SYSTEMIC EFFECTS IN RABBITS RECEIVING INJECTIONS OF PAPAIN AND CHONDROITIN SULFATE*

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The collapse of rabbit ears following intravenous injection of crude papain originally observed by Thomas,[1] has been found to be associated with the escape of an acid polysaccharide from the cartilage matrix into interstitial perichondrial spaces, lymph vessels, and regional lymph nodes.[2] Several observations and considerations indicate that this acid material is chondroitin sulfate. In this report the histologic changes in kidney, liver and blood, following papain injection, are described and compared with different manifestations resulting from the injection of commercially available chondroitin sulfate.

Methods

Rabbits treated with crude papain as previously described[1,2] were sacrificed at 7 hours and at 1, 3, 9 and 22 days following injection and necropsies were performed immediately.

Specimens from these animals and three controls were fixed in one or more solutions including: acetic alcohol formalin (A.A.F.), Carnoy's mixture, Bouin's fluid and calcium acetate formalin. The fixatives were prepared and a variety of staining procedures were carried out as summarized by Lillie.[3]

Results

After papain, sections of the A.A.F.-fixed tissue stained with azure A at pH 5.0 revealed frequent clumps of finely to coarsely granular, globular or fibrillar orthochromatic material in the lumens of many small to large arteries and veins throughout the body. Sections of clotted blood from these animals similarly fixed and embedded in paraffin showed comparable dark blue staining aggregates (Fig. 1). However, when stained with azure-eosin mixture, these bodies were eosinophilic. They stained red with the periodic acid-Schiff (PAS) procedure for polysaccharide and pink with the ninhydrin Schiff method for amino groups. They did not stain with the Hale dialyzed iron method for acid mucopolysaccharides.

Although fine and occasionally coarse orthochromatic granules are

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seen infrequently in vessels of normal rabbits, no aggregates morphologically similar to those illustrated have been found in these animals. The abnormal polymorphic material was found in abundance in the blood of rabbits 7 hours and 1 and 3 days after the administration of papain. Aggregates were seen occasionally in small hepatic and renal arcuate vessels at 9 days and in a very few renal vessels at 22 days.

The type of fixative utilized influenced both the morphologic and tinctorial appearances of the aggregates. The morphologic features appeared most conspicuously after A.A.F. fixation. With formalin fixatives (except Bouin's) there was clear distinction between the orthochromatic staining of the blood clumps and the metachromatic staining of the material previously described in lymph vessels.[2] In tissue fixed in Bouin's or Carnoy's fluid this distinction disappeared because the blood material also stained metachromatically (Figs. 2, 10, 11). With these two fixatives normal animals showed no blue or purple material in the blood so that there was greater contrast between unstained material in control animals and the metachromatic aggregates in those receiving papain.

Electrophoretic analysis of the plasma from a rabbit one day after the injection of papain revealed a strongly anionic abnormal component migrating far ahead of the normal serum proteins (Fig. 3). The appearance of the acid component coincided with a comparable diminution in the basic portion of the pattern; i.e., the β globulin plus fibrinogen peak.

Dried plasma smears of the experimental animals differed in an unusual and as yet unexplained manner from those of controls. They had the opaque whitish appearance of frosted glass as compared with the translucent normal smear. Viewed under the phase contrast microscope the frosty smear showed irregularly rounded vesicular bodies not seen in normal plasma smears (Figs. 5 and 6).

Severe tubular hemorrhage occurred in the kidney after papain administration. This involved the straight segments principally, and largely although not completely spared the convoluted portion of the proximal tubules (Fig. 7). Collecting tubules and glomeruli showed no hemorrhage. Distal tubules frequently were found to enclose fused metachromatic casts (Fig. 8). Animals sacrificed 1 to 3 days after the introduction of papain showed these lesions.

