for PP and media MPS (*supra vide*). Uronic acid was determined on these reprecipitated polysaccharide fractions as previously described. Glucose consumption and lactate production were expressed in μM/3 hr/100 mg wet cartilage incubated. Radioactivity was expressed in CPM/3 hr/100 mg wet cartilage. Analysis of PP for protein and CSA (by calculation) was similar to our previous report(10).

Results. The effect of papain addition *in vitro* on the metabolism of REC was evaluated in 2 experiments (Table I). A response was evident by both an increase in media MPS and a decrease in the MPS of the incubated cartilage as judged by the uronic acid content in these two fractions. Associated with this observation was a minor increase in glucose consumption and lactate production. No significant alteration was observed in S^{35} incorporation into the two MPS fractions, although, in both experiments, a greater percent of the total incorporated radioactivity appeared in the cartilage residual MPS.

In Figure 1 the results obtained with variable concentrations of media papain on the fractionation of the incubated MPS are presented. The increase in the media MPS is paralleled by an associated decrease in the PP fraction with a very slight change in the residual cartilage MPS with papain concentrations of from 10 to 40 μg/flask. The gradu-



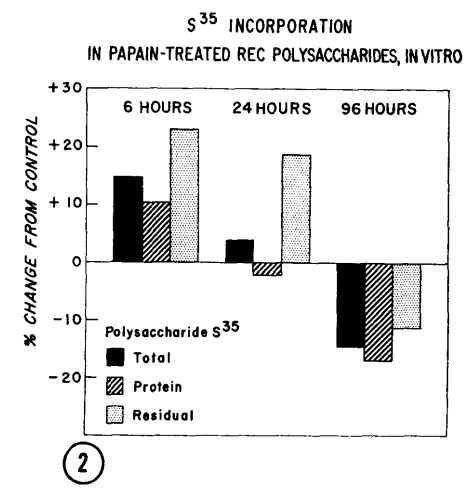


FIG. 1. REC segments were incubated in Krebs Ringer bicarbonate solutions containing variable concentrations of papain. At the end of incubation, the 3 mucopolysaccharide fractions were isolated as described in *Methods*. Data represent the mean concentration and 1 S.D. observed in each fraction and obtained from 3 flasks testing each concentration.

FIG. 2. S^{35} incorporation into total tissue mucopolysaccharides, and the 2 contained fractions (PP and residual MPS) of papain treated REC, *in vitro*. Data presented as % change in radioactivity observed in comparison to control (0 baseline). Data derived from observations on 4 control and 4 experimental flasks for each time interval after injection of papain (2.0 mg/0.1 kg) or a control solution (albumin).

al loss of tissue MPS into the media was accompanied by a gradual increase in glycolysis which only reached significance at a papain concentration of 40 μg/flask. At this concentration, glucose consumption and lactate production were significantly increased ($p < 0.05$) in comparison to the control by 14% and 20%, respectively. S^{35} incorporation into the total tissue MPS was depressed at all 3 papain concentrations (Table II). This depression in S^{35} uptake could not be completely attributed to a loss of labeled PP if calculations based on the specific activity (CPM/μg uronic acid) of this fraction were corrected for the increased MPS appearing in the media. Conclusive proof could not be obtained due to the presence of excessive extraneous radioactivity in this media fraction which negated any direct measurement. As observed in the initial experiments, the depression of radioactivity occurred predominantly in the PP fraction with little change in the residual cartilage MPS fraction except at 20 μg/flask.

The inability to demonstrate a reciprocal association between the loss of tissue MPS and S^{35} incorporation, *in vitro*, and the rather large loss of matrix polycations led to several experiments studying the effect of papain administration, *in vivo*. In Table III the data obtained on the incubated REC of animals treated with 10 mg papain per 0.1 kg body weight 24 hours prior to incubation are presented with appropriate controls. This dosage was found to have a remarkable effect on the content of MPS in this tissue with a 49% loss of the PP fraction and a 10% loss of the residual cartilage fraction. MPS release into the media was not essentially different from baseline observations, although the treated REC released a smaller quantity in comparison to the controls and probably was related to the reduced tissue MPS pool present in the treated animals. In contradistinction to the *in vitro* observations, both glucose consumption and lactate production were decreased significantly (16% and 17% respectively). S^{35} incorporation was similarly affected with a 19% reduction in total tissue MPS radioactivity, of which 100% occurred in the PP fraction as opposed to no change in the residual MPS fraction. As observed with the addition of papain *in vitro* a larger fraction of the total S^{35} incorporation appeared in the cartilage residual MPS.

