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The Sugar Content of Blood

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To the Graduate Council:

I am submitting herewith a thesis written by Ben King Harned entitled "The Sugar Content of Blood." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Chemistry.

, Major Professor

We have read this thesis and recommend its acceptance:

ARRAY(0x7f6ffe157e98)

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

THE SUGAR CONTENT OF BLOOD

A Thesis

Submitted in partial fulfillment of the

requirements for the

Degree of

33

Master of Science

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By

Ben King Harned

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Original

Historical.

The presence of a sugar-like substance in diabetic urine was recognized by Thomas Willis in 1647. It is probable that ancient literature would reveal the fact that this sugar-like substance was recognized long before the time of Thomas Willis, since the Jews, for example, are known to have a predisposition to diabetes, and diabetes was unquestionably a disease common among them from their earliest history. This sugar-like substance was identified as glucose by Chevreul in 1815.

Although it is an obvious proposition that components of the urine must either exist in the blood or be elaborated in the kidney, it was not until 1775 that Dobson recognized the presence of a sugar-like substance in diabetic blood; and it was not until seventy years later that its presence was demonstrated in normal blood by the great French Physiologist, Claude Bernard.

Criterian of Accuracy for Blood Sugar Determinations.

The early methods for determining blood sugar were all macro-chemical methods. Bierry and Portier (1) introduced one of the best early methods; but their technique requires 50 ml. of blood, thus precluding any clinical application. It remained for Lewis and Benedict (2) in 1913 to introduce a micro-chemical method applicable both to clinical and scientific purposes. Early in the same year Bang described a very ingenious gravimetric -volumetric procedure requiring only a few drops of blood. The exacting technique of the Bang method made any extensive clinical application impractical. Since 1913 many studies have been made on blood sugar and at present there are more recognized accepted and popular methods

for determining blood sugar than for any component of the blood. It is unfortunate that most of these methods give widely varying results, and especially is this true of the most popular clinical methods.

It is very common to find two laboratories almost side by side,using different methods, one reporting normal values to be from 80 - 100 mg. of glucose per 100 ml. of blood, the other reporting normal values to be from 100 - 125 mg. This is a very considerable difference, and consequently requires a statement of the method used, in order to avoid an erroneous conclusion by the clinician. In our investigations we have attempted to throw some light on the absolute accuracy of the best known methods and their clinical applicability.

The best criterion we have for the accuracy of these methods is the recovery of added sugar. This is a very poor test of accuracy, however, because the reactions used for sugar estimations are not specific, and there are many interfering substances in the blood. For example, creatinine, adrenalin, uric acid, and many other substances found in the blood give similar reactions to glucose and consequently cause blood sugar values to be much too high. We may, by a certain method, find that a blood contai ns per 100 ml., 125 mg. of glucose, of which 30 mg. may be due to some interfering substance. When glucose is added the error on this blood remains constant and the glucose is quantitatively recovered. The method apparently is acceptable, but upon critical examination may prove to be almost worthless, due to the fact that the error of interfering substances does not remian constant for different bloods. This interfering error is emphasized in abnormal blood. The ultimate criterion in quantitative

chemical analysis is the balance. Thus the ideal method for estimating blood sugar would be to precipitate the glucose and then employ a gravimetric determination. This procedure has been used in ~~the~~ blood determinations, particularly in the estimation of acetone bodies by Van Slyke and Fitz (66). A gravimetric-titrimetric procedure after precipitation, such as the method employed by Clark (67) in his determination of blood calcium, is also acceptable. Even a colorimetric determination after precipitation such as employed by Folin (68) in the determination of uric acid is not objectionable. We do not, however, have any specific reagent that will completely remove glucose,and consequently we have to rely on indirect determinations, most of which are subject to many errors. In the early part of our work we attempted to precipitate the glucose as the osazone and thus make a gravimetric determination. We chose this reaction because it is more nearly specific,than any other reaction we have for glucose. After many attempts under various conditions, we found that we were unable to obtain more than 85 per cent of the theoretical osazone, and since this percentage varied slightly, we abandoned this phase of the problem.

Classification of Methods.

The micro-chemical methods for blood sugar estimations may be divided into five groups: (1) polarimetric determinations; (2) refractometric determinations; (3) determinations dependent upon the reduction of alkaline ~~the~~ copper solutions; (4) determinations dependent upon the reduction of alkaline picric acid; and (5) determinations dependent upon the reaction between glucose and some compound not included in the above groups.

Polarimetric methods have been used to estimate glucose

in blood, but these methods are little better than qualitative tests. This is quite obvious when we consider that the blood contains many substances which are optically active and would necessarily intefere. If the optically active substances other than glucose remained constant the problem would be simplified, but these other optically active substances do not remain constant. It might be suggested that a determination of the optically active substances other than glucose be made, and this figure substracted from the total figure. When we consider that there are possibly hundreds of these substances in the blood, many of which have never been determined, it is evident that this procedure is not only impractical but impossible with our present knowledge.

Refractometric methods have also been used but they are subject, even more seriously, to the same criticisms as the polarimetric methods, because there are more substances in the blood which will affect the refractometer than the polarimeter. In 1923 Lloyd (62) suggested a refractometric method adapted to rapid determinations. The impraticability of this method was conclusively proven by Adams and Payne (4).

In discussing the determinations dependent upon the reduction of alkaline copper solutions it is necessary to subdivide this group into titrimetric and colorimetric determinations. There are many acceptable titrimetric determinations. Most of these methods are open to some criticism, but the clinical applicability of even a perfect titrimetric method may be seriously questioned. In these methods the cupric copper is reduced to cuprous copper by the glucose. The cuprous copper formed or the cupric copper remaining, is titrated with a standard iodine solution, and the sugar thus

calculated. Most of the workers prefer to titrate the reduced copper. This method is the basis for the widely used determinations of Bang (6), Maclean (7), Scales (8), Cammidge modification of the Scales method (9), and Shaffer-Hartman (10), which is a modification of the Shaffer method (11). There are some methods which employ the titration of the cupric copper left in the solution, but none of these seems to be very widely used. The determination of glucose by titrating the cupric copper was used by Lehman (12) in 1897, and Shaffer-Hartman (10) also give this titration but state that they prefer the cuprous titration. The reactions between copper and iodine have been studied very extensively by Shaffer and Hartman and their blood sugar determination is probably the most accurate titrimetric method. L. Michalis (23) has modified Bertrand's method (5) so as to determine sugar in small amounts of blood. In this method the reduced duprous oxide is dissolved in ferric sulphate-sulphuric acid solution and titrated with a standard solution of potassium permanganate. The colorimetric alkaline copper determinations depend upon the development of a color when the reduced copper is mixed with a special phosphomolybdic acid reagent. This is the basis of the determination of Folin and Wu (16). This method is simple, apparently accurate, and very widely used.

The methods dependent upon the color developed when picric acid is reduced to picramic acid by glucose, are the most widely used of all the methods. The picric acid methods are all colorimetric, most of them have a very simple technique, which may be easily mastered by the average technician, and are apparently accurate. Among these methods must be mentioned the determination of Lewis and Benedict (2),

with its modifications by S.R.Benedict (13), Myers and Bailey (14), and Pearce (63).

