# *Studies on Amino-hexoses. IV. N-Deacylation with Hydrazine and Deamination with Nitrous Acid, a Clue to the Structure of Aminopolysaccharides*[1]

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Considerable confusion is observed in the older literature concerning the nitrous deamination products of glucosamine. However, the formation of chitose, a 2, 5-anhydro-hexose[2], from glucosamine is understood in terms of intramolecular rearrangement[3,4] which accompanies the deamination, and a few years ago one of present authors demonstrated experimentally that the same mechanism operated in the nitrous deamination both of glucosaminol and 2-amino-erythro-pentitols which form 2-deoxy-glucose[3] and 2-deoxy-ribose[5] respectively.

The nitrous deamination of chitosan (deacetylated chitin or polyglucosamine) has also resulted in conflicting conclusions[6], but it is noteworthy that every worker reports the rupture of glucosaminidic linkage and the formation of highly reducing saccharides. More recently, it was reported[7] that ψ-heparin (the desulfated heparin) also produced reducing saccharides with nitrous acid. In these instances, it is not conceivable that the hydrolytic cleavage of amino-glycosidic linkages preceede the deamination, since it is known[8] that glucosaminidic linkage strongly resists to acid hydrolysis (see Fig. 1) and nitrous acid is so weak an acid. Therefore, some special mechanism must operate.

It may be expected that the cleavage of glucosaminidic linkage (or probably 2-amino-glycosidic linkages in general) with nitrous acid will provide a clue to the structure of amino-polysaccharides. However, it seems desirable to obtain more experimental evidences and to establish the theory according to which the 2-amino-glycosidic linkage cleaves.

The authors have isolated from the deamination mixture of chitosan a diphenylhydrazone which is identical with the long known diphenylhydrazone[9] which was obtained from the deamination mixture of glucosamine. Although, as we

1) A Part of this article was delivered at the 9th annual meeting of the Chemical Society of Japan, April 1956.

2) E.Fischer and F.Tiemann, *Ber.*, **27**, 138 (1894); E.Fischer and F. Andreae, ibid., **36**, 2587 (1903); W. N.Haworth, E.L.Hirst and V.S.Nicholson, *J. Chem. Soc.*, **1927**, 1513.

3) Y.Matsushima, *Bull. Chem. Soc. Japan*, **24**, 144 (1951).

4) A.B.Foster, *Chem. and Ind.*, **1955**, 627.

5) Y.Matsushima and Y.Imanaga, This Bulletin, **26**, 506 (1953).

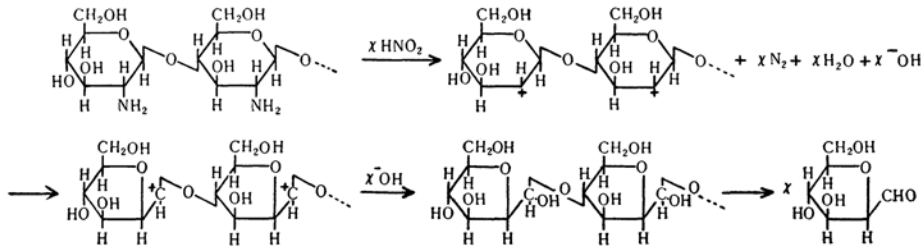
6) W.Armbrecht, *Biochem. Z.*, **95**, 108 (1919); P. Karrer and S.M.White, *Helv. Chim. Acta*, **13**, 1105 (1930); K.H.Meyer and H.Wehrli, *Helv. Chim. Acta* **20**, 353 (1937).

7) A.B.Foster, E.F.Martlew and M.Stacey, *Chem. and Ind.* **1953**, 825.

8) J.C.Irvine and A.Hynd, *J. Chem. Soc.*, **101**, 1128 (1912).

