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REFERENCES

Armstrong, F. H. & Fisher, K. C. (1947). *J. gen. Physiol.* **30**, 279.

Adler, E., Günther, G. & Everett, J. E. (1938). *Hoppe-Seyl. Z.* **255**, 27.

Beevers, H. (1953). *Amer. J. Bot.* **40**, 91.

Bonner, J. (1949). *Amer. J. Bot.* **36**, 429.

Braunstein, A. E. (1947). *Advanc. Protein Chem.* **3**, 1.

Burris, R. H. & Wilson, P. W. (1952). *Biochem. J.* **51**, 90.

Clayton, J. C., Hems, B. A., Robinson, F. A., Andrews, R. D. & Hunwicke, R. F. (1944). *Biochem. J.* **38**, 452.

Clifton, C. E. (1946). *Advanc. Enzymol.* **6**, 269.

Conway, E. J. (1950). *Microdiffusion Analysis and Volumetric Error*, 3rd ed. London: Crosby Lockwood.

Daubney, C. G. & Smedley-Maclean, I. (1927). *Biochem. J.* **21**, 373.

Dixon, M. (1943). *Manometric Methods*, 2nd ed. Cambridge University Press.

Dixon, M. (1949). *Multi-enzyme Systems*, Cambridge University Press.

Dohan, J. S. & Woodward, G. E. (1939). *J. biol. Chem.* **129**, 393.

Elliot, W. H. (1951). *Biochem. J.* **49**, 106.

Fenn, W. O. (1927). *Amer. J. Physiol.* **83**, 309.

Folkes, B. F. (1953). *Analyst*, **78**, 496.

Folkes, B. F., Willis, A. J. & Yemm, E. W. (1952). *New Phytol.* **51**, 317.

Fruton, J. S. (1950). *Yale J. Biol. Med.* **22**, 263.

Grunert, R. R. & Phillips, P. H. (1951). *Arch. Biochem.* **30**, 217.

Haas, E., Harrer, C. J. & Hogness, T. R. (1942). *J. biol. Chem.* **143**, 341.

Hanes, C. S., Hird, F. J. R. & Isherwood, F. A. (1950). *Nature, Lond.*, **166**, 288.

Krebs, H. A. (1948). *Biochem. J.* **43**, 51.

Krebs, H. A., Gurin, S. & Eggleston, L. V. (1952). *Biochem. J.* **51**, 614.

Lindan, O. & Work, E. (1951). *Biochem. J.* **48**, 337.

Lipmann, F. (1941). *Advanc. Enzymol.* **1**, 99.

Lipmann, F. (1949). *Fed. Proc.* **8**, 597.

Loomis, W. F. & Lipmann, F. (1948). *J. biol. Chem.* **173**, 807.

McIlwain, H. (1950). *Brit. med. Bull.* **6**, 301.

McLean, D. J. & Fisher, K. C. (1947). *J. Bact.* **54**, 599.

Massart, L. & Heymans, P. (1950*a*). *Bull. Soc. Chim. biol., Paris*, **32**, 879.

Massart, L. & Heymans, P. (1950*b*). *Bull. Soc. Chim. biol., Paris*, **32**, 888.

Milthorpe, J. & Robertson, R. N. (1948). *Aust. J. exp. Biol. med. Sci.* **26**, 189.

Northcote, D. H. & Horne, R. W. (1952). *Biochem. J.* **51**, 232.

Robertson, R. N., Wilkins, M. J. & Weeks, D. C. (1951). *Aust. J. sci. Res.* B**4**, 248.

Roine, P. (1947). *Ann. Acad. Sci. fenn.* Ser. A, **2**, *Chem.* no. 26.

Runnström, J., Brandt, K. & Marcuse, R. (1940). *Ark. Kemi Min. Geol.* B**14**, No. 8.

Simon, E. W. (1953*a*). *J. exp. Bot.* **4**, 377.

Simon, E. W. (1953*b*). *J. exp. Bot.* **4**, 393.

Simon, E. W. (1953*c*). *Biol. Rev.* **28**, 453.

Simon, E. W. & Beevers, H. (1952). *New Phytol.* **51**, 163.

Smedley-Maclean, I. & Hoffert, D. (1923). *Biochem. J.* **17**, 720.

Smythe, C. V. (1939). *Enzymologia*, **6**, 9.

