A MICROMETHOD FOR THE COLORIMETRIC DETERMINATION OF N-ACETYL GROUPS IN ACID MUCOPOLYSACCHARIDES

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SUMMARY

Deacetylation of mucopolysaccharides and other N-acetylated compounds with 2 *N* HCl–methanol is a useful and simple procedure for determining N-acetyl groups in these compounds. The acetyl groups are converted to methyl acetate which is colorimetrically determined as a hydroxamic–ferric complex. This method permits the determination of N-acetyl groups in a range of 1 to 10 μmoles and is more sensitive than previously described methods. The reproducibility and some of the factors affecting the deacetylation and colorimetric analysis have been studied.

INTRODUCTION

The determination of acetyl groups is of considerable importance in the study of mucopolysaccharides. Available methods are tedious and require relatively large amounts of material. Earlier procedures have been comprehensively reviewed by MEYER[1]. More recently a number of modifications including variation of conditions of hydrolysis and methods of determination of liberated acetic acid[2,3] have been studied.

This paper describes the quantitative microdetermination of acetic acid present as an N-acetyl group in the hexosamine moiety of certain mucopolysaccharides, amino acids and aromatic amines. The methyl acetate formed by acid methanolysis is distilled *in vacuo* and determined colorimetrically by transformation to acetohydroxamic acid which yields a purple color with ferric salts. The intense color of the hydroxamic–ferric complex permits determination of acetyl groups in the range of 1 to 10 μmoles. The residue after deacetylation can be used for further analyses or isolations. This method is relatively simple and rapid.

EXPERIMENTAL

Apparatus

Fig. 1 illustrates the distilling apparatus. It consists of a chamber (A) with a side arm (B) so bent that the latter extends to about 20 mm above the bottom of a suction tube (C). This suction is connected to a desiccator tube filled with $CaCl_2$. Between the desiccator tube and the water pump there is a T tube which can be opened to the outside.

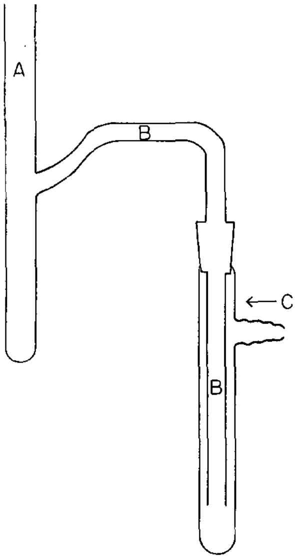

Fig. 1. Apparatus for the colorimetric microdetermination of N-acetyl groups. Tube A is 190 × 11 mm. All other dimensions are drawn to scale. A Polyethylene cap is used for chamber A, but a rubber stopper is preferable for chamber C.

Reagents

(a) Hydroxylamine HCl, 0.35 *M*. (b) NaOH 1.5 *M*. (c) Alkaline hydroxylamine. This solution is prepared just before using by mixing equal volumes of 0.35 *M* hydroxylamine HCl and 1.5 *M* NaOH. (d) Perchloric acid, 0.75 *M*. (e) Ferric–perchloric acid solution is prepared by dissolving 1.9 g of $FeCl_3 \cdot 6H_2O$ in 5 ml of concentrated HCl. To this solution is added 5 ml of 70 % perchloric acid. It is then evaporated almost to dryness and diluted to 100 ml with H_2O. The acidity of this solution is between 0.55 and 0.65 *M*. The ferric–perchloric acid solution also can be conveniently prepared by dissolving the equivalent amount of $Fe(ClO_4)_3 \cdot 6\,H_2O$* in 0.4 *N* perchloric acid.

The reagent for the deacetylation is prepared by absorbing dry HCl into refrigerated absolute methanol. An aliquot of the HCl–alcohol solution is titrated with standard alkali and a 2 *M* solution is prepared by dilution. This solution is stable for a few months in the cold if protected from moisture.

The 0.35 *M* hydroxylamine hydrochloride and ferric perchloric solutions are stable up to one month in the cold.

