THE REMOVAL OF CARTILAGE MATRIX, IN VIVO, BY PAPAIN

Identification of Crystalline Papain Protease as the Cause of the Phenomenon*

By ROBERT T. McCLUSKEY, M.D., and LEWIS THOMAS, M.D.

(From the Department of Pathology, New York University–Bellevue Medical Center, New York)

Plates 26 and 27

(Received for publication, May 21, 1958)

In a previous communication from this laboratory (1), it was shown that the intravenous injection of crude papain into young rabbits results in rapid depletion of the basophilic component of cartilage matrix throughout the body, resulting in loss of the normal rigidity of the rabbits' ears and consequent collapse of these structures. Reconstitution of cartilage matrix, with restoration of the ears to their usual appearance, occurs within several days. Recovery is prevented by the administration of cortisone.

These observations were extended by Spicer and Bryant (2), who observed loss of metachromasia of cartilage and the appearance of amorphous, basophilic material in the pericartilaginous interstitial spaces, lymphatics and blood vessels; subsequently these workers described metachromatic material in other blood vessels and in the renal tubules (3). Bryant, Leder, and Stetten (4) reported the isolation of a mucopolysaccharide with the characteristics of chondroitin sulfate from the blood and urine of rabbits following an injection of papain.

Our earlier investigations of the effect on cartilage failed to reveal the identity of the responsible component in crude papain. Injections of large amounts of crystalline papain protease containing cysteine, or of crystalline papain lysozyme, did not cause collapse of rabbit ears, and it was assumed that neither was the active constituent (1). Recently, however, it was noted that some of the animals given crystalline protease developed histological changes suggesting slight depletion of the matrix, and the possibility that this preparation contained traces of active material was therefore investigated. In the course of this study, solutions of crystalline papain protease were inactivated by prolonged dialysis against distilled water with removal of cysteine and consequent oxidation of the enzyme, or by the addition of two thiolantagonists, *p*-chloro-

* Aided by grants from the United States Public Health Service (H-2022 and A-1395) and the Research and Development Division, Office of the Surgeon General, Department of the Army, (under Contract No. DA-49-007-MD-590).

mercuribenzoate and iodoacetamide, prior to injection. Paradoxically, such treatment was found to change the protease, so that all of the gross and histological changes caused by crude papain could now be reproduced with small amounts of the crystalline enzyme.

The present report is concerned with a description of these findings, together with other evidence indicating that the component of crude papain which is responsible for the action on cartilage *in vivo* is, in fact, crystalline papain protease.

Materials and Methods

Young, hybrid albino rabbits of both sexes, weighing between 800 and 1000 gm., were used in all experiments, since it had been previously observed that mature animals are relatively refractory to the effects of papain (1).

Crude papain was obtained from several commercial sources in the form of dry, powdered latex. An approximate 1 per cent solution was prepared by stirring the powder in warm physiological saline and removing insoluble residue by paper filtration. In most instances, the final solution contained approximately 4 mg./ml. of non-dialysable protein (as measured by biuret and Kjeldahl determinations).

Crystalline papain protease (2 × crystallized), prepared according to the method of Kimmel and Smith (5), was obtained from Worthington Biochemical company, Freehold, New Jersey, and from Nutritional Biochemicals Corporation, Cleveland. The enzyme was received in saline solution containing 0.03 M cysteine.

Assays of proteolytic activity of papain preparations were performed by the alcohol titration technique of Grassman and Heyde (6), as employed by Davis and Smith (7). The substrate was alpha benzoyl-L-argininamide (BAA),[1] which is deaminated by papain. Reactions were run at pH 7.0, at 37°C., using 0.05 M BAA. Under the conditions of assay, the hydrolysis of BAA is a first order reaction. Specific activity (C_1) is expressed as K_1 (first order rate constant calculated in decimal logarithms) per milligram of protein nitrogen per milliliter of reaction mixture. Cysteine, versene (ethylenediamine-tetraacetic acid dihydrate), iodoacetamide or *p*-chloromercuribenzoate were added to the reaction mixtures under conditions described below.

Bromelin, a crude protease preparation derived from pineapple, was obtained from Nutritional Biochemicals Corporation. Solutions of this material were prepared in the same manner as for crude papain.

Rabbit tissues were fixed in 10 per cent formalin and stained with hematoxylin and eosin. In certain instances, toluidine blue was also used for staining.

