Myocardial Connective Tissue Metabolism
in Response to Injury

II. INVESTIGATION OF THE MUCOPOLYSACCHARIDES INVOLVED IN ISOPROTERENOL-INDUCED NECROSIS AND REPAIR IN RAT HEARTS

By Joseph T. Judd, Ph.D., and Bernard C. Wexler, Ph.D.

With the Technical Assistance of George Williamson and Jacob Boyer

ABSTRACT

Myocardial necrosis was produced in rats by administering isoproterenol, and the increase in hexosamine-containing materials in the injured myocardium was investigated. Total hexosamine, galactosamine, neutral sugars, and uronic acids were determined on various fractions isolated from the hearts. Mucopolysaccharides were precipitated as the cetyl pyridinium chloride complex and fractionated according to their solubility in NaCl solutions. Of the hexosamine in the heart, approximately 50% was retained after proteolytic digestion and dialysis. Approximately one-fourth to one-third of the nondialyzable hexosamine was in mucopolysaccharides. The hyaluronic acid fraction of the mucopolysaccharides increased during the early edematous reaction observed in the hearts. The chondroitin sulfate fraction increased in conjunction with the beginning fibrinogenic processes and remained elevated during repair. There was evidence that the glycoproteins may be involved in the myocardial necrosis and repair.

ADDITIONAL KEY WORDS necrosis scar tissue formation edema hexosamine metabolism glycoproteins myocardial infarction

In a previous study on connective tissue changes following isoproterenol-induced myocardial infarction, a marked fluctuation in myocardial hexosamine was found (1). The myocardial hexosamine content increased rapidly for the first 3 days following treatment, and then declined to a relatively constant but elevated level. The initial increase in hexosamine was correlated with both the change in the weight of the heart and with histological changes thought to be related to myocardial edema. During the period of the increase in hexosamine, there was also an increase in a Hale stain-positive material, which was also digested by testicular hyaluronidase and was believed to be mucopolysaccharide. In connection with these changes in myocardial ground substance early collagenization of the necrotic foci was also observed.

The present study was undertaken to determine the extent of involvement of mucopolysaccharides in the change in hexosamine observed previously (1), to determine if other hexosamine-containing substances contribute to such change, and to identify the types of mucopolysaccharides involved in each phase of the healing of the damaged cardiac muscle.

Materials and Methods

Mature virgin male Sprague-Dawley rats weighing 350-400 g were used in all experiments.
The hearts were digested with papain and trypsin. Extraction with diethyl ether. The sample was centrifugation and analyzed for hexosamine.

The supernatant fluid from the digestion mixture was compared with the hexosamine content of the Supernatant fluid from the digestion mixture was compared with the hexosamine content of the supernatant fluid from the digestion mixture with the dried heart powder. Trichloroacetic acid was then added to a final concentration of 10%, and any precipitate was removed by centrifugation and analyzed for hexosamine. Excess trichloroacetic acid was removed by extraction with diethyl ether. The sample was then placed in cellulose tubing (average pore size, 4.8 μm) and dialyzed for 48 hours, first against running tap water and then against several changes of distilled water. The material remaining in the dialysis bag was recovered by lyophilization and diluted to a known volume with 0.02 M Na₂SO₄. Aliquots of this solution (subsequently referred to as the nondialyzable fraction) were used for analysis.

Two precipitation procedures were used for recovering the mucopolysaccharides from the nondialyzable fraction. The first involved direct precipitation with cetylpyridinium chloride (CPC) from the 0.02 M Na₂SO₄ solution, followed by collection of the precipitate on a 10 X 70 mm cellulose column. In the second procedure, the mucopolysaccharide (MPS) was precipitated from the 0.02 M Na₂SO₄ solution by addition of ethanol to a final concentration of 75% followed by collection of the precipitate on a 10 X 70 mm cellulose column saturated with 75% alcohol. The precipitate was then converted to the MPS-CPC complex on the column by washing with 15 ml of 0.02 M Na₂SO₄ containing 30 mg cetylpyridinium chloride. The eluate from this wash was recycled through the column three times to remove any precipitate (cloudiness) which formed after the previous passage through the column.

