The fact that heparin is a polymer of sulfated \( \text{n-glucosamine} \) and \( \text{uronic acid} \) has been established for a number of years (2-4). It has been proposed that the uronic acid is glucuronic as a result of the finding that hydrolysis in the presence of bromine yielded \( \text{n-glucaric} \) (\( \text{n-glucosanearic} \)) acid (5). This conclusion required further substantiation because of the drastic conditions employed in the hydrolysis. Furthermore, the isolation of \( \text{d-glucaric} \) acid did not eliminate the possibility that the uronic acid is \( \text{l-guluronic} \). It has also been reported that glucuronic acid or its lactone could be identified in heparin hydrolysates by paper chromatography (6). This, however, did not establish whether the configuration is \( \text{D} \) or \( \text{L} \) and whether glucuronic acid is a major component of heparin. Also, since the values of mobility of all the possible hexuronic acids have not been compared, paper chromatography cannot yield a definite conclusion.

In a recent communication, Foster et al. (7) reported the identification of glucuronic acid from heparin hydrolysates on the basis of the crystalline acetamide that they obtained. On the other hand, Helbert and Brown (8) and Brown, Rosenthal, and Helbert (9) presented evidence that the uranic acid of heparin was primarily not an aldohexuronic acid. The color intensity for heparin in the Dische carbazole reaction (10) also raised some doubts as to whether the uronic acid was glucuronic. The present study was therefore made to ascertain the identity of the hexuronic acid component of heparin.

The major difficulty in the identification of uronic acids in polysaccharides stems from the fact that they are unstable under the conditions of acid hydrolysis. In fact, it has been shown that the hydrolytic conditions which liberate uranic acid from heparin will also destroy glucuronic acid (11, 12). To obviate this difficulty, it was desired to reduce the carboxyl groups of the uronic acid in heparin. Identification of the hexose obtained on hydrolysis of reduced heparin would then give evidence as to the structure of the original uronic acid. The present paper describes experiments on the reduction of heparin and the isolation and identification of the hexose obtained upon hydrolysis of the reduced product.

**EXPERIMENTAL PROCEDURE AND RESULTS**

**Materials and Methods**—The methyl ester of desulfated acetamido heparin (Fraction M-III) was prepared as described in a previous publication (12) except that the methanolyzes were carried out at room temperature.

Reducing groups were determined by the Nelson method (13) and hexosamine by the Boas modification of the Elson-Morgan reaction (14). The carbazole (10), deacarbonylation (15), and orcinol (16) methods were employed for uronic acid determinations. Glucose was analyzed by the glucose oxidase reaction (17) with the Glucostat reagent purchased from Worthington Biochemical Company.

Whatman No. 1 filter paper was used for paper chromatography and the following solvent systems were employed: butanol-acetic acid-water, 6:1:2; butanol-pyridine-water, 6:4:3; and butanol-ethanol-water, 4:1:1. The chromatograms were run approximately 18 hours at 20-25° in the descending manner. Known hexoses, pentoses, uronic acids, and hexosamines served as reference compounds. Papers were dried at room temperature and stained with the following: (a) ammoniacal silver nitrate, (b) aniline hydrogen phthalate, (c) Glucostat (18), and (d) ninhydrin.

Infrared spectra were obtained with the Perkin-Elmer Infracord spectrophotometer by the KBr pellet technique.

**Reduction of Desulfated Acetamido Heparin**—To a solution of 19.9 g of the methyl ester of desulfated acetamido heparin (Fraction M-III in 400 ml of water were added dropwise 100 ml of a 10% aqueous solution of sodium borohydride. The reaction was conducted in an ice bath with constant stirring. Small amounts of acetic acid were added at intervals to maintain a pH of 8 to 9. The reaction mixture was kept for 48 hours at 5°. Acetic acid was then added to bring the pH to 6 and the solution was passed through a column (300 x 20 mm) of IR-120 resin (H-form). The column was washed with 2 volumes of water and the combined eluate and washings were evaporated to dryness at 50°. Boric acid was removed as the methyl ester by adding 50 ml of methanol to the residue and evaporating to dryness. This was repeated 5 times; there was no loss in weight on repetition of the process. The product obtained (18.2 g) was then treated with 0.06 N methanolic hydrogen chloride to esterify any saponified unreduced carboxyl groups. Analysis of the methylated product (Fraction M-III-R-1) for uronic acid content indicated that about 50% of the uronic acid had been reduced (Table I). Reduction was also evidenced by the sharp decrease in infrared absorption at 1,740 cm\(^{-1}\). The unreduced ester has a peak at this frequency which is attributable to the carbomethoxy group.