A procedure has been developed for demonstrating more effectively and for localizing hemorrhage in tissue fixed in Bouin's fluid. The method involves staining with azure A in the pH 2.5 to 3.5 range after only partial removal of the picrate by a 1 to 2 minute rinse in running water. This short rinse replaces the conventional thorough 30 minute

rinse to remove all picrate. It is assumed that in this procedure, picric acid is not removed by the brief rinse and forms an addition compound[4] with a blood constituent. This, being strongly acid, reacts with azure A at low pH. The dye colors the blood in vessels a dark reddish purple against a pale blue or colorless background and stains the blood in the tubules even more conspicuously. Sections stained by this method demonstrate the extent of the tubular hemorrhage more clearly and reveal its localization principally in the outer medulla (Fig. 9).

There was marked variation in response to papain. In less affected animals only the small vessels of the renal medulla contained the aggregates of abnormal material. Thus 2 of the 3 rabbits examined at 24 hours showed large amounts of material in vessels of all organs examined. The third animal (the one used for the electrophoretic analysis of plasma) was less affected in that the cartilage lost only part of its metachromasia, no hemorrhage occurred in the renal tubules, and a loss of liver glycogen was limited to the portal zones. The vessels in this animal did not reveal abnormal material except in the small vessels of the renal medulla, which contained numerous globular aggregates. Moreover, the more severely affected animals showed the greatest amount of material in these vessels, those of the papilla being filled particularly (Figs. 10 to 12). These vessels often appeared occluded by the foreign matter. In fact, in the rare fortunate instances where the section followed the course of an involved vessel, embolic plugging of the lumen was indicated by erythrocyte engorgement proximal to the obstruction (Fig. 13). Although the aggregates in blood vessels in A.A.F.-fixed material were orthochromatic elsewhere, they occasionally stained metachromatically in the renal *arteriolae rectae.*

In the animals examined 3 and 9 days after receiving papain, renal tubule casts were seen in the outer medulla. After neutral formalin fixation these stained green with pH 5 azure A and red with the PAS method. From their structure and the absence of associated fresh hemorrhages, these appeared to represent a phase of resolution of the hemorrhagic lesion (Fig. 14). The collecting tubules at 9 days contained PAS-positive, azure A nonreactive casts (Fig. 15), contrasting with the infrequent, weakly PAS-positive casts seen in control animals. In the animal examined at 22 days the tubules appeared normal.

Liver glycogen, characterized as the diastase digestible, PAS-positive, cytoplasmic constituent, was absent in the periportal regions in moderately affected animals (Fig. 16), and was altogether absent in severely affected animals 24 hours after papain administration. However, there was periportal glycogen depletion at 11 hours and complete

depletion at 24 hours in fasted controls. It may be significant, however, that the decrease of the periportal glycogen persisted in the animals examined 9 and 22 days after the introduction of papain. Moreover, in all the papain-treated rabbits, the liver cell cytoplasm, particularly in the periportal regions, stained in basophilic fashion with azure A (Fig. 18). The liver parenchymal cells of one rabbit which succumbed 20 hours after the intracardiac administration of papain contained numerous, dense, orthochromatic, PAS-positive bodies, usually seen in vacuoles.

Two rabbits given intravenous injections of 3.0 gm. of chondroitin sulfate prepared from pig nasal cartilage (General Biochemicals, Inc.) and two rabbits treated similarly with chondroitin sulfate derived from beef tracheal and nasal cartilage (Nutritional Biochemicals Corp.) were sacrificed 4 and 5 hours later respectively. Plasma smears were prepared from an animal treated with beef chondroitin and were found not to reveal a frosty appearance upon drying. Tissues fixed in the battery of fixative solutions showed no polymorphous basophilic material in blood vessels and no renal hemorrhages. However, after the introduction of pig chondroitin, when fixed with A.A.F. or Bouin's fluid, the epithelium of the proximal and distal straight renal tubules exhibited metachromatic staining (Fig. 19). Only a few isolated tubules stained metachromatically in animals treated with beef chondroitin. After the introduction of beef chondroitin and with Bouin's fluid fixation, the renal cortex revealed very numerous dark red PAS-positive globules, 1 to 6 μ in diameter in glomerular and postglomerular capillaries, in Bowman's capsular spaces and in the proximal convoluted tubules at their points of origin (Figs. 20 and 21). In control animals similar bodies were seen in Bowman's capsular spaces but were much less abundant. Medullary capillaries contained fewer such bodies; and there were none in the large vessels. A few of the bodies were seen in the animals treated with pig chondroitin. In sections from both groups of animals there were metachromatic or orthochromatic granular casts in the collecting tubules (Fig. 22) as well as in an occasional dilated cystic collecting tubule. The epithelium of the collecting tubules, mainly in the inner stripe of the medulla, included scattered densely basophilic cells.

