soluble fraction were globulin X, myosin, F-actin and G-actin.

3. The bound nucleotide of the myofibril remained associated with the myofibril on prolonged extraction with 0.078M-borate at pH 7.1, but passed into solution on extraction with salt solutions of higher ionic strength.

4. Exhaustive extraction with potassium chloride

and lithium chloride failed to dissolve 9–17% of the myofibrillar protein, whereas extraction with Weber's and potassium iodide solutions reduced the insoluble residue to 2–4%.

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A New Colorimetric Reagent for Carbohydrates

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Methods for the estimation of carbohydrates, and particularly polysaccharides, have been somewhat tedious till the introduction by Dreywood (1946) and Morris (1948) of the reagent anthrone in concentrated sulphuric acid. This provided a sensitive, facile and general method for carbohydrate analysis, since simple dilution of the reagent (2 volumes) by the test sample (1 volume) produces a strongly acid medium sufficiently hot to hydrolyse a polysaccharide, which then gives a colour reaction with the anthrone equivalent to that of its constituent units. Thus the reagent may be calibrated against standards of the constituent monosaccharide, since the colorimetric yield is known to be quantitative.

Unfortunately the anthrone reagent is somewhat unstable, necessitating frequent preparations, including standards for calibration. A search was therefore made for a similar but stable reagent. Various simple polyphenols, substituted phenols and aromatic amines were tried, but those which were chromogenic were also unstable in sulphuric acid. In an endeavour to stabilize resorcinol, one of the most promising reagents when used in place of anthrone, the hydroxyl groups were protected

by benzylation. When resorcinol dibenzoate in ethanolic solution and sulphuric acid were added in succession to test samples, the benzoyl groups were removed by hydrolysis with the development of a satisfactory colour, but benzoic acid crystallized out on cooling. The ditoluene-*p*-sulphonate proved resistant to hydrolysis and gave scarcely any colour. *O*-Substitution having failed, the nuclear-substituted derivative resorcinol-4:6-disulphonic acid (RDSA) was prepared, and found to be stable in aqueous solution, although insoluble in concentrated sulphuric acid. The disulphonic acid was about as sensitive a colorimetric reagent as the parent phenol, giving an intense golden-brown colour with carbohydrates. This reaction has been investigated so as to provide a reliable and simple method for carbohydrate estimation which would be useful when only very small volumes or very dilute solutions were available for analysis. Under the conditions of the reaction all the isomeric hexoses tested gave a peak at 490 m μ , but the sensitivity varied with the individual hexose (Fig. 1). For any series of determinations, therefore, the appropriate hexose must be used as standard

when calibrating the reagent. The reagent has been used chiefly for determining glucose and fructose and their polysaccharides.

Fig. 1. Absorption spectra of reaction products of: A, 250 μ g. glucose; B, 250 μ g. fructose; C, 250 μ g. galactose; each with RDSA (0.5%, w/v). Curves for sorbose and mannose are very similar to B and C, respectively.

Since somewhat different colours are given with different sugar derivatives, and since sulphonation appears to stabilize phenols in solution, other sulphonated phenols were tested, first for greater discrimination between different sugars, e.g. aldoses and ketoses, and secondly in an attempt to find a substance combining the stability of such compounds with the higher specificity and colorimetric yield of the anthrone reagent.

EXPERIMENTAL

Reagent

Resorcinol-4:6-disulphonic acid was prepared according to the procedure of Piccard & Humbert (1876) as modified by Kaufmann & de Pay (1904). Resorcinol (22 g.) was added in small portions with stirring to fuming H_2SO_4 (150 g., sp.gr. 1.875) kept at 60° in a water bath. After stirring at this temperature for 1 hr., the mixture was cooled to 0°. The calculated amount of water required to dilute the acid to 96% (13 ml.) was then added very slowly and the product which separated filtered off on to sintered glass, washed well with concentrated H_2SO_4 and drained as much as possible. The resulting paste may be used directly for the preparation of the reagent as described below, but further purification was carried out as follows: the paste (10 g.) was dissolved in 100 ml. water, and excess CaCO_3 added with vigorous stirring. After 30 min. the precipitated CaSO_4 and excess CaCO_3 were filtered off and washed, and the combined washings and filtrate evaporated to about 20 ml. After cooling to 0° the calcium salt together with some CaSO_4 was precipitated by addition of ethanol and filtered off. The precipitate was then dissolved in 20 ml. hot water and a little residual CaSO_4 removed by filtration. The filtrate was treated with charcoal, filtered, cooled and precipitated with