EXPERIMENTAL

The Conditions under Which Cartilage Matrix is Removed by Crystalline Papain Protease, in Vivo

In our previous report (1), it was stated that crystalline papain protease failed to produce collapse of rabbit ears, even when injected in amounts as large as 10 mg. All of the preparations tested contained cysteine, to insure activation of the enzyme. In the present study, it was learned that when the cysteine was removed, or the enzyme inactivated by treatment with thiol

[1] BAA, alpha benzoyl-L-argininamide.

antagonists, the same *in vivo* action on cartilage as had been encountered with crude papain became readily demonstrable with the crystalline protease. The point is illustrated by the following experiments.

The in Vivo Activity of Dialyzed Preparations of Crystalline Papain Protease.—

A solution of crystalline protease, originally containing 0.03 M cysteine, was dialyzed for 16 hours against repeated changes of distilled water. At the end of this time, the preparation showed a tenfold diminution of proteolytic activity *in vitro*. Four rabbits were injected by vein with 4 mg. of the dialyzed material. Four others received the same preparation, but with the addition of 0.03 M cysteine and 0.001 M versene.

TABLE I

The in Vivo Effect on Cartilage of Dialyzed Crystalline Papain

Dose	Substance added to enzyme solution	C_1^*	No. of rabbits injected	No. of rabbits with ear collapse	No. of rabbits with loss of basophilic staininge of cartilage
mg.					
5	None	0.04	4	4	4
5	0.03 M cysteine 0.001 M versene	0.4	4	0	0

Enzyme solution dialyzed against distilled water for 16 hours.

* $C_1 = K_{1/E}$; $K_1 = 1/t \log \frac{100}{100 - X}$ in which X is the per cent of substrate (α-benzoyl-L-argininamide) hydrolyzed at time t. E = enzyme concentration in milligrams of protein N per milliliter.

Solutions incubated for 15 minutes at 37° before injection. Results recorded at 24 hours.

The results are shown in Table I. All of the rabbits receiving the dialyzed enzyme exhibited collapse of the ears (Fig. 1) and histological sections showed depletion of cartilage matrix. In contrast, none of the animals given dialyzed enzyme to which cysteine and versene were added developed ear collapse or cartilage changes, indicating that reduction, or "activation," of the protease before injection results in loss of its capacity to affect cartilage *in vivo*.

The in Vivo Activity of Crystalline Protease after Treatment with Sulfhydryl-Blocking Agents.—Results compatible with the above were obtained when crystalline papain protease was inactivated by *p*-chloromercuribenzoate (PCMB)[2] or iodoacetamide (IAA)[3] before being injected, as is shown by the following experiment.

Solutions of crystalline protease were incubated in the presence of various concentrations of PCMB or IAA for 15 minutes at 37°C. Groups of four rabbits each were injected intra-

[2] PCMB, *p*-chloromercuribenzoate.

[3] IAA, iodoacetamide.

venously with the treated enzyme, while similar groups of control animals received untreated material. The results are summarized in Table II.

Ear collapse, accompanied by typical histological alterations in cartilage, occurred in all the animals receiving crystalline protease treated with the thiol antagonists, while no changes were seen in the rabbits given untreated protease.

Assays of proteolytic activity *in vitro* yielded results comparable with those obtained with protease before and after dialysis. For example, as is shown in Table II, the C_1 value of untreated enzyme was 0.4 (this preparation caused

TABLE II

The in Vivo Effect on Cartilage of Crystalline Papain Treated with Iodoacetamide or p-Chloromercuribenzoate

Dose	Substance added to enzyme solution	C_1	No. of rabbits	No. of rabbits with ear collapse	No. of rabbits with loss of basophilic staining of cartilage
mg.					
10	0.03 M cysteine 0.001 M versene	0.4	4	0	0
5	None	0.1	4	0	0
5	0.005 M IAA	0.01	4	4	4
5	0.06 M IAA		4	4	4
5	0.001 M PCMB		4	4	4
2	None	0.1	4	0	0
2	0.3 M IAA		2	2	2
1	0.0005 M IAA	0.01	4	2	2
1	0.0005 M PCMB		4	0	0

Crystalline papain diluted in 0.05 M phosphate buffer pH 7 so as to contain 5 mg./ml of enzyme and 0.006 M cysteine. All solutions incubated for 15 minutes at 37° prior to injection. IAA = iodoacetamide; PCMB = *p*-chloromercuribenzoate. Results recorded at 24 hours. $C_1 = K_{1/E}$.

no changes in cartilage *in vivo*), while the C_1 after treatment with 0.005 M IAA was decreased to 0.01 (this preparation produced typical ear collapse).