Discussion

The available evidence favors the conclusion that acid polysaccharide released from cartilage by papain, combines with basic plasma protein to form an acid circulating component. Thus the appearance of

an abnormal acid peak in the plasma electrophoretic pattern coincides with a comparable decrease in one or more basic plasma proteins. The presence of orthochromatic PAS-positive, pleomorphic material in blood vessels coincides with the occurrence of structurally similar metachromatic aggregates in lymph vessels. The blood vessel material, while staining orthochromatically in tissues prepared with neutral or weakly acid formalin fixatives, stains metachromatically in tissues prepared with Carnoy's or Bouin's solutions. This could be explained on the basis of a dissociation of the protein-polysaccharide complex under the conditions of fixation. Thus the blood material is rendered metachromatic with the Carnoy fixative which, lacking formalin, is well known to dissolve rather than to precipitate or fix basic proteins.[3] Another example of the failure of these two fixatives to demonstrate basic tissue proteins is their inability to preserve the eosinophilic granules of Paneth cells.[5] Dissociation of the polysaccharide protein complex in the Bouin fixative, with restoration of the metachromasia of blood aggregates, in all probability depends on the strong acidity of this solution. In this case the pH at which the polysaccharide-protein complex dissociates, correlates well with the pH at which metachromasia of chondroitin sulfate is suppressed; i.e., below pH 2. This suggests that combination of the acid polysaccharide with either the basic dye or basic proteins involves a similar type of linkage, possibly an ionic bond.

The disparity in the manifestations following the introduction of papain on the one hand and chondroitin sulfate on the other is surprising. In particular, the absence of blood aggregates and the alterations in the kidney in rabbits treated with chondroitin raise several questions. The difference may be due to impurities in the commercial chondroitin preparations. The possibility remains that there are differences between the mucopolysaccharides themselves, perhaps reflecting species variations or modifications resulting from the commercial preparative procedures. It is known that there are at least three types of chondroitin sulfate and that various mammalian tissues differ in composition with respect to these substances.[6]

The pathogenesis of the renal lesions is an interesting problem which may be better understood in the light of recent knowledge of the physiology of the renal circulation. Evidence from various sources[7,8] indicates that hemoconcentration increases progressively in renal medullary vessels toward the tip of the papilla. It seems reasonable that the predilection of the globular aggregates for the vessels of the papilla in the papain-treated rabbits is related to the twofold increase

in plasma osmotic pressure known to occur there.[7] The embolic occlusion of these vessels by precipitated material has been demonstrated (Fig. 13). Such embolism might be related to the development of the tubular hemorrhages. It is difficult to explain the absence of casts in the collecting tubules in sections showing massive hemorrhages in proximal tubules except on the basis of some obstruction to the progress of the blood down the tubules. The dense metachromatic casts observed in some distal tubules (Fig. 8) may be important in this respect.

Hemoconcentration may also be a factor in the deposition of PAS-positive globules in glomerular and post-glomerular capillaries after the injection of beef chondroitin. However, no plausible explanation can be suggested for the cortical distribution of this material as compared with the predominantly medullary distribution of the intravascular material after papain administration. The metachromatic casts in collecting tubules of rabbits treated with chondroitin resemble those noted by Oliver in collecting tubules of animals with proteinuria arising presumably from the formation of a protein-sulfated mucopolysaccharide complex.[9]

Summary

Histologic changes occur in the blood, kidneys and liver following the intravenous administration of papain. In blood vessels, polymorphous, PAS-positive aggregates are seen which appear orthochromatic with selected stains following formalin fixation and metachromatic after fixation with Carnoy's or strongly acid Bouin's solution. The plasma contains an abnormal acid component in the electrophoretic pattern; and the plasma smears dry with a frosty appearance. The kidneys show severe tubular hemorrhages which are more clearly demonstrated and localized by a new procedure for staining blood in tissue sections. Depletion of glycogen and basophilia in periportal liver cells persists many days after the administration of papain. Rabbits injected with chondroitin sulfate do not reveal aggregates in the blood vessels but do show renal changes which differ from those observed following the introduction of papain.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Orthochromatic fibrils in blood clot 1 day after papain administration. Fixed in A.A.F., embedded in paraffin. Azure A stain at pH 4.5. × 260.