Somogyi, M. (1945). *J. biol. Chem.* **160**, 61.

Sussman, M. & Spiegelman, S. (1951). *Arch. Biochem.* **29**, 85.

Syrett, P. J. (1953*a*). *Ann. Bot.* **17**, 1.

Syrett, P. J. (1953*b*). *Ann. Bot.* **17**, 21.

Tatum, E. L. (1949). *Fed. Proc.* **8**, 511.

Trevelyan, W. E. & Harrison, J. S. (1952). *Biochem. J.* **50**, 298.

Virtanen, A. I., Csaky, T. Z. & Rautanen, N. (1949). *Biochim. biophys. Acta*, **3**, 208.

Willis, A. J. (1950). Nitrogen assimilation and respiration in barley. Ph.D. Thesis, University of Bristol.

Willis, A. J. (1951). *Biochem. J.* **49**, xxvii.

Willis, A. J. & Yemm, E. W. (1954). *New Phytol.* (in the Press).

Winzler, R. J., Burk, D. & du Vigneaud, V. (1944). *Arch. Biochem.* **5**, 25.

Yemm, E. W. (1949). *New Phytol.* **48**, 315.

Yemm, E. W. & Folkes, B. F. (1953). *Biochem. J.* **55**, 700.

The Estimation of Carbohydrates in Plant Extracts by Anthrone

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The green colour produced when carbohydrates are heated with anthrone in acid solution was first used as a qualitative test by Dreywood (1946); since then anthrone has been extensively employed as a convenient and specific reagent for the colorimetric estimation of a variety of carbohydrates (see, for example, Viles & Silverman, 1949; Seifter, Dayton, Novic & Muntwyler, 1950; Trevelyan & Harrison, 1952). There has, however, been no systematic investigation of the reaction with the different hexose and pentose carbohydrates which may be encountered in biological material. Moreover, different conditions have been used for the reaction and the extent to which sugars may interfere with one another when present in mixtures is uncertain. Johanson (1953*a*) has, in fact, recently stated that

arabinose, although itself giving rise to relatively little colour under the conditions employed for the estimation of glucose, nevertheless interfered seriously in the estimation of a mixture of these two sugars. An investigation of the reaction has therefore been carried out with a range of naturally occurring sugars and sugar mixtures, with a view to assessing the reliability of the anthrone reagent for the analysis of carbohydrates found in plant tissues.

MATERIALS AND METHODS

Anthrone reagent. This was prepared as described by Trevelyan & Harrison (1952) by dissolving 0·2 g. of anthrone in 100 ml. of H_2SO_4, made by adding 500 ml. of conc. acid to 200 ml. of water. The reagent was allowed to stand for 30–40 min. with occasional shaking until it was perfectly clear. Recrystallization from benzene and light petroleum was necessary with some commercial samples of anthrone. The reagent was freshly prepared each day and used within 12 hr.

Sugars. The purest commercially available preparations of sugars (British Drug Houses Ltd., L. Light and Co. Ltd., Roche Products Ltd.) were used. Water contents were determined on samples of the sugars by drying to constant weight at 80° or, for rhamnose hydrate, for short periods at 105°.

Reaction conditions. The reaction was carried out under conditions similar to those used by Trevelyan & Harrison (1952). The anthrone reagent (5 ml.) was pipetted into thick-walled Pyrex tubes (150 × 25 mm.) and chilled in ice water. The solution under test (1 ml.) was layered on the acid, cooled for a further 5 min. and then thoroughly mixed while still immersed in ice water. The tubes were loosely fitted with corks, heated as required in a vigorously boiling, constant-level water bath and then cooled in water for 5 min.

Colorimetry. For routine purposes a photoelectric colorimeter (Evans Electroselenium Ltd.) was used, with $\frac{5}{8}$ in. diameter tubes and with an Ilford Spectrum Orange filter no. 607 (max. transmission at 600 mμ.). The measurements of test solutions and of reagent blanks were made against water as a reference. The relation between scale readings and amounts of sugars was not strictly linear, and it was necessary to use calibration curves for the different sugars. Absorption spectra were determined in a spectrophotometer (Unicam SP. 500) with a 1 cm. cell.

