

The absorption spectrum of the haemoprotein or the nature of the ligands co-ordinated with it would then be irrelevant. All the components revealed by this study would then be 'peroxidases' if they are indeed separate, naturally occurring species. The term 'paraperoxidase' with its implication of the modification of an original enzyme could be applied in the sense of Keilin & Hartree (1951) to the insoluble or less-soluble derived materials formed from peroxidases on storage. Component A_1 may thus have formed a 'paraperoxidase' in our experiments on the ageing of horse-radish juice.

SUMMARY

1. The peroxidases of horse-radish juice and the latex serum of *Ficus macrophylla* have been examined by filter-paper electrophoresis.

2. Horse-radish contains possibly five components with peroxidase activity; *Ficus macrophylla*, one.

3. There is great seasonal variation in the relative amounts of the peroxidase components present in horse-radish juice; one of the two major components is absent in the late spring.

4. A major component is always present in horse-radish juice which corresponds to the peroxidase II of Theorell; other material probably corresponding to his peroxidase I is also present.

5. Two components have been discovered which are negatively charged at pH 5.0 and cannot be equated with either peroxidase I or II.

6. One of these components has a positive peroxidatic activity against benzidine and little or none against guaiacol; all the components show some differences in reactivity towards various substrates.

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REFERENCES

- Boman, H. (1952). *Nature, Lond.*, **170**, 703.
 Chance, B. (1951). *The Enzymes, their Chemistry and Mechanism of Action*, vol. II, chap. 56C. New York: Academic Press.
 Cremer, H. P. & Tiselius, A. (1950). *Biochem. Z.* **320**, 273.
 Fišer-Herman, M. & Davorin, P. (1953). *Biochem. Z.* **324**, 96.
 Foter, M. J. (1940). *Food Res.* **5**, 147.
 Franklin, A. E., Quastel, J. H. & Van Straten (1952). *Nature, Lond.*, **168**, 687.
 Gillespie, J. M., Jermyn, M. A. & Woods, E. F. (1952). *Nature, Lond.*, **169**, 487.
 Gillespie, J. M. & Woods, E. F. (1953). *Aust. J. Biol. Sci.* **6**, 447.
 Hall, D. A. & Wewalka, F. (1952). *Nature, Lond.*, **168**, 685.
 Jermyn, M. A. (1952a). *Nature, Lond.*, **169**, 488.
 Jermyn, M. A. (1952b). *Aust. J. Sci. Res. (Ser. B)*, **5**, 433.
 Jermyn, M. A. (1953). *Aust. J. Biol. Sci.* **6**, 77.
 Jermyn, M. A. & Thomas, R. (1953). *Nature, Lond.*, **172**, 728.
 Keilin, D. & Hartree, E. F. (1951). *Biochem. J.* **49**, 88.
 Kondo, K. & Morita, Y. (1953). *Chem. Abstr.* **47**, 3893.
 Maehly, A. C. (1951). *Enzymes and Enzyme Systems, their State in Nature*, p. 79. Cambridge, Mass.: Harvard University Press.
 Moore, S. & Stein, H. W. (1952). *Annu. Rev. Biochem.* **21**, 530.
 Smith, E. L. (1952). *Nature, Lond.*, **169**, 60.
 Strange, R. E. & Harkness, N. (1953). *Nature, Lond.*, **171**, 77.
 Sumner, J. B. & Somers, G. F. (1947). *Chemistry and Methods of Enzymes*, 2nd ed., p. 219. New York: Academic Press.
 Theorell, H. (1940). *Ark. Kemi Min. Geol.* **14 B**, no. 20.
 Theorell, H. (1942). *Ark. Kemi Min. Geol.* **16 A**, no. 2.
 Theorell, H. (1947). *Advanc. Enzymol.* **7**, 265.
 Theorell, H. & Åkeson, Å. (1942). *Ark. Kemi Min. Geol.* **16 A**, no. 8.
 Van Fleet, D. (1947). *Biodynamica*, **6**, 125.
 Weber, R. (1953). *Helv. chim. acta*, **36**, 424.
 Woods, E. F. & Gillespie, J. M. (1953). *Aust. J. Biol. Sci.* **6**, 130.

A Colorimetric Micro-method for the Determination of Glucose

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When a dilute solution of glucose is heated with concentrated sulphuric acid, a bluish pink colour develops, the intensity of which is proportional to the concentration of glucose (Mendel & Bauch, 1926). This reaction has served as the basis for simple methods of determining glucose in blood (Mendel & Bauch, 1926; Mendel & Hoogland, 1950) and glycogen in tissues (Kemp & Kits van Heijningen,

1954). Both methods depend on the formation of 5-hydroxymethylfurfural (HMF) from glucose in hot acid, a reaction which has been studied by Holzman, MacAllister & Niemann (1947), Singh, Dean & Cantor (1948), Wolfrom, Schuetz & Cavalieri (1948) and Ikawa & Niemann (1949). The coloured compound seems to be produced in a subsequent reaction between HMF and one of its pre-

cursors. This reaction is highly dependent on the concentration of sulphuric acid and appears to be specific for glucose, fructose and related carbohydrates.