In the determinations based upon the action of glucose and some substance not mentioned in any of the preceding groups, attention must be called to the method of Hagedorn (17). Hagedorn uses an idometric titration, but employs potassium ferrocyanide in place of alkaline copper. Benedict and Osterberg (18) describe a method for sugar in urine, dependent upon the reaction between glucose and an unknown decomposition product of acetone and picric acid. They claim that this method is the most specific test yet used in the determination of glucose. We have adapted this method to the determination of sugar in blood, with very satisfactory results. Attention should also be called to the reaction between dinitrosalicylic acid and glucose. This reaction has been used to determine sugar in urine by Summer (19), and might also be applied to sugar in blood.

Review of Methods.

The Shaffer-Hartman method is probably the most popular titration method, because of its accuracy, and simplicity, for a titration method. All titration methods have too many chances for error, except in the hands of an experienced chemist, and laboratory technicians are too seldom experienced chemists. This objection precluded any extensive study of this method by us. The values by this method are essentially the same as those by the Folin-Wu method (16). The two most popular picric acid methods are the Myers-Bailey (14), and the Benedict (13),. Criticisms applicable to one of these methods will also apply to the other, for the most part. In this laboratory we have accumulated some experimental evidence

that will account for part of the difference between these two methods. The Folin-Wu method (16) is simple and very popular in clinical laboratories.

Our problem is to study the three most widely used clinical methods: the Folin-Wu (16), which gives normal blood sugar values from 80 to 100 mgs. of glucose per 100 ml. of blood; the Myers-Bailey (14), which gives normal values from 90 to 100 mgs; and the Benedict method (13), which gives normal values from 100 to 125 mgs. It has been our experience in working with the Benedict method that bloods with no known metabolic abnormalities give figures considerable higher than 125, the upper limit of normality. Our picric acid was purified by recrystallizing from benzene as suggested by Benedict (20), and satisfied his requirements of purity. The picrate mixture was titrated and found to be of the proper acidity, Benedict (21). These higher values for normal bloods have also been found by other workers. Thalhimer and Updegraff (22) state that in 24 cases with no known metabolic abnormalities the average of blood sugar values by the Benedict method was 141 mgs. per 100 ml. of blood.

In attempting to account for the differences in the results obtained by the above methods, the first possibility is that the differences are due to different reagents used in precipitating the blood proteins. The Myers-Bailey method and also the Benedict method precipitate the blood proteins with picric acid. This precipitation is very convenient, since the picric acid remaining in the filtrate is the reagent used in determining the sugar. The Folin-Wu method employs tungstic acid to precipitate the proteins. Part of

the difference in the results of these methods may be due to the possibility that picric acid does not properly precipitate the proteins, and the protein fraction left in the filtrate is responsible for the higher values obtained by the picric acid methods. The most satisfactory way to approach this question is to precipitate the blood proteins by different reagents, and then by using the same further procedure on all of the filtrates, to find the influence of the various protein fractions left in the filtrates, on the final sugar values. There are a large number of reagents which have been used to precipitate the blood proteins but it is possible to mention only a few of them here. Claude Bernard (24) used acetic acid and sodium sulphate; M. Abeles (25), alcoholic zinc acetate; Schenck (26), mercuric chloride and hydrochloric acid; E. W. Reid (27), phosphotungstic acid in hydrochloric acid; Lepine and Boulud (28), and Bierry and Portier (1), mercuric nitrate; Herzfeld (30), meta-phosphoric acid; and Michaelis and Rona (31), hundredth normal acetic acid and colloidal iron or kaolin. Aluminium cream has also been recommended for use with hundredth normal acetic acid, and gives results almost identical with those obtained by dyalized iron and kaolin.

A few of our results showing that differences in the final sugar values may be caused by different precipitating reagents are included in table 1. We have compared the original precipitation of the Folin-Wu method with an acetic acid precipitation and also the original precipitation of the Benedict method with an acetic acid precipitation. Acetic acid is a very poor protein precipitant. In applying the Benedict technique to filtrates other than the picrate - picric acid filtrate, we have used 4 ml. of the filtrate and treated in

the same way that Benedict treats his standard solutions. In applying the Folin-Wu technique to filtrates other than the tungstic acid filtrate, we have used 2 ml. of the filtrate and treated as Folin-Wu treat their standard. We chose the Benedict method because it is the latest and supposedly the most accurate picric acid method; criticisms of the Benedict method are also applicable to the Myers-Bailey method, for the most part.

TABLE 1.

Influence of Precipitant on Sugar Values.

Method of precipitation	Method of determination	Mg. of glucose per 100ml. of blood.		
		Blood #1 Fresh	Blood #2 48 hrs.	Blood #3 Fresh
N/100 acetic acid and heat.	Benedict	144	111	164
N/100 acetic acid heat, Al.cream.	Benedict			138
Picrate-picric acid.	Benedict	135	94	125
N/100 acetic acid and heat.	Folin-Wu	105	80	106
N/100 acetic acid heat, Al.cream.	Folin-Wu			98
Tungstic acid.	Folin-Wu	98	65	88

It can readily be seen that the method of precipitation affects the results in both methods. It seems probable then that the different methods of precipitation used might be responsbile for the differences between the two methods. We have inserted some figures taken from a table by Csonka and Taggart (32) who have worked on this particular phase of the question. These workers determined the sugar content of a

tungstic acid filtrate by the Folin-Wu and Benedict techniques and then repeated the work on picrate-picric acid filtrates. No difficulty of technique was experienced with the tungstic acid filtrate because it was colorless. For the determination 4 ml. of the filtrate was treated as Benedict treats his standard. In applying the Folin-Wu technique to the picrate-picric acid filtrate it was necessary after adding the molybdic acid reagent and allowing the blue color to develop to add from 1 to 1.2 ml. of picrate-picric acid to the Folin-Wu standard before dilution in order to counteract the color of the picrate-picric acid in the filtrate. Csonka and Taggart state that the color produced could be matched with ease. They found that the presence of picric acid did not influence the copper reduction.

TABLE 11.

Comparison of Benedict and Folin-Wu Techniques.

Tungstic acid Filtrate			Picrate-Picric Acid Filtrate	
Blood No.	Folin-Wu method	Benedict Technique	Folin-Wu Technique	Benedict Method
1-normal	73	86	72	120
2-normal	83	82	81	117
3-normal	87	100	86	121
4-normal	88	91	87	118
5-normal	89	94	87	120
6-normal	87	104	81	130
7-normal	91	106	89	122
8-diabetic	97	110	96	150
9-diabetic	200	230	200	263

It is obvious from an inspection of the table that when the Folin-Wu technique is used, tungstic acid filtrates and

picrate-picric acid filtrates yield the same results (columns 1 & 3). It is generally conceded that tungstic acid is a better precipitating reagent for blood proteins than picric acid. The non-carbohydrate fraction not precipitated by the picric acid does not affect the reduction of the alkaline copper solution employed in the Folin-Wu technique. It can also be seen from an inspection of the table of columns 2 & 4, that when the Benedict technique is employed, the tungstic acid filtrates are distinctly lower than the picrate-picric acid filtrates. This means that the non-carbohydrate fraction not precipitated by the picrate-picric acid does interfere with the Benedict technique. Tungstic acid does not precipitate some of the sugar because added sugar is recovered. It may also be observed that when the tungstic acid filtrate is employed with the Folin-Wu and Benedict techniques (columns 1 & 2) that the Benedict technique gives higher results than the Folin-Wu technique. This seems to indicate that even the tungstic acid fails to precipitate all of the non-carbohydrate substances which interfere with the picric acid method.

