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A Spectrophotometric Study on the Orcinol Reaction with Carbohydrates

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The colour reaction of carbohydrates with orcinol and sulphuric acid was studied in detail by Sørensen and Haugaard [1]. They heated the samples for 5 to 30 minutes at 80° in a water bath and the developed colours were read in a Pulfrich step photometer, usually with filters S 43 and S 53 (maximum transmission at 430 and 530 mμ resp.). The ratios of the densities with these filters were plotted against the time of heating. The curves obtained were considered characteristic for the different carbohydrates.

The present study was carried out with the Warburg and Negelein objective spectrophotometer [2] and with the Beckman spectrophotometer model DU. The absorption spectra thus obtained allowed both a qualitative and a quantitative characterisation of many different carbohydrates, and proved to be useful for the study of the chemistry of the egg coat in sea-urchins.

METHODS

In a detailed investigation of the orcinol reaction, Sørensen and Haugaard [1] studied the effect of variations in the orcinol and sulphuric acid concentrations, the temperature, and the time of heating. It was accordingly not judged necessary to deviate from the conditions which they found to be optimum. However, since it is difficult to prevent a 2 per cent solution of orcinol in 20 per cent sulphuric acid (by volume) from crystallising, a 1 per cent orcinol solution in the same strength of acid was used (Rimington [3] employed a 1.6 per cent orcinol solution in 30 per cent sulphuric acid). The concentration of the stock sulphuric acid was changed from 6 : 4 to 7 : 3, *i. e.*, 7 volumes of concentrated sulphuric acid to 3 volumes of distilled water, in order to maintain as far as possible the same final concentrations of orcinol and sulphuric acid

as used by Sørensen and Haugaard and to employ simple volume quantities of the components. Furthermore, 2 ml instead of 1 ml of the carbohydrate solutions were used, as a convenience in testing mixtures of different sugars. The approximate final orcinol and sulphuric acid concentrations according to the modifications of Sørensen and Haugaard (S-H), Rimington (R), and the author (V) are as follows:

	S-H	R	V
H_2SO_4 concentration in milliequiv. per ml sample	20.0	19.6	19.4
Orcinol concentration in mg per ml sample	2.22	2.16	2.00

Reagents

Carbohydrate solutions. Solutions of the various sugars were made up to contain 10 micromoles of monosaccharide per 2 ml, *e. g.*, for glucose, 90.0 mg per 100 ml; for dextran (a polysaccharide consisting solely of glucose units, $(C_6H_{10}O_5)_n$, $M = n \cdot 162$), 81.0 mg per 100 ml. These were the stock solutions from which other concentrations were prepared.

Orcinol solution. 1.000 g orcinol was dissolved in about 50 ml distilled water, a cooled mixture of 20 ml of concentrated sulphuric acid (sp. gr. 1.84) and 30 ml of distilled water was added, and the solution adjusted to 100 ml with distilled water. Titration gave approximately 7.4 equivalents of H_2SO_4 per liter.

Sulphuric acid solution. 700 ml of concentrated sulphuric acid was mixed with 300 ml of distilled water under careful chilling. Sp. gr. 1.699 at 20°; titration gave approximately 26.8 equivalents per liter.

Procedure

Into an ice-chilled test tube* provided with a stirrer** was pipetted 2 ml of the carbohydrate solution, followed by 3 ml of the orcinol solution. When the mixture had cooled, 10 ml of the sulphuric acid solution was added, and the sample was cautiously stirred to keep the temperature as low as possible***. The sample, still provided with the stirrer, was heated in a water bath at $80 \pm 0.5°$ for exactly 2, 5, or 20 minutes. While heating, the samples may not be stirred. In order to quickly and thoroughly cool the sample, the tube was circulated about in ice water while occasionally stirring the contents. Photo-

* Jena Geräte glass; 180 × 18 mm.

** 5 mm glass tubing with a bulb of about 12 mm blown at one end.

*** After thorough cooling, the sample may be kept in ice water, preferably in the dark, for as long as 12 hours without deterioration. After heating, the density is the same as for freshly prepared samples.

meter measurements of the 2 and 5 minute samples were taken as soon as possible after heating, whereas the 20 minute samples could be kept in ice water for some time without any appreciable change of the density.

The colour displayed by fructose is especially labile and gradually fades. By cooling the 2 minute samples for 5 minutes after heating, and measuring every two minutes at 485 mμ for some time, the density values obtained by extrapolation to zero time were directly proportional to the concentration of fructose.