It is to be noted that these results were seen with very high concentrations of iodoacetamide, indicating that in addition to binding cysteine in the enzyme diluent, the IAA reacted directly with the enzyme. This was shown more directly in the following experiment.

Solutions of crystalline papain were dialyzed against distilled water for 20 hours to remove the cysteine. To such solutions, IAA was added in concentrations of 0.005 or 0.01 M. *In vitro* assays using BAA hydrolysis showed that such preparations possessed no activity, nor was there any return of activity upon addition of 0.1 M cysteine and 0.001 M versene.

The results following injection of IAA-treated dialyzed crystalline papain are shown in Table III, from which it can be seen that the changes in cartilage

occurred following such treatment. This indicates that reversal of IAA inactivation of crystalline papain takes place in the rabbit.

The Effects of Thiol Antagonists and Cysteine, on the in Vivo Activity of Crude Papain Solutions.—The foregoing experiments indicated that crystalline papain protease does not bring about depletion of cartilage matrix *in vivo* if injected in the reduced state and it therefore became of interest to determine whether

TABLE III

In Vivo Effect on Cartilage of IAA-Treated Dialyzed Crystalline Papain

Dose	IAA added	C_1	C_1 after addition of 0.03 M cysteine*	No. of rabbits	No. of rabbits with ear collapse	No. of rabbits with loss of basophilic staining cartilage
mg.						
5	0.005 M	0.01	0.02	4	4	4
5	0.01 M	0.00	0.00	4	4	4

Crystalline papain solution dialyzed against distilled water for 20 hours. IAA = iodoacetamide. Enzyme solutions incubated for 15 minutes at 37°C. before injection. Results recorded at 24 hours.

* Cysteine not added to enzyme solution injected.

TABLE IV

The in Vivo Effect on Cartilage of Cysteine-Versene-Treated Crude Papain

Dose	Cysteine	Versene	No. of rabbits injected	No. of rabbits with ear collapse	No. of rabbits with loss of basophilic staining of cartilage
*mg.**					
10	0.1 M	0.001 M	4	0	0
10	None	None	4	4	4

* Milligrams protein crude papain. Enzyme solutions incubated 15 minutes at 37°C. before injection. Results recorded at 24 hours.

the same is true for a solution of crude papain. Accordingly, the following experiment was performed:—

A 1 per cent solution of crude papain was divided into two aliquots. To one, cysteine HCl (0.1 M) and versene (0.001 M) were added. Each aliquot was then injected by vein into a group of 4 rabbits.

As is shown in Table IV, ear collapse did not occur in any of the animals injected with the reduced crude papain, while all of the controls showed typical reactions.

It was also found that the addition of PCMB or IAA to crude papain solutions did not interfere with the capacity to produce ear collapse. Indeed, in several experiments, the impression was gained that ear collapse occurred

somewhat more rapidly and completely in such rabbits, as might be predicted on the basis of the observed effects of thiol antagonists on the crystalline protease.

Studies on the Action of Crystalline Papain Protease on Isolated Rabbit Ear Cartilage

The Direct Action of Crystalline Papain Protease on Rabbit Ear Cartilage.—

Rabbit ear cartilage was isolated and placed in solutions of crystalline papain protease containing thiol or antithiol agents, in order to learn whether changes occurred similar to those observed in the living animal.

Within 2 hours after immersion at 37°C. in solutions containing 0.1 mg. crystalline papain, 0.005 M cysteine and 0.001 M versene, the ear cartilage had become completely flaccid and collapsed. In contrast, cartilage placed in papain containing 0.001 M IAA or 0.0001 M PCMB remained erect and without loss of normal tone.

Histological sections of the ear cartilage undergoing collapse *in vitro* showed changes strikingly similar to those seen *in vivo*. The basophilic component of the cartilage matrix disappeared completely, while the cartilage cells remained intact. The cartilage samples placed in papain containing thiol antagonists showed no loss of basophilia in the matrix.

These results may seem at first to conflict with those observed in the living rabbit, since here the reduced papain protease is active against cartilage matrix while the enzyme treated with antithiol compounds is ineffective. However, it will be shown in the sections to follow that the conflict is not a real one, since the *in vivo* action of papain protease also requires that the enzyme undergo reduction once it has succeeded in reaching cartilage.