The MPS-CPC complex was eluted from the cellulose column with 0.04 M NaCl containing 0.1% CPC, 0.4 M NaCl containing 0.1% CPC, 1.2 M NaCl containing 0.1% CPC, 2.1 M NaCl, and 4 M NaCl. The eluant was allowed to flow by gravity giving a flow rate of 1 to 3 ml per hour. The fractions obtained with each of the eluting solutions and the sample eluate were analyzed for uronic acids using the Dische method (9, 10), and for hexoses, hexosamines, and galactosamine as described above.

The alcohol supernatant fluid remaining after the second precipitation procedure described above was diluted with water, frozen, and lyophilized. The residue was dissolved in water and aliquots taken for determination of total hexosamine, galactosamine, and hexoses. The remaining portion of the alcohol residue was hydrolyzed using Dowex-50 as a catalyst, in a manner similar to that described by Anastassiadis and Common (11) and Jeffrey and Rienits (12) for the isolation of hexuronic acid from tissues or mucopolysaccharides. To 2 ml of sample containing approximately 0.5 mg of hexosamine was added 2.2 ml of a 1:2 w/v suspension of Dowex-50 X 2, 200-400 mesh, hydrogen form, in 0.1 N HCl. Two-tenths ml of glucuronic acid-6-C¹⁴

---

1Isoproterenol was furnished by the Sterling-Winthrop Research Institute of the Sterling Drug Corp., Rensselaer, N. Y.

2Union Carbide Corp., obtained from Matheson Scientific.

3Whatman Cellulose Powder CF11.
(specific activity 2.4 mc/mxt) containing 10^8 dpm was added to each sample to estimate the extent of destruction of liberated hexuronic acids. The destruction of the labeled material was considered to represent only the maximum, and not the average for the sample, which was assumed to be less because of time required to liberate the hexuronic acid from its conjugates. After hydrolysis, the samples were successively fractionated on Dowex-50 and Dowex-1 columns as described by Jeffrey and Rienits (12), and the fractions were analyzed for hexosamine, uronic acid, and neutral sugars. The extent of hydrolysis of the hexosamine-containing materials was estimated by comparison of the hexosamine value with that obtained on the same sample hydrolyzed with 2N HCl at 95°C for 18 hours.

Results

In conjunction with the dual purpose of the present work, (a) the identification of the mucopolysaccharides involved in the repair of isoproterenol-induced myocardial necrosis, and (b) the determination of the extent to which changes in the mucopolysaccharide concentration are responsible for the overall fluctuation in myocardial hexosamine (1), total hexosamine was determined after each step in the proteolytic digestion and dialysis involved in the isolation procedure. In Figure 1, the results of such determinations are presented for five samples, each containing five hearts. Two samples are from placebo-injected control rats (designated 0 days after injection in Figure 1), and one sample is from isoproterenol-treated animals on the second, third, and fifth days following the initial injection of isoproterenol. The control animals received injections of distilled water equal to the volume of diluent used for the isoproterenol (1 ml) and were killed and examined along with the 2- and 5-day treated animals. No difference was found between un.injected normal animals and placebo-injected controls. The fluctuation in total hexosamine (as determined on the acetone-extracted, dried heart) followed the same pattern described previously (1). Digestion of the dried heart material with papain resulted in no loss in hexosamine, while accomplishing virtually complete solubility of the tissue. However, dialysis of the samples in cellulose tubing for 48 hours against water, resulted in considerable loss of hexosamine. The amount of hexosamine removed at this step was relatively constant in the control and isoproterenol-treated animals, and thus the hexosamine-containing materials removed by this procedure, while representing an appreciable portion of the total, appear to contribute very little to the overall hexosamine increase during repair. The samples were subsequently digested with trypsin and simultaneously dialyzed for 5 days as described by Schiller et al. (5), with the consequent loss of hexosamine shown in Figure 1. Addition of trichloroacetic acid to a final concentration of 10%, removal of the precipitate formed, and ether extraction and dialysis to remove the trichloroacetic acid resulted in a small additional loss of hexosamine.

The cumulative loss of hexosamine through this procedure was 1.5 mg/g dry weight of heart for the controls (average of both pooled
samples), and 2.5 mg/g dry weight for the samples from the isoproterenol-treated animals, regardless of the time elapsed after treatment. The pattern of hexosamine fluctuation following myocardial injury was still evident in the purified material remaining in the nondialyzable fraction.