ethanol as before. The product, now almost free from SO_4^{2-} , was finally recrystallized from aqueous ethanol. (Found: C, 22.6; H, 2.3; S, 19.4; Ca, 11.5. Calc. for $\text{C}_6\text{H}_4\text{O}_6\text{S}_2\text{Ca} \cdot \text{H}_2\text{O}$. C, 22.1; H, 1.9; S, 19.6; Ca, 12.2%. Analysis by Weiler and Strauss, Oxford.) A solution of this salt may then be used as reagent. A 0.6% (w/v) aqueous solution of the Ca salt is made up (the equivalent of a 0.5% (w/v) solution of the free acid), and filtered free from dust. A solution of the free acid may also be prepared directly from the paste (1%, w/v), decolorized with charcoal and filtered, but this may contain as little as 0.5% (w/v) RDSA.

Method

The most satisfactory method is described first, followed by the studies which led up to this choice.

To 5 ml. samples containing up to 250 μ g. carbohydrate in closely similar stoppered tubes, 1 ml. of 0.5% RDSA (as the Ca salt) in water is added. Conc. H_2SO_4 (A.R., 10 ml.) is added from a fast-running pipette (or burette) with shaking. After standing for 1 hr. in the dark to cool, the optical densities are read at 490 $\text{m}\mu$, using 1 cm. cells, against the least of several blanks run concurrently, using 5 ml. distilled water. The mean value of these blanks is then subtracted from the mean of the observations.

The reagent is calibrated with standard solutions of the monosaccharide to be determined, taking every precaution to exclude dust. (All optical measurements were made on a Unicam Spectrophotometer S.P. 500.) The optical density at 490 $\text{m}\mu$ is directly proportional to the quantity of hexose in the 5 ml. sample up to 200 μ g. ($E=0.570$), though it falls off slightly at higher densities. The concentration range 0–200 μ g. is readily measured using 1 cm. cells. Starch and inulin gave results equivalent to 100% of their monosaccharide units; the more resistant polysaccharide dextran, as used clinically for transfusions, gave colours representing a 95.5% yield, the standard error of the method being $\pm 1.0\%$.

It is hoped to publish further details concerning the estimation of dextran.

Variation of experimental conditions

The effect of a number of experimental conditions on the reaction was investigated.

Effect of calcium. The Ca salt behaved in exactly the same way as its equivalent of free acid. No precipitation of CaSO_4 was observed. A 1.2% (w/v) solution of the Ca salt gave the same intensity of colour with 120 μ g. glucose as an identical solution from which the Ca had been precipitated with the calculated quantity of oxalic acid. (Optical densities 0.633 ± 0.006 and 0.638 ± 0.004 , respectively.)

Concentrations of reagent and acid. The sensitivity increases with reagent concentration to a plateau at about 3% (Fig. 2), when 100 μ g. glucose gives an optical density of about 0.8. This is so high that the dilution required makes it extremely difficult to eliminate scatter due to minute dust particles, and for ordinary use a 0.5% reagent solution is more satisfactory.

No variation was found when the stock conc. H_2SO_4 contained up to 3% (v/v) water.

Vessels. All reagents and apparatus must be scrupulously clean and free from dust, cotton wool strands, etc., otherwise the blanks will be appreciably coloured and the optical densities erratic. Since the validity of the optical measure-

ments rests on the quality of the blanks, it is advisable to prepare three or more blanks. The test and standard solutions were read against the lowest blank, and from these results the mean of the other blanks, which should be small (optical density against distilled water <0.050 at $490\text{ m}\mu$.), was subtracted.

The vessels should be stoppered and closely similar in shape and thickness. Since the heat of dilution of the acid brings about reaction, the rate of cooling affects the final optical density, so that the geometric form of the vessel is important, though it does not affect the initial maximum temperature appreciably (cf. Van Slyke & Sinex, 1952). Further, the optical densities are increased to a maximum by 20 min. heating in a boiling-water bath, when the spectrum is little changed (Figs. 3, 4). If the heating is continued beyond 20 min. the optical density at $490\text{ m}\mu$ falls rapidly and the spectrum changes with the development of a new peak at $440\text{ m}\mu$ (Fig. 4). With the anthrone reaction, similar heating changes the colour visibly to grey-green.