In Vitro Action of Papain Protease in the Cartilage of Isolated Rabbit Ears, Amputated after Intravenous Injection of Enzyme.—Earlier experiments had suggested that the active component of crude papain is rapidly cleared from the circulating blood, presumably being taken up in considerable part by cartilage. When the arterial blood supply to one ear was clamped off just before the intravenous injection of papain into a vein elsewhere, and the clamp released 15 minutes later, this ear failed to undergo collapse, indicating that insufficient papain remained in the blood after this lapse of time (1).

In the light of this finding, it became of interest to learn whether the enzyme had actually been taken up by cartilage soon after injection. One way to do this was to determine whether the same change in cartilage that occurs *in vivo* would proceed *in vitro* if an ear were surgically removed and incubated. The following experiment was therefore performed:

Three groups of rabbits were injected in the femoral vein with 5 mg. crude papain. Thirty minutes later the ears were removed. At this time they showed no loss of rigidity. One set was propped upright at 37°C. and observed at frequent intervals, the second set at 20°C., and the third at 4°C. The results are illustrated in Fig. 2.

All of the ears kept at 37°C. showed curling of the distal portion at 3 or 4 hours, and were completely collapsed and limp by the end of 18 hours. Histological sections at this time showed disappearance of all basophilia from the cartilage matrix, and amorphous accumulations of basophilic, metachromatic material in the connective tissue adjacent to cartilage. The ears held at 20°C. showed much less collapse, with curling only of the distal 2 or 3 cm. portion, and histologically a larger amount of basophilic matrix was still present. The ears placed at 4°C. showed no collapse, and no microscopic alteration in the cartilage.

Control ears, from animals not injected with papain, did not exhibit collapse nor histological alterations when kept for 20 hours at any of the temperatures employed in the experiment.

Similar *in vitro* collapse was demonstrated in rabbit ears removed 30 minutes after intravenous injection, into the femoral vein, of dialyzed crystalline papain protease (2 mg.) and protease treated with IAA or PCMB, suggesting that the purified enzyme had become incorporated into the cartilage after injection. With this information, it now became possible to carry out more direct experiments into the nature of the events involved in ear collapse.

The Effect of Thiol and Antithiol Compounds on Ear Cartilage Removed after Intravenous Injection of Papain Protease.—In order to learn whether the injected enzyme was dependent on SH groups in producing the change in cartilage matrix, the effects of cysteine, IAA, and PCMB brought into contact with the cartilage were studied in the following experiments:—

Rabbits were injected, in the femoral vein, with 5 mg. crude papain. Thirty minutes later the ears were removed and the skin and connective tissues were stripped away from the cartilage. One isolated cartilage plate from each animal was then placed upright in wide test tubes containing either of the following: cysteine HCl (0.005 M) and versene (0.001 M), IAA (0.002 M), PCMB (0.002 M), each in 0.05 M phosphate buffer at pH 7. As controls, cartilage plates from the opposite ears were placed in phosphate buffer alone. The cartilage plates were kept at room temperature and observed at intervals during the next 20 hours.

The results are illustrated in Tables V and VI. It will be seen that the presence of cysteine and versene greatly enhanced the rate and severity of collapse of the cartilage plate *in vitro*, while both IAA and PCMB completely prevented collapse from occurring. These results were confirmed by histological study of the cartilage; gross collapse and limpness were accompanied by loss of basophilic matrix, while retention of rigidity was associated with normal matrix.

The same experiment was performed with isolated cartilage plates from rabbits injected 30 minutes previously with crystalline papain protease treated with IAA or PCMB, and similar results were obtained, as shown in Tables V and VI. Immersion of the cartilage plates in solutions containing cysteine and versene caused accelerated collapse and loss of matrix, while exposure to IAA or PCMB prevented collapse of the cartilage.

Absence of in Vitro Cartilage Effect after Intravenous Injection of Reduced Papain Protease.—Having previously demonstrated that crystalline papain protease containing cysteine failed to produce ear collapse *in vivo*, while dialyzed protease preparations were active in this respect, it was of interest to learn whether corresponding events occurred *in vitro*, in the isolated ear cartilage.

