PAPAIN-INDUCED CHANGES IN RABBIT CARTILAGE

Alterations in the Chemical Structure of the Cartilage Matrix

By THEODORE T. TSALTAS,* M.D.

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

(Received for publication, June 9, 1958)

Injection of a crude papain solution intravenously into rabbits produces a marked softening of their ears which, within a few hours, eventually collapse and rest on either side of the head. A profound depletion of the basophilic components of the cartilage matrix has been demonstrated histologically in such collapsed ears. Both gross and histological changes are completed within 24 hours after the injection of papain. Thereafter the rigidity of the ears is gradually restored and a concomitant increase of the basophilic components of the cartilage matrix is seen. The details of this phenomenon have been described by Thomas (1). Further descriptions of the histological changes in various organs have been given in subsequent publications (2, 3).

It is known that the basophilic properties of the cartilage matrix are ascribable to the mucoproteins. One such mucoprotein, chondromucoprotein (M.C.S.), which accounts for the major fraction of the hexosamine in cartilage, has been isolated in high yields from bovine nasal septal cartilage (4). In this report some biochemical aspects of the changes produced by intravenously injected papain in rabbits are considered. Attention was given particularly to changes in the content of chondromucoprotein (M.C.S.) in cartilage and a possible alteration of its composition.

Materials and Methods

All experiments were performed with matched pairs of young hybrid albino rabbits weighing 1300 gm. (±30 gm.). The solution of papain was prepared according to the method of Thomas (1)[1]. In all the experiments described here, 1 ml. of a 1 per cent solution of papain was injected intravenously 7 days after the intravenous injection of S^{35}–sulfate[2] (2 mc. in 1

* Present address: Charlotte Drake Cardeza Foundation and the Department of Pathology, Jefferson Medical College of Philadelphia, Philadelphia.

[1] Papain was obtained as the crude powder derived from the dried latex, from the Nutritional Biochemical Corporation, Cleveland.

[2] The S^{35} was supplied by the Oak Ridge National Laboratory on allocation from the United States Atomic Energy Commission.

ml. of solution at pH 7.5). At various intervals of time, as indicated subsequently, blood samples were collected either by venous or cardiac puncture. The blood was allowed to clot and the serum was separated immediately by centrifugation. The urine excreted in the given periods of time was collected by the use of metabolic cages.

At the time of sacrifice, cartilage from the ears, trachea, and ribs was taken for analysis. Portions of each tissue were used in the preparation of autoradiograms; a detailed report of these will be published separately. Small portions of ear cartilage were dried to determine their water content, as described by Malawista and Schubert (5). The remainder of the ear cartilage was used for the preparation of chondromucoprotein (M.C.S.)[3] according to the most recent directions of Malawista and Schubert (5).[4] The sera and the chondromucoprotein (M.C.S.) samples were analyzed for hexosamine by the method of Schloss (6). Nitrogen was determined by micro-Kjeldahl. The content of glucuronic acid in the M.C.S. samples and in the urine was determined by the use of the Dische (7) carbazole method.

Total S^{35} activity in the M.C.S. samples was determined by counting the barium sulfate precipitates isolated on filter paper disks after oxidation of 10 to 12 mg. portions of the materials with Denis' reagent (8) as described by Dziewiatkowski (9). The S^{35} activity in the sera was measured similarly, using 0.2 ml portions. For the determination of the S^{35} in some components in the sera, 1 ml. portions of the sera were mixed with 2 ml. of water and to this 3 ml. of a 20 per cent solution of trichloroacetic acid (T.C.A.) was added with vigorous shaking. The tubes were set aside for 30 minutes at 0°C. and then centrifuged. Inorganic and total S^{35} were determined in the supernatant fluid and in the urine samples (10). All measurements of radioactivity were made with a gas flow counter and automatic recording device for a minimum of 30,000 counts.

RESULTS

The maximum collapse of the rabbit ears occurs approximately 24 hours after the injection of papain. On the basis of this observation the experiments described here can be divided into two groups: (1) Experiments in which the animals were sacrificed 24 hours after the injection of papain so as to determine the chemical changes at the time of the observed maximum gross alterations, (2) experiments in which animals were sacrificed after intervals of time following injection of papain so that the progress and direction of these changes could be determined.