EXPERIMENTAL

Reagents

Sulphuric acid. The only reagent necessary for the determination of glucose in pure solution is concentrated H_2SO_4 . The samples of 96% (w/w) H_2SO_4 (sp.gr. 1.84) obtained from Canadian Industries Ltd. (Canada), Grasselli (U.S.A.), Brocades-Stheeman (Holland), Merck and Co. (Germany) and May and Baker (England) proved to be suitable. Samples supplied by a number of other firms often gave a yellowish pink instead of the bluish pink colour. The concentration of H_2SO_4 determined by titration should be approximately 36N. If the concentration in commercial samples is too high, the H_2SO_4 has to be diluted and should then be allowed to stand 2-3 days in a stoppered bottle before use.

Deproteinizing solution. For determining the glucose concentration in blood samples, the blood is deproteinized with a 5% (w/v) solution of trichloroacetic acid (TCA) (AnalaR Reagent) containing 0.1% (w/v) Ag_2SO_4 (A.R.) (Mendel & Hoogland, 1950).

Procedure

One ml. of an aqueous glucose solution, containing not more than 15 mg. glucose/100 ml., is added to 3 ml. of 96% H_2SO_4 in a wide test tube, the contents of which should be mixed immediately by vigorous shaking. After mixing, the concentration of H_2SO_4 in the solution is 82% (w/w); the temperature of the solution rises temporarily to about 85°. The mixture is heated for exactly 6.5 min. in a boiling-water bath; the water should be boiling vigorously to prevent a decrease in temperature when the test tubes are inserted. The test tubes are subsequently cooled under tap water and the intensity of the pink colour is measured at 520 m μ . in a Beckman Spectrophotometer, model DU. The method can easily be adapted for the Coleman Junior or any other colorimeter. Should it be inconvenient to measure the optical density immediately, the tubes can be stoppered and allowed to stand at room temp. for several hours, since the pink colour is stable in 82% H_2SO_4 .

Various modifications of this procedure have been tested but they did not appear to offer any advantage. The same colour intensity is obtained when (a) the H_2SO_4 is added to the glucose solution, (b) the glucose solution and H_2SO_4 are cooled to 0° while being mixed, and (c) the mixture is not heated at 100° immediately but kept for 30 min. before being heated.

For determining the blood-sugar level, 0.1 ml. blood is pipetted into 1.9 ml. of the TCA: Ag_2SO_4 solution. The pipette should be rinsed well with the deproteinizing solution and the suspension mixed briefly. The suspension is centrifuged at 3000 rev./min. for 5 min. to obtain a clear supernatant fluid. The precipitate can also be removed by filtration through a small filter paper (cf. Mendel & Hoogland, 1950), but special care must be taken to ensure that the paper is free of chlorides. One ml. of the clear supernatant fluid or filtrate is added to 3 ml. of conc. H_2SO_4 and the colour reaction is carried out as described above.

The concentration of glucose in the TCA extract can be read from a standard curve. Up to a concentration of 15 mg./100 ml., the relationship between the glucose concentration and colour intensity is linear, an extinction of about 0.160 at 520 m μ . being obtained with an aqueous solution containing 10 mg. glucose/100 ml. The same standard curve can be used as long as the concentration and brand of H_2SO_4 remain unchanged.

RESULTS

Absorption spectrum

Figs. 1 and 2 show the absorption spectrum of the coloured solution which is obtained after heating a dilute solution of glucose for 6.5 min. at 100° in 82% sulphuric acid. Absorption maxima are found at 520 m μ . in the visible range (Fig. 1) and at 318 and 255 m μ . in the ultraviolet (Fig. 2). The absorption peak at 520 m μ . is due to the bluish pink compound which is formed in the reaction. The maxima at 318 and 255 m μ . are due to the formation of HMF from glucose in heated sulphuric acid, their positions and relative optical densities being almost identical with those of HMF dissolved in sulphuric acid of the same concentration (Fig. 2).

Upon dilution of the coloured reaction mixture with water, the absorption maxima in the ultraviolet shift their positions in precisely the same way as do the absorption maxima of HMF. The bluish pink colour diminishes when the sulphuric acid is diluted, and disappears completely when the concentration of sulphuric acid is decreased to approximately 55%. Since the absorption spectrum of HMF is still present under these conditions, it seems probable that the coloured compound dissociates into simpler molecules in less concentrated sulphuric acid.

The bluish pink compound is not formed when HMF is heated with concentrated sulphuric acid, though a slight yellowish colour with a maximal absorption at 430 m μ . is produced. However, the intensity of the pink colour is not diminished when up to 25% of the glucose is replaced by HMF (Fig. 3). It appears, therefore, that the formation of the coloured compound in heated sulphuric acid is dependent on the presence of HMF and a compound intermediate between glucose and HMF.

