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Inducible Collagenolytic Activity in Isolated Perfused Rat Hearts

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There is an extensive collagen network in the heart. The precise anatomy and function of this system has not been fully elucidated. The system does appear to contribute to diastolic compliance, and evidence indicates that the system may be important in directing the stress generated by sarcomeres to the ventricular cavity. Little is known about the mechanisms controlling collagen deposition and resorption in the heart. In this paper the authors demonstrate that disulfide reagents are capable of inducing a collagenolytic reaction in the isolated perfused heart that removes all components of the collagen matrix of the heart as visualized by scanning electron microscopy. The expression of collagenolytic activity requires perfusion of the heart for 1 hour with a disulfide reagent followed by 2 hours with Krebs–Hensleit alone. These results suggest that an inducible and active collagenolytic system exists in cardiac tissue and that this system may be expressed under conditions of oxidative stress. (Am J Pathol 1988, 131: 199–205)

THERE IS an extensive collagen network in the heart.[1–4] This network has been subdivided into various components, but the functional interaction of the components and the myocytes has not been fully delineated. All contiguous myocytes are interconnected by a series of collagen struts, which insert near the Z line in rat hearts. Insertion is into the basal lamina surrounding the myocytes. Capillaries are tethered to adjacent myocytes by struts that also insert into the basal lamina of the vessels and myocytes.[5] A weave of collagen bundles surrounds groups of myocytes, and the weave complexes of one group of myocytes are connected through long tendonlike structures.[6] There is an array of surface cables arranged parallel to the long axis of the myocytes termed coiled perimysial fibers.[7] Also on the surface of the myocytes is a series of small bundles, parallel to the long axis of the cell, that extends about 1–3 sarcomeres.[8] These structures, visible by scanning electron microscopy (SEM), are complimented by a series of fine fibrils visible only by transmission electron microscopy (TEM).[9]

The various components of the collagen matrix of the heart are markedly reduced to absent 2.5–3 hours after coronary artery ligation in the ischemic area.[10,11] Although no change is detectable by SEM in the normally perfused regions, this rapid loss of collagen before any appreciable influx of leukocytes suggests the presence of a collagenolytic activity within the myocardium. In general, collagenase-type proteins have been demonstrated to be secreted from cells as proenzymes. Activation of extracellular collagenase by limited proteolysis or by sulfhydryl-active agents has been extensively reported.[12] We performed experiments designed to test the possibility that a disulfide-sensitive collagenolytic system exists in cardiac tissue and that this system could be activated by exogenous disulfides. We demonstrate that this system could be activated by exogenous disulfides. We demonstrate that perfusion of myocardium *in vitro* with either disulfide reagent for 3 hours results in total disappearance of the collagen network. The loss of collagen is not accompanied by cell necrosis. This paper will describe the methods used and discuss possible mechanisms that result in the loss of the collagen matrix.

Materials and Methods

Rats (200–250 g Sprague–Dawley) are anesthetized with a Ketamine–Rompun mixture given intramus-

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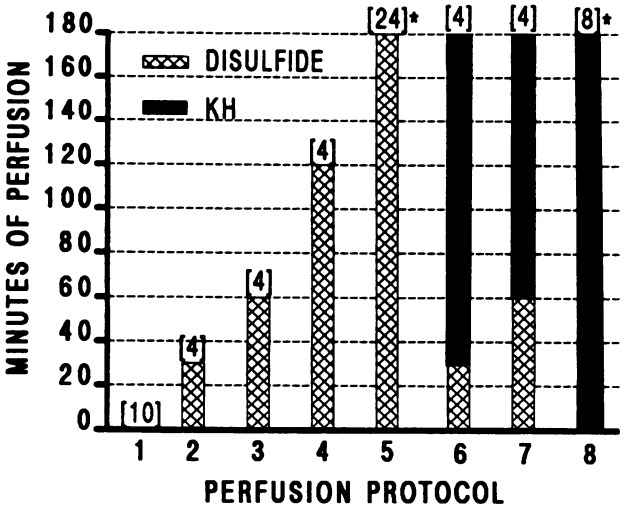
Table 1—Perfusion



The numbers at the top of the bars are the number of hearts examined by light microscopy and SEM for each perfusion time. The asterisks are at times that four hearts were examined by TEM as well. The hatched bars indicate perfusion time with a disulfide reagent in Krebs-Hensleit (KH) solution, and the solid bars indicate perfusion time with KH alone. Approximately one-half of the disulfide hearts were perfused with 1 mM DTNB, and the others with 2 mM GSSG.

cularly. The heart is exposed, packed with ice, removed, and placed in chilled Krebs-Hensleit. Extraneous tissue is removed, a cannula is fixed in the aorta, and the heart flushed with chilled Krebs-Hensleit. When the effluent is clear, the heart is perfused on the Langendorff apparatus at 90 cm H_2O pressure at 35–36 C.