Previous reports on the effect of papain administration have suggested a dose dependency and, in high concentrations, irreparable

TABLE II

Flask content	Concentration, μg/flask	S^{35} incorporation into: Proteinpolysaccharide, CPM/3 hr	S^{35} incorporation into: Cartilage residual polysaccharides, CPM/3 hr (% of total)
Control	0	3218 ± 229	1013 ± 91 (23.9 ± 2.0)
Papain	10	3121 ± 40	1056 ± 79 (25.1 ± 1.9)
"	20	2655 ± 260*	1286 ± 50† (32.8 ± 2.2)*
"	40	1410 ± 542‡	1059 ± 70 (44.1 ±10.6)†

Significance based on comparison to control: * $p < .02$, † $p < .01$, ‡ $p < .005$.

REC isolated as described and incubated for 3 hr in media containing different concentrations of papain. Data represent the mean plus I S.D. of the radioactivity incorporated into the 2 tissue polysaccharide fractions observed in 3 flasks/group.

TABLE III

	Glucose consumption, μM/3 hr*	Lactate production, μM/3 hr*	Media polysaccharides (uronic acid), μg/3 hr*	S^{35} incorporation into: Protein-polysaccharide CPM/3 hr*	S^{35} incorporation into: Cartilage residual polysaccharides CPM/3 hr*
Control	2.74 ± .09	5.64 ± .27	41.5 ± 3.3	2903 ± 201	1914 ± 152
Papain treated	2.29 ± .14	4.79 ± .33	31.9 ± 2.3	1687 ± 115	1914 ± 100
p	<.005	<.01	<.01	<.001	N.S.

* Expressed per 100 mg wet cartilage incubated.

REC from control and papain-treated animals were isolated 24 hr after injection of control and papain solutions (10 mg/0.1 kg body wt) and incubated as described. Data presented represent the mean and I S.D. observed in 4 flasks for each group.

tissue damage(3). The excessive loss of MPS observed in these experiments suggested that the failure to elicit an increase in S^{35} incorporation might be related to such an occurrence. In the subsequent study one-fifth the dosage of papain (2.0 mg/0.1 kg body weight) was employed and the response of the isolated tissue was studied at several time intervals after treatment (Fig. 2). Six hours after papain administration total MPS-S^{35} incorporation was significantly ($p<0.05$) higher in comparison to the controls by 12%. PP-S^{35} increased to 8% and the residual cartilage polysaccharide-S^{35} increased to 20% ($p<0.01$). At 24 hours, the total S^{35} incorporation remained elevated, but the increase was entirely confined to the residual fraction (+18%) ($p<0.01$) with the PP fraction actually decreased (—5%) in comparison to the controls. S^{35} incorporation was depressed in both the PP fraction (—20%) and the residual fraction (—9%) at 96 hours. Glucose consumption and lactate production paralleled the changes observed in S^{35} incorporation although the changes were not statistically significant. In Table III the data on the fractionation of the incubated tissue MPS are presented. No significant difference was noted in the total MPS at either 6 or 24 hours after papain administration (Table IV). At 24 hours, PP content was significantly reduced while at 96 hours both total tissue polysaccharides and the residual polysaccharides were significantly increased. The papain treated tissue was also observed to release more MPS into the media than the control after 96 hours.

The alterations induced in the chemical analysis of PP by administration of both high and low doses of papain 24 hours prior to analysis are presented in Table V. In both instances the loss of MPS (uronic acid) exceeded that of the protein obtained in this fraction with a resultant decrease in the polysaccharide/proteinpolysaccharide ratio. The higher dose was associated with a more profound change although in both conditions the differences were statistically significant. Calculations based on the weight of the unincubated cartilage segments demonstrated an as-