For each carbohydrate solution investigated, a blank without orcinol was also measured. The orcinol solution was replaced by 20 per cent by volume sulphuric acid. Similarly, when new orcinol or sulphuric acid solutions were employed, a blank without carbohydrate was also determined. It was found advisable to use orcinol and sulphuric acid of as pure a grade as possible in order to minimise the values of samples without carbohydrate. It was also expedient to prepare large volumes of sulphuric acid solution at a time and to store it in vessels free of dust. The orcinol solution could be used for at least six months if stored in a brown bottle kept in the dark except when adding to a sample. In this manner the blank values could be kept constant.

Since the colour in the blank without carbohydrate, if the orcinol is pure, is probably due to traces of pentosans in the sulphuric acid, the analyses must be corrected accordingly. On the other hand, it is not quite certain that the whole density for the blank without orcinol should be subtracted from the density of the sample. Upon heating fructose and rhamnose, and to a lesser extent sorbose and mannose, without orcinol (2 ml of carbohydrate solution, 3 ml of 20 per cent sulphuric acid, and 10 ml of sulphuric acid solution), a colour was formed with maximum absorption at 460 mμ. This colour appears to be suppressed in the presence of orcinol (*cf.* also Sørensen [4]). Very probably these carbohydrates contain the same kind of impurity, probably of aromatic character, which produces a colour with the furfural derivative formed, but more slowly than does orcinol. The correct procedure in this case would be to subtract only a fraction of the density of the blank without orcinol. Since it is hardly possible at present to calculate this fraction, it is advisable to omit this correction for the blank in most cases.

EXPERIMENTAL

From the theory of the absorption of light in a coloured solution [5], $\log \frac{I_0}{I} = D = k \cdot c \cdot d$, and $\log D = \log k + \log c + \log d$ (where I_0 = incident intensity, I = transmitted intensity, D = density, k = specific density = $\frac{D}{c \cdot d}$, c = concentration of the coloured substance, and d = thickness of the liquid layer) it becomes evident that the curve sof $\frac{I}{I_0}$ or $\log \frac{I_0}{I}$ plotted against wavelength, λ, or frequency, ν, change their shape depending on the concentration, c, and thickness, d. If however, $\log D$ or $\log k$ is plotted as ordinate, curves of the same shape are obtained, regardless of the values of c or d.

The colour curves for 20 minutes heating are based on experiments using 1 micromole of monosaccharide as measured in the Warburg and Negelein apparatus. The measurements of the densities were generally performed with a cell thickness sufficient to produce density values ranging from about 0.160 to 0.600, with $d = 0.1$ cm for the strongest densities, and up to 3 cm for the weakest. Concentrations of 0.5 micromoles and, in general, 5 micromoles of carbohydrate as well gave curves in accordance with these standard curves, at least between 470 and 530 mμ; 10 micromoles gave a more or less pronounced turbidity which elevated the density at 560 mμ but depressed it at 425 mμ. It was therefore advantageous to employ a concentration of carbohydrate in the neighbourhood of 1 micromole per sample for the 20 minute curves. On the other hand, 5 micromoles was convenient for the 5 minute curves of the aldohexoses, while 10 micromoles was most useful for their 2 minute curves; under these conditions cells of about 1 cm thickness could be used. For the curves of the ketohexoses and pentoses and for the 5 minute and 20 minute curves of the methylpentoses, 1 micromole was satisfactory and 5 micromoles for the 2 minute curves of the methylpentoses. The curves obtained with the Beckman apparatus during the later part of the study were in good agreement with those obtained with the Warburg and Negelein apparatus.

The maximum at 425 mμ of the 20 minute curves is too sharp to be used for quantitative measurements when photometers with filters are employed (*e. g.*, Pulfrich, Klett, Spekker) since the density values vary too much with variations in concentration and cell thickness. On the other hand, portions of the curves between 480 and 520 mμ, especially of the aldohexoses, slope more gradually and may be used for the estimation of carbohydrate concentration. The Pulfrich step photometer, for example, gives good results with filter S 50 (maximum transmission at 500 mμ) in the determination of blood glucose.

Table 1. Density values with one micromole glucose per sample; 20 minutes heating; measurements with the Beckman apparatus.

λ mμ	Date							Mean
	21/1	22/1	23/1	24/1				
490	0.236	0.230	0.225	0.241	0.246	0.222	0.241	0.2344 ± 0.0034
510	0.225	0.215	0.217	0.230	0.237	0.215	0.232	0.2244 ± 0.0034
520	0.214	0.205	0.208	0.218	0.220	0.208	0.218	0.2130 ± 0.0023

Table 2. Density values with one micromole rhamnose per sample; 5 minutes heating; measurements with the Beckman apparatus.

