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5. **Makoto Yokoo**: Application of Azotometry. XVII.* Determination of Glycosamine.

(*Research Laboratories, Takeda Pharmaceutical Industries, Ltd.***)

Amino sugars are widely distributed in the animal and vegetable kingdom and majority of the sugar portions of polysaccharides in the animal in particular consists of glycosamine and glucuronic acid, with the exception of glycogen. For instance, chondroitinsulfuric acid which is found in mucin and mucoid, and heparin which is found in the heart, liver, and muscles, and is said to have the action of preventing coagulation of blood, are polymerization product of glycosamine and glucuronic acid. Further, amino sugars are also contained in various kind of antibiotics, e.g. N-methylglucosamine in streptomycin, 3-amino-3-deoxy-D-ribose in puromycin, and 3-dimethylamino-3,4,6-trideoxyhexose in erythromycin. Since the essential substance of the resistant factor advocated by Kuhn was recently found to be the amino sugar, amino sugars have come to be studied with much interest in the biochemical field.

Therefore, analytical methods of these substances, especially of glycosamine, are being studied by many researchers and numerous reports[1~6] have been published, not only on the determination of free glycosamine but also on separative determination of the substance present in polysaccharides and tissues. When glycosamine in polysaccharides or tissues is determined, however, protein and other impurities interfere and this effect is particularly great in colorimetric method. To offset these obstacles, separative methods utilizing ion-exchange resin[7, 8] and electrophoresis[9] have been employed but results were not satisfactory.

The present author, who has already reported the quantitative determination of glycosamine by Iwasaki's amino-N Azotometry,[10] which utilizes generation of nitrogen gas by reaction between glycosamine and nitrous acid, succeeded in the separative determination of glycosamine in polysaccharides. Pauly and Ludwig reported[11] that glycosamine reacts with acetylacetone to produce the pyrrole ring according to the scheme shown in Chart 1.

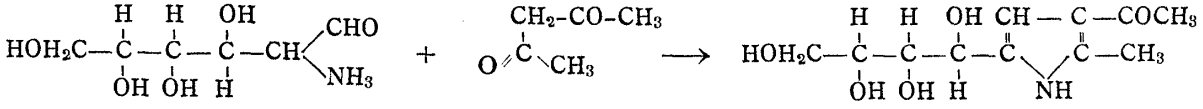

Chart 1.

In the case of microgram order, this reaction can be completed by heating for 20 minutes at 100°, and the resultant pyrrole derivative no longer reacts with nitrous acid and therefore nitrogen gas is generated no more. This reaction was combined with Iwasaki's amino-N Azotometry for the present determination of glycosamine. A sample was subjected to this Azotometry before and after treatment with acetylacetone, and the difference between

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** Juso-Nishino-cho, Higashiyodogawa-ku, Osaka (横尾 亮).

1) E. Baumann: Ber., **19**, 3218(1886).
2) H. Stuedel: Z. physiol. Chem., **33**, 223(1901).
3) F. Zuckerhandl: Biochem. Z., **236**, 19(1931).
4) L. A. Elson, W. T. J. Morgan: Biochem. J., **27**, 1824(1933).
5) S. Roseman: Anal. Chem., **28**, 1743(1956).
6) B. Drake: Arkiv. Chem., **4**, 469(1952).
7) N. F. Boas: J. Biol. Chem., **204**, 553(1953).
8) S. Roseman: Z. Anal. Chem., **157**, 217(1957).
9) O. W. Newhaus, M. Latzring: Anal. Chem., **29**, 1230(1957).
10) M. Yamagishi, M. Yokoo: Yakugaku Zasshi, **74**, 278(1954).
11) H. Pauly, E. Ludwig: Z. physiol. Chem., **121**, 176(1922).

