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Note

An evaluation of the Morgan–Elson assay for 2-amino-2-deoxy sugars

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The most commonly used quantitative assay[1,2] for 2-amino-2-deoxy sugars and their derivatives, including their phosphate esters, involves acetylation by acetic anhydride at alkaline pH, heating, and treatment of the chromogenic substance(s) with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde in conc. hydrochloric acid–glacial acetic acid) to yield a colored product[3]. Although there is general agreement as to the three-step mechanism[4–6], different structures for the chromogens have been proposed[4,7–9]. The Morgan and Elson assay has been modified at various times to increase color yield[2,5,10] or to improve its reliability[4,6]. In particular, the work by Reissig *et al.*[2] has demonstrated the effect of varying reaction parameters. In the course of studying the enzymes, L-glutamine:D-fructose 6-phosphate aminotransferase (EC 2.6.1.16) and 2-amino-2-deoxy-D-glucose 6-phosphate aldo–ketoisomerase (EC 5.3.1.10), in salivary glands of the fruitfly *Drosophila virilis*, we were in need of a simple, reproducible method to determine 2-amino-2-deoxy-D-glucose 6-phosphate in nmol amounts, with many samples to be measured simultaneously. Therefore, we have re-examined the Morgan–Elson assay and studied conditions to fulfil our requirements and further increase the utility of the test.

RESULTS

The *N*-acetylation of 2-amino-2-deoxy-D-glucose (**1**) and 2-amino-2-deoxy-D-glucose 6-phosphate (**2**) in acetic anhydride solution resulted in ten-fold higher absorption readings than the solution of 2,4-pentanedione used by Boas[11], and by Rondle and Morgan[12].

The color yield was dependent on the pH of the borate solution, the optimum being at 8.7. Routinely, a borate solution of pH 9.0 was used because borate has its maximal buffering capacity at this pH. Color yield was optimal after heating for about

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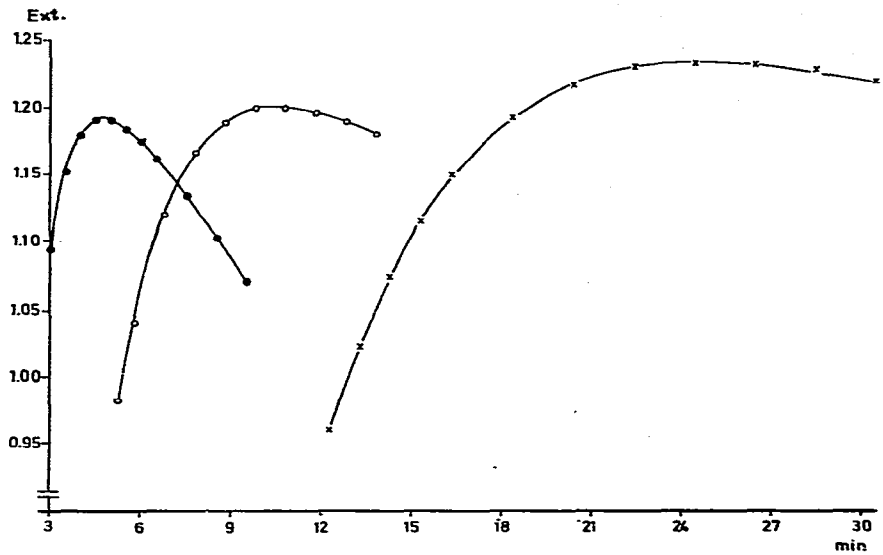


Fig. 1. Time course of color formation as a function of various *p*-dimethylaminobenzaldehyde concentrations. Zero time was the moment to add the reagent: ●, undiluted stock solution; ○, stock solution, diluted 1:1 (v/v) with glacial acetic acid; ×, stock solution, diluted 1:3 (v/v); 60 nmol of **1** were condensed with *p*-dimethylaminobenzaldehyde at 25°; 50 μL of water instead of 2-ethoxyethanol. The remaining procedure was as described in the standard assay. Average of two experiments.

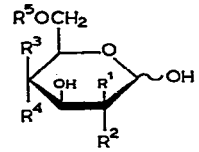
8 min. Subsequent to heating, the samples had to be cooled in ice for at least 5 min, and may be kept for 2.5 h without reduction in color yield.

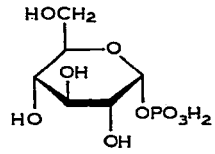
Fig. 1 shows the time course for color development and subsequent fading as a function of *p*-dimethylaminobenzaldehyde concentration. Whereas maximal color was reached within 4.5–5 min with the undiluted stock solution, the time interval was 10 min for a 1:1 (v/v, diluted with glacial acetic acid) and 25 min for a 1:3 (v/v) diluted solution. Accordingly, the time interval, within which maximal absorbance remains fairly constant, was prolonged from 0.5 to 5 min (Fig. 1). At the same time, bleaching decreased from 11% per 5 min to 1.5%. The *p*-dimethylaminobenzaldehyde concentration appears to have a slight effect on the height of maximal absorbance: use of a 1:3 (v/v) diluted instead of an undiluted solution resulted in a 4% increase (Fig. 1).

