EPIMERIZATION OF D-GLUCURONIC ACID TO L-IDURONIC ACID BY DEAMINATION OF HEPARINS

Fumio Yamauchi, Masashi Kosakai and Zensaku Yosizawa

Department of Biochemistry, Tohoku University School of Medicine
Sendai, Japan

Received October 15, 1968

Deamination procedure with nitrous acid has been shown to be a useful tool for the structural study of heparins (Foster et al., 1963; Yosizawa, 1964; Cifonelli, 1965; Lindahl, 1966; Horner, 1967) and heparitin sulfate (Knecht et al., 1965, 1967). It has been shown that N-sulfated glucosamine residues of these substances are converted to 2,5-anhydromannose with the concomitant rupture of adjacent glycoside bonds by the deamination. Although any change of D-glucuronic acid moiety has not been reported, we found recently a large amount of L-iduronic acid in the deamination products of heparins. Since L-iduronic acid was not detected in the starting materials, this observation implied that there might be an epimerization of D-glucuronic acid to L-iduronic acid by the deaminative cleavage of the glycosidic linkages between C_4 of D-glucuronic acid molecules and C_1 of D-glucosamine residues of heparins. This communication presents an evidence of the formation of L-iduronic acid from D-glucuronic acid of heparins and 4-O-(2-amino-2-deoxy-α-D-glucopyranosyl)-D-glucuronic acid by the deamination with nitrous acid.

Materials---- α-Heparin from porcine intestine and ω-heparin from whale intestine were the specimens reported previously (Kotoku et al., 1967). 4-O-(2-Amino-2-deoxy-α-D-glucopyranosyl)-

D-glucuronic acid was prepared from an acid hydrolysate of porcine α-heparin by a gel filtration with Sephadex G-10 and by preparative paper chromatography on thick paper. This oligosaccharide was shown to contain equimolar amino-free glucosamine and glucuronic acid without sulfate by analysis. Moreover, glucuronic acid molecule of this oligosaccharide was reduced completely with sodium borohydride without any change of glucosamine residue.

Deamination of α-heparin and ω-heparin----Each heparin (0.5 g) in 50 ml of 20% acetic acid was kept for 72 hours at 27°. To this solution were mixed 50 ml of 5% aqueous sodium nitrite. The reaction mixture was kept at 27° overnight, then lyophilized. The neutralized solution of the deamination products was passed twice through a column (2.5 x 140 cm) of Sephadex G-10, and the effluent containing the deamination products was lyophilized. The yield of the products was 90%, in an average, of the weight of the starting materials.

Analysis of the deamination products----As can be seen in Table I, uronic acid values of the deamination products estimated by the Bitter and Muir (1962) modification of the carbazole method of Dische (1947) were higher than those determined by the original method, contrary to the reverse relation between the corresponding values of the starting materials. This observation suggests a structural change of D-glucuronic acid by the deamination. All the glucosamine residues remained in the deamination products were found to be N-acetylated.

Characterization of uronic acid in the deamination products---The deamination products were hydrolyzed with 2.5 N hydrochloric acid in a boiling water bath for 1.5 hours and the hydrolysate was passed through a column (1 x 6 cm) of Dowex 50 (H^+ form). The effluent was concentrated to a small volume under reduced pressure.

The paper chromatograms (Fig. 1) on the concentrate showed the presence of L-iduronic acid as the major uronic acid. However,

TABLE I

Analytical data[1] of α-heparin and ω-heparin and their deamination products

	Starting material		Deamination Products	
	α-Heparin	ω-Heparin	α-Heparin	ω-Heparin
Glucosamine (%)[2]	30.9	35.5	0.7	7.6
Hexuronic acid (%)[3]				
Dische[4]	44.2	50.9	10.0	19.9
Bittler-Muir[5]	36.1	41.3	21.8	28.7
2,5-Anhydromannose (%)[6]	0	0	19.5	11.2
Sulfur (%)[7]	12.3	8.5	10.4	7.7

1) Expressed as free compound. 2) Total glucosamine calculated by the method of Yosizawa (1964). 3) Expressed as D-glucuronic acid. 4) Determined by the carbazole method of Dische (1947). 5) Determined by the Bitter and Muir (1962) modification of the carbazole method of Dische (1947). 6),7) Determined by the procedures reported previously (Yosizawa, 1964).

the starting α-heparin and ω-heparin were found to contain D-glucuronic acid exclusively, by the same procedures.

