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Analytical Chemical Studies on Amino Sugars.[1] IV.[2] Determination of 2-Deoxy-2-sulfamido-D-glucose and 2-Acetamido-2-deoxy-D-glucose in Heparin

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A novel spectrophotometric method for microdetermination of 2-deoxy-2-sulfamido-hexose and 2-acetamido-2-deoxyhexose residues in heparin is described. Application of MBTH method to heparin and acid hydrolysate of heparin gives sulfamidohexose content and total hexosamine content, respectively. The difference of the two values corresponds to acetamidohexose content of the mucopolysaccharide.

Several studies have shown that there are at least two modes of N-substituent on amino sugar units in heparin, namely, sulfate and acetate.[4–6] Estimation of these groups often becomes important in the studies of heparin. For this purpose, Lagunoff and Warren[7] reported a method using indole–hydrochloric acid reagent.[8] According to their method, hexosamine moiety is converted into 2,5-anhydrohexose by deamination with nitrous acid and the product is then treated with the above reagent to give red color. This color reaction is, however, interfered with by several biological materials such as neutral sugars and uronic acids.[9]

Employment of 3-methyl-2-benzothiazolone hydrazone (MBTH) in place of indole–hydrochloric acid reagent overcame this disadvantage. In the preceding paper,[10] application of MBTH to assay of free hexosamines was described. In the present paper, we show that MBTH method is suitable for determination of variously substituted hexosamines in heparin. This method can also give degree of deacetylation of chitin. Undesirable side reactions of the polysaccharides are minimized because MBTH reacts with 2,5-anhydrohexose under mild conditions.

Materials and Methods

Heparin from bovine lung and whale intestine were the gift of Dr. T. Yamaha of National Institute of Hygienic Sciences. Chondroitin sulfate-C from shark cartilage (ChS-C)[11] was supplied by Dr. A. Idani of Taiyo Gyogyo Co., Ltd. Chitosan was provided by Dr. H. Fujii of Kyowa Oil and Fat Co. Heparin samples 1, 2, 3 and chondroitin sulfate A (ChS-A)[11] were purchased from Daiichi Pure Chemicals Co., Ltd. (lot No. 302A), Novo Industri A/S (lot No. D-629), Wako Pure Chemicals Co., Ltd. (lot No. EP-6603) and Seikagaku Kogyo Co., Ltd. (lot No. 3361), respectively.

1) This work was presented at the 89th Annual Meeting of the Pharmaceutical Society of Japan, Nagoya, April 1969.
2) Part III: M. Maeda, T. Kinoshita and A. Tsuji, *Anal. Biochem.*, submitted.
3) Location: *a) Hatanodai 1–5–8, Shinagawa-ku, Tokyo; b) Akasaka 3–2–6, Minato-ku, Tokyo.*
4) A.S. Perlin, M. Mazurek, L.B. Jaques and L.W. Kavanaugh, *Carbohyd. Res.*, **7**, 369 (1968).
5) S. Inoue and Y. Inoue, *Biochem. Biophys. Res. Commun.*, **23**, 513 (1966).
6) B. Rhadakrishnamurthy, E.R. Dalferes, Jr. and G.S. Berenson, *Anal. Biochem.*, **26**, 61 (1968).
7) D. Lagunoff and G. Warren, *Arch. Biochem. Biophys.*, **99**, 396 (1962).
8) Z. Dische and E. Borenfreund, *J. Biol. Chem.*, **184**, 517 (1950).
9) Z. Dische, "Method of Biochemical Analysis," Vol. 2, ed. by D. Glick, Interscience Publishers, New York, 1954, p. 355; S. Gardell, *ibid.*, Vol. 6, 1958, p. 289.
10) A. Tsuji, T. Kinoshita and M. Hoshino, *Chem. Pharm. Bull.* (Tokyo), **17**, 1505 (1969).
11) The abbreviations in parenthesis are used in Table I.

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Absorbances were measured by Hitachi Model 139 Spectrophotometer. NMR spectra were obtained by Hitachi Model R-20 spectrometer operating at 60 MHz. Signal integrations were carried out with the integrator attached to Hitachi Model R-20 spectrometer.