In Figure 2, data from the analysis of the nondialyzable fraction isolated from rat hearts following myocardial injury are presented. Hearts from five animals were pooled at each point, digested with papain and trypsin, treated with trichloroacetic acid, and dialyzed. To facilitate the handling of a large number of samples, trypsin digestion was not performed with simultaneous dialysis, but was instead performed in flasks, followed by trichloroacetic acid precipitation and dialysis. Prior testing of this procedure showed little difference in either the recovery of hexosamine or the removal of protein from the sample. Both glucosamine and galactosamine increased to a maximum 48 hours after the initial isoproterenol injection, and then declined. The major increase in hexosamine during this early period of the repair process was due to glucosamine. The galactosamine, however, appeared to remain elevated, compared to the controls, throughout the period of study, while the glucosamine was more variable. The pattern of fluctuation of neutral hexoses was similar to that of the hexosamines but differed greatly in the magnitude of the increase and in the timing of the change. The initial increase was not observed until the second day, when the hexosamines had already reached their peaks. The peak of the increased neutral hexose concentration occurred on the fourth day, 2 days after the peak hexosamine level. The level of hexose in the heart remained elevated throughout the later stages of healing, with a very slow return to near normal by the third week.

In Table 1 and Figure 3 are presented the results from the two procedures used to precipitate the mucopolysaccharides from the nondialyzable fraction. In the experiment summarized in Table 1, the mucopolysaccharides were precipitated by adding cetylpyridinium chloride to the nondialyzable fraction, after reducing its volume to 10 ml by lyophilization and adding Na₂SO₄ to a final concentration of 0.02 M. Figure 3 shows the results of precipitating the mucopolysaccharides with alcohol, followed by conversion to the cetylpyridinium chloride complex on a cellulose column. The same elution scheme was used to fractionate the MPS-CPC complex.

In Table 1, the effect of myocardial injury induced by isoproterenol is demonstrated by an increase in uronic acid in both the 0.4 M and 1.2 M NaCl fractions of the MPS-CPC.
TABLE 1

Effect of Isoproterenol-Induced Necrosis on Myocardial Mucopolysaccharides Two Days after Injury (Fractionation of the MPS-CPC Complex by Solubility in NaCl)

<table>
<thead>
<tr>
<th></th>
<th>Glucuronic acid (mg/g dry wt of heart)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.4 M NaCl fraction</td>
</tr>
<tr>
<td>Control</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>(4)</td>
<td>(4)</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>0.58 ± 0.08</td>
</tr>
<tr>
<td>(3)</td>
<td>(3)</td>
</tr>
<tr>
<td>% Increase</td>
<td>262</td>
</tr>
<tr>
<td>P</td>
<td>(&lt;0.005)</td>
</tr>
</tbody>
</table>

Values are means ± SE. Number in parentheses is number of samples. Each sample contained hearts from five animals.

% Increase = \( \frac{\text{isoproterenol} - \text{control}}{\text{control}} \times 100 \).

\( P \) values calculated from a \( t \)-test between the control and isoproterenol-treated groups.

Complex. The sample eluate, a 0.04 M NaCl fraction, and a final 4 M NaCl fraction contained no uronic acid. A 2.1 M NaCl fraction contained an occasional trace of uronic acid. The uronic acid-containing material obtained from control hearts was almost equally distributed between the 0.4 M and 1.2 M NaCl fractions. In the isoproterenol-treated animals, the necrotic hearts, taken 2 days after injury, showed an increase in uronic acid in the 0.4 M fraction equal to 3.6 times that in the control hearts. In the 1.2 M NaCl fraction, an increase in uronic acid due to injury to twice the control level was observed.

