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Analytical Chemical Studies on Amino Sugars. II.[1] Deterimation of Hexosamines using 3-Methyl-2-benzothiazolone Hydrazone Hydrochloride[2]

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A novel method for determination of hexosamines was established utilizing 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH) and ferric chloride. This method was highly sensitive and specific. All the procedure was carried out in mildly acidic aqueous solution at room temperature. The hexosamine content of chondroitin sulfates and hyaluronic acid estimated by this method showed good agreement with that calculated from nitrogen contents of the polysaccharides. Moreover, only 2 hr of hydrolysis with 2N hydrochloric acid was sufficient for the assay of these mucopolysaccharides.

The analytical procedures most frequently used for determination of hexosamines are those based on the Elson–Morgan[4] and Morgan–Elson[5] methods for free hexosamines and N-acyl hexosamines, respectively. The former method has also been recommended as the most effective tool in the assay of hexosamine residue in mucopolysaccharides[6] and glycoproteins.[7] However, Elson–Morgan reaction is not specific for hexosamine and requires strict observance of reaction conditions.[8] The indole–hydrochloric acid method reported by Dische Borenfreund[9] gives too low values when applied to chondroitin sulfates.[10] 2,4,6-Trinitrobenzenesulfonic acid method[11] and *p*-nitrobenzenediazonium salt method[12] were recently published but these methods are interfered by amino acids. Previously, the authors have reported a colorimetry[13] using *p*-nitrobenzaldehyde and a fluorometry[14] employing pyridoxal. The former is highly specific for 2-aminosugars and about equally sensitive as

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2) Preliminary communication of this work has been published, A. Tsuji, T. Kinoshita, and M. Hoshino, *Chem. Pharm. Bull.* (Tokyo), **17**, 217, (1969).
3) Location: *a*) *Hatanodai, Shinagawa-ku, Tokyo*; *b*) *Akasaka, Minato-ku, Tokyo.*
4) L.A. Elson and W.J.T. Morgan, *Biochem. J.* **26**, 1824 (1933).
5) W.J.T. Morgan and L.A. Elson, *Biochem. J.* **28**, 988 (1934).
6) J.S. Brimacombe and J.S. Webber, "Mucopolysaccharides," Elsevier Publishing Co., Amsterdam, 1964, p. 6.
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12) S. Ogawa, M. Morita, A. Nakajima, and A. Yoshida, *Yakugaku Zasshi*, **88**, 866, 871 (1968).
13) A. Nakamura, M. Maeda, K. Ikeguchi, T. Kinoshita, and A. Tsuji, *Chem. Pharm. Bull.* (Tokyo), **16**, 184 (1968).
14) A. Tsuji, T. Kinoshita, and M. Maeda, the 41st Annual Meeting of the Japanese Biochemical Society, Oct. 29, 1968; *idem*, *Seikagaku*, **40**, 495 (1968).

Elson-Morgan method. The latter is far more sensitive than any other analytical method for aminosugars although not exclusively specific for hexosamines.

The method described in this paper is based on the findings that 2,5-anhydrohexoses produced by deamination of hexosamines react with 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH)[15] to yield an intense blue color. Although this procedure utilizes deamination of hexosamines in the first stage like Dische–Borenfreund method and its modifications,[16,17] the principle of color development from the anhydrosugars is completely different from those of these methods. A probable mechanism of this color reaction has been published in the preliminary communication.[2] The present method was found to be sensitive, specific and simple. Satisfactory results were obtained on the estimation of hexosamine content of mucopolysaccharides.

Materials

Glucosamine hydrochloride, mannosamine hydrochloride and N-acetylglucosamine are the gift of Dr. Y. Hirasaka and Dr. S. Takanashi, Chugai Pharmaceutical Co., Ltd. Galactosamine hydrochloride and 3-methylbenzothiazolone hydrazone hydrochloride (MBTH) were purchased from Tokyo Kasei Kogyo Co., Ltd. Chondroitin sulfates were the products of Seikagaku Kogyo Co., Ltd. Hyaluronic acid is the gift of Prof. G. Matsumura, Showa University.

Methods

Reagents——(a) 5% $NaNO_2$, (b) 5% $KHSO_4$, (c) 12.5% $NH_4SO_3NH_2$, (d) 0.5% 3-Methylbenzothiazolone hydrazone hydrochloride (0.5% MBTH), (e) 0.5% $FeCl_3$, 100 ml of this reagent contains 0.83 g of $FeCl_3 \cdot 6H_2O$.