It has been suggested that probably the higher values obtained by the picric acid method are due to the fact that picric acid not only gives the free but also the combined glucose. Scott (43) believes that some of the blood sugar is combined with lecithin, and Kleiner (44) believes that in diabetes some of the sugar is in a combined state. McGuigan and Ross (41), Van Hess and McGuigan (42), and Macleod (40) present evidence showing that blood sugar exists in the uncombined state and indeed the best authorities hold this opinion. We decided to compare the results obtained by the Folin-Wu method, The Benedict method, and the Benedict technique applied to a

tungstic acid filtrate and also a mercuric nitrate filtrate. Mercuric nitrate precipitates all of the polyphenols and organic nitrogen thus giving a filtrate free from practically all of the interfering substances. If the Benedict technique applied to the mercuric nitrate filtrate gives higher results than the Folin-Wu method then the difference must be due to some source, other than interfering substances.

TABLE 111.

Error in Benedict Method Due to Interfering Substances.

Method of Precipitation	Technique	Mg. of Glucose per 100 ml. of Blood						
		#1.	#2.	#3.	#4.	#5.	#6.	#7.
		Age hrs. 18	72	18	18	0	0	0
Picrate-Picric Acid	Benedict	121	83	132	123	119	234	119
Tungstic Acid	Benedict		72	107	111	103	229	117
Mercuric Nitrate	Benedict	94	55	83	95	89	210	113
Tungstic Acid	Folin-Wu	76	48	85	84	88	109	87

We see that as the nitrogen fraction decreases, the sugar values also decrease but on a filtrate free from nitrogen and polyphenols, the sugar values by the Benedict technique are higher than those obtained by the Folin-Wu method. Evidently some factor or factors other than the interfering substances is responsible for part of the difference between the Benedict and Folin-Wu methods. We have discovered in our work that one of these is due to the use of impure picric acid. It is practically impossible to work with pure picric acid because of the rapidity and readiness of the decomposition of picric acid. These decomposition products of picric acid give an intense color with sodium carbonate or sodium hydroxide. The color is identical with that obtained by the action of glucose

on alkakline picric acid and consequently causes sugar values to be much too high. We purified our picric acid by recrystallizing from benzene according to Benedict (20) and met his requirements for purity. His test for purity was as follows; take two 10 ml. portions of a saturated picric acid solution, and to one portion add ½ ml. of water and to the other ½ ml. of ten per cent sodium hydroxide, allow to stand for ten minutes and then compare in a colorimeter. Set the picric acid plus water at 20 mm., and the picric acid plus sodium hydroxide should read from 11 to 14 mm. We have included some of our data showing that picric acid deteriorates when dry, damp, or in solution and whether in the light or dark. A sample of picric acid was purified according to Benedict (20). A saturated solution was made and tested with the following results:

TABLE IV.

	Colorimeter reading
10 ml.of saturated picric acid plus ½ ml.of HOH	20
10 ml.of saturated picric acid plus ½ ml. of 10% NaOH	13

The solution was divided into two portions, one portion was kept in the dark and one exposed to the laboratory light. After nineteen months the two portions were tested.

Saturated picric acid solution kept in the dark.

10 ml.of saturated picric acid plus ½ ml.of HOH	20
10 ml.of saturated picric acid plus ½ ml.of 10% NaOH	10.5

Saturated picric acid solution kept in laboratory light.

10 ml.of picric acid saturated sol.plus ½ ml.of HOH	35
10 ml.of picric acid saturated sol.plus ½ ml.of 10% NaOH	0.9

Another sample of picric acid was purified and the experiment repeated on a little larger scale.

10 ml.of saturated picric acid plus ½ ml.of HOH	20
10 ml.of saturated picric acid plus ½ ml.of 10% NaOH	11

The solution was divided into two portions, one portion was placed in the dark, the other exposed to laboratory light. After ten days the solutions were tested.

The portion remaining in the dark.

Colorimeter reading

10 ml.of saturated picric acid plus ½ ml. of HOH -------20

10 ml.of saturated picric acid plus ½ ml. of 10% NaOH---10.5

Portion exposed to laboratory light.

10 ml.of saturated picric acid plus ½ ml. of HOH -------20

lo ml.of staurated picric acid plus ½ ml. of 10% NaOH--- 7.2

Sixteen days later the solutions were again read.

Portion remaining in dark.

10 ml.of saturated picric acid plus ½ ml. of HOH---------20

10 ml.of saturated picric acid plus ½ ml. of 10% NaOH---- 9.2

Portion remaining in light.

10 ml.of saturated picric acid plus ½ ml. of HOH---------20

10 ml. of saturated picric acid plus ½ ml. of 10% NaOH---6

Four portions of the second sample of purified picric acid were taken on the same day that it was purified. Two portions remained dry and one was placed inthe dark and the other in the light; two portions were dampened with 20% water (this percentage of water is required of all picric acid shipped by rail) and one placed in the dark and the other in laboratory light. Twenty-six days later the purity of each sample was tested.

10 ml.of sat.sol.of dry, dark, picric acid plus ½ ml.HOH------20

10 ml.of sat.sol.of dry, dark, picric acid plus ½ ml.10%NaOH--9.8

10 ml.of sat.sol.of dry, light, picric acid plus ½ ml.10% NaOH----8.2

10 ml.of sat.sol.of wet,dark, picric acid plus ½ ml.10% NaOH--8

10 ml.of sat.sol. of wet,light picric acid plus ½ ml.10% NaOH-7

Picrate-picric acid solutions deteriorate even more rapidly than picric acid solutions. In most laboratories a sample of

picric acid is purified and used for the next six months or more. Our data show that this introduces a serious error. We are continuting this work on the decomposition of picric acid. There are other errors due to the fact that the colors do not develop at the same rate in the standard and unknown, and the fact that the final color is a mixture of the colors of picric acid and picramic acid. We do not know the proportion of the mixture and so we can not expect an accurate sugar value.

Literature on the Picric Acid Methods.

There is an abundant literature criticising the picric acid methods on the ground that there are too many substances which produce color with picric acid, thereby interfering with the picric acid sugar determination. Only a few of these papers can be mentioned. We have included a table, taken from the work of Morgulis and Jahr (33), showing the error due to creatinine in Benedict's latest modification (13).

TABLE V.

5 mg. of creatinine per 100 ml.of Blood produce an error of 6.6.mg. of glucose.

10 mg.of creatinine per 100 ml.of Blood produce an error of 20 mg. of glucose.

20 mg.of creatinine per 100 ml.of Blood produce an error of 70 mg. of glucose.

30 mg.of creatinine per 100 ml.of Blood produce an error of 94 mg. of glucose.

Cowie and Parsons (34) found the Benedict method very useful but after several adverse criticisms had been made of it, they started an investigation. They found that the picrate-picric acid solution was:

4 times as sensitive to creatinine as to glucose.
50 times as sensitive to diacetic acid as to glucose.
500 times as sensitive to acetone as to glucose.
1000 times as sensitive to adrenalin as to glucose.

They state that the picrate-picric acid reacts with much smaller quantities of acetone than are normally present in the blood and that adrenalin may also cause an error. They found that urea in large quantities would react with picrate-picric acid solutions. This will not produce an error in blood sugar determinations but it does increase the data showing that picrate-picric acid reacts with a large number of substances, other than glucose, found in the blood.

From a study of some figures comparing different blood sugar methods we find much evidence supporting the contention that there are a large number of substances which interfere with the picric acid methods. Many of these figures were taken from work not intended to show the errors of the picric acid methods. Host and Hatlehol (45) compare the alkaline copper method of Bang (6), the potassium ferricyanide method of Hagedorn (17), the old Folin-Wu method (15) and the Myers-Bailey picric acid method (14). Part of this table is included below. (See next page).