*This study was supported by a research grant (H-2823) from the National Institutes of Health, United States Public Health Service. 
† For Paper II, see reference (1). 
‡ The authors are indebted to Dr. Grant H. Barlow and the Abbott Corporation for generous gifts of heparin.

**EXPERIMENTAL PROCEDURE AND RESULTS**

**Materials and Methods**—The methyl ester of desulfated acetamido heparin (Fraction M-III) was prepared as described in a previous publication (12) except that the methanolyzes were carried out at room temperature.

Reducing groups were determined by the Nelson method (13) and hexosamine by the Boas modification of the Elson-Morgan reaction (14). The carbazole (10), deacarbonylation (15), and orcinol (16) methods were employed for uronic acid determinations. Glucose was analyzed by the glucose oxidase reaction (17) with the Glucostat reagent purchased from Worthington Biochemical Company.

Whatman No. 1 filter paper was used for paper chromatography and the following solvent systems were employed: butanol-acetic acid-water, 6:1:2; butanol-pyridine-water, 6:4:3; and butanol-ethanol-water, 4:1:1. The chromatograms were run approximately 18 hours at 20-25° in the descending manner. Known hexoses, pentoses, uronic acids, and hexosamines served as reference compounds. Papers were dried at room temperature and stained with the following: (a) ammoniacal silver nitrate, (b) aniline hydrogen phthalate, (c) Glucostat (18), and (d) ninhydrin.

Infrared spectra were obtained with the Perkin-Elmer Infracord spectrophotometer by the KBr pellet technique.

**Reduction of Desulfated Acetamido Heparin**—To a solution of 19.9 g of the methyl ester of desulfated acetamido heparin (Fraction M-III) in 400 ml of water were added dropwise 100 ml of a 10% aqueous solution of sodium borohydride. The reaction was conducted in an ice bath with constant stirring. Small amounts of acetic acid were added at intervals to maintain a pH of 8 to 9. The reaction mixture was kept for 48 hours at 5°. Acetic acid was then added to bring the pH to 6 and the solution was passed through a column (300 x 20 mm) of IR-120 resin (H-form). The column was washed with 2 volumes of water and the combined eluate and washings were evaporated to dryness at 50°. Boric acid was removed as the methyl ester by adding 50 ml of methanol to the residue and evaporating to dryness. This was repeated 5 times; there was no loss in weight on repetition of the process. The product obtained (18.2 g) was then treated with 0.06 N methanolic hydrogen chloride to esterify any saponified unreduced carboxyl groups. Analysis of the methylated product (Fraction M-III-R-1) for uronic acid content indicated that about 50% of the uronic acid had been reduced (Table I). Reduction was also evidenced by the sharp decrease in infrared absorption at 1,740 cm\(^{-1}\). The unreduced ester has a peak at this frequency which is attributable to the carbomethoxy group.

*This study was supported by a research grant (H-2823) from the National Institutes of Health, United States Public Health Service. 
† For Paper II, see reference (1). 
‡ The authors are indebted to Dr. Grant H. Barlow and the Abbott Corporation for generous gifts of heparin.