Paper chromatography of sugars. This was carried out by descending filter-paper chromatography according to the methods of Partridge (1948) and Hough, Jones & Wadham (1950). The solvents employed were *n*-butanol:pyridine:water (10:3:3, v/v), *n*-butanol:ethanol:water (40:11:19, v/v) and acetic acid:ethyl acetate:water (2:9:2, v/v). *p*-Anisidine hydrochloride (1%, w/v) in *n*-butanol was used to locate the sugars. In some cases glycosides were eluted with warm water from the paper, hydrolysed with N-H_2SO_4 for 3 hr. at 100°, neutralized with $BaCO_3$ and rechromatographed for the identification of the sugar components.

Plant extracts. Extracts were prepared as described by Yemm & Willis (1954). Leaves were exhaustively extracted with 70% (v/v) ethanol and the extracts evaporated to dryness *in vacuo*. They were then taken up in warm water and cleared with aluminium hydroxide.

RESULTS

The course of the colour reaction. With different sugars the intensity of colour was measured after different periods of heating, and the results for hexoses, 6-deoxyaldohexoses and aldopentoses are shown in Figs. 1–3. Of the hexoses, the ketoses fructose and sorbose react most quickly giving maximum colour development after about 1·5 min. and thereafter a marked decrease in colour with further heating. The rapid reaction with ketoses has been exploited by Johanson (1953*b*) to give a specific colour test for these sugars. The aldoses react more slowly and give a much lower colour production which reaches a maximum after about 6–7 min. for galactose and mannose and about 10 min. for glucose. It is clear that the rate of decrease of colour is approximately the same for both ketohexoses and aldohexoses. A solution of sucrose (calculated as invert sugar) gave colour densities agreeing closely with those expected from a mixture of equal parts of glucose and fructose, when the necessary correction for the non-linear calibration of the colorimeter had been made. 6-Deoxyaldohexoses (rhamnose and fucose) were very similar with regard to colour production and destruction (Fig. 2); for both sugars colour was developed more slowly than for ketoses and maximum absorption was observed after 3 min. heating. The aldopentoses tested, xylose, ribose and arabinose, produced much less colour than the hexoses or 6-deoxyaldohexoses. All three aldopentoses showed rapid colour production which reached a maximum after heating for about 2–2·5 min. (cf. Trevelyan & Harrison, 1952), and then the blue-green colour very quickly disappeared, there being only little absorption after 10 min. heating. At the time of maximum absorption xylose gave a much more intense colour than either ribose or arabinose. The initial rate of colour destruction was very much greater than that of the hexoses, the 6-deoxyaldohexoses being intermediate in this respect. In view of the rapid disappearance of colour with pentoses, the reaction was investigated at lower temperatures with xylose (Fig. 4). Progressive improvement in the stability of the colour was obtained, but the maximum absorption was substantially reduced at lower temperatures. Similar results were obtained with arabinose.

Absorption spectra. For several of the sugars detailed absorption spectra were investigated, both at the time of maximum colour production and after more prolonged reaction. The results are shown in Fig. 5*A*, *B*. With pentoses and hexoses the maximum optical density is at approximately 630 mμ., and is slightly displaced in the case of rhamnose. On more prolonged heating the characteristic absorption band of arabinose disappeared

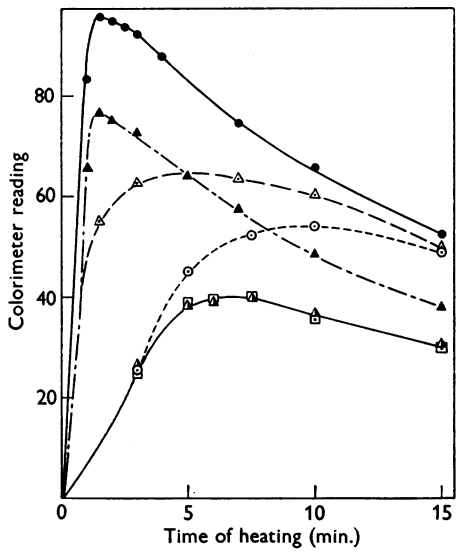

Fig. 1. Colour production during anthrone reaction with hexoses. Colour measurements are corrected for reagent blanks; 75 μg. of each sugar in 1 ml. of water was added to 5 ml. of anthrone reagent. ●—●, D-Fructose; ▲-·-▲, L-sorbose; ⊙---⊙, D-glucose; ◭—◭, D-galactose; ⊡—⊡, D-mannose; △---△, sucrose (calculated as invert sugar).