When the course of the reaction is followed by means of the absorption spectra, the concentration of HMF is found to reach a maximum after approximately 2 min. at 100°, as judged by the optical density at 318 m μ . (Fig. 4). It can be calculated that about 65% of the glucose originally present must have been converted into HMF at this point. With continued heating the HMF concentration decreases. The bluish pink colour appears more slowly than the HMF and reaches its maximum after about 10 min. at 100° (Fig. 4).

Further heating fails to cause an appreciable change in the intensity of the bluish pink colour; however, a brownish colour with an absorption maximum at $430\text{ m}\mu$. appears, the intensity of which increases the longer the glucose is heated with concentrated sulphuric acid (Fig. 4). The heating time of 6.5 min. at 100° was selected to permit a

maximal development of the bluish pink colour with minimal interference by the brownish decomposition products.

Sulphuric acid concentration

Fig. 5 shows the results obtained when a dilute glucose solution is heated for 6.5 min. at 100° in sulphuric acid of different concentrations. Pro-

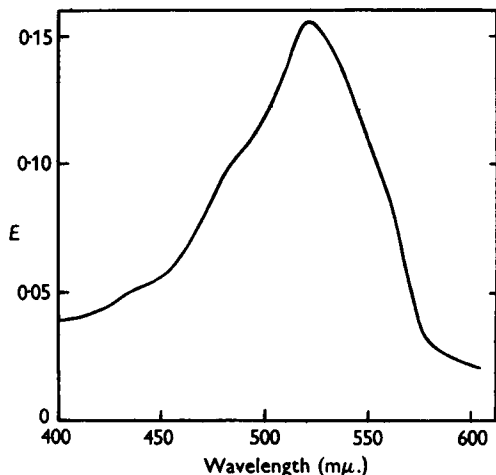


Fig. 1. Visible spectrum obtained after heating 1 ml. of an aqueous glucose solution (0.01 %) with 3 ml. of 96 % (w/w) H_2SO_4 at 100° for 6.5 min. The extinctions were measured in a 1 cm. cuvette with a Beckman Spectrophotometer.

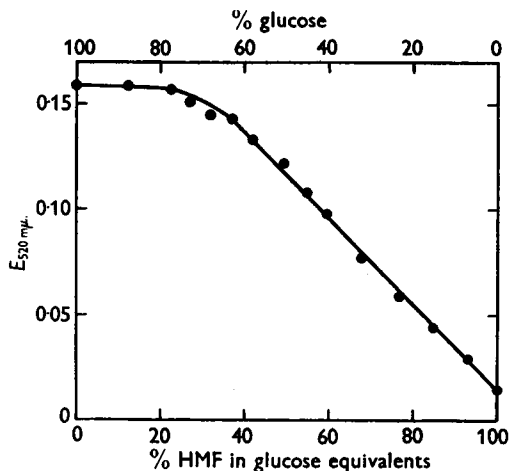


Fig. 3. The extinction of solutions containing glucose plus 5-hydroxymethylfurfural (HMF) in various proportions was measured at $520\text{ m}\mu$. after the solutions had been heated with three times their volume of 96 % (w/w) H_2SO_4 at 100° for 6.5 min. The total concentration of glucose plus HMF in the aqueous solutions was $5.56 \times 10^{-4}\text{ M}$, equivalent to 0.01 % glucose.

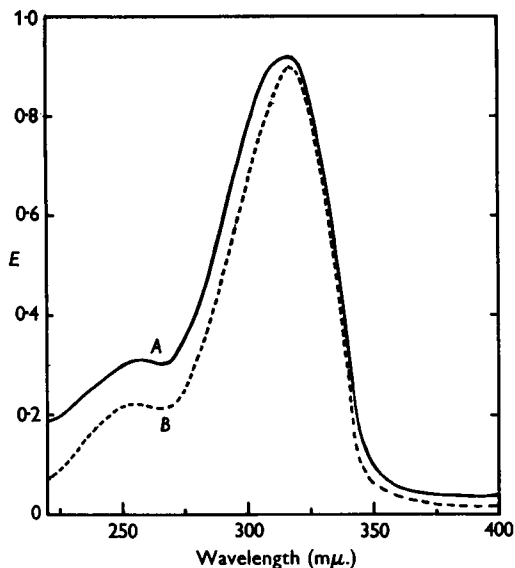


Fig. 2. Ultraviolet spectra obtained (A) after heating 1 ml. of an aqueous glucose solution (0.01 %) with 3 ml. of 96 % (w/w) H_2SO_4 at 100° for 6.5 min., and (B) after mixing 1 ml. of an aqueous solution containing $29.6\text{ }\mu\text{g}$. of 5-hydroxymethylfurfural with 3 ml. of 96 % H_2SO_4 at 0° .

