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Harold Ellis Gentry
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BLOOD SUGAR DETERMINATIONS: A COMPARISON OF DIFFERENT METHODS -- THEIR VALIDITY AND FALLACY

Harold Ellis Gentry Jr.

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INTRODUCTION

A disease process characterized by 'honey urine' and a melting away of tissues (diabetes mellitus) had been known and described by physicians dating back to the Hindus of remote times. But it was not until Sylvius (1680) that any notion was advanced of a morbid state of the blood associated with the disease. (1) One hundred and fifty years elapsed following Sylvius' theory of blood morbidity before sugar was finally detected in blood by chemical analysis; this credit being attributed to an apothecary Ambrosian in 1835. Attempts by chemists prior to this time were ineffacacious, so it is not surprising that the history and significance of blood sugar determinations dates back and progresses with a history and knowledge of the disease entity, diabetes mellitus. Many other morbid processes manifest an altered glycemia although none have been as influential in stimulating a desire to learn about blood sugar and investigate methods of determination as has diabetes mellitus.

Although blood sugar determinations have been possible since the middle of the ninteenth century until the last thirty years such studies were largely confined to the physiological laboratory because of

the lack of suitable methods. However the discovery of insulin by Banting and Best in 1921 inspired a tremendous advancement in the development of clinical procedures for blood sugar analysis. With such a potent material at the hands of the physician for treatment of the diabetic patient it became a matter of considerable importance to be able to measure the actual glucose content of the blood as closely as possible. Today blood sugar determination is one of the most common of all chemical procedures in clinical medicine.(2) In the absence of obvious clinical signs and symptoms of diabetes mellitus, this procedure is the major criterion for diagnosing the disease.

PURPOSE AND SCOPE OF STUDY

It has been estimated by Spiegelman and Marks (3) that the total population of the United States will be at a maximum in 1965. By that time one may expect an increase of 74% in the diabetic population, while the general population will have increased only 22%.

Diabetes is one of the most prevalent of human diseases. The malady is a disorder of carbohydrate metabolism and is characterized by hyperglycemia and glycosuria.(4) Glycosuria, however, is not a constant feature of the disease particularly in mild cases and

diabetics of long standing who develop a high renal threshold to the spilling of sugar in the urine. Hyperglycemia, on the other hand, is always manifested in some way in the disease process whether it be a high fasting level, high glucose tolerance curve, or a prolongation in the return to a normal level during the glucose tolerance test. The delayed return to normal is the most adequate criterion for diagnosis on a glucose tolerance basis. (5) It is evident the importance of blood analysis for sugar in the diagnosis of a disease process which is rapidly increasing in incidence throughout the world.

Management of the diabetic patient is likewise best carried out by following the blood sugar as adequately and accurately as possible. A knowledge of the blood picture frequently over a 24 hour period is very desirable in knowing what type of insulin to prescribe and when to give it as well as how much to give. In conjunction with this manner of thinking in diabetic management, many 'rapid method' blood sugar tests have recently been devised and are gradually becoming more in demand. Agreement is perhaps universal that a frequent check on the blood picture is much more important and far more accurate than the

urine analysis.

Blood sugar determinations have become of utmost value in the diagnosis of renal glycosuria. This condition is a lowering of the renal threshold to blood sugar in the absence of an abnormal hyperglycemia resulting in a harmless elimination of sugar in the urine. It is sometimes called orthoglycemic glycosuria or diabetes innocens. (6)

Mosenthal (5) stresses the point that the renal threshold should always be determined using arterial or capillary blood and not venous blood. This is rather obvious as tubular excretion is in direct proportion to arterial concentration. Glucose is found in the urine normally when arterial blood sugar concentration is at about 200 mg %. This is a fairly constant relationship.

Joslin et al., (7) feel that glycosuria must be present during the fasting state before a diagnosis of renal glycosuria can be made. However, Lawrence (6) does not agree with this concept, but feels that the condition is proved whenever glycosuria occurs with a normal blood sugar.

The condition is not rare having been observed in 65% of 800 cases of glycosuria among selectees

during the last war. It is a life long condition in most cases and is inherited in some families as a mendelian dominant characteristic, so that half of the children are affected. Also it is a common temporary occurrence during pregnancy. No treatment is required as it does not develop into true diabetes. (6)

Another symptomless glycosuria known as the Lag-storage curve, oxyhyperglycemia or alimentary hyper-glycemia is characterized in individuals by glycosuria after meals. It is explained on the basis that from a normal fasting level the sugar rises rapidly after glucose ingestion to over 200 mg% (arterial), but returns to the fasting level within the normal time. The glycosuric condition is commonly seen in persons with a rapidly emptying stomach or after gastro-enter-ostomy. The high blood sugar level and glycosuria is a result of rapid absorption of glucose into the blood stream. (6)

Other conditions that upset the normal blood sugar picture and produce 'pseudodiabetic curves' (6) include: 1. Toxic and septic conditions. 2. Endocrine, a. thyrotoxicosis b. hyperpituitarism c. chromaffin suprarenal tumors. 3. Old age, where carbohydrate tolerance is commonly reduced, although Blotner (6)

feels that the decrease in carbohydrate tolerance is not due to age itself, but is the result of prolonged inactivity. 4. Obesity. 5. Previous starvation or carbohydrate restrictions when ketonuria is present. 6. Liver disease especially hepatitis. In all cases of the above conditions diagnosis as to diabetes or a non-diabetes condition is ultimately dependent upon the history and blood sugar picture during periods of fasting and stimulation.