λ mμ	Sample		Deviation of sample 1 from sample 2 %
	1	2	
370	1.057	1.080	2.1
375	.958	.990	3.2
380	.998	1.025	2.6
385	1.060	1.100	3.6
390	1.140	1.170	2.6
400	1.260	1.300	3.1
410	1.380	1.400	1.4
420	1.350	1.370	1.5
430	1.170	1.200	2.5
440	.880	.919	4.2
450	.598	.621	3.7
460	.378	.393	3.8
470	.271	.280	3.1
480	.233	.238	2.1
490	.230	.235	2.1
500	.239	.241	0.8
510	.229	.234	2.1
520	.195	.200	2.5
530	.154	.160	3.7
540	.115	.122	5.7
550	.084	.090	6.7
560	.063	.069	8.7
580	.035	.039	10.2
Mean of deviation (per cent)			2.8

The accuracy and reproducibility of the method is fairly good as seen from Tables 1 and 2. Table 1 represents some values on glucose obtained with the Beckman spectrophotometer by a probationer.

The carbohydrates investigated in this work were glucose, mannose, galactose, fructose, sorbose, xylose, arabinose, rhamnose, potassium gluconate, sodium glucuronate, mannitol, glucosamine, dextran *, mannan from yeast **, and nucleic acid from yeast. The results obtained with glucose, mannose, galactose, rhamnose, xylose, and fructose are summarised in Fig. 1.

* Generously supplied by Dr. Björn Ingelman.

** Generously supplied by Dr. Gösta Lindstedt.

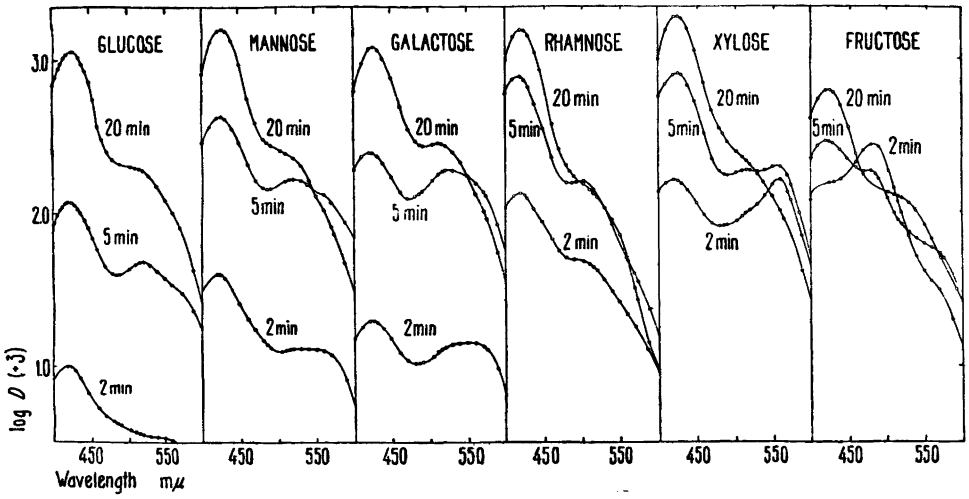

Fig. 1. Colour curves between 400 and 600 mμ for 1 micromole of glucose, mannose, galactose, rhamnose, xylose, and fructose in 15 ml orcinol and sulphuric acid sample after 2, 5, and 20 minutes heating.

Naturally, potassium gluconate and mannitol, like glucosamine, failed to give any colour because the colour development depends on the transformation to a furfural derivative which reacts with orcinol. The preparation of sodium glucuronate tested was a brown mass exhibiting a strong brownish colour when dissolved in water. In spite of this, the blank without orcinol was almost colourless. The colour curves obtained were somewhat similar to both the pentose and the galactose curves. For more reliable values, purer samples of glucuronic acid are necessary; the values obtained in the present investigation are therefore not shown. The ketohexose, sorbose, behaved similar to fructose, and the pentose, arabinose, similar to xylose. The results with dextran, mannan, and yeast nucleic acid are discussed below.