Observations 24 Hours after Injection of Papain

Tables I and II show the results from one of the pairs of animals (12 pairs were used) in experiments of the first type. It can be seen in Table I that the amount of M.C.S. extracted from the ear cartilage of the injected animals, expressed as milligrams of M.C.S., was 42 per cent lower than that of the control animals. This decrease ranged from 40 to 48 per cent in the animals tested. The hexosamine content of the isolated M.C.S. was also reduced. In parallel

[3] This material was designated in a previous publication (*Fed. Proc.*, 1958, **17**, pt. I, 461) as muco-chondroitin sulfate. In order to conform with nomenclature more commonly used, the name chondromucoprotein is presently used.

[4] We wish to thank the authors for communicating the details of this method before its publication.

with this there was a tenfold increase in the excretion of glucuronic acid in the urine of all animals tested.

Of significance is the difference in the $\frac{N}{\text{Hexosamine}}$ molar ratio of the M.C.S. isolated from the papain-injected animals and the controls. This increase ranged from 42 to 45 per cent (44 per cent in the figures shown) in all the animals tested.

In Table II the results of the S^{35} measurements on the M.C.S. samples, blood, and urine of the same animals are tabulated. As can be seen, the S^{35} activity

TABLE I

	Control animals	Papain-injected animals
Chondromucoprotein (M.C.S.), mg. per gm. of cartilage	37.4	22.0
Hexosamine, per cent in M.C.S.	8.6	6.6
Nitrogen (N), per cent in M.C.S.	7.7	9.2
N/hexosamine, molar ratio in M.C.S.	11.5	17.8
Weight ratio, wet/dry cartilage	3/1	3.1/1
Glucuronic acid, mg. per 24 hr. urine	0.83	9.42

TABLE II

	Control animals	Papain-injected animals
	C.P.M.	*C.P.M.*
S^{35} activity, per mg. of chondromucoprotein	62,800	32,120
Total S^{35} activity, per ml. of whole serum	16,190	32,550
Inorganic S^{35} activity, per ml. of deproteinized serum	1,176	7,776
Organic S^{35} activity, per ml. of deproteinized serum	148	2,604
Inorganic S^{35} activity, per 24 hr. urine	715,200	2,214,000
Organic S^{35} activity, per 24 hr. urine	97,600	302,750

of M.C.S. from the ear cartilage of the papain-treated animals was only 49 per cent as great as that obtained from the controls. In many other experiments the values ranged from 35 to 50 per cent of the control values. Thus not only was the amount of M.C.S. reduced but in addition the specific activity of M.C.S. remaining in the cartilage was less than that of the control value.

Marked changes in the S^{35} activity of the sulfur-containing components of serum and urine also were found. The activity per milliliter of whole serum was twice as great in the papain-treated animals as in the controls; the activity present as inorganic sulfate was five times as great. Even more striking was the increased activity of organically bound sulfur, soluble in T.C.A. In various

experiments there was 17 to 100 times as much organically bound S^{35} in the sera of the papain-treated animals as in the sera of the controls.

Observations at Various Intervals Following Injection of Papain

The progress of the changes indicated in Tables I and II was investigated as follows. Each of sixteen rabbits received 2.5 mc. of S^{35}-sulfate intravenously. 7 days later papain was injected into twelve of the animals; the remaining four animals were not given papain. Two of the rabbits that received no papain were sacrificed at this time. Every 12 hours thereafter a pair of papain-treated animals was sacrificed. The second pair of control animals was sacrificed at the same time as the last pair of papain-treated animals. The second pair of control animals differed only slightly from the first pair of control animals sacrificed at the begin-

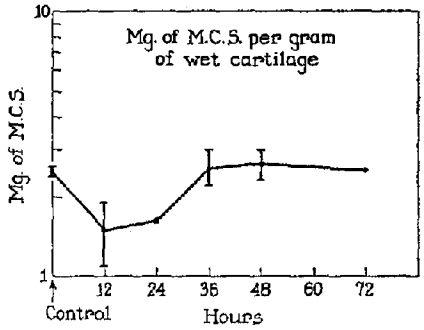

FIG. 1

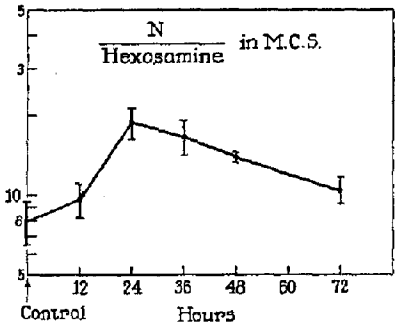

FIG. 2

FIG. 1. Mg. of isolated chondromucoprotein (M.C.S.) per gram of wet ear cartilage.

