A MODIFIED COLORIMETRIC METHOD FOR THE ESTIMATION OF N-ACETYLAMINO SUGARS*

By JOSÉ L. REISSIG, JACK L. STROMINGER, and LUIS F. LELOIR

(From the Instituto de Investigaciones Bioquímicas, Fundación Campomar,† Buenos Aires, Argentina, and the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland)

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The usefulness of the method of Aminoff, Morgan, and Watkins (1) for the estimation of *N*-acetylamino sugars is limited by interference resulting from susceptibility to minor variations in the pH of the sample and from the presence of sugars plus amino acids (1, 2) or magnesium ions. With the present method, as the result of substituting a concentrated borate buffer for the original carbonate buffer and introducing several concomitant changes, such limitations are largely overcome. In addition, the color yield is increased about 2-fold, and the time required for full color development is shortened from 1½ hours to 20 minutes. The method has been applied in a number of analytical situations.

An enhancing effect of borate upon color production had been noticed by Aminoff *et al.*, who, however, did not take advantage of it in their standard assay method.

Materials and Methods

Apparatus—Optical density of the solution was measured with a Beckman quartz spectrophotometer (model DU), with Corex cells and a 1 cm. light path. Wave-length calibration was performed with reference to the 656 mμ of the hydrogen lamp. A further check on the calibration was afforded by the location of an absorption band at 550 mμ in a solution of cytochrome *c* plus sodium dithionite.

Reagents—

Potassium tetraborate (Fisher Scientific Company). A solution 0.8 M in borate is prepared, and the pH is adjusted to 9.1 with KOH (pH 8.9 when diluted 6-fold). For many of the experiments reported here, the borate was prepared by adding the calculated amount of KOH to a H_3BO_3 solution. The solution was then concentrated, and the crystalline mass was washed with cold water and dried. The pH was 9.2 (when diluted).

p-Dimethylaminobenzaldehyde (DMAB) reagent. 10 gm. of DMAB

* Similar methods were developed independently in Buenos Aires and Bethesda, and it was decided to combine experiences in a single publication.

† J. Alvarez, 1719.

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(Eastman Kodak Company, Rochester, New York, or Amend Drug and Chemical Company, Inc., New York)[1] are dissolved in 100 ml. of analytical reagent glacial acetic acid which contains 12.5 per cent (volume per volume) 10 N HCl (analytical reagent). This reagent could be stored at 2° for a month without significant deterioration. Shortly before use it was diluted with 9 volumes of reagent grade glacial acetic acid.

N-Acetylglucosamine. A commercial sample (Nutritional Biochemicals Corporation, Cleveland), which contained 5 to 10 per cent glucosamine, was purified by passing it through Dowex 50 H^+ and recrystallizing from the effluent according to White (3). Another sample was prepared from glucosamine hydrochloride as indicated by White (3) and purified as above.

N-Acetylgalactosamine. Samples were kindly supplied by Dr. C. E. Cardini (prepared according to Roseman and Ludowieg (4)) and Dr. S. Roseman.

Assay Procedure

To the sample, blank and standard, each contained in a volume of 0.5 ml. in a 13 × 100 mm. test-tube, 0.1 ml. of potassium tetraborate is added. The tubes are heated in a vigorously boiling water bath for exactly 3 minutes and cooled in tap water. 3 ml. of DMAB reagent are then added, and, immediately after mixing, the tubes are placed in a bath at 36–38°. After precisely 20 minutes the tubes are cooled in tap water and read without delay at 544 or 585 mμ. In the experiments to be reported, the wavelength 544 mμ was used (according to Aminoff *et al.* (1)) unless otherwise indicated. However, the wave-length 585 mμ may be preferred, as some increase in sensitivity results at no disadvantage and the color due to interfering substances is considerably reduced at this wave-length.

The method has been adapted to the semimicro and micro scales, with final volumes of 0.5 and 0.05 ml., respectively. Volumes of all reagents were proportionally reduced. Readings were made in cells of 5 and 1.5 mm. width, respectively (1 cm. light path), obtained from the Pyrocell Manufacturing Company, New York.

For comparison, the procedure of Aminoff *et al.* (1) was carried out essentially as described, except that the final volume was reduced to 5.5 ml.