A few rare instances where melituria is due to reducing glucids other than glucose occur from time to time and must be ruled out. This is easily accomplished by doing a fermentation test on the urine using baker's yeast since only glucose is fermented by yeast.(8) Somogyi (10) however, emphasized the fact that whenever yeast is used in the fermentation process it must be purified by repeated washings. Variable amounts of reducing substances are given up by yeast in the fermentation process when not thoroughly cleansed before use. Other tests helpful in diagnosing melituria, not due to glucose, include: Seliwanoff's test which gives a positive result for fructose; the Bial test which is positive for pentoses; and the characteristic osazone formed by the various

sugars when reacted with phenylhydrazine.(11)

A knowledge of the blood sugar, particularly in the morning before breakfast, is of extreme importance in the diagnosis of hypoglycemic states whether it be due to hyperinsulinism, hypofunction of other endocrines or for any other cause known to produce hypoglycemia. The glucose tolerance test has not been of value in diagnosing this state unless the test is performed over a 5 to 6 hour period. Hypoglycemia usually does not become manifested until that much time has elapsed.(12)

The above evidence is conclusive regarding the importance of a knowledge of the blood sugar. It is imperative not only in the diagnosis and management of the diabetic patient but also in many other disorders characterized by either melituria, hyper- or hypoglycemia.

UNIVERSALITY AND ACCURACY

The primary purpose for writing this paper is not just to emphasize the importance of doing blood sugar determinations but to discuss the need for a 'universal' clinical method with unquestionable accuracy. Multiplicity of procedures usually means that none are fool-proof. Kleeberg (13) in reviewing the literature

found no less than 69 different methods of examining monosaccharides and 24 different clinical procedures.

In the 34 years since Hamman and Hirschman's suggestion (14) of following the blood sugar level after ingestion of glucose, numerous variations of the glucose tolerance test have been devised. There are at present four popular methods, each with its own criteria. Likewise each of the numerous laboratory methods for blood sugar determination has its own separate standards for the so called 'norms' of the particular procedure.

Table I illustrates the multiplicity of criteria resulting from several different clinical procedures now in vogue.

TABLE I

A comparison of criteria for normal glucose tolerance curves showing the marked variations resulting from different methods and different authors interpretations.

Author	BLOOD SUGAR mg%											
	Venous Blood						Capillary Blood					
	True B.S.			B.S. +ngrs			True B.S.			B.S. +ngrs		
	F	H	2h	F	H	2h	F	H	2h	F	H	2h
Am. Diab. Ass. (15)	110	150	110	130	200	130	120	200	120	140	240	140
Mosenthal (16)	100	150	100	120	170	120	120	200	120	120	200	120

True B.S.-----True Blood Sugar.
B.S. +ngrs----Blood sugar plus non-glucose reducing substances.
F------Fasting.
H-----Height of glucose tolerance curve.
2h----2 hours after glucose ingestion.

From the Table it will be noted that differences as high as 80 mg% exist depending on the method used and which author's criteria is accepted.

As stated previously, a return to the fasting level in two hours or less is the most important diagnostic feature of the glucose tolerance curve, but the fasting level and height of curve must not be overlooked. Therefore, it is imperative that we have some idea of what the normal values are, which are all too confusing at the present time.

Muhberg (17) found the following inconsistency among thirty insurance agencies: Practically all companies used the Folin-Wu laboratory procedure or some modification of it. Eighteen companies used 100 grams of glucose; six used 75 grams; and six used 50 grams. Two companies utilized the Exton-Rose method. There was very little agreement as to the time intervals used in doing the tests. Thirteen companies used venous blood; ten used arterial; and seven employed either one. In cases where renal glycosuria existed, twelve companies issued policies with extra premiums. 120 mg% was the tendency for fasting normal, and one half an hour after glucose ingestion figures varied from 225 mg% down to 160 mg%.

The finding of so many variations in laboratory procedure and standards of such an important diagnostic aid, further emphasizes the dire need for study into the problem with a goal set at universality and accuracy.

SOURCES OF ERROR

Sources of error in determining blood sugars (17) are many and include: 1. Laboratory technique. 2. Use of the tourniquet when obtaining venous blood. 3. Arterial versus venous blood. 4. True blood sugar versus non-glucose reducing substances, and 5. Blood sugar stability in the fasting state.

Laboratory technique is invariably a source of error in any clinical procedure. It must always be taken into consideration for a just evaluation of the results. 8 mg% has been arbitrarily set as the maximal figure arising from error in technique.(5)

Use of the tourniquet in obtaining venous blood has been a controversial subject for some time. Mosenthal (17) found that the application of a tourniquet before drawing venous blood could cause a rise or fall in venous blood sugar amounting to 20 to 25 mg%. Haunz and Keranen (2) ascribed the increase in venous blood sugar to a forcing of arterial blood with its higher glucose content into the occluded veins. However, they found that in the fasting state the arterio-venous

blood sugar difference was negligible so a tourniquet applied at that time made no significant difference. The general opinion in vogue is that a tourniquet should not be used in obtaining blood for venous blood sugar determinations.

Arterial versus venous blood for blood sugar determinations is one of the primary factors causing the controversy in this subject. At the present time most workers in this field agree that arterial and capillary blood is for all practical purposes identical.(17) Likewise, as previously stated, arterial and venous blood sugar concentrations are the same during the fasting state. An investigation of A-V blood sugar differences carried out by Mosenthal (17) revealed enormous variations during the course of glucose tolerance tests. In 35.5% the A-V difference exceeded 20 mg% of blood sugar which is a significant variation in evaluating sugars. Both Mosenthal (17) and Fabrykant (12) found a reversal of the A-V ratio (venous blood sugar higher than arterial) in a few patients. Fabrykant ascribes this to a 'back diffusion' of glucose from the tissues or tissue spaces into the circulation. He noted that hypoglycemic episodes occurred when a negative A-V ratio existed (usually

5 to 6 hours postprandial). His explaination was that it represented a response on the part of the venous system to correct a critically low arterial concentration when a fall in arterial blood sugar occurred. Mosenthal found that the reversal of the A-V ratio in diabetics is not as prevalent as the literature reports. He reported a reversal in only 8% of his diabetic patients. Somogyi (18) agrees there is a marked variation in the A-V ratio and feels it is proportional to the supply of insulin (increased insulin gives an increase in the A-V ratio). This being true the A-V ratio should be lower in the diabetic person, but in clinical studies it is often higher in such persons. On the other hand Somogyi does not agree with the concept of a negative A-V ratio and feels that such a state does not exist for such a ratio disregards the established fact that muscle cells cannot yield free glucose. Thus, he maintains, that whenever a negative A-V ratio is attained it is due to one of two things: Either there are certain conditions under which muscle cells secrete into the circulation some reducing matter other than glucose or else the analytical techniques employed are unsatisfactory.