FIG. 2. Metachromatic material in vessels of the liver 1 day after introduction of papain. Bouin's fixation. Azure A stain at pH 4.5. × 130.

FIGS. 3. & 4. Electrophoretic plasma pattern of rabbit 1 day after papain administration (Fig. 3) and control rabbit (Fig. 4), showing abnormal acid component and decreased basic protein in experimental animal. Oxalated plasma is diluted 3-fold with, and dialyzed 18 hrs. against 0.1 ionic pH 8.5 veronal buffer in the cold. Descending boundary recorded at 2 hours.

FIGS. 5. & 6. Phase contrast photomicrographs showing structural detail of the frosty dried plasma smear from papain treated animal (Fig. 5) and the transparent dried plasma smear of control rabbit (Fig. 6). × 515.

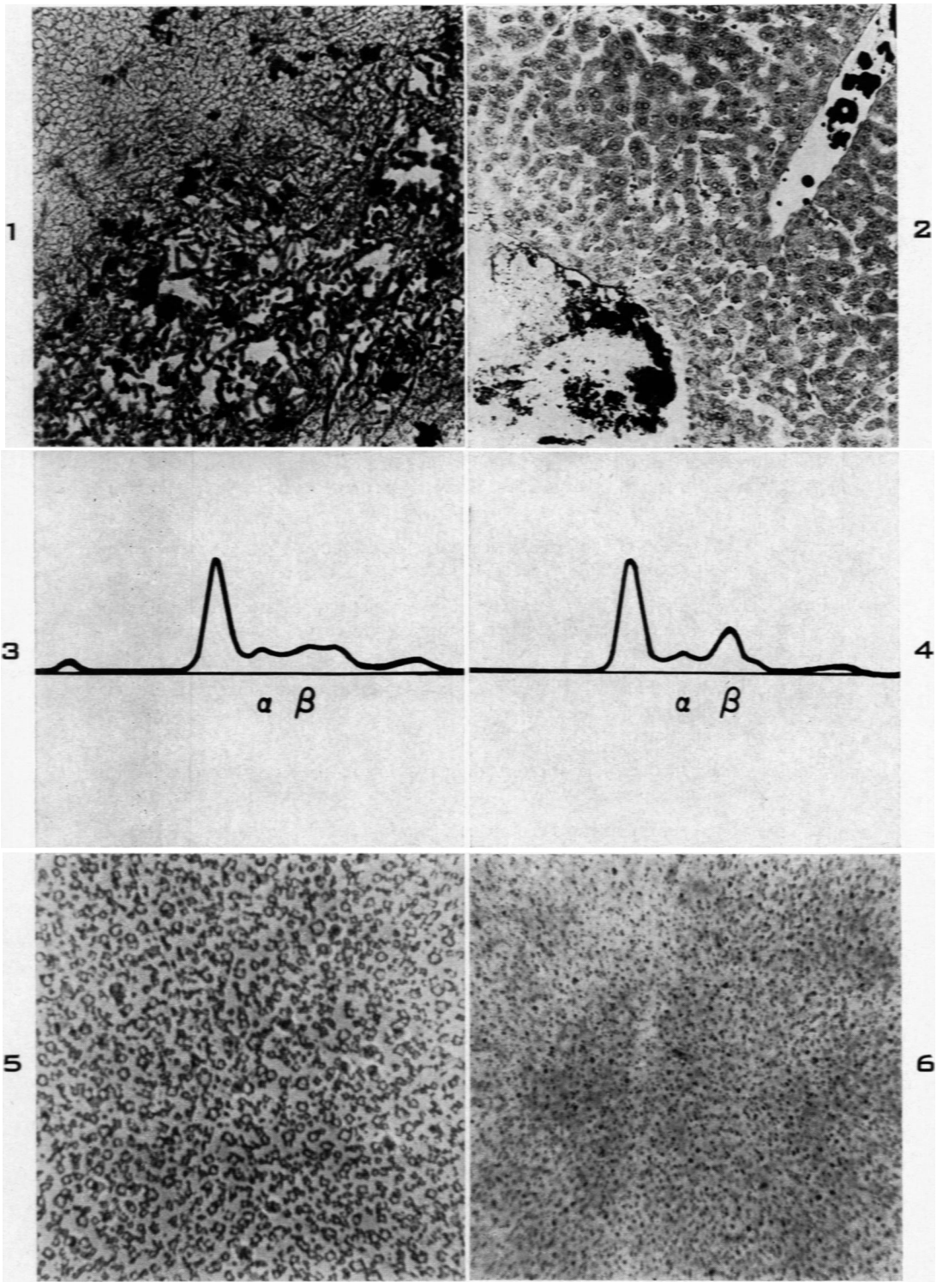
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FIG. 7. Outer renal medulla of rabbit 1 day after administration of papain, showing hemorrhage and metachromatic debris in proximal straight tubules and metachromatic material in capillaries. Bouin's fixation. Azure A stain at pH 4.8. × 205.

