THE REMOVAL OF CARTILAGE MATRIX BY PAPAIN

Factors Affecting the Distribution of Crystalline Papain in Vivo*

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In a previous communication from this laboratory it was shown that the intravenous injection of crude papain into young rabbits was followed by reversible depletion of cartilage matrix throughout the body with loss of the basophilic staining property of cartilage (1). Subsequently, Spicer and Bryant described loss of metachromasia of cartilage and the appearance of basophilic material in pericartilaginous tissues shortly after an injection of crude papain (2). Bryant, Leder, and Stetten demonstrated increased amounts of chondroitin sulfate-like material in the blood and urine of similarly treated animals (3). Tsaltas reported that in rabbits previously given sulfur-35, which is incorporated largely into sulfated mucopolysaccharides, the administration of crude papain resulted in a rapid rise in blood and urine levels of S^{35}. Autoradiographs of cartilage from such animals showed reduction in activity compared with controls (4). All of these findings indicate that crude papain causes the *in vivo* liberation of chondroitin sulfate from cartilage.

It was found that the purified crystalline papain protease could produce the same changes in cartilage, provided that the enzyme was injected in an inactive form. Active (reduced) papain protease, on the other hand, was found to have little or no effect on cartilage *in vivo* following intravenous injection. It was postulated that active papain reacts with one or more substrates in the blood and as a result fails to enter cartilage following intravenous injection; inactive papain, in contrast, remains free to diffuse out of the circulation and into cartilage where it is activated (5, 6).

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Commercially available crystalline papain is supplied in a suspension containing cysteine. It was found that the same *in vivo* effects on cartilage occurred following injection of this material whether inactivated by dialysis, which removes the cysteine and leads to oxidation to the disulfide form, or inactivated by treatment with iodoacetamide. Following treatment with iodoacetamide, no *in vitro* enzymatic activity is restored to crystalline papain by the subsequent addition of a reducing agent such as cysteine, and the inactivation of papain by iodoacetamide is considered to be irreversible. In view of the findings that depletion of cartilage matrix could be produced by the intravenous administration of papain treated with excess iodoacetamide, it was postulated that there existed in the rabbit a mechanism which could reactivate papain acetamide (5).

In the present report, the problem of the reactivation of papain treated with iodoacetamide has been studied, and an alternative explanation has been found for the *in vivo* effects of this material. In addition, a study has been made of the factors determining the difference in behavior of active and inactive papain in rabbits. In this study a new method has been employed for quantitating the amount of chondroitin sulfate released from cartilage.

Materials and Methods

Albino rabbits of either sex, weighing between 800 and 1000 gm. were used.

Crystalline papain protease (recrystallized 2 times) was obtained from Nutritional Biochemicals Corp., Freehold, New Jersey. The enzyme was supplied in phosphate buffer, pH 6.9, containing approximately 20 mg. of protease/ml. and 0.03 M cysteine.

Antiserum to crystalline papain was prepared in rabbits immunized by two subcutaneous injections of 5 mg. crystalline papain with Freund's complete adjuvant at an interval of 4 days, followed 1 week later by intravenous injections of 1 to 2 mg. of active crystalline papain thrice weekly for 4 weeks. The animals were bled 10 days after the last injection. After preliminary titration, the most active sera were pooled and analyzed by the quantitative precipitin technique (7).

Two pools of serum were prepared and these contained respectively 1.0 mg. and 4.0 mg. antibody/ml. The antigen:antibody ratio at equivalence was 1:11. The amount of material precipitated following addition of papain was the same for active and inactive papain in equal quantities, whether in antigen or antibody excess. On double diffusion in agar, a single precipitin line formed between the antiserum and several different preparations of active or inactive papain. Thus it appeared that the antigenic determinants on the papain molecule were not significantly affected by procedures modifying the enzymatic activity of papain, and that the enzyme was a pure antigen. However, this antiserum formed several distinct precipitin lines on double diffusion with crude papain, suggesting that the crystalline material contained sufficient traces of other antigens at least to stimulate antibody formation.

For certain experiments papain was labelled as follows with iodine ^{131} (I^{131}). A solution of 100 mg. of crystalline papain diluted in normal saline to 20 ml. was dialyzed for 24 hours at 4°C. with normal saline containing 0.001 M ethylene diamine tetraacetic acid (EDTA), resulting in removal of cysteine and oxidation of the enzyme to the inactive disulfide form. To the dialyzed papain was added 1 ml. of 0.05 M carbonate buffer, pH 10. A solution of carrier-free I^{131}, 2 mc. in 2 ml., was added to 1 ml. KI_3 solution (I_2, 0.123 gm., KI, 0.157 gm. in 100 ml.

H_2O) and the resulting mixture transferred to the buffered solution of papain. After 20 minutes at 4°C., the solution of papain was dialyzed for 3 days with four changes of normal saline (1 liter) at 4°C. This preparation was stored at 4°C.; aliquots used in experiments were dialyzed for 18 to 24 hours and after warming to 37°C. were centrifuged for 15 minutes at 2500 R.P.M. to remove traces of insoluble material.

In vitro tests showed that 70 to 86 per cent of the original proteolytic activity could be restored to papain, following iodination, by addition of cysteine and EDTA. Solutions of papain reactivated by cysteine and EDTA or treated with iodoacetamide, or following incubation at 37°C. for 30 minutes, contained only insignificant amounts of dialyzable radioactivity. Of the radioactivity in aqueous solutions of labeled papain, 85 to 89 per cent was present in the material precipitated by a specific antiserum to crystalline papain.

Two methods were used to measure the enzymatic activity of papain *in vitro*. The first was the method of Davis and Smith (8), using alpha benzol-L-argininamide (BAA) as the substrate. Reactions were run at pH. 7.0, at 37°C. using 0.05 M BAA. Under the conditions of assay the hydrolysis of BAA behaves as a first order reaction. Specific activity (C_1) is expressed as K_1 (first order rate constant calculated in decimal logarithms) per milligram of enzyme protein nitrogen per milliliter of reaction mixture. Because of the difficulty in detecting and measuring by this method small amounts of enzymatically active material, an alternative method of assay using I^{131}-labeled human serum albumin (HSA I^{131}) was devised. This procedure differs in detail from the method of assay for trypsin and chymotrypsin, using casein labeled with I^{131} described recently by Katchman, Zipf, and Homer (9). I^{131}-labelled HSA was obtained from Abbott Laboratries, Inc., Teterboro, New Jersey, and was mixed with unlabeled HSA so that the radioactivity of 1 mg. of the protein mixture was approximately 20,000 C.P.M. The protein was made up in normal saline in a concentration of 5 mg./ml. At the start of the assay, 0.2 ml. of this solution was added to 1.8 ml. of 0.1 M phosphate buffer, pH. 7, containing some or all of the reagents, papain, cysteine, and EDTA, in amounts described below. The reaction mixtures were warmed to 37°C. prior to addition of the substrate. Aliquots of 0.2 ml. were withdrawn from the reaction mixture immediately after addition of the substrate, and thereafter at intervals up to 60 minutes. These samples were transferred to 12 × 75 mm. test tubes containing 2 ml. of 2.5 per cent trichloracetic acid (TCA) in water, and mixed. The tubes were then centrifuged for 15 minutes at 2,500 R.P.M. (1,380 R.C.F.). The supernatants were poured into 12 × 75 mm. tubes and the tubes containing the precipates were inverted on filter paper for 20 minutes. The radioactivity was counted in all the tubes containing supernatant or precipitate, using a well scintillation counter. Enzymatic breakdown of the substrate was measured as the increase in the amount of radioactive material soluble in 2.5 per cent TCA. In each sample the percentage of TCA-soluble material was calculated from the expression

$$\frac{\text{C. P. M. supernate}}{\text{C. P. M. supernate plus precipitate}} \times 100.$$

This measurement was unaffected by small volumetric errors in pipetting the samples. Initially all assays were run in duplicate, and it was found that the duplicate determinations did not differ by more than 1 or 2 per cent.

