UDC 612.122 : 545.822

124. **Michio Ui** : Microdetermination of Reducing Sugars in Blood.

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On determining blood sugar in animals, the colorimetric method first described by Somogyi[1] and later modified by himself[2] and Nelson[3] is now widely used. This is based on the reduction of arsenomolybdate.

This Somogyi-Nelson method, though excellent in its accuracy, reproducibility, and stability of color developed, is poor in sensitivity to determine less than 10 γ of glucose. When repeated measurement of blood sugar of rat is desired, 0.1 cc. of blood to be drawn each time is so large in quantity that the withdrawal itself may exert some undue effect on physiological state of the rat. While some observations in fact revealed that repeated sampling of 0.1 cc. of blood would cause a hemodilution, the amount of glucose contained in 0.02 cc. of normal blood lies outside the range of measurable amount by Somogyi-Nelson method.

In order to follow the change of blood sugar in a rat over a long period of time, therefore, a method is required which makes certain an accurate determination of glucose for amounts as low as 2~3 γ.

In this paper, a highly sensitive colorimetric method for the determination of reducing sugars is presented which is based on the reduction of ferricyanide and color formation with ferric ion.

Folin[4] was the first to introduce such a principle, but his method of estimating Prussian Blue formed at 520 mμ suffers from low sensitivity. Later, Park and Johnson modified Folin's method and determined as low as 1~9 γ glucose successfully.[5] In Park-Johnson method, however, cyanide ion employed to enhance oxidation of sugar is liberated in gaseous form as the color develops on acidification with sulfuric acid. For this reason, utmost care is required and this method is not suitable for routine use, and in fact has scarcely been employed for measurement of blood sugar.

Methods

Reagents

(1) 100 mg% $K_3Fe(CN)_6$: Analytical grade $K_3Fe(CN)_6$, free from $Fe(CN)_6^{4-}$, is dissolved in distilled water and stored in a dark glass-stoppered bottle.

(2) NaOH-Na_2CO_3 solution : In about 0.25*N* NaOH, checked to be equivalent to 2% trichloroacetic acid, anhyd. Na_2CO_3 is dissolved to make 2.1% solution.

(3) $NH_4Fe(SO_4)_2$ solution : 0.1% $Fe_2(SO_4)_3 \cdot (NH_4)_2SO_4 \cdot 24H_2O$ in 0.3*N* H_2SO_4.

Withdrawal of Blood from Rat—The tip of tail of rat under pentobarbital-anesthesia is slightly injured with a razor blade and a drop of blood obtained by gently rubbing the tail is sucked into a 0.02-cc. pipette. Repetition of rubbing brings forth a drop of blood each time and 2~3 drops amount to 0.02 cc. Blood specimen thus taken is expelled into 1.78 cc. of distilled water and the pipette is rinsed thoroughly with this colorless supernatant. After hemolysis by shaking, 0.2 cc. of 20% trichloroacetic acid is slowly added under continuous agitation and the precipitate formed is centrifuged off. An aliquot of the solution is then transferred to a test tube and determined for glucose.

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1) M. Somogyi : J. Biol. Chem., **117**, 771(1937).
2) *Idem.* : *Ibid.*, **160**, 61(1945).
3) N. Nelson : *Ibid.*, **153**, 375(1944).
4) O. Folin : *Ibid.*, **77**, 421(1928).
5) J. T. Park, M. J. Johnson : *Ibid.*, **181**, 149(1941).

Results and Discussion

Standard Procedure

(1) 0.5 cc. of the solution containing 2~20 γ/cc. glucose and 2% trichloroacetic acid is placed in a test tube of 18 mm. in diameter.

(2) One cc. of the mixture of $K_3Fe(CN)_6$ solution and NaOH–Na_2CO_3 solution (1:1) are added and the tube is immersed in a boiling water bath for 15 minutes.

(3) The tube is then cooled in water for 3 minutes and added with 1 cc. of NH_4-$Fe(SO_4)_2$ solution.

(4) Distilled water is added to make a total volume of 6 cc.

(5) The color developed is instantly measured at 660 mμ.

Oxidation of Glucose—In the presence of 2.1% Na_2CO_3, incomplete oxidation of glucose occurred during the heating step when the concentration of $K_3Fe(CN)_6$ was below 50 mg% and time of heating was shorter than 10 minutes.