1413
TABLE I

Analysis of heparin derivatives

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Heparin</th>
<th>M-III*</th>
<th>M-III-R-1</th>
<th>M-III-R-2</th>
<th>M-III-R-3</th>
<th>M-III-R-3A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Hydrogen</td>
<td>22.87</td>
<td>40.60</td>
<td>40.54</td>
<td>40.60</td>
<td>40.53</td>
<td>49.29</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>3.41</td>
<td>6.26</td>
<td>6.73</td>
<td>6.65</td>
<td>6.57</td>
<td>6.90</td>
</tr>
<tr>
<td>Sulfur</td>
<td>1.93</td>
<td>2.84</td>
<td>3.27</td>
<td>3.26</td>
<td>3.40</td>
<td>3.26</td>
</tr>
<tr>
<td>Methoxyl</td>
<td>12.91</td>
<td>3.26</td>
<td>3.13</td>
<td>2.67</td>
<td>2.76</td>
<td>1.89</td>
</tr>
<tr>
<td>Glucosamineb</td>
<td>22.4</td>
<td>29.4</td>
<td>33.0</td>
<td>32.3</td>
<td>32.2</td>
<td>33.8</td>
</tr>
<tr>
<td>Uronic acidc</td>
<td>25.2</td>
<td>38.8</td>
<td>16.7</td>
<td>12.5</td>
<td>11.2</td>
<td>10.3</td>
</tr>
<tr>
<td>Carboxole</td>
<td>39.5</td>
<td>52.9</td>
<td>21.1</td>
<td>16.1</td>
<td>17.8</td>
<td>15.4</td>
</tr>
<tr>
<td>Orcinol</td>
<td>10.4</td>
<td>31.4</td>
<td>18.1</td>
<td>12.6</td>
<td>11.8</td>
<td>11.5</td>
</tr>
<tr>
<td>Acetyl</td>
<td>7.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8.5</td>
</tr>
<tr>
<td>Molar ratio of glucosamine to uronic acid6</td>
<td>1:1</td>
<td>1:1</td>
<td>1:0.5</td>
<td>1:0.4</td>
<td>1:0.3</td>
<td>1:0.3</td>
</tr>
<tr>
<td>$[\alpha]_{D}^{20}$</td>
<td>+47.6°</td>
<td>+70.6°</td>
<td>+77.5°</td>
<td>+78.9°</td>
<td>+80.3°</td>
<td>+79.4°</td>
</tr>
</tbody>
</table>

* The specific materials to which these designations are assigned are described in the text.
* Determined as anhydroglucosamine.
* Determined as anhydroglucuronic acid.
* Decarboxylation method.
* Uronic acid based on decarboxylation method.
* As 2% solutions in water.

Fig. 1. Rate of hydrolysis of reduced desulfated acetamido heparin in 1 N H₂SO₄ at 100°, as measured by release of reducing groups and increase in hexosamine color.

whereas the reduced product shows only slight absorption. The reduced ester (18.1 g) was subjected to further treatment with sodium borohydride as described above. Subsequent conversion to the methyl ester with methanolic hydrogen chloride yielded 15.3 g of Fraction M-III-R-2. The reduction and methylation were repeated a third time to give 13.5 g of Fraction M-III-R-3. To insure that the product employed in subsequent hydrolyses was completely N-acetylated, Fraction M-III-R-3 was acetylated as previously described (12). The acetylated material is designated as Fraction M-III-R-3A.

Hydrolysis—Preliminary studies were made on the rate of hydrolysis of the reduced heparin. Solutions of 1% Fraction M-III-R-3A in 1 N H₂SO₄ were heated at 100° for 16 hours and aliquots were withdrawn at different time intervals and analyzed for reducing groups and hexosamine. It was found that the rate of hydrolysis is greatest within the first two hours and that both increase of reducing groups and release of hexosamine becomes comparatively slow after 6 hours (Fig. 1). A 6-hour period of hydrolysis was chosen in the subsequent experiments because it was desired to obtain disaccharides and oligosaccharides, in addition to monosaccharides.

Fractionation No. 1—After refluxing 12 g of Fraction M-III-R-3A in 480 ml of 1 N H₂SO₄ for 6 hours, the solution was cooled and neutralized to pH 4 with barium carbonate (Fig. 2). Barium sulfate was removed by centrifugation and the supernatant passed through a column (30 x 2 cm) of Dowex 50 X8, 200 to 400 mesh (H⁻ form). The column was washed with water until no reducing material came through and the combined effluent was concentrated to a sirup (Fraction W). Paper chromatography of Fraction W revealed a major spot with the same mobility as glucose, and trace components having higher values of mobility.