The rate of the reactions, R, was calculated by expressing the increase in TCA-soluble radioactivity between any successive pair of samples as a percentage of the precipitable radioactivity in the first of the pair, and dividing this percentage by the number of minutes in the interval between the samples.

The values of R obtained by this approximation remained fairly constant over a period of 60 minutes, and were closely related to the concentration of papain (C) in the reaction mixture. Over a range of concentrations of papain between 25 micrograms and 1.0 mg. per ml., the rela-

tionship between $\frac{1}{R}$ and $\frac{1}{\sqrt{C}}$ was linear. On this basis, the residual *in vitro* activity of papain following inactivation procedures was recorded as the weight of fully active papain having equivalent activity.

The amount of chondroitin sulfate released from cartilage was measured by a turbidimetric procedure using hexamminecobaltic chloride. This trivalent cation combines with certain anionic materials such as chondroitin sulfate to form insoluble complexes in media of low ionic strength. It has been shown that when chondroitin sulfate is added to serum or water the turbidity produced by the addition of hexamminecobaltic chloride is proportional to the concentration of chondroitin sulfate within the range of 0.025 to 2.500 mg./ml. The specificity and limitations of this method have been described elsewhere (10). The procedure may be described briefly as follows: 0.4 ml. of the solution to be tested is dialyzed for 48 hours with 2 changes of distilled water; the contents of the bag are transferred to a 10 × 75 mm. test tube and diluted to 2.2 ml. with distilled water; after centrifugation at 2000 R.P.M. for 10 minutes, the supernatant is transferred to a Coleman Jr. spectrophotometer tube[1] and the optical density at 615 mm. is measured using distilled water as a blank; 5 minutes after the addition of 0.2 ml. of a 5 per cent *w/v* solution of hexamminecobaltic chloride, the optical density is again determined, and the difference between the first and second readings is recorded as the result in absorbance units.

Rabbits were bled by cardiac puncture and the blood was either transferred to tubes containing dry ammonium potassium oxalate mixture or allowed to clot in tubes containing no anticoagulant. The animals were sacrificed by intracardiac injection of nembutal. Blocks of tissue for histologic examination were fixed in 10 per cent buffered formalin and stained as routine with hematoxylin and eosin.

The distribution of papain in serum was studied by electrophoresis in 0.8 per cent agar gel. Following electrophoresis, the serum proteins were identified by goat antiserum to whole rabbit serum, and papain by rabbit antiserum to papain.

In subsequent studies using papain labeled with I^{131}, the sera were fractionated by starch block electrophoresis, following the method described by Kunkel (11). Barbital buffer, pH 8.6, ionic strength 0.05, was used. In the eluates from the starch the concentration of protein was determined by a modified Folin technique (11) and the I^{131} activity was measured in a well scintillation counter, using 2 ml. aliquots in 12 × 75 mm. tubes.

RESULTS

Reactivation in Vitro of Papain Treated with Iodoacetamide

The chemical nature of the active site of papain has been discussed recently by Smith (12). For the purpose of the present report the activity of the enzyme can be regarded as dependent on a sulfhydryl group. Simple oxidation of the active, reduced form of papain (PSH) leads to reversible inactivation by conversion to the disulfide form (PS—SP). This product can be reactivated by reducing agents such as cysteine. Reduced papain is also susceptible to alkylation by agents such as iodoacetamide, with the formation of papain acetamide ($PS—CH_2CONH_2$), which cannot be reactivated by cysteine. In the disulfide form, papain is resistant to alkylation by iodoacetamide.

When a solution of crystalline papain containing cysteine is treated with

[1] Coleman Instruments, Inc., Maywood, Illinois.

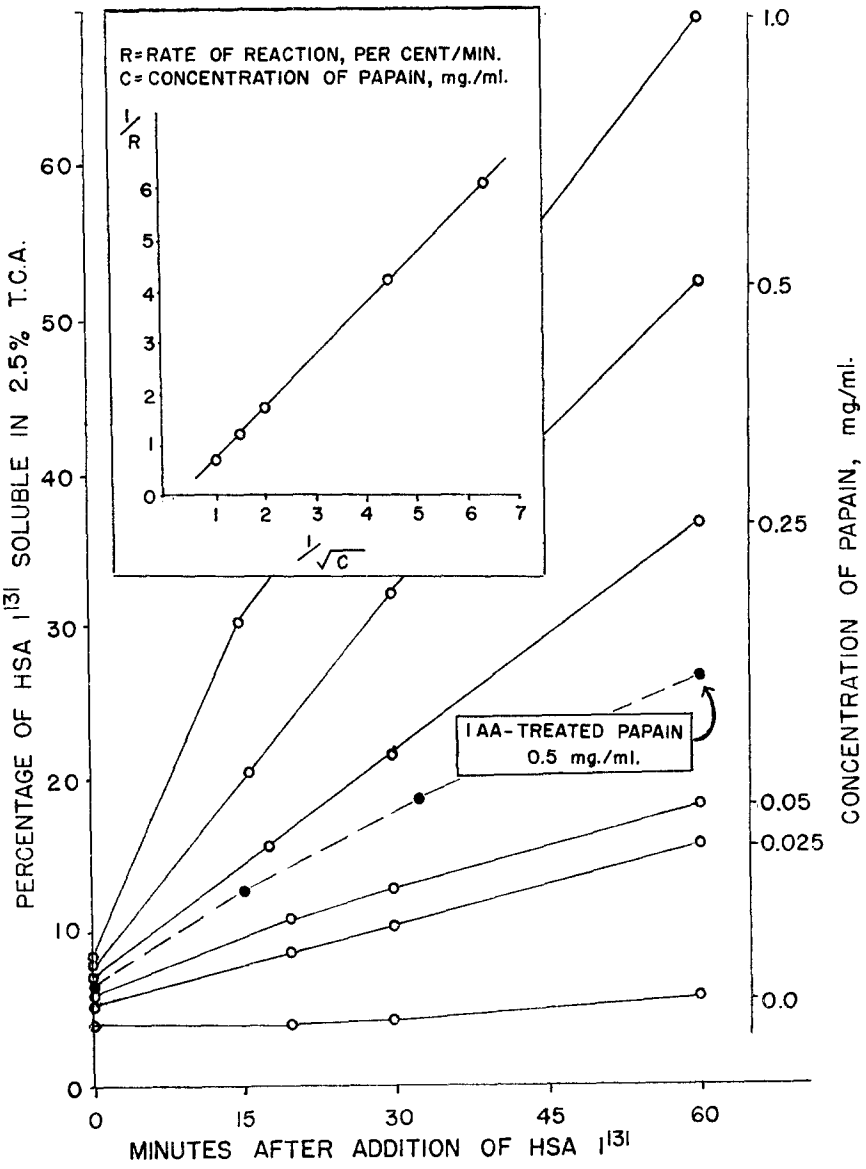
excess iodoacetamide, and the mixture injected intravenously in a rabbit weighing less than 1000 gm., widespread depletion of cartilage matrix occurs. This mixture, however, shows no *in vitro* enzymatic activity, even following addition of excess cysteine. As a tentative interpretation of these findings, it was previously suggested that papain acetamide was reactivated in the rabbit (5). Subsequently an alternative explanation was formulated, according to which the *in vivo* depletion was not the result of reactivation of papain acetamide, but instead was due to reactivation, in cartilage, of a portion of the injected enzyme which was in the disulfide form. This implied that following intravenous injection of the mixture, the excess iodoacetamide was in effect neutralized or distributed elsewhere from the enzyme.