The perfusate is composed of NaCl, 118 mM; $NaHCO_3$, 27.2 mM; KCl, 4.8 mM; KH_2PO_4, 1.0 mM; $MgSO_4 \cdot 7H_2O$, 1.2 mM; glucose, 11.1 mM; $CaCl_2$, 1.75 mM (pH 7.4), to which is added 2 mM oxidized glutathione (GSSG) or 1 mM 5,5′-Dithio (2-nitrobenzoic acid) (DTNB). The mixture is gassed with 95% O_2 and 5% CO_2 for 1 hour before use. The perfusion schedules are described in Table 1. After perfusion, those hearts that receive a disulfide reagent were perfused with Krebs-Hensleit for 10 minutes for removal of the reagent.

At completion of the defined perfusion time, the hearts, while beating, were perfused with 2% glutaraldehyde in phosphate buffer for 10–15 minutes. The hearts were then demounted from the Langendorff apparatus and sliced in rings 1–2 mm thick. These slices were made parallel to the atrioventricular groove; a slice from the midventricular region provided the blocks for SEM. The blocks for SEM were taken from endocardium to epicardium. The blocks were trimmed to form a trapezoid with the epicardium forming the broad base for easy identification of the epi- and endocardium. Two sets of blocks were obtained from the anterior, lateral, posterior, and septal regions. One set of blocks was oriented such that the cut surface parallel to the atrioventricular groove would face the electron beam in the SEM. The second set of blocks was oriented such that the face exposed to the electron beam was parallel to the long axis of the heart. The blocks were fixed overnight in 2% glutaraldehyde in phosphate buffer. The next day, the blocks were fixed with 2% OsO_4 in phosphate buffer, dehydrated in graded alcohols, critical point-dried with CO_2, affixed to stubs with conductive glue, and given a thin coating of gold with a Technics sputter device.[1,13]

One slice of the heart was transferred to 10% neutral-buffered formalin and processed for light microscopy. A slice adjacent to the one used for SEM was sampled and processed for TEM.[13]

Results

The collagen matrix of normal rat hearts has been described.[1–4] After 3 hours' perfusion of rat hearts with Krebs-Hensleit in a Langendorff apparatus, there is no change in the extracellular matrix by SEM. The struts between myocytes appear normal, the capillary–myocyte struts show no alterations, and the weave complex surrounding groups of myocytes appears unaltered (Figures 1 and 2). By SEM, the basal lamina surrounding myocytes is intact (Figure 1). By transmission electron microscopy, the 3-hour control hearts resemble those previously described after perfusion in a Langendorff apparatus.[17] There are occasional myelin figures in the mitochondria, but no appreciable swelling is seen (Figure 3). The sarcomeres are in register and maintain normal morphologic features. The basal lamina is intact; and banded collagen fibrils, both single and in groups, are visible in the interstitial space. There is separation of myocytes and an expansion of the extracellular space, which is common with perfused hearts.

Three hours' perfusion with either disulfide reagent added to the Krebs-Hensleit results in a totally different picture. The struts between myocytes are extremely rare, and the struts that connect myocytes to capillaries are seen infrequently and in many preparations not at all (Figure 4). The weave component and its associated tendon-like structures are absent (Figure 5). At higher magnification, the surface cables described by Orenstein et al are not visible (Figure 6).[8] The coiled perimysial fibers running parallel to the long axis of the myocytes are visible and do not appear to be seriously altered (Figure 5). In spite of this marked alteration in the extracellular matrix, the basal lamina appears intact. The myocardium, after

the disulfide reagents, closely resembles the appearance of neonatal rat hearts before the collagen matrix has developed.[14] The perfusion with disulfide reagents results in an expansion of the extracellular space, as is seen with perfused control hearts (Figure 7). The important observation is the absence of collagen bundles associated with either capillaries or myocytes. There are occasional collagen fibrils, generally widely spaced. There does not appear to be any alteration of either myocyte or capillary basement membrane. There is some dilation of the myocyte tubular elements, but this is common in the control perfused hearts.

