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A SIMPLE SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF BLOOD SUGAR BY PHENYLHYDRAZINE HYDROCHLORIDE

by

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Fischer discovered the reaction of phenylhydrazine with sugars in 1884 (1). Later, the reaction was used to establish the structure of carbohydrates. Rodillon (2) was the first to propose a quantitative technique for determining the blood sugar by osazone formation but his method was designed only for rough clinical tests and could not be used for accurate determinations. Herzfeld (3) and Butkevich et al (4) suggested other methods based on the same reaction which were fairly accurate, except that they required prolonged filtration, extraction and other processes. In an attempt to develop a new technique, Wahba et al (5) modified Rodillon's method and adopted colorimetry in their estimations. In Wahba's technique the conditions for optimum color-production seemed to be unfavorable because an insufficient amount of acetic acid was added to the reaction mixture.

I found that by adding an excess of acetic acid to the reaction mixture, the rate of osazone-formation was greatly accelerated, thus reducing considerably the time needed for heating. The technique described below also has the advantages that the duration of heating of the mixture is not critical, that the specificity for sugars is improved and that the color produced is stable for several days.

MATERIALS AND METHODS

Reagents

Phenylhydrazine-HCL reagent: 8 gr. of pure phenylhydrazine-HCI (6) and 20 gr. of sodium metabisulphite are dissolved completely in 100 ml. of distilled water in one liter beaker. Care must be taken to stir the solution to ensure that the solids are completely dissolved. Into this solution, 900ml. of pure aldehyde-free glacial acetic acid (7) are thoroughly stirred. The mixture is stored in a refrigerator overnight and then filtered through a carbohydrate-free filter paper in buchner funnel. The filtrate, which should be stored in a dark bottle in a refrigerator, can be used for several months. .....

Standard solutions of glucose: 100 mg. of pure glucose, dried in vacuum oven at 60° - 70°c are dissolved in 100 ml. of saturated benzoic acid solution in water. From this stock solution, working standards of glucose in saturated benzoic acid are made containing 20, 40, 60, 80, 100 and 120 micrograms glucose per ml.

Procedure: Blood protein-free filtrate was prepared by three methods: Folin-Wu (8, 9, 10), Somogyi (11) and the cadmium sulfate-sodium hydroxide method (12, 13, 14). The best dilution of blood with any of these various reagents was found to be 1: 10.

One ml. of protein-free filtrate is transfered to a 10 ml. marked test tube (2 cm. x 22 cm.), and 9 ml. of phenylhydrazine-HCI reagent are added. One ml. of distilled water, instead of protein-free filtrate, is used in another tube as a blank. The contents of each tube are mixed and the tubes heated for thirty minutes in vigorously boiling water. They are removed, cooled to room temperature and made up to the 10 ml. mark with distilled water. The optical density of each solution is determined with a Beckman spectrophotometer (model DU) at wave length 400 millimicron and the amount of sugar present read off from the standard.

Preparation of standard graph: One ml. of each working standard solution is transfered to the 10 ml. marked tube and the procedure outlined above is followed exactly. The standard graph is prepared by plott-

ing the observed optical densities against the various concentrations of the working standard solutions.

RESULTS AND DISCUSSION

When a Beckman spectrophotometer (model DU) was used, the relationship between optical density and glucose concentration was linear (Fig. 1), but this relationship was linear only at the lower glucose concentrations when a Colman instrument (model 6A) was used. A Beckman was therefore used in all subsequent estimations.

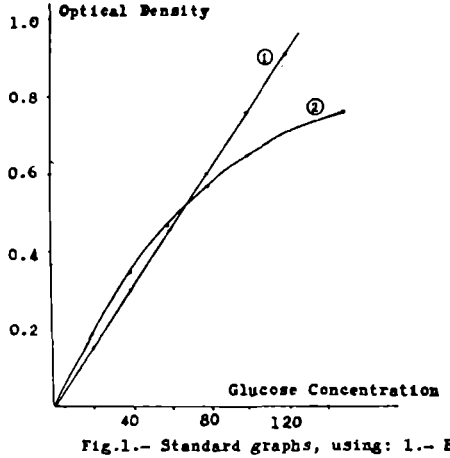

Fig.1.- Standard graphs, using: 1.- Beckman (Model DU) & 2.- Colman (Model 6A) spectrophotometers.

