THE COLORIMETRIC DETERMINATION OF HEXOSES WITH CARBAZOLE

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The difficulty of applying classical procedures to the qualitative and quantitative determination of carbohydrate in certain biological materials has led to the development of colorimetric methods in which the carbohydrate-containing material is treated with strong mineral acids, causing the formation of substances which will react with compounds such as diphenylamine, resorcinol, orcinol, indole, carbazole, etc., to give distinctively colored products. The rate at which these colored products are formed (1) and the nature of their absorption spectra are frequently sufficiently distinctive to allow their use in differentiating the various sugars.

One of the most widely used of the above colorimetric methods is the carbazole-sulfuric acid method first described by Dische (2–4) and further developed by Gurin and Hood (5, 6) for the identification and estimation of hexoses and pentoses. The latter procedure was used by Seibert and Atno (7) for the analysis of the polysaccharides present in serum and by Knight (8) for the identification of the sugars present in influenza virus. Dische (9) has recently described a modification of the carbazole method for the analysis of uronic acids.

Although the carbazole-sulfuric acid method has been used extensively, no systematic study of the variables influencing this method appears to have been reported. Difficulties encountered in the quantitative application of the carbazole method have been commented upon (7) and indeed the significance of the carbazole reaction, or other color tests, for the qualitative identification of sugars has been questioned (10). Recently in the course of a study of the polysaccharide fractions from hog gastric mucin, we have had occasion to investigate the more important variables associated with the carbazole reaction as applied to the determination of hexoses. In the course of this study the optimum conditions for the quantitative determination of hexose were determined and certain aspects of the qualitative identification of hexoses were examined.

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EXPERIMENTAL

Reagents—Eastman White Label carbazole was precipitated three times from a concentrated sulfuric acid solution by dilution with cold water, and the dried product recrystallized from toluene. The sugars were recrystallized from aqueous ethanol according to conventional methods (11). Technical furfural was fractionally distilled and the fraction boiling at 80–81° and 50 mm. reserved for use. In order to obtain lower blanks, C.P. sulfuric acid was refluxed with potassium persulfate (20 mg. per liter) until a negative test for oxidizing agents was obtained with starch-iodide.

Apparatus—A Klett colorimeter, green filter No. 54, was used for all colorimetric analyses. Duplicate or triplicate analyses were always performed and the results reported are average values. A Beckman model DU spectrophotometer, equipped with a 1 cm. cell, was used in determining spectral absorption. Intensities were measured at 10 mμ intervals except

TABLE I

Effect of Sulfuric Acid Concentration

Concentrated H_2SO_4 added	Klett value*
per cent by weight	
80	215
82	230
84	230
86	220
89	180

* Corrected for blank on 1 ml. of water.

in the region of maxima and minima where the interval was reduced to 2 to 5 mμ.

Effect of Sulfuric Acid Concentration—With the amount of carbazole set at 1.5 mg., the quantity of hexose at 100 γ of glucose per ml., and 10 minutes for the time of heating, variation of the sulfuric acid concentration gave rise to the values shown in Table I. Similar results were obtained with galactose.

Effect of Carbazole Concentration—With the concentration of the added sulfuric acid maintained at 84 per cent (by weight), the amount of hexose at 100 γ of glucose per ml., and the time of heating at 10 minutes, variation of the carbazole concentration between the limits of 1.5 and 4.5 mg. gave rise to the values presented in Table II. Similar results were obtained with galactose.

Effect of Time of Heating—Test solutions containing 9 ml. of 84 per cent

sulfuric acid, 3 mg. of carbazole, and 100 γ of glucose in 1 ml. of water were heated in a boiling water bath for varying periods. It was found that 80 per cent of the maximum color intensity was attained after heating for 7½ minutes, 99 per cent after 10 minutes, 100 per cent after 15 minutes, and 97 per cent after 20 minutes. As in other experiments galactose gave similar results.

TABLE II

Effect of Carbazole Concentration

Carbazole present	Klett value*
mg.	
1.5	240
2.5	316
3.0	363
3.5	402
4.5	438

* Corrected for blank on 1 ml. of water.

TABLE III

Determination of Glucose with Modified Procedure

Glucose	Klett value*	Average deviation, Klett units
γ		
140	682	6
120	607	12
100	527	11
80	425	10
60	349	3
40	272	4
20	185	7
10	144	4
5	129	5

* Average of six separate determinations.