Exposure to disulfide reagents for a half-hour, followed by 2½ hours of Krebs-Hensleit perfusion, showed at best questionable loss of the collagen matrix. However, perfusion with either disulfide for 1 hour, followed by 2 hours' of Krebs-Hensleit, provided an appearance identical with the 3-hour perfusions with either disulfide (Figure 8).

Figure 8 is of a heart perfused with 1 mM GSSG for 1 hour and then 2 hours' of perfusion with Krebs-Hensleit. The collagen struts are no longer visible. Arrows indicate elongate structures commonly seen after disulfide perfusion. These structures closely resemble those described by Canale, et al and identified as nerves.[15] Dissolution of the collagen with preservation of nerves and myocytes was effected by Canale et al, subjecting fixed tissue to hydrolysis with 8 N HCl.

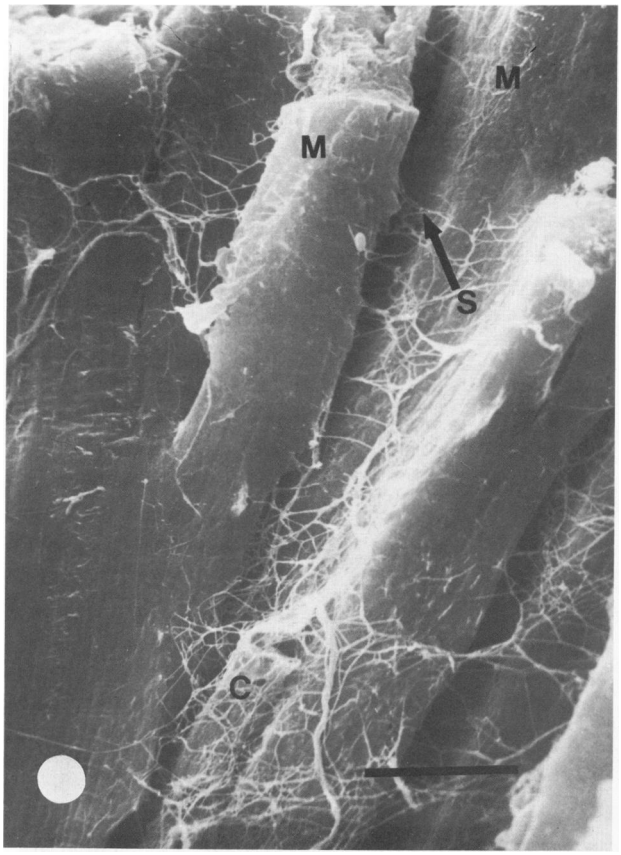


Figure 1—SEM of a rat heart perfused with Krebs-Hensleit for 3 hours. The struts interconnecting myocytes are indicated by an arrow. A capillary (c) with struts connecting it to adjacent myocytes (*M*) is visible in the lower part of the picture. The *bar* represents 10 μ. (×2000)

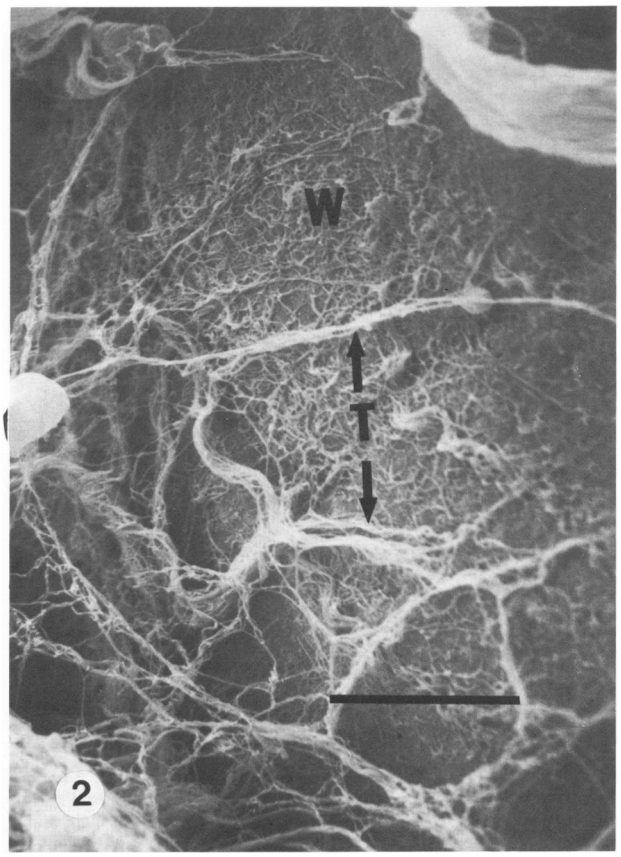


Figure 2—SEM of a rat heart perfused with Krebs-Hensleit for 3 hours. This demonstrates a weave (*w*) that surrounds a group of myocytes. The long tendonlike (*T*) structures that interconnect adjacent weaves are visible. The *bar* represents 10 μ. (×2400)