TABLE Vl.

Comparison of Popular Blood Sugar Methods.

METHOD	MG. of Glucose per 100 ml. of Blood.												
	non-diabetic							diabetic					
	Blood No.1	2	3	4	5	6	7	8	9	10	11	12	13
Bang	100	97	84	84	91	70	97	214	172	187	101	101	321
Hagedorn	100	91	94	77	97	80	82	181	181	191	103	121	lost
Myers-Bailey	102	118	90	98	118	89	97	208	208	222	150	150	351
Folin-Wu	95	105	102	89	105	91	93	176	176	182	121	121	333

The two titration methods seem to agree very closely. The Folin-Wu method is slightly higher than the titration methods but this work was done with the old Folin-Wu method which has since been modified, to eliminate certain interfering substances, and in its present form gives results which would parallel the titration methods. The Myers-Bailey picric acid method gives distinctly the highest results of the four. It may also be observed that whereas the difference between the titration and picric acid methods is about 12 for normal bloods it is about 29 for diabetic bloods. How may this increase in the difference between the methods be accounted for? It has already been shown that acetone and diacetic acid cause a disturbing error in the picric acid methods. The interfering substances which increase in the blood in diabetes are evidently responsible for the great increase in the difference between the methods. Our conclusion is that blood sugar values in diabetes, by the picric acid methods, are incorrect due to acetone and diacetic acid and the more advanced the case of diabetes the greater the error.

We call attention again to the tables of Csonka and Taggart (32) found on page 10 of this article. The Folin-Wu

method gives results averaging 36 mg. per 100 ml. lower than Benedict's method for normal bloods and 58 lower for diabetic bloods. These results indicate that the picrate-picric acid method is even more sensitive to the interfering substances found in diabetes than the Myers-Bailey method, where the concentration of the picric acid radical is less.

Thalhimer and Updegraff (22) working with the latest Folin-Wu modification (16), the latest Benedict modification (13) and the Myers-Bailey method (14) give a comparison of the methods. Some of their figures are given below.

TABLE V11.

MG. of Glucose per 100 ml. of Blood.

METHOD	non-diabetic								diabetic		
	Blood No.1	2	3	4	5	6	7	8	9	10	11
Benedict	125	121	136	125	115	115	125	117	200	316	263
Myers-Bailey	114	110	115	115	107	103	115	100	183	273	234
Folin-Wu	100	100	100	107	97	97	94	80	154	261	219

The Folin-Wu method gives results averaging about 15 mg. per 100 ml. lower than the Myers-Bailey method for diabetic and non-diabetic blood but the data on the diabetic blood is too limited. A more extensive data would doubtless reveal the same results as found by Host and Hatlehol (45). The Folin-Wu method averages about 30 mg. lower than the Benedict method for normal bloods but about 50 mg. lower for diabetic bloods. The conclusion might be drawn that the Myers-Bailey method is not so seriously affected by the interfering substances which accumulate in diabetes as the Benedict method. Work has been done proving that the Myers-Bailey method is not as sensitive to interfering substances

as the Benedict method because the concentration of the picric acid radical is less. Thalhimer and Updegraff find the blood sugar values by the Benedict modification (13) at a distinctly higher level than the original Lewis-Benedict method (2). Their conclusion is that as the concentration of the picric acid radical is increased in a blood filtrate there is an increased sensitiveness to substances other than glucose. Wesselow (38) and Myers (39) agree with Thalhimer and Updegraff that this is true.

Myers- Bailey (39) recognize the fact that their method is affected by certain substances which accumulate in nephritis. We include a table taken from their work showing the effect of creatinine.

TABLE Vlll.

5 mg.of creatinine per 100 ml.of Blood	cause an error in blood sugar of 7 mg.
10 mg.of creatinine per 100 ml.of Blood	cause an error in blood sugar of 15 mg.
20 mg.of creatinine per 100 ml.of Blood	cause an error in blood sugar of 25 mg.

The Myers-Bailey method is also open to other serious criticism, which may account for most of the difference between the values obtained by it and the Benedict method. In the Myers-Bailey method the blood is laked with water and then sufficient solid picric acid is added to the hemolyzed blood to precipitate the proteins and saturate the filtrate with picric acid. The directions state that the mixture should be stirred for several minutes until it is thoroughly yellow, then centrifuged and filtered. Picric acid dissolves very slowly in cold water and it is our belief as well as the belief of others that the filtrates are not saturated with

picric acid in many determinations. The standard contains glucose in a saturated solution of picric acid and if the standard contains more picric acid than the unknown it will develop a disproportionate amount of color, causing the unknown to give a low value. Myers and Bailey state that their standard which is made up in a saturated picric acid solution keeps permanently. So far as the deteridation of the glucose is concerned it doubtless is permanent, but it is a known fact from our data, previously presented, and also from other workers, that picric acid solutions deteriorate rapidly, especially when not kept in a brown bottle in the dark; and Myers and Bailey make no mention of this precaution. If the color of the standard is due both to the color developed by the glucose and also to the decompostion products of picric acid, it will be much stronger than it should be, and the unknowns consequently give too low values. We maintain then that in view of these observations, the Myers-Bailey method is subject to greater error than the Benedict method in many instances.

Myers and Killian (46) using the Myers-Bailey method report 23 cases of sugar in nephritis and find sugar values ranging from 110 to 320 with an average of 157. They conclude that high blood sugar values accompany high urea values. This is evidently true, judged by the picric acid methods, but it is a question as to whether or not it is true to anything like the same degree by other methods less sensitive to interfering substances.

We are including a paragraph taken from the work of Williams and Humphreys (47) who are working with the Myers-Bailey method. "In the early stages of nephritis when the

general metabolism of the body is but little disturbed, blood sugar values as a rule are normal. In the last stages of nephritis when the patient is uremia, blood sugar values are very high, often equalling the severe stages of diabetes. Cardio-vascular cases characterized by high blood pressure and little or no evidence of renal disturbance usually exhibit blood sugar values slightly above normal. In severe cases of nephritis patients may excrete small quantities of sugar in the urine, frequently giving rise to the misapprehension that true diabetes exists. In such cases the blood sugar level is inappreciably influenced by carbohydrate restriction and the patient should not be subjected to the discomforts of a rigorous diabetic diet." High blood sugar values in nephritis are to be questioned when the picric acid methods are used. High values in cardio-vascular cases are questionable in view of the great sensitivity of picric acid solutions to adrenalin as shown by Cowie and Parsons (34). The fact that diabetic diets did not affect the sugar in the urine might also open the question of the accuracy of the sugar-in-urine determination. Williams and Humphreys use the Benedict copper sulphate method (58), which is not specific for sugar in urine. They should have used the quantitative acetone method of Benedict (18).

Bailey (49) using the Myers-Bailey method states that the blood sugar level at which glycuresis occurs is 125 mg. of glucose per 100 ml. of blood in early mild diabetes, 290 in nephritis, and 300 in cases of diabetes with renal involvement. In view of the interfering substances in the picric acid methods these values are to be seriously questioned.

Addis and Shevky (35) working with the Lewis-Benedict method (2) found that when dextrose and picrate are heated in the presence of an alkali, the rate of color production is different for each concentration and does not change in direct proportion to the change in dextrose concentration. This means that the method is accurate only when the unknown contains the same amount of glucose as the standard and this is of course impractical. Added sugar would be quantitatively recovered because a special standard would be prepared to match the color produced by the added sugar. Benedict also recognized this difficulty and supposedly overcame it in his latest method (13), but this may be seriously questioned.