9) P.Schorigin, and N. N. Makarowa-Semljanskaya, *Ber.* **68**, 965 (1935).

lacked the structural proof, the diphenylhydrazone was not necessarily thought to be the diphenylhydrazone of chitose until recently, a report[10] proving the 2,5-anhydromannose structure of the diphenylhydrazone has appeared. Therefore, the isolation



of the diphenylhydrazone provides a proof of the existence of chitose in the deamination products of chitosan. How chitose is produced may be seen in the following scheme:

Here also the mechanism of intramolecular rearrangement operates and the formation of the intermediate half-acetal which may be highly labile explains the result. This theory has its counterparts in the anomerism of free sugars[11] and in the ease of hydrolysis of fructofuranosidic linkage[11,12]. The ease of hydrolysis of fructofuranosidic linkages, e. g. of sucrose, was attributed to the foregoing hydrolysis of the furanoid ring of fructose moiety, thus forming an intermediate half-acetal structure.

Unfortunately, since the amino residue of natural amino-polysaccharides is acylated without exception, nitrous acid can not attack it as such. Common deacylating agents, strong acid or alkali, may cause too far reaching degradation with two known exceptions of chitin and heparin. Therefore, the authors adopted the method of hydrazinolysis which was successfully introduced[13] into the protein chemistry. With anhydrous hydrazine, the amide linkage, or ester linkage if present, in polysaccharides may be cleaved, liberating free amino residue and the glycosidic or other linkages may remain intact.

To begin with, the authors applied the hydrazinolysis method to chondroitin sulfate. The ratio of amino to total nitrogen reaches about 0.9 when barium chondroitin sulfate is treated with anhydrous hydrazine for 10 hours at 100°. The hydrazine-treated specimen, only after treatment with nitrous acid, gives several spots on paper chromatograms, thus indicating the cleavage of galactosaminidic linkage with nitrous acid (see Fig. 2). Though no quantitative conclusion about the structure of chondroitin sulfate can be drawn so far, this method seems to be promising for the structural study of amino-polysaccharides. The work will be continued.

## Experimental Part

**Appearance of Reducing Power when Chitosan is treated with Nitrous Acid.**—500 mg. of chitosan was dissolved in 25 cc. of acetic acid (ca. 15%) and a solution of 230 mg. of sodium nitrite in 25 cc. of water was added. 5 cc. aliquots were pipetted off and the reducing power was determined by Bertrand's method. A comparison run with hydrochloric acid was carried out similarly.

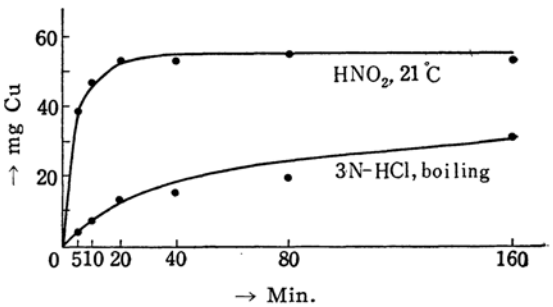



Fig. 1. The velocity of the appearance of reducing power in nitrous deamination and acid hydrolysis respectively of chitosan. Ordinate: Reducing power in mg. Cu. Abscissa: Time in minutes.

**Isolation of Chitose Diphenylhydrazone from the Nitrous Deamination Mixture of Chitosan.**—To the highly viscous solution which was prepared by dissolving 2 g. of chitosan into a mixture of 1N hydrochloric acid (13.2 cc.) and water (20 cc.), 4.6 g. of silver nitrite was added at room temperature. The evolution of gas and

10) S. Akiya and T. Osawa, *J. Pharm. Soc. Japan*, **74**, 1259 (1954).
11) S. Akabori and K. Uehara, "Discussion on Enzymes," Tokyo, (1942).
12) S. Matsumoto, *J. Chem. Soc. Japan*, **61**, 387, 395 (1940).
13) S. Akabori, K, Ohno and K. Narita, This Bulletin, **25**, 214 (1952).