The sirup was applied to a column of powdered cellulose (60 x 3 cm), and eluted with water-saturated butanol. This procedure yielded two minor fractions (W-1 and W-2) followed by a major fraction (W-3). Paper chromatography of Fraction W-3 indicated the presence of only one component which was identified as glucose on the basis of Rf and reaction with glucose oxidase (18). The component in Fraction W-2 had the same Rf and staining properties (19) as N-acetyl-β-glucosamine. Fraction W-3 stained only with silver nitrate and gave negative reactions for pentoses, ketoses, and uronic acid.

To identify the glucose definitely, Fraction W-3 was evaporated to dryness and the residue was acetylated. The residue was heated at 140° with 2 ml of acetic anhydride and 125 mg of anhydrous sodium acetate for 2 hours. The mixture was shaken with 5 volumes of water and the resulting solution was extracted with four 4-ml portions of chloroform. The chloroform extract was evaporated to dryness and the residue recrystallized from...
**HEPARIN**

1. Methanolation
2. N-Acetylation
3. Methanolation

**DESULFATED ACETAMIDO-HEPARIN METHYLESTER**

M-III

1. Reduction
2. Esterification
3. Repeat steps 1 and 2 twice
4. N-Acetylation

**PARTIALLY REDUCED DESULFATED ACETAMIDO-HEPARIN**

M-III-R-3A

1. IN H₂SO₄ - 6 hours
2. Removal of sulfate as the barium salt

---

**FIG. 2. Scheme for fractionation No. 1**. The diagram shows the conversions beginning with heparin ether-petroleum ether to yield 263 mg of β-D-glucopyranose pentaacetate.

**C₅₁H₄₂O₄₃**

Calculated: C 49.23, H 5.68, CH₃CO, 55.14

Found: C 49.34, H 5.60, CH₃CO, 55.24

The product had a melting point of 131.2° which was not depressed upon admixture with authentic β-D-glucopyranose pentaacetate, [α]D +2° (c, 1 in chloroform).

Elution of the Dowex 50 column containing the original hydrolysate, with 0.04 N HCl, yielded a fraction (A) having the characteristics of a disaccharide. This step was followed by elution with 0.3 N HCl (Fraction B) and then 1.0 N HCl (Fraction C). Fraction B was evaporated to dryness and the residue recrystallized from aqueous ethanol. The solid obtained was identified as glucosamine by its mobility upon paper chromatography and by the fact that it was converted to arabinose upon treatment with ninhydrin (20). Fraction C contained a mixture of oligosaccharides.

Since there was an appreciable loss of reducing material with each fractionation and purification, it seemed possible that, in addition to glucose, another hexose was present in the hydrolysate and that it was not detected. The major loss in yield occurred in the step when the sulfate was removed as the barium sulfate precipitate. Repeated experiments in which the precipitation technique was varied did not result in an increased yield of reducing material. An alternate fractionation was therefore performed.

**Fractionation No. 2**—Polysaccharide that had been submitted to only one reduction and acetylation (Fraction M-III-R-1A) was employed since most of the reduction of the desulfated methyl ester took place during the first treatment with sodium borohydride. After hydrolyzing 9.0 g of Fraction M-III-R-1A in 320 ml of 1 N H₂SO₄ for 6 hours, the solution was shaken with Dowex 1-X8 (200 to 400 mesh) (carbonate form) until there was no further evolution of gas. The mixture was then poured into a chromatographic column containing additional Dowex 1-carbonate and washed with water until the washings were free from reducing sugar. The combined eluates and washings were concentrated under reduced pressure to 250 ml (Fraction A, Fig. 3).

**FIG. 3. Scheme for fractionation No. 2**
eluted in a column with 0.1 N H₂SO₄ until the effluent was free of reducing material. The acid solutions were neutralized with Ba(OH)₂ and the BaSO₄ was removed by filtration (the filtrate was designated as Fraction Z).