Folin and Wu (15) working with Benedict's latest modification (13) and the Myers-Bailey modification (14) state that the rate of color development in blood filtrates by the picrate methods seems not to proceed at the same rate of speed as the color derived from a corresponding amount of glucose. If the heating is interrupted at the end of two or three minutes the blood sugar values will be hearly 50% higher than when heating is continued for ten minutes or more. If this is true at the beginning of the determination, it is doubtless true to some extent at the end of determination. Such quantitative variations are not encountered in the Folin-Wu method.

Falk and Nayes (36) show that picric acid sugar methods involve a mixture of two colors, the color of the picric acid and of the picramic acid formed. They show that picrate-sugar determinations are accurate only when the standard and unknown are very nearly alike in composition and concentration

thereby giving the same proportionate mixture of colors.

Rohde and Sweeney (37) purified a sample of picric acid and divided it into two parts. One part they placed in a brown bottle in the dark. The other part they dampened and placed in a clear bottle in the laboratory light. Ten months from this time picrate-picric acid solutions were made from both samples. They found that the ~~damp~~ damp sample in the colorless bottle failed to properly precipitate the blood proteins and in addition gave a highly colored filtrate. It was also found that the acidity of the sample had decreased. The dry sample in the dark bottle in the dark apparently hadn't deteriorated appreciably. Benedict (21) thinks that the failure to properly precipitate the proteins was due to the low acidity of the picrate-picric acid and suggests titrating the picrate-picric acid to be sure that its acidity is between 0.05 N. and 0.04 N. If the acidity is too low he suggests building up the acidity with acetic acid. He does not explain the highly colored filtrate. Work done by us has corroborated the work of Rohde and Sweeney. Errors in creatinine determinations due to impure picric acid have been called attention to by Hunter and Campbell (64), and McCrudden and Sargent (65). We are of the opinion that the picric acid sugar determinations are open to almost as serious criticisms.

Wallis and Gallagher (50) have worked extensively with the Lewis-Benedict method and its modifications, the MacLean method, the Bang method, and the Folin-Wu method, and prefer the Folin-Wu method to any.

Investigation with The Folin-Wu Method.

In view of the work done on the picric acid methods and of the fact that no titration method is adapted to clinical use, we decided to investigate the Folin-Wu method with the view of determining its absolute accuracy.

This method has the advantage of using tungstic acid as the protein precipitant but even in the tungstic acid filtrate there are substances other than glucose which will reduce the alkaline copper solution of Folin and Wu.

Errors in The Folin-Wu Procedure.

We have found that the reagent is reduced by uric acid, creatinine, and adrenalin. There are probably a large number of other substances which affect the reagent but these illustrate our point sufficiently, namely that the method is not specific for glucose. We have included some of our tables showing the error produced by the above named substances.

TABLE IX.

Blood No.	MG. of Uric Acid Added per 100 ml. of Blood	MG. of Glucose per 100 ml. of Blood	Error Due to Uric Acid.
1	0	97	
1	10	99	2
1	20	100	3
2	0	93	
2	10	96	3

Large quantities of uric acid will cause a marked reduction of the alkaline-copper solution. In this work we dissolved the uric acid in a phosphate solution such as recommended for uric acid determinations by Benedict (51). Insufficient phosphate was introduced to affect the reaction. Care must be taken to use a fresh uric acid standard and not one which

has been preserved by chloroform. The decompostion products of chloroform will introduce an error many times greater than that due to uric acid.

TABLE X.

Error Due to Creatinine.

Blood No.	MG.of Creatinine added per 100 ml.of Blood	MG.of Glucose per 100 ml.of Blood	Error Due to Creatinine
1	0	93	
1	10	97	4
11	0	107	
11	100	133	26

The error due to creatinine is negligible for the quantities found in normal blood, but it is quite probable that the creatinine found in the blood is not identical with the substance we call creatinine, though evidently very closely related to it. This work on the creatinine of blood has been done principally by Behre and Benedict (52) and Greenwald and McGuire (59). The form of creatinine found in the blood is probably a more active reducing agent. Creatinine either does not occur in the same form in the blood in which we know it or there is something associated with it which causes an error because we have found that in bloods with a very high non-protein-nitrogen that the error in the Folin-Wu sugar method was increased. It is significant that uric acid and creatinine do produce an error, even though it is small.

We have found the alkaline copper solution very sensitive to adrenalin, 0.008 mg.of adrenalin will produce a distinct error. It is quite possible that the alkaline copper solution is sensitive to the other internal secretions.

Folin-Wu Technique Applied to Nitrogen Free Filtrates.

It is significant that all of the above mentioned substances, which produce an error, are nitrogenous compounds.

The formulas for only two of the internal secretions are known (adrenalin and thyroxin) but it is possible that all of the other hormones are also nitrogenous compounds. We decided to work with nitrogen free filtrates and thus avoid any error due to interfering nitrogenous substances. We precipitated the blood proteins with mercuric nitrate, which not only eliminates all of the nitrogenous compounds but also the polyphenols. Johnson (57) was the first to use mercuric salts preliminary to sugar determinations. In 1887 he used mercuric chloride to remove creatinine previous to determining sugar in urine. We employed at first the mercuric nitrate solution of Benedict (13). This solution was made as follows: To 160 ml. of concentrated nitric acid in a beaker, add in small portions 220 gms. of mercuric oxide. When all has dissolved, heat the mixture to boiling, cool, and add 60 ml. of 5% sodium hydroxide, make up to 1 liter and filter. Keep in a brown bottle. The solution probably originated with Patein and Defau (53). When 5 ml. blood is diluted 1 to 6 or 1 to 10, 2 ml. of this solution will ordinarily give a nitrogen free filtrate. We removed the excess mercury with sodium bicarbonate as recommended by Benedict instead of with sodium hydroxide employed by Patein and Defau (53) and Schondorff (29). The last trace of mercury could not be removed by zinc as recommended by Benedict (13) and also by Deniges (56) because of the fact that zinc formed nitrites, which when made acid with the Folin-Wu molybdate solution, liberated nitrous acid and thus destroyed the blue color

of the final blue solution. We attempted to employ iron in place of zinc to remove the last trace of mercury but this caused the same trouble as zinc. Copper was thought of but was not used because of the fact that it would upset the delicate copper balance in the Folin-Wu alkaline copper reagent. We attempted to remove the mercury by electrolysis but this also caused a fading of the final blue color. The fact that zinc liberates nitrites was also observed by Hartman-Shaffer (10) in their method for sugar in urine. Schondorff (29) also observed that zinc interfered with the Pflugers copper method for sugar. Schondorff in his urine determination removed the last trace of mercury with hydrogen sulphide and then removed the excess hydrogen sulphide with an air current. We found that this procedure caused a slight fading of the final blue color in the Folin-Wu method. We attempted to remove the excess hydrogen sulphide with hydrogen but this apparently did not decrease the fading. Hartman and Shaffer in their urine method removed the last trace of mercury with sodium sulphide and then removed the excess sulphide with copper sulphate. This procedure does not interfere with the titration method of Hartman and Shaffer but in the Folin-Wu method the alkaline copper solution contains a definite amount of copper and any excess copper would interfere with the determination. We attempted to use sodium sulphide to remove the mercury and then remove the sodium sulphide by hydrochloric acid and an air current. We made the filtrate from the sodium bicarbonate precipitation acid with hydrochloric acid and then added sodium sulphide, again made the solution acid with hydrochloric acid and removed the hydrogen sulphide with a current of hydrogen. This procedure also

caused fading. We repeated the work adding sodium sulphide so carefully that no excess sulphide remained in the solution after precipitating all of the mercury but the final blue color of the Folin-Wu method continued to fade.