It is evident that ideas on the A-V ratio are still controversial. Some advancement has been made though in that most workers agree that arterial blood sugar should be used in determining 'renal threshold'. Mosenthal (17) and others feel the venous blood should be used for guidance in treatment of diabetes and carrying out glucose tolerance tests. In those cases the primary concern is with the utilization of carbohydrate, which is better revealed by the venous blood. In commenting on Mosenthal's work, Powers (17) was of the opinion that arterial blood sugar was quite as important as venous blood sugar. He felt this to be so because arterial blood reveals the function of the liver which is intimately associated with glucose levels of the blood. One might conclude the last word has not been said concerning A-V differences and their usefulness.

Perhaps an even more disturbing subject than A-V differences is the so called non-glucose reducing substances or 'saccharoids' of the blood. Their presence in the blood has been a tremendous stimulus for continued interest in improving laboratory methods to selectively determine glucose concentration.

The presence of saccharoids became evident when

it was noted that one technique of blood sugar determination consistently yielded lower results than another. Most authorities believe the saccharoid fraction is due largely to glutathione, ergothionine, creatinine, cysteine, levulose and glucuronic acid.(2) (20) Benedict (19) believed the high reducing values might also be the result of substances in the blood filtrate capable of causing an alteration in the copper complex employed (when copper is used as the oxidizing agent). The alteration allows the copper to be more readily reduced by glucose or other reducing materials present.

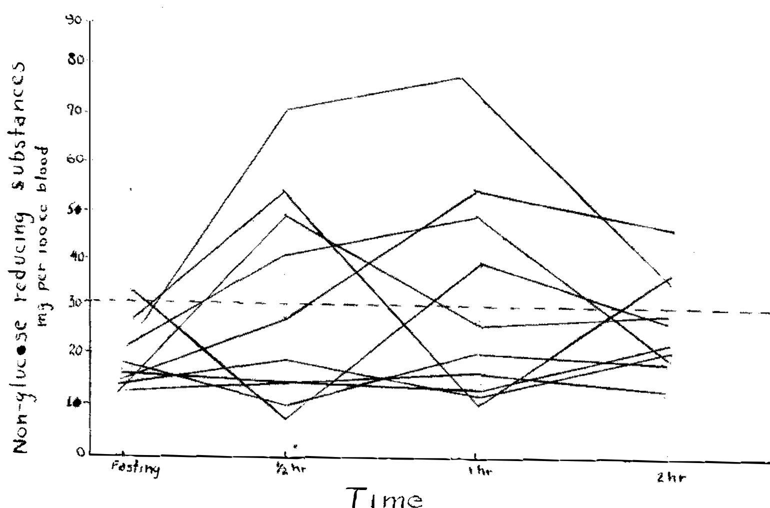
Otto did some of the original research in determining the 'residual reduction' (saccharoids). He subjected the blood to alcoholic fermentation, converting the glucose to alcohol, and determined the remaining reducing substances by other methods. His results were quite varied and Somogyi believed this to be due to reducing substances within the impure yeast suspensions. By purifying the yeast Somogyi found the saccharoids present to be very constant, averaging 27 mg%.(10)

Investigations by Benedict (48), Mosenthal (5) and Haunz (2) revealed the saccharoids to be quite unpredictable, ranging from 0 to 78 mg%. Mosenthal

found values exceeding 30 mg% in 38% of 200 determinations. Chart 1 shows the irregularity of non-glucose reducing substances found in ten patients during glucose tolerance tests.

CHART 1

Non-glucose reducing substances (difference between true blood sugar method and Folin-Wu method) in normal glucose tolerance tests.



Johnson in 1887 was the first to point out that the interfering reducing substances in blood were precipitated out with the protein by salts of heavy metals. Mercury chloride was used extensively by Johnson. Later Patein and Dufau improved upon the method by using mercury nitrate neutralized by alkali.(21) Somogyi in recent years has found the use of zinc sulfate and barium hydroxide to be very successful in removing the saccharoids during the deproteinizing process.(22) Apparently a generally accepted conclusion is that a

'true' glucose value is obtained by alcoholic fermentation with purified yeast or, by reduction methods when salts of heavy metals (zinc, mercury, iron etc.,) are used in deproteinization to eliminate the majority of non-glucose reducing substances.

Mosenthal (17) cites a case (Table II) where failure to realize the amount of non-glucose reducing substances present led to a wrong diagnosis and consequent refusal of a life insurance policy for several years. The patient revealed glycosuria on examination of the urine. The Folin-Wu method used in the laboratory procedure (which includes non-glucose reducing substances evaluated at a constant of 30 mg%) produced a mild diabetic glucose tolerance curve. However, the same glucose tolerance curve determined by a true blood sugar method was well within normal limits. Non-glucose reducing substances were tabulated above 50 mg% in all four blood sugar determinations.

TABLE II

High values for non-glucose reducing substances in a sugar tolerance test.

TIME	VENOUS BLOOD SUGAR mg.%		
	True B.S. met'd	Folin-Wu method	N.G.R.S.
Fasting	87	138	51
½ hour	102	176	74
1 hour	108	176	68
2 hour	90	168	78

True B.S. met'd------True blood sugar method.
N.G.R.S.---------Non-glucose reducing substances.