Modified Procedure[1]—A reagent was prepared by adding 10 ml. of a 1.0 per cent solution of carbazole in absolute ethanol to 300 ml. of 84 per cent sulfuric acid. 9 ml. portions of this reagent were chilled in an ice bath, 1 ml. of the hexose solution poured onto the reagent, and the solutions

[1] The procedure of Gurin and Hood (5) consists of heating 1.5 mg. of carbazole (0.3 ml. of a 0.5 per cent solution in ethanol), 1 ml. of hexose solution, and 9 ml. of 89 per cent sulfuric acid (8:1 concentrated sulfuric acid and water) for 10 minutes in a boiling water bath.

thoroughly mixed and heated in a boiling water bath for 15 minutes. After cooling in an ice bath, the intensity of the color produced was determined in the Klett colorimeter. Typical results, obtained with glucose, are given in Table III.

DISCUSSION

It is seen from Table I that the Klett values are particularly dependent upon the acid concentration and that maximum color intensity is obtained with 82 to 84 per cent added sulfuric acid. It was shown, by means of extinction curves, that the differences noted in Table I were due to differences in intensity alone. While extinction values varied widely with sulfuric acid concentration, the position of the maxima lay between 540 and 550 mμ in every instance. However, it should be pointed out that qualitative observations of Dische (2) suggest that significant changes in respect to the position of the maxima may occur also if the sulfuric acid concentration is varied widely.

The dependence of the Klett values upon the carbazole concentration (Table II) emphasizes the necessity of precision in adding the carbazole to the reaction mixture. An error of 3 per cent in the addition of 1.5 mg. of carbazole (0.3 ml. of a 0.5 per cent solution in ethanol) would cause a corresponding variation of about 4 units in Klett values. It is obvious that the addition of small volumes of a carbazole solution is undesirable, especially since the solvent is ordinarily absolute ethanol, which is difficult to pipette accurately. While large amounts of carbazole undoubtedly increase the sensitivity of the procedure, especially since the blank values are practically constant over the range studied, the low solubility of carbazole in the diluted sulfuric acid limits the upper concentration.

In contrast to other variables the time of heating is not particularly critical, provided the time is not less than 10 minutes or more than 20 minutes. A period of heating of 15 minutes, with 84 per cent sulfuric acid, appears to be a reasonable choice.

In order to simplify the procedure of Gurin and Hood (5) the carbazole was dissolved in a relatively large quantity of 84 per cent sulfuric acid and aliquots of this reagent were used for analysis. This reagent simplified the procedure for routine analysis as well as eliminated errors arising from the addition of carbazole to individual tubes. The reagent is prepared by mixing an ethanolic solution of carbazole with 84 per cent sulfuric acid, because solid carbazole dissolves very slowly in sulfuric acid of this concentration. Although the reagent is known to be stable for at least 6 hours, occasionally a green color has appeared after standing for more than 24 hours. It is recommended that the reagent be prepared daily, as was suggested by Seibert and Atno (7).

Nitrate and ferric iron are presumed to interfere in the carbazole procedure of Gurin and Hood (5). No interference from these constituents, or from nitrite, was observed with the modified procedure. The same Klett values were obtained, within the limits of precision discussed below, in the presence or absence of 5 γ of sodium nitrate, sodium nitrite, or ferric chloride when the amount of hexose present was 100 γ of glucose. Sodium