It may be observed that in making the mercuric nitrate solution employed by Benedict, that there is a large excess of sodium nitrate present. Since we believe that the nitrates under certain conditions liberate nitrites, it is desirable to reduce the nitrates present to a minimum. In order to do this we modified the solution employed by Benedict. The mercuric nitrate solution employed by Benedict contains 347.9 gms. of mercuric nitrate in one liter of solution. In our modification we put 347.9 gms. of solid mercuric nitrate in a liter flask, added about 900 ml. of water, shook thoroughly for about fifteen minutes and then added nitric acid a few ml. at a time and finally drop by drop until the mercuric nitrate was dissolved and then the solution was diluted to one liter. The solution contained no excess nitrate and gave nitrogen free blood filtrates. We removed the excess mercury with hydrogen sulphide and the excess hydrogen sulphide with a current of hydrogen. We found that if the filtrate from the sodium bicarbonate precipitation was made acid to tropaedin 00, before adding the hydrogen sulphide that there was no fading of the final blue color of the Folin-Wu method.

Mercuric Nitrate Method.

Lake one volume of blood with eight volumes of water in a small erlenmeyer flask. To the completely hemolyzed blood add slowly while rotating the hemolyzed blood one volume of the mercuric nitrate solution. The blood proteins are pre-

cipitated in a jellylike mass. The flask in which the precipitation was made is then thoroughly stoppered and shaken vigorously for two minutes and the shaking continued, though not so vigorously, for about eight minutes. The jellylike precipitate breaks up almost immediately. This prolonged shaking is probably a little excessive but it precludes the possibility of any sugar being enclosed in the precipitate and thus lost. The precipitated proteins are then filtered off and the filtrate never fails to be water clear and colorless.

Sodium bicarbonate is added to the filtrate until it is slightly alkaline to litmus thus precipitating most of the mercury in the filtrate. Care must be exercised to avoid adding an appreciable excess of sodium bicarbonate. The precipitated mercury compound is then completely filtered off by means of a double filter of fine paper. Difficulty will often be experienced unless this precaution is heeded. This procedure precipitates practically all of the mercury from a pure glucose solution but does not precipitate it nearly so completely in a blood filtrate. Potassium bisulphate which has been thoroughly dried and powdered is added to the filtrate, from the bicarbonate precipitation, until the filtrate gives a distinctly red solor with tropaeolin OO. The solution is then put in test tube and heated in a water bath until its temperature is between 50° and 60° C. It is then removed from the water bath and hydrogen sulphide is passed in rapidly. The complete precipitation of the mercury is very rapid. The mercuric sulphide is filtered off on a rapid filter and the excess hydrogen sulphide removed with a fast current of hydrogen.

The hydrogen is washed with water, alkaline potassium permanganate solution, and then water again. The hydrogen sulphide is usually removed from the warm filtrate in about thirty seconds but a few drops of the filtrate must be tested to be sure that all of the hydrogen sulphide has been removed. We used a silver nitrate solution for this test. The blood filtrate is then cooled and 2 ml. taken for the determination and the regular Folin-Wu technique followed. In preparing the standard it is necessary to add potassium bisulphate to the standard until its pH is the same as the pH of the mercuric nitrate filtrate.

Discussion of the Mercuric Nitrate Method.

The dilution of the blood in the mercuric nitrate precipitation is one to ten, the same as in the tungstic acid precipitation of Folin and Wu. It was our idea to follow as closely as possible the Folin-Wu technique. The final blue color does not fade in fifteen minutes. Longer standing will produce a slight fading but this is true of practically all colorimetric methods.

In the early part of the work the filtrate from the bicarbonate precipitation was acidified with hydrochloric acid before precipitating the excess mercury with hydrogen sulphide. Potassium bisulphate has two advantages over hydrochloric acid; it is a solid and does not affect the volume appreciably and it also gives us the sulphate ion in the filtrate, thus more nearly paralleling the Folin-Wu method which has the sulphate ion in the filtrate. We found, however, that the sulphate ion does not affect the reduction or the final color.

We found before our technique was perfected that if the hydrogen sulphide precipitation was made in a warm filtrate instead of a cold that the fading of the final blue color was much reduced. Later when we found that the fading could be controlled by the acidity, we continued to heat the filtrate because the mercury precipitation was much more rapid and also very little hydrogen sulphide was dissolved by the filtrate and this small amount was easily dispelled from the warm filtrate. Bierry and Portier (1) removed the hydrogen sulphide from their filtrate by boiling but this procedure was not practicable in our method.

The Folin-Wu filtrate has a definite acidity. The pH of the Folin-Wu precipitation is discussed by Merrill (54). The alkaline copper solution is adjusted for the acidity of the Folin-Wu filtrate and any change in the acidity of the filtrate will produce a change in the reduction of the alkaline copper. It was necessary to have the acidity of the Folin-Wu filtrate the same as the acidity of the mercuric nitrate filtrate if we were to have an accurate comparison between the two methods. The acidity of the filtrates must be such that when a drop of the filtrate is added to a drop of tropaeolin 00 a distinctly red color is meediately produced. The acidity at this point is about 0.05 normal. We should call attention to the fact that the acidity of the mercuric nitrate filtrate can not be materially reduced without causing a slight fading of the final blue color. Folin-Wu state that when 2 ml. of alkaline copper solution is titrated, 1.4 ml. of 0.1 normal acid is required to neutralize it. Our filtrate is 0.05 normal and we use 2 ml., so the alkalinity of the copper solution is not materially affected, but is affected enough to cause the pH

of the tungstic acid filtrate to have to be adjusted until it is the same as that of the mercuric nitrate filtrate in order to get an accurate comparison. The red color of the tropaeolin 00 used by us in adjusting the acidity of the mercuric nitrate filtrate is exactly matched in adjusting the acidity of the tungstic acid filtrate and of the sugar standard. We are of the opinion that the pH of the solutions varies only slightly if at all. At any rate it would not vary enough to cause any error because the alkaline reagent is not sensitive to the maximum variation in pH possible under the conditions. The tungstic acid filtrate is slightly acid, the sugar standard of Folin -Wu is neutral, consequently if the alkaline copper solution were so very sensitive to small variations in pH, Folin and Wu would have suggested making their standard slightly acid before proceeding with their determination. We have also found that the alkaline copper solution is not sensitive to very slight changes in the pH of the filtrates. The pH of the tungstic acid filtrates undoubtedly varies much more than our adjusted acidities.

Attention must be called to the fact that the precipitation of the residual mercury must be made in two steps; 1- by adding sodium bicarbonate and filtering, 2- by adding hydrogen sulphide to the filtrate. There is apparently a loose combination between some nitrogenous compounds and the mercuric oxide. When the residual mercury is precipitated by bicarbonate the nitrogenous compounds are also precipitated. When the bicarbonate precipitation is omitted and hydrogen sulphide used to remove all of the mercury at one time, all the nitrogenous compounds are not precipitated and consequently the filtrates are not nitrogen free. The small amount of mercury remaining

after the bicarbonate precipitation apparently is not in combination with any nitrogenous substances. An attempt was made to clear the tungstic acid filtrates of nitrogen by mercuric nitrate, but this was impracticable due to the formation of a mercuric tungstate, which interfered. Below will be found a table giving the efficiency of our mercuric nitrate solution in precipitating nitrogenous compounds. We made a great many precipitations with the mercuric nitrate solution employed by Benedict and found that it readily gave nitrogen free filtrates. These results are not included in our table but since our mercuric nitrate solution is practically as efficient as the solution of Benedict it was necessary for us to test only a few of our filtrates to determine that they were invariably nitrogen free.

