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166. Tsutomu Momose, Yoshiko Yano (née Mukai), and Katsuko Ohashi : Organic Analysis. XLIV.*1 A New Deproteinizing Agent for Determination of Blood Sugar.

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Several deproteinizing agents are now available for the determination of blood sugar. Those involve zinc sulfate and sodium hydroxide,[1] sodium tungstate and sulfuric acid,[2] and zinc sulfate and barium hydroxide.[3] These reagents consist of two components, respectively, which should exactly match with each other in a definite ratio, and should carefully be prepared, preserved and used. Among all, zinc sulfate and barium hydroxide (Somogyi's reagent) is preferable to give the "true sugar value" in blood, yet a more simple reagent is desirable for wide use in clinical tests. In the writers' laboratory, sodium tungstate and alum proved to fit for the purpose in the determination of blood sugar with 3,6-dinitrophthalic acid as the color developing agent.

Experimental

Reagents

Sodium Tungstate Solution——10.0 g. of sodium tungstate, $Na_2WO_4 \cdot 2H_2O$, is dissolved in sufficient H_2O to measure 100 ml.

Alum Solution——9.6 g. of alum, $KAl(SO_4)_2 \cdot 12H_2O$, is dissolved in sufficient H_2O to meaure 100 ml. The color developing agents[4] are modified as follows.

3,6-Dinitrophthalic Acid Solution——1.50 g. of 3,6-dinitrophthalic acid monopyridinium salt (Ishizu Seiyaku Kabushiki Kaisha) is dissolved in sufficient H_2O to measure 500 ml., and stored in a light-resistant bottle, to which an automatic burette, 10 ml., is attached.

Alkaline Solution——125 g. of anhyd. K_2CO_3 and 25.0 g. of sodium thiosulfate are successively dissolved in H_2O and made up to 500 ml., stored in a bottle, to which an automatic burette, 10 ml., is attached, and protected from CO_2.

Standard Procedure for Clinical Tests——0.100 ml. of blood is haemolysed with 2.90 ml. of H_2O in a test-tube, and 0.50 ml. of sodium tungstate solution is added. To the mixture, 0.50 ml. of alum solution is added, and the test-tube shaken vigorously. The mixture is then transferred to a centrifuge tube, stoppered with a piece of aluminium foil, and centrifuged. 2.00 ml. of the supernatant solution is pipetted into a test-tube, 1.00 ml. of 3,6-dinitrophthalic acid solution and 1.00 ml. of alkaline solution are successively added, developed, and diluted in the same way as in the previous paper.[4] The absorption intensity is measured at 450 mμ with the reagent blank, and the blood sugar value is obtained from the calibration curve which is prepared by developing 2.00 ml. of standard glucose solutions, 10~100 γ/ml. The deproteinizing operation should be avoided for glucose solutions.

In this study, blood was first diluted to an appropriate volume with H_2O, and a definite volume of the solution was pipetted which corresponded to 0.100 ml. of the blood to eliminate the error caused by using small pipettes. The pipetting of reagents and sample solutions was carried out by the rapid automatic pipettes.[5]

Results and Discussion

Aqueous sodium tungstate and alum precipitate very slightly soluble aluminium tungstate.[6] This compound removes proteins in blood in the presence of sulphate.

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When the concentration of sodium tungstate is fixed as 10 g./dl., the equivalent concentration of alum is 9.6 g./dl. to match with each other in the same volume. In the deproteinization of blood or serum, however, the concentration of alum can be varied from 9 to 10 g./dl. for blood and from 8.5 to 10 g./dl. for serum to give the same blood sugar value as shown in Fig. 1. This large allowance proves the easiness of preparation and preservation of the reagents. Furthermore, it is important to note that there may be no possibility of failure in the deproteinization in clinical tests.

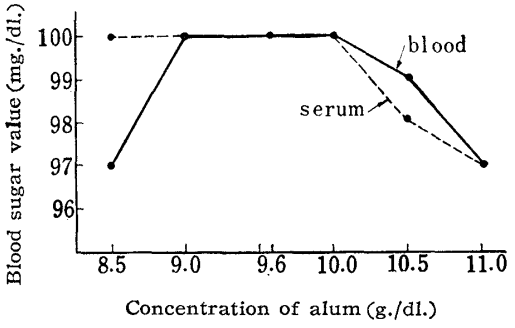

Fig. 1. Relationship between Blood Sugar Value and the Concentration of Alum which corresponds to Sodium Tungustate Solution (10 g./dl.) Blood sugar value is converted into 100.