FIG. 2. $\frac{N}{\text{Hexosamine}}$ ratio in chondromucoprotein (M.C.S.) isolated from ear cartilage.

ning of the experiment. The experiment was repeated three times using sixteen rabbits each time. The analyses were as those in the first type of experiments.

Figs. 1 through 5 show the results obtained from one of these groups of animals. In Fig. 1 it is seen that following the injection of papain there was a rapid depletion of the M.C.S. in the ear cartilage; a minimum value was reached at about the 12th hour postinjection and this continued for an additional 12 hours. Thereafter the content of M.C.S. increased to its initial values, normal levels being attained 36 to 48 hours after the beginning of the experiment.

Fig. 2 shows the alterations in the molar ratio of $\frac{N}{\text{Hexosamine}}$ in the M.C.S. isolated from the ear cartilage of the papain-treated animal. This ratio increased steadily to a maximum 24 hours after the injection of papain, and decreased slowly thereafter to approximately normal values. It should be noted that the highest value on this curve approximately coincided with the minimum value for M.C.S. in the cartilage.

Fig. 3 shows the decrease in S^{35} activity of the isolated M.C.S. samples. A minimal value was reached 12 hours after the injection of papain. Values obtained thereafter remained at approximately the same level.

Fig. 4 shows the S^{35} activities of the serum fractions. The activity of all fractions of the papain-treated animals increased markedly, reaching a maxi-

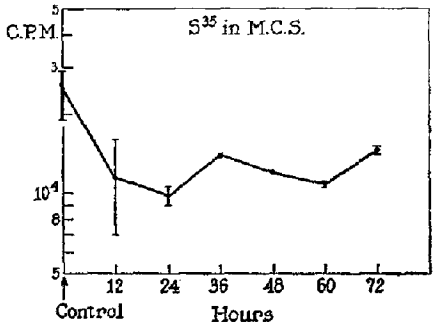

FIG. 3. S^{35} activity (in C.P.M.) in chondromucoprotein (M.C.S.) isolated from ear cartilage.

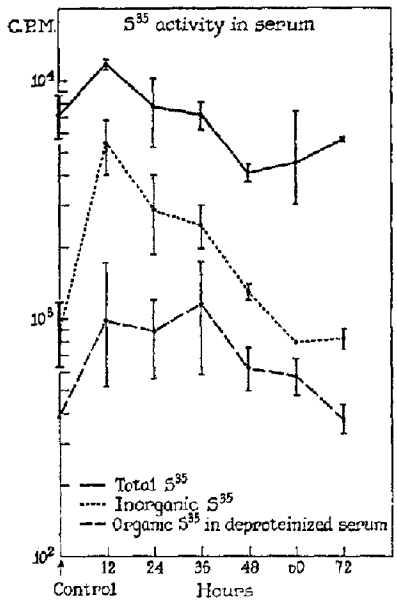

FIG. 4

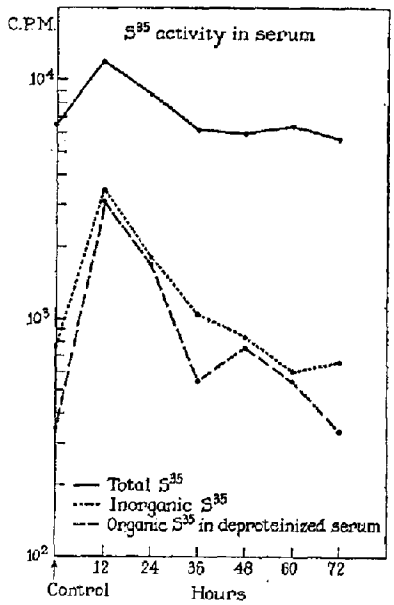

FIG. 5

FIG. 4. S^{35} activity (in C.P.M.) in various serum fractions (16 animals used.)

FIG. 5. S^{35} activity (in C.P.M.) in various serum fractions. (Values shown were obtained from serial bleedings of one animal.)

mum at a time when there was the smallest amount of M.C.S. in the cartilage (Fig. 3).