TABLE V

Effect of Cysteine and Versene on Rate of Collapse in Isolated Rabbit Ears

Enzyme preparation injected	Ear in cysteine and versene	Ear in buffer
Crude papain 5 mg	++	—
Crystalline papain, 4 mg. with 0.005 M IAA	++	—
Crystalline papain, 4 mg. with 0.001 M PCMB	++	—
Crystalline papain, 4 mg. with 0.03 M cysteine	—	—
None	—	—

Ears excised 30 minutes after injection and kept at room temperature. Results recorded at 4 hours. ++ collapse distal 3 to 4 cm. of ear; — ear erect. Test ear placed in 0.05 M phosphate buffer pH 7 which contained 0.005 M cysteine and 0.001 M versene; opposite ear placed in phosphate buffer alone.

TABLE VI

Effect of Iodoacetamide or p-Chloromercuribenzoate on Rate of Collapse in Isolated Rabbit Ears

Enzyme preparation injected	Ear in IAA or PCMB	Ear in buffer
Crude papain, 5 mg	IAA—*	++‡
Crude papain, 5 mg	PCMB—	++
Crystalline papain, 4 mg. with 0.005 M IAA	IAA—	++
Crystalline papain, 4 mg. with 0.005 M IAA	PCMB—	++

Ears excised 30 minutes following injection and kept at room temperature. Results recorded at 20 hours.

* — ear erect.

‡ ++ collapse of distal 3 to 4 cm. of ear. Test ear placed in 0.05 M phosphate buffer pH 7 which contained either *p*-chloromercuribenzoate (PCMB) 0.002 M or iodoacetamide (IAA) 0.002 M; opposite ear placed in plain buffer.

Three rabbits were therefore injected by vein with 4 mg. crystalline protease in a solution containing 0.03 M cysteine, while three others received the same quantity of protease from which the cysteine had been removed by dialysis. Thirty minutes later, the ears were removed and the cartilage plates placed in buffer containing 0.03 M cysteine.

No changes occurred in the cartilage plates from the rabbits injected with papain containing cysteine, while complete collapse took place within a few hours in all samples from the animals given dialyzed papain.

The observations indicated that the reduced papain had failed to be taken up by cartilage after intravenous injection. The significance of this will be dealt with in the Discussion section below.

Demonstration of in Vivo Depletion of Cartilage Matrix with IAA-Treated Bromelin

In earlier experiments, solutions of crude bromelin had been found to cause no changes in cartilage when injected intravenously. In the light of the findings with papain described above, new experiments along comparable lines were undertaken with bromelin.

TABLE VII

In Vivo Effect on Cartilage of IAA Treated Bromelin

Enzyme preparation injected	No. of rabbits	No. of rabbits with ear collapse	No. of rabbits with loss of basophilic staining of cartilage
Crude bromelin, 6 mg.....	4	0	0
Crude bromelin with 0.01 M IAA 6 mg....	4	4	4

Solutions incubated for 15 minutes at 37°. Results recorded at 24 hours. IAA = iodoacetamide.

A 1 per cent solution of crude bromelin was prepared, and 0.005 M iodoacetamide was added to an aliquot. The IAA-treated enzyme was then injected intravenously into rabbits, and the results compared with those in animals injected with untreated bromelin.

The outcome is shown in Table VII. All of the rabbits given IAA-treated bromelin showed complete collapse of their ears within several hours, and histological sections revealed the same changes in cartilage matrix as with papain. It is evident that bromelin contains an enzyme, or enzymes, with the same property with respect to cartilage as papain protease. Further studies on this material are now in progress.

DISCUSSION

The intravenous injection of crystalline papain protease produces rapid depletion of cartilage matrix in young rabbits, provided the enzyme is inactivated by oxidation or sulfhydryl blocking agents prior to injection.

The paradox of the production of marked *in vivo* changes in cartilage following injection of inactivated, but not of activated, crystalline papain is clarified by study of the activity of the enzyme in cartilage. Such studies can be performed on plates of ear cartilage removed shortly after injection, since the enzyme is rapidly taken up by cartilage and continues to produce its characteristic gross and histological changes when the cartilage is incubated *in vitro*. By exposing the isolated cartilage to solutions containing either cysteine or the sulfhydryl blocking agents *p*-chloromercuribenzoate or iodoacetamide, it can be shown

that once the enzyme has been taken up by cartilage it is active in the reduced state and is inhibited by antithiol agents. In these respects, its behavior now resembles the proteolytic activity of crystalline papain *in vitro* (5). Exposure of normal rabbit cartilage *in vitro* to solutions containing crystalline papain produces the same morphological changes in cartilage as are seen *in vivo*, and in this situation the effect is also accelerated by cysteine and inhibited by sulfhydryl blocking agents.