Examination of control hearts by light microscopy using H & E or trichrome stains shows no difference between perfused and nonperfused hearts. Similarly, the hearts perfused with either disulfide were comparable to Krebs-Hensleit-perfused controls by light microscopy. This lack of difference is due to the fact that most of the collagen matrix as seen with SEM is not visible in the light microscope. The periarterial connective tissue seems little affected by exposure to the disulfide reagents. We did not use silver stains, which may have shown a difference.[16]

Discussion

Most of our results were obtained by SEM. This instrument has a resolution of 10–15 nm and a very

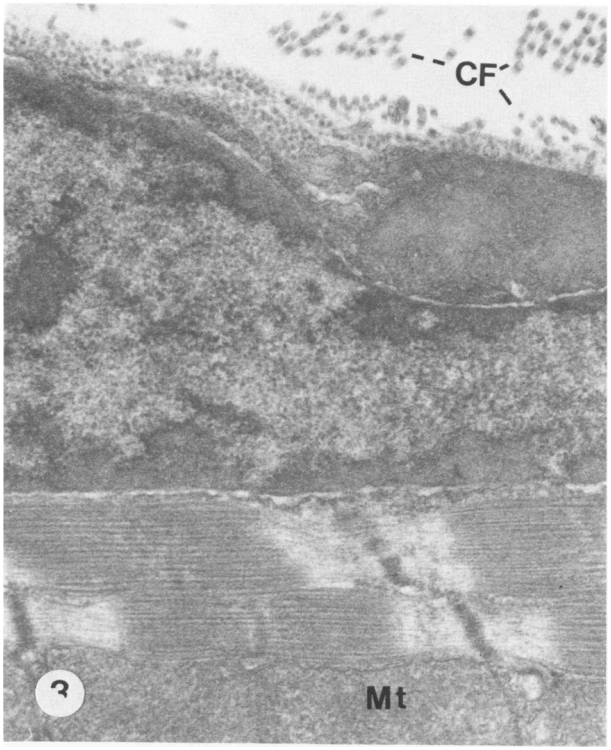


Figure 3—TEM of a rat heart perfused with Krebs-Hensleit for 3 hours. Collagen fibrils (*CF*) associated with the basement membrane and extending into the interstitial space are present. The sarcomeres are stretched because this heart was perfused fixed at 90 cm H_2O pressure. Mitochondria (*Mt*) are present and reasonably normal. (×33,800)

great depth of focus, which permits good visualization of the three-dimensional collagen matrix. A limiting factor with SEM is its inability to quantitate. For it to detect a change, it must be of major proportions. The collagen network of normal rat hearts is not visible by light microscopy using H & E or trichrome stains. Portions are visible with silver stains, but the appearance is not nearly as complete as that seen by SEM.[16] TEM requires thin sections, and this procedure prevents demonstration of the complex network because most of the collagen bundles do not follow a straight course.[1] In our work, unless loss of the matrix is extensive in examined areas, we consider no change to have taken place. This precludes detection of early mild or subtle changes in the extracellular matrix. Although this problem can be overcome by chemical analysis of the collagen in the hearts, such analysis provides no positional or structural data essential to interpreting the results.

Proteins that have collagenolytic activity are normally secreted from cells as pro-enzymes.[12] These zymogens can be activated by a number of mechanisms, including limited proteolysis, and compounds known to modify protein sulfhydryls.[12,18,19] The exact mechanism responsible for collagenase activation is unclear; however, in general, collagenase activation results in a decrease in the molecular weight of the collagenase zymogen. This observation indicates possible removal of an inhibitor peptide by proteolysis or chemical treatment (mercurials, etc.).[12] A collagenase isolated from neutrophil granules has been reported to be reversibly activated by a number of disulfide reagents known to modify protein sulfhydryls.[20] This activation process was likewise accompanied by changes in the apparent molecular weight of the zymogen. Collagenase activation by sulfhydryl reagents produced a dissociation of an inhibitor–collagenase complex and a decrease in the apparent molecular weight of the collagenase. Although the protein chemistry involved in the collagenase activation process in general remains obscure, we tested the possibility that cardiac tissue might contain a native collagenase zymogen as part of a collagenolytic system and that it can be activated by disulfide reagents. Our results (Figures 4–8) show that perfusion of the isolated rat heart with 1 mM DTNB or 2 mM GSSG in Krebs-Hensleit produced dramatic dissolution of the collagen matrix. Other disulfides may work equally well. On the basis of our observations, we are not able to distinguish between the two

