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Modifications of the Park–Johnson Ferricyanide Submicromethod for the Assay of Reducing Groups in Carbohydrates

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The Park–Johnson method for the analysis of reducing groups in carbohydrates has been modified using oxalic acid as a solvent for ferric ferrocyanide in place of sodium dodecyl sulfate which was used in the original procedure and which in our experiments gave solutions with persistent turbidity. Differences in the course of the reactions involved, due to the reducing power of oxalic acid, were considered and kept under control. The modified method proved to be reproducible and precise, with a degree of sensitivity which was generally higher than that of the original method.

The Park–Johnson method is known to be accurate and highly sensitive for the assay of reducing sugars (1–3). The reaction is based on the reduction of potassium ferricyanide by the aldehyde groups of a carbohydrate and the subsequent formation of ferric ferrocyanide (Prussian blue)[1] after addition of Fe^{3+}. A tensioactive agent, sodium dodecyl sulfate (SDS), is used to keep the Prussian blue in suspension in order to allow its colorimetric determination.

In our experiments with bacterial polysaccharides and with their acid hydrolysates, this procedure proved unsatisfactory due to the marked turbidity which always developed in the final mixture. Moreover, in the case of some carbohydrates, the turbidity persisted even though the acid concentration of the system was increased, as has been suggested (2). We therefore examined the possibility of substituting SDS with oxalic acid, which has the property of dissolving Prussian blue (4–6). However, a more complex mechanism of reaction was to be expected, in view of the reducing power of oxalic acid and its property of forming complexes with Fe^{3+} ions (7). The original method was therefore modified in order to obtain adequate control of unwanted side reactions.

[1] Abbreviations used: Prussian blue, ferric ferrocyanide; SDS, sodium dodecyl sulfate.

MATERIALS AND METHODS

The original method was performed as described (1–3). The component solutions and reagents of the modified method were as follows: (a) the alkaline ferricyanide solution contained 1.5 mM potassium ferricyanide, 50 mM sodium carbonate, and 10 mM potassium cyanide and was stored in dark glass bottles; (b) the ferric iron solution contained 1.36 mM ferric ammonium sulfate and 25 mM sulfuric acid; and (c) the oxalic acid solution contained 125 mM oxalic acid. All chemicals used were of reagent grade.

The procedure of the modified method was as follows: 10-ml test tubes with screw caps were used and 1 ml of alkaline ferricyanide solution was added to a 0.5-ml sample containing sugars in amounts ranging from 4.5 to 66 nmol. The tubes were heated for 15 min in a boiling water bath. The samples were brought to room temperature and, after the addition of 2.5 ml of ferric iron solution, they were heated in a water bath at 50°C for 15 min. They were then cooled to room

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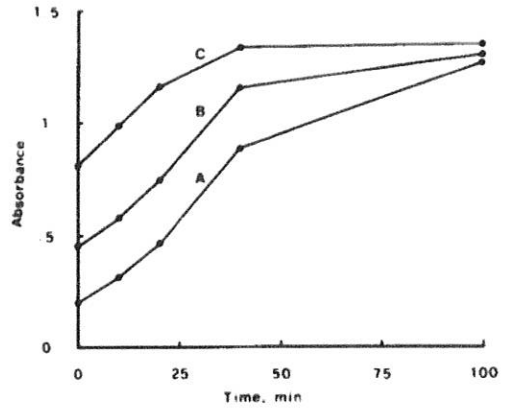

FIG. 1. Time course of the absorbance at 690 nm after addition of oxalic acid in the conditions of the original method; i.e., 5.6 mM Fe^{3+}. Carbohydrate used: (A) 15.2 nmol, (B) 30.4 nmol, and (C) 60.8 nmol rhamnose.

temperature; 0.5 ml of 125 mM oxalic acid solution was added to each sample and they were shaken. Spectrophotometric readings were promptly taken at 690 nm against a parallel reagent blank.

A computerized, double-beam spectrophotometer (Perkin-Elmer Model 5547, Palo Alto, Calif.) was used to follow the kinetic mechanisms and a membrane electrode type P 502 (Orion Research, Cambridge, Mass.) was used for the analysis of carbon dioxide.

RESULTS

When oxalic acid was used as a solvent for Prussian blue in substitution for the SDS used in the Park-Johnson method, the different carbohydrate concentrations showed an increase in absorbance over time and all eventually reached the same value. Under these conditions it was impossible to distinguish between the various concentrations of reducing sugars (Fig. 1).