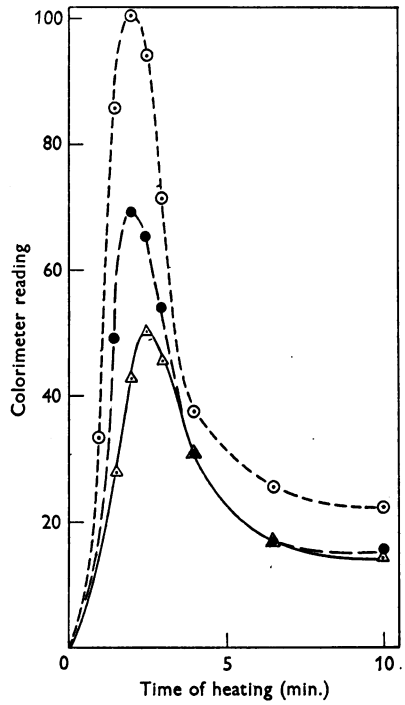

Fig. 3. Colour production during anthrone reaction with aldopentoses. Reaction conditions and colour measurements as for Fig. 1, except that 300 μg. of sugar in 1 ml. of water was added to 5 ml. of anthrone reagent in all tests. ⊙---⊙, D-Xylose; ●---●, D-ribose; △—△, L-arabinose.

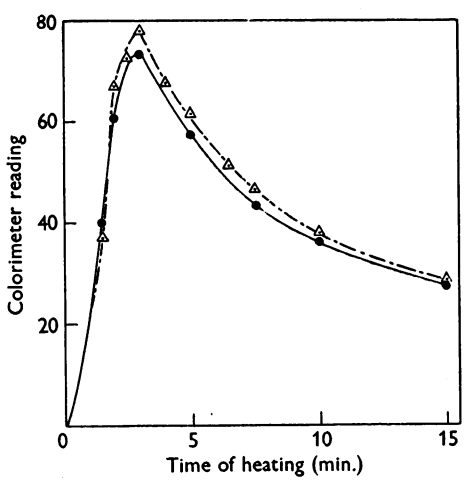

Fig. 2. Colour production during anthrone reaction with 6-deoxyaldohexoses. Reaction conditions for each sugar (75 μg./ml.) and measurements as for Fig. 1. △-·-△, L-Rhamnose; ●—●, L-fucose.

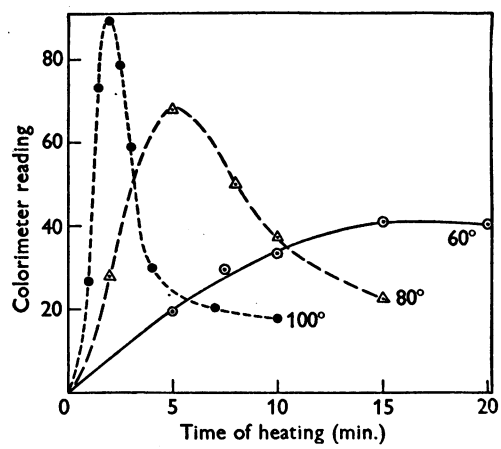

Fig. 4. The effect of temperature on the anthrone reaction with D-xylose. The pentose (240 μg. in 1 ml. of water) was added to 5 ml. of anthrone reagent and allowed to react in a boiling-water bath (●---●), at 80 $\pm$ 1° (△---△) and at 60 $\pm$ 1° (⊙—⊙). The readings are corrected for reagent blanks.

and there was an increase of optical density in the region of 500 mμ. Essentially similar changes, although less pronounced, occurred with hexose sugars.

Analysis of sugar mixtures. When mixtures of sugars were examined it was observed that the absorption curves were consistent with those expected from a summation of the individual sugars. In view of the findings of Johanson (1953*a*) a special test was made with mixtures of glucose and arabinose. The absorption spectra of the individual sugars and of a mixture of the two after 10 min. heating are shown in Fig. 5*C*. At all the wavelengths investigated the optical density of the sugar mixture agreed satisfactorily with that of the sum of the individual sugars, and there was no evidence of appreciable interaction such as that described by Johanson (1953*a*).