Presumptive diagnoses made from fasting blood sugars has caused many errors as pointed out by Mosenthal. (17) In normal fasting individuals the blood sugar remains fairly constant. However in fasting diabetics, 63% showed considerable variation in blood sugar concentration. Approximately 48% showed a drop in blood sugar (maximal 127 mg%), while 15% had a rise (maximal 115 mg%). One should be cautioned against making a diagnosis on fasting blood sugars alone.

DESCRIPTION AND DISCUSSION OF PRESENT METHODS OF BLOOD SUGAR DETERMINATION

It has been known for many years that sugars having a free aldehyde or ketone group (free carbonyl group) are unstable in the presence of alkali and are readily fragmented into smaller units having strong reducing properties. This is the basis for the vast majority of methods employed for blood sugar determinations. In addition, blood sugar determinations may also be carried out by polaroscopic studies or by alcoholic fermentation with baker's yeast. Polaroscopic studies have never gained popularity in clinical laboratories for they are not adapted for determining small amounts of blood sugar and in addition, involve the use of expensive equipment. Likewise, methods of determination by fermentation processes

have never attained widespread use in the laboratory for it is a rather awkward procedure. It is interesting to note that of the three general methods of blood sugar determination, the two methods showing more specificity for glucose have never come into practical clinical use.

A discussion of the various methods will be confined mostly to those procedures involving reduction of metallic salts in hot alkaline solutions as they are the principle methods in vogue.

The first alkaline metallic salt used for sugar determinations was a copper sulfate solution introduced by Fehling in 1848. Picric acid, a nitroaromatic acid, was used as a sugar oxidizing agent by Lewis and Benedict (23), but has fallen into disfavor by the popularity of copper and iron (ferricyanide) salts. Benedict himself abandoned the picric acid for copper reagents.

As stated previously, most methods involve the reduction of hot alkaline metallic salts by fragments of the sugar molecule (containing free carbonyl group). It has been demonstrated if sugars (containing the free carbonyl group) are heated for a longer period of time or with stronger alkali for a short time (20 to 30 minutes), the reducing substances formed by the initial

action of the alkali are either destroyed or polymerized so they will no longer have reducing properties. It is therefore desirable to establish optimum conditions of alkalinity and heating time to obtain maximum reduction.(24)(33) This fact is another reason for the frequent modifications and continued improvement of various laboratory methods.

The method by which the blood filtrate is prepared has become an extremely important phase of improved technique. A wide variety of deproteinizing agents have been used among which are tungstic acid, trichloracetic acid, picric acid, metaphosphoric acid, alcohol, colloidal iron, as well as mercury, zinc, copper, cadmium and iron salts. The selection of the precipitant depends upon the particular analysis to be made. Blood sugar requires a faintly acid, neutral or slightly alkaline filtrate. Folin and Wu (25) made use of tungstic acid for the removal of blood proteins in their system of blood analysis introduced in 1919. By their technique blood is diluted ten times in the process of deproteinization. The filtrate from 10 ml of blood permitted the determination of the non-protein nitrogen, urea, creatinine, creatine, uric acid, amino acid nitrogen, sugar and chlorides. Because of the fact the method of depro-

teinization was well adapted for so many different blood chemistry determinations, it has become the most popular method in the majority of clinical laboratories. However, it has since been proven that other methods of deproteinization, particularly in regard to blood sugar determinations, are much superior as they also rid the filtrate of practically all non-glucose reducing substances. This desirable feature is unfortunately not present in Folin's tungstic acid filtrate.(26) Salts of heavy metals are particularly adapted to rid the filtrate of non-glucose reducing substances.(21),(26),(27)

A description of the laboratory methods forthcoming will be with emphasis not on laboratory technique but rather on the relative merits of each method.

Folin-Wu Method (25),(28)

This method was introduced by the co-authors in 1920 after an unsuccessful attempt to employ a phenol color reagent to the cuprous oxide, formed by reduction with blood glucose, for colorimetric determination. It was found that phenol reactions introduced unavoidable errors and was replaced by a new reagent, molybdic acid.

The principle involved employs the use of the Folin-Wu tungstic acid blood filtrate heated with an

alkaline copper solution using a special tube to prevent reoxidation. The cuprous oxide formed is treated with a phosphomolybdic acid solution, and a blue color being obtained by the reaction is then compared with a standard solution colorimetrically. The alkaline copper solution contains sodium carbonate for an alkaline medium, tartaric acid to prevent formation and precipitation of copper hydroxide and copper sulfate the oxidizing agent. Phosphomolybdic acid reacts with the reduced copper ion forming colloidal oxides of molybdium. The resulting color intensity is proportional to the amount of copper reduced in the reaction.

<u>Advantages (24)(31)</u>

1. Laboratory technique simple.

2. Laboratory equipment inexpensive.

3. Readily adapted to multiple simultaneous determinations.

4. Oxidizing agent readily soluble, minimizing difficulty in preparing the solution.

5. Adaptation of tungstic acid filtrate for blood analyses other than glucose.

<u>Disadvantages (2)(17)(28)(32)</u>

1. Autoreduction of copper in alkaline tartrate solution with distortion of results.

2. Modification of course of the reaction by the tartrate.

3. Deterioration of the alkaline copper reagent.

4. Longer heating time than previously recommended needed for complete reduction.

5. Deterioration of molybdic acid reagent.

6. Failure of tungstic acid to remove saccharoids in deproteinization of blood.

7. Need of special equipment (constricted test tubes) to prevent reoxidation.

8. Requires venipuncture to obtain blood.

9. Increase in error at higher blood sugar concentrations.

10. Instability of tungstic acid solution.

Folin, realizing many of the pitfalls of the method made modifications in an effort to eliminate some of the undesirable features. A new alkaline copper reagent was prepared by decreasing the concentration of sodium carbonate and adding sodium bicarbonate, thereby, decreasing the alkalinity to a more neutral state. Folin found the specific merit of the reagent was attained from the fact that it yielded lower blood sugar values. It was by keeping close to the lowest permissable degree of alkalinity that more accurate determinations were obtained.(29) However a disadvantage encountered with the new reagent was the instability with loss of carbon dioxide from the solution (30) unless kept in small, well filled, tightly corked bottles. Likewise, with the decrease in alkalinity, an increase in the heating period was required for

more complete reduction. Along with the new alkaline copper reagent, Folin developed a new molybdate reagent possessing a deeper color and greater alkalinity which could be used with most alkaline copper reagents.

