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A SPECIFIC METHOD FOR D-GALACTOSE QUANTITATIVE DETERMINATION: A MODIFICATION OF THE D-GALACTOSE OXIDASE ASSAY*

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ABSTRACT

After treatment with D-galactose oxidase to form an aldehyde group, D-galactose or 2-acetamido-2-deoxy-D-galactose reacted with indole–hydrochloric acid to give a colored compound having a spectrum very similar to that of D-galacturonic acid, but with a maximum at 500 nm and a shoulder at 480 nm. The reaction is linear between 16.6 and 83 nmol of sugar per mL of final solution. 2-Amino-2-deoxy-D-galactose gave no reaction, even when 5 μmol were used, and 2-deoxy-D-*lyxo*-hexose did not interfere either.

INTRODUCTION

D-Galactose is one of the most important sugars of living tissues, but a convenient and specific technique for its quantitative determination was lacking until the isolation of D-galactose oxidase[1,2]. This enzyme catalyzes the oxidation of D-galactose at C-6 to give D-*galacto*-hexodialdose, with simultaneous reduction of molecular oxygen to hydrogen peroxide. The evolution of stoichiometric quantities of hydrogen peroxide during the reaction affords a simple way for the determination of D-galactose, through reaction with any of a number of reducing agents and dyes. These compounds, in the presence of another enzyme (peroxidase), produce a variety of substances that absorb light in the visible or u.v. region. A large number of reducing agents has been used, *o*-dianisidine and benzidine[3,4] giving the best results with regard to linearity of color response and sensitivity. The method suffers, however, from two main disadvantages. It does not distinguish between D-galactose, 2-acetamido-2-deoxy-D-galactose, 2-amino-2-deoxy-D-galactose, or any other D-galactose derivative[5], as the enzyme recognizes[2,6] C-4 and -6. Also, the presence of hydrogen peroxide, or

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of significant amounts of catalase or other agents that consume hydrogen peroxide, will interfere with the accuracy or sensitivity of the assay.

In this paper, we present a modification of the D-galactose oxidase technique that eliminates these disadvantages. It is based on the observation that D-galacturonic acid gives a color reaction with indole–hydrochloric acid, with a typical spectrum[7] having a maximum at 492 nm and a shoulder at 470 nm. Under the conditions used for the uronic acid assay, D-galactose gave only 9% of the color. The product of the oxidation, D-*galacto*-hexodialdose, reacted with indole–hydrochloric acid to give a compound absorbing light in the visible range and having a spectrum very similar to that of D-galacturonic acid, but with a maximum at 500 nm and a shoulder at 480 nm. The reaction presents a high degree of specificity for D-galactose and 2-acetamido-2-deoxy-D-galactose, as 2-amino-2-deoxy-D-galactose and -D-glucose, and 2-deoxy-D-*lyxo*-hexose do not interfere with the reaction.

EXPERIMENTAL

Unless otherwise stated, materials and methods were as described previously[7]. D-Galactose oxidase Type IV (D-galactose oxygen 6-oxidoreductase, EC 1.1.3.9) and peroxidase Type I (donor: hydrogen peroxide oxidoreductase; EC 1.11.1.7) were purchased from Sigma Chemical Co. (St. Louis, MO 63178). Other reagents were of analytical grade.