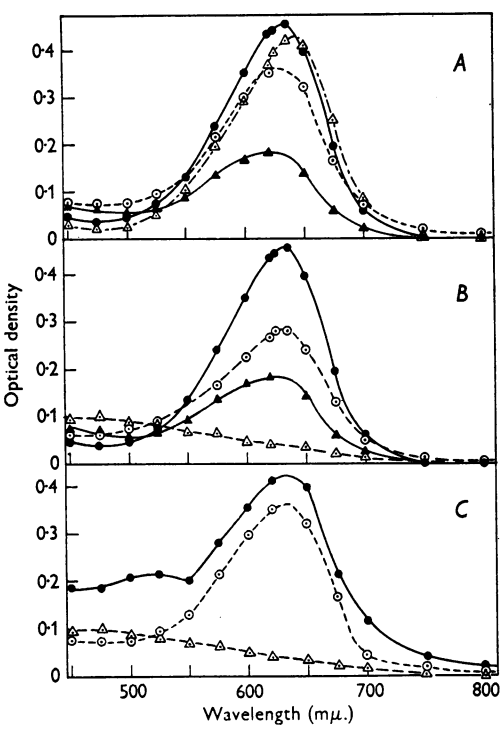

Fig. 5. Absorption spectra of pure sugars and their mixtures after varying periods of heating with anthrone reagent. Measurements of optical density are corrected for reagent blanks. *A*. Pure sugars after optimum heating period. ●—●, D-Fructose (35 μg.; 3 min. heating); △-·-△, L-rhamnose (40 μg.; 3 min. heating); ⊙- - -⊙, D-glucose (50 μg.; 10 min. heating); ▲—▲, L-arabinose (150 μg.; 3 min. heating). *B*. The effects of prolonged heating. D-Fructose (35 μg.): ●—●, 3 min. heating; ⊙- - -⊙, 10 min. heating. L-Arabinose (150 μg.): ▲—▲, 3 min. heating; △- -△, 10 min. heating. *C*. Absorption spectra of glucose, arabinose and their mixture after 10 min. at 100°. △- - -△, L-Arabinose (150 μg.); ⊙- - -⊙, D-glucose (50 μg.); ●—●, L-arabinose (150 μg.) + D-glucose (50 μg.).

More complex mixtures of sugars have been tested with both the spectrophotometer and the colorimeter. The results of one such test carried out with arabinose, rhamnose, fructose and glucose and a mixture of these sugars are given in Table 1. It will be seen that the optical density of the mixture agreed within ±3% of the sum of the optical densities of the individual sugars after heating periods of 3 and 10 min. Several mixtures of hexoses, 6-deoxyhexoses and pentoses have also been investigated by means of the colorimeter and in all cases satisfactory agreement with the expected sum was observed.

The estimation of pure sugars and their recovery from plant extracts. The effective ranges of sugar concentration over which the method described here can be used are indicated in Table 2, together with the heating times adopted for analysis of different sugars. The reproducibility of colour production has been judged from a number of calibrations carried out with different samples of reagent over several months. The results for glucose, fructose and sucrose are based on more than ten determinations;

Table 1. *Reaction of anthrone with sugars and sugar mixture*

In each test the sugar (or mixture) in 1 ml. of solution was added to 5 ml. of anthrone reagent and the optical density measured after heating at 100° for 3 and 10 min.

Sugar	Weight of sugar (μg.)	Optical density at 620 mμ. 3 min.	10 min.
L-Arabinose	51·1	0·064	0·012
L-Rhamnose	22·8	0·204	0·095
D-Fructose	24·8	0·310	0·193
D-Glucose	25·0	0·074	0·169
Mixture of above	123·7	0·635	0·485

Table 2. *The range of sugar concentration and reproducibility of colorimeter readings*

The maximum variation was estimated from calibrations made on different occasions and calculated as a percentage of the colorimeter reading for the middle of the effective range.