The failure of reduced crystalline or crude papain to produce *in vivo* changes in cartilage suggests that the enzyme does not reach cartilage in sufficient concentration to cause detectable changes. This interpretation is strengthened by the observation that when rabbits are injected with papain containing cysteine, their cartilage is not only unaffected *in vivo*, but also fails to undergo change when immersed in solutions containing cysteine and versene.

On these grounds, it is postulated that shortly following injection, the reduced enzyme reacts with some component in the blood, perhaps a protein substrate, and this reaction somehow prevents passage of the enzyme from the blood to cartilage. If injected in an inactive state, this reaction in the blood does not take place, and the enzyme diffuses into cartilage where it becomes reactivated.

The method by which the enzyme is inactivated prior to injection does not appear to be of importance. Similar results were obtained by removing cysteine from the enzyme solution by dialysis, by mercaptide formation with PCMB or by alkylation with IAA. It is rather surprising that treatment of crystalline papain with a large excess of iodoacetamide fails to eliminate its activity *in vivo*. The inactivation of crystalline papain by iodoacetamide is not reversed *in vitro* upon addition of reducing substances such as cysteine, and it is generally stated to be irreversible. It was found that IAA-treated crystalline papain, which showed no return of activity *in vitro* following addition of cysteine and versene, produced the characteristic gross and histological changes in cartilage following injection. This indicates that IAA-treated papain is reactivated in the rabbit. The mechanism by which this occurs is not known, but warrants further investigation. It has been reported that iodoacetamide does not cause destruction of the enzyme and that following precipitation of iodoacetamide-inactivated papain with alcohol, the activity may be restored almost to its original level with cysteine (8).

The high state of purity of crystalline papain (9) makes it unlikely that the effects are due to an impurity in the preparations used. However, if an impurity is, in fact responsible for the effects on cartilage, its properties must be remarkably similar to those of papain protease itself, in view of the findings with respect to activation by thiol and inactivation by antithiol substances in the *in vitro* studies on isolated cartilage plates.

The gross and histological changes in cartilage which follow injection of inactive crystalline papain protease are indistinguishable from those pro-

duced by crude papain. The amount of crystalline papain required to produce the change, however, is approximately the same as the amount of crude papain in terms of protein, using the relatively crude index of ear collapse as a measure of the effect on cartilage. With both crude and crystalline papain, ear collapse generally occurs with injections of 2 mg. of protein, but usually not with 1 mg. The failure to obtain increase in activity with the crystalline enzyme may be due to two factors, aside from the possibility that an impurity in the preparation of crystalline papain is the active factor. First, there may be inhibitors in crude papain which are more effective in "protecting" the enzyme in the blood than those tried with crystalline papain, thus permitting a larger percentage of the injected material to diffuse into cartilage. Secondly, the possibility that other components of crude papain may produce the changes seen in cartilage cannot be excluded. The fact that another plant enzyme, bromelin, causes identical changes demonstrates that crystalline papain is not the only enzyme which can do so.

The chemical nature of the change produced in cartilage by papain has not been determined. It is known that chondroitin sulfate exists in cartilage in large part in the form of a mucoprotein, the protein moiety of which is not collagen (10). Tsaltas has shown, in this laboratory, that there is a considerable reduction in the chondroitin sulfate content of cartilage following an injection of crude papain (11). The appearance of metachromatic material in pericartilaginous connective tissue, which occurs in the first several hours after injection, suggests that chondroitin sulfate is being liberated from cartilage in a state of relatively high polymerization. The isolation by Bryant, Leder, and Stetten (4) of a mucopolysaccharide with the characteristics of chondroitin sulfate from the blood and urine of papain-injected rabbits strengthens this interpretation. The latter workers suggest that papain acts on the protein moiety of the mucoprotein of cartilage, with dissociation of chondroitin sulfate and its diffusion out of cartilage. It has been reported that crystalline papain causes reduction in the viscosity of a protein-chondroitin sulfate complex *in vitro* (12). Further *in vitro* studies on the effect of crystalline papain on cartilage and on the mucoprotein of cartilage are in progress.

