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Note

A colorimetric method for the determination of neutral and acidic carbohydrates

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The colorimetric methods for the assay of carbohydrates are based chiefly on the reaction of 2-furaldehyde or its derivatives (produced on heating carbohydrates with strong acids) with, for example, phenol, indole, diphenylamine, anthrone, *etc.*, to yield coloured complexes[1–6]. The structures of the relevant chromogens are not well established, but their characteristic absorption properties are useful in devising sensitive, quantitative, and reliable assays of microquantities of carbohydrates in biological material. Certain chromogens may be modified[7] by chelation with a metal cation such as Fe^{3+}. The carbohydrate content in biological material is usually determined by the phenol–sulphuric acid[8] and anthrone–sulphuric acid[2] procedures. These methods are also used[9,10] for the determination of such polysaccharides as starch, cellulose, glycogen, agar, *etc.* Our studies[11] of the ethanol fractionation of polysaccharides dispersed in buffered urea showed that the presence of urea (30–180 mg/ml) gave markedly high values for carbohydrate content determined by the phenol–sulphuric acid method. Similarly, ethanol (5–30%) interfered in the anthrone–sulphuric acid method. Based on the earlier observations of the reaction of carbohydrates with thymol–sulphuric acid[12] and thymol–ferric chloride–hydrochloric acid[13], we have now standardised conditions for the latter reagent which proved to be suitable for the required carbohydrate determination.

EXPERIMENTAL

Reagents. — *A*, Thymol (5%) in ethanol, which is stable for 5–6 months at room temperature (26–28°) when preserved in an amber-glass bottle; *B*, 0.5% of ferric chloride in conc. hydrochloric acid.

Solutions of sugar (20–250 μg) in distilled water (1.0 ml) were well mixed with reagent *A* (0.5 ml), followed by reagent *B* (5.0 ml). The mixtures, contained in stoppered tubes to minimise evaporation, were heated on a boiling water-bath for 40 min and then rapidly cooled to room temperature. The mixtures at this stage had

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a turbid appearance with a floating, green complex. Dilution with ethanol (10 ml) gave a clear green solution, the absorbance of which was determined at 635 nm on a Bausch and Lomb Spectronic 20 instrument, using a 12.5-mm path length and a reagent blank (Figs. 1–4, Tables I–III). All other determinations (Figs. 5–7, Table IV) were effected with a Beckman DU 2 spectrophotometer.

RESULTS AND DISCUSSION

The optimal concentrations of thymol and Fe^{3+} ions, and the optimal period for the development of the colour-complex, are illustrated for glucose in Figs. 1 and 2. The formation of the coloured product is proportional to the amount of carbohydrate present in the reaction mixture. The plots of absorbance against concentration for glucose, ribose, galactose (Fig. 3), agar, and starch (Fig. 4) obey Beer's law. The absorption spectra of the green complex for different sugars exhibit slight differences in λ_{max} (Fig. 5), and the colour yields are also different, as reflected in the slopes of the standard curves.

The coloured complex was stable at room temperature (26–28°) in daylight during 6 h, but after 16–18 h, even in the dark, the absorbance increased by a factor of 1.33, and the value of λ_{max} was shifted to 615 nm (Fig. 6). Thus, the latter changes cannot be attributed to any photochemical reaction.

Under conditions identical to those used for Fe^{3+} ions, other metal cations, namely, Zn^{2+}, Cd^{2+}, Hg^{2+}, Al^{3+}, Mg^{2+}, and Mn^{2+}, yielded complexes which differed in colour from violet to blue-green (Table I).

TABLE I

INFLUENCE OF METAL CATIONS ON THE CHROMOGEN DERIVED FROM D-GLUCOSE

	Zero time			*After 1 h*		
	λ_{max}	*Absorbance*	*Colour yield*[a]	λ_{max}	*Absorbance*	*Colour yield*[a]
Zn^{2+}	610	0.14	28	583	0.145	29
Cd^{2+}	630	0.25	50	610	0.215	43
Hg^{2+}	630	0.285	57	610	0.240	48
Al^{3+}	625	0.195	39	600	0.185	37
Mg^{2+}	610	0.23	46	600	0.205	41
Mn^{2+}	630	0.38	76	625	0.37	74
Fe^{3+}	635	0.50	100	635	0.50	100

[a]Expressed as a percentage of the colour produced with Fe^{3+}.