It was also found that the addition of 2-ethoxyethanol instead of water during the condensation reaction increased maximal absorbance and shortened the time for maximal color development.

The rate of absorbance increase or decrease is also dependent on the temperature at which the condensation is performed. At 27°, maximal color was observed 3 min after addition of *p*-dimethylaminobenzaldehyde; at 18°, the maximum was reached after ~16 min; and at 14°, only after 25 min.

Each of the substances tested [**1**, **2**, 2-acetamido-2-deoxy-D-glucose (**3**), and 2-acetamido-2-deoxy-D-glucose 6-phosphate (**4**)] showed a straight line when con-





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1 $R^1 = R^3 = R^5 = H, R^2 = NH_2 \cdot HCl, R^4 = OH$
2 $R^1 = R^3 = H, R^2 = NH_2, R^4 = OH, R^5 = PO_3H_2$
3 $R^1 = R^3 = R^5 = H, R^2 = NHAc, R^4 = OH$
4 $R^1 = R^4 = R^5 = H, R^2 = NHAc, R^3 = OH$
5 $R^1 = NHAc, R^2 = R^3 = R^5 = H, R^4 = OH$
6 $R^1 = R^3 = H, R^2 = NHAc, R^4 = OH, R^5 = PO_3H_2$

TABLE I

COLOR FORMATION OF VARIOUS 2-AMINO-2-DEOXY SUGARS IN THE STANDARD TEST, AND COMPARISON TO OTHER STUDIES OF THE MORGAN–ELSON REACTION

Compound	*Absorbance/μmol*[a]	*Ref.*
1	19.5 ±0.37	*b*
2	13.0 ±0.54	*b*
3	19.3 ±0.37	*b*
4	7.0 ±0.33	*b*
5	9.5 ±0.50	*b*
6	13.6 ±0.66	*b*
7	13.0 ±0.72	*b*
1	1.6	10
1	2.1	5
3	5.0	2
1, 3	6.5	6

[a]Mean values ± s. d. [b]This study.

centrations of 5–200 nmol were plotted against absorbance values. Day to day variations did not exceed ±3%. Absorbance values, based on the molar concentrations of the compounds in the final assay volume, are listed in Table I. Whereas *N*-acetylation does not affect the molar absorbance, phosphorylation at C-6 leads to a decrease of about 30%. 2-Acetamido-2-deoxy-α-D-glucopyranosyl phosphate (**7**) had first to be hydrolyzed, which also resulted in a decrease in absorbance. 2-Acetamido-2-deoxy-D-galactose (**4**) and -D-mannose (**5**) produced even less color. Compound **2** obtained from various sources gave, in our hands, substantial differences in absorbance.

When **2** was tested in the presence of increasing amounts of L-glutamine, the absorbance decreased drastically between 2 and 3 μmol (from 100% to 45%, Fig. 2). As previously noticed by Benson[6], primary amines interfere by removing acetic

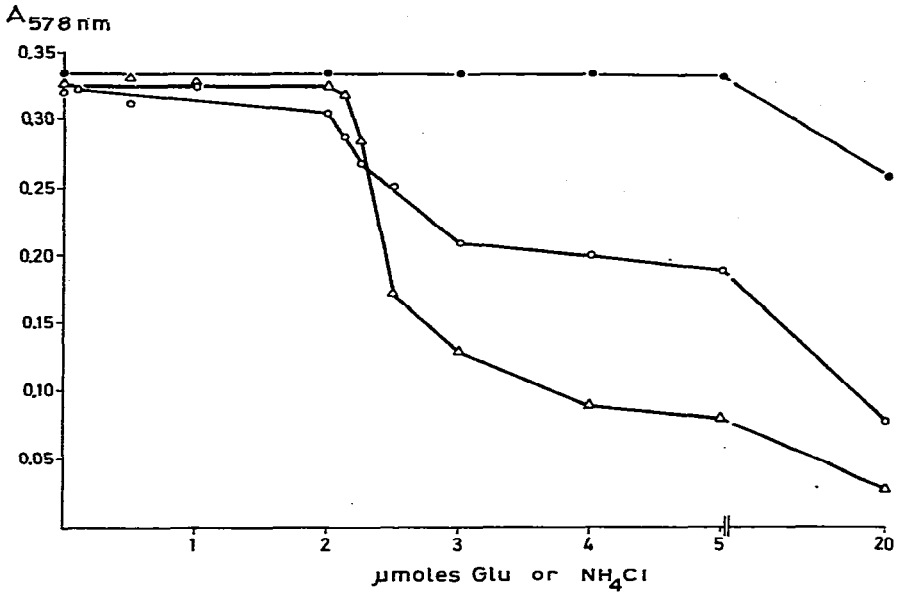

Fig. 2. Effect of L-glutamine and ammonium chloride on the color developed by **2** and **6**: ●, **6** (25 nmol) and L-glutamine; △, **2** (25 nmol) and L-glutamine; and ○, **2** (25 nmol) and ammonium chloride. Each point represents the average of three determinations.

