SUMMARY

1. The use of a new colorimetric reagent, resorcinol-4:6-disulphonic acid, for the determination of hexoses and their polysaccharides, is described. The reagent has been used chiefly for the determination of glucose, fructose and their polysaccharides.

2. From 10 to 250 μ g. of glucose in 5 ml. may be determined with errors of less than 1 %.

3. Inulin and starch give 100 % and dextran 95 % of the colour intensity given by their constituent monosaccharides.

We are indebted to Prof. J. R. Squire for his encouragement of this work; to various members of the Chemistry Department of this University for specimens of some of the carbohydrate derivatives tested; to Dr C. R. Ricketts for help with the manuscript, and to the Medical Research Council for studentship grants.

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Spectroscopic Studies of Carbohydrates

1. THE ACTION OF SULPHURIC ACID ON SUGARS

By R. M. LOVE

Department of Scientific and Industrial Research, Torry Research Station, Aberdeen

(Received 15 November 1952)

The conversion by acids of pentoses to furfural and of hexoses to 5-hydroxymethylfurfural (HMF) has been extensively studied, principally by Bandow (1937), Holzman, MacAllister & Niemann (1947) and Ikawa & Niemann (1949, 1951), but little is known of the mechanism of these reactions. The subject has been recently reviewed (Newth, 1951).

Concentrated solutions of glucose in 28N-sulphuric acid blacken rapidly on heating, owing to the conversion of HMF to 'humic substances' of unknown structure, but very dilute solutions under similar conditions become rose-pink (Mendel & Bauch, 1926). This colour development was utilized by the latter authors in a method for the determination of blood glucose. The mechanism again was obscure.

It was the object of the present work to follow spectroscopically the conversion of a number of sugars to the corresponding furfural derivatives in sulphuric acid at 60°, and to note the changes in the visible spectrum that occurred when they were heated at 100°, in an attempt to throw some light on the nature of the reaction.

MATERIALS AND METHODS

Reagents. 98 % H_2SO_4 , A.R. grade (sp.gr. 1.840), was used. All the sugars were the best commercial samples available, and were used without further purification, apart from being dried to constant weight over P_2O_5 . Furfural was 'specially purified' (British Drug Houses Ltd.); 5-hydroxymethylfurfural was prepared according to the method of Haworth & Jones (1944), and was twice distilled at 0.1 mm. pressure, yielding almost colourless crystals, m.p. 30°.

Apparatus. Electrically heated water baths, maintained at 60° ($\pm 1^\circ$) and 100° were used for heating the samples. A pipette was calibrated to deliver 6 ml. (11.04 g.) conc. H_2SO_4 in a delivery time of 1 min. The absorption spectra of the solutions were determined with a Unicam photoelectric spectrophotometer.

Procedure. For experiments using 28.3N- H_2SO_4 , 6 ml. conc. H_2SO_4 were pipetted into wide (6 $\times$ 0.75 in.) test tubes containing 2 ml. sugar solution, undue rise of temperature being avoided by swirling in ice-cold water. Blanks were prepared by mixing 6 ml. conc. H_2SO_4 with 2 ml. water under the same conditions. The tubes were placed in racks in the water bath and cooled at intervals in ice-cold water. The solutions were then poured into 1 cm. quartz cells for absorption measurements, the blank solution being placed in the compensator cell.

RESULTS

Ultraviolet spectra

Spectra in 28.3N-sulphuric acid. The conversion of pentoses to furfural and of hexoses to HMF is not quantitative, and varies from sugar to sugar. However, the extinction due to the furfural formed from a given weight of sugar is constant (Dunstan & Gillam, 1949). The latter authors determined the yield of furfural or its derivatives from several sugars by heating 0.5–1.5 ml. of 0.1–0.5 % sugar solution at 170° with 5 ml. 85 % phosphoric acid (sp.gr. 1.689), and steam-distilling off the product. Under these conditions, considerable decomposition of HMF occurred, so that although pentoses yielded reproducible results, data from hexoses were variable and low. Ikawa & Niemann (1949) heated sugars in 79 % (w/v) sulphuric acid at 100°, and measured the absorption after 15 min. In this case, both furfural and HMF had undergone some decomposition, so values of molecular extinction coefficients (ϵ) for both pentoses and hexoses were low.