Results

Optimal Conditions for Color Production

The production of color in this test depends upon two independent processes: the formation of an intermediate compound, possibly a glucoxazo-

[1] With the present samples of DMAB, it was not necessary to recrystallize as indicated by Aminoff *et al.* (1).

line (1, 3, 5), formed by heating the acetylhexosamine with alkali, and the reaction of this intermediate with DMAB during the development of color in an acid medium.

Borate Heating Step—The effects of varying the time of heating and the pH and concentration of the borate solution are depicted in Figs. 1, 2, and 3 (Curve A), respectively. The optimal heating time is 3 minutes, and, if prolonged to 10 minutes, the color yield is reduced by 10 per cent.

The optimal pH is 8.9. This is conveniently close to the pH at which borate has maximal buffering power (the pK for the dissociation of the first H^+ in H_3BO_3 is 9.2).

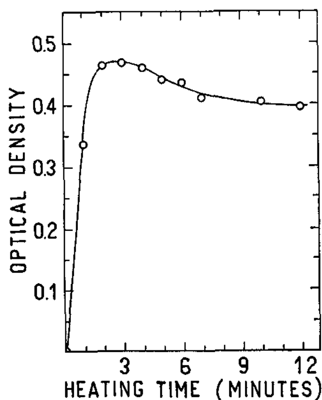

FIG. 1

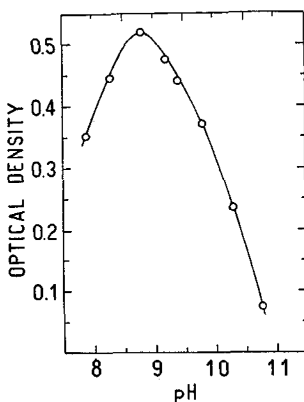

FIG. 2

FIG. 1. Effect of time of heating on color yield from 0.1 μmole of acetylglucosamine assayed by the present method.

FIG. 2. Effect of pH of the borate solution on color yield. The samples contained 0.1 ml. of 0.8 M borate, 0.1 μmole of acetylglucosamine, various amounts of HCl or KOH, and water to make up 0.6 ml.

Color Development Step—The rate of color development is dependent upon the incubation temperature and also upon the acidity and the water content of the final mixture. At room temperature full color development requires 90 to 120 minutes. At 36–38°, as employed in this study, full color develops in 20 minutes (Fig. 4) and then begins to fade at a rate of 1.5 per cent loss every 5 minutes. The loss can be brought down to 0.5 per cent every 5 minutes by cooling to room temperature (22–25°). At higher temperatures (60° or 100°) color develops in a shorter time, but fades so rapidly as to make the use of these temperatures impractical.

Borate concentration, above a certain threshold, has little effect on the formation of the intermediate compound (Fig. 3, Curve A), but the amount of borate does influence markedly color development (Curve B). If too

much borate is added to the sample, color development (both rate and yield) is interfered with. Borate interferes with color development in part, at least, because it lowers the acidity of the final mixture; the higher the HCl content of the DMAB reagent, the lower the borate interference.

The influence of each of the above variables on color intensity was followed quantitatively. It was concluded that the conditions chosen for

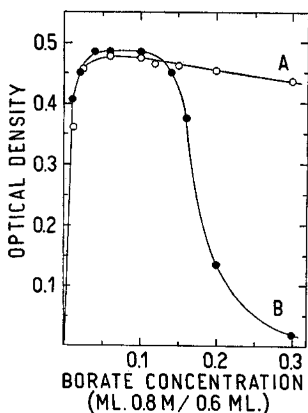

FIG. 3

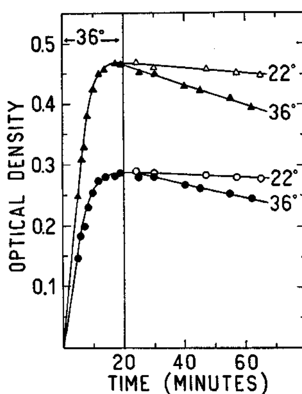

FIG. 4

FIG. 3. Effect of borate concentration on color yield from 0.1 μmole of N-acetylglucosamine assayed by the present method. For Curve A, the borate concentration was maintained only during heating at 100°, by using either less than 0.1 ml. of borate or less than 0.5 ml. of sample. Before the DMAB was added, the mixture was made up to standard conditions by adding either borate solution or water. For Curve B, borate was added initially up to the indicated concentration in a total volume of sample plus borate of 0.6 ml.