Fig. 2. Variation in optical density ($490\text{ m}\mu$.) of RDSA/glucose reaction mixture with various concentrations of RDSA (Ca salt); $100\text{ }\mu\text{g}$. glucose.

In these experiments B.19 Quickfit stoppered test tubes of closely similar size and weight ($41 \pm 1\text{ g.}$) were used. There was a statistically significant difference in optical density between batches treated in light (33 g.) and heavy (41 g.) tubes (heavy 0.400 ± 0.004 ; light 0.366 ± 0.001 ; $t=12.22$, $n=5$, $P<0.001$).

Sensitivity to light. This is greater than the experimental error. Of ten replicates, five were kept for 1 hr. in the dark, while five stood 30 cm. from a 60 w lamp. The optical densities were 0.439 ± 0.004 and 0.411 ± 0.003 , respectively ($P<0.001$). Accordingly, all reaction mixtures should be allowed to cool in the dark, and since after the initial rapid development the colour continues to increase very slowly (approx. $1\%/hr.$), the cooling should occupy a standard time (e.g. 1 hr.).

Temperature of reaction. This passes through a broad maximum when the volume of acid is approximately twice the volume of the aqueous solution. For 5 ml. of water, the addition of 8, 9, 10, 11, 12 ml. acid gave a maximum temperature of 108, 112, 118, 117.5 and 116.5° , respectively. Nevertheless, when the volume of acid added to 5 ml. of

sample plus 1 ml. of reagent is increased from 10 to 12 ml. there is a decrease in optical density (corrected for dilution) of nearly 40%. The cause of this is not apparent. The proportional decrease was identical for glucose and for dextran, i.e. the degree of reaction of dextran was not increased as compared with glucose.

Fig. 3. Variation in optical density ($490\text{ m}\mu$.) of RDSA (0.5% , w/v) in glucose ($250\text{ }\mu\text{g}$.) due to heating reaction mixture at 100° for various periods.

Fig. 4. Absorption spectra of RDSA/glucose reaction mixture. $250\text{ }\mu\text{g}$. glucose, 0.5% (w/v) RDSA. A, normal; B, after heating at 100° for 20 min.; C, after heating at 100° for 40 min.

Presence of chloride. Readings are increased in the presence of chlorides, as shown in Fig. 5, the shape of the absorption curve being little changed. This cannot be overcome by adding NaCl to the reagent, since crystallization occurs on cooling before the plateau of Fig. 5 is reached. The high sensitivity of the method often necessitates dilution, which normally eliminates this effect. Otherwise a correction may be made or polysaccharide material may be precipitated out with ethanol, as in the application of the anthrone method by Bloom & Wilcox (1951) to the estima-

tion of dextran in blood and urine. The sensitivity of the anthrone reagent was found to be increased (by 17%) by the presence of NaCl (1%). The acid procedure of Schaffer & Hartmann (1921) was found to give results 2% low for dextran when the hydrolysis was conducted in the presence of 1% NaCl.

Specificity of coloration. RDSA gives no reaction with the amino acids except tryptophan (pale yellow), nor with carboxylic acids, acetone, inositol, cholestan-3-one, cholest-4-en-3-one, gelatin, sodium ethylmercurithiosalicylate (British Drug Houses Ltd.) or barbitone. Various shades of yellow-brown were given by aldehydes and lactic acid and also by alcohols in much larger concentrations (probably owing to oxidation to aldehydes), and by products of sugar degradation (furfural, laevulinic acid). Ethyl acetoacetate gave a slight reaction, ninhydrin a strong claret red and dioxan a characteristic cherry red which could probably be

Fig. 5. Variation in sensitivity of RDSA (0.5%, w/v) with glucose due to presence of chloride. (Sensitivity = optical density (490 m μ .)/glucose present (μ g.).)

used for the detection of traces of this substance. The intense golden-brown obtained with hexoses was distinguishable from the more orange colour with pentoses, and the yellow-brown colour with aldehydes (e.g. acetaldehyde, $\lambda_{\max}$ = 390 m μ .). Glucosamine gave a feeble red-brown colour only on long standing, hexuronic acids a characteristic yellow becoming black-brown, rhamnose and 6-iodofucose, red-brown, similar to the hexoses, galactal yellow-brown, 2-deoxygalactose yellow-brown and 1:2-dideoxyglucose a faint pink.