Under the present conditions, the extinction at $\lambda_{\max}$ was measured at frequent intervals during the heating of the solutions, which usually contained 5 μg . sugar/ml., and the maximum extinction noted before appreciable decomposition had occurred. As decomposition of HMF at 60° was slow, data from hexoses were as reproducible as those from pentoses. Results are illustrated in Table 1, the 328 $m\mu$. maximum of rhamnose being due to 5-methylfurfural.

The exact position of $\lambda_{\max}$ depends on the concentration of sulphuric acid used, e.g. for glucose it is 324 $m\mu$. in conc. acid (Bandow, 1937), and 320, 316 and about 300 $m\mu$. in 28.3, 26.4 and 25.3N-acid, respectively.

It was observed that sorbitol, mannitol and gluconic acid did not develop selective absorption, even after several hours, which confirms a similar observation made by Bandow (1937).

Fig. 1. Absorption spectra of decomposition products of arabinose in H_2SO_4 . $\odot$, D-arabinose (5 μg ./ml.) after heating 25 min. at 60° in 28.3N- H_2SO_4 ; $\bullet$, L-arabinose (5 μg ./ml.) after heating 25 min. at 60° in 28.3N- H_2SO_4 ; $\square$, D-arabinose (5.5 μg ./ml.) after heating 10 min. at 60° in 37.1N- H_2SO_4 ; $\triangle$, D-arabinose after heating 10 min. at 60° in 37.1N- H_2SO_4 , then diluting six times with water (final concn. 5.2 μg ./ml.).

Spectra in 37.1N-sulphuric acid. In addition to the main absorption band at about 320 $m\mu$., furfural and HMF in 25–37.1N-sulphuric acid also exhibit a characteristic lesser band at about 260 $m\mu$. Bandow (1937), using concentrated acid, observed

Table 1. *Formation of furfural or derivatives from sugars*

(28 μM solutions of sugars in 28.3N- H_2SO_4 were heated at 60° until the ultraviolet extinction reached a maximum. The pure furfural and HMF used as references were also in 28.3N-acid, but not heated.)

Substance	$\epsilon_{\max}$	$\lambda_{\max}$ ($m\mu$.)	No. of determinations	Time taken to reach maximum extinction (min.)
Furfural	22 180	315	5	—
D-Xylose	21 270	315	13	10
D-Ribose*	11 430	315	5	40
D-Arabinose	11 220	315	10	25
L-Arabinose	11 220	315	5	25
D-Lyxose*	8 970	315	5	7
5-Hydroxymethylfurfural	22 750	320	4	—
D-Glucose	16 200	320	10	80
D-Galactose	13 600	320	5	50
D-Mannose	12 000	320	5	40
D-Fructose	19 550	320	8	3
L-Sorbose	14 830	320	5	3
L-Rhamnose	14 300	328	10	25

* Lyxose shows an additional maximum at 290 $m\mu$. which exceeds that at 315 $m\mu$. Ribose shows an inflexion at 290 $m\mu$.

that although most sugars gave rise to such spectra, with some the relative strengths of the two bands were reversed. In the present work, no such variation was observed when 28.3N-sulphuric acid was used, but in 37.1N-sulphuric acid some striking changes occurred in the spectra of ribose, arabinose and lyxose. As shown for arabinose in Fig. 1, bands existed at 271 m μ . and subsidiary bands at 315 m μ . On dilution with water, the spectra showed maxima at 276 m μ . (greater) and 220 m μ .

Complete spectra of ribose in 37.1N-sulphuric acid were plotted after 1, 2, 5, 10 and 35 min. at 60° and,

Fig. 2. Absorption spectra of decomposition products of glucose, obtained by heating in 28.3N-H₂SO₄. ○, glucose heated 70 min. at 60° then diluted fifty times with water (final concn. 5 μg./ml.); □, laevulinic acid (0.6 mg./ml.) in 28.3N-H₂SO₄; △, glucose (5 μg./ml.) heated 210 min. at 100°; ●, glucose heated 200 min. at 100°, then diluted ten times with water (final concn. 25 μg./ml.).

although the intensity of absorption increased with time, the general picture remained the same. The minimum between the two peaks became progressively less pronounced, however, and after 35 min. the 315 m μ . maximum was little more than an inflexion.