From this hypothesis, it follows that when excess iodoacetamide has been removed from a solution of papain, some enzymatic activity should be restored *in vitro* by addition of cysteine. Moreover, the *in vivo* properties of material so treated should be identical with those of reduced papain and it should not produce depletion of cartilage matrix following intravenous injection. Finally, repeated treatment with iodoacetamide should not progressively diminish the restorable enzymatic activity of papain, unless preceded by measures to convert the disulfide portion to the reduced form, and thus make it susceptible to alkylation. These predictions were tested as described below.

A solution of commercial crystalline papain containing 20 mg./ml. of the protease and 0.03 M cysteine was diluted with an equal volume of phosphate buffer containing 0.02 M iodoacetamide, and incubated at 37°C. for 15 to 30 minutes. The solution was then dialyzed for 24 hours at 4°C. with a 200-fold volume of 0.1 M phosphate buffer, pH 7.0, which contained 0.001 M EDTA, to minimize combination of the enzyme with trace metals. The proteolytic activity of this material was measured on I^{131} labeled HSA. The results of a typical experiment are shown in Text-fig. 1.

After treatment with iodoacetamide followed by dialysis, papain showed restorable activity equivalent to 30 per cent of the untreated enzyme. When this material was again treated with iodoacetamide, and then dialyzed, assay on I^{131}-labeled HSA showed no further loss of restorable activity. However, when the second treatment with iodoacetamide was preceded by incubation with cysteine, 0.01 M, the restorable activity was further diminished (Table I, lines 1 to 4). The proteolytic activity of a mixture of untreated and iodoacetamide-treated papain was significantly less than the sum of the two components, indicating that irreversibly inactivated papain may inhibit the active enzyme in this system (Table I, line 6). The reaction was also retarded by other proteins, such as chondromucoprotein (Table I, lines 7 and 8).

Although the amount of potentially active enzyme remaining after treatment with iodoacetamide may thus be greater than indicated by assay on I^{131}-labeled HSA, these results do indicate that when treated with iodoacetamide under the conditions described a substantial proportion of papain may remain in a



TEXT-FIG. 1. Assay of papain protease with iodine^{131}-labeled human serum albumin as substrate. Each point on the line relating the rate hydrolysis to the concentration of papain (inset) represents the average calculated from successive determinations of the percentage of radioactivity soluble in T.C.A.

reversibly oxidized disulfide form. If this portion is responsible for the depletion of cartilage following injection of iodoacetamide treated papain, the addition of cysteine to the mixture prior to injection should abolish the *in vivo* effect on cartilage, as in the case of dialyzed papain.

TABLE I

Enzymatic Activity of Papain Preparations Measured with I^{131}-Labeled Human Serum Albumin as Substrate

Enzyme preparation assayed on HSA-I^{131}	Rate of reaction	Untreated papain having equivalent activity	
		Amount	Percentage of amount assayed
	per cent/min., R	*mg/ml., C.*	
1. Untreated papain	0.94	0.5	100
2. Papain (A) treated once with IAA*	0.45	0.15	30
3. Papain (A) treated twice with IAA*	0.45	0.15	30
4. Preparation 2, treated with cysteine, then with IAA*	0.074	0.005	1.0
5. Untreated papain (B)	0.52	0.19 (A)	100
6. Preparation 4, 0.5 mg. Preparation 5, 0.5 mg.	0.40	0.12	62
7. Untreated papain (C)	1.22	0.69(A)	100
8. Untreated papain (C) with CMP,‡ 0.5 mg/ml.	0.99	0.50	72

The reaction mixtures contained papain, 0.5 mg./ml., I^{131}-labeled albumin, 0.5 mg./ml., cysteine, 0.005 M, EDTA, 0.001 M in phosphate buffer, 0.1 M, pH 7.0; all were maintained at 37°C. without agitation.

* IAA, iodoacetamide. For procedure see text.

‡ CMP, bovine nasal chondromucoprotein.

Accordingly, a solution of crystalline papain was treated with 0.01 M iodoacetamide for 30 minutes at 37°C. and dialyzed for 24 hours to remove the excess iodoacetamide. Cysteine (0.01 M) was then added to a portion of the material and this and the remaining portion were incubated for 15 minutes at 37°C. The results of intravenous injection of the preparations are shown in Table II.

In the majority of rabbits, unequivocal depletion of cartilage matrix occurred following injection of iodoacetamide-treated papain; this effect was abolished by the addition of cysteine to the mixture prior to injection.

These findings indicate that papain acetamide is not reactivated in the rabbit and can best be explained by assuming that the effects observed after injection result from the activation in cartilage of a portion of the injected enzyme in the disulfide form. If it were possible to treat papain with iodoacetamide in such a way as to form a product consisting entirely of papain acetamide with none of the enzyme in the disulfide form, such material should have no restorable activity *in vitro* and no effect on cartilage *in vivo*.

Accordingly, a solution of crystalline papain was incubated for 15 minutes after addition of cysteine (0.01 M) and EDTA (0.001 M); following addition of iodoacetamide (0.02 M) the mixture was incubated for a further 60 minutes and then dialyzed for 24 hours at 4°C. with a 200-fold volume of phosphate buffer. This whole procedure was then repeated. The final product was tested for *in vitro* proteolytic activity after addition of cysteine and EDTA and the results are shown in Table I, line 4.

An injection consisting of 5 mg. of this preparation without cysteine was given to each of 6 rabbits. There was no subsequent loss of rigidity of the ears, and the sections of cartilage obtained from 5 of the animals sacrificed 24 hours later showed normal basophilic staining of the matrix. In the sixth rabbit some hematoxylin and eosin-stained sections of cartilage showed moderate loss of basophilia. There was a moderate increase in the amount of cobalt-precipitable material in the sera obtained 24 hours after injection of this preparation of papain, indicating that it did have a slight effect on cartilage *in vivo*.

TABLE II

Effects of Iodoacetamide-Treated Papain on Cartilage in Vivo

Papain preparation injected intravenously	No. of rabbits with loss of rigidity of ears*			No. with loss of basophilia in cartilage matrix‡			Average cobalt turbidity of serum 18–24 hrs. after papain (OD at 615 mμ) (No. tested)
	− to 1+	2+ to 3+	4+ to 5+	− to 1+	2+ to 3+	4+ to 5+	
Papain treated once with IAA§ 5 mg.	9	4	9	4	3	4	0.239 (22)
Papain treated once with IAA, then with cysteine 5 mg.	9	0	0	3	0	0	0.097 (9)
Papain treated once with IAA, then with cysteine, and again with IAA 5 mg.	6	0	0	5	1	0	0.165 (6)

* Loss of rigidity: − to 1+, no loss, or collapse of distal 1 cm.
2+ to 3+, collapse of distal 2 to 5 cm.
4+ to 5+, collapse of distal 6 cm. or more.