In an experiment similar to the ones described above, animals were sacrificed at 2, 4, 8, 12, and 24 hours after the injection of papain. The amount of

M.C.S. and S^{35} in cartilage between 0 and 12 hours decreased uniformly and reached the same values as shown in Fig. 1. The same was true as regards the S^{35} activities in the serum fractions.

Finally, another type of experiment was performed to define specific activity of the various serum fractions more closely. For this purpose four animals were injected with papain 7 days after administration of S^{35}. The animals were bled just before the injection of papain and these blood samples were used as controls. They were bled again at 12, 24, 36, 48, 60, and 72 hours. The S^{35} activities in the different fractions were practically identical; therefore the curves for only one of them are shown in Fig. 5. The curves in Figs. 4 and 5 are very similar, except that the curves in Fig. 5 are much smoother in appearance. It can be seen that the most marked increase was in the organic fraction of the serum soluble in T.C.A., a finding similar to that shown in Table II.

DISCUSSION

The results of the experiments presented here indicate the nature of the changes previously reported (1). Early in these experiments, an effort was made to determine whether collapse of the ear was due to loss of water from the cartilage. As is shown in Table I, the ratio of the wet to dry weight of the cartilage was identical for control and treated animals, thus excluding this possibility.

Several investigators have shown that approximately 80 per cent of intravenously injected inorganic S^{35} is excreted within the first 24 hours after its administration, and that after 4 to 5 days only small amounts continue to be excreted. The S^{35} activity in the blood of such animals falls rapidly to low levels by 24 hours after the injection and thereafter continues to decrease slowly. In contrast, in the present study a marked increase in the S^{35} of the sera and urine was produced by the injection of papain. The maximum depletion of the cartilage of its M.C.S. (and S^{35}), coincides with the maximal increase of S^{35} activity in the serum (Figs. 3 and 4). Since no S^{35} was available to the animal at that time, apart from that already in the various tissues, it seems most likely that the increase in serum S^{35} came from cartilage.

The changes in the $\frac{N}{\text{Hexosamine}}$ ratio of cartilage are of considerable importance. They indicate that the chemical composition of M.C.S. was temporarily altered by the action of papain. The finding that this ratio was increased at the time that serum and urine S^{35} were increased, suggests that the papain acted in such a way as to release chondroitin sulfate from M.C.S., and that this escaped into the blood and passed into the urine. It would therefore seem that the restoration of cartilage to its normal state requires restoration of M.C.S. to normal composition as well as to normal amount.

The increase of S^{35} in the urine can be accounted for on the basis of the

marked increase of the organic and inorganic sulfur components in the blood, which were present in much greater than normal amounts.

SUMMARY

Some biochemical aspects of the collapse of the rabbit ears produced by the intravenous injection of papain have been studied. A marked depletion of chondromucoprotein (M.C.S.) and a reduction of the S^{35} content of cartilage matrix were found to coincide with the gross and histologic changes in the cartilage. At the same time there was a marked increase in the amount of S^{35} in the serum and an increase of S^{35} and glucuronic acid excreted in the urine. Alteration in the composition of the M.C.S. remaining in the cartilage of the papain-injected animals was detected.

The findings indicate that the collapse of the rabbit ears is due to loss of chondromucoprotein from cartilage and reduction of chondroitin sulfate in the chondromucoprotein that remains.

All these changes were reversed in recovery.

The author wishes to thank Dr. D. D. Dziewiatkowski for many helpful suggestions.

The author also wishes to thank Miss G. Folkhart, and Miss C. Weissenborn for technical assistance.

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. Shatton, J., and Schubert, M., Isolation of mucoprotein from cartilage, *J. Biol. Chem.*, 1954, **211,** 565.
5. Malawista, I., and Schubert, M., Chondromucoprotein; new extraction method and alkaline degradation, *J. Biol. Chem.*, 1958, **230,** 535.
6. Schloss, B., Colorimetric determination of glucosamine, *Anal. Chem.*, 1951, **23,** 1321.
7. Dische, Z., A new specific color reaction of hexuronic acids, *J. Biol. Chem.*, 1947, **167,** 189.
8. Denis, W., The determination of total sulfur in urine, *J. Biol. Chem.*, 1910, **8,** 401.
9. Dziewiatkowski, D. D., Sulfate-sulfur metabolism in the rat fetus as indicated by sulfur-35, *J. Exp. Med.*, 1953, **98,** 119.
10. Folin, O., On sulfate and sulfur determinations, *J. Biol. Chem.*, 1905-06, **1,** 131.