Procedure

An amount of the compound corresponding to 1.0 to 10 µmoles of acetyl is placed in a 70 × 7 mm pyrex tube. This solution is evaporated to dryness in a vacuum desiccator over anhydrous $CaCl_2$ by use of an oil pump. To the dried residue is added 0.5 ml of 2 *N* HCl–methanol solution. The acid methanol solution is cooled for a few seconds with a mixture of solid CO_2–methanol and the tube is rapidly sealed with an oxygen torch. The tube is heated at 100° in a water bath for 4 h, cooled, and opened close to the end of the sealed tip.

Distillation

The deacetylation tube is carefully placed in the bottom of chamber A of the

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* The G. Frederick Smith Chemical Co., Columbus, Ohio.

distillation apparatus, which is maintained in an upright position. Suction tube (C) is cooled to the level of the sidearm with a solid CO_2–methanol mixture during the entire distillation. After evacuation (30 to 40 mm Hg), chamber A is submerged to a depth of within 10 mm of the sidearm in a water bath at 35 to 45°. Distillation under these conditions proceeds smoothly. After all the acid alcohol solution is distilled and collected in the suction tube (C), the polyethylene cap is removed from chamber A and 0.5 ml of absolute methanol is carefully added to the deacetylation tube and distillation is repeated. This complete operation requires 20 min.

Determination of methyl acetate

After complete distillation, tube C is removed and placed in a water bath at 20–25°. After 2 min 1.0 ml of H_2O is added, followed by 2.0 ml of alkaline hydroxylamine solution. The tube is shaken and allowed to stand for 10 min. 2 ml of 0.75 *M* perchloric acid is added, the mixture is shaken, and 1.0 ml of ferric perchloric acid solution is added.

The color is read in 5 to 10 min at 520 mμ in the Coleman Junior Spectrophotometer. (For a large number of samples it has been found convenient to keep the distillates in a freezer after stoppering and covering the sidearms with parafilm until they are to be analyzed.) The quantity of methyl acetate is estimated by comparison with ethyl acetate standards.

Preparation of the standard curve

The standard solution is 0.005 *M* ethyl acetate in methanol–H_2O (50:50, v/v).

Suitable aliquots of this standard are diluted to a volume of 2.0 ml with methanol–H_2O (50:50, v/v) to yield amounts varying from 1 to 10 μmoles. An HCl–methanol blank is used.

RESULTS

Factors affecting deacetylation

HCl–methanol was chosen as a deacetylation agent. Advantages of this reagent are the rapid methanolysis of acetyl groups and the ease of removal of HCl. During methanolysis, methyl chloride is formed, but this compound does not interfere with the colorimetric analysis.

Table I represents the results of experiments designed to evaluate the extent of reaction of HCl with methanol. Several concentrations of HCl in absolute methanol were heated at 100° in sealed tubes for varying lengths of time. This table shows that a rapid drop of acidity occurs during the first minutes of methanolysis.

Fig. 2 indicates the effect of HCl concentration on deacetylation. The results obtained with several compounds indicate that the maximum deacetylation is obtained with 2 *N* HCl–methanol. In the case of S-tribromo N-acetanilide, a compound in which the N-acetyl group is difficult to remove, the determination of acetyl content was accomplished by the use of 3.5 *N* HCl–methanol.

Fig. 3 illustrates the rate of deacetylation of several compounds with 2 *N* HCl–methanol. The results indicate that with these compounds, complete deacetylation is accomplished in less than 4 h.

It should be emphasized that o-acetyl groups are also liberated; consequently,

TABLE I

RATE OF CHANGE OF THE ACID MOLARITY

The acid molarity was determined by alkali titration using phenolphthalein as indicator

Time (h)	*HCl–methanol Molarity*				
0	3.67	2.76	2.08	1.50	1.00
0.25	1.16	0.94	0.75	0.50	0.43
0.50	0.71	0.63	0.48	0.35	0.28
1.0	0.40	0.35	0.28	0.20	0.15
2.0	—	0.24	0.18	0.10	0.06
5.0	—	0.08	0.06	0.05	0.04