The spectrum of glucose showed no great difference in the proportions of the two bands between the concentrations of 28–37N-acid, neither did the spectrum of furfural alter appreciably. However, when glucose was heated for a long time in

28.3N-acid at 100°, much of the HMF was destroyed, but the residual spectrum showed a principal maximum at 255 m μ ., which exceeded E_{310} by 27% (Fig. 2). On dilution with water, the 310 m μ . maximum shifted to about 280 m μ ., but the 255 m μ . maximum shifted slightly the other way, to form an inflexion at 260 m μ .

The observation made by Holzman *et al.* (1947) that in mannose the 258 m μ . peak was the greater of the two was not confirmed here at 60° in sulphuric acid of 28.3, 31, and 37.1N concentration, nor when the conditions of these workers were repeated, although an increase in the 258 m μ . band was seen.

Fig. 3. Visible spectra of glucose under various conditions. □, in 28.3N-H₂SO₄ after heating for 4 min. at 100° (0.1 mg./ml.); ●, after 6.5 min. heating; △, after 10 min. heating; ⊙, after 50 min. heating.

Visible spectra

More concentrated sugar solutions were used than for ultraviolet spectra; the final mixture in 28.3N-sulphuric acid usually contained 0.25 mg. sugar/ml. The samples were heated at 100° and cooled in ice-water at various time intervals. The spectra were plotted from about 350 to 550 m μ ., and although there was often much 'ground absorption', the spectra also showed well-defined bands.

An example of such a group of spectra is shown in Fig. 3 (glucose), and spectra from other sugars are described in Table 2.

The rose-pink colour described by Mendel & Bauch (1926) was given solely by glucose. The fact

Table 2. *Description of visible spectra of sugars and related substances after heating in 28.3N-sulphuric acid*(Two ml. 5.6 mm.-aqueous sugar solution were mixed with 6 ml. 98% H₂SO₄ and heated at 100° for various times. Wavelengths in italics denote inflexions.)

Time of heating (min.)	2		4		8		10		15		30		35	
	$\lambda_{\max.}$ (m μ .)	ϵ	$\lambda_{\max.}$ (m μ .)	ϵ	$\lambda_{\max.}$ (m μ .)	ϵ	$\lambda_{\max.}$ (m μ .)	ϵ	$\lambda_{\max.}$ (m μ .)	ϵ	$\lambda_{\max.}$ (m μ .)	ϵ	$\lambda_{\max.}$ (m μ .)	ϵ
D-Glucose	—	—	510 475	554 524	—	—	430 510	1033 921	—	—	430 510	2000 1250	—	—
D-Galactose	—	—	430 470	399	430	1093	—	—	430	1650	—	—	430	2215
D-Mannose	—	—	approx. 430 470	316	approx. 420	589	—	—	approx. 420	895	—	—	—	—
D-Fructose	—	—	470 510	1150	470 510	1041 941	—	—	470 515 440	1250 1100	—	—	470 435 515	1440 1425 1365
L-Sorbose	470	354	430 470	870	—	—	430 470 510	1660	—	—	—	—	—	—
L-Arabinose	—	—	430	79	—	—	430	199	—	—	430 510	443	—	—
D-Lyxose	—	—	approx. 430	96	—	—	430	181	—	—	430 510	331	—	—
D-Ribose	—	—	420	138	—	—	420	294	—	—	430 510	501	—	—
D-Xylose	—	—	430	100	—	—	430	222	—	—	430 520	308 209	—	—
5-Hydroxymethyl furfural	470	128	470 440 510	609	—	—	470 440 510	1040	—	—	—	—	—	—
Furfural	—	—	465 520	62	465 520	92 34	—	—	465 520	172 55	—	—	465 520	346 135
L-Rhamnose	—	—	460	116	460	262	—	—	455	502	—	—	440	1440

that several sugars showed inflexions and even maxima at 510–520 $m\mu$. suggested that the colour might be produced by other sugars under different experimental conditions. Evidence for the nature of the pink colour was obtained from the following observations.

