

## A COMPARISON OF BANG'S MICROMETHOD FOR DETERMINING BLOOD SUGAR WITH BERTRAND'S METHOD \*

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It is recognized that the constituents of the blood are of as great a clinical significance as the constituents of the products of excretion, but heretofore blood analysis has not been undertaken as a matter of routine in the clinic, on account of the technical difficulties in the methods employed or because of the large amounts of blood required. Folin and Denis,<sup>1</sup> however, have recently described relatively simple and accurate ways for estimating the non-protein nitrogen, urea, ammonia, and uric acid of the blood, and Marshall<sup>2</sup> has published a method for determining blood urea. These various tests are proving of great value, because clinicians are now able to estimate the nitrogen-containing elements of blood as part of the ordinary study of different types of disease.

Since Claude Bernard<sup>3</sup> recognized that glucose could be found in the circulating blood, a large literature on blood-sugar has accumulated. Two methods for blood-sugar determinations have been generally used. After the proteins from the blood have been removed, the sugar in the filtrate has been quantitated by polarization, or by one of the metallic reduction tests.

The blood proteins have been taken out by numerous reagents. Bernard and Röhmnn<sup>4</sup> chose sodium sulphate, Abeles<sup>5</sup> selected an alcoholic solution of zinc acetate or chlorid, precipitating the zinc by sodium carbonate and washing the sugar into the filtrate. According to Schenck,<sup>6</sup> the proteins could be precipitated with mercuric chlorid, which was removed by hydrogen sulphid. MacLeod<sup>7</sup> concluded in later experiments, however, that certain substances in addition to the proteins were brought down by the mercury and that therefore the results so obtained were not absolutely accurate. He advised the use of

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1. Folin and Denis: *Jour. Biol. Chem.*, 1912, xi, 527.

2. Marshall: *Jour. Biol. Chem.*, 1913, xv, 487.

3. Bernard: *Leçons de physiologie*, 1855, Baillière, Paris.

4. Röhmnn: *Centralbl. f. Physiol.*, 1890, iv, 12.

5. Abeles: *Ztschr. f. physiol. Chem.*, 1891, xv, 495.

6. Schenck: *Arch. f. d. ges. Physiol. (Pflüger's)*, 1894, Iv, 203.

7. MacLeod: *Jour. Biol. Chem.*, 1909, v, 443.

phosphotungstic acid as described by Reid<sup>8</sup> to obtain the sugar solution. A disadvantage of all these methods lay in the fact that a certain amount of sugar was held back from the filtrate during precipitation. As a result the precipitates had to be washed sugar-free to obtain accurate estimations and this procedure required a large bulk of fluid, and considerable time was occupied in later concentrating this fluid.

Edie and Spence<sup>9</sup> attempted to obviate this by dialysis of the sugar from the blood into saline solution. Michaelis and Rona<sup>10</sup> introduced the use of colloidal iron in the presence of an electrolyte. They were able to precipitate the blood proteins completely by these reagents without absorption of sugar and therefore they could use aliquot parts of the filtrate instead of washing the precipitate. Furthermore since the iron and salt were precipitated with the protein, no necessity existed for repeated filtering. Thus this method was more rapid and simple than the older ones. Bang,<sup>11</sup> however, has preferred alcohol and animal charcoal as a means of obtaining the sugar filtrate.

The polarization of blood-sugar solutions has been advocated by Michaelis and Rona and their pupils. The majority of workers on the various means of quantitative sugar analysis have used one of the reduction tests. These are all based on the same underlying principle. It is known that a cupric or mercuric salt is reduced quantitatively by sugar and boiling, and if the amount of copper or mercury involved in the reaction can be estimated, the total amount of sugar in a given solution can be determined. The various tests most generally used have been gravimetric, titrimetric or colorimetric.

Allihn devised a method, modified by Pflüger<sup>12</sup> in which a solution of copper sulphate was boiled with an unknown sugar solution. The reduced copper was filtered, ignited and weighed as pure copper. From this figure the amount of sugar present was estimated from especially prepared tables giving the value of varying amounts of sugar in terms of copper. This test or one slightly modified has been used by Bönniger,<sup>13</sup> Edie and Spence, Harley,<sup>14</sup> MacLeod and Reid. Bertrand,<sup>15</sup> instead of weighing the reduced copper, dissolved it in a solution of ferric sulphate and sulphuric acid. The amount of ferrous salt so formed was determined by titration against potassium permanganate

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8. Reid: *Jour. Physiol.*, 1896, xx, 316.