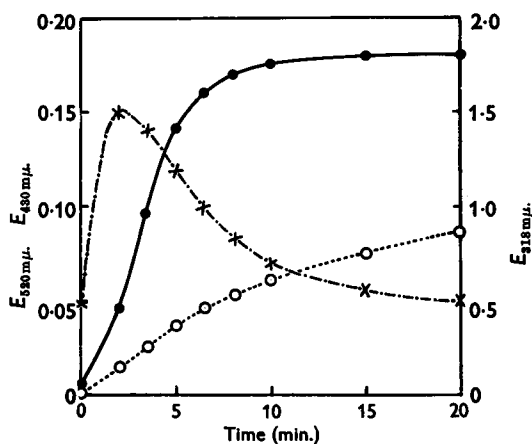


Fig. 4. The extinctions obtained after heating 0.01 % glucose solutions with three volumes of 96 % H_2SO_4 at 100° for various intervals of time were measured at $520\text{ m}\mu$. (●—●), $430\text{ m}\mu$. (○---○) and $318\text{ m}\mu$. (×---×). The figures obtained for the extinction at $318\text{ m}\mu$. are plotted on one-tenth of the scale for the values at 520 and $430\text{ m}\mu$.

gressively larger amounts of HMF and brownish decomposition products are formed from the glucose as the concentration of the sulphuric acid is increased. The intensity of the pink colour, on the other hand, reaches a maximum when the solution

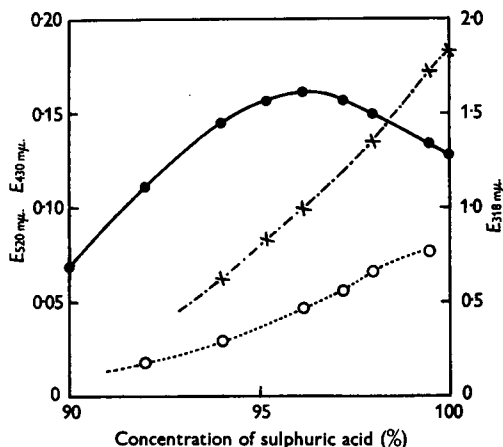


Fig. 5. Glucose solutions (0.01 %) were heated at 100° for 6.5 min. with 3 vol. of H_2SO_4 of various concentrations. The extinctions were measured at 520 $\text{m}\mu$. (●—●), 430 $\text{m}\mu$. (O---O) and 318 $\text{m}\mu$. (x---x); the figures obtained for the extinction at 318 $\text{m}\mu$. are plotted on one-tenth of the scale used for the values at 520 and 430 $\text{m}\mu$.

contains 82–83 % sulphuric acid. When much more concentrated or more dilute solutions of sulphuric acid are used, the pink colour fails to appear.

A final concentration of sulphuric acid within the optimum range is conveniently obtained by mixing 1 ml. aqueous glucose solution with 3 ml. of commercial 96 % sulphuric acid. Under these conditions the ratio of the optical densities at 520 and 430 $\text{m}\mu$. after heating for 6.5 min. should be about 3 and the resulting solution should have a bright pink colour. However, it has been found that some samples of sulphuric acid may give a considerably lower optical density at 520 $\text{m}\mu$. and a higher density at 430 $\text{m}\mu$., even though the concentration has been adjusted to 96 %. In this case the ratio of the optical densities at 520 and 430 $\text{m}\mu$., after heating with the glucose solution, may be as low as 1.6 and the pink solution will have a distinct brownish tinge. Although this latter type of sulphuric acid is much less suitable for the colorimetric determination of glucose, both types will give a linear relationship between glucose concentration and optical density at 520 $\text{m}\mu$.

Specificity of the colour reaction

The colour reaction appears to be specific for certain hexoses. Fructose gives about 20 % more colour than glucose, while the saccharides derived from these two hexoses, e.g. sucrose, glycogen,

Table 1. *Colour reaction of various carbohydrates and derivatives in hot sulphuric acid*

One ml. of a solution containing 0.1 mg. of the compounds listed below was heated with 3 ml. of 96 % (w/w) H_2SO_4 at 100° for 6.5 min. The intensity of the pink colour produced was measured at 520 $\text{m}\mu$. in a 1 cm. cuvette with a Beckman spectrophotometer and is given below as $E_{520 \text{ m}\mu}$. In the last column of the table, the colour intensity has been expressed as a percentage of that obtained with an equimolar amount of glucose for the compounds 1–5, 13, 14 or, in the case of the di-, tri- and poly-saccharides, 19–23, as a percentage of the colour obtained with an equivalent amount of the constituent monosaccharides.