Reagent (d) and (e) should be prepared freshly every three days and stored in a refrigerator.

Determination of Hexosamines——To 1 ml of the sample solution containing 1 to 30 μg of hexosamine, 1 ml of 5% $KHSO_4$ and 1 ml of 5% $NaNO_2$ are added. The mixture is then left standing with occasional shaking for 15 min, upon which the deamination is completed. The excess nitrous acid is then removed by adding 1 ml of 12.5% $NH_4SO_3NH_2$ and repeatedly shaking the mixture for periods of 5 min. To the deaminated mixture is added 1 ml of 0.5% MBTH, and the reaction mixture is allowed to stand for 60 min. Finally, 1 ml of 0.5% $FeCl_3$ is added and the absorbance at 650 mμ is read after 30 min against the reagent blank.

Determination of Hexosamines in Mucopolysaccharides——The mucopolysaccharide solution in 2 N HCl (about 0.3 mg/ml) is heated in a sealed tube on a boiling water-bath for 2 hr and then cooled under tap water. One milliliter aliquot of the sample is pipetted into a 5 ml volumetric flask. Into the flask is added 1 drop of 0.5% alcoholic solution[18] of phenolphthalein followed by careful addition of 1 N NaOH until the solution turns pink. The solution is back-titrated dropwise with 1% $KHSO_4$ until the color just disappears and the resultant colorless mixture is made up to 5 ml with water. Into a test tube is pipetted 1 ml of this solution and the hexosamine content is estimated in the manner as described above, using either glucosamine hydrochloride or galactosamine hydrochloride as the standard hexosamine. Treatment of the standard hexosamines under hydrolytic conditions before assay is not necessary.

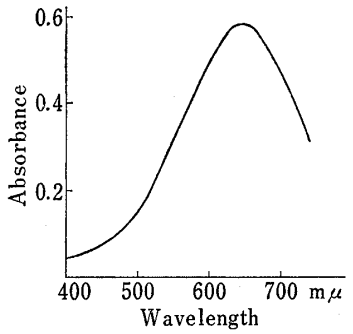

Fig. 1. Absorption Spectrum of the Reaction Product of Glucosamine by the Standard Procedure against Reagent Blank

final concn. of glucosamine: 9.27×10^{-5} M

Results and Discussion

Absorption Spectra and Molar Extinction Coefficients of the Reaction Products of Deaminated Hexosamine with MBTH

Fig. 1 shows the absorption spectrum of the reaction product obtained from glucosamine taken with the

15) E. Sawicki, T.R. Hauser, T.W. Stanley, and W. Elbert, *Anal. Chem.*, 33, 93 (1961).
16) D. Exley, *Biochem. J.* **67**, 52 (1957).
17) Y.C. Lee and R. Montgomery, *Arch. Biochem. Biophys.* **93**, 292 (1961).
18) The alcohol should be free of aldehyde.

Hitachi Model EPU-2 Spectrophotometer. The curve has a single maximum at 653 mμ. The absorption maxima and apparent molar extinction coefficients of hexosamine and their derivatives are shown in Table I. All these materials show a single peak in the range of 652±5 mμ.

Table I. Absorption Maxima and Apparent Molar Extinction Coeffcients of Hexosamines and Their Derivatives

Compounds	λ_{max}, mμ	ε_{max} (app.) $\times 10^{-4}$
D-Glucosamine	653	3.86
D-Galactosamine	653	3.62
D-Mannosamine	657	0.80
Methyl α-D-glucosaminide	651	3.89
Acid hydrolysate of chondroitin sulfate A[a)]	647	—
Acid hydrolysate of chondroitin sulfate C[a)]	649	—

a) Hydrolysis was carried out in a boiling water-bath for 2 hr with 2N hydrochloric acid.

Specificity of Reaction

Various sugars and amino acids were submitted to the color reaction and the results are shown in Table II. Neutral sugars, N-acylglucosamines, ascorbic acid and glucuronic acid yield no color. Most of amino acids give no color or very faint color which is negligible in the usual hexosamine determination. Although tryptophan, threonine and methionine exhibit color, the color intensities are far lower than those of hexosamines.