Sensitivity with various hexoses. The sensitivity to RDSA varied with the hexose as is evident from Fig. 1. The curve obtained with mannose is very similar to that shown for galactose and with sorbose to that shown for fructose. The calibration curve for fructose has a slope of about 60% of that for glucose, which is surprising since resorcinol is used in the Seliwanoff test for ketoses.

Effect of other carbohydrates. Pentoses and uronic acids in all amounts interfere with the quantitative determination of the hexoses; the reagent is more sensitive to pentoses than

to hexoses. ($E_{\max}$ for 100 μ g. xylose = 1770 at 470 m μ .). Glucosamine does not interfere with the determination of hexoses in the concentration range tested (up to 100 μ g./100 μ g. glucose).

Other reagents

N-1-Naphthylethylenediamine. This reagent (0.2% in conc. H₂SO₄) gave an intense crimson colour with hexoses ($\lambda_{\max}$ = 540 m μ .; optical density proportional to weight of glucose), but the reagent deteriorated as rapidly as anthrone.

Orcinol. This reagent, used similarly, showed a major peak at 435 m μ . and a lesser one at 470 m μ .; that at 435 m μ . showed a higher sensitivity than any other reagent but the calibration plot was appreciably curved. This reagent was unstable, as were the other common phenols. The relative optical densities of these compounds for 100 μ g. glucose/5 ml. were as follows (all %, w/v): with anthrone 0.2% at 625 m μ ., 0.25; with orcinol 1% at 470 m μ ., 0.31; with resorcinol 1% at 495 m μ ., 0.43; with RDSA 0.5% at 490 m μ ., 0.28; RDSA 3% at 490 m μ ., 0.83.

4-Hydroxydiphenyl-4'-sulphonic acid (Latschinow, 1873). The solution used was prepared by adding *p*-hydroxydiphenyl (twice recrystallized, 20 g.) to conc. H₂SO₄ (A.R., 200 ml.) stirred at 90° in a water bath, over a period of 30 min. After stirring for a further 30 min. at 100° all the solid had dissolved; the clear solution was cooled to 0° and cautiously added to 1800 ml. ice-water. The solution was decolorized with animal charcoal and filtered free from dust. Used as described for RDSA, the reagent gave a characteristic orange-pink colour with fructose which was absent with glucose. The fructose curve shows a sharp peak at 470 m μ ., while that for the same amount of glucose shows only a low continuous absorption with no peak in the wavelength range studied (350–550 m μ .). Rhamnose and fucose gave a peak at 420 m μ . and xylose at 450 m μ . Galactose and mannose gave spectra similar to glucose. The reagent appears to be stable and calibration curves for fructose were easily reproducible.

Chromotropic acid (1:8-dihydroxynaphthalene-3:6-disulphonic acid). The recrystallized sodium salt (1% aqueous solution), used as RDSA, gave a purple coloration with glucose, yellowish red with fructose and yellow with rhamnose. It is, however, insensitive and the aqueous solution of the reagent changes colour in a few days.

Anthrone-1- and -2-sulphonic acids. These were prepared by the reduction of the corresponding anthraquinone-sulphonic acids with alkaline sodium dithionite by the method of Battegay & Hueber (1923), the sodium salts recrystallized from aqueous ethanol and were used similarly to RDSA in 1% aqueous solution. It was hoped that these might combine the higher carbohydrate specificity of anthrone with the stability of RDSA. Unfortunately, neither gave any appreciable colour with glucose or fructose under the conditions of the experiment.

Anthrone. This reagent was tested with some substances not reported by Dreywood (1946) or Morris (1948). It gave an intense scarlet colour with lactic acid, brown colours with aldehydes and more feebly with alcohols, and a reddish colour with tryptophan in large quantities: sodium alginate (polymannuronic acid) and potassium hyaluronate gave grey becoming brown and pale green respectively. Fucose, as the diacetone derivative, gave an intense blue colour.

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.

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Spectroscopic Studies of Carbohydrates

1. THE ACTION OF SULPHURIC ACID ON SUGARS

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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.