No.	Compound	Visible colour	$E_{520 \text{ m}\mu}$	Relative colour intensity (%)
1	D-Glucose	Bluish pink	0.160	100
2	L-Glucose	Bluish pink	0.160	100
3	D-Fructose	Bluish pink	0.188	117
4	Sorbose (D and L)	Bluish pink	0.144	90
5	D-Mannose	Yellowish pink	0.045	28
6	D-Galactose	Yellow	—	—
7	DL-Glyceraldehyde	None	—	—
8	Arabinose	Faint yellow	—	—
9	Xylose	Faint yellow	—	—
10	Rhamnose	Faint yellow	—	—
11	Glucoheptose	Yellow brown	—	—
12	Glucosotose	Yellow brown	—	—
13	Glucose 1-phosphate (NH_4K salt)	Bluish pink	0.091	99
14	Glucose 6-phosphate (Ba salt)	None	0.009	12
15	Glucose 1:6-diphosphate	None	—	—
16	Fructose 1:6-diphosphate	None	—	—
17	Glucuronic acid	Faint yellow	—	—
18	Sorbitol	None	—	—
19	Sucrose	Bluish pink	0.177	107
20	Raffinose	Bluish pink	0.134	101
21	Inulin	Bluish pink	0.201	96
22	Glycogen	Bluish pink	0.178	101
23	Starch	Bluish pink	0.170	96

inulin and starch, give a pink colour of proportionate intensity (Table 1); little pink colour is developed from mannose. Glucose 1-phosphate is readily hydrolysed in acid (cf. Umbreit, Burris & Stauffer, 1945) and gives the same colour intensity as an equivalent amount of glucose. Glucose 6-phosphate, on the other hand, is relatively resistant to acid hydrolysis (cf. Umbreit *et al.* 1945) and does not give any pink colour under these conditions (Table 1), although the characteristic absorption maximum at 318 $m\mu$. develops upon heating with sulphuric acid.

A large number of non-carbohydrate substances which might occur in deproteinized blood filtrates were also heated in 82% sulphuric acid at 100° for 6.5 min.; in no case was a pink colour formed. The effect of these same compounds on the colour reaction given by glucose was investigated; little or no interference was observed in the presence of urea (80 mg./100 ml.) uric acid, creatine, creatinine, ascorbic acid, β -hydroxybutyric acid (all in concentrations of 10 mg./100 ml.), acetone (40 mg./100 ml.), alanine, arginine, aspartic acid, cystine, glutamic acid, glutamine, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, norleucine, phenylalanine, proline, serine, threonine and valine (all in concentrations of 25 mg./100 ml.). The only substances tested which were found to interfere appreciably with the formation of the bluish pink compound from glucose were sodium chloride (cf. Mendel & Bauch, 1926), ergothioneine, glutathione, tryptophan, methionine and tyrosine (Table 2). The concentrations of the last three amino acids in blood are relatively low (Johnson & Bergeim, 1951) so that the amounts found in a 20-fold diluted blood filtrate would not interfere with the colorimetric determination of glucose.

Effect of chloride

Mendel & Bauch (1926) reported that the presence of chloride ion interfered with the production of the

pink colour from glucose in heated sulphuric acid. This effect is illustrated in Fig. 6. The formation of the bluish pink compound with an absorption maximum at 520 $m\mu$. is inhibited when the concentration of sodium chloride in the aqueous glucose solution is greater than 2.5 mg./100 ml. The amount of HMF formed, on the other hand, is increased in the presence of chloride. Apparently more glucose is converted into HMF in the presence of chloride, while the intermediary reaction leading to the coloured compound is prevented.

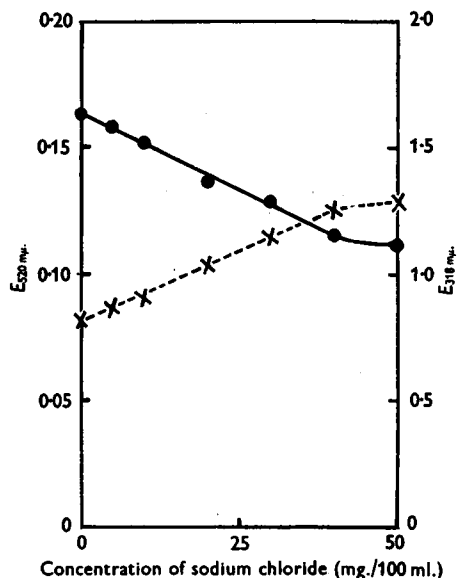


Fig. 6. Glucose solutions (0.01%) containing various concentrations of NaCl were heated with three volumes of 96% (w/w) H_2SO_4 at 100° for 6.5 min. The extinctions were measured at 520 $m\mu$. (●—●) and at 318 $m\mu$. (x---x); the figures obtained for the extinction at 318 $m\mu$. are plotted on one-tenth of the scale used for the values at 520 $m\mu$.