Uronic acids in the above concentrate were also checked by gas-liquid chromatography. As shown in Fig. 2, large amounts of L-iduronic acid and small amounts of D-glucuronic acid were detected. The unaltered D-glucuronic acid residues in the deamination products were probably substituted by the N-acetylated glucosamine molecules (Kotoku *et al.*, 1967).

The uronic acid giving the spots corresponding to L-iduronic acid on paper chromatograms was isolated by preparative paper chromatography on thick paper with butylacetate-acetic acid-*n*-butanol-methanol-water (3:2:1:1:1, by volume) and *n*-butanol-pyridine-water (5:3:2, by volume). Carbazole-to-orcinol ratio of this uronic acid was 0.28, which was in accord with that of L-iduronic acid reported by Hoffman *et al.* (1956).

Infrared spectrum (Fig. 3) of this uronic acid was identical with that of L-iduronic acid prepared from chondroitin sulfate B,

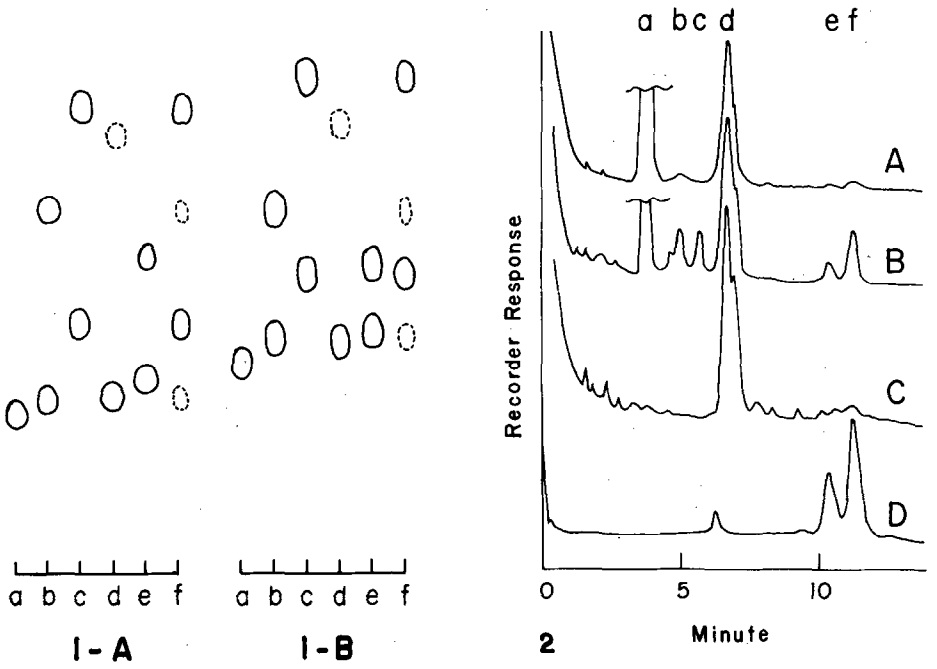

Fig. 1. Tracing of a paper chromatogram of uronic acids in the deamination product of α-heparin (f).
Paper chromatography was carried out on Toyo filter paper No. 51 by the descending technique, using ter-amylalcohol-90% formic acid-water (8:2:3, by volume) (1-A) and butylacetate-acetic acid-n-butanol-methanol-water (3:2:1:1:1, by volume) (1-B) for 16 hours at room temperature. Staining reagent, alkaline-silver.
References: a, D-galacturonic acid; b, D-glucuronic acid; c, L-iduronic acid; d, L-guluronic acid; e, D-mannuronic acid.
D-Mannuronic acid and L-guluronic acid were kindly supplied by Miss S. Fujibayashi. L-Iduronic acid was prepared from chondroitin sulfate B, which was a generous gift from Dr. K. Tsurumi. D-Glucuronic acid and D-galacturonic acid were of commercial source.

Fig. 2. Gas-liquid chromatograms (g.l.c.) of uronic acids in the deamination products of α-heparin (A) and ω-heparin (B).
G.l.c. of the trimethylsilyl derivatives of the methyl glycosides was carried out by the procedure of Yamakawa and Ueta (1964), using one-meter column packed with 5% Ucon LB 550 on Chromosorb W at 205°.
References: C, L-iduronic acid; D, D-glucuronic acid. Peaks: a, 2,5-anhydromannose; b, c, unknown; d, L-iduronic acid; e,f, D-glucuronic acid.