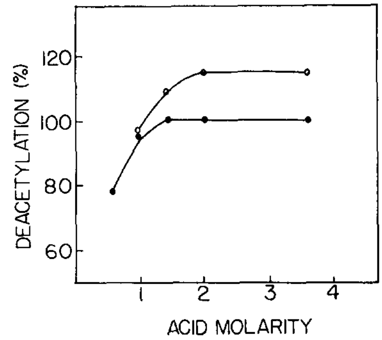

Fig. 2. Percent deacetylation of hyaluronic acid (○), and N-acetylglucosamine (●), in 4 h at 100° in sealed tubes at different acid molarities. Percent deacetylation of HA calculated on the basis of the colorimetric analysis for glucosamine[28]. Per cent deacetylation of N-acetylglucosamine calculated on the basis of N-acetylglucosamine analysis[25].

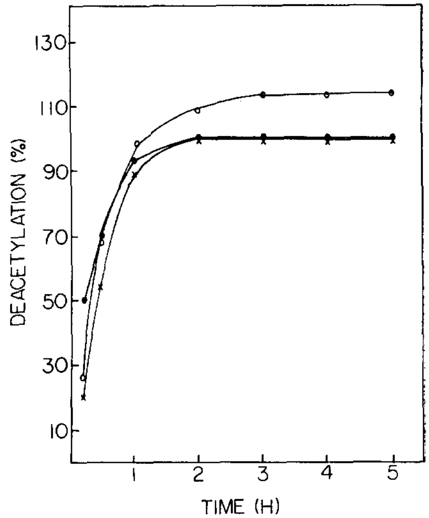

Fig. 3. Percent of deacetylation of N-acetylglucosamine (●), N-acetylglycine (×), and hyaluronic acid (○), versus time using 2 *N* HCl–methanol at 100° in sealed tubes. Percent deacetylation was calculated on the basis of colorimetrically determined hexosamine content[28]. Percent deacetylation of N-acetylglycine calculated on the basis of nitrogen determined colorimetrically by a micro-Kjeldahl procedure.

under the conditions of this method differentiation between these two groups is not possible.

Color development

The usefulness of the qualitative "spot test" first described by Feigl[4,5] has been established in the quantitative colorimetric estimation of carboxylic esters[6–10]. Similar reactions have been found for anhydrides[5,11], nitrites[12], amides[13,14], and lactones[15,16]. Colorimetric hydroxamic acid determinations have been described for acetylated pectic acid[17] and o-acetylated proteins[18,19].

The reagents used in this determination are essentially the alkaline hydroxylamine reagent of Hestrin[9] and the Hill ferric perchloric acid solution[7,16] suitably modified for this purpose.

Previous reports have indicated that hydroxamic acid formation occurs most rapidly in strongly alkaline solutions[9, 10]. However, since an acid solution is necessary for color development it is important that sufficient perchloric acid be added. A sharp decrease in the color intensity was observed when the molarity of the added $HClO_4$ was below 0.6 M. No changes in color intensity were obtained with $HClO_4$ solution up to 0.85 M.

A study has been carried out of certain other variables which influence this method. The effect of temperature and time on the formation of the hydroxamic acid in alkaline solution has been studied[16]. As previously reported[15], variations in temperature between 20° and 30° have little effect on color intensity; however, at —15° there is a marked increase in color intensity. It is, therefore, important that color development be carried out at 20–30°.

GODDU *et al.*[20], observed that changes of solvent result in change of absorption intensity. Higher values were observed when H_2O was replaced by alcohol. We found an increase of about 20 % in color intensity when color development was carried out in a final concentration of 75 % methanol rather than in the final concentration of 15 % methanol. The latter concentration is preferred in this method because the color is more stable.

The fading of the color is less than 1 % between 5 and 10 min. At 30 and 60 min, a 4 and 8 % decrease in color intensity, respectively, was observed.

Reproducibility

In order to determine the reliability of the method, duplicate determinations on standards were carried out. The average values and standard deviations of six such determinations are given in Table II.