When mixtures of sugars and HMF were heated in 28.3N-sulphuric acid, the spectra were often different from those of either constituent alone. Glucose (0.25 mg./ml.) at 100° showed a pale-pink colour after 3 min. heating which deepened and reached its fullest intensity before becoming brown in 6–8 min. HMF in 28.3N-sulphuric acid became yellow-brown on heating, but a mixture of glucose and HMF became deep pink after 3 min., with a spectrum showing maxima at 470 and 515 $m\mu$.

In a quantitative experiment, 6 ml. conc. sulphuric acid were mixed with (a) 1 ml. glucose + 1 ml. water, (b) 1 ml. HMF + 1 ml. water, (c) 1 ml. glucose + 1 ml. HMF. All three solutions were heated for 3 min. at 100°, when $E_{515 m\mu}$ was found to be 0.142, 0.161 and 0.600 respectively. Thus the absorption of the mixture was almost twice that of the sum of the components. Sorbose and fructose, however, did not show any increase in pink colour with HMF. In a similar experiment, substituting fructose for glucose, $E_{510 m\mu}$ was 0.305, 0.390 and 0.700, respectively, the last value being just that expected for a simple mixture. However, the 470 $m\mu$. absorption was greatly increased in both ketoses on addition of HMF. Conc. sulphuric acid was mixed with fructose plus water, HMF plus water and fructose plus HMF, and allowed to stand at room temperature for 35 min., when the extinctions at 470 $m\mu$. were 0.020, 0.096 and 0.248, respectively.

Mannose and galactose, which when heated with sulphuric acid alone (Table 2) did not even show inflexions at 515 $m\mu$., became pink on heating for 1 min. with HMF, and then rapidly went brown (3 min.). Spectra showed sharp maxima at 515 $m\mu$. and (more intense) at about 460 $m\mu$.

Rhamnose plus HMF became greenish brown after 3 min. heating owing to a broad absorption band at 560–580 $m\mu$. Arabinose and furfural showed no change.

Some sugars when mixed with HMF were found to develop the pink colour in 28.3N-acid at room temperature. Mannose, alone among the hexoses, possessed this property. In a solution containing 0.25 mg./ml. and an equal amount of HMF, the colour was just discernible immediately after mixing with the acid, and developed further on standing. The spectrum showed maxima at about 480, 525 and 560 $m\mu$. Lyxose rapidly became deep brown-pink (λ_{max} . 445 and 525 $m\mu$.) and ribose deep pink (525 $m\mu$. only). Both spectra also showed inflexions at about 560 $m\mu$. On heating, the

colour changed rapidly and gave place to brown in 2 min. Xylose showed a pale-pink colour after standing 45 min. at room temperature.

On diluting the acid solutions threefold with water, the pink colour was destroyed. Visible spectra merely showed a gradual rise in absorption towards the ultraviolet.

Laevulinic acid in water has been shown to absorb maximally at about 265 $m\mu$. (Singh, Dean & Cantor, 1948). In 28.3N-sulphuric acid, a shift to 255 $m\mu$. was observed (Fig. 2), and the spectrum did not alter on heating at 100°, apart from the transitory appearance of a weaker band at about 315 $m\mu$.

DISCUSSION

Practical implications. Several workers have described methods for the estimation of glucose or glycogen which depend on the formation of the pink colour, measurement of which is carried out after heating for an exact time at 100°. The data presented in this paper show that the method is fundamentally unsuitable for accurate determinations because (a) the optical density at 515 $m\mu$. is relatively low, (b) the visible spectrum consists of several absorption bands, and (c) the value of $E_{515 m\mu}$ after 6 min. heating is increasing rapidly (Fig. 3), thus lowering the accuracy of the method. If heating is continued beyond 6 min., the band at 515 $m\mu$. ceases to predominate, and the pink colour becomes masked by a deep brown which makes visual estimation impossible. Measurement of the absorption at 320 $m\mu$. is more satisfactory as a method of estimation, since the absorption curve consists of one main maximum only, the molecular extinction coefficient is about 20 times as great, and under suitable conditions the absorption rises to a steady value (Love, 1953).

Products other than furfural or HMF. Some of the foregoing data indicate that, although sugars are converted to furfural or HMF at 60°, there is also a tendency to form other compounds with similar absorption spectra, to an extent which probably depends on the configuration of the sugar.