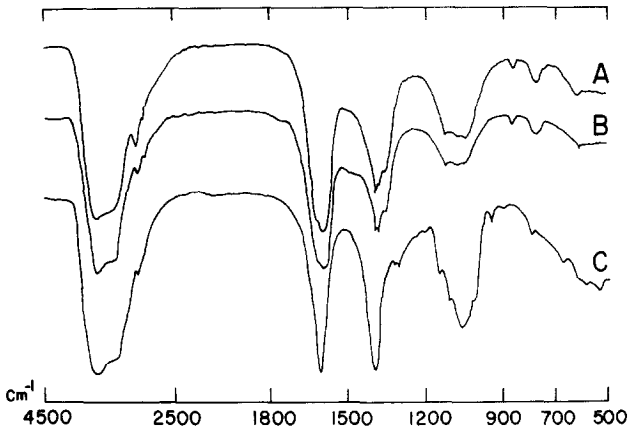

Fig. 3. Infrared spectra of the uronic acid isolated from the deamination product of α-heparin (A), L-iduronic acid (B) and D-glucuronic acid (C).
Infrared spectra were made by examination of KBr pellets in a Hitachi Model EPI-G2 infrared spectrophotometer.

---

but differed from that of D-glucuronic acid in the region between 700 cm^{-1} and 1,200 cm^{-1}, specifically at 775 cm^{-1} and 865 cm^{-1}.

Formation of L-iduronic acid from 4-O-(2-amino-2-deoxy-α-D-glucopyranosyl)-D-glucuronic acid by deamination----The disaccharide (0.30 mg) in 0.5 ml of 1 N acetic acid was treated with the same volume of 1 N aqueous sodium nitrite at 27° overnight. The deamination product was subjected to preparative high voltage paper electrophoresis at 3,000 volts for 20 min with pH 2.0 buffer (formic acid-acetic acid-water = 150:100:750, by volume). The substance remained on the starting band was eluted with water and the eluat was concentrated *in vacuo*. The paper chromatograms showed that L-iduronic acid was only the detectable uronic acid in the deamination product. On the other hand, the deamination of the disaccharide was also carried out in a refrigerator, followed by the same treatment as above. The resulting product was

also revealed to contain L-iduronic acid and 2,5-anhydromannose. The deamination of this disaccharide at -15° in the presence of glyme by the procedure of Lindahl (1966) showed that about one-thirds of D-glucuronic acid molecule was converted to L-iduronic acid. Contrary to this observation, Knechet et al. (1965, 1967) and Lindahl (1966) reported that under these conditions free amino group of hexosamine did not react with nitrous acid.

Summary----Porcine α-heparin, whale ω-heparin and 4-O-(2-amino-2-deoxy-α-D-glucopyranosyl)-D-glucuronic acid were deaminated with nitrous acid. Uronic acid in the deamination products was identified as L-iduronic acid by paper chromatography, gas-liquid chromatography, infrared spectrophotometry and carbazole-to-orcinol ratio of the color reaction. The present data indicate that the deaminative cleavage of the glycosidic linkage between C_4 of D-glucuronic acid and C_1 of D-glucosamine results in the concomitant epimerization of D-glucuronic acid to L-iduronic acid.

References

Bitter, F., and Muir, H. M., Anal. Biochem., 4, 330 (1962).
Cifonelli, J. A., Federation Proc., 24, 354 (1965).
Dische, Z., J. Biol. Chem., 167, 189 (1947).
Foster, A. B., Harrison, R., Inch, T. D., Stacey, M., and Webber, J. M., J. Chem. Soc., 2279 (1963).
Hoffman, P., Linker, A., and Meyer, K., Science, 124, 1252 (1956).
Horner, A. A., Can. J. Biochem., 45, 1015 (1967).
Knecht, J., and Dorfman, A., Biochem. Biophys. Res. Commun., 21, 509 (1965).
Knecht, J., Cifonelli, J. A., and Dorfman, A., J. Biol. Chem., 242, 4652 (1967).
Kotoku, T., Yosizawa, Z., and Yamauchi, F., Arch. Biochem. Biophys., 120, 553 (1967).
Lindahl, U., Biochim. Biophys. Acta, 130, 368 (1966).
Yamakawa, T., and Ueta, N., Japan. J. Exp. Med., 34, 37 (1964).
Yosizawa, Z., Biochem. Biophys. Res. Commun., 16, 336 (1964).