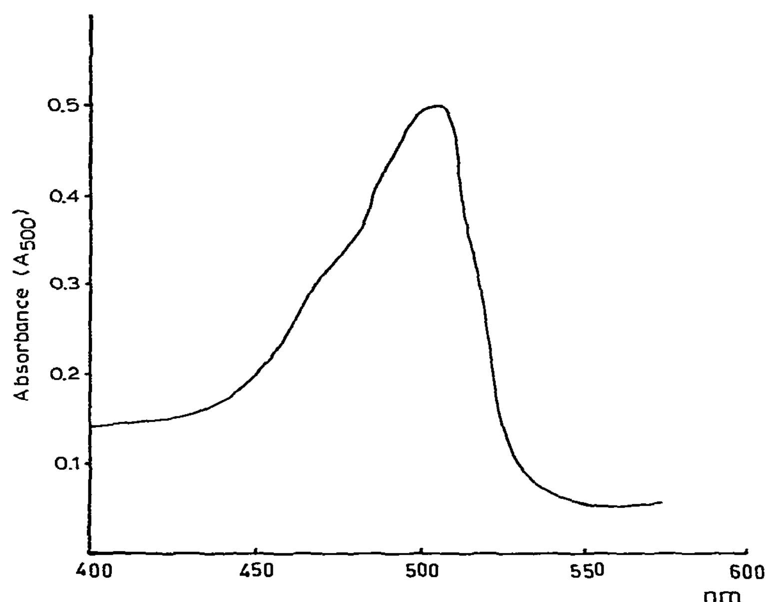

Fig. 1. Absorption spectrum of the colored product formed in the reaction of D-galactose oxidase-treated D-galactose with 1.25 mM indole–3M hydrochloric acid for 10 min at 100°.

RESULTS AND DISCUSSION

The requirement for an aldehyde group for the indole-condensation reaction suggested that the 6-aldehyde derivative of D-galactose that is formed after treatment of the sugar with D-galactose oxidase could react with indole and hydrochloric acid under the conditions for D-galacturonic acid dehydration[7]. Fig. 1 shows the spectrum of the colored product formed, which is very similar to that obtained with D-galacturonic acid, but with the absorption maximum changed from 490 to 500 nm.

Reaction times ranging between 1 and 60 min were tested, and Figure 2 shows the curve obtained with an optimum time between 10 and 20 min. The optimum time for D-galacturonic acid was found to be between 30 and 40 min, as shown in the superposed curve.

The optimum concentration of hydrochloric acid was found to be 1–2M. D-Galacturonic acid required higher concentrations (see Fig. 3). The optimal concentration of indole was found to be between 6 and 9mM, but 4mM was selected as a compromise between high absorbance and a colorless blank.

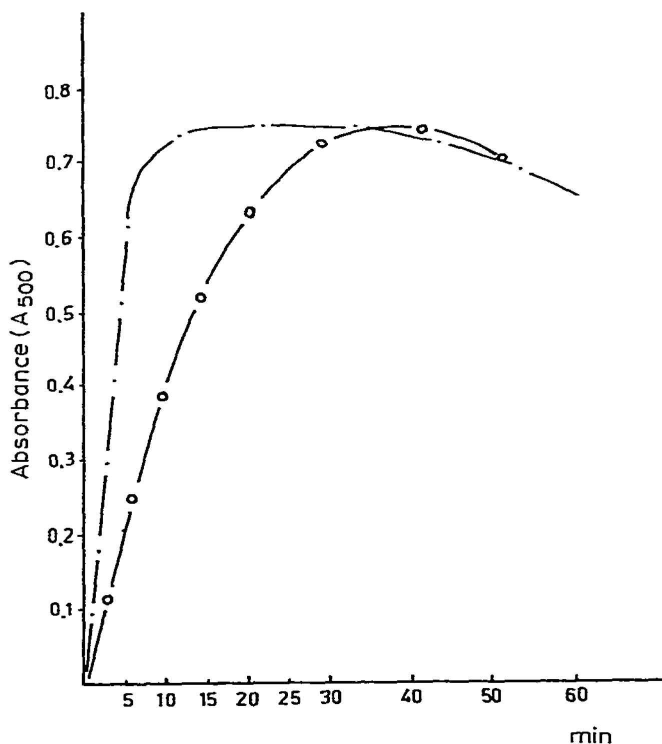

Fig. 2. Time course of the reaction of D-galactose oxidase-treated D-galactose with indole–hydrochloric acid, as compared with the direct reaction of D-galacturonic acid with indole–hydrochloric acid. Other conditions as described in legend to Fig. 1: (–.–.–) D-galactose; (–O–O–) D-galacturonic acid.