The maximum spectral absorbtion curve was determined for two different solutions of glucosazone and that obtained from the reaction product of blood filtrate with phenylhyrazine-HCI reagent (Fig. 2). Maximum absorbtion was at wave length 400 millimicrons for all three solutions.

The concentration of glacial acetic acid necessary for maximum color production was determined by varying the amount of acid in the final 10 ml. of the reaction mixture, all other components remaining unchanged (Fig. 3). When 3 ml. of acid was added to each tube, as recom-

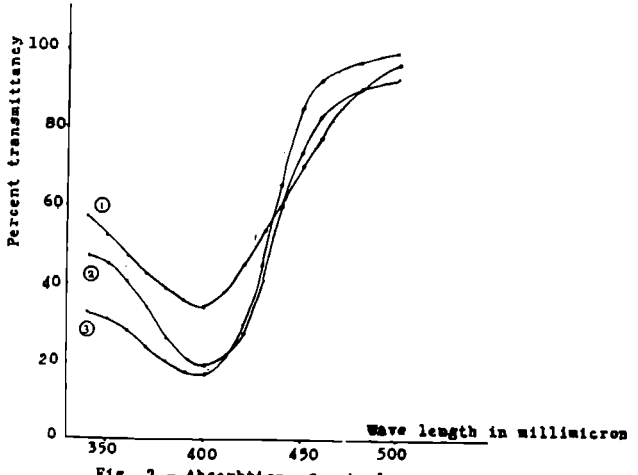

Fig. 2.- Absorbtion spectral curves using Beckman Spectrophotometer: 1.- Solution of glucosazone in ethanol acidified with a drop of conc. HCl, 2.- Solution of glucosazone with 80% acetic acid and 3.- Reaction product of blood filtrate with phenylhydrazine-HCl reagent.

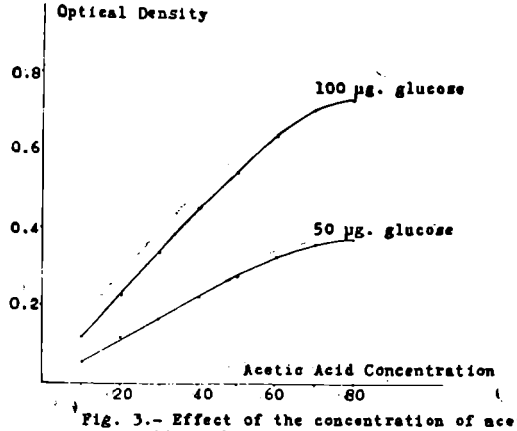

Fig. 3.- Effect of the concentration of acetic acid on the reaction rate.

mended by Wahba et al (5), a period of heating of 3 hours was necessary for maximum color production. By adding 8.1 ml. of acetic acid to each tube, however, the time of heating to attain maximum color was reduced to 30 minutes (Fig. 4).

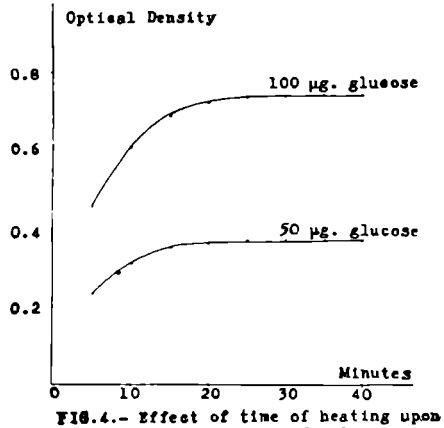

FIG.4.- Effect of time of heating upon the production of color.

The color reaction seems to be specific for blood sugar. Some non-carbohydrate substances, normally present in blood filtrate, might interfere with the intensity of the color produced. A number of such substances were, therefore, tested (amino-acids, glutathione, uric acid, creatine, creatinine and ascorbic acid) but only ascorbic acid was found to interfere with the color reaction. The optical density produced by 1000 micrograms of ascorbic acid, at wave length 400 millimicrons was equivalent to that of 100 micrograms of glucose. The amount of ascorbic acid present in blood varies from 6-25 micrograms per ml. of blood, whose effect is negligible in comparison with that of the amount of sugar normally present (55-109 micrograms per ml.).