If a mole ratio of 1:1 for hexosamine to hexuronic acid is assumed for the MPS-CPC complex isolated from these hearts, the hexuronic acid present in the NaCl fractions represents one-fourth to one-third of the theoretical value obtainable if all of the hexosamine present in the nondialyzable material isolated from the hearts was precipitated as the MPS-CPC complex. (The uronic acid values were corrected for the effect of NaCl on the colorimetric test.) Attempts to obtain further mucopolysaccharide precipitate from the eluate of these samples using cetylpyridinium chloride or other compounds of the quaternary ammonium type were unsuccessful. The analysis of the MPS-CPC fractions using the anthrone method (4) gave a positive test for hexoses. These were generally distributed through all fractions, without any apparent relationship to the uronic acid-containing material, which, in contrast, had a very sharp elution pattern.
TABLE 2

Distribution of the Hexosamine and Neutral Hexoses in the Various Fractions Obtained in the Isolation of Mucopolysaccharides from Rat Hearts

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Hexosamines (mg/g dry weight)</th>
<th>Hexoses (mg/g dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dialysate after proteolytic digestion</td>
<td>2.19 ± 0.13</td>
<td>4.91 ± 0.74</td>
</tr>
<tr>
<td>MPS-CPC fractions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.02M NaCl supernatant after removal of the CPC precipitate</td>
<td>0</td>
<td>0.58 ± 0.12</td>
</tr>
<tr>
<td>0.4M NaCl</td>
<td>0.06 ± 0.006</td>
<td>*</td>
</tr>
<tr>
<td>1.2M NaCl</td>
<td>0.20 ± 0.013</td>
<td>*</td>
</tr>
<tr>
<td>Alcohol supernatant after removal of the precipitate containing the MPS</td>
<td>2.08 ± 0.06</td>
<td>1.31 ± 0.14</td>
</tr>
</tbody>
</table>

Values are means ± SE. Each value is the mean of 11 fractions, each obtained from a pool of five rat hearts, at various stages of repair following myocardial injury. MPS = mucopolysaccharides; CPC = cetylpyridinium chloride.

*Hexoses distributed throughout all fractions, including those of higher NaCl concentration, which contain no hexosamine or uronic acid.

Although hexoses present minor interference (approximately 7%) in the colorimetric method used for uronic acid determination, the contribution to the total color formed in any one fraction was negligible. However, the presence of this hexose in the cetylpyridinium chloride precipitate along with the MPS-CPC complex, together with the failure to precipitate an amount of mucopolysaccharide equivalent to the hexosamine in the nondialyzable fraction, presented further need for investigation.

A second experiment, from which data was presented in Figures 2 and 3, was conducted in an attempt to evaluate the completeness of the precipitation of the mucopolysaccharides by cetylpyridinium chloride and to investigate the anthrone-positive material found in the previous mucopolysaccharide fractionation. At the same time, the heart samples were taken serially after isoproterenol injury in order to define the changes in the mucopolysaccharides throughout the healing processes in this type of myocardial injury. After determination of glucosamine, galactosamine, and neutral hexoses in the nondialyzable fraction after proteolytic digestion (Fig. 2), the remainder of each sample was precipitated by the addition of alcohol to a final concentration of 75%. The mucopolysaccharide obtained in that precipitate was converted to the cetylpyridinium chloride complex and eluted from a cellulose column as described in Methods. The alcohol supernatant fluid was diluted with water, frozen, lyophilized to dryness, and analyzed for uronic acid (see below).

The distribution of the hexosamine and neutral sugar in the various fractions in this second experiment is presented in Table 2. The values are the average for all fractions at the same step in the isolation procedure. The major portion of the hexosamine remaining in the nondialyzable fraction after proteolytic digestion is recovered in the alcohol supernatant fluid following removal of the mucopolysaccharide-containing precipitate. Small amounts of hexosamine, consistent with (but lower than theoretically obtainable) the amount of mucopolysaccharide, indicated by the uronic acid content are recovered in the sodium chloride fractions. Of the hexose present in the nondialyzable fraction, approximately one-fourth remained in the alcohol supernatant fluid. One-sixth of the hexose in the material precipitated with alcohol remained in the 0.02M NaCl supernatant fluid after precipitation with cetylpyridinium chloride. The remaining hexose, 40% of that in the original dialysate, appeared generally distributed throughout the NaCl fractions of the cetylpyridinium chloride precipitate, making
quantitative determination difficult and of little value.