TABLE II

CALCULATED AVERAGE ABSORBANCY AND STANDARD DEVIATION AT EACH CONCENTRATION

Concentration µmoles/7 ml	*Average absorbancy*	*Standard deviation*
2	0.281	0.007
4	0.570	0.012
6	0.837	0.012
8	1.100	0.017
10	1.375	0.016

Analyses

Although this method was devised primarily to determine quantitatively the N-acetyl groups in mucopolysaccharides, its usefulness is not limited to these compounds. Good results have also been obtained for N-acetyl amino acids and N-acetylated aromatic amines.

Table III shows the results obtained with several N-acetylated compounds. Some of these results are correlated with the chromic acid oxidation method of WIESENBERGER[29].

It is observed that in both methods the acetyl content of mucopolysaccharides is higher than the colorimetrically determined hexosamine content. This is due to the fact that the colorimetric method which was used to determine hexosamines con-

TABLE III

ANALYTICAL RESULTS*

Explanation of the letters in the Table: [a] 14-h hydrolysis with 4 *N* HCl[28] at 100°, without using Dowex 50 H^+. [b] Micro-Kjeldahl procedure. [c] As obtained from Dr. A. WINTERSTEIN. [d] Purity of N-acetyl amino acids and aromatic amines were checked by their melting points. [e] Determination of N-acetyl content by using 3.5 *N* HCl–MeOH. [f] Hexosamine ratio determined by N-acetylglucosamine analysis[25].

	Hexosamine[a]	*Glucuronic acid*[21]	*Nitrogen*[b]	*N-acetyl (colorimetric method)*				*N-acetyl (chromic acid method*[29]*)*
CSA** from cartilage[22]	1.0	1.13		1.11	1.09	1.10		1.10
CSA from cartilage[22]	1.0	1.12		1.16	1.16	1.15		1.12
Umbilical cord HA*** [23]	1.0	1.12		1.10	1.11	1.12	1.10	1.13
Streptococcus HA[23]	1.0	1.13		1.16	1.15	1.16	1.15	1.10
CSA-B[24]	1.0			1.22	1.20	1.24	1.24	1.20
CSA-B [c]	1.0			1.26	1.26	1.28		
N-acetylglucosamine[25]	1.0[f]			1.01	1.00	1.02		0.99
N-acetyltryptophan [d]			1.0	1.05	1.02			
N-acetyl leucine			1.0	0.98	0.97			
N-acetyl phenylalanine			1.0	1.01	1.00			
N-acetyl glycine			1.0	1.02	1.00			
Acetanilide			1.0	1.06	1.04			
S-tribromoacetanilide[26] [e]			1.0	0.92	0.94			

* All analyses are given as molar ratios with respect to hexosamine or nitrogen.
** Chondroitin sulfuric acid-A.
*** Hyaluronic acid.

sistently gave slightly low results. Similar effects have been noticed by other investigators when hexosamine containing samples were hydrolyzed with strong acid for long periods of time[28]. However, it is advantageous to relate N-acetyl groups with the colorimetrically determined glucosamine analysis in samples which are too small to be weighed.

Compounds containing no acetyl groups, such as glycogen, heparin, glucosamine HCl, glucuronolactone and glycine gave the same color intensity as the blank. Samples containing small amounts of salts of inorganic volatile acids do not affect the N-acetyl group analysis. Salts of volatile organic acids interfere with this method.

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THE EFFECT OF OXYGEN UPON THE MICRO DETERMINATION OF HISTIDINE WITH THE AID OF THE PAULY REACTION

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SUMMARY

The colour obtained upon the addition of diazosulphanilic acid to histidine in alkaline medium (Pauly reaction) is bleached rather suddenly after a certain lag. This lag is shorter the more oxygen is present in the alkaline reaction medium.

A method of determining histidine on a micro scale, consisting of an improved Pauly reaction after paperchromatographic separation, is described in detail.

INTRODUCTION

Although accurate chromatographic methods for determining the amino acid content of protein hydrolysates are available, methods requiring but simple equipment are still useful, especially where only a limited number of amino acids need to be determined. The determination of histidine by a combination of paper chromatography and Pauly reaction, as described by Fraenkel-Conrat[1], is an example of such a method.