A STUDY OF HEPARIN FRACTIONS

S. M. Bychkov and
V. N. Kharlamova

UDC 612.115.35.015.2

When a complex of a total unfractionated heparin preparation and hexammine–cobalt(III) is treated with 2-3 M KCl solution a heparin fraction containing 3 sulfuric acid residues to 1 glucosamine residue passes into solution. A heparin fraction containing 4 sulfuric acid residues to 1 glucosamine residue remains in the precipitate thereby freed from hexammine–cobalt(III). The two fractions were isolated in a chemically individual form.
KEY WORDS: *heparin; heparin fractions; hexammine–cobalt(III) chloride.*

In the modern view, heparin exists in animal tissues as several fractions which differ in certain physical, chemical, and biological characteristics [1, 4, 5]. However, these fractions have received little study. [6].

Two chemically individual heparin fractions were studied.

EXPERIMENTAL METHOD

Heparin was separated into two fractions on the basis of differences in the solubility of complexes of these fractions with hexammine–cobalt(III) in KCl solutions, discovered by the writers. The fraction containing fewer sulfuric acid residues passes into solution under these conditions whereas the fraction containing more of these residues, freed from hexammine–cobalt(III), remains undissolved.

The original total heparin preparation (Spofa, Czechoslovakia, anticoagulant activity 110 i.u.) was dissolved in water (5 g in 100 ml) and centrifuged (28,000g, 60 min, 2-4°C, at which temperature all subsequent centrifugations were carried out). A solution of hexammine–cobalt(III) chloride was then added gradually to the transparent solution, with vigorous stirring, so that its concentration (in percent) in the final mixture was twice the concentration of heparin. The mixture was allowed to stand at 20°C for 1-2 h. The precipitate of the complex [hexammine–cobalt(III)]·n-heparin, dark orange in color, was collected by centrifugation (2300g). The wet precipitate was washed several times with 0.2 M KCl to remove chondroitin sulfates and other such impurities, and the residue was separated at 2300g. The washed residue was immediately treated with small portions (100-150 ml each) of 2-3 M KCl, the solution being separated from the residue each time by centrifugation (2300g), until the residual precipitate was completely decolorized, for which 700-800 ml of 2 M and 500-600 ml of 3 M KCl were required.

The pooled salt solutions of the complex [hexammine–cobalt(III)]·n-heparin were dialyzed against 2 M or 3 M KCl (depending on the concentration taken to fractionate the solution) until complete removal of the hexammine–cobalt(III) chloride, as shown by decolorization of the solution. KCl was removed from the colorless solution by dialysis against water. The purified solution was treated with 5 g of cation-exchange resin (Amberlite IR-120, K^+ form). The mixture was allowed to stand for 18-20 h at 2-4°C. The resin was removed by centrifugation (5800g), the solution was frozen, and the heparin precipitated

(Presented by Academician of the Academy of Medical Sciences of the USSR S. S. Debov.)
Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 81, No. 3, pp. 289-291, March 1976. Original article submitted August 11, 1975.

TABLE 1. Analysis of Heparin Fractions (in mmoles/g anion of fraction)

Component and ratio between components	Sample						
	I	II	III	IV	V	VI	VII
	fraction						
	soluble				insoluble		
Nitrogen	1,04	1,20	1,16	1,19	1,23	1,17	1,19
Aminosugar (A)	1,41	1,40	1,41	1,41	1,32	1,32	1,32
Sulfate (B)	4,19	4,51	4,50	4,46	5,33	5,17	5,20
Hexuronic acids (C)	1,45	1,43	1,51	1,54	1,24	1,43	1,42
B/A	2,97	3,22	3,19	3,17	4,04	3,92	3,94
C/A	1,03	1,02	1,07	1,09	0,94	1,08	1,08
Aspartic aid	0,207	0,200	—	—	0,070	0,081	—
Threonine	0,300	0,241	—	—	0,070	0,090	—
Serine	0,200	0,200	—	—	0,073	0,084	—
Glutamic acid	0,207	0,200	—	—	0,100	0,110	—
Proline	0,140	0,176	—	—	0,070	0,070	—
Glycine	0,160	0,160	—	—	0,100	0,111	—
Alanine	0,140	0,160	—	—	0,061	0,078	—
Valine	0,082	0,090	—	—	0,023	0,044	—
Isoleucine	0,038	0,041	—	—	0,014	0,021	—
Leucine	0,043	0,080	—	—	0,021	0,033	—
Tyrosine	0,207	0,384	—	—	0,114	0,100	—
Phenylalanine	0,464	0,400	—	—	0,146	0,129	—
Arginine	0,041	0,030	—	—	Traces	Traces	—
Lysine	Traces	0,025	—	—	0,016	0,018	—
Histidine	Traces	0,020	—	—	0,007	0,008	—
Total amino acids	2,229	2,407	—	—	0,885	0,977	—

Legend. —) No analysis was undertaken.

by two volumes of ethanol, and cooled to between − 10 and − 15°C, in the presence of 10% potassium acetate. After the mixture had stood at 2-4°C for 18-20 h the residue was collected by centrifugation (2300g) and dissolved in 40 ml water. To remove possible traces of hexammine–cobalt(III) in the specimen, the solution was treated again with the cation-exchange resin as described above, after which it was centrifuged (28,000g, 60 min) and freeze-dried.