Sugar	Time of heating (min.)	Effective range (μg./ml.)	Max. variation (±%)
D-Glucose	10	10–100	3·8
D-Fructose	10	10– 80	2·0
Sucrose	10	10– 80	3·2
L-Sorbose	10	10–100	2·0
D-Galactose	10	10–125	2·8
D-Mannose	10	10–125	3·4
L-Rhamnosé	3	10– 70	2·0
L-Fucose	3	10– 70	2·0
D-Xylose	3	30–300	10·7
D-Ribose	3	35–350	4·5
L-Arabinose	3	40–400	5·0

more limited data are available for other sugars. The maximum variation in calibration did not exceed ±4% except for pentoses, where, owing to the instability of the colour complex, the heating and cooling conditions are of critical importance. It has been observed consistently that a higher degree of reproducibility can be obtained in a series of analyses carried out with the same solution of anthrone. For example, replicate estimates made in this way with a solution of glucose containing 50 μg./ml. had a standard error of ±0·30 μg./ml., with a maximum variation of ±1·6%, and at a concentration of 100 μg./ml., the standard error was ±0·58 μg./ml. and maximum variation ±1·4%. It has therefore been our general practice to include at least one determination of a known amount of sugar in each series of analyses. One important source of error arises from the variation shown in blank determinations, and as this variation appears to be largely due to contamination by dust, care in cleaning glassware is essential.

The effect of a number of compounds on colour production has been examined by Scott & Melvin (1953), and, with the exception of chlorides, only little interference was found. Increased colour production of sugars in the presence of chloride has been confirmed in this laboratory; when solutions containing sodium chloride are used, calibration with pure sugars with the same salt concentration is required.

The reliability of the anthrone method for the analysis of plant extracts has been tested by determining the recovery of sugars added to a leaf extract. The results of this investigation are given in Table 3. Satisfactory recovery of all the sugars tested was obtained. As found above, the greatest variation was observed when arabinose was added, in all probability owing to the instability of the anthrone colour complex with this sugar.

The analysis of plant extracts. A further test of the anthrone method has been made by comparing it with a copper reagent (Somogyi, 1945) for the determination of soluble carbohydrate in leaf tissues. It has already been shown (Yemm & Willis, 1954) that for extracts of leaves of Shasta daisy (*Chrysanthemum maximum*) estimations by the anthrone method agree substantially with those by means of copper reagent, after correction has been made for the substances not removed by rapid fermentation by yeast. Similar agreement has now been found for extracts of leaves of broad bean (*Vicia faba*) and here it is highly probable that the well-defined anthrone reaction obtained with leaf extracts after fermentation is due to the presence of stable glycosides. It was found chromatographically that sucrose, glucose and fructose were the only detectable free sugars in the leaves, but that pigment glycosides were also present. After hydrolysis of one of these pigments, obtained by elution from paper, approximately equal quantities of rhamnose and arabinose together with small amounts of glucose were detected on chromatograms. Support for this finding was obtained from a more detailed study of the course of the anthrone reaction with the carbohydrates remaining in the leaf extract after fermentation. Colour developed to a maximum after about 3 min. heating and then declined to a value of about 64% of its maximum

Table 3. *Recovery of sugars added to leaf extract*

Weighed quantities of sugars were added to an extract of broad bean leaves. The initial sugar content of the leaf extract was calculated as a mixture of equal parts of glucose and fructose, and the recovery of added sugars estimated from calibration graphs for the individual sugars.

Sugar added to extract	Time of heating (min.)	Wt. of sugar added (μg./ml.)	Total sugar found (μg./ml.)	No. of observations	Mean recovery and max. variation (%)
None	3	—	36·0±0·9	4	—
	10	—	38·6±0·3	4	—
Sucrose	10	40	78·5	3	99·7±1·4
Glucose	10	40	79·1	3	101·2±3·2
Fructose	10	40	78·8	3	100·5±2·6
Rhamnose	3	40	76·1	5	100·3±2·1
Arabinose	3	150	187·0	4	100·7±4·7

Table 4. *Analysis of extracts of broad bean leaves with anthrone*

These estimates, made by heating 1 ml. of extract with 5 ml. of anthrone reagent for 10 min., are expressed as equivalent mg. glucose/g. dry wt.