DISCUSSION

The colour curves of the aldohexoses, ketohexoses, pentoses, and methylpentoses differ distinctly from each other. Within each group of carbohydrates, however, the form of the curves is very similar. The aldohexoses have maxima at about 425, 515, and 560 mμ, and a minimum at about 485 mμ (at 470 mμ for galactose after 5 minutes heating). The ketohexoses exhibit a pronounced maximum at about 485 mμ after 2 minutes heating; after 20 minutes heating, their curves are more similar to those of the aldohexoses but

with lower intensities. The pentoses are characterised by well-defined maxima at about 560 mμ for heating times of 2 and 5 minutes, whereas the 20 minute curves are still steeper between 470 and 530 mμ than those of the aldohexoses. The methylpentose, rhamnose, is almost devoid of the maximum at 560 mμ, even for the shorter heating times. The other maxima are displaced toward the short wave region of the spectrum. They are situated at 418 instead of 425 mμ, and at 505 instead of 515 mμ.

The 20 minute curves of the three aldohexoses differ from each other in the following respects. The mannose curve has the steepest slope between 470 and 530 mμ, the glucose curve slopes less steeply in this region, whereas the galactose curve actually rises between 490 and 510 mμ. With the aid of these dissimilarities it is easy to distinguish between them and also between their derivatives. Mannan, for example, gives a 20 minute curve which almost completely corresponds with that of mannose, and the dextran curve nearly parallels that of the glucose analogue. It is remarkable, however, that the densities of these polysaccharides only amount to about 90 per cent of those of the monosaccharides. This may partially depend upon the time required for hydrolysis of the polysaccharides. Furthermore, their 5 minute curves are too low and closely resemble the 2 minute curves of the monosaccharides. If however, the samples are heated for 6 or 7 minutes, the curves nearly attain the shape of the 5 minute curves of mannose and glucose, respectively. The 21 and 22 minute curves rather than the 20 minute curves of these polysaccharides are in good agreement with the 20 minute curves of the monosaccharides. Yeast nucleic acid shows curves similar to those of the pentoses, but the colour development is even more delayed than in the case of mannan and dextran. For the quantitative determination of carbohydrates in macromolecular compounds, this retardation must be kept in mind.

Another fact worth pointing out is that aldohexoses produce no significant colour after 2 minutes heating, even in concentrated solutions, whereas fructose even after 1 minute exhibits a yellow colour of measureable density. After 2 minutes heating, samples containing methylpentoses also show a yellow colour which however is paler than that given by the same amount of ketohexoses. Finally, pentoses give a purple colour after 2 minutes heating. As compared with the density values at 485 mμ for fructose after 2 minutes heating, the values, under the same conditions, for xylose are only 1/3, for rhamnose 1/5, for galactose 1/25, for mannose 1/18, and for glucose 1/60 as intense. On the other hand, xylose has a strong absorption at about 560 mμ after 2 minutes heating, compared to which fructose is 1/5, rhamnose 1/7, galactose 1/10, mannose 1/13, and glucose 1/50 as great. Unfortunately, the colours developed under these conditions are not very stable and their intensities vary

with small differences in the dimensions of the test tubes. The 2 minute extinctions are therefore difficult to use for quantitative determinations. If sufficient care is taken to avoid variation in size of tubes, etc., the use of the 2 minute or even the 1 minute extinctions may be very useful. Thus, by using this method with only 1 minute heating and reading the densities with different filters of the Pulfrich step photometer, Dr. Anders Westgren demonstrated that the reducing sugar in human semen consists chiefly of fructose *.

With this method, it is possible to characterise small amounts of carbohydrates such as are present in limited amounts of biological material. With only a few milligrams of pure carbohydrate, a complete set of colour curves for 2, 5, and 20 minutes heating can be obtained. In more complicated cases, it is advantageous to employ the principle of Dische [6] using different colorimetric reactions. In this way, various sugars exhibit greater dissimilarities than is possible to obtain with only one method. A detailed study of other colorimetric reactions would well compliment the usefulness of the method of Sørensen and Haugaard [1] and the modifications outlined in this paper.

SUMMARY

The colour reaction of carbohydrates with orcinol and sulphuric acid has been studied with the Warburg and Negelein and the Beckman spectrophotometers. The colour curves obtained after 2, 5, and 20 minutes heating are characteristic for aldohexoses, ketohexoses, pentoses, and methylpentoses. These differences permit a distinction of the kind of sugar present even in small samples of biological material. In most cases, a quantitative estimation of the carbohydrate is also possible with this method. The carbohydrates investigated were glucose, mannose, galactose, fructose, sorbose, xylose, arabinose, rhamnose, potassium gluconate, sodium glucuronate, mannitol, glucosamine, dextran, mannan, and yeast nucleic acid.

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* This was demonstrated independently of the work by T. Mann, *Nature* **157** (1946) 79.

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