TABLE X1.

Nitrogen Free Filtrates after Mercuric Nitrate Precipitation.

Blood No.	Dil.of Filtrate	No. of ml. Digested	Mg.of N.P.N. After Tungstic Acid Precipitation	Mg.of N.P.N. After $HgNO_3$ Precipitation.
1	1-10	10	119	0
2	1-10	10	31	0
3	1-10	10	31	0
4	1-10	10	36	0
5	1-10	10	36	0
6	1-10	10	36	0

Nesslers solution was used in estimating the nitrogen. Smaller quantities of mercuric nitrate will clear normal blood of nitrogen but the quantities used by us in our determination will clear practically any blood of nitrogen. We were not able to try our solution on any blood with an N.P.N. higher than 119 but while using the Benedict solution we were fortunate in

obtaining a blood with an N.P.N. of 281 and found that one volume of mercuric nitrate per one volume of blood yielded nitrogen free filtrates, and our solution is practically as efficient as the Benedict solution. We applied our determination to a muscle extract containing 130 mg. of creatinine per 100 ml. and 60 mg. of N.P.N. per 100 ml. and found that using the same proportions of mercuric nitrate that we employed in our blood determinations, volume per volume, nitrogen free filtrates were obtained.

We found that no sugar was lost in our mercuric nitrate precipitation. 10 ml. of a sugar standard containing 0.4 mg. of glucose in 2 ml. was diluted with 8 ml. of water and 2 ml. of mercuric nitrate solution was added. The portion of mercuric nitrate added was the same as employed in our blood precipitation. The mercury was removed in the usual way and the sugar content of the solution determined and no sugar was lost in the procedure. A blank determination was made employing 18 ml. of water and 2 ml. of mercuric nitrate and it was found that the treatment yielded a blank. The treatment then does not cause any increase in the final sugar value.

TABLE XII.

Recovery of Glucose Added to the Blood using the Mercuric Nitrate Precipitation.

Blood No.	Kind	Mg. of Glucose added per 100 ml. of Blood	Mg. of Glucose found per 100 ml. of Blood	Glucose Recovered.
1	Phlor.* dog	0	38	
1	"	96	132	94 of 96
1	"	144	180	142 of 144
2	Human#	0	48	
2	" #	80	133	85 of 80
3	"	0	84	
3	"	100	186	102 of 100
4	"	0	79	
4	"	100	184	105 of 100
5	"	0	70	
5	"	250	313	243 of 250
6	"	0	113	
6	"	400	492	379 of 400
7	"	0	154	
7	"	100	253	99 of 100
8	"	0	87	
8	"	96	182	95 of 96
9	Phlor.* dog	0	36	
9	"	98	132	96 of 98

*Phlorhizinized.
#48 hours old.

It might be said that some of the sugar is in the form of an amino sugar and would thus be precipitated. We have mentioned before that the best evidence is in favor of the opinion that blood sugar exists as glucose in the uncombined form. Denis and Giles (60) present evidence that the blood sugar in diabetes

tends to be in the form of gamma glucose. This fact is disputed by Tolstoi (61) and others. Whatever may be the true solution, gamma glucose would not be precipitated by mercuric nitrate and our method would not decrease in accuracy in diabetic bloods.

Blood contains small amounts of fructose, aldehydes, ketones, and glycuronic acid, all of which are reducing agents and might cause a small error. With our present knowledge it is impossible to eliminate these but it is quite probable that the error produced by them is very small if accountable at all.

In comparing the Folin-Wu method with our method it was unnecessary to adjust the pH of the standard and so this step was omitted. It was necessary, however, to be sure that the pH of the Folin-Wu filtrate was the same as the pH of our filtrate. Before the method was perfected we had a blood with an N.P.N. of 281, Folin-Wu sugar 158 and mercuric nitrate sugar 126, per cent difference between the two sugar methods, 20.3. We also had another blood uric acid 18, creatinine 35, Folin-Wu sugar 101, mercuric nitrate sugar 71, per cent difference 30.6. These differences were nearly twice as great as we had been finding and seemed to indicate that the Folin-Wu method was less accurate for bloods with very high N.P.N. than it was for normal bloods. The N.P.Ns. for the bloods found in table XIII ranged from 119 to 30 . For this range of N.P.Ns. there was no consistent increase in the per cent difference between the Folin-Wu and the mercuric nitrate method.

TABLE XIII.

Comparison of Folin-Wu and Mercuric Nitrate Methods.

Blood No.	Age	Kind	Mg. of Glucose per 100 ml. of Blood.		Per Cent Difference
			Folin-Wu	Mercuric Nitrate	
1	Fresh	Human	100	86	14
2	"	"	113	99	12.3
3	"	"	93	77	17.2
4	"	"	87	71	18.3
5	"	"	68	59	13.2
6	"	"	121	107	11.5
7	"	Phlor. dog	74	60	18.9
8	"	Human	101	86	14.8
9	24 hrs.	"	67	48	28.3
10	Fresh	Phlor. dog	73	48	34.2
11	"	Human	98	84	14.2
12	"	"	120	106	11.6
13	"	"	115	101	12.1

It will be observed that the Folin-Wu method gives results that are from 12 to 30 per cent higher than our method which is more specific because of the elimination of most and possibly all of the interfering substances.

In attempting to verify the results obtained with our mercuric nitrate precipitation we have adapted the acetone method of Benedict (18) for determining sugar in urine, to a determination of sugar in blood. We selected this reaction because Benedict claims that it is the most specific reaction known for glucose. So far as we have been able to find, this method has never been adapted to blood sugar determinations. We attempted to apply this method to a tungstic acid filtrate and also to a mercuric nitrate filtrate but found that results

much too high were obtained in both cases. The results are to be found in the following table.

TABLE XlV.

Comparison of Folin-Wu method and Acetone Method on Tungstic Acid and Mercuric Nitrate Blood Filtrates.

Blood No.	Method of Precipitation	Mg. of Glucose per 100 ml. of Blood	
		Folin-Wu Technique	Acetone Technique
1	Tungstic acid	74	81
1	Mercuric Nitrate	0*	107
2	Tungstic Acid	85	102
2	Mercuric Nitrate	0*	99

*No determination made.

A sugar standard was treated with the mercuric nitrate solution and the acetone method employed and much more sugar was recovered, than was present in the original standard. Salts found in the tungstic acid filtrate and also in the mercuric nitrate filtrate evidently interfere with the reaction. These methods of precipitation had the disadvantage of being too dilute to be employed accurately in the acetone method. We decided to precipitate the blood in such a way as to eliminate the salts in the filtrate and also to avoid too great a dilution. We precipitated the blood proteins with N/100 acetic acid, heat and kaolin.

Acetone Method for Determining Sugar in Blood.