FIG. 4. Rate of color development and fading with the present method. ● and ▲, samples kept throughout at 36°; ○ and △, placed at 22° after 20 minutes at 36°. Upper curves, 0.1 μmole of acetylglucosamine; lower curves, 0.06 μmole. Zero time was taken as the time of addition of the DMAB reagent. The temperature was controlled by means of the thermostatic attachment on the spectrophotometer.

the standard procedure are optimal and that deviations of 10 per cent in the volume of the sample, the borate solution, or the DMAB reagent have no effect on the assay besides that which results from fluctuations in the final volume.

Higher readings can be obtained by increasing the concentration of DMAB in the reagent (*e.g.*, 5 per cent higher with a 30 per cent increase in concentration). This had also been noticed by Aminoff *et al.* (1) who, however, used one-fifth as much DMAB as is employed in the present test. They had to limit the DMAB concentration to avoid exceedingly high blank readings, a difficulty not encountered in our experiments with the borate

method. A higher DMAB concentration also widens the range in which optical density is proportional to the amount of acetylglucosamine.

Sensitivity and Specificity

Plots of the color given by standard solutions of *N*-acetylglucosamine and *N*-acetylgalactosamine are shown in Fig. 5. With acetylglucosamine there is a slight departure from linearity for quantities greater than 0.13 μmole. The $\epsilon_{1\text{ cm.}}^{585\text{ m}\mu}$ for *N*-acetylglucosamine[2] ranges from 18,000 to 21,000 by the present procedure, and from 9000 to 10,000 with the method of Aminoff *et al.* Corresponding values for *N*-acetylgalactosamine are about 7400 with the present method and 2100 with the method of Aminoff *et al.* (35

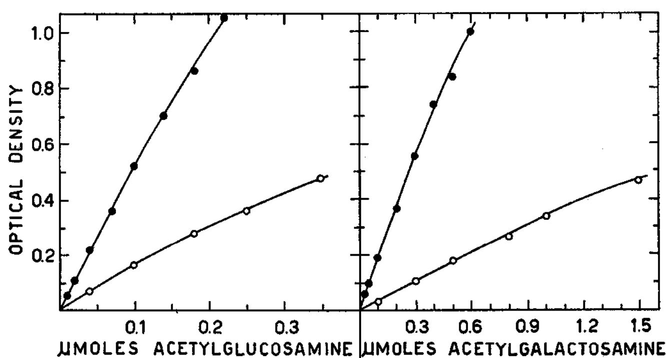

FIG. 5. Relationship between quantity of the acetylhexosamine and color yield. ●, as assayed by the present method; ○, by the method of Aminoff *et al.*, performed in a final volume of 5.5 ml.

and 21 per cent, respectively, of the color developed with *N*-acetylglucosamine).

With the micromodification of this method, as little as 3×10^{-10} mole of acetylglucosamine can be determined.

The *N*-acetylamino uronic acids of Park (6) have been reported to give 2 to 3 times the color yielded by *N*-acetylglucosamine in the original procedure. However, with the present method the color given by these compounds is equivalent to that produced by *N*-acetylglucosamine.[3] With either method, *N*-acetylglucosamine-6-phosphate gives the same extinction coefficient that *N*-acetylglucosamine has at 544 and 585 mμ[4] (7, 8).

[2] $\epsilon_{1\text{ cm.}}^{585\text{ m}\mu}$, the molar extinction coefficient for the chromogen at the indicated wavelength and light path, has been calculated on the basis of the molarity in the final reaction volume of the substance being analyzed. Since the nature and the molarity of the colored compound are unknown, this value is not necessarily the extinction coefficient of the colored compound.

[3] Strominger, J. L., in preparation.

[4] Reissig, J. L., in preparation.