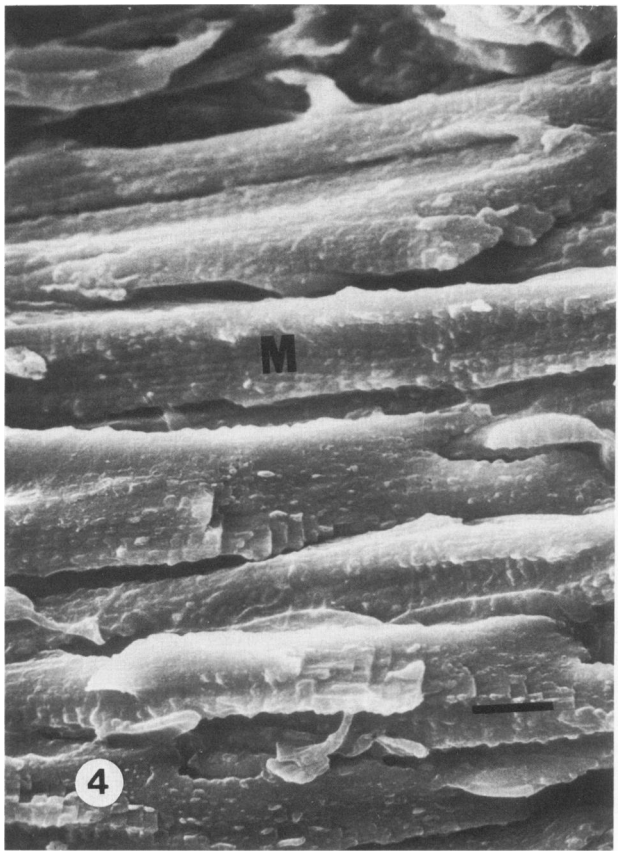


Figure 4—SEM of a rat heart perfused with 1 mM DTNB for 3 hours. The normal collagen matrix is no longer visible. The *bar* represents 10 μ. (×1100)

possibilities of collagenase zymogen activation presented by Cawston and Murphy.[12] That is, does sulfhydryl treatment of isolated perfused rat heart activate a protease which in turn activates the collagenase zymogen, or does the disulfide reagent directly activate the collagenase activity (see Gilbert[19] for example)?

One half-hour exposure to either disulfide at the concentration tested, followed by 2½ hours of Krebs-Hensleit perfusion, resulted in, at best, very questionable loss of the collagen matrix. One hour's perfusion with disulfide with immediate fixation was ineffectual in altering the collagen matrix. Two hours' perfusion with either disulfide with immediate fixation again showed no definite evidence of collagen loss. One hour's perfusion with a disulfide followed by 2 hours of Krebs-Heinsleit alone induced changes comparable in all respects to 3 hours' perfusion with the disulfide reagents. This suggests that the dissolution of collagen required about 1 hour's exposure to the activator and another 2 hours for sufficient proteolysis of the collagen matrix to be clearly demonstrable by SEM. The 1-hour disulfide/2-hour Krebs-Hensleit treatment indicates that the disulfide reagents probably are not having a direct effect on the collagen matrix, because after 1 hour's perfusion, little change is visible, and the subsequent 2 hours' perfusion with Krebs-Hensleit would wash the small water-soluble disulfides out of the tissue.