the diminution of viscosity was observed immediately. After two days standing at room temperature, the silver chloride formed and the residual silver nitrite was filtered off, and the silver ion in the solution was removed by hydrochloric acid. A small amount of urea could exclude residual nitrous acid. To the solution the pH of which was adjusted to 4, were added 1.5 g. of diphenylhydrazine and a necessary amount of ethanol to obtain a clear solution. After heating at 80° for one hour the solution was concentrated in vacuo and the residue was agitated with a small amount of benzene and seed crystals. White needles began to crystallize in an hour. The crystals were washed thoroughly with water and benzene. The crude crystals thus obtained melted at 140-141° and weighed 0.97 g. Recrystallization was carried out as follows: the crude crystals were dissolved in 10 cc. of ethanol, and 40 cc. of water was added. Fine crystals came out immediately and the mixture was agitated thoroughly with a small amount of benzene-petroleum ether (1 : 1) in order to make free from coloring matter, filtered and washed with ethanol-water (1 : 4) and benzene. The recrystallized specimen weighed 0.67 g. and melted at 143.4-144.0° alone or at 145-145.5° with an authentic specimen of chitose diphenylhydrazone melting at 145.8-146.2°.

*Anal.* Found: C, 65.39; H, 6.05; N, 8.28. Calcd. for $C_{18}H_{20}O_4N_2$: C, 65.85; H, 6.09; N, 8.54%.

**Hydrazinolysis of Barium Chondroitin Sulfate.**—4.57 g. of barium chondroitin sulfate (N, 2.7%) was heated with 11.1 g of anhydrous hydrazine at 100° for ten hours. The excess hydrazine was evaporated off in vacuo and the residue was dissolved in 20 cc. of water and dialysed to running water of nineteen hours using collodion membrane. The inorganic precipitate was discarded and the solution was dried up in vacuo yielding 0.5 g. of a faintly yellow substance.

*Anal.* Found: N, 3.02 (Kjeldahl method); 2.65 % (van Slyke method).

**Deamination of the Hydrazine-treated Specimen with Nitrous Acid.**—0.4 g. of the specimen was dissolved in 4.6 cc. of water and to this solution 0.87 g. of silver nitrite and 3.4 cc. of 1N hydrochloric acid was added. A gas evolved immediately. After standing for one day at room temperature, the silver chloride formed and the residual silver nitrite was filtered off and the silver ion was removed with 1N hydrochloric acid. A small amount of urea was added in order to decompose the residual nitrous acid, and after the pH was adjusted to 5.8 the solution was concentrated in vacuo. The residue was paper-chromatographed. It is to be noted that color develops with such ketose reagents as resorcinol-hydrochloric acid or urea-hydrochloric acid[14]. These facts suggest that every spot in chromatograms contains 2,5-anhydro-hexose (Hydrofurfural) structure.

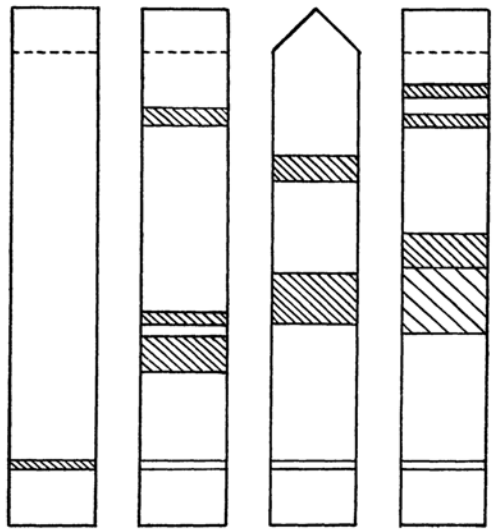



Fig. 2. Diagrammatic representation of the paper chromatograms.
I: The specimen without the treatment with nitrous acid. II: Urea-HCl development. III: Long run chromatography with urea-HCl development. IV: Resorcinol-HCl development. The moving solvent is butanol-ethanol-water-acetic acid (35 : 35 : 30 : 10) in all the run.

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14) A.I. Oparin, *J. Japanese Chem.* **10**, 506 (1956).