The yield of the coloured complex was dependent on the structure of the carbohydrate substrate (Tables II and III). Hexoses, pentoses, di- and oligosaccharides, and polysaccharides with free or potentially free reducing-groups gave a good yield of green complex, whereas rhamnose, fucose, 2-deoxy-D-*erythro*-pentose (DNA), and 2-amino-2-deoxy-D-glucose gave low colour yields. Mannitol and glycerol gave no colour.

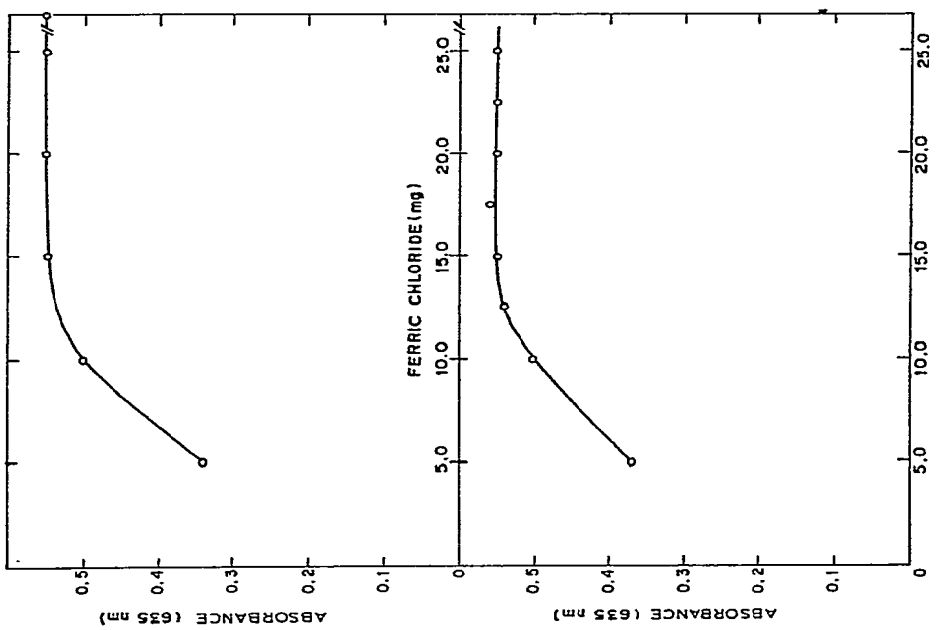

Fig. 1. Effect of concentration of Fe^{3+} ions and thymol on the formation of the coloured complex.

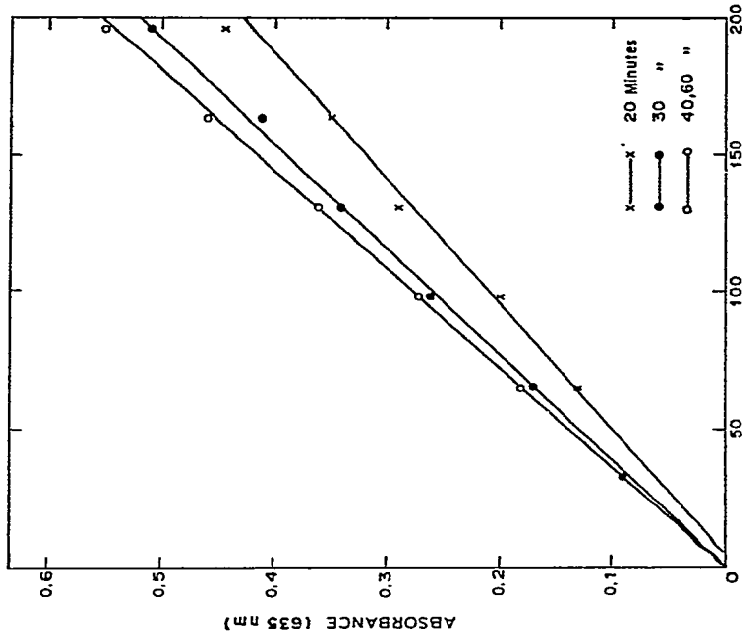

Fig. 2. Influence of time on the formation of the coloured complex. The reaction mixture contains 200 µg of D-glucose.