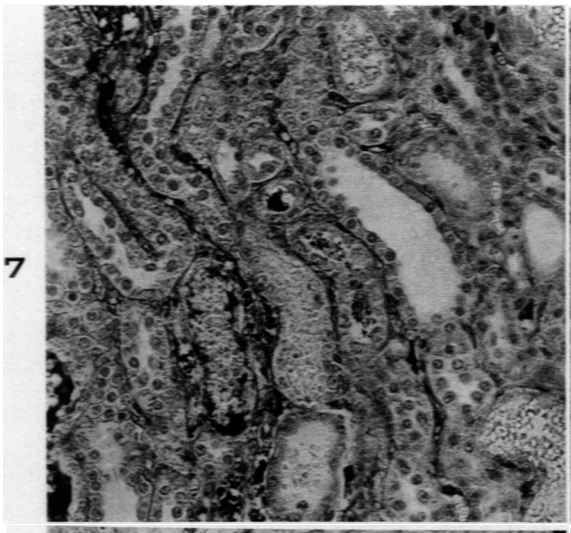
FIG. 8. Renal cortex of same rabbit, showing metachromatic cast in distal tubule. Carnoy's fixation. Azure A stain at pH 4.5. × 285.

FIG. 9. Outer renal medulla 1 day after injection of papain, showing tubule hemorrhages. Sections fixed in Bouin's fluid were rinsed 90 seconds in water and stained with azure A at pH 2.5. × 63.

FIG. 10. Renal medulla of same rabbit, showing metachromatic material in blood vessels. Bouin's fixation. Sections were rinsed thoroughly as usual prior to azure A staining at pH 4.8. × 60.

FIG. 11. Renal medulla 1 day after administration of papain, showing metachromatic globules in small vessels. Bouin's fixation. Azure A pH 4.5 stain. × 260.

FIG. 12. Renal papilla of the same animal, showing globules in vessels. PAS stain. × 230.



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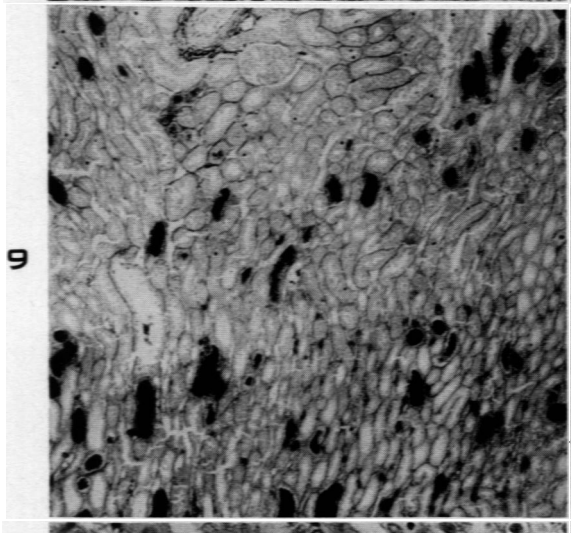