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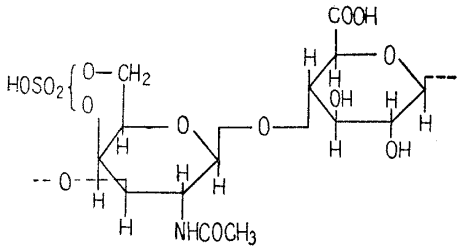
the two values of the nitrogen gas generated was regarded as the N-value ascribable to glycosamine.

When glycosamine in the hydrolyzate of polysaccharides or tissue is determined by the usual method, amino acid is especially obstructive. In the present method, however, glycosamine can be easily determined in the presence of amino acids, because the latter do not react with acetylacetone. Iwasaki's Azotometry before treatment with acetylacetone gives the value of nitrogen generated from glycosamine and amino acids, and Azotmetry after the treatment furnishes the value of nitrogen generated only from amino acids. Therefore, the quantity of glycosamine is calculated from this difference.

The method used for the separative determination of glycosamine in chondroitinsulfuric acid and heparin will now be described. As shown in Chart 2, chondroitinsulfuric acid consists of acetylgalactosamine, glucuronic acid, and sulfuric acid, and heparin is composed of glycosamine, glucuronic acid, and sulfuric acid.



Chondroitinsulfuric acid

Heparin

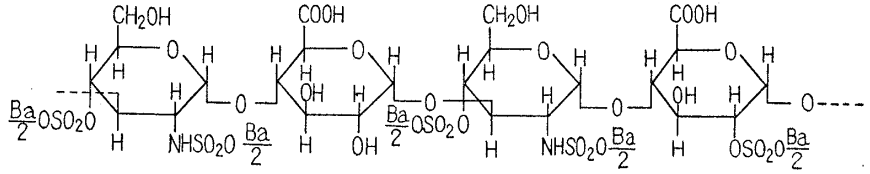


Chart 2.

These substances must be hydrolyzed before the determination and they were found to be hydrolyzed by heating with 4*N* hydrochloric acid in a sealed tube for 4~5 hours at 100°. As the samples used were not pure, consideration for nitrogen-containing impurities other than amino acids was necessary. Therefore the total nitrogen of each sample measured by Kjeldahl method was compared with the total amino-nitrogen measured after hydrolysis. As the values agreed with each other, the nitrogen of the sample was ascertained to be all amino-nitrogen.

Methods

About 50~150 mg. of chondroitinsulfuric acid or heparin is weighed exactly in an ampule and, after addition of 5 cc. of 4*N* HCl, the ampule is sealed and heated for 5 hrs. in a boiling water bath. After cool, the content is neutralized with NaOH solution and diluted exactly to 100 cc. to make a test solution.

A 1-cc. portion of the test solution is used for amino-N determination by Iwasaki's Azotometry. The volume of nitrogen gas generated is measured and the value is converted to that of its standard (normal) state (designated as V). To another 1-cc. portion of the test solution in a beaker, 1 cc. of an acetylacetone solution (ca. 1 cc. of acetylacetone in 50 cc. of 0.5*N* Na_2CO_3 solution) is added and the beaker is heated for 20 mins. on a water bath. The solution thus treated is used for Iwasaki's Azotometry to obtain V′ against V.

Since the molecular weight of glycosamine is 179 and 179 γ of this substance generates 22.4 mm^3 of nitrogen gas, the quantity of glycosamine is calculated by the following equation:

$$\text{Glycosamine } (\gamma) = \frac{(V - V') \times 179}{22.4}$$

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Experimental

1) Period of the reaction between glucosamine and acetylacetone: Each of 50, 200, and 400 γ of glucosamine in 1 cc. solution was mixed with the acetylacetone reagent, heated at 100° for various lengths of time, and the quantity of residual glycosamine was determined. From the results shown in Table I, it was found that the reaction was completed in 20 mins. when the quantity of glucosamine was 50~400 γ.

TABLE I.