Table 2. Effect of substances found to interfere with the colour reaction

One ml. of an aqueous solution containing 0.1 mg. glucose and various concentrations of the substances listed below was heated with 3 ml. of H_2SO_4 at 100° for 6.5 min.; the intensity of the pink colour produced was measured at 520 $m\mu$.

Compound added	Amounts occurring in a 20-fold diluted blood filtrate (mg./100 ml.)	Amount added to the glucose solution (mg./100 ml.)	$E_{520\text{ m}\mu}$
None	—	—	0.160
Sodium chloride	28-31	30.0	0.125
Ergothioneine	0.15-0.6	1.5	0.150
Glutathione	0.8-2.0	5.0	0.119
Tryptophan	0.024-0.05	2.5	0.093
		0.5	0.130
		0.1	0.160
Methionine	0.017-0.035	25.0	0.020
		0.5	0.161
Tyrosine	0.04-0.07	25.0	0.080
		0.5	0.162

Experiments with blood samples

Since TCA was without influence on the production of the pink colour from glucose in heated sulphuric acid, it was selected as deproteinizing agent instead of the unstable 'metaphosphoric acid' used by Mendel & Bauch (1926). By the addition of silver sulphate to the TCA solution the chlorides in the blood sample are precipitated simultaneously with the protein. When 0.1 ml. of blood is pipetted into 1.9 ml. of TCA solution containing 0.1% (w/v) silver sulphate, chlorides are only rarely detectable in the filtrate and never in concentrations higher than 2 mg./100 ml. If desired, 0.2 ml. of blood can be pipetted into 1.8 ml. of the 5% (w/v) TCA solution; however, the concentration of silver sulphate should then be increased to 0.2% (w/v). The excess of silver ions in the filtrate, amounting to less than one-fifth of the amount added, has no effect on the intensity of the pink colour (Table 3).

It appeared that the silver sulphate in the deproteinizing solution also removes the two other interfering substances in the blood, namely glutathione and ergothioneine. Addition of large amounts of glutathione and ergothioneine to normal blood failed to exert any significant influence on the colour reaction (Table 4).

Table 3. *Effect of silver ions on the colour reaction with glucose*

The aqueous solution contained 10 mg. glucose/100 ml. and various concentrations of Ag_2SO_4 ; the colour reaction was carried out in the usual way.

Concn. of Ag_2SO_4 in the glucose soln. (mg./100 ml.)	$E_{520 \text{ m}\mu}$
0	0.162
10	0.162
50	0.163
100	0.165
200	0.171

Table 4. *Determination of glucose in blood to which glutathione or ergothioneine had been added*

Glutathione or ergothioneine were added to defibrinated human blood, and 0.2 ml. of the blood was deproteinized with 1.8 ml. of 5% (w/v) TCA containing 0.2% (w/v) Ag_2SO_4 . One ml. of the clear supernatant fluid obtained after centrifuging was added to 3 ml. of H_2SO_4 and the colour reaction was carried out in the usual way.

Substance added	Amount added to the blood (mg./100 ml.)	$E_{520 \text{ m}\mu}$	Blood glucose found (mg./100 ml.)
None	—	0.153	94
Glutathione	25	0.152	94
Glutathione	50	0.156	96
Ergothioneine	15	0.153	94

Apart from the carbohydrates, other substances present in blood do not give the colour reaction with hot sulphuric acid. No pink colour was formed after the glucose in blood had been removed by fermentation with yeast.

Table 5 shows that the usual anticoagulants do not affect the colorimetric determination of blood sugar. Sodium fluoride in a concentration of 10 mg./ml. appeared to be the most suitable anticoagulant since it prevents glycolysis (Lovatt Evans, 1922) as well as clotting.

Repeated determinations of the blood sugar on the same sample of blood showed that the reproducibility of the results was very satisfactory. The average of eight determinations on one sample of normal human blood was 85.1 mg. glucose/100 ml. (s.d. 1.63 mg./100 ml. or 1.9%). Five determinations on a second sample of normal human blood gave an average of 92.8 mg. glucose/100 ml. (s.d. 1.65 mg./100 ml. or 1.8%). Glucose added to blood could always be recovered completely from the blood filtrates (Table 6).

Table 5. *Determination of the glucose in blood to which anticoagulants had been added*

The anticoagulants listed below were added to defibrinated human blood; 0.1 ml. of the blood was deproteinized with 1.9 ml. of 5% (w/v) TCA containing 0.1% (w/v) Ag_2SO_4 . After centrifuging, the colour reaction was carried out in the usual way.

Anti-coagulant added	Amount added to the blood (mg./ml.)	$E_{520 \text{ m}\mu}$	Blood glucose found (mg./100 ml.)
None	—	0.069	85
Sodium oxalate	2	0.069	85
Sodium fluoride	10	0.068	84
Sodium fluoride	25	0.069	85
Heparin	10	0.070	86

Table 6. *Determination of the glucose in blood to which known amounts of glucose had been added*

Known amounts of glucose were dissolved in human blood containing 10 mg. NaF/ml. as anticoagulant; 0.1 ml. of the blood was deproteinized with 1.9 ml. of TCA: Ag_2SO_4 solution, centrifuged, and the colour reaction carried out in the usual way.