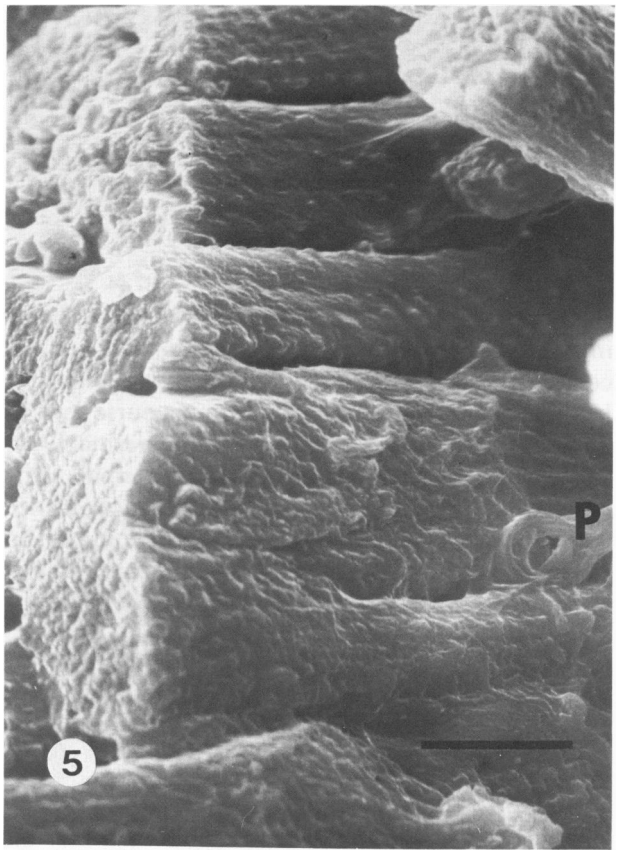


Figure 5—SEM of a rat heart perfused with 2 mM GSSG for 3 hours. This area should have a weave surrounding groups of myocytes. The weave as well as the associated tendons are not visible. A coiled perimysial fiber (*P*) is present. The *bar* represents 10 μ. (×1900)

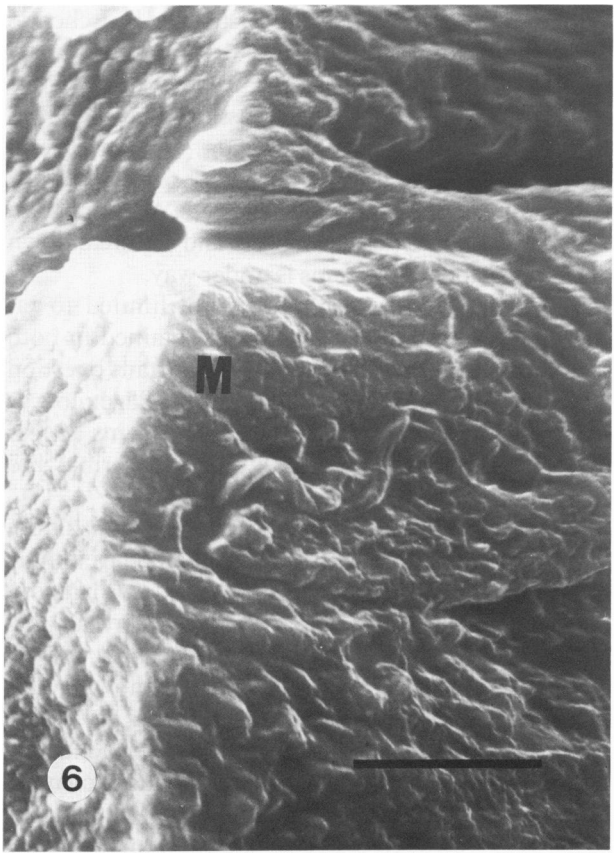


Figure 6—Higher magnification of region depicted in Figure 5. The surface cables described by Orenstein et al should be visible at this magnification, but are not. The *bar* represents 5 μ. (×4800)

By light, scanning, and transmission electron microscopy, no evidence of toxic change was present in the myocytes exposed to either disulfide for 3 hours. Toxicity associated with DTNB is not recorded in the data base of the Chemical Information Service. We saw no evidence of myocyte damage associated with DTNB as we used it at 1 and 2 mM concentrations. Similarly, GSSG at 2 mM concentration caused no evidence of morphologic damage to the myocytes by light or electron microscopy.

The time for loss of cardiac collagen in the *in vitro* system used is consistent with the time sequence of collagen loss after ligation of a coronary artery.[10,11] Whether the mechanism in the two systems is the same or not is not known. Oxidized glutathione is increased in ischemic myocardial cells and may be released to the extracellular space.[21] However, the

amount of oxidized glutathione produced by ischemic myocytes is considerably lower than the concentration used in these experiments. At lower concentrations of GSSG (0.5 mM), some loss of collagen matrix was observed at 3 hours of perfusion. The *in vitro* and *in vivo* systems are different in many respects, and it is difficult to interpret these differences. A comparison by chemical analysis of collagen and its breakdown products in the two systems is under way.