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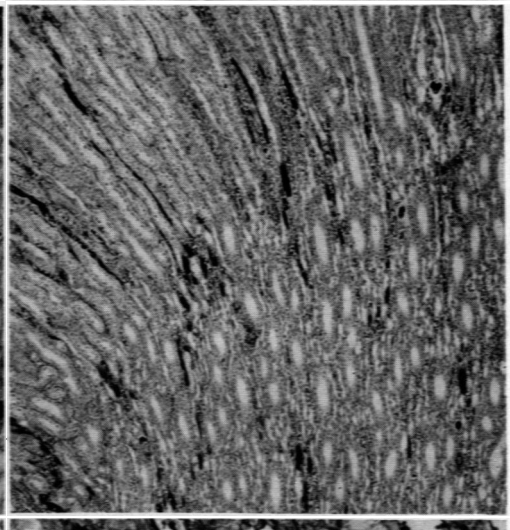

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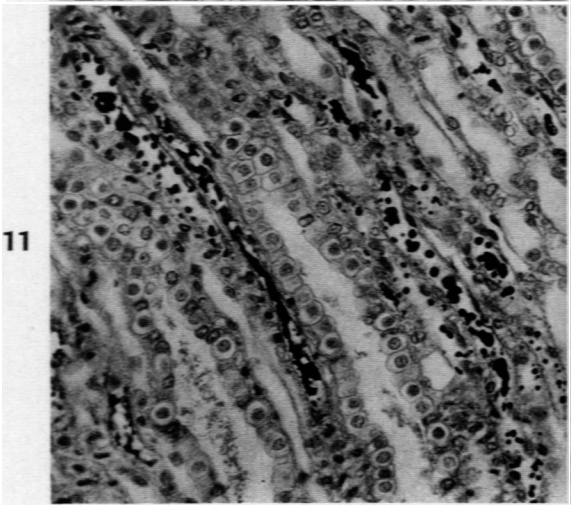

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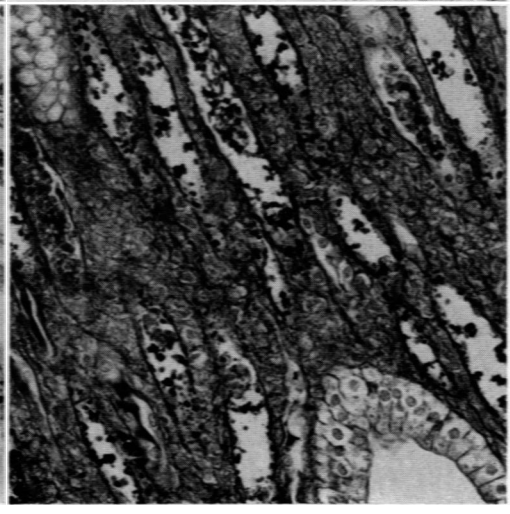

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FIG. 13. Renal papilla 1 day after introduction of papain, showing embolization of small vessel by metachromatic debris. Bouin's fixation. Azure A pH 4.8 stain. × 310.

FIG. 14. Outer medulla 3 days following injection of papain, showing greenish hyalinized casts. Neutral formalin fixation. Azure A stain at pH 4.5 × 200.

FIG. 15. Renal papilla 9 days after introduction of papain showing casts in collecting tubules. PAS stain. × 105.

FIG. 16. Liver of moderately affected rabbit 1 day after papain injection. Note striking depletion of periportal glycogen. PAS stain. × 70.

FIG. 17. Liver of control rabbit, showing clear cytoplasm in periportal region. Azure A pH 4.5 stain. × 260.

FIG. 18. Liver of rabbit 22 days after papain administration, showing persistent basophilia. Azure A pH 4.5 stain. × 260.