9. Edie and Spence: *Biochem. Jour.*, 1907, ii, 103.

10. Michaelis and Rona: *Biochem. Ztschr.*, 1908, vii, 329.

11. Bang: *Ztschr. f. physiol. Chem.*, 1910, lxxv, 497.

12. Pflüger: *Arch. f. d. ges. Physiol.* (Pflüger's), 1898, lxxix, or 1902, xcvi, 1.

13. Bönniger: *Deutsch. med. Wchnschr.*, 1908, xxxiv, 780.

14. Harley: *Jour. Physiol.*, 1891, xii, 391.

15. Bertrand: Quoted from Grube, Abderhalden's *Handbuch der biochemischen Arbeitsmethoden*, 1910, ii, 181, Urban and Schwarzenberg, Berlin and Vienna.

standardized with ammonium oxalate. Knowing this, the amount of copper present in the reaction was found, and the amount of sugar in an unknown solution was estimated by tables giving different copper-sugar values.

Bernard used Fehling's method. An alkaline copper solution was made of such strength that it was perfectly reduced on boiling with a fixed amount of sugar. In making an analysis, a part of the sugar solution was added to the copper until no further reduction occurred and the ratio which this bore to the entire solution gave information from which it was possible to discover the total quantity of sugar present. The difficulty with the test lay in the fact that the end-point was uncertain. Pavy<sup>16</sup> modified the reaction by making a solution of ammoniacal copper which held the reduced copper in solution but lost its blue color after complete reduction occurred. A known amount of copper was reduced by a part of the unknown sugar solution, and from this titration the entire amount of sugar present could be estimated. Kumagawa and Suto<sup>17</sup> have obtained good results by its use.

Bang's<sup>18</sup> test is on somewhat the same principle. The reduced copper is held in solution by potassium rhodamate in the presence of carbonates. In performing the test an excess of copper solution is added to the sugar, and is later reduced to a colorless solution by hydroxylamin. The amount of sugar taking part in the reaction is estimated by tables prepared from known sugar solutions and the reagents.

Instead of using copper as a reduction test, Knapp<sup>19</sup> selected mercuric cyanid which is reduced into metallic mercury by glucose. A solution was made which could be reduced by a known amount of sugar, and titration was carried out until no mercury salt was left in solution. Röhmann, Schenck and Liefmann and Stern<sup>20</sup> used the method in their studies on blood-sugar, while Tachau<sup>21</sup> modified it to serve as a rapid test for clinical purposes.

Wacker<sup>22</sup> introduced colorimetric means for determining blood-sugar. The albumin in the blood was precipitated by iron alum and sodium carbonate and the amount of sugar in an aliquot part of the filtrate was found by a sensitive color reaction between the sugar and a solution of phenylhydrazin sulphacid in concentrated sodium hydrate.

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16. Pavy: Pavy, *Chem. News*, 1880, ix, 98, Abs. *Ztschr. f. anal. Chem.*, 1880, xix, 98.

17. Kumagawa and Suto: *Salkowski's Test*, Salkowski's *Festschrift*, 1904, p. 211, Hirschwald, Berlin.

18. Bang: *Biochem. Ztschr.*, 1906, ii, 271.

19. Knapp: Quoted from Hammarsten. *A text-book of physiological chemistry*, 1893, 417, John Wiley & Sons, New York.

20. Liefmann and Stern: *Biochem. Ztschr.*, 1906, i, 299.

21. Tachau: *Deutsch. Arch. f. klin. Med.*, 1911, cii, 597.

22. Wacker: *Ztschr. f. physiol. Chem.*, 1910, lxxvii, 197.

Quantitative readings were obtained by comparison of the color of unknown solutions with that of known sugar solutions mixed with the reagents. Autenrieth and Tesdorpf<sup>23</sup> boiled sugar solutions with Bang's copper solution, diluted the mixture to a known amount and estimated the amount of reduced copper by colorimetry. The copper solution lost color in proportion to the amount of sugar present, so that Autenrieth and Tesdorpf were able to construct a color scale of sugar values which gave accurate readings. Forschbach and Severin<sup>24</sup> and Borchardt and Bennigson<sup>25</sup> used this method satisfactorily. Reicher and Stein<sup>26</sup> depended on Molisch's reaction as a quantitative test. Tablets of alphanaphthol and sulphuric acid were added to a sugar solution from which the albumin was precipitated and the color changes were compared with tubes containing similar amounts of reagent and increasing amounts of sugar. The readings obtained were higher than by the other methods, which made Forschbach and Severin believe that the test was not sufficiently delicate for general use.