Finally, a word should be said about the general implications of the observations described above. It has been shown that a substance with potent biological properties, capable of producing extensive morphologic changes in a vulnerable tissue widely distributed throughout the body, can only do so if it is injected in an inactive state. If it is activated before injection, it never succeeds in reaching the target tissue. Its effects seem to be dependent, to a crucial degree, on the capacity of the tissue itself to bring about activation of the enzyme. The possibility that this seemingly paradoxical situation may have a counterpart in other mechanisms of tissue damage is one which deserves mention.

SUMMARY

The intravenous injection of crystalline papain into young rabbits results in depletion of cartilage matrix throughout the body, with loss of rigidity and collapse of the ears, provided the enzyme is inactivated by oxidation or sulfhydryl blocking agents prior to administration. Cysteine-activated crystalline papain, when injected intravenously, produces little or no change in cartilage.

The changes which occur in cartilage following an injection of inactivated crystalline papain are indistinguishable from those produced by crude papain.

Activation of crude papain by cysteine prior to injection results in loss of its capacity to produce *in vivo* changes in cartilage.

The progressive changes which take place in cartilage *in vivo* also occur *in vitro* in isolated rabbit ears removed shortly after an injection of crude papain or inactivated crystalline papain. *In vitro* ear collapse occurs rapidly at 37°C. and does not occur at 4°C. Collapse is enhanced by exposing the cartilage to cysteine and prevented by exposure to iodoacetamide or *p*-chloromercuribenzoate.

The direct action of crystalline papain on plates of normal cartilage, *in vitro*, results in the same gross and histological changes which were observed *in vivo*. The direct action is accelerated by cysteine and inhibited by iodoacetamide or *p*-chloromercuribenzoate.

The intravenous injection of iodoacetamide-treated bromelin produces the same *in vivo* changes in cartilage as papain. Untreated bromelin has no demonstrable effect on cartilage.

It is suggested that the reason for the failure of activated papain to enter cartilage, after being injected intravenously, is that it probably reacts with a substrate or substrates in the blood. Oxidized or otherwise inactivated papain, in contrast, is readily taken up by cartilage and there converted to its active form.

BIBLIOGRAPHY

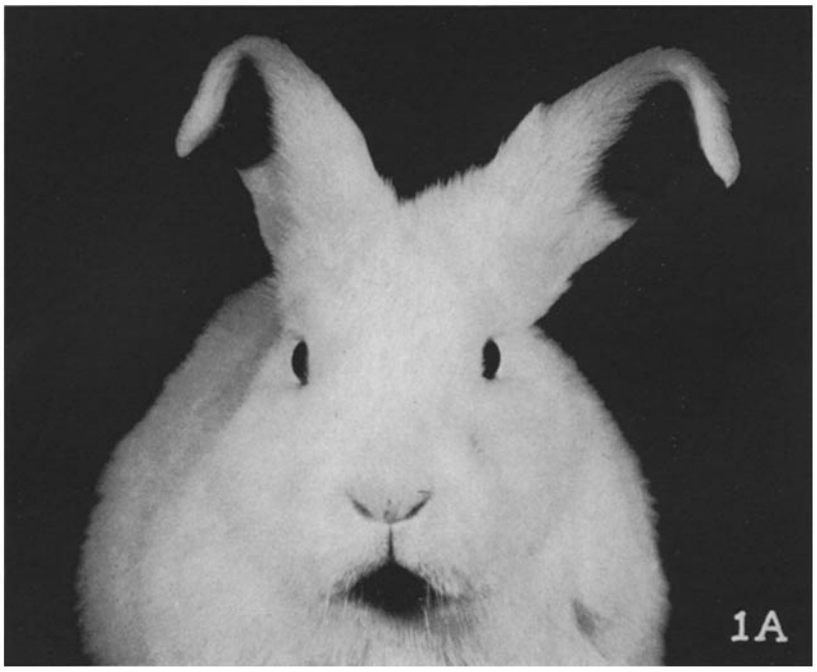
1. Thomas, L., Reversible collapse of rabbit ears after intravenous papain, and prevention of recovery by cortisone, *J. Exp. Med.*, 1956, **104,** 245.
2. Spicer, S. S., and Bryant, J. H., Cartilage changes in papain treated rabbits, *Am. J. Path.*, 1957, **33,** 1237.
3. Spicer, S. S., and Bryant, J. H., Systemic effects in rabbits receiving injections of papain and chondroitin sulfate, *Am. J. Path.*, 1958, **34,** 61.
4. Bryant, J. H., Leder, I. G., and Stetten, D., Jr., The release of chondroitin sulfate from rabbit cartilage following the intravenous injection of crude papain, *Arch. Biol. Chem. and Biophysics*, in press.
5. Kimmel, J. R., and Smith, E. L., Crystalline papain, I. Preparation, specificity and activation, *J. Biol. Chem.*, 1954, **207,** 515.
6. Grassman, W., and Heyde, W., Alkalimetrische Mikrobestimmung der Aminosaeuren, *Z. physiol. Chem.*, 1929, **183,** 32.