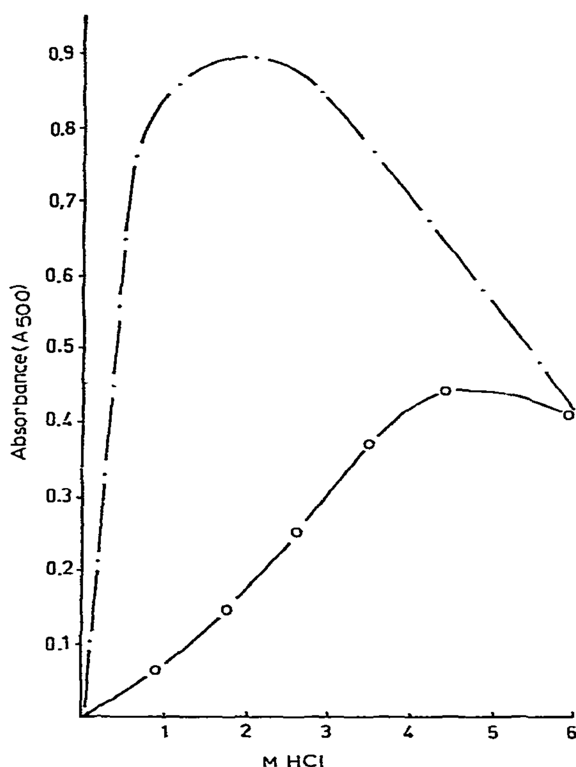

Fig. 3. Concentration of hydrochloric acid and color development. Indole concentration: 1.25mM; heating time: optimum reported in Fig. 2; (–.–.–) D-galactose; (–O–O–) D-galacturonic acid.

In a study of the amount of enzyme necessary to give a complete reaction, a plateau was reached with 1.35 unit of D-galactose oxidase per 250 nmol of D-galactose, and an optimum incubation time between 60 and 90 min; 1 h was selected for all experiments.

Under the conditions just determined [4mM indole and a heating time of 15 min at 100° (see Fig. 4)], a standard calibration curve for D-galactose was established for 2M hydrochloric acid. The reaction was found to be linear between 16.6 and 83 nmol/mL of final solution.

2-Acetamido-2-deoxy-D-galactose and 2-amino-2-deoxy-D-galactose react with D-galactose oxidase[2], but, under the conditions described here, 2 amino-2-deoxy-D-galactose (5 μmol) gave no reaction. In order to test whether the presence of OH-2 is required for the reaction of indole–hydrochloric acid with CHO-6, 2-deoxy-D-*arabino*- and 2-deoxy-D-*lyxo*-hexose were treated with indole–hydrochloric acid, with and without previous treatment with D-galactose oxidase. As expected, a previous treatment with D-galactose oxidase did not change the results for 2-deoxy-D-*arabino*-hexose, because this sugar is not a substrate for the enzyme. However, for 2-deoxy-D-*lyxo*-hexose, the absorbance at 490 nm and at 500 nm was reduced to less than one

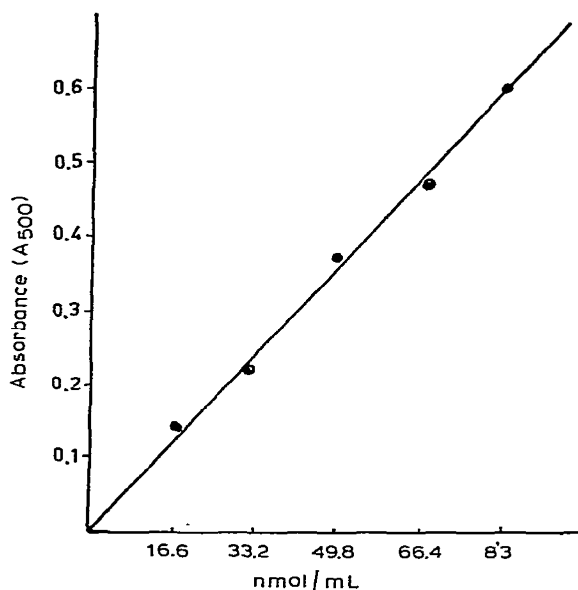

Fig. 4. Standard calibration curve for the reaction of D-galactose oxidase-treated D-galactose with 4mM indole–2M hydrochloric acid with a heating time of 15 min at 100°.