The residue isolated from the total complex [hexammine–cobalt(III)]·n-heparin obtained initially by treatment with 2-3 M KCl was dissolved in 60 ml water and then dialyzed against water to remove the chlorine ions. The solution was treated with cation-exchange resin and centrifuged, after which the heparin was precipitated from it with ethanol (see above). The precipitate was treated again in the manner used to obtain the first fraction. Yield: soluble fraction 28%, insoluble fraction 52% of the original total heparin preparation. The specimens were analyzed quantitatively for nitrogen (micro-Kjeldahl), glucosamine [2], sulfates [3], hexuronic acids [3], and in some cases, the amino acid composition of the protein component was determined by means of the Hitachi (model 037-0004) automatic amino acid analyzer. When the results of the analysis showed that the preparations were sufficiently pure, the procedure of obtaining the complex of the fraction with hexammine–cobalt(III) and all subsequent purification operations were repeated.

EXPERIMENTAL RESULTS AND DISCUSSION

The two heparin fractions contained virtually the same amount of nitrogen, not more than 1.23 mmole/g anion (Table 1). The glucosamine content in the soluble fraction was somewhat higher than in the insoluble fraction, but the first fraction contained fewer sulfate groups than the second. The ratio between the number of sulfate groups and glucosamine in the soluble and insoluble fractions was 3 and 4 respectively. The ratio between the content of hexuronic acids and aminosugar in both fractions was 1.

The total amino acid content in the soluble fraction was about 2.5 times that in the insoluble fraction (Table 1). Both fractions contained predominantly dicarboxylic amino acids (17.0-18.0 and 12.0-19.0%), hydroxyamino acids (19.0-22.0 and 16.0-18.0%), tyrosine (9.0-16.3 and 10.0-13.0%) and phenylalanine (17.0-21.0 and 13.0-16.0%). Arginine, lysine, and histidine were found only as traces. The small discrepancy between the contents of individual amino acids in different samples of the same fractions can be attributed to errors of analysis unavoidable when the quantity of these acids was so small.

During preparation of the total sample of heparin with hexammine–cobalt(III) considerable purification from various ballast impurities took place. All stages of isolation and purification of the fractions were carried out at neutral pH, so that any change in the end products was completely excluded. The protein (polypeptide) component present in the heparin fractions was joined to the polysaccharide by a covalent bond [1]. The existence of electrovalently bound protein in samples of these fractions could be ruled out, because it was completely removed from them during preparation of the heparin–hexammine–cobalt(III) complex and subsequent treatments with the cation-exchange resin. The difference between the amino acid content in the fractions can be attributed only partly to differences in the content of sulfate, for the difference observed was greater than would follow from this fact. This problem requires further study.

By obtaining a total complex of [hexammine–cobalt(III)]·n-heparin and subsequently fractionating this complex with 2-3 M KCl it is thus possible to isolate two chemically individual heparin fractions, the first of which contains three and the other contains four sulfuric acid residues to one glucosamine residue. The chemical individuality of these fractions was confirmed by the constancy of composition of all samples of them. Previous investigations [4] showed that the anticoagulant activity of the heparin fraction which contains four sulfuric acid residues is 1.4 times greater than that of the fraction containing three such residues to one residue of aminosugar.

LITERATURE CITED

1. S. M. Bychkov, Uspekhi Sovr. Biol., 65, 323 (1968).
2. S. M. Bychkov and M. F. Kolesnikova, Biokhimiya, 31, 533 (1966).
3. S. M. Bychkov and V. N. Kharlamova, Byull. Éksperim. Biol. i Med., No. 12, 28 (1974).
4. S. M. Bychkov and V. N. Kharlamova, Byull, Éksperim. Biol. i Med., No. 2, 61 (1975).
5. H. Danishefsky, H. Steiner, and A. Bella, Jr., J. Biol. Chem., 224, 1741 (1969).
6. J. E. Scott, T. E. Stacey, and M. J. Tigwell, Biochem. J., 108, 50 (1968).