Stability of Color—The concentration of the reagent adopted in the standard method was decided to minimize the change of color on standing. For instance, the lower the pH of the final solution, i.e., the higher the concentration of H_2SO_4 in $NH_4Fe(SO_4)_2$ solution, the faster the color becomes dark. Use of a more concentrated $K_3Fe(CN)_6$ solution results in a variable color of the blank. The change in concentration of $NH_4Fe(SO_4)_2$ seems to have no effect on the stability of color in the standard procedure.

Effect of Light—The characteristics of the color formed in this procedure was markedly influenced by exposure to light. Not only direct exposure to light but also leaving in a bright room intensified the color instantly. A dim light in the room on a cloudy day or electric light intensifies the color somewhat (Fig. 1).

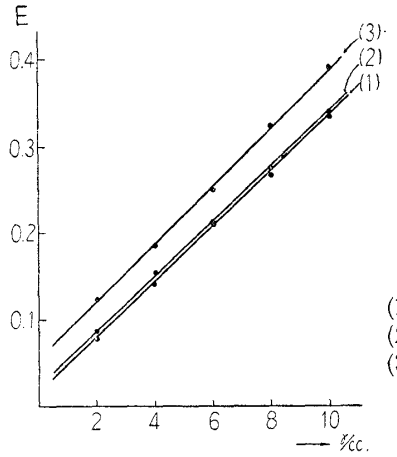

Fig. 1.
Effect of Exposure to Light

(1) Measured immediately after color developed.
(2) Measured after being stored in dark for 2 hr.
(3) Measured after being stored in light for 2 hr.

Effect of Sodium Laurylsulfate—Park and Johnson[5] reported that sodium laurylsulfate had been employed to keep Prussian Blue in suspension. Even in the absence of sodium laurylsulfate, however, Prussian Blue was found to be well suspended and the optical density was proportional to glucose concentration up to 20 γ/cc., if 0.5 cc. of glucose solution was employed as in the standard procedure. When the amount of glucose solution to be tested was increased to 1.0 cc., the proportionality was observed only below the concentration of 10 γ/cc. Addition of sodium laurylsulfate failed to restore the bending of the straight line as shown in Fig. 2. Tween 80 was also tested but was found ineffective.

When water added to make the final volume to 6 cc. was replaced with conc. Na_2SO_4 or $(NH_4)_2SO_4$ solution, the solution containing large amount of glucose was safely determined; i.e. the proportionality of optical density to the amount of glucose was observed

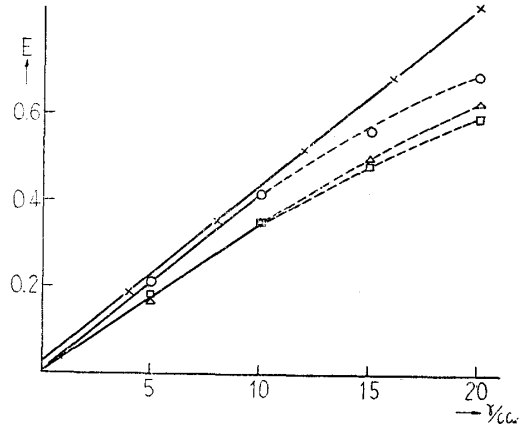

Fig. 2.
Bending of Dose-response Curve at High Glucose Level

○-○ Na laurylsulfate added
△-△ Tween 80 added
□-□ no addition
×-× Na_2SO_4 added
glucose solution employed: 1.0 cc.

in the wide range of glucose concentration (Fig. 2). Addition of Na_2SO_4 or $(NH_4)_2SO_4$, however, gave a color so unstable that its use may be restricted to a small-scale assay. Na_2SO_4 was preferred to $(NH_4)_2SO_4$ because of the high viscosity of the latter.

Effect of Deproteinization—Somogyi's deproteinizing reagents consisting of $Ba(OH)_2$ and $ZnSO_4$ lowered the optical density. Though a lowered value can be restored by increasing the concentration of $NH_4Fe(SO_4)_2$ to some extent as is illustrated in Fig. 3, these deproteinizing reagents reduce the stability of color. Trichloroacetic acid was, therefore, employed as deproteinizing reagent.