Reagents——(a) 5% $NaNO_2$, (b) 5% $KHSO_4$, (c) 12.5% ammonium sulfamate, (d) 0.5% 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH), (e) 0.5% $FeCl_3$. Reagents (d) and (e) should be prepared freshly every three days and stored in a refrigerator.

Method A for Determination of 2-Deoxy-2-sulfamido-D-glucose in Heparin——To 1 ml of the sample solution containing 50—100 μg of heparin are added 1 ml of 5% $KHSO_4$ and 1 ml of 5% $NaNO_2$. The mixture is then left standing with occasional shaking for 2 hr upon which the deamination is completed. The nitrous acid is then destroyed by adding 1 ml of 12.5% ammonium sulfamate and repeatedly shaking the mixture for periods of 5 min. To the solution is added 1 ml of 0.5% MBTH and the reaction mixture is allowed to stand for 1 hr. Finally, 1 ml of 0.5% $FeCl_3$ is added and the absorbance is measured at 650 mμ after 30 min against the reagent blank. 2-Amino-2-deoxy-D-glucose hydrochloride is used as the standard. Determination of 2-amino-2-deoxy-D-glucose moiety in chitosan is carried out in the same manner except that chitosan requires 1 hr for deamination (Fig. 1).

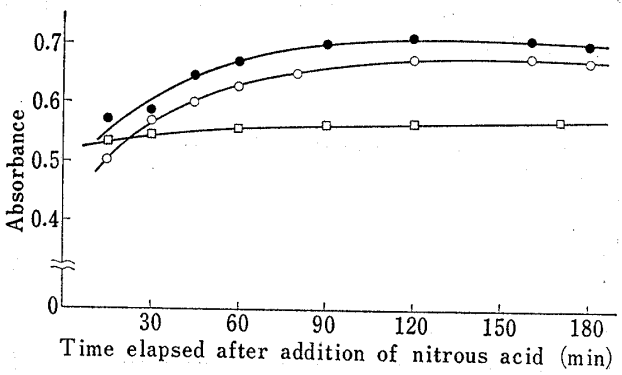

Fig. 1. Formation of 2,5-Anhydrohexose from Heparin (●), De-N-sulfated Heparin (○) and Chitosan (□)

final concentration of heparin (heparin-1 in Table I): 17.1 μg/ml; de-N-sulfated heparin (prepared from heparin-1): 17.1 μg/ml as intact heparin; chitosan; 3.8 μg/ml

Method B for Determination of Total 2-Amino-2-deoxy-D-glucose in Heparin——The solution of heparin (200—400 μg) in 1 ml of 2N HCl is heated in a sealed tube on a boiling water-bath for 5 hr and, after cooling, the mixture is transferred into a 5 ml volumetric flask with the aid of 2 ml of water. Into the flask is added 1 drop of 0.5% alcoholic solution of phenolphthalein followed by careful addition of 2N NaOH until the solution turns pink. The solution is back-titrated dropwise with 1% $KHSO_4$ until the indicator color just disappears and the resultant colorless mixture is made up to 5 ml with water. One milliliter aliquot of this solution is taken and the amino sugar is estimated in the same manner described in method A except that the deamination time is 15 min in this case. Treatment of the standard hexosamine under the hydrolytic conditions before assay is not necessary because no appreciable decomposition of the standard hexosamine occurs under these conditions.

NMR Method for Determination of 2-Acetamido-2-deoxy-D-glucose in Heparin——Heparin (about 50 mg) is dissolved in 0.5 ml of D_2O containing 4 mg of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) and the mixture is submitted to NMR spectroscopy. The peak area under the signal at 2.0 ppm of methyl protons of N-acetyl group is estimated with an integrator. 2-Acetamido-2-deoxy-D-glucose is used as the standard. Acetamidohexose content can be calculated by the following equation:

$$\frac{W_s \times P \times PD_s \times 100}{W \times P_s \times PD} = \text{per cent of 2-acetamido-2-deoxy-D-glucose in heparin}$$

where W is the weight in mg of heparin in sample solution, W_s is the weight in mg of 2-acetamido-2-deoxy-D-glucose in standard solution, P is the peak area under the signal of N-acetyl group in sample, P_s is the peak area under the signal of N-acetyl group in standard, PD is the peak area under the signal of DSS in sample, and PD_s is peak area under the signal of DSS in standard.