The values of the normal redox potentials of the couples $CO_2/C_2O_4^{2-}$ ($E_0 = -0.49$ V) and $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ ($E_0 = 0.36$ V) clearly show that oxalic acid may act as a reducing agent with respect to the ferricyanide ion. In fact, the development of CO_2 in the reaction mixture was demonstrated by an electrochemical assay with the electrode described under Materials and Methods. The results shown in Fig. 1 could therefore be explained by assuming that the reducing effect of the oxalic acid was added to that of the reducing carbohydrate. Under these conditions, the same amount of Prussian blue developed in all of the samples and was equivalent to the amount of potassium ferricyanide present. In other words, the proposed equilibria are

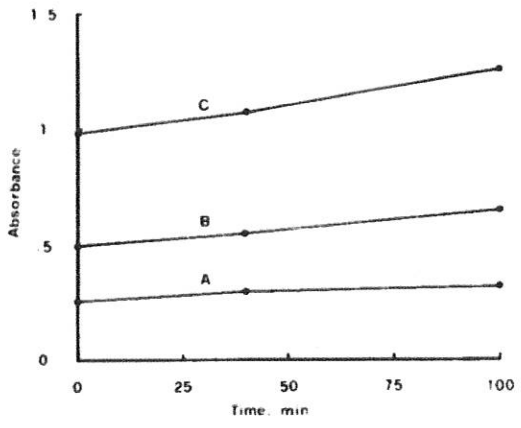

FIG. 2. Time course of the absorbance at 690 nm after addition of oxalic acid using 1.36 mM Fe^{3+}. Carbohydrate used: (A) 15.2 nmol, (B) 30.4 nmol, and (C) 60.8 nmol rhamnose.

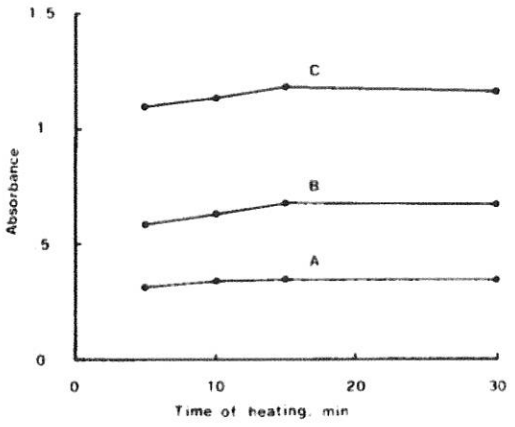

FIG. 3. Effect of heating on the absorbance at 690 nm using 1.36 mM Fe^{3+}. After addition of $FeNH_4(SO_4)_2$ the mixtures were heated at 50°C for the times shown in the figure. Spectrophotometric readings were taken after addition of 125 mM oxalic acid solution.

Principal reaction:

$$Fe^{3+} + [Fe^{II}(CN)_6]^{4-} \rightleftarrows [Fe^{III}Fe^{II}(CN)_6]^{-} \quad [1]$$

Prussian blue

Side reactions:

$$2[Fe(CN)_6]^{3-} \text{ (excess)} + C_2O_4^{2-} \rightleftarrows 2[Fe^{II}(CN)_6]^{4-} + 2CO_2 \quad [2]$$

$$Fe^{3+} \text{ (excess)} + [Fe^{II}(CN)_6]^{4-} \rightleftarrows [Fe^{III}Fe^{II}(CN)_6]^{-} \quad [3]$$

On the basis of the proposed scheme, it was necessary to control the course of reaction [1] by limiting the interference of the competitive reaction [2]. For this purpose, we examined the possibility of reducing the amount of added Fe^{3+} to the lowest level compatible with reaction [1] taking place.

We performed various analyses using rhamnose as a reference sample with different concentrations of ferric ammonium sulfate, starting with the stoichiometric amount for reaction [1]. As expected, at low concentrations of Fe^{3+} reaction [1] was very slow and required solutions in the range 1–1.5 mM Fe^{3+} in order to attain a sufficient sensitivity and linearity of the absorbance between the different amounts of carbohydrates in comparison with the original method (the original method requires 5.6 mM Fe^{3+}). However, reaction [1] was still very slow at room temperature using Fe^{3+} in this concentration range and the absorbance increased slowly over an interval of 100 min although values proportional to the different carbohydrate concentrations were reached (Fig. 2). These results confirmed the hypothesis that the smaller amount of Fe^{3+} did, in fact, reduce interference by the competitive reaction [3]. It was necessary, however, to increase the rate of reaction [1], e.g., by raising the temperature. Thus, after addition of 1.36 mM Fe^{3+} solution, the reaction mixtures were heated in a water bath at temperatures ranging from 37 to 50°C. As

TABLE 1

COMPARISON BETWEEN THE TWO METHODS IN THE ANALYSIS OF VARIOUS CARBOHYDRATES

Carbohydrate	Absorbance (690 nm)/μmol carbohydrate	
	Park–Johnson	Modified method
Glucose	23.6	26.7
Mannose	23.6	25.2
Galactose	22.0	24.7
Glucosamine	23.1	26.9
N-Acetylglucosamine	27.8	29.0
Sodium glucuronate	15.6	23.4
Rhamnose	17.2	19.2
Ribose	12.9	19.1