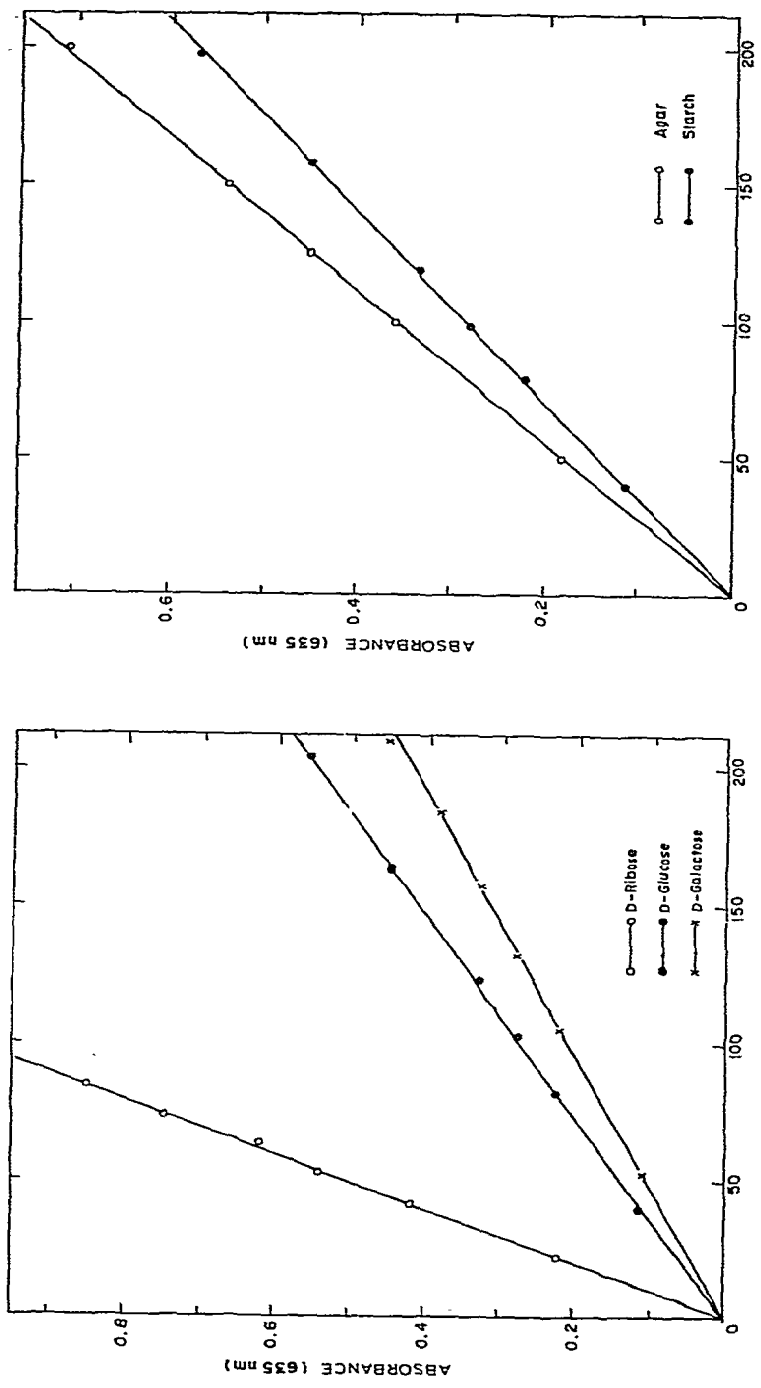

Fig. 3. Standard curves for the determination of D-ribose, D-glucose, and D-galactose.