The action of the disulfides is not limited to rat hearts. Dissolution of collagen was obtained in both rabbit and dog hearts. An equally important question is whether these disulfides can induce collagen loss in other tissues. In preliminary work, the agents appear to be equally effective in breaking down dermal and pulmonary collagen *in vivo*.

The collagenolytic activity induced by the disulfides is similar in degree and timing to the collagenolytic activity seen after coronary artery ligation. What conditions, other than the severe ischemia of coronary occlusion and perfusion with disulfides, might induce the loss of the collagen matrix are not known. With loss of the collagen matrix *in vivo*, the stress developed by the sarcomeres would be delivered to the ventricles in an inefficient manner resulting in global changes essentially identical to poor contractility.[16] Another important consideration is that after removal of the collagen matrix, presumably collagen would return. If the replacement follows the normal course seen in neonates,[14] the ventricle would return to normal function. If the collagen is deposited in an abnormal distribution around the viable myocytes, the appearance would be of diffuse fibrosis, a situation common to cardiomyopathies and the diffuse subendocardial fibrosis seen with ischemic and hypertensive heart disease.

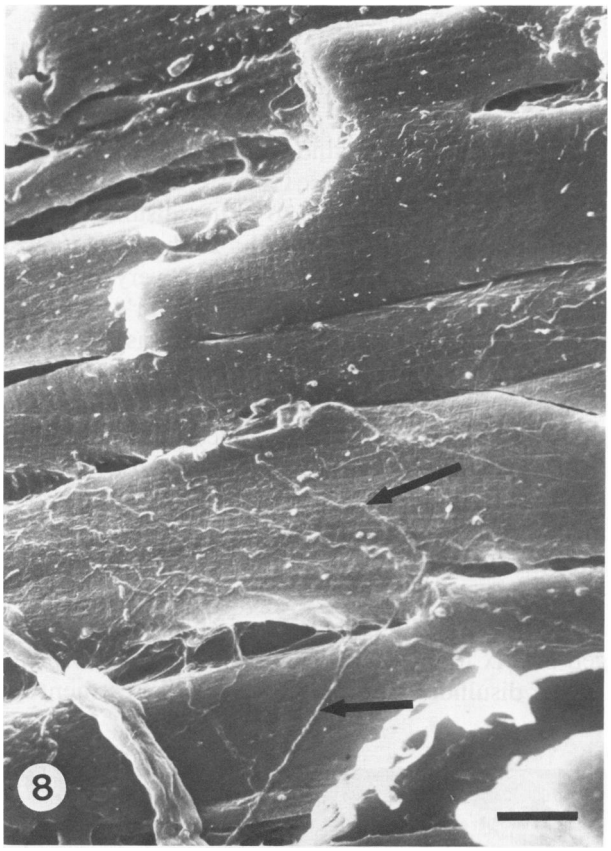


Figure 8—SEM of a rat heart perfused with 1 mM GSSG for 1 hour and then with Krebs-Hensleit for 2 hours. The *arrows* indicate structures that are identical to those identified by Canale et al in scanning micrographs of a heart that had the collagen removed after fixation by hydrolysis with 8 N HCl. The normal collagen matrix is not visible. The *bar* represents 10 μ. (×1000)

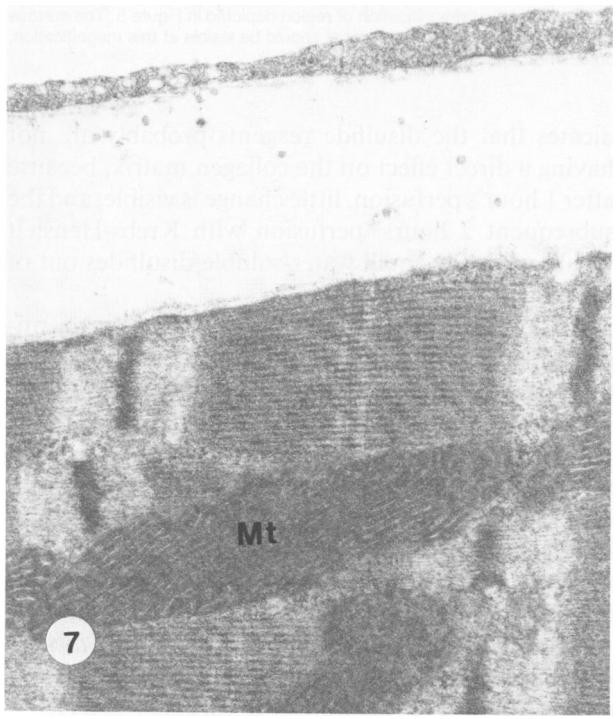


Figure 7—TEM of a rat heart perfused for 1 hour with 1 mM GSSG and then 2 hours with Krebs-Hensleit. The capillary and myocyte basement membranes are visible. There are very few collagen fibrils associated with the basal laminae and few in the interstitial space. Components of the myocytes, including mitochondria (*Mt*), are normal in appearance. (×27,000)