Our procedure was as follows: Thoroughly lake one volume of blood with three volumes of N/100 of acetic acid and heat slowly to boiling. This precipitation is best carried out in a very small Kjeldahl flask, with a long neck. A wet cloth is wrapped around the neck of the flask acting as a condenser for any water vapor which might otherwise

escape and thus introduce an error. After heating to boiling the flask is stoppered, cooled in cold water to prevent excess evaporation and concentration. The blood is then filtered into a small centrifuge tube. The filtrate is not always perfectly clear but this does not interfere. To the filtrate is added a small amount of purified charcoal (for method of purification see Benedict, 18) and kaolin in the proportion of 1 gram of each to 15 ml.of filtrate. The mixture is shaken for five minutes and allowed to stand for ten minutes, then centrifuged and filtered. Measure 3 ml.of the filtrate into a pyrex test tube graduated in 5,10, and 15 ml. and into each of two other similar test tubes add 3 ml. of a standard glucose solution containing respectively 0.6 mg. of glucose in 3 ml. and 1.2 mg. in 3 ml. These standards will give accurate determinations for blood sugar values ranging from 60 to 250 mg. per 100 ml. of blood. Treat both the unknown and standards as follows: Add exactly one ml. of 0.6% picric acid, 0.5 ml. of 5% sodium hydroxide and 5 drops of a freshly prepared 50% acetone solution. After adding the acetone rotate the tubes gently and immediately place in a boiling water bath and allow to remain for 15 minutes after which they are removed, cooled, diluted and read in a colorimeter. It is necessary that the unknown and the standard be diluted the same. The 0.6 standard is usually diluted to 10 ml. and the 1.2 standard to 15 ml.

Discussion of Picric Acid-Acetone Method.

Attention must be called to the fact that the 50% acetone solution prepared by diluting equal quantities of acetone with water must be prepared fresh every day. The picric acid solution must be made from purified picric acid and must be kept in a brown bottle in the dark and prepared fresh every 4 weeks. In

adding the reagents to the test tube care must be taken to have the solutions dropped into the bottom of the tube instead of permitting them to run down the sides. This is not a picric acid method and is not subject to the errors of the picric acid methods since the glucose reacts with an unknown decomposition product of picric acid and acetone and not with the picric acid. Our technique is essentially the same as that employed by Benedict(18) in his determination of sugar in urine. If attempts have been made before to apply this method to blood they must have been abandoned because of the interference of salts found in the filtrates. The success of our modification depended on the fact that we used acetic acid and kaolin as our precipitants for the blood proteins. Benedict claims that this is one of the most specific reactions for glucose and we were glad to find that this method gave the same results as our mercuric nitrate method,as may be seen from the following table. Both of the methods are approximately 15% lower than the Folin_Wu method.

TABLE XV.

Comparison of the Acetone and Folin_Wu Methods.

Blood No.	Kind	Age	Mg. of Glucose per 100 ml. of Blood		% Difference.
			Folin-Wu Method.	Acetone Method.	
1	Human	Fresh	94	78	17.0
2	"	"	93	76	18.2
3	"	"	125	113	9.6
4	"	"	81	71	12.3

TABLE XVl.

Recovery of Added Sugar by Acetone Method.

Blood No.	Kind	Mg.of Glucose per 100 ml.of Blood	Mg.Found	Recovery
1	Human	0	76	
1	Human	125	206	130 of 125
2	Human	0	77	
2	Human	125	207	130 of 125
3	Human	0	88	
3	Human	100	188	100 Of 100

Tissue Extracts.

Both the acetone and mercuric nitrate methods are applicable to tissue extracts but we prefer the mercuric nitrate method and believe that it is slightly more accurate. We applied both methods to muscle extracts, each containing 60 mg. of N.P.N., and 130 mg. of creatinine per 100 ml., and compared these results with the regular Folin-Wu procedure. For the tungstic acid precipitation we used 4 ml. of extract, 15.2 ml. of water, 0.4 ml. of sodium tungstate and 0.4 ml. of 2/3 N. sulphuric acid. For the mercuric nitrate precipitation we used 8 ml. of extract, 24 ml. of water, 8 ml. of mercuric nitrate solution. For the acetone method we treated with charcoal and used 3 ml. of the extract. The results were as follows:

TABLE XVll.

Comparison of Folin-Wu, Mercuric Nitrate, and Acetone Methods on Tissue Extracts.

Muscle Extract No.l.

Folin-Wu method..... 48 mg.of Glucose per 100 ml.of extract.

Mercuric Nitrate..... 19 mg.of Glucose per 100 ml.of extract.

Acetone..... 16 mg.of Glucose per 100 ml.of extract.

Muscle Extract No.2.

Folin-Wu Method..... 41 mg.of Glucose per 100 ml.of extract.

Mercuric Nitrate..... 19 mg.of Glucose per 100 ml.of extract.

These methods were also used in determining the glucose content of a liver extract. The same quantities of precipitating reagents were employed as in the muscle extract.

Liver Extract No.1.

Folin-Wu Method..... 55 mg.of Glucose per 100 ml.of extract.

Mercuric Nitrate..... 56 mg.of Glucose per 100 ml.of extract.

Liver Extract No.2.

Folin-Wu Method.....106 mg.of Glucose per 100 ml.of extract.

Mercuric Nitrate.....107 mg.of Glucose per 100 ml.of extract.

All mercuric nitrate filtrates were found to be nitrogen free. It is apparent that for sugar in muscle extract the Folin-Wu method is very inaccurate. The liver extracts contained only 4 mg.of creatinine per 100 ml. and we can see from the table that the Folin-Wu method gave results identical with our mercuric nitrate method. Cori and Cori (69) in determining tissue sugar treated the extract with Lloyd's reagent before applying the Folin-Wu method. They found that this reduced the muscle sugar value 44 per cent. They make no mention of the amount of creatinine left in the extract and we are inclined to think that it is considerable, since their sugar values are considerably higher than ours which were tested and found to be nitrogen free. We found that our mercuric nitrate method gave sugar values 57 per cent lower than the Folin-Wu values on muscle extracts.

Summary and Conclusions.

1 We have shown that the picric acid methods are not accurate.

2 We have shown that the titrimetric methods are not adapted to clinical use.

3 We have demonstrated that the Folin-Wu method, which gives the same results as the titrimetric methods, and is the most accurate method used in clinics, gives results approximately 15 per cent too high, because of interfering substances.

4 We have developed a mercuric nitrate method, specific for blood sugar by virtue of the fact that it precipitates interfering substances before making the determination.

5 We have developed an acetone method, specific for blood sugar, which gives the same results as the mercuric nitrate method.

6 We have adapted our mercuric nitrate method and acetone method for determining glucose in tissue extracts.

Addendum.

The most laborious and time consuming part of this work was in the mastering of the fading of the final blue color of the mercuric nitrate method. The mastering of this point required nearly nine months time and over a thousand determinations.

After this work had been completed and while the report was being written a progress report by Benedict (55) appeared, in which he stated that he had developed a copper solution specific for glucose and that this method gave results from 15 to 30 per cent lower than the Folin-Wu method. This corroborates the blood sugar values found by our two methods. While this report was being written we also noticed in Chem. Abstracts, Feb.10,1925, a brief mention of some work done by Bierry and Moquet (69) who adapted a mercuric nitrate precipitation to the Folin-Wu method. We have been unable to obtain the original article, but from an abstract published in the Berichte Uber Die Gesamte Physiologie und experimentelle Pharmakologie, Nov.1924, Vol.38,P.103, we obtained some information on the article. These workers use a mercuric nitrate solution very

similar to our modification. They precipitate with sodium hydroxide in place of sodium bicarbonate and remove the last trace of mercury with copper shavings in an acid filtrate. Their method is somewhat different from our method although the principle is the same. We were unable to find any values reported by this method.

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