In the pentose series, xylose (Table 1) shows a much greater absorption at λ_{max} . than the other sugars. When the spectra were examined, only that of xylose resembled the furfural spectrum in having a sharp maximum at 315 $m\mu$. and a smaller sharp peak at 256 $m\mu$. In arabinose (Fig. 1) and ribose, there was only an inflexion at 260 $m\mu$., and in lyxose, which had by far the lowest absorption at 315 $m\mu$., the spectrum showed an additional maximum at 290 $m\mu$. Further, it can be seen that $\epsilon_{315 m\mu}$ was in all cases less than that of furfural. Similarly, in the hexose series, only the spectra of glucose and fructose resembled HMF; galactose, mannose and sorbose merely showed inflexions at

260 $m\mu$. In addition, the chief maxima of galactose and mannose were much broader than in HMF. There does not seem to be any correlation between the final optical density and the rate of reaction (Table 1), but it is evident that the only sugars producing spectra resembling furfural or HMF are those which show the greatest absorption at 315 or 320 $m\mu$., respectively.

The conclusion to be drawn is that sugars in 28.3N-sulphuric acid may form two other compounds besides furfural or HMF, one absorbing maximally at 290 $m\mu$., the other at about 315 $m\mu$.. The presence of the latter compound is deduced from the broadness of the main band shown in mannose and galactose and not in the pentoses. The compounds are the result of side reactions in the dehydration of the sugars and not decomposition products of furfural or HMF, since they were not observed after heating the latter substances at 60°.

Bandow (1937) showed that the furfural molecule was not radically altered in concentrated sulphuric acid, since after dilution the spectrum was identical with that of furfural in water: the shape was the same as in concentrated acid solutions, but λ_{max} . had shifted to about 280 $m\mu$., and the 258 $m\mu$. band to 228 $m\mu$. (Fig. 2). Thus the relatively high absorption at 255 $m\mu$. obtained by the prolonged heating of glucose at 100° cannot be due entirely to HMF, since on dilution it apparently remains at about 260 $m\mu$., although the 315 $m\mu$. maximum moves to 280 $m\mu$.. A similar effect occurs in HMF. Identification of the substance absorbing at 255 $m\mu$. is not possible on the strength of the available data, but laevulinic acid (Fig. 2) seems the most likely. However, the molecular extinction coefficient of the latter is too low for it to account for all the absorption.

Similarly, the high absorption at 271 $m\mu$. produced by heating certain pentoses in 37.1N-sulphuric acid (Fig. 1) is not all due to furfural, since it does not move towards the far ultraviolet on dilution, but to 275 $m\mu$.. This band is thus probably made up of two components, furfural, which shifted from 315 $m\mu$. on dilution, and another compound of unknown structure. The latter is not a breakdown product of furfural, since the 271 $m\mu$. band exceeds the 315 $m\mu$. band at all stages of heating. It will be noted that xylose again yields only furfural.

To sum up, therefore, it appears that the differences in appearance of the spectra of sugars after heating in sulphuric acid are caused by varying amounts of at least four other substances which absorb in the region 255–315 $m\mu$.. It is the formation of these substances which explains why some sugars yield less furfural or HMF than others.

The nature of the pink compound. The discovery by Blanksma & Egmond (1946) that HMF was an intermediate product in the formation of humin

from hexoses might lead one to suppose that since the pink compound described by Mendel & Bauch (1926) was observable only in dilute glucose solutions, it might be formed by the condensation of just a few HMF molecules. However, this would not explain why it was formed in smaller amounts in fructose, and not formed at all in mannose, galactose or sorbose. Further, more pink colour was produced by furfural than by HMF, although neither produced much.

The result of adding pure HMF to the sugars before acidifying indicated the nature of the pink compound in a striking way. Since none of the four pentoses produced the colour alone or when mixed with furfural, but three of them did so when mixed with HMF, the $-\text{CH}_2\text{OH}$ group of HMF evidently played a part in the reaction. Also, since the pink colour developed in the cold immediately the sulphuric acid was added in lyxose, ribose and mannose, these sugars must have a structure particularly favourable for its formation. The only discernible feature common to all three sugars is that the hydroxyl groups on carbon atoms 2 and 3 are *cis* to one another. This suggests that the pink compound is formed by condensation between these groups and HMF. The fact that galactose, glucose and xylose all formed the pink colour with HMF after some delay (galactose and glucose required heating), suggested that some rearrangement of the molecules was necessary, such as epimerization, if it were possible in 28.3N-sulphuric acid.