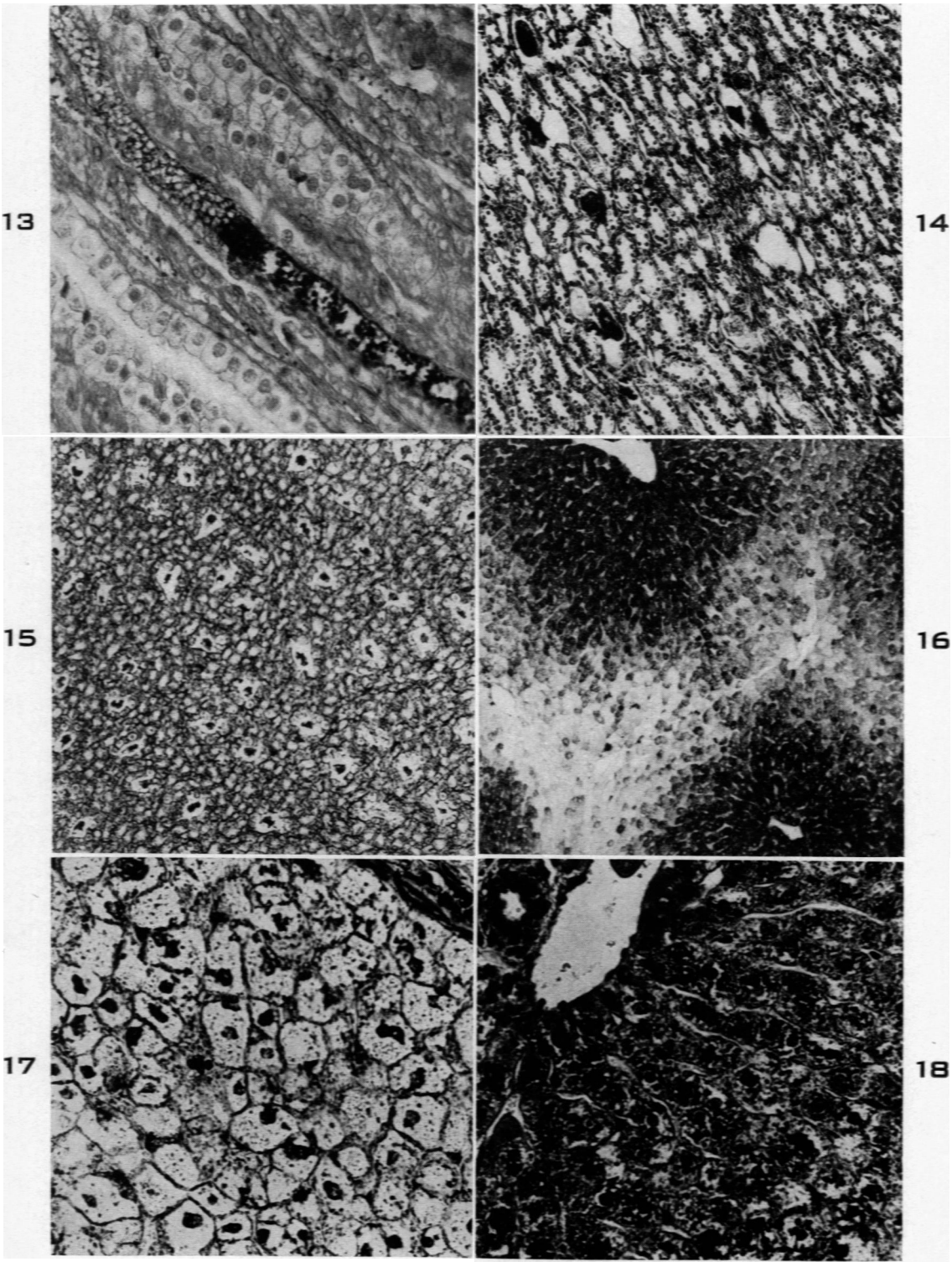
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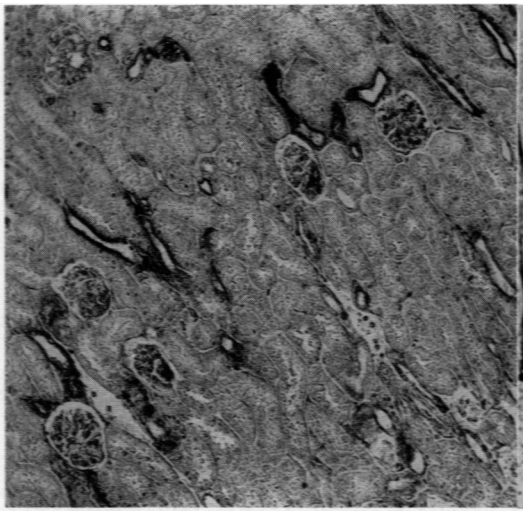
FIG. 19. Kidney 4 hours after injection of 3 gm. of pig cartilage chondroitin sulfate. Note metachromatic epithelium of distal tubules. A.A.F. fixation. Azure A pH 4.5 stain. × 100.