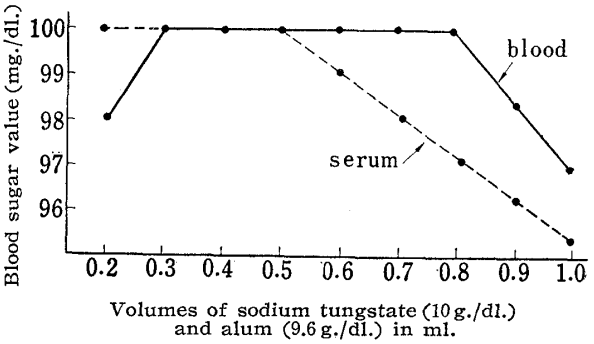

Fig. 2. Relationship between Blood Sugar Value and the Volume of Deproteinizing Agent for 0.1 ml. of Blood or Serum. Blood sugar value is converted into 100.

The volumes of sodium tungstate and alum solutions which need for deproteinizing 0.1 ml. of blood or serum are shown in Fig. 2. A constant sugar value is given in the range of 0.3 to 0.8 ml. of the reagents for blood, and a little smaller volumes for serum. Therefore, each 0.5 ml. of the solutions was used in the standard method. The pH value of the deproteinized solution is about 4.7, and gives no effect on the color development.

A recovery test was carried out by adding known amount of glucose to blood solutions. Table I shows that the found values are in good agreement with the added amount. The mean value of recovery in per cent, 99.5, proves that the deproteinizing agent adsorbs no glucose in the reaction.

Table I. Recovery Test (Mean value of 4 determinations)

Initial blood sugar value (mg./dl.)	Glucose added (mg./dl.)	Total glucose found (mg./dl.)	Glucose recovered (%)
100	50	149	98
	100	200	100
	200	303	102
92	50	142	100
	100	193	101
	200	286	98
78	50	126	96
	100	179	101
	200	277	100
100	50	150	100
	100	200	100
	200	295	98
113	50	163	100
	100	211	98
	200	313	100

Mean 99.5%

Now, blood sugar tests were carried out with the deproteinizing agent by adding some substances in blood solutions. Some other reducing substances other than glucose which are present in blood give practically no influence on the determination as shown in Table II. The amount of ascorbic acid in the table, which shows a little higher

Table II. Influence of Reducing Substances on Blood Sugar Value
(Mean value of 4 determinations)

Initial blood sugar value (mg./dl.)	Blood sugar value found (mg./dl.)			
	Creatinine added (10 mg./dl.)	Creatine added (10 mg./dl.)	Glutathione added (10 mg./dl.)	Ascorbic acid added (10 mg./dl.)
93	93	93	94	95

sugar value, is a large excess than the usual value in blood. The influence of some preservatives which may be added in blood is shown in Table III, and sodium fluoride only gives a lower value. This fact may be caused by the formation of soluble complex ion $(AlF_6)^{3-}$ in the deproteinized solution.

Table III. Influence of Preservatives on Blood Sugar Value
(Mean value of 4 determinations)

Initial blood sugar value (mg./dl.)	Blood sugar value found (mg./dl.)			
	Sodium citrate added (5 mg./ml.)	Sodium oxalate added (2 mg./ml.)	EDTA added (1.5 mg./ml.)	Sodium fluoride added (10 mg./ml.)
153	153	152	153	146

To test the precision of this method, each 16 aliquots of a sample blood solution were deproteinized and developed for three times. All data are summarized in Fig. 3. The standard deviation of the test is 1.6 mg. for a mean sugar value of 188 mg./dl.

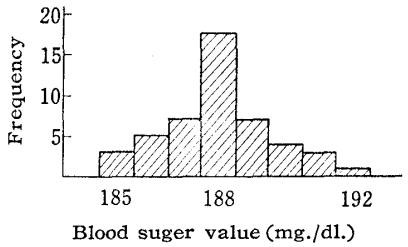

Fig. 3. Graph of Frequency for 48 Analyses by Standrad Method

The results of parallel tests with other deproteinizing agents are shown in Table IV. The present deproteinizing agent gives "true sugar values" which agree with Somogyi's reagent in an experimental error. The sugar values determined by the other methods than 3,6-dinitrophthalic acid are also shown in the table for reference.

Micro-Method

The deproteinizing agent can successfully be used in a micro-method of determination of blood sugar.

Reagents

Sodium tungstate solution——5 g./dl.
Alum solution——4.8 g./dl.
Color developing agent——The same volumes of 3,6-dinitrophthalic acid solution and alkaline solution under Standard method are mixed before use.