Result and Discussion

Heparin and chitosan give color absorbing at the same wavelength (653 mμ) with those of hexosamine[10] by method A. Modifications of procedures in the present methods brought about no change in specificities of the color reaction and linearity of the working curve reported in the previous paper.[10]

Fig. 1 shows that heparin is deaminated in a particularly slow rate while chitosan is deaminated in 30 min. Free hexosamines are deaminated in only 15 min.[10] Interestingly, Fig. 1 suggests that removal of amine sulfate from heparin molecule does not affect the rate of deamination although Lagunoff and Warren[7] menthioned that the de-N-sulfated heparin is more readily deaminated than the intact one.

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Fig. 2 exhibits the time profile of hydrolysis of heparin and chitosan with 2N HCl. Highest recovery of hexosamine is obtained after at least 4 hr of hydrolysis, and the hexosamine value given at this stage is in good agreement with nitrogen content of each polysaccharide. These facts provided the bases for method B. As already described,[10] hydrolysis for 1 hr is sufficient for chondroitin sulfates and hyaluronic acid. These discrepancies in hydrolysis time may be due to the stability of heparin and chitosan to acid hydrolysis compared with other mucopolysaccharides.[12]

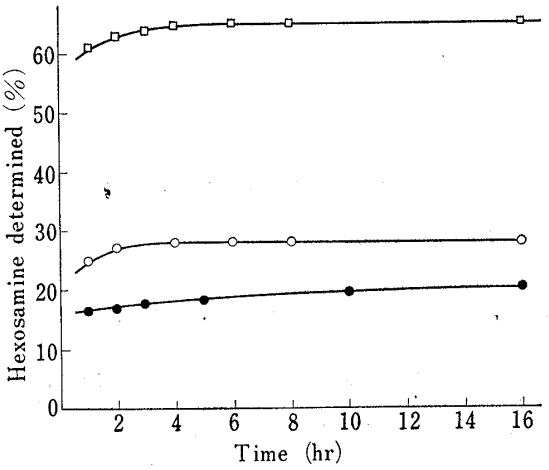

Fig. 2. Effect of Hydrolysis Time of Heparin[a] with 2N HCl (○), with 0.04N HCl (●), and Chitosan with 2N HCl (□)

a) Heparin-1 in Table I was used.

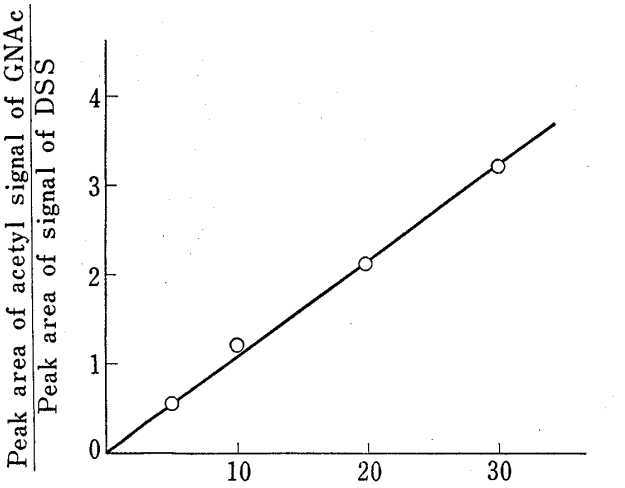

Fig. 3. Working Curve for 2-Acetamido-2-deoxy-D-glucose (GNAc) in NMR Method

On the other hand, method A gives lower hexosamine value than method B for both heparin and chitosan and de-N-sulfation of heparin with dilute acid has little effect on the results (Fig. 2). Since the difference between two methods were expected to correspond to the amount of N-acetyl group, acetamidohexose content was determined by nuclear magnetic resonance (NMR) spectroscopy. Working curve as shown in Fig. 3 was given by the standard procedure.

