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Note

Assay for 2-amino-2-deoxy-D-glucose in the presence of other primary amines

ROBERT L. BENSON

Department of Entomology, Washington State University, Pullman, WA 99163 (U. S. A.)

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2-Amino-2-deoxyhexoses may be assayed by the Elson–Morgan reaction[1–3], by the *p*-nitrobenzaldehyde reaction of Nakamura[4,5], by reaction with ninhydrin after separation from amino acids on an amino acid analyzer[6], and by the Levvy–McAllan[7] modification of the Morgan–Elson reaction[8]. Accurate determination of 2-amino-2-deoxyhexoses requires separation from other components by ion-exchange chromatography[2,6,9], except with the less sensitive Nakamura method.

During investigations of D-glucosamine phosphate isomerase (E.C. 5.3.1.10)[10,11], we preferred the Levvy–McAllan assay to the Nakamura assay because of its greater sensitivity. As with work in other laboratories[9], inhibition of the Levvy–McAllan assay by glycine and other primary amines has been noted, and a simple procedure to avoid this problem is described here.

The Morgan–Elson[8] assay for 2-amino-2-deoxy-D-glucose, as modified by Levvy and McAllan[7], involves (Step 1) conversion into 2-acetamido-2-deoxy-D-glucose with acetic anhydride at alkaline pH, and borate may then be used to complex the *N*-acetylated sugar in the furanose form; (Step 2) heating at alkaline pH to decompose the excess of acetic anhydride, and to cause dehydration of the proposed furanose–borate complex to monoanhydro sugar derivatives; and (Step 3) treatment of the 5-membered ring monoanhydro sugars with Ehrlich reagent (*p*-dimethylaminobenzaldehyde in HCl–glacial acetic acid) to cause additional dehydration to furan derivatives that subsequently react with *p*-dimethylaminobenzaldehyde with the formation of a colored complex (reviewed by Horton[12]). Primary amines interfere during Step 1 by competing with 2-amino-2-deoxy-D-glucose for acetic anhydride.

Procedures A and B described in the experimental section detect 2-amino-2-deoxy-D-hexoses, 2-acylamido-2-deoxy-D-hexoses[13], and their 6-phosphates (but not 1-phosphates[14]). Whereas several D-glucose derivatives (2-amino-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-glucose, and their 6-phosphates) give equal amounts of color per mole by these procedures, other amino sugars, such as 2-amino-2-deoxy-D-mannose or 2-amino-2-deoxy-D-galactose, do not give equivalent amounts of color per mole when compared to 2-amino-2-deoxy-D-glucose[7,15].

Procedures A and B utilize 26.5 and 53 μmol of acetic anhydride respectively, and allow quantification of up to 100 nmol of 2-amino-2-deoxy-D-glucose in mixtures containing up to 20 and 40 μmol of other primary amines, respectively. Procedure C does not utilize acetic anhydride and detects only 2-acylamido-2-deoxy-D-hexose sugars and their 6-phosphates, the foregoing amounts of primary amines having little effect upon this assay.

If 2-amino-2-deoxy-D-glucose is assayed by Procedure A with the lower amount (26.5 μmol) of acetic anhydride, and increasing amounts of glycine are added, the optical density observed decreases sharply between 20 and 22 μmol of glycine (Fig. 1). In the presence of 40 μmol of glycine, no characteristic purple color is evident, and the solution is bright yellow.

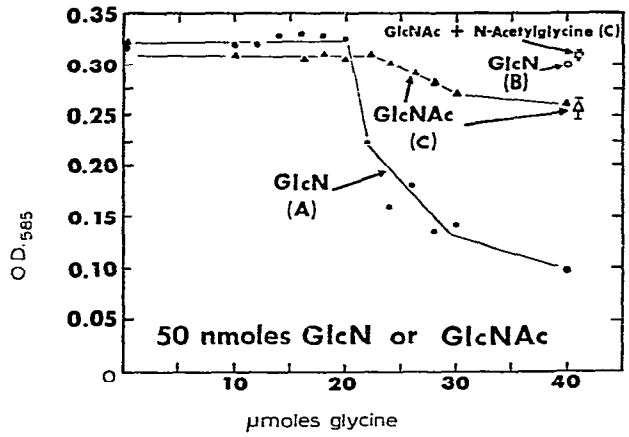

Fig. 1. Effects of glycine and *N*-acetylglycine on the assay of 2-amino-2-deoxy-D-glucose (GlcN) and 2-acetamido-2-deoxy-D-glucose (GlcNAc). 2-Amino-2-deoxy-D-glucose (50 nmol) was assayed by Procedure A (●) with 26.5 μmol of acetic anhydride and by Procedure B (○) with 53 μmol of acetic anhydride, each in the presence of various amounts of glycine. 2-Acetamido-2-deoxy-D-glucose (50 nmol) was assayed by Procedure C (▲) (no acetic anhydride) in the presence of increasing amounts of glycine. In addition, 2-acetamido-2-deoxy-D-glucose was assayed by Procedure C in the presence of 40 μmol of glycine (△) or 50 nmol of *N*-acetylglycine (□), the bars representing the average deviations of 8 replicates.