FIGS. 20 and 21. Kidney 5 hours after injection of beef cartilage chondroitin sulfate, showing dark red bodies at the origin of proximal convoluted tubules and in glomerular and postglomerular capillaries. Bouin's fixation. PAS stain. × 420.

FIG. 22. Renal papilla 4 hours after injection of pig cartilage chondroitin sulfate, showing metachromatic granules in collecting tubules. A.A.F. fixation. Azure A pH 4.5 stain. × 260.



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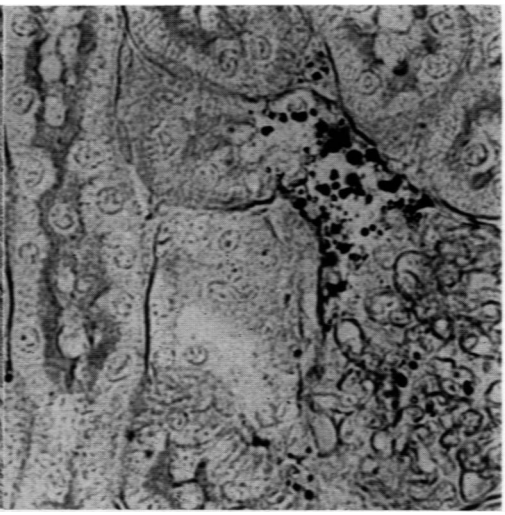

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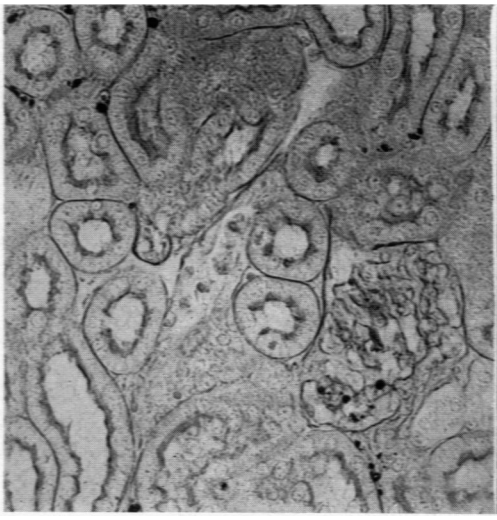

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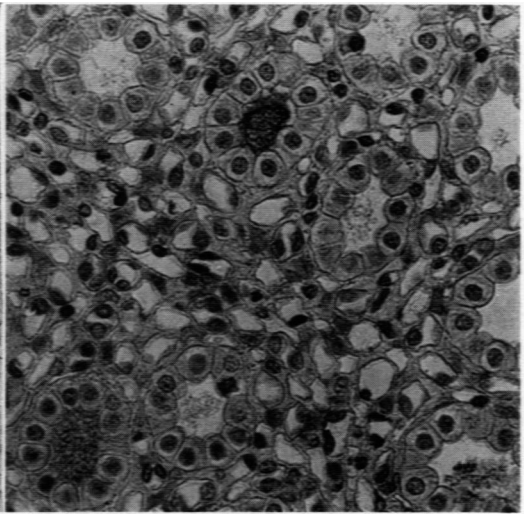

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