TABLE IV

	Time after inj, hr	Total polysaccharides (uronic acid)	Protein-polysaccharides (uronic acid)	Residual polysaccharides (uronic acid)	Media polysaccharide (uronic acid)
		μg‡			
Control		589 ± 14	349 ± 12	212 ± 4	26.6 ± 1.9
Papain	6	588 ± 26	343 ± 13	218 ± 13	27.8 ± 3.6
Control		678 ± 38	413 ± 17	236 ± 20	30.5 ± 2.9
Papain	24	629 ± 21	359 ± 26*	241 ± 10	29.7 ± 1.2
Control		588 ± 11	324 ± 4	240 ± 11	24.9 ± 2.0
Papain	96	615 ± 11†	324 ± 6	262 ± 8*	29.6 ± 1.1†

Significance in comparison to control: * $p < .02$, † $p < .01$.
‡ μg/100 mg wet cartilage.

REC from control and papain-treated animals were isolated at several time intervals after administration of papain (2 mg/0.1 kg body wt) or a control solution and incubated for 3 hr. Polysaccharides were isolated as described in *Methods* and uronic acid determined. Data represent the mean and I S.D. observed in 4 flasks for each group.

TABLE V

Exp	Proteinpolysaccharide: Uronic acid	CSA (calculated)	Protein	% CSA
	mg			
A. Control	1.504 ± .031	4.890 ± .100	3.880 ± .400	55.9
Papain-treated (low dose)	1.292 ± .081	4.200 ± .250	3.680 ± .210	53.1
p	<.01	<.01	N.S.	<.05
B. Control	1.056 ± .079	3.432 ± .257	3.792 ± .105	47.5
Papain-treated (high dose)	.534 ± .048	1.736 ± .156	3.530 ± .081	32.9
p	<.001	<.001	<.01	<.001

REC from control and papain-treated animals was isolated 24 hr after injection of papain and proteinpolysaccharide isolated and analyzed. Data represent the mean and I S.D. observed in a minimum of 3 animals. In Exp A (low dose) 2.0 mg/0.1 kg body wt of papain was employed; in Exp B (high dose) 10.0 mg/0.1 kg body wt of papain was given. Data have been expressed in mg per 100 mg acetone dried cartilage.

sociation between a decreasing water content (dry wt/wet wt minus 100%) in the treated tissue and the loss of PP. This finding was consistent with the well known observation that a relationship exists between the water phase of tissue and its (relatively) high MPS content. In addition, at both high (24 hr) and low doses (96 hr) of papain there was a significant increase in the dry weight of the isolated REC ($p<0.05$), but whether this represented an increase in the organic or the ash portion of dry cartilage was not further evaluated.

Discussion. The mechanism underlying MPS synthesis is not discernible in this study, although it would appear from the data that the loss of matrix MPS into the media (*in vitro*) or into serum and urine (*in vivo*) is intimately related. The inability to demonstrate a reciprocal increase in S^{35} incorporation commensurate with the papain-induced loss of MPS may have a partial explanation relative to the liberation of a sizable portion of matrix polycations into the media which, in turn, affect intracellular metabolism. These matrix polycations may depress oxidation and the increased glycolysis observed in these experiments is consistent with this possibility as well as the associated decrease in S^{35} incorporation(10). High doses of papain *in vivo* were also associated with a significant loss of matrix MPS and decreased S^{35} incorporation. These findings suggest that with profound loss of matrix constituent, there is sufficient damage to this tissue impairing its ability to compensate by increasing MPS synthesis. Papain itself, however, has been demonstrated to have a direct effect on cell morphology and these observations on glycolysis and/or

MPS synthesis might be attributed to this factor(3).

The administration of small doses of papain was accompanied by an increase in MPS synthesis. This increase was evident at 6 hours and remained elevated, although of lesser magnitude, at 24 hours. Determination of the total tissue MPS content after treatment revealed no significant decrease after papain treatment in comparison to the controls over a 24-hour period and, indeed, a slight increase at the end of 96 hours which is consistent with a compensatory mechanism. Minor changes were observed in glycolysis, reflecting either the direct action of papain on intracellular metabolism or its action causing matrix MPS loss but further studies are necessary for delineation of this point. The marked depression in these parameters noted at 96 hours is unexplained. It would appear that papain treatment had altered the metabolism of this tissue, and the possibility that some aspects of the biologic processes of REC had been altered, even at these lower doses, is reasonable. Hulth and Westerborn(3,11) have demonstrated that endochondral bone formation was affected by papain administration, and, that with suitable doses in immature animals, premature closure of the epiphyses and dwarfism resulted. The significant changes in the compositional analysis of this tissue produced by alterations in MPS is consistent with the speculation that MPS may be directly involved in calcification processes.