From this review it is seen that there are a variety of ways for estimating blood sugar, none of which are free from criticism. Difficulties have been encountered not only in precipitating the proteins from the blood but also in getting the sugar values. Constant readings, however, which have been valuable in comparative studies over long intervals of time, have been obtained by using any one method. The objections to the various tests from the point of view of the clinician have been the large amounts of blood required to conduct the analyses, the length of time for their completion, and the difficulty of technic.

Any test, to be of use in clinical studies on blood analysis, must require small amounts of blood, so that it can be repeated at will, must be accurate enough for comparative readings, and must be so simple that it can be performed by individuals without specialized training in chemical technic. Bang<sup>27</sup> has described a method for blood-sugar analysis which fulfills these requirements. Thannhauser and Pfitzer,<sup>28</sup> Götzky,<sup>29</sup> Bing and Windelöw,<sup>30</sup> Bing and Jacobsen<sup>31</sup> and Hirsch and Reinbach<sup>32</sup> have confirmed the value of this test, but have

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23. Autenrieth and Tesdorpf: *München. med. Wchnschr.*, 1910, lvii, 1780.

24. Forschbach and Severin: *Zentralbl. f. d. ges. Physiol. u. Path. d. Stoffwechs.*, 1911, n. f. vi, 54, 177, 665.

25. Borchardt and Bennigson: *München. med. Wchnschr.*, 1913, ix, 2275.

26. Reicher and Stein: *Biochem. Ztschr.*, 1911, xxxvii, 321.

27. Bang: *Der Blutzucker*, 1913, J. F. Bergmann, Wiesbaden.

28. Thannhauser and Pfitzer: *München. med. Wchnschr.*, 1913, ix, 2155.

29. Götzky: *Ztschr. f. Kinderh.*, 1913, Orig., ix, 64.

30. Bing and Windelöw: *Ztschr. f. Kinderh.*, 1913, Orig., ix, 64.

31. Bing, L., and Jakobsen, B.: *Ugeskr. f. Laeger*, 1913, lxxv, 1627; *Abst. Jour. Am. Med. Assn.*, 1913, lxi, 1946.

32. Hirsch and Reinbach: *Ztschr. f. physiol. Chem.*, 1913, lxxxvii, 122.

compared it with other methods in a comparatively small number of cases. In this country no systematic use of Bang's test has been reported.

This paper gives the results of a series of blood-sugar determinations made by Bang's micromethod and controlled by Bertrand's method to determine if the micromethod could be used with sufficient accuracy and sufficiently constant readings for clinical use. The technic of the two methods as used by the observer is given and the results are tabulated.

Twenty-five c.c. of blood were withdrawn from a vein according to usual technic into a small flask containing a few crystals of potassium oxalate to prevent clotting or were defibrinated by shaking. This blood was used for subsequent analysis either immediately, or after standing for a few hours to induce glycolysis. Each analysis by the two methods, however, was made at the same time. Two or three readings were made by the micromethod, and one by the Bertrand. Unfortunately enough blood for double determinations throughout was not obtained.

#### BERTRAND METHOD

The proteins were precipitated according to Rona and Michaelis. From 15 to 20 c.c. of blood were pipetted into a flask and were laked with 100 c.c. of distilled water; 110 c.c. of dialyzed iron solution<sup>33</sup> were added and 10 c.c. of saturated sodium sulphate solution. The mixture was diluted to 350 c.c. with distilled water, and was shaken for five or ten minutes. On filtering, a clear filtrate was usually obtained. In a few cases, re-filtration was necessary after the addition of a few additional cubic centimeters of the iron solution. An aliquot part of the filtrate, representing from 10 to 15 c.c. of blood was measured off, made acid with acetic acid and evaporated to 10 c.c. bulk on the water-bath. The mixture was then washed carefully into a 200 c.c. Erlenmeyer flask and the sugar estimation was conducted according to Bertrand's technic described by Grube.<sup>34</sup>