The MPS-CPC complex formed from the alcohol precipitate was eluted from a cellulose column as described in Methods. Uronic acid was determined on all fractions. In addition, those fractions within each eluting solution which gave a positive response to the uronic acid test were pooled and analyzed for glucosamine and galactosamine and reanalyzed for uronic acid. The major hexosamine in the 0.4M NaCl fraction was glucosamine, which averaged 71% of the total; in the 1.2M NaCl fraction, galactosamine, which also averaged 71% of the total, was the major hexosamine. The galactosamine in the 0.4M fraction and the glucosamine in the 1.2M fraction did not vary significantly during the repair processes. The sum of the hexosamines in either fraction was lower than would be expected when compared to uronic acid content, assuming the methods used gave strictly quantitative results. However, as shown in Figure 3, as healing of the heart proceeded, there was good correlation between the relative change in the major hexosamine in each fraction and in the change in the uronic acid in the same fraction. The mucopolysaccharide in the 0.4M fraction increased to twice the control level within 24 hours, continued to increase for the next 3 to 4 days, and then declined precipitously to levels near that of the controls. The mucopolysaccharide in the 1.2M fraction did not increase as early as that in the 0.4M fraction, but instead, remained relatively normal for the first 48 hours following induction of myocardial injury, then increased rapidly to levels 1.5 to 2 times that of the controls, and remained elevated throughout the healing processes.

The hexosamine-containing material remaining in the alcohol supernatant fluid was analyzed for uronic acid after hydrolysis with Dowex-50 resin in a manner similar to that described by Anastassiadis and Common (11) and Jeffrey and Rienits (12). Conditions for the resin-catalyzed hydrolysis were established by comparison of the amount of hexosamine liberated with the amount obtained from samples hydrolyzed with 2N HCl for 15 hours at 100°C, which had been shown to afford maximum recovery of hexosamine. D-glucuronic acid-6-C$^{14}$ (10$^5$ dpm/sample) was added to each sample prior to hydrolysis to determine the maximum extent of destruction of the liberated hexuronic acid. Standard glucuronic acid samples to which the same labeled material had been added were treated with resin and acid without heating and then used to test recovery of the uronic acid in the 2N acetic acid fraction eluted from the Dowex-1-acetate column (12). Based on either the colorimetric or radiochemical determination, 98 to 100% of the glucuronic acid was recovered from standards. Following hydrolysis with the resin in 0.5N HCl for 48 hours at 90°C, hydrolysis of the hexosamine-containing material was virtually complete. However, less than 25% of the added activity was recovered in the acetic acid fraction. It was decided to use the above resin and acid concentration in triplicate samples, and to hydrolyze each for a different length of time. Hydrolysis of a typical set of such samples for 6, 18, and 42 hours resulted in 30, 70, and 84% liberation of the hexosamine with 50, 45, and 38% recovery of the labeled uronic acid. However, no uronic acid was detected in this eluate or in that from any samples of the alcohol supernatant fluid. While the results of the hydrolysis and recovery of uronic acid, using this method, make quantitative assessment of the uronic acid content difficult or impossible, the failure to detect any uronic acid in the alcohol fraction of the hexosamine-containing material supports the conclusion that the prior precipitation of the mucopolysaccharide with alcohol was essentially complete.

Discussion

We previously reported (1) that intensive mucopolysaccharide accumulations, indicated by histological and histochemical methods, occurred during the edematous phase of isoproterenol-induced myocardial infarction. The mucopolysaccharide entered the edematous areas after the accumulation of fluid had begun, remained prominent throughout the
edematous reaction in the heart, and then disappeared as the edema was resolved. In the later stages of repair, foci of healing containing mucopolysaccharide and collagen were evident within the myocardium. Determination of the amount of hexosamine present in the heart following myocardial injury, showed that an accumulation of materials containing hexosamine and their subsequent removal from the heart occurred concomitantly with the histologically observed changes in the mucopolysaccharides. The present work shows that while the major hexosamine-containing materials in the heart are glycoproteins, important changes in the mucopolysaccharides do occur, as indicated previously. In addition, changes in the glycoprotein-hexosamine and neutral sugars of the glycoproteins also occur during the edematous reaction and during the later stages of healing.