half after treatment with D-galactose oxidase. Apparently, when CHO-6 is formed, a colored compound with a maximum at 490 nm cannot result, but one with a maximum at 500 nm may be obtained when OH-2 is present [as shown by the results with D-galactose (Table I)] or when NH_2-2 is replaced by NHAc-2, as for 2-acetamido-2-deoxy-D-galactose.

As D-galactose is a better substrate for D-galactose oxidase than is 2-deoxy-D-*lyxo*-hexose[2], increasing amounts of enzyme were used to ensure that the substrate

TABLE I

REACTION OF D-GALACTOSE, 2-DEOXY-*lyxo*-HEXOSE, AND 2-DEOXY-*arabino*-HEXOSE WITH INDOLE–HYDROCHLORIC ACID, WITH AND WITHOUT PREVIOUS TREATMENT WITH D-GALACTOSE OXIDASE.

Sugar[a]	*Galactose-oxidase treatment*	A_{500nm} *after indole–hydrochloric acid treatment*
D-Galactose	+	0.482
D-Galactose	–	0.080
2-Deoxy-D-*lyxo*-hexose	+	0.377
2-Deoxy-D-*lyxo*-hexose	–	0.843
2-Deoxy-D-*arabino*-hexose	+	0.419
2-Deoxy-D-*arabino*-hexose	–	0.418

[a]Sample of 250 nmol.

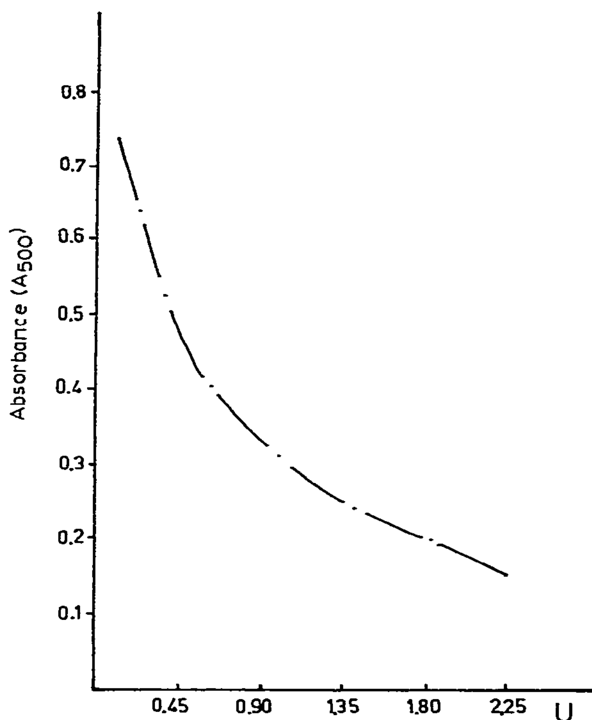

Fig. 5. Reaction of 2-deoxy-D-*lyxo*-hexose (25 nmol) with indole–hydrochloric acid after a treatment with increasing amounts of D-galactose oxidase.

had been completely oxidized (see Fig. 5). The color reaction of 2-deoxy-D-*lyxo*-hexose with indole–hydrochloric acid decreased with the increase of the enzyme concentration. With an enzyme at saturation level for D-galactose (2.25 U/250 nmol) the absorbance was reduced to a minimum (see Fig. 6). Thus, an excess of enzyme allows the dosage of D-galactose in the presence of 2-amino-2-deoxy-D-galactose and 2-deoxy-D-*lyxo*-hexose.