Fig. 4. Calibration curves for the determination of total polysaccharide content of agar and starch.

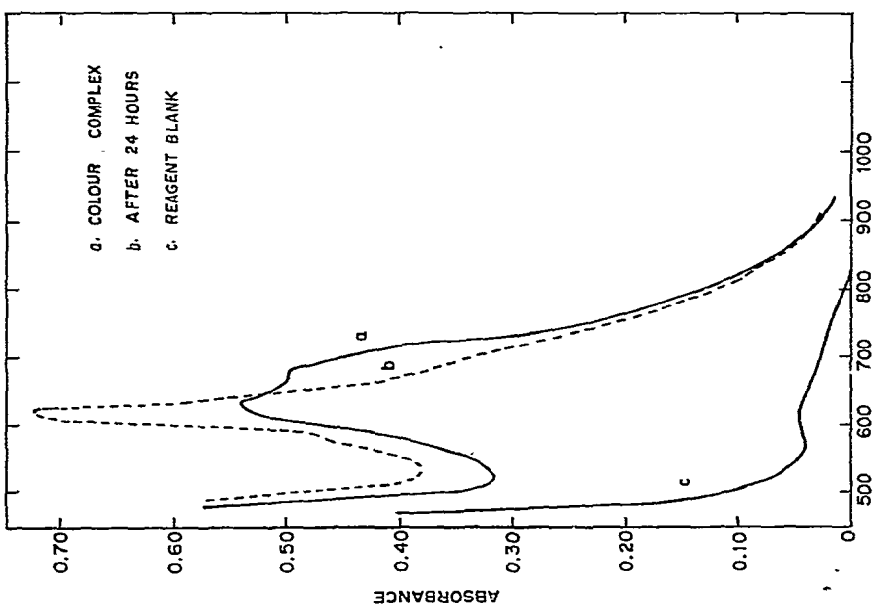

Fig. 5. Absorption spectra of the coloured complexes for D-glucose (60 μg), L-arabinose (60 μg), and sucrose (60 μg) (Beckman DU-2 spectrophotometer, 10-mm quartz cuvettes, against ethanol as a blank).

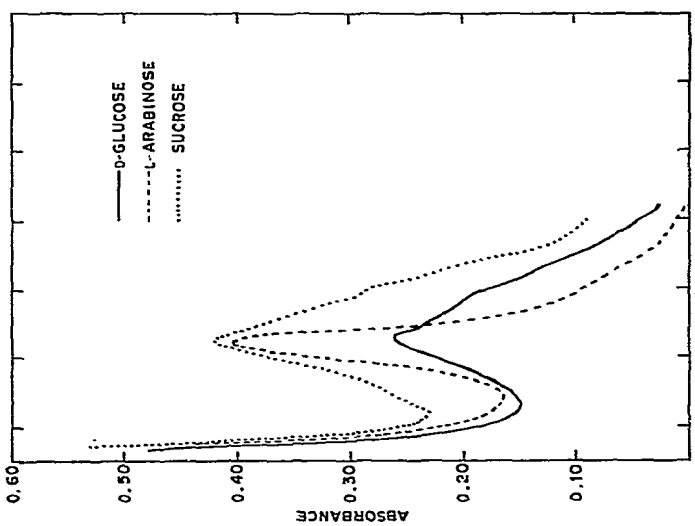

Fig. 6. Absorption spectra of the coloured complex for D-glucose (150 μg).

TABLE II

COLOUR YIELDS FOR SUGARS

Sugar	*Colour yield*[a]
D-Glucose	1.00
D-Fructose	1.82
D-Galactose	0.61
D-Mannose	1.00
L-Arabinose	1.47
D-Xylose	2.99
D-Ribose	2.30
L-Rhamnose	0.20
D-Fucose	0.12
α-D-Glucopyranosyl-1-phosphate (dipotassium salt, dihydrate)	1.00
2-Amino-2-deoxy-D-glucose hydrochloride	0.05
D-Galacturonic acid	1.61
Cellobiose	2.20
Maltose	2.13
Sucrose	3.16
Lactose	1.70
Raffinose (hydrate)	3.11
Mannitol	0
Glycerol	0

[a]Expressed as a percentage of that for D-glucose on a molar basis.