While the procedures used in this investigation were directed primarily toward isolation of the mucopolysaccharides, the glycoproteins were simultaneously separated into several nonspecific fractions. Digestion of the lipid-free, dried heart muscle with proteases produced a dialyzable fraction of the hexosamine, which contained as much as 50% of the total myocardial hexosamine. However, the amount of dialyzable hexosamine was relatively constant in injured hearts even though the total hexosamine in the heart fluctuated greatly as necrosis and healing progressed. In addition, the presence of a similar fraction of the hexosamine in normal hearts in amounts comparable to that in injured hearts, on a relative (percent) basis, leads to the conclusion that the constituents of this hexosamine fraction represent a normal portion of the myocardial glycoproteins, and possibly, metabolic intermediates of the amino sugars.

The hexosamine remaining in the nondialyzable fraction following proteolytic digestion was subject to the same pattern of fluctuation as the total myocardial hexosamine following myocardial infarction. Precipitation of the mucopolysaccharide from this fraction and analysis of the precipitate for uronic acid indicated that only 25 to 35% of the hexosamine present was in the mucopolysaccharide; the remainder is assumed to be bound to glycopeptides. The rapid increase in hexosamine to maximum levels within 48 hours after injury may suggest that serum glycoproteins entering the injured area account for much of the observed increase. However, while glucosamine, which is the major hexosamine (two to three times higher than galactosamine) declined in a pattern corresponding to the disappearance of the edematous accumulations, the galactosamine appeared to remain elevated for several weeks. In subjecting the hexosamine in this fraction to colorimetric analysis for the determination of galactosamine, and the determination of glucosamine by difference from the total hexosamine, it is assumed that these hexosamines occur exclusively in the polymerized carbohydrates of the glycopeptides retained after dialysis.

Determination of the neutral sugars remaining in the nondialyzable fraction following proteolytic digestion, indicated that glycoproteins rich in such constituents increase dramatically in the healing myocardial tissue, and remain elevated for 2 to 3 weeks. However, the increase in neutral sugars was considerably delayed when compared to that of the hexosamine in the same fraction. Assuming that both represent glycoproteins, the difference in the timing of the increase in the two constituents may reflect not only the complex nature of the different glycoproteins involved, but also that the glycoproteins may arise from different sources. The increase in the neutral sugar contained in the glycoproteins occurred after the major edematous reaction had subsided and fibrinogenic processes had begun. While it has long been recognized that the early appearance of an amorphous matrix in an injured area is characteristic of wound healing in general, the accumulation of hexosamine in wound tissue has been variously ascribed not only to the mucopolysaccharides of such a matrix, but also to the accumulation of serum glycoproteins containing hexosamines (13, 14), and in a few cases to glycoproteins arising in situ in the necrotic and healing tissues.

Circulation Research, Vol. XXVI, January 1970
Precipitation of the mucopolysaccharides from the nondialyzable fraction with cetylpyridinium chloride and analysis of the fractions eluted with solutions of varying sodium chloride concentrations separated the mucopolysaccharides into two major fractions: (1) the hyaluronic acid fraction (soluble in 0.4M NaCl and containing glucosamine as the major hexosamine) and (2) the chondroitin sulfate fraction (soluble in 1.2M NaCl and containing galactosamine as the major hexosamine). The hyaluronic acid was elevated in the injured heart within the first 24 hours after induction of necrosis, remained elevated throughout the edematous reaction, and then declined to near normal levels. The well-known occurrence of hyaluronic acid in a variety of normal tissues containing large amounts of fluids leads us to speculate that the occurrence of this material in the edematous heart is related to physiological processes which protect the muscle tissue from damage due to the pathological infusion and to the restoration of normal tissue-fluid balance with the blood. Increased chondroitin sulfate levels in the injured heart were delayed until approximately the third day following treatment with isoproterenol, and then were apparent for 2 to 3 weeks, when the major fibrinogenic processes were evident.

References
Myocardial Connective Tissue Metabolism in Response to Injury: II. INVESTIGATION OF THE MUCOPOLYSACCHARIDES INVOLVED IN ISOPROTERENOL-INDUCED NECROSIS AND REPAIR IN RAT HEARTS

JOSEPH T. JUDD, BERNARD C. WEXLER, George Williamson and Jacob Boyer

doi: 10.1161/01.RES.26.1.101

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/26/1/101