Glucosamine (γ)	Reaction time (mins.)					
	0	10	15	20	25	30
50	50	0	0	0	0	0
200	200	24	0	0	0	0
400	400	123	20	0	0	0

2) Effect of amino acids on the determination of glucosamine: Glucosamine was determined in the presence of glutamic acid, tryptophan, leucine, lysine, histidine, alanine, or proline. A 39.6-mg. portion of glucosamine and ca. 30 mg. of each amino acid were dissolved in 100 cc. of water and 1 cc. of the solution was used for the determination. The results are shown in Table II.

TABLE II.

Glucosamine-HCl (γ)	Amino acids* (γ)	Amino-N found (mm^3)		Amino-N of glucosamine	Glucosamin-HCl found	
		Total amino-N	Amino-N from amino acids		(γ)	%
396	300	70.7	29.3	41.4	398	100.5
396	300	70.6	29.0	41.6	400	101.0
396	300	69.8	28.9	40.9	394	99.4

* Glutamic acid, tryptophan, leucine, lysine, histidine, alanine, and proline.

3) Hydrolysis of chondroitinsulfuric acid and heparin: Chondroitinsulfuric acid and heparin were each heated with 4*N* HCl in a sealed tube and the resulting glycosamine was determined at regular intervals. The values in Table III show the percentage of glycosamine. As shown in Table III, heating for 4 hrs. seems sufficient, because the value of glucosamine became constant at that time and increased no longer by additional heating.

TABLE III.

Sample	Time of heating (hrs.)					
	2	3	4	5	7	9
Chondroitinsulfuric acid	22.3	26.6	29.6	29.5	30.8	30.0
Heparin	13.4	15.5	19.7	19.4	19.0	19.9

4) Determination of galactosamine in 4 samples of chondroitinsulfuric acid: The determination was repeated twice for each sample and the results are shown in Table IV.

TABLE IV.

Sample	Quantity (mg.)	Kjeldahl-N (%)	Amino-N found			Amino-N of galactosamine (mm^3)	Galactosamine found	
			Total (%)	Amino-N (mm^3)	Amino-N from amino acids (mm^3)		(mg.)	(%)
1	1.301	2.39	2.30	48.0	0	48.0	0.384	29.5
	1.443		2.47	57.0	0	57.0	0.456	31.5
2	1.326	3.02	2.96	63.0	28.7	34.3	0.273	20.6
	1.117		3.06	54.8	25.5	29.3	0.233	20.9
3	1.108	3.38	3.35	59.4	28.3	31.1	0.248	22.4
	0.982		3.40	53.4	27.5	25.9	0.207	21.1
4	0.890	1.97	1.83	26.0	0	26.0	0.208	23.4
	0.890		1.86	26.5	0	26.5	0.212	28.3

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5) Determination of glucosamine in a sample of heparin: The results are shown in Table V.

TABLE V.

Sample (mg.)	Kjeldahl-N (%)	Amino-N found			Amino-N of glucosamine (mm^3)	Glucosamine found	
		Total (%)	Amino-N (mm^3)	Amino-N from amino acids (mm^3)		(mg.)	(%)
1.178	1.84	1.78	33.5	4.8	28.7	0.230	19.5
1.178		1.81	34.2	5.4	28.8	0.231	19.5
0.920		1.80	26.5	4.1	22.4	0.179	19.4
0.920		1.82	26.8	4.0	22.8	0.182	19.8

The author wishes to express his grateful thanks to Dr. Satoru Kuwada for his encouragement throughout the present work.

Summary

A new method for the separatory determination of glycosamine present in polysaccharides was established. Glycosamine generates nitrogen gas by reaction with nitrous acid, but it no longer produces nitrogen gas after reaction with acetylacetone, because the reaction converts it into the corresponding pyrrole derivative. Combination of the two reactions can determine the amount of glycosamine present in the hydrolyzate of polysaccharides. The values of nitrogen gas generated by the reaction of a sample with nitrous acid before and after treatment with acetylacetone are measured by Iwasaki's amino-N Azotometry, and the quantity of glycosamine in the sample is calculated from the difference between the two values. In this method the presence of amino acids is not obstructive.

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