Morgulis (34) described a modification based on Benedict's method of uric acid determination whereby sugar could be determined by the Folin-Wu method. The reagent involved, arsenomolybdate, was claimed by the author to give a deeper color for better colorimetric determination.

<u>Folin Micro Method (35)</u>

Folin introduced this method in 1928. The procedure utilizes a very small amount of blood (0.1 ml) and depends on the reduction of potassium ferricyanide to ferrocyanide in the presence of a cyanide-carbonate buffer. The ferrocyanide is then converted to Prussian blue by a solution of ferric sulfate. Gum arabic is added to keep the Prussian blue (ferri-ferrocyanide) in colloidal solution. The resulting solution is measured colorimetrically.

<u>Advantages (35)(39)</u>

1. Utilization of small amount of blood.
2. No special equipment needed.
3. Less heating time (5-8 min).
4. Ferrocyanide more stable (no reoxidation) than

cuprous oxide in old Folin-Wu method.

5. No venipuncture required.

Disadvantages (35)(36)(48)

1. Micro method less accurate than macro because of greater possibilities for error in laboratory technique.

2. Very inaccurate at high glucose levels; therefore not well adapted in diabetic clinic.

3. Instability of tungstic acid solution.

4. Impurities (ferrocyanide) in ferricyanide reagent.

5. Ferric ion less specific for reaction with glucose than is cupric ion.

6. Inconvenience in keeping all reagents separate before use to prevent deterioration.

Folin (37) modified the ferric sulfate solution by substituting gum arabic with gum ghatti attaining a better colloidal suspension. Potassium permanganate was added to neutralize any unwanted reducing agents in the gum ghatti. Folin and Malmros (38) further modified the method to insure accuracy within a range from 50 to 600 mg%. Previously it was only accurate from 50 to 200 mg% being of no practical use in diabetic clinics where blood sugar values often run extremely high. The problem involved was how to measure the Prussian blue in the presence of a large surplus of yellow ferricyanide. The bad effects were easily removed by constructing a light screen using a filter

paper soaked with picric acid. By doing this they found they could use four times as much ferricyanide as in the original method. Also by increasing the amount of ferric sulfate they decreased a 5 minute waiting period for Prussian blue to develop shortening the total length of time required for the procedure.

Harvath and Knehr (40) adapted the method for determination by photoelectric colorimetry and substituted a commercial product (Duponal) for gum ghatti which later proved not to be superior.(41) Reincke (42) also adapted the method for utilization with extremely small quantities of blood (0.01 ml) which has been of considerable value in small animal experimental work.

Lewis-Benedict Method (43)

Lewis and Benedict's original method depended upon the reduction of picric acid by glucose to picramic acid with the formation of a red color. Picric acid is also used to deproteinize the blood. The reaction takes place in a hot alkaline medium of sodium carbonate by heating to dryness. A colorimeter is employed to compare the color with a standard solution of picramic acid. In 1918 Benedict modified the method (44) by eliminating the heating to dryness of the blood filtrate-picric acid solution. In addition the blood filtrate

was decreased in dilution so the reaction between glucose and picric acid would be more concentrated producing a better color.

Advantages (43)

1. Simple laboratory procedure.
2. No special laboratory equipment needed.

Disadvantages (25)(31)

1. Results very inaccurate in abnormal bloods e.g. in diabetes.

Duggan and Scott (31) expound on the inaccuracy of Benedict's method with abnormal blood. They state that a reduced amount of protein in the blood will leave an excess of picric acid in the filtrate over and above that assumed by the author to be present. Therefore an increase in either the oxidizing or reducing agent, the other remaining constant, would lead to an increase in one or more of the end products. Blood containing an abnormally large amount of sugar or abnormally small amount of protein could be presumed to indicate a higher concentration of sugar with this method than would be the case with other methods. This is so, for with other methods the balance between the oxidizing and reducing agents is not so easily disturbed.

Benedicts Copper-citrate method (45)

Benedict, apparently realizing the failure of his

picrate method on abnormal bloods, gave it up in preference to a procedure using an alkaline copper citrate-carbonate solution with a small amount of sodium bisulfite incorporated into the mixture. The action of bisulfite by combining with fragments of the sugar molecule prevented the destruction of the molecule by the carbonate present until they have reduced the copper. For development of color with the reduced cuprous oxide, arsenophosphotungstic acid and formalin was used. In the several years following, Benedict made numerous small modifications (46)(47) including a micro method. Also adjustments of the sulfite-carbonate concentration were made. Arsenophosphotungstic acid was replaced by phosphomolybdic acid. Alanine was added to the alkaline solution as the amino acid appeared to form a complex with the cupric salts. The complex is relatively unaffected by non-glucose substances that reduce other copper reagents.

Advantages (45)(48)

1. Simple laboratory technique.

2. No special laboratory equipment.

3. Low blood sugar readings particularly when using zinc filtrates. Saccharoids not reduced to an appreciable extent.
4. Bisulfite allows greater reduction of copper by sugar.

Disadvantages (28)(33)

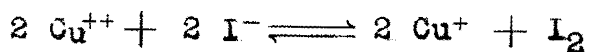
1. Citrate exerts powerful depressive action on the oxidative properties of dissolved copper, thus the reason for lower blood sugar readings.

2. Bisulfite solutions spontaneously oxidize to sulfate altering the efficiency of bisulfite.

Although Benedict's method repeatedly gave lower results than most other alkaline copper methods, it never gained popularity in clinical laboratories.