References

1. Caulfield JB, Borg TK: The collagen network of the heart. Lab Invest 1979, 40:364–371
2. Borg TK, Caulfield JB: The collagen matrix of the Heart. Fed Proc Fed Am Soc Exp Biol 1984, 40:2037–2041
3. Robinson TF, Cohen-Gould L, Factor SM: The skeletal framework of mammalian heart muscle: Arrangement

of inter and pericellular connective tissue structures. Lab Invest 1983, 49:482–498
4. Robinson TF, Cohen-Gould L, Remily RM, Capasso JM, Factor SM: Extracellular structures in heart muscle, Advances in Myocardiology. Vol 5. Edited by P Harris, PS Poole-Wilson. New York, Plenum, 1985, pp 243–255
5. Caulfield JB, Borg TK, Abel FL: The effects of systole on left ventricular blood flow, Advances in Myocardiology. Vol 4. Edited by E Chazov, V Saks, G Rona. New York, Plenum, 1983, pp 379–393
6. Borg TK, Ranson WF, Moslehy FA, Caulfield JB: Structural basis of ventricular stiffness. Lab Invest 1981, 44:49–54
7. Robinson TF, Cohen-Gould L, Factor SM, Eghbali M, Blumenfield OO: Structure and function of connective tissue in cardiac muscle: Collagen type III in endomysial struts and perimysial fibers. Scanning Microscopy International (In press)
8. Orenstein J, Hogan D, Bloom S: Surface cables of cardiac myocytes. J Molec Cell Cardiol 1980, 12:771–780
9. Robinson TF, Winegrad S: A variety of intercellular connections in heart muscle. J Mol Cell Cardiol 1981, 13:185–195
10. Caulfield JB, Sun BT, Nachtigal M: Ventricular collagen matrix and alterations,[4] pp 257–269
11. Sato S, Ashraf M, Millard RW, Friginora H, Schwartz A: Connective tissue changes in early ischemia of porcine myocardium: An Ultrastructural study. J Mol Cell Cardiol 1983, 15:261–275
12. Cawston TE, Murphy G: Mammalian collagenases. Methods Enzymol 1981, 80:711–722
13. Arborgh B, Bell P, Brunk V, Collins VP: The osmotic effect of glutaraldehyde during fixation: A transmission electron microscopy, scanning electron microscopy and cytochemical study. J Ultrastruct Res 1976, 56: 339–350
14. Borg TK, Caulfield JB: Collagen in the heart. Texas Rep Biol Med 1979, 39:321–333
15. Canale ED, Campbell GR, Smolich JJ, Campbell JH: Cardiac muscle, Handbook of Microscopic Anatomy. Vol 2. New York, Springer-Verlag, 1986, pp 7, 12, 16, 129
16. Factor SM, Robinson TF: Comparative connective tissue structure function relationships in biological pumps. Lab Invest (In press)
17. Weissler AM, Krugen FA, Baba N, Scarpelli DG, Leighton RF, Gallimore JK: Roles of anaerobic metabolism in the preservation of anoxic myocardium. J Clin Invest 1968, 47:403–416
18. Harper E, Bloch KJ, Gross J: The zymogen of tadpole collagenase. Biochem 1971, 10:3035–3041
19. Gilbert HF: Biological disulfides: The third messenger. J Biol Chem 1982, 257:12086–12091
20. Tschesche H, MacCartney HW: A new principle of regulation of enzymic activity: Activation and regulation of human polymorphonuclear leukocyte collagenase via disulfide-thiol exchange as catalyzed by the glutathione cycle in a peroxidase-coupled reaction to glucose metabolism. Eur J Biochem 1981, 120:183–190
21. Curello S, Ceconi C, Bigoli C, Ferrari R, Albertini A, Guarnien C: Changes in the cardiac glutathione status after ischemia and reperfusion. Experientia 1985, 41: 42–43

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