Sample no. (collected 18. vii. 53)	Time collected (G.M.T.)	Epidermis		Mesophyll	
		Free sugars	Glycosides	Free sugars	Glycosides
1	2 a.m.	31·5	35·7	51·7	19·6
2	10 a.m.	51·3	38·4	71·5	18·1
3	5.30 p.m.	84·1	35·8	106·9	18·1

after 10 min. heating. Both the formation and destruction of colour are consistent with the reaction of a mixture of sugars containing mainly rhamnose and arabinose. The analytical results suggest that in certain tissues of the leaf these glycosidic pigments may constitute a considerable part of the total soluble carbohydrate. Some typical results are summarized in Table 4. In the sample collected during the night the major part of the carbohydrates reacting with anthrone in extracts of the epidermis is derived from glycosides, and, calculated as equal amounts of arabinose and rhamnose, would constitute about 75 % of the total carbohydrate of the extract. Despite large fluctuations in the other carbohydrates (mostly sucrose) during the day, the glycoside carbohydrates remain relatively constant in both the epidermis and the mesophyll.

DISCUSSION

As pointed out by Sattler & Zerban (1948), it is very probable that the reaction between sugars and anthrone depends on dehydration and ring formation to form furfural or furfural derivatives which may then react with anthrone. Evidence of these changes in polysaccharides and sugars heated with strong sulphuric acid has been given by Black (1951) and by Love (1953) from measurements of ultraviolet absorption. There is a general parallel between the rate of formation of 5-hydroxymethylfurfural from different hexoses (Love, 1953) and the rate of development of the anthrone colour as shown in Fig. 1. Furthermore, the relative colour yield with anthrone from different hexoses generally corresponds to the amounts of hydroxymethylfurfural formed as judged by Love's absorption measurements at 320 mμ. The parallel seems to hold also for the production of methylfurfural from rhamnose, although here the destruction of the colour during heating is somewhat greater than with other hexoses. With pentoses, on the other hand, the yield of colour is much less than with hexoses, and this can be related to the very high rate of destruction under the conditions normally employed for the reaction. Xylose gives the most intense colour (Fig. 3), corresponding to the most rapid and greatest yield of furfural (Love, 1953). However, the low colour yields of arabinose relative to ribose do not appear to reflect the amounts of furfural produced from these sugars. It is clear that the anthrone method as applied to pentoses is seriously limited by the stability of the furfural–anthrone complex. Although the stability is greater at lower temperatures the sensitivity of the reagent progressively decreases, so that little advantage can be gained by carrying out the reaction at 60° or 80° (Fig. 4). It seems unlikely, therefore, that the anthrone reaction with different hexoses and pentoses can be fully interpreted in terms of the yield and stability of their respective furfural derivatives, especially since furfural and methylfurfural are considerably more stable than hydroxymethylfurfural in acid solution (see Newth, 1951).

For the estimation of soluble sugars in plant materials, a reaction period of 10 min. at 100° has several advantages, and has been adopted for routine analysis of tissue extracts. Under these conditions glucose and fructose, which together make up the bulk of the hexoses, give approximately the same colour density so that fairly accurate estimates of total sugars can be obtained without separate determination of the constituents. Pentoses do not interfere seriously in these estimations; even if present in equal amounts with hexoses the colour density produced by the pentose at 620 mμ. would represent only about 10 % of that of the hexose alone. No evidence has been found of interaction of pentoses and hexoses of the type described by Johanson (1953*a*), and it seems possible that the discrepancy is due to different experimental conditions. In the present work with both a disaccharide (sucrose) and artificial mixtures of monosaccharides the colour reaction has been shown to agree closely with that expected from the behaviour of the individual sugars. Similar findings with lactose and maltose have been described by Morris (1948), who further showed that glycogen behaved in the same way as its hydrolysate. Purified starches have also been found, in this laboratory, to react in agreement with their equivalent of glucose. The method has therefore been shown to give reproducible results with a wide range of hexoses and with polysaccharides. However, as a test for pentoses the reaction is much less sensitive, although satisfactory estimates can be made with carefully standardized heating periods of 2–3 min. A useful feature of the method is that some information may be gained regarding the type of carbohydrate present in extracts under test by studying the course of the anthrone reaction.

The high specificity of the anthrone reaction for carbohydrate (Morris, 1948; Sattler & Zerban, 1948) makes it particularly useful for the analysis of tissue extracts. It has been shown in the present work that, under the routine conditions employed, uronic acids give a small but appreciable colour formation. For example, D-galacturonic acid gives about 10 % of the colour produced by an equal weight of glucose after 10 min. reaction. In connexion with other investigations it has been established that lactic acid and glycerol do not produce green colours with anthrone, but that, in agreement with Shetlar (1952), carbohydrates in the presence of tryptophan give low optical densities

at 620 mμ. Under our conditions, 50 μg. of glucose with 40 μg. of DL-tryptophan gives a colour density of about 95 % of that of the sugar alone.

