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Analytical Chemical Studies on Amino Sugars. I. New Color Reaction of Hexosamines using *p*-Nitrobenzaldehyde and Tetraethylammonium Hydroxide[1]

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It was found that glucosamine-HCl reacted with *p*-nitrobenzaldehyde in pyridine–methanol solution to yield Schiff base and gave a blue color by addition of tetraethylammonium hydroxide solution. This new color reaction is selective for 2-amino-2-deoxy sugars and α-amino carbonyl compounds, such as aminoacetaldehyde, δ-aminolevulinic acid and α-aminoacetophenone. The microdetection methods for 2-amino-2-deoxy sugars and their derivatives were proposed and the reaction mechanism was investigated.

The most widely used procedures for the determination of hexosamines are those derived from the method originated by Elson and Morgan.[3] The Elson–Morgan reaction has been widely investigated and modified to increase its sensitivity, specificity and reproducibility.[4] The other color reaction of hexosamines based on their deamination to 2,5-anhydrohexoses was described by Dische.[5] Ninhydrin reaction for amino acids is also used for the determination of amino sugars.[6] Recently, two new spectrophotometric method for hexosamines were reported, one of them involved the reaction with trinitrobenzene-1-sulfonic acid,[7] the other one is based on the color reaction with *p*-nitrobenzenediazonium salt in alkaline medium.[8] In the course of our studies on amino sugars, a new color reaction has been devised for the microdetection and determination of hexosamines, based on a principle completely different from that of the Elson–Morgan or Dische reaction and having the advantage of being considerably more simple and selective. *p*-Nitrobenzaldehyde (*p*-NBA) reacted readily with hexosamines in pyridine solution to yield Schiff bases (*p*-nitrobenzylidene hexosamines), and gave a blue color by addition of tetraethylammonium hydroxide solution. By the use of glucosamine, the microdetection method for hexosamines and related compounds with *p*-NBA and its reaction mechanism were examined.

Experimental

Reagents——(1) 1% and 2% *p*-NBA solution in pyridine, (2) 10% aqueous Et_4NOH, (3) 0.3% Et_4NOH; 3.0 ml of 10% Et_4NOH was diluted to 100 ml with ethanol. (4) 10% HCl and 1% HCl ethanol solution, (5) Various amino sugars and their derivatives were synthesized or commercial preparations. (6) Other

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2) Location: *Hatanodai, Shinagawa-ku, Tokyo.*
3) L.A. Elson and W.T.J. Morgan, *Biochem. J.*, **26**, 1824 (1933).
4) Z. Dische, in R.L. Whistler and M.L. Wolfrom (Eds.), "Methods in Carbohydrate Chemistry," Vol. I, Academic Press, New York, 1962, p. 507; A. Gottschalk, "Glycoproteins," Elsevier Publishing Co., Amsterdam, 1966, p.210, 225.
5) Z. Dische and E. Borenfreund, *J. Biol. Chem.*, **184**, 517 (1950).
6) K. Brendel, N.O. Roszel, R.W. Wheat and E.A. Davidson, *Anal. Chem.*, **18**, 147, 161 (1967).
7) J.T. Galambos and R. Shapira, *Anal. Biochem.*, **15**, 334 (1966).
8) S. Ogawa, M. Morita, A. Nakajima and T. Yoshida, *Yakugaku Zasshi*, **88**, 866, 871 (1968).

sugars, amino acids and various amines were of reagent grade and used without further purification. (7) N-*p*-Nitrobenzylidene glucosamine (*p*-NB-GN) and various aldimines of glucosamine were prepared by the method described in the literature.[9] (8) Standard solution of glucosamine HCl; 10 mg of glucosamine HCl was dissolved in 0.5 ml of water and diluted to 20 ml with pyridine. 3.0 ml of this solution was diluted again to 20 ml with pyridine (75 μg/ml).

Procedure——Method A: To 1 drop of the aqueous test solution containing hexosamine 0.3 ml of pyridine and 3 drops of 2% *p*-NBA solution were added, mixed thoroughly, and allowed to stand for several minutes in ice water. The mixture was made alkaline with a drop of 10% Et_4NOH. A blue color appeared instantly. A blank solution was faint brown.