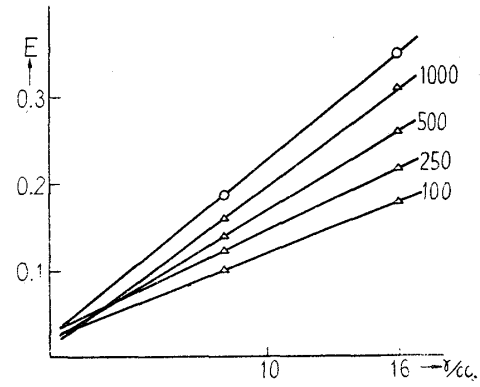

Fig. 3. Effect of $Ba(OH)_2$-$ZnSO_4$ Deproteinization
Figures denote the concentration of $NH_4Fe(SO_4)_2$ used.
○-○ Without deproteinization
△-△ With deproteinization

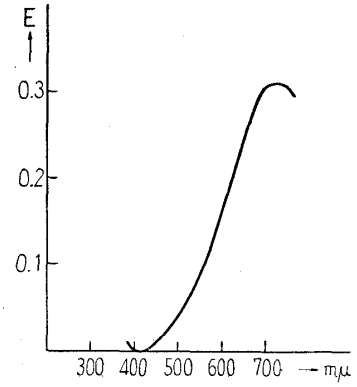

Fig. 4. Absorption Spectrum

Absorption Spectrum—The maximum absorption existed near 700 mμ as shown in Fig. 4.

Standard Curve—When the standard glucose solutions were treated according to the standard procedure, plotting of optical density against glucose concentration gave the standard curve shown in Fig. 5.

Comparison with Somogyi-Nelson Method—The blood sugar values obtained by this method are higher than those by Somogyi-Nelson method, presumably because reducing substances such as phosphorylated sugars and glutathione in blood do not precipitate on addition of trichloroacetic acid. For example, the blood sugar level of rabbits determined by this method as 115, 112, 109, and 128 was found to be 104, 104, 101, and 96, respectively, according to Somogyi-Nelson method.

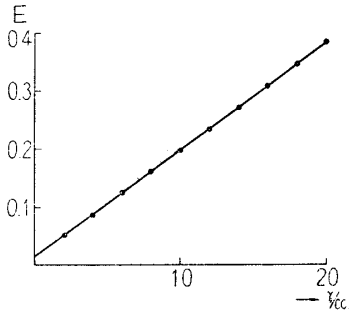

Fig. 5.

Standard Curve

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Summary

An assay method suitable for reducing sugars present in 0.02 cc. of normal blood was described.

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125. Nobusuke Kawano : Studies on the Structure of Sciadopitysin, a Flavonoid from the Leaves of *Sciadopitys verticillata* SIEB. ET ZUCC. VII.*1

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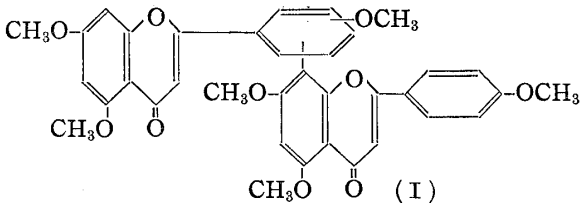
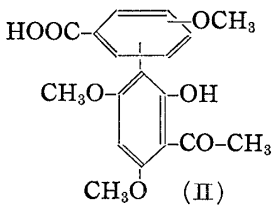
In the preceding paper,[1] it was deduced that sciadopitysin trimethyl ether, $C_{36}H_{30}O_{10}$, consists of xanthoxylin, substance B (a phenolic acid, $C_{18}H_{18}O_7$), and anisic acid, and that its structure must be represented by a tentative formula (I) because it gave these three fragments in considerably high yield when treated with methanolic barium hydroxide solution. Consequently, substance B must have a biphenyl structure (II) instead of a desoxybenzoin derivative as suggested earlier.[2]





On oxidation with alkaline potassium permanganate solution, substance B monomethyl ether produced a monoketodicarboxylic acid (III), $C_{19}H_{18}O_9$, m.p. 216~217°(decomp.), which gave *p*-nitrophenylhydrazone of m.p. 223~224°(decomp.) and 2,4-dinitrophenylhydrazone of m.p. 230~231°(decomp.). When kept at 215~220°, this acid (III) decomposed rapidly to give a monocarboxylic acid (IV), $C_{17}H_{18}O_6$, m.p. 231°, which was subsequently

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*1 This paper constitutes Part XXXVI of "Chemical Consituentes of the Plants of Coniferae and Allied Oders" by T. Kariyone. Part XXXV : Ref. 1).

*2 Showa-machi, Nagasaki (河野信助).

1) Part VI : Yakugaku Zasshi, **79**, 1182(1959).

2) N. Kawano : *Ibid.*, **76**, 457(1956) [C.A., **50**, 16759(1956)].