The sugar solution was boiled with Bertrand's two solutions for three minutes. On cooling the cuprous oxid was filtered and washed on a Gooch crucible, and was re-dissolved in a solution of ferric sulphate and sulphuric acid. The total copper present was determined by titration with potassium permanganate, and from the amount present the sugar was calculated by Bertrand's tables. From beginning to

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33. The solution used was Merck's dialyzed iron (liquid 5 per cent.  $\text{Fe}_2\text{O}_3$ ), the estimation was conducted according to Bertrand's technic described by Grube.<sup>34</sup>

34. Grube: Abderhalden's Handbuch der Biochemischen Arbeitsmethoden, 1910, ii, 181, Urban and Schwarzenberg, Berlin and Vienna.

end, the entire test took from three to four hours. In the few cases in which double determinations were made, the results checked with accuracy, so that on the whole the method appeared to give constant readings and was perfectly satisfactory as a control.

#### BANG'S MICROMETHOD

The tests were made according to Bang's original method as modified by Thannhauser and Pfitzer. The test depends on the assumption that copper is reduced by sugar without regard for the amounts present. In other words, a sugar solution from 100 mg. of blood will reduce as much copper proportionately as a solution from 100 c.c. of blood. If the microscopic amount of copper involved in the reaction can be determined the results will be as accurate as if larger amounts of the metal are used. Accordingly an albumin-free sugar solution is made from a small amount of blood and is boiled with copper solution. The cuprous oxid which is formed is held in solution by the method which Bang has described previously, and is reoxidized by iodine in the presence of potassium carbonate. Cupric carbonate and potassium bicarbonate are formed while potassium iodide is set free and can be tested for by starch as an indicator. The reaction is quantitative and extremely delicate. The technic for the test is given in detail.

The copper solution is made by dissolving 160 gm. of potassium bicarbonate in from 600 to 700 c.c. of distilled water, 100 gm. of potassium carbonate are added, 66 gm. of potassium chloride and 100 c.c. of a 4.4 per cent. copper sulphate solution ( $\text{CuSO}_4 + 5\text{H}_2\text{O}$ ). When everything is dissolved the mixture is diluted to one liter, and is ready for use after it is allowed to stand for twenty-four hours. In making an analysis this solution is diluted with a 20 per cent. potassium chloride solution made from 150 c.c. of saturated solution plus 70 c.c. of water. Titration is made with one-hundredth normal iodine solution prepared from 1 c.c. of 2 per cent. potassium iodate solution, 2 gm. of potassium iodide and 5 c.c. of one-tenth normal hydrochloric acid, the mixture being diluted to 50 c.c. This solution is unstable and must be renewed every three or four days.

Two or three drops of blood are placed on a small piece of thick blotting-paper, which previously has been boiled in dilute acetic acid and water to remove traces of albumin and sugar. The blotting-paper should be cut in oblongs 10 by 20 mm. and weighed before and after the blood is added. In this way the weight of blood to within 1 mg. is found. In these experiments small weighing flasks were used to hold the blood and paper. After the blood is weighed, 7 c.c. of boiling hot potassium chloride solution is added and acidified with 2 or 3

drops of 40 per cent. acetic acid. The albumin in the blood precipitates on the blotter, while the sugar diffuses through into the solution.

After half an hour's cooling the sugar solution is transferred to a 50 c.c. Jena flask with a straight neck and is washed in by 4 c.c. of the potassium chlorid solution. In this way all the sugar in the blood is removed from the weighing flask and is ready for titration.

Three c.c. of the copper solution are now added and the mixture is boiled over a micro-burner. The heating is an important factor in the test. The solution must be warmed so that it begins to boil in between one minute and fifteen seconds and one minute and a half, and must be boiled for exactly two minutes, since the copper solution can be reduced by longer or more vigorous boiling and will give improper readings. At the end of this time the flask is immersed in cold water and titrated.

A second source of error in the determination is air oxidation of the reduced copper. During boiling this is discounted. But after cooling, air must be kept out of the solution by manipulating it under carbon dioxid gas. For this purpose Bang introduced into the neck of the flask a hooked glass tube which was connected to a carbon dioxid tank. The gas was turned on as soon as the flask was put into cold water and in this way air oxidation was prevented. Thannhauser and Pfitzer used small flasks with side-arms which were easier to handle. Similar flasks were used in these experiments.