SUMMARY

1. The anthrone reaction has been investigated with a range of naturally occurring hexoses and pentoses.

2. The course of colour production varies widely with different sugars, and the test is much less sensitive for pentoses than for hexoses.

3. Sugar mixtures give results agreeing closely with those expected from the colour production of the individual sugars. Under the conditions used here there is no evidence of serious interaction of hexoses and pentoses.

4. For the estimation of soluble sugars in plant extracts the method yields results comparable with those obtained with the copper reagent, but includes the sugars of stable glycosides which may constitute a large proportion of the soluble carbohydrates in some plant tissues. Used in conjunction with chromatographic identification, the anthrone method is of particular value when limited quantities of tissue are available.

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REFERENCES

Black, H. C. (1951). *Analyt. Chem.* **23**, 1792.
Dreywood, R. (1946). *Industr. Engng Chem. (Anal.)*, **18**, 499.
Hough. L., Jones, J. K. N. & Wadham, W. H. (1950). *J. chem. Soc.* p. 1702.
Johanson, R. (1953*a*). *Nature, Lond.*, **171**, 176.
Johanson, R. (1953*b*). *Nature, Lond.*, **172**, 956.
Love, R. M. (1953). *Biochem. J.* **55**, 126.
Morris, D. L. (1948). *Science*, **107**, 254.
Newth, F. H. (1951). *Advanc. Carbohyd. Chem.* **6**, 83.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Sattler, L. & Zerban, F. W. (1948). *Science*, **108**, 207.
Scott, T. A. & Melvin, E. H. (1953). *Analyt. Chem.* **25**, 1656.
Seifter, S., Dayton, S., Novic, B. & Muntwyler, E. (1950). *Arch. Biochem.* **25**, 191.
Shetlar, M. R. (1952). *Analyt. Chem.* **24**, 1844.
Somogyi, M. (1945). *J. biol. Chem.* **160**, 61.
Trevelyan, W. E. & Harrison, J. S. (1952). *Biochem. J.* **50**, 298.
Viles, F. J. & Silverman, L. (1949). *Analyt. Chem.* **21**, 950.
Yemm, E. W. & Willis, A. J. (1954). *New Phytol.* (in the Press).

Separation of the Bile Pigments of Serum, Bile and Urine

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Van den Bergh & Müller (1916) described two types of diazo reaction for bile pigment in serum. The sera of normal persons, and of patients with haemolytic jaundice, gave a red colour with diazotized sulphanilic acid only in the presence of ethanol. This was called an 'indirect' reaction, and was distinguished from the 'direct' reaction of bile, or sera from patients with obstructive jaundice, for which ethanol was unnecessary. The direct- and indirect-reacting components of serum were separated chromatographically by Cole & Lathe (1953). While the indirect-reacting pigment appeared to be bilirubin, it was suggested that the direct-reacting fraction was a mixture of two pigments. This has now been confirmed by separating these pigments on reverse-phase chromatograms. These pigments have also been found in human necropsy bile, and in the urine of patients with obstructive jaundice.

METHODS

Extraction of pigments. Pigments were prepared from the sera of jaundiced patients by adding, with stirring, to 1 vol. of serum, 0·18 vol. of saturated $(NH_4)_2SO_4$ and 2·5 vol. of ethanol. After standing for 30 min. the precipitate was removed by centrifuging. The supernatant was stored at −12° and was used as soon as possible, since the polar pigments were found to be altered after storing for several days at this temperature. Before use the supernatant was taken to dryness *in vacuo* at <40°.

The pigments were separated from specimens of bile by the addition of 70 g. of $(NH_4)_2SO_4$/100 ml. of bile. After vigorous mechanical stirring, the mixture was centrifuged and the resulting pellet of protein, pigment, etc., was lifted from the surface of the solution. It was stored in this form at −70°. Pigments and lipids were separated from the protein and salt of the pellet by grinding it with *n*-butanol: methanol (1:1, v/v) and re-extracting until an almost colourless solution was obtained. This procedure was then