2-Acetamido-2-deoxy-D-galactose reacted with indole–hydrochloric acid, after D-galactose oxidase treatment, to give a colored solution with a spectrum identical with that obtained with D-galactose, and of the same intensity. If 2-acetamido-2-deoxy-D-galactose was *N*-deacetylated with 0.25M hydrochloric acid for 2 h at 100°, no reaction with indole–hydrochloric acid was detected, even with 5 μmol of sugar.

A possible reaction of D-galactose in a combined state was studied with melibiose (6-*O*-α-D-galactopyranosyl-D-glucose) and raffinose [*O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-β-D-fructofuranoside], which gave 100% yield of color as compared with D-galactose, but lactose (4-*O*-β-D-galactopyranosyl-D-glucose) gave only 5.5%. These results reflect the specificity of the glycosidic linkage for the reaction with D-galactose oxidase. 2-Furylmethanol, levulinic acid, and D-galactitol gave almost no color, even when 2.5 μmol were used, and the same amount of D-glucuronic acid gave 3.5% of the color given by D-galactose. Several pentoses,

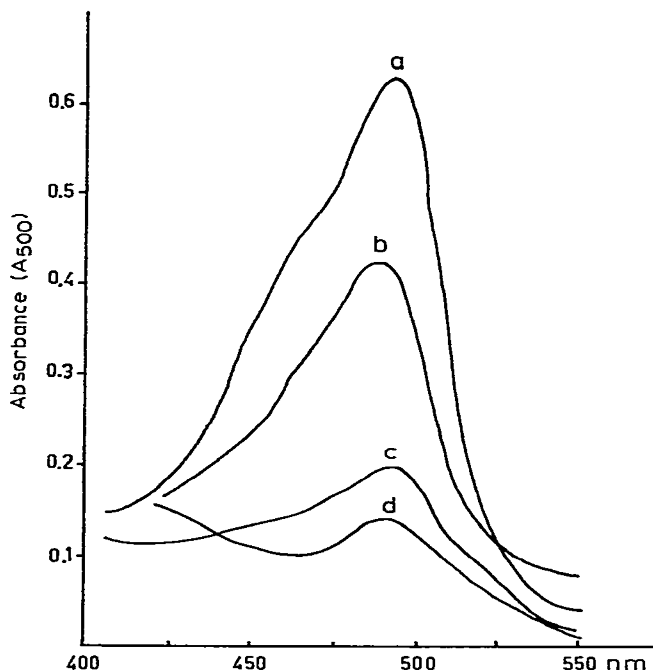

Fig. 6. Absorption spectra of the colored compounds formed when 2-deoxy-D-*lyxo*-hexose (250 nmol), previously treated with various amounts of D-galactose oxidase, was heated with indole–hydrochloric acid for 10 min at 100°: (a) 0, (b) 0.45, (c) 1.35, and (d) 2.25 units.

hexoses, ketoses, hexosamines, and 6-deoxyhexoses gave very low color intensities with indole–hydrochloric acid after D-galactose oxidase treatment (Table II).

The method was tested with a mixture of equimolar amounts of D-galacturonic acid, 2-deoxy-D-*lyxo*-hexose, D-galactose, and 2-amino-2-deoxy-D-galactose in the following way: (a) for D-galacturonic acid determination, an aliquot was heated for 30 min with 3M hydrochloric acid and then treated with 3mM indole–3M hydrochloric acid for another 30 min at 100°; (b) for 2-deoxy-D-*lyxo*-hexose determination, an aliquot of the mixture was treated with 4mM indole–0.5M hydrochloric acid for 10 min at 100°; (c) for D-galactose determination, an aliquot of the mixture was treated with an excess of D-galactose oxidase, and then with 4mM indole–M hydrochloric acid for 15 min at 100°; and (d) for 2-amino-2-deoxy-D-galactose determination, an aliquot of the mixture was deaminated with sodium nitrite and acetic acid[8], and then treated with 2.75mM indole–1.65M hydrochloric acid for 5 min at 100°. The results are shown in Table III.