Uridine diphosphate *N*-acetylglucosamine, uridine diphosphate *N*-acetylgalactosamine, and *N*-acetylglucosamine-1-phosphate fail to give the test unless they are previously hydrolyzed with acid. The nucleotides can be hydrolyzed by heating at 100° in 0.01 N acid for 15 minutes (9, 10), but the sugar phosphate requires more rigorous conditions, *e.g.* 5 minutes at 100° in 0.1 N HCl.[5]

Interfering Substances

Glucose Plus Lysine—The color produced in the original and in the present method by an equimolar mixture of glucose plus lysine (pH 10.8) was compared. The ratio of $\epsilon_{1\text{ cm.}}^{585\text{ m}\mu}$ for acetylglucosamine to $\epsilon_{1\text{ cm.}}^{585\text{ m}\mu}$ for the glucose-lysine mixture is about 4500 by the present method and about 100 by the original method (for the glucose plus lysine concentrations used in the experiment of Fig. 6, which are not high enough to inhibit color development). With either method, the spectra[6] of the chromogens formed by the sugar plus amino acid mixture and by *N*-acetylamino sugars are distinctly different (Fig. 6), as has been already reported by Aminoff *et al.* (1). At 585 mμ the sugar plus amino acid interference is at its minimum. This is particularly marked in the borate method (Fig. 6), and the interference is, therefore, greatly reduced.

Magnesium Ions and Salts—The color given by acetylglucosamine in the present test is inhibited only 2 per cent by 0.03 M magnesium ions, but 67 per cent in the original method. No inhibition was observed when the sample was made up in 1 M NaCl.

Acids and Bases—The advantages of the higher buffering capacity afforded by the borate in the new test are brought out by Fig. 7, inspection of

[5] However, Kuhn *et al.* (5) have recently reported that with the Morgan-Elson method not all 1 substituents require acid hydrolysis, and also that substitution at the 4 position abolishes the color reaction. Attention may also be directed to the fact that with the original procedure the reaction may be given by a number of *N*-acetyl-2-amino sugars of varying chain length, that *N*-acetyl can be replaced by other *N*-acyl groups, and that other compounds (chiefly pyrroles) give the reaction, although the characteristics of these reactions reportedly differ from the reaction given by *N*-acetylamino sugars. These data have been recently reviewed by Kent and Woodhouse (11). No attempt has been made to evaluate these factors by the present procedure.

[6] If the spectra reported here (Fig. 6), obtained both in Bethesda and in Buenos Aires, and those of Cabib *et al.* (9) are compared with the spectra presented by Aminoff *et al.* (1), it will be noticed that they are shifted about 5 mμ relative to each other. We have no explanation to offer for this discrepancy, except that an inaccurate wave-length calibration of the spectrophotometer might be involved. If a spectrophotometer of greater band width is used in the assay of acetylglucosamine, the spectrum of the chromogen should be checked with that apparatus. The Coleman junior spectrophotometer (model 6A), for instance, yields a spectrum with a single maximum located at about 565 mμ.

which shows that in assaying acid-labile N-acetylhexosamine it is often unnecessary to neutralize the acid used for hydrolysis.

Provision of Blanks—In dealing with crude biological materials, it is sometimes found that color is produced even if the sample has not been heated with borate. An appropriate blank, in which the 3 minute boiling is omitted, should then be included.

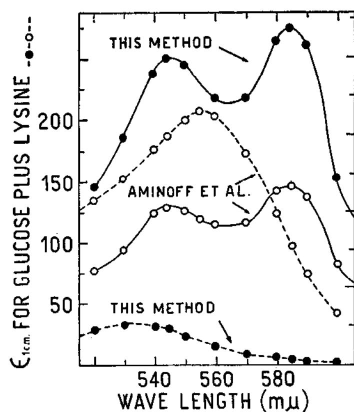

FIG. 6

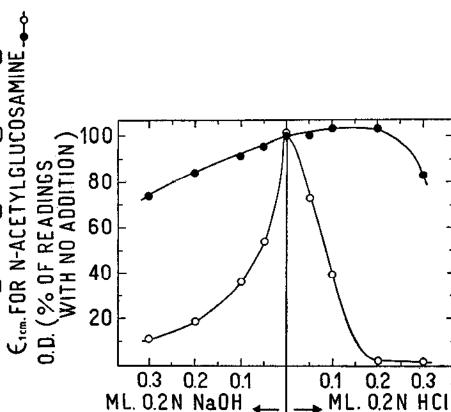

FIG. 7

FIG. 6. Absorption spectra of the color developed. ●, present method; ○, method of Aminoff *et al.* Molar extinction coefficients are plotted on the ordinates; the scale on the right and the solid lines correspond to the spectra of N-acetylglucosamine (0.2 μmole assayed with the original method or 0.05 μmole by the present method); the scale on the left and the dotted lines correspond to the spectra of lysine plus glucose (8 μmoles of each by the original method or 20 μmoles by the present one).