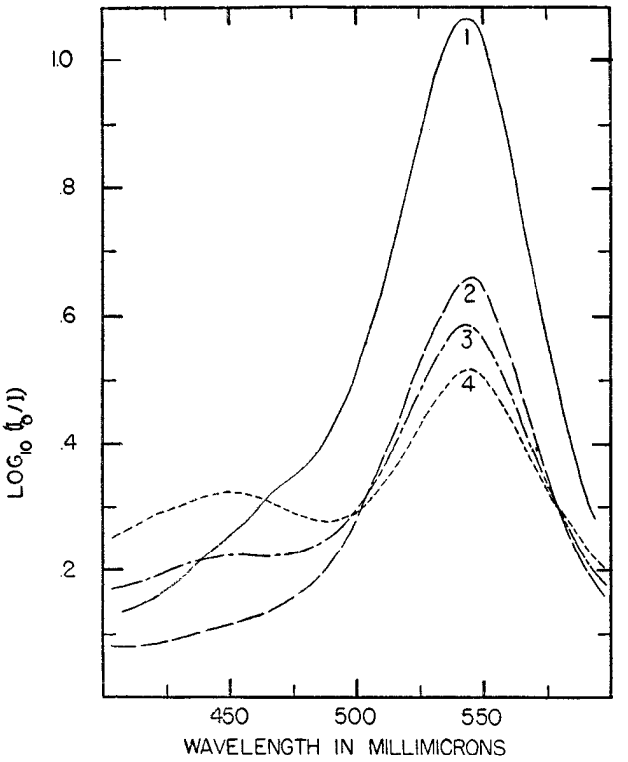

FIG. 1. Absorption curves for colors obtained with various sugars on reaction with carbazole. Curve 1, 100 γ of fructose; Curve 2, 100 γ of glucose; Curve 3, 100 γ of galactose; Curve 4, 100 γ of mannose.

nitrite imparted a faint green color, as did ferric chloride at higher concentrations, to the cold carbazole-sulfuric acid solution. However, this color generally disappears on heating.

While the modified procedure possesses the advantages of convenience and reliability, the precision would appear to be no greater than that of the original when the latter is applied with extreme care. The modified procedure has a precision of 2 to 5 per cent (Table III) in the range of 50 to 150

γ of glucose. The relatively low precision is still unexplained, although the complexity of the reactions occurring in sulfuric acid, revealed by the extinction curves discussed below, suggests that experimental conditions may not still be sufficiently reproducible.

The extinction curves for the carbazole-hexose colored products obtained by the modified procedure are shown in Fig. 1. The curves resemble qualitatively those obtained previously (7, 8). However, the color ob-

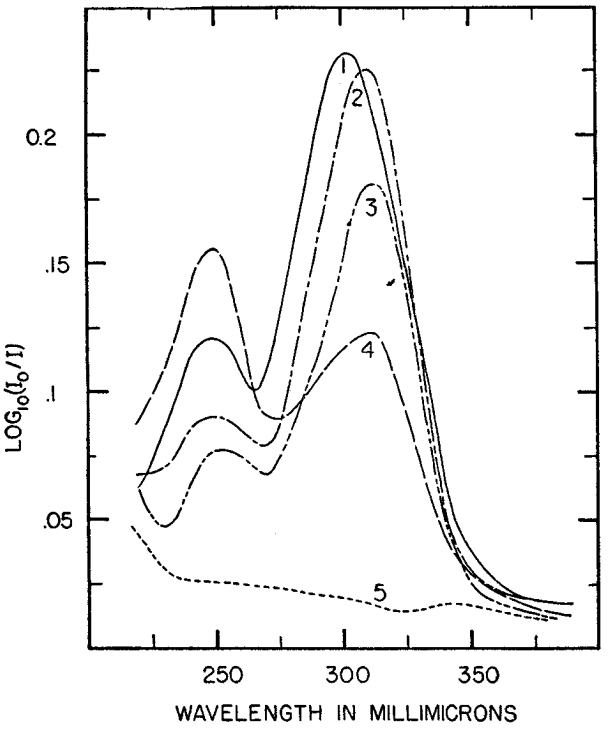

FIG. 2. Absorption curves for various sugars heated in sulfuric acid solution. Curve 1, 100 γ of galactose; Curve 2, 100 γ of glucose; Curve 3, 100 γ of fructose; Curve 4, 100 γ of mannose; Curve 5, 100 γ of N-acetylglucosamine.

tained with mannose does not appear to be as markedly different from that of the other hexoses as has been observed by others (7, 8). It would appear that precise control of the sulfuric acid concentration is of utmost importance, not only for quantitative procedures, but also in the qualitative interpretation of absorption spectra. Owing to the similarity of the curves for glucose, fructose, mannose, and galactose, the low precision of the carbazole method, and because of possible interferences from other

types of compounds (5), the qualitative identification of sugars by means of the modified procedure would appear to be dubious.