Table II. Compounds Tested for Interference (Chromogenicity of D-Glucosamine-HCl=100%)

	Compounds	Chromogenicity (%)
Sugars	D-glucose	0.1
	D-galactose	0.0
	D-xylose	0.1
	N-acetyl-D-glucosamine	0.0
	methyl N-carbobenzoxy-α-D-glucosaminide	0.0
	L-ascorbic acid	0.1
	D-glucuronic acid	0.0
Amino Acid	L-alanine	0.7
	L-arginine-HCl	0.1
	L-citrulline	1.0
	L-glutamine	0.2
	L-ornithine-HCl	0.7
	L-histidine-HCl	0.2
	L-leucine	0.7
	L-tyrosine	1.0
	L-glycine	1.6
	L-methionine	2.8
	L-threonine	4.7
	L-tryptophan	15.9
	L-serine	1.2

Color Development

Use of the initial deamination of hexosamines followed by reagents for reducing sugar was introduced by Dische and Borenfreund[9,10)] and subsequently had become the bases for some modifications. In these methods, 2,5-anhydrohexoses produced by deamination are heated in the presence of strong acid to give hydroxymethylfurfural or δ-hydroxylevulinalde-

hyde which reacts then with indole,[9] pyrrole[16] or phenol.[17] However, these reaction conditions are drastic and the reagents react also with neutral sugars. On the other hand, MBTH, proposed by Sawicki, *et al.*[15] for the microdetermination of aliphatic aldehydes, was found to react directly with 2,5-anhydrohexose at room temperature in a mildly acidic aqueous solution to give color.

Deamination——The deamination procedures hitherto published[9,16,17] for the determination of hexosamines employed acetic acid as a reagent for liberation of nitrous acid from sodium nitrite, but acetic acid often contains aldehydes. Potassium hydrogen sulfate was found to be superior to acetic acid because it gave lower blank value. Fig. 2 shows relationship between concentration of potassium hydrogen sulfate and color intensity. Highly concentrated reagent gives rise to decrease of color intensity. But the reagent should not be too dilute, otherwise excess nitrite can not completely be destroyed by sulfamate in the subsequent procedure. Five per cent is therefore selected as the most appropriate concentration of the reagent. As shown in Fig. 3, the decomposition of nitrous acid with ammonium sulfamate at room temperature is complete within 5 min and slight decrease in color intensity occurs with increase of time course, presumably due to the instability of 2,5-anhydrohexose. Five minutes are therefore chosen as the most suitable for this step.

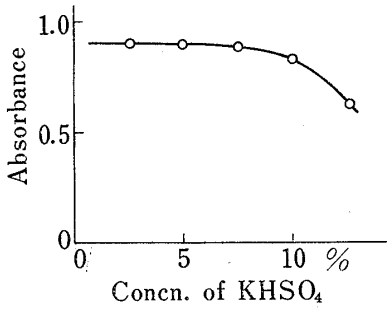

Fig. 2. Effect of Concentration of Potassium Hydrogen Sulfate on Color Intensity at 25°

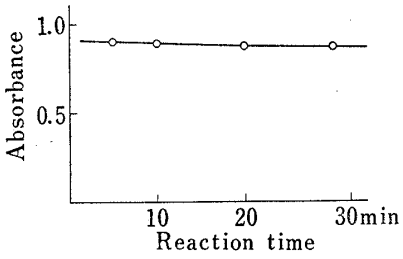

Fig. 3. Effect of Time Course for Decomposition of Nitrous Acid on Color Intensity at 25°

final concn. of glucosamine: 5.03 μg/ml as hydrochloride

Color Reaction——As shown in Fig. 4, the influence of concentration of MBTH on intensity and stability of color was evaluated by allowing the color to develop for period ranging from 0 to 100 min after addition of ferric chloride reagent. Maximum intensity and stability is obtained by the use of 0.5% MBTH and this concentration is employed in the standard procedure. Fig. 5 shows the effect of reaction time of MBTH and deaminated glucosamine on