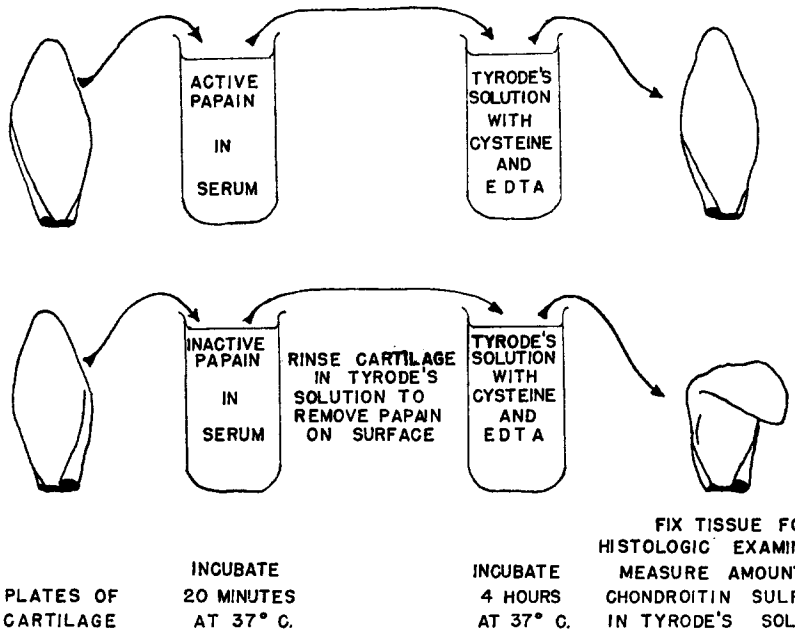
‡ Loss of basophilia: − to 1+, no loss or slight loss.
2+ to 3+, moderate loss.
4+ to 5+, marked or complete loss.

§ IAA, iodoacetamide.

Factors Which Determine the Difference between Active and Inactive Papain in Their Effect on Cartilage Following Intravenous Administration

On the basis of the preceding observations, it can be assumed that whatever method is used to inactivate papain prior to injection, the ensuing changes in cartilage result from reactivation of enzyme in the disulfide form. The question remains as to why papain injected in the active form fails to have any effect on cartilage. It was postulated that in this form the enzyme rapidly combines with

substrates in the circulation and is thereby prevented from diffusing into cartilage. This possibility was tested by means of an *in vitro* system designed to reproduce the probable sequence of events occurring *in vivo*. The procedure is shown schematically in Text-fig. 2.



TEXT-FIG. 2. Effect of serum upon entry of papain into cartilage *in vitro*. The cartilage initially exposed to active papain in serum remained intact (upper), whereas the cartilage exposed to inactive papain subsequently lost rigidity and showed other changes indicating depletion of matrix (lower). See also text and Table III.

The skin and subcutaneous tissue were stripped from the lateral surface of ear cartilage plates immediately after removal from rabbits weighing between 800 and 1000 gm. The plates of cartilage were immersed for 20 minutes at 37°C. in one of the 6 solutions shown in Table III. In the mixtures containing papain, the enzyme was mixed with the prewarmed serum or Tyrode's solution just before the plates of cartilage were inserted. After the initial 20 minute period of incubation, the plates were washed with Tyrode's solution to remove traces of papain on the surface, and were then transferred to tubes containing Tyrode's solution with cysteine, 0.005 M, and EDTA, 0.001 M. After 4 hours' incubation at 37°C. the plates were examined for loss of rigidity, and blocks of tissue were fixed for histologic examination. The media surrounding the plates of cartilage were analyzed for material precipitable by hexamminecobaltic chloride. The results are shown in Table III.

It was found that the plates of cartilage exposed initially to either active or inactive papain in Tyrode's solution both showed considerable loss of rigidity and depletion of matrix, as judged by loss of basophilic staining in hematoxylin

and eosin sections. Approximately equal amounts of cobalt-precipitable material were released into the surrounding media. Similar effects were produced in the cartilage exposed to inactive papain in serum. In contrast, the cartilage initially exposed to active papain in serum subsequently showed only slight loss of rigidity and depletion of matrix, and released substantially less cobalt-precipitable material into the medium. With minor modifications, this experiment was repeated twice with essentially the same outcome.

These results show that normal rabbit serum has the property of hindering the entry of active papain into cartilage *in vitro* and they support the interpre-

TABLE III

Influence of Rabbit Serum upon Entry of Papain into Cartilage in Vitro

Solution in which cartilage was incubated for initial 20 min. period	Changes in Cartilage on subsequent incubation for 4 hrs. in Tyrode's solution*		Increase in cobalt turbidity of surrounding medium at 4 hrs. Δ *O.D.*
	Loss of rigidity‡	Loss of basophilia in matrix§	
Tyrode's solution	−	−	0.016
Serum	−	−	0.005
Inactive papain‖ in Tyrode's solution	+++	++++	0.166
Inactive papain‖ in serum	+++	++++	0.169
Active papain‖ in Tyrode's solution	+++	++++	0.183
Active papain‖ in serum	+	+	0.080

* Containing cysteine 0.005 M and EDTA 0.001 M.

‡ Loss of rigidity; See Table II.

§ Loss of basophilia; See Table II.

‖ Concentration of papain, 100 micrograms/ml.

tation that intravenously injected active papain fails to produce changes in cartilage because it interacts with one or more components of serum.

Rates of Disappearance of Papain from the Circulation Following Intravenous Injection in Rabbits

It was postulated that because of interaction with material in the blood, active papain would leave the circulation more slowly than inactive papain following intravenous injection.

The problem was investigated using papain labeled with I^{131}. This material was assayed on BAA before and after iodination, and the amount of dialyzable I^{131} was measured following activation or inactivation procedures. Samples were precipitated with specific antiserum to test the specificity of the I^{131} label. The results obtained with two preparations of labelled papain are listed in Table IV.

Amounts of active or inactive papain in the range of 1 to 4 mg./kg. were injected by vein into rabbits which weighed approximately 1000 gm. Samples of blood were obtained by cardiac puncture at intervals thereafter, and were mixed with dry anticoagulant in tubes immersed in an ice bath. The plasma was separated by centrifugation at 4°C. and the radioactivity of 0.5 ml. samples of plasma was measured in a well scintillation counter. In a number of samples, the radioactivity was also measured in the washed cells and in the fibrin clot produced by recalcification of the plasma. Since it was found that the serum contained virtually all of the radioactive material present in samples of whole blood, the data from these experiments are presented as concentrations of papain in serum in micrograms/milliliter.