Shaffer-Hartman method (49),(50)

This method was introduced in 1921 and patterned after the initial procedure of DeHaen in 1854 as adapted by Bang 1913, Scales 1915 and Maclean 1916. The principle involved depends on measuring either the cuprous oxide formed in the oxidation of blood sugar or, knowing the total copper, the residual cupric salt left over by means of iodometric titration with thiosulfate using starch as an indicator. The reaction being reversible, direction depends upon the concentration of ions.



$$2\ Cu^{++} + 2\ I^{-} \rightleftharpoons 2\ Cu^{+} + I_2$$

For the complete oxidation of cuprous salts by iodine the concentrations of cupric and iodide ions must be maintained at very low values; while for the complete conversion of cupric to cuprous salt a very high concentration of iodide must be maintained. The usual method is by oxidizing the cuprous ion back to cupric

(equation from right to left) and insuring completion of the reaction by removing cupric ions with oxalate. Thus the remaining iodine, after the above reaction, is titrated with thiosulfate. The amount of copper oxidized reveals how much glucose was present.

Somogyi (51) modified this original procedure by adjusting the alkaline copper reagent to a more constant degree of alkalinity, thus giving better reduction and higher values.

Advantages (33)(48)(50)(51)

1. Accurate when technique adhered to.
2. Laboratory procedure simple.
3. No special laboratory equipment.
4. Iodides decrease autoreduction of copper.
5. Iodides decrease sensitivity to non-glucose reducing substances.

Disadvantages (31)(33)(50)

1. Not accurate in lower ranges as potassium iodide renders a small part of the precipitated cuprous oxide soluble. This increases the surface exposed to reoxidation and limits the sensitiveness of the method in low blood sugar ranges.
2. Thiocyanate solution deteriorates.
3. Reoxidation of cuprous oxide by air.
4. Long heating period (15 - 20 min).
5. Iodide makes method unsuitable for colorimetric work.

Somogyi-Nelson Method (39)(52)

Somogyi's modification of the old Shaffer-Hartman method was further improved when he found the presence of sulfate in the copper solution depressed the solubility of oxygen in the solution. This prevents the reoxidation of cuprous oxide by the air with the result that much smaller amounts of sugar can be determined. However sulfate also depresses the ionization of carbonate decreasing the alkalinity and slowing down the reaction velocity requiring a longer heating period.(36)

In 1945 Somogyi came forth with a new reagent, still using copper as the reduction ion, and including sodium sulfate to prevent reoxidation of the cuprous oxide. The principle change from the old Shaffer-Hartman reagent is that the carbonate-bicarbonate buffer has been replaced by a phosphate buffer containing di- and tribasic sodium phosphate. The solution contains no potassium iodide so that it may be analized colorimetrically or, by the addition of potassium iodide after reduction, can be determined iodometrically. The phosphate, being more alkaline, shortens the heating period and depresses the self-reduction of copper when exposed to room temperature or sunlight. In addition the increased alkalinity

adapts the reagent for use with slow-acting sugars e.g., maltose, that takes longer to oxidize.(38) Nelson, working with Somogyi's new copper-phosphate solution developed a color producing reagent using arsenomolybdic acid which was well adapted for photometric determination.(52) Iodometric titrations have been proven to be more accurate than visual colorimetric procedures but are less accurate than photometric colorimetry. Thus the new Somogyi-Nelson method is adapted for colorimetric (Photo and visual colorimeter) or iodometric procedures as well as macro or micro methods. In addition Somogyi utilizes the use of zinc sulfate and barium hydroxide for deproteinization of blood.(22) It was noted that salts of heavy metals remove not only protein but also non-glucose reducing substances.(26) The use of barium hydroxide in place of sodium hydroxide further increases its usefulness by adapting the reagent for both serum and plasma as well as whole blood.(38) Sodium hydroxide made the reagent useful for whole blood deproteinization, but was inadequate for precipitation of proteins in plasma. Barium hydroxide also precipitates anti-coagulants such as oxalate or flouride which often interfer with deproteinization.

It was noted by Benedict (48) that zinc filtrates of blood, which contain slight quantities of zinc yielded lower sugar values than the same filtrates after removal of the zinc. Somogyi (36) feels this is due to the zinc salts contained in the filtrate forming with the copper reagents a fine flocculent precipitate. Cuprous oxide settles out on the flocculent precipitate in a fine state of dispersion; convection currents during the heating period move the precipitate to the surface and expose cuprous oxide to the air and thereby to reoxidation. Therefore all precipitates and suspended particles in the zinc filtrate must be removed before the blood sugar determination is carried out.

<u>Advantages (19)(22)(36)(38)</u>

1. Laboratory technique simple.
2. No special laboratory equipment.
3. No reoxidation of cuprous oxide.
4. Stability of copper solution, even in sunlight.
5. Adapted for slow and rapid reducing sugars.
6. Adapted for both macro and micro determinations using the same reagent.
7. Adapted for colorimetric or iodometric determinations.
8. Non-glucose reducing substances removed with use of zinc as a deproteinizer.

Disadvantages (36)(48)

1. Seven minute longer procedure than Folin-Wu method.
2. Any residual zinc in protein free filtrate depresses reduction value of copper reagent.

Van Slyke-Hawkins Method (53)(54)

These co-authors introduced their method of blood sugar determination in 1927 using a Folin-Wu blood filtrate. The filtrate is subjected to an alkaline ferricyanide solution for reduction, and the excess ferricyanide measured by the amount of nitrogen gas given off when reacted with hydrazine. A Van Slyke-Neil manometric blood gas apparatus is needed to measure the amount of nitrogen produced. A blank is run with all solutions except the blood filtrate to standardize the apparatus prior to running determinations. The difference in pressure readings between the blank and the solution containing blood sugar reveals the amount of ferricyanide reduced, and therefore, the amount of blood sugar present.