Fraction A was passed through a column (30 × 2.5 cm) of Dowex 50 (H⁺ form) and the resin was washed with water until no further reducing material was obtained (Fraction AW). Subsequent elution with 0.04 N HCl gave two distinct peaks (Fractions BA and BB in Fig. 3). Elution with 0.3 N HCl yielded Fraction X and 1.0 N HCl, Fraction Y.

Analysis of Fraction AW showed that it contained 405 mg of reducing sugar of which a large portion (352 mg) was glucose (as measured by the action of glucose oxidase). A portion of this fraction, containing 200 mg of reducing material, was evaporated to dryness and acetylated as described above. The crude pentaacetate was dissolved in a minimal amount of ethyl ether and adsorbed on a column of silicic acid. The adsorbent was eluted with mixtures of petroleum ether (b.p. 30–60°) and ethyl ether, in which the proportion of the latter was gradually increased. The pentaacetate was removed from the column with solvent containing 35 to 50% ethyl ether. The solvent was evaporated and the residue was recrystallized from chloroform and petroleum ether. The yield of β-D-glucopyranose pentaacetate was 152 mg.

Chromatography of Fraction AW on paper revealed the presence of three components but glucose was by far the major one. The mobility values of the other components with butanol-acetic acid-water, 6:1:2, were Rₙ glucose 2.01 and Rₙ glucose 2.81. The slower of the two was identified as N-acetyl-D-glucosamine on the basis of its mobility and staining reaction with the Ehrlich reagent (19). To determine the relative amounts of the different components in Fraction AW, a portion containing 8.0 mg of reducing material was applied as streaks to six sheets of filter paper and developed with butanol-acetic acid-water, 6:1:2. After drying the paper, the zones containing reducing material were identified by staining the ends of the sheets with silver nitrate. The corresponding areas were cut out and eluted with water. Determination of reducing material in each zone indicated that the ratio of reducing material was 6.6:0.7:0.2 for glucose, N-acetyl-D-glucosamine, and the fastest component, respectively.

Evaporation of Fraction X and recrystallization from ethanol yielded 920 mg of glucosamine hydrochloride.

The residue obtained on evaporating Fraction Y to dryness was soluble in methanol. Ethanol was added to incipient turbidity and the mixture was kept in the refrigerator for a day. This yielded a crystalline precipitate (Fraction BA-1) which was separated by filtration. The filtrate was evaporated to dryness and the residue washed with ethanol and ether (Fraction BA-2).

Evaporation of Fraction BB to dryness gave a granular material which was insoluble in methanol. This was dissolved in water and precipitated with ethanol (Fraction BB-1). Similarly, dissolving the residue from Fraction Y in water and precipitating with increasing amounts of ethanol yielded Fractions Y-1 and Y-2, respectively. The residue from Fraction Z was dissolved in water and precipitated with ethanol to yield Fraction Z-1. Evaporation of the supernatant to dryness yielded Fraction Z-2.

Analysis of Fractions—The results of analyses on the above fractions are given in Table II. It can be seen that except for Fractions BB-1 and Z-2, the uronic acid in these oligosaccharides had not been reduced to an appreciable extent. Paper chromatography of acid hydrolysates of Fractions BB-1 and Z-2 showed glucose to be the only hexose. Similarly, Fractions BA-1, BA-2, and BB-1 were esterified with methanolic HCl and reduced with sodium borohydride. About 50% reduction was effected. Hydrolysis of the reduced products and paper chromatography of the eluates again revealed the presence of glucose.

**DISCUSSION**

Sodium borohydride reacts with the methyl ester of desulfated acetamido heparin to reduce most of the carbethoxy groups to alcohol groups. On the basis of uronic acid determinations by the Tracey decarboxylation method, 55% of the uronic acid groups are reduced after the first treatment with borohydride (Table I). Esterification of the remaining carbonyl groups and repeated treatment with the reducing agent causes the reduction of an additional 10 to 15% of the uronic acid groups. Yoshizawa reported that only 16% of the carboxyl groups of heparin could be reduced with sodium borohydride (21). However, in his experiments the heparin was not N-acetylated and was not desulfated to the same extent as in the present work. These differences in the reactant may account for the resistance to reduction that he encountered.