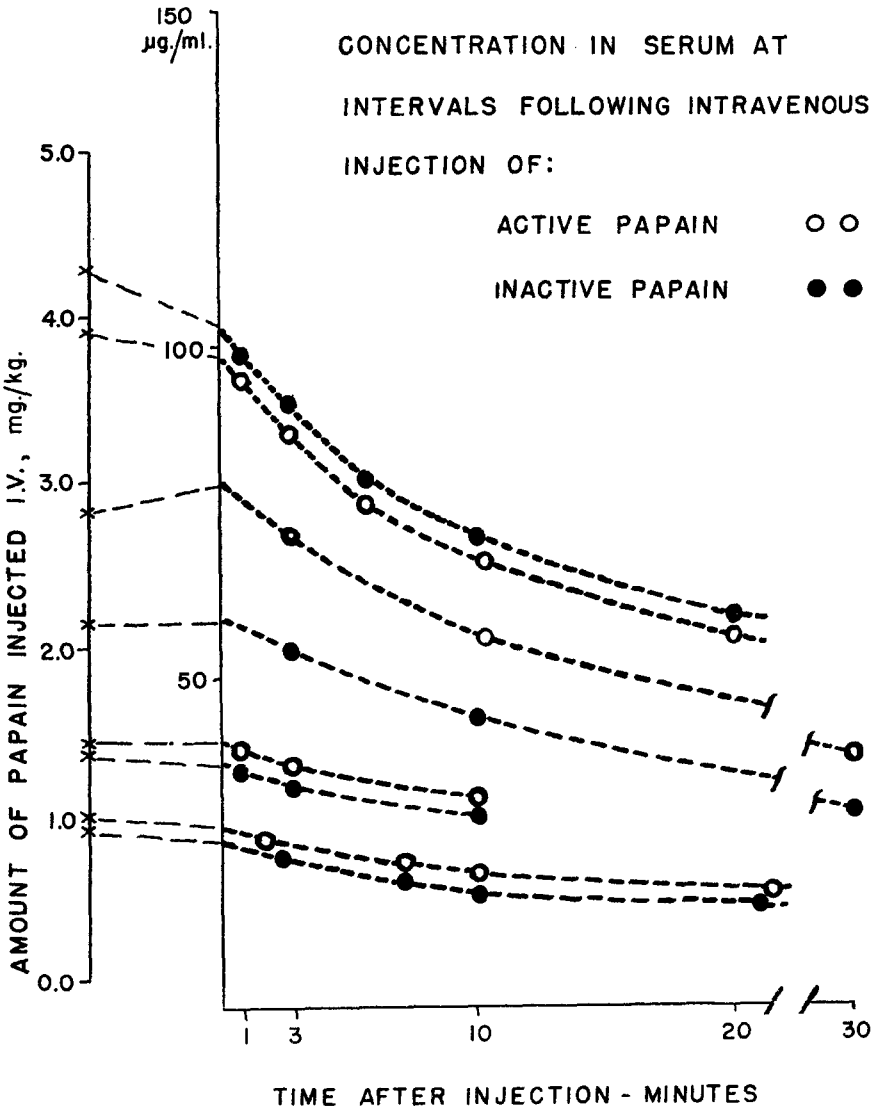
TABLE IV

Properties of I^{131}-Labeled Papain

	Specific enzymatic activity on BAA (C_1)	
	Before labeling	After labeling
Preparation I	0.269	0.188 (70 per cent)
Preparation II	0.151	0.130 (86 per cent)
	Distribution of radioactivity after dialysis following activation or inactivation procedures	
	Non-dialyzable	Dialyzable
	c.p.m./ml.	*c.p.m./ml.*
Active papain	417,345	1,655
Inactive papain	420,035	2,965
	Distribution of radioactivity after precipitation with specific antiserum in antibody excess	
	Precipitate	Supernatant
	c.p.m./ml.	*c.p.m./ml.*
Preparation I	5,828 (85 per cent)	1,029
Preparation II	2,100 (89 per cent)	270

As shown in Text-figs. 3 and 4, the rate of disappearance of papain was a function of the concentration, and the rates were the same whether the papain was active or inactive at the time of injection. It is evident that any tendency for inactive papain to diffuse into cartilage more readily than the active enzyme was not reflected by a measurable difference in the rates of disappearance from the circulation. In view of the possibility that a difference might be apparent if the concentration of papain in serum exceeded a threshold level, 5 rabbits were given injections of active or inactive I^{131}-labeled papain, 5 mg./kilo. The three animals given the fully active form of this batch of papain died very soon after injection, and it was therefore impossible to compare the rate of dis-

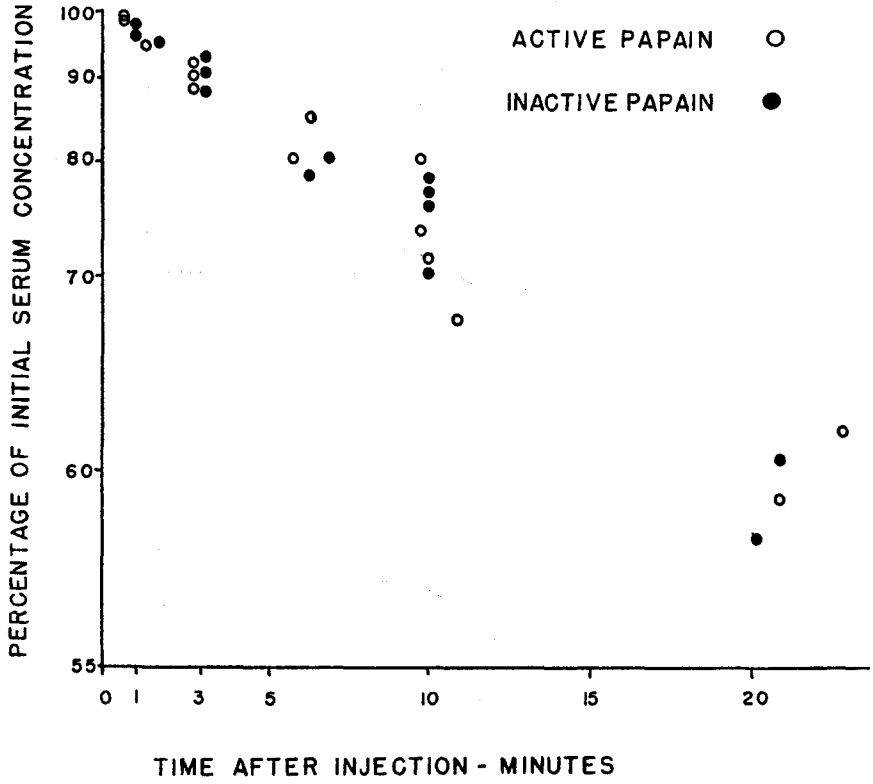
appearance of the inactive preparation with that of an equivalent dose of fully active papain. Acute toxic manifestations following administration of fully active papain in quantities exceeding 5 mg./kg. have been observed frequently; the majority of animals have shown no immediate untoward reaction after



TEXT-FIG. 3. Concentration in rabbit serum of papain after intravenous injection in the active and inactive form. Each point represents the concentration of I^{131}-labeled papain in micrograms/ml., calculated from the radioactivity of samples of serum obtained by cardiac puncture. See also Fig. 4.

administration of lesser amounts of active papain, or after inactive papain in amounts up to 10 mg./kg.

In 2 rabbits, the amount of radioactive material in ear cartilage was measured 30 minutes after an injection of 4 mg. of I^{131}-labeled inactive papain; the concentration of papain was estimated to be of the order of 2 micrograms/gm.