FIG. 7. Effect of adding acid or base on color yield. ●, present method (0.06 μmole); ○, method of Aminoff *et al.* (0.2 μmole). The N-acetylglucosamine and the acid or base added were contained in the standard sample volume (0.5 ml.) prior to heating with borate. The pH of the borate buffer (diluted plus sample volume) was 9.2.

Applications of Method

The method has been employed in a number of analytical situations: (*a*) Study of the enzymatic conversion of acetylglucosamine-1-phosphate into acetylglucosamine-6-phosphate (7). (*b*) Assay of the N-acetylglucosamine and N-acetylgalactosamine content of mixtures of uridine diphosphate acetylglucosamine and uridine diphosphate acetylgalactosamine. Pontis (10) has differentiated both acetylhexosamines, taking advantage of the fact that after deacetylation they give equivalent readings per mole in the Elson-Morgan (12) reaction, while acetylgalactosamine produces lower readings by the present test. Mixtures of standard solutions of both ace-

tylhexosamines give additive values by the present procedure. (*c*) Analyses of the amounts of easily hydrolyzable *N*-acetylamino sugars in crude extracts of animal and bacterial tissues. With the extracts of hen oviduct, rabbit liver, *Staphylococcus aureus*, and *Lactobacillus helveticus* 335 the validity of the estimation was checked by chromatographing the extracts on anion exchange resins and estimating the recovery of *N*-acetylamino sugar-containing compounds. Recoveries were 85 to 95 per cent (13). The method was also satisfactorily employed for analysis of the effluents from the anion exchange columns (8). (*d*) The *N*-acetylamino sugars in the uridine nucleotides from *S. aureus* (6), yeast (9), and hen oviduct (8) have been estimated quantitatively.

SUMMARY

1. A modification of the method of Aminoff, Morgan, and Watkins for the estimation of *N*-acetylamino sugars has been described and compared with the original procedure. It is less time-consuming and affords enhanced sensitivity, more stringent specificity, and less susceptibility to factors which might interfere with color development.

2. With the present procedure the molar extinction coefficient of the chromogen (based on acetylhexosamine concentration) is about 21,000 for *N*-acetylglucosamine and about 7400 for *N*-acetylgalactosamine at the wave-length of maximal absorption (1 cm. light path). With a micro-adaptation of the method 3×10^{-10} mole of *N*-acetylglucosamine can be determined.

3. Several applications of the method are described.

BIBLIOGRAPHY

1. Aminoff, D., Morgan, W. T. J., and Watkins, W. M., *Biochem. J.*, **51**, 379 (1952).
2. Vasseur, E., and Immers, J., *Ark. Kemi*, **1**, 253 (1949).
3. White, T., *J. Chem. Soc.*, 428 (1940).
4. Roseman, S., and Ludowieg, J., *J. Am. Chem. Soc.*, **76**, 301 (1954).
5. Kuhn, R., Gauhe, A., and Baer, H. H., *Chem. Ber.*, **87**, 1138 (1954).
6. Park, J. T., *J. Biol. Chem.*, **194**, 877, 885, 897 (1952).
7. Reissig, J. L., *Actes bioquím.*, Rosario, **2**, 52 (1953).
8. Strominger, J. L., *Biochim. et biophys. acta*, in press.
9. Cabib, E., Leloir, L. F., and Cardini, C. E., *J. Biol. Chem.*, **203**, 1055 (1953).
10. Pontis, H. G., *J. Biol. Chem.*, **216**, 195 (1955).
11. Kent, P. W., and Woodhouse, M. W., Biochemistry of the amino sugars, London, 311 (1955).
12. Elson, L. A., and Morgan, W. T. J., *Biochem. J.*, **27**, 1824 (1933).
13. Strominger, J. L., *J. Pharmacol. and Exp. Therap.*, **110**, 47 (1954).