Although the values for uronic acid content obtained by the carbazole method do not agree with those by the decarboxylation procedure (Table I), the figures for percentage reduction calculated by each method are within the same order of magnitude. Contrary to a previous report (22), desulfation of heparin did not decrease the relatively high values obtained by the carbazole method. On the other hand, the results by the ozimolar method are low only for heparin and are close to those obtained by the decarboxylation method for the desulfated heparin and the reduced products. This suggests that the low uronic acid value given for heparin by the ozimolar method is due to the number or position of the sulfate groups rather than to the presence of some unidentified uronic acid.

The uronic acid groups which had been reduced with boro-

---

**Table II**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Reducing sugar</th>
<th>Uronic acid</th>
<th>Glucosamine</th>
<th>Nitrogen</th>
<th>Sulfur</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BA-1</td>
<td>11.9</td>
<td>29.2</td>
<td>6.1</td>
<td>26.1</td>
<td>2.50</td>
</tr>
<tr>
<td>BA-2</td>
<td>21.9</td>
<td>29.7</td>
<td>9.3</td>
<td>37.2</td>
<td>2.82</td>
</tr>
<tr>
<td>BB-1</td>
<td>36.7</td>
<td>10.8</td>
<td>17.6</td>
<td>32.5</td>
<td>3.14</td>
</tr>
<tr>
<td>Y-1</td>
<td>11.2</td>
<td>23.1</td>
<td>6.7</td>
<td>35.4</td>
<td>3.43</td>
</tr>
<tr>
<td>Y-2</td>
<td>18.6</td>
<td>22.7</td>
<td>15.1</td>
<td>36.3</td>
<td>3.42</td>
</tr>
<tr>
<td>Z-1</td>
<td>13.2</td>
<td>30.5</td>
<td>3.2</td>
<td>19.6</td>
<td>1.71</td>
</tr>
<tr>
<td>Z-2</td>
<td>9.1</td>
<td>15.4</td>
<td>2.8</td>
<td>12.1</td>
<td>1.22</td>
</tr>
</tbody>
</table>

* The specific materials to which these designations are assigned are shown in Fig. 3 and described in the text.

1 Determined as anhydroglucose.

2 Determined as anhydrouronic acid by the decarboxylation method.

3 Elson-Morgan reaction carried out directly with no prior hydrolysis.

4 Elson-Morgan reaction after hydrolysis for 16 hours with 4 N HCl. Determined as anhydroglucosamine.

5 Analyses by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.
Hydride are identified as \( \nu \)-glucuronic since \( \nu \)-glucose was the only hexose isolated from the hydrolysates by two fractionation procedures. Reduction of the uronic acid-containing oligosaccharides from the hydrolysates (Table II) and identification of only glucose in these products, indicates that a major portion of the uronic acid originally refractory to reduction is also glucuronic. These results thus confirm previous conclusions that glucuronic acid is present in heparin (5, 7, 23) and, in addition, demonstrate that this is the major uronic acid in heparin. The high uronic acid value for heparin, obtained by the carbazole method, may be characteristic of a specific glycosidic linkage by which heparin may differ from other mucopolysaccharides. A previous paper from this laboratory (1) gave evidence for the presence in heparin of uronicidic linkages to carbon 6 of glucosamine. This linkage may be the cause of some of the unique chemical properties of heparin and may confer upon it certain of its specific physiological properties.

**SUMMARY**

The conversion of the methyl ester of desulfated acetamido heparin, to a product in which approximately 70% of the carboxyl groups are reduced, is described. Hydrolysis of the reduced polysaccharide and fractionation of the hydrolysate by two procedures reveals no other hexose but \( \nu \)-glucose. It is concluded that \( \nu \)-glucuronic acid is the principal uronic acid component of heparin.

**REFERENCES**

Investigations on the Chemistry of Heparin: III. REDUCTION AND HYDROLYSIS
I. Danishefsky, Harold B. Eiber and Esther Langholtz