Recent studies on the MPS of cartilage have demonstrated the presence of these compounds physiologically in protein complexes and that these protein moieties confer some degree of separation of these macromolecules into various pools based on extractability and sedimentation(12,13). The data presented in this study implicate the importance of the above considerations in studying the action of papain. Both *in vivo* and *in vitro* the fraction most affected (lost) appeared to be PP, the fraction with the greatest water solubility and which, in this tissue, represented only 60%(9) of the total MPS (uronic acid). Previous studies have indicated that the decreasing response of cartilage to papain with aging is a function of total decreasing tissue MPS and/or tissue permeability(14). Our observations suggest that the complexed protein determines or is related to the susceptibility of MPS to the action of this protease in a viable state. The decreasing response observed may be related not only to a decrease in PP quantitatively but also to the increase in protein content and sedimentibility of these PP complexes found with aging (15). In addition, the permeability of this tissue has been related to these complexes, and the diminished penetrability of papain into the enclosed water space might be explained by the Osgood-Phelps phenomenon(16).

The changes observed in PP in this tissue are similar qualitatively to those reported in rabbit cartilage. Essentially, there was a decrease in total PP content with the MPS loss of greater magnitude than the protein resulting in a decrease in the percent CSA (this study) or an increase in the nitrogen/hexosamine molar ratio(17). These findings suggest that a protease of endogenous origin(7) could lead to changes in the character of PP not unlike that observed in diseased cartilage where loss of CSA was the predominant finding and attributed to a hyaluronidase enzyme present in lysosomes(18).

The effect of papain on the MPS of this tissue was to produce an increase in the residual cartilage fraction when compared to the total tissue uronic acid and S^{35} incorporation. While this observation could, in part, be explained by a loss of PP, its occurrence with low doses of papain, *in vivo,* at 96 hours suggests some alterations in the structure of the remaining PP complexes. In a similar manner, although papain administration was associated with losses of some soluble MPS fractions in skin, a net increase in the residual insoluble MPS fraction was observed(19). It is possible that the liberation of MPS residues from these individual water-soluble macromolecules may not have been complete and resulted in altered complexes with different physical properties(*i.e.,* solubility, state of aggregation). This possibility, however, necessitates further study.

Conclusions. The effect of papain on REC MPS and S^{35} MPS incorporation has been

studied *in vitro*. Papain treatment, both *in vivo* and *in vitro*, has been demonstrated to have a greater effect on the water-soluble fraction (PP) of the total MPS from this tissue. Small doses of papain, administered *in vivo*, were associated with an increased S^{35} MPS incorporation, evident within 6 hours and present up to 24 hours. These studies suggest that extracellular alterations of MPS in their physiologic state (protein complexes) have affected the biologic processes and matrix integrity of REC.

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Rat Epiphyseal Cartilage: V. Glucose-C^{14} Metabolism as Related to Growth and to Various Anatomical Areas, *in vitro*.* (31747)

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Studies of the processes involved in endochondral bone formation and remodeling have revealed the significance of cellular kinetics (1-3). However, the relation between these functions and glycolysis, recently reviewed (4,5), is not well understood. While studies in this laboratory have been directed towards proteinpolysaccharide metabolism in rat epiphyseal cartilage (REC)(6), the data(7) on the metamorphotic and growth properties of this tissue have suggested that it might afford an opportunity to explore the relationship of intermediary carbohydrate metabolism to various aspects of cellular kinetics and remodeling processes.

Utilizing glucose-C^{14} and REC of constant cellular composition, the alterations in C^{14} incorporation produced by modifications of the incubation media *in vitro* have been reported(8). Studies employing the same *in vitro* technique in varying anatomic areas of cartilage as well as during the formation of the ossification center might apply in correlating different cellular populations and/or their predominant remodeling process to glucose metabolism. The incorporation of glucose-C^{14} into CO_2, lactate, and five tissue fractions (TCA soluble, lipid, alkali extract-

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