anhydride. This is clearly shown by Fig. 2: the inhibition becomes noticeable when the amount of L-glutamine approaches the amount of acetic anhydride present in the standard assay (3 μmol). Glycine had the same effect, whereas ammonium chloride or L-glutamic acid produced a smaller decrease in absorbance. If, however, **6** was assayed (whereby acetic anhydride is omitted) with increasing amounts of L-glutamine or ammonium chloride, no fading in absorbance was observed up to 7 μmol, and even at 20 μmol an absorbance over 80% could still be observed (Fig. 2). This, too, agrees with earlier observations by Benson[6].

DISCUSSION

One advantage of the present procedure is greater sensitivity (Table I). This is partly due to the smaller assay volume. The positive effect of addition of 2-ethoxyethanol is obscure at present. By scaling down the procedure to a final volume of 50 μL, 0.1 nmol of 2-amino-2-deoxy sugar can be measured with accuracy, *i.e.*, less than half the limit value stated for the micromodification of the Reissig *et al.*[2] procedure. It must be emphasized that our assay conditions were not optimal, some sensitivity being lost for greater convenience and reliability by working at pH 9.0 (the optimum pH is at 8.7), employing the undiluted *p*-dimethylaminobenzaldehyde stock solution, and measuring the absorbance at the 578-nm mercury line, outside the color maximum (585 nm).

The greatest advantage for routine application was the "freezing" of the

chromogens after the heating step, so that many samples could be accumulated, and the subsequent color assay could be performed under identical measuring conditions. Thus, in tests, sources of error that may arise from handling the samples at different times can be avoided. Possibly, this waiting period may be prolonged far beyond 150 min, the maximal period that we tested.

In the assay of L-glutamine:D-fructose 6-phosphate aminotransferase and related enzymes, inhibition of the color reaction by L-glutamine and other primary amines is a potential source of error (D-fructose 6-phosphate does not react). This is due to competition of the amine with 2-amino-2-deoxy-D-glucose 6-phosphate for acetic anhydride; obviously it does not interfere in the determination of *N*-acetylated sugars. Benson[6] demonstrated that this interference could be overcome by using higher amounts of acetic anhydride in the test; when acetic anhydride was increased twenty-fold, the amount of glycine could be increased ten-fold for the same inhibition. L-Glutamine or ammonium salts are also inhibitory, but to a lesser extent. With regard to the high reactivity of *p*-dimethylaminobenzaldehyde, interference by a broad range of compounds must be expected[13].

EXPERIMENTAL

Materials. — Compounds **1–5** were obtained from Merck (Darmstadt, W. Germany); Sigma Chemical Co. (St. Louis MO 63178), and ICN Nutritional Biochem. (Cleveland OH 44128). Compounds **6** and **7** were synthesized and purified according to Enghofer *et al.*[14].

Assay procedure. — Determination of **1** *and* **2**. A solution (100 μL) of the sample (5–50 nmol) was mixed with acetic anhydride solution (20 μL; 1.5%, v/v, acetic anhydride in acetone) and borate buffer (100 μL; 1.12M boric acid and 0.56M potassium hydroxide; pH 9.0). The reaction mixture was kept for 8 min at 95°. The samples were cooled to 0°; they could be kept for 150 min at least at this temperature without appreciable effect on the absorbance. Then, 750 μL of *p*-dimethylaminobenzaldehyde reagent (9 g in 100 mL of glacial acetic acid, which contained 12.5%, v/v, conc. hydrochloric acid; prepared fresh daily), and 2-ethoxyethanol (50 μL) were added. The sample was placed in a thermostat at 20–21° and, after 15 min, the color formed was recorded at 578 nm in an Eppendorf photometer. Of the two absorption maxima, at 545 and 585 nm, measurement near the latter was chosen, because of less interference by other substances, like amino acids, was expected[2].

Compounds **3** and **6** were determined by the same method, but replacing the acetic anhydride reagent by an equal volume of acetone. Compound **7** was not detected by this procedure. It was assayed as **3** after hydrolysis[15] with 0.1M hydrochloric acid for 10 min at 100°.

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