Procedure

0.020 ml. of blood is haemolysed with 0.58 ml. of H_2O in a centrifuge tube, and 0.20 ml. of sodium tungstate solution is added. To the mixture, 0.20 ml. of alum solution is added, and the tube shaken

Table IV. Parallel Test with Other Methods and Other Deproteinzing Agents
(Mean blood sugar value (mg./dl.) of 3 determinations)

Method of determination	3,6-Dinitrophthalic acid		Somogyi-Nelson	Hagedorn-Jensen
Deproteinizing agent	Sodium tungstate and alum	Barium hydroxide and zinc sulphate	Barium hydroxide and zinc sulphate	Sodium hydroxide and zinc sulphate
Blood				
1	130	129	131	135
2	124	122	123	131
3	153	153	155	164
4	203	205	206	212
5	76	77	79	91
6	149	152	148	159
7	71	72	67	83
8	110	110	108	126
9	75	77	77	94
10	173	169	173	192
11	93	90	—	92[a]
12	89	87	—	89[a]
Serum				
1	68	67	68	—
2	88	90	89	—
3	69	68	69	—
4	77	79	81	—
5	84	84	84	—
6	110	113	116	—
7	78	77	75	—
8	94	93	97	—

a) Deproteinized with barium hydroxide and zinc sulphate

vigorously. The tube is stoppered with a piece of aluminium foil, and centrifuged. 0.50 ml. of the supernatant clear solution is pipetted into a small test tube of about 11×120 mm., 0.50 ml. of the color developing solution is added, and mixed well. The test tube is packed in a proper heating basket with the reagent blank, and heated in a boiling water bath for exactly 10 min. After cooling in running water, the mixture is diluted to 5.0 ml., and its absorption intensity is measured at 450 mμ with the reagent blank. The blood sugar value is read from a calibration curve which is prepared by developing 0.50 ml. of glucose solutions, 10~100 γ/ml.

The precision of this method was examined by developing each 16 aliquots of a blood solution for three times. The data are summarized in Fig. 4. The standard deviation, 2.0 mg. for a mean blood sugar value of 190 mg./dl., shows that the method is accurate enough for usual clinical tests.

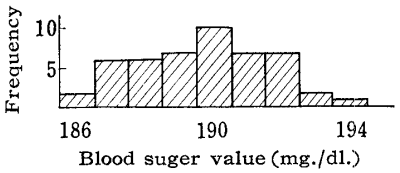

Fig. 4. Graph of Frequency for 48 Analyses by Micro-Method

Powdered Deproteinizing Agent

The new deproteinizing agent can also be used in a powdered state as follows.

Reagent

To a hot solution of 50 g. of sodium tungstate in 200 ml. of H_2O, a hot solution of 48 g. of alum in 250 ml. of H_2O is added in one portion, and the mixture is heated on a water bath. 25 g. of K_2SO_4 is added in the mixture, and the mixture evaporated to dryness. The white mass is then dried at 100~105° for 5 hr., powdered, and shifted with a 100-mesh sieve. It is stable in the air.

Procedure

0.100 ml. of blood is heamolysed with 3.90 ml. of H_2O in a test-tube, about 0.1 g. of the deproteinizing agent is added, and the test-tube shaken vigorously. The mixture is then transferred in a cen-

trifuge tube, centrifuged, and treated in the same way as in the standard method. For deproteinizing 0.100 ml. of serum, about 0.05 g. of the reagent is used.

The powdered deproteinizing agent gives the same blood sugar value when used in the range of 0.08 to 0.12 g. for 0.1 ml. of blood, and 0.04 to 0.06 g. for 0.1 ml. of serum. The blood sugar values obtained by this method agree with those of the standard method as shown in Table V. The powdered reagent is very simple to use, and may sometimes be suitable for clinical tests.

Table V. Parallel Test of Powdered Deproteinizing Agent with the Standard Method
(Mean blood sugar value (mg./dl.) of 4 determinations)

Blood		Serum	
Standard method	Powdered deproteinizing agent (0.1 g.)	Standard method	Powdered deproteinizing agent (0.05 g.)
95	94	204	203
94	94	83	84
57	58	99	98
151	149	127	128
70	71	136	135

The authors extend their gratitude to Dr. Junji Nagai, Central Clinical Laboratory of the Kyushu University Hospital, for the preparation of blood and serum samples.

Summary

Aqueous sodium tungstate and alum were successfully used in the deproteinization of blood or serum to determine the sugar value with 3,6-dinitrophthalic acid. They were easily prepared and preserved, and fitted for clinical tests. The deproteinizing agent could also be used in a powdered state.

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