TABLE 2

PERCENT RECOVERY OF CARBOHYDRATES IN REPLICATE ANALYSES

Carbohydrate	Recovery (%)
Glucose	99–104
Mannose	96–101
Galactose	98–102
Rhamnose	98–100
Glucosamine	100–102
N-Acetylglucosamine	98–101
Sodium glucuronate	99–102

TABLE 3

RELATIVE REDUCING POWERS OF DIFFERENT CARBOHYDRATES

Carbohydrate	Reducing power
Glucose	1
Mannose	0.94
Galactose	0.93
Glucosamine	1.01
Rhamnose	0.72
N-Acetylglucosamine	1.09
Ribose	0.72
Lactose	1.22
Sodium glucuronate	0.88
Maltose	0.90

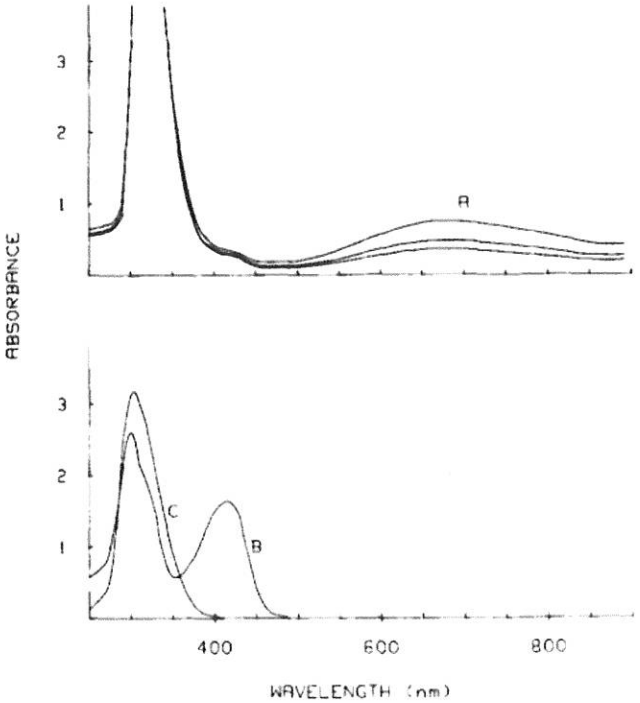

FIG. 4. Absorption spectra of the final reaction mixture in the original method compared with the spectra of the reagents used; (A) 15.2, 30.4, and 60.8 nmol rhamnose, (B) potassium ferricyanide, and (C) ferric ammonium sulfate.

shown in Fig. 3, a temperature of 50°C proved satisfactory since after 15 min constant values for the absorbance were achieved. Thus, reducing the amount of Fe^{3+} compared with that used in the classical method, heating at 50°C, and adding the oxalic acid solution gave a completely controlled reaction.

The results obtained with the original method are compared in Table 1 with the results of the modified version for several carbohydrates. The comparison shows that generally the modified method presents a greater sensitivity than the original technique. In the case of *N*-acetylglucosamine, there is an increase in sensitivity of about 4%, while for sodium glucuronate and ribose there is a rise of 50% in sensitivity compared with the original method.

The recovery of carbohydrates using the modified method, with the conditions described under Materials and Methods, is shown in Table 2. The average value found for the analyses performed was 98.5% of the theoretical value. The relative reducing powers of the carbohydrates used with the present method, calculated on a molar basis, are shown in Table 3.

As the Park–Johnson method is often used after hydrochloric acid hydrolysis of polysaccharides, we checked the influence of chloride ions on the modified method. No noticeable differences were found in the sensitivity up to a concentration of 0.2 M chloride ion in the final reaction, in contrast to the results reported by some authors with the original method (2).

Ca^{2+} and Mg^{2+} ions, which are often present in biological materials, inhibited the development of color in our experiments using the original method. In the present method, on the contrary, Ca^{2+} did not affect the results at a concentration of 50 mM, while Mg^{2+} caused a reduction in the absorbance

of about 50%. Phosphate ions did not interfere in either of the two methods.

In our experience, the present method gave satisfactory results with meningococcal and pneumococcal polysaccharides and their hydrolysates, whereas in the original procedure an excessive turbidity was present. We therefore suggest use of the present method for cases in which the Park–Johnson procedure gives rise to similar problems.

DISCUSSION

In order to clarify the course of the reactions that interfere in the modified method, the absorbance spectra of the reaction mixtures were compared using different concentrations of Fe^{3+}.