To asses the effect of the method of deproteinization on the amount of blood sugar present in the filtrate, three different methods of preparing protein-free filtrate were used, Folin-Wu (8-10), Somogyi (11) and the cadmium sulfate-sodium hydroxide method (12-14), and the filtrates

were treated in the way described above. As Table 1 shows, the method of deproteinization had no significant effect on the final estimates of blood sugar.

TABLE 1

Comparative values of blood sugar analysis, using three different methods of deproteinization

Blood No.	mg. of sugar per 100 ml. blood		
	Na-tungstate-H2SO4 (Folin-Wu)	ZnSO4-Ba (OH)2 (Somogyi)	CdSO4-NaOH
1	72.0	72.0	74.0
2	88.6	86.1	86.1
3	99.0	98.0	97.0
4	70.0	74.0	74.0
5	104.0	103.5	103.5
6	94.6	93.1	93.1
7	305.0	304.0	304.0
8	94.0	93.0	94.0
9	86.0	83.5	82.5
10	71.0	70.0	70.0
11	210.0	210.0	210.5
Mean	117.65	117.02	117.75

Three different methods for estimating blood sugar were compared: the anthrone method (15), the copper-reduction method (16-18) and the method described in this paper. The slightly lower values of blood sugar obtained by present technique may have been due to the high specificity of the method (Table 2).

TABLE 2.

Comparative analysis of blood sugar by three different methods

Blood No.	mg. sugar per 100 ml. of blood		
	Anthrone method	Copper-reduction method	Osazone method
1	60.0	63.6	58.0
2	100.6	108.0	99.0
3	81.2	85.0	80.0
4	63.0	70.0	63.0
5	73.1	72.5	72.7
6	182.3	183.5	180.5
7	317.0	313.0	304.0
8	73.0	74.0	70.0
9	69.0	69.0	69.0
10	147.0	150.0	145.0
Mean	116.6	118.9	114.2

SUMMARY

An improved method has been developed for the estimation of blood sugar. Protein-free filtrate, mixed with phenylhdrazine-HCI reagent, was heated for thirty minutes, cooled and measured with a spectrophotometer.

The duration of heating of the mixture was found not to be critical and the color that appeared was stable for a long time. The reaction was more specific for carbohydrates than the anthrone and copper-reduction reactions.

Concentrations of blood sugar ranging from 10 to 120 mg. per 100 ml. of sample could be determined.

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STATISTIQUE DES PRODUITS BIOLOGIQUES MEDICAUX ET VETERINAIRES

Espèce de produits						Quantité délivrée en 1959
						(F'lacons)
Sérum antidiphtérique		purifié et concentré		5000	unités	16.554
"	"	"	"	10000	"	79.274
"	"	"	"	40000	"	2.162
"	antitétanique	"	"	3000	"	25.138
"	"	"	"	5000	"	43.947
"	"	"	"	10000	"	13.109
"	anticharbonneux					1.755
"	polyvalent contre la morsure des serpents					3.414
"	antirabique					1.000
						(doses)
Anatoxine diphtérique purifiée et adsorbée sur phosphate d'alumine						334.650
Vaccine mixte antidiphtérique et antitétanique						599.587
Vaccin mixte antidiphtérique, antitétanique et anticoquelucheux						71.382
Vaccin anticoquelucheux						24.658
Anatoxine tétanique pure						1.018
Vaccin anticharbonneux						19.002.200
"	anticlaveleux et antivariole caprine					8.121.200
"	anti-entérotoxémie					3.354.583
"	antinewcastle des volailles					2.423.050
"	antisymptomatique					1.325.500
"	antipasteurellique					546.500
"	antipestique					400.000
"	antivariole aviaire					111.500
"	mixte charbonneux et tétanique					21.200
"	antisalmonellique					20.000
"	antibrucellique					4.535
"	antispirochettose					7.000

Espèce de produits	Quantité délivrée en 1959
ANTIGENS DIVERS	
	44.878cc
Malléine	40cc
Antigène brucellique	1.975cc
Antigène pullorum	13.000cc
Réactif de Schick	12.900cc
Antigène pertussis	2.900cc
PRODUITS ANTIPARASITAIRES	
Antidistomatose	3.130.121
Antipiroplasmose des bovins et ovins (ampoues de 6cc)	111.681
Sérum normal	12.900 ml

SOMMAIRE DU FASCICULE 12