After the two minutes' boiling, the gas is introduced and the flask is immediately cooled. This can be done in less than three seconds. A few drops of 1 or 2 per cent. soluble starch solution are now added as an indicator. The titration is made from a 1 or 2 c.c. differential pipet reading in hundredths of a cubic centimeter, and the iodine solution is added until the light blue color of the copper solution takes on the deep blue color of the starch and iodide.

Bang has tested the method carefully with known sugar solutions. The amount of iodine used by increasing amounts of sugar is so nearly constant that he has been able to make a formula for determining the amount of sugar present. The amount of iodine used in titration is divided by 2.2. This result minus 0.01 will give the milligrams of sugar present in the solution. In these experiments a special table was constructed, however, which showed the iodine readings for varying amounts of sugar from which the blood sugar readings per 100 c.c. of blood were calculated.

The entire test takes less than three-quarters of an hour, requires so little blood that it can be repeated on the same individual at will, and is very easy to perform after short practice.

The results of forty-two blood examinations are given in the accompanying table. The Bang figures represent the average of duplicate readings unless otherwise noted. All but one or two read within 0.02 of a gram of each other per 100 c.c. of blood. The amounts of blood used, varied between 50 and 120 mg., although most constant readings were obtained when from 80 to 100 mg. were taken.

RESULTS OF FORTY-TWO BLOOD EXAMINATIONS

Blood Number	Methods		Blood Number	Methods	
	Bertrand* Per Cent.	Bang† Per Cent.		Bertrand* Per Cent.	Bang† Per Cent.
1	0.04	0.06	22	0.11	0.11
2	0.04	0.03	23	0.12	0.14*
3	0.05	0.06‡	24	0.14	0.12
4	0.05	0.085	25	0.14	0.15
5	0.06	0.08	26	0.15	0.12
6	0.06	0.075	27	0.16	0.145
7	0.06	0.07	28	0.16	0.15
8	0.06	0.05	29	0.18	0.17*
9	0.07	0.055	30	0.18	0.205
10	0.07	0.07	31	0.18	0.17
11	0.07	0.09	32	0.19	0.16
12	0.075	0.08	33	0.20	0.17
13	0.075	0.065	34	0.20	0.195
14	0.08	0.075	35	0.21	0.20
15	0.08	0.12	36	0.21	0.20
16	0.08	0.095	37	0.22	0.21
17	0.09	0.085	38	0.25	0.245
18	0.09	0.105	39	0.28	0.26
19	0.10	0.085	40	0.30	0.295
20	0.10	0.10	41	0.30	0.31
21	0.10	0.125	42	0.32	0.305‡

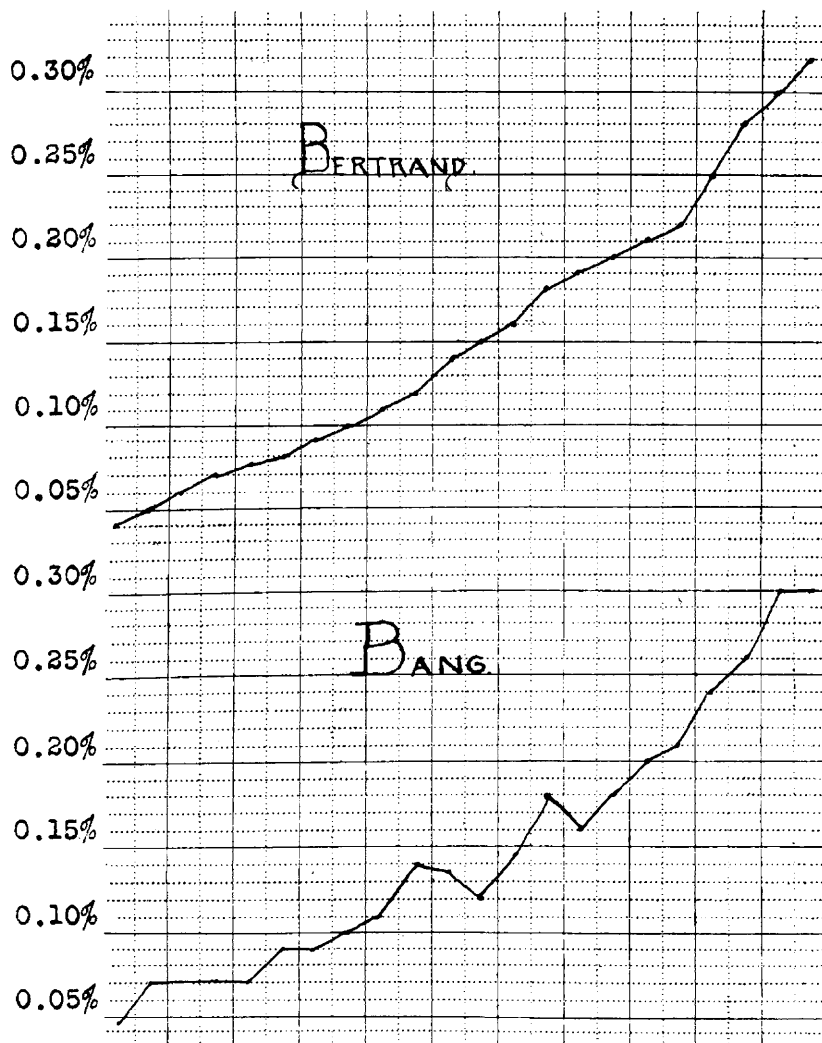
\* One reading. † Two readings throughout this column except where the star (\*) and double dagger (§) occur.

If the Bertrand method is considered as the more accurate it is seen that the Bang readings were not correct. With two exceptions, however, the average Bang reading at any of the sugar concentrations encountered was within 0.03 per 100 c.c. of blood of the Bertrand. This, of course, makes the actual percentage of error large. But as the essential feature of blood sugar determinations is not so much the absolute accuracy of the figures encountered—since objections to any one method can be found—as it is the relative value in a series of comparative determinations, the Bang method is perfectly satisfactory. This is illustrated by the accompanying chart made from the average of all readings encountered at all the different concentrations.

While the curve is not absolutely parallel, yet for comparative studies it is sufficiently similar. Furthermore, as control determinations were not made by the Bertrand method, it is possible that the differences occurring may have been considerably less.



Since his first publication, Bang<sup>35</sup> has modified the method slightly. The copper solution is made up as before, but 1 c.c. is used instead of 3 c.c. The protein is precipitated by hydrochloric acid in potassium chlorid instead of by acetic acid. For this purpose 1,360 c.c. of saturated potassium chlorid are diluted with 640 c.c. of distilled water and



1.5 c.c. of 25 per cent. hydrochloric acid is added. Of this mixture 6.5 c.c. are used for the precipitation and added boiling hot; 6.5 c.c. are added to wash the solution into the boiling flask. Titration is done with two-hundredths normal iodine made by diluting the original

35. Bang: *Biochem. Ztschr.*, 1913, Ivii, 300.

iodin solution to 100 c.c. instead of 50. The reagent is added until a permanent blue color appears. The amount of iodin solution used minus 12 divided by 4 gives the sugar values. In a few comparisons with Fehling's and Knapp's titration methods these results were found to be from 0.01 to 0.15 per cent. higher. This agrees with the results of Hirsch and Reinbach and Thannhauser and Pfitzer. Therefore to obtain most accurate results, Bang advises the subtraction of 0.015 from the estimated reading.

These modifications were used in a series of ten more bloods. In my experience, the results were no more accurate than by the first series. Furthermore so sharp an end-point in titration could not be obtained by the diluted iodin solution and therefore the former method is on the whole more satisfactory.

#### CONCLUSION

These comparative experiments show that the results obtained by the micromethod were not absolutely accurate if the results obtained by the Bertrand method are considered correct. In a series of forty-two determinations by both methods, however, in only two cases was there a difference of more than 0.03 gm. per one hundred c.c. of blood. This means that, while the absolute error was large, the relative error was slight. The curve of blood sugar determinations made from all the examinations was closely parallel. Hence for comparative and repeated studies in blood sugar estimations the micromethod is admirable. Furthermore, since the amount of blood required to make the test is so small, and the technic so simple, the method affords an excellent clinical means for the estimation of blood-sugar.

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