A comparison of their method with the Folin modification of the Folin-Wu method and Somogyi's modification of the Shaffer-Hartman method showed their values to be higher than Folin's and lower than Somogyi's.(54) It was noted by the authors that in-

accurate results often occur when the tungstic acid filtrate is too strong to be neutralized by the alkaline ferricyanide solution.

Advantages (53)

1. Well adapted for speed in multiple determinations.
2. Accuracy comparable with other methods.
3. No standard solutions needed as in colorimetric methods.

Disadvantages (36)(54)

1. Special laboratory equipment needed.
2. High acid filtrate will alter result.
3. Ferric ion less specific for glucose than copper ion.

Hagedorn-Jensen method (55)

The method, introduced in Denmark in 1918, depends on the quantitative reduction of alkaline potassium ferricyanide by sugar. Excess ferricyanide is titrated iodometrically using potassium iodide, thiosulfate and starch. The authors use zinc sulfate and sodium hydroxide in preparing the protein free filtrate, a method which was later modified by Somogyi.(26)

Advantages (31)(32)(55)

1. No special laboratory equipment needed.
2. Laboratory procedure simple.
3. Ferric ion not reoxidized by oxygen.

4. Non-glucose reducing substances removed with use of zinc as a deproteinizer.

Disadvantages (31),(32),(36)

1. Reagents not stable.

2. Errors can creep in in filtration process.

3. Ferric ion less specific than cupric ion.

4. Not accurate in blood sugar range over 200 mg%.

Fermentation Method (10)

The principle underlying this procedure involves the determination of reducing material in two samples of the same blood, one of which has been subjected to fermentation by yeast for the removal of blood sugar. The difference between the two determinations represents 'true' blood sugar by alchohic fermentation of glucose. Conflicting results were noted by Hiller et. al., (56) and Somogyi (10) felt this to be due to impurities (reducing substances) within the yeast which he disposed of by thoroughly washing the yeast suspension prior to use. By this method Somogyi also noted the non-glucose substances were very stable averaging 27 mg%. Folin and Svedberg showed the fermentation could be carried out on Folin-Wu blood filtrates as well as on whole blood.

The procedure has been used a great deal in experimental laboratories but has not become popular in

clinical laboratories.

Advantages (10)

1. Measures only fermentable blood sugars (glucose).

2. No special laboratory equipment.

3. Laboratory technique simple.

Disadvantages (10)(56)

1. Time consuming (requires 2 samples of blood and 2 procedures).

2. Difficult to purify yeast.

Within recent years many rapid 'bedside' methods of blood sugar determination have been appearing in the literature. These undoubtedly are the result of a realization that management of the diabetic patient by following the blood sugar picture is much more accurate than relying on the glycosuria. Likewise they are useful in 'on the spot' treatment of diabetic coma and for screening purposes as a diagnostic aid. Most of the methods have several things in common in that they are rapid and simple, small amounts of blood are utilized and accuracy is sacrificed for rapidity. The following are a few that have appeared in the literature.

Hawkins and Van Slyke (57) base their method on the rate which sugar reduces yellow ferricyanide to colorless ferrocyanide. The amount of sugar is meas-

ured by the time required (100 to 300 sec.) for the disappearance of the yellow color. Accuracy, according to the authors is plus or minus 5%. Extreme speed and simplicity of operation plus no requirement of special equipment are the advantageous features when time is essential.

Leech and Woodford (58) presented a method of blood sugar estimation by the reduction of dinitrosalicylic acid. The determinations were carried out by using Somogyi blood filtrates. Results were obtained by color matching with standards or by the photometer. They found their results to be quite accurate when the blood sugar was within 75 to 300 mg%. Color changes were not adequate outside of this range.

Mendel and Hoagland (59) base their procedure on the fact that when a dilute solution of glucose is heated in the presence of sulfuric acid a pink color develops, the intensity of which is proportional to the concentration of glucose. The method does not depend on reducing properties of glucose, as in other methods, but rather on the dehydration of glucose with the subsequent formation of hydroxymethyl furfural. The results are read photometrically. Accuracy of the method compares favorably with results by the Somogyi

method. It is a simple procedure and requires only two reagents which are stable for a long time. A determination takes about ten minutes to complete. In cases where further speed is desired color scales can be used instead of the photometer.

Wilkerson and Heftmann (60) devised a very useful and extremely simple method based on the original Hagedorn-Jensen ferricyanide procedure. However, they have practically eliminated the laboratory work and technique by standardizing the reagents in the form of tablets. The heating period has likewise been controlled by the use of specific time-burning tablets. All the equipment necessary for the procedure (tablets for 50 determinations, capillary pipette, test tube, asbestos heating stove, and glass spoon) comes complete in a carton the size of an ordinary cigar box.

The test is carried out by adding 0.1 ml of blood to a test tube filled to a designated mark with tap water, mix and place on the asbestos stove. Two tablets are added to the solution: Tablet No. 1 contains zinc hydroxide to precipitate the protein; tablet No. 2 alkalinizes the solution and adds potassium iodide to the mixture. Two heating tablets (timed) are placed

in the stove and the solution is allowed to boil until the fire burns out (2 min). During this period of boiling the proteins are precipitated and rise to the top of the tube where they are scraped off with the glass spoon. To the protein free filtrate is added tablet No. 3 containing a definite amount of potassium ferricyanide and the solution is again brought to a boil using just one heating tablet this time. After boiling has ceased the test tube is cooled (place under tap water) and tablet No. 4, containing starch and tartaric acid, is added to the solution. The resulting color of the solution is either blue or colorless depending upon the amount of glucose present in the blood. A blue solution signifies that more ferricyanide was present than could be reduced by glucose. The excess ferricyanide reacts with potassium iodide, in an acid solution (tartaric acid), forming free iodine which turns the solution a blue color in the presence of starch. A colorless solution is evidence that glucose is in excess and has completely reduced ferricyanide so there is none left to react with the iodide. The tablet containing ferricyanide (No. 3) comes in two quantities, one containing ferricyanide equivalent to 130 mg% of glucose and the other equivalent

to 180 mg%. Thus by the color of the solution at the end of this very simple procedure, we have a knowledge of the range of the blood sugar. The entire process takes less than five minutes and certainly does not require an experienced laboratory technician to perform.