The different behavior of 2-acetamido-2-deoxy-D-galactose and 2-amino-2-deoxy-D-galactose in the reaction with indole–hydrochloric acid after treatment with D-galactose oxidase allow the determination of the ratio of 2-acetamido-2-deoxy-D-galactose to 2-amino-2-deoxy-D-galactose, and of D-galactose to 2-acetamido-2-deoxy-D-galactose. D-Galactose and 2-acetamido-2-deoxy-D-galactose are determined by direct reaction with D-galactose oxidase and indole–hydrochloric acid. If a previous

TABLE II

SPECIFICITY OF THE REACTION WITH INDOLE–HYDROCHLORIC ACID AFTER TREATMENT WITH D-GALACTOSE OXIDASE

Sugar	*Amount (µmol)*	A_{500nm}	*Color formed*[a]
D-Ribose	1	0.07	3
D-Arabinose	1	0.09	4
L-Arabinose	1	0.11	5
D-Xylose	1	0.03	1.3
D-Lyxose	1	0.09	4
D-Glucose	1	0.01	0.45
D-Mannose	1	0.04	1.8
D-Fructose	1	0.01	0.45
L-Sorbose	1	0.01	0.45
D-Fucose	1	0.01	0.45
L-Rhamnose	1	0.01	0.45
D-Glucosamine	1	0.00	0
D-Galactosamine	1	0.02	0.9
2-Acetamido-2-deoxy-D-glucose	1	0.02	0.9
2-Acetamido-2-deoxy-D-galactose	0.1	0.22	100
D-Glucuronic acid	2.5	0.141	3.4
D-Galacturonic acid	2.5	0.457	11
D-Galactono-1,5-lactone	1	0.02	0.9
Ascorbic acid	1	0.04	1.8
2-Furylmethanol	2.5	0.04	0.7
Galactitol	2.5	0.05	0.9
Levulinic acid	2.5	0.05	0.9
Lactose	2.5	0.299	5.5

[a]In percent relative to the color produced by D-galactose.

TABLE III

ANALYSIS OF AN EQUIMOLAR MIXTURE OF D-GALACTURONIC ACID, 2-DEOXY-D-*lyxo*-HEXOSE, D-GALACTOSE, AND 2-AMINO-2-DEOXY-D-GALACTOSE

Compound determined	*Amount (nmol)*	
	Theor.	*Found*
D-Galacturonic acid[a]	200	175
2-Deoxy-D-*lyxo*-hexose[b]	100	110
D-Galactose[c]	100	92
2-Amino-2-deoxy-D-galactose[d]	75	72[e]

[a]For this determination, aliquots of the mixture were heated for 30 min at 100° with 3M hydrochloric acid, and then with 3mM indole–3M hydrochloric acid for 30 min at 100°. The optical absorbance was read at 490 nm. [b]For this determination, aliquots of the mixture were heated for 10 min at 100° with 0.5M hydrochloric acid and 4mM indole. [c]For this determination, aliquots of the mixture were treated with D-galactose oxidase, and then with 2M hydrochloric acid and 4mM indole for 15 min at 100°. The optical absorbance was read at 500 nm. [d]For this determination, aliquots of the mixture were deaminated[8], and then treated with indole–hydrochloric acid[8] for 5 min at 100°. Standard calibration-curves for 2-amino-2-deoxy-D-galactose and 2-deoxy-D-*lyxo*-hexose were established simultaneously. [e]The optical absorbance corresponding to 75 nmol of 2-deoxy-D-*lyxo*-hexose (A_{490} 0.22) was subtracted from the optical absorbance obtained with the mixture (A_{490} 0.76). The difference (A_{490} 0.54) corresponds to 72 nmol of 2-amino-2-deoxy-D-galactose in the standard calibration curve of this sugar.

N-deacetylation step is performed, only D-galactose is measured. Finally, by applying the Dische–Borenfreund reaction[8,9], only 2-amino-2-deoxy-D-galactose is determined. Thus, by a combination of the three procedures, the three sugars may be determined in a mixture.

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