Several methods are available for analysis of N-acetyl groups on glycosaminoglycans. Ludowieg and Dorfman[13] reported colorimetry using hydroxylamine–ferric complex. Rhadakrishnamurthy and co–workers developed gas chromatographic assy.[6] In comparison with these methods, NMR methods is very simple and rapid because it requires no pretreatment of samples. Samples can be recovered by dialysis after measurements.

The analytical data obtained in the way described above are summarized in Table I. Hexosamine values given on hydrolysis of polysaccharides coincide well with those calculated from nitrogen contents. There is satisfactory agreement between acetamidohexose contents determined by MBTH method and that by NMR method. Exceptionally, heparin from bovine lung shows no signal around 2 ppm but gives a small amount of acetamidohexose by MBTH method. The cause of this fact remains uncertain.

Although the hexosamine–hexuronic acid ratio in heparin has been believed to be 1:1 on the basis of chemical analysis, Perlin and co–workers[4] suggested from their NMR investigations that heparin consists of approximately equal number of D-hexosamine, D-glucuronic acid and L-iduronic acid moieties, and the hexosamine–hexuronic acid ratio is, therefore, 1:2. They claimed that chemical methods might not provide an adequate ratio. On the contrary,

12) J.A. Cifonelli, *Carbohyd. Res.*, **2**, 150 (1966).
13) J. Ludowieg and A. Dorfman, *Biochim. Biophys. Acta*, **38**, 212 (1960).

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Table I. 2-Deoxy-2-sulfamido-D-hexose (HNS), 2-Amino-2-deoxy-D-hexose (HN) and 2-Acetamido-2-deoxy-D-hexose (HNAc) Content of Mucopolysaccharides

Sample[a]	Nitrogen content (%)	HNS content or HN content (%)[b] Calcd. from nitrogen content	Found by Method A	Found by Method B	HNAc content (%)[b] Found by B-A[c]	Found by NMR method
Heparin sample						
1	2.37	30.3	18.3	28.0	9.7	10.1
2	2.43	31.1	17.3	27.8	10.5	10.9
3	2.44	31.2	18.0	26.1	8.1	8.6
from bovine lung	2.23	28.5	21.8	27.1	5.3	0.0
from whale intestine	2.70	34.5	25.0	33.3	8.3	9.7
Chitosan	5.93	75.8	45.0	68.1	23.1	19.0
ChS-A	2.78	35.6	0.0[d]	32.2[d]	32.2	31.5
ChS-C	2.60	33.3	2.4[d]	31.9[d]	29.5	30.1

a) For sources of the mucopolysaccharides and abbriviations, refer to the text.
b) Caculated as 2-amino-2-deoxy-D-glucose for heparins and chitosan and 2-amino-2-deoxy-D-galactose for chondroitin sulfates.
c) Difference between the 2-amino-2-deoxy-D-hexose contents obtained by method B and that by method A.
d) 2-Amino-2-deoxy-D-galactose was used as the standard.

Yoshizawa and co-workers[14] obtained a result which supports 1:1 ratio employing indole-hydrochloric acid reagent and, in addition, pointed out that L-iduronic acid is an artifact produced by deamination with nitrous acid.[15] Total hexosamine values demonstrated by the present procedure agree well with those by Elson-Morgan method. Among the samples listed in Table I, heprin[16] from bovine lung and one from whale intestine showed total hexosamine value of 27.1% and 33.3%, respectively, by MBTH method, an 25.0% and 33.3%, respectively, by Elson-Morgan method. These results also support 1:1 ratio. High acetamidohexose content of whale heparin given by both MBTH method and NMR method is in accord with the result of Yoshizawa and co-workers.[14] Acetamidohexose in commercial heparin may partly arise from impurities.

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14) T. Kotoku, Z. Yoshizawa and F. Yamauchi *Arch. Biochem. Biophys.*, **120**, 553 (1967).
15) F. Yamauchi, M. Kosakai and Y. Yoshizawa, *Biochem. Biophys. Res. Commun.*, **33**, 721 (1968).
16) Purification and Elson-Morgan assay of these two heparin samples were carried out by Dr. Yamaha and co-workers: T. Yamaha, Private communication; K. Nagasawa, T. Yamaha, T. Kimura and T. Takahashi, *Seikagaku*, **36**, 29 (1964).

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