Relevant to the problem of spectral identification of sugars are the extinction curves in the ultraviolet region that have been obtained for sugars in sulfuric acid solution in the absence of carbazole. Fig. 2 shows curves obtained for 100 γ of sugar in 1 ml. of water and 9 ml. of 84 per cent sulfuric acid solution after heating 15 minutes on the water bath. The hex-

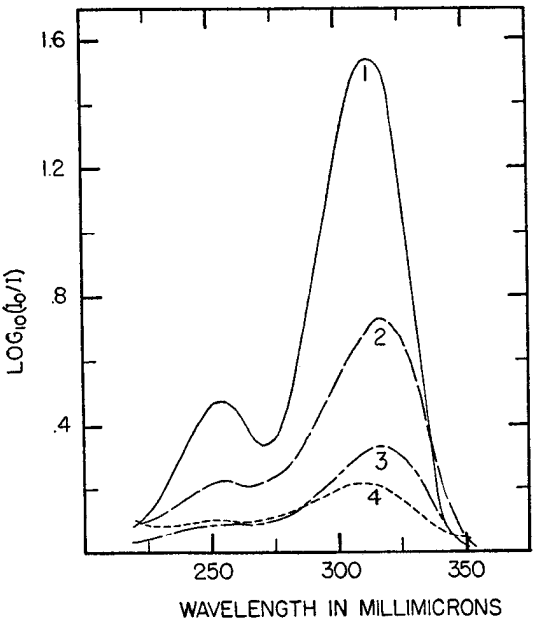

FIG. 3. Absorption curves for furfural and various sugars in sulfuric acid solution. Curve 1, 100 γ of furfural; Curve 2, 100 γ of fructose after standing 40 minutes; Curve 3, 100 γ of fructose after standing 5 minutes; Curve 4, 100 γ of fructose after standing 40 minutes and then heating 15 minutes in a water bath. Curves for galactose, mannose, glucose, and N-acetylglucosamine are not shown, since the densities are below 0.03 throughout the wave-length range.

oses studied exhibit maxima at about 250 and 320 mμ. Fructose, glucose, and galactose show similar curves, while mannose differs in having less absorption at 320 mμ than at 250 mμ. The positions of the maxima correspond closely to those observed for furfural under the same conditions; the apparent conversion of the hexoses to a furfural derivative would appear to be only 10 to 20 per cent as judged from the spectra. While the mechanism of the carbazole reaction and similar color tests is not clearly

understood, presumably the formation of aldehyde intermediates is important (2, 9). It is significant that N-acetylglucosamine, which does not give a color test with carbazole, also shows no specific absorption in the ultraviolet in sulfuric acid solution.

The relative heights of the maxima in the ultraviolet are not correlated with the intensity of the colors produced in the carbazole reaction, a fact which might argue for the unimportance of the compounds showing ultraviolet absorption in the subsequent color reaction. However, extinction curves of unheated sulfuric acid solutions of the hexoses (Fig. 3) suggest a complicating feature. The ultraviolet spectra were found to be sensitive to time of heating, and the extinction values increased and then decreased on heating for successively longer periods. Glucose, fructose, mannose, and N-acetylglucosamine show no appreciable absorption in the cold, while fructose is converted rapidly to an intermediate showing specific absorption in the region 250 to 320 mμ; maximum absorption is reached after about 40 minutes at 25°. When the fructose solution is then heated, the specific absorption decreases markedly, indicating other reactions leading to decomposition. Fructose also shows characteristic behavior in the carbazole reaction in that a color appears several times faster and with greater intensity than for any of the other hexoses. It is apparent that the marked difference in behavior of fructose from other sugars in cold sulfuric acid could readily be adapted to its detection under suitable conditions. Since it is known that heated acid solutions of the hexoses will not react appreciably with carbazole in the cold (9), the carbazole reaction would appear to consist of at least two series of reactions, (1) the conversion of hexoses to intermediates showing specific ultraviolet absorption and the simultaneous decomposition of these intermediates in hot acid solution, and (2) the reaction of some or all of the products with carbazole in hot acid solution to yield a stable visible color.

SUMMARY

The optimum conditions for the colorimetric estimation of hexoses by reaction with carbazole in hot sulfuric acid solution have been determined and a convenient procedure, giving results with a precision of 2 to 5 per cent in the range of 50 to 150 γ of glucose, is described. The colors obtained with glucose, galactose, fructose, and mannose are not sufficiently distinctive to allow their ready differentiation and identification by spectral measurements. The significance of the ultraviolet spectra of heated and unheated sulfuric acid solutions of hexoses to the problem of estimation and identification of hexoses is discussed.

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