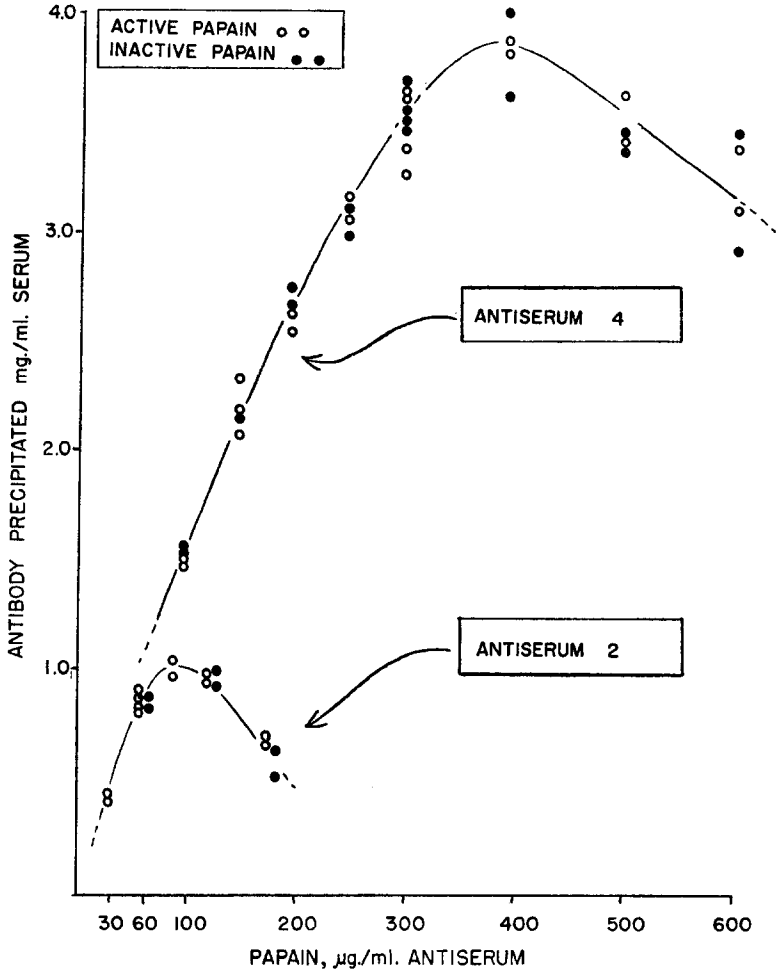


TEXT-FIG. 4. Concentration in rabbit serum of papain after intravenous injection. The data shown in Fig. 3 are here plotted as percentages of the initial concentration of papain, estimated by extrapolation to zero time on a logarithmic scale of concentration.

wet weight, and was more than double the estimated amount in cartilage from 2 rabbits given fully active papain. However, the low level of radioactivity did not permit more precise quantitation.

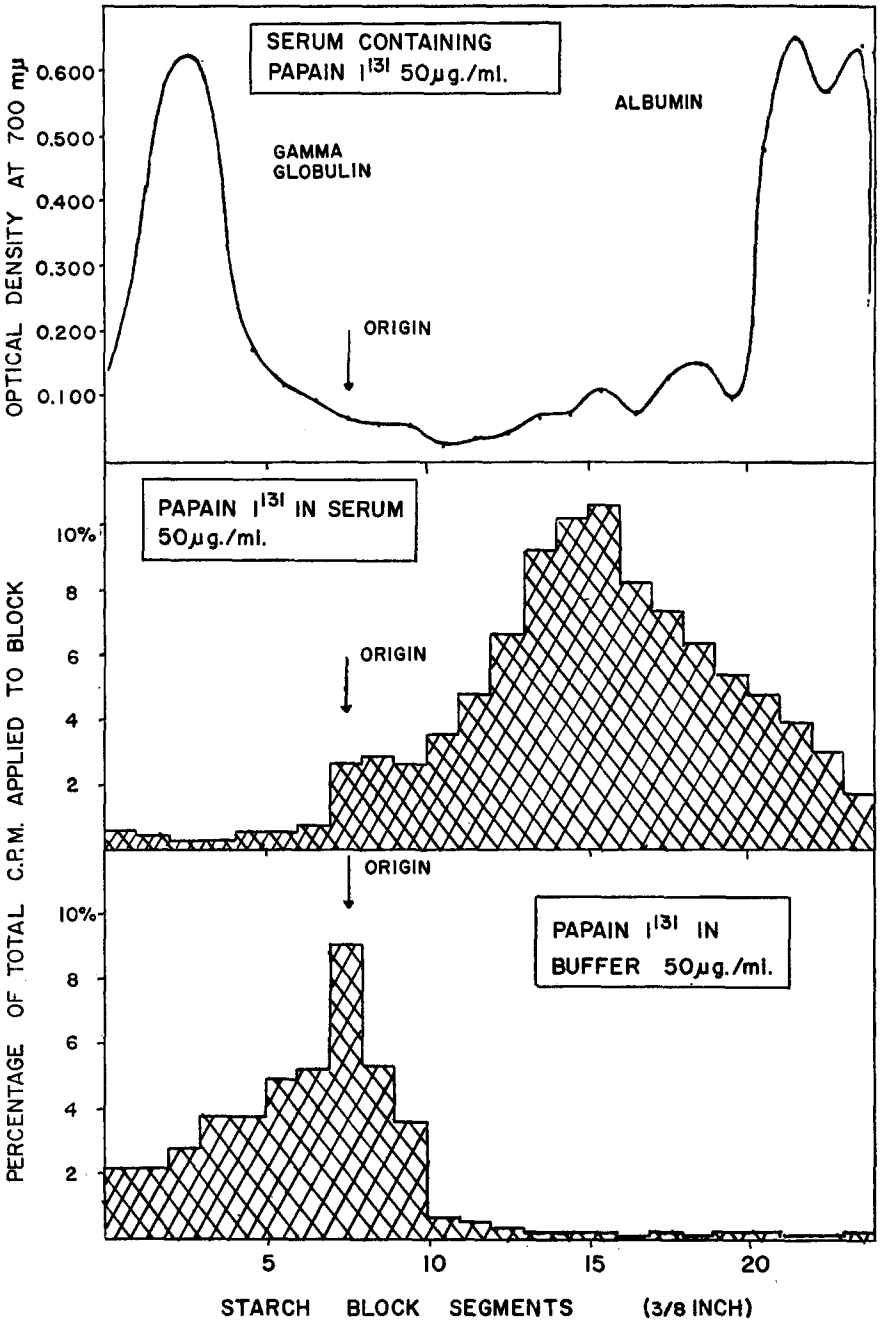
To test the validity of the data on disappearance rates obtained with I^{131}-labeled papain, specific antisera were used to demonstrate the presence of papain in sera withdrawn at intervals following intravenous administration of active and inactive papain, labeled or unlabeled. As shown in Text-fig. 5, the antigenic similarity between active and inactive papain was established by the quantitative precipitin technique. By double diffusion in agar, it was possible

to demonstrate the presence of papain in rabbit serum up to 24 hours after a single intravenous injection. Quantitation based upon the relative intensity and position of the precipitin lines corresponding to wells filled with sera ob-



TEXT-FIG. 5. Analysis of rabbit anti-papain serum by the quantitative precipitin technique. The amount of protein in the washed precipitates redissolved in 0.25 M acetic acid was measured as O.D. at 280 mu. Active and inactive papain precipitated equal amounts of antibody.

tained at various intervals after the administration of papain provided a crude measure of rates of disappearance. These rates were entirely compatible with the results obtained with I^{131}-labeled papain. A typical example of double diffusion in agar is shown in Fig. 1.