The typical absorption spectrum of the reaction in the original method compared with the spectra of the reagents adopted is shown in Fig. 4. The peak at 690 nm, which is typical of Prussian blue, is accompanied by another peak at 420 nm, typical of potassium ferricyanide, and still another peak at 295 nm corresponding to Fe^{3+}.

The comparison between the spectra of reaction mixtures in the modified method with concentrations of 0.68, 1.36, or 2.8 mM Fe^{3+} is shown in Fig. 5. In the first two cases, the color remains constant over a period of time and all three peaks are present at the indicated wavelengths. When the 2.8 mM Fe^{3+} solution is employed, however, the peak at 420 nm is still faintly visible at zero reading time, but disappears after 6 h, with a consequent increase in color up to the common value with the three different carbohydrate concentrations used. It is significant that the peak at 295 nm is also considerably smaller at 6 h compared to the same peak at zero reading time.

Further tests were performed to confirm that oxalic acid was, in fact, able to reduce

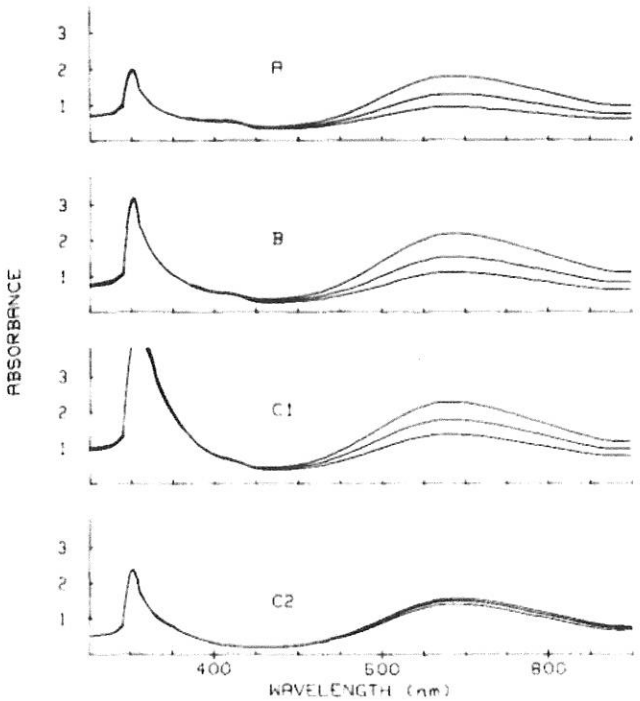

FIG. 5. Absorption spectra of the final reaction mixture in the modified method at different concentrations of Fe^{3+}; (A) 0.68 and (B) 1.36 mM Fe^{3+}. Spectra (A) and (B) did not alter appreciably over a period of 24 h; (C1) 2.8 mM Fe^{3+} at time 0 and (C2) 2.8 mM Fe^{3+} after 6 h. Carbohydrate used: 15.2, 30.4, and 60.8 nmol rhamnose.

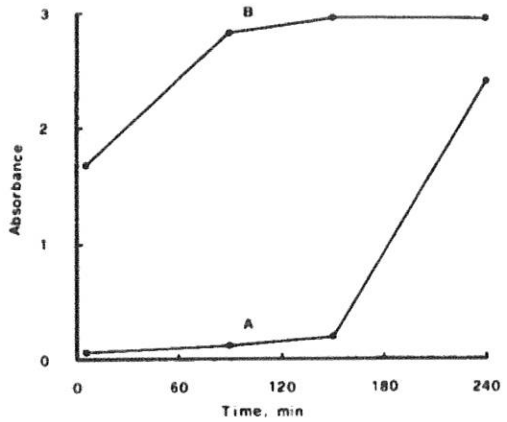

FIG. 6. Formation of Prussian blue by reaction of potassium ferricyanide with oxalic acid in the absence of reducing carbohydrates and at different concentrations of Fe^{3+}; (A) 2.8 and (B) 5.6 mM Fe^{3+}. A 1.36 mM Fe^{3+} solution did not develop any appreciable absorbance.

$[Fe^{III}(CN)_6]^{3-}$ with the consequent formation of Prussian blue, in the presence of Fe^{3+} at concentrations higher than 1.36 mM. Reactions were performed with the present method in the absence of reducing carbohydrates and with different concentrations of Fe^{3+}, and the relative absorbances in an interval of time were compared. As shown in Fig. 6, when 5.6 mM ferric ammonium sulfate solution was used, the blue color developed immediately in the presence of oxalic acid, reaching a plateau value after about 2 h; a high increase in absorbance was also observed using 2.8 mM Fe^{3+} solution. In contrast, at the concentration of Fe^{3+} required by the present method, no color developed in the final mixture even after 24 h.

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