TABLE III

CARBOHYDRATE CONTENT OF SOME BIOPOLYMERS

Biopolymer	*Colour yield*[a]
Starch (potato)	104.7
Amylose (potato)	104.7
Amylopectin (potato)	104.8
Cellulose	105.0
Agar	127.0
Pectin	17.0
Inulin	252.0
DNA (buffalo liver)	0.01[b]
RNA (yeast)	20.0[b]
Gelatin (pig skin)	0.51
Casein	1.73
Human hair	0

[a]Expressed as a percentage of the colour given by D-glucose, on a weight basis. [b]As for *a*, but with D-ribose as the reference.

Preliminary observations show that the thymol–ferric chloride–hydrochloric acid reagent (**1**) can be used for the determination of carbohydrates in proteins. Urea, methyl sulfoxide, cetylpyridinium chloride, 1-butanol, glutamic acid, cystine, lactic acid, and pyruvic acid did not interfere. Tryptophan and vitamin C in high concentrations (2–5 mg/ml) yielded a red colour (λ_{max} 515 nm).

The thymol reagent **1** can be used for the determination of micro quantities (1–14 μg) of sugars (Fig. 7). On the basis of molar extinction coefficients of the

TABLE IV

EXTINCTION COEFFICIENTS FOR VARIOUS COLOURED COMPLEXES OF SOME SUGARS

Reagent	*Molar extinction coefficient*		
	D-Glucose	*Sucrose*	*L-Arabinose*
Thymol–$FeCl_3$–HCl	6.03×10^3 (635 nm)	2.053×10^4 (635 nm)	9.45×10^3 (625 nm)
Phenol–H_2SO_4 (Ref. 2)	1.26×10^4 (488 nm)	2.156×10^4 (488 nm)	9.450×10^3 (480 nm)
Anthrone–H_2SO_4 (Ref. 8)	4.536×10^3 (620 nm)	1.109×10^4 (620 nm)	3.960×10^2 (610 nm)

coloured complexes formed by D-glucose, sucrose, and D-arabinose (Table IV), reagent 1 compares well with phenol–sulphuric acid[2]. The convenience of the experimental procedure and the stability of the component reagents offer some advantages over the anthrone–sulphuric acid and phenol–sulphuric acid procedures.

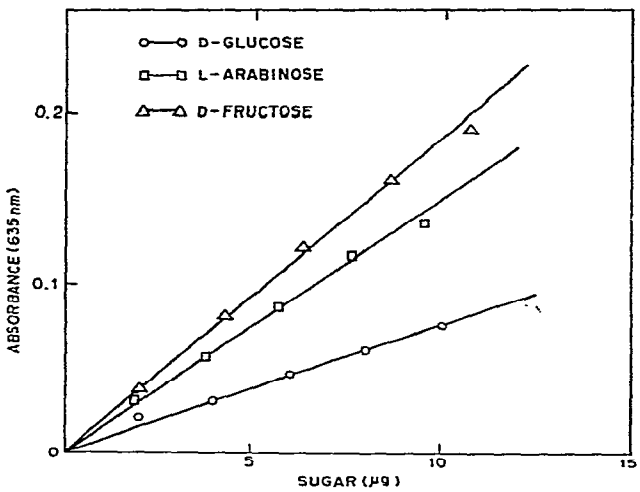

Fig. 7. Standard curves for the determination of D-glucose, L-arabinose, and D-fructose on a microscale. The reaction mixture contained sugar (2–10 μg) in 0.50 ml of solution. Reagents *A* (0.20 ml) and *B* (2.0 ml) were added, and the mixtures were heated on a boiling water-bath for 40 min and then cooled rapidly to 26–28°, and ethanol (1.30 ml) was added.

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