TEXT-FIG. 6. Effect of serum upon electrophoretic mobility of I^{131}-labeled papain. On electrophoresis at pH. 8.6, labeled papain alone either remained at the origin or moved towards the cathode. In normal rabbit serum, the papain moved towards the anode, the radioactive material being distributed around a peak corresponding with alpha$_2$ globulin.

It was shown that normal rabbit serum impaired the entry of active papain into isolated cartilage. Furthermore, in blood withdrawn following intravenous injection of I^{131}-labeled active or inactive papain, almost all of the radioactive material remained in serum, little or none being present in the washed cells or fibrin clot. As these findings indicated that normal rabbit serum might contain one or more components which could combine with papain, the distribution of papain in serum was studied by measuring the radioactivity in the fractions eluted from starch block electrophoresis of sera obtained following intravenous injection of the labeled protease. In addition, similar studies were made on normal rabbit serum to which labeled papain had been added *in vitro*. It was found that the peak concentration of radioactive material was present in the fractions corresponding to the alpha globulin zones, and that this distribution was the same for both active and inactive labeled papain, whether mixed with serum *in vitro*, or in serum obtained following intravenous administration of the labeled protease (Text-fig. 6). In parallel electrophoresis on starch at pH 8.6, and in the absence of serum, papain showed quite different electrophoretic mobility, and moved toward the cathode rather than the anode. These findings were confirmed by immuno-electrophoresis of unlabeled papain in saline and in serum, using specific antisera to papain and to rabbit serum to identify respectively the protease and the components of rabbit serum. Thus it appeared that both active and inactive papain in concentrations up to 50 micrograms/ml. in serum were bound to protein in the alpha globulin region. Additional studies with more prolonged electrophoresis, and the preparation of a larger number of fractions from the same block, have shown a peak concentration corresponding to the $alpha_2$ globulin fraction. In some experiments papain was incubated with fractions of normal serum which were then subjected to electrophoresis on starch. It was found that the mobility of papain was modified by the $alpha_2$ globulin fraction, and not by gamma globulin; the albumin fraction retained only 20 per cent of the radioactivity.

Concentration of Papain Required for in Vitro Depletion of Cartilage Matrix

In view of the evidence indicating that the amount of papain entering cartilage after intravenous injection might not exceed 2 micrograms/gm. wet weight, it was necessary to determine the amount of papain which *in vitro* would produce changes in cartilage comparable with those seen *in vivo*.

The skin and connective tissue were stripped from ears removed from rabbits weighing approximately 800 gm. The plates of cartilage were incubated at 37°C. for 4 hours in 0.1 M phosphate buffer, pH. 7.0, 40 ml., containing 0.005 M cysteine, 0.001 M EDTA., and crystalline papain in concentrations in the range of 0.2 to 10.0 micrograms/ml. The plates were then examined for loss of rigidity, and blocks of tissue were fixed for histologic examination. The media were analyzed for material precipitable with hexamminecobaltic chloride (Table V).

It was found that a detectable loss of rigidity occurred in cartilage exposed to papain in concentrations as low as 2 micrograms/ml.

A more detailed and quantitative study of the diffusion of papain into isolated cartilage is in progress, and preliminary findings indicate that, in circumstances similar to the foregoing experiment, the concentration of papain in cartilage attains levels only slightly in excess of those in the surrounding media.

TABLE V

Effects of Active Crystalline Papain on Cartilage in Vitro

Concentration of papain in medium	Changes in cartilage after 4 hrs. at 37°C.		Increase in cobalt turbidity of surrounding medium at 4 hrs. Δ O.D.
	Loss of rigidity*	Loss of basophilia in matrix‡	
micrograms/ml.			
10	+++	++	0.069
8	++	++	
6	+	+	0.048
4	+	±	
2	+	±	
1	±	±	
0.5	−	−	0.017
0.2	−	−	
Nil	−	−	0.015

* Loss of rigidity, see Table II.

‡ Loss of basophilia, see Table II.

DISCUSSION

On the basis of the observation that depletion of cartilage matrix occurred in rabbits following the intravenous administration of papain treated with excess iodoacetamide, it was previously postulated that reactivation of papain acetamide occurred in the intact animal. However, the inactivation of crystalline papain by iodoacetamide is not reversed *in vitro* upon addition of reducing agents such as cysteine, and is generally stated to be irreversible. For this reason, it was decided that some alternative explanation should be found. The possibility was considered that when papain is incubated with iodoacetamide, a portion of the enzyme which is in the disulfide form escapes alkylation, and that this portion is responsible for the effects observed *in vivo*. The validity of this explanation was confirmed by the results of three lines of investigation. First, it was shown that following dialysis to remove the excess iodoacetamide from a solution of papain, some proteolytic activity was restorable upon the addition of cysteine. Second, it was found that when cysteine was added to a solution of papain from which excess iodoacetamide had been removed by

dialysis, the product did not produce *in vivo* depletion of cartilage, and thus behaved in this respect like fully active papain. Third, when steps were taken to increase the percentage of enzyme converted to the acetamide form by repeated exposure to cysteine and excess iodoacetamide, the product obtained had considerable less restorable proteolytic activity and its effect on cartilage *in vivo* was diminished.

It is relevant to note that the residual *in vivo* effectiveness of papain treated with iodoacetamide was greater than might be expected on the basis of restorable activity of the same preparations measured by assay on HSA I^{131}. A possible explanation for this disparity could be the observation that the enzymatic degradation of this substrate by active papain was retarded by the addition of iodoacetamide-treated papain. While the exact mechanism of this inhibitory effect is not known, it is evident that the proportion of active papain present in a mixture with inert protease may be considerably greater than indicated by assay on HSA I^{131}. Alternatively, it is possible that the simultaneous injection of inert papain acetamide may facilitate the entry of the potentially active protease into cartilage *in vivo*.

The demonstration that normal rabbit serum impaired the entry into cartilage of active papain, and not of inactive, provides a basis for explaining the difference between the effects of active and inactive papain on cartilage *in vivo* when given intravenously. As was shown previously, the intravenous injection of inactive papain into young rabbits results in widespread depletion of cartiage matrix, whereas an injection of fully active papain has virtually no effect on cartilage. It is thus likely that following intravenous injection, active papain rapidly interacts with one or more protein components of serum and as a result the passage of the enzyme into cartilage in amounts sufficient to produce depletion is prevented. On the other hand, a larger amount of inactive papain remains free, following intravenous injection, to leave the circulation and enter cartilage; the widespread depletion of cartilage matrix observed is presumed to be the result of reactivation of the enzyme in this tissue. Using papain labelled with I^{131}, it has been demonstrated that a significantly greater amount is present in cartilage following injection in the inactive than in the active form. While the amount of enzyme found in cartilage following injection of inactive papain represented at most 2 micrograms of enzyme/gm. wet weight of cartilage, it has been shown that such a concentration is sufficient to produce depletion of isolated cartilage.