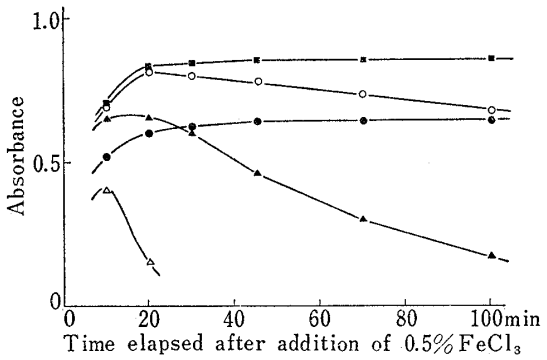

Fig. 4. Effect of Concentration of MBTH on Color Intensity at 25°

concn. of MBTH
●: 0.25% ■: 0.5% ○: 0.75% ▲: 1.0% △: 2.0%
final concn. of glucosamine: 5.02 μg/ml

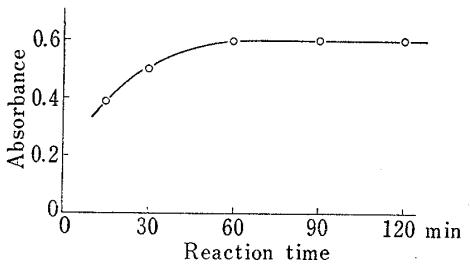

Fig. 5. Effect of Reaction Time of MBTH with Deaminated Glucosamine on Color Intensity at 25°

final concn. of glucosamine: 3.65 μg/ml as hydrochloride

color intensity. Maximum intensity is given in 60 min. Sixty minutes is therefore chosen as the most appropriate reaction time. Fig. 6 exhibits the influence of concentration of ferric chloride reagent on color intensity. This result indicates that the concentration of 0.5% or more gives highest intensity but highly concentrated reagent increases the blank value. Considering these facts, 0.5% $FeCl_3$ is employed.

Under these conditions, color intensity reaches its maximum in 30 min after addition of ferric chloride solution and is stable for more than 20 hr as ahown in Fig. 7.

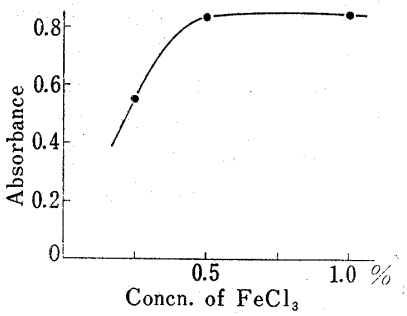

Fig. 6. Effect of Concentration of $FeCl_3$ on Color Intensity at 25°

final concn. of glucosamine: 5.52 μg/ml as hydrochloride

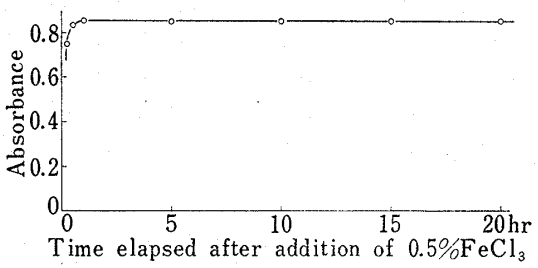

Fig. 7. Stability of Color developed by the Standard Procedure at Room Temperature

final concn. of glucosamine: 5.02 μg/ml as hydrochloride

Working Curves for Hexosamines

Fig. 8 shows the working curves for glucosamine and galactosamine. Galactosamine has a little smaller molar extinction coefficient than glucosamine (Table I). Both sugars give linear relationships in the range of 1 to 30 μg per ml. Mannosamine also gives linear working curve but its molar extinction coefficient is smaller than that of other hexosamines. Standard deviation estimated at 20 μg per ml glucosamine hydrochloride is 1.0 μg per ml (n=6).

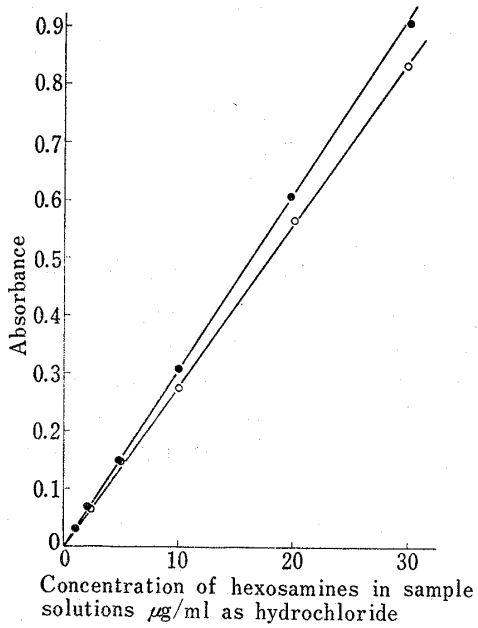

Fig. 8. Working Curves for Glucosamine (●) and Galactosamine (○)