Method B: One drop of the aqueous test solution or a little amount of the solid sample containing N-substituted hexosamine, hexosaminide or acid mucopolysaccharide was mixed with a drop of 10% HCl, and heated in a boiling water bath for about 20 minutes. After cooled, the mixture was then neutralized by addition of $NaHCO_3$, and examined in the same manner as method A above mentioned. Spectrophotometric Method: To 1.0 ml of the standard solution 0.5 ml of 1% *p*-NBA solution was added, mixed thoroughly and allowed to stand for 20 minutes at 27—28°. The mixture, after cooling for about 5 minutes in an ice water bath, was diluted to 10 ml with ice cold 0.3% Et_4NOH. The maximum absorbance of the resultant red color was measured at 504 mμ against the reagent blank between 10 and 25 minutes.

Absorption Spectra of N-*p*-NB-GN under Various Conditions——(1) Two ml of *p*-NB-GN solution in ethanol (containing *p*-NB-GN 0.103 mg/ml) was mixed with 10 ml of 0.3% Et_4NOH and diluted to 20 ml with ethanol and then the absorption spectra were measured (Fig. 2), (2) Two ml of *p*-NB-GN solution in ethanol (containing *p*-NB-GN 0.10056 mg/ml) was mixed with 5 ml of 0.3% Et_4NOH. After standing for 20 minutes, the equivalent amount of 1% HCl ethanol solution was added to the alkaline solution and ethanol was added to make up to 20 ml. The changes of absorption spectra were measured immediately (Fig. 3).

C-2 Epimerization and Determination of Mannosamine——Ten ml of *p*-NB-GN solution in methanol (containing 5 mg of *p*-NB-GN) was mixed with 0.3 ml of 1N sodium methoxide methanol solution and allowed to stand for 90 minutes. The resultant red solution was acidified by addition of excess 10% HCl. Ethanol was evaporated under reduced pressure at room temperature. The remaining solution was diluted to 10 ml with water, extracted with chloroform (10 ml × 3) and then mannosamine in the aqueous layer was determined by the method described in the literature.[10]

Results and Discussion

The general applicability of this new test for hexosamines has been investigated for various hexosamines. As shown in Table I, 2-amino-2-deoxy sugars showed a similar blue color under this condition, while various N-substituted derivatives, hexosaminides, acid mucopolysaccharides, most of the other sugars and amino acids gave no color by this method. Compounds giving 2-amino-2-deoxy sugars on hydrolysis, such as N-acetyl-2-amino-2-deoxy sugars and acid mucopolysaccharides, gave positive results with Method B as shown in Table II. Methyl 3-amino-3-deoxy-, and methyl 6-amino-6-deoxy-α-D-glucopyranosides gave negative results by both methods, therefore, this color reaction is selective for 2-amino-2-deoxy sugars. In order to confirm the selectivity of this color reaction, Elson–Morgan and ninhydrin reaction have been compared with this method. Elson–Morgan reaction gives positive results with N-acetyl amino sugars and 3-amino-3-deoxy sugars as well as 2-amino-2-deoxy sugars. Ninhydrin reaction gives no color with N-acetyl amino sugars but positive results with various amino compounds. The relationship between the chemical structures and this color reaction was examined. Aminoacetaldehyde, δ-aminolevulinic acid and α-aminoacetophenone give also blue color by this method, and serine, glucosaminol, glucosaminic acid do not react. From the above results, it seems probable that this reaction is selective for α-aminocarbonyl groups in 2-amino-2-deoxy sugars and related compounds.

Then, we wish to discuss on the mechanism of this color reaction. Glucosamine condenses with *p*-NBA to yield *p*-NB–GN. *p*-NB-GN, prepared by the procedure reported in the literature,[9] gave an intensive blue color in an alkaline pyridine medium and the absorption spectrum in an alkaline pyridine–methanol solution was similar to that of the colored solution

9) A. Neuberger, *Biochem. J.*, 32, 1435 (1938); Z.E. Jolles and W.T.J. Morgan, *Biochem. J.*, 34, 1183 (1940).
10) J. Ludowieg and J.D. Benmaman, *Anal. Biochem.*, 19, 80 (1967).

Table I. Color Reaction of Hexosamines and Various Compounds

Compound	*p*-NBA	Elson–Morgan	Ninhydrin
Glucosamine HCl	+++	+++	+++
Galactosamine HCl	+++	+++	+++
Mannosamine HCl	+++	+++	+++
Fructosamine acetate	+++	+++	+++
3-O-Methyl-D-glucosamine	+++	+	+++
3,4,6-Tri-O-methyl-D-glucosamine	+++	+	++
Hyalobiuronic acid	++	++	++
Muramic acid	++	++	++
Chondrosine	++	++	++
Tetra-O-acetyl-D-glucosamine	+	+++	++
N-Acetylglucosamine	−	+++	−
N-Acetylgalactosamine	−	+++	−
N-Acetylmannosamine	−	+++	−
Penta-acetyl-D-glucosamine	−	++	−
Methyl α-D-glucosaminide	−	−	+++
Ethyl N-acetyl-β-D-glucosaminide	−	−	−
Phenyl N-acetyl-β-D-glucosaminide	−	−	−
3-Amino-3-deoxy-D-allouronic acid	−	++	++
Methyl 3-amino-3-deoxy-α-D-glucopyranoside	−	−	++
Methyl 3-amino-3-deoxy-α-D-mannopyranoside	−	−	++
Methyl 6-amino-6-deoxy-α-D-glucopyranoside	−	−	++
Glucosaminic acid	−	−	++
Glucosaminol	−	−	++
Chondroitine sulfate A (Na-salt)	−	−	−
Chondroitine sulfate C (Na-salt)	−	−	−
Hyaluronic acid (K-salt)	−	−	−
Heparine (Na-salt)	−	−	−
Glucose, Xylose, Mannose, Glucuronic acid	−	−	−
Amino acid (19 samples)	−	−	+++

Table II. Color Reaction of N-Acetyl Hexosamines and Acid Mucopolysaccharides

Compound	Method A	Method B
N-Acetylglucosamine	−	+++
N-Acetylmannosamine	−	+++
N-Acetylgalactosamine	−	+++
Methyl α-D-glucosaminide	−	+++
Ethyl N-acetyl-β-D-glucosaminide	−	+++
Phenyl N-acetyl-β-D-glucosaminide	−	+++
Tetra-O-acetyl-D-glucosamine	+	+++
Tetra-O-acetyl-N-methyl-D-glucosamine	−	+++
Penta-acetyl-D-glucosamine	−	+++
Chondroitine sulfate A (Na-salt)	−	+++
Chondroitine sulfate C (Na-salt)	−	+++
Hyaluronic acid(K-salt)	−	+++
Heparine (Na-salt)	−	+++

produced with glucosamine HCl and *p*-NBA by spectrophotometric method as shown in Fig. 1. Therefore, the first intermediate compound of this color reaction is N-*p*-nitrobenzylidene 2-amino-2-deoxy sugar.

Spectral changes of *p*-NB-GN in methanol solution occurred by addition of an alkaline reagent. As shown in Fig. 2, the intensity of the 285 mμ band of the aldimine decreased with time and the intensity of the newly formed absorption band at 485 mμ increased gradually.

The distinct isosbestic point was observed on the absorption curve. When the alkaline colored solution was neutralized by addition of the equivalent amount of acid, the color of the solution changed to yellow immediately and then faded gradually. This spectral changes are shown in Fig. 3. The isosbestic points were also observed at 308 and 262 mμ. The same reversible color changes were observed in the case of the colored solution obtained by the spectrophotometric method.

Aldimines of glucosamine with benzaldehyde, *p*-chlorobenzaldehyde, salicylaldehyde, *p*-dimethylaminobenzaldehyde, 2,4-dichlorobenzaldehyde and *p*-methoxybenzaldehyde were examined in the same manner. The results are shown in Table III. Each of them formed aldimine but did not give distinct color in alkaline medium. Therefore, the electron-withdrawing nitro group is essential in this color reaction. In view of these facts,

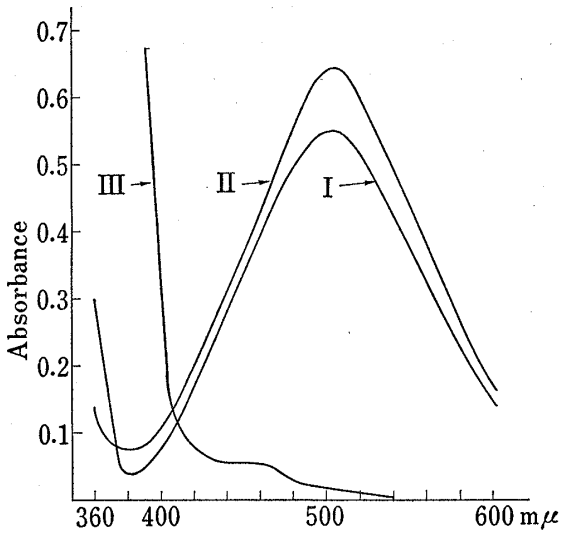

Fig. 1. Absorption Spectra

I: reaction solution of glucosamine HCl with *p*-nitrobenzaldehyde and tetraethylammonium hydroxide by spectrophotometric method (III was used as reference. final concentration: *ca.* 7.5 μg/ml)
II: reaction solution of N-*p*-nitrobenzylidene glucosamine with tetraethylammonium hydroxide in pyridine–methanol (1:9) (III was used as reference. final concentration: *ca.* 10 μg/ml)
III: reagent blank solution of 1 (H_2O was used as reference)

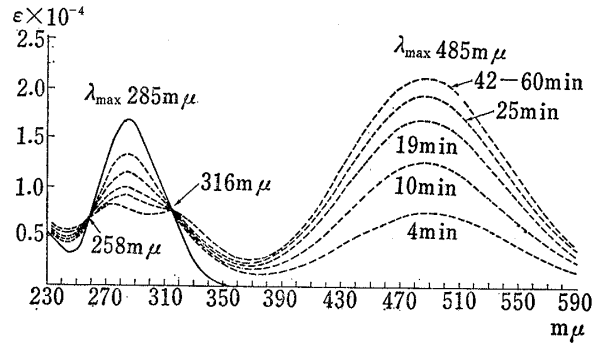

Fig. 2. Absorption Spectra of N-*p*-Nitrobenzylidene Glucosamine in Methanol at Various Times after Addition of Tetraethylammonium Hydroxide

concentration: N-*p*-nitrobenzylidene glucosamine; 3.30 ×10⁻⁵M tetraethylammonium hydroxide; 0.15%

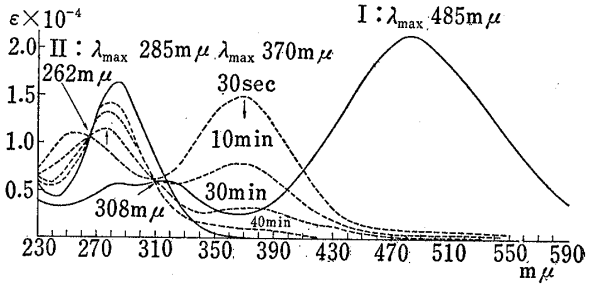

Fig. 3. Absorption Spectral Changes *vs.* Time Course of N-*p*-Nitrobenzylidene Glucosamine neutralized with Equivalent Amount of HCl in Methanol after Standing for 20 Minutes in Alkaline Methanol

concentration: N-*p*-nitrobenzylidene glucosamine; 3.22 ×10⁻⁵M
I: N-*p*-nitrobenzylidene glucosamine in alkaline methanol
II: N-*p*-nitrobenzylidene glucosamine in neutral methanol

TABLE III. Absorption Maxima and Molar Extinction Coefficients of Various Aldimines of Glucosamine

Aldehyde	In neutral		In alkaline	
p-Nitrobenzaldehyde	285 mμ	(1.33)[a]	485 mμ	(2.12)[a]
Benzaldehyde	248	(0.35)	362	(0.07)
p-Chlorobenzaldehyde	255	(0.69)	375	(0.38)
2,4-Dichlorobenzaldehyde	258	(0.42)	392	(0.44)
p-Dimethylaminobenzaldehyde	328	(1.27)	327	(1.24)
p-Methoxybenzaldehyde	265	(0.44)	366	(0.10)
o-Hydroxybenzaldehyde	256	(1.23)	382	(0.57)
	317	(0.39)		

a) ( ); ε×10⁻⁴

the chromogen exhibiting an absorption maximum at 485 mμ may be the resonance structure of the aldimine anions in which the pyranose ring is opened. The structure of the compound having the absorption maximum at 370 mμ in Fig. 3 may be the neutral enol form which is more conjugated system because the wave length of the absorption maximum is longer than that of the original aldimine. As shown in Fig. 3, this compound gradually returns to the original aldimine by changing to the aldehyde type and closure of the pyranose ring. If the enol form is one of the intermediates, C-2 epimerization might occur to yield mannosamine derivatives in closure of the pyranose ring. On the other hand, it would be expected that glucosamine will be produced by the reaction of mannosamine and *p*-NBA. Then, the experiment was undertaken to detect and determine mannosamine in the reaction mixture. The results are shown in Table IV. Small amount of mannosamine was found in the solution obtained

Table IV. Percentage of C-2 Epimerization

Compound	Starting material		
	N-*p*-Nitrobenzylidene glucosamine	Glucosamine HCl + *p*-Nitrobenzaldehyde	Mannosamine HCl + *p*-Nitrobenzaldehyde
Mannosamine [a]	8.1 %	4.7 %	6.7 %
Glucosamine [b]	49.4	55.3	30.1
Total hexosamine [c]	57.5	60.0	38.7

a) % of mannosamine $= \frac{\text{determined amount of mannosamine } (\mu g)}{\text{amount of sample } (\mu g)} \times 100$

b) % of glucosamine = total hexosamine (%) − mannosamine (%)

c) % of Total hexosamine $= \frac{\text{determined amount of total hexosamine } (\mu g)}{\text{amount of sample } (\mu g)} \times 100$

by hydrolysis after acidifying the alkaline methanol solution of *p*-NB-GN, and glucosamine was found in the reaction mixture of mannosamine and *p*-NBA. The compound having the absorption peak at 370 mμ, thus, seems to be an enol form.

In view of these facts, the most reasonable mechanism of the reaction of glucosamine with *p*-nitrobenzaldehyde and alkali was proposed in Chart 1.

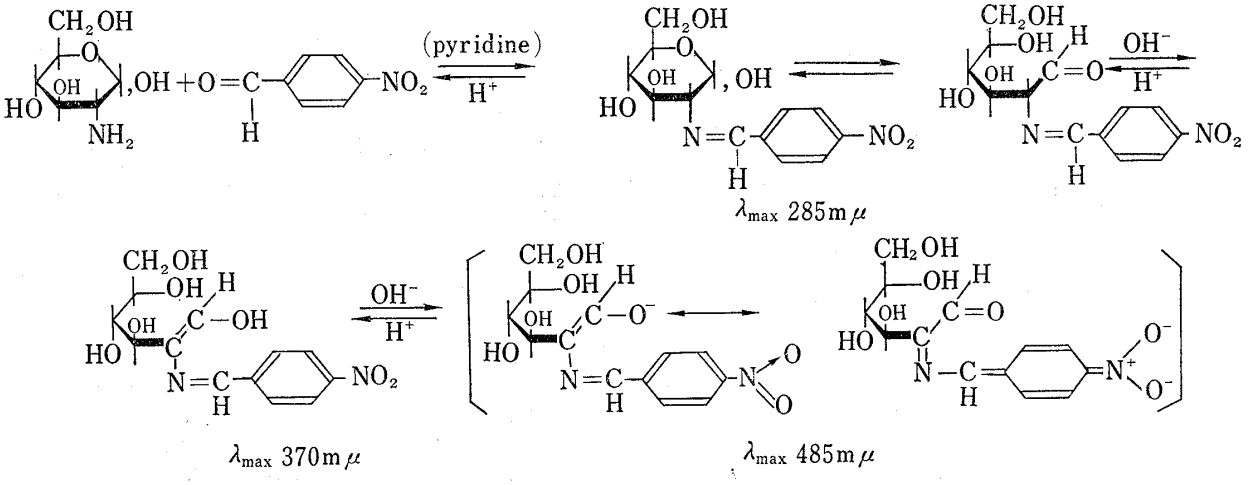

Chart 1. Mechanism of Color Reaction

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