The concentration of 2 micrograms of papain/gm. wet weight of cartilage was demonstrated at a time when the concentration in the circulation was over 50 micrograms/ml. of serum. Thus it is evident that the enzyme is not selectively taken up by cartilage despite the observation that histological changes occur regularly in this tissue only.

On the basis of the marked change produced in the viscosity of bovine nasal

chondromucoprotein by very small amounts of the enzyme (13), it would appear that this substrate undergoes profound physical changes as a result of the cleavage of relatively few bonds. Evidence that the change in the viscosity of chondromucoprotein observed under these conditions actually results from enzymatic splitting of the molecule has been obtained by differential alcohol fractionation of the products. It is noteworthy that papain may continue to circulate for several hours in such a high concentration without apparent harm to the animal, in spite of the fact that an intradermal injection of comparable amounts of the enzyme has been found to produce localized necrosis of skin (14).

From the foregoing evidence that only a small fraction of the injected material enters cartilage it is not surprising that no difference was demonstrable in the rate of disappearance from the circulation of the active and inactive forms of the enzyme. On the basis of the data obtained using papain labeled with I^{131}, it was found that about 40 per cent of the material left the blood within the first 20 or 30 minutes following injection and it is reasonable to assume that this loss represents equilibration with the extracellular space. If this is true, it is necessary to postulate that the failure of active papain to enter cartilage depends on interaction not only with serum components within the circulation but also with components within the extracellular space.

The interaction of papain with components of serum was studied by means of immuno-electrophoresis and zone electrophoresis in starch. It was found that the electrophoretic mobility of papain was altered in serum and that in concentrations up to 50 micrograms/ml. of serum, the enzyme moved with protein in the alpha globulin region. Since this effect was the same whether papain was mixed with serum in either the active or inactive form, it may well be that this localization in serum has no direct bearing on the observed differences in effects between active and inactive papain following intravenous injection. It seems likely that the portion of the enzyme which disappears from the circulation in the first few minutes is not bound by this fraction of serum and that it is the difference in handling of the unbound portion of the enzyme that accounts for the difference in the effects on cartilage of active and inactive papain injected by vein. As already stated, it would appear that this difference depends on the interaction between the active enzyme and proteins in the interstitial space, perhaps as the result of enzyme-substrate combination.

While the foregoing observations on the fate and distribution of papain in the rabbit are concerned with the way in which a particular foreign enzyme is handled, they may have bearing on the way in which other potentially harmful enzymes are prevented from causing damage to tissues. Following entry into the circulation, bacterial or tissue enzymes may also selectively combine with alpha globulin or other protein and as a result may be prevented from entering and injuring tissue.

SUMMARY

In rabbits, the depletion of cartilage matrix which occurs following intravenous administration of papain treated with iodacetamide is attributable to a portion of the enzyme in the disulfide form which has not undergone alkylation. It is this portion that is reactivated in cartilage *in vivo* and initiates the enzymatic breakdown of the protein-polysaccharide complex which forms a major component of the matrix. Evidence presented in support of these conclusions indicates that, contrary to an earlier hypothesis, papain acetamide is not reactivated *in vivo*.

Following intravenous injection in amounts up to 4 mg./kg., active and inactive papain leaves the circulation at a rate proportional to the concentration, and it is likely that the initial rate of disappearance represents equilibration with the extracellular space. Following injection in the active or inactive form, a high proportion of papain in serum is bound to protein in the alpha globulin fraction. It is believed that in the case of fully active papain, the proportion which is not bound to alpha globulin becomes attached to other proteins of serum in extracellular fluid, such as albumin, by a process of enzyme substrate combination, and is thus prevented from diffusing into cartilage. In the case of inactive papain, a comparable excess remains free to enter cartilage, where it initiates depletion of matrix following reactivation within the tissue.

These conclusions provide an explanation for the failure of fully active papain to cause depletion of cartilage matrix *in vivo*; the widespread changes seen after the injection of papain inactivated by iodoacetamide or by simple reversible oxidation are attributable to a small proportion of the injected material which enters cartilage in the disulfide form in a concentration of approximately 2 micrograms/gm. wet weight. The possibility that such a small amount of protease, when reactivated, can produce changes in cartilage matrix has been confirmed by studies on the effects of papain on isolated cartilage and chondromucoprotein *in vitro*.

It has been shown that severe local injury results when active papain is injected into the skin of a rabbit in low concentrations. Since a much higher concentration of papain can be attained in the circulation without obvious adverse effects, it is evident that binding of the protease by alpha globulin and possibly other serum proteins may exemplify a mechanism whereby the tissues are protected from injury following entry into the circulation of other potentially harmful agents, such as proteolytic enzymes derived from cells or bacteria.

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EXPLANATION OF PLATE 96

FIG. 1. Double-diffusion in agar of serum obtained from rabbits following intravenous papain. Center wells, 12: rabbit anti-papain serum. Peripheral wells, 1, 2, 3, and 4: sera obtained 5 minutes, 1 hour, 4 hours, and 12 hours after injection of inactive papain. Peripheral wells, 7, 8, 9, and 10: sera obtained 5 minutes, 1 hour, 4 hours, and 12 hours after injection of active papain. Peripheral wells 5 and 11: sera obtained just before injection of papain. Peripheral wells 6: normal rabbit serum containing papain, 40 micrograms/ml., mixed *in vitro*.

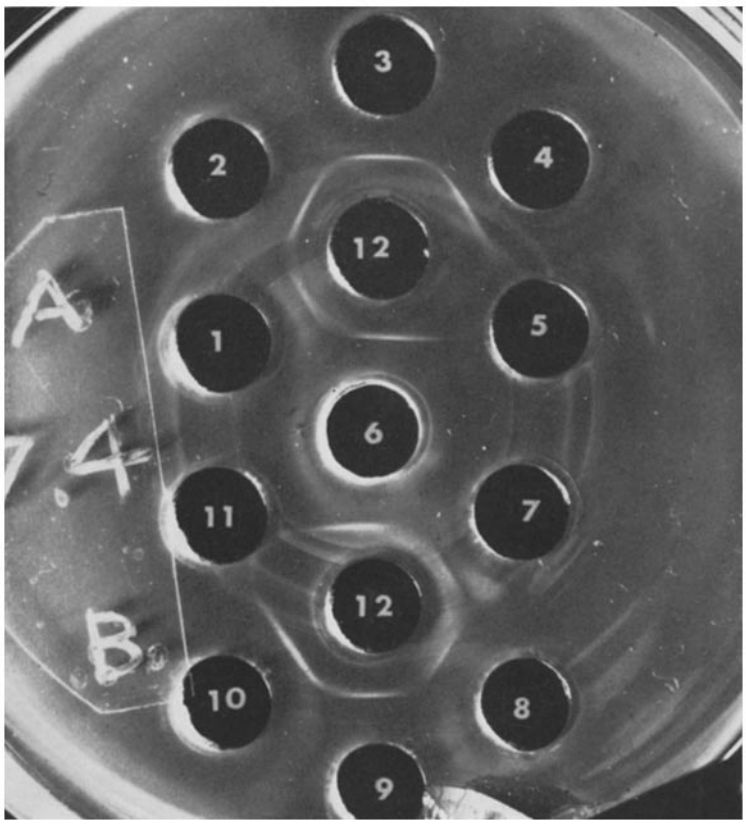

FIG. 1

(Potter *et al.*: Removal of cartilage matrix by papain)