In contrast, if 2-acetamido-2-deoxy-D-glucose is assayed by Procedure C (no acetic anhydride) in the presence of various amounts of glycine, a smaller decrease in optical density is observed (Fig. 1) with the highest amounts of glycine. Most solutions display the characteristic purple color, but the solution containing 40 μmol of glycine is more reddish. Absorption spectra show that this is caused by the bright-yellow background produced by the reaction of glycine and the assay reagents. This smaller decrease in optical density with high content of glycine results from an unknown cause. It appears not to be brought about by conversion of 2-acetamido-2-deoxy-D-glucose into 2-amino-2-deoxy-D-glucose via transfer of acetyl groups to glycine because *N*-acetylglycine (50 μmol) does not donate acetyl groups to 2-amino-2-deoxy-

D-glucose (50 nmol) during attempted assay by Procedure C. *N*-Acetylglycine (50 μmol) does not greatly affect assay of 2-acetamido-2-deoxy-D-glucose by Procedure C.

If 2-amino-2-deoxy-D-glucose is assayed by Procedure B with the larger amount (53 μmol) of acetic anhydride in the presence of 40 μmol of glycine, the normal optical density is observed (Fig. 1). This is consistent with the lack of effect of *N*-acetylglycine on 2-acetamido-2-deoxy-D-glucose assay by Procedure C.

Amines such as the buffer tris(hydroxymethyl)aminomethane, and ammonium chloride, also inhibit assay for 2-amino-2-deoxy-D-glucose by Procedures A and B when present in quantities larger than the amounts of acetic anhydride used. Neither of these two compounds interferes as strongly as does glycine, when equimolar amounts of each compound are compared. If 2-amino-2-deoxy-D-glucose is assayed in the presence and absence of 35 μmol of ammonium chloride by Procedure B with 53 μmol of acetic anhydride, no difference in optical density is seen (without ammonium chloride 0.293 ± 7, with ammonium chloride 0.288 ± 6; $n = 8$, average deviations).

The standard curves obtained by Procedures A, B, and C for 2-amino-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-glucose are linear and compare favorably. Nevertheless, the optical densities obtained by these procedures vary from day to day from unknown causes. Generally, on a given run, all of the standards fall on a linear plot, the optical density obtained with 50 nmol of amino sugar varying from 0.285 to 0.330. Occasionally, the highest standard (100 nmoles) yields an optical density that is low compared with that expected on the basis of the other standards.

In conclusion, this paper describes the manner whereby primary amines inhibit the Levvy–McAllan assay for 2-amino-2-deoxy-D-glucose. By limiting the total amount of primary amines to 40 μmol, amino sugars may be accurately determined by Procedure B. This procedure is rapid and avoids time-consuming separations of 2-amino-2-deoxy-D-glucose from other primary amines prior to the assays described by others[2,6,9]. Occasionally, the Levvy–McAllan reaction (Procedure A) has been used to determine 2-amino-2-deoxy-D-glucose in the presence of quantities of other primary amines that exceed the limit of 20 μmol. The quantitative errors in these papers may be corrected by following the guidelines contained here.

EXPERIMENTAL

Procedure A. — The modified[10,11] Levvy–McAllan assay[7] for 2-amino-2-deoxy-D-glucose and its 6-phosphate in the absence of large amounts of primary amines is as follows. A sample containing up to 100 nmol of 2-amino-2-deoxy-D-glucose or its 6-phosphate in 0.40 ml is treated with 0.25 ml of 1.12M H_3BO_3–0.56M potassium hydroxide solution. Next, 0.050 ml of freshly prepared 5% aqueous acetic anhydride at O-2° is added, and the tubes are shaken and incubated for at least 4 min at room temperature. The tubes are then capped with marbles and placed in a boiling-water bath for at least 9 min while the tops of the tubes are cooled with a stream of air. The

tubes are then placed in ice–water, and then warmed to room temperature. Three ml of freshly prepared Ehrlich reagent (1.00 g of *p*-dimethylaminobenzaldehyde, 1.25 ml of 10M hydrochloric acid, and 100 ml of glacial acetic acid) is added to each tube, the contents are mixed, and then the tubes are incubated for 20 min at 37°. The optical densities are read at 585 nm on a suitable spectrophotometer. The aliquot of acetic anhydride solution contains about 26.5 μmol of acetic anhydride or 53 μmol of acetic acid upon hydrolysis. This assay is accurate if no more than approximately 20 μmol of other primary amines is present.

Procedure B. — The foregoing procedure may be remodified to permit assay for 2-amino-2-deoxy-D-glucose and its 6-phosphate in the presence of up to approximately 40 μmol of other primary amines. Tubes containing up to 100 nmol of 2-amino-2-deoxy-D-glucose in 0.35 ml are treated with borate buffer as before, and then 0.050 ml of freshly prepared 10% acetic anhydride (ice cold) is added. The tubes are incubated for at least 6 min, and 0.050 ml of M sodium hydroxide (50 μmol) is added. The tubes are capped with marbles and treated as already described. The aliquot of 10% acetic anhydride contains 53 μmol of acetic anhydride which yields 106 μmol of acetic acid. The purpose of the sodium hydroxide is to neutralize the excess acid and maintain the proper pH during the step of boiling with borate. Failure to add sodium hydroxide results in erroneously low optical densities. If more than 40 μmol of primary amines is present, low optical densities will result, the decrease depending upon the amounts of and the pK values of the amines.

Procedure C. — 2-Acetamido-2-deoxy-D-glucose and its 6-phosphate may be determined in the presence of 2-amino-2-deoxy-D-glucose by Procedure A provided that acetic acid solution is substituted for acetic anhydride in order to maintain the proper pH during the step of boiling with borate. Hydrolyzed 5% acetic anhydride solution (5 min, 100°) is suitable for this purpose.

Procedure D. — 2-Amino-2-deoxy-D-glucose may be detected in the presence of small amounts (no more than 50 nmol) of 2-acetamido-2-deoxy-D-glucose if the assay for the latter is used as a control for the assay of the former.

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