Blood sample	Amount of glucose added to the blood (mg./100 ml.)	Blood glucose found (mg./100 ml.)	Glucose recovered (mg./100 ml.)
A	0	65	—
	25	91	26
	50	113	48
	100	160	95
	250	315	250
B	0	79	—
	50	129	50
C	0	71	—
	50	122	51

Table 7. *Comparison of the blood-sugar values determined by the methods of Somogyi and Mendel & Hoogland*

Blood samples were taken from non-fasted experimental animals. Three of the samples were obtained from dogs which had been rendered diabetic by pancreatectomy. The blood-sugar value of each sample was determined by the iodometric method of Somogyi (1945) and by the colorimetric method of Mendel & Hoogland (1950) as described in the text.

Sample no.	Species	Blood sugar (mg./100 ml.)	
		Somogyi	Mendel & Hoogland
1	Rat	120	121
2	Guinea pig	117	115
3	Rabbit	106	100
4	Sheep	39	39
5	Duck	130	130
6	Diabetic dog A	284	280
7	Diabetic dog B	302	300
8	Diabetic dog C	324	312

Finally a comparison was made between the blood-sugar values determined by the present method and the values found on the same samples of blood by the iodometric macro-method of Somogyi (1945). The results obtained with blood from experimental animals are given in Table 7. It can be seen that there is a good agreement between the two methods in almost every case.

DISCUSSION

Most of the accepted methods for the determination of blood sugar, e.g. Folin & Wu (1922) and Hagedorn & Jensen (1923), are based on the reducing properties of glucose. Since blood also contains 'non-sugar' reducing substances, it is difficult to determine its 'true glucose' content with these methods, though more reliable results have been obtained with the techniques developed by Somogyi (1937, 1945, 1952). The colorimetric method described here, on the other hand, does not depend on the reducing power of glucose but on the formation of 5-hydroxymethylfurfural. The colour reaction appears to be specific for the hexoses which occur in blood, i.e. glucose and fructose (Bacon & Bell, 1948). 'Non-sugar' reducing substances are not determined by this method.

Another method which depends on the formation of furfural derivatives from carbohydrates in hot sulphuric acid has been described by Dische (1930, 1947) (cf. Gurin & Hood, 1939, 1941). This method does not appear to be specific for hexoses and in addition requires very pure carbazole as a chromogenic agent. No chromogenic agent is necessary for the formation of the pink colour in the reaction described by Mendel & Bauch (1926). Moreover, the new method is simple, and requires only two re-

agents which can easily be obtained and which are stable over long periods.

After this paper was completed, Love (1953) reported studies on the spectra which are obtained when carbohydrates are heated with concentrated sulphuric acid. It will be noted that Love (1953) used a higher concentration of sulphuric acid (98 %) and high concentrations of glucose (40–100 mg. 100 ml.) in his investigation of the visible spectra. Both factors lead to an early formation of humic substances which, by their yellow-brown colour, would mask the characteristic bluish pink colour obtained with glucose and fructose under the conditions described in the present paper. Since our method can easily be adapted for measurements with a colour scale (Kemp & Zuidweg, 1952) or with simple colorimeters, it should be suitable for routine determinations of blood glucose or tissue glycogen.

SUMMARY

1. A colorimetric micro-method for the determination of glucose has been described. This method is based on the observation that a bluish pink colour develops when one volume of a dilute solution of glucose is heated with three volumes of 96 % (w/w) sulphuric acid; the intensity of the pink colour is proportional to the concentration of glucose.

2. The glucose is dehydrated in hot sulphuric acid to 5-hydroxymethylfurfural, which reacts with a compound intermediate between glucose and hydroxymethylfurfural to produce the pink colour. For practical purposes, this reaction appears to be specific for glucose, fructose and saccharides containing these two hexoses.

3. The true glucose content of blood can be determined by this simple method after the blood has been deproteinized with a trichloroacetic acid solution containing silver sulphate.

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REFERENCES

- Bacon, J. S. D. & Bell, D. J. (1948). *Biochem. J.* **42**, 397.
 Dische, Z. (1930). *Mikrochemie*, **8**, 4.
 Dische, Z. (1947). *J. biol. Chem.* **167**, 189.
 Folin, O. & Wu, H. (1922). *J. biol. Chem.* **51**, 209.