It may be asked why among all the sugars only glucose and fructose formed the pink compound when heated alone in 28.3N-sulphuric acid. The answer is clear in the case of pentoses, for furfural, the end-product, does not condense with the hydroxyl groups. However, it was surprising that mannose, which reacted in the cold in the presence of HMF, exhibited no selective absorption at all at 515 $m\mu$. when heated without it.

The explanation lies probably in the rate of reaction. Table 1 shows that of the three aldohexoses, glucose took the longest to be converted to HMF. If this were due to its greater stability, then, when some HMF had formed, there would still remain sufficient undecomposed glucose to react with it, whereas in the other sugars the reactive grouping would have disappeared.

Other absorption bands in the visible spectrum. The 470 $m\mu$. band seen in the visible spectra of hexoses is probably due to the condensation of HMF with itself, since it alone is produced when HMF is heated in 28.3N-sulphuric acid (Table 2), and is the first band to form in fructose and sorbose, which are converted to HMF very rapidly (Table 1).

The remaining important band, at 430 $m\mu$., appears last in glucose (Fig. 3) and the ketohexoses, and is the main pentose band. Since it is not formed

by furfural alone, it is probably the product of condensation of the —CHO group of furfural (HMF in hexoses) with the sugar molecule.

SUMMARY

1. The conversion of several sugars to furan derivatives at 60° in 28.3N-sulphuric acid was followed spectroscopically. Variations in the apparent yields and in the shapes of the absorption spectra were attributed to two other absorbing compounds, which were formed at the expense of the furan derivatives in amounts dependent on the configuration of the sugars.

2. Another by-product was formed by three of the sugars when 37.1N-sulphuric acid was used. Its absorption exceeded that of the furfural, and caused a complete change in the appearance of the spectra.

3. The colours formed when sugars were heated at 100° in 28.3N-sulphuric acid were found to be due to three main absorption bands. The nature of the absorbing substances is discussed.

The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

The author is indebted to Dr A. Banks of this Department, and to Prof. R. A. Morton and Dr F. D. Collins, of Liverpool University, for helpful discussion and advice.

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Studies on Influenza Virus Receptor-Substance and Receptor-Substance Analogues

I. PREPARATION AND PROPERTIES OF A HOMOGENEOUS MUCOID FROM THE SALIVARY GLAND OF SHEEP

By J. F. MCCREA*

The Lister Institute of Preventive Medicine, London, S.W. 1

(Received 16 December 1952)

Agglutination of red cells by influenza virus, first described by Hirst (1942), may be inhibited by specific antibody, by non-specific serum inhibitors (Burnet & McCrea, 1946; McCrea, 1946) and by a wide range of mucoid substances. Relatively weak inhibition of viral haemagglutination by apple pectin was described by Green & Woolley in 1947, but this apparently non-specific effect has not been confirmed by other workers. Inhibition by serum mucoid and crude human 'mucins' (Burnet, McCrea & Anderson, 1947) appears, however, to be a more specific reaction, since the inhibitory power of such substances is destroyed on incubation with living influenza virus or the bacterial receptor-

destroying enzymes (Burnet, McCrea & Stone, 1946; Burnet & Stone, 1947) in the same highly characteristic fashion as are the 'true' receptors on the erythrocyte surface.

Although purified human serum mucoid (McCrea, 1948*a*; Hirst, 1949) and ovarian-cyst mucopolysaccharide (McCrea, 1948*b*) show high activity in inhibiting haemagglutination, it has not yet been definitely established that such activity is an integral part of the mucoid molecule. Other mucoid substances such as ovomucin (Lanni & Beard, 1948; Gottschalk & Lind, 1949), human cervical mucin (Burnet, 1948*a*) and the allantoic-fluid inhibitor (Svedmyr, 1948), which show high antihæmagglutinin activity, have generally been used in a relatively crude form. Until a virus-inhibitory mucoid has been obtained in a homogeneous state,

* Australian National University Scholar. Present address: Department of Microbiology, Yale University School of Medicine, New Haven, Connecticut, U.S.A.