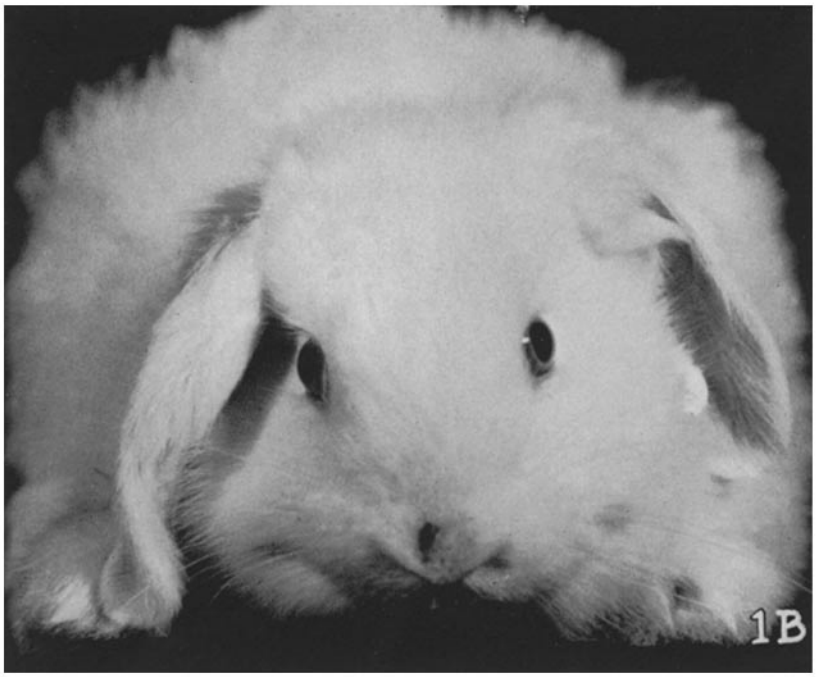
7. Davis, N. C., and Smith, E. L., *Methods Biochem. Anal.* 1955, Vol. **2,** 215.
8. Purr, A., Studies on the reversible inactivation of papain and cathepsin, *Biochem. J.*, 1935, **29,** 5.
9. Smith, E. L., Kimmel, J. R., and Brown, D. M., Crystalline papain. II. Physical studies; the mercury complex, *J. Biol. Chem.*, 1954, **207,** 533.
10. Shatton, J., and Schubert, M., Isolation of a mucoprotein from cartilage, *J. Biol. Chem.*, 1954, **211,** 565.
11. Tsaltas, T., Papain-induced changes in cartilage: alterations in the chemical structure of the cartilage matrix, *J. Exp. Med.*, 1958, **108,** in press.
12. Muir, H., The effect of papain on the viscosity of chondroitin sulphate, *Biochem. J.*, 1956, **62,** 26.

EXPLANATION OF PLATES

PLATE 26

FIGS. 1 A and 1 B. Appearance of rabbits given 4 mg. dialyzed crystalline papain.
FIG. 1 A. 4 hours after injection. × ⅗.
FIG. 1 B. 16 hours after injection. × ¾.





(McCluskey and Thomas: Removal of cartilage matrix, *in vivo*, by papain)

PLATE 27

FIGS. 2 A and 2 B. Appearance of isolated rabbit ears removed 30 minutes after injection of crude papain. Photographed 18 hours after injection. × 1.

FIG. 2 A. Erect ear kept at 4°; opposite ear partially collapsed, kept at 20°.

FIG. 2 B. Erect ear kept at 4°; opposite ear, totally collapsed, kept at 37°.

(McCluskey and Thomas: Removal of cartilage matrix, *in vivo*, by papain)