- Gurin, S. & Hood, D. B. (1939). *J. biol. Chem.* **131**, 211.
 Gurin, S. & Hood, D. B. (1941). *J. biol. Chem.* **139**, 775.
 Hagedorn, H. C. & Jensen, B. N. (1923). *Biochem. Z.* **135**, 46.
 Holzman, G., MacAallister, R. V. & Niemann, C. (1947). *J. biol. Chem.* **171**, 27.
 Ikawa, M. & Niemann, C. (1949). *J. biol. Chem.* **180**, 923.
 Johnson, C. A. & Bergeim, O. (1951). *J. biol. Chem.* **188**, 833.
 Kemp, A. & Kits van Heijningen, A. J. M. (1954). *Biochem. J.* **56**, 646.
 Kemp, A. & Zuidweg, M. (1952). *Acta physiol. pharmacol. neerl.* **2**, 280.
 Lovatt Evans, C. (1922). *J. Physiol.* **56**, 146.
 Love, R. M. (1953). *Biochem. J.* **55**, 126.
 Mendel, B. & Bauch, M. (1926). *Klin. Wschr.* **5**, 1329.
 Mendel, B. & Hoogland, P. L. (1950). *Lancet*, **2**, 16.
 Singh, B., Dean, G. R. & Cantor, S. M. (1948). *J. Amer. chem. Soc.* **70**, 517.
 Somogyi, M. (1937). *J. biol. Chem.* **117**, 771.
 Somogyi, M. (1945). *J. biol. Chem.* **160**, 69.
 Somogyi, M. (1952). *J. biol. Chem.* **195**, 19.
 Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1945). *Manometric Techniques and Tissue Metabolism*, 1st ed. pp. 164-9. Minneapolis: Burgess Publ. Co.
 Wolfrom, M. L., Schuetz, R. D. & Cavaliere, L. F. (1948). *J. Amer. chem. Soc.* **70**, 514.

A Colorimetric Micro-method for the Determination of Glycogen in Tissues

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A new method for the determination of blood sugar has recently been described by Mendel & Hoogland (1950) and Mendel, Kemp & Myers (1954). This method is based on a colour reaction which occurs when a dilute solution of glucose is heated with concentrated sulphuric acid (Mendel & Bauch, 1926). Since glycogen is hydrolysed to glucose in hot sulphuric acid, this reaction can also be used to determine glycogen.

The deproteinizing solution used by Mendel & Hoogland (1950) contains trichloroacetic acid and, in addition, a small amount of silver sulphate to precipitate free chloride and other interfering substances. Although pure glycogen dissolves readily in this deproteinizing solution, only part of the glycogen can be extracted from the tissues with a cold solution of trichloroacetic acid (Willstätter & Rhodewald, 1934; Young, 1937; Bloom, Lewis, Schumpert & Shen, 1951; cf. Przylecki & Majmin, 1934; Mystkowski, 1937). However, it was found in the present investigation that all of the glycogen can be brought into solution by grinding the tissue with trichloroacetic acid solution and then heating the suspension for 15 min. at 100°.

Any glucose present in the tissues will of course be extracted and determined, together with the glycogen by this method. The amount of glucose in the tissues is usually small in comparison with the amount of glycogen present, but under certain experimental conditions it might be desirable to remove the glucose. Procedures will, therefore, be described for the determination of both glycogen and glucose in muscle and liver. Of the glycogen metabolites containing a hexose molecule, only

glucose 1-phosphate gives the colour reaction (Mendel *et al.* 1954); however, the concentration of glucose 1-phosphate in tissues (cf. Umbreit, Burris & Stauffer, 1945) is within the limits of error of this determination.

METHODS

Reagents

Deproteinizing solution. Trichloroacetic acid (5 g., A.R.) and Ag_2SO_4 (100 mg., A.R.) are dissolved in water and made up to 100 ml. (cf. Mendel *et al.* 1954). The solution is stored in an amber bottle in the cold.

Sulphuric acid, 96% (w/w, approx. 36N) (cf. Kemp & Zuidweg, 1952; Mendel *et al.* 1954).

Methanol, 80% (v/v).

Procedure

Determination of glycogen plus glucose. Muscle or liver (25-75 mg.) are ground with 5 ml. of the deproteinizing solution in a centrifuge tube; a stainless-steel pestle with longitudinal grooves which fits closely into a cylindrical centrifuge tube has proved useful for this purpose. The fluid level is marked on the centrifuge tube and the tube, covered with a glass cap, is placed in a boiling-water bath for 15 min. Then the tube is cooled in running water, filled up to the mark with deproteinizing solution to compensate for evaporation, and centrifuged at 3000 rev./min. for 5 min. One ml. of the clear supernatant fluid is added to 3 ml. of H_2SO_4 in a wide test tube and mixed by vigorous shaking. The mixture is heated in a boiling-water bath for exactly 6.5 min. and subsequently cooled in running tap water. The intensity of the pink colour produced is measured spectrophotometrically at 520 m μ and the glycogen concentration read from a standard curve in terms of glucose equivalents. As noted previously (Mendel *et al.* 1954), glycogen gives the same colour intensity as does an equivalent amount of glucose.