Although the method does not give a definite value for blood sugar, it does give three important ranges, below 130 mg%, 130 to 180 mg% and above 180 mg% which are of great practical value in presumptive diagnosis and in diabetic management. Haunz and Keranen (2) in evaluating the test found only seven discrepancies in 200 tests using capillary blood as compared with a true sugar test using venous blood. They compared the procedure with the time honored Folin-Wu method and found it to be more accurate than the 40 minute Folin-Wu method. Margolin and Gentry (61) in comparing the test with the Folin-Wu method found only one discrepancy in 32 determinations on 1■ diabetic patients (Table III). In evaluating the results it must be kept in mind that all Wilkerson-Heftmann determinations should be lower than the Folin-Wu readings. This is so because the W-H method uses a heavy metal for deproteinization, removing non-glucose reducing sub-

stances. It is therefore classified among the 'true' blood sugar methods. The Folin-Wu method, on the other hand, includes non-glucose reducing substances in the final reading and allows 30 mg% for the discrepancy. In only one case (No. 6) is the W-H reading higher than the Folin-Wu. Either method may be at fault in this discrepancy.

Table III

A comparison of sugar values obtained by the Folin-Wu and Wilkerson-Heftmann methods on the same samples of fasting venous bloods

CASE	AGE	FOLIN-WU mg.%	WILKERSON-HEFTMANN	
			130 mg.%	180 mg.%
1	63	162	130+	180-
2	40	140	130+	180-
3	63	170	130+	180-
4	17	100	130-	180-
5	71	154	130+	180-
6	68	114	130+	180-
7	60	126	130-	180-
8	32	78	130-	180-
9	52	207	130+	180-
10	41	145	130±	180-
11	51	170	130+	180-
12	48	185	130+	180-
13	58	123	130-	180-
14	25	78	130-	180-
15	48	106	130-	180-
16	25	100	130-	180-

Margolin (61) found the test to be extremely helpful in the clinical management of diabetic patients by obtaining a blood sugar determination 'on the spot' while evaluating the patient. Most patients were arranged to be seen and evaluated about 2 to 3 hours

after eating their last meal. An extremely valuable knowledge of the patient's management was gained by this procedure and alterations in insulin dosage, diet, etc. were given to the patient before leaving the clinic.

This simple method is of unquestionable value in diabetic coma where time is at a premium. Likewise it is a most valuable aid to the general practitioner in areas where adequate laboratory facilities are unavailable.

CONCLUSIONS

1. Tourniquets should not be used in obtaining venous blood for blood sugar determinations except during the fasting state.

2. Marked changes occur in the magnitude of the arterio-venous difference in blood sugar concentration during carbohydrate stimulation but it is still controversial as to whether a negative A-V ratio (arterial less than venous) actually exists.

   a. Arterial blood is best for renal threshold determinations.

   b. Venous blood is more reliable for a knowledge of the ability of the tissues to utilize glucose.

3. Non-glucose reducing substances (saccharoids) likewise fluctuate in magnitude and cause considerable error

in blood sugar determinations.

a. The Folin-Wu, most popular clinical method in vogue, is not the most accurate as it includes non-glucose reducing substances.

4. Methods of deproteinization employing salts of heavy metals (zinc, mercury, iron etc.,) are far superior in blood analysis for glucose as they rid the filtrate of non-glucose reducing substances.

5. Establishment of a correct diagnosis has proved to be more reliable by true blood sugar methods (those using heavy metals for deproteinization).

a. The Somogyi-Nelson, by a comparison of advantages and disadvantages is unquestionably the best adapted for colorimetric or iodometric or for macro or micro methods. Its accuracy as a true blood sugar is unexcelled among methods in vogue.

6. Rapid methods, though less accurate, have their place in presumptive diagnosis and diabetic management as well as in diabetic coma where time is at a premium.

a. The Wilkerson-Heftmann method, a 5 min. procedure is on par with the 40 min. Folin-Wu laboratory method.

b. Although rapid methods are useful they should never be used exclusively for definitive diagnosis.

Definitive diagnosis must always be established with a clinical method of unquestionable accuracy.

SUMMARY

1. The history of blood sugar determinations dates back to the apothecary Ambrosian in 1835, but it did not become an important clinical procedure until the discovery of insulin in 1921.

2. A knowledge of the blood sugar picture is important not only in the diagnosis and management of diabetes mellitus but also in renal glycosuria, alimentary glycosuria, conditions causing hyper- or hypoglycemia and in melliturias caused by sugars other than glucose.

3. Multiplicity of methods of blood sugar determination resulting in innumerable variations in standards of 'norm' has led to many a wrong diagnosis causing the patient undue mental and financial suffering. Therefore it is imperative that a universal method of unquestionable accuracy be strived for in an effort to eliminate such occurrences.

4. Sources of error in blood sugar determination have been thoroughly investigated in an effort to eliminate the causative factors wherever possible. Such errors include laboratory technique, use of tourniquets, arterio-venous ratios, non-glucose reducing substances, and stability in the fasting state.

5. Many of the modern methods of blood sugar determination have been presented giving the general principle involved as well as the advantages and disadvantages of each method. Most methods in vogue are based on the reducing properties of glucose in an alkaline solution of copper or iron.

6. Within the last few years many 'bedside' methods of blood sugar determination have come into practical use. Several are presented and one method in particular, with which the author has had some experience, is discussed in more detail. The results accrued were compared with a standard laboratory method.

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