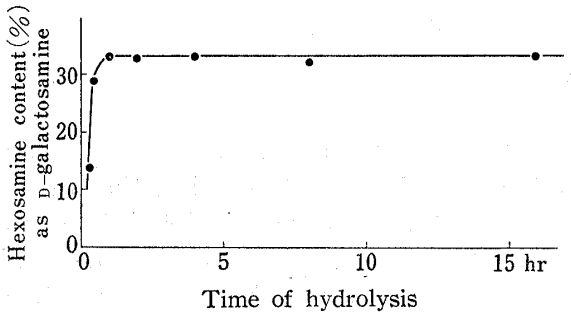

Fig. 9. Effect of Time of Hydrolysis of Chondroitin Sulfate A on the Hexosamine Value obtained in the Standard Procedure

Hydrolysis was carried out with 2N HCl in a boiling water-bath.

Determination of Hexosamines in Mucopolysaccharides

Chondroitin sulfate A was hydrolysed with 2N HCl in a boiling water-bath in various times and hexosamine content was estimated by the present method. As shown in Fig. 9, a plateau

was reached after 1 hr of hydrolysis and the hexosamine value at this stage was in good agreement with that calculated from nitrogen content of the mucopolysaccharide. This indicates that the polysaccharide is completely deacetylated in the course of 1 hr. Hydrolysis for 1 hr is, of course, not sufficient for complete cleavage of glycosidic linkages. Complete hydrolysis, however, does not seem to be necessary for the present method, because methyl glucosaminide gives almost identical molar extinction coefficient with free glucosamine as shown in Table I. Hydrolysis for 2 hr was therefore selected as the most suitable condition. Under the hydrolysis–condition employed, practically no collapse of hexosamine was observed. Recovery test of glucosamine from N-acetylglucosamine was carried out at three levels under the standard procedure. As listed in Table III, the recovery is almost complete. The working curve obtained from these data is identical, after correction according to the molecular weights, with that of glucosamine hydrochloride which is shown in Fig. 8. Hexosamine hydrochlorides can therefore be employed as the standard in the determination of hexosamines in N-acetylated mucopolysaccharide and treatment of the standard under the same hydrolysis condition is not required.

Table III. Recovery of Glucosamine after Hydrolysis of N–Acetylglucosamine with 2N Hydrochloric Acid for 2 hr in a Boiling Water–Bath

N–Acetylglucosamine (μg/ml)	No. of determination	Recovery of glucosamine (μg/ml as N–acetylglucosamine) (%)	
36.5	5	36.3±0.4	99.2±1.1
18.3	5	18.0±0.2	98.8±1.1
9.1	5	9.6±0.2	104.8±0.2

Table IV. Hexosamine Content in Mucopolysaccharides determined by the Present Method

Mucopolysaccharides	Nitrogen content (%)	Hexosamine content (%) calcd. from nitrogen content	Hexosamine content (%) found	Found / Calcd.
Chondroitin sulfate A	2.65	33.9	32.8	0.97
Chondroitin sulfate C	2.65	33.9	32.0	0.94
Hyaluronic acid	3.36	42.9	40.9	0.95

Table IV exhibits the hexosamine content in chondroitin sulfate A, chondroitin sulfate C and hyaluronic acid estimated by the present procedure. The observed values of hexosamine were over 94% of those calculated from nitrogen content of these materials.

The present method provides a more rapid and accurate tool for the estimation of hexosamines in mucopolysaccharides than other proper methods. Elson–Morgan method has generally been used but requires about 16 hr for hydrolysis at 100° with 2N hydrochloric acid and gives erroneous result when time of hydrolysis is shortened. Dische–Borenfreund method gives only 55% of the value found with Elson–Morgan reaction when applied to chondroitin sulfates.

In addition, free hexosamine can be exactly estimated by the present method in the presence of N-acylhexosamines. This advantage may positively be utilized in elucidation of structure of mucopolysaccharides and